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SYNTHESIS AND CHARACTERIZATION OF LIQUID-SILICATE FERTILIZER FROM TREATED AND UNTREATED ASH RICE HUSK

(Sintesis dan Kajian Sifat-Sifat Baja Cecair-Silikat daripada Abu Sekam yang Dirawat dan Tidak Dirawat)

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Abstract

Rice husk ash (RHA) is an agricultural waste material that is abundantly available in all rice-producing Asian countries. The RHA is rich in silica (Si), which can be extracted as silicate sources for many industries, such as fertilizer, building material, insulation material, or fuel. In this research, organic acid, which is a phosphoric acid (H_3PO_4), was used to treat RHA. RHA was treated with H_3PO_4 at various temperatures (60°C, 80°C, and 100°C) and molarity (1 M, 2 M, and 3 M), and followed by an extraction process of untreated and treated RHA using potassium hydroxide (KOH) at 2 and 3 hours of extraction time and 1 M and 2 M of KOH. The extracted liquid-silicate was determined by using Fourier Transfer Infra-Red (FTIR), X-ray fluorescence (XRF), and Particle Size Analyzer (PSA). The highest silica content (40.910%) in liquid-silicate fertilizer (LSF) was recorded for the treatment condition of 2 M H_3PO_4 at 80 °C based on the XRF result. The highest wave number was 1008.78 cm^{-1} from treated RHA at 2 M H_3PO_4 80 °C. According to PSA data, the smallest particle size (1465.9µm) was recorded after leaching with H_3PO_4 at 2 M 80 °C as compared to untreated RHA at 1639.75µm.

Keywords: rice husk ash, liquid-silicate fertilizer, acid leaching, phosphoric acid, silica extraction

Abstrak

Abu sekam padi (RHA) adalah bahan buangan pertanian yang banyak terdapat di semua pengeluar beras untuk negara Asia. RHA kaya dengan silika (Si), yang boleh diekstrak sebagai sumber silikat untuk banyak industri seperti baja, bahan binaan, bahan penebat atau bahan api. Dalam penyelidikan ini, asid organik, iaitu asid fosforik (H_3PO_4) digunakan untuk merawat RHA. RHA dirawat dengan H_3PO_4 pada pelbagai suhu (60 ℃, 80 ℃, dan 100 ℃) dan kemolaran (1 M, 2 M dan 3 M). diikuti dengan proses pengekstrakan RHA yang tidak dirawat dan dirawat menggunakan kalium hidroksida (KOH) pada 2 dan 3 jam masa pengekstrakan dan 1 M dan 2 M KOH. Cecair-silikat yang diekstrak ditentukan dengan menggunakan Inframerah transformasi Fourier (FTIR), pendaflour sinar-X (XRF) dan penganalisa saiz partikel (PSA). Kandungan silika tertinggi (40.910%) dalam baja silikat cecair (LSF) direkodkan untuk keadaan rawatan 2 M H_3PO_4 pada 80 °C berdasarkan keputusan XRF. Nombor gelombang tertinggi ialah

1008.78 cm^{-1} daripada RHA yang dirawat pada 2 M H_3PO_4 80 °C. Menurut data PSA, saiz zarah terkecil (1465.9µm) direkodkan selepas larut lesap dengan H_3PO_4 pada 2 M 80 °C berbanding RHA yang tidak dirawat pada 1639.75µm.

Kata kunci: abu sekam padi, baja cecair-silikat, asid larut lesap, asid fosforik, pengekstrakan silika

Introduction

In 2019, the world's total paddy rice production was estimated to be 757 million tons [1]. China is the world's leading producer of rice and paddy. As of 2019, China's paddy rice production was 211 million tons, accounting for 27.92% of global paddy rice production. The remaining top five countries which account for 71.54% of the production are India, Indonesia, Bangladesh, Vietnam, and Thailand. Malaysia's paddy rice production in 2019 was 2.91 million tons. From the production of paddy rice, about 1.2 million tons per year of agricultural waste is disposed of in Malaysia's landfills [2]. During the paddy rice cultivation, several by-products are also generated such as broken rice, the husk, and the bran layer. Hence, the utilization of these by-products or agricultural solid waste has been done to save the environment. In this study, rice husk ash (RHA) was the targeted waste material from paddy rice cultivation that can be converted into a potential liquid-silicate fertilizer (LSF).

The RHA can be obtained from the combustion process of RH in the furnace under a controlled temperature, which is below 700 ℃. A suitable temperature and burning environment can provide a better quality of RHA as its particle size and specific surface area are dependent on burning conditions. For every 1000 kg of milled paddy, it will produce around 200 kg (20%) of RH, and when this husk undergoes a burning process in the boilers or incinerators, around 50 kg (25%) of RHA is produced [3]. Usually, a completely burnt RHA will be observed in physical color changes from grey to white. From the burning of RH, the ash contains mainly amorphous silica (Si) and carbon black residue, which is also called carbonaceous siliceous material [4]. The silica group minerals are framework silicates with the composition of SiO_2. They belong to the silicate mineral class. The most common silica mineral is quartz. Besides quartz, other silica polymorphs include cristobalite, tridymite, coesite, and stishovite [5].

The burning conditions can affect the amount of silica and carbon black in the ash. As proven by many researchers, RHA has a high content of silica, which is 94% of the total content, while the remaining 6% contains potassium oxide (K_2O), calcium oxide (CaO), magnesium oxide (MgO), aluminum oxide (Al_2O_3), and phosphorus pentoxide (P_2O_5) in decreasing concentrations [2]. Because of that, it has been utilized in many industries such as fertilizer, coating, and paint industries.

To obtain RHA, RH will undergo a carbonization process. Carbonization is a method by which solid residues with increasing carbon element content are produced from organic material in an inert atmosphere, typically by pyrolysis. Mehta proposed that essential amorphous silica can be obtained by holding the temperature for prolonged periods below 500℃ and under oxidizing conditions or the temperature can be increased up to 680 ℃ but with a hold-time of not more than 1 minute [6]. Other researchers have claimed that the burning of RH in extreme temperatures (i.e. high temperature) or long duration of time would result in the formation of crystalline phases [7]. The partially crystalline phase of RHA is caused by burning the RH for more than 1 hour in a temperature range between 973 and 1073 Kelvin. Burning RH at temperatures above 1073 Kelvin for more than 1 hour has been documented to lead to the formation of a complete crystalline phase.

Good quality biogenic silica can be produced by the treatment process. Several researchers have carried out the experiments by using a leaching process with hot acid treatment with inorganic acids such as hydrochloric acid (HCl), nitric acid (HNO_3), and sulfuric acid (H_2SO_4) or hot basic treatment with inorganic bases such as ammonium hydroxide (NH_4OH) and sodium hydroxide (NaOH) [8-11]. Acid leaching treatment of RHA by using organic acid is highly encouraged because it has many economic and ecological benefits compared to a strong acid [12-13]. It is a proper route for silica extraction to remove substances from solid organic

materials via the liquid extraction method. Based on the results obtained from the previous paper, an organic acid solution such as citric acid was considered a more environmentally friendly process during the acid-leaching treatment. However, phosphoric acid (H_3PO_4) was used as a pH adjustor in liquid fish fertilizers, as an inert ingredient in pesticide products, and as a sanitizer for dairy equipment [14].

A method based on acid treatment before the alkaline extraction is developed to produce high-purity silicate with minimal contaminants. This process does not provide any harm to the life of organisms, moreover, it is more economical. In addition, a chelating reaction occurs between the carboxyl group (-COOH) and metal cations, which leads to the removal of metal impurities in the RHA. Conventionally, the removal of metallic impurities is done by using inorganic acids such as HCL, HNO_3, and H_2SO_4. However, the use of inorganic acid has caused harm to human beings and the environment. Furthermore, the expensive materials with corrosion resistance needed for the leaching process using inorganic acid can cause an economic problem. There is also a high consumption of water to remove the acid, as well as the need for a special disposal system [12]. That is the reason why organic acid should be used as the substitute for inorganic acid, to comply with the environmental requirements for less exposure or disposal of hazardous or corrosive chemical waste. For this paper, organic acid which is H_3PO_4 was used to treat the RHA before the liquid-silicate extraction process. This study aimed to synthesize LSF from treated (acid leaching) and untreated RHA and to compare the results of the characterization of LSF by using XRF, FTIR, and PSA techniques for both treated and untreated RHA.

Materials and Methods

Materials

In this experiment, the RHA was obtained from a rice mill, DIBUK Sdn. Bhd., located in Perlis, Malaysia. Chemicals such as 45% potassium hydroxide (KOH) and 85% phosphoric acid (H_3PO_4) were obtained from Reichle & De-Massari (R&M), Switzerland.

Material preparation

An amount of 3 kilograms of RHA was obtained directly from the factory. The rice husk ash (RHA) had already undergone the carbonization process at the factory at 650 °C. After that, RHA was cleansed by using distilled water and dried in the oven to remove any impurities and reduce the moisture content. This was to avoid any effects during the treatment and extraction. The sample was dried at 60 °C for 60 mins [15]. Once the RHA was completely dried, the RHA was grounded using a mechanical grinder until it turned powdery. A few grams of the sample were kept without further treatment process as an untreated sample (blank sample).

Acid leaching treatment process

200 g of RHA were soaked in each beaker containing H_3PO_4 at various molarities (i.e.1 M, 2 M, and 3 M) to undergo acid-leaching treatment. During the treatment process, these acid-treated samples were placed on the hot plate magnetic stirrers to heat the mixture until they reached various temperatures at 60, 80, and 100 °C and were kept constant for 2 hours for each sample [16]. After the acid leaching treatment was completed, each sample was washed using distilled water to neutralize the sample. The pH of the sample must be monitored until it reaches pH 7. After that, all samples were desiccated in an oven at 100 °C for 2 hours.

Extraction process of treated and untreated RHA

100 g of treated and untreated RHA were soaked in beakers containing various molarities of KOH (i.e., 1 M, 2 M, and 3 M) for the extraction process. During the extraction process, each sample was placed on a hot plate magnetic stirrer to heat until the temperature reached a constant 100 °C at 2 and 3 hours for each sample, respectively. Next, the samples were desiccated in an oven at 100 °C for 2 hours.

Chemical composition analysis and characterization of treated and untreated rice husk ashes

Fourier transform infrared (FTIR) spectroscopy, X-ray fluorescence (XRF) spectroscopy, and particle size analyzer (PSA) were used to determine and compare the difference between the composition of silica in treated and untreated RHA in their potential as an LSF. The FTIR technique was used to characterize the major chemical groups present within the LSF using an FTIR spectrometer model L1280044 manufactured by Perkin

Elmer USA. After launching the spectrum software, the sample was placed on the sample holder followed by the operation of the spectrometer, and each spectrum was finally collected until complete spectra were projected on the monitor.

Next, the XRF spectrometer, PAN analytical MiniPAL 4 X-ray (Philips, UK) was used to characterize the number of chemical compounds within the liquid silicate [2]. Each sample was placed in a cup that was covered using a suitable foil, usually Kapton material. After that, the sample was placed into the XRF spectrometer.

Finally, RHA characterization was performed by using PSA Malvern Mastersizer 2000 (Malvern, UK). PSA was used to determine the size and distribution of the particles that comprise a material. The characterization and analysis of the composition of RHA were conducted using the Standard Operation Procedure (SOP) instructions. Then, the sample was added into the water to ensure an adequate amount of sample was aliquoted (one drop at a time) to turn the Laser Obscuration bar green (i.e. until it achieved the mid-value) [17-18]. The sample was measured and the outcome (size distribution) was computed. The recorded measurement was saved to a measurement file automatically.

Results and Discussion

Acid leaching treatment with phosphoric acid (H_3PO_4)

Acid leaching treatment on rice husk ashes was done by using H_3PO_4. The purpose of this treatment is to remove some amounts of metal impurities [15]. Based on the XRF data in Table 1, the highest silica content was at 1 M 60 °C sample with 57.676% and had the lowest potassium (K) and Iron (Fe) content, which are 1.413% and 39.357%, respectively. To proceed with the next stage of the experiment, which was the extraction process, the highest silica content from each proposed molarity was chosen. Hence, 1 M at 60 °C, 2 M at 80 °C, and 3 M at 60 °C were chosen to undergo the extraction process later. From previous studies, it has been demonstrated that the phosphoric acid activation of lignocellulosic [19] precursors produced carbons having not only a well-developed porosity, but also considerable amounts of acid surface groups. [20]

Table 1. XRF data for acid leaching treatment of RHA

Samples	Temperature (°C)	Si (%)	K (%)	Fe (%)	Traces Element (%)
	60	57.676	1.413	39.357	1.554
1 M	80	55.363	1.825	41.176	1.636
	100	54.400	1.758	42.205	1.637
	60	53.835	1.877	42.633	1.655
2 M	80	54.360	1.874	42.107	1.659
	100	53.593	1.754	43.002	1.651
3 M	60	51.378	1.885	45.061	1.676
	80	51.323	1.806	45.248	1.623
	100	51.139	1.731	45.487	1.643

Note: Data are given in mass percentage. The trace elements are Na, Mg, Al, P, Ca, Cu, and Zn

Extraction process using potassium hydroxide (KOH)

The extraction process was done by using KOH for treated and untreated RHAs. The purpose of extraction is to produce LSF. Further characterization was carried out to observe the content, such as silica content in the LSF.

XRF spectroscopy

Two types of sample conditions were tested, which are untreated and treated RHA. The chemical composition was analyzed by using an XRF spectrometer. Table 2 shows the chemical composition of treated and untreated RHA. Untreated RHA recorded the lowest silica content. Meanwhile, treated RHA at 1 M 2 H yielded the highest silica content, which is 40.910% at the treatment

condition of 2 M 80 °C, compared to untreated RHA, in which the silica content was 29.157%.

The other metallic elements such as iron and potassium are presented in the LSF of both treated and untreated RHA. The results indicated that most iron was reduced when the RHA underwent leaching treatment. Similar results were exhibited for the effect of acid-leaching treatment on the potassium content. The removal of these metallic cations after chelating with H_3PO_4 improved the chemical composition of a leached sample to be rich in alkaline metals, which are water-solubles such as potassium. Hence, the unwanted impurities were washed away by the distilled water used in the neutralization process. The impurities in the husk were removed using a chelate reaction between carboxyl groups (-COOH) and metal elements [2]. This is because the majority of elements absorbed during the leaching process were silica which was extracted from RHA. As a result of the strong covalent bond that exists between silicon and oxygen in the properties, silica becomes very hard and rigid [2], which makes it the end product of this leaching process for its useful real-life application as an agricultural fertilizer.

As shown in Table 2, H_3PO_4 leaching treatment was significantly useful and effective in removing impurities and increasing silica purity in RHA. However, the silica content for treated 2 M 2 H and 2 M 3 H was observed to decline from 21.317 to 18.477% and 19.148 to 18.212%, respectively when undergoing the treatment process. As shown in Table 2, the iron content was the highest compared to silica and potassium content. This correlates with the literature finding that, iron is very essential in plant growth. Iron is required for many vital plant functions, including enzyme and chlorophyll production, nitrogen fixation, development, and metabolism. Even though iron is abundant in soil, plants frequently suffer from iron deficiency due to its low solubility, particularly in alkaline calcareous soils. Hence, our liquid silicate fertilizer is very suitable for the plant as it has a high content of iron.

Table 2. XRF data for treated and untreated RHA

Sample (RHA)	Treatment Condition	Si (%)	Fe (%)	K (%)	Traces Element (%)
Untreated Sample (Blank)	-	29.157	52.350	15.164	3.329
Treated 1 M 2 H	1 M 60 °C	37.685	51.053	9.769	1.493
Treated 1 M 2 H	2 M 80 °C	40.910	46.401	11.014	1.675
Treated 1 M 2 H	3 M 60 °C	29.777	50.891	13.906	5.426
Untreated 1 M 3 H	-	28.516	54.098	14.993	2.393
Treated 1 M 3 H	1 M 60 °C	39.285	45.704	13.417	1.594
Treated 1 M 3 H	2 M 80 °C	38.223	49.182	10.905	1.690
Treated 1 M 3 H	3 M 60 °C	34.004	49.943	13.555	2.498
Untreated 2 M 2 H	-	21.317	55.121	21.973	1.589
Treated 2 M 2 H	1 M 60 °C	21.711	55.087	21.680	1.522
Treated 2 M 2 H	2 M 80 °C	22.501	54.454	21.636	1.409
Treated 2 M 2 H	3 M 60 °C	19.477	56.551	22.794	1.178
Untreated 2 M 3 H	-	19.148	58.881	20.276	1.695
Treated 2 M 3 H	1 M 60 °C	27.767	50.150	20.857	1.226
Treated 2 M 3 H	2 M 80 °C	28.073	48.720	22.114	1.093
Treated 2 M 3 H	3 M 60 °C	18.212	52.906	27.465	1.417

Note: Data are given in mass percentage. The samples are from two conditions which are treated and untreated RHA. The trace elements are Na, Mg, Al, P, Ca, Cu, and Zn

FTIR spectroscopy

Figure 1 and Table 3 represent the infrared spectra of treated and untreated RHA at 1 M 2 H. Based on the XRF data tabulated in Table 2, the highest silica can be extracted from 1 M 2 H. Hence, the selected samples were used to further analyze the functional groups in treated and untreated RHA by using FTIR spectroscopy. The peak from 1095 to 1075 cm^{-1} or 1055 to 1020 cm^{-1}

showed the stretching of silicone, Si-O- [21]. Meanwhile, the peak from 1100 to 900 cm^{-1} showed the stretching band of silicate ions [21]. Because of the asymmetric stretching of the silicate ion, it demonstrated a peak at wave number 1006.88, 1001.13, 1008.78, and 1003.34 cm^{-1} for (a) untreated, (b) treated with 1 M 60 °C, (c) treated with 2 M 80 °C, and (d) treated 3 M 60 °C, respectively. The silicate ion present was due to the liquid silicate produced from the extraction process. Based on Table 3, it shows that the highest wavenumber was treated RHA at 2 M 80 °C. This can be proven based on the XRF results as it exhibited the highest silica content in the liquid silicate from the treated RHA of 2 M 80°C (1008.78 cm^{-1}) [22].

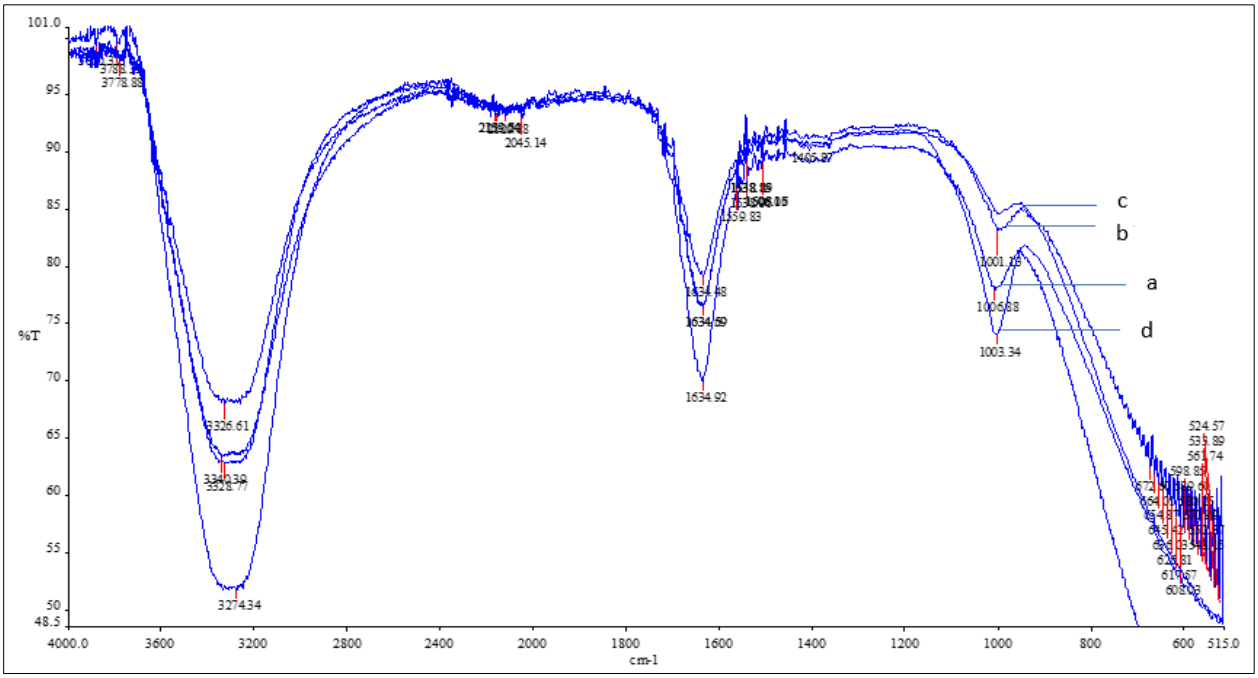


Figure 1. FTIR spectra of treated and untreated RHA of 1M 2H; (a) untreated RHA, (b) treated RHA with 1M 60°C, (c) treated RHA with 2 M 80 °C, and (d) treated RHA with 3 M 60 °C

The presence of O-H stretching was represented by both treated and untreated RHA spectrum, which shows broad characteristic peaks at 3274.34, 3326.61, 3328.77, and 3340.39 cm^{-1} for (a) untreated, (b) treated with 1 M 60 °C, (c) treated with 2 M 80 °C, and (d) treated 3 M 60 °C, respectively (Refer to Table 3). This O-H stretching indicates the presence of phenols and alcohols [23]. The reason for O-H stretching was due to the moisture content in the fibers, which contains hydroxyl groups in the form of lignin, hemicelluloses, and cellulose [24, 25].

Table 3. Wavenumber functional groups identified from treated and untreated RHA at 1M, 2H & 3M

	Wavenumber (cm^{-1})			
Vibration Band Assignment	**Untreated RHA**	**1M 60 °C**	**2M 80 °C**	**3M 60 °C**
Asymmetric stretching of silicate ion	1006.88	1001.13	1008.78	1003.34
O-H	3274.34	3326.61	3328.77	3340.39
O=C=O	2120.18	2155.60	2159.24	2045.14

The presence of O=C=O stretching was represented by both treated and untreated RHA spectrum, which is CO_2 at the wavelength of 2120.18, 2155.60, 2159.24, and 2045.14 cm^{-1} for (a) untreated, (b) treated with 1 M 60

°C, (c) treated with 2 M 80 °C, and (d) treated 3 M 60 °C, respectively (refer to Table 3). Major functional groups of O-H and O=C=O gave rise to bands above ~1500 cm^{-1}. However, the precise pattern of bands seen in the fingerprint region was specific to each compound. The presence of CO_2 in treated and untreated RHA was due to the carbonization process, which produced a gaseous product high in CO_2. However, as shown on the spectrum of treated RHA, the CO_2 formed was present after acid-leaching treatment. This shows that liquid silicate cannot remove volatile materials such as CO_2 [26].

PSA analysis

Table 4 displays the results of the PSA of the treated and untreated RHA at 1 M 2 H. The mean particle diameter decreased with the increase in treatment temperature. The mean particle diameter of H_3PO_4 at 2M 80 °C was recorded as the lowest as compared to other treatments.

Table 4. PSA data for treated and untreated RHA

Component	Untreated	Treated 1 M 60 °C	Treated 2 M 80 °C	Treated 3 M 60 °C
$D_{0.1}$	722.184	878.156	38.975	743.054
$D_{0.5}$	1165.935	1237.355	645.737	1168.995
$D_{0.9}$	1639.751	1645.679	1465.954	1642.913

Note: $D_{0.1}$, $D_{0.5}$, $D_{0.9}$ are the mean diameter for the smallest 10%, 50%, and 90% of the analyzed liquid silicate of untreated RHA, treated RHA at 1 M 60 °C, treated RHA at 2 M 80 °C and treated RHA at 3 M 60 °C.

The particle size was reduced after leaching with H_3PO_4 at 2 M 80 °C before the liquid silicate extraction process. This finding has proven that high temperature is suitable to produce a good small particle size. The temperature has a significant impact on particle size, with lower temperatures producing smaller particles with a larger surface area than higher temperatures [27]. As a result, as the particle size decreases, a greater proportion of the particles are found at the material's surface. Hence, acid leaching treatment is the most suitable method for extracting silica from the rice husk ashes for its application as a fertilizer.

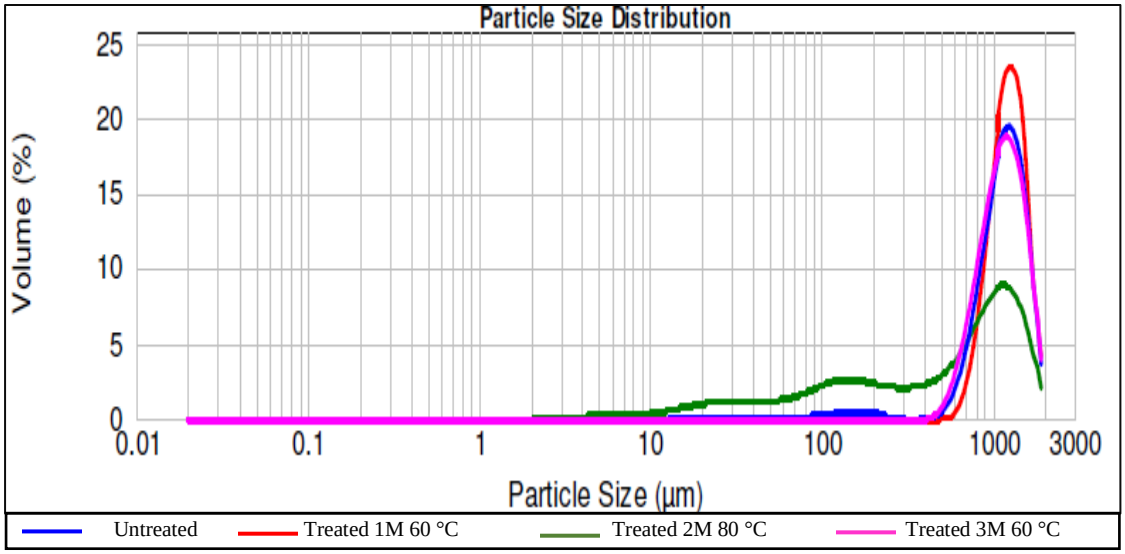


Figure 2. Particle size distributor of treated and untreated RHA at 1M 2H

All organic components dissolve in water, resulting in slow precipitation and small particles forming droplets. The removal of all organic compounds causes more silicate, iron, and potassium ions to be present. Hence, the high amount of silicate particles in the liquid silicate solution of treated RHA at 2 M 80 °C resulted in a decrease in the mean particle diameter.

Figure 2 demonstrates the comparison of particle size distributors between both treated and untreated RHA. As a result, the peak of the treated RHA at 2 M 80 °C was the broadest compared to the rest of the samples as a smaller particle size is preferable as a silicate particle. Besides, the rest of the peak only had a slightly different mean particle diameter. This proved that an acid-leaching treatment process somehow affects the formation of silicate particles.

Conclusion

The characterization of treated and untreated RHA via XRF spectroscopy, FTIR spectroscopy, and PSA of this study has demonstrated that acid leaching treatment using H_3PO_4 produces promising LSF as they contain silica, potassium, iron, and other trace elements, which are deemed to be suitable for plant growth. H_3PO_4 leaching treatment is significantly useful and effective in removing impurities and increasing the silica purity in RHA. Besides that, the alkaline extraction by 1M 2H KOH also exhibits the highest results of silica (40.91%), iron (46.40%), potassium (11.01%), and trace element (1.675%), respectively. These results are correlated with PSA and FTIR analysis. If RHA undergoes suitable treatment such as acid leaching treatment before the extraction process, it will produce high-quality LSF with the addition of other minerals such as iron and potassium.

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PEROXYMONOSULFATE ACTIVATION USING NITROGEN-DOPED BIOCHAR FOR ACID ORANGE 7 REMOVAL IN WATER

(Pengaktifan Peroksimonosulfat dengan Bioarang Terdop Nitrogen untuk Penyingkiran Asid Oren 7 dalam Air)

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Abstract

In this study, N-doped plastic-derived biochar was synthesized at different synthesis temperatures (600, 700 and 800 ºC) using a facile one-pot pyrolysis technique. The as-prepared N-doped biochars were employed to investigate the effect of pyrolysis temperature on the biochar characteristics and catalytic performance as peroxymonosulfate (PMS) activator for acid orange 7 (AO7) removal. The X-ray Diffraction (XRD) analysis indicated that the N-doped biochar consists of amorphous carbon whereas the field emission scanning electron microscope (FESEM) images illustrated that the catalysts have microparticles morphology with porous features. The N-doped biochar synthesized at 800 ºC (N-800) displayed the best performance with 98% AO7 removed in 30 min (pseudo first-order rate constant, k_{app} = 0.211 min^{-1}). The k_{app} was positively affected by increasing the PMS dosage and N-800 loading while neutral condition (pH = 7) was optimum for AO7 removal. The scavenger study suggests that singlet oxygen ($^1O_2$) was the dominant reactive oxygen species (ROS) in this reaction system as sodium azide (NaN_3) scavenger inhibited the AO7 removal by 80%. N-800 catalytic performance in removing AO7 was reduced from 97 to 22% after four consecutive cycles due to irreversible adsorption and oxidation of active sites. Overall, the N-800 was able to activate PMS effectively with a promising potential for sustainable azo dye removal. Future-wise, the employment of co-doping other heteroatoms (such as S, B) into N-doped plastic-derived biochar for synergistic effects should be investigated.

Keywords: advanced oxidation process, peroxymonosulfate, acid orange 7, nitrogen doping, biochar

Abstrak

Dalam kajian ini, bioarang terdop N telah disintesis daripada plastik pada suhu sintesis yang berbeza (600, 700 dan 800 ºC) dengan menggunakan teknik pirolisis satu periuk. Bioarang terdop N yang disediakan telah digunakan untuk menyiasat kesan suhu pirolisis ke atas ciri-ciri bioarang dan prestasi sebagai pemangkin bagi pengaktifan peroksimonosulfat (PMS) untuk penyingkiran asid oren 7 (AO7). Analisa pembelauan sinar-X menunjukkan bioarang terdop N terdiri daripada karbon amorfus manakala mikroskop elektron pengimbasan pelepasan medan (FESEM) menggambarkan bahawa pemangkin mempunyai morfologi mikrozarah dengan ciri berliang. Bioarang terdop N yang disintesis pada 800 ºC (N-800) menunjukkan prestasi terbaik dengan penyingkiran 98% AO7 dalam 30 min (pemalar kadar pseudo tertib pertama, k_{app} = 0.211 min^{-1}). Didapati k_{app} meningkat dengan peningkatan dos PMS dan pemuatan N-800 manakala pH 7 merupakan pH terbaik bagi penyingkiran AO7. Kajian mengunakan perencat radikal natrium azida (NaN_3) menunjukkan bahawa oksigen singlet ($^1O_2$) adalah spesies oksigen reaktif (ROS) yang dominan dalam sistem tindak

balas ini. Prestasi pemangkin N-800 dalam penyingkiran AO7 telah berkurangan daripada 97 kepada 22% selepas empat kitaran berturut-turut disebabkan oleh penjerapan tidak berbalik dan pengoksidaan tapak aktif. Secara keseluruhan, N-800 dapat mengaktifkan PMS dengan berkesan dan menunjukkan potensi penyingkiran pewarna azo yang mampan. Dalam masa hadapan, pendopan bioarang terdop N terbitan plastik bersama heteroatom lain (seperti S, B) perlu disiasat untuk kesan sinergistik.

Kata kunci: proses pengoksidaan lanjutan, peroksimonosulfat, asid oren 7, nitrogen terdop, bioarang

Introduction

Industrialization and population development led to an increase in the usage of water and water pollution. This is predicated on the quality of the water deteriorating and the quantity of hydric resources decreasing, and the world is expected to experience a 40% water shortage by 2030 [1]. Synthetic dyes are widely employed in a variety of industries. Azo dyes are common dyes used in this dyeing industry. Due to the tremendous demand in diverse industries such as pulp and paper, textiles, pharmaceuticals and photo-electrochemical cells, huge amounts of azo dyes are produced globally (about 1 million tons per year) [2]. Acid orange 7 (AO7) is a commonly recognized azo dye since it is low in cost and can be applied rapidly in weakly acidic solutions. Exposure to AO7 has adverse health effects and its consumption is potentially lethal as it is carcinogenic and mutagenic [3]. There are several ways to eliminate AO7 from wastewater including adsorption, biodegradation, and advanced oxidation processes (AOPs). AOPs are chemical treatment procedures designed to remove organic and inorganic materials in wastewater.

Recently, persulfate-based advanced oxidation processes (PS-AOPs) emerged as a viable option for the decontamination of organics in water [1]. The activation of PS can yield strong oxidative species such as sulfate radical ($SO_4^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) [4, 5]. These reactive species can be employed to decompose organic pollutants in wastewater. PS is commercially available as peroxymonosulfate (PMS) and peroxydisulfate (PDS) depending on the number of SO_3^- groups in its structure. Heterogenous catalytic activation of PS is highly preferred over other methods (e.g. heat activation, photoactivation) as no external energy sources are required, making heterogenous catalytic PS activation an economic choice [6]. Between PMS and PDS, the former is more easily activated due to it asymmetricity of structure [7]. Also, PMS is cheaper than PDS [8] making PMS a more advantageous choice of PS to be activated for organic pollutants degradation. Metal-based catalysts are effective in activating PMS, however, the inevitable leaching of metallic ions requiring the need for secondary treatment is a serious drawback [9]. Hence, carbon-based catalysts are emerging as sustainable alternatives. Carbon-based catalysts have preferable characteristics such as excellent specific surface area, microporosity, pore volume, chemical inertness, and stability. Examples of carbon-based catalysts are carbon nanotubes (CNTs), graphene, biochar and nanodiamonds. Among all, biochar is considered as the most economically viable option as it can be prepared directly from biomass waste. The catalytic activity of biochar can be increased by doping with a heteroatom such as nitrogen, sulfur and phosphorus [10]. N is the most compatible heteroatom dopant compared to others (e.g., B, S, P) due to its similar atom size with carbon, high electronegativity, and ability to provide effective active sites for PMS activation [10]. Moreover, the physiochemical properties of the biochar (i.e. thermal stability, functional groups) and the bonding configuration of the N-dopants are dependent on the synthesis temperature of the biochar [11]. These changes affect the overall catalytic performance of the N-doped biochar. Therefore, it is pivotal to study the effects of the synthesis temperature on the catalytic activity of N-doped biochar for PMS activation.

In this study, we aim to prepare and investigate the characteristics of N-doped plastic-derived biochar at different pyrolysis temperatures (600, 700, and 800 ℃). Thereafter, the catalytic performance of the as-prepared N-doped carbon-based catalysts was evaluated as PMS activator for AO7 degradation. The effects of different operational condition (i.e., PMS dosage, catalyst loading, pH) on AO7 removal via PMS activation were evaluated. Chemical scavenging study was employed to

investigate the mechanism of PMS activation by N-doped biochar. Lastly, the reusability of the catalyst was investigated.

Materials and Methods

Chemicals

The disposable vinyl gloves were purchased and used as the precursor in this study. The chemicals employed in this study were urea (QRëC), PMS (as Oxone®, $2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$, Acros Organics), AO7 (Sigma-Aldrich), sodium hydroxide (NaOH, Fisher Chemical), ethanol (EtOH, 99.7%, QRëC), sodium perchlorate ($NaClO_4$, Sigma-Aldrich), tert-butyl alcohol (TBA, Supelco), and sodium azide (NaN_3, Merck). All experiments were carried out with deionized (DI) water.

Synthesis of N-doped biochar

In this study, the N-doped biochar was synthesized from disposable plastic gloves using a simple one-pot pyrolysis method. Firstly, the plastic gloves were cut into dimension of 1.4×1.4 cm, rinsed several times using DI water and dried in an oven at 60 ℃. Then, the gloves and urea were mixed at a weight ratio of 1:2 (3g of gloves with 6g of urea), respectively, and the mixture was transferred into a crucible. The crucible was closed tightly and wrapped with aluminum foil to produce an oxygen limited atmosphere. Thereafter, the crucible was placed in the furnace (Carbolite Gero Ltd.) and heated to a selected temperature with a heating rate of 10 ℃ min^{-1} for 2 h. The calcination temperature was varied from 600, 700 to 800 ℃. Thereafter, the resultant N-doped biochar was allowed to cool under room temperature, washed several times with DI water and dried in an oven at 60 ℃. The resultant N-doped biochar was labelled as N-600, N-700, and N-800 according to the calcination temperature.

Characterization

The attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy (Frontier, Perkin Elmer) was used for the determination of the functional groups of N-doped biochar. The crystal structure/s of the as-prepared N-doped biochar samples were analyzed using the powder X-ray diffraction (XRD) patterns (Bruker D8 Advance diffractometer). Field Emission Scanning Electron Microscope (FESEM, Quanta FEG650, FEI) equipped with energy dispersive X-ray spectroscopy (EDX) were used to analyze the samples' surface morphologies and elemental composition. Thermogravimetric analysis (TGA) was conducted using PerkinElmer thermal analyzer in nitrogen atmosphere (ramping rate = 10 ℃ min^{-1}) to analyze the thermal properties of the as-prepared N-doped biochar samples.

Catalytic performance evaluation

Briefly, a solution containing PMS (0.2 g L^{-1}) and AO7 (10 mg L^{-1}) was prepared in a 250 mL reaction vessel (mole ratio of PMS:AO7 of 1:23). To initiate the catalytic degradation, 0.025 g L^{-1} of the N-doped biochar catalyst was added into the solution mixture mentioned above. At 5 min intervals, 3 mL aliquots of the solution were drawn from the solution and filtered using 0.45 μm cellulose acetate filter to remove the catalyst particles and avoid misleading results. The concentration of AO7 was determined by a calibration curve using a UV-Vis spectrophotometer (Shimadzu 2600 UV–Vis) at $\lambda = 485$ nm. The effects of various operating parameters including initial pH (3-9), catalyst loading (0.01-0.1 g L^{-1}) and PMS dosage (0.1-0.25 g L^{-1}) for AO7 degradation were also studied. To identify the dominant ROS, chemical scavengers were used. TBA (30 mM), NaN_3 (3 mM), $NaClO_4$ (485 mM) and EtOH (2 M), were used as scavengers for $^{\bullet}OH$, $^1O_2$, nonradical electron-transfer, and both $SO_4^{\bullet-}$ and $^{\bullet}OH$, respectively. The degradation tests were performed in duplicates to ensure reproducible results. The reusability of N-800 was studied by conducting four consecutive catalytic cycles in which the N-doped biochar catalyst was recovered using gravitational method, dried and scaled out after each cycle and reused. The total organic carbon (TOC) removal of AO7 by N-800 was measured by using a TOC analyzer (Shimadzu TOC-VCPH).

Results and Discussion

Characterization of biochar catalyst

The crystal structure of the as-synthesized N-800 was investigated using XRD and the results are presented in Figure 1a. In general, the presence of short-range order in the carbon structure is characterized by diffuse and broad bands in XRD patterns, whereas highly crystalline phases with a high degree of long-range order are

confirmed by sharp and distinct peaks. Notably, several diffraction peaks typical of amorphous carbon can be observed at 2θ = 26.2° and 43.9°, representing the (002) and (101) planes, respectively, for N-800. These two planes show the presence of amorphous carbon after the pyrolysis suggesting that the plastic residue has been successfully carbonized into biochar [9]. Apparently, the diffraction peak at 2θ = 26° shows the presence of a crystalline phase with a well-ordered structure produced through calcination and N-doping [12,13]. A sharp peak at 2θ = 29° indicating the existence of crystalline inorganic elements which can be identified as calcium ($CaCO_3$) in plastic residue [14]. The presence of Ca was minimally present in EDX mapping. Other researchers that studied the pyrolysis of other wastes also noted the existence of inorganic elements [15]. Overall, the result showed that the biochar was successfully synthesized.

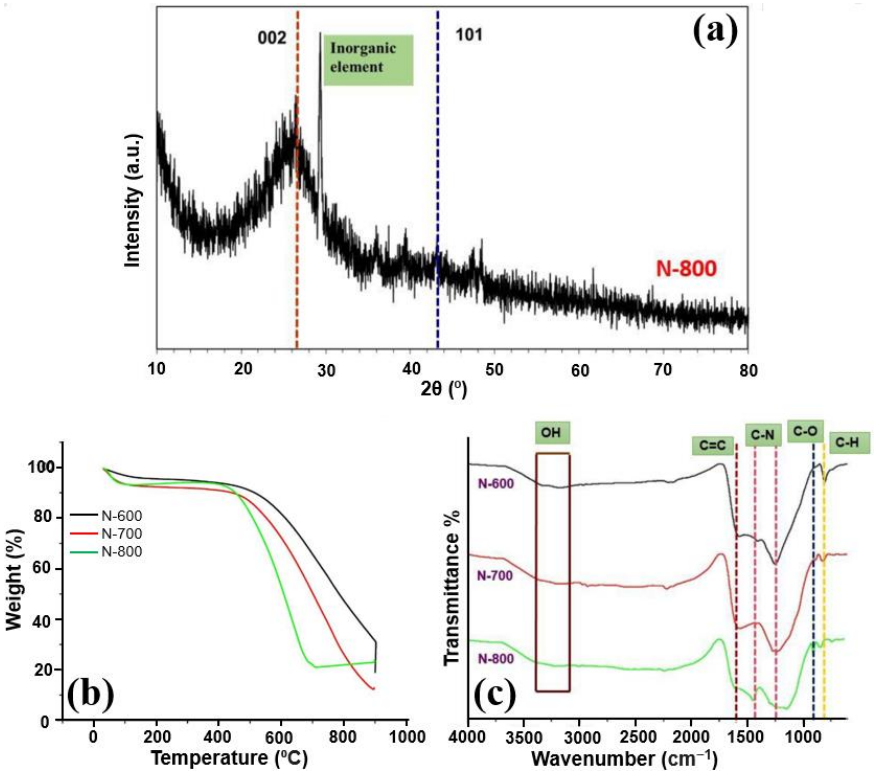


Figure 1. (a) XRD diffractogram, (b) TGA patterns, and (c) FTIR spectra of the as-prepared catalysts

The TGA analysis was carried out to evaluate the thermal stability of N-600, N-700, and N-800 by observing the weight change of the samples with respect to increasing temperature (Figure 1b). All the TGA curves displayed an initial weight loss between 10-30% corresponding to the evaporation of the adsorbed H_2O molecules. There is a continuous drop in weight over a wide temperature range between 500 to 900 °C. Generally, N doping may improve heat tolerance in contrast to normal carbon biochar since N doping induced greater thermodynamic stability of the catalyst against combustion reaction [12]. Furthermore, a larger percent weight/weight inorganic content, which primarily consists of Ca, could increase the proportion of inert components in the carbocatalyst, reducing overall catalytic activity.

The functional groups in the N-doped biochar were analyzed using FTIR and the results are illustrated in Figure 1c. The presence of an absorbance peak at 3250 cm^{-1} is observed signifying -OH bond from hydroxyl and carboxylic groups, and adsorbed water [16]. The broad peaks at the range of 950-1100 cm^{-1} show C-O bond of hydroxyl and alkoxy groups [17]. Several peaks

have appeared at the range of 740-825 cm^{-1} after the thermal treatment indicating the presence of C-H bending vibration (aromatic) while the peak at 1500 cm^{-1} shows that C=C bonding is present. This bonding denotes a higher degree of graphitization [18,19]. Two broad peaks form at 1185 cm^{-1} and 1381 cm^{-1} which shows C-N bond due to the doping of N on the catalyst [20].

The structure and elemental mapping of the synthesized N-800 were characterized using FESEM-EDX (Figure 2). It was observed that N-800 has a morphology consisting of microparticles with porous features. The N-800 displayed a dispersive pore structure making it an effective platform for adsorption and catalysis [10]. The exterior surface of N-800 illustrated roughness with wrinkles and folds suggesting that N doping procedure caused the creation of defective sites. Other than that, N-800 has smooth interior surface indicating that N-doping is limited to the external surface. A possible reason might be the N-doping method where the urea acting as nitrogen precursor will be unable to diffuse into the interior pores implying that adjusting particle size could improve the surface coverage of N species [12]. Besides that, the elemental mapping shows that the N and O atoms are uniformly distributed on N-800. The presence of Ca was also detected in EDX analysis albeit at lower concentration.

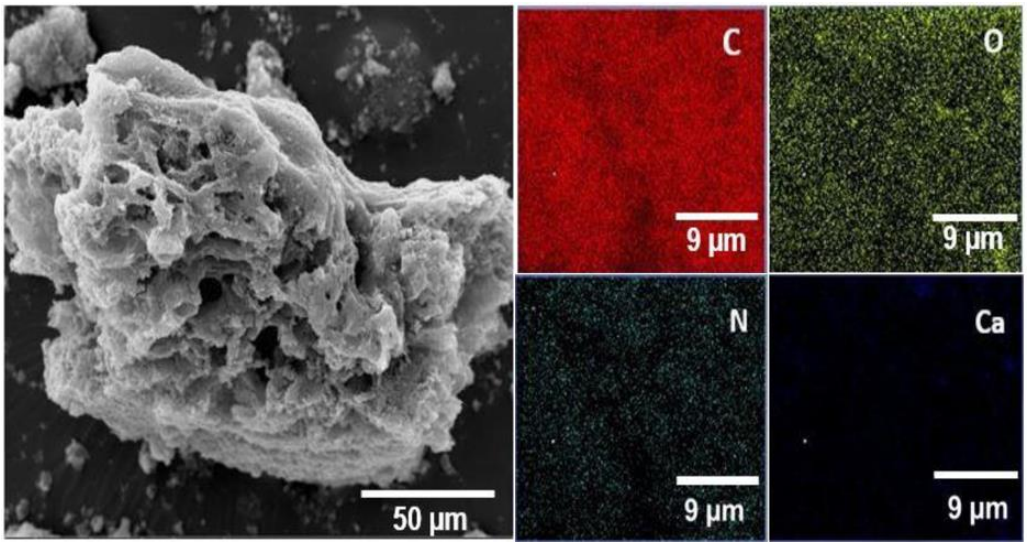


Figure 2. SEM image and elemental mapping of N-800.

Catalytic performance of different catalysts

Figure 3a shows the effect of synthesis temperature of N-doped biochar on the performance of AO7 removal by PMS activation. The control study shows that the AO7 adsorption at 30 min was ~32%. Notably, almost no AO7 degradation was observed in the presence of PMS alone indicating that AO7 is stable to direct PMS oxidation. However, when PMS was activated in presence of N-600, N-700, and N-800, rapid AO7 degradation can be observed. The AO7 degradation by N-doped biochar was evaluated using the pseudo first order kinetics as follows:

$$[AO7]_t = [AO7]_0 \, e^{-kapp\, t} \quad (1)$$

where $[AO7]_t$, $[AO7]_0$, and k_{app} are the AO7 concentration at selected time interval, initial AO7 concentration, and pseudo-first order rate constant (min^{-1}), respectively. Apparently, N-800 displays the best catalytic performance where degradation efficiency of AO7 reached 98% in 30 min while N-600 shows the slowest AO7 removal with only 75% of AO7 removed in 30 min. The obtained k_{app} was in order: N-600 (k_{app} = 0.044 min^{-1}) < N-700 (k_{app} = 0.083 min^{-1}) < N- 800 (k_{app} = 0.211 min^{-1}). While higher calcination temperature will cause the C-N bond to break resulting in the loss of nitrogen, it promotes incorporation of N atoms into the graphitic carbon network as graphitic N [21]. The

graphitic N has been previously identified as the main active site for PMS activation. Hence, N-800 was used in additional degradation studies since it demonstrated the greatest catalytic performance to activate PMS for AO7 degradation.

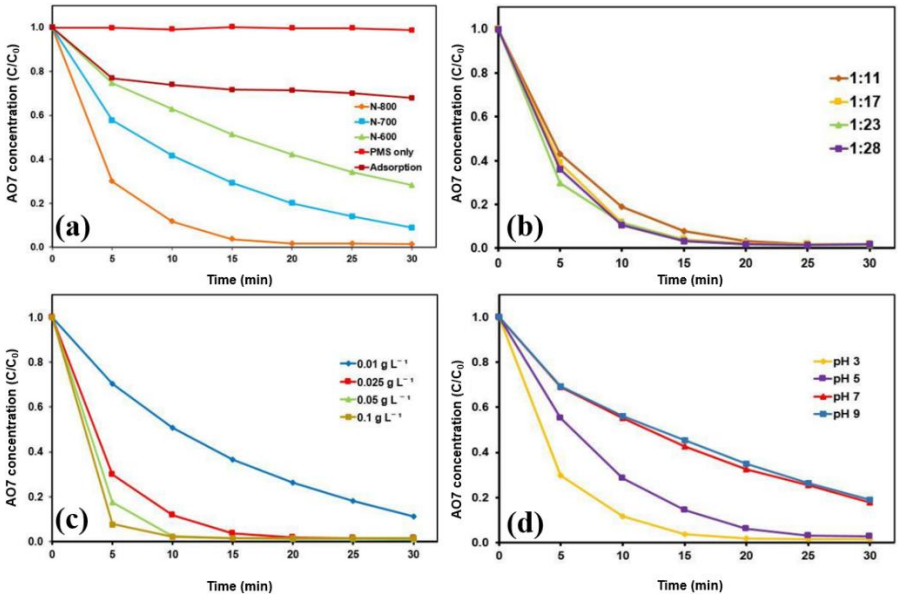


Figure 3. (a) Catalytic degradation of AO7 by different catalysts. Effects of (b) AO7:PMS ratio, (c) catalyst loading, and (d) initial pH

Effect of reaction parameters

Figure 3b shows the effect of PMS dosage on AO7 removal. The k_{app} increased when the ratio of AO7:PMS was decreased in following order: [0.1 g L^{-1} of PMS] 1:11 (k_{app} = 0.155 min^{-1}) < [0.15 g L^{-1} of PMS] 1:17 (k_{app} = 0.161 min^{-1}) < [0.20 g L^{-1} of PMS] 1:23 (k_{app} = 0.170 min^{-1}). However, when the ratio of AO7:PMS was decreased from 1:23 to 1:28 [0.25 g L^{-1} of PMS], the k_{app} decreased to 0.168 min^{-1}. From these results, it can be observed that 1:23 of AO7: PMS shows the highest AO7 degradation rate and optimum rate for AO7 removal. Usually, higher PMS dosage can promote greater interaction between catalyst and PMS for effective AO7 degradation. However, the rate decreased slightly when AO7: PMS was decreased to 1:28 due to the $SO_4^{\bullet-}$ consumption by excessive PMS leading to unproductive $SO_4^{\bullet-}$ use. Excessive PMS could scavenge $SO_4^{\bullet-}$ and produce less reactive $SO_5^{\bullet-}$ (Eq. (2)) [22].

$$HSO_5^- + SO_4^{\bullet-} \rightarrow SO_5^{\bullet-} + SO_4^{2-} + H^+ \quad (2)$$

Figure 3c shows the effect of catalyst loading on the AO7 removal. The degradation efficiency and rate increased linearly with increasing catalyst loading in the following order: 0.01 g L^{-1} (k_{app} = 0.067 min^{-1}) < 0.025 g L^{-1} (k_{app} = 0.211 min^{-1}) < 0.05 g L^{-1} (k_{app} = 0.260 min^{-1}) < 0.1 g L^{-1} (k_{app} = 0.264 min^{-1}). Increasing the catalyst loading increases the concentration of active sites available for PMS activation to produce ROS for AO7 degradation [12,23]. As the catalyst can be reused, modifying the catalyst loading is a more effective strategy to manage the reaction than adjusting PMS dosage. The effect of pH on the degradation of AO7 is presented in Figure 3d. From the figure, the catalytic activity of N-800 is much better achieved under acidic conditions compared with neutral and alkaline conditions. The k_{app} for AO7 removal increased in the following order: pH 3 (k_{app} = 0.170 min^{-1}) > pH 5 (k_{app} = 0.130 min^{-1}) > pH 7 (k_{app} = 0.057 min^{-1}) > pH 9 (k_{app} =0.054 min^{-1}). This observation might be due to the repulsion effect between the surface of the catalyst (pH_{pzc} = 7.4), AO7 (pKa = 10.7) and PMS (pKa_2 = 9.4) [12,23]. Both radical and nonradical routes require PMS

to be in contact with the surface of the catalyst to facilitate the direct electron transfer process. Therefore, this surface repulsion will be detrimental to the reaction, decreasing the overall rate of degradation of AO7 [24,25]. Moreover, the initial pH may have an impact on how much AO7 is adsorbed by the catalyst, causing it to adsorb more AO7 in moderately acidic conditions and increasing the AO7 degradation rate [26].

Identification reactive species and possible catalytic mechanism

For PMS activation using carbocatalysts, there are two activation pathways that have been reported which are the radical and nonradical pathways. These pathways have an impact on the system's performance. Chemical scavengers such as EtOH, TBA, NaN_3 and $NaClO_4$ were used to identify the dominant ROS. EtOH was used to selectively scavenge $^{\bullet}OH$ and $SO_4^{\bullet-}$ ($k_{SO4^{\bullet-}+EtOH}$ = 1.6 × 10^7 M^{-1} s^{-1}; $k_{^{\bullet}OH+EtOH}$ = 1.9 × 10^9 M^{-1} s^{-1}) [12]. As shown in Figure 4, the addition of EtOH into the reaction system shows 48% inhibition and reduced the k_{app} from 0.170 min^{-1} to 0.088 min^{-1}. This suggests that $SO_4^{\bullet-}$ and $^{\bullet}OH$ might be involved in degradation of AO7. To determine the dominant radical, TBA was used to quench $^{\bullet}OH$ because TBA has larger value of reaction rate constant with $^{\bullet}OH$ compared to $SO_4^{\bullet-}$ ($k_{SO4^{\bullet-}+TBA}$ = 4–9.1 × 10^5 M^{-1} s^{-1}, $k_{^{\bullet}OH+TBA}$ = 3.8–7.6 × 10^8 M^{-1} s^{-1}) [12]. TBA shows a minor inhibitory effect in this reaction system. This suggests that the involvement of $^{\bullet}OH$ was limited in degradation of AO7. Next, $NaClO_4$ was employed to reduce the interaction between the catalyst and PMS which decreases the formation of surface complexes for the nonradical pathway. $NaClO_4$ addition into the reaction system shows a 30% inhibition and reduced the k_{app} from 0.170 min^{-1} to 0.119 min^{-1}. $NaClO_4$ can reduce the amount of the nonradical pathway without significantly influencing the radical pathway because it can hinder the surface contact between PMS and the catalyst [27]. Finally, NaN_3 is used to scavenge $^1O_2$. When NaN_3 was employed, the percentage of inhibition was 80% and k_{app} was reduced from 0.170 min^{-1} to 0.034 min^{-1}. The highest inhibitory effect shown by the NaN_3 signifies that $^1O_2$ was dominant in the system.

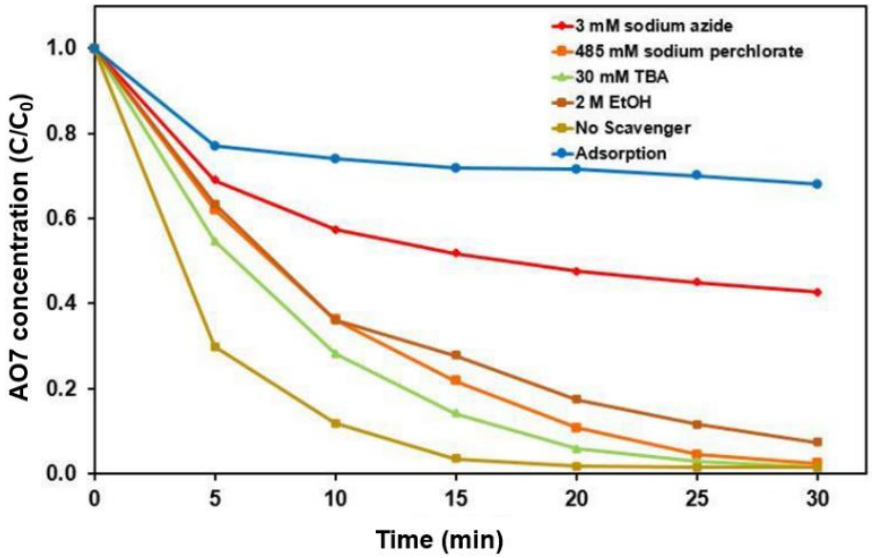


Figure 4. Effect of chemical scavengers on AO7 degradation efficiency by N-800

The results show that there was coexistence between radical and nonradical pathway in this system. Sun et al. proposed that the radical route occurs when the abundant free-flowing electrons from sp^2 carbons conjugate with the lone-pair electrons of N dopants found at Lewis basic sites containing lone-pair electrons such as pyridinic N, pyrrolic N, and ketonic groups. These functional groups can operate as electron donors, transferring electrons to activate PMS by cleaving the O-O bond resulting in production of $SO_4^{\bullet-}$ and $^{\bullet}OH$

[28,29]. Oh et al. proposed that N-doped biochar from biomass waste can be considered as an optimal surface for electron transfer reactions to take place since it has a high concentration of graphitic N and sp^2 hybridized carbon. An electron-deficient environment for the carbon atom is produced when the highly electronegative N atom in the graphitic plane attracts an electron from the less electronegative carbon next to it. Through an electron abstraction process, the electron-deficient carbon may interact strongly with PMS, resulting in the production of $^1O_2$ [30]. The overall results are in consistent with this observation. Hence, it can be speculated that Lewis base group like pyridinic N, pyrrolic N and ketonic groups involved in free radical pathway, while $^1O_2$ formation involves in PMS activation by electron deficient carbon induced by the graphitic N (dominant). The mechanism of PMS activation by N-doped plastic-derived biochar for AO7 degradation is illustrated in Figure 5.

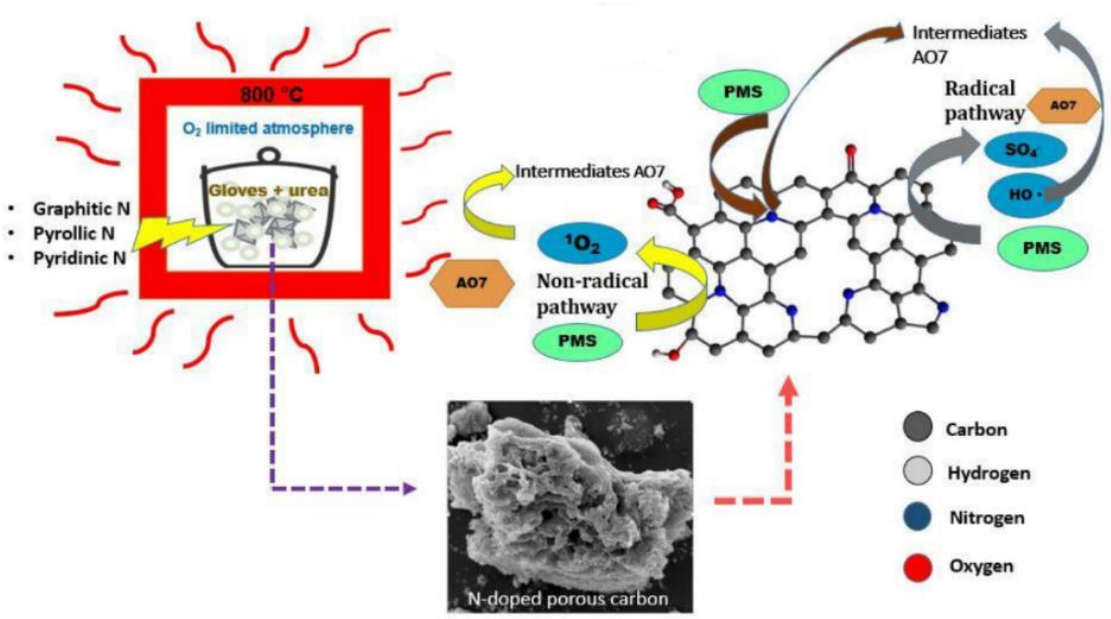


Figure 5. Mechanism of PMS activation for AO7 removal by N-800

Reusability of N-800

In practical applications, stability of N-800 in the PMS system for degradation of pollutant is important. The degradation of AO7 was carried out 4 times using recycled N-800 biochar and the results are presented in Figure 6. In the first cycle, the degradation rate was 97% in 30 min. The removal efficiency was reduced to 89%, 56%, 22% within 30 min in the second, third and fourth cycles, respectively. The N-800 deactivation could be ascribed to the change of configuration of nitrogen bonding and intermediates coverage of catalyst which blocked the PMS interaction with active sites and minimized production of ROS [21]. In short, the N-800 can still efficiently remove AO7 after being used repeatedly. TOC removal of ~5% was achieved in the first 60 min of the oxidation reaction. After 3h, TOC removal reached a 10% suggesting that AO7 can be mineralized by the oxidation processes (Eq. (3)) provided that prolong treatment time was employed.

$$AO7 + ROS \rightarrow CO_2 + H_2O + \text{inorganic ions} \quad (3)$$

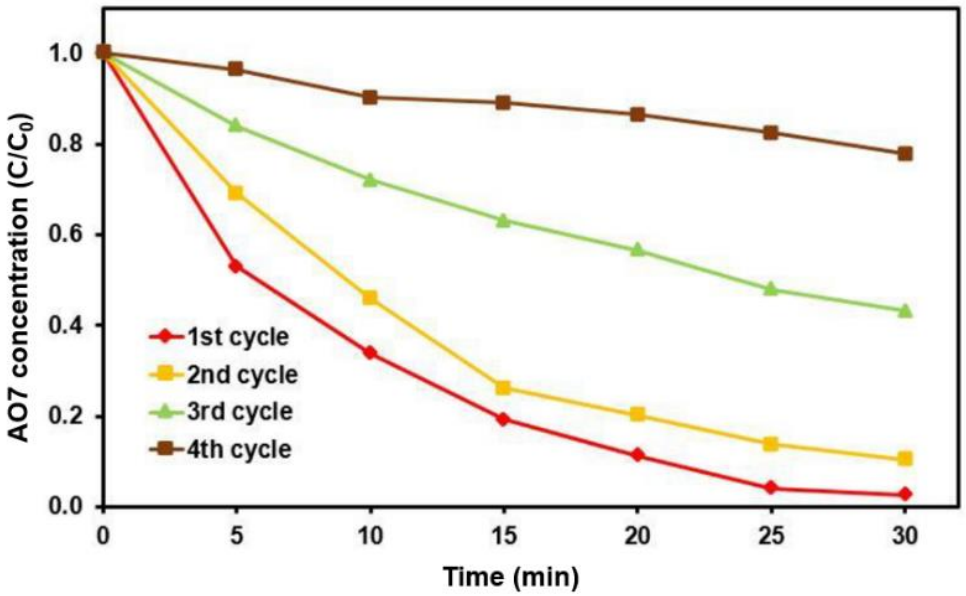


Figure 6. Reusability of N-800 in AO7 removal

Conclusion

In conclusion, N-doped biochar was successfully synthesized by utilizing a thermal calcination approach in an oxygen-limited environment for AO7 degradation by PMS activation. The obtained FTIR spectra of all N-doped biochar and XRD patterns of N-800 proved that the catalysts were successfully synthesized in this study. Through FESEM-EDX, it can be confirmed that N-800 consisted of microparticles with a porous feature with uniform distribution of the N atoms. From effect of synthesis temperature on the performance of AO7 removal by PMS activation, N-800 exhibited the highest efficiency in degrading AO7 and was used for further degradation experiments. About 98% of AO7 was successfully eliminated within 30 min employing N-800/PMS. The catalytic activity of N-800 in terms of PMS activation for AO7 removal with various operating variables (i.e. initial pH, catalyst loading, and PMS dosage) were investigated. The k_{app} values were found to be increasing with the increasing catalyst loading. An AO7: PMS ratio of 1:23 was found to be the optimum ratio for the degradation of AO7. The lower dosage of PMS lowered the k_{app} due to the limited PMS molecules available for activation and ROS formation. Excessively higher dosage of PMS also lowered the k_{app} because it could scavenge $SO_4^{\bullet-}$ and produce less reactive $SO_5^{\bullet-}$. On the other hand, the study on initial pH indicated that the ideal operating pH is found in acidic conditions. This can be explained by the surface repulsion between catalyst, PMS and AO7 at alkaline pH. The TOC removal efficiency by using N-800 was 10% indicating that mineralization of AO7 was achieved. Moreover, the catalyst was able to be reused for four consecutive cycles. Scavenger studies showed that the $^1O_2$ pathway was the prevailing pathway in the degradation of AO7. Overall, it can be inferred that SR-AOPs is a promising technology which can be used effectively to treat organic contaminants in the wastewater.

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SPECTROSCOPIC, CRYSTAL STRUCTURE, HIRSHFELD SURFACE AND DFT STUDIES OF 2-AMINO-4-CHLOROBENZONITRILE

(Kajian Spektroskopi, Struktur Hablur, Permukaan Hirsfeld dan DFT bagi 2-Amino-4-Klorobenzonitril)

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Abstract

This research concentrates on a summary of the global reactivity parameters on the single crystal structure of 2-amino-4-chlorobenzonitrile, which can be utilised in a wide range of applications. The commercial 2-amino-4-chlorobenzonitrile in light yellow (Sigma-Aldrich) [ACBN] was recrystallized from ethanol. The compound crystallizes in the triclinic, *P-1* space group with unit cell parameters *a* =3.8924 (9) Å, *b* = 6.7886 (15) Å, *c* = 13.838 (3) Å, *α* =77.559 (16)°, *β* = 8.898 (17)° and γ = 83.021 (17)°. The presence of intermolecular interactions was further analyzed via the Hirshfeld Surface analysis. The electronic properties of the compound were investigated via the Density Functional Theory (DFT) using the CIF file of the single crystal. Full geometry optimization was carried out using the DFT at B3LYP with 6- 311++G (d, p) basis set level. The structure of the recrystallized ACBN was confirmed via single crystal X-ray Crystallography, FTIR, and UV-Vis. The IR spectrum showed three main stretching bands namely nitrile, C≡N (2211 cm^{-1}), C-Cl (782 cm^{-1}), and 1° NH (3452 and 3363 cm^{-1}). The UV-Vis analysis showed two main absorption peaks namely $\pi \rightarrow \pi*$ and $n \rightarrow \pi*$ which occured in an aromatic ring and nitrile, C≡N. The compound crystallized in the triclinic system with the space group Pī. Nitrile (C≡N) and C-N have bond lengths of 1.146(4) and 1.369(4) Å, which are less than the theoretical values of 1.160 and 1.470 Å due to the conjugation of the aromatic ring. The intrinsic molecular characteristics analysis showed that the compound has the capability to donate electrons although it is categorized as a hard compound. The exploration of intrinsic molecular features revealed that, despite the fact that ACBN is stable and less reactive, it still has the ability to transfer and accept electrons. Furthermore, the Mulliken analysis revealed that the compound has the capability to function as a nucleophilic agent due to the nitrogen atoms (highly negative charges). As recommendation, the DFT study revealed that the compound can be investigated more thoroughly in terms of reaction kinetics and mechanisms such as corrosion inhibition and biological activities.

Keywords: benzonitrile, density functional theory, mulliken, molecular electrostatic potential, Hirshfeld surface

Abstrak

Kajian ini menumpukan kepada ringkasan parameter kereaktifan global bagi struktur hablur tunggal 2-amino-4-klorobenzonitril, yang boleh diaplikasikan dalam pelbagai kegunaan. Komersial 2-amino-4-klorobenzonitril yang berwarna kuning cair (Sigma-Aldrich) [ACBN] telah dilakukan penghabluran semula daripada etanol. Sebatian tersebut menghablur dalam triklinik, kumpulan ruang P-1 dengan parameter sel unit a =3.8924 (9) Å, b = 6.7886 (15) Å, c = 13.838 (3) Å, α =77.559 (16)°, β = 8.898 (17)° and γ = 83.021 (17)°. Kewujudan interaksi antara molekul dianalisis selanjutnya dengan Permukaan Hirshfeld. Sifat elektronik sebatian telah dikaji dengan Teori Fungsian Ketumpatan (DFT) menggunakan fail CIF hablur tunggal. Pengoptimuman geometri penuh sebatian telah dilakukan menggunakan B3LYP dengan tahap asas 6- 311++G (d, p). Struktur hablur ACBN telah disahkan melalui Kristalografi sinar-X hablur tunggal, FTIR, dan UV-Vis. Spektrum IR menunjukkan tiga jalur regangan yang utama iaitu nitril, C≡N (2211 cm^{-1}), C-Cl (782 cm^{-1}), dan 1° NH (3452 dan 3363 cm^{-1}). Analisis UV-Vis menunjukkan dua puncak serapan utama iaitu $\pi \rightarrow \pi*$ dan $n \rightarrow \pi*$ yang wujud dalam gegelang aromatik dan nitril, C≡N. Sebatian itu terhablur dalam sistem triklinik dengan kumpulan ruang Pī. Nitril (C≡N) dan C-N mempunyai panjang ikatan iaitu 1.146(4) dan 1.369(4) Å di mana kurang daripada nilai teori iaitu 1.160 dan 1.470 Å disebabkan oleh konjugasi dalam gegelang aromatik. Analisis ciri molekul intrinsik menunjukkan bahawa sebatian ACBN mempunyai keupayaan untuk menderma elektron walaupun dikategorikan sebagai sebatian keras. Penerokaan ciri molekul intrinsik mendedahkan bahawa, walaupun hakikatnya ACBN adalah stabil dan kurang reaktif tapi masih mempunyai keupayaan menderma dan menerima elektron. Tambahan pula, analisis Mulliken mendedahkan bahawa sebatian tersebut mempunyai keupayaan untuk berfungsi sebagai agen nukleofilik disebabkan oleh atom nitrogen mempunyai cas negatif yang tinggi. Sebagai cadangan, analisis DFT mendedahkan bahawa sebatian ACBN boleh dikaji lebih teliti dari segi kinetik tindak balas kimia dan mekanisma seperti perencatan kakisan dan aktiviti biologi.

Kata kunci: benzonitril, teori fungsian ketumpatan, Mulliken, potensi elektrostatik molekul, permukaan Hirshfeld

Introduction

Benzonitrile (BN) is produced primarily by heating benzoic acid and theocyanate at 191°C. The liquid benzonitrile smells of almond and is clear and colorless. Due to its properties as an aprotic polar solvent, BN has been widely employed as a solvent in chemistry. In headspace and aqueous extract aromas, it is a powerful odorant. Derivatives of benzonitrile are used in the food preservation business as well as the dye industry to create aniline blue [1]. Besides that, in medicine, the primary byproducts of benzonitrile-benzoic acid are used as urinary antiseptics in salt form and bronchial tube disinfectants in vapor form. Nitrile derivatives have a wide range of industrial applications; for instance, phthalonitrile has been utilized as a precursor to phtalocyanide, a crucial component of dyes [2]. Lacquers, polymers, anhydrous metallic salts as well as medicinal, agrochemical, and other organic chemical intermediates are all generated from benzoinitrile (BN) and its derivatives [3]. Benzonitrile, which features an electron-donor CN substituent, is referred to as being bi-functional since it has the capability to interact with both the CN lone pair and the π-conjugated ring surfaces [4]. The cyano (C≡N) group is a versatile ligand that can serve as both an acceptor and a donor [5]. It is known that the C≡N stretching frequency of the nitrile group changes from low to high depending on whether or not it interacts with a solvent that donates hydrogen bonds [6]. For the re-crystallized compound, Density Functional Theory (DFT) calculations utilizing the B3LYP level of theory and the LANL2DZ basis set were reported, including geometry optimization, vibrational frequency analysis, and electronic structures. This work concentrates on the DFT analysis, with data generated using single crystal data of 2-amino-4-chlorobenzonitrile (CIF format). Although a previous study on DFT analysis by Sudha et al. (2011) was discussed, the researchers employed the proposed structure rather than single crystal data. It is clear that the DFT and Hirshfeld Surface analysis data generated using the CIF file is very different from the previous study. Besides that, the spectroscopic study of ACBN is also discussed in this work. The goal of studying the intrinsic molecular features of ACBN by DFT is to determine its potential to be used in corrosion inhibition, biological activity, energy storage, energy conversion, and other applications.

Materials and Methods

The commercial 2-amino-4-chlorobenzonitrile (Sigma-Aldrich) was recrystallized from ethanol.

Physical measurements

Using a Shimadzu FTIR-8900 spectrophotometer with KBr pellets, the IR spectrum of the compound was recorded in the range 4000-400 cm^{-1}. The ultraviolet absorption spectra of the compound were analyzed in the range 200-400 nm using a Unicam UV-Vis spectrophotometer device with a 10-mm quartz cell. The UV model was taken from a 2×10^{-5} M solution of the compound, dissolved in ethanol at 20°C.

Crystal structure determination

A yellow crystal with dimensions of 0.044 × 0.285 × 0.672 mm was mounted on the goniometer of a STOE IPDS II diffractometer. At room temperature (296 K), measurements were performed using graphite monochromate MoKα radiation (λ = 0.71073 Å). Reflections were gathered in the rotation mode and cell parameters were determined using the X-AREA software [7]. Absorption correction was achieved by the integration method via X-RED32 software [7]. SHELXS 97 [8] was used in the solvation of the structure by direct methods. All atoms except for hydrogens were refined anisotropically by the full-matrix least squares procedure based on F2 using SHELXL-97 [8]. All H atoms were positioned geometrically and treated using a riding model, fixing the bond lengths at 0.86, 0.93, and 0.96 Å for NH, CH and CH_3, respectively. The program ORTEP-3 for windows [9] and MERCURY [10] was used in the preparation of the figures. The crystal data collection and refinement parameters are given in Table 2.

Hirshfeld surface analysis

The Hirshfeld surface analyses were done using the CrystalExplorer17.5 program [11]. The normalized contact distances (dnorm) were mapped into the Hirshfeld surface, which allowed the visualization of intermolecular interactions. The red color represents the closest contact because it indicates the sum of di (the distance from the surface to the nearest nucleus internal to the surface) and de (the distance from the surface to the nearest nucleus external to the surface) whereby de is shorter than the sum of the relevant van der Waals radii. The blue and white color signify the negligible and weak intermolecular interactions, respectively. The two-dimensional fingerprint plots were used to quantify the various intermolecular interactions in the title compound.

Density functional theory

All computations were performed using the Gaussian09 software package. Full geometry optimization was carried out using the Density Function Theory at B3LYP with 6-311++G (d, p) basis set level. The Mullikan charge distributions of the investigated compound was also computed at the same level of method. The GaussView 5.0 program was used to construct the optimized molecular geometry, HOMO and LUMO electron distributions, and the HOMO-LUMO energy gap. The TD-DFT calculation was performed on the ethanol solvent using the IEPCM method.

Results and Discussion

Fourier transform infrared

Nitrile (C≡N) is allotted as a single, typically intense stretching band at 2211 cm^{-1} (for cyano compounds). The C-Cl stretching vibration in the ACBN at 782 cm^{-1} confirms that the halogen group in the para position is highlighted to the aromatic CN group [12]. Geethapriya et al. claimed that the CH in plane bending vibration is typically evident at 1300-1000 cm^{-1} and the CH out-plane is captured at 1000-750 cm^{-1} [13]. The CH in plane and out-plane for the ACBN is displayed in Figure 1. The ACBN's FTIR spectroscopic results show that it has distinctive peaks at 3452 and 3363 cm^{-1}, which refer to the primary NH functional group [14]. Due to the presence of benzene ring C=C stretching vibrational bands, there is significant stretching frequency at 1433 and 1485 cm^{-1}. The stretching bands at 3100–3000 cm^{-1} range is where pure C–H stretching vibrations of aromatic compounds predominate. However, the stretching frequency of the ACBN is slightly different from the report, caused by the presence of withdrawing group Cl at para position.

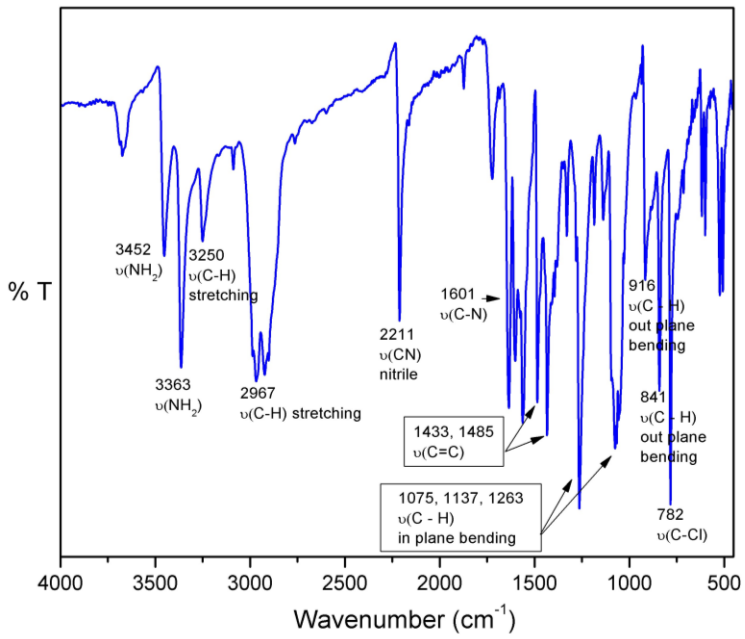


Figure 1. FTIR spectrum of ACBN

Ultraviolet-Visible

The ACBN absorption peak values are depicted in Figure 2. It is commonly perceived that the absorption peak approximately at 328 nm involve transition of n→π* in the C≡N group. In contrast, the aromatic ring of π → π∗ and n → π∗ transitions may be accountable for the absorption peaks in the 210–400 nm range [15]. Figure 6 depicts the B3LYP frontier orbitals of ACBN, demonstrating that the HOMO and LUMO electrons were primarily localized across the whole ACBN where the excitation process occurred. Besides that, based on the experimental and theoretical spectra, there are a slightly different number of absorption peaks. Changes in the geometry of the ACBN in the polar solvent (ethanol) would be associated with the creation of hydrogen bonds between ACBN-Ethanol. Hence, this will influence the electron localization in the HOMO-LUMO regions, causing the early absorption peak (TD-DFT) to be split into two peaks, as shown in Figure 2(a).

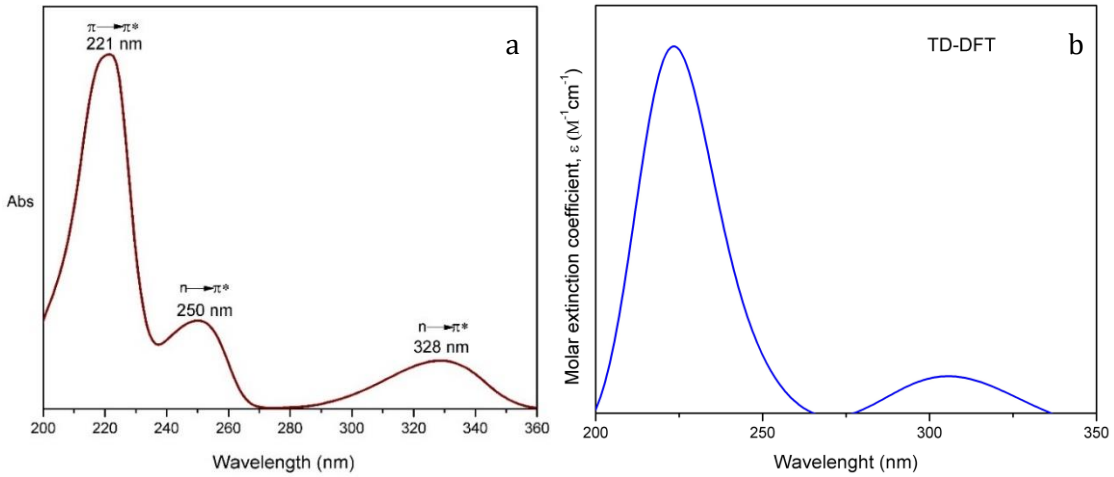


Figure 2. UV-Vis spectrum of ACBN; experimental (a) and theoretical (b)

Single crystal X-ray diffraction

This compound was obtained from the re-crystallization in ethanol. Figure 3 depicts the compound's crystal structure along with an atom-labelling system. The compound crystallized in the triclinic system with the space group Pī. Table 1 summarizes the key crystallographic information, in addition to the bond lengths and angles. Nitrile (C≡N) and C-N have bond lengths of 1.146(4) and 1.369(4) Å, which are less than the theoretical values of 1.160 and 1.470 Å due to the conjugation of the aromatic ring. Furthermore, the presence of the electron-withdrawing group, Cl, in the aromatic ring also led to the reduction of bond lengths. In the crystal structure of BN, the compounds were linked by intermolecular hydrogen bonds, N1---H1A---N2 and N1---H1B—N2 to form 1D chain along a axis as shown in Figure 4 whilst the hydrogen bond geometries are presented in Table 2.

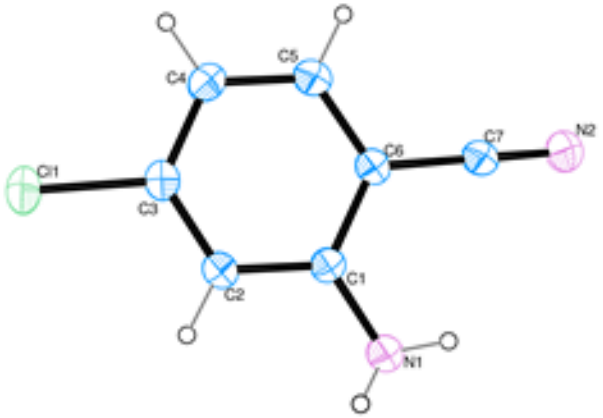


Figure 3. The molecular structure of ACBN with the atom labelling scheme. Displacement ellipsoids are drawn at the 50% probability level

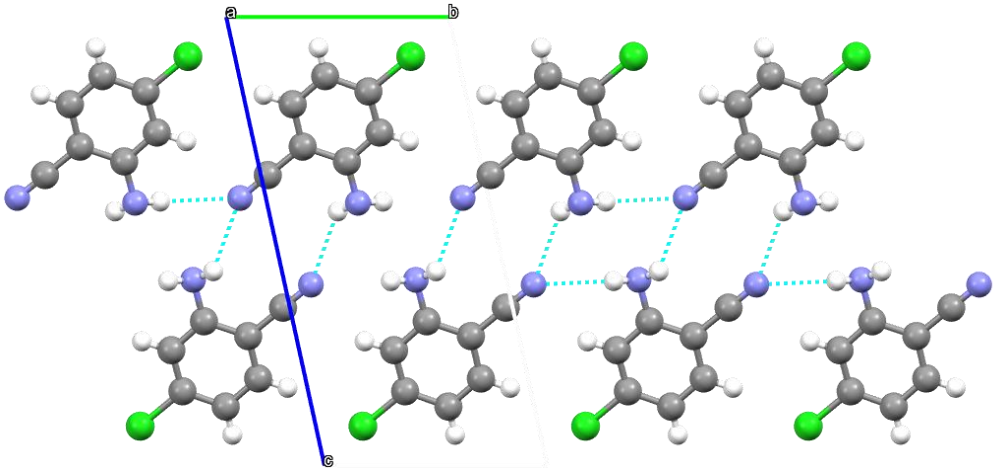


Figure 4. H-bonding view along a-axis

Table 1. Crystallographic data and structural refinement details of ACBN

Crystal Data	
Chemical Formula	$C_7H_5ClN_2$
Molecular mass (gmol^{-1})	152.58
Crystal system, space group	Triclinic, P-1
Temperature (K)	293
a, b, c (Å)	3.8924 (9), 6.7886 (15), 13.838 (3)
α, β, γ (°)	77.559 (16), 88.898 (17), 83.021 (17)
V (Å^3)	354.41 (14)
Z	2
Radiation type	M_o K_α
μ (mm^{-1})	0.45
Crystal size (mm)	0.54 x 0.52 x 0.26
T_{min}, T_{max}	0.243, 0.267
No. of measured, independent and observed [I> 2σ(I)] reflections	6340, 2450, 1033
R_{int}	0.080
(sin θ/λ)max (Å $^{-1}$)	0.750
Refinement R[F^2 > 2σ(F^2)], wR(F ^{2}), S	0.072, 0.158, 1.02
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.20, −0.24

Table 2. Hydrogen bond geometries of **ACBN**

D–H···A	**D–H (Å)**	**H···A (Å)**	**D···A (Å)**	**D–H···A (°)**	**Symmetry Codes**
N1–H1A···N2	0.86	2.5	3.166(4)	135	2-x,-y,1-z
N1–H1B···N2	0.86	2.53	3.375(4)	168	x,1+y,z

Hirshfeld surface analysis

The Hirshfeld Surface Analysis was performed to quantify the nature of the intermolecular interactions [16]. Figure 5 presents the Hirshfeld Surface mapped over dnorm. The deep red spots on the dnorm Hirshfeld surfaces represent the close contact interactions, which are mainly responsible for the significant intermolecular N–H···N interactions. The Hirshfeld surfaces mapped over the shape index (Figure 6a) shows comparatively weak interactions involving the phenyl rings represented by the yellowish triangular-shaped regions, which indicate that π···π stacking interactions are present in the crystal packing. The π···π stacking interaction is significant as the *Cg*1-to-*Cg*1 separation ranges from 3.892(2) to 3.893(2) Å [17]. The Cg1 represents the centroid of C1, C2, C3, C4, C5 and C6. The presence of this π···π stacking interactions is also evident by the map of curvedness (Figure 6b) which shows flat surface patches. Two-dimensional fingerprint plots from the Hirshfeld surface analysis of this title compound were calculated to gain quantitative data on the percentage contributions of different intermolecular interactions to the overall Hirshfeld surfaces. The overall two-dimensional fingerprint plot that represents intermolecular contacts are illustrated in Figure 7. The most dominant contribution (26.7%) originates from N···H/H···N contacts which signify the presence of N–H···N interactions. The Cl···H/H···Cl contacts are observed at 21.3% while H···H contacts at 17.9%. The other contacts which are H···C/C···H, C···C, Cl···Cl, N···C/C···N, Cl···C/C···Cl and N···N, show the percentage contributions of 12.3%, 10.8%, 4.2%, 2.6%, 2.3% and 2%, respectively. All these interactions contribute a significant role in crystal packing.

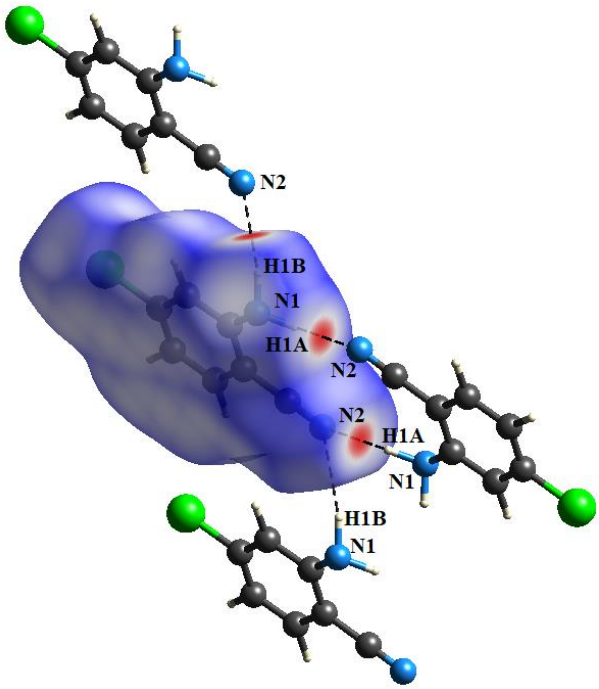
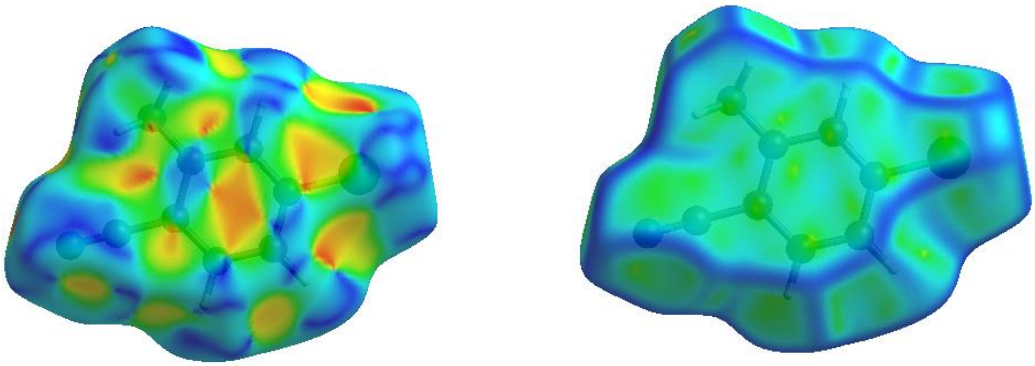


Figure 5. Hirshfeld surfaces mapped over dnorm showing hydrogen-bonding interactions (black dash line) with their neighbouring molecules



(a) (b)

Figure 6. Hirshfeld surfaces mapped over (a) shape index and (b) curvedness

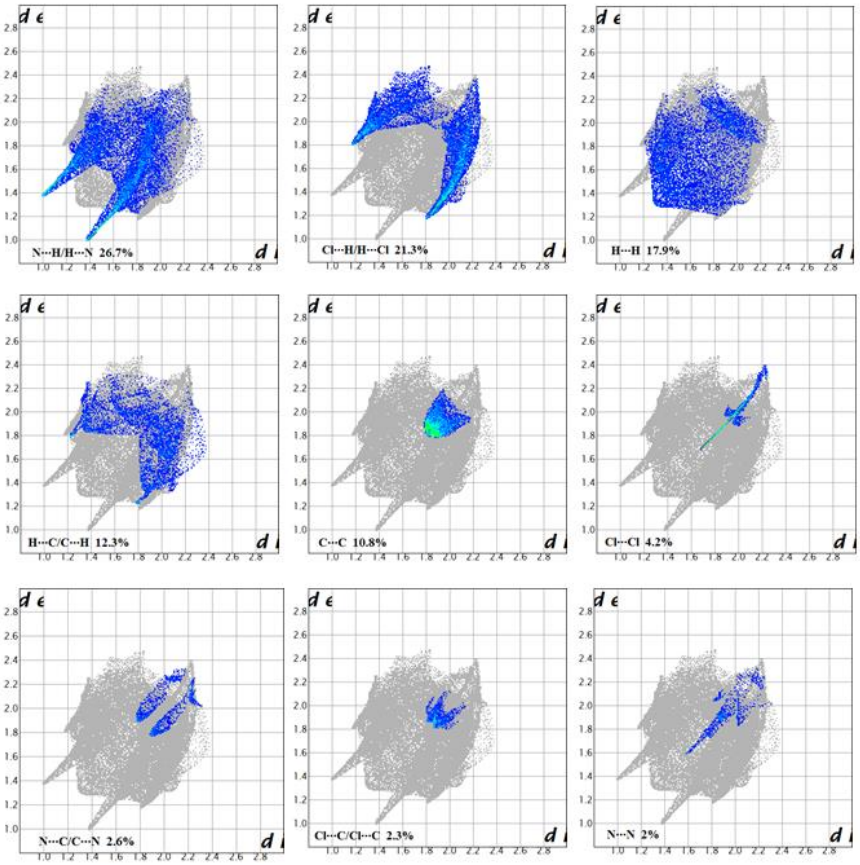


Figure 7. Two-dimensional fingerprint plots of the title compound with their respective contributions

Density functional theory: Mulliken population method

The Mulliken population method using B3LYP/6-311++G(d,p) level of calculation was successfully calculated to obtain the total atomic charge distribution of ACBN. As shown in Figure 8, the 7 atoms from ACBN exhibit negative charges namely 1C, 3C, 4C, 6C, 11N, 14C, and 15N. Positive charges are found in 2C, 5C, 7H, 8H, 9H, 10Cl, 12H, and 13H. The result showed that atom 15N and 11N have a high tendency to act as a nucleophilic atom for the compound as they have negative charges with values of -0.202 and -0.237, respectively.

Intrinsic molecular characteristics

The global reactivity parameters of ACBN are shown in Table 3. The HOMO and LUMO electrons were primarily localized across the whole ACBN. The greater values of ionization potential, I and electron donating capability (ω^-) showed that the ACBN has the capability to donate rather than accept the electrons [18]. Hence, it has the capability to form complexes easier. In comparison to literature, the energy gap value, ΔE, resolved from the crystal data is lower at 3.4988 eV. Sudha et al. [19] generated DFT data using the proposed structure, while this study used crystal data. Calculated ΔE is a crucial stability indicator that aids in defining the compound's chemical reactivity and kinetic stability [20]. As the ΔE value of ACBN is lower than that of the previous study, the reactivity rises with a greater degree of electronic charge transfer from the ACBN as a free ligand to the central metal ion, making it easier to provide electrons to an acceptor. Sherif and Abdel-Kader et al. [21] stated that the ACBN undergoes conjugation, which contributes to the low value of the

energy gap as well. The ACBN has very strong electrophilic properties since the global electrophilicity value is more than 1.5 eV [22]. Global hardness (η) and softness (S) are frequently employed to assess the stability and reactivity of compounds. The ACBN is categorized as a hard compound because the value of global hardness (η) is larger than global softness (S). The high hardness value consistently suggests greater stability and lessened reactivity. It is also consistent with the high value of chemical potential. But despite the high value of ω^-, the ACBN still shows potential to donate electrons to other compounds for specific applications.

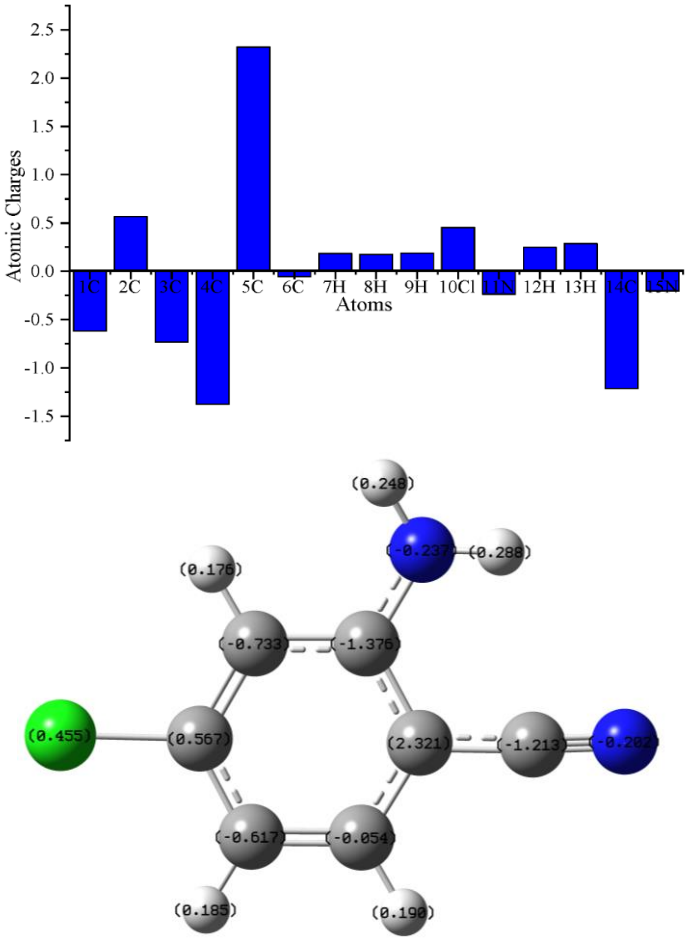


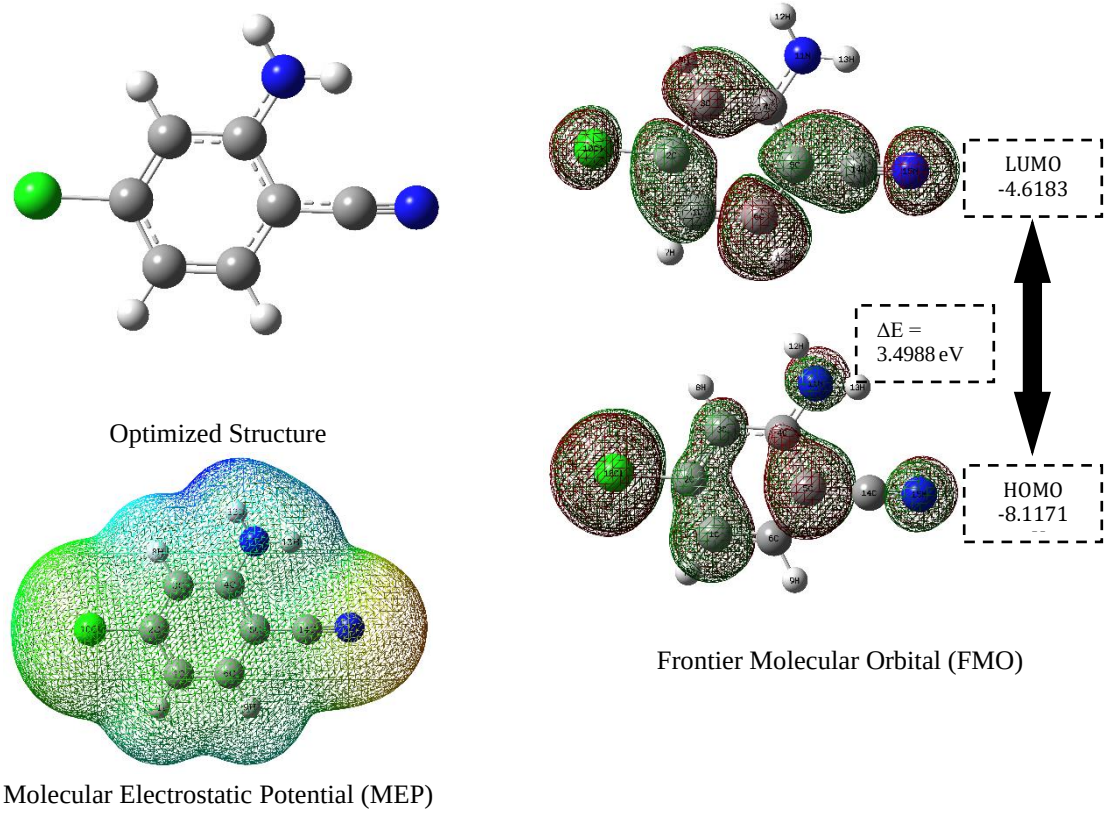
Figure 8. Optimized structure and bar diagram representing the charge distribution of ACBN

Table 3. Global reactivity parameters of ACBN

Molecular energy (eV)	**ACBN**	**Molecular energy (eV)**	**ACBN**
E_{HOMO}	-8.1171 eV	Global Softness (S)	0.1570 eV^{-1}
E_{LUMO}	-4.6183 eV	Chemical potential (μ)	-6.3677 eV
Energy gap (ΔE)	3.4988 eV	Electronegativity (χ)	6.3677 eV
Ionization potential (I)	8.1171 eV	Global electrophilicity (ω)	11.589 eV
Electron affinity (A)	4.6183 eV	Electron donating capability (ω^-)	14.992
Global hardness (η)	1.7494 eV	Electron accepting capability (ω^+)	8.6238

Molecular electrostatic potential

The nitrile (C≡N) is identifiable as a high electron density region as shown in Figure 9, indicating the most preferable reactivity for nucleophilic site. Besides that, the proton of NH_2 has a very noticeable low electron density, which indicates the most preferable reactivity for an electrophilic site [23].



Optimized Structure

Molecular Electrostatic Potential (MEP)

Frontier Molecular Orbital (FMO)

Figure 9. The optimized structure, MEP and FMO of ACBN

Conclusion

The single crystal structure of 2-amino-4-chlorobenzonitrile (ACBN) was successfully confirmed using spectroscopic and single crystal X-ray crystallographic studies. The Hirshfeld Surface analysis revealed that N···H/H···N contacts are the most dominant contributions to the total Hirshfeld surfaces which signify the presence of N–H···N interactions. The intrinsic molecular characteristics analysis showed that although the ACBN is stable and less reactive, the compound still shows a potential in transferring and accepting electrons. Besides that, the Mulliken study showed that the compound has the potential to act as a nucleophilic agent through the nitrogen atoms (highly negative charges). As recommendation, the DFT study revealed that the compound could be investigated more thoroughly in terms of reaction kinetics and mechanisms such as corrosion inhibition and biological activities.

Acknowledgement

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Supplementary Data

CCDC-2224917 contains the supplementary crystallographic data for the compound reported in this paper. These data can be obtained free of charge at

www.ccdc.cam.ac.uk/conts/ retrieving.html [or from the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ, UK; fax:+44(0)1223-336033; e-mail: deposit@ccdc.cam.ac.uk].

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PHYTOCHEMICALS ISOLATION, ANTIBACTERIAL AND ANTIBIOFILM ACTIVITIES OF MALAYSIAN *Uncaria tomentosa* AGAINST ORAL STREPTOCOCCI

(Pengasingan Fitokimia, Aktiviti Antibakteria dan Antibiofilem *Uncaria tomentosa* dari Malaysia terhadap *Streptococci* Oral)

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Abstract

Uncaria tomentosa (Rubiaceae), locally known as 'kait-kait' has long been used for medicinal purposes to heal various diseases, including those related to bacterial infection. In an attempt to search for new antimicrobial agents, the present study was undertaken to evaluate the antibacterial and antibiofilm potentials of hexane (Hx), dichloromethane (DCM), and 80% ethanolic (EtOH) stem extracts of Malaysian *U. tomentosa* against five oral *streptococci* bacteria namely *S. salivarius*, *S. mitis*, *S. uberis*, *S. mutans* and *S. anginosus*. The antibacterial assay employed the agar-well diffusion method where cycloheximide was used as the positive control, while minimum inhibitory concentration (MIC) was determined through resazurin microtiter assay using 96-well microtiter plates. On the other hand, the antibiofilm activity was investigated using a crystal violet assay. The DCM stem extract was found to be active against all tested bacterial strains with ZOI ranging from 10.3±0.6 to 16.0±1.0 mm, and a MIC value of 234.4 µg/mL. Compared to average biofilm growth, the extract reduced biofilm formation by at least 50%. Phytochemical work on the DCM extract afforded four compounds elucidated as isopteropodine (**1**), pteropodine (**2**), β-sitosterol (**3**), and scopoletin (**4**). These four compounds have been previously reported to exhibit antibacterial activity against different bacterial strains. The findings provide evidence to support the plant's traditional use and could be the source of antibacterial and antibiofilm agents for further studies.

Keywords: *Uncaria*, dichloromethane extract, oral bacteria, streptococci, antimicrobial

Abstrak

Uncaria tomentosa (Rubiaceae), yang dikenali sebagai 'kait-kait' telah lama digunakan untuk tujuan perubatan dalam menyembuhkan pelbagai penyakit, termasuk yang berkaitan dengan jangkitan kuman. Dalam usaha mencari agen antimikrob baharu, kajian ini telah dijalankan untuk menilai potensi antibakteria dan antibiofilem ekstrak heksana (Hx), diklorometana (DCM) dan 80% etanol (EtOH) daripada batang *U. tomentosa* dari Malaysia terhadap lima bakteria *streptokokus* oral iaitu *S. salivarius, S. mitis, S. uberis, S. mutans* dan *S. anginosus*. Ujikaji antibakteria menggunakan kaedah ujian peresapan telaga agar di mana sikloheksimida digunakan sebagai kawalan positif, manakala kepekatan perencatan minimum (MIC) ditentukan melalui ujian mikrotiter resazurin dengan menggunakan plat mikrotiter 96 telaga. Ekstrak batang DCM didapati aktif terhadap semua strain bakteria yang diuji dengan ZOI dalam julat 10.3±0.6 - 16.0±1.0 mm, dan nilai MIC 234.4 µg/mL. Berbanding purata pertumbuhan biofilm normal, ekstrak ini dapat mengurangkan pembentukan biofilm sekurang-kurangnya 50%. Kajian fitokimia pada ekstrak DCM menghasilkan empat sebatian yang dikenalpasti sebagai isopteropodine (**1**), pteropodine (**2**), β-sitosterol (**3**), dan scopoletin (**4**). Empat sebatian ini sebelum ini telah dilaporkan mempamerkan aktiviti antibakteria terhadap strain bakteria yang berbeza. Penemuan ini memberikan bukti untuk menyokong penggunaan tradisional tumbuhan dan boleh menjadi sumber agen antibakteria dan antibiofilem untuk kajian lanjut.

Kata kunci: *Uncaria*, ekstrak diklorometana, bakteria oral, streptokokus, aktiviti antimikrob

Introduction

Oral bacteria are an example of an opportunistic human species capable of causing biofilm-related infections. Epithelial cells, dental surfaces, and orthodontic prostheses are some of the many surfaces in the oral cavity that are prone to the formation of multispecies biofilms [1]. In the form of supragingival and subgingival plaque, biofilm is the etiologic agent in dental caries (tooth decay) and periodontal (gum) disease [2]. Furthermore, studies have demonstrated an association between periodontitis and various systemic diseases and conditions in recent years, including cardiovascular disease, diabetes mellitus, respiratory disease, adverse pregnancy outcomes, obesity, pancreatic cancer, and Alzheimer's disease [3].

Uncaria (Rubiaceae) is a flowering woody climber plant. The genus comprises 34 species that distributed mainly in tropical regions, including Southeast Asia, Africa, and South America. Cultivation varies based on the species and region [4,5]. *Uncaria tomentosa* was given the name una de gato (cat's claw) because of hook-like thorns that resemble the claws of a cat. Phytochemical studies of the Peruvian *U. tomentosa* have led to the isolation of numerous constituents including quinovic acid glycosides, oxindole alkaloids, proanthocyanidins, polyphenols, triterpenes and numerous sterols [6-8]. Although there was no literature on the existence of the species in Malaysia, the species has been discovered in Malaysian forests.

The extracts of *U. tomestosa* is also widely studied compared to other species of the genus and they have been found to display multiple biological activities including anti-inflammatory, antioxidant, cytotoxicity, immunostimulant, and antibacterial [9,10]. So far, there is a lack of scientific reports indicating this plant's antimicrobial activities against oral pathogens. Moreover, no study has been published to indicate the potential antibiofilm activities of the plant. Hence, this study aimed to investigate the antibacterial and antibiofilm potentials of the hexane (Hx), dichloromethane (DCM), and 80% ethanol (EtOH) stem extracts of *U. tomentosa* against oral bacteria and to isolate its chemical constituents.

Materials and Methods

Plant material

The plant material was collected from Pasir Raja, Dungun, Terengganu in December 2011. The plant sample was identified by Dr. Shamsul Khamis of Universiti Putra Malaysia. The plant materials were air-dried in the dark at room temperature before being ground with a mechanical grinder.

Preparation of extracts

The dried samples were cut into small pieces and ground into a fine powder with a mechanical grinder. The fine powder of the stems (5 kg) was successively extracted with solvents ranging in different polarities: hexane (nonpolar), dichloromethane (medium polar) and 80% ethanol (polar) solvents by continuous hot extraction

technique using a Soxhlet extractor. The solvents were then evaporated under reduced pressure at a temperature lower than 40 ºC using a vacuum rotary evaporator. The dried extracts were stored in the refrigerator at a temperature of 4 °C until further analysis.

Antibacterial activity

The extracts of stems were prepared by dissolving in 10% dimethyl sulphoxide (DMSO) to a final concentration of 100 mg/mL. As a solvent for all the test samples, DMSO was first tested against bacteria to ensure that it did not contribute to the antibacterial activity of the samples. Then, the extracts were diluted to reach the concentration of 50, 35, and 15 mg/mL. These concentrations of test samples were subjected to an agar well-diffusion assay screening test. The lowest concentration of the test samples showed inhibition activity in the screening test and were chosen for further evaluation of MIC using the resazurin microtiter-plate dilution assay.

Bacterial strains and growth condition

A total of 5 oral bacteria were employed in the study, including *Streptococcus salivarius* (ATCC 13419), *Streptococcus mitis* (ATCC 6249), *Streptococcus uberis* (ATCC 19436), *Streptococcus mutans* (ATCC 25175) and *Streptococcus anginosus* (ATCC 33397). All bacterial strains were purchased from Oxoid (UK). The bacterial strains were grown in brain heart infusion broth (BHI) and were maintained on brain heart infusion agar (BHI) at 4 °C, and subcultures were freshly prepared before being subjected to bioassay.

Antibacterial screening

The agar well-diffusion assay was performed in this study to screen the antibacterial activity of the extracts against the bacteria tested. Briefly, 24 hours of bacterial suspension was adjusted to absorbance reading within the range of 0.08-0.10 at OD625 nm, equivalent to 1-2 x 10^8 CFU/mL [11] that matched the turbidity 0.5 McFarland standards [12]. Liquefied (25 mL) sterile brain heart infusion agar (BHI) at 45 °C was aseptically mixed with 1 mL standardized inoculum and poured immediately into previously labeled sterile petri dishes. Then, the plates were left to solidify in a laminar flow cabinet for 20 minutes. Once the agar hardened, four wells (8 mm) were bored using a sterile cork borer. A 100 µL volume of each extract was introduced into the wells. Chlorhexidine (CHX) of 0.12 % was used as a positive control, while a 10% DMSO was used as a negative control. The extracts were allowed to pre-diffuse at room temperature for 1 hour before the agar plates were incubated at 37 °C for 24 hours. The antibacterial activity was expressed as the average diameter of a clear zone to the nearest millimeter. The tests were performed in triplicate and repeated twice to reduce the possibility of a test error.

Determination of minimum inhibitory concentration (MIC)

Resazurin microtiter-plate assay was used to determine the MIC. The assay was performed according to the procedure described by Gahlaut and Chhillar [13] with some modifications. Under aseptic conditions, a flat bottom sterile 96-well polystyrene tissue culture plate was labeled, and each well was filled with 100 µL of BHI broth. Each extract (100 µL) was then added to the first well, and two-fold serial dilutions were performed so that each well had 100 µL of test extracts in serially descending concentrations. Then, the standardized bacterial culture suspension (50 µL) was added to each well. The final volume in each well was 150 µL. The following controls were prepared: wells containing broth only (sterility control), bacterial strain with no extract (inoculum viability), serial dilution of CHX (positive control), and DMSO (negative control). The plates were incubated at 37 °C overnight. A 30 µL resazurin solution was added to each well, and the plates were incubated for another one to two hours. The MIC was determined as the lowest sample concentration at which no pink color (signifying live growth) appeared.

Antibiofilm activity: Preparation of biofilm development

Preliminary work has been done to determine the optimum growth condition applied to the biofilm formation assay. This work was adopted according to the protocol designed by Stepanović et al. [14]. The standardized bacterial suspension (~1 x 10^8 CFU/mL) was vortexed, 50 µL of the cell solution was then diluted 1:100 by adding to 4950 µL of desired medium [BHI,

BHI+ 1% (w/v) glucose, BHI+ 1% (w/v) sucrose]. These diluted bacteria (~1 x 10^6 CFU/mL) were then vortexed for 30 s to 1 min and transferred to a flat bottomed 96-well polystyrene tissue culture treated plate (200 µL per well). The plates were prepared in triplicates and placed in an incubator shaker at 37 °C for 24 hours. After incubation, the attached biofilm was quantified by measuring the absorbance of the solution at 570 nm [15].

Determination of minimum biofilm inhibitory concentration

The crystal violet (CV) assay was used to investigate the effect of *U. tomentosa* extracts on biofilm formation by adopting the protocol by Stepanović et al. [14]. Briefly,100 µL of fresh BHI supplemented with 1% (w/v) sucrose was distributed into all the wells of the flat bottom 96-well tissue culture plate. In sequence, 100 µL of extracts in a concentration used in the antibacterial activity (MIC) were added to the wells. Serial dilution was performed and followed by adding 100 µL bacterial suspension (1 x 10^6 CFU/mL). The plates were then incubated at 37 °C for 24 hours. After 24 hours of incubation time, the supernatant was discarded. Then, the plates were gently washed thrice with phosphate buffer saline (PBS) solution to remove the remaining planktonic cells, followed by air drying for 30 minutes. The adherent biofilm in the plates was fixed with methanol for 20 minutes, the solution was then removed, and the plates were air-dried. The attached bacterial cells were then stained with 150 µL of 2% crystal violet and left at room temperature for 15 minutes. Following staining, the stains were discarded and rinsed off by placing the plates under running tap water. The plates were blotted on paper towels and air-dried at room temperature. The bound dye was extracted from the stained cells using 150 µL of 95 % EtOH and left at room temperature for 30 minutes. The content's optical density (OD) was measured at 570 nm using a microtiter plate reader. The intensity of staining is directly proportional to the number of biofilms adhering to the 96-well plate. The percentage biofilm inhibition was calculated by the following formula, according to:

$$\%\ inhibition = \frac{OD\ (mean)^{control} - OD\ (mean)^{test\ sample}}{OD\ (mean)^{control}} \times 100\% \quad (1)$$

Where OD control is the mean OD measured for 24 hours bacterial biofilm grown without the extracts, while ODtest samples is the mean OD measured for 24 hours bacterial biofilm grown in the presence of extracts. The minimum biofilm inhibitory concentration ($MBIC_{50}$) was defined as the lowest concentration of the tested extracts required to prevent the formation of ≥50% of the biofilm [16].

Phytochemical work: General procedures

Column chromatography was conducted using silica gel 60, 70-230 mesh ASTM (Merk 7734 packed by slurry packing method. Radial chromatography was carried out using a glass plate with Merck's silica gel (Kieselgel 60 PF254 Merck Art 7749). Preparative TLC was performed using glass-supported silica gel 60 F254 (1.0 mm thickness). TLC was used to monitor the eluates was performed using pre-coated aluminium-backed supported silica gel 60 F254 (0.2 mm thickness). Identification of the spots on the TLC plate was carried out by spraying Dragendorff's reagent for alkaloid and P-anisaldehyde-sulfuric acid for terpenes, sterols, and steroids. Spots and bands for compounds on TLC and PTLC were viewed individually under UV lights (254 and 365 nm). The mass spectrum was recorded with an Agilent1100 series LC/MS ion trap. The infrared (IR) data was obtained on a Perkin Elmer model FT-IR spectrometer as a KBr disc. ^1H and ^{13}C NMR spectra were recorded with a Bruker 300 Ultra shield NMR spectrometer measured at 300 and 75 MHz, respectively.

Isolation and elucidation of compounds

The DCM extract was subjected to an acid-base extraction yielded an alkaloid mixture (3g) and a non-alkaloid mixture (18g). The alkaloid and non-alkaloid mixture was then subjected to column chromatography over silica gel, eluted with Hx:DCM (1:0-0:1) and subsequently DCM:MeOH (1:0-0:1) yielding 30 and 9 fractions, respectively. Based on their TLC profiles, the fractions from the alkaloid mixture were combined together into 9 major subfractions (F1-F9). Subfraction

F7 (40 mg) was subjected to radial chromatography using DCM and EtOAc in an increasing polarity manner to give compounds **1** (20 mg) and **2** (15 mg). Column chromatography on the targeted fraction of non-alkaloid mixture using solvent system Hx:DCM (9.5:0.5) successfully yielded compound **3** (9.4 mg). Another targeted fraction of the mixture using preparative TLC on silica gel using solvent system DCM: EtOAc (8.0:2.0) afforded 2.7 mg of compound **4**. The structure of isolated compounds was elucidated as isopteropodine (**1**), pteropodine (**2**), β-sitosterol (**3**), and scopoletin (**4**) based on spectroscopic data analysis as well as by comparing with the literature values.

Statistical analysis

All the experiments were run in triplicates and were repeated at least twice. The values obtained were analyzed using IBM SPSS Statistics v.22. The replicate absorbance readings were calculated as the mean of individual experiments in triplicate. The significant difference between the means of the test samples and the control group was performed using a Bonferroni post-test. A difference was considered significant when p values ≤ 0.05.

Result and Discussion

Antibacterial activity

The antibacterial activity possessed by extracts of increasing polarity against five oral bacteria is reported in Table 1. From the results obtained, the antibacterial activity of the extracts was greatly affected by solvent polarity. Out of the three extracts, the DCM extract exerted the most pronounced activity. This extract exhibited an inhibitory effect against all tested bacterial strains (*S. mutans*, *S. mitis*, *S. salivarius*, *S. anginosus*, and *S. uberis*). Meanwhile, Hx and 80% EtOH extracts were effective against only two strains (*S. salivarius* and *S. uberis*). The results obtained from this study indicate that the extracts showed varying degrees of antibacterial activity as the extracts were prepared by successive extraction of different polarities. Separating the constituents into nonpolar (Hx), medium polar (DCM), and polar fractions (80% EtOH) is expected to concentrate each extract's antibacterial constituents and enhance their inhibitory ability. The screening test using the agar well-diffusion assay was not a definitive method in detecting the inhibition of the test bacterial because many of the antimicrobial compounds in plant extracts are relatively nonpolar, and these compounds do not diffuse well into the surrounding agar around the wells [17]. This could be the reason for the negative activity shown by the Hx extract.

The test samples were then subjected to the microplate dilution method with resazurin as an indicator to determine the extracts' minimum inhibitory concentration (MIC) because it is more sensitive and provides reproducible results. The results are shown in Table 2. The blue color or the color before pink in the microtitre plates after adding the resazurin dye indicator was determined as the lowest concentration that completely inhibited the microorganism's growth. The MIC values obtained in this study from the extracts tested ranged from 234.4 µg/mL to 937.5 µg/mL. The antimicrobial potential of plant extracts is classified as follows: MIC < 100 µg/mL – strongly active; MIC 100-500 µg/mL – moderately active; MIC 500-1000 µg/mL – weakly active and MIC > 1000 µg/mL – inactive [18]. Based on the criteria given, generally, the extracts of this plant had moderate to low antibacterial activity against the bacteria tested. The strongest (MIC = 234.4 µg/mL) and the weakest (MIC = 937.5 µg/mL) antibacterial activity was shown by the DCM against *S. mutans*, and *S. anginosus*, respectively. In addition, the Hx and DCM extracts showed similar growth inhibitory activity against *S. salivarius*, *S. mitis* and *S. uberis* at a MIC value of 468.8 µg/mL. Meanwhile, no antibacterial activity was observed with the Hx extract against *S. mutans* and *S. anginosus*, and none by the 80% EtOH extract against either bacterial tested.

The difference in the MIC values obtained suggests a varying degree of susceptibility of the microorganisms, as was determined by comparing the MIC values of the test extracts against each bacterial tested. The lower the MIC value, the more susceptible the bacteria tested to the antimicrobial agent and vice-versa. Of the five bacterial tested, *S. mutans* was the most susceptible, as indicated by the lowest MIC values obtained from the DCM extracts (234 µg/mL). The trend in susceptibility to the extracts was *S. mutans* > *S. salivarius*, *S. mitis*, *S. uberis* > *S. anginosus*. In contrast, *S. anginosus* was

found to be less sensitive as only inhibited by the DCM extract. The activity of the extract tested can be ranked as DCM > Hx > EtOH 80% extracts. These results indicated considerable variation in antibacterial activity between extracts obtained with the different polarities of extracting solvents.

Table 1 Inhibition zone diameter of stem extracts against oral bacteria

Extract Type	**Conc. (mg/mL)**	**Zone of Inhibition (mm)**				
		S. mutans	**S. mitis**	**S. salivarius**	**S. uberis**	**S. anginosus**
Hx	50	0	0	12.0±0.0	11.0±0.0	0
	35	0	0	12.0±0.0	10.7±0.6	0
	15	0	0	12.0±0.0	10.3±0.6	0
DCM	50	12.0±1.0	11.0±0.0	15.0±0.0	16.0±1.0	11.3±0.6
	35	11.3±0.6	10.7±0.6	14.3±0.6	13.0±1.0	11.0±0.0
	15	11.0±0.0	10.3±0.6	14.0±1.0	12.3±0.6	10.7±0.6
80% EtOH	50	0	0	15.0±0.0	14.7±0.6	0
	35	0	0	15.0±0.0	14.3±0.6	0
	15	0	0	13.5±0.6	14.0±1.0	0
DMSO	0	0	0	0	0	0
CHX	29.0±1.0	27.0±1.0	29.7±0.6	27.7±0.6	25.3±0.6	29.0±1.0

Table 2. Minimum inhibitory concentration (MIC) of extracts against oral bacteria

Extract Type	**Minimum Inhibitory Concentration (µg/mL)**				
	S. mutans	**S. mitis**	**S. salivarius**	**S. uberis**	**S. anginosus**
Hx	-	468.8	468.8	468.8	-
DCM	234.4	468.8	468.8	468.8	937.5
80% EtOH	-	-	-	-	-

Different solvent extracts showed a different activity level, with DCM extract showing the most pronounced activity against all tested bacteria. It seems that extracts with an intermediate polarity had a much higher antibacterial activity than more polar (80% EtOH) or nonpolar (Hx) extracts. This agrees with the study by Eloff et al. reported that *Melianthus comosus* extract from the intermediate polarity solvent (acetone) had the best activity against fungal pathogens [19]. This has been attributed to more polar compounds being either less or completely inactive against the bacteria tested. It is worth notifying that DMSO (negative control) used as diluents did not affect the bacteria tested. Reports are available on the antibacterial activity of different concentrations of *U. tomentosa* extracts against several microorganisms [20-22]. But a few studies have

investigated the antibacterial activity of *U. tomentosa* against microorganisms frequently found in the human oral cavity. Ccahuana-Vasquez and co-workers employed MIC assay to determine the antimicrobial activity of 0.25% to 5% of micropulverized *U. tomentosa* extract on different strains of microorganisms isolated from the human oral cavity [23]. The authors reported that concentrations of 3% *U. tomentosa* inhibited 52 % of the *S. mutans* strain, which agrees with our findings. On the other hand, different authors reported 2% CC (cat's claw) gel prepared from freeze-dried extract inhibited *Enterococcus faecalis*, *Staphylococcus aureus*, and *Candida albicans* which are microorganisms commonly found in infected root-filled teeth [24].

Antibiofilm activity

The effects of adding alternative carbohydrates, glucose, and sucrose, in the BHI media frequently used to grow oral biofilm were examined to determine the best medium for the subsequent biofilm assays. For this purpose, three different culture media, BHI, BHI + 1% glucose, and BHI + 1% sucrose, was employed in a static biofilm setup to evaluate the best-suited medium for oral biofilm development under in vitro conditions. Biofilm formation of the oral *Streptococci* was assayed and categorized based on the A575 of the crystal violet-stained biofilm: OD570 >2.0, a good biofilm former; OD570 = 1.0 to 2.0, moderate biofilm former and OD570< 1.0, poor biofilm former [15].

As shown in Figure 1, when culture media supplemented with or without glucose were used, the biofilm of the bacteria tested grew poorly (OD570 < 1.0). The results point to the optimal media for simultaneous growth of at least three bacteria to be BHI with 1% sucrose, hence was used for all subsequent experiments. This result is in line with previous findings where sucrose is considered the most cariogenic carbohydrate not only it is being fermented by oral bacteria, but is also a substrate for the synthesis of extracellular polysaccharides which will then promote changes in the composition of the biofilm's matrix [25]. Out of the five bacteria tested, only *S. mutans* was found to be a good biofilm former. Thus, this bacterium was then subjected to a biofilm inhibition assay. The three remaining bacteria, which were found to be poor biofilm formers, were all of the following strains: *S. uberis*, *S. mitis*, and *S. anginosus*.

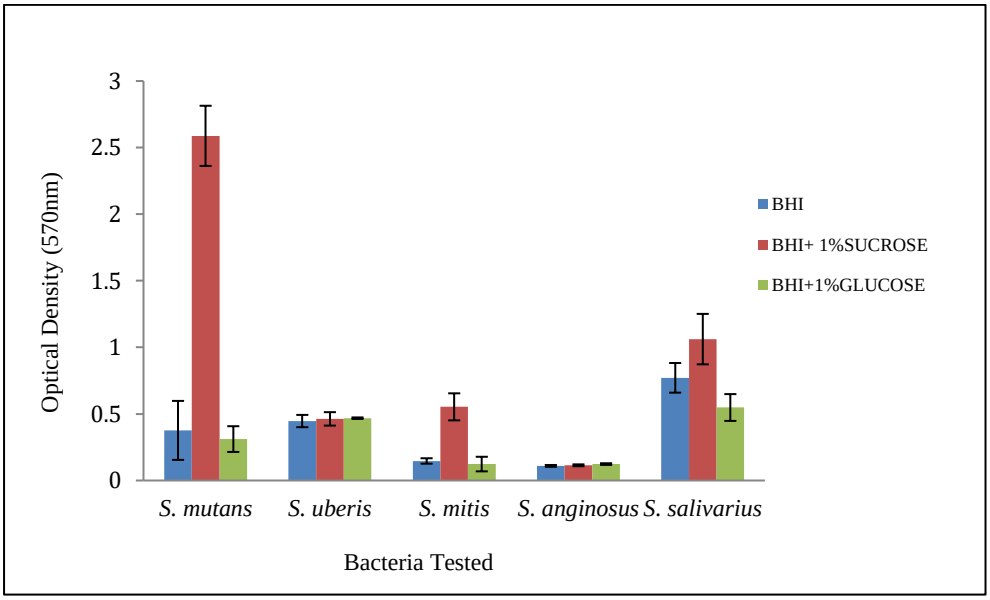


Figure 1. Results from Growth Optimization Assay. Blue, red, and green bars represent bacteria grown in different growth conditions. All assays were performed triplicate, and the data represent the average of 3 or more wells with standard deviations denoted by the error bars

Due to the highest antibacterial activity recorded by the DCM extract, it was further subjected to antibiofilm activity produced by *S. mutans*. As shown in Figure 2, *U. tomentosa* DCM extract showed an increasing ability to inhibit the biofilm by increasing its concentrations evidenced by the reduced absorbance values compared to the control biofilm (no extracts). Inhibition was expressed as percentages, with the extracts exhibiting good antibiofilm activity ≥ 50% inhibition. In this study, the lowest $MBIC_{50}$ reported was at a concentration of 58.6 µg/mL, indicating moderate antibiofilm activity. Higher MBIC (93.21% and 95.36%) was observed for the extract at the higher tested concentrations of 234.4 µg/mL and 468.8 µg/mL, respectively. Perusal the literature value, there are several ways in which natural products execute their antibiofilm activity which include preventing the formation of the polymer matrix, suppressing cell adhesion, interrupting extracellular matrix (ECM) generation, and reducing the production of virulence factors. This, in turn, blocks the quorum sensing (QS) network and hinders biofilm development [26]. The activities here could be contributed by some moderate polarity compounds that work synergically or singularly in the extract. The stem of the plant has been reported to contain a moderate polarity of compounds including coumarins, terpenes, flavonoids and alkaloids that may be responsible for the *U. tomentosa's* medicinal effects [9]. While these compounds may be responsible for the antibacterial activities reported in this study, only flavonoids and an alkaloid had been reported to possess good antibacterial activity [27,28]. In order to have more insight on the possible chemical constituents of the active extract, the DCM extract was subjected to phytochemical study.

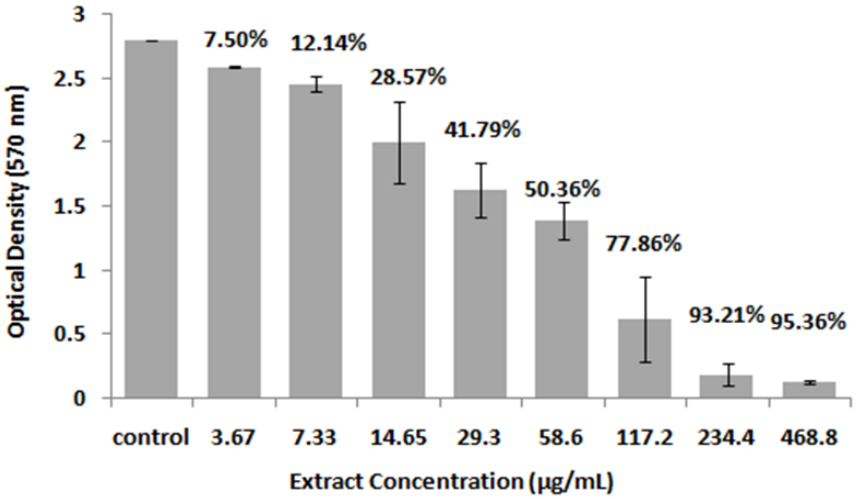


Figure 2. Inhibitory activity towards *S. mutans* biofilm formation by DCM extract. Percent biofilm formation inhibition, shown at the top of each column, was calculated by comparing the absorbance values of test samples with the absorbance of the control biofilm

Phytochemical work

Repeated chromatographic techniques and purification on the DCM extract of the stems of *U. tomentosa* afforded four compounds where two from the alkaloidal [isopteropodine (**1**) and pteropodine (**2**)] and two from the non-alkaloidal [β-sitosterol (**3**) and scopoletin (**4**)]. The structures were determined by spectroscopy particularly extensive NMR analyses as well as by comparison of their spectral data with the literature data [29-32]. The significant spectroscopic characteristics of the compounds are as follows, while their structures are shown in Figure 3.

Isopteropodine (**1**): ESIMS m/z 368 $[M+H]^+$ for $C_{21}H_{24}N_2O_4$; IR (KBR) υ_{max}: 3368 (NH), 1718 (ester C=O), 1657 (amide C=O) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ ppm: 8.30 (1H, *s*, N-H), 7.43 (1H, *s*, H-17), 7.29 (1H, *d*, *J* = 7.5 Hz, H-9), 7.20 (1H, *ddd*, *J* = 7.5, 1.2 Hz,

H-11), 7.03 (1H, *ddd*, *J* = 7.5, 1.2 Hz, H-10), 6.89 (1H, *d*, *J* = 7.5 Hz, H-12), 4.36 (1H, *m*, H-19), 3.60 (3H, *s*, OCH_3), 3.33 – 3.20 (2H, *m*, H-21β, H-5β), 2.60 – 2.30 (5H, *m*, H-15, H-21α, H-6β, H-5α, H-3), 2.00 (1H, *m*, H-6α), 1.60 (2H, *m*, H-14α, H-20), 1.43 (3H, *d*, *J* = 6 Hz, H_3-18), 0.89 (1H, *m*, H-14β); ^{13}C NMR ($CDCl_3$, 75 MHz) δ ppm: 181.25 (N-C=O), 167.64 (O-C=O), 155.00 (C-17), 140.96 (C-13), 133.69 (C-8), 127.58 (C-11), 124.07 (C-9), 122.04 (C-10), 109.85 (C-16), 109.43 (C-12), 72.16 (C-19), 71.34 (C-3), 57.09 (C-7), 54.15 (C-5), 53.53 (C-21), 50.99 (OCH_3), 37.90 (C-20), 34.86 (C-6), 30.49 (C-15), 30.19 (C-14), 18.65 (CH_3).

Pteropodine (**2**): ESIMS m/z 368 $[M+H]^+$ for $C_{21}H_{24}N_2O_4$; IR (KBR) υ_{max}: 3206 (NH), 1721 (ester C=O), 1629 (amide C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm: 8.66 (1H, *s*, N-H), 7.49 (1H, *s*, H-17), 7.22 (1H, *d*, *J* = 7.5 Hz, H-9), 7.19 (1H, *ddd*, *J* = 7.5, 1.2 Hz, H-11), 7.07 (1H, *ddd*, *J* = 7.5, 1.2 Hz, H-10), 6.83 (1H, *d*, *J* = 7.5 Hz, H-12), 4.57 (1H, *m*, H-19), 3.63 (3H, *s*, OCH_3), 3.37 – 3.19 (2H, *m*, H-21β, H-5β), 2.50 – 2.31(5H, *m*, H-15, H-21α, H-6β, H-5α, H-3), 2.03 (1H, *m*, H-6α), 1.74 (1H, *m*, H-14α), 1.61 (1H, *m*, H-20), 1.51 (1H, *m*, H-14β), 1.43 (3H, *d*, *J* = 6 Hz, H_3-18); ^{13}C NMR ($CDCl_3$, 75 MHz) δ ppm: 181.0 (N-C=O), 167.77 (O-C=O), 155.29 (C-17), 140.48 (C-13), 133.41 (C-8), 127.98 (C-11), 123.27 (C-9), 122.74 (C-10), 109.25 (C-12), 109.22 (C-16), 74.42 (C-3), 72.23 (C-19), 56.08 (C-7), 55.17 (C-5), 53.63 (C-21), 50.93 (OCH_3), 37.88 (C-20), 34.59 (C-6), 30.98 (C-15), 29.59 (C-14), 19.0 (CH_3).

β-sitosterol (**3**): ESIMS m/z 414 $[M+H]^+$ for $C_{29}H_{50}O$; IR (KBR) υ_{max}: 3381 (OH), 2934 - 2833 (aliphatic C-H), 1668 (C=C), 1450 (H-C-H) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm: 5.37 (1H, *t*, *J* = 5.4 Hz, H-6), 3.54 (1H, *m*, H-3), 2.24 (1H, *m*, H-4), 2.00 (2H, *m*, H-7, H-4), 1.83 (1H, *m*, H-25), 1.75 – 1.60 (4H, *m*, H-7, H-20, H-15, H-16), 1.55 – 1.45 (6H, *m*, H-2, H-12, H-11, H-28, H-24, H-17), 1.44 – 1.40 (2H, *m*, H-9, H14), 1.35 – 1.30 (5H, *m*, H-1, H-15, H-16, H-12, H-2), 1.25 – 1.00 (5H, *m*, H-11, H-22, H-23, H-1, H-8), 1.02 (3H, *s*, H_3-19), 0.93 (3H, *d*, *J* = 6.0 Hz, H3-21), 0.94 (3H, *t*, *J* = 6.6 Hz, H_3-29), 0.88 (3H, *d*, *J* = 6.3 Hz, H_3-27), 0.85 (3H, *d*, *J* = 6.3 Hz, H_3-26), 0.75 (3H, *s*, H_3-18); ^{13}C NMR (CDCl3, 75 MHz) δ ppm: 140.76 (C-5), 121.73 (C-6), 71.80 (C-3), 56.76 (C-14), 56.04 (C-17), 50.12 (C-9), 45.82 (C-24), 42.31 (C-13), 42.27 (C-4), 39.76 (C-12), 37.24 (C-1), 36.49 (C-10), 36.14 (C-20), 33.93 (C-22), 31.89 (C-7), 31.89 (C-8), 31.64 (C-2), 29.13 (C-25), 28.24 (C-16), 26.05 (C-23), 24.30 (C-15), 23.05 (C-28), 21.07 (C-11), 19.82 (C-26), 19.39 (C-19), 19.02 (C-27), 18.77 (C-21), 11.98 (C-29), 11.85 (C-18).

Scopoletin (**4**): ESIMS m/z 192 $[M+H]^+$ for $C_{10}H_8O_4$; IR (KBR) υ_{max}: 3350 (OH), 1670 (C=O), 1629 (amide C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm: 7.86 (1H, *d*, *J* = 9.3 Hz, H-4), 7.12 (1H, *s*, H-5), 6.79 (1H, *s*, H-8), 6.22 (1H, *d*, *J* = 9.3 Hz, H-3), 6.20 (1H, *br s*, OH), 3.92 (3H, *s*, OCH_3); ^{13}C NMR (CDCl3, 75 MHz) δ ppm: 161.46 (C=O), 150.25 (C-6), 149.70 (C-9), 144.01 (C-7), 143.30 (C-4), 113.40 (C-3), 111.49 (C-10), 107.49 (C-5), 103.19 (C-8), 56.41 (OCH_3).

As has been mentioned previously, these alkaloids (**1** and **2**), coumarin **4**, and terpene **3** have been reported to exhibit antibacterial activity against different bacterial strains [9,27,28]. Thus, these compounds are presumably responsible for the antibacterial and antibiofilm activities of the DCM extract in the present study. This is the first report of antibiofilm activity of *U. tomentosa* extracts to the best of our knowledge. The findings provide evidence to support the usage of *U. tomentosa* in microbial infections and could be the source of antibacterial and antibiofilm agents for further studies.

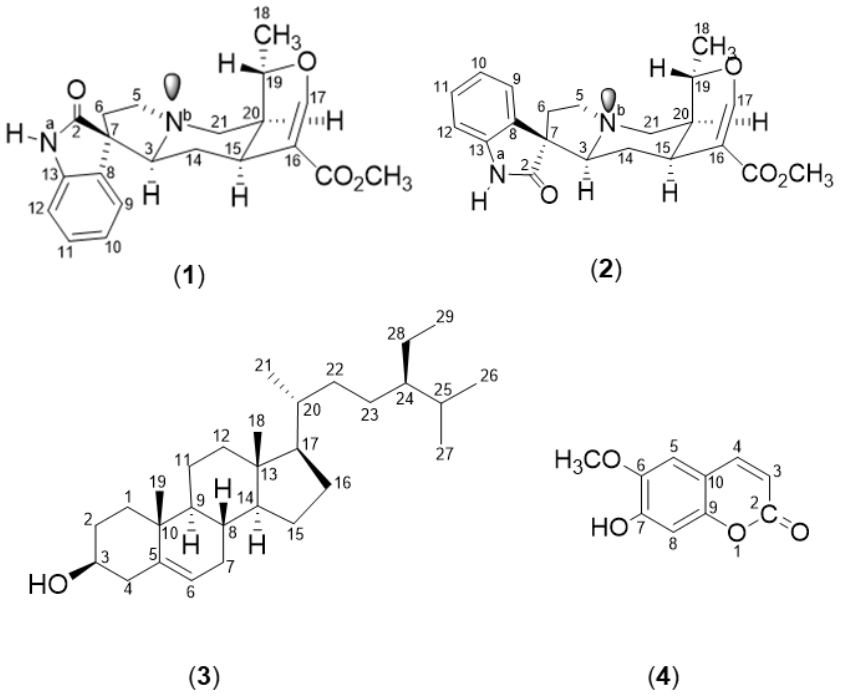


Figure 3. Structure of the isolated compounds: (**1**) pteropodine, (**2**) isopteropodine, (**3**) β-sitosterol and (**4**) scopoletin

Conclusion

The present data suggest that Malaysian *U. tomentosa* extracts exhibited antibacterial and antibiofilm activities against oral bacteria. The isolated phytochemicals namely isopteropodine, pteropodine, and β-sitosterol, scopoletin, are presumably responsible synergically and singularly for the activities of the active DCM extract. However, further research is needed to evaluate these compounds to ensure their contribution. The findings provide evidence to support the plant's traditional use and could be the source of antibacterial and antibiofilm agents for further studies.

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SYNTHESIS OF CARRAGEENAN-BASED BIOCOMPOSITE PLASTICIZED WITH DEEP EUTECTIC SOLVENT AND CHARACTERIZATION OF ITS MECHANICAL PROPERTIES

(Sintesis Biokomposit Berasaskan Karaginan Diplastikkan dengan Pelarut Eutektik Dalam dan Penyifatan Ciri-Ciri Mekanikal)

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Abstract

Carrageenan from seaweed tends to be brittle in the formation of hard capsules. In this study, a carrageenan-based biocomposite was synthesized to provide an alternative to gelatin hard capsules. This study aims to characterize the mechanical properties of carrageenan biocomposite plasticized with a deep eutectic solvent (DES) of choline chloride (ChCl) and glycerol. The carrageenan biocomposite was formulated at varying concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1.0 v/v%) of DES to improve the strength and elasticity of carrageenan biocomposite films and hard capsules. The absence of the ChCl band at 1348 cm^{-1} and the reduced intensity of the C–O glycerol band at 1107 cm^{-1} in the ATR-FTIR spectra of DES were regarded as evidence for the formation of the eutectic mixture. This can be explained by the hydrogen bond donor and acceptor interaction between the DES constituents, which are chloride ions (Cl^-) of ChCl and the hydroxyl group (–OH) of glycerol ($Cl^-\cdots OH$). The highest viscosity of Carra-DES 0.2 at 504.9 mPa·s reflects the improved film tensile strength up to 60.1 MPa, which gives a positive effect after the addition of DES. The capsule loop strength reached its peak at 31.7 N for Carra-DES 0.4. A significant increase in the elongation at break of Carra-DES film was observed at DES concentrations of 0.2–0.6%. However, the concentration of DES should be controlled to achieve high tensile and loop strengths in hard capsule application. In conclusion, the incorporation of DES in carrageenan biocomposite can reduce its brittleness while improving its elasticity and strength in the production of hard capsules.

Keywords: Biocomposite, carrageenan, choline chloride, deep eutectic solvent, plasticizer

Abstrak

Karaginan daripada rumpai laut cenderung menjadi rapuh dalam pembentukan kapsul keras. Dalam kajian ini, biokomposit berasaskan karaginan telah disintesis sebagai alternatif kepada kapsul keras gelatin. Kajian ini bertujuan menyifatkan ciri-ciri mekanikal biokomposit karaginan yang ditambah pemplastik pelarut eutektik dalam (DES) yang diperbuat daripada kolina klorida

(ChCl) dan gliserol. Biokomposit karaginan telah dirumuskan dengan DES menggunakan kepekatan yang berbeza (0, 0.2, 0.4, 0.6, 0.8, dan 1.0 v/v%) untuk meningkatkan kekuatan dan keanjalan filem biokomposit karaginan dan kapsul keras. Ketiadaan jalur penyerapan ChCl pada 1348 cm^{-1} dalam analisis ATR-FTIR dan peralihan jalur getaran C–O dalam gliserol ke arah 1728 cm^{-1} dianggap sebagai bukti pembentukan campuran eutektik. Ini boleh dijelaskan oleh interaksi penderma dan penerima ikatan hidrogen antara konstituen DES, iaitu ion klorida (Cl^-) ChCl dan kumpulan hidroksil (–OH) gliserol ($Cl^{-}\cdots OH$). Kelikatan tertinggi Carra-DES 0.2 pada 504.9 mPa·s mencerminkan kekuatan tegangan filem yang lebih baik sehingga 60.1 MPa dan memberikan kesan positif selepas penambahan DES. Kekuatan gelung kapsul mencapai kemuncaknya pada 31.7 N untuk Carra-DES 0.4. Peningkatan ketara pemanjangan filem Carra-DES semasa putus berlaku pada kepekatan DES 0.2% hingga 0.6%. Walau bagaimanapun, kepekatan DES harus dikawal untuk mencapai kekuatan tegangan filem dan gelung kapsul yang tinggi dalam aplikasi kapsul keras. Kesimpulannya, penggabungan DES dalam biokomposit karaginan dapat mengurangkan kerapuhan di samping meningkatkan keanjalan dan kekuatan dalam menghasilkan kapsul keras.

Kata kunci: Biokomposit, karaginan, kolina klorida, pelarut eutektik dalam, pemplastik

Introduction

A high interest in sustainable polymers from plants has been shown to replace gelatin hard capsules from animal sources due to consumers' dietary restrictions. Carrageenan, a versatile natural polysaccharide, emerges as a promising alternative material for hard capsules with characteristics similar to gelatin, including thermal reversibility and film-forming properties. Derived from seaweed, this resource abounds in Malaysia, particularly in East Malaysia, Sabah [1]. Unveiling superior gelling and high viscosity properties, carrageenan is able to form a biopolymer film, finding widespread application in the pharmaceutical industry as a thickener, stabilizer, and emulsifier [2]. The distinctive attributes of this material have assured its market demand. Despite its film-forming ability, an inherent brittleness limits its application as a hard capsule, presenting an intriguing challenge for further exploration. In order to overcome the shortcomings of carrageenan, formulations of carrageenan biocomposite are thoroughly studied. A biocomposite is a blend of two or more naturally derived materials to develop a reinforced product with improved performance over individual constituent materials. The formulation of carrageenan biocomposite has been modified by incorporating isovanillin as the crosslinker [3] with cellulose nanocrystals (CNCs) and cellulose nanofibers as the reinforcing agents [4, 5]. Thickening agents have also been incorporated, such as gum Arabic [6, 7], hydroxypropyl methylcellulose [8], and carboxymethyl starch [9]. In the application of green bioplastics, *Chlorella vulgaris* microalgae have also been blended into the formulation to improve the hydrophobicity of the carrageenan film [10].

Plasticizer is an additive introduced in polymers to produce more flexible films, increase elasticity, and reduce friction during the manufacturing process. Deep eutectic solvent (DES) has properties that are suitable as a potential plasticizer to improve the mechanical properties of biocomposite films [11]. It is a novel solvent system that can be prepared from a range of anionic and/or cationic chemicals that are commonly available to form an eutectic mixture [12]. Deep eutectic solvent, an analog to ionic liquid (IL), is also utilized as a solvent, reagent, catalyst, and crosslinker in various applications [13]. Deep eutectic solvent is safe and non-toxic as opposed to IL based on its non-volatility and the alleged benign nature of its main constituents [14]. Studies on DES have begun with choline chloride (ChCl) as a hydrogen bond acceptor (HBA), and it is one of the most commonly used compounds in synthesizing DES [12]. Glycerol, the hydrogen bond donor (HBD) used in this study, is a simple natural polyol that is inexpensive, biodegradable, non-toxic, and extensively used in the food industry [15]. The simplicity of DES synthesis, achieved by the precise mixing of HBA and HBD components in specific molar ratios, as well as low toxicity, make it a more viable and safer choice for numerous applications, such as edible biocomposites [16].

In comparison to conventional plasticizers, DES (ChCl and glucose/glycerol/urea) are highly promising plasticizers for cellulose-based biocomposites, with an increase in elongation at break (EAB) up to 81%, while the tensile strength is comparable to that of a single component plasticizer [13]. Furthermore, DES (ChCl/urea) has also been proven to refine film

uniformity and smoothness with only 0.01% of DES, preserving the structural stability and optical properties of the CNC film while significantly reducing film brittleness [17]. The addition of DES (ChCl/lactic acid) with lignin also increased the tensile strength of the chitosan film to 31.6 MPa and effectively increased the plasticity, giving a maximum EAB of 62.6% [18]. The EAB of the gelatin/polyvinyl alcohol composite film reached up to 654% when the mass fraction of DES (sodium acetate trihydrate/urea) was 60% [19]. Most studies reported the advantages of DES for polysaccharides and better plasticizing ability than conventional plasticizers [20,21]. Currently, it is difficult to find research on the influence of DES as a plasticizer for carrageenan biocomposite and the plasticization effect on the carrageenan biocomposite film and hard capsules. This work aimed to synthesize carrageenan biocomposite plasticized with DES (ChCl/glycerol) at different concentrations and to characterize the mechanical properties of the resulting biocomposite films and hard capsules. It was hypothesized that the addition of DES as a plasticizer can reduce the brittleness and increase the carrageenan hard capsule strength when incorporated in an appropriate amount.

Materials and Methods

Materials

Refined carrageenan as the main material of the biocomposite was bought from CV Simpul Agro Globalindo, Indonesia. Hydroxypropyl methyl cellulose (84 kDa) as a thickening agent, 4-methoxybenzyl alcohol (98%) as a crosslinker, and calcium alginic acid (584.5 Da) as a toughening agent were obtained from Sigma-Aldrich (USA). Meanwhile, CNCs for reinforcement were prepared from the microcrystalline cellulose (Avicel PH-101) purchased from Sigma-Aldrich (St. Louis, MO, USA). The DES composed of ChCl (98%, 139.62 Da) and glycerol (99.5%, 104.06 Da) as a plasticizer were obtained from Merck (Darmstadt, Germany). All chemicals used are of analytical grade. Deionized water was used throughout the entire experiment.

Preparation of DES as a plasticizer

Pure ChCl was pretreated to remove any possible moisture by drying in a vacuum oven overnight at 80 °C and was stored in a dry place for further use [22]. Accordingly, ChCl and glycerol were mixed with gentle stirring at a molar ratio of 1:2 at 60 °C for 30 min until homogeneous with no evidence of solid particles [23, 24]. The eutectic mixture of ChCl and glycerol was designated as the DES solution.

Preparation of CNC as a reinforcement agent

The CNC solution was prepared according to Mohd. Amin et al., where 5.0g of microcrystalline cellulose was stirred overnight in 500 mL of deionized water using a hot plate [25]. A QSonica (Q700, USA) ultrasonicator was used to sonicate the cellulose dispersion for 20 min at a 20% amplitude. This process was carried out with 8 s pulsed on and 2 s pulsed off. After resting for 10 min, the bottom layer of the solution was removed, and the solution was left overnight. The top clear layer of the solution was removed to collect 300 mL of the concentrated portion, which was identified as the CNC solution.

Synthesis of carrageenan biocomposite

The biocomposite solution was prepared by dissolving 3.0g of refined carrageenan in 150 mL of deionized water. The solution was heated for 30 min at 60 °C under continuous stirring using a hot plate and a magnetic stirrer. Then, 4-methoxybenzyl alcohol (0.7 w/v%), CNCs (1.6 w/v%), hydroxypropyl methylcellulose (0.8 w/v%), and calcium alginic acid (0.07 w/v%) were added gradually to the biocomposite solution. These concentrations were incorporated into the carrageenan biocomposite following previous research from Hamdan et al. [4] and Ramli et al. [8]. The DES, which composed of ChCl and glycerol, was added to the biocomposite solution at varying concentrations from 0 to 1.0 v/v%, as shown in Table 1. The solution was poured on a round stainless-steel tray with a diameter of 20 cm after 3 h of mixing, while the remaining solution was dipped using the stainless-steel mold pins (size 1) to form the hard capsule. All samples of biocomposite films and hard capsules were dried overnight at room temperature.

Table 1. Sample formulation of carrageenan biocomposite plasticized with DES at various concentrations

Samples	Concentration of Carrageenan (w/v%)	Concentration of DES (v/v%)
Control	2.0	0
Carra-DES 0.2	2.0	0.2
Carra-DES 0.4	2.0	0.4
Carra-DES 0.6	2.0	0.6
Carra-DES 0.8	2.0	0.8
Carra-DES 1.0	2.0	1.0

Functional group determination

The presence of different functional groups in ChCl and glycerol was analyzed by conducting Fourier transform infrared (FTIR) spectroscopy to recognize any potential shifting of the chemical bonds in the formation of DES. The samples were subjected to an attenuated total reflection (ATR)-FTIR spectrometer (Perkin Elmer, Frontier, USA) equipped with OMNIC software within the range of 400-4000 cm^{-1}. Overall, 32 scans were collected with a spectral resolution of 8 cm^{-1} at a rate of 0.15 s/scan [26].

Rheological analysis

The viscosity is a crucial characteristic of a biocomposite as it will affect the mechanical properties of the carrageenan biocomposite. Higher viscosity relates to stronger intermolecular forces between the materials, where the molecules are strongly bonded together. The viscosity and shear stress of the biocomposite solution were measured using a rotational viscometer (Brookfield, Rheo 3000, USA) equipped with LCT 25 4000010 geometry. Approximately 16.5 mL of the biocomposite solution was poured into the cylindrical compartment of the viscometer for each measurement. The sample was evaluated in triplicate at a constant temperature of 40 °C using a preset speed of 300 rpm with 100 MPoints [27].

Mechanical strength evaluation

The mechanical evaluation was carried out to investigate the deformation of the produced films and hard capsules in response to a load or force applied. A texture analyzer (CT3, Brookfield, USA) was used to measure the tensile strength and EAB of the biocomposite films. The analyzer was outfitted with TexturePro CT V1-8 Build 3.1 software and a 5-kN load cell. The tensile strength was measured following the standard method of ASTM D882-12. The biocomposite films were cut into strips with a dimension of 20 × 60 mm^2. The film thickness was calculated as the average of three distinct positions of the film samples measured with Vernier calipers. The analysis was controlled by setting up the texture analyzer with a baseline grip width of 80 mm and a crosshead speed of 30 mm/min to a maximum distance of 15 mm [28]. The tensile strength and the EAB for each sample were calculated as in Equation 1 and Equation 2, respectively. The load at break and the elongation at rupture were collected from the results of the texture analyzer, and the initial gauge length was the set-up value for the crosshead speed used for the measurement.

$$\text{Tensile strength (MPa)} = \frac{\text{Load at break}}{\text{Initial width} \times \text{Initial thickness}} \quad (1)$$

$$\text{Elongation at break (\%)} = \frac{\text{Elongation at rupture}}{\text{Initial gauge length}} \times 100\% \quad (2)$$

The same texture analyzer (CT3, Brookfield, USA) was used to conduct the capsule loop test, but with a capsule loop fixture. A hard capsule was attached to a pair of separating rods, with the lower rod positioned on the stationary platform and centrally beneath the probe. The upper rod gradually went higher at a speed of 0.50 mm/s with a target value of 5.0 mm until the hard capsule was torn apart. [29]. The applied force (N) to break the hard capsule was reported as the capsule loop strength. The analysis was conducted with at least three measurements taken for each sample to calculate the average result.

Moisture content determination

Moisture content can affect the mechanical qualities of a sample. Accordingly, a moisture analyzer (MS70, A&D Japan) was used to measure the moisture content in the biocomposite film. About 0.1 g of the sample was placed on the heating pan of the moisture analyzer [30]. The moisture in the sample would evaporate after air-drying at 30–200 °C. The sample was heated by a halogen lamp for fast and uniform heating to give a quick measurement. When the mass of the sample reached a constant value, the analysis was automatically stopped. The electric balance weighed the sample before and after heating to determine the moisture content, as shown in Equation 3.

$$\text{Moisture content (\%)} = \frac{\text{Initial weight - Dried weight}}{\text{Initial weight}} \times 100\% \qquad (3)$$

Results and Discussion

Functional groups of DES

The formation of the eutectic mixture was analyzed by ATR-FTIR spectroscopy for DES and its pure constituents. The spectra of DES together with ChCl and glycerol are shown in Figure 1. Pure ChCl exhibited vibrational bands at 3223 cm^{-1} and 1200–880 cm^{-1}, corresponding to stretching vibrations of –OH or N–H bonds. The vibrational bands at 3024 cm^{-1} and 1479–1348 cm^{-1} corresponded to alkyl groups or N–H vibrations in quaternary ammonium groups. Other bands in the wavelength ranged from 1273 to 864 cm^{-1}, with the most intense band at 950 cm^{-1}, also showed the existence of hydroxyl and ammonium group characteristics of ChCl. The most prominent group of CH_2 bending was detected at 1479 cm^{-1}. This is comparable to the band at 1482 cm^{-1} from the study by Jakubowska et al. [31].

The most important band in the glycerol spectrum is the typical O–H stretching of the hydroxyl group, which was represented by the vibrational band at 3384 cm^{-1}. The vibrational bands at 2932 and 2878 cm^{-1} corresponded to C–H stretching and 1412 cm^{-1} to CH_2 bending of the alkyl group. The bands at 1107, 1028, and 849 cm^{-1} corresponded to different functional groups, namely C–O stretching, asymmetric C–C–O stretching, and symmetric C–C–O stretching, respectively. An additional band that appeared at 1208 cm^{-1} referred to C–O–H bending. These functional groups are absorbed at a wavelength similar to that reported by Delgado et al. [32].

A few peaks related to ChCl coexisted after the formation of DES. The absence of a ChCl band at 1348 cm^{-1} is consistent with previous results pertaining to the solvent systems of ChCl/malonic acid [33], ChCl/lactic acid [31], and ChCl/acrylic acid [34]. The reduced intensity around 1475 and 1107 cm^{-1}, which indicated CH_2 bending of ChCl and C–O stretching of glycerol, could be interpreted as evidence for the formation of the eutectic mixture of DES. This can be explained by the hydrogen bond donor-acceptor interaction between the constituents of DES, which are chloride ions of ChCl and the –OH group of glycerol ($Cl^{-}\cdots OH$). The absorption band of glycerol at 3293 cm^{-1} was discovered to shift down to 3287 cm^{-1} in the DES, indicating the formation of a hydrogen bond between ChCl and glycerol, such as $Cl^{-}\cdots OH$ and $CH_3\cdots OH$. A similar trend of shifted peaks between the DES constituents was also observed by Sun et al., who synthesized 25 types of ChCl-based DES for cellulose [35].

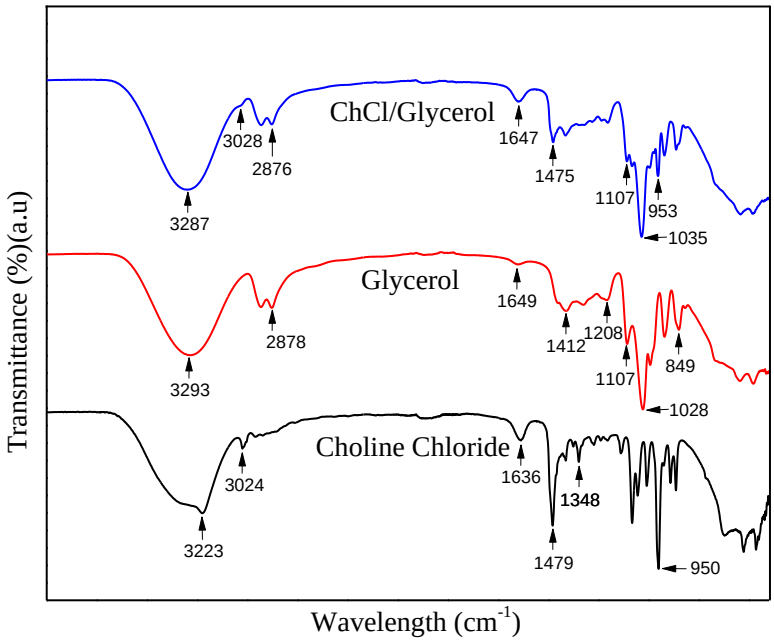


Figure 1. FTIR spectra of DES formation from ChCl and glycerol

Rheological analysis

Viscosity, a property of rheology, is defined as the measure of the resistance of a fluid to gradual deformation by shear stress. Figure 2 presents the relationship between shear stress and viscosity of the carrageenan biocomposite at different DES concentrations, ranging from 0 to 1.0 v/v%. The increase in shear stress between the layers of the biocomposite solution would also increase the viscosity of the solution [36]. The biocomposite without DES had a viscosity of 442.5 mPa·s and a shear stress of 171.2 Pa. The inclusion of 0.2 v/v% DES increased the viscosity and shear stress to their highest values of 504.9 mPa·s and 201.4 Pa, respectively. This increase is due to the rheological complexity of the carrageenan matrix when introduced with DES as a plasticizer. The trend for both plots of viscosity and shear stress fluctuated. It is worth noting that a significant reduction in the viscosity of Carra-DES 0.6 by 16.1% occurs because an excess amount of plasticizer enhances the molecular mobility of the biocomposite, reducing friction force or shear stress, thereby increasing the flowability of the biocomposite [37]. Defining the optimal concentration of a plasticizer in a formulation is crucial, as the viscosity of the biocomposite influences its mechanical properties.

Film tensile strength and EAB

The biocomposite materials used for hard capsules possess high mechanical properties to restrain the load applied, resulting in lower rejection rates during capsule filling. Although carrageenan has good film-forming properties, it is fragile and exhibits poor flexibility. The best concentration of DES as the plasticizer has to be quantified to produce hard capsules with low brittleness. In the mechanical evaluation, tensile strength and EAB were used as indicators for the biocomposite films, while loop strength was assessed for the hard capsules.

Figure 3 presents the tensile strength and EAB of carrageenan biocomposite at different DES concentrations. A significant peak in tensile strength was achieved by Carra-DES 0.2 with an increase of 67.1% from 19.8 to 60.1 MPa. This increase indicates that the addition of DES to the biocomposite film can effectively improve its flexibility and reduce the brittleness of the film. This enables its application as a plasticizer for hard capsules to be exploited. Moreover, the increase is directly related to the increase in viscosity of the biocomposite, as shown in Figure 2. The HBD and HBA of the DES were able to form hydrogen bonds with the hydroxyl groups on the glycosidic moiety of the polysaccharide chains of carrageenan. The increased

intermolecular interaction between DES and carrageenan strengthens the biocomposite film. This is applicable to the lower concentration of DES as the tensile strength gradually decreased with the increasing DES concentration from 0.4 to 0.8 v/v%. The newly formed hydrogen bonds were able to disrupt the intermolecular interactions between the polysaccharide chains, leading to an increase in chain movement and greater flexibility of the biocomposite [19].

In contrast, the EAB of the carrageenan biocomposite film depicted an opposite trend, which was consistent with previous studies [19, 38]. The EAB of the control film gradually increased from 6.1% and reached its maximum value of 45.5% with the addition of DES at a concentration of 1.0 v/v%. The directly proportional relationship between the EAB value and the DES concentration indicated that the DES had a significant plasticizing effect on the biocomposite films. The DES made from ChCl and glycerol was a viable plasticizer as it could significantly increase the percentage of EAB, even at a low volume. At a concentration of 0.6 v/v%, the EAB almost reached a constant value because the excess DES concentration disrupted the intermolecular hydrogen bonding between the polymer chains of carrageenan. This trend was comparable to the study by Zhao et al. in which a high concentration of natural DES was added to the chitosan/zein films [38]. In general, there is an inverse correlation between tensile strength and elongation. The best and most desirable biocomposite films for hard capsule application are those that exhibit both strength and flexibility in an optimal balance. These films would be able to withstand external forces while still being flexible enough to undergo deformation without breaking. Therefore, the amount of DES used in the formulation should be controlled to achieve high physical strength in hard capsule application.

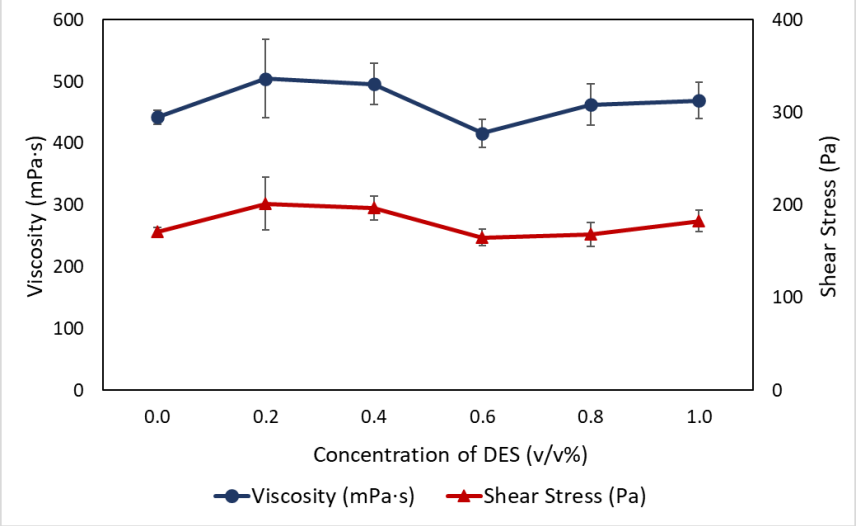


Figure 2. Viscosity and shear stress of carrageenan biocomposite at various DES concentrations

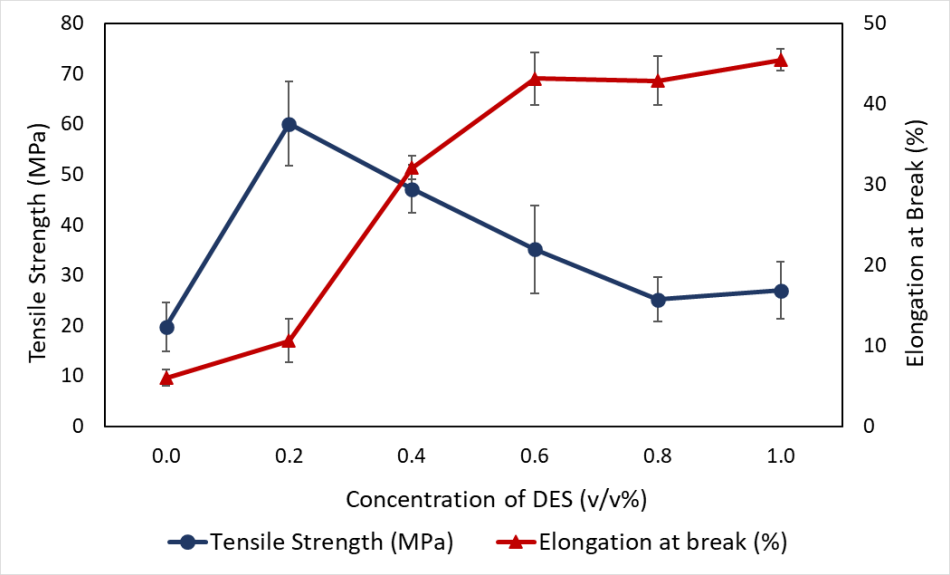


Figure 3. Tensile strength and EAB of carrageenan biocomposite films at various DES concentrations

Capsule loop strength

The loop strength for each carrageenan hard capsule sample at different DES concentrations is shown in Figure 4. The capsule loop strength increased significantly after the addition of DES, up to 35.4% for Carra-DES 0.4. The strength increased to 31.6 N as opposed to the control sample with 20.4 N. As discussed in the tensile strength result, the new hydrogen bond formed between the DES constituents and the carrageenan matrix improved the strength of the capsule loop due to the increased intermolecular interaction. The trend of the graph abruptly declined after the addition of DES at concentrations higher than 0.4 v/v%. The capsule loop strength decreased to 12.8 N in the sample of Carra-DES 1.0. The polar groups of DES made it easy to form hydrogen bonds with carrageenan. However, excessive plasticization weakened the carrageenan hard capsule as it could destroy the existing intermolecular hydrogen bonds between the carrageenan molecules [18]. Therefore, upon adding a higher concentration of DES, the capsule loop strength began to decrease.

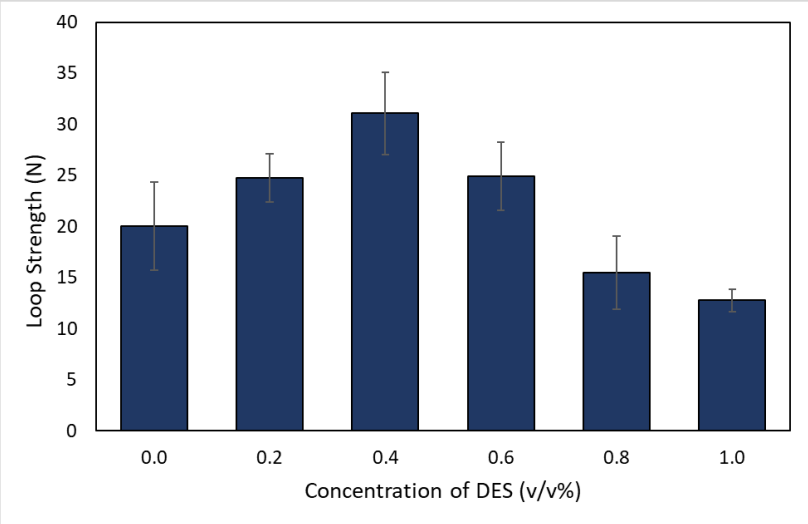


Figure 4. Loop strength of carrageenan hard capsules at various DES concentrations

Film thickness and moisture content

Table 2 presents the film thickness and moisture content of Carra-DES samples at varied concentrations. The control sample without DES was reported to be the thickest film compared to other samples with 0.23 mm. The addition of plasticizer increased the molecular mobility of the carrageenan matrix, which promoted the flowability of the biocomposite solution during film casting and thus reduced the film thickness. The thickness of the biocomposite films with the addition of DES from 0.2 to 1.0 v/v% presented a slight discrepancy, which was around 0.11–0.15 mm.

Table 2. Film thickness and moisture content of carrageenan biocomposite films at various DES concentrations

Samples	Film Thickness (mm)	Moisture Content (%)
Control	0.23 ± 0.02	14.5 ± 0.3
Carra-DES 0.2	0.12 ± 0.02	13.5 ± 0.5
Carra-DES 0.4	0.14 ± 0.01	15.4 ± 0.6
Carra-DES 0.6	0.11 ± 0.04	15.8 ± 0.6
Carra-DES 0.8	0.15 ± 0.01	15.9 ± 0.7
Carra-DES 1.0	0.12 ± 0.03	14.8 ± 0.1

Water can also act as a plasticizer for a biocomposite film, which in excess impairs its mechanical properties. Consequently, the moisture content of the carrageenan biocomposite film should be considered for the development of hard capsules. As shown in Table 2, the moisture content of all tested samples was around 13-15%, indicating that the influence of DES concentration was insignificant in this study. Moreover, these values were comparable to those of commercial gelatin capsules with a moisture content of 13% [39]. Similar results were also found by Hamdan et al., who reported that the moisture content of Carra-CNC biocomposites ranged from 15.5% to 17.5% [4]. However, it is noteworthy that high concentrations of DES at 0.4-0.8 v/v% had the highest moisture content, surpassing 15%. The hygroscopic characteristics of DES likely affect the ability of the film to absorb moisture from the surroundings when a high DES concentration is introduced into the formulation [40].

Physical appearances of films

Figure 5 presents the images of carrageenan biocomposite films at different DES concentrations with a black panel as the background. Based on the observation under natural light, the control sample appears transparent and has a rough surface due to the insoluble solid crystals of CNCs. The addition of the DES to the formulation improved the poor uniformity observed in the control sample. The transparency gradually decreased with increasing DES concentrations. Under the same conditions, Carra-DES 0.8 and Carra-DES 1.0 were observed to be almost opaque. These two samples have a relatively smooth surface compared to the control sample. Higher DES concentrations can improve the appearance of the carrageenan biocomposite film, where the smoothness increases differently. Nevertheless, a higher DES concentration would decrease the mechanical properties of the carrageenan biocomposite film, as discussed in Figure 3 and Figure 4. Wei et al. speculated that a higher DES concentration directly destroys the structure of CNCs, resulting in a loss of optical and mechanical properties of CNC/DES films [17].

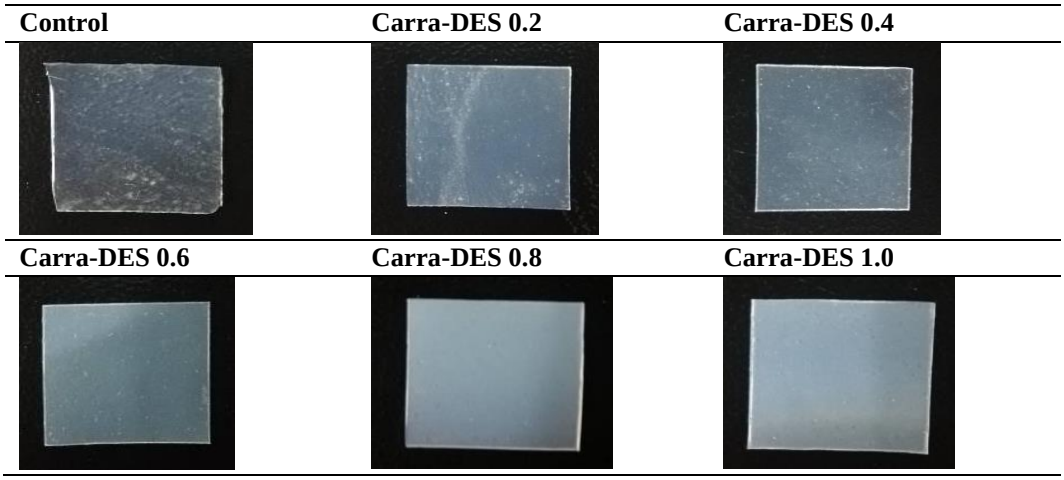


Figure 5. Images of carrageenan biocomposite films at various DES concentrations

Conclusions

In summary, DES has been successfully applied as a new type of plasticizer in the synthesis of carrageenan biocomposite films and hard capsules. The DES, which consists of ChCl and glycerol, is a good and feasible plasticizer because it can significantly increase the flexibility of carrageenan biocomposites even at low concentrations. The EAB of the carrageenan film greatly increased from 6.1% to 45.5% when the DES concentration increased to 1.0 v/v%. Meanwhile, Carra-DES at 0.2 v/v% gave the highest tensile strength of 60.1 MPa, which was the best concentration of Carra-DES biocomposite with a capsule loop strength of 24.8 N. The formation of hydrogen bonds between DES and carrageenan improves the mechanical strength of the biocomposite films. Despite that, an excess concentration of DES could have a detrimental effect on the film tensile and capsule loop strengths of the carrageenan biocomposite. In addition, the hygroscopic characteristics of DES would lead to poor stability of the hard capsule with increased moisture content. For that reason, it should be controlled at an appropriate concentration to achieve high mechanical strength in hard capsule application.

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BUTTERFLY PEA FLOWER-INFUSED QUINOA STARCH FILM AS A pH INDICATOR FOR SHELLFISH FRESHNESS ASSESSMENT

(Filem Kanji Kuinoa yang Diserapkan dengan Bunga Telang sebagai pH Indikator untuk Penilaian Kesegaran Kerang-Kerangan)

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Abstract

The pH indicator film has been utilised in food packaging to guarantee quality and safety as well as to track the freshness of perishable food in real-time. However, previous studies have concentrated on synthetic dyes, so this study investigated the use of butterfly pea flower (BPF) which contains anthocyanin, a natural pigment that is sensitive to a wide pH range. Quinoa starch (QS) contains 10-21% amylose with a small starch granule size of around 1 µm that allows for easier dispersion, making it a promising material to produce pH indicator films. BPF extract was incorporated into QS matrix films as a pH indicator to monitor the freshness of different types of shellfish (blood cockles and short-necked clams) stored at 4°C. The quinoa starch-fish gelatine-butterfly pea flower extract (QS/F-BPF) films were developed with different concentrations of BPF (5% and 10%), and then their characteristics including physical appearance, colour, thickness, water vapour permeability (WVP), moisture content, and colour responses were evaluated at different pH values (pH 1-12). The addition of different concentrations of BPF to the quinoa-gelatine-based film did not significantly affect the thickness, moisture content, and WVP. Meanwhile, the micrograph of the films showed good surface morphology. The colour and pH of the QS/F-BPF film were examined every two days for six days of storage of shellfish at 4°C, with the colour changing from blue to green with increasing pH on day 6, indicating spoilage. In conclusion, the QS/F-BPF pH indicator film is effective for monitoring the freshness of different types of shellfish stored in chiller conditions.

Keywords: butterfly pea flower, quinoa starch, pH indicator, anthocyanin, shellfish

Abstrak

Filem penunjuk pH telah digunakan dalam pembungkusan makanan untuk menjamin kualiti dan keselamatan serta untuk mengesan kesegaran makanan mudah rosak dalam masa nyata. Walaubagaimanapun, kajian lepas tertumpu kepada pewarna sintetik, jadi kajian ini menyiasat penggunaan bunga telang (BPF) yang mengandungi antosianin, pigmen semulajadi yang sensitif kepada julat pH yang luas. Kanji kuinoa (QS) mengandungi 10-21% amilosa dengan saiz butiran kanji kecil sekitar 1 µm yang membolehkan penyebaran lebih mudah, menjadikannya bahan yang berpotensi untuk menghasilkan filem penunjuk pH. Ekstrak BPF telah

dimasukkan ke dalam matriks filem QS sebagai penunjuk pH untuk memantau kesegaran pelbagai jenis kerrang-kerangan (kerang dan lala kepah) yang disimpan pada suhu 4°C. Filem kanji kuinoa-gelatin ikan-ekstrak bunga telang (QS/F-BPF) telah dihasilkan dengan kepekatan BPF yang berbeza (5% dan 10%), dan kemudian ciri-cirinya termasuk rupa fizikal, warna, ketebalan, kebolehtelapan wap air (WVP), kandungan lembapan dan tindak balas warna dinilai pada nilai pH yang berbeza (pH 1-12). Penambahan kepekatan BPF yang berbeza kepada filem berasaskan kuinoa-gelatin tidak menjejaskan ketebalan, kandungan lembapan dan WVP dengan ketara. Sementara itu, mikrograf filem menunjukkan morfologi permukaan yang baik. Warna dan pH filem QS/F-BPF diperiksa setiap dua hari selama enam hari penyimpanan kerrang-kerangan pada suhu 4°C, dengan warna berubah daripada biru ke hijau dengan peningkatan pH pada hari ke-6, menunjukkan kerosakan. Kesimpulannya, filem penunjuk pH QS/F-BPF berkesan untuk memantau kesegaran pelbagai jenis kerrang-kerangan yang disimpan dalam keadaan suhu dingin.

Kata kunci: bunga telang, kanji kuinoa, pH indikator, antosianin, kerang-kerangan

Introduction

Food spoilage is the deterioration of food caused by microbial and biochemical spoilage, and this will lead to changes in food pH [1]. Food spoilage can be detected through smell, touch or sight and is easily detected on perishable foods such as raw chicken, beef, and seafood products. Thus, to ensure the food is safe for human consumption, intelligent packaging made from polymer immobilized with active compounds such as anthocyanin can help identify the changes in food pH through a colour response due to reactions between the anthocyanin and volatile amines produced by bacteria and the enzymes [2].

Anthocyanins are coloured polar pigments that belong to the phenolic group [3]. Anthocyanins have a colour-sensitive characteristic after being applied to a wide pH range. The changes in different colours occur due to the anthocyanins' chemical structure change along with the different pH values. Thus, Anthocyanin is a natural colour pigment that can act as an environment-friendly pH indicator [4]. *Clitoria ternatea* or butterfly pea is known as a perennial herbaceous plant from the Fabaceae family [5]. The butterfly pea flower is well known for its stability and application as a natural food colourant as well as an antioxidant.

Recently, several studies used various sources of anthocyanins and biodegradable polymers to form the solid matrix pH film including butterfly pea with sago (Metroxylon sagu) [6], blackberry pulp with arrowroot starch [7] and mulberry with k-carrageenan [8]. Anthocyanin and starch combined create a smooth and homogeneous surface with an intermolecular hydrogen bond that extends the wavelength of the biopolymer [9]. In addition, the primary biopolymers used in the creation of biodegradable films are proteins and polysaccharides. Carboxymethyl cellulose, starch, and chitosan are the polysaccharides most frequently used for food-based biodegradable films [10]. Proteins have a higher gas barrier and mechanical properties like fish gelatine. Meanwhile, polysaccharides such as quinoa starch can enhance the functionality of films [11].

Quinoa is a flowering plant that is grown as a crop primarily for its edible seeds which are rich in protein, dietary fibre, B vitamins and dietary minerals in higher amounts than many other grains. Most of the dry matter in quinoa grains which is up to 70% is made up of starch [12]. Araujo-Farro et al. [13] reported that quinoa starch contains about 10-21% amylose with a small starch granule size of around 1µm that allows for easier dispersion which is a promising material to produce pH films. To immobilise the natural dyes from butterfly pea flower extract, a solid matrix made from natural and biodegradable polymers, which is quinoa starch, was used in this research as it has better mechanical properties and barrier properties compared to corn and amaranth starch [12]. Although there were many studies on butterfly pea extract with other starch, quinoa starch has not been used as a pH film indicator. Thus, it is the main interest in researching BPF extract incorporated with quinoa starch as a solid matrix of pH indicator films.

Shellfish is a marine life that has a shell and can be categorised as crustaceans, molluscs or echinoderms. Molluscs are divided into three categories, which are bivalves such as cockle, clam, mussel, oyster or scallop, univalves such as abalone, conch or snail and

cephalopods such as octopus or squid. Shellfish that completely seal their shells can be kept for up to seven days except for the storage life of mussels is three to four days [14]. Perishable foods such as shellfish can easily deteriorate and cause biochemical degradation, enzymatic activities, and microbial growth, which will affect the quality characteristics. By developing intelligent film for packaging, where the colour changes from blue to green colour that indicating the spoilage (volatile nitrogenous) of shellfish, the observation of shellfish freshness can be done just using the naked eye in real-time. To the authors' knowledge, limited studies were found to observe the freshness of different types of shellfish using pH indicator films, specifically, films made up of quinoa starch. Therefore, the main objectives of this study were 1) to develop a pH indicator film by incorporating different concentrations of BPF extract into the quinoa starch matrix and determine its physicochemical properties and 2) to evaluate the potential of the developed film for monitoring the freshness and quality characteristics of blood cockles (*Tegillarca granos*a) and short-necked clams *(Paphia undulata*) during storage at 4 °C.

Materials and Methods

Materials

The butterfly pea flower was collected from a local area in Negeri Sembilan. Quinoa flour and fish gelatine were purchased from a bakery supplier in Bangi, Selangor. The blood cockles and short-necked clams were purchased from the Seri Kembangan wet market in Selangor, Malaysia.

Extraction of anthocyanin from butterfly pea flower

The extraction was carried out through ultrasound–assisted extraction of fresh petals. Butterfly pea flower petals were separated from the pods and leaves prior to immersion in 50% ethanol as solvent. A water bath was used to regulate the temperature and was positioned beneath the extraction setup. The solvent containing butterfly pea flowers was submerged completely with the ultrasonic probe and sonicated for 30 mins at 40 °C with 160W [15]. The extracted solution was then gone through a rotary evaporator (Eyela 1L Rotary Evaporator with Water/Oil Bath, N-1001V-WD, China) to remove solvent and will undergo lyophilized for 48 h. Then, the freeze-dried BPF powder was wrapped with aluminium foil and stored in the freezer for further analysis.

Colour responses of anthocyanin in BPF extract to pH change

The colour changes were carried out by adding 0.01g of freeze-dried butterfly pea flower extract to 4 mL of distilled water. The buffer solutions containing different pH of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 were prepared with an aqueous solution of 0.1 M HCl and 0.1 M NaOH and distilled water, then checked by a digital pH meter. The BPF extract (1 mL) was mixed in buffer solution (4 mL) ranging from pH values of 1.0 to 12.0 and the colour changes were photographed using a digital camera [4].

Development of quinoa starch films by immobilizing the anthocyanin pigment

The quinoa starch films were developed through solution casting with slight modification [4]. Solution with 100 mL of an aqueous dispersion containing 2.5g of quinoa starch (Q), and 2.5g of fish gelatine was prepared. Then, 30% glycerol was added as the plasticiser on a starch-dry basis. The solution was mixed well using a magnetic stirrer (MS-H280-Pro, DLAB Scientific Inc, Malaysia) which was then heated to 90 °C for 1 hour. Following that, the BPF extract amount (5% and 10% w/w) that corresponded to the weight of starch was added after the solution cooled down to at least 50 °C. The control film (QF) was made without adding BPF extract. The film-forming solution was immediately poured into a petri plate and film hydrogels were formed. Then, the hydrogels were dried to film at room temperature under a fan for 12 hours. After that, the film was peeled from the Petri plate and dried quinoa starch films were preserved in controlled conditions for further use. The films were labelled as QF, Q/F-BPF 5% and Q/F-BPF 10%.

Appearance and colour of film

The colour of the films was measured by a colourimeter (Chroma Meter CR-410, Japan) with L* (light/dark), a* (red/green), and b* (yellow/blue) values in triplicate and the average values were taken [4]. The total colour

difference (ΔE) of the films was calculated according to the following equation (1):

$$\Delta E = \sqrt{(L* - L)^2 + (a* - a)^2 + (b* - b)^2} \quad (1)$$

Where L*, a* and b* were the colour parameters of the BPF incorporated with quinoa starch films while L, a and b were the colour indices of the control film (QF).

Film thickness

The thickness of the films was determined by a digital micrometre (C112XBS, Mitutoyo, Japan) by randomly selecting five different positions on each film with a precision of 0.001 mm [4].

Moisture content

The moisture content of the film was determined using a moisture analyser (SHS AND MX-50, Japan) until it reached constant weight. The analyses were performed in triplicate and the results were recorded.

Water vapour permeability (WVP)

The WVP for BPF incorporated with composite QF films was performed by cutting the films into (7 cm x 7 cm) and then mounted on the top of the 50 ml crucible that contains 6 ml distilled water. The initial weight was recorded prior to being in a desiccator with 55% RH at 25°C. Then, the crucible weight was measured and recorded at 1-hour intervals for 8 hours [16]. WVP was calculated according to the following equation (2):

$$Water\ vapour\ permeability = \frac{W \times X}{t \times A \times \Delta P} \quad (2)$$

Where W is the test tube weight (g), X is the film thickness (m), t is the time for the gain in test tube weight, A is the area of film sample for permeation (m2) and ΔP is the water vapour pressure difference between the two sides of the film (Pa).

pH sensitivity

The films were cut into (2 cm x 2 cm) and then immersed in 9 ml of buffer solutions consisting of different pH values ranging from 1.0–12.0 for around 5 min. The colour changes of the films were photographed with a white background [4, 16].

Microstructural characterization of the film

The morphology of the QF composite films was observed through a Scanning Electron Microscope (SEM) at room temperature. The films were cut into (13 mm x 13 mm), then placed on an aluminium base and covered with a gold layer then observed for the upper surface and the cross-section of films operated at 10kV [7].

Application of quinoa starch films on observing shellfish freshness

The pH films were cut into a square shape (1 cm × 1 cm) and then fixed on the inner lid of each polypropylene container's headspace containing different types of shellfish (cockles and clams). The container was stored in a chiller at 4 °C for 2 to 6 days. Six samples were taken randomly for analysis every 2 days. The changes in the colour of pH indicator films were measured by a hand-held colourimeter (Chroma Meter CR-410, Konica Minolta Inc., Tokyo, Japan) and recorded for triplicate until the end of storage [4].

Determination of pH on shellfish sample

The pH values of the ground cockles and clam samples were measured by homogenizing the sample in distilled water and determined with a pH meter (Eutech pH 700, Thermo Fisher Inc., USA). The pH values of the samples were observed on days 0, 2, 4 and 6.

Statistical analysis

The results obtained from the samples were reported using one-way analysis of variance (ANOVA) from Minitab version 19.1.1.0 and reported by the average values ± standard deviation. The significance of the values was defined at $p < 0.05$.

Results and Discussion

Colour change of butterfly pea flower extract and film

The BPF extract displayed various colour changes after exposure to a wide pH range (Figure 1), turning to pinkish-red colour in a buffer with a pH of 1 and 2 or a strong acid, then to purple or purplish-blue at pH 3 until pH 6 which is slightly acidic and displayed in its original blue colour at natural pH 7. The colour of the BPF extract turned bluish-green at pH 8, greenish at pH 9 and

deep green at pH 10 until pH 12. These colour changes were similar to the previous studies by Hidayati et al. [17] and Hashim et al. [18].

Anthocyanin colour changes are influenced by increasing pH from acidic to alkaline conditions [2] and involve broad values (pH 1.0 to 12.0) with four different stages (flavylium cation, quinoidal base, carbinol pseudobase, and chalcone). Anthocyanins extracted from BPF consist of glycosides which contain flavylium cations that produce the red colour in acidic pH due to the formation of a double bond [19]. The red colour fades at higher pH due to the hydrolysis of the flavylium cation [20], with a slightly higher pH causing the dominance of carbinol, a purplish colour that was perceptible at pH 3-5. At neutral pH, carbinol does not provide any colour with the high abundance of polyacylated anthocyanins being responsible for the pigment's stable blue colour [21]. As the pH increases (pH >8), the colour changes from green to yellow because of chalcone formation [2]. Taken together, BPF is a promising anthocyanin pigment that can be used to develop pH indicator films.



Figure 1. Colour response of BPF extracted in different pH buffer solutions (pH 1.0-12.0)



Figure 2. Colour response of Q/F-BPF 10% films in different pH buffer solutions (pH 1.0-12.0)

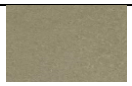
Figure 2 shows that the visible colour of the Q/F-BPF films changed due to the response to different pH and were slightly leached after immersing for 5 min in buffer solution (pH 1.0−12.0). The colour gradually changed from red (pH 1) to pink (pH 2) to violet (pH 3) to purple (pH 4−5) to deep blue (pH 6) to original blue (pH 7) to greenish-blue (pH 8) to greenish (pH 9) and darken green (pH 10−12). A previous study reported similar colour changes from pink to yellow of starch BPF films after immersion in pH 1−14 which indicates that the films have a prominent colour response to pH [4]. The response of the pH indicator films is practical and effective for their application in determining the freshness of food products. In addition, the range (pH 1.0−12.0) in this study was sufficient as the colour changing of the film is usually from neutral to acidic or alkaline involving small shifts of pH values when the food deteriorates.

Thickness of Films

Table 1 presents the thickness of the films showing no significant ($p < 0.05$) difference between different concentrations of anthocyanin. The anthocyanins are evenly distributed throughout the solid mass of the film

matrix due to abundant OH groups in BPF, starch and fish gelatine. This mixture of anthocyanin and starch can strongly bond like a bridge with a protein matrix from gelatine through intermolecular interactions [22].

Table 1. Physical properties of the composite films.

Films	Appearance	Colour				Thickness (mm)	Moisture Content (%)	WVP (x 10^{-10} g/m.Pas)
		L^*	a^*	b^*	ΔE			
QF (control)		71.01 ± 1.46[a]	1.52 ± 0.15[a]	6.22 ± 0.18[a]	0	0.23 ± 0.023[a]	18.74 ± 5.40[a]	1.40 ± 0.00[a]
Q/F-BPF 5%		33.80 ± 5.83[b]	15.71 ± 0.70[b]	-29.36 ± 5.93[b]	53.40	0.23 ± 0.02[a]	14.22 ± 0.91[a]	1.41 ± 0.00[a]
Q/F-BPF 10%		29.06 ± 0.76[b]	18.51 ± 0.84[c]	-28.39 ± 0.41[b]	56.98	0.24 ± 0.01[a]	16.25 ± 2.17[a]	1.64 ± 0.00[a]

Note: All values are mean ± standard deviation of three replicates. Means that do not share the same letter are significantly different ($p < 0.05$) in the same column.

Film moisture content

Moisture content is an important factor for food packaging material because high moisture is likely to encourage microbial growth on the surface of packaged foods [23]. The moisture content of QF and QF composite indicator films are listed in Table 1 showing no significant difference in moisture content because gelatine is highly hydrophilic and the interaction between the hydrophilic BPF anthocyanin results in more polar groups which can absorb moisture from the surroundings. However, the control film exhibits a slightly high moisture content possibly because the interaction between the hydroxyl group of starch and anthocyanins present in BPF could lower the number of free hydroxyl groups of starch to integrate with moisture during the drying process [24].

Film water vapour permeability

WVP is a crucial measure of application performance and indicates the barrier property which reflects the ability to modulate the flow of moisture [25]. The WVP values are tabulated in Table 1 showing no significant ($p < 0.05$) differences between films. However, the composite film with BPF anthocyanin shows a slightly higher increase than the control film, possibly due to the good solubility of BPF anthocyanin extract. Q/F-BPF 10% had a slightly increased WVP value of 1.64 ×10-10 g/m.Pa.s. because it has a higher concentration of BPF anthocyanin and anthocyanin helped in the transportation of water vapour due to its hydrophilic nature [26]. Therefore, it can be concluded that the quinoa starch and fish gelatine immobilised with BPF had a greater impact on the film WVP

Colour appearance of films

Colour is a crucial parameter for developing pH film indicators. Moreover, colour illustrates the different appearance of the control film and the films immobilised with BPF as shown in Table 1. The QF film was transparent and colourless whereas Q/F-BPF films were opaque and dark blue with the increased BPF concentration. The L* (lightness) of the films was significantly ($p < 0.05$) decreased after immobilised with BPF in the starch matrix. This could be due to the white properties of quinoa starch without any anthocyanin colour. The a* (redness) value significantly ($p < 0.05$) increased from 1.52 to 18.51 while the b* (yellowness) value significantly ($p < 0.05$) decreased along with the increase of BPF concentration. The results indicated that the colour tends to be redder and bluer on Q/F-BPF films.

The colour difference (ΔE) of the films presented in Table 1 increases with the increase in BPF concentration. When the total colour difference (ΔE) was higher than 3, the colour changes are visible to the naked eye [4]. According to Table 2, the (ΔE) values for Q/F-BPF films were above 3 where the lowest was 53.40 and the highest was 56.98, indicating that the colour portrayed by each Q/F-BPF film was visually

discernible. A study by Husin et al. [27] distinguished significant changes in L*, a* and b* of delphinidin-based colour-indicator films coalesced with butterfly anthocyanin. Therefore, these results indicate that Q/F-BPF films are potential indicator films and that the colour changes are visible to the naked eye.

Film microstructure

Figure 3 indicates the surface microstructure as well as the cross-section of QF and Q/F-BPF films. The microstructures are crucial to determining the effect of BPF on the surface and cross-section of the starch gelatine matrix. The QF films exhibited smooth and homogeneous surfaces which demonstrate good compatibility as well as dispersion. The two polymers are very compatible with hydrogen bonding between the carboxyl groups (COO) of the protein and the -OH groups of the polysaccharide, especially when used in the same ratio [28].

The Q/F-BPF film surface was a little rough with a homogeneous and dense structure. Starch and BPF significantly interact, and the anthocyanin extract was thoroughly mixed. The development of hydrogen bonds with the starch matrix is facilitated by the addition of phenolic compounds like anthocyanin [4]. According to Kim et al. [28], although they tended to aggregate in some areas of the film, overall, they were evenly dispersed throughout the polymer matrix. The cross-section specifies the compact and dense surface with the dispersion of the polymers.

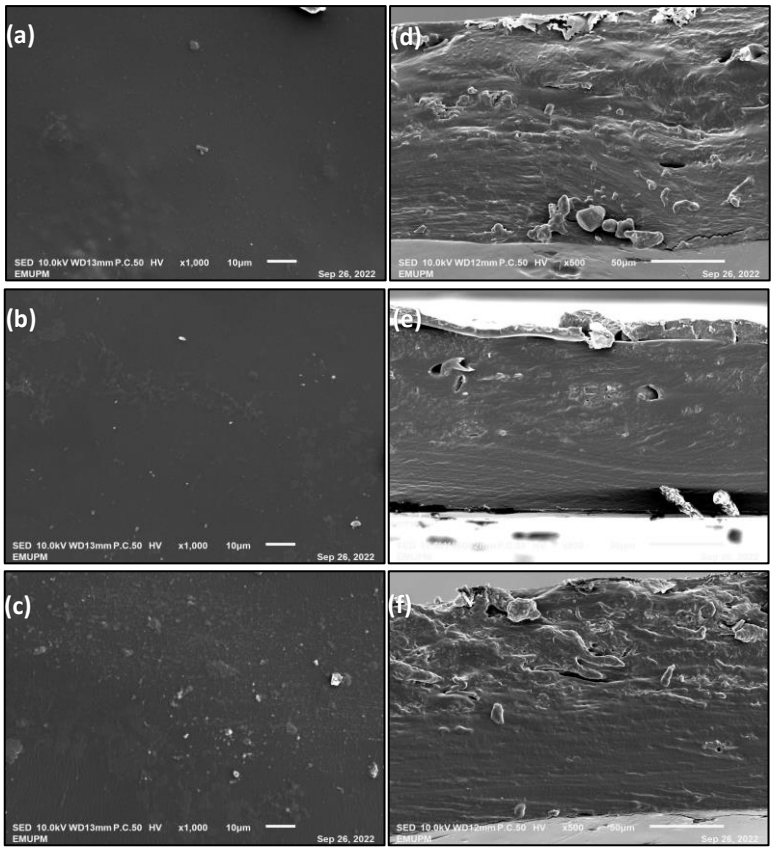


Figure 3. SEM images of the surface of the QF (A), Q/F-BPF 5% (B), and Q/F-BPF 10% (C) indicators and cross sections of the QF (D), Q/F-BPF 5% (E), and Q/F-BP 10% (F) indicators.

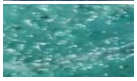
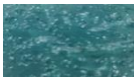
Application of Q/F-BPF films to monitor shellfish freshness

The spoilage of seafood products is initially caused by microbes as well as biochemical amine reactions which lead to freshness decline and the release of volatile nitrogenous compounds. Ammonia, trimethylamine, and dimethylamine are volatile foul-smelling gases released by microbial activity and enzyme degradation of proteins in seafood [25]. The pH rises when these volatile nitrogenous compounds accumulate in the headspace of the air-tight containers during extended storage. Thus, pH indicator films are used to detect the shellfish freshness. The Q/F-BPF films were implemented to observe the shellfish freshness under chiller storage (4 °C for 6 days).

The results showed the Q/F-BPF film colour changes indicated shellfish spoilage, with the odour becoming obvious towards the end of the storage. The initial colour of the Q/F-BFE 5% and Q/F-BPF 10% indicator was used as a reference for ΔE calculation and changes in ΔE during storage are presented in Table 3. The ΔE value after 2 days of storage on Q/F-BPF 5% was 5.57 for blood cockles and 7.79 for short-necked clams, indicating an increase in spoilage parameters. The ΔE value of the Q/F-BPF 5% indicator sharply increased to 24.33 for blood cockles and 16.70 for short-necked clams after 6 days of storage and the indicator colour changed from blue to green. Thus, the colour of the Q/F-BPF 5% indicator changed according to the changes in the quality of blood cockles and short-necked clams. The colour changes of the Q/F-BPF films at the end of the storage were visible to the naked eye when the ΔE was more than 3 [4].

Taken together, Q/F-BPF films showed a significant colour change throughout the 6 days of storage of blood cockles and short-necked clams due to the spoilage and the formation of volatile nitrogenous compounds. A previous study reported that the colour of S-BPF films turned to intense green from pink on the 6th day of shrimp storage [4]. Similarly, the recognisable anthocyanin colour changes during storage showed that the quality of the shrimp had declined [28]. The colour changes were more distinct in the Q/F-BPF 10% film which had a higher concentration of BPF extract. These results depicted that BPF immobilised with quinoa starch and fish gelatine has the potential to monitor shellfish freshness and other food samples.

Table 3. The colour response of Q/F-BPF films at different time intervals (0, 2, 4, 6 days) for shellfish freshness

Day	Films	Colour				Films Appearance
		L^*	a^*	b^*	ΔE	
Blood Cockles Sample						
0	Q/F-BPF 5%	53.90 ± 1.54[a]	3.31 ± 0.39[a]	-6.28 ± 2.49[a]	0	
2		57.60 ± 5.03[a]	3.54 ± 0.34[a]	-10.43 ± 1.23[b]	5.57	
4		62.21 ± 4.36[a]	3.87 ± 0.64[a]	-11.51 ± 1.24[a]	9.84	
6		65.62 ± 2.32[a]	0.91 ± 2.77[a]	14.90 ± 29.30[a]	24.33	
0	Q/F-BPF 10%	55.98 ± 2.96[a]	3.45 ± 0.40[a]	-6.73 ± 1.72[a]	0	
2		64.50 ± 4.64[a]	2.59 ± 0.06[b]	-6.11 ± 0.84[a]	8.59	
4		68.20 ± 4.59[a]	2.63 ± 0.85[a]	-7.86 ± 3.47[a]	12.30	

Day	Films	Colour				Films Appearance
		L^*	a^*	b^*	ΔE	
6		63.20 ± 4.74[a]	0.547 ± 0.61[a]	-3.38 ± 0.21[a]	8.47	
Short-necked Clams Sample						
0	Q/F-BPF 5%	51.94 ± 5.25[a]	3.81 ± 0.55[a]	-8.51 ± 1.46[b]	0	
2		59.44 ± 1.95[a]	3.71 ± 0.19[a]	-10.58 ± 1.58[b]	7.79	
4		66.91 ± 2.26[a]	0.93 ± 1.14[a]	-6.70 ± 1.12[a]	15.35	
6		66.93 ± 2.98[a]	-1.55 ± 2.18[a]	-3.46 ± 0.04[a]	16.70	
0	Q/F-BPF 10%	60.94 ± 2.04[a]	3.07 ± 0.37[a]	-5.41 ± 0.22[a]	0	
2		64.07 ± 3.17[a]	2.95 ± 0.095[b]	-5.66 ± 0.77[a]	3.15	
4		69.73 ± 0.38[a]	2.95 ± 0.09[a]	-5.66 ± 0.77[a]	8.87	
6		66.28 ± 1.84[a]	0.08 ± 0.99[a]	-3.39 ± 0.36[a]	6.44	

Note: All values are mean ± standard deviation of three replicates. Means that do not share the same letter are significantly different ($p < 0.05$) in the same column.

pH as an indicator of shellfish freshness

Table 4 presents a summary of the pH values of blood cockles and short-necked clams. The pH for both shellfish initially was low, 5.8 for blood cockles and 5.9 for short-necked clams, then increased significantly ($p < 0.05$), reaching 8.2 for blood cockles and 8.1 for short-necked clams by day 6. This was due to the accumulation of volatile nitrogenous compounds released by those shellfish [29]. This trend was in line with the significant increase in the pH of mackerel (*Scomber scombrus*) from day 0 to day 15 when stored in a chiller [30]. Hence, the increase in pH of the blood cockles and short-necked clams on day 6 depicted that the freshness and quality of the shellfish declined, and that spoilage had begun.

Table 4. pH value of blood cockles and short-necked clams during storage

Fish Sample	Day	pH
Blood cockles	0	5.83 ± 0.15[d]
	2	6.60 ± 0.10[c]
	4	7.30 ± 0.36[b]
	6	8.22 ± 0.25[a]
Short-necked clams	0	5.97 ± 0.21[d]
	2	6.73 ± 0.21[c]
	4	7.50 ± 0.20[b]
	6	8.20 ± 0.14[a]

Note: All values are mean ± standard deviation of three replicates. Means that do not share the same letter are significantly different ($p < 0.05$) in the same column.

Conclusion

In conclusion, the anthocyanin extract from BPF incorporated into QS solid matrix films produces a good pH film indicator which has good colour intensity, and physical properties, and can be applied to seafood products. The addition of BPF to the quinoa starch and fish gelatine base did not greatly affect the thickness,

moisture content or WVP. The film has good surface morphology with an even dispersion of quinoa starch, fish gelatine, BPF and glycerol. The Q/F-BPF film colour changed from blue to green indicating the spoilage (volatile nitrogenous) of shellfish during storage. The freshness assessment confirmed the shellfish spoilage started on day 1 and corresponded to the colour changes of the Q/F-BPF films on day 6, which were visible to the naked eye. Therefore, Q/F-BPF pH indicator films have promising potential to be used as a pH indicator to monitor shellfish freshness.

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ENCAPSULATION OF A MIXTURE OF NONI (*Morinda citrifolia* L.) FRUIT EXTRACT AND PATCHOULI (*Pogostemon cablin*) OIL WITH MALTODEXTRIN AND ITS ANTIOXIDANT ACTIVITY

Enkapsulasi Campuran Ekstrak Buah Noni (*Morinda Citrifolia* L.) dan Minyak Nilam (*Pogostemon cablin*) Berasaskan Maltodextrin dan Aktiviti Antioksidannya

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Abstract

Excessive free radical compounds in the human body can cause various dangerous diseases. This can be prevented by consuming antioxidant-rich foods, one of which is noni fruit with its pungent odor and taste that many people dislike. To overcome this issue, noni fruit can be encapsulated. In this study, noni fruit extract was encapsulated with maltodextrin with the addition of patchouli oil. The extraction of noni fruit was done by the maceration method. Research maltodextrin in this study was produced from partial hydrolysis of cassava starch using 0.5% α-amylase enzyme, achieving a Dextrose Equivalent (DE) value below 20. However, as it is insoluble in water, this maltodextrin cannot be used as an encapsulating agent. Therefore, the encapsulation stage was carried out using the freeze-drying method on with commercial maltodextrin as a coating agent, tween 80 as an emulsifier, and noni fruit extract and patchouli oil as the core ingredients in a ratio of 4:1 and 1:1. The highest encapsulation efficiency of 91.35% was achieved in the encapsulation of noni fruit extract without the addition of patchouli oil. Nevertheless, the organoleptic test results showed that patchouli oil positively reduced the unpleasant odor of noni fruit extract. Furthermore, the SEM test results indicated irregular particle shapes and fractures without any pores, while the results of the antioxidant activity test using the DPPH method revealed that the addition of patchouli oil in a ratio of 1:1 made a positive contribution to increasing the antioxidant activity of encapsulated noni extract, with an IC_{50} value of 50.80 µg/mL.

Keywords: antioxidant, encapsulation, maltodextrin, noni, patchouli oil

Abstrak

Sebatian radikal bebas yang berlebihan dalam tubuh manusia boleh menyebabkan berbagai penyakit berbahaya. Ini boleh dicegah dengan pengambilan makanan yang kaya dengan antioksidan, salah satunya ialah buah mengkudu dengan bau dan rasa yang menyengat yang tidak disukai orang ramai. Untuk mengatasi masalah ini, buah mengkudu boleh dikapsulkan. Dalam kajian ini, ekstrak buah mengkudu dikapsulkan dengan maltodekstrin dengan penambahan minyak nilam. Pengekstrakan buah mengkudu dijalankan dengan kaedah maserasi. Maltodekstrin penyelidikan dalam kajian ini dihasilkan daripada hidrolisis separa kanji ubi kayu menggunakan enzim α-amilase 0.5%, mencapai nilai *Dextrose Equivalent* (DE) di bawah 20. Namun, kerana ia tidak larut dalam air, maltodekstrin ini tidak boleh digunakan sebagai agen pengekapsul. Oleh kerana itu, enkapsulasi dijalankan

menggunakan kaedah pengeringan beku dengan maltodekstrin komersial sebagai agen salutan, tween 80 sebagai pengemulsi, dan ekstrak buah mengkudu, dan minyak nilam sebagai bahan teras dalam nisbah 4:1 dan 1:1. Kecekapan enkapsulasi tertinggi iaitu 91.35% dicapai dalam pengkapsulan ekstrak buah mengkudu tanpa penambahan minyak nilam. Namun begitu, keputusan ujian organoleptik menunjukkan bahawa minyak nilam secara positif mengurangkan bau yang tidak menyenangkan daripada ekstrak buah mengkudu. Tambahan pula, keputusan ujian SEM menunjukkan bentuk zarah yang tidak teratur dan patah tanpa sebarang liang, manakala keputusan ujian aktiviti antioksidan menggunakan kaedah DPPH mendedahkan bahawa penambahan minyak nilam dalam nisbah 1:1 memberi sumbangan positif kepada peningkatan aktiviti antioksidan kapsul ekstrak mengkudu, dengan nilai IC_{50} 50.80 µg/mL.

Kata Kunci: antioksidan, enkapsulasi, maltodekstrin, mengkudu, minyak nilam

Introduction

Free radicals are reactive and unstable molecules with one or more unpaired electrons in their outermost orbitals that play a role in oxidative tissue damage and pathological processes in the human body by attacking lipid compounds, proteins, and DNA, thus causing various dangerous diseases such as cancer, cardiovascular diseases, and diabetes mellitus. Therefore, antioxidants are essential for the human body as they can inhibit oxidation through reactions with free radicals to form non-reactive and stable free radicals. One of the antioxidant-rich fruits in Indonesia is noni fruit (*Morinda citrifolia* L.), which is known to contain more than 160 phytoconstituents. One of the antioxidants contained in noni fruit is ascorbic acid, reaching 24-158 mg per 100 grams of noni fruit [1]. This content is higher than that of several other fruits such as tangerine (3.716 mg/100 grams), persimmon (2.878 mg/100 grams), and tamarillo (11.328 mg/100 grams) [2].

However, the caproic acid content in noni fruit produces an unpleasant odor and bitter taste, making people reluctant to consume it. This can be circumvented by encapsulation, a method of coating compounds with certain wall materials to reduce damage to the compounds and improve their characteristics and organoleptic properties [3]. The encapsulating agent used in this study is maltodextrin, which has low viscosity, high solubility, and high binding capacity in forming a microcapsule matrix [3]. Maltodextrin can be produced through enzymatic processes or acid hydrolysis of starch. In Indonesia, one of the extremely abundant sources of starch is cassava. Due to its high starch content, namely around 85%, cassava has the potential to be developed for use in various applications. Further utilization of cassava starch in the industry is expected to increase the economic value of this root vegetable [4]. This study attempts to produce maltodextrin from cassava starch for use in the encapsulation of noni fruit extract.

In this study, encapsulation was performed using the freeze-drying method with the addition of patchouli oil to suppress the unpleasant odor of noni fruit extract and increase the antioxidant activity of the encapsulation results. The use of the freeze-drying method is considered the most suitable for encapsulating compounds that are volatile and sensitive to heat or temperature, such as compounds found in essential oils. When compared with other methods, freeze drying has the advantage of not using high temperatures like other methods so that it can protect sensitive bioactive compounds, the implementation steps are limited compared to other methods and the product can be reshaped quickly and easily. Meanwhile, other methods such as spray drying, extrusion and pan-coating have the disadvantage of removing a lot of material because they use high temperatures during the process and produce a lot of residue [22] This study focuses on changes in the organoleptic properties of noni fruit extract in three different encapsulation variations, especially the decrease in the unpleasant odor and bitter taste of noni fruit extract and the increase in antioxidant activity of the encapsulation results with *freeze drying* method for encapsulation.

Materials and Methods

Materials

Noni (*Morinda cirrifolia* L.) fruit powder, which is the main ingredient in this study, was purchased at Pasar Gede Surakarta, Central Java, Indonesia and macerated

with 96% ethanol solvent (Merck). Commercial patchouli oil was obtained from Happy Green Patchouli Oil e-commerce store. The encapsulation process used Tween 80 and commercial Maltodextrin with DE value of 10-12 purchased from Indoplant e-commerce store. Meanwhile, the alternative maltodextrin in this study was produced from partial hydrolysis of cassava starch and distilled water (Aquadest) with 500 grams of α-amylase enzyme from Micro Bio e-commerce store, D(+)-Glucose anhydrous (Merck), Fehling A or $CuSO_4$ (Merck), Fehling B or KOH and $NaKC_4H_4O_6.4H_2O$ (Merck), n-Hexane (Merck), 200 ppm anhydrous $CaCl_2$ (Merck), 0.1 N Hydrochloric acid or HCl (Merck), 0.1 N Sodium hydroxide or NaOH (Merck), and universal pH paper. Furthermore, the antioxidant testing employed the DPPH method with methanol p.a. (Merck), 2,2-diphenyl-1-picrylhydrazyl or DPPH (Merck), and dimethylsulfoxide ($(CH_3)_2SO$) (Merck).

Instrumentations

This study utilized the following instruments: Scanning Electron Microscope (Jeol JSM-60 SEM), UV-Vis Spectrophotometer (Hitachi UH5300), Particle Size Analyzer (Malvern Zetasizer Nano ZS), GC-MS Spectrophotometer (Shimadzu QP2010S), and Freeze Dryer (Telstar LyoQuest).

Making maltodextrin from cassava starch

Maltodextrin was made based on the literature with several changes to the parameters such as the concentration of the enzyme used. First step is mixing 30% of fine-textured cassava starch in a 200 ppm $CaCl_2$ solution. After that, 0.1 N NaOH was added to the solution to reach pH 7. In the previous method, 0.1% enzyme was used. Meanwhile, in this study, α-amylase enzymes were used at 0.5% of the solution volume. The mixture was heated at 85°C for 120 minutes with a stirring process and cooled rapidly until the temperature dropped to 30 °C. The pH was readjusted to 3-4 by adding 0.1 N HCl. After leaving it at room temperature for 15-30 minutes, 0.1 N NaOH was added to the mixture until the pH became 4.5-6. The maltodextrin obtained was dried in an oven at 50–60 °C for 24 hours [5]. Afterwards, it was crushed and sieved with a 100-mesh sieve to obtain fine maltodextrin. Lastly, an analysis of the Dextrose Equivalent (DE) value and particle size was carried out using Particle Size Analyzer (PSA).

Determination of the physiochemical properties of maltodextrin

The DE value of the maltodextrin was determined by Lane-Eynon Titration based on the SNI 01-2892-1992 standard as a quantitative method of testing reducing sugars. Meanwhile, the solubility of the maltodextrin in water was determined by a solubility test [6], and the results were adjusted to the quality requirements for maltodextrin solubility by the National Standardization Agency of Indonesia (*Badan Standardisasi Nasional*/BSN).

Encapsulation of a mixture of noni fruit extract and patchouli oil

The ratio between encapsulating agent, core ingredients, and Tween 80 in this study is 4:1:1. A total of 30 mL of maltodextrin solution was added to 0.943 mL of Tween 80 then homogenized at a speed of 750 rpm for 15 minutes. Next, comparative modifications were carried out at the stage of adding the core ingredients to determine the effect of the amount of core ingredients on the antioxidant activity and organoleptic properties. Some of these variations are: (1) 100% noni fruit extract without the addition of patchouli oil; (2) 0.8 grams of noni extract and 0.2 grams of patchouli oil in a ratio of 4:1; and (3) 0.5 grams of noni extract and 0.5 grams of patchouli oil in a ratio of 1:1. After that, the mixture was homogenized at a speed of 750-1000 rpm for 30 minutes at room temperature until an emulsion phase was formed. The final stage was drying with a freezer dryer for 3 days. The encapsulation results were then analyzed for yield, organoleptic properties, encapsulation efficiency, microcapsule morphology, and antioxidant activity [7, 8].

Analysis of encapsulation

Changes in the organoleptic properties of noni extract before and after encapsulation with the addition of patchouli oil were determined by carrying out organoleptic tests in the form of observing the taste, color, aroma, texture and overall hedonic test (liking test) which was attended by 15 panelists who had fulfilled the requirements of the Quality Management

Module. Organoleptic Physics (2013). Then, an Encapsulation efficiency analysis was carried out to determine the success of the encapsulation process for noni extract and patchouli oil which was carried out by calculating the percentage of the core ingredients of each noni fruit extract and patchouli oil encapsulated by maltodextrin using the division formula between the difference in total extract used and surface oil content by total extract [10]. The encapsulation results were also analyzed using SEM instruments to compare the morphology of the particles resulting from freeze drying between the encapsulation results without the addition of patchouli oil and with the addition of patchouli oil. Then the particle size was analyzed using PSA to compare the particle size of the maltodextrin before and after encapsulation [14].

Testing of antioxidant activity with the DPPH method

In this study, the positive control was vitamin C, whereas the blank solution was methanol. The concentrations of the test sample solutions were 5, 10, 15, 20, and 25 ppm, prepared by dissolving 100 ppm of test samples in 5 mL of DMSO. Each concentration variation was added to the reagent solution of 0.2 mM DPPH and incubated for 30 minutes in a dark room. The absorbance was then measured using a UV-Vis spectrophotometer at a wavelength of 517 nm [11].

Results and Discussion

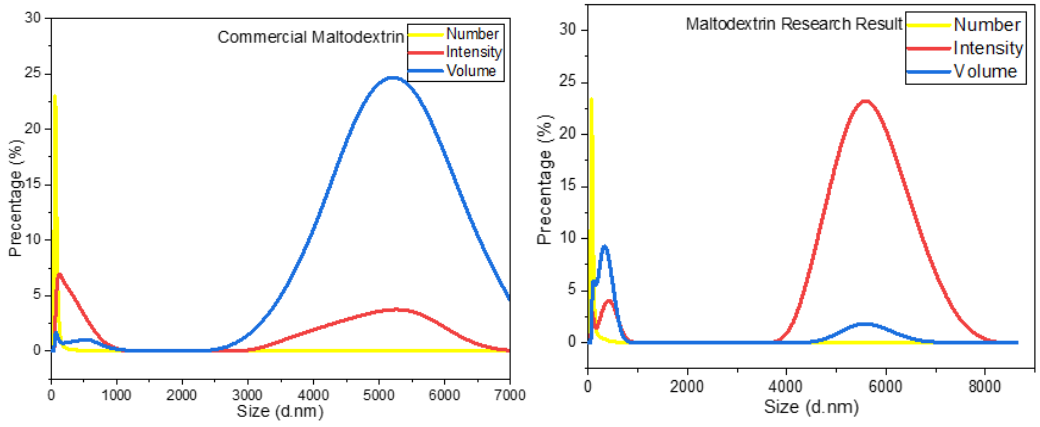
Making maltodextrin

Hydrolysis of cassava starch with α-amylase enzyme at a concentration of 0.5% of the solution volume in this study produced maltodextrin with different physical properties from those of commercial cassava starch-based maltodextrin. The differences in the physical properties of the two maltodextrins can be seen in Table 1.

Table 1**.** Physical properties of maltodextrin

Parameter	**Research Maltodextrin**	**Commercial Maltodextrin**
Color	Cream	White
Flavor	No flavor	No flavor
Solubility in water	63%	98.85%
Texture	Rugged and not sticky	Smooth and slightly sticky

According to the National Standardization Agency of Indonesia (BSN), the standard for maltodextrin solubility is in the range of 97-98%. This means that the maltodextrin produced in this study does not meet the specified quality requirement. Maltodextrin's solubility can be affected by its particle size; the larger the particle size, the smaller the surface area, and the longer it takes for water to absorb the starch particles. In other words, if the particle size is smaller, the hydration rate of the flour will increase [12]. Based on the PSA results, the average particle diameters for commercial maltodextrin and research maltodextrin were 214.3 and 415.9 nm, respectively.



(a) (b)

Figure 1. Graph of particle size distribution in commercial maltodextrin (a) and research maltodextrin (b)

From the PSA results for research maltodextrin (Figure 1b), the highest distribution peak was based on intensity in the particle size range of 5000-6000 nm, signifying that the intensity of photon absorption at these particle sizes is higher than at other particle sizes. Meanwhile, for commercial maltodextrin (Figure 1a), the highest distribution peak was based on volume in the particle size range of 3500-5500 nm, indicating that these particle sizes have a larger total volume than other particle sizes. The presence of larger particles with high intensity can cause the solubility of maltodextrin to decrease. The polydispersity index (Pd index) values of commercial maltodextrin and research maltodextrin were 0.380 and 0.424, respectively. The Pd index value of below 0.5 suggests that the homogeneous dispersion system formed is monodisperse and does not settle. The dispersion results of both maltodextrins are relatively good since these values reflect a fairly narrow particle size distribution and a homogeneous particle size diameter.

Furthermore, based on the analysis results, the DE values of research maltodextrin and commercial maltodextrin were 9.2 and 9.6, respectively. The difference in DE values for the two maltodextrins can be influenced by the concentration of the α-amylase enzyme and the hydrolysis time. With increasing hydrolysis time and α-amylase enzyme concentration, the DE value also increase as more substrates are hydrolyzed over a longer time and at higher enzyme concentrations, thus increasing the numbers of short polymers (monosaccharides, disaccharides, and oligosaccharides) formed in the maltodextrin. Based on the Food and Drug Administration (FDA) standards, maltodextrin has a DE value range of <20 [13]. Hence, the hydrolyzed maltodextrin and commercial maltodextrin in this study have met the FDA standards in terms of their DE values. However, as the maltodextrin produced in this study did not meet the BSN standards for its solubility, it could not be used as encapsulating agent in the encapsulation of noni fruit extract. Therefore, commercial maltodextrin was used as the encapsulating agent in this study.

Compound content of patchouli oil

This study used commercial patchouli oil with the following specifications:

Botanical name : *Pogostemon cablin*
Product Code : 150028
Batch Number : 220408/177366
Appearance : Clear liquid
Color : Yellow-Brown
Smell : Leafy, Earthy, Sweet, and Woody
Production Time : 08 April 2022

Based on its specifications presented in Table 2, the density and color of commercial patchouli oil have met the SNI 06-2385-2006 standards, namely 0.9328-0.9652 g/mL at the same temperature and light yellow to reddish brown.

Table 2**.** Specifications of commercial patchouli oil

Test Item	**Specification**	**Result**
Density (20°C)	0.9328-0.9652	0.9427
Specific Gravity (20°C)	0.9345-0.9669	0.9444
Refractive Index (20°C)	1.4921-1.5225	1.5041
Solubility	Not Soluble in Water	Not Soluble in Water

The results of GC-MS analysis of patchouli oil compounds are displayed in Figure 2 and Table 3.

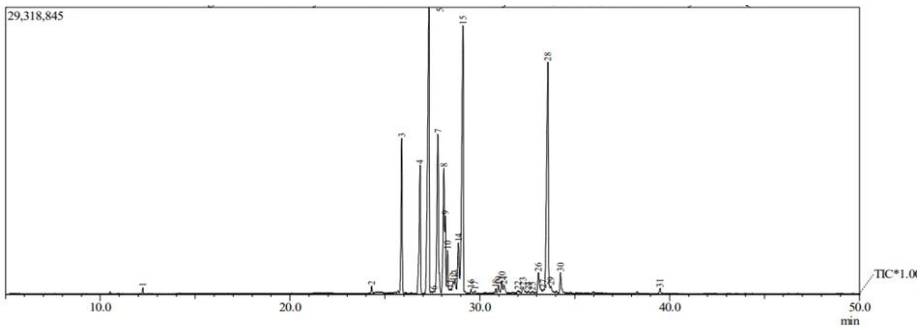
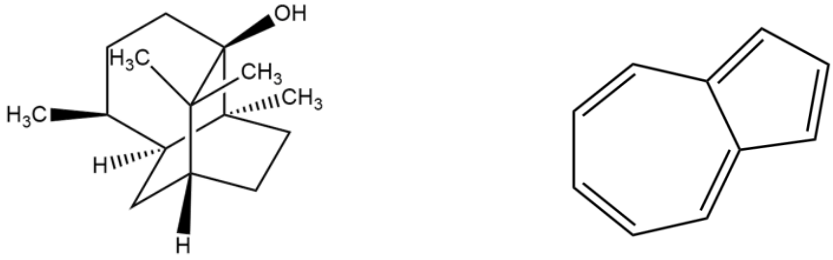


Figure 2. Chromatogram of commercial patchouli oil compounds

Table 3**.** GC-MS analysis of commercial patchouli (*Pogostemon cablin*) oil

RT	Compounds	Area (%)	SI
27.320	Azulene	19.52	92
29.113	delta-Guaiene	16.69	94
33.587	Patchouli Alcohol	16.62	92
27.794	Seychellene	10.03	86
28.104	1H-3a,7-Methanoazulene	7.00	87
25.884	1H-Cyclopropa[a]naphthalene	6.85	91
26.854	trans-Caryophyllene	6.82	93
28.872	Alloaromadendrene	3.11	88
33.090	1H-Cycloprop[e]azulen-4-ol	1.47	84
34.253	2H-Pyran-2,4(3H)-dione	1.08	80
28.690	2,4-Diisopropenyl-1-methyl-1-vinyl-cyclohexane	0.81	90
8.602	1-Iodo-2-methylnonane	0.69	81
31.160	(-)-Caryophyllene oxide	0.63	90
31.273	Pentadecane (CAS) n-Pentadecane	0.59	96
28.439	gamma-Gurjunene	0.30	92
39.497	Octadecanoic Acid	0.27	92

As seen in Table 3, commercial patchouli oil has a patchouli alcohol content of 16.62%, and the highest compound is azulene with a percentage of 19.52%. The chemical structures of patchouli alcohol and azulene compounds are presented in Figure 3.



(a) (b)

Figure 3. Chemical structures of patchouli alcohol (a) and azulene (b)

Encapsulation efficiency of a mixture of noni fruit extract and patchouli oil

Encapsulation efficiency states the percentage of noni fruit extract and patchouli oil that can be encapsulated from the total core ingredients used [14]. The yield and efficiency of the encapsulation samples in this study are shown in Table 4.

The decrease in encapsulation efficiency in the three variations can be caused by the increase in encapsulation surface area in each variation. Overall, all encapsulation samples are considered efficient as they range from 80% to 97% [14].

Table 4. Encapsulation yields and efficiencies

Encapsulation Sample	Yield (%)	Encapsulation Efficiency (%)
Noni Fruit Extract	91.35	91.77
Noni Fruit Extract and Patchouli Oil (4:1)	88.03	Noni Fruit Extract: 83.5% Patchouli Oil: 90.78%
Noni Fruit Extract and Patchouli Oil (1:1)	83.51	Noni Fruit Extract: 90.34% Patchouli Oil: 87.15%

Organoleptic test

Organoleptic tests with 15 panelists were carried out on five samples: (1) noni fruit extract before encapsulation; (2) encapsulation of noni fruit extract; (3) encapsulation of a mixture of noni fruit extract and patchouli oil (4:1); and (4) encapsulation of a mixture of noni fruit extract and patchouli oil (1:1). The results of the organoleptic tests show differences in taste, aroma, color, and texture between noni fruit extract before and after being encapsulated together with patchouli oil. After encapsulation, the bitter taste of noni fruit extract is reduced, but the addition of patchouli oil brings back the bitterness because patchouli oil has a bitter taste (*pungent taste*) [15]. Furthermore, the unpleasant odor of noni fruit extract also decreases after encapsulation with the addition of patchouli oil. Meanwhile, based on the results of the favorability test, 15 panelists preferred the encapsulation of pure noni fruit extract (without the addition of patchouli oil) and the encapsulation of noni fruit extract with the addition of patchouli oil in the smallest ratio of 4:1. The results of the organoleptic tests are presented in graphs as displayed in Figure 4.

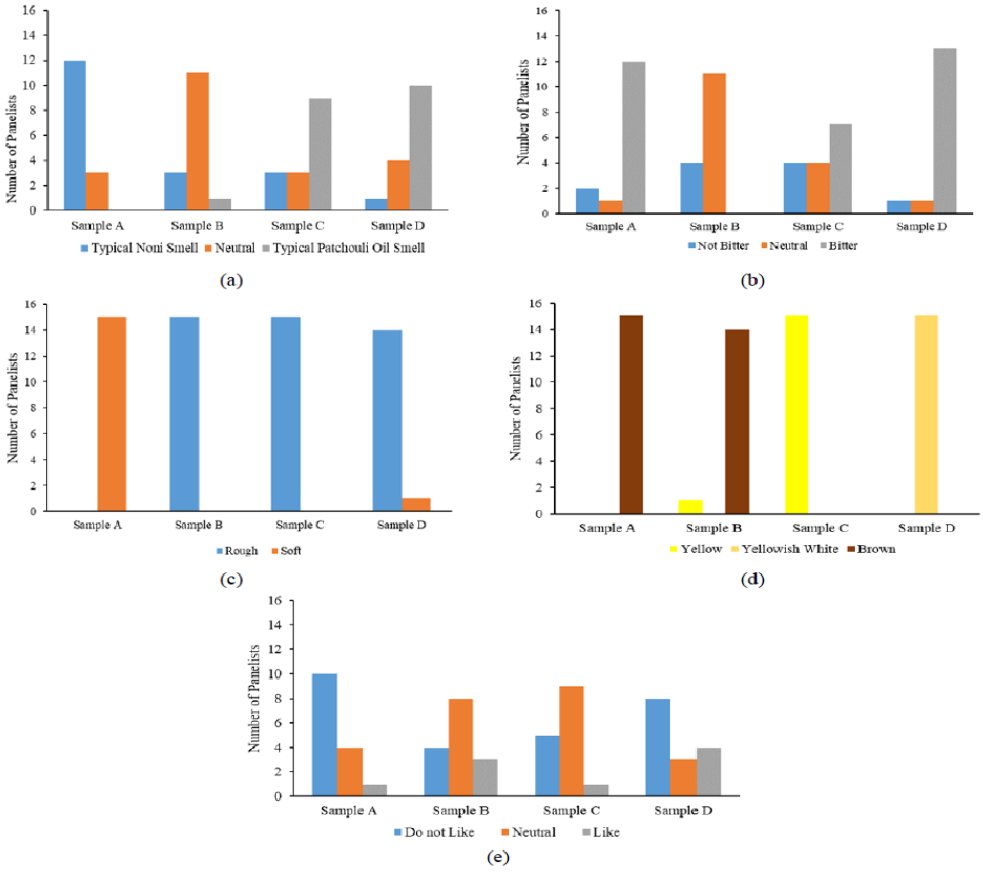


Figure 4. Graphs of organoleptic test results for aroma (a), taste (b), texture (c), color (d), and favorability (e)

Microcapsule morphology using SEM

The morphological analysis results of the microcapsules using SEM reveal an appearance that is not much different from one another at 500x magnification with a scale of 50 μm, as displayed in Figure 5. The resulting microcapsules are irregular, shaped like fractures, and have diverse sizes. This can be caused by incomplete freeze-drying process (the result is not completely dry) and uneven stirring with a magnetic stirrer. Wrinkles formed in the encapsulation of noni fruit extract and patchouli oil may occur due to the inappropriate composition and the lack of emulsifying ability of the maltodextrin used as encapsulating agent [7]. In addition, the particle surface of the three microcapsules did not show detectable pores, signifying that the solvent did not evaporate too quickly during the drying process. The results of the morphological analysis in this study are consistent with those of a previous study that encapsulated cold-pressed seed oils with various unsaturated fatty acids using the same freeze-drying method [16].

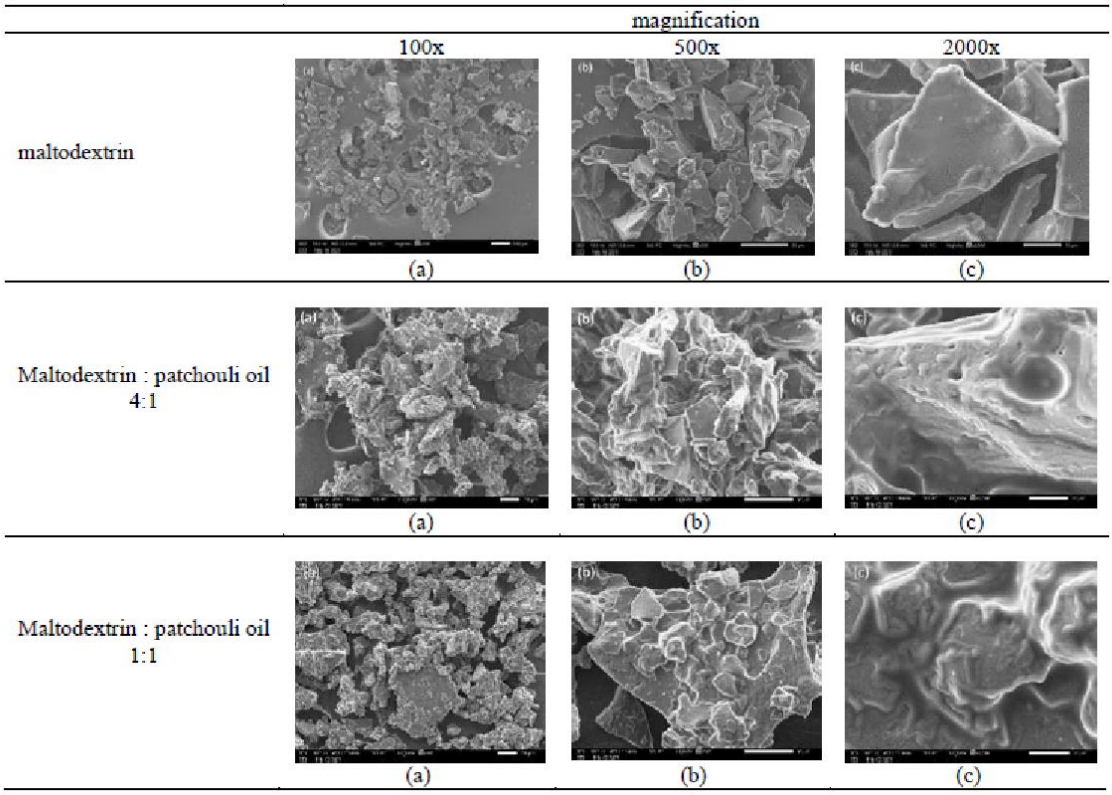


Figure 5. Morphology of noni fruit extract microcapsules with maltodextrin

Antioxidant activity test of the encapsulation

The success of an encapsulation process can be seen from several parameters, i.e., organoleptic properties, encapsulation efficiency, micro-particle characteristics, stability, release after consumption [17], and the antioxidant activity [18]. In this study, the antioxidant activity test was performed using the DPPH method, whose is that antioxidant compounds will donate hydrogen atoms and bond with free electrons in radical compounds, causing the free radical (diphenylpicrylhydrazyl) to change into a more stable non-radical compound (diphenylpicrylhydrazine) [19]. The sample absorbance results are used to determine the IC_{50} values of the encapsulation samples, which are shown in Table 5.

Table 5. IC_{50} values of encapsulation samples

Sample	IC_{50} (µg/mL)	Category
Noni Fruit Extract	61.74	Intermediate
Maltodextrin	1083346.9	Very weak
Encapsulation of Noni Fruit Extract with Maltodextrin	553.24	Very weak
Encapsulation of a Mixture of Noni Fruit Extract and Patchouli Oil (4:1)	88.30	Intermediate
Encapsulation of a Mixture of Noni Fruit Extract and Patchouli Oil (1:1)	50.80	Intermediate
Ascorbic Acid	1.10	Very strong

After encapsulation, the IC_{50} value of noni fruit extract experienced a significant increase. This indicates weak antioxidant activity as maltodextrin encapsulates the noni fruit extract and its active compounds. In this regard, the ratio of the core ingredients greatly influences the antioxidant activity of encapsulation result; the higher the concentration of maltodextrin compared to the core ingredients, the less fruit extract is encapsulated, resulting in the decreased amount of active compounds that act as antioxidants [20]. In this study, the ratio of coating material to core ingredients is 80:20, producing a higher IC_{50} value after encapsulation and resulting in lower antioxidant activity. However, the addition of patchouli oil has been found to contribute positively to increasing the antioxidant activity of encapsulated noni fruit extract. Due to the presence of patchouli alcohol which has hydroxy groups in phenols and terpenes, patchouli oil is known to have high antioxidant properties. As mentioned previously, the patchouli oil used in this study contains 16.62% Patchouli alcohol. Additionally, the patchouli oil also has an IC_{50} value of 22.45 µg/mL, which falls into the 'strong' category [21]. The results of this research indicate that the addition of patchouli oil to the encapsulation of noni extract is able to reduce the unpleasant aroma of noni extract and is able to increase the antioxidant activity of noni extract when combined. In previous research, modifications were also made to the encapsulation of noni extract, namely by adding lemon peel and it gave positive results to eliminate the unpleasant aroma of noni extract. However, this research did not include the antioxidant activity of the encapsulation results so it cannot be said to have potential as an antioxidant [23].

Conclusion

Based on the research that has been carried out, it can be concluded that Maltodextrin with a Dextrose Equivalent (DE) value of less than 20 can be produced through the partial hydrolysis process of cassava starch using the α-amylase enzyme with a concentration of 0.5% of the solution volume. The addition of patchouli oil to the encapsulation process of noni extract with maltodextrin has a positive effect on reducing the unpleasant aroma of noni extract and there is an increase in the antioxidant activity value of noni extract. Therefore, this research can be continued and developed as a solution for consuming noni fruit and preventing diseases caused by free radicals.

Acknowledgement

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MICROPLASTICS POLLUTION MITIGATION IN WASTEWATER TREATMENT: CURRENT PRACTICES, CHALLENGES, AND FUTURE PERSPECTIVES

(Pengurangan Pencemaran Mikroplastik dalam Rawatan Air Sisa: Amalan Semasa, Cabaran dan Perspektif Masa Depan)

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Abstract

The growing attention to microplastics stems from their significant environmental and human impacts. Microplastic accumulation in the environment also contributes to the spread of micropollutants. Daily human activities involving the use of plastics, especially synthetic materials, lead to their eventual presence in wastewater treatment plants (WWTPs). Although WWTPs play a crucial role in removing microplastics during the treatment process, the technologies currently in use are not entirely effective in filtering out all microplastic particles. As a result, WWTPs are recognized as major contributors to microplastic release into the environment. This review delves into the sources and prevalence of microplastics, the methods used for their removal in WWTPs, and the potential risks they pose to human health. Several removal methods are discussed, including sedimentation and flotation, activated sludge and sedimentation, reverse osmosis, and rapid sand filtration. The efficiency of each method is critically assessed, highlighting their strengths and weaknesses in addressing microplastic contamination. Moreover, this review underscores the importance of ongoing comprehensive research and development to improve the removal efficiency of microplastics in WWTPs. Efforts to optimize existing removal techniques and investigate new technologies should be intensified to achieve more holistic microplastic removal. By tackling the microplastics issue at the WWTP level, we can reduce their release into the environment, thereby diminishing potential health risks. In conclusion, the environmental presence of microplastics and their associated micropollutants demands robust removal strategies within WWTPs.

While existing technologies offer some level of effectiveness, there is a need for further advancements to ensure more comprehensive microplastic removal. This review offers valuable insights into the current state of microplastic removal in WWTPs, emphasizing the need for continued research to protect both the environment and human health.

Keywords: microplastics, chemical contaminants, wastewater treatment plants, removal techniques

Abstrak

Mikroplastik telah menjadi tumpuan kerana kesannya yang ketara terhadap alam sekitar dan manusia. Pengumpulan mikroplastik di persekitaran membawa kepada penyebaran bahan pencemar mikro . Aktiviti harian manusia yang melibatkan penggunaan plastik, terutamanya bahan sintetik, mengakibatkan mereka masuk ke dalam loji rawatan air sisa (WWTP). WWTP memainkan peranan penting dalam membuang mikroplastik semasa proses rawatan, Walaubagaimanapun, teknologi sedia ada yang digunakan tidak berkesan sepenuhnya dalam menapis semua zarah mikroplastik. Kajian semula ini memfokuskan kepada sumber dan kewujudan mikroplastik, kaedah semasa yang digunakan untuk penyingkiran mikroplastik di WWTP, dan risiko terhadap kesihatan manusia. Pelbagai kaedah penyingkiran telah dipertimbangkan, termasuk pemendapan dan pengapungan, enap cemar dan pemendapan diaktifkan, osmosis terbalik, dan penapisan pasir pantas. Kecekapan dan keberkesanan setiap kaedah diperiksa secara kritikal, menjelaskan keupayaan dan batasannya dalam mengurangkan pencemaran mikroplastik. Kajian semula ini juga menekankan keperluan untuk penyelidikan dan pembangunan komprehensif yang berterusan untuk meningkatkan keberkesanan penyingkiran mikroplastik dalam WWTP. Strategi untuk mengoptimumkan kaedah penyingkiran sedia ada dan meneroka teknologi baru harus diteruskan untuk mencapai penyingkiran mikroplastik yang lebih komprehensif. Dengan menangani isu mikroplastik di peringkat WWTP, pembebasan zarah ini ke alam sekitar dapat diminimumkan, seterusnya mengurangkan potensi risiko kepada kesihatan manusia. Kesimpulannya, kehadiran mikroplastik dan bahan pencemar mikro yang berkaitan dalam alam sekitar memerlukan strategi penyingkiran yang mantap. Walaupun teknologi semasa menunjukkan beberapa keberkesanan, kemajuan selanjutnya diperlukan bagi memastikan penyingkiran mikroplastik yang lebih cekap dan menyeluruh. Oleh itu, semakan ini memberikan pandangan yang berharga tentang keadaan semasa penyingkiran mikroplastik di WWTP, menekankan kepentingan penyelidikan berterusan untuk menjaga integriti alam sekitar dan kesejahteraan manusia.

Kata kunci: mikroplastik, bahan cemar kimia, loji rawatan air sisa, teknik penyingkiran

Introduction

Plastic is a ubiquitous material that has revolutionized modern life since its inception in 1907 [1]. Its versatility, low cost, and moldability make it an ideal choice for a myriad of applications. One of the primary advantages of plastics is their lightweight nature, which is particularly beneficial in the transportation industry and consumer goods [2]. The chemical structure of plastics is based on long chains of molecules, known as polymers, which consist of repeating units. These units can be tailored to produce plastics with a diverse range of physical and chemical properties, such as transparency, flexibility, and resistance to heat, moisture, and chemicals. Packaging is one of the primary applications for plastics, owing to their exceptional barrier properties. They can be engineered to be impermeable to gases, moisture, and light, making them perfect for safeguarding food, pharmaceuticals, and other delicate materials [3]. According to a 2021 survey in Europe, polypropylene (PP), low-density polyethylene (LDPE), and high-density polyethylene (HDPE) are the three most widely used polymers, as cited by [4]. PP is in high demand, making up roughly 19% of total demand, because of its superior strength, chemical resistance, and recyclability. This makes it apt for a variety of applications, including packaging, automotive, and textiles. Conversely, LDPE and HDPE, which account for about 18% and 12% of the total plastic demand, respectively, also have a significant footprint in the European polymer industry [4]. Figure 1 presents the statistics of plastic demand in Europe in 2021.

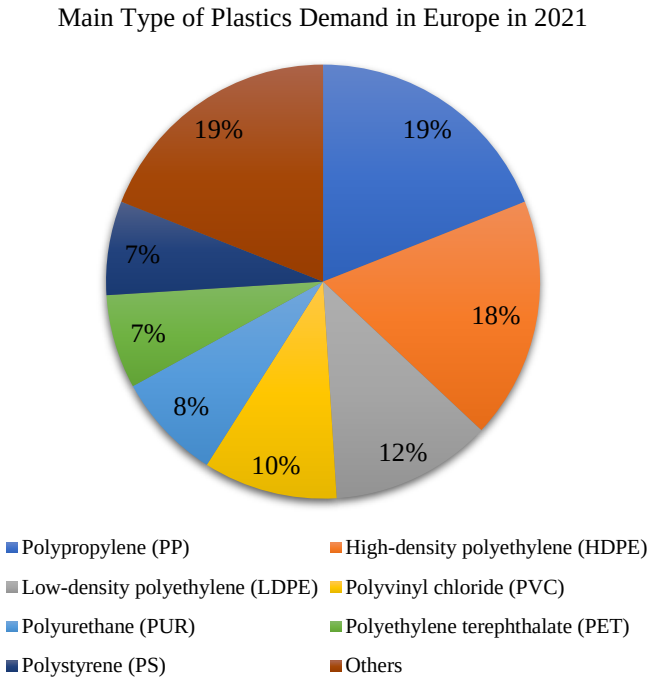


Figure 1. Plastics demand statistics in Europe in 2021

Despite their numerous advantages, the extensive use of plastics has led to significant environmental challenges, notably plastic pollution in oceans and waterways. Plastic waste varies in size from minuscule micrometre-sized particles to large meter-sized items and can originate from diverse sources, including both domestic and industrial [5]. Prior research indicates that domestic plastic production has consistently risen over the past decade, with only a minor fraction being recycled or incinerated [2]. In fact, about 79% of plastic waste either finds its way into landfills or is directly released into the environment, where it accumulates and can persist for centuries [6]. The build-up of plastic waste in aquatic environments is especially alarming due to its potentially detrimental effects on marine life. For instance, marine animals can become entangled in plastic debris, leading to suffocation, or they might ingest it, resulting in health complications or even death [7]. Moreover, plastic waste can release toxic chemicals into water, further jeopardizing the health of aquatic ecosystems [8].

The enduring presence of plastic waste in the environment results in the release of microplastics (MPs) – tiny plastic particles less than 5 mm in size. MPs are divided into two categories: primary and secondary MPs. Primary MPs are deliberately produced in small sizes, such as microbeads in personal care products, plastic pellets in feedstock or plastic manufacturing, and plastic powders for moulding. These particles are inherently small and can linger in the environment for prolonged periods, accumulating in various ecosystems, including oceans. According to Hwang et al. (2020), primary MPs constitute approximately 10% of global plastic waste, equating to 1.5 million metric tons annually [10]. Secondary MPs, conversely, form from the degradation of larger plastic items. They arise from various human activities like textile washing, tire wear, and paint chipping. These particles are minute enough to bypass wastewater treatment plants, eventually entering the environment. The scientific community has increasingly focused on these materials, believed to accumulate in the environment either due to direct discharge or from the fragmentation of larger particles [10].

The potential adverse health implications of microplastic exposure, whether through physical or

chemical pathways in daily life, are becoming a pressing concern. In vivo studies have shown that certain microplastics, especially polystyrene ones, can infiltrate body cells like macrophages, erythrocytes, and rat alveolar epithelial cells, causing intracellular structure damage [11]. The mechanisms by which microplastics enter cells remain elusive, but they are thought to penetrate through various pathways, including phagocytosis, endocytosis, and passive diffusion [12]. Once inside, microplastics can disrupt standard cellular functions, leading to cellular stress and inflammation. This can negatively impact human health and might even play a role in the onset of various diseases. Furthermore, the diminutive size of microplastics allows them to migrate to different organs and tissues in the body, potentially causing more health issues [13]. The accumulation of microplastics in tissues and organs can result in chronic inflammation, tissue damage, and disruption of regular organ function [14].

Additionally, the propensity of MPs to adsorb persistent organic pollutants, metals, and pathogenic microorganisms is alarming, as it can lead to the build-up of these noxious substances in the environment, posing potential risks to humans and other organisms. MPs have a high surface area to volume ratio, making them particularly adept at absorbing and concentrating both organic and inorganic pollutants in the environment [15]. This can happen through various physical and chemical mechanisms, such as electrostatic attraction, van der Waals forces, and hydrophobic interactions [16]. The adsorption of pollutants onto MPs can lead to aggregate formation, which, when ingested by organisms, can induce toxicity. The leaching of chemical additives from microplastics can further amplify their toxic effects [8]. Furthermore, MPs can contain various chemical additives like plasticizers, flame retardants, and pigments, which might be released into the environment as the plastic degrades [17]. These chemical additives can adversely affect both human and environmental health, leading to endocrine disruption, neurotoxicity, and even carcinogenicity [18]. While the potentially harmful effects of microplastics on human health are still debated, several studies have reported alarming findings. For instance, Prata noted an elevated risk of respiratory symptoms in workers exposed to polypropylene flocks compared to their non-exposed counterparts [19]. Chronic inhalation exposure to fine particles has also been associated with gene mutations. Synthetic textile workers exposed to polypropylene for 10-20 years had a higher cancer incidence. Moreover, polyvinyl chloride workers exhibited an increased lung cancer risk, correlated with age, years of work, and exposure duration at the factories [20].

Microplastic sources and occurrence

The widespread presence of microplastics in aquatic ecosystems is a growing concern due to their potential adverse effects on the environment and its inhabitants. Microplastics can be transported globally by various means, including ocean currents, wind, and other environmental factors. This has led to the detection of microplastics in all ocean basins and numerous freshwater systems.

The primary sources of microplastics are diverse, encompassing household sewage, polymeric particles from cosmetics and cleaning products, feedstocks for plastic product manufacturing, and plastic pellets or powders used for air blasting [21]. These sources discharge microplastics directly into the environment, contributing to their accumulation in aquatic ecosystems. Additionally, microplastics can form through the degradation of larger plastic items due to mechanical forces or UV light exposure [22]. This process results in the creation of microplastics that can accumulate in the environment. The fragmentation of larger plastic items into smaller microplastics can occur through various processes, such as photodegradation, thermal degradation, and mechanical abrasion [10]. Once released, microplastics can persist for extended periods due to their resistance to degradation.

The global ingestion of microplastics by a variety of aquatic organisms has unveiled new and concerning environmental threats, as reported by [23]. The consumption of microplastics has been extensively documented in fish, shellfish, and other aquatic creatures, raising concerns about potential adverse

effects on their health and the broader food chain. Microplastics can enter wastewater treatment plants (WWTPs) through various sources, eventually finding their way into water bodies and, subsequently, the wastewater treatment system. Recent research underscores the role of WWTPs in mitigating microplastic presence in the environment. According to Bretas Alvim et al., conventional WWTPs can remove up to 90% of microplastics. However, these systems remain a primary source of microplastic introduction into the environment. This is partly due to the direct release of microplastic-containing skincare products and toothpaste into wastewater from daily human activities [24]. Additionally, during washing, synthetic garments made of polyester and nylon can shed vast quantities of fibers, which can accumulate in WWTPs and contribute to microplastic release [19, 25].

The release of synthetic textile fibers during washing is a significant source of microplastic pollution in oceans. According to Boucher and Friot, about 35% of oceanic microplastics originate from synthetic textile fibers shed during washing [27]. The number of fibers released per wash can vary dramatically, with some studies reporting up to a million fibers shed by a single garment. For instance, Acharya et al. estimated the annual release of polyester and cotton microfibers during washing in Finland to range from 154,000 to 411,000 kg [28].

Several factors influence the release of microplastic fibers from synthetic textiles during washing. Textile properties, such as polymer type and knit structure, can affect fiber shedding [29]. For example, polyester fabrics tend to release more fibers per wash than acrylic fabrics [30]. Washing conditions, including temperature, friction, velocity, and duration, also play a role [30, 31]. Higher washing temperatures and extended durations can increase fiber release [30, 32], as well as mechanical friction and water velocity [31]. Additionally, the use of certain detergents and softeners can influence fiber release. Enzymatic or high-pH detergents can degrade fibers, increasing their release, while fabric softeners can coat fibers, reducing friction and shedding [33].

Microplastics entering WWTPs are typically captured and removed by designed systems. However, some microplastics still find their way into aquatic ecosystems, leading to environmental pollution and potential human health risks. The release of microplastics from WWTPs has garnered increased attention from researchers [34]. Studies have shown that while larger plastic particles are efficiently removed, microplastics can bypass the system and accumulate in aquatic ecosystems [35]. Given that many WWTPs are located near coastal areas and water sources, the discharge of microplastics can pose significant challenges. For example, in mainland China, 1,873 of the 3,340 WWTPs, with a treatment capacity of 78×106 m^3/day, are situated in coastal regions. Their effluents can directly or indirectly enter aquatic ecosystems [36]. To address this issue, researchers are examining the sources, occurrence, and removal of microplastics in WWTPs [21, 37, 38]. Table one summarizes the sources and types of plastics entering WWTPs.

Table 1. Source and type of plastics entering WWTPs

Source	**Type**	**References**
Domestic	Polyethylene (PE)	[39, 40]
	Polyethylene terephthalate (PET)	
	Polyester (PES)	
	Polyacrylate (PAC)	
	Polyamide (nylon)(PA)	
	High-density polyethylene (HDPE)	
	Low-density polyethylene (LDPE)	

Source	Type	References
Landfill	Polyacrylate (PAC)	[41–43]
	Polyimide (PI)	
	Polyethylene terephthalate (PET)	
	Polyoxymethylene (POM)	
	Polystyrene (PS)	
	Polyester (PES)	
	Polypropylene (PP)	
Stormwater runoff	Styrene-butadiene-rubber (SBR)	[44]
	Synthetic rubber (SR)	
Industry	Polyethylene terephthalate (PET)	[45]
	Polyester (PES)	
	Polyacrylate (PAC)	
	Polyamide (nylon)(PA)	
	Polystyrene (PS)	
	Polyvinyl chloride (PVC)	

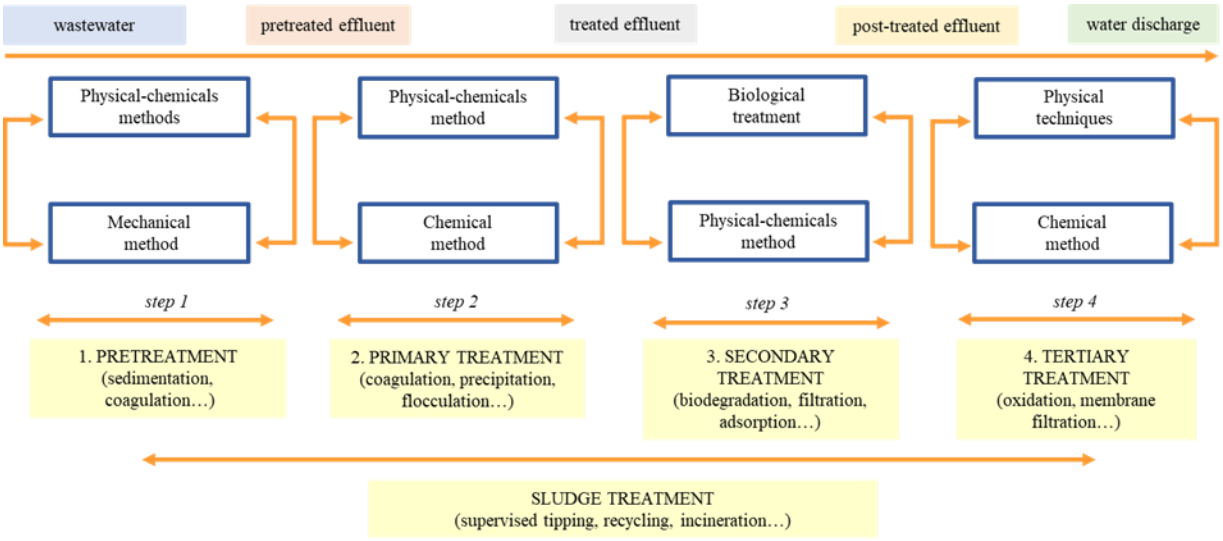


Figure 2. The schematic diagram of general process of WWTPs

Introduction to wastewater treatment plant

Efficient wastewater treatment is essential to maintain a clean and healthy environment and to comply with legal regulations that define decontamination objectives. Typically, conventional wastewater treatment combines physical, chemical, and biological processes to remove solids, including colloids, organic matter, nutrients, and soluble contaminants (e.g., metals, organics) from effluents. Figure 2 provides a comprehensive overview of available technologies for removing various contaminants from wastewater. Conventional methods encompass physical, chemical, and biological processes such as coagulation/flocculation, precipitation, biodegradation, filtration, and adsorption using activated carbon. These methods, established for contaminant removal in wastewater treatment, are widely employed in conventional treatment plants [46].

Besides conventional methods, established recovery processes, including solvent extraction, evaporation, oxidation, electrochemical treatment, membrane

separation, membrane bioreactors, ion exchange, and incineration, have also been utilized for contaminant removal [47]. Emerging removal methods under development and investigation include advanced oxidation, adsorption onto non-conventional solids, biosorption, biomass, and nanofiltration [47]. These methods are gaining popularity due to their high efficiency and ability to treat contaminants challenging to address using conventional methods. Advanced oxidation processes are explained in Tufail et al. [48]. Adsorption onto non-conventional solids, like agricultural waste or biochar, shows promise for contaminant removal, as discussed in Wang et al. [49]. Biosorption and biomass methods utilize microorganisms or plants to remove contaminants from wastewater through absorption or uptake. Nanofiltration, which uses membranes with pore sizes between ultrafiltration and reverse osmosis, effectively removes organic and inorganic compounds and has proven efficient in removing pharmaceuticals and endocrine disruptors, as mentioned in Tufail et al. [48].

The wastewater treatment process generally consists of five steps, as illustrated in Figure 2. The first step, preliminary treatment, uses physical and mechanical methods to remove large solids and debris. The second step, primary treatment, employs physicochemical and chemical processes to remove suspended solids and organic matter. Chemical coagulation or precipitation may enhance particle settling, as discussed in Turan et al. [37]. The third step, secondary treatment or purification, uses chemical and biological methods to degrade organic matter and nutrients with microorganisms. Efrin et al. [50] details the use of activated sludge systems, trickling filters, and other biological reactors for this purpose. Chemical treatments, like disinfection, may also be used to eliminate pathogens and other harmful microorganisms. The fourth step, tertiary or final treatment, uses physical and chemical methods such as filtration, adsorption, or reverse osmosis to remove any remaining contaminants. Advanced treatment methods like membrane filtration and ozonation may also be employed, as discussed in Tufail et al. [48]. The fifth step addresses the treatment of sludge generated during the process, which may involve supervised disposal, recycling, or incineration, as mentioned in Ngo et al. [51].

The first two steps of the wastewater treatment process are often referred to as pre-treatment or preliminary steps, depending on the context. Pre-treatment involves removing large solids and debris that might damage downstream equipment or block pipes, typically using screens or grit chambers, as described in Efrin et al. [50]. Primary treatment focuses on removing suspended solids and organic matter, often through sedimentation or flotation. It may also involve chemical coagulation or precipitation to enhance particle settling, as discussed in Turan et al. [37].

Secondary treatment, the subsequent step, relies on microorganisms to degrade organic matter and nutrients in the wastewater. This can be achieved using activated sludge systems, trickling filters, or other biological reactors, as explained in Enfrin et al. [50]. Chemical treatments, like disinfection, may also be used to eliminate pathogens and other harmful microorganisms. The tertiary or final treatment step typically employs physical or chemical methods, such as filtration, adsorption, or reverse osmosis, to further purify the wastewater. This step might also involve adding chemicals to adjust pH or remove specific contaminants. Enfrin et al. [50] discusses using advanced treatment methods like membrane filtration and ozonation for tertiary treatment. Lastly, treating the sludge generated during the process is crucial. This might involve landfill disposal or specialized techniques like composting, anaerobic digestion, or incineration to convert the sludge into a stable and safe material, as mentioned in [37].

Microplastic removal efficiency in WWTP

Sedimentation and flotation

Two technologies commonly used for MP removal are sedimentation and air flotation. The sedimentation process involves the settling of heavier MPs, such as those with high densities, to the bottom of the tank due to gravity. Concurrently, some MPs might get trapped in the solid flocs that form during the process. The effectiveness of the sedimentation process depends on several factors, including the size, shape, and density of

the MPs. The shape of the MPs can significantly influence their settling velocity, with spherical particles generally settling more slowly than irregularly shaped ones, as discussed in Li et al. [52]. The density of the MPs also affects their settling velocity, with higher-density MPs settling faster than those with lower density. Figure 3 below illustrates the process of flocculation and sedimentation in WWTPs.

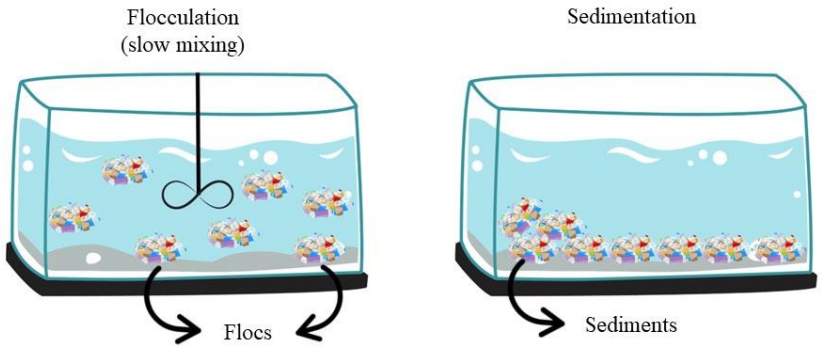


Figure 3. Flocculation and sedimentation process in WWTPs

The efficiency of sedimentation in removing MPs varies, with some studies reporting removal rates as low as 40.7% and others as high as 91.7%. This variation in removal efficiency can be attributed to differences in polymer types, and the size and shape of MPs in the wastewater flow [51, 53]. Polymer types such as PES, PET, and POM, which are denser than wastewater, are more readily removed by physical sedimentation, leading to higher removal efficiency [51]. The natural buoyancy of pollutants in the wastewater environment can also influence the effectiveness of sedimentation technology. Chemical compounds adsorbed onto the surface of MP particles can change their buoyancy, making them harder to remove [54]. The shape and size of the MPs also play a role in their removal efficiency, with fragments and granules being more easily removed than fibers [34].

A study by Liu et al. found that a combination of a coarse and fine grid, aerated grit chamber, and primary settlement tank reduced MPs from 79.9 to 47.4 nL^{-1}, achieving a removal rate of 40.7% [55]. However, another study by Turan et al. discovered that approximately 45% of influent MPs were trapped in the grit and grease fractions of the WWTP, while around 34% were retained within the primary sedimentation tank [37]. This indicates that a total of 80% of MPs accumulated in the primary sludge fraction, also referred to as biosolids. Using biosolids as fertilizer can introduce MPs into the environment, posing a potential risk to human health [56, 57].

Air flotation technology, in contrast, capitalizes on the density difference between the microplastics and the surrounding water. During this process, air bubbles are introduced, allowing MPs with a lower density to attach to them and rise to the water's surface, where they can be skimmed off. The shape and size of the MPs can also affect their removal efficiency in air flotation. Generally, MPs with larger sizes and irregular shapes exhibit higher removal efficiencies, as cited in literatures [51, 58]. Moreover, the presence of surfactants, which can stabilize the MPs and reduce their density, can diminish the effectiveness of air flotation, as noted by Swat et al. [59].

Activated sludge and sedimentation

Activated sludge is a prevalent technology in municipal WWTPs, typically employed after primary treatment processes such as sedimentation tanks, aerated grit chambers, or dissolved air flotation. Figure 4 illustrates the activated sludge and sedimentation process in WWTPs. During the growth phase, sludge flocs or bacterial extracellular polymers assist in accumulating

MPs contaminants in sewage, which are then removed during the sedimentation process. However, the intricate interaction between MPs and microorganisms, as well as the degree to which the process can capture MPs, remains ambiguous and requires further research [60].

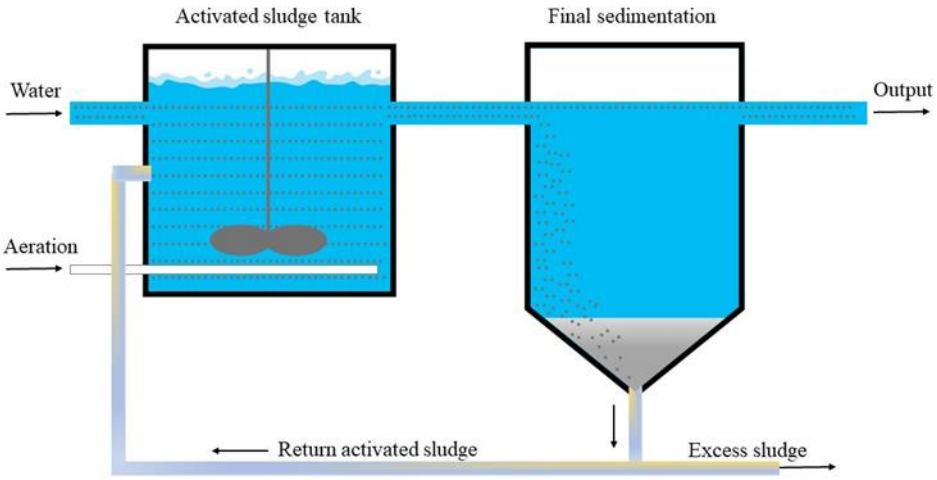


Figure 4. Example of activated sludge and sedimentation process

Ziajahromi et al. [61], Liu et al. [62], and Yang et al. [45] have reported MPs removal rates in activated sludge ranging from 28.1% to 66.7%. This variation is attributed to factors such as retention time and nutrient levels [61]. Extended contact times enhance the likelihood of surface biofilm formation on MPs, altering the surface, size, and relative densities of contaminants. Magni et al. reported that the number of MPs in WWTP sludge was 113 ± 57 MPs/g sludge dry weight (dw), with MPPs/g and MPFs/g accounting for 59.5 ± 21.6 and 53.3 ± 48.9, respectively [63]. The estimated daily accumulation of 3.4 billion MPs in sewage sludge underscores the importance of effective MP removal methods in WWTPs. While WWTPs can mitigate the direct introduction of MPs into aquatic environments by transferring some MPs to sludge during treatment, applying this sludge can introduce MPs into the soil. Li et al. (2018) estimated the total amount of MPs in dry sludge produced by W- and X-WWTP to be 1.64 × 108 and 1.88 × 108 particles per year, respectively [64].

Despite the potential of activated sludge technology to remove MPs, its inconsistent removal rate emphasizes the need for supplementary methods for effective MP removal. Combining sedimentation and air flotation with activated sludge might achieve optimal MP removal efficiency. Additionally, the role of microorganisms in accumulating MPs in sludge flocs, as highlighted by Scherer et al., stresses the significance of studying MPs removal mechanisms in wastewater treatment processes [60]. By understanding these mechanisms, we can pinpoint potential strategies to enhance MP removal in WWTPs, thereby reducing MP pollution in the environment.

Reverse osmosis (RO)

The application of RO (Reverse Osmosis) technology for MP (microplastics) removal has garnered attention as a potential solution to mitigate microplastic pollution in water sources. RO, a method widely employed for desalination and water purification, works by using a semipermeable membrane to separate contaminants based on their size and charge, as depicted in Figure 5. While RO is highly effective in removing dissolved substances, its efficiency in eliminating microplastics is contingent on various factors. This discussion offers an overview of the application of RO technology for microplastics removal, bolstered by pertinent statistical data.

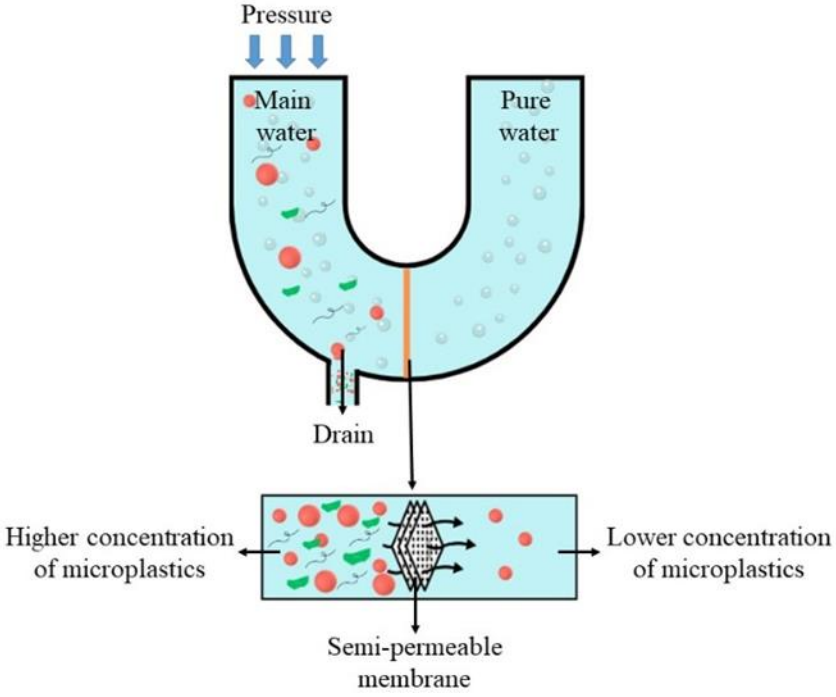


Figure 5. Semipermeable membrane used to separate contaminants

Several studies have evaluated the efficacy of RO in removing microplastics from water sources, yielding invaluable statistical insights. For instance, a study by Liu et al. explored the removal efficiency of RO membranes for microplastic particles ranging from 10-200 μm in size [64]. The research showed that RO achieved a microplastic removal efficiency spanning from 99% to 100% for microplastics of varying sizes. Another study by Ziajahromi et al. centered on the removal efficiency of RO for microplastic particles sized between 25 μm and 100 μm, revealing that RO could effectively eliminate microplastics with removal efficiencies reaching 98% [61].

Other studies have highlighted the impact of specific factors on the efficiency of microplastics removal via RO. Membrane characteristics, such as pore size and surface properties, are pivotal. Mohana et al. delved into the effect of different RO membrane pore sizes on microplastics removal, finding that membranes with tinier pore sizes displayed higher removal efficiencies, achieving 99% for microplastic particles larger than 0.4 μm [65]. The concentration of microplastics in the feedwater also significantly influences RO efficiency. Krishnan et al. assessed the effect of initial microplastic concentration on removal efficiency using RO, determining that higher initial microplastic concentrations resulted in diminished removal efficiencies, with rates ranging from 88% to 97% for initial concentrations from high to low [66].

While RO technology demonstrates the potential for microplastics removal, it is not without challenges and limitations. Fouling of the RO membrane by organic matter and particulate substances can compromise its efficiency. For example, a study referenced as [67] noted that microplastic removal efficiency was higher in tap water samples compared to raw water samples, both spiked with MPs (1 mg/L, 1 μm PS). The raw water sample exhibited a higher TMP value of 74.0 kPa, in contrast to 13.5 kPa for the tap water sample. An increase in the TMP value indicates more severe membrane fouling. Moreover, the energy demands of RO systems warrant consideration, as elevated energy consumption can lead to increased operational costs and environmental repercussions [68].

Rapid sand filtration

Rapid sand filtration is a widely used and economically viable technology for water treatment in various applications, including water supply and sewage treatment. This technique effectively removes impurities from water, making it a preferred choice globally [69, 70]. Within wastewater treatment plants (WWTPs), rapid sand filtration is often employed as a tertiary treatment stage to enhance water quality. Figure 6 illustrates the use of rapid sand filtration in WWTPs. The process works by passing water through a bed of sand, which serves as a filtration medium, removing suspended particles, pathogens, and organic matter. Both physical and biological mechanisms, such as adsorption, sedimentation, and microbial activity, contribute to contaminant removal and water quality improvement. One of the primary benefits of rapid sand filtration is its cost-effectiveness. The technology incurs relatively low operation and maintenance costs, making it an economically attractive choice for water treatment facilities [70]. Additionally, the system's simplicity promotes its broad adoption across various settings.

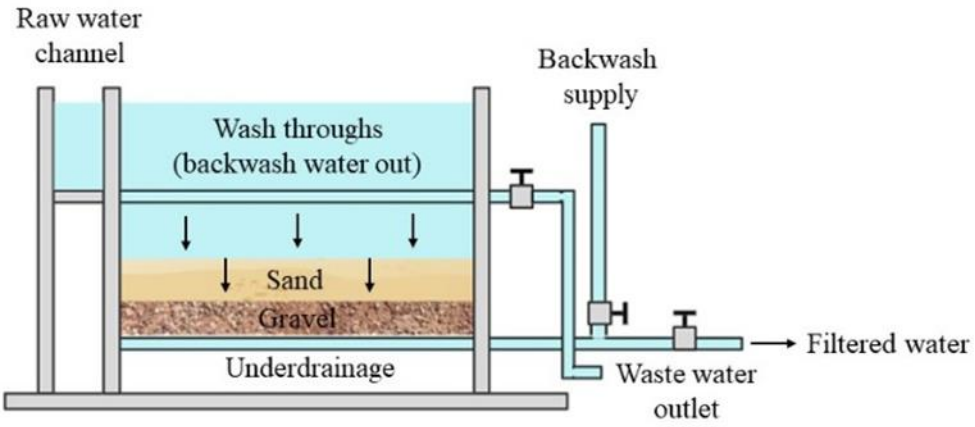


Figure 6. Rapid sand filtration in WWTPs

While rapid sand filtration effectively removes many impurities, its efficacy in MP removal has garnered significant attention. Several studies have explored the capability of rapid sand filtration in this regard, providing insights into its performance and limitations. When comparing the removal efficiency of MPs in different water treatment processes, rapid sand filtration seems to have a slightly lower efficiency than membrane bioreactors (MBRs). Egea-Corbacho et al. reported a removal efficiency of 97.1% for MPs in MBRs, while rapid sand filtration achieved 99.9% [38]. However, some inconsistencies in MP removal rates using rapid sand filtration have been observed. For instance, a study referenced as [71] found that a sand and anthracite coal filtration system in a WWTP in Western New York, USA, achieved only a 15% removal rate for MPs. The reduced efficiency in sand filters might be due to the potential mixing of anthracite coal and sand during filtration, possibly increasing the filter material's porosity over time. This diminished porosity might lead to bypassing or incomplete MP removal, affecting the system's overall efficiency. In contrast, filtration units using alternative methods have shown higher MP removal efficiencies, with several studies reporting rates exceeding 95% [51, 61, 72]. These findings underscore the effectiveness of such filtration units in removing MPs from water sources. Further research by Sembiring et al. identified a trend in MPs removal, indicating a decrease in removal percentages with increasing filtration rates. Their study revealed that the highest MP removal, at 95.5%, occurred at a filtration rate of 6 m/h after one hour of reactor operation. In contrast, the lowest removal, at 77.8%, was observed at a rate of 8 m/h

after five hours of operation [73]. This pattern suggests that as the filtration rate rises, the efficiency of microplastic removal decreases.

Several factors can influence the efficiency of MP removal during rapid sand filtration. One such factor is the MPs' particle size, as removal efficiency may differ based on the size distribution of these particles in the influent water. Different MP sizes might interact uniquely with the sand filtration medium, influencing their capture and removal [74]. A study by Umar et al. reported that smaller microplastics exhibited the highest removal rates by sand filtration compared to larger sizes. Furthermore, the composition of MPs can also affect their removal efficiency. MPs can consist of various polymers, additives, and contaminants, each potentially interacting differently with the sand filtration media [75]. Some MPs might have surface properties that enhance their adhesion to sand particles, leading to better removal, while others might exhibit properties that reduce such interactions, resulting in lower efficiencies.

Operational conditions of the filtration system can also impact MP removal efficiency. Factors like filtration rate, contact time between microplastics and the filtration media, and the filter bed's condition can all influence the process's overall effectiveness [75]. For example, an excessively high filtration rate might reduce the contact time between MPs and the filtration media, limiting removal efficiency [73]. Over time, the accumulation of organic matter and biofilm on the filter bed's surface can alter the sand's pore size and porosity, potentially diminishing the system's ability to capture and retain MPs [76].

Limitations of current technology in WWTPs in removing MPs

All current technological approaches have their limitations in removing MPs. Sedimentation, flocculation, activated sludge, reverse osmosis, and sand filtration are effective existing technologies in WWTPs, but their efficiency in removing microplastics is limited. The small size and buoyancy of microplastics make their removal through sedimentation and flotation challenging due to difficulties in efficient capture. Activated sludge, which can inadvertently break plastic particles into MPs, may contribute to the release of secondary microplastics into the environment. While reverse osmosis has been touted as efficient in removing MPs, its effectiveness depends on several factors. Moreover, it is expensive and energy-intensive. Sand filtration, on the other hand, has limitations in removing MPs, especially those of sub-micron size. Over time, particles can pass through the filter material. These challenges underscore the need for more advanced and specific technologies, such as advanced oxidation processes, membrane filtration techniques, and emerging separation methods. Based on findings from relevant studies, these approaches aim to address the size, density, and efficiency issues inherent in conventional treatment techniques.

Conclusion and future perspectives

In conclusion, the efficiency of MP removal in WWTPs is influenced by variations in design and operational characteristics. While existing technologies predominantly achieve retention of microplastics in sludge and solid waste, they do not offer a comprehensive solution for complete removal. The presence of micropollutants, including pharmaceuticals and additives from cosmetics, poses a significant challenge, as biological treatment processes alone are insufficient for their complete removal. Consequently, WWTPs are increasingly opting to upgrade their facilities by incorporating downstream treatment stages, specifically aimed at the elimination of micropollutants.

Future studies should perform comprehensive investigations into various treatment stages aimed at enhancing the efficiency of MP elimination in WWTPs. These studies should encompass the exploration of various technologies and approaches, considering the intricate characteristics, interactions, and removal mechanisms associated with MPs. Additionally, it is crucial to address the simultaneous presence of micropollutants alongside MPs during treatment processes, as both contaminants necessitate comprehensive and integrated treatment strategies. The findings derived from such studies should be

thoughtfully considered when implementing downstream treatment stages in WWTPs. The objective should be to develop and implement integrated treatment systems that not only target effective MP removal but also demonstrate efficacy in addressing micropollutants. This multidimensional approach may contribute to the realization of more efficient and sustainable wastewater treatment practices, consequently mitigating the release of MPs and micropollutants into the environment.

To achieve these goals, research efforts should focus on the following areas:

Advanced treatment technologies: Further exploration and evaluation of innovative treatment technologies, such as advanced oxidation processes, membrane filtration techniques, and emerging separation methods, should be pursued. In the advanced oxidation process, powerful oxidants are used to break down and remove various contaminants, including MPs present in the WWTPs. Meanwhile, membrane filtering techniques effectively capture and retain small particles of MPs, preventing their discharge into the environment. Emerging separation methods should be studied more since these approaches are continually evolving to offer more effective and focused removal. Therefore, these technologies hold promise for enhanced MP removal and micropollutant control in WWTPs.

Process optimization: Process optimization is a crucial strategy to overcome challenges and increase the effectiveness of MP removal and micropollutant control. Optimal operating conditions for MP removal should be identified through systematic optimization of process parameters such as contact time, filtration rates, and dosing of coagulants or adsorbents. The potential for MPs and micropollutants to interact with treatment agents may be increased by longer contact time, which would increase the effectiveness of removal. The removal efficiency may also increase when the filtration rates are adjusted to achieve a balance between the removal process and system capacity. Chemicals and their dosages used in a process also need to be optimized. Insufficient dosing may result in incomplete removal, while excessive dosing may increase costs and raise potential environmental issues. Therefore, process optimization in WWTPs is essential to help maximize the removal efficiency of MPs and micropollutants, save costs, and protect the environment.

Material development: Research should be dedicated to the development of novel materials with enhanced adsorption or filtration properties specifically designed for MP and micropollutant removal. This includes exploring the use of bio-based materials, nanomaterials, and hybrid composites. Bio-based materials from renewable sources such as agricultural waste, algae, and bacterial byproducts can exhibit good adsorption properties. The development of nanomaterials with a high surface area and reactivity will have a high potential for trapping MPs and micropollutants. Hybrid composites consist of various materials that benefit from their complementary properties. They may be designed to excel at trapping MPs and micropollutants while maintaining structural stability. By developing novel materials, the efficiency of MPs and micropollutant removal will be improved.

Fate and transport studies: Comprehensive investigations into the fate and transport of MPs within WWTPs, including their behaviour during different treatment stages and potential release pathways, should be conducted. Sample collection can be obtained at different stages of WWTPs, enabling the tracking of the movement and fate of MPs within the WWTPs. To ensure the accuracy of the data collected, a consistent methodology for the sampling step for each stage must be established. This will provide valuable insights for designing targeted removal strategies.

Monitoring and analysis: Advancements in analytical techniques and monitoring methodologies should be pursued to accurately quantify and characterize MPs in wastewater, treated effluent, and receiving water bodies. Monitoring will allow the assessment of the effectiveness of each stage of WWTPs. This will aid in assessing the efficiency of treatment processes and the environmental impact of released contaminants.

By focusing on these research directions, the researchers can further contribute to the development of more efficient and sustainable treatment solutions for MPs in WWTPs. This will in turn facilitate the implementation of comprehensive treatment strategies to safeguard water resources and protect environmental and human health.

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INSIGHTS INTO THE STRUCTURAL, MORPHOLOGICAL, AND OPTICAL PROPERTIES OF V_2O_5 CATHODE MATERIAL SYNTHESIZED BY SELF-PROPAGATING COMBUSTION METHOD UNDER VARIED ANNEALING TEMPERATURES

(Pembelajaran ke atas Ciri-Ciri Struktur, Morfologi, dan Optik V_2O_5 yang Disintesis Melalui Kaedah Pembakaran yang Menyebar Sendiri di Bawah Pelbagai Suhu Pemanasan)

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Abstract

In this study, we report the novel synthesis of V_2O_5 cathode material utilizing the self-propagating combustion (SPC) method, marking the first-ever successful application of this technique for V_2O_5 production. Nonetheless, a thorough examination of the synthesis of V_2O_5 cathode material is required, with a special emphasis on the impact of annealing temperatures. The insufficient examination of the structural, morphological, and optical properties of V_2O_5 produced by the self-propagating combustion method in the current literature impedes the capacity to enhance its synthesis for application in energy storage devices, catalytic systems, and optoelectronic devices. In order to close this information gap, this study will carefully investigate how different annealing temperatures affect the final V_2O_5 material properties by providing comprehensive insights into the structural, morphological, and optical properties of V_2O_5 at varying annealing temperatures. The thermal profile was studied using Simultaneous Thermal Analysis (STA) providing measurement of multiple thermal properties of the sample as a function of time or temperature. The structural characterization was carried out using X-ray diffraction (XRD) analysis, revealing the crystal structure and phase purity of the synthesized V_2O_5 samples. Field emission scanning electron microscopy (FESEM) was employed to examine the morphological features, including particle size, shape, and surface morphology. Additionally, Energy Dispersive X-ray Spectroscopy (EDX) provides elemental composition to further substantiating the synthesis method's efficacy. The optical properties were investigated through UV-Visible (UV-Vis) spectroscopy, providing information on the bandgap energy and optical absorption behavior of the synthesized V_2O_5. The results revealed that the annealing temperature significantly influenced the

structural, morphological, and optical properties of V_2O_5. With increasing annealing temperature, the crystallinity and phase purity of V_2O_5 improved. The morphological analysis indicated variations in particle size, shape, and surface texture as a function of annealing temperature. Moreover, the optical characterization demonstrated that the annealing process influenced the bandgap energy and optical absorption properties of V_2O_5. In this study, it was found that, the optimum annealing temperature is at 600ºC, which gives a single-phase crystal structure with uniform thickness of nano-sheets like appearance.

Keywords: self-propagating combustion method, annealing temperatures, X-ray diffraction, field emission scanning electron microscopy, UV-Visible spectroscopy

Abstrak

Dalam kajian ini, kami melaporkan sintesis V_2O_5 sebagai bahan katod baru dengan menggunakan kaedah pembakaran penyebaran sendiri (SPC), menjadikan ianya laporan pertama penghasilan V_2O_5 menggunakan kaedah ini. Walau bagaimanapun, kajian menyeluruh sintesis bahan katod V_2O_5 diperlukan, dengan penekanan khusus kepada kesan suhu pemanasan. Penyelidikan yang tidak mencukupi mengenai sifat struktural, morfologi dan optik V_2O_5 yang dihasilkan oleh kaedah pembakaran yang menyebarkan diri dalam kajian semasa, menghalang keupayaan untuk meningkatkan sintesisnya untuk aplikasi dalam peranti penyimpanan tenaga, sistem katalis, dan peranti optoelektronik. Untuk menutup jurang maklumat ini, kajian ini akan mengkaji dengan teliti bagaimana suhu pemanasan yang berbeza mempengaruhi sifat bahan V_2O_5 dengan menyediakan konsepsi yang komprehensif ke atas sifat struktural, morfologi, dan optik V_2O_5 pada suhu pemanasan yang berlainan. Profil haba telah dianalisa menggunakan Analisis Terma Serentak (STA) yang menyediakan pengukuran pelbagai sifat haba sampel sebagai fungsi masa atau suhu. Bagi mengkaji struktur kristal dan fasa asli bahan, analisis pembelauan sinar-X (XRD) telah dijalankan ke atas sampel V_2O_5 yang telah disintesis. Mikroskopi Imbasan Elektron Pancaran Medan (FESEM) digunakan untuk mengkaji ciri-ciri morfologi, termasuk saiz partikel, bentuk, dan morfologi permukaan. Selain itu, Spektroskopi Sinar-X Tenagan Serakan (EDX) menyediakan komposisi elemental untuk lebih membuktikan keberkesanan kaedah sintesis. Ciri-ciri optik telah dipelajari melalui spektroskopi UV-Vis, yang menyediakan maklumat mengenai tenaga jurang dan tingkah laku penyerapan optik V_2O_5 yang disintesis. Hasilnya mendedahkan bahawa suhu pemanasan secara signifikan mempengaruhi sifat struktural, morfologi dan optik V_2O_5. Dengan meningkatnya suhu pemanasan, kristaliniti dan kemurnian fasa V_2O_5 meningkat. Analisis morfologi juga menunjukkan perubahan dalam saiz partikel, bentuk, dan tekstur permukaan. Selain itu, sifat optik menunjukkan bahawa proses pemanasan mempengaruhi tenaga jurang dan sifat penyerapan optik V_2O_5. Dalam kajian ini, ia telah ditemui bahawa, suhu pemanasan optimum adalah pada 600ºC, yang memberikan struktur kristal satu fasa dan morfologi denagn permukaan yang sekata serta ketebalan berbentuk lapisan bersaiz nano yang seragam.

Kata kunci: kaedah pembakaran penyebaran sendiri, suhu pemanasan, pembelauan sinar-X, mikroskopi imbasan elektron pancaran medan, spektroskopi UV-cahaya nampak

Introduction

The need for effective electrical energy storage has arisen because of the increasing demand for cleaner energy and the swift expansion of the global economy [1, 2]. Among the various commercialized technologies, batteries have attracted enormous attention due to their relatively high energy density and long cycle life [3]. Rechargeable lithium-ion batteries (LIBs) have been widely used as the most popular energy storage technology in electronic devices and vehicles [4]. It is widely used in the field of portable energy storage due to its long life, without memory effect and environmental toxicity [5, 6]. Nevertheless, some problems including low safety, high cost and lithium resources shortage still hinder its development for large-scale application [5]. Aluminum ion batteries are viewed as practical substitutes for large-scale energy storage devices due to their high theoretical capacity (2980 mAh g^{-1}, 8063 mAh cm^{-3}), abundance, and safety, with aluminum metal anodes being the primary contributing factor [7, 8]. On the other hand, developing cathode materials *via* intercalation reaction seems challenging due to the high charge density of trivalent aluminum ions. Therefore, in recent years, there have been many reports on the cathode materials of aluminum batteries, including carbon-based materials [9-11], oxides [12, 13], sulfides [14, 15], and selenides [16]. Among these cathode materials, vanadium (V) oxide, also known as V_2O_5, is a widely recognized transitional metal oxide (TMO) due to its layered structure, low toxicity, low

cost, and ability to exhibit multiple oxidation valences (V^{2+}, V^{3+}, V^{4+}, and V^{5+}).

V_2O_5 stands as a prominent cathode material in advanced energy storage systems, due to its exceptional redox properties and high theoretical capacity [17]. Despite its appealing features, V_2O_5 has limitations that hinder its performance in certain applications. For example, the material has poor electronic and ionic conductivities, leading to lower reversible capacity and reduced rate capability [18]. To address these limitations and improve the performance of V_2O_5, researchers are actively exploring various synthesis methods for V_2O_5 nanomaterials. Those methods are including the sol-gel method, hydrothermal synthesis, electro-spinning technique and dispersion method [19]. However, based on our current understanding, no prior attempts have been made to utilize the self-propagating combustion (SPC) method for the synthesis of V_2O_5 cathode material. SPC method has emerged as a good choice due to its simplicity, cost-effectiveness, and potential for precise control over material properties [17]. The low electrical and ionic conductivities of V_2O_5 can be ascribed to its crystal structure and morphology, which can be altered by annealing at various temperatures. By manipulating the annealing temperature, one can exert control over the crystal structure, phase purity, and morphology of V_2O_5, hence influencing its electronic and ionic conductivities [20]. Therefore, in this study, we introduce the SPC method as a novel synthesis approach and emphasizes the importance of annealing temperature variation in tailoring material characteristics. The insights garnered herein not only contribute to the fundamental understanding of V_2O_5, as a cathode material but also hold significant implications for the design and fabrication of high-performance energy storage devices. Simultaneous thermal analysis (STA), X-ray diffraction (XRD), field-emission scanning electron microscopy (FESEM), Energy Dispersive X-ray Spectroscopy (EDX) and Ultraviolet-Visible Spectroscopy (UV-Vis) were used to study the thermal, structural, morphological and optical characteristics of the obtained V_2O_5 cathode materials.

Materials and Methods

Preparation of V_2O_5 material

A self-propagating combustion method was used to prepare vanadium(V) oxide, V_2O_5 nanoparticles. The raw material and fuel are crucial components in combustion process. The weight of each materials used was calculated using stoichiometry method. Citric acid (fuel) and the starting material, ammonium metavanadate, NH_4VO_3 was stirred in 15 ml and 200 ml deionized water respectively before the fuel was poured into the NH_4VO_3 solution. The poured solution was vigorously agitated for 25 minutes on a magnetic stirrer to achieve a homogenous mixture. The solution is held on the hot plate at 300 ºC until combustion occurs, and then the material is calcined in a prepared furnace at different temperatures of 500 ºC, 600 ºC and 700 ºC for 6 hours. The materials were removed after the material is allowed to cool in the furnace in off mode resulting in the production of black powder product.

Thermal analysis

Thermal profile of the precursors was carried out using a simultaneous thermogravimetric analyzer (STA; SETARAM SETSYS Evolution 1750, Caluire, France). It means that the thermogravimetric analysis (TGA) and the differential scanning calorimetry (DSC) were measured simultaneously. In this study, about 10-15 mg of each precursor was heated from 30 °C to 700 °C with a heating rate of 10 °C per minute under air atmosphere. The precursors were placed in alumina crucibles which was suitable for heating to high temperatures.

Characterization

The XRD of the samples was obtained by a diffractometer (PANalytical X'pert PRO) with Cu kα radiation. The morphology of cathodes was characterized *via* FESEM. The EDX was used to characterize the composition of the cathode materials. The optical band gap characteristics of the sample were obtained using UV-Vis spectroscopy.

Results and Discussion

Thermal profile

Figure 1 shows the decomposition behavior of the precursor. There are three weight loss occurring on the TGA profile marked as I, II and III. The first weight loss in region I is around 6.5% at the temperature range of 30 °C to 160 °C accompanied by an exothermic peak that

is attributed to the evaporation of water from the sample surface [21]. NH_4VO_3 began to transform into ammonium vanadate ($(NH_4)_2(V_6O_{16})$) with the escape of NH_3 [22]. The abrupt weight loss of 30.8% from ~200 °C to 430 °C period may correspond to the bound water in the structure [23] and it is related to the exothermic process occur at that point. The third region, between 420 °C and 560 °C relates to weight loss of 27.20%. It is correlated with the high and broad exothermic peak on DSC curve at ~540 °C which indicates the releasing of NH_3, O_2 and H_2O [24]. The endothermic peak at 680 °C was attributed to the recrystallization of V_2O_5 materials physical process, which did not affect the vanadium valence of the final products, in facts it involves breaking and reforming intermolecular bonds [23]. This can be related to the TGA graph, where there is no weight loss in the compound at above 600°C, while the endothermic peak may be due to the structural phase transition of V_2O_5 crystals. Consequently, according to the acquired thermal analysis, all samples were annealed at 500 °C, 600 °C and 700 °C. From here, it was suggested that varying the annealing temperature may lead to distinct material characteristics.

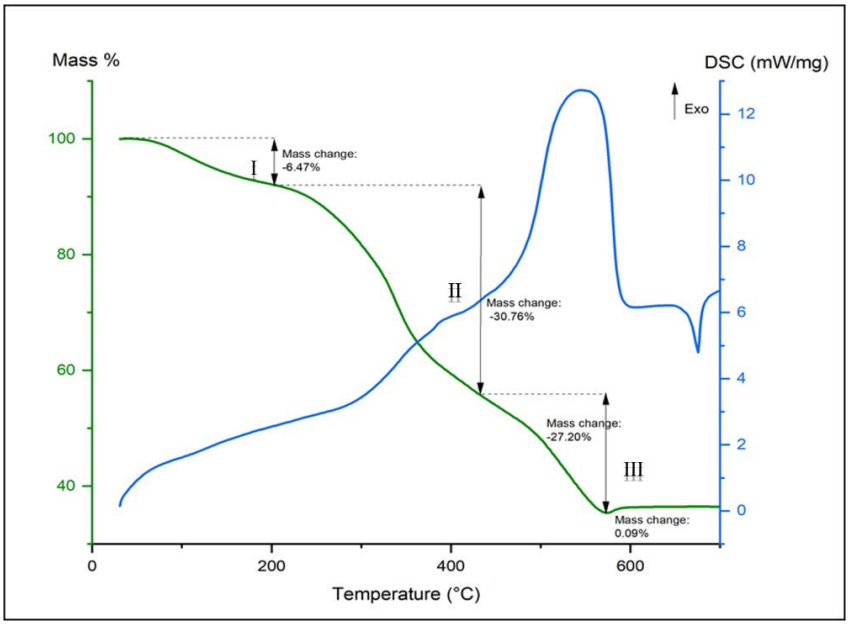


Figure 1. Simultaneous thermogravimetric analyzer (STA) of the V_2O_5 precursor

Structural characterization

Figure 2 displays the XRD patterns of various materials. The annealed sample at a temperature of 500 °C (as shown in Figure 2(a)) exhibits XRD peaks of low intensity, suggesting the existence of orthorhombic and monoclinic crystal structures attributed to vanadium (V) oxide and vanadium (IV) oxide, respectively. The orthorhombic crystal structure of V_2O_5 can be achieved through the process of annealing at temperatures of 600 °C and 700 °C, as depicted in Figure 2(b) and (c) correspondingly. Both samples have a high level of purity and possess an orthorhombic crystal structure system with the Pmmm space group, as validated by the JCPDS reference number 00-041-1426. The augmentation of the annealing temperature results in a corresponding augmentation of the XRD peak intensities. This observation suggests enhancements in the crystallinity of the V_2O_5 material, as well as the production of well-organized crystals. Nevertheless, an abrupt increase in magnitude is noted in the specimen that underwent annealing at a temperature of 700 °C. This increase aligns with the recrystallization peak identified in the STA analysis, which occurs at around 680 °C during the thermogravimetric analysis's stable phase (refer to Figure 1). FESEM micrographs can provide additional evidence to corroborate this condition.

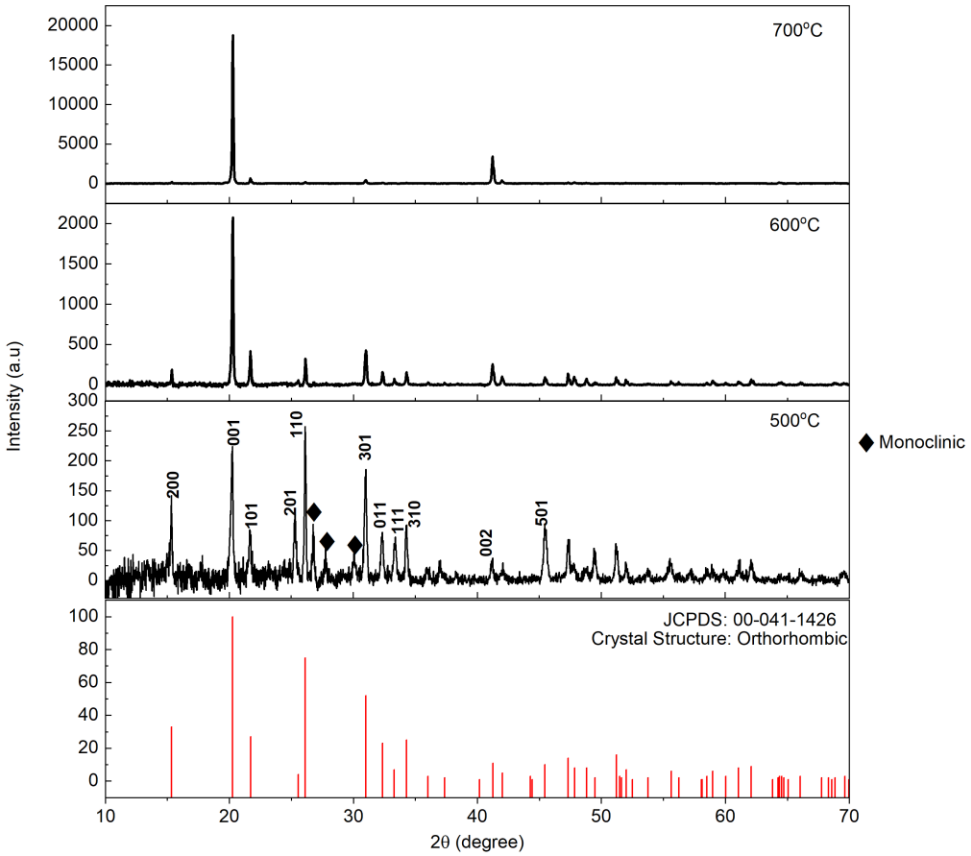


Figure 2 XRD patters of V_2O_5 samples annealed at 500 °C, 600 °C and 700 °C

Morphological characterization

The shape of V_2O_5 at various annealing temperatures is depicted in the FESEM pictures presented in Figure 3(i) and (ii). For the sample annealed at 500 °C, the observed structures exhibit a high degree of agglomeration, forming flake-sheet-like arrangements. These structures possess an average thickness of 196 nm. The flakes have a slender and flat configuration reminiscent of a two-dimensional plane, characterized by a sleek exterior and intermittent protrusions and boundaries. The nano-sheets depicted in sample annealed at 600 °C exhibit a homogeneous thickness spanning 48 nm, accompanied by a regular arrangement of layers while sample annealed at 700 °C shows that the compound displayed prominent bulky flakes with a sheet-like structure. This observation suggests that the compound maintained a constant flaky sheet shape as the annealing temperature was raised. Significantly, the flaky sheets exhibited an increase in size while retaining their characteristic layered structure, as depicted in Figure 3(ii) under a magnification of 50,000x. The observed morphology aligns with the findings of the XRD investigation, which suggests an increase in crystallinity with elevated annealing temperatures. The structures' large aspect ratio is believed to contribute to their possibly improved characteristics in the context of battery applications. The reduced thickness of micro- and nano-sheets facilitates shorter diffusion paths inside the electrode material, leading to enhanced ion transport and improved utilization of the active material. Consequently, this enables faster charge and discharge cycles. The utilization of this data can facilitate the optimization of the annealing temperature to achieve the desired V_2O_5 compound, which serves as an optimal cathode material for usage in aluminum batteries. The compositional atomic percent of the elements obtained by EDX are shown in Table 1 with percentage difference less than ~4%.

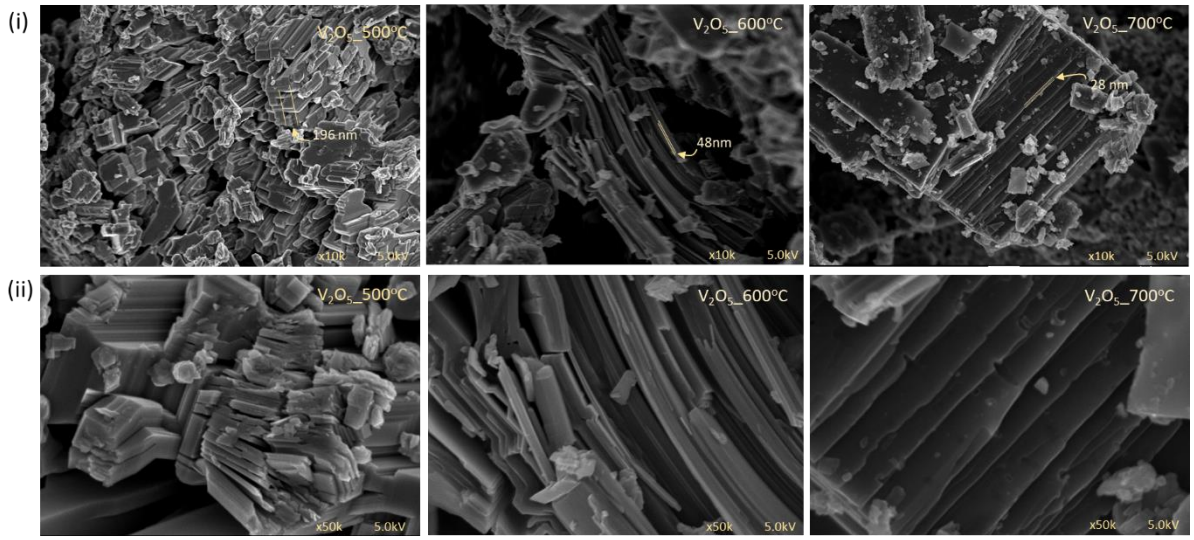


Figure 3. (i) FESEM images of pure V_2O_5 at 10K magnification with annealing temperature of 500ºC, 600ºC and 700ºC and (ii) 50K magnification for each sample

Table 1 Compositional distribution from EDX of the constituent elements for the V_2O_5 at different annealing temperatures.

		Experimental Atomic %					
Elements	**Theoretical Atomic %**	**V_2O_5_ 500ºC**	**% Difference**	**V_2O_5_ 600ºC**	**% Difference**	**V_2O_5_ 700ºC**	**% Difference**
Vanadium	28.6	27.5	3.9	29.7	3.8	29.0	1.4
Oxygen	71.4	72.5	1.5	70.3	1.6	71.0	0.6

Optical characterization

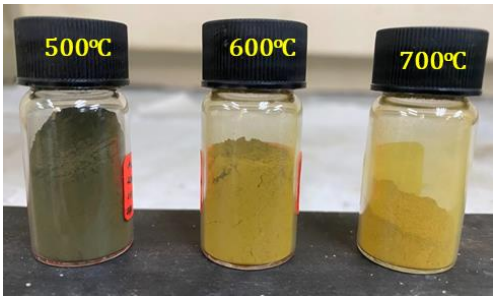


Figure 4. V_2O_5 samples at different annealing temperature

As in Figure 4, the samples of synthesized V_2O_5 were observed at different annealing temperatures. An interesting observation was made as the annealing temperature increased, the colour of the synthesized V_2O_5 became lighter. For optical band gap evaluation, reflectance spectra of all samples were obtained by UV-Vis spectroscopy and further analysed using the Tauc equation (equation (1) [25],

$$(\alpha h\nu) = A(h\nu - Eg)^x \quad (1)$$

where α is the absorption coefficient of the material at a certain value of the wavelength λ, h is the Planck's constant, A is a proportionality constant, υ is the frequency of light, Eg is the band gap energy and x = 1⁄2 (for direct transition mode materials). The absorption coefficient is evaluated using,

$$\alpha = k \ln \left(\frac{R_{max} - R_{min}}{R - R_{min}}\right) \quad (2)$$

where k is a constant, R_{max} is the maximum reflectance and R_{min} is the minimum reflectance.

Consideration of Eqs. (1) and (2) gives,

$$(\alpha h\nu)^2 = A'(h\nu - Eg) \quad (3)$$

where A′ is a constant. From Eq. (3), a Tauc plot can be drawn of $(\alpha h\nu)^2$ versus $h\nu$. The band gap energy is obtained by extrapolation of the linear part and the point that meets the abscissa will give the value of the band gap energy of the material. Upon careful analysis using Tauc plots (Figure 5), it was evident that the band gap values experienced a significant reduction as the annealing temperature increased. Specifically, the band gap values were measured to be 3.896 eV, 3.878 eV, and 3.870 eV for annealing temperatures of 500°C, 600°C, and 700°C respectively. These changes indicate the alterations in the electronic structure of the material. Specifically, it points towards a reduction in the band gap [26]. This correlation between the colour of the sample and the band gap is in line with expectations. Lighter colours are associated with lower band gap values. This is because a smaller band gap implies that less energy is required to promote an electron from the valence band to the conduction band. As a result, the material absorbs and reflects light differently, giving rise to the observed colour change. In battery operation, band gap governs charge transfer mechanisms, dictating the efficiency with which charge carriers the electrons and holes are generated and facilitating smooth charge transfer during electrochemical reactions. Moreover, the band gap significantly influences the voltage and energy density of the battery, ensuring compatibility with other components for optimal performance. Furthermore, identifying a suitable band gap contributes to cycling stability, offering insights into the material's durability over numerous charge-discharge cycles, a pivotal factor for the battery's long-term reliability [27].

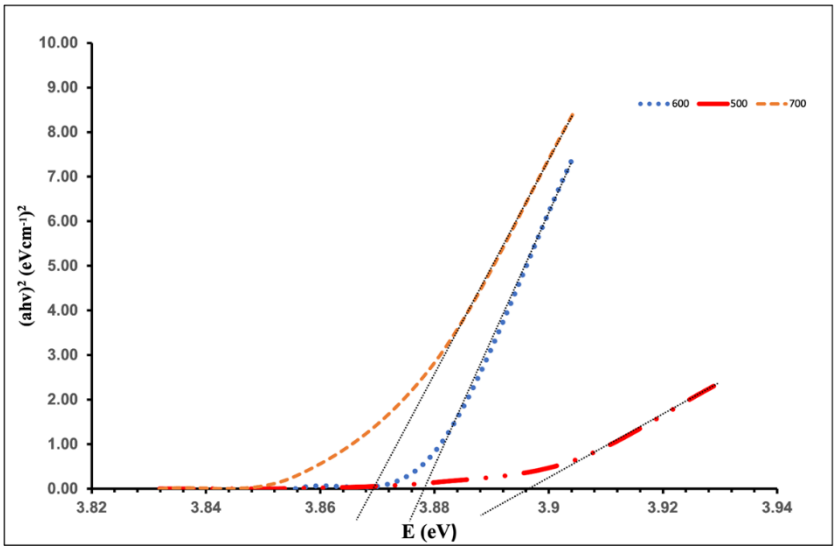


Figure 5. Tauc plots for V_2O_5 powder annealed at 500 ºC, 600 ºC and 700 ºC

Conclusion

Structural, morphological and optical properties of single-phase V_2O_5 powder are synthesized by self-propagating combustion method. XRD results show that the compound is polycrystalline with orthorhombic symmetry structure. After the annealing process, the crystallization was improved by increasing temperature. FESEM images show a highly agglomerated flakes-sheet-like structure for sample annealed at 500 ºC. All flakes exhibited thin and planar structure that resemble a two-dimensional sheet. The surface of the flakes was found to be predominantly smooth, with occasional ridges and edge. Upon increased annealing temperature, the compound exhibited consistent flaky sheet morphology. The flaky sheets grew in dimensions while maintaining their distinct layered appearance as well as

the ridges and edges. This is consistent with the XRD analysis where it is profound that the crystallinity was enhanced at higher annealing temperature. Sample anneals at 600 ^oC gives a single-phase orthorhombic crystal structure with uniform thickness of nano-sheets like appearance and smooth surface suggesting that the optimum annealing temperature was at 600 ^oC. Optically, it was found that lighter colors of V_2O_5 powder associated with lower band gap values. The observed changes in color and band gap values provide valuable insights into how annealing temperatures influence the electronic properties of the synthesized V_2O_5, which in turn can have significant implications for its performance as a cathode material in energy storage applications.

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SIZE AND ORGAN-SPECIFIC HEAVY METAL ACCUMULATION PATTERNS IN LONGTAIL TUNA (*Thunnus tonggol*) FROM TERENGGANU

(Saiz dan Organ-Khusus Corak Pengumpulan Logam Berat dalam Ikan Aya Hitam (*Thunnus tonggol*) dari Terengganu)

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Abstract

Longtail tuna, *Thunnus tonggol*, belongs to the *Scrombidae* family and is commonly served in special Terengganu delicacies, such as in *Nasi Dagang* gravy. Primarily, the predator fish is found in shallow waters. The Terengganu state is located on the east coast of Peninsular Malaysia, where the oil and gas industry is a primary economic bolster. The rapid urbanisation, large-scale developments, and tourist attraction in the area might contribute to the heavy metal pollution in Terengganu waters. The present study was conducted to determine the concentration of six heavy metals, such as copper (Cu), zinc (Zn), cadmium (Cd), lead (Pb), arsenic (As), and mercury (Hg) in longtail tuna muscle, gills, liver, and stomach, by employing inductively coupled plasma-mass spectrometry (ICP-MS). The highest concentration of Cu, Cd, and Pb was recorded in the liver. The muscle samples revealed the highest As and Hg concentrations, while the gills documented the maximum Zn concentration. The results also revealed that the heavy metals possessed a significant positive relationship with the tuna size (p <0.05). For instance, Hg demonstrated a strong positive association with fish size, recording a 0.577 r-value. Conversely, the longtail tuna samples indicated apparent growth dilution effects for Cu and Pb, proved by the negative correlations between their concentrations and the fish length. This study successfully outlined comprehensive approaches to better understand the controlling factors, including fish size and targeted organs in determining the concentration of heavy metals in longtail tuna.

Keywords: heavy metal, inductively coupled plasma-mass spectrometry, pollution, South China Sea, *Thunnus tonggol*

Abstrak

Ikan Aya hitam, *Thunnus tonggol*, dikategorikan dalam keluarga *Scrombidae* dan lazimnya dihidangkan sebagai hidangan istimewa di Terengganu seperti dalam kuah Nasi Dagang. Lazimnya, spesis pemangsa ini dijumpai di kawasan air cetek. Terengganu terletak di Pantai Timur Semenanjung Malaysia yang mana industri minyak dan gas merupakan penyumbang ekonomi utama. Pembandaran yang pesat, pembangunan berskala besar, dan tarikan pelancongan di negeri tersebut mungkin menjadi antara punca pencemaran logam berat dalam perairan Terengganu. Kajian ini dijalankan untuk menentukan kepekatan enam logam berat; kuprum (Cu), zink (Zn), kadmium (Cd), plumbum (Pb), arsenik (As) dan merkuri (Hg) di dalam isi, insang, hati, dan perut ikan

Aya hitam menggunakan ICP-MS. Keputusan menujukkan bahawa kepekatan Cu, Cd dan Pb paling tinggi dicatatkan di dalam hati. Isi ikan merekodkan kepekatan As dan Hg paling tinggi, manakala insang mencatatkan kepekatan Zn yang paling tinggi. Keputusan kajian ini juga menunjukkan bahawa semua logam berat mempunyai hubungan positif yang ketara dengan saiz ikan Aya hitam (p <0.05). Contohnya, Hg mempunyai hubungan positif yang kukuh dengan saiz ikan dengan nilai r ialah 0.577. Sebaliknya, ikan Aya hitam menunjukkan kesan pencairan pertumbuhan untuk Cu dan Pb dengan hubungan negatif antara kepekatan logam berat dengan saiz ikan. Kajian ini telah Berjaya menunjukkan pendekatan menyeluruh dan kefahaman yang lebih mendalam terhadap faktor-faktor yang mempengaruhi kepekatan logam berat di dalam ikan Aya hitam, termasukalah saiz ikan dan organ sasaran.

Kata kunci: logam berat, spektroskopi jisim- plasma gadingan aruhan, pencemaran, laut China Selatan, *Thunnus tonggol*

Introduction

Recently, heavy metal pollution has attracted global attention. Uncontrolled heavy metal contamination could pose critical threats to ecosystems and detrimental health impacts on humans. Increasing levels of heavy metal effluents in marine environments from several anthropogenic sources, such as industrial activities, domestic sewage, and agriculture decreased the quality of fish and other aquatic organisms. Moreover, the growing number of vehicles and metal leachings from various sources, including landfills, waste dumps, and runoffs, could contribute to the increasing heavy metal concentrations in marine environments, which might harm marine life organisms and the ecosystem as a whole [1].

Metals could bioaccumulate and biomagnify in food chains and food webs, posing risks to aquatic organisms and human health [2, 3]. Heavy metals also damage multiple organs, even at low exposure concentrations. Fish in metal-polluted environments could be exposed to metals through food chains or via uptake from contaminated water. The introduction of heavy metals into fish organs is predominantly through adsorption and absorption, hence the accumulation rate is defined by the uptake and depuration rates [4].

In Terengganu, longtail tuna, locally known as *ikan Aya hitam* (*Thunnus tonggol*), is one of the most popular fish. The fish is commonly served in special delicacies, including the gravies for *Nasi Dagang* and *Nasi Lemak* and as *ikan singgang*. A survey documented that the Terengganu locals consumed approximately 437.4 g/person of longtail tuna weekly [5], which constitutes 62.49 g of their daily intake. The consumption rate was slightly higher than the average amount of fish consumed by other Malaysians in 2009 at 60.0 kg/person/day [6].

Longtail tuna is a species belonging to the *Scomberoidae* family and the *Thunnus* genus. The fish could reach a maximum total length of 142 cm and is the second smallest of eight *Thunnus* species [7]. The longtail tuna has a relatively slower growth rate than other tuna species. The species is also considered a suitable indicator of environmental pollution by wastewater, microplastic, or heavy metals as they reportedly accumulate higher concentrations of heavy metals [8, 9].

Heavy metal concentrations in longtail tuna could indicate exposure time and the metal contents in the sediments and water columns of the areas from which the fish are sourced. The size of the tuna emulates the exposure period to the contaminated water. At the same time, the concentrations of heavy metals in their organs allow health condition assessments of the area from which they were collected. This study emphasised the relationship between the accumulation of heavy metals and the size of the longtail tuna and targeted organs.

Materials and Methods

Sampling area

The longtail tuna, *Thunnus tonggol*, samples employed in this study were bought from local fishermen of vessel Class B operating in Kuala Terengganu waters (see Figure 1). The specimens were purchased from the fishermen on the same day of capture and promptly brought to the laboratory on ice before being dissected (see Figure 2).

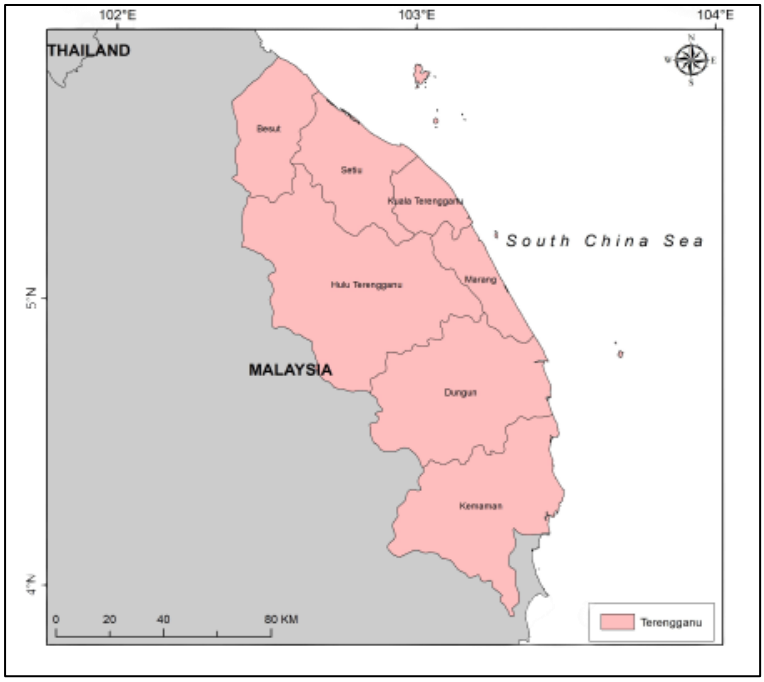


Figure 1. The longtail tuna samples were caught in Kuala Terengganu waters and landed at Lembaga Kemajuan Ikan Malaysia (LKIM) Complex, Pulau Kambing, Kuala Terengganu



Figure 2. The longtail tuna (*Thunnus tonggol*), or locally known as *ikan Aya*, *ikan kayu*, or *ikan tongkol hitam* in Malaysia

Sample collection and preparation

The present study obtained 75 longtail tuna specimens from fishermen before measuring and recording the total length and weight of the samples (see Table 1). Subsequently, the gills, liver, stomach, and muscles were dissected with clean ceramic equipment (see Figure 3). The tissue samples were oven-dried at 60°C for three days or until the samples recorded a constant weight. The dried specimens were then homogenised with mortar and pestle before digestion.

Table 1. The total length and weight ranges of the longtail tuna samples

Location	***n***	**Total length (cm)**	**Total weight (g)**
Terengganu waters	75	21.1–49.5	339–1537

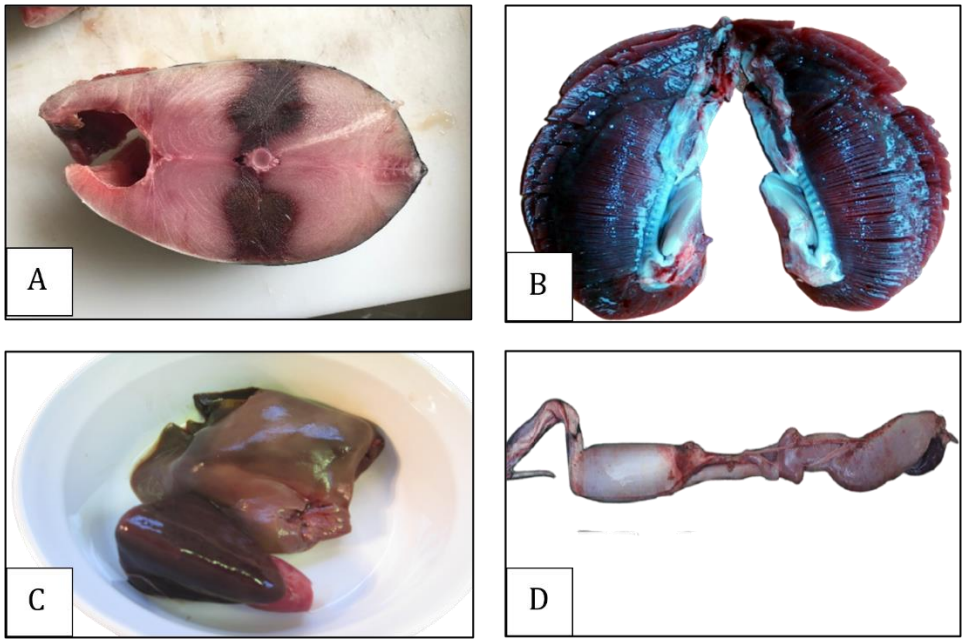


Figure 3. The A: muscle, B: gills, C: liver, and D: stomach samples of the longtail tuna

Samples digestion and heavy metal detection

During the digestion process conducted in this study, 0.05 g of the homogenised dried sample was weighed and mixed with 1.5 mL of Suprapur nitric acid (HNO_3; Merck KGaA, Germany) in an acid-washed Teflon beaker. The Teflon beaker was closed with a cap in a Teflon bomb before being placed into an oven for 8 h at 100 °C to allow complete digestion of the samples [10-12]. The digested samples were cooled to room temperature, transferred into centrifuge tubes, and diluted with deionised water up to 10 mL. Finally, the samples were stored at under 4 °C before selected metal element (Cu, Zn, Cd, Pb, As, and Hg) measurements with inductively coupled plasma-mass spectrometry (ICP-MS) (ELAN 9000) [13].

The standard reference material employed in this study, Dolt-4 Fish Liver SRM 1946 (National Research Council Canada), and blank specimens were digested following a procedure similar to the longtail specimens. The blank samples were prepared with the Suprapur HNO_3 without adding the fish samples. The standard solutions utilised in the present study were yielded via mixing the Suprapur HNO_3 with 0.05 g of DOLT-4 fish liver [14]. The current study constructed the standard to enhance confidence in the levels of heavy metals determined in the longtail tuna samples evaluated.

Statistical analysis

A Shapiro-Wilk (p-value <0.05) normality assessment is conducted to assess the distribution of data before constructing reference intervals and variables. Normality evaluations are required for accurate and reliable conclusions. Generally, non-normally distributed data are subjected to non-parametric assessments. This study applied Friedman and Spearmank's rank, which are non-parametric evaluations, based on the variables involved.

Heavy metal concentrations in the muscle, gills, liver, and stomach of the longtail tuna samples

The present study obtained non-normally distributed data. Accordingly, a Friedman evaluation, which is a non-parametric assessment, was conducted to determine the differences between the independent groups (muscle, gills, liver and stomach) when the dependent criterion (heavy metal concentration) was measured as a continuous parameter (ratio). The employment of the Friedman evaluation was appropriate for the present study, as the concentration of the heavy metals was measured in triplicates or more. The variables also recorded a significant difference when the p-value was under 0.05.

The selected heavy metal concentrations in longtail tuna of different sizes

The data in the present study did not assume normality. Consequently, a non-parametric linear correlation analysis, Spearman's rank, was applied to determine the strength and direction of the associations between two ranked variables: the size of the tuna samples (independent variable) and the concentration of the heavy metals (dependent variable) detected. The non-parametric evaluation was employed as two parameters in this study were continuous (ratio). The Spearman's rank assessment was also utilised to determine the strength of following monotonic relationships.

(i) Increasing longtail tuna size improves the concentration of heavy metals.
(ii) Improving longtail tuna size decreases the concentration of heavy metals.

The Spearman's correlation coefficient, r_s, which might be between +1 and −1, were defined as follows.

(i) A +1 r_s indicates a perfect association of ranks
(ii) A zero r_s zero denotes no association between ranks
(iii) A −1 n r_s demonstrates a perfect negative association of ranks

Results and Discussion

Heavy metal contents in the muscle and target organs of the longtail tuna from Terengganu waters

Heavy metal concentrations (Cu, Zn, Cd, Pb, As, and Hg) in the muscle, gills, stomach, and liver of the longtail tuna (*Thunnus tonggol*) specimens collected caught in Terengganu waters are listed in Table 2. The heavy metals were statistically significant (*p*-value <0.05) to the tissues of the longtail tuna samples, including the muscle, gills, liver, and stomach.

Based on the results, the muscle and organ samples recorded variations in the levels of heavy metal accumulated. For example, the muscle tissues recorded the highest accumulation of As and Hg than the other metals, while Zn demonstrated a higher tendency to accumulate in the gills. On the other hand, Cu, Cd, and Pb were highly concentrated in the liver.

Table 2. The average concentration of heavy metals detected in the muscle, gills, stomach, and liver of the longtail tuna (*Thunnus tonggol*) samples

	The Average Concentration of Heavy Metals (mg/kg) Dry Weight			
Metal	**Muscle**	**Gills**	**Stomach**	**Liver**
Copper	4.46 ± 6.67	1.98 ± 1.81	4.32 ± 5.32	11.2 ± 7.34
Zinc	13.3 ± 18.6	56.1 ± 71.4	31.4 ± 41.5	32.3 ± 22.2
Cadmium	0.060 ± 0.183	0.086 ± 0.129	0.310 ± 0.384	4.73 ± 5.30
Lead	0.075 ± 0.222	0.076 ± 0.118	0.026 ± 0.022	0.087 ± 0.117
Arsenic	10.5 ± 10.2	2.44 ± 1.41	7.98 ± 5.59	7.54 ± 4.90
Mercury	0.114 ± 0.16	0.020 ± 0.023	0.052 ± 0.048	0.080 ± 0.217

Heavy metals could accumulate in most organs in a fish, including the skin, gills, intestine, and kidneys, which might harm the fish physically and internally [15]. Moreover, even trace amounts of heavy metals could be toxic to fish. Heavy metal toxicity in marine organisms depends on several factors, such as exposition routes, nutritional status of the exposed organism, chemical species, genetics, age, and gender [16, 17]. The direct absorption of heavy metals in fish occurs through water columns, while an indirect route is via food chains. Nevertheless, heavy metal concentration variabilities in fish organs could also be due to the types of tissues and organs, which significantly depend on the concentration of heavy metals in the surrounding waters [18, 19].

Among the six elements evaluated in the present study, Hg and As recorded higher concentrations in fish muscle than the other targeted organs. Previous reports hypothesised that the higher tendency of Hg to accumulate in fish muscle is related to its high affinity

to the thiol groups in amino acids, a protein component in muscle tissues [20-22] also suggested that the metals bioaccumulated in muscle tissues could be linked to the feeding habits of the species, contributing to metal level variations [23]. Large predatory fish, such as longtail tuna, consume numerous smaller fish, accumulating Hg in their muscles. Post-Hg assimilation in fish, the contaminant is distributed throughout the organism via blood and stored in various tissues, specifically the muscles [24]. Similarly, this study recorded the highest As levels in the muscle samples. The observations might be due to its diet since longtail tuna have a high feeding rate and are carnivorous and active predators [25].

Besides food, heavy metals could also be absorbed into fish organs via mechanical captures of suspended hydroxide particles in the gills and chemical ion absorptions via membranes [26, 27]. In this study, Zn recorded the highest concentration in fish gills at 56.1 ± 71.4 mg/kg dry weight. Fish gills consist of epithelial membranes primarily containing mucus-covered phospholipids. The gill surfaces are negatively charged, thus providing potential sites for interactions with positively charged metals [27]. Fish also assimilate metals through ionic exchanges across lipophilic membranes in their gills during respiration [28].

Dural et al. [29] found that the accumulation of heavy metals predominantly occurred in metabolically active tissues, such as gills and the liver. A higher metal accumulation in fish liver correlates with the presence of natural protein binding, known as metallothionein (MTs), which stores the metals to fulfil enzymatic and other metabolic demands in the fish [30]. The MTs are small cysteine-rich proteins that are critical in metal homeostasis and protection against heavy metal toxicity, DNA damage, and oxidative stress [30].

Metalloregulatory processes involve MTs by binding the natural proteins to heavy metals through the thiol groups in their cysteine residues. The MTs have a high affinity for heavy metals, hence possessing the ability to bind to xenobiotic heavy metals to protect against metal toxicity, especially Cd [31]. Furthermore, MTs attaching to physiological heavy metals, such as Cu, enable their participation in regulating cell growth and proliferation and protecting the fish against oxidative stress [32]. The MTs also act as an essential metal store to meet enzymatic and other metabolic demands [33]. Nonetheless, higher toxic element concentrations, including Cd and Pb, could lead to adverse health effects as they compete with calcium (Ca^{2+}) at enzymatic locations [34].

The relationship between the selected heavy metal concentrations and the size of the longtail tuna samples caught in Terengganu waters

Generally, the size of a longtail tuna could affect the heavy metal concentration in its muscles. The length of the longtail tuna is commonly considered the fundamental measure as its probability to change considerably is low [35]. The standard length of a fish is the measurement from its closed mouth to the end of the fleshy part of its body [36].

This study assessed the strength of associations between the variables, which were the size of the specimen and the concentration of heavy metals detected. Resultantly, three relationship strength ranks, low, medium, and strong, were obtained (see Table 3). A linear correlation analysis, the Spearman's rank correlation assessment, was employed in the current study to determine the relationship between heavy metal concentration and the longtail tuna size (standard length).

Table 4 demonstrates the relationship value (*r*) that indicates the strength of the correlation between the parameters evaluated. The *p*-values obtained represent the relationship significance between the variables. *r*-values approaching 1 indicated the crucial role of size and strong correlation with heavy metal accumulation.

Table 3. The *r*- and *r* squared (R^2)-values of the linear correlation between the longtail tuna size and the heavy metal concentration

***r*-value**	**R^2-value**	**Indication**
0.1–0.29	0.01–0.084	Low relationship association
0.3–0.49	0.09–0.24	Medium relationship association
0.5–1.0	0.25–1.0	Strong relationship association

Table 4. The Spearman's rank correlation coefficient (*r)* and significance levels (*p*) of the association between heavy metal concentrations in the muscle of the longtail tuna and their size

Element	**Muscle**	
	***r*-value**	***p*-value**
Cu	−0.435	0.000*
Zn	0.353	0.000*
Cd	0.237	0.001*
Pb	−0.192	0.006*
As	0.469	0.000*
Hg	0.577	0.000*

In this study, positive relationships were observed between the fish size and metal levels. Nevertheless, negative associations were demonstrated by Cu and Pb with the longtail tuna muscle samples. The results paralleled a report that documented positive correlations between the size of *Branchiostoma belcheri* with the concentration of Zn, Hg, and Cd [37]. Farkas et al. [32] also found a positive correlation in Hg, Cu, and Zn with the size of *Abramis brama* L. In another report, fish length was positively associated with the concentrations of Cr, Cu, Fe, and Pb but recorded a negative relationship with Cd, Cu, Cr and Pb levels [38].

Typically, increasing fish size reflects the age of the fish [30]. Heavy metal concentration assessments of marine fish from the Oman Gulf found that Hg levels increased with the age and size of the fish [39]. Douben et al. [40] suggested that metal accumulation in fish reaches a steady state after a certain age. Nonetheless, if metal concentrations in the surrounding water are higher than the capacities of growth associated-tissues and lowered metabolic activities in older (larger) organisms, it might not affect the dilution of the metal levels in the fish. Thus, continued metal accumulation might occur, and a positive relationship could be observed. Conversely, the significant negative relationship between Cu and longtail tuna size documented in this study could be due to the slow uptake rates of Cu in muscles compared to other tissue [41]. Furthermore, Cu elimination is reportedly efficient in muscle tissues [41].

Heavy metal concentration could also be affected by environmental contamination and exposure duration [42, 43]. Therefore, larger or older longtail tunas are expected to have accumulated higher metal concentrations than their smaller or younger counterparts. Since longtail tuna is a predator fish, rising heavy metal levels with increasing fish size could also be related to the greater consumption of contaminated prey from higher trophic levels [44].

Conclusion

The concentration of heavy metals in the longtail tuna fish specimens assessed in this study varied significantly. The differences were not only attributable to the size of the specimen but also to the targeted organs. The functions and properties of the organs, such as the presence of natural protein binding, metallothioneins, in fish liver, also influenced the remarkable ability of the organs to absorb more heavy metals. Heavy metal concentrations in the environment, such as sediment and the surrounding water, also affect heavy metal contamination degrees in fish. Nevertheless, the heavy metal levels in the areas were

not measured in the current study.

The findings reported in this study provided the current status of heavy metal contents in longtail tuna from Terengganu waters. The data could assist the Malaysian Environmental Department in evaluating, monitoring, and controlling metal pollutant discharges into marine environments. The information also documents the quality of longtail tuna, which could be beneficial to the Department of Fishery Malaysia (DOF) in enhancing the quality of seafood sources.

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EXTRACTION OF β-KERATIN FROM POULTRY FEATHER WASTE USING SODIUM METABISULFITE AND SODIUM DODECYL SULFATE

(Pengekstrakan β-Keratin dari Sisa Bulu Ayam Menggunakan Natrium Metabisulfit dan Natrium Dodesil Sulfat)

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Abstract

Environmental contamination due to the disposal of poultry feather waste requires careful attention and management. As poultry feathers are a rich source of keratin protein, this study aims to determine the most efficient reducing agents for extracting β-keratin from chicken and duck feather waste as well as their optimal concentrations. For this purpose, sodium metabisulfite ($Na_2S_2O_5$) and sodium dodecyl sulfate ($NaC_{12}H_{25}SO_4$) at different concentrations, namely 0.2 M, 0.5 M, and 1 M, were utilized as the reducing agents. The results showed that the optimal concentration of reducing agents for extracting β-keratin from both chicken and duck feather waste was 0.2 M. Sodium metabisulfite (SMB) was proven to be the most effective reducing agent for chicken feather waste, with a yield of 76.9%. For duck feather waste, on the other hand, the use of sodium dodecyl sulfate (SDS) resulted in β-keratin extraction of 39.8%.

Keywords: β-keratin, feather waste, chicken feathers, duck feathers, sodium dodecyl sulfate

Abstrak

Pencemaran alam sekitar yang disebabkan oleh sisa bulu unggas memerlukan perhatian dan ditangani secara serius. Bulu unggas dapat dimanfaatkan sebagai salah satu sumber protein keratin. Kajian ini bertujuan untuk menentukan agen penurunan yang paling berkesan dan kepekatan optimum untuk pengekstrakan β-keratin dari sisa bulu unggas (bulu ayam dan bulu itik). Natrium metabisulfit ($Na_2S_2O_5$) (SMB) dan natrium dodesil sulfat ($NaC_{12}H_{25}SO_4$) (SDS) dengan pelbagai kepekatan 0.2 M, 0.5 M, dan 1 M telah digunakan sebagai agen penurunan. Kepekatan yang berkesan untuk pengekstrakan β-keratin dari sisa bulu ayan dan itik adalah 0.2 M. Agen penurunan yang berkesan untuk mengekstrak β-keratin daripada sisa bulu ayam adalah SMB menghasilkan sebanyak 76.9%. Manakala penggunaan SDS dalam sisa bulu itik menghasilkan 39.8% β-keratin.

Kata kunci: β-keratin, sisa bulu, bulu ayam, bulu itik, natrium dodesil sulfat

Introduction

As a global problem that requires careful attention and management, environmental pollution is caused by various contaminants, one of which is poultry feather waste [1]. It is estimated that around 8.5 billion tons of feathers are produced by the poultry processing industry

as waste every year throughout the world [2]. This waste is usually disposed of through incineration and landfilling. Human population growth, increasing income, and changes in lifestyle and diet have caused a rapid increase in livestock production [3]. However, the growth in poultry production also contributes to environmental pollution, especially because the feathers are underutilized in various applications.

Poultry feathers are rich in keratin protein which can be used in various fields, including green chemistry, food science, pharmaceutical industry, biomedicine, hair cosmetics, and composite materials [4]. Keratin is the third most abundant polymer after cellulose and chitin. Due to its unique and non-toxic biodegradability and biocompatibility properties, keratin can be modified and developed into various forms, such as gels, films, beads, and nano/microparticles. Feather keratin is a small and uniform protein with a molar mass of 10-36 kDa. The structure of this keratin is stabilized by various covalent (e.g., disulfide bonds) and non-covalent interactions (e.g., electrostatic forces, hydrogen bonds, hydrophobic forces) [5]. Meanwhile, the high strength and stiffness of keratin are due to the high proportion of cysteine residues in the polypeptide backbone, which are held together by disulfide bonds [4].

The average poultry feather consists of 32.2% α-helix, 53.6% β-sheet, and 14.2% random coils that form turns [3]. Chicken feathers contain more than 85% β-keratin, while duck feathers are an excellent source of β-keratin-based protein comprising around 90% β-keratin [6]. β-keratin can be extracted through hydrolysis, reduction, and oxidation [7]. Among the three, the reduction process is more commonly used for β-keratin extraction owing to its high efficiency. Reducing agents decrease the stability of β-keratin fibers by disassociating disulfide bonds and hydrogen bonds as well as allowing protease access to the polypeptide backbone to dissolve proteins into solution. In the presence of reducing agents, the solubility of β-keratin increases at high temperature [4].

Numerous prior studies have demonstrated the successful extraction of β-keratin from chicken feathers through the application of various reducing agents, namely sodium metabisulfite (SMB), sodium bisulfite ($NaHSO_3$), sodium sulfide (Na_2S), and thioglycolic acid ($HSCH_2COOH$), with respective extraction yields of 87%, 84%, 53%, and 75% [8]. Other reducing agents, such as 2-mercaptoethanol and dithiothreitol (DTT), have also been reported to produce extraction efficiencies of 83.8% and 77.6%, respectively [9]. A mixture of SMB and urea ($CO(NH_2)_2$) with SDS extracted 28.1%, sodium hydroxide (NaOH) yielded 11.8%, cysteine resulted in 17.4% of β-keratin, and a mixture of SMB, urea, and Na_2S obtained 19.5% [10]. Furthermore, a urea-based mixture showed an extraction efficiency of 50.6% for β-keratin, a combination of Na_2S and NaOH yielded 41.5%, while an SDS and $NaHSO_3$mixture achieved an extraction of 18.3% [11]. These findings highlight the potential of utilizing poultry feather waste as an eco-friendly source of β-keratin through an extraction process involving SMB and SDS as reducing agents to minimize the negative impact of this waste on the environment.

Materials and Methods

Materials

In this study, β-keratin was extracted from chicken and duck feather waste collected from the Silir Chicken Market in Surakarta, Central Java on December 2-5, 2021. Sodium dodecyl sulfate (SDS) and sodium metabisulfite (MBS) used for the reduction process were acquired from Merck. The pH of the solution was maintained in the range of 10-13 using sodium hydroxide (NaOH) obtained from Sigma Aldrich. Universal pH paper was utilized for this purpose. Furthermore, the precipitation procedure employed a commercial ammonium sulfate ($(NH_4)_2SO_4$) purchased from the Muda Berkah Jogja e-commerce store, along with deionized water from the One Lab WaterOne brand.

Instrumentations

Several instruments were utilized in this study, namely: ATR-FTIR spectrometer (Agilent Cary 630), HPLC system (Thermo Fisher), and Freeze Dryer (Telstar LyoQuest).

Extraction of β-keratin from chicken and duck feathers

The collected poultry (chicken and duck) feather wastes were washed with soapy water and dried at room temperature. The dried poultry feathers were weighed and crushed with a blender. About 10-50 mL of SDS at various concentrations of 0.2 M, 0.5 M, and 1 M was added to 0.25-1.25g of poultry feathers, then the solution was heated to a temperature of 30 ºC while the pH was maintained at around 10-13. After that, the solution was stirred for 6 hours and filtered using filter paper until a clean filtrate was obtained, which was then centrifuged at 10000 rpm for 5 minutes to form a supernatant liquid. The whole process was repeated using SMB at the same concentration variations, namely 0.2 M, 0.5 M, and 1 M, as the reducing agent.

The $(NH_4)_2SO_4$ solution was added slowly to the resulting chicken feather filtrate with a 1:1 ratio, then this mixture was centrifuged at 10.000 rpm for 5 minutes to isolate solid particles. This procedure was repeated using duck feather filtrate. Next, deionized water was added to the obtained solid particles and stirred until dissolved. The mixture was then freeze-dried until a solid was obtained. The brown-colored β-keratin in the solid particles was separated using a spatula from the remaining white $(NH_4)_2SO_4$.

Yield analysis

The yield is calculated by dividing the weight of β-keratin by the weight of the feather waste sample and expressed in percent (%) as shown in the following equation 1.

$$yield = \frac{(mass\ of\ \beta\text{-}keratin\ obtained)}{(mass\ of\ feathers\ used)} \text{ x } 100\% \quad (1)$$

Identification of β-keratin structures by ATR-FTIR

The chemical structure of β-keratin was analyzed using Attenuated Total Reflectance Fourier-transform Infrared Spectroscopy (ATR-FTIR) at Sebelas Maret University, in Surakarta, Indonesia. The ATR-FTIR analysis was carried out in transmission mode to collect spectra in the wavenumber range of 4000 cm^{-1} to 650 cm^{-1}. The wavelength of β-keratin obtained in previous study was compared with the FTIR spectra of keratin extracted from feathers using sodium sulfite (Na_2S).

Identification of amino acid composition of β-keratin

The amino acid composition of β-keratin can be determined using High-Performance Liquid Chromatography (HPLC). For this purpose, 4 mL of 6 N HCl was added to 60 mg of β-keratin sample, then the mixture was heated for 24 hours at 110 ºC. Afterwards, the mixture was cooled to room temperature and neutralized (pH 7) with 6 N NaOH. Aquabides was then added to the sample to a volume of 10 mL and filtered with 0.2 mm Whatman filter paper. Next, 50 µL of the sample was added to 300 mL of OPA (Orthophalaldehyde) solution and stirred for 5 minutes, then 10 µL of the mixture was inserted into the HPLC injector L. The HPLC instrument was operated at room temperature, LiChrospher 100 RP-18 (5mm) column, an eluent flow rate of 1.5 mL/min, and mobile phases of A= CH_3OH:50 mM CH_3COONa:THF (2:96:2) pH 6.8 and B = 65% CH_3OH using isocratic elution. Furthermore, detection was performed using a Thermo Ultimate 3000 RS Fluorescence Detector, with an excitation wavelength of 300 nm and an emission wavelength of 500 nm.

Results and Discussion

Extraction of β-keratin from chicken and duck feathers

β-keratin is insoluble in water with low chemical reactivity, thereby requiring high temperature and reducing agents to increase its solubility. In the process of reducing β-keratin in poultry feathers, 2 M NaOH needs to be added so that the pH can be maintained at 10-13. NaOH functions to form an alkaline atmosphere needed in the reduction process that cannot occur in an acidic environment; without an alkaline state, protons cannot be released, thus being unable to break ionic bonds. In an alkaline state, on the other hand, protons are removed from the amino group, and the ionic bonds formed by the electrostatic attraction of the NH_3^+ group of the diamino acid and the COOH group of the dicarboxylic acid can be broken [7].

Severe extraction conditions, such as extremely high/low pH at prolonged exposure and high temperature, produce β-keratin with low molecular weight. Conversely, extraction of β-keratin under mild conditions (i.e., at a temperature of 30 ºC) will break the

disulfide bonds of the protein fibers without significantly breaking the polypeptide bonds, resulting in a stable micromolecular structure of β-keratin with a high molecular weight [8]. To extract β-keratin properly, the reduction process takes 6 hours so that the reducing agent can penetrate deeper into the cell wall. An extraction time of 6 hours has a better effect on the yield of β-keratin compared to an extraction time of 4 hours. The longer the extraction time, the higher the yield of β-keratin [12]. In this study, the reduction reaction of β-keratin is shown in equation 2 and Figure 1 [13].

$$SMB \rightarrow 2Na^{+} + SO_3^{2-} + SO_2 \quad (2)$$

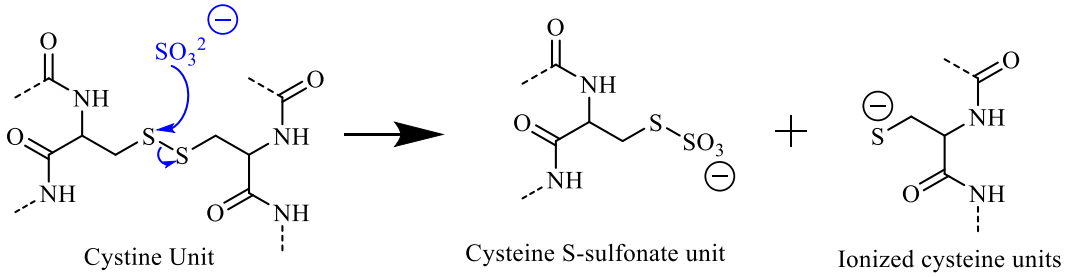


Figure 1. Reduction reaction of β-keratin from poultry feathers using SMB

During the reduction process, β-keratin undergoes heterolytic cleavage of disulfide bonds (-S-S-). The disulfite anion (SO_3^{2-}) is a weak nucleophile, whereas the sulfur atom is a relatively weak reaction center. Nucleophiles must react covalently with electrophilic groups in the protein to become sensitized. The only significant electrophilic groups in proteins are the disulfide bonds of the cystine units that can act as weak electrophiles. The SO_3^{2-} anion attacks one of the sulfur atoms in the S–S bond and breaks it heterolytically, with the thiolate anion of the cysteine unit acting as the leaving group [13].

Reduction process is closely related to a decrease in the oxidation number due to the breaking of disulfide bonds that can affect the structure of the cysteine obtained. In the reduction process of β-keratin, the S atom in the cysteine unit, which has an oxidation number of +4, will experience a decrease in its oxidation number to -1 in the ionized cysteine unit. Disulfide bonds that are disrupted by sulfites will produce cysteine thiols (reduced β-keratin) and cysteine-S-sulfonate residues (Bunte salt), where β-keratin-Cys-S- is reduced keratin and β-keratin-Cys-SSO_3- is Bunte salt [9]. In this study, precipitation of the extracted filtrate was carried out with $(NH_4)_2SO_4$ in high concentration. The addition of salt in high concentrations causes water molecules that were originally bound to the hydrophobic surface of the proteins to bind to the salt. The large number of water molecules that bind to salt ions results in the proteins interacting with each other, aggregating, and precipitating (salting out). In this regard, divalent salts such as $MgCl_2$, $MgSO_4$, and $(NH_4)_2SO_4$ are more effective for protein precipitation than monovalent salts such as NaCl and KCl. $(NH_4)_2SO_4$ is often used in protein precipitation because of its high level of solubility, non-toxicity, and low price [14].

Centrifugation of poultry feathers reduced with SMB produces a brown precipitate at the bottom of the tube, which is β-keratin. Meanwhile, centrifugation of feathers reduced with SDS will form 2 layers; the thick brown liquid in the upper layer is β-keratin, whereas the particle-free supernatant liquid in the lower layer is clear yellow, indicating that β-keratin has been separated from impurities. Additionally, the result of centrifugation of feathers reduced with SDS has a different shape from that of feathers reduced using SMB since the surfactant molecules form a micelle structure on the hydrophobic side of the protein. With the formation of the β-keratin-SDS complex and the prevention of protein aggregation, reduction process using SDS is considered faster and more efficient [9]. SDS prevents the aggregation of polypeptide chains by

blocking the formation of new cross-links, thus increasing the extraction rate and stability of the extracted β-keratin [8]. In this present study, the reduction process was repeated 3 times to increase accuracy; the more reduction processes carried out, the higher the accuracy and validity of the results obtained. The resulting β-keratin solid is brownish white, as presented in Table 1.

Table 1. Extracted β-keratin solids

Chicken Feathers						**Duck Feathers**					
SMB			**SDS**			**SMB**			**SDS**		
0.2 M	0.5 M	1 M	0.2 M	0.5 M	1 M	0.2 M	0.5 M	1 M	0.2 M	0.5 M	1 M
											

Yield analysis

The yield of β-keratin is influenced by several factors, including the concentration of the reducing agent used in the extraction process. SMB concentrations above 0.2 M will decrease the percentage of β-keratin yield as most of the disulfide bonds are broken [16]. There is also a loss of β-keratin during the extraction process, which can be caused by β-keratin not settling completely or remaining in the container when changing or washing. Furthermore, when the pH is too high or too alkaline, β-keratin typically cannot maintain its structure. In addition, hair removal with hot water (high temperature) causes non-reversible denaturation where β-keratin does not regain its original structure [17]. The yields of β-keratin extracted from chicken and duck feathers in this study can be seen in Table 2.

Table 2. Yields of β-keratin extraction at various concentrations of SMB and SDS

SMB Concentration (M)	**Yield (%)**			
	Chicken Feathers		**Duck Feathers**	
	SMB	**SDS**	**SMB**	**SDS**
0.2	76.9	25.4	37.4	39.8
0.5	32.7	19.3	14.3	38.6
1	45.6	15.3	32.5	10.8

In this study, the highest yield was achieved in the extraction of β-keratin from chicken feathers using 0.2 M SMB, which reached 76.9%. This finding is consistent with a previous study which found that chicken feathers reduced using 0.2 M SMB produced a yield of 87.6% [15]. On the other hand, the yield of β-keratin extracted from duck feathers with SDS at a concentration of 0.2 M as the reducing agent had the largest percentage (39%). Therefore, SDS was proven more effective as a reducing agent for extracting β-keratin from duck feathers.

Identification of β-keratin structures by ATR-FTIR

To measure the effect of the extraction process using SMB, the FTIR spectra of β-keratin extracted from chicken and duck feathers using SMB in different concentrations of 0.2 M, 0.5 M, and 1 M were compared, as shown in Figure 2.

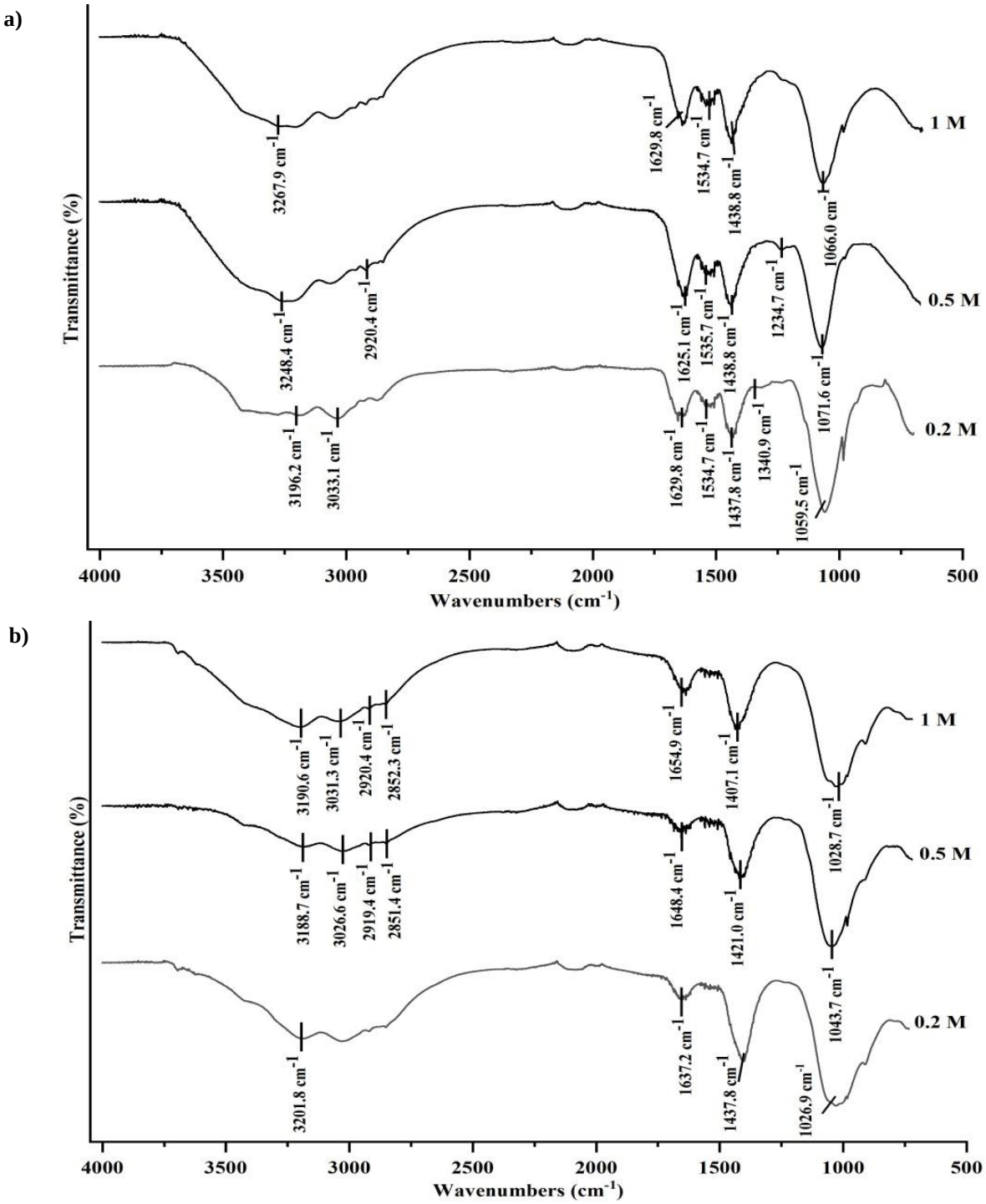


Figure 2. FTIR spectra of β-keratin using SMB reducing agent; a) chicken feathers; b) duck feathers

Similarly, to assess the effects of the extraction process using SDS, the FTIR spectra of β-keratin extracted with SDS in different concentrations of 0.2 M, 0.5 M, and 1 M were compared, as shown in Figure 3.

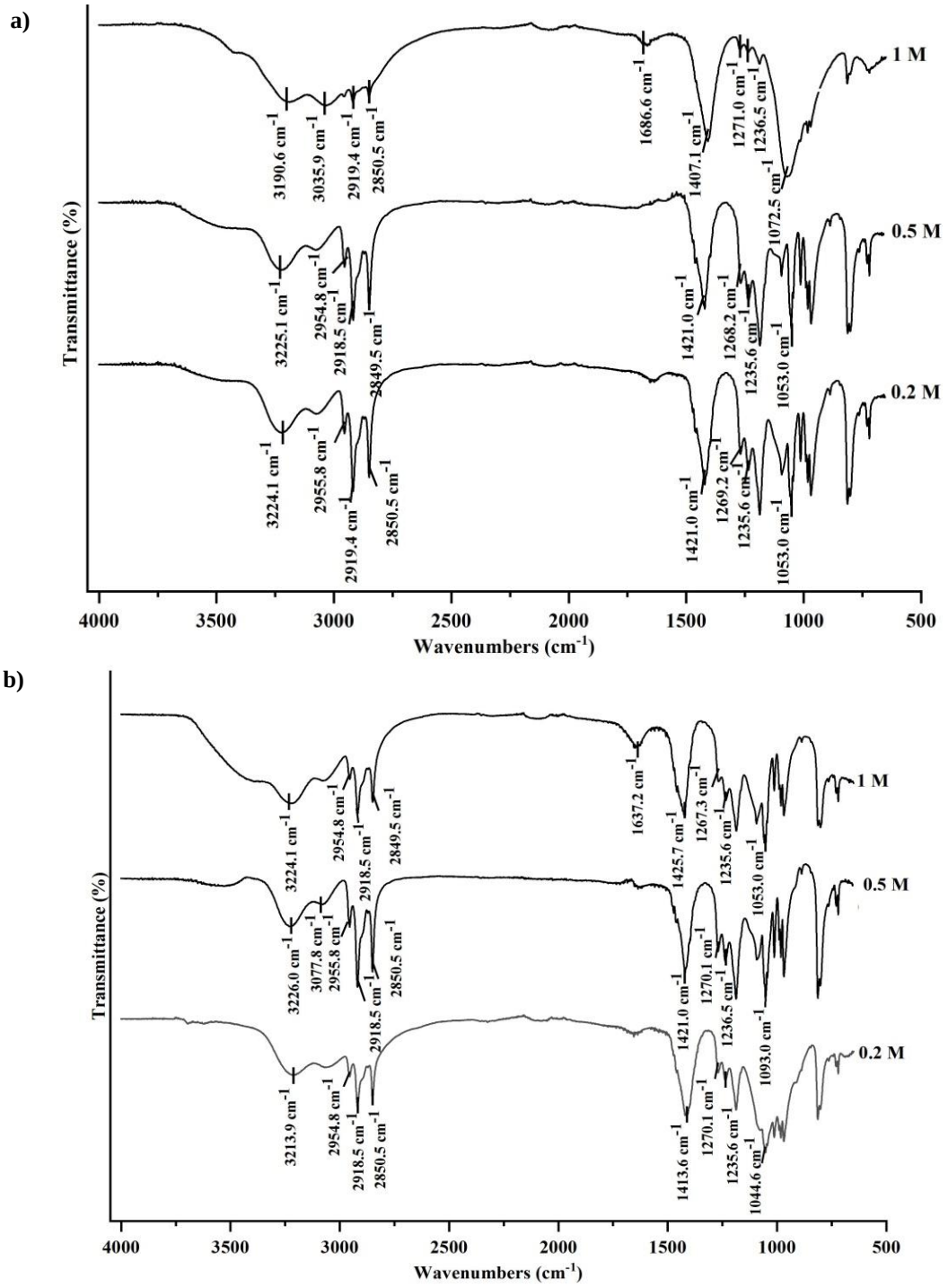
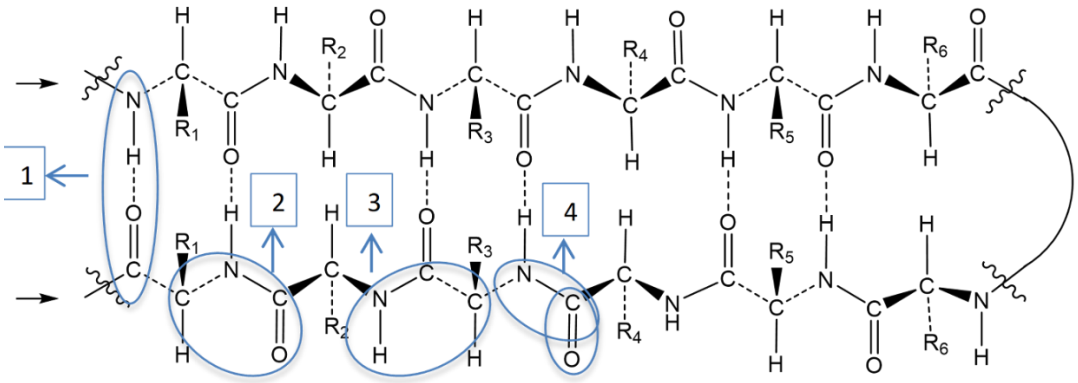


Figure 3. FTIR spectra of β-keratin using SDS reducing agent; a) chicken feathers and b) duck feathers

As seen in Figures 2 and 3, the absorption peaks of β-keratin extracted from chicken and duck feather waste using both SMB and SDS had similar characteristics at the three different concentrations. β-keratin is a protein with unique absorption properties of the peptide bonds (-CONH), and the vibrations of these peptide bonds come from amide A,

amide I, amide II, and amide III bands. The β-sheet structure of β-keratin is displayed in Figure 4.



Notes: Amide A = 1; Amide I = 2; Amide II= 3; Amide III= 4

Figure 4. β-sheet structure of β-keratin

The peaks that appear in the wavenumber range of 3200 to 3500 cm^{-1} indicate amide A and an α-helix structure. N-H and O-H stretching vibrations are insensitive to the backbone of β-keratin, but susceptible to the strength of hydrogen bonds [18]. On the other hand, the absorption bands in the range of 3000 to 2800 cm^{-1} exhibit C-H stretching vibrations and are associated with symmetric CH_3 strains. Such absorption is antisymmetric from the C-H bond in CH_3, where the side chains of amino acid residues in the primary protein chain are saturated, thus being insensitive to structural changes [19].

The extraction process affects the chemical structure of β-keratin. In this regard, the use of SMB at higher concentrations (1 M) to extract β-keratin from chicken and duck feathers causes structural damage to β-keratin as shown by the absence of an absorption pattern at wavenumbers ranging from 2800 to 3000 cm^{-1}. Contrarily, in the extraction process with SDS at high concentrations (1 M), an absorption pattern appears because the reaction using surfactants leads to the formation of by-products in the form of C=O groups [20].

Amide bands I–III provide crucial information about the conformation and structure of the protein backbone. The split peak from 1700-1600 cm^{-1} (Amide I region) corresponds to the C=O stretching vibrations and originates from the combination of α-helix and β-sheet [4]. C=O stretching vibrations are the most sensitive part of proteins in determining secondary structure. These bonds are directly related to the conformation of the backbone, and each type of secondary structure has different C=O strains that can change the conformation of proteins and polypeptides [21].

The amide II region which originates from N-H bending and C-H stretching, signifying absorption bands in the range of 1580 and 1480 cm^{-1}, gives information about the vibration bands of the protein backbone and is related to the β-sheet structure by resulting in lower sensitivity to changes in protein conformation, compared to the amide I band [22]. The absorption band with a weak peak of around 1300 to 1220 cm^{-1} is in the amide III region, which is caused by a combination of C-N stretching and N-H in-plane bending as well as some contributions from C-C stretching and C=O bending [4]. The absorption pattern of amide III as shown in Figure 4 is not visible because most of the disulfide bonds have been broken at SMB concentrations above 0.2 M [16].

The absorption band in the range of 1000 to 1200 cm^{-1} is associated with the asymmetric and symmetric S–O stretching vibrations of the cysteine-S-sulfonate residues formed from the reaction of sulfite and cysteine during the protein extraction process [23]. The sharp

peak also indicates an increase in the content of cysteine-S-sulfonate residues in β-keratin [24]. However, because signals from the disulfide bonds (S–S) cannot be detected by FTIR spectroscopy, the use of Raman spectroscopy is highly necessary to verify these bonds. Absorption of disulfide bonds (S–S) in Raman will appear at 510 cm^{-1} [25]. Based on the FTIR spectra of β-keratin above, β-keratin extracted from both chicken and duck feathers using SDS has a better absorption pattern as SDS itself can prevent the aggregation of polypeptide chains by blocking the formation of new cross-links in the protein, thus increasing the extraction rate and structural stability of the extracted β-keratin [8].

Identification of amino acid composition of β-keratin by HPLC

The process of analyzing the amino acid composition of β-keratin using HPLC was carried out in several stages: sample preparation, standard solution preparation, derivatization, and injection. The preparation of β-keratin began with a hydrolysis process using HCl. The data obtained from this process were in the form of area, retention time, and amino acid levels. The Figure 5 displays the chromatograms of β-keratin extracted from chicken and duck feathers.

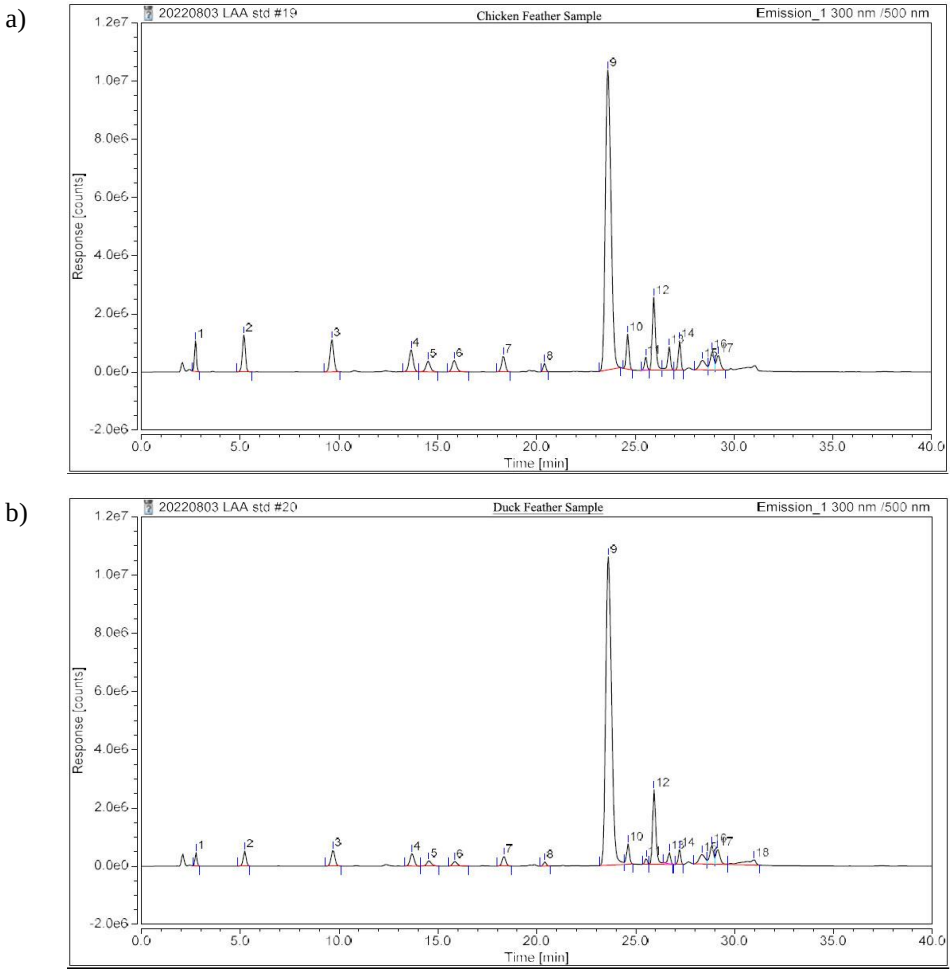


Figure 5. HPLC chromatograms of β-keratin from; a) chicken feathers and b) duck feathers

Based on the chromatograms, the type of amino acids contained in β-keratin in chicken feathers can be determined by comparing the obtained retention time with amino acid standards. Meanwhile, to find out the levels, calculations were carried out using the standard curve functions for amino acids. The amino acids that

make up β-keratin in chicken and duck feathers are: 1) aspartic acid; 2) glutamic acid; 3) serine; 4) glycine; 5) threonine; 6) arginine; 7) alanine; 8) tyrosine; 10) valine; 11) phenylalanine; 13) ileucine; and 14) leucine. The calculation results showed that the highest concentration of amino acids in chicken feathers is glutamic acid at 24.61 ppm. This is in line with a previous study which mentioned that glutamic acid is the most abundant amino acid in chicken feathers [11]. Meanwhile, the highest amino acid content in duck feathers is serine at 11.15 ppm. This finding also supports another prior study which stated that serine is the most abundant amino acid in duck feathers [26]. However, peaks 9, 12, 15, 16, 17, and 18 in Figure 5 do not indicate the types of amino acids that make up β-keratin in chicken and duck feathers but are rather considered as impurities.

Conclusion

The optimal concentration of reducing agent for the extraction of β-keratin was 0.2 M for both chicken feather waste and duck feather waste. The utilization of SMB in the extraction process of β-keratin from chicken feather waste produced the highest yield of 76.9%. Meanwhile, extracting β-keratin from duck feather waste using SDS resulted in a maximum yield of 39.8%. These findings indicate that SMB is preferred as reducing agent for the extraction of β-keratin from chicken feathers, whereas SDS is more effective for duck feathers.

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RESPONSE SURFACE METHODOLOGY FOR OPTIMISING CROSS-LINKING CONDITIONS OF UNRIPE BANANA (*Musa balbisiana* VAR. Abu) STARCH

(Kaedah Tindak Balas Permukaan untuk Pengoptimuman Keadaan Penghubung Silang Kanji Pisang Belum Masak (*Musa balbisiana* Var. Abu))

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Abstract

Starch is the most abundant organic compound found in nature after cellulose. However, native starch has undesirable properties which could limit its application in food industries. Therefore, starch modification is necessary to enhance its positive attributes and to eliminate the limitations of its specific application in food manufacturing. This study aims to optimize the conditions of the cross-linking of unripe banana (*Musa balbisiana* var. Abu) starch using response surface methodology (RSM). The optimization was conducted using the central composite design (CCD) approach. The extracted unripe banana (*Musa balbisiana* var. Abu) starch was cross-linked with sodium trimetaphosphate (STMP) and sodium tripolyphosphate (STPP) mixture as the cross-linking agent. The two independent variables selected were temperature (40.0- 60.0 ℃) and pH (9.00-11.00). The maximum degree of cross-linking was obtained at the following optimum conditions: temperature 50.57 ℃ and pH 9.52. The degree of cross-linking obtained at the optimum conditions of RSM was verified, and the value obtained was 81.73%. There was no significant difference (*p*-value >0.05) between the predicted and verified degree of cross-linking values. Thus, it indicated that the predicted optimum condition by RSM can be accepted. These outcomes of this study are very important as they would assist the food industries in developing and designing the process of optimal cross-linking of starch from unripe bananas, which can be used in the future.

Keywords: response surface methodology, unripe banana, banana starch, starch modification, cross-linking

Abstrak

Kanji adalah sebatian organik yang paling banyak ditemui dalam alam semula jadi selepas selulosa. Walau bagaimanapun, kanji semula jadi yang belum diproses mempunyai sifat yang tidak diingini yang boleh mengehadkan penggunaannya dalam industri makanan. Oleh itu, pengubahsuaian kanji adalah perlu untuk meningkatkan kebaikan justeru dapat menghapuskan had penggunaan khususnya dalam pembuatan makanan. Matlamat kajian ini adalah untuk mengoptimumkan keadaan ikatan silang kanji pisang belum masak (*Musa balbisiana* var. Abu) menggunakan kaedah tindak balas permukaan (RSM). Pengoptimuman telah dijalankan dengan pendekatan reka bentuk komposit pusat (CCD). Kanji pisang belum masak (*Musa balbisiana* var. Abu) yang diekstrak telah diikat silang dengan campuran natrium trimetafosfat (STMP) dan natrium tripolifosfat (STPP) sebagai agen

penghubung silang. Dua pembolehubah bebas yang dipilih ialah suhu (40.0-60.0 °C) dan pH (9.00-11.00). Darjah maksimum penghubung silang diperoleh pada keadaan optimum berikut: suhu 50.57 °C dan pH 9.52. Darjah silang yang diperoleh pada keadaan optimum RSM telah disahkan dan nilai yang diperoleh ialah 81.73%. Tidak terdapat perbezaan yang signifikan (nilai p >0.05) antara tahap ramalan dan disahkan nilai silang silang, oleh itu ia menunjukkan bahawa keadaan optimum yang diramalkan oleh RSM boleh diterima. Hasil kajian ini adalah sangat penting kerana ia akan membantu industri makanan untuk membangunkan dan mereka bentuk proses pengikatan silang kanji yang optimum daripada pisang yang belum masak, yang boleh digunakan pada masa hadapan.

Kata kunci: kaedah tindak balas permukaan, pisang belum masak, kanji pisang, pengubahsuaian kanji, penghubung silang

Introduction

Starch, a storage carbohydrate in plants, can be found abundantly in leaves, flowers, fruits, seeds, stems, and roots [1]. It has wide functions and applications in food industries and is commonly used as a thickener, binder, gelling agent, foam stabilizer and texture improver [2]. Starch can be basically manufactured by the combination of the following steps: grinding of the starch-rich crop and wet separation technique to sediment the starch granules in water. Starch has gained much attention due to its usefulness in food and non-food industries, wide availability, low cost of extraction and purification, edibility and total composability without toxic residues [3, 4]. Most of the starches are obtained from cereals such as corn, wheat and various rice, tubers, and roots such as potatoes and cassava. In this regard, unripe bananas have been studied as a potential source of starch [5-7].

Banana, a climacteric fruit that belongs to the *Musaceae* family and *Musa balbisiana*, is the most widely distributed banana in tropical and subtropical regions [8]. The banana fruits were usually harvested at the unripened stage for long handling and transportation because they are perishable fruits that could lead to serious economic losses during maturation [9-11]. The starch content from unripe bananas is between 70-80%, depending on their variety and maturity index, and its starch content is similar to other starches obtained from the endosperm of corn and potatoes [8, 12-14]. Apart from digestible starch, it also contains a high amount of resistant starch and amylose and amylopectin ratio that has potential in diabetes treatment since it contributes to low glycaemic index [7, 15]. Most of the starch obtained from different sources is in its native state. The native starches were found incompetent for industrial use, leading to the least suitability in processing [16] and posing many limitations with regards to their application and functionality that related to the processing conditions; for example-weak-bodied and they produce a cohesive and rubbery paste [16] and undesirable gel upon cooling, easily retrograded, having a lower solubility in cold water, lower viscosity and thickening characteristic after cooking and low shear stress and thermal resistance at higher temperature [17-20]. However, these limitations can be overcome and improved through starch modification treatments [20, 21].

There are several methods of starch modification treatments, which can be classified as physical and chemical methods. Chemical methods involve the use of chemical agents, whereas physical methods involve the application of several physical factors (temperature, pressure, shear, moisture, radiation, ultrasonic wave, and etc). The examples of physical methods include pre-gelatinization, extrusion, heat-moisturizing, annealing, and microwave treatments [22]. These methods are able to change particle size, surface properties, solubility index and functional properties such as water absorption [23]. The most common method for chemical modification is cross-linking treatments. Cross-linking treatment involves the introduction of functional groups to the native starch in the presence of cross-linking agents that are responsible for the formation of the intra or inter-ester or ether linkages between the hydroxyl (-OH) groups in the starch amorphous region [20, 21, 24-26]. The most commonly cross-linking agents used in food industries are phosphorus oxychloride or phosphoryl chloride ($POCl_3$), sodium trimetaphosphate (STMP),

epichlorohydrin (EPCH) and sodium tripolyphosphate (STPP) [26-28]. STMP is the most widely used cross-linking agent as it is non-toxic and has a slow penetration rate [29]. According to Xie et al. [26], chemical modification is usually employed to enhance the mechanical strength of the starch. This method limits the starch swelling to a greater extent because the linkages formed restrict the mobility of the starch molecules in the amorphous region [30]. As a result, it reduces subsequent disintegration during hydrothermal processing [31]. In addition, it also increases resistance to high temperature, shear resistance and the stability of the swollen starch granules [32, 33]. Cross-linking not only alters the chemical and physical properties but also the thermal transition characteristic of the starch. However, its effect depends on the botanical sources of the starch and the cross-linking agent [21].

Cross-linking has been previously performed by many researchers using different temperatures and pH on different starch sources, including porous wheat starches, rice starches, tapioca starches and corn starches [20, 26, 34, 35]. Since the starch modification involves more than one factor to affect the desired response, therefore Response Surface Methodology (RSM) is the most suitable and effective tool to be used since it can provide relevant information in the shortest time with the least number of experimental runs [36]. According to Bambang et al. [37], optimization is a method used to identify and improve the best solution based on specific quality criteria such as process efficiency. It involves a few steps, including the selection of independent variables and responses, choosing of experimental design strategy, running of experiments and data collection, fitting of the model equation to experimental data, verifying the response graph and model (ANOVA), and lastly determining the optimal conditions for the design the possible approaches available in RSM is central composite design (CCD).

Previously, researchers have used RSM to optimize different starch modification processes in various studies. For example, the application of RSM for the pre-gelatinization process of sweet potato and walur starches [37, 38], to modify sago starch through octenyl succinic anhydride (OSA) process [39] and also the cross-linking of corn and wheat starches [40]. Hence, this study aims to optimize the conditions (temperature: 40-60 ℃ and pH value: 9.00-11.00) for unripe banana (*Musa balbisiana* var. Abu) starch modification using the cross-linking method to obtain cross-linked starch with improved physicochemical properties.

Materials and Methods

Materials

An unripe banana (*Musa balbisiana* var. Abu) at maturity index 2 (peel color: green with a trace of yellow) was purchased from a local seller/farmer in Kota Kemuning, Shah Alam. Sodium sulphate, sodium trimetaphosphate (STMP), sodium tripolyphosphate (STPP), sodium hydroxide (NaOH), hydrochloric acid (HCl) and sodium sulphate (Na_2SO_4) used were purchased from Sigma-Aldrich

Flour preparation

Banana flour was prepared by using the method from Ayo-Omogie et al. [41]. The banana was washed, peeled and sliced to 2-3mm thickness using a slicer and was frozen in a freezer (Sanyo, MDF-U5412, Japan) at the temperature of -40 ℃. The frozen samples were then transferred into the freeze dryer (Martin Christ, Alpha 1-4 LD plus), which operated at -50 ℃ and 0.1m Pa. After the drying was completed, dried samples were mashed using a pestle and mortar and kept in an air-tight container for further analysis.

Starch extraction

Agama-Acevedo et al. [42] used the starch extraction method with slight modifications. 100g of banana flour was suspended in 1 L of 1% sodium sulfate solution (pH 4.5), and the suspension was divided into several glass jars. All the glass jars were capped and kept in an incubator shaker at room temperature (27 ℃) for 12 hours. After that, the suspension was centrifuged at 3000 rpm for 15 minutes, and the supernatant was discarded. The sediment was then re-suspended with distilled water, filtered through 200 US mesh to remove the fibre in the solution and rinsed several times to ensure the maximum amount of

starch could be collected. The residue was discarded, and lastly, the filtrate was centrifuged at 3000 rpm for 15 minutes. The white starch sediment obtained was collected and dried in a drying oven (Memmert, Germany) at 45 ℃ for 24 hours.

Experimental design

In this experiment, RSM was used to optimize the cross-linking of banana starch to achieve an optimal degree of cross-linking (%). The experiment was optimized using central composite design (CCD) and conducted in a randomized manner using the Design Expert 12 statistical package. The degree of cross-linking (%) was chosen as the response value, while the temperature (X_1) and pH (X_2) were selected as the independent variables. The ranges for both independent variables were temperature (40-60 ℃) and pH (9.00-11.00), as shown in Table 1.

Table 1. Coded and un-coded table for range and level of independent variables for cross-linking method

Independent Variables		**Factor Level**				
		$-\alpha$	**-1**	**0**	**+1**	**$+\alpha$**
Temperature, (℃)	X_1	30.00	40.00	50.00	60.00	70.00
pH	X_2	8.00	9.00	10.00	11.00	12.00

About 5g of unripe banana starch was dispersed in distilled water (75 mL) containing sodium sulphate (10%, w/w starch basis) and a 99:1 mixture of STMP/STPP (12%) as described by Park et al. [43]. The dispersion was adjusted to various pH (8.59-11.41) with 0.1 N NaOH solution and was kept at different temperatures (35.86-64.14 ℃) in a shaking water bath for 2 hours at 200 rpm. After cooling to ambient temperature, 0.1 N HCl solution was added until the dispersion reached pH 6.0. The starch was recovered by centrifuging at 3,600 rpm for 10 minutes (Kubota 5420, Japan), and the precipitate was washed with 100 mL distilled water (3 times). This sample was dried at 35 ˚C for 24 hours, ground in a mortar, sieved and later stored in a glass jar.

Determination of degree of cross-linking of the cross-linked unripe banana starch

The degree of cross-linking of the modified starch was determined using the method of Kaur et al. [44] with slight modification by using a rheometer (Anton Paar MCR 301). About 5 mL of distilled water was added into the rheometer cell containing 0.6 g of sample and was held at a temperature of 45℃ for 2 minutes. Next, it was heated up to 95℃ at the heating rate of 10℃/min and held for 3 minutes, then cooled to 45℃ at the cooling rate of 10℃/min. The peak viscosity values of the native and cross-linked starch were recorded, and the degree of cross-linking was calculated according to the following formula [45]:

$$\text{Degree of cross-linking, \%} = \frac{A-B}{A} \times 100 \quad (1)$$

Where A is the peak viscosity of the native starch, and B is the peak viscosity of the modified starch.

Swelling power and solubility index of native and modified starches

The swelling power and solubility of native and modified starches were conducted according to the Kittipongpatana and Kittipongpatana methods [46]. About 0.4g (dry basis) of starch samples were mixed with 12.5 mL of distilled water in a 15.0 mL centrifuge tube (pre-weighed). The mixture was equilibrated for 5 minutes at 25 ℃ and heated to 60 ℃ in a shaking water bath for 30 minutes. Next, the samples were cooled in an ice water bath for 1 minute, kept equilibrated at 25 ℃ for another 5 minutes and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected, placed in an evaporating dish and dried at 130 ℃ for 4 hours. The swollen starch sediment was weighed. The swelling power

and solubility of the sample was calculated as follows:

$$\text{Swelling power, (g/g):} \quad \frac{weight\ of\ sediment}{initial\ weight\ of\ dry\ sample} \tag{2}$$

$$\text{Solubility index, (\%)} \quad : \frac{weight\ of\ dried\ supernatant}{initial\ weight\ of\ dry\ sample} \times 100 \tag{3}$$

Results and Discussion

Optimization of cross-linking condition using response surface methodology

Cross-linking of starch is a method that involves the addition of functional groups into the starch molecules, which affects the physicochemical properties of this starch. Response Surface Methodology (RSM) was conducted at different temperatures (40-60 ℃) and pH values (9.00-11.00) (Table 2) to obtain the optimum condition for the cross-linking. Table 2 shows the experimental values (Y) and the predicted values (FITS) of the response generated by the software. The highest experimental and predicted response values were at run number 13, which were 79.70% and 79.27%, respectively, under the temperature of 50 ℃ and pH 10.00 (Table 2). On the other hand, the lowest experimental and predicted response values were at run number 4, which were 35.83% and 35.73%, respectively, at the temperature of 60℃ and pH 11.00. According to Almonaityte et al. [47], natural starches are unstable to changes in pH and temperature. Therefore, minor changes in pH and temperature could influence its degree of cross-linking. Furthermore, different cross-linking agent also have different optimal pH and temperature ranges [48, 49]. In certain conditions, some level of alkalinity can facilitate a reaction by transforming the hydroxyl groups within starch molecules into alkoxide anions through ionization [50]. The response obtained shows that the degree of cross-linking was in the range of 35.83% to 79.70% (Table 2).

Table 2. Full RSM experimental design and the comparison between experimental and predicted values of the degree of cross-linking (%)

Run	Factors		Response (Degree of cross-linking, %)	
	X_1	X_2	Y	FITS
1	40.00	9.00	62.40	62.65
2	60.00	9.00	60.08	60.33
3	40.00	11.00	42.75	42.66
4	60.00	11.00	35.83	35.73
5	35.86	10.00	48.50	48.42
6	64.14	10.00	41.95	41.88
7	50.00	8.59	71.62	71.30
8	50.00	11.41	39.61	39.77
9	50.00	10.00	79.56	79.27
10	50.00	10.00	79.37	79.27
11	50.00	10.00	79.16	79.27
12	50.00	10.00	78.54	79.27
13	50.00	10.00	79.70	79.27
R^2: 0.9997%, R^2 (adj): 0.9995%, CV: 0.65%				

Note: X_1: Temperature (℃), X_2: pH value, Y: Experimental value, FITS: Predicted value, CV: Coefficient of Variation

The goodness of fit of the regression model was defined by determining the R^2 and adjusted R^2, which provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. Theoretically, the R^2 values should be at least 0.8 to have a good fit model.

The value closer to 1.0 shows a better fitting of the model with the actual data and a good correlation between experimental and predicted values. The R^2 value obtained was 0.9997, which means 0.9997 of the variability in the observed response could be explained by the model, and the remaining 0.0003 of the variability cannot be explained by the model obtained due to other factors that are not included in the model (Table 2). It indicated a strong and reasonable fit of the models to the experimental data [51].

The adjusted R^2 is the corrected value for R^2 after the elimination of unnecessary model terms. The adjusted R^2 obtained should not be more than 0.2 difference from the R^2 value [52] and it was observed that the adjusted R^2 value was 0.9995. The smaller difference between the adjusted R^2 and the R^2 shows that there are many non-significant terms included in the model, and it signifies the adequacy of model accuracy [53]. The coefficient of variation (CV) is the ratio of the standard error of estimation to the mean value of the observed response [54] and expressed as percentage (%). The CV of the model was 0.65% (Table 2). Firatligil-Durmus and Evranuz [55] stated that a model can be considered reasonably reproducible if its CV is not more than 10%. Therefore, the obtained CV has good reproducibility for this model and can be employed for the optimization process. A response surface regression analysis was performed, and the result for both regression and coefficient for the optimization of cross-linking of unripe banana starch can be presented as a coded equation at a 5% level:

$$Y_1 = 79.27 - 2.31X_1 - 11.15X_2 - 1.15X_1X_2 - 17.06X_1^2 - 11.86X_2^2$$

Where, Y1 = Degree of cross-linking (%), X_1 = Temperature (°C), and X_2 = pH value

Analysis of variance for the optimization of cross-linking condition of unripe banana starch

The effects of experimental variables on the linear, square and interaction terms were tested for adequacy and fitness by analysis of variance (ANOVA). The summary of the results obtained is presented in Table 3. The p-value is the probability of the factors having a very little or insignificant effect on the response. The larger F-value signifies the better fit of the RSM model to the experimental data. The F-value should be larger, and the p-value should be lower than 0.05 in order for the model to be statistically significant [56]. Therefore, all the regression, linear, square, and interaction models found in this study are highly significant as they have p-values lower than 0.05.

The lack-of-fit test measures the variation of the data concerning the fitted model and is one of the important aspects of a reduced model. If the lack of fit is significant, it means that the model does not fit the data well. The data should be rejected if the results show any significance in the lack-of-fit test. Insignificant lack-of-fit indicates a good model, which means that the p-value must be higher than 0.05. The F-value of the lack-of-fit can be obtained by dividing the lack-of-fit mean square by its pure error mean square. In this study, the lack-of-fit of the selected model showed a non-significant result (*p*-value >0.05) with a *p*-value of 0.7270. Thus, the non-significant p-value indicates that the model is good and fits well with the experimental data, and the parameters have a significant effect on the response value [57].

Table 3. Analysis of variance (ANOVA) of overall effects of test variables on the optimization of cross-linking

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value	Status
Model	3723.98	5	744.80	4712.63	< 0.0001	Significant.
X_1	42.80	1	42.80	270.79	< 0.0001	Significant
X_2	993.89	1	993.89	6288.73	< 0.0001	Significant.
X_1X_2	5.29	1	5.29	33.47	0.0007	Significant.
X_1X_1	2024.47	1	2024.47	12809.66	< 0.0001	Significant.
X_2X_2	979.20	1	979.20	6195.81	< 0.0001	Significant
Residual	1.11	7	0.1580			
Lack of Fit	0.2824	3	0.0941	0.4570	0.7270	Not Significant
Pure Error	0.8239	4	0.2060			
Total	3725.09	12				

Note: X_1: Temperature (℃), X_2: pH value, df: Degree of freedom

Contour and surface plot

The previously mentioned model was used for the optimization process, and the contour and surface plot was generated as the graphical representation of the response factor based on the regression equation above. The response variable was set to a maximum while both temperature and pH variables were kept in range all the time.

The desirability value was used to determine the optimum combination of the variables for the cross-linking conditions of the starch that leads to the maximized response while satisfying the restriction of the model [58,59]. The value for desirability is between 0.000 and 1.000 for a set of responses [51], and usually, the factor setting/combination that maximized the desirability for the optimization was chosen. The ideal optimum desirability in the RSM model is 1.000, with a value between 0.6 and 0.8 is considered acceptable. The highest desirability value of 1.000 (Figure 1) was obtained at a temperature of 50.57℃ with pH 9.52 **(**these values have been rounded to the nearest integer**)** and was identified as the optimum condition for the cross-linking of the unripe banana starch in this study. A high desirability value signified that the optimized model had high potential in producing the desired outcome aimed for the model.

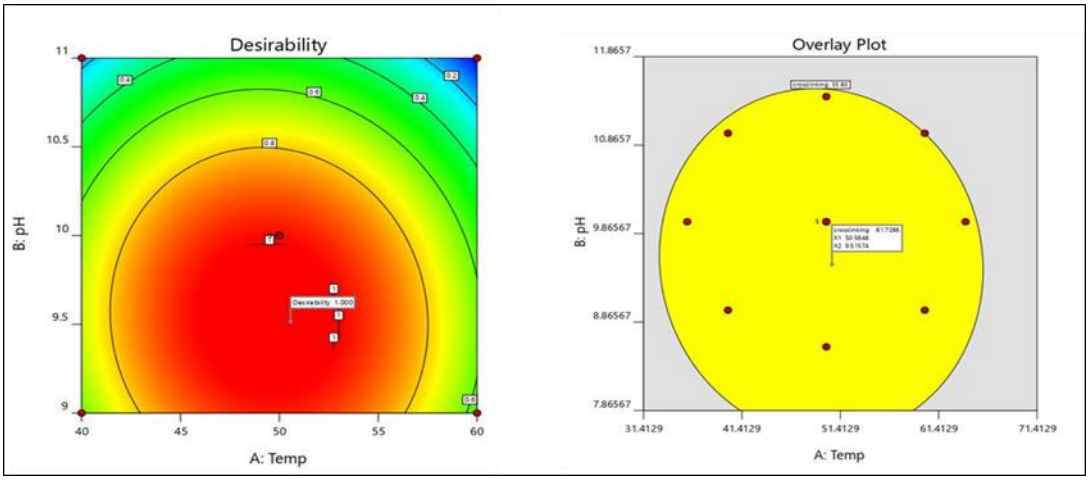


Figure 1. Desirability and overlaid contour plot for the degree of cross-linking (%) at the predicted optimum conditions

The overlaid contour plot is a technique used to combine the variables and response in a similar graph, and the conclusion can be made according to the interaction between the variables and response [60]. The overlaid contour plot is presented in Figure 1 in order to identify the feasibility of the response value at the optimum condition of the variables. The overlaid contour plot showed that the suggested optimum conditions for the temperature (50.57 ℃) and pH (9.52) fell in the yellow region, with the predicted value of the response (degree of cross-linking) of 81.73%. The yellow region means the entire range of all the intervals meets the specified criteria, which indicates that the conditions are feasible to be carried out. On the contrary, the grey region represents the area in which the conditions are not fit to be experimentally done.

The two-dimensional (2D) contour plot and three-dimensional (3D) surface plot (Figure 2) affirmed the effect of both temperature and pH as independent variables towards the degree of cross-linking. The red zone in both 2D and 3D plots represents the higher degree of cross-linking, while the blue zone indicates the lowest. The temperature and pH have greater interaction and also highly contribute to the response, as indicated by the steeper and more curved surface [61] (Figure 2). The 3D plot explained that the increase in temperature and time led to a substantial increase in the response until it achieved the optimal conditions at a temperature of 50.57 ℃ and pH 9.52. However, the response decreased significantly at the point higher than the optimal conditions, which means the variables have a negative impact on the degree of cross-linking. Relatively, the optimal conditions for this experiment correspond to the maximum degree of cross-linking of 81.73%.

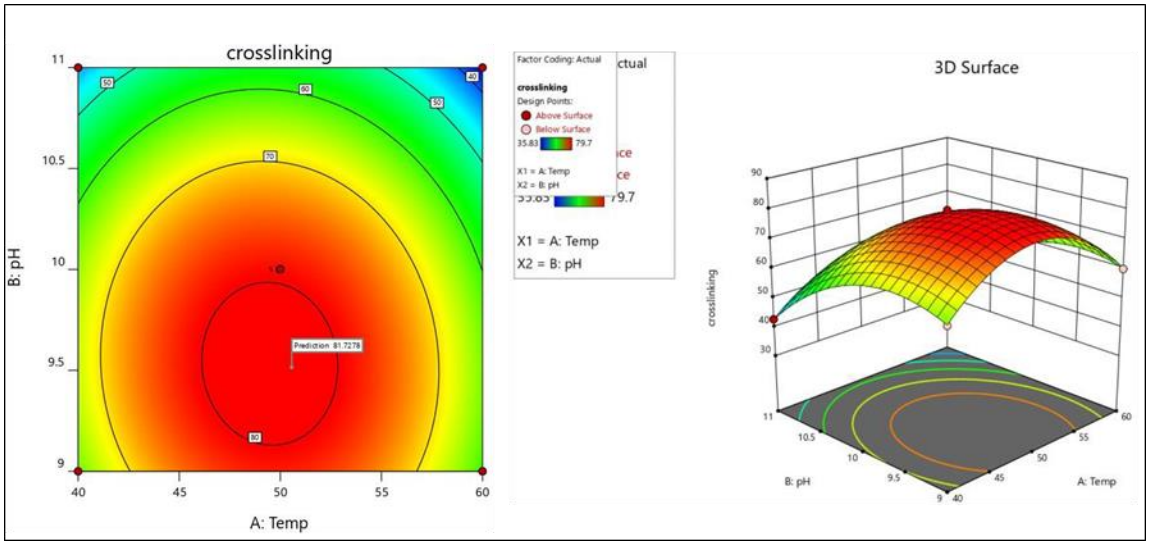


Figure 2. Two-dimensional (2D) contour plot and three-dimensional (3D) surface plot of the degree of cross-linking (%) at the predicted optimum conditions

Verification of the feasible optimum condition

In order to achieve a precise estimation of the optimum condition of all the variables and a prediction of the model fitness, both numerical and graphical validation plays a definite role in deciding the optimum variable combination for a maximum output [62]. The suitability of the model equation for predicting the optimum response values was further evaluated and tested using an independent sample t-test by SPSS between the experimental and predicted values. The result in Table 4 shows that there is no significant difference (p-value>0.05**)** between the experimental values (82.38%) and predicted values (81.73%) for all dependent variables. Therefore, the validation of the model was confirmed, and the mentioned model can be applied for the optimization of the cross-linking of

unripe banana starch.

Table 4. Verification of the experimental and predicted value of the degree of cross-linking (%) at the optimum condition

Optimum Condition		Degree of cross-linking (%)	
X_1	X_2	Y	FITS
50.57	9.52	82.38[a]	81.73[a]

Note: X_1: Temperature (℃), X_2: pH Value, Y: Experimental Value, FITS: Predicted Value. Means with same letters within a column are not significantly different (p >0.05) between experimental and predicted values.

Degree of cross-linking and its effects on swelling and solubility

The degree of cross-linking of the cross-linked starch was measured and calculated using its peak viscosity value. Yin et al. [63] have mentioned that peak viscosity is the most useful property to measure the degree of cross-linking. As shown in Table 5, the peak viscosities obtained are between 8.40 and 18.99 cP, at 35 to 75% degree of cross-linking. It was observed that the peak viscosities of this modified starch decreased significantly as the degree of cross-linking was above 75%, with peak viscosity ranging between 6.01 and 6.35 cP. The highest degree of cross-linking obtained from this study was 79.70%, with a peak viscosity of 6.01 cP. Almonaityre et al. [47] explained that the degree of cross-linking affects the peak viscosity of the cross-linked starch paste, where starch paste with a higher level of cross-linking will show a lower peak viscosity [21]. The findings of this study agree with Babu et al. [64] (Table 5). The presence of an STMP/STPP mixture in an alkaline condition caused a large number of hydrophilic phosphorous groups to react with hydroxyl moieties of starch chains, enabling di-starch phosphate interaction in the inner of the granules [62, 63]. Kou et al. [65] also stated that a higher amount of phosphorous will produce a higher degree of cross-linking, and an increase in the alkalinity of the cross-linking medium will accelerate this reaction [48]. The introduction of the covalent bonds or the cross-links in the granules, therefore, decreased the mobility of the molecules and strengthened the glucose chain [47]. This outcome will inhibit the granular breakdown, producing more stable starch toward thermal and mechanical energy and resulting in a lower peak viscosity value.

Table 5. Peak viscosity and degree of cross-linking (%) of each RSM run order

Run	Factor		Peak viscosity (cP)	Degree of Cross-Linking (%)
	X_1	X_2		
1	40.00	9.00	11.13	62.40
2	60.00	9.00	11.82	60.08
3	40.00	11.00	16.95	42.75
4	60.00	11.00	18.99	35.83
5	35.86	10.00	15.24	48.5
6	64.14	10.00	17.18	41.95
7	50.00	8.59	8.40	71.62
8	50.00	11.41	17.88	39.61
9	50.00	10.00	6.05	79.56
10	50.00	10.00	6.11	79.37
11	50.00	10.00	6.17	79.16
12	50.00	10.00	6.35	78.54
13	50.00	10.00	6.01	79.70

Note: X_1: Temperature (℃), X_2: pH, cP: Centipoise

Table 6 shows the swelling and solubility results for native and cross-linked starches at the optimum conditions suggested by RSM. Swelling is the hydration capacity of starch and the extent of starch granules interaction within both amorphous and crystalline regions [66]. In contrast, solubility is associated with the leaching of amylose from the starch granules during swelling [67]. The results revealed that both swelling, and solubility of native starch were reduced from 2.0528 g/g and 13.17% to 1.9011 g/g and 8.56%, respectively, after the cross-linking treatment at the optimum conditions (temperature: 50.57℃, pH: 9.52) with 81.73% degree of cross-linking. According to Carmona-Garcia et al. [68], a higher degree of cross-linking resulted in a decrease of hydration properties and inhibition of water penetration for swelling, thus lowering swelling of the starch [48, 65] and restricting the starch molecules (mainly amylose) to leach out from the starch granule [66, 67] and reduce the starch solubility [69].

Table 6. Swelling power and solubility index of native and cross-linked starch

Starch Sample	Native	Cross-Linked
Swelling power (g/g)	$2.0528^{a} \pm 0.0008$	$1.9011^{b} \pm 0.0011$
Solubility index (%)	$13.17^{a} \pm 0.0200$	$8.56^{b} \pm 0.0200$

Note: Means with different letters within the row are significantly different (p <0.05) between native and cross-linked samples

Conclusion

The optimisation of starch modification through cross-linking by using RSM was successfully investigated, and the data was systematically interpreted. The optimisation of the present work through CCD revealed that the best optimum conditions for a maximum degree of cross-linking (81.73%) were temperature 50.57 ℃ and pH 9.52. Both variables (temperature and pH) had a significant effect on the response (degree of cross-linking). It was also observed that the degree of cross-linking affects the peak viscosity of the modified starch in the RSM run. The peak viscosity decreased significantly when the degree of cross-linking was higher than 75. It was also observed that cross-linking has significant effects on the swelling power and solubility of the starch, whereby both the swelling power and solubility index of cross-linked starch were significantly decreased. Swelling power decreased from 2.0528 g/g to 1.9011, and the solubility index decreased from 13/17% to 8.56%. This was due to the new linkages created during the cross-linking process that strengthened the starch intermolecular interaction and integrity. Thus, this prevents chain disruption and breakage and reduces swelling power and solubility index by limiting swelling and amylose leaching. As additional covalent bonds were introduced through the cross-linking process, cross-linked starch exhibits higher tolerance to acid, heat, and shear forces and improves its viscosity properties. Therefore, cross-linked starch can be used as a thickening, texturizing and stabilizing agent in foods such as yoghurt, sauces and dairy products.

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MODIFIED CHRYSIN-BASED BIOSORBENT FOR THE DISPERSIVE MICRO-SOLID PHASE EXTRACTION OF SELECTED PHENOLIC ACIDS FROM STINGLESS BEE HONEY

(Biopenjerap yang Diubahsuai berasaskan Krisin untuk Pengekstrakan Fasa Pepejal-mikro Asid Fenolik Terpilih secara Serakan daripada Madu Lebah Tanpa Sengat)

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Abstract

This study focuses on the preparation, characterisation, and application of a chrysin-based biosorbent (TA-CHY) for extracting protocatechuic acid (PCA) and vanillic acid (VA) from stingless bee honey (SBH) using a dispersive micro-solid phase extraction method. The extracted analytes were separated using high-performance liquid chromatography and detected using an ultraviolet detector. TA-CHY was prepared by depositing tannic acid onto the amino-functionalized chrysin. Its point of zero charge was determined at pH 6.8. Fourier transform infrared spectroscopy analysis had identified the presence of C=N, C=C, O-H, SiOR and C-O groups in TA-CHY, while aggregate formation was observed on TA-CHY through scanning electron microscopy. Energy dispersive X-ray study revealed the elemental composition of TA-CHY was 52.9% of C, 34.0% of O, 6.80% of Si and 6.3% of N. Its specific surface area was found to be 5.913 m^2/g via Brunauer-Emmett-Teller surface area analysis. Optimised extraction parameters were 1 min extraction time, 300 µL methanol as desorption solvent, 15 min desorption time, sample pH of 2, no NaCl salt addition, 0.20 g biosorbent mass and 2 mL ethanol as dispersant solvent. The developed method was validated using matrix-match calibration where real SBH samples spiked with PCA and VA in the concentration range of 0.5-50 mg/L. The method exhibited satisfactory analytical performance characteristics, with a limit of detection ranging from 0.81 to 1.37 mg/kg, a limit of quantification ranging from 2.69 to 4.56 mg/kg, relative recoveries ranging from 79.38% to 113.38%, and precision with relative standard deviation values ranging from 0.29% to 10.83%. Importantly, the proposed method obtained a score of 71 on the analytical Eco-Scale, indicating its acceptability as a green method.

Keywords: chrysin, biosorbent, dispersive micro-solid phase extraction, phenolic acids, stingless bee honey

Abstrak

Kajian ini memberi tumpuan pada penyediaan, pencirian, dan penggunaan biopenjerap berasaskan krisin (TA-CHY) untuk mengekstrak asid protokatekuik (PCA) dan asid vanillik (VA) daripada madu lebah (SBH) tanpa sengat dengan menggunakan

kaedah pengekstrakan fasa pepejal-mikro secara serakan. Analit yang diekstrak telah diasingkan menggunakan kromatografi cecair berprestasi tinggi dan dikesan menggunakan pengesan ultraungu. Biopenjerap TA-CHY telah disediakan dengan mendepositkan asid tannik pada krisin yang telah diberikan fungsi amino. Cas sifar titiknya telah ditentukan pada pH 6.8. Analisis spektroskopi inframerah transformasi Fourier telah mengenalpasti kehadiran kumpulan C=N, C=C, O-H, SiOR dan C-O dalam TA-CHY manakala pembentukan agregat diperhatikan pada TA-CHY melalui mikroskop pengimbas electron. Kajian tenaga penyerakan sinar-X mendedahkan komposisi unsur bagi TA-CHY ialah 52.9% C, 34.0% O, 6.80% Si dan 6.3% N. Luas permukaan spesifiknya didapati sebanyak 5.913 m^2/g melalui analisis kawasan permukaan Brunauer-Emmett-Teller. Parameter pengekstrakan yang dioptimumkan ialah 1 minit masa pengekstrakan, 300 µL metanol sebagai pelarut desorpsi, 15 minit masa desorpsi, pH sampel 2, tiada penambahan garam NaCl, 0.20 g jisim biopenjerap dan 2 mL etanol sebagai pelarut penyerakan. Kaedah yang dibangunkan telah disahkan menggunakan kalibrasi padanan-matriks dengan sampel SBH tulen dengan menambah PCA dan VA dalam lingkungan kepekatan 0.5-50 mg/L. Kaedah ini menunjukkan ciri prestasi analisis yang memuaskan, dengan had pengesanan antara 0.81 dan 1.37 mg/kg, had kuantitatif antara 2.69 dan 4.56 mg/kg, pemulihan relatif antara 79.38% dan 113.38%, dan ketepatan dengan sisihan piawai relatif, antara 0.29% dan 10.83%. Kaedah yang dicadangkan telah memperoleh markah 71 pada skala-Eko analitik yang menunjukkan kebolehterimaannya sebagai kaedah hijau adalah sangat penting.

Kata kunci: krisin, biopenjerap, pengekstrakan fasa pepejal-mikro secara serakan, asid fenolik, madu lebah tanpa sengat

Introduction

Stingless bee honey (SBH) is a remarkable natural sweetener that possesses several unique physicochemical characteristics, namely high acidity (pH 3.07-3.19), high moisture content (20.00-33.24 g/100 g honey), and low sugar content (50.35-62.38 g/100 g honey) [1, 2]. It is well-known for its unique formulation of bioactive compounds, notably phenolic acids (PAs). PAs are bioactive compounds with potent anti-inflammatory and anti-oxidant properties [3, 4], making their analysis in honey crucial for assessing honey quality and potential health-promoting effects. However, the complicated matrix of the viscous and hygroscopic honey sample makes it difficult to determine the presence of PAs in a direct way. This challenge inspired researchers and thereby promoted the development of more effective and specialized sample preparation methods for SBH [5]. As the search for eco-friendly and sustainable analytical methods has gained prominence, and green analytical chemistry ideas are now more widely acknowledged [6]. It is critical to develop efficient, innovative and environmentally friendly sample preparation procedures for assessing PAs in honey.

Sample preparation methods, such as solvent extraction (SE), liquid-liquid extraction (LLE) and solid-phase extraction (SPE), have been commonly employed for PA analysis [7]. These methods are relatively cheap and simple, without the need of expensive, complicated and specialized instrumentation. Nonetheless, these methods are often associated with drawbacks, including the use of large volumes of organic solvents, which are not environmentally friendly and may pose potential health hazards. For instance, the main disadvantages of LLE are the high volume of solvents used (from a few mL to L of ethyl acetate and diethyl ether) and the emulsion formation, which makes it challenging to recover some phenolic compounds effectively. To overcome emulsion problem, addition of surfactant (methanol and ethanol), the need of ultrasonication, freezing out after separation of phases and gentle sample shaking might be needed. While these solutions are less favorable as addition of surfactant may affect the partition equilibrium between phases, additional steps like shaking, ultrasonication and freezing out could be laborious and may need a longer time to perform extraction. More organic solvent also may be needed for rinsing after the freezing out process.

In SPE, commercial SPE cartridges often require smaller quantities of samples and low amounts of organic solvents while its vacuum manifold and vacuum pumps might be costly for the analysis of a reduced number of samples. The number of stages should be kept to a minimum because each stage or transfer might result in loss of analytes. These limitations motivated analytical chemists to improve and develop new mode of SPE methods and one of these methods is dispersive-micro-solid phase extraction (d-µ-SPE). Furthermore, various analytical approaches have been used to analyze PAs, each with its own set of limitations. Thin-layer chromatography, which employs silica gel, cellulose, or polyamide layers, provides a quick and cost-effective separation process. However, its disadvantage is that it has limited quantitation capabilities due to the loss of analytes during sample processing. The Folin-Ciocalteu reagent, which has traditionally been used for colorimetric measurement of total phenolic content has difficulty in identifying individual phenolic components. This is mostly due to the involvement of other components in food extracts that function as reducing agents. However, employing gas chromatography for

the separation of PAs is challenging as these phenolic compounds have low volatility and derivatisation is necessary. Reversed phase high-performance liquid chromatography (HPLC) provides effective separation of PAs, nevertheless, there were limited studies that focused on PAs. Hence, HPLC was employed for the separation of PAs in this study.

The first d-μ-SPE was developed by Anastassiades et al. [8]. It is one of the desirable methods for sample preparation due to its capacity to offer improved sensitivity, adaptability, and compatibility with automation [9]. When compared to SE, LLE and SPE, d-μ-SPE usually uses far less organic solvent (few μL to mL), making it a greener and a more economical option. It also tends to be quicker than SE and LLE as only few minutes needed for its sorption-desorption process [10]. Besides that, d-μ-SPE may give high selectivity for target analytes by using the right sorbent and experimental conditions. Therefore, sorbent is a core element of this method, facilitating the efficient separation of analytes from complicated matrices. Dispersive mode of SPE typically employs sorbent materials based on silica, layered double hydroxides, magnetic nanomaterials, porous polymers, metal organic framework, and even carbonaceous materials such as activated carbon, graphene, graphite and carbon nanotubes [11]. Furthermore, the development of biosorbents has become imperative in order to make full use of natural resources which includes magnetic cork composites [12], seed [13], tannic acid [14] and many others. Careful sorbent selection is significant in obtaining optimum analytical performances. However, ensuring the high efficiency and effectiveness of an analytical method is equally important as assuring its environmental sustainability.

Flavonoids, which are plentiful in nature, provide a sustainable and biocompatible alternative to traditional sorbents [10, 15]. In this study, d-μ-SPE was developed and combined with a novel chrysin-based biosorbent, TA-CHY for the analysis of vanillic acid (VA) and protocatechuic acid (PCA) in SBH. Chrysin, a natural flavonoid present in propolis and honey in large quantities [16] and shares structural similarities with PAs (benzene rings and hydroxyl groups). The modification of chrysin as a biosorbent which offer numerous advantages, including eco-friendliness, simplicity, and high potential to extract PAs. Since the chrysin biosorbent has remarkable affinity and selectivity towards PAs, it enables effective preconcentration of PAs from difficult matrices like SBH. The distinctive structural characteristics, such as its surface morphology and the presence of functional groups (hydroxyl groups, carbonyl groups, benzene ring), play a key role in increasing its ability to bind analytes, resulting in better analyte recovery and sensitivity [17]. The analysis of PAs in SBH is of great importance for assessing its quality. Therefore, this innovative approach is expected to contribute to the advancement of green analytical practices and provides researchers with a reliable method for the analysis of PAs in SBH. The eco-friendliness of the newly developed method was assessed by analytical Eco-scale metric proposed by Gałuszka et al. [18] to ensure the method align with the principles of green analytical chemistry.

Materials and Methods

Chemicals and reagents

Potassium dihydrogen phosphate, sodium nitrate, sodium hydroxide pellets, ortho-phosphoric acid (85%), nitric acid (65%), sodium chloride and tannic acid were supplied by Merck (Darmstadt, Germany). Analytical grade of toluene, *n*-hexane and acetonitrile were purchased from Thermos Fisher Scientific (Massachusetts, USA), Qrec (Selangor, Malaysia) and Qrec (Selangor, Malaysia), respectively. VA (97%) and PCA were obtained from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol used in this study was obtained from VWR International S.A.S. (Fontenay-sous-Bois Cedex, France). Chrysin (99+%) and 3-aminopropyltriethoxysilane (APTES, 99%) were obtained from Acros Organics B.V.B.A. (Geel, Belgium). Methanol and isopropanol were provided by J.T. Baker (Center Valley, PA, USA). The ultrapure water (18.2 MΩ) was collected from Milli-Q gradient water system (Millipore, Massachusetts, USA). All analytical grade chemicals were used without further purification. 1000 mg/L stock solution of each analyte (PCA and VA) were separately prepared in methanol. After that, the stock solution of each analyte was utilized to make their respective single standard solutions of 100 and 10 mg/L. A sample solution was always made fresh before use. When not in use, the stock standard solutions were stored at 4 °C.

Instruments and apparatus

Fourier-transform infrared (FTIR) characterisation was carried out utilizing a PerkinElmer FrontierTM FT-IR spectrometer paired with a polarized UATR (Massachusetts, USA). Surface morphology was studied using a Jeol JSM-IT300LV scanning electron microscopy (SEM) from Essex County, Massachusetts, at 10-15 kV, 1000-2000× magnification, and a working

distance of 10.4-11.1 mm. The elemental composition was investigated using an energy dispersive X-ray spectroscopy (EDX) from Oxford Instrument X-max in Oxford, UK. The *Brunauer-Emmett-Teller* (BET) surface areas of the biosorbent were investigated using a NOVAtouch surface area and pore size analyser acquired from Quantachrome Corporation, Boynton Beach, FL, USA. The pH of the solution was measured using a Eutech pH meter, model pH 700 (Paisley, UK). The sorption equilibration of the biosorbent during point of zero charge measurement (pH_{pzc}) was facilitated by the use of a PROTECH orbital shaker (model 722). A ZX4 vortex mixer was purchased from VELP SCIENTIFICA, Italy for extraction purpose. A FAVORIT stirring hotplate (HS0707V2) was used to stir the solution. A KUBOTA 2420 centrifuge machine, made in Tokyo, Japan, was used for liquor separation. A BRANSON CPX3800H ultrasonicator (Danbury, CT, USA) was used to desorb analytes from the biosorbent surface and to degas the buffer solution. Mass of biosorbent was measured by using a Sartorius ENTRIS224-1S analytical balance (Göttingen, Germany).

The VA and PCA analyses were performed using a reversed phase HPLC (model 1220 Infinity LC) combined with a ultraviolet detector (G4288C). The HPLC was equipped with a 5 m, 2.1×150 mm Eclipse Plus C_{18} separating column. Both HPLC instrument and the separating column were purchased from Agilent Technologies, Santa Clara, CA, USA. The wavelength of detection for VA and PCA was fixed at 280 nm. An isocratic mobile phase, buffer-methanol (90:10) (v/v), was utilized for the chromatographic separation. First, 3.4022 g of potassium dihydrogen phosphate was dissolved in ~480 mL of ultrapure water. Next, the buffer pH was carefully adjusted to 3 using ortho-phosphoric acid or/and sodium hydroxide solution, followed by diluting the buffer to desired volume mark (500 mL) for the making of 0.05 M buffer solution. The prepared buffer was filtered using an LC solvent filter assembly (Agilent technologies, Santa Clara, CA, USA) paired with a 47 mm diameter nylon membrane filter (Whatman, Buckinghamshire, UK). The injection flow rate and volume were set at 0.5 mL/min and 20 µL, respectively. ChemStation software was used to process the chromatographic data. The peaks for PCA and VA showed on the chromatogram at ~4.2 and ~13.25 min, respectively.

Preparation of TA-CHY biosorbent

0.8 g of chrysin was weighed and dispersed in 80 mL of ethanol via stirring. Then, APTES (3%, v/v) prepared in 20 mL ethanol was added dropwise to the stirring mixture, followed by the addition of 2 g of tannic acid to the mixture. The mixture was stirred at room temperature for 24 hours. After that, the solid product was separated from the solvent mixture via filtration. It was rinsed with ethanol and deionized water for several times. At last, the solid product was collected and dried for 24 hours at 60°C.

Point of zero charge determination

Salt addition method was used to determine the pH_{pzc} of TA-CHY. First, initial pH value ($pH_{initial}$) of a 0.1M $NaNO_3$ solution (10 mL) was set at 3, adjusted with nitric acid or sodium hydroxide solutions. Then, in a 50 mL conical flask, 0.1 g of TA-CHY was dispersed uniformly in the $NaNO_3$ solution. The mixture was equilibrated for 24 hours using a mechanical orbital shaker at 200 rpm. At last, final pH, pH_{final} of the $NaNO_3$ solution was measured and recorded. The experiment was repeated with different pHi_{nitial} values (pH 5, 7, and 9), their corresponding pH_{final} values were determined as well.

Dispersive-micro solid phase extraction

A 45 mL of sample solution (pH 5) was poured to a centrifuge tube containing 0.15 g of TA-CHY dispersed in 2 mL of dispersant solvent, ethanol. Then, a vortex mixer was used to extract the analytes, PCA and VA from the sample for one min at 900 rpm. After that, TA-CHY was separated via centrifugation. The collected biosorbent was added with 300 µL of methanol as desorption solvent, followed by ultrasonically desorbed for 15 min. The extract was then separated again via centrifugation and dried under nitrogen flow. The dried sample was added with 150 µL of mobile phase (buffer: methanol at 9:1 ratio) as sample solvent. Finally, the reconstituted extract (20 µL) was injected to the HPLC instrument.

Real sample preparation

Four raw samples of SBH were collected from Alor Gajah (Malacca), Batang Kali, Hulu Selangor (Selangor), Mata Air, Chuping (Perlis) and Taiping (Perak) in Malaysia. Next, 10 g of honey was dissolved in 100 mL of ultrapure water to make the sample. Sodium hydroxide or ortho-phosphoric acid were used to adjust the pH of the diluted sample (~95 mL) to 2. Then, the pH-adjusted sample was diluted to the desired volume, 100 mL. The prepared sample was then readied for further study. When not in use, the SBH samples were kept in a dark, dry area at room temperature.

Method validation

The developed d-μ-SPE was verified using matrix-matched calibration by assessing a series of genuine SBH samples spiked with different levels of analytes. Its analytical characteristics, namely linearity, sensitivity in terms of limits of quantification (LOQ, 10(standard deviation of the response, σ/slope of the calibration curve, S)) and limits of detection (LOD, 3(σ/S)), accuracy in terms of relative standard deviation (RSD), and recovery in terms of relative recovery (RR) were investigated. The linearity ranges of both PVA and VA were 0.5-50 mg/L, and their model fitnesses were assessed through the coefficient of determination, R^2. Three replicate samples were examined on the same day (n=3) and different days (n=9) for intra- and inter-day precision investigations. The percentage of the recovered analyte in the spiked sample vs the known analyte in the spiked sample was expressed as the RR in this study.

Results and Discussion

Biosorbent characterization

A multidisciplinary approach was used to study the properties of TA-CHY such as studying pH_{pzc} using salt addition method, molecular compositions using FTIR, surface morphology and elemental analysis using SEM-EDX, and specific surface area using BET which revealed comprehensive insights into the structural and chemical characteristics of TA-CHY.

Surface morphology, elemental composition and surface area study

The SEM images of chrysin, tannic acid, and TA-CHY are shown in Figure 1. Figure 1(a) depicts pristine tannic acid as spherical with a smooth surface. In contrast, pristine chrysin has a cylindrical form with a rough surface, as seen in Figure 1(b). Figure 1(c) depicts SEM images of TA-CHY with some aggregate formation on the surface of tannic acid, demonstrating that the deposition of chrysin on the tannic acid surface was effective with the aid of APTES. Additional confirmation was obtained in the EDX results (Table 1), where the weight percentages of components changed when compared to the pristine chrysin and tannic acid. The presence of Si and N components in TA-CHY demonstrated that APTES bounded to the biosorbent surface. The surface areas of chrysin, tannic acid and TA-CHY were 5.262, 2.78 and 5.913 m^2/g, respectively.

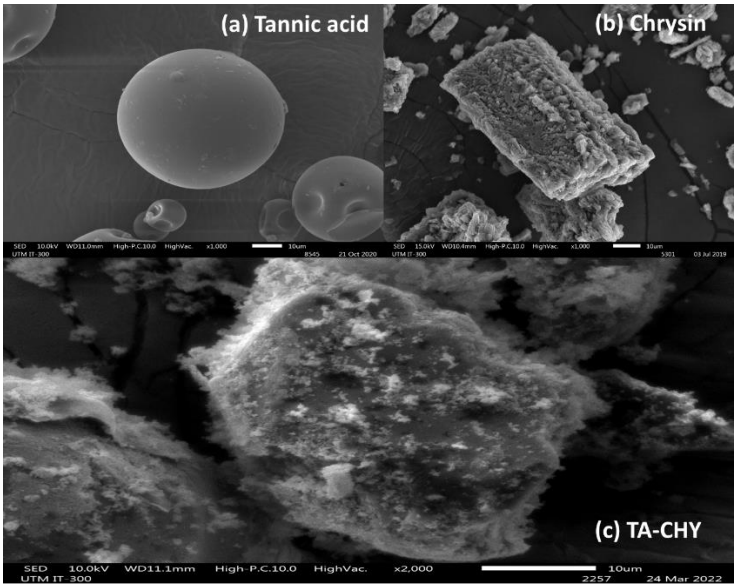


Figure 1. SEM images of (a) tannic acid at ×1000 resolution, (b) chrysin at ×1000 resolution and (c) TA-CHY at ×2000 resolution

Table 1. EDX results of TA-CHY, chrysin and tannic acid

Element	Weight Percentage (%)		
	TA-CHY	Chrysin	Tannic Acid
C	52.9	71.6	55.4
O	34.0	28.4	44.6
Si	6.80	-	-
N	6.30	-	-

Fourier transform infrared spectroscopy (FTIR) study

FTIR spectroscopy is a powerful analytical method that provides useful information on the functional groups that exist in the materials under investigation. The FTIR method was utilized in this investigation to analyze the functional groups of TA-CHY, chrysin, and tannic acid, as shown in Figure 2 (A). The FTIR spectra of chrysin showed multiple peaks at 1447.23-1496.74 and 3006.96 cm^{-1}, which were ascribed to aromatic ring C=C stretching and C-H stretching, respectively. Tannic acid and TA-CHY, on the other hand, displayed multiple C=C stretching bands of aromatic ring with vibration wavelengths of 1444.48-1607.43 and 1447.47-1600.36 cm^{-1}, respectively [19]. Furthermore, the C-O stretching, =C-H bending, C=O stretching, and C-O-C stretching were related with the peaks of chrysin at 1243.55, 839.19, 1649.34, and 1164.63 cm^{-1}, respectively. Many functional groups, including C=O, O-H, O=C-O-C, and C-O, were detected in tannic acid, with peaks emerging at 1703.14 (C=O stretching), 3273.99 (O-H stretching), 1310.56 (aromatic O=C-O-C stretching), and 1181.64 cm^{-1} (C-O stretching of aromatic alcohol). Several notable vibration frequencies were also detected in the FTIR spectra of TA-CHY: Sp^2 C=N double bond stretching measured at 1700.9 cm^{-1}, phenolic O-H bending measured at 1329.74 cm^{-1}, Si-OR stretching measured at 1038.77-1071.21 cm^{-1}, O-H stretching measured at 3152.64 cm^{-1}, and aromatic alcohol C-O stretching measured at 1198 cm^{-1}. This showed that some significant functional groups found in TA-CHY include the benzene ring, the O-H, the C=N, and the C=O groups, may improve the contacts between the sorbent and the sorbate via hydrophobic interaction, cation-π interaction, π-π interaction/aromatic stacking, and hydrogen bonding [20, 21]. Furthermore, the presence of a silanol group in TA-CHY showed that APTES were attached to the biosorbent surface to aid in the deposition of chrysin on tannic acid, as shown in Figure 1 (c). The discussed FTIR data are listed and tabulated in Table 2.

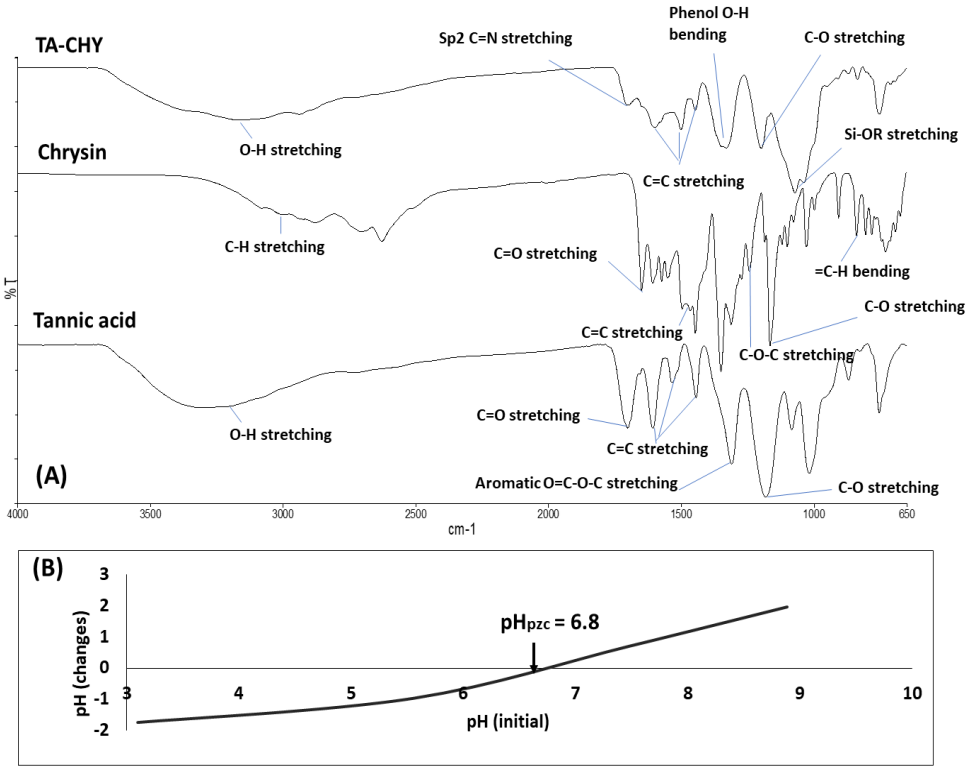


Figure 2. FTIR spectra of TA-CHY, chrysin and tannic acid (A); Graph of point of zero charge (B)

Table 2. FTIR results of chrysin, TA-CHY and tannic acid

Sample	Wavenumber (cm^{-1})	Functional group	Vibration
Chrysin	1243.55	Aromatic alcohol	C-O stretching
	839.19	Alkene	=C-H bending
	1447.23, 1465.50, 1496.74	Aromatic ring	C=C stretching
	3006.96	Aromatic ring	C-H stretching
	1649.34	Ketone	C=O stretching
	1164.63	Ether	C-O-C stretching
TA-CHY	1700.9	Imine	Sp^2 C=N double bond stretching
	1447.47, 1501.12, 1600.36	Aromatic ring	C=C stretching
	1329.74	Aromatic alcohol	Phenol O-H bending
	1038.77, 1071.21	Silanol	Si-OR stretching
	3152.64	Carboxylic acid	O-H stretching
	1198	Aromatic alcohol	C-O stretching
Tannic acid	1310.56	Ester	Aromatic O=C-O-C stretching
	1607.43, 1444.48, 1533.71	Aromatic ring	C=C stretching
	1703.14	Ester	C=O stretching
	1181.64	Aromatic alcohol	C-O stretching
	3273.99	Carboxylic acid	O-H stretching

Point of zero charge (pH_{pzc}) determination

It is vital for scientists to investigate the pH_{pzc} of a biosorbent surface in order to identify its relative surface charges. When the surface charge of a biosorbent surface is thoroughly studied, interactions (mostly electrostatic interactions) between analytes and the biosorbent

surface may be easily manipulated. When the solution pH is greater than pH_{pzc}, the biosorbent surface showed negative charges, and vice versa when the solution pH falls below the pH_{pzc}. When the pH solution was equal to pH_{pzc}, the biosorbent surface was in a neutral condition. Figure 2 (B) depicts a curve formed by graphing $pH_{changes}$ vs $pH_{initial}$. pH_{pzc} of TA-CHY (pH 6.8) which was identified using the intersection point of the curve at axis-x. At solution pH of 6.8, TA-CHY exhibited a neutral charge. Positive charges were present on the biosorbent surface when the solution pH is less than 6.8. The biosorbent surface, on the other hand, exhibited negative charges when the solution pH exceeded 6.8.

Method optimisation

A series of water samples loaded with PCA and VA at a concentration of 0.5 mg/L were prepared and employed for method optimisation. The involved parameters included type of desorption solvent, extraction time, desorption time, solution pH, NaCl addition, biosorbent mass and dispersant solvent. One variable at a time method was used to optimise these parameters.

Effect of type of desorption solvent

In this study, a total of four different organic solvents, including toluene, hexane, ethanol, and methanol, were tested for their potential to desorb PCA and VA. The solvents were then divided into two groups: non-polar solvents (toluene and hexane) and polar solvents (methanol and ethanol). Figure 3(a) demonstrates that methanol has the greatest desorption ability when compared to other solvents. According to Musa et al. [22], methanol was the optimum desorption solvent for PAs with moderate polarity (log P of PCA= 0.82; log P of VA= 1.35 [23]. During this study, the following extraction variables were kept constant: ethanol as the dispersant solvent, solution pH of 8, extraction duration of 1 min, desorption time of 15 min, biosorbent mass of 0.1 g, and 0% NaCl addition (w/v).

Effect of extraction time

Vortex agitation was used for this extraction procedure with the goal to enhance the mass-transfer rate of analytes to the biosorbent surface and shorten the equilibrium period. The extraction time was varied between 0.5 and 4 min with the desorption time set at 15 min whereby methanol was used as the desorption solvent, 0.1 g of biosorbent mass, solution pH 8, 0% NaCl addition (w/v), and ethanol as the dispersant solvent. As seen in Fig. 3 (b), PCA recovery increased from 0.5 to 1 min. The PCA recovery responses decreased and stayed virtually consistent in the 2 to 4 min range. In contrast, VA recovery improved dramatically from 0.5 to 1 min. After that, VA recovery responses fell and stayed steady between 2 and 3 min, followed by another decline at 4 min. The extracted analytes then reached their maximum levels at 1 min. After 1 min, the biosorbent may suffer from active site saturation, resulting in a decline of analyte recovery responses. As a result, 1 min was chosen as the optimal extraction time.

Effect of desorption time

Ultrasonication was utilized to help in the desorption of sorbates from a sorbent by enhancing mechanical agitation and improving mass transfer at the solid-liquid interface. Between 5 and 20 min, the effect of desorption time on sorption recovery was examined (Fig. 3(c)). Other experimental settings were held constant during this study: extraction time was 1 min, desorption solvent was methanol, solution pH was 8, dispersant solvent was ethanol, and the amount of NaCl added was 0% (w/v). Under these conditions, PCA's responses were steady for the first 10 min, then increased at 15 min, followed by a slight decrease at 20 min. The recovery responses for VA climbed gradually from 5 to 15 min, then dropped at 20 min. Both VA and PCA responses decreased after 15 min of desorption processes, suggesting that some of the analytes were re-sorbed by the TA-CHY. Therefore, 15 min was chosen as the desorption time for this research.

Effect of solution pH

As solution pH can have a substantial influence on the existing form of analytes and surface charges of a biosorbent surface which is crucial in an extraction process. The effect of solution pH on PCA and VA recovery was examined in this study throughout a pH range of 1 to 8 (Fig. 3(d)). Other experimental parameters had 15 min of desorption time, methanol as the desorption solvent, 0.1 g of biosorbent mass, 1 min of extraction time, 0% NaCl addition (w/v), and ethanol as the dispersant solvent. Particularly, when the pH increased from 1 to 2, the VA recovery responses increased significantly. The recovery response of VA reduced dramatically and progressively after pH 3 solution. The recovery responses of PCA, on the other hand, were practically consistent from pH 1 to 4. It showed an increasing trend at pH 6 solution before dropping at pH 8 solution. As a result, VA and PCA responded optimally at solution pH levels of 2 and 6, respectively. When compared to PCA (lower hydrophobicity with log P of 0.82 [23], VA (greater hydrophobicity with log P of 1.35 [23] indicated a substantial preference for the positively charged TA-

CHY biosorbent at solution pH 2. This might be attributed to greater cation-π, hydrophobic interactions and π-π interactions between them [24]. In research performed by Musa et al., a higher hydrophobicity analyte, *p*-hydroxybenzoic acid (log P of 2.27) was also found more favorable to a hydrophobic graphene-based adsorbent than a lower hydrophobicity analyte, PCA [22]. In addition, the presence of an extra methyl group in VA might increase electron density in the aromatic ring, resulting in a greater electrostatic affinity with the positively charged biosorbent [25]. This stronger attraction led to a more stable and beneficial cation-π interaction. Other possible interaction mechanisms were primarily driven by hydrogen bonding between the biosorbent's silanol/carbonyl/hydroxyl/amino groups and the analytes' carbonyl/hydroxyl groups. Furthermore, both PCA and VA have pK_a values around 4.2. This might explain why the best solution pH for PCA recovery was pH 6, since there was an electrostatic contact between the deprotonate PCA and the positively charged TA-CHY biosorbent.

Effect of NaCl addition

Salt addition may have a major influence on the sorption process. As salt increases a solution's ionic strength, it may impact the sorbate sorption efficiency and selectivity. For example, when the ionic strength of a solution changed, the electrostatic connections between the analytes and biosorbent were disrupted and weakened. Figure 3(e) shows that when no NaCl salt (0%, v/w) was added to the sample solution, both PCA and VA showed the best recovery responses. Their recovery responses decreased considerably when 1% and 2% (w/v) of NaCl were added to the sample solutions. This is because the additional NaCl salt may compete with the analytes for binding sites on the TA-CHY surface. Therefore, 0% (v/w) NaCl salt addition was set for this method. Other experimental settings used in this optimisation were 1 min of extraction time, 15 min of desorption time, methanol as the desorption solvent, solution pH 2, ethanol as the dispersant solvent and 0.1 g of biosorbent mass.

Effect of mass of biosorbent

The effect of TA-CHY mass on PCA and VA recovery was investigated by increasing the mass from 0.10 g to 0.25 g while keeping the other experimental parameters constant: extraction time = 1 min, desorption solvent = methanol, desorption time = 15 min, solution pH = 2, dispersant solvent = ethanol and NaCl addition = 0% (w/v). The recovery responses of VA indicated an increasing trend from 0.10 to 0.20 g, followed by a drop at 0.25 g in Fig. 3(f). PCA showed a rising tendency from 0.10 to 0.15 g, followed by a drop after 0.20 g. The availability of more analyte sorption sites resulted in an increase in recovery responses with increasing amounts of biosorbent. Therefore, the best biosorbent mass for VA and PCA were 0.20 g and 0.15 g, respectively. The biosorbent mass for the subsequent study was determined to be 0.15 g. The observed reduction in the recovery response of PCA and VA at larger biosorbent mass ranges may be due to the saturation of the TA-CHY adsorption sites and the desorption of analytes back to the bulk phase.

Effect of type of dispersant solvent

In this study, a dispersant solvent was used to promote biosorbent dispersion in a sample solution. Extraction time, desorption solvent, desorption time, solution pH, NaCl addition, and mass of biosorbent were all designated as 1 min, methanol, 15 min, pH 2, 0% (w/v), and 0.15 g biosorbent, respectively. Acetonitrile, ethanol, and methanol were chosen to investigate their potential as dispersant solvents in the newly designed d-μ-SPE (Figure 3(g)). Among these solvents, the use of ethanol as a dispersant solvent provided the highest analyte recovery results. Ethanol, isopropanol, and acetonitrile had log P values of -0.34, 0.05, and -0.54, respectively. The hydrophilicity of these solvents was organized based on their Log P values: isopropanol, ethanol and acetonitrile in ascending order. Since isopropanol is less hydrophilic, it may cause inadequate dispersion of TA-CHY in the sample, thus resulting in unequal interaction between the biosorbent and the analytes. While acetonitrile is more hydrophilic, it may interact negatively with the hydrophobic component of TA-CHY, resulting in poorer biosorbent-analyte interactions. However, because of the modest hydrophilicity, ethanol was able to adequately disseminate the CHT-TA in the sample. The enhanced dispersion was due to its high compatibility with TA-CHY, which enhanced the interaction of TA-CHY with analytes. Therefore, ethanol was chosen as the dispersant solvent.

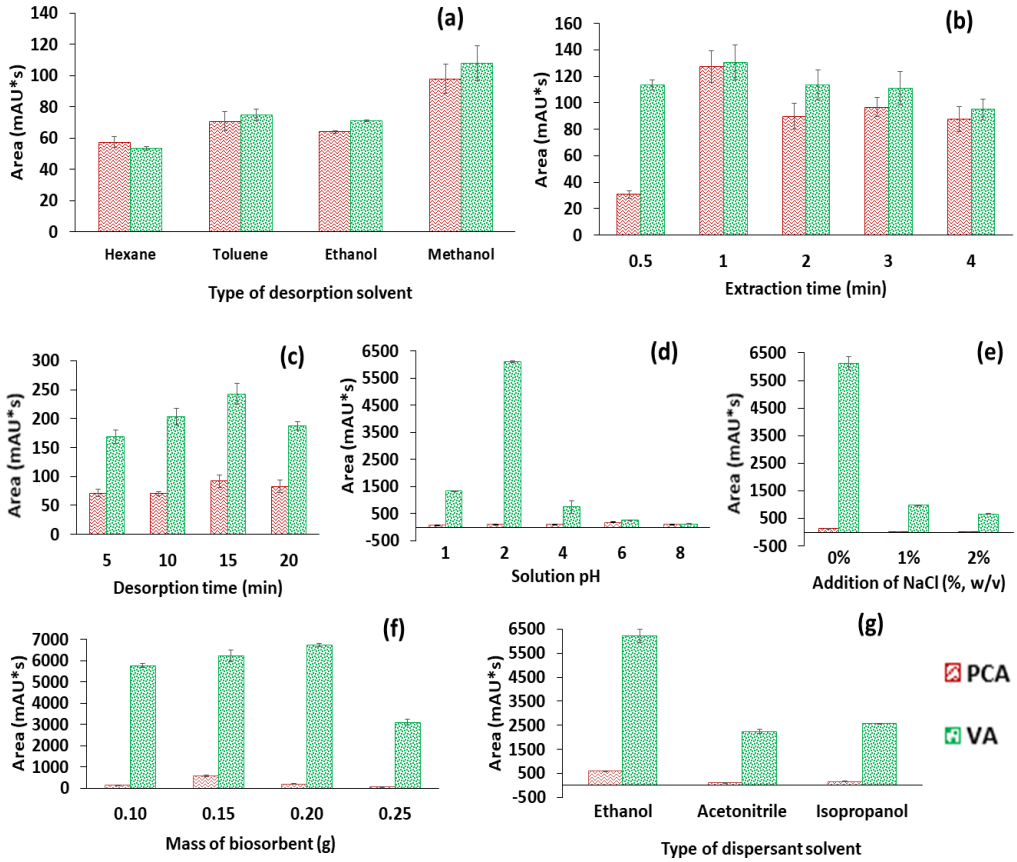


Figure 3. Effects of (a) type of desorption solvent, (b) extraction time, (c) desorption time, (d) solution pH, (e) addition of NaCl, (f) mass of biosorbent and (g) type of dispersant solvent on TA-CHY-d-μ-SPE of VA and PCA

Method validation

Matrix-matched calibration was used to match the matrices of SBH samples and calibration standards to equalize the analyte responses. A series of genuine samples were prepared by spiking PCA and VA in concentrations ranging from 0.5 to 50 mg/L. Both analytes displayed good linearity, with R^2=0.9929 for PCA and 0.9962 for VA (Table 3). LOD values of PCA and VA were 1.37 and 0.81 mg/kg, respectively; while LOQ values of PCA and VA were 4.56 and 2.69 mg/kg, respectively. The approach achieved good RRs and RSDs in the range of 79.38-113.38% and 0.29-9.83%, respectively. To summarize, the approach was shown to be adequate for the determination of PCA and VA in SBH samples with good analytical features.

Table 3. Analytical parameters of the developed method

Analytical Parameter	PCA	VA
Linearity range (mg/L)	0.5-50	0.5-50
LOD (mg/kg), S/N= 3	1.37	0.81
LOQ (mg/kg), S/N= 10	4.56	2.69
Intraday study (*n*=3)		
Spiked concentration at 5 mg/L		
RR (%); RSD (%)	79.38; 2.53	88.88; 0.29
Spiked concentration at 40 mg/L		

Analytical Parameter	PCA	VA
RR (%); RSD (%)	113.12; 9.83	113.38; 7.07
Intraday study (*n*=9)		
Spiked concentration at 5 mg/L		
RR (%); RSD (%)	80.58; 3.01	87.86; 3.43
Spiked concentration at 40 mg/L		
RR (%); RSD (%)	90.49; 9.92	99.45; 10.83

Real sample analysis

The d-μ-SPE approach, which used TA-CHY as a biosorbent, was expanded to analyze PCA and VA in genuine SBH samples collected from Malaysia's Malacca, Perlis, Perak, and Selangor states. Table 4 summarizes the measure quantities of both analytes reported for these SBH samples and their comparison with other honey products. According to the data presented in Table 4, there appears to be a trend showing that SBH samples had greater levels of PCA and VA [26, 27]. The greatest amount of PCA were found in H9 (Greece), SBH sample 2 (Perak, Malaysia), and SBH sample 3 (Perlis, Malaysia), with amount of 16.777, 7.69, and 10.67 mg/kg, respectively. SBH samples 2 (Perak, Malaysia), B and D (Santa Catarina State, Brazil) had the greatest levels of VA, at 11.97, 4.21, and 3.46 mg/kg, respectively. SBHs showed to benefit honey consumers because VA has anti-obesity, anti-diabetic and cardio-protective properties, whilst PCA has anti-inflammatory, antioxidant, anti-microbial, anti-apoptotic, anti-hyperglycemic and anti-proliferative properties [28].

Table 4. Results of PCA and VA analysis in real SBH sample and their comparison with other honey products

Sample, Source	Sample Code	Found Amount (mg/kg ± SD)		Reference
		PCA	VA	
SBH, Malacca, Malaysia	1	2.31±0.26	ND	This study
SBH, Perak, Malaysia	2	7.69±0.86	11.97±1.33	
SBH, Perlis, Malaysia	3	10.67±1.20	ND	
SBH, Selangor, Malaysia	4	3.88±0.43	ND	
SBH, Santa Catarina State, Brazil	A	0.38±0.5	ND	[29]
	B	0.07±0.1	4.21±1.0	
	C	ND	ND	
	D	ND	3.46±0.7	
	E	0.53±1.0	2.78±1.4	
	F	ND	3.36±1.4	
	G	ND	0.96±0.1	
	H	0.4±0.1	ND	
Honey and SBH, different states of Brazil	*Apis mellifera* honey (control)	$0.016\pm0.002\times10^{-3}$	NM	[30]
	SBH1	ND	NM	
	SBH2	ND	NM	
Honey, different regions of Greece	H1	0.649±0.015	0.225±0.006	[31]
	H5	0.346±0.025	0.236±0.002	
	H11	0.300±0.022	0.245±0.011	
	H8	0.590±0.021	0.103±0.001	
	H9	16.777±0.780	0.307±0.014	
	H7	3.258±0.024	0.149±0.002	
	H4	4.140±0.131	0.262±0.008	
	H10	5.967±0.057	0.376±0.002	
	H6	3.058±0.111	0.340±0.013	
	H3	2.394±0.050	0.237±0.007	
	H2	1.046±0.027	0.201±0.005	

Sample, Source	Sample Code	Found Amount (mg/kg ± SD)		Reference
		PCA	VA	
	H12	0.303±0.007	0.092±0.002	
Honey, Xinjiang, China	1	NM	0.042±0.001	[32]
	2	NM	0.021±0.001	
	3	NM	0.031±0.001	
	4	NM	0.052±0.001	
	5	NM	0.031±0.001	
	6	NM	ND	
	7	NM	ND	
	8	NM	0.022±0.001	
	9	NM	0.038±0.001	
	10	NM	ND	

ND= not detectable; NM= not mentioned

Method comparison

Table 5 compares several sample preparation techniques, including this approach, solvent extraction, G-Fe_3O_4-MSPE, and HLB SPE. These techniques were reviewed and compared in terms of LOD, LOQ, linearity range, RSD, and RR. Remarkably, HLB SPE exhibited an outstanding sensitivity with the lowest LOD (0.0002 µg/kg) and LOQ values (0.0006 µg/kg), allowing the reliable detection and quantification of trace VA concentrations. Secondly, G-Fe_3O_4-MSPE allowed for facile sorbent collection by using an external magnetic field while retaining its good analytical properties. Moreover, G-Fe_3O_4-MSPE also used the least amount of toxic organic solvent (~500 µL) during the sample preparation stage, as compared to solvent extraction (>100 mL), HLB SPE (>15mL) and this method, TA-CHY-d-µ-SPE (<4 mL). However, TA-CHY-d-µ-SPE demonstrated comparable sensitivity and accuracy to solvent extraction but used less hazardous organic solvent than solvent extraction. All techniques mentioned in Table 5 demonstrated adequate accuracy, with RSD values of less than 10.83%, thus emphasizing their precision and consistency between replicates. Overall, each technique has its own set of benefits and drawbacks. TA-CHY-d-µ-SPE is considered sufficient and effective in accomplishing its intended purpose in the analysis of PAs in SHB.

Table 5. Comparison with other published sample preparation methods

Method	Sample	Analyte	LOD (µg/kg)	LOQ (µg/kg)	Linearity range	RSD (%)	RR (%)	Reference
TA-CHY-d-µ-SPE	SBH	PCA	1.37	4.56	0.5-50 mg/L	2.53-9.92	79.38-113.12	This study
		VA	0.81	2.69		0.29-10.83	87.86-113.38	
HLB SPE	Honey	PCA	NM	NM	NM	NM	NM	[32]
		VA	0.0002	0.0006	0.001-0.50 mg/L	2.4-5.1	78.5-82.9	
Solvent extraction	Honey	PCA	0.99	3.00	NM	NM	>85	[33]
		VA	0.89	2.71	NM	NM		
G-Fe_3O_4-MSPE	SBH	PCA	0.14	0.46	3-50 mg/kg	0.3-8.5	78.8-107.5	[22]
		VA	NM	NM	NM	NM	NM	

*NM= not mentioned; SPE= solid phase extraction; HLB= Hydrophilic-Lipophilic Balance; G-Fe_3O_4= graphene-magnetite composite; MSPE= magnetic solid phase extraction

Analytical Eco-scale

An analytical Eco-scale calculation [18] was performed to evaluate the eco-friendliness of the developed d-μ-SPE method combined with the use of TA-CHY biosorbent. This covered several stages of a single sample analysis, namely biosorbent preparation, sample preparation and instrumental detection. The calculation was based on the total score obtained from a deduction of total penalty points from 100. The penalty points were given for using less green reagent, instrument, high waste volume and occupational hazard. 100 score of analytical Eco-scale indicated a method as ideal green analysis, whereas score above 75 was excellent, score between 74 and 50 suggested the method as an acceptable green analysis, and score at below 50 indicated inadequate method. This method received a total score of 71, suggesting that it is an acceptable green analytical approach. The calculation details were shown in Table 6.

Table 6. Analytical Eco-scale calculation

Reagent	**Penalty Point**			
	Amount (Point)	**Number of Pictogram (Point)**	**Signal Word (Point)**	**Calculated Point**
Chrysin	<10 g(1)	0(0)	None(0)	1×0×0=0
3-aminopropyltriethoxysilane	<10 mL(1)	2(2) 	Danger(2)	1×2×2=4
Tannic acid	10 g(1)	0(0)	None(0)	1×0×0=0
Sodium hydroxide	<10 g(1)	1(1) 	Danger(2)	1×1×2=2
Ethanol	10-100 mL (2)	2(2) 	Danger(2)	2×2×2=8
Phosphoric acid	<10 mL(1)	2(2) 	Danger(2)	1×2×2=4
Methanol	<10 mL(1)	3(3) 	Danger(2)	1×3×2=6
Potassium dihydrogen phosphate	<10 g(1)	0(0)	None(0)	1×0×0=0
Water	10-100 mL(2)	0(0)	None(0)	2×0×0=0
Total point				**24**
Instrument		**Energy used per sample (kWh) (point)**		**Calculated point**
HPLC-UV		≤1.5(1)		1
Centrifuge		≤1.5(1)		1
Vortex mixer		≤0.1(0)		0
Vacuum pump		≤0.1(0)		0
Sonicator		≤0.1(0)		0
Total point				**2**
Other				
Waste		<10 mL(3)		3
Occupational hazard		Hermetization in analytical process(0)		0
Total point				**3**
Total penalty point				24+2+3=29
Analytical Eco-Scale total score				100-29=71 **(acceptable)**

Conclusion

In conclusion, our investigation on the newly developed biosorbent, TA-CHY for the extraction of PAs offered substantial insights. CHY-TA had been successfully prepared, characterised and applied for d-μ-SPE of VA and PCA, with higher selectivity towards VA via strong cation-π, hydrophobic interactions and π-π interactions with CHY-TA. The method was extended for the analysis of PCA and VA in Malaysian SBHs. It demonstrated good analytical performance, with LODs ranging from 0.81 to 1.37 mg/kg, LOQs ranging from 2.69 to 4.56 mg/kg, RRs ranging from 79.38% to 113.38%, and RSDs ranging from 0.29% to 10.83%. It also proved its capability to stand alone as a new green analytical method (analytical Eco-scale = 71; acceptable green analysis). This research also contributed to the advancement of analytical methods for studying phenolic acids in stingless bee honey, emphasizing the potential for broader applications in honey analysis and quality assessment.

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ANALYTICAL TECHNIQUES OF BIOACTIVE COMPOUNDS FROM MEDICINAL PLANTS WITH THE POTENTIAL TO ADDRESS ALZHEIMER'S DISEASE AND ASSOCIATED SYMPTOMS: A BRIEF OVERVIEW

(Teknik Analisis bagi Sebatian Bioaktif daripada Tumbuhan Ubatan dengan Potensi untuk Merawat Penyakit Alzheimer dan Gejala Berkaitan: Ulasan Ringkas)

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Abstract

Alzheimer's disease (AD) is a progressive neurological disorder of the brain, named after the German physician Alois Alzheimer, who first documented it in 1906. AD is the most prevalent form of dementia, affecting approximately ten million individuals worldwide. The literature on the extraction and available analytical methods is very helpful in treating AD owing to the diverse and synergistic effects of bioactive compounds. The extraction methodologies allow researchers to concentrate and isolate specific compounds, optimizing their potency and purity for effective treatment. By reviewing these isolated compounds, scientists could have better understanding of their mechanisms, and tailor the dosage and formulation for precise treatments with fewer side effects. This method utilizes the natural qualities of plants and provides a hopeful path for creating safer and more focused treatments for AD. While certain herbal remedies may aid in enhancing brain function, scientific evidence supporting their effectiveness in treating AD remains limited. Thus, the main objective of this review is mainly to provide insight pertaining to AD as well as the analytical techniques of bioactive compounds from medicinal plants that show the potential to treat AD and its associated symptoms.

Keywords: Alzheimer's disease, medicinal plants, analytical techniques, natural products

Abstrak

Penyakit Alzheimer (AD) adalah gangguan neurologi progresif dalam otak, dinamakan sempena doktor Germany, Alois Alzheimer, yang pertama kali mendokumentasikannya pada tahun 1906. AD merupakan bentuk demensia yang paling meluas, memberi kesan

terhadap lebih kurang sepuluh juta individu di seluruh dunia. Kajian literatur mengenai kaedah pengekstrakan dan analisis yang sedia ada amat membantu dalam rawatan AD disebabkan kesan pelbagai dan sinergistik sebatian bioaktif ini. Kaedah-kaedah pengekstrakan membolehkan saintis mengumpul dan mengasingkan sebatian khusus, mengoptimumkan keberkesanan dan ketulenan mereka untuk rawatan yang berkesan. Dengan mengkaji sebatian-sebatian yang diasingkan ini, saintis dapat memahami mekanisme mereka dengan lebih baik, menyesuaikan dos dan rumusan rawatan yang tepat dengan kesan sampingan yang kurang. Kaedah ini menggunakan sifat semula jadi tumbuhan dan kaedah yang boleh digunakan untuk mencipta rawatan yang lebih selamat dan tertumpu untuk AD. Walaupun beberapa ubatan herba mungkin membantu meningkatkan fungsi otak, bukti saintifik yang menyokong keberkesanan mereka dalam merawat AD masih terhad. Oleh itu, objektif utama kajian ini adalah untuk memberikan pandangan ringkas tentang AD serta teknik analisis sebatian bioaktif daripada tumbuhan ubatan yang menunjukkan potensi dalam merawat AD dan gejala yang berkaitan dengannya.

Kata kunci: penyakit Alzheimer, tumbuhan ubatan, teknik analisis, produk semulajadi

Introduction

Neurodegenerative diseases have become one of the most serious health issues worldwide. It is because the development of the first symptoms tends to show up late and the continual aging process of society contributes to the increasing number of these diseases [1]. One of the neurodegenerative diseases includes dementia. Dementia can be defined as a clinical syndrome that can be categorized by a progressive reduction in two or more cognitive abilities which is memory, attention, visual/auditory processing, and reasoning [2]. This leads to loss of functions and inability to perform activities of daily living. According to WHO, dementia influences each person differently, based on the main causes, health conditions, and cognitive functions before getting ill [3]. Study shows that there would be 131 million dementia patients globally by 2050 [4]. Dementia can be broadly classified as primary or secondary dementia. Neurodegenerative dementia is primary, while dementias with underlying systemic or brain diseases are considered secondary dementias [5]. There are many types of primary dementia but the three most common are Alzheimer's disease (AD), vascular dementia, and dementia with Lewy bodies.

Alzheimer's disease

AD is the most common cause of dementia in up to 80% of all dementia cases [2]. AD is a complex neurodegenerative illness that develops slowly over time with symptoms from minor forgetfulness to serious mental disability [6]. The highest number of people with AD is found in the older age group of 65 years and above. According to studies, the number of AD patients will reach 74.7 million by 2030 due to the number of older people increasing all over the world [7]. In the past few years, a significant increase in the exploration and investigation of the benefits of natural products and their bioactive compounds due to the preventive effects against a wide range of illnesses have been documented including neurological diseases [8]. The bioactive compounds in a well-balanced diet have been claimed to have an impact on the mechanisms of AD [9]. Bioactive compounds that can have the potential to treat AD include curcumin, isoflavones, and berberine. The main goal of this review is to offer insights related to AD, focusing on the analytical techniques of bioactive compounds from medicinal plants. These compounds have shown promising potential in treating AD and its related symptoms.

Other types of dementia

In Europe and North America, vascular dementia is estimated to account for 15% to 20% of dementia cases, with slightly higher numbers of roughly 30% in Asia and developing nations. Vascular dementia is generally recognized as the second most common type of dementia after AD. Cognitive deterioration can often happen suddenly in vascular dementia after a stroke that affects vital brain blood arteries. The recorded actual risk of dementia after stroke varies significantly, from less than 7% in population-based studies of first-time stroke in patients who had never had dementia, to over 40% in hospital-based analysis of recurrent stroke with pre-stroke dementia involved [10]. Memory loss that resembles AD may result from a vascular injury that first develops in the parts of the brain that are critical for storing and retrieving information. The pathophysiology of vascular dementia is variable involving interactions between risk factors and alterations to the cerebral blood vessels that lead to pathological events such as infarcts,

white matter ischemia, hemorrhage, thrombosis, and vasospasm [11]. Lewy bodies dementia is an umbrella term that covers dementia with Lewy bodies and Parkinson's disease dementia. This type of dementia has become the third most common type of neurodegenerative dementia. The main protein component that causes Lewy bodies dementia is the abnormalities of alpha-synuclein metabolism [12]. According to clinical criteria, the person with Lewy bodies dementia will have symptoms such as repeated complex visual hallucinations, Parkinsonism, and rapid eye movement sleep disorder. Compared to AD, Lewy bodies dementia confers poorer diagnosis and greater caregiver's stress [13]. Frontotemporal dementia contains a group of health conditions that can be detected by persistent changes in behavior, lack of self-regulation skills, or language [14]. This condition appears due to nerve cell damage in the frontotemporal region of the brain resulting in loss of functions in these brain functional areas. Frontotemporal dementia consists of a few clinical syndromes. Primary progressive aphasia (PPA) is a type of frontotemporal dementia that contain two subgroups which are semantic variant PPA (svPPA) and non-fluent variant PPA (nfvPPA) [15].

Mixed dementia is a combination of AD and vascular dementia. It contributes up to 13% to 17% of all dementia cases worldwide. Researchers also used the term 'mixed dementia' to refer to combinations of any two kinds of dementia [16]. Heavy alcohol consumption has been demonstrated to be both a contributing factor and a required factor in which the disease only exists in the presence of alcohol. Decreasing in rational thinking, physical abilities and also memory are the characteristics of alcohol-related dementia. Due to its links to cardiovascular risk factors and conditions like high blood pressure and stroke, heavy alcohol use is indirectly linked to vascular dementia [17]. Every individual with Down syndrome has the neuropathology of AD by the time they are forty years old, and the majority of them go on to develop AD. It is challenging to spot early signs of dementia because individuals with Down syndrome have different standard functions [18]. According to this interpretation, almost every individual with Down syndrome has amyloid plaques and tau neurofibrillary tangles and their lifetime risk of dementia is more than 90% [19]. As a result, Down syndrome is now recognized as an AD that is caused by genetics. AIDS dementia complex or HIV-associated dementia is the neurocognitive decline linked to HIV [20]. According to epidemiological research, HIV-associated dementia is uncommon (2-4%), with the majority of patients exhibiting milder forms of HIV-associated neurocognitive disorders, such as moderate neurocognitive disorder and asymptomatic neurocognitive impairment [21]. Traumatic brain injury also leads to increased occurrences of dementia, including AD. The probability of having AD, Parkinson's disease, and chronic traumatic encephalopathy will increase by traumatic brain injury resulting from closed head trauma [22]. Childhood trauma such as parental abuse and financial problems is more likely to develop cardiovascular disease and mental disorders. Study shows that childhood trauma can be caused by paternal death before a child is 15 years old which is linked to dementia risk among Aboriginal Australian [23]. Neuronal ceroid lipofuscinoses are the main cause of childhood dementia. It is because in up to 7–8 out of 100 000 births, neuronal ceroid lipofuscinoses are reported to be the major source of childhood dementia globally [24]. Creutzfeldt-Jakob disease (CJD) can be classified as a human prion disease. This disease is rare and can affect both human and non-human mammals [25]. Mad cow disease is unrelated to classic CJD. Additionally, "variant CJD" is another prion disease that is connected to mad cow disease but differs from classic CJD [26].

Causes and risk factors of Alzheimer's disease

There are a lot of biological processes such as oxidative stress, neuroinflammation, excitotoxicity, mitochondrial dysfunction, abnormal protein misfolding and aggregation, and apoptosis that are linked to neurodegeneration [8]. Different types of dementia may be traced to cell damage in specific regions of the brain. The two abnormal proteins that are related to AD are tau and amyloid-β (Aβ). Pathologically, AD is described by the development of amyloid plaques made of aggregated amyloid beta (Aβ), neurofibrillary tangles, which are intraneuronal clusters of hyperphosphorylated tau protein, and brain atrophy due to the loss of neurons and

synapses [27]. The hippocampus is the brain region that has the most impact on AD, followed by degeneration of the association cortices and subcortical structures [28]. Several risk factors can cause AD. A family history of Alzheimer's dementia is a risk factor for late-onset Alzheimer's dementia. Those who have first-degree relatives with Alzheimer's dementia have a high possibility of developing dementia, but it is not a guarantee [29]. The development of AD also appears to be influenced by behavioral factors such as social interactions, sleep patterns, and diet and nutrition. AD and cardiovascular disease also have a strong correlation. The same goes for traumatic brain injury which elevated the risk of dementia [30].

Sign and symptoms of Alzheimer's disease

The most common symptoms of AD are loss of short-term memory, followed by a gradual decline in explicit and implicit memory. People who have the disease also experience neuropsychiatric symptoms and eventually lose the ability to carry out basic activities of daily living. Acetylcholinesterase inhibitors such as donepezil, rivastigmine, galantamine, and the N-methyl-D-aspartate (NMDA) (NMDA) receptor antagonist memantine, are being given to reduce these symptoms [28]. Three stages are linked to AD which are mild stage, moderate stage, and severe stage. Table 1 shows the signs and symptoms linked to AD in the three stages [31].

Table 1. Signs and symptoms according to stages.

No.	Stage	Description	Signs/ symptoms
1	Mild (early)	As a result of the signs and symptoms being rarely seen, dementia's early stages are usually overlooked. Anxiety, apathy, irritability, and depression are examples of neuropsychiatric disorders that can be observed in the mild stage of AD.	• Memory difficulties • Language disturbance • Misplacing or losing things
2	Moderate	The signs and symptoms become clearer and more noticeable. These can be persistent for years.	• Forgetfulness of recent events and people's names • Increasing difficulty with communication • Changes in sleep patterns • Roaming and frequent questioning
3	Severe (late)	It is become close to complete inactivity and reliance. The signs/symptoms become more noticeable. All prior abilities keep getting worse from time to time.	• Need for assistance with everyday tasks and personal care • Loss of capability to handle their movement • Mute, incontinence, and becoming bedridden • Numerous complications

Medicinal herbs to treat Alzheimer's diseases

In the field of science, interest in treating AD has increased. Numerous scientific studies have shown that AD is not an independent illness. This condition is triggered by various metabolic abnormalities brought on by the interaction with multiple pathogenic factors. The cholinergic theory, the -amyloid hypothesis, the tau protein theory, the metal ion issue hypothesis, and the neuroinflammation hypothesis are the five pathogenesis hypotheses that have been presented [32]. Table 2 summarizes the medicinal plants with the potential to treat Alzheimer's and associated symptoms and their analytical methods/ profiling.

Extraction methods

The extraction method is a technique used to separate one or more specific substances from a mixture using suitable solvents. When applying extraction, the choice of methods depends on factors such as the nature of the compounds being extracted. Curcumin, obtained from

Sigma Aldrich, and Rosiglitazone maleate can improve mitochondrial function, potentially leading to positive effects on synapses [33]. Many types of bioactive compounds, such as flavonoids, alkaloids, and phenolic acids, are polar. Therefore, these compounds can be extracted using ethanol or methanol, depending on the polarity of each compound. Before extraction, these compounds need to be made homogeneously through processes like washing, drying, and grinding to form a powder. Subsequently, they are soaked in the extraction solvent, followed by filtration to obtain the desired analyte. In research by Benedec et al. [34], Chlorogenic acid was successfully extracted from four *Amaryllidaceae species*, which is known for its antimicrobial activity. In the research conducted by Eruygur et al. [35] *Achillea cucullata* was extracted using 80% ethanol, and the extracted compounds were identified as phenolic acids and flavonoids. It was found that in *Achillea cucullata*, there were more phenolic acids than flavonoids. Fujimori et al. [36], extracted *Centella Asiatica* using 50% ethanol, yielding araliadiol components that could reduce oxidative stress and endoplasmic reticulum stress, as well as improve cognitive impairment in short-term memory. The concentration of ethanol is determined by the selected target compound class intended for separation. Yu et al. [37] conducted the extraction of macamides from *Lepidium meyenii* Walp, yielding N-benzyl- by soaking dried maca powder in ethanol.

Kumaran et al. [38] used 70% methanol in water to extract *N. nucifera* seed embryo, and the extraction yielded flavonoids. El-Hawary et al. [39] applied high-purity methanol (100% methanol) with no water or impurities to extract *Opuntia ficus-indica* cladode peel and fruits, resulting in an abundance of phenolic acids, flavonoids, and fatty acids. This is because high purity reduces the risk of interferences from impurities, as these compounds were analyzed using HPLC-MS, thus ensuring accurate and reproducible results. A group of researchers [40-42] used methanolic extraction to extract the fruit of *Phyllanthus emblica*, the plant *Indigofera sessiliflora*, and the stem of *Tinospora cordifolia*, respectively. Methanolic fruit extraction of *Phyllanthus emblica* (MFEPE) yields flavonoids, polyphenols, and tannins, possessing antioxidant properties that can reduce damage caused by free radicals. Extraction of phenols and flavonoids from the plant *Indigofera sessiliflora* reduces anxiety behavior and reverses scopolamine-induced amnesia in rats. Rosmarinic acid was extracted from *Salvia fruticose* using methanol [43]. The compound pseudoginsenoside RT8 (PG-RT8) in *Salvia fruticose* contains anti-inflammatory properties that can improve cognitive performance. Rho et al. [44] extracted triterpene saponins and three steroidal saponins from *Panax Ginseng* using methanol extract.

Several flavonoids were extracted from dried *Ginkgo biloba* leaves using 60% w/w acetone [45]. In their research, Ionita et al. [46] and Naseri et al. [47] used hydroalcoholic extract to extract *Matricaria chamomilla L.* and *Melissa officinalis L.,* respectively. The plant extract was prepared using a mixture of water and alcohol (ethanol) as solvents, followed by the evaporation of the solution. The extraction of *Matricaria chamomilla L*. yields flavonoids and polyphenolic carboxylic acid, while the extraction of *Melissa officinalis L.* yields rosmarinic acid, rutin, and apigenin. Callizot et al. [48] used traditional extraction and microwave-assisted extraction to extract huperzine A and two phenolic acids, caffeic acid, and ferulic acid from *Huperzia serrata*. Traditional extraction involves using conventional methods such as soaking, boiling, and cooling to extract compounds from the plant. Microwave-assisted extraction is a modern technique that utilizes microwave energy (Milestone Ethos EX) to heat the sample, accelerating the extraction process of *Huperzia serrata*.

In terms of extraction, solvent extraction or solvent fractionation stands out as the most commonly used technique for preparative separation. Modern extraction methods, often referred to as green extraction methods such as ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and pressurized liquid extraction, have gained significant attention in recent years. However, their application is still limited to extracting bioactive compounds from medicinal plants with the potential to address AD.

Analytical methods (quantitative and qualitative/characterization analysis): Quantitative analysis

Chromatography is a widely used method for the separation and identification of complex mixtures. When choosing the chromatographic technique, the choice depends on the type of sample, the compound to be analyzed, and the sensitivity of the measurement. Chromatographic techniques, such as vacuum liquid chromatography (VLC), are used for the isolation of Tinosporide and 8-hydroxytinosoride from *Tinospora cordifolia (Mernispermaceae)*. Meanwhile, Thin-layer chromatography (TLC) is used to monitor the reaction progress and analyze product mixtures. Based on the study, Tinosporide and 8-hydroxytinosoride can serve as key components for developing new drugs for the treatment of AD [41]. Column chromatography is a separation technique used to isolate a mixture of substances by allowing the mixture to flow through a tube packed with finely divided solid coated with an adsorbent liquid. The different components in the mixture separate at different rates as they travel through the column due to their distinct interactions with the stationary phase. For the study of medicinal plants, column chromatography was used for the separation and analysis of *Nelumbo nucifera (Nelumbonaceae)*, in which reverse-phase silica gels were used as the adsorbent, and a water/methanol mixture (50:50-0:100, v/v) was used as the solvent to elute the extract. After further purification by column chromatography, five flavonoids namely rutin, orientin, isoorientin, isoquercetin, and hyperoside were obtained [38].

High-performance liquid chromatography (HPLC) is the most widely used analytical method for the analysis of bioactive compounds in medicinal plants that have the potential to treat AD. Plant extracts usually contain a combination of bioactive compounds with different polarities, making it difficult to separate them. Fortunately, HPLC is a versatile analytical technique that allows its application to a wide range of compounds with varying polarity and molecular mass. Moreover, it enables the analysis of complex plant matrices with minimal sample preparation, thereby reducing the risk of compound degradation during the analysis. Additionally, HPLC offers high sensitivity, enabling the detection of even trace amounts of bioactive compounds in intricate samples. HPLC analysis is quick, and its system can be automated, allowing for rapid separation when analyzing multiple samples [49, 50].

In the study of AD, HPLC is utilized for the analysis of bioactive compounds in 10 medicinal plants, including the analysis of Araliadiol (C33) in *Centella asiatica L. (Umbelliferae)*. Acetonitrile and 0.1% phosphoric acid are employed as the solvent, revealing the neuroprotective properties of the compound against cognitive impairment. [36]. N-benzyl-(9Z,12Z)-octadecadienamide (M18:3) found in *Lepidium meyenii* Walp (*Brassicaceae*) was examined using UV-Vis detection with a mobile phase composed of 90% acetonitrile. Qualitative identification was achieved by comparing the retention times of macamides with the standards, and peak areas were analyzed to obtain the concentration. The results suggest that macamides and maca extract improve cell adaptability in CORT-induced PC12 cells [37]. In *Nelumbo nucifera (Nelumbonaceae)*, a mobile phase composed of water containing 0.5% acetic acid and methanol, with a gradient program, was used to obtain the major fractions of flavonoids in the seed embryo of *Nelumbo nucifera*. These fractions were determined to be rutin, orientin, isoorientin, isoquercetrin, and hyperoside, detected at a wavelength of 254 nm. The concentrations found to inhibit Aβ1-40 toxicity on dPC-12 cells were in the range of 10–50 µg/mL [38].

Analysis of flavonoids, polyphenols, and tannins, which are bioactive compounds present in Phyllanthus emblica (Euphorbiaceae), was also conducted by injecting filtered samples into the HPLC column for the identification and quantification of glutathione (GSH). This confirmed the excellent antioxidant and radical-scavenging properties, which could be significant in addressing AD [40]. HPLC-MS/MS and HPLC with water and acetonitrile (ACN) as a mobile phase were used to characterize phenolic acids, flavonoids, and fatty acids present in *Opuntia ficus-indica L. (Cactaceae).* Based on the findings, several phenolic acids and flavonoids were detected, with flavonoids as the major compound. However, no betalains were detected due to their low concentration in the extract. This bioactive

compound offers the potential to address various neurological conditions due to its antioxidative properties [39]. HPLC-MS was used to study the antioxidant and microbial properties of phenolic compounds in the extract of *Galanthus nivalis L. (Amaryllidaceae)*. The fingerprint of the polyphenolic compounds was obtained, revealing quantitative analysis surpassing 15 mg/g [34]. *Panax ginseng Meyer (Araliaceae)* was analyzed using HPLC coupled with electrospray-ionization-quadrupole-time-of-flight-mass spectrometry (ESI-Q-TOF-MS), through which the presence and identification of 10 compounds in the extract of P. ginseng seeds were achieved. The retention time obtained was compared with the ion chromatograms (EICs) using the expected theoretical mass-to-charge ratio (*m/z*) of the sodiated pseudomolecular ion from the authentic sample, confirming the presence of the 10 compounds. A new saponin, three known protopanaxatriol saponins, three sterol glycosides, two phenolic glycosides, flavonoid, and three primary metabolites, along with 15 ginsenosides, were expected to be present, but due to a lower concentration of saponin, it could only be observed in the n-butanol extract [44].

Salvia fruticose (Lamiaceae)'s blend, which contained rosmarinic acid, was analyzed utilizing LC-DAD-ESI-MS/MS detection. This helped uncover the underlying neuroprotective properties of the blend for the discovery of new drugs to treat AD [43]. In another study conducted by Naseri et al. [47], the bioactive compound present in the hydroalcoholic extract of *Melissa officinalis L. (Lamiaceae)* was determined using reversed-phase (RP) liquid chromatography, and it was confirmed that the extract was composed of flavonoids and polyphenols, which had antidiabetic effects, inflammation and oxidative stress reduction, and increased insulin secretion. The retention time was used to detect the bioactive compound, and the flavonoid groups were determined to be rutin and apigenin, constituting 9.36% and 0.56% of the extract, respectively. Meanwhile, rosmarinic acid was the main phenolic compound in the extract, composing 4.87% of the extract.

Ultra-high performance liquid chromatography (UHPLC) coupled with mass spectrometry (MS) was used for the analysis of bioactive compounds in two medicinal plants. When compared to HPLC, UHPLC offered the advantage of a shorter analysis time. When the crude extract of *Indigofera sessiliflora (Fabaceae)* was analyzed for the presence of secondary metabolites, eighteen distinct phytocompounds belonging to the flavonoid group were observed. These compounds were found to possess antioxidant characteristics and abilities to participate in cholinergic neuromodulation. [42]. Huperzine A, along with two phenolic acids, caffeic acid and ferulic acid, found in the extract of *Huperzia serrata (Lycopodiaceae)*, was identified as the three major compounds in the extract. This combination has the potential to treat AD and memory loss [48]. A study by Ionita et al. also used HPLC to analyze the photochemical composition of the compound (flavonoids and polyphenolic carboxylic acid) in *Matricaria recutita (Asteraceae)* extract that was administered to the rat model to induce memory impairment. DAD was utilized as a detector, and acetonitrile and water containing 0.1% acetic acid were used as the mobile phase [46].

Qualitative and characterization analysis: Transmission Electron Microscopy

Transmission electron microscopy (TEM) is an advanced imaging technique that involves the interaction of electron beams with atoms in a specimen, enabling the visualization of the specimen's microstructure at an extremely high resolution. This technique permits the study of a specimen's structure and allows observation at the nanometer (nm) scale. In a study conducted by Chen et al. [33], Curcumin (diferuloylmethane), a bioactive component found in *Curcuma longa L. (Zingiberaceae)* was analyzed using TEM. The extract was administered to the mice, and the resulting examination of synapses in the hippocampal CA1 area revealed the promising potential of curcumin in the initial stages of AD.

Electron paramagnetic resonance

Electron paramagnetic resonance (EPR) also known as electron spin resonance (ESR) is a type of spectroscopic technique that is commonly used for the study of the

interaction of atoms or molecules containing unpaired electron with an external magnetic field. EPR spectroscopy can provide information about the electronic structure and bonding of a paramagnetic species. *Galanthus nivalis L. (Amaryllidaceae)* is one of the medicinal plants with potential in the treatment of AD, which uses EPR for the study of its bioactive compounds, specifically chlorogenic acid. Although the extract of *Galanthus nivalis L. (Amaryllidaceae)* contains the highest amount of chlorogenic acid, the EPR signal was revealed not to be dominated by chlorogenic acid. Instead, it is a mixture of isoquercitrin, quercitrin, and chlorogenic acid free radicals [34].

ELISA MRX micro-plate reader

ELISA MRX micro-plate reader is an instrument that can be used for the detection and quantification of specific proteins, antigens, antibodies in a biological sample. It relies on the principle in which the antibodies are bound to a specific antigen, or a target molecule and the measurement of absorbance or fluorescence of samples can then be obtained. Gargouri et al. [45] used ELISA MRX micro-plate reader for the analysis of flavonoids in *Ginkgo biloba L. (Ginkgoaceae)* extract EGb 761 in which the production of pro-inflammatory and cytokines were measured, and the results showed a decrease in neuro-inflammatory activity in primary microglial cells.

Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) is an analytical technique that is used for the identification and quantification of molecules based on their mass-to-charge ratio. The sample solution containing the analyte will be introduced into a mass spectrometer and subjected to an electrospray process which leads to the formation of gas-phase ions in ESI-MS. Flavonoids in *Nelumbo nucifera (Nelumbonaneae)* such as rutin, orientin, isoorientin, isoquercetrin, and hyperoside's chemical structure can be confirmed using ESI-MS's spectral obtained with the published data [38].

UV-Vis spectrophotometer

Two medicinal plants were analyzed by UV as the analytical for the determination of bioactive compounds. UV spectra are useful for the detection of a flavonoid compound due to these compounds having typical chromophoric groups which exhibit characteristics UV profile. In a study conducted by Kumaran et al. [38], extracts of *Nelumbo nucifera (Nelumbonaneae)* were visualized under UV lights at a wavelength of 254 nm and 365 nm and the spectral data were compared with the reported value of the compound. Similarly, Eruygur et al. [35] employed a UV-Vis spectrophotometer to measure the optical density of flavonoids in *Achillea cucullate (Asteraceae)* at 517 nm. The study highlighted the significance of *A. cucullate* aerial parts due to their antioxidant, anticholinesterase, antidiabetic, and moderate antibacterial properties which could be useful as anti-AD agents.

NMR spectroscopy

NMR is an analytical technique to study the magnetic properties of specific atomic nuclei for the determination of their molecular structure and monitor their composition in a mixture. NMR can also be used to study molecular dynamics and interaction. The principle of NMR spectroscopy is based on the absorption and emission of radio waves by the nuclei due to other atoms or molecules bonded to them and the waves are then detected by a detector. For the study of three medicinal plants, the bioactive compounds were determined using NMR spectroscopy. Adib et al. [41] and Kumaran et al. [38] both used the 1H and 13C NMR for the detection of the bioactive compound in *Tinospora cordifolia (Menispermaceae)* and *Nelumbo nucifera (Nelumbonaceae)* respectively. Tinosporide and 8-hydroxytinos oride were isolated from *Tinospora cordifolia (Menispermaceae)* and it is molecular structure was identified and confirmed using proton and carbon NMR. Then, the two bioactive compounds are studied for their acetyl-cholinesterase (AChE) and butylcholinesterase (BuChE) inhibitory activities which it was determined to have anti-AChE activities for treating cognitive impairments in AD. For *Nelumbo nucifera (Nelumbonaceae),* the flavonoid groups that are found that include rutin, orientin, isoorientin, isoquercetrin, and hyperoside is confirmed by obtaining the NMR spectrum and comparing it with the spectrum of the standard that is available commercially [38]. Araliadiol that was isolated from the extract of *Centella*

asiatica L. (Umbelliferae) was determined using proton NMR and the study on neuroprotective effects of *C. asiatica* and Araliadiol shows that cognitive dysfunction can be shielded by the extract (Fujimori et al., 2022).

The combination of chromatographic and spectroscopic techniques, such as LC-MS and LC-NMR, may result in elucidating the structure of natural products without the need for isolation. Using more selective tools in the extraction, fractionation, and purification processes can expedite the time from collecting biological material to obtaining the final purified compound.

Table 2: Medicinal plants with the potential to treat Alzheimer and associated symptoms and their analytical methods/ profiling.

Plant	Bioactive Compounds	Extraction Method	Analytical Methods / Profiling	Experimental Models	Results	Ref
Bacopa monniera Wettst. (Scrophulariaceae)	Alkaloid & Bacoside A	Extracted with chloroform & ethanolic extract	1. HPTLC	In-vitro	Bioactive compounds prevent the activation of N9 microglial cells by inhibiting the release of TNF-α and IL-6. This shows that bioactive compounds proved that they could help with the treatment that focuses on neuroinflammation and have the potential to cure a variety of central nervous system disorders including Alzheimer's disease.	[51]
Rosmarinus officinalis (Lamiaceae)	Nepitrin	Extracted with butanol	1. Column chromatography 2. Thin layer chromatography	In-vivo	Nepitrin has considerable anti-amnesic effects in vivo, inhibits choline esterase enzymes in vitro, and has antioxidant properties. Nepitrin may therefore be a valuable tool in the creation of therapeutics that can help with Alzheimer's disease's short-term and long-term memory loss.	[52]
Urtica dioica L. (Clusiaceae)	Rutin, 2-O-caffeoyl malic acid, and chlorogenic acid	Extracted with ultrasound-assisted extraction technique	1. HPLC	In-vivo	This extract increased antioxidant defense and reduced oxidative stress while also enhancing cardiac performance and hypertension.	[53]
Withania somnifera (Solanaceae)	Withanone	Ethanolic extract	1. HPLC 2. IR 3. NMR	In-vitro and In-vivo	Withanone showed potential for AD cure because of cognitive advantages and mechanisms of action related to the basic pathophysiology of the disease. It has a lot of benefits other than inhibiting AChE, such as modification of Aβ process, decreasing oxidative stress, and reducing inflammation.	[54]
Salvia officinalis (Lamiaceae)	Polyphenols and flavonoids	Methanolic extract	1. HPLC 2. UV-Vis	In-vivo	These findings provide more proof of the effectiveness of S. officinalis extract in the cure of cognitive deficits and may provide a natural treatment for Alzheimer's disease.	[55]
Curcuma longa L. (Zingiberaceae)	Curcumin (diferuloylme	Curcumin was provided by Sigma–	1. Transmission Electron Microscopy	In vivo	Curcumin seems promising as a novel treatment for AD.	[33]

Plant	Bioactive Compounds	Extraction Method	Analytical Methods / Profiling	Experimental Models	Results	Ref
	thane)	Aldrich				
Galanthus nivalis L. (Amaryllidaceae)	Chlorogenic acid	Extracted with 70% ethanol	1. HPLC-MS 2. electron paramagnetic resonance (EPR) spectroscopy	In vitro	The contents of bioactive compounds could be important sources to prevent various diseases associated with oxidative stress including Alzheimer's diseases.	[34]
Ginkgo biloba L. (Ginkgoaceae)	Flavonoids	Extraction solvent (acetone 60% (w/w))	1. ELISA MRX micro-plate reader	Transgenic animal models of AD were used	By targeting PGE2 release and cytokines, gingko biloba extract decreases neuro-inflammatory activity in primary microglial cells.	[45]
Matricaria recutita (Asteraceae)	Flavonoids and polyphenol carboxylic acids	The hydroalcoholic extract	1. HPLC/DAD	A rat model of amnesia	The extract enhanced the scopolamine-induced memory impairments in rats by modulating AChE activity, promoting BDNF, and inhibiting IL1 expression in the hippocampus.	[46]
Nelumbo nucifera (Nelumbonaceae)	Flavonoids (rutin, orientin, isoorientin, isoquercetrin and hyperoside)	70% methanol extract	1. Column chromatography 2. UV 3. ESI-MS 4. 1H, and 13C NMR 5. HPLC	In-vitro assay models	The flavonoid-rich Nn-M-B-VII and Nn-M-B-IX active fractions from N. nucifera seed embryos showed significant reductions in Ab1e40-induced toxicity in dPC-12 cells along with strong radical scavenging activities. It is important to evaluate the optimal concentrations for both the prevention and cure of AD, however, the active fractions indicated cytotoxicity at higher dosages (above 50 mg/mL).	[38]
Phyllanthus emblica (Euphorbiaceae)	Flavonoid, polyphenols, and tannin	Methanolic extract	1. HPLC column	In vitro	Fruit extract from P. emblica has excellent antioxidant and radical-scavenging properties. In the human brain cell line PC12, P. emblica has been shown to have neuroprotective properties.	[40]
Lipidium Meyenii Walp (Brassicaceae)	N-benzyl-(9Z,12Z)-octadecadienamide (M 18:3)	Ethanol extract of maca	1. HPLC	PC12 cells culture using the method described by RuilePan	In PC12 cells, macamides can reduce CORT-induced neurotoxicity, especially M18:3.	[37]
Achillea cucullata (Asteraceae)	Flavonoids are phenolic	Extracted with 80% ethanol	1. UV-Vis spectrophotometer	In vitro	According to this study, A. cucullata's aerial parts have substantial antioxidant,	[35]

Plant	Bioactive Compounds	Extraction Method	Analytical Methods / Profiling	Experimental Models	Results	Ref
	compounds				anticholinesterase, antidiabetic, and moderate antibacterial properties. Therefore, this plant has the potential to serve as a source of naturally occurring anti-AD, anti-diabetic, and anti-inflammatory agents.	
Panax Ginseng (Araliaceae)	Triterpene saponins and three steroidal saponins	Extracted with methanol	1. HPLC-ESI-Q-TOF-MS	In vitro	P. ginseng seeds are a useful natural resource for finding new bioactive compounds, and pseudoginsenoside RT8 has the potential for use in pharmaceuticals as an anti-inflammatory drug.	[44]
Opuntia ficus-indica L. (Cactaceae)	Phenolic acids, flavonoids and fatty acids	Extracted with 100% methanol	1. HPLC-MS/MS 2. HPLC	In vitro and In vivo	Opuntia ficus-indica prevents oxidative stress and may be a promising treatment alternative for a variety of neurological conditions.	[39]
Huperzia serrata (Lycopodiaceae)	Huperzine A and two phenolic acids, caffeic acid and ferulic acid	Traditional extract (NSP01-TE) and the microwave-assisted extract (NSP01)	1. UHPLC-MS	Cell culture of primary rat cortical neurons	Huperzia serrata extract is very potential to cure Alzheimer's disease and loss of ability.	[48]
Indigofera sessiliflora (Fabaceac)	Phenols/flavonoids	Methanolic extract	1. UHPLC-MS	In-vitro, in-vivo & in silico	The phytocompounds in IS.CR have antioxidant characteristics and their capabilities to participate in cholinergic neuromodulation explain why Indigofera sessiliflora has the neuroprotective effects that have been associated with it in traditional uses.	[42]
Melissa officinalis L. (Lamiaceae)	Rosmarinic acid, rutin, and apigenin	Hydroalcoholic extract	1. Reversed phase (RP) liquid chromatography	Diabetic rat model	M. officinalis can improve learning and memory in diabetic rats.	[47]
Salvia fruticose (Lamiaceae)	Rosmarinic acid	Extracted with methanol	1. LC-DAD-ESI-MS/MS	In vitro	Due to its ability to inhibit glycogen synthase kinase 3β CK-1 and β-secretase and increase p-GSK-3 beta protein levels, Salvia fruticosa infusion has a good	[43]

Plant	Bioactive Compounds	Extraction Method	Analytical Methods / Profiling	Experimental Models	Results	Ref
					potential for multipurpose neuroprotective agents for further investigations into the discovery of new anti-drugs Alzheimer's from natural sources.	
Tinospora cordifolia (Menispermaceae)	Tinosporide and 8-hydroxytinosoride	Methanolic extract of the stem of T. cordifolia	1. vacuum liquid chromatography (VLC) 2. 1H and 13C 3. thin-layer chromatography (TLC)	In vitro	These two bioactive compounds could be used as main to develop new drugs for AD.	[41]
Centella asiatica L. (Umbelliferae)	Araliadiol (C33)	Extracted with 50% ethanol	1. NMR spectroscopy. 2. HPLC	In vivo and in vitro	These findings imply that araliadiol has neuroprotective properties that may shield against cognitive impairment.	[36]

Conclusion

Herbal remedies hold promising potential for early-stage treatment of Alzheimer's and similar conditions marked by memory impairment and dementia. A key advantage lies in their comparatively lower toxicity when contrasted with pharmaceutical agents. Comparing the use of herbal medicines in AD treatment with the current pharmacological approaches is essential. These investigations should involve pinpointing the active compounds to enhance clinical trial validation. Additionally, multicenter research is imperative to establish the efficacy of these substances in mitigating cognitive decline in AD. Until such conclusive results are obtained, this review furnishes preliminary evidence of the potential advantages offered by a diverse array of herbs encompassing various traditional medicine systems, including Malay, Indian, Chinese, and European, for AD treatment. The time-consuming and labor-intensive extraction and isolation processes have been a barrier to using natural products in drug development. However, advancements in technology have led to the creation of faster and automated techniques for extracting and separating natural products. These new methods may meet the needs of high-throughput screening in drug development.

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POTENTIAL GREEN LIQUID FROM TERNARY DEEP EUTECTIC SOLVENT COMPOSED OF GALLIC ACID, UREA, AND ZINC CHLORIDE: CHARACTERIZATION OF THEIR PHYSICOCHEMICAL AND THERMAL PROPERTIES

(Potensi Cecair Hijau daripada Pelarut Eutektik Terdalam Ternari daripada Asid Galik, Urea, dan Zink Klorida: Pencirian Sifat Fisikokimia dan Termanya)

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Abstract

Deep eutectic solvents (DESs) have attracted wide attention due to their cheaper cost, ease of manufacture, lower toxicity, and higher biological compatibility. In this study, we offer a more affordable and adaptable path to similar systems using gallic acid (GA), urea (U), and zinc chloride ($ZnCl_2$). The mixture produced, called GA-based DES, was prepared at varying molar ratios of 1:5:1, 1:6:1, 1:7:1, and 1:8:1 (GA:U:$ZnCl_2$). The eutectic liquid form of the GA-based DES mixture was obtained when heated at an operating temperature of 120 °C, below the melting temperature of each individual chemical. The structural and physicochemical properties of the DESs were studied via Fourier transform infrared spectroscopy, thermogravimetric analysis, and viscosity test. Different molar ratios of the DES mixture affected the hydrogen bond interaction formed between GA and U as the hydrogen bond donors and $ZnCl_2$ as the hydrogen bond acceptor in the DES mixture with the presence of O–H stretching and N–H stretching vibration bands as an association effect of GA, U, and $ZnCl_2$. An increase in the U ratio weakened the hydrogen bond and reduced the viscosity of the liquid in the DES mixture due to an increase in O–H stretching. The current findings provide a potential justification for the viscosity of DES mixtures and aid in tailoring the design and development of new DES mixtures for further applications, such as extraction, separation, conversion to functional porous carbon, and biochemical technology.

Keywords: chemical properties, deep eutectic solvent, gallic acid, urea, physical properties

Abstrak

Pelarut eutektik terdalam (DES) telah menarik perhatian luas kerana kosnya yang lebih murah, kemudahan pembuatan, ketoksikan yang lebih rendah, dan keserasian biologi yang lebih tinggi. Dalam kajian ini, kami menawarkan laluan yang lebih berpatutan dan boleh disesuaikan kepada sistem serupa menggunakan asid galik (GA), urea (U), dan zink klorida ($ZnCl_2$). DES berasaskan GA yang dihasilkan dalam kajian ini adalah daripada campuran GA-U-$ZnCl_2$ yang terdiri daripada empat nisbah molar berbeza iaitu 1:5:1, 1:6:1, 1:7:1, dan 1:8:1. Cecair eutektik campuran DES berasaskan GA diperoleh apabila dipanaskan pada suhu operasi 120 °C di bawah suhu lebur setiap bahan kimia. Sifat struktur dan fizikokimia DES dikaji melalui spektroskopi inframerah transformasi Fourier, analisis termogravimetri, dan ujian kelikatan. Nisbah molar campuran DES yang berbeza mempengaruhi interaksi ikatan

hidrogen yang terbentuk antara GA dan U sebagai penderma ikatan hidrogen dan $ZnCl_2$ sebagai penerima ikatan hidrogen dalam campuran DES dengan kehadiran regangan O–H dan jalur getaran regangan N–H sebagai kesan persatuan GA, U, dan $ZnCl_2$. Peningkatan nisbah U melemahkan ikatan hidrogen dan mengurangkan kelikatan cecair dalam campuran DES disebabkan oleh peningkatan dalam regangan O–H. Penemuan semasa memberikan justifikasi yang berpotensi untuk kelikatan hasil daripada campuran DES dan membantu dalam menyesuaikan reka bentuk dan perkembangan campuran DES baharu untuk aplikasi selanjutnya.

Kata kunci: sifat kimia, pelarut eutektik dalam, asid galik, urea, ciri-ciri fizikal

Introduction

Green chemistry stands at the forefront of scientific endeavors, focusing on creating sustainable procedures and substances. With recent industrial growth causing substantial harm to the environment, various substitutes for processes and solvents have emerged to reduce this adverse impact. Ionic liquids (ILs) were introduced as one such substitute solvent in the past few decades and have increasingly gained attention. Ionic liquids have obtained significant interest, particularly in the realms of catalysis, material chemistry, and preprocessing of biomass [1]. The creation of a liquid-phase product, known as a eutectic mixture, arises from the combination of specific precursor components during the synthesis of ILs. Nevertheless, it has been confirmed that these solvents come with specific disadvantages, such as costly synthesis, purification demands, and toxicity [2]. The claimed environmental friendliness of these modern solvents is currently a subject of considerable debate in existing literature [3]. Numerous reports have highlighted the potentially harmful toxicity and the notably low biodegradability of the majority of ILs [4, 5, 6]. Moreover, their synthesis is far from environmentally friendly, as it typically necessitates a substantial quantity of salts and solvents for complete anion exchange. These shortcomings, coupled with the elevated cost of conventional ILs, regrettably impede their industrial advancement. Consequently, there is a pressing need for innovative approaches to employ these systems in a more judicious manner [1].

Therefore, in recent years, a fresh class of so-called green solvents known as deep eutectic solvents (DESs) has emerged as a promising alternative. Deep eutectic solvents are formed by combining two or more salts, incorporating both hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs), such as amides, amines, alcohols, and carboxylic acids [7, 8, 9], which then could be capable of engaging in hydrogen bonds and electrostatic interactions. This combination leads to the formation of a eutectic mixture [6, 10, 11]. Moreover, many HBDs and HBAs have been identified as capable of forming DESs through self-association. The distinctive solvent characteristics of DESs enable a broad spectrum of solutes to exhibit significant solubility in such solvents under certain specific conditions [12]. These properties collectively offer the potential to utilize DESs as an environmentally friendly and effective alternative to traditional methods for synthesizing functional materials. For instance, they can serve as solvent precursors and even contribute to the production of functional carbon material sources. The synthesis of DESs has gained wide attention in numerous literatures. Wang et al. [9] produced DES from different sources of HBAs and HBDs (i.e., tyrosine, urea, and zinc chloride), while Wei et al. [13] applied novelty in synthesizing DES from the mixture of boron acid, choline chloride, and urea. Besides, the key characteristic that sets DESs apart is that their melting point is notably lower than that of the individual components. In some cases, this reduction in melting point can be quite substantial, leading to a liquid mixture even at room temperature.

Furthermore, DESs embody all the favorable attributes of an environmentally friendly and relatively safe solvent [14]. This family of compounds provides numerous benefits, such as straightforward preparation with high levels of purity and yield [15]. Furthermore, DESs are also recognized for their non-toxicity, biodegradability, biocompatibility, and minimal vapor pressure [3, 16]. In addition, DESs hold significant promise in various industrial applications, including organic synthesis, extraction processes,

electrochemistry, enzymatic reactions, and the purification of biodiesel and flavonoids [17, 18, 19]. Some eutectic systems have already demonstrated their ability to dissolve complex lignocellulosic materials like lignin, hemicellulose, cellulose, and starch, yielding successful outcomes.

In this specific context, we introduce a cost-effective and adaptable approach to develop DES systems. This innovative method involves combining gallic acid (GA), urea (U), and zinc chloride ($ZnCl_2$) to create a DES. The inclusion of GA and U, along with $ZnCl_2$ salt, results in a solvent with unique properties. The presence of the amino group ($-NH_2$) in U influences the stretching of O–H bonds, thus impacting the hydrogen bond interaction between the HBD and HBA of $ZnCl_2$ salt in the DES. Luo et al. [20] reported on the synthesis of DES consisting of phenols/ketones, specifically utilizing tannic acid and U. This approach was identified as a favorable pathway for hydrogen bond interaction, with tannic acid serving as the carbon precursor and U as the nitrogen precursor. Carriazo et al. [21] also prepared DES from a phenolic compound, specifically a resorcinol-based DES acting as both a soft template and carbon source. Consequently, drawing inspiration from these previous studies, GA, which is categorized under the phenolic acid group, was chosen as a precursor in this investigation due to its natural origin, cost-effectiveness, and ready availability.

The experimental conditions, including the molar ratio of precursors, were varied to emphasize their impact on bond interaction, composition, morphology, and structural characteristics of the resulting DES. Varied molar ratios of DES mixtures influence hydrogen bond interactions among GA, U (HBD), and $ZnCl_2$ (HBA). This is evident through the O–H and N–H stretching vibration bands, representing the association effect between the individual components. The optimized sample exhibits exceptional solvent and functional performance when utilized as a solvent or other functional materials. This research aims to comprehensively characterize DES systems derived from GA, U, and $ZnCl_2$, providing insights into their physical, chemical, and thermal properties. The ultimate goal is to deepen our understanding of their potential applications, with a specific focus on producing custom porous carbon in future studies.

Materials and Methods

Preparation of des samples at different molar ratios

Chemically pure $ZnCl_2$ and silicone oil were purchased from R&M Chemicals (Selangor, Malaysia), while GA (98%) and U (99.5%) were purchased from Acros Organics (Selangor, Malaysia). The DESs were prepared using various molar ratios of GA, U, and $ZnCl_2$ mixture with GA as a carbonaceous precursor and U as a nitrogen precursor. Zinc chloride was also added as a porogen and activating agent. Figure 1 presents the raw materials used in the synthesis of the DES samples. A round-bottom flask immersed in an oil bath heated by a heating plate was used to prepare the DES mixture. The physical mixture of GA, U, and $ZnCl_2$ in a molar ratio of 1:5:1 was heated at 120 °C with steady stirring until a homogeneous liquid was formed. To investigate the influence of precursor material ratios on the hydrogen bond formation in the synthesized DESs, the molar ratios of GA to U and $ZnCl_2$, designated as Ga:U:$ZnCl_2$, were established at 1:6:1, 1:7:1, and 1:8:1. Figure 2 depicts the experimental set-up.

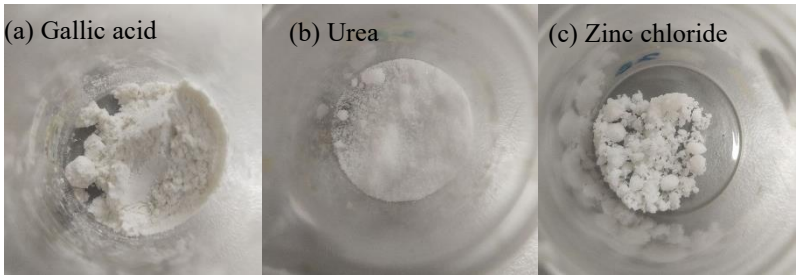


Figure 1. The raw materials of a) gallic acid, b) urea, and c) zinc chloride

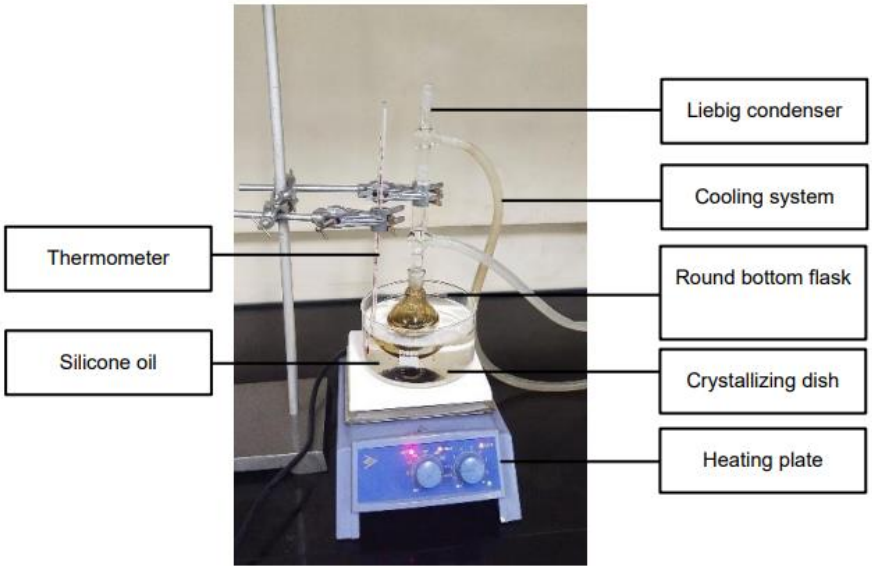


Figure 2. The experimental set-up for DES preparation

Characterization of DESs: The structural and physicochemical properties

The effect of different molar ratios of U content on the structural and physicochemical properties of GA-based DES samples was investigated in depth using viscosity test and Fourier transform infrared (FTIR) spectroscopy analysis. The viscosity test was conducted in this study using a stainless steel digital viscometer (ATAGO VISCO) for each four different molar ratios of DESs. The test was conducted under the operating temperature of 46 °C at 1.0 rpm for each run. Three measurements of the viscosity were taken for each DES sample, and the average values and standard deviations are shown as results. Besides, studies on the chemical structure and functional groups present in the GA-based DES samples produced from four different molar ratios were conducted via FTIR spectroscopy analysis. The wavenumber scans of the FTIR were adjusted to the range 4000–500 cm^{-1}.

Results and Discussion

Physical properties of DESs

To explore the optimal parameters for the formation of DESs, various molar ratios of GA, U, and $ZnCl_2$ were examined. The criteria for assessment included the time required for DES formation, the highest temperature reached during the process, the appearance of the mixture during heating, and the emergence of a homogeneous liquid state. Table 1, Figure 3, and Figure 4 present some physical characteristics of DES mixtures at different molar ratios when subjected to heating in an oil bath.

The evolution of DES formation at the tested ratios was monitored over a 20-min duration at a temperature of 120 °C. Based on visual observations, DESs at various ratios exhibited the formation of highly viscous and adhesive dark brownish liquids, with no apparent color differences among them. However, it was noted that the viscosity of the samples varied, with DES-1:8:1 being the least viscous among the investigated ratios. To validate this finding, viscosity analysis was conducted.

The physical properties of the GA-U- $ZnCl_2$ DES mixtures were determined via the viscosity test for each sample at different molar ratios of DESs using a digital viscometer set at 1.0 rpm. Table *2* presents the data obtained from the viscosity test at different molar ratios of Ga:U:$ZnCl_2$.

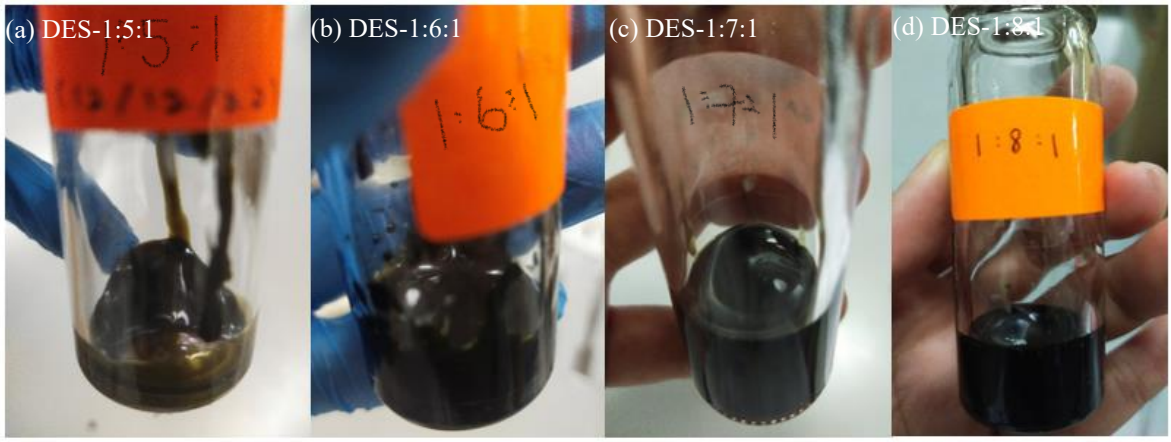


Figure 3. Visual observation of the DES samples at different ratios: a) DES-1:5:1, b) DES-1:6:1, c) DES-1:7:1, and d) DES-1:8:1

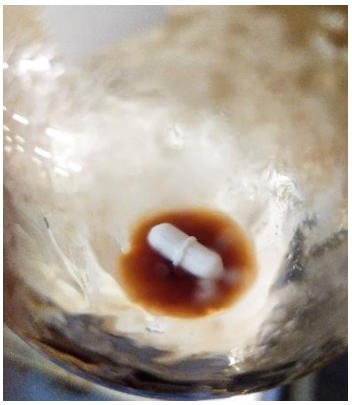


Figure 4. The initial appearance of the DES mixture which turned into a homogeneous brown liquid during heating

Table 1. Properties of DES formation at different molar ratios

Molar Ratio (GA:U:$ZnCl_2$)	**Time (min)**	**Temperature (˚C)**	**Appearance during Heating**	**Appearance at Room Temperature**
1:5:1	15	120	Melts into a brown liquid	Highly viscous mixture with a sticky texture
1:6:1	15	120	Melts into a brown liquid	Highly viscous mixture with a sticky texture
1:7:1	15	120	Melts into a brown liquid	Highly viscous mixture with a sticky texture
1:8:1	15	120	Melts into a brown liquid	Highly viscous brownish liquid with a sticky texture

Table 2. Viscosity of DESs at different molar ratios

Molar Ratio of Ga:U:$ZnCl_2$	**Viscosity Reading (cP)**			
	1st	2nd	3rd	Average
1:5:1	1,400.80	1,399.50	1,400.50	1,400.50 ± 0.89
1:6:1	1,363.60	1,363.20	1,362.10	1,362.97 ± 0.78
1:7:1	1,317.70	1,315.70	1,316.20	1,316.53 ± 1.04
1:8:1	1,063.40	1,062.80	1,063.37	1,063.37 ± 0.55

The interactions between DES components and their macroscopic properties are well-recognized to be strongly connected. Determining the physical characteristics of the DES would therefore be greatly influenced by the hydrogen bond interactions [22] and this statement is aligned with other researchers, in which the hydrogen bonding, van der Waals force, and electrostatic force interaction are the main factors that viscosity is highly dependent on [23, 24, 25]. Viscosity is considered to establish a link between the potency of hydrogen bonds in DESs and their viscosities, which denotes the intrinsic resistance to flow and reflects the intensity of intramolecular interactions within the liquid. *Table 2* illustrates that an increase in the molar ratio, indicating a higher U content in the DESs, results in a decrease in viscosity readings. The sequence of DES-1:5:1 > DES-1:6:1 > DES-1:7:1 > DES-1:8:1 corresponds to a decrease in viscosity.

In this system, U and GA functioned as HBDs, while $ZnCl_2$ acted as a HBA. Increasing the HBD content in the DESs resulted in a reduction in viscosity, primarily due to a decrease in hydrogen bond interactions formed between the chloride ion of salt as HBA and HBD components in the DES mixture. This finding aligns with the results reported by Ghaedi et al. [7], where they observed a similar trend of decreasing viscosity with an increasing amount of HBDs. As the HBD content increases, the hydrogen bonding between the HBD and HBA weakens, which results in an increase in OH stretching bonds, ultimately resulting in lower viscosity [7]. According to Yang et al. [26], one of the major challenges in producing DESs is their typically high viscosity, which can limit mass transfer when used as a solvent. For practical solvent applications, achieving low viscosity in DESs is desirable to enhance mass transfer [7, 27, 28]. Therefore, based on the viscosity test results presented in *Table 2*, DES-1:8:1 can be considered the optimal molar ratio when compared to the other three molar ratios (DES-1:5:1, DES-1:6:1, and DES-1:7:1) because it exhibits the lowest viscosity. Figure 5 depicts the structural elements of GA, U, and $ZnCl_2$ in the synthesis of DES. The precursor components exhibit hydrogen bonding resulting from the interaction between the HBA and HBD.

Chemical properties of DESs

In this study, we formulated and examined newly developed natural DESs. It is common knowledge that DESs comprise HBDs and HBAs. These blends were prepared using a naturally occurring aromatic organic acid, GA, and U as the HBDs, with $ZnCl_2$ as the HBA, and their corresponding spectra are shown in Figure 6a -Figure *6*c. Figure 6d also displays the FTIR spectra of DESs with varying compositions. In accordance with the research by Pena-Solorzano et al. [29], the presence of vibrational bands associated with the NH_2 group is denoted by a peak falling within the range of 3500–3200 cm^{-1}. The peaks located at 1623 and 1461 cm^{-1}, as depicted in Figure 6a, can be attributed to the NH and C-N bending characteristics observed in the pure U sample. Furthermore, a C=O stretching peak is evident at a wavenumber of 1678 cm^{-1}, which aligns with the findings reported by other researchers [30]. Regarding the standard GA depicted in Figure 6b, the spectral region spanning from 3600 to 2500 cm^{-1} is associated with the stretching vibrations of the OH group, and a distinct and sharp OH band is noticeable at 3471 cm^{-1}. Furthermore, the presence of a carbonyl group is indicated by a robust and narrow peak at 1635 cm^{-1}. In addition, the bands appearing at 1635, 1535, and 1431 cm^{-1} are characteristic of stretching vibrations within the C–C bonds present in the aromatic ring of GA. These frequencies closely resemble those previously reported in the literature [31]. Additionally, several other peaks observed in the range of 136–1030 cm^{-1} can be attributed to stretching vibrations associated with the C–O bonds and the bending vibration of the O–H bond within GA. In contrast, the spectra for $ZnCl_2$ (Figure 6c) reveal a vibrational band at 3519 cm^{-1}, which is characteristic of the O–H stretching bond. Furthermore, the spectrum at a wavenumber of 1605 cm^{-1} indicates the presence of O–H bending vibrations in the pure $ZnCl_2$ sample. According to the findings of Pena-Solorzano et al. [29], the occurrence of the O–H bond can be attributed to the presence of water molecules, potentially resulting from the retention of moisture in the sample during analysis. Zinc chloride is known to be hygroscopic, meaning it tends to form hydrates when exposed to water molecules.

Subsequently, FTIR analyses were conducted on various DES samples at different molar ratios, as illustrated in Figure 6d. In general, it can be observed that the four different molar ratios of DESs exhibit minimal variation in the detected peaks and possess relatively similar spectral patterns. The strong C=O band appears distinctly at 1620 cm^{-1} (narrow) and exhibits peaks at 3435 cm^{-1} (broad) in the characteristic bands of DES samples, which can be attributed to the interactions between hydrogen bonds involving the HBD urea, as well as the presence of water content. The stretching vibrations of the C–O bond at 1030 cm^{-1} suggest that the structure of GA remains largely intact within the DES system, demonstrating that the fundamental structural characteristics of GA are preserved in the DESs. When comparing the spectra of pure U (Figure 6a) with the DES samples, a shift in the carbonyl vibration of U is evident, moving from 1678 cm^{-1} to peak wavenumbers at 1573.30, 1573.79, 1572.99, and 1573.11 cm^{-1} for the DES mixtures in Figure 6d. These shifts can be attributed to the formation of coordination bonds primarily between the metal ion and the oxygen atom within the carbonyl group of U [32]. Additionally, Mariappan et al. [30] suggested that these peaks are associated with the C=O stretching vibrations present in the DES samples. Furthermore, the peaks at 1452.06, 1450.34, 1445.35, and 1447.24 cm^{-1} for DES ratios of 1:5:1, 1:6:1, 1:7:1, and 1:8:1 indicate C-N bending attributed to U, as reported.

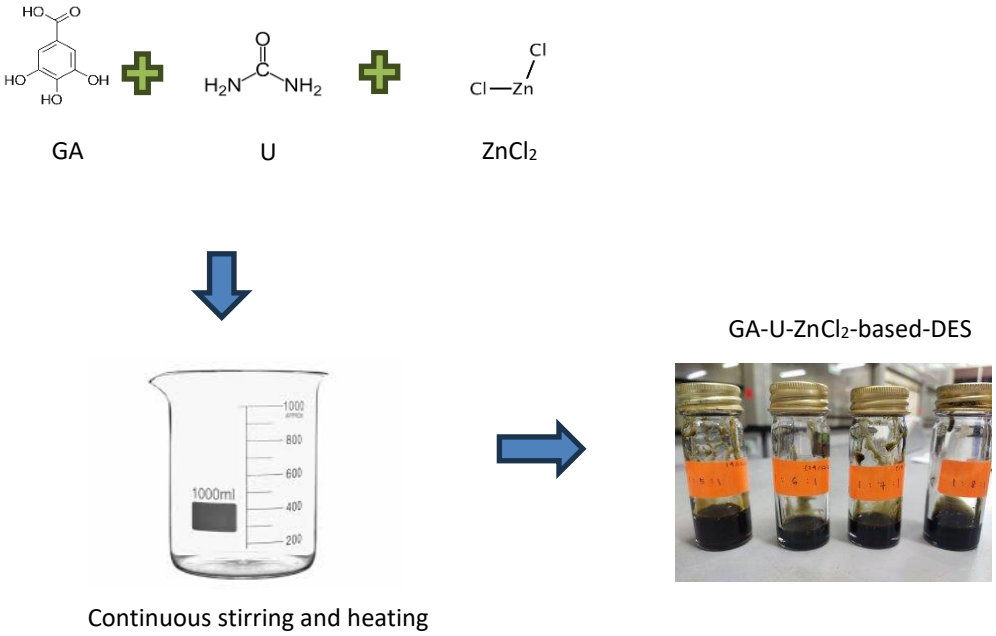


Figure 5. Structural formation of DES from precursor components

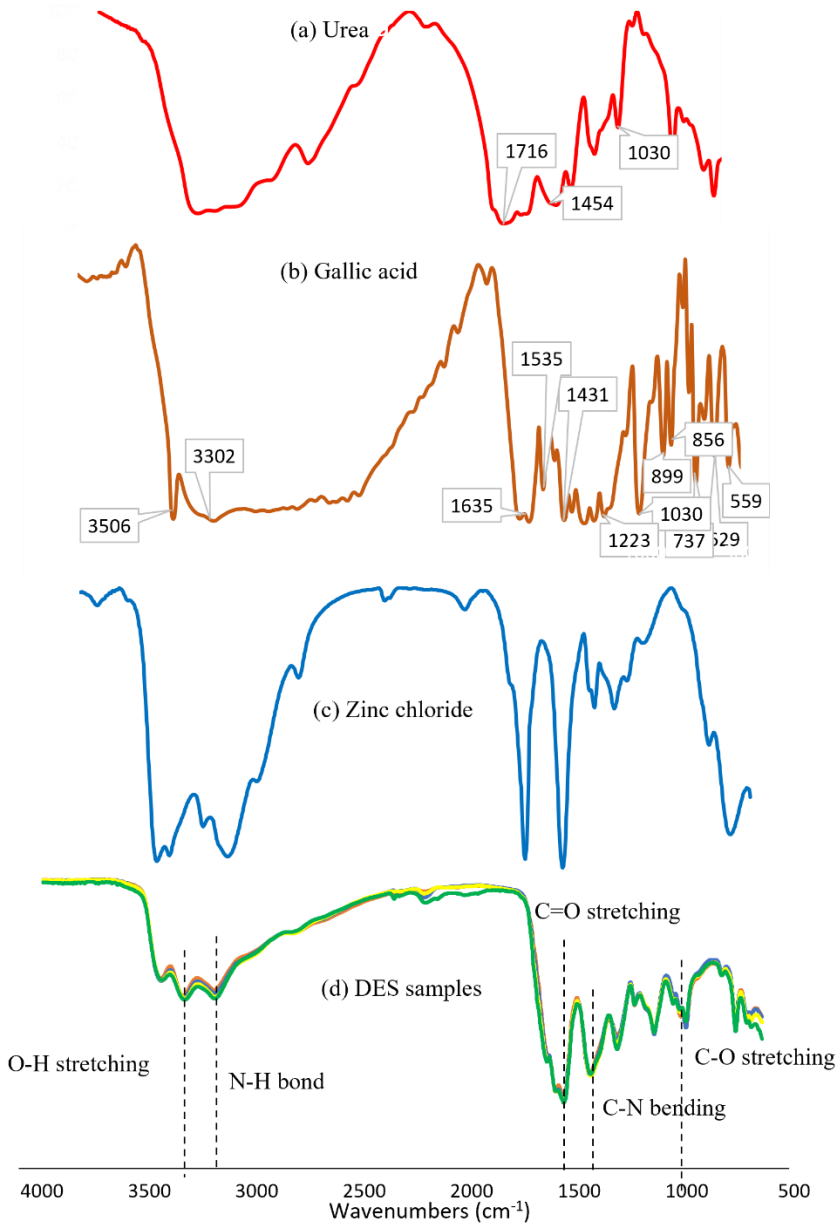


Figure 6. FTIR spectra of DES samples

Thermal properties of DESs

In most cases, hydrogen bond interactions between HBD and HBA result in the formation of homogeneous DES mixture [33]. Hence, the thermal stability of DES was evaluated via thermogravimetric analysis (TGA). Throughout this analysis, the decomposition of HBD and HBA in the investigation at different molar ratios can be determined and studied. According to Chen et. al. [33], at high temperatures, DESs often initially break down into HBDs and HBAs due to the weakening of the hydrogen bond interactions. The volatilization or decomposition of HBA with relatively low boiling points or weak stabilities occurs at a lower temperature than that at which the corresponding HBD occurs [34].

The thermal behavior study of the neat components used in the production of DES is illustrated in thermogravimetric (TG) and differential

thermogravimetric (DTG) thermograms shown in Figure 7. Urea has the lowest boiling point in comparison to GA and $ZnCl_2$. Thus, from Figure 7a, U starts to decompose at an early temperature of 154 °C. Additionally, U shows no further decomposition after 650 °C, which is earlier than the temperatures at which GA (839 °C) and $ZnCl_2$ (723 °C) no longer exhibit decreases in sample weight. The findings from the experiments are aligned with Chen et. al [33], who stated that an HBA generally has poor thermal stability and low boiling temperature, which can cause them to break down or volatilize early during TGA analysis, where U is the HBA in the preparation of the DES sample.

From Figure 7a, the TGA curves demonstrate the first step of weight loss at 86 °C, which might be due to the elimination of crystalline water [35]. The DTG curves show the onset of weight loss at 264 and 320 °C. On the other hand, the initial stage of weight percentage loss for U (Figure 7b) is at 154 °C. The TG thermogram of $ZnCl_2$ shows two steps of the thermal degradation process, where the material had lost around 1.025% and 85.72% in the first and second steps of degradation, respectively, of their total original weight during this process (Figure 7c). The pattern of thermal degradation of $ZnCl_2$ was nearly identical to one of the reported data [35]. The thermogram of the sample disclosed the temperature of the maximum derivative weight loss (T_{max}) at 570 °C. This TG/DTG thermal characteristic information would be helpful for the preparation of eutectic systems.

The thermal stability of DES mixtures can be investigated through TGA and DTG thermal decomposition profiles, which are presented in Figure 8. The TGA and DTG curves extend over the temperature interval (25–900 °C) within which the TGA was conducted. From Figure 8, it can be seen that an initial mass loss (stage A) occurred between the temperature of 25 °C and 110 °C for all samples due to moisture loss. Another peak (stage B) exists in a range of 110–280 °C, which corresponds to the decomposition of U [23]. The mass loss in stage C (from 280 to 400 °C), on the other hand, is due to the decomposition attributed to polymer formation (e.g., monoester and diester) due to the complex reaction among chloride, GA, and U [36]. The onset temperature of sample thermal degradation (T_{onset}), usually defined by 10% mass loss curve, is also a relevant parameter to be investigated through the TGA-DTG thermograms [36]. This temperature establishes the temperature range at which the samples can be utilized without degrading. The values of T_{onset} for DES-1:5:1, 1:6:1, 1:7:1, and 1:8:1 are 180, 181, 176.8, and 175 °C, respectively. These results portray an enhanced thermostability of HBD, particularly U, which initially has a low T_{onset} (154 °C). From the thermal stability point of view between the DES mixture prepared at different molar ratios, $ZnCl_2$ is more heat stable than U; hence, the T_{onset} of DES mixture would decrease with the increased ratio of U. This phenomenon can be ascribed to the surplus U present in the mixture prior to the formation of DES with $ZnCl_2$ (HBA). Overall, the composition appears to resemble a combination of DES and impurities (excess U), indicating that it is not an entirely DES-based system despite the formation of certain hydrogen bonds and the preparation of an approximate eutectic mixture. Additionally, from the TGA and DTG curves, when the U concentration increased, the maximum decomposition temperature (T_{peak}) shifted to higher temperatures. The coordinating nature of the ions, intermolecular interaction, and excess U might explain the rise in thermal decomposition temperatures [27]. These results show that the change in molar ratio has a significant impact on the thermal stability of ternary DES.

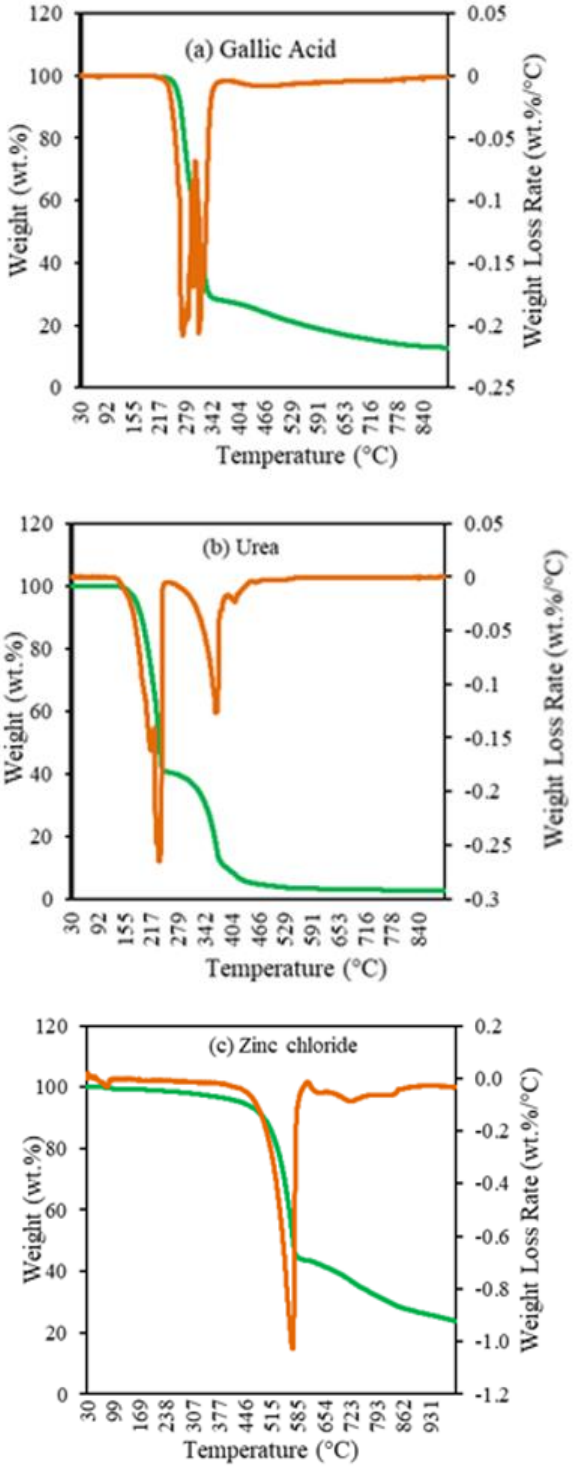


Figure 7. TGA-DTG of the components in DES

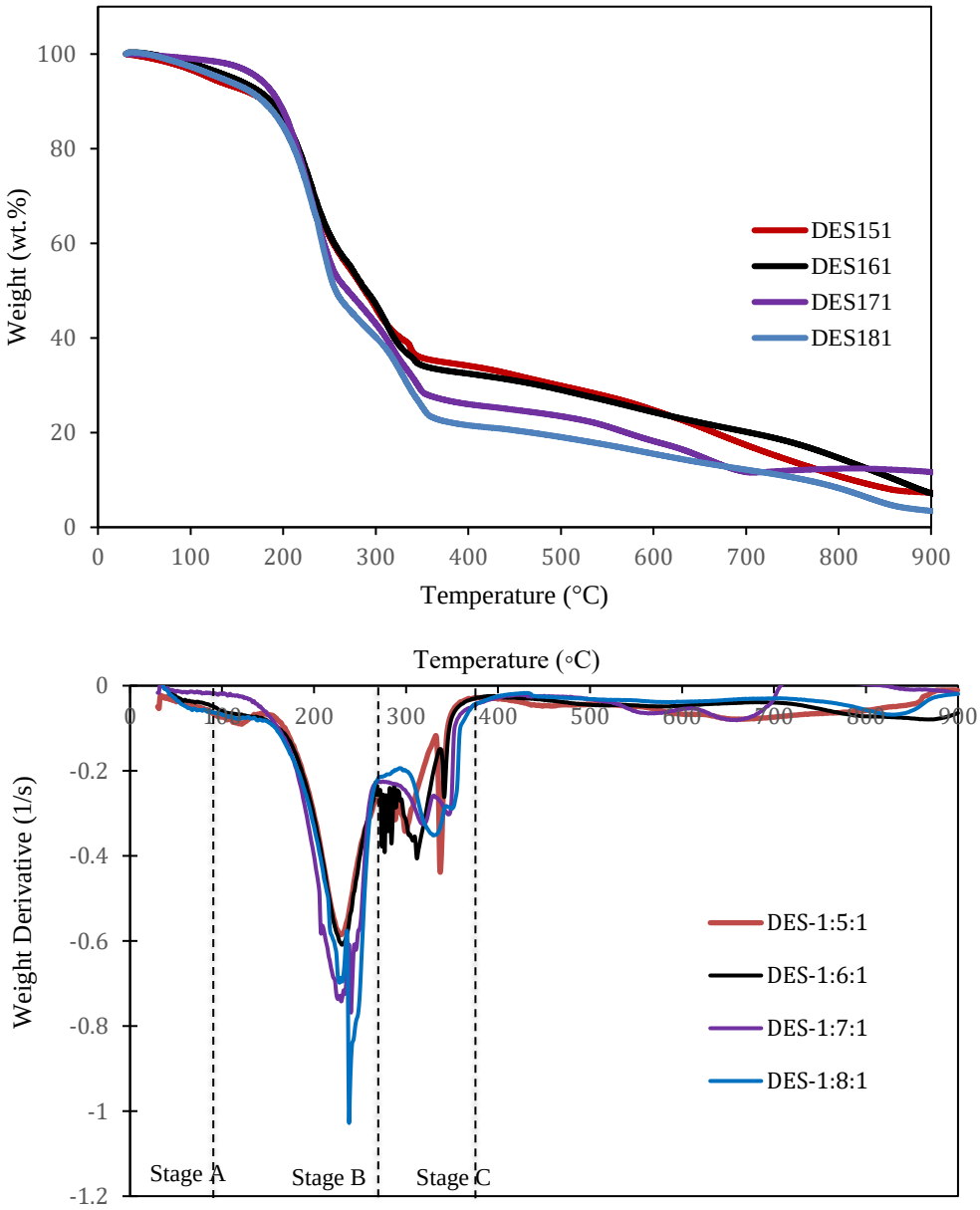


Figure 8. TGA-DTG of the DES samples

Conclusions

In this investigation, a series of DESs was successfully produced using natural components, including GA and U as HBDs, and $ZnCl_2$ as both the salt and HBA. Four different molar ratios, denoted as DES-1:5:1, DES-1:6:1, DES-1:7:1, and DES-1:8:1, were employed for the synthesis. The viscosity results indicated that DES-1:8:1 exhibited the lowest viscosity, suggesting that an increased U (HBD) content led to a reduction in viscosity due to weakened hydrogen bonding resulting from increased O–H stretching during DES synthesis. Furthermore, the FTIR analysis revealed that the GA structure predominantly maintained its integrity within the DES system, as evidenced by the C–O stretching. Additionally, the TGA showed that the thermal stability depended on the molar ratio for the ternary eutectic

mixtures. By carefully choosing an appropriate molar proportion of HBDs and HBAs, particularly focusing on the HBDs, it becomes feasible to synthesize DESs possessing the desired level of thermal stability. This study introduces a novel approach to synthesizing environmentally friendly ternary DESs, showcasing noteworthy properties that hold promise for applications in functional solvents and other materials. The findings not only contribute to advancing the formulation of novel ternary natural DESs but also offer insights for future applications and the customization of formulations in this field.

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A REVIEW ON THE SYNTHESIS STRATEGIES AND FACTORS CONTRIBUTING TO THE FORMATION OF ZEOLITIC IMIDAZOLATE FRAMEWORKS (ZIFs) AND THEIR APPLICATIONS

(Ulasan Strategi Sintesis dan Faktor yang Menyumbang Pembentukan Kerangka Zeolit Imidazolat (ZIFs) dan Aplikasi)

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Abstract

Zeolitic Imidazolate Frameworks (ZIFs) are a subclass of Metal-Organic Frameworks (MOFs) that have attracted significant attention due to their unique properties such as high surface area, tunable pore size, and excellent thermal and chemical stability. The review aims to discuss reported single and mixed-linker of ZIFs. The synthesis of ZIFs can be achieved through various methods such as solvothermal, hydrothermal, ionothermal, microwave-assisted, sonochemical, contra-diffusion synthesis, and mechanochemical. The review discusses the benefits and disadvantages of each method as well as a critical analysis of their effectiveness. The synthesis factors of ZIFs are classified into several types such as organic solvents, additives, concentration, temperatures, metal salt variations and the reaction time. Furthermore, the paper highlights several applications of ZIFs, including their impact on adsorption of pollutants, catalysis and drug delivery system. The paper concludes by summarising the recent advances in ZIFs research which leads to future directions for further research in this field.

Keywords: zeolitic imidazolate frameworks (ZIFs), method, factors, application

Abstrak

Kerangka kerja zeolit imidazolat (ZIF) adalah subkelas kerangka kerja logam organik (MOF) yang telah menarik perhatian kerana sifat uniknya seperti permukaan yang luas, saiz liang yang boleh dilaraskan dan mempunyai kestabilan terma dan kimia yang sangat baik. Ulasan ini bertujuan untuk membincangkan ZIF dengan satu dan pelbagai penyambung yang telah dilaporkan sebelum ini. Sintesis ZIF boleh dicapai melalui pelbagai kaedah seperti solvoterma, hidroterma, ionoterma, bantuan gelombang mikro, sonokimia, sintesis kontra-resapan dan mekanokimia. Kajian ini membincangkan kelebihan dan kekurangan setiap kaedah serta

menyediakan analisis yang kritikal tentang keberkesanan sesuatu kaedah. Faktor sintesis ZIF juga dikelaskan kepada beberapa jenis seperti pelarut organik, bahan tambahan, kepekatan, suhu, variasi garam logam dan masa tindak balas. Tambahan pula, kertas kerja ini membincangkan beberapa aplikasi ZIF, termasuk kesan ZIF terhadap penjerapan bahan pencemar, pemangkin dan sistem penghantaran ubat. Kertas kajian ini juga meringkaskan kemajuan terkini dalam penyelidikan ZIF yang membawa ke menyerlahkan hala tuju masa depan untuk bidang ini dengan lebih lanjut.

Kata kunci: kerangka zeolit imidazolat, kaedah, faktor, aplikasi

Introduction

ZIFs emerges as a promising subfamily of MOFs that combines the advantages of zeolites and MOFs. ZIFs shows high thermal and chemical stability due to the tetrahedral metal-nitrogen bonds, high porosity, and tunable surface area which is achieved by simple modification of the imidazolate linkers or combined mixed linkers. ZIFs are constructed from transition metals like Zn^{2+} and Co^{2+} bridged to N-donor atoms of the imidazolate linkers in a tetrahedral cluster to form a larger and neutral framework (Figure 1). ZIF structures closely resemble aluminosilicate zeolites, where the tetrahedral metal centres of ZIFs mimic the role of silicon, while the N-donor atoms of the imidazolate linkers replace the function of oxygen in zeolites. It was found that the bonding angle of ZIFs was similar to that of zeolites, Si-O-Si, at 145° [1–3].

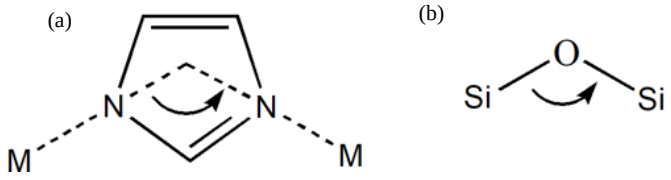


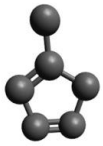
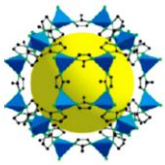
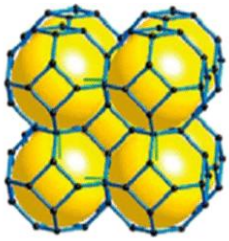
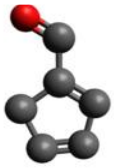
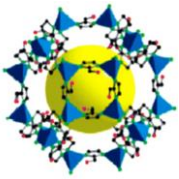
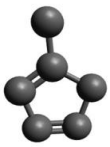
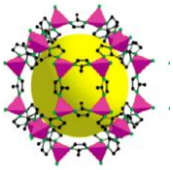
Figure 1. (a) The bond angle in the imidazolate linker, M-Im-M is 145° (b) Tetrahedral arrangement of ZIFs where blue: Metal (Zn, Co) green: imidazolate [3]

Therefore, it is not surprising that ZIFs showed advantages of both zeolites and MOFs. Most ZIF materials demonstrated high thermal stability between 200 to 400 °C under nitrogen. The frameworks also remained stable in various organic solvents at high temperatures and ambient conditions. For example, a chemical stability experiment for ZIF-300, ZIF-301, and ZIF-302 was conducted by immersing the materials in boiling water for seven days. The PXRD patterns of these materials were in good agreement with the simulated PXRD patterns generated from SCXRD [4]. Moreover, the frameworks did not show any compositional changes in organic solvents such as methanol and benzene at 65 and 85 °C within the seven days, indicating that ZIFs were chemically stable materials [1]. The coordination ability between organic linkers and metal centres influenced the formation of ZIFs and offered more controllability and flexibility compared to zeolites. The pore size of ZIFs can be modified by incorporating more than one type of imidazolate linker into the framework along with multiple functional groups. It is also possible to synthesise ZIFs with a wide range of imidazolate linkers and metal centres to create similar topologies. For example, ZIF-8 was synthesised using 2-methylimidazole (2mIM) and zinc nitrate tetrahydrate ($Zn(NO_3)_2.4H_2O$), shared the same sodalite (sod) topology as ZIF-67, which is composed of 2mIM and Co^{2+} centres [5] (Table 1). A similar case occurred with ZIF-90, where this framework was synthesised using 2-imidazolecarboxaldehye (ICA) and ($Zn(NO_3)_2.4H_2O$), resulting in a **sod** topology [6].

In this review, we pay full attention to the research developments in the synthesis and possible applications of ZIFs. We first consider the previously reported single and mixed-linkers of ZIFs, a range of preparation methods (synthetic routes) of ZIFs, encompassing both solvent and solvent-free approaches. In addition, the effects of synthesis conditions on the formation of ZIFs including the main applications in some promising areas

were also highlighted in the write-up. The review is proposed to provide guidance and highlight the significant to the readers for the future development of ZIF materials.

Table 1. Comparison between crystal structures of ZIF-8, ZIF-90 and ZIF-67 with **sod** topology [5]

Metal centre	Linker	Structure	Topology
Zn^{2+}	 2mIM	 ZIF-8	 **sod** topology
Zn^{2+}	 ICA	 ZIF-90	
Co^{2+}	 2mIM	 ZIF-67	

Solvent methods of ZIFs

The solvothermal method is a well-known technique to synthesise MOFs and ZIFs by mixing organic linkers and metal ions in a specific reaction solvent such as dimethylformamide (DMF), diethylformamide (DEF) and *N*-methyl-2-pyrrollidone (NMP). The synthesis reaction is conducted in a closed space environment, such as NMR tube, microplates and vials that can withhold high temperatures (80 to 140 ^{o}C) to generate autogenous pressure [7]. These conditions enhance the solubility of reactants and accelerate the interaction between metal centre and organic linker during the reaction. The synthesis of ZIFs typically takes place over a period of 12 to 96 hours [1–4,6,8]. High boiling point solvents may degrade at high temperatures, forming amine as side product that act as nucleophilic bases, facilitating the crystallisation process [3]. Consequently, ZIFs synthesised via the solvothermal method consistently yield highly crystalline products. The synthesis of ZIF-1 to ZIF-12 was reported in 2006 in which the initial development of ZIFs were focused with Zn^{2+} and Co^{2+} as the metal centres, and the different types of imidazolate linkers such as imidazole (IM), benzimidazole (bzIM) and 2mIM). In 2008, a group of researchers introduced two types of imidazolate linkers bonded in a framework which was used to successfully synthesise ZIF-60 to ZIF-80 in different solvents at the reaction temperature beyond 80 ^{o}C [1,5].

Undeniably, the solvothermal method allows the formation of highly crystalline and phase-purified ZIFs by increasing the solubility of reactants [9]. However, organic solvents are not environmental-friendly and cost-redundant for large-scale synthesis of ZIFs. In addition, large solvent molecules are difficult to remove from the framework's pores. Therefore, the hydrothermal synthesis method has been applied to reduce the usage of toxic organic solvents by replacing them with water [10,11]. This method is usually performed after the successful development of established ZIFs through solvothermal method. Several factors that need to be considered before synthesising

ZIFs using the hydrothermal method includes the concentration of imidazolate linkers, the addition of additives and zinc source [12]. These factors are crucial to ensure crystallinity of ZIFs can be achieved.

from solvothermal synthesis methods, the ionothermal method has been employed to produce ZIF material that utilises ionic liquid or eutectic mixtures [13,14]. It should be mentioned that an ionic liquid can act simultaneously as a solvent and a structure-directing agent (template) to avoid the competition between the solvent framework and the template-framework that existed in hydrothermal preparation due to the presence of hydrogen bonding. This method is performed under ambient pressure in an open system, which eliminates the risks of high autogenous pressure in a sealed autoclave [15]. In addition, the advantage of using ionic liquids as solvent is that they can be reused for further synthesis reactions [14]. ZIF-8 was also synthesised under the ionothermal method using a deep eutectic solvent by heating the mixture of $Zn(NO_3)_2.6H_2O$, 2mIM linker, and tetrabutylammonium bromide (TBAB) while stirring at 40 ^oC [16]. The coordination between the 2mIM linker and Zn^{2+} was initiated by the hydrogen bond interaction between the N-H bond of 2mIM linker and the halide ion of TBAB, which promoted the deprotonation of the 2mIM linker. The obtained material possessed a high BET surface area of 1486 $m^2 g^{-1}$, higher than the ZIF-8 material synthesised under the hydrothermal method (460 $m^2 g^{-1}$). The crystallinity of synthesised ZIF-8 under the ionothermal method was also comparable with the simulated PXRD pattern of ZIF-8 [16]. ZIF-67 was synthesised under the same method in 1-ethyl-3-methylimidazolium bromide ([emim])Br ionic liquid. The mixtures of $Co(NO_3)_2.6H_2O$, 2mIM linker, and ([emim])Br ionic liquid were stirred, and the solution was transferred to a 30 mL Teflon-line autoclave at 140 ^oC for several minutes. The PXRD pattern of ZIF-67 synthesised from the ionothermal method was in good agreement with the PXRD pattern of ZIF-67 synthesised from the solvothermal method [17].

Microwave-assisted synthesis is an alternative method that is analogous to conventional synthesis methods. The fundamental concept behind microwave-assisted synthesis is that microwave heating is produced from the interaction of electromagnetic radiation with mobile electrical charges between solid or soluble conducting ions and polar solvent molecules [15]. In the solid phase, the material's electrical resistance produced the electric current, which contributed to the heating effect on conducting or semiconducting materials [18–20]. While in solution phase, high temperatures could increase the collisions between aligned polar molecules with the rapidly changing electromagnetic field, hence increasing the kinetic energy of these molecules [15,18]. This has led to the generation of microwave heating due to molecular friction. The microwave-assisted method is carried out at a temperature above 100 ^oC in a short time within one hour. The feasibility of the microwave-assisted method has been explored for the preparation of ZIF-11. In the first step, bzIM linker and zinc acetate dihydrate ($Zn(OAc)_2.2H_2O$) were dissolved in the solvent mixture (MeOH, toluene and ammonium hydroxide). The solution was placed in a Teflon liner and microwave- heated at 100 ^oC for 60 minutes. ZIF-11 displayed a regular hexagonal shape with an average size of 5.0 μm and possessed high BET surface area of 745 $m^2 g^{-1}$ [21]. ZIF-8 synthesised under this method exhibited good crystallinity and BET surface area, which is consistent with the results obtained from solvothermal and hydrothermal methods. This material also showed good performance as an adsorbent for the adsorption of Congo Red dye (CGR) and Rhodamine B (RhB), with a removal efficiency achieved beyond 87% [22].

The third synthesis method of ZIFs is the sonochemical method, which is related to the molecules' chemical and physical changes after ultrasonic irradiation. The alternate pressure from ultrasonic irradiation is responsible for generating acoustic cavitation. The hot spots around the liquid were developed in the acoustic cavitation and provided extraordinary high temperature and pressure, which initiated the chemical reaction of ZIFs [18,23,24]. This is an eco-friendly method for producing ZIFs over a short period of time. The sonochemical method is performed using a direct immersion ultrasonic horn or ultrasonic cleaning bath. ZIF-8 was obtained under ultrasound radiation with various power settings (98 kHz, 300 kHz, and

1 MHz) and frequencies (10, 20, and 30 watts) [25]. The crystallinity, purity, and yield of ZIF-8 were maintained using this method. However, ultrasound has a significant impact on the nucleation step and contributes to the fast production of the nucleation centre. This resulted in a reduction of the particle size inZIF-8 [25]. Thin film ZIF-8 was prepared under this method by dissolving 2mIM linker in a mixture of deionised water and TEA while stirring for 10 minutes at room temperature [26]. Then, the zinc hydroxide solution was immersed in the respective 2mIM linker solution before being sonicated at room temperature. The frequency and power of the sonicator was set at 40 kHz and 100 watts. The crystal size of ZIF-8 was found to increase as the reaction time proceeded from 5 minutes to 60 minutes. Moreover, the PXRD patterns of ZIF-8 became more sharp and intense, resulting from the prolonged sonication time [26].

Contra-diffusion is another method that can be used to produce existing ZIFs. During the first stage of the reaction, the metal and organic linker solutions are diffused in opposite directions along the support or the defects in the membrane grains, forming a thin membrane layer [27]. Moreover, the contra-diffusion approach is an alternative method for reducing the reaction rate and producing a thin and defect-free membrane. A well-intergrown and continuous ZIF-8 layer is formed on the metal ion side surface of the poly(4-vinylpyridien) brushes as a result of a combination of surface graft and the optimal addition of sodium formate into the ligand. ZIF-8/poly(4-vinylpyridien) membrane demonstrated excellent reusability as an adsorbent material for iodine, with a removal efficiency of 73.4% after five cycles [28]. In 2019, a group of research successfully formed a uniform layer of ZIF-8 on porous polyvinylidene fluoride (PVDF) ultrafiltration membranes. The membranes were separated and filled with solutions of 2-methyl imidazole and zinc nitrate. Both the processes of solution diffusing into the membranes crystallisation took place at room temperature which resulted in an excellent performance of the ultrafiltration process [29]. ZIFs can be synthesised using various methods and it is evident that further development can be undertaken to reduce the reaction time, increase production yield and improve the porosity of the framework.

Solvent-free method of ZIFs

Mechanochemical synthesis is another method that could produce ZIFs. Compared with conventional synthesis methods, mechanochemical synthesis is an approach that uses little solvent and energy and reduces reaction time. This synthesis method uses a ball mill to grind metal salt and organic linker without the involvement of solvent or heat [18]. The mechanical energy generated from grinding would minimise the particle size to promote the interaction between the organic linker and metal ions [15]. Mechanochemical synthesis was conducted at room temperature by converting ZnO to ZIF-8. The solution of ZnO and 2mIM linker were placed in a 300 mL ceramic pot before the ceramic pot was rotated at 100 ppm [30]. The transformation of ZnO to ZIF-8 produced a lower yield product after prolonged milling exposure. The addition of $(Zn(OAc)_2.2H_2O)$ was essential to promote acid dissolution of ZnO and facilitate the complex formation between ionised Zn and the 2mIM linker [30]. Thus, adding a small liquid could promote the formation of ZIF and improve the morphology of ZIF-8 under mechanochemical synthesis from agglomerated amorphous ZIF to dendritic porous architecture (Chen *et al.*, 2014) . The glass-forming ZIF-62 was synthesised by the mechano-synthetic route using liquid-assisted grinding. All starting materials, that are ZnO, 2mIM, and IM linkers, were mixed together in a 10 mL stainless steel grinding jar and was shaken for 30 minutes. In this synthesis method, ZnO was used instead of $Zn(NO_3).2H_2O$ or $ZnCl_2$ to avoid the formation of HNO_3 or HCl by-products that would have resulted from the metathesis of Zn salts with IM and bzIM linkers. This is because the acid by-products would digest ZIF to its starting materials. The mechanochemical method would reduce the amount of starting materials and reaction time for the production of materials compared with the solvothermal synthesis method. Although the crystal size of ZIF-62 obtained from the mechanochemical method is smaller than the conventional synthesis method [31].

Synthesis of single linker ZIFs

Up until 2023, the synthesis and characterisation of ZIFs had been achieved. The pioneering work in this field was led in 2006, when they successfully synthesised 12 ZIFs.

These included ZIFs with Zn^{2+} centres, namely ZIF-1 to ZIF-10, as well as those with Co(II) centres, specifically ZIF-9 and ZIF-11. This achievement was accomplished through the use of a variety of imidazolate linkers, such as IM, bzIM, and 2mIM, resulting in the formation of novel ZIF materials with distinct topological structures [3]. There are six ZIF structures, all composed of Zn^{2+} centres with imidazolate linkers, each displaying different three-dimensional (3D) topologies, including **crb, dft, cag,** and **mer** s depicted in Figure 2.

Notably, ZIF-1, ZIF-2, ZIF-3, ZIF-4, ZIF-10, and ZIF-14 were crystallised in various space groups and crystal systems, which were influenced by the molar composition of the reaction conditions. Consequently, this variance resulted in the formation of diverse topologies. In particular, ZIF-10 was found to crystallise within a tetragonal crystal system with a space group of *I4/mmm. A*mong these ZIFs, ZIF-10 exhibited a notably high crystal volume of approximately 14,211(2) $Å^3$. This exceptional volume can be attributed to the high concentration of imidazolate linker which led to the formation of bigger framework. All the nitrogen atoms from imidazolate are coordinated to Zn^{2+} centres in a tetrahedral geometry resulting in the formation of an 8-membered ring and a 4-membered ring of **mer** and **dft** topology [3,5]. The pore diameter of **mer** topology is 12.12 Å, which is more significant than **dft** topology, 8.02 Å [32]. ZIF-8 and ZIF-67 are well-known frameworks that consist of a similar linker, 2mIM. They adopted the **sod** topology with 4- and 6-membered rings, despite having different kind of metal centres. This topology featured one kind of vertice and edge with a pore diameter of 11.6 Å, which received a considerable attention from many researchers to utilise these frameworks in many promising applications. The surface areas of ZIF-8 and ZIF-67 are 1947 $m^2 g^{-1}$ and 1676 $m^2 g^{-1}$, respectively, surpassing those of other ZIFs with single linker [3,5].

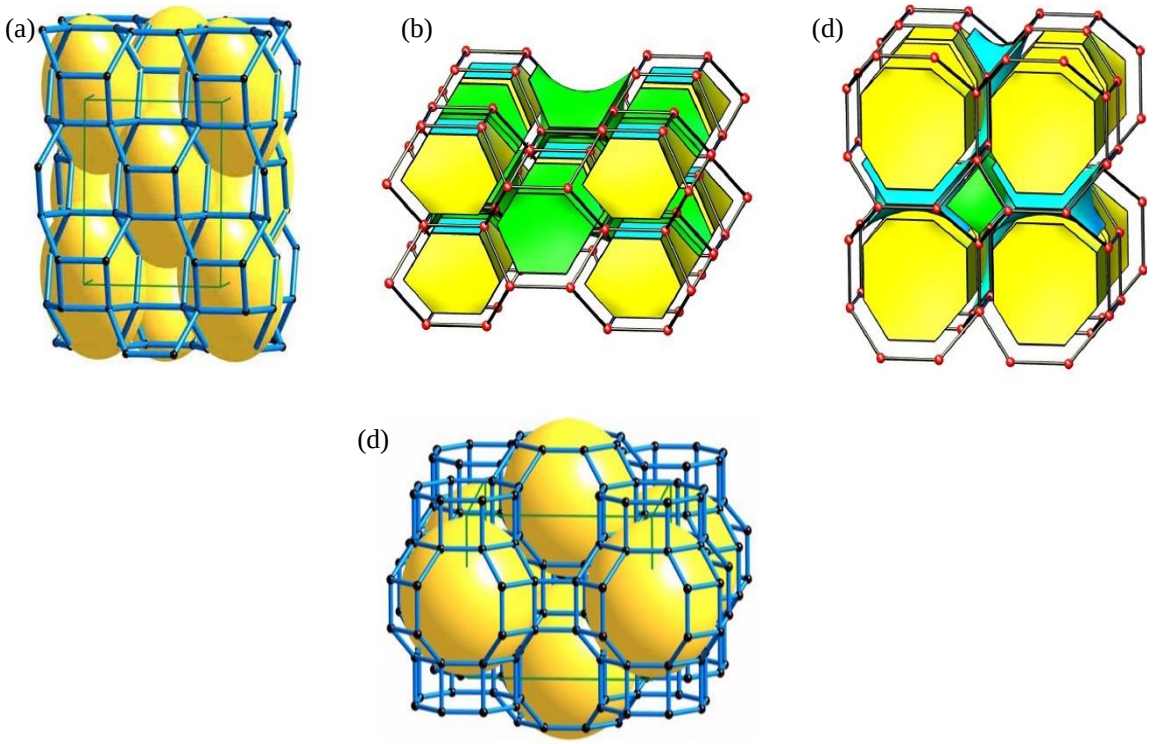


Figure 2. Topologies of the network structures; (a) ZIF-4, **cag**; (b) ZIF-1 and ZIF-2, **crb**; (c) ZIF-3, **dft**; (d) ZIF-10, **mer** [3]

Meanwhile, ZIF-7 and ZIF-11 are comprised of similar metal centres joined by bzIM linkers, but these frameworks adopted different topologies (ZIF-7: **sod and** ZIF-11: **rho),** as shown in Figure 3. The introduction of bzIM in the framework allows for the formation of a larger topology due to the functionalisation of the imidazole linker with benzene rings at 4th and 5th positions. The crystal systems for ZIF-7 and ZIF-11 are hexagonal and cubic, with space groups of $R\bar{3}$ and $Pm\bar{3}m$, respectively. Based on SCXRD results, ZIF-11 was proven to have a high crystal volume of about 23,787(14) Å^3, in contrast to ZIF-7 (7,214(14) Å^3). The topology of ZIF-11 (**rho**) is considered a large topology in the presence of 4-, 6-, and 8-membered rings, compared to the **sod** topology. Therefore, ZIF-11 was successfully crystallised in a larger framework, over longer reaction time and medium temperature of 100 °C, which led to a slow crystallisation process and higher surface area of the framework. Both the ZIFs have exhibited high chemical stability and the crystallinity of the frameworks were maintained after immersing them in boiling benzene, water, methanol, and ethanol for seven days.

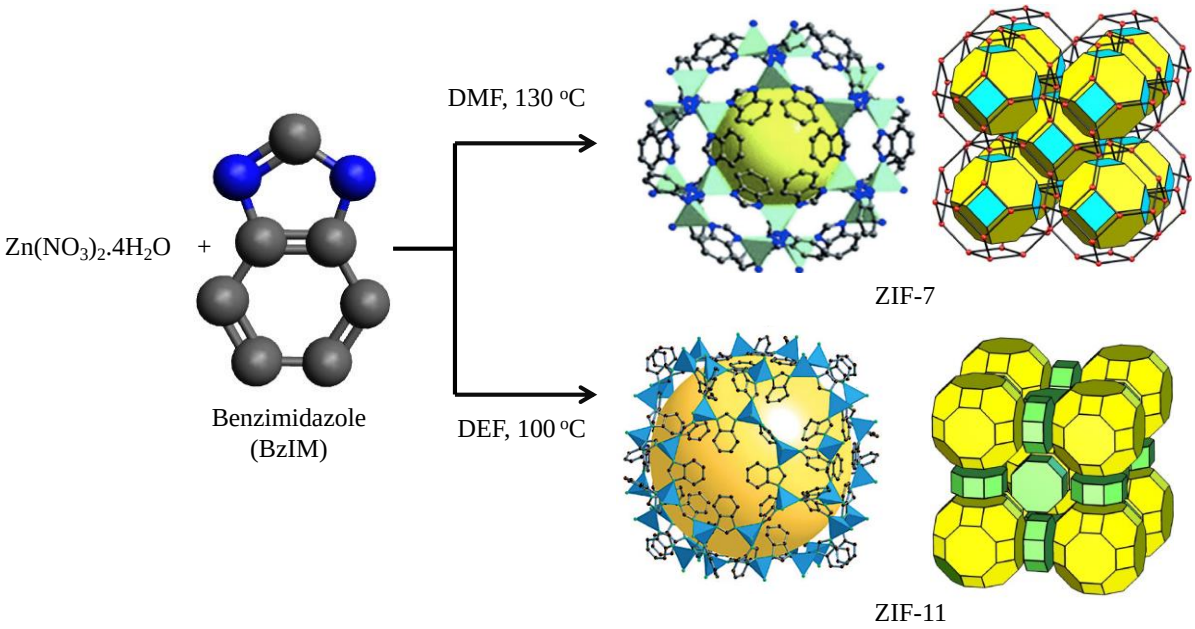


Figure 3. The formation process of ZIF-7 and ZIF-11 which produced different kind of topology [3,5]

In 2008, new porous ZIFs with high surface area were reported, namely ZIF-95 and ZIF-100 (Figure 4). ZIF-95 was constructed using zinc nitrate tetrahydrate ($Zn(NO_3).4H_2O$) and 5-chlorobenzimidazole (5cbIM). ZIF-95 crystallises within the tetragonal space group, displaying *I4/mmm* symmetry, resulting in an underlying net topology symbolised as **poz**. In contrast with ZIF-95, ZIF-100 featured a tetrahedral metal centre with zinc trimethafluorosulfanate ($Zn(O_3SCF_3)_2$). SCXRD results revealed that ZIF-100 shared the same crystal system as ZIF-11 and has $Im\bar{3}$ symmetry, with an extensive cell volume of approximately 372,932(4) Å^3, which proved ZIF-100 to be a large inorganic framework. It is believed that the incorporation of 5cbIM into the framework led to the formation of a new topology, facilitated by the functionalisation of bzIM with a chlorine atom at the 5th position. Figure 4 illustrates the ZIF-100 underlying net topology, denoted as the **moz** topology, with an inner diameter of the framework exceeding that of the faujasite (**fau**) topology, measuring approximately 35.6 Å [2].

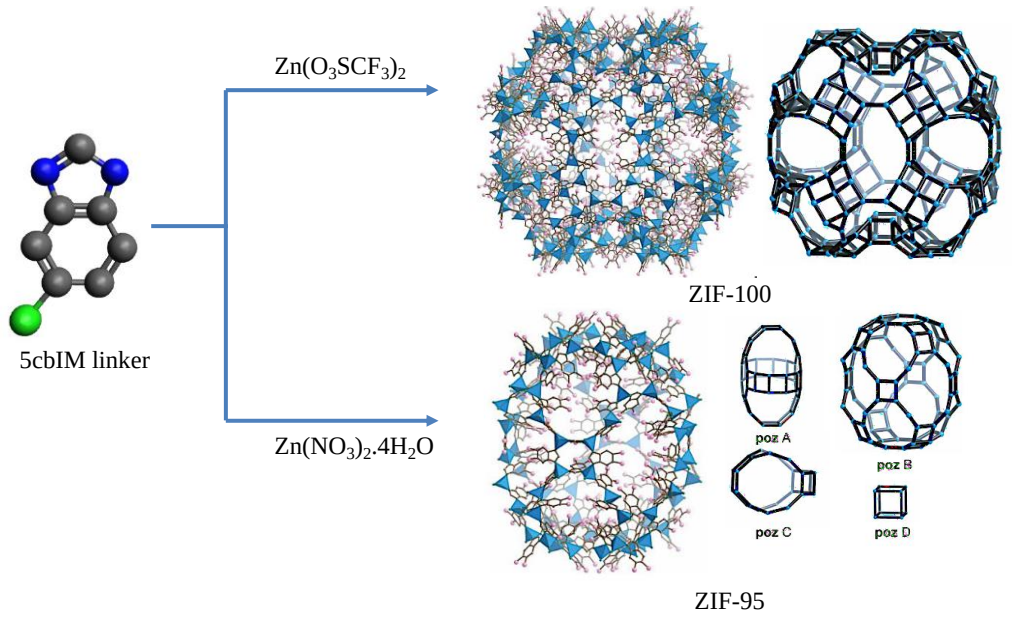


Figure 4. The structure of ZIF-95 and ZIF-100. Both the ZIFs were made up of different kind of metal salts with 5cbIM linker , forming different types of topologies [2].

Notably, ZIF-95 encompasses two distinctive types of cages with varying inner diameters. The initial cage, known as **poz A**, possesses an inner diameter measuring 25.1 x 14.3 Å, making it roughly the same size as the super cage in the **fau** topology. The second cage of ZIF-95 is **poz B** with 30.1 x 20.0 Å of inner diameter, significantly greater than the diameter of the **fau** topology, which is around 14.1 Å. The extended framework was linked by the tetra coordinates of Zn^{2+} centre for ZIF-95, resulting in the creation of an exceptionally expansive **poz** topology. This topology accommodates two large cages, with the first **poz** cage (**poz** A) followed by the second **poz** cage (**poz** B), both cages containing 8-, 10-, and 12-membered rings. Importantly, each imidazolate linker in ZIF-95 exhibits specific steric indexes (δ), as indicated in Figure 5.

The steric index is used to measure the size and shape of imidazolate linkers, taking into account their van der Waals volume and length. Imidazole linkers with increased bulkiness exhibit a higher steric index value (δ) compared to smaller imidazole linkers,. The strategic introduction of bulkiness at the 4th and 5th positions of imidazolate linkers has profound impact, resulting in the formation of large cages containing more than 8-membered rings within the frameworks. This led to the formation of entirely new and extensive underlying net topologies, which is a direct outcome of incorporating imidazolate linkers with high steric indices [8].

Synthesis of mixed-linker ZIFs bearing two different imidazolate linkers

There are several advantages in mixed-linker ZIFs compared to single linker ZIFs. Incorporating two or more different imidazolate linkers in the framework enables the fine-tune of surface area and pore size by selecting imidazolate linkers of varying size and length. For instance, the gas separation efficiency is significantly enhanced in mixed-linker ZIF-7-8, whereas the incorporation of the bulkier bzIM linker, along with the 2mIM linker, results in the formation of a larger aperture [33]. The introduction of different functional groups derived from distinct imidazolate allows for the tailored design of functionality within the frameworks, providing a variable surface environment. This mixed-linker strategy is exemplified by introducing two different imidazolate linkers with diverse functional group, such as combining 2mIM with 2-aminobenzimidazole (2amBzIM) in the ZIF-8 framework [34]. This approach serves to improve the permeability and selectivity for CO_2 capture. Moreover, the frameworks that contained three imidazolate linkers

leads to formation of a substantial topology, as observed in ZIF-412, -413, and -414, generating a novel underlying topology [8]. This unique topology demonstrated capability in capturing large molecules, such as xylene, which further proves the capability of mixed-linker ZIFs to capture molecules like xylene which is comparably difficult to attain in a single linker ZIFs.

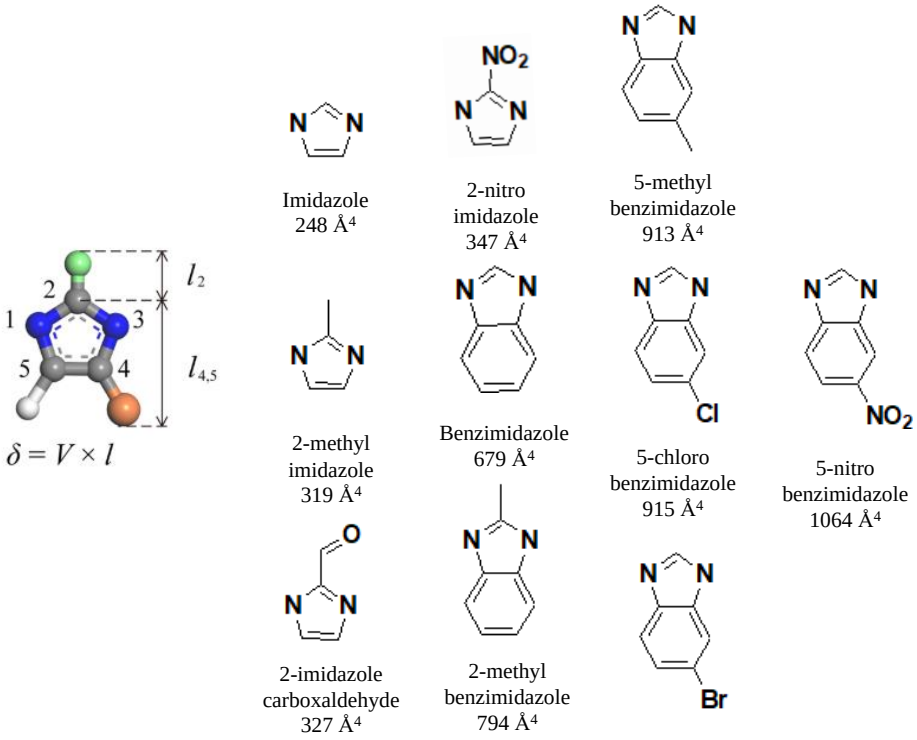


Figure 5. Steric index for imidazolate linkers based on the van der Waals volume (V) and length (*l*) [8]

Incorporating multiple linkers within a single framework proves to be an efficient strategy for the development of novel ZIFs with diverse functionalities, adjustable porosity, customisable topology, and enhanced performance tailored for specific applications. Mixed-linker ZIFs were synthesised based on the chabazite, **cha** topology, which involves the utilisation of bulky imidazolate derivatives such as 5-methylbenzimidazole (5mbIM), ZIF-302; 5-chlorobenzimidazole (5cbIM), ZIF-301; and 5-bromobenzimidazole (5bbIM) for ZIF-300. These derivatives were employed with 2mIM to form coordination bonds with Zn^{2+} centres [4]. ZIF-300 and ZIF-302 were crystallised in trigonal crystal system, and the space group associated with the crystals was *R*-3. Consequently, these ZIFs were classified as part of the isoreticular series of mixed-linker ZIFs.

The isoreticular framework constitutes of a series of compounds sharing the same topology while featuring organic linkers with varying functionalities and sizes [35,36]. Based on NMR analysis, bzIM derivatives were the dominant organic linkers in the frameworks compared to the 2mIM linker, even though the molar composition of metal to organic linkers maintained an equivalent ratio for both ZIF-300 and ZIF-301. This finding was further supported by SCXRD results, demonstrating that all the organic linkers were bound to the tetrahedral Zn^{2+} clusters. The resulting 3-periodic frameworks exhibit the **cha** topology, where the use of water as a co-solvent plays a crucial role in forming specific cavities, as shown in Figure 6. These cavities were composed of two distinct types: a *cha* $[4^{12}6^{2}8^{6}]$ cavity (the symbol $[m^n]$, signifying the presence of n faces with *m*-membered rings), which is described as an 8-membered ring in this topology. Here 5mbIM, 5cbIM

and 5bbIM linkers are situated within this cavity. In contrast, derivatives of 2mIM or bzIM linkers are positioned at the edge of **cha** cavities, connecting to the two 6-membered rings of *hpr* cavities [$4^6 6^2$] (small cage). In addition, all of these frameworks remained stable when subjected to boiling water for seven days, maintaining their crystallinity, as evident from experimental PXRD patterns. Furthermore, these ZIFs demonstrated type-III isotherms as a result of incorporating hydrophobic functionalities into the frameworks, thereby enhancing the overall stability of the frameworks [4].

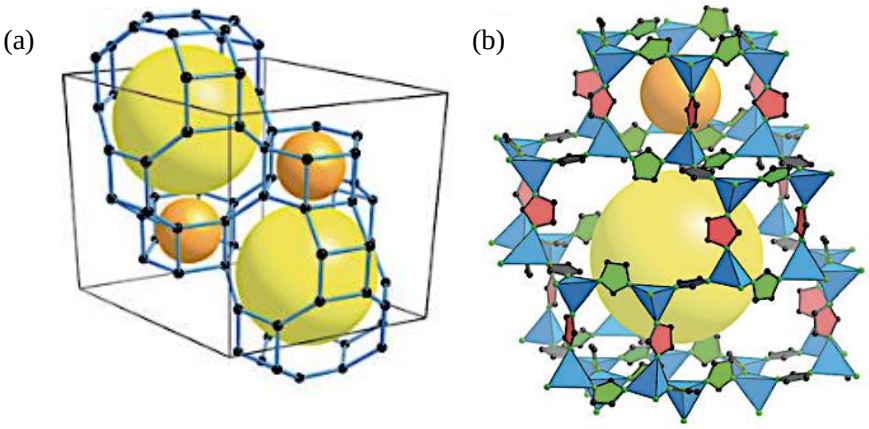


Figure 6. (a) Ball and stick model represent **cha** topology composed of two types of cavities *hpr* (large) and *cha* (small). (b) The **cha** topology of ZIF-300 and ZIF-301. Greyish-blue and red imidazolate linkers represent 2mIM and bulky imidazolates (5bbIM, 5cbIM and 5mbIM) respectively whereas green imidazolate linkers indicated the position for non-specific linkers either 2mIM or bzIM derivatives can occupy in this position [4, 37].

The isoreticular series of ZIFs were successfully synthesised using 2-nitroimidazole (2nIM) with different types of imidazolate derivatives ranging from polar (-CN, ZIF-82; -NO_2, ZIF-78) to non-polar functionalities (-CH_3, ZIF-79; -Br, ZIF-81; -Cl, ZIF-80). These ZIFs formed definite topology with broad channels and large pore diameter [1]. In particular, the exact topology of the framework structures was constructed with different functionalities of cavities and different sizes of organic linkers, which determined the isoreticular series of ZIFs, as illustrated in Figure 7. ZIF-79, ZIF-80, ZIF-81, ZIF-82 and ZIF-78 were synthesised by solvothermal method using different solvent and period of reactions. These ZIF compounds crystallised in a hexagonal crystal system, sharing the common space group of $P6_3/mmc$. Intriguingly, despite the introduction of diverse functionalities of the imidazolate linkers, these five mixed-linker ZIFs exhibited a gmenilite (**gme**) topology [2,3,5]. As shown in Figure 7, the **gme** topology consists of *hpr* cages where the 2nIM linker occupies along the cage edges, while the imidazolate linker with varying functionalities are within the cavities of *kno* cages with pore diameter ranging from 7.1 Å to 15.9 Å.

Remarkably, functionalised imidazolate linkers found in ZIF-78 and ZIF-79, such as 5-nitrobenzimidazole (nbIM) and 5mbIM contributed to smaller pore diameters around 7.1 Å and 7.5 Å, respectively, achieving a slightly tilted orientation in the topology. ZIF-82 containing smaller imidazolate linkers, 4-cyanoimidazole (cnIM) and 2nIM, featured larger pore diameter around 12.3 Å [1]. In addition, PXRD of these ZIFs remained unchanged even after exposure to boiling benzene, water and methanol. This demonstrated that ZIFs are extremely stable under diverse environmental conditions. The integration of multiple imidazolate linkers within a single framework emerges as an efficient strategy for attaining variable functionalities, larger cages and varying pore sizes of the frameworks.

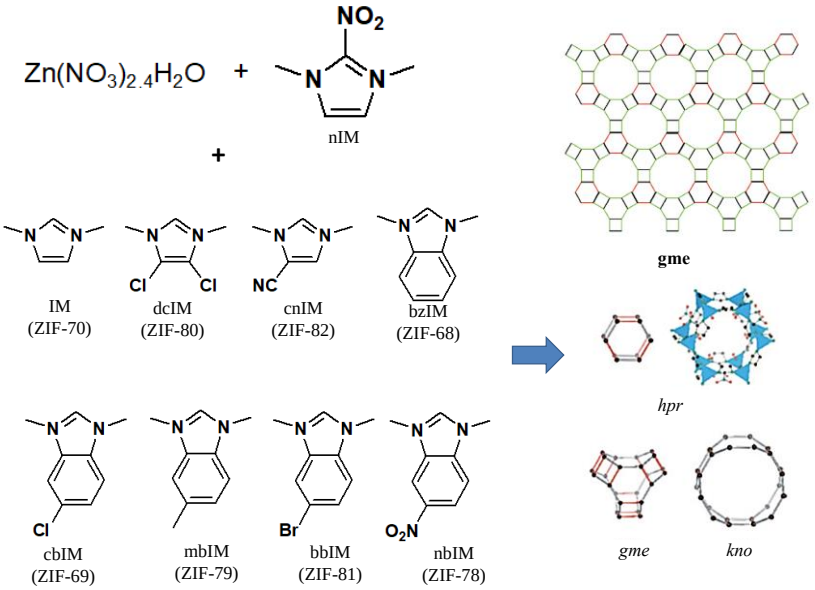


Figure 7. Imidazolate linkers and their abbreviations (IM: Imidazole; dcIM: 4,5-dichloroimidazole; 4cnIM: 4-cyanoimidazole; 5cbIM: 5-chlorobenzimidazole; 5mbIM: 5-methylbenzimidazole; 5bbIM: 5-bromobenzimidazole; 5nbIM: 6-nitrobenzimidazole) reacted with Zn^{2+} and 2-nitroimidazole (2nIM) to form **gme** topology. The **gme** topology consist of several cavities such as *hpr*, *kno* and *gme* as illustrated on the left side [2,3,5].

In 2017, a group of researchers obtained new ZIFs by introducing two bulky imidazolate linkers into the framework, named ZIF-516 and ZIF-586. The crystal structure of ZIF-516 consisted of 5bbIM and 5mbIM linkers, while ZIF-586 featured 5mbIM and 2mbIM linkers, linked together by tetrahedral Zn^{2+} clusters. These ZIFs were crystallised in a tetragonal crystal system; however, both ZIFs were arranged into different space groups (ZIF-516: $P4_32_12$ and ZIF-586: $P4_2/ncm$). Despite the distinct space groups, these frameworks were classified under the same underlying net topology, **ykh,** containing large cages ranging from 22.1 to 22.3 Å. Figure 8 shows the **ykh** and **moz** topology of ZIF-516, ZIF-586 and ZIF-408. The introduction of 5bbIM, 5mbIM, and 5cbIM with functionalised imidazolate linkers at 4th and 5th positions resulted in the formation of large and small cages comprising of 3-, 4-, 10-, 12-, and 14-membered rings, generating large topologies such as **ykh**, **poz**, **moz,** and **zea** [8].

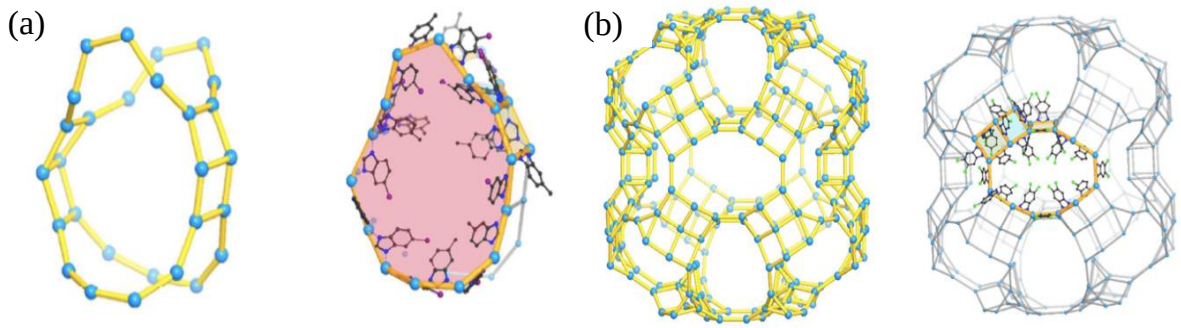


Figure 8. (a) **ykh** topology of ZIF-516 with 3-, 4-, 10-, 12-, and 14-membered rings. (b) **moz** topology of ZIF-408 consists of 4- and 12-membered rings. C: black, N: blue, O: red, Cl: green and Br: violet [8]

ZIF-412, ZIF-413, and ZIF-414 formed a new underlying topology, **ucb**, featuring several types of membered rings ranging from 4-, 6-, 8- to 24-membered rings. The cage size was measured at 45.8 Å, making it the largest cage within the topology (Figure 9). The building units for ZIF-412, ZIF-413, and ZIF-414 was a combination of multiple bulky imidazolate linkers (6nbIM, bzIM, 5mbIM) with small imidazolate linkers (2nIM and IM). These compounds crystallised in a cubic system with the space group, $Fm\bar{3}m$. The combinations of small and large steric indices facilitate the formation of large and small cages in ZIFs, resulting in topologies like **moz** (41.2 Å) and **ucb** (45.8 Å). Therefore, it is important to incorporate small imidazolate linkers like imidazole alongside other bzIM derivatives with high steric index to enable the creation of extensive crystal structures [8].

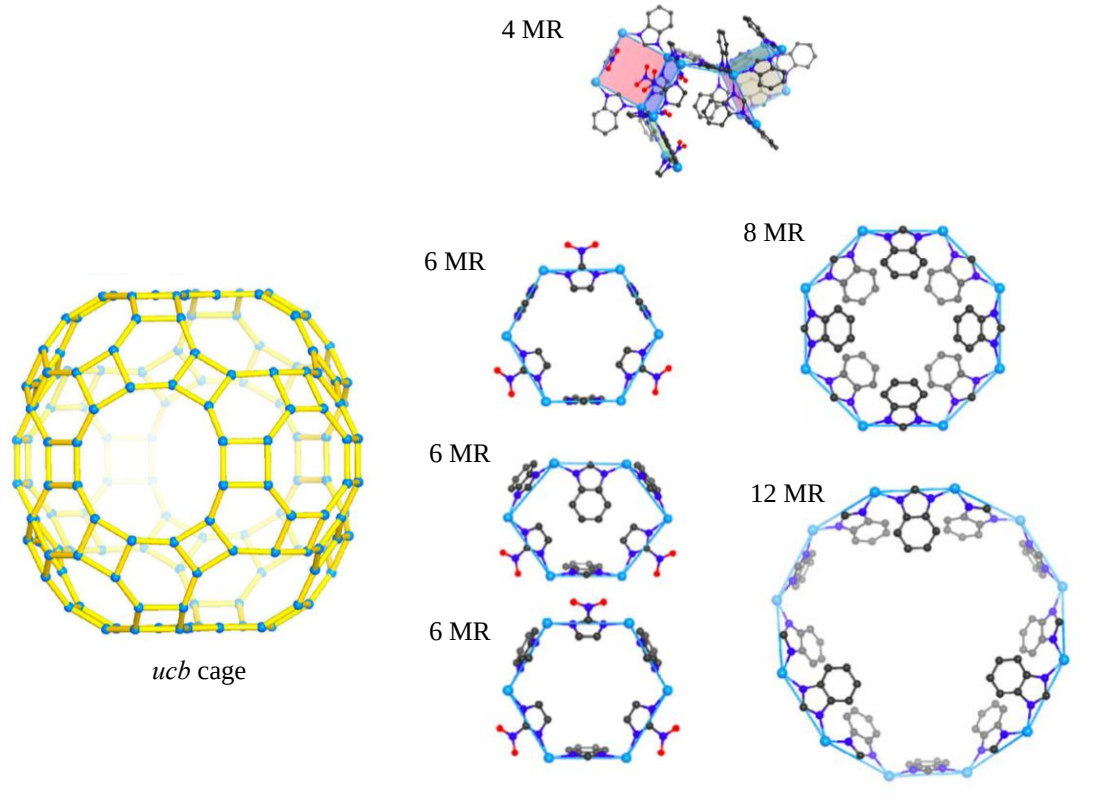


Figure 9. The **ucb** topology of ZIF-412, ZIF-413 and ZIF-414 [8]

Factors in the formation of ZIFs

There are several prevalent factors affecting the formation of ZIFs, such as (i) the effect of organic solvents, (ii) effect of additives, (iii) the effect of concentration, (iv) the effect of temperature, (v) the effect of metal salt variation and (vi) the effect of reaction time on synthesis. Among these, it is believed that the reactant concentration and the type of solvent significantly impact the crystallinity of newly synthesised ZIFs.

Effect of organic solvents

Solvents play a critical role in the deprotonation process of imidazolate linkers, influence the rate of reactions, and affect the size of ZIF crystals. High-boiling-point solvents such as dimethylformamide (DMF), dimethylacetamide, and diethylformamide are widely employed as the primary solvents for the synthesis of ZIFs. The boiling points allow hydrolysis to occur at elevated temperatures, converting them into primary amines. This, in turn, facilitates the deprotonation of imidazole linkers and influences the bonding formation

between metal centres and imidazolate linkers, resulting in the formation of large crystals [4]. The use of DEF for the synthesis of ZIF-11 is due to its high boiling point solvent (178.3 ^{o}C), which allows for control of the nucleation growth of crystals and reduces the formation of cracks in the crystals [38,39].

Dimethylsulfoxide (DMSO) has also been employed as a solvent for synthesizing ZIF-8 and ZIF-67. These frameworks were synthesised at room temperature using DMSO, marking the first synthesis involving DMSO as a solvent. They found that the crystal sizes of ZIF-8 and ZIF-67 obtained using DMSO were smaller compared to those produced in the presence of methanol [40]. For instance, the average particle size of ZIF-67 in DMSO after 30 minutes was only 30 to 50 nm, whereas ZIF-67 in methanol had an average particle size of approximately 306 nm (Figure 10) [41]. Consequently, methanol has been chosen as the primary solvent for the room-temperature synthesis of ZIFs. In addition, ZIF-71, comprising of Zn^{2+} centres and 4,5-dichloroimidazole (dcIM), was synthesized at room temperature in 15 ml of methanol [42]. The precursors were mixed, and the reaction was allowed to proceed for 24 hours at room temperature. A centrifugation process was applied to recover the crystals. Methanol-based ZIF-71 exhibited a higher surface area of approximately 1118.65 m^2 g^{-1} compared to ZIF-71 synthesised with DMF solvent.

Furthermore, ZIF-8 was synthesized at room temperature in the presence of methanol. The mixture solution of $Zn(NO_3)_2.6H_2O$ and 2mIM linker was stirred at 200 rpm for 60 minutes, resulting in an average crystal size of 1 μm. Another ZIF-8 synthesis was conducted at room temperature with the same solvent at 1000 rpm for five minutes. However, the crystal size of ZIF-8 obtained at a higher stirring rate was smaller [43]. This is because the stirring rate influences the rate of crystallisation, which in turn determines the ultimate crystal sizes of ZIFs [44].

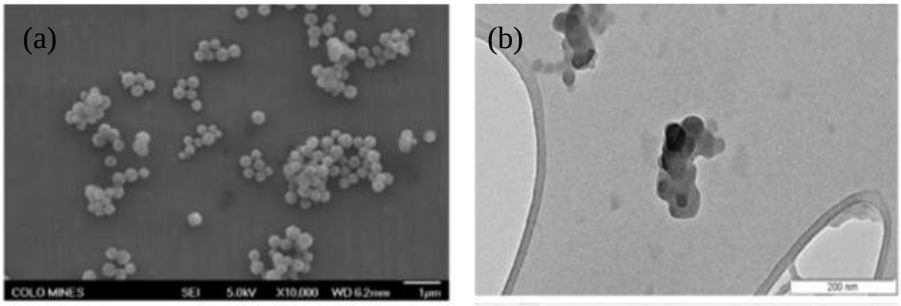


Figure 10. SEM images of ZIF-67 crystals synthesised using (a) methanol and (b) DMSO [41]

Effect of additives

Water is considered an eco-friendly and ideal solvent for the green synthesis method to fabricate ZIFs. However, a high concentration of water in the reaction system can lead to the hydrolysis of Zn^{2+} ions, leading to an increase in by-products of the reaction. Besides, using a high amount of water in the ZIF formation can slow down the crystallisation of ZIF due to the high pK_a value in the aqueous solution. This situation typically occurs when the concentration of imidazolate linkers is lower in the system, slowing down the reaction rate between metal centres and imidazolate linkers [43, 44].

In addition, it can be challenging to synthesise ZIF with a low concentration of precursors in water at room temperature without a base [45, 46]. Therefore, some base agents have been introduced in the synthesis reactions of ZIFs to deprotonate imidazole linkers and accelerate the reaction rate in the water-based synthesis method [47]. ZIF-8 was prepared using an aqueous mixed base, employing ammonium hydroxide (NH_4OH) and triethylamine (TEA) as deprotonating agents. [48]. The mixed base system was believed to have high potential for promoting the formation of ZIF framework at room temperature within several minutes, based on previous works. The synthesis reaction was completed

by stirring the solution mixture for 10 minutes at room temperature [49,50]. As shown in Figure 11, ZIF-8 particles in the presence of TEA formed aggregated sub-micrometer crystals. ZIF-8 particles synthesized without TEA showed a more uniform cubic shape and had a similar morphology to those synthesized using the solvothermal method.

The use of water as a solvent significantly enhances the thermal stability and morphology of the framework. The addition of bases as deprotonation agents in chemical reactions involving organic linkers has resulted in rapid nucleation rates, leading to the formation of small ZIF crystals [51]. The inclusion of additives in the ZIF synthesis is crucial for reactions that use water as the primary solvent. In contrast to solvothermal reactions, these additives serve as deprotonating agents that initiate the crystallisation process, thereby accelerating the reaction and achieving a high yield. Although most of the products are formed within the nanometre size range, they are well-suited for applications requiring nanosized materials, such as catalysts and drug delivery [52].

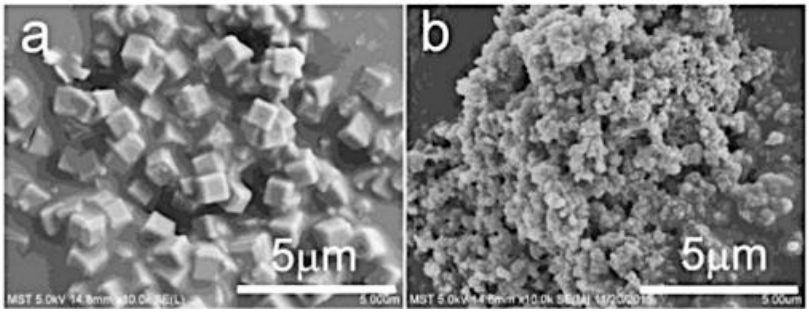


Figure 11. SEM images of ZIF-8 (a) in the absence of TEA and (b) in the presence of TEA [53]

Effect of concentration

The solvothermal method is as well-established as the conventional method to synthesise highly crystalline ZIFs. In this method, a low-molar composition of Zn^{2+} and imidazolate linkers is used to form the framework, with organic amine solvents (DMF, DEF, and DMA) acting as nucleophilic agents in the reaction. Most of the mixed-linker ZIFs were synthesised within the range of 2 to 4 moles under the solvothermal conditions. Pure microcrystals of ZIFs were formed using a low-molar composition of Zn^{2+}:2mIM, demonstrating that a high concentration of imidazolate linkers is not required for the solvothermal method [5]. The presence of organic amine solvents is sufficient to initiate the nucleation process in the synthesis reaction [51]. Despite the promising development of ZIFs, the use of organic solvents contributes to environmental pollution. Therefore, there is a strong desire to use water as a solvent to address the environmental issues and reduce costs.

Nonetheless, using a high amount of water as a solvent can lead to the hydrolysis of Zn^{2+}, increasing the production of by-products and slowing down the rate of deprotonation of the 2mIM linker [45, 47]. To accelerate the reaction rate, a high amount of imidazolate linkers is required in hydrothermal synthesis to increase the basicity of the aqueous solution. The morphology and sizes of crystals heavily depend on the molar composition of metal ions and organic linkers. Thus, conducting multiple synthesis reactions with different concentrations is crucial to obtain ZIF, as determined by SEM and PXRD analysis. In a previous study in 2018, the effect of the molar composition of 2mIM/Zn^{2+} on the properties of ZIF-8 was investigated [44]. ZIF-8 was synthesised with five different molar compositions of 2mIM/Zn^{2+}, ranging from 1, 2, 4, 8, to 16 in 50 mL of methanol at room temperature [44]. Good yield and crystallinity of ZIF-8 were obtained with molar composition of Zn^{2+}/2mIM below 8. Molar compositions of 2 and 4 resulted in ZIF-8 with a truncated dodecahedral morphology. In contrast, different morphologies were observed with higher molar compositions (8 and 16) of 2mIM/Zn^{2+}, as shown in Figure 12.

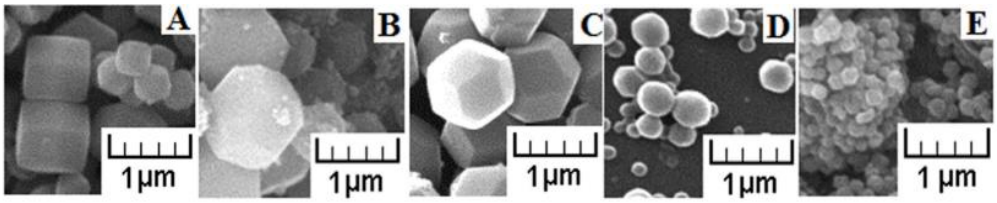


Figure 12. SEM images of different ZIF-8 samples with different molar ratios of Zn^{2+}/2mIM of (a) 1, (b) 2, (c) 4, (d) 8 and (e) 16 [44].

One limitation was that high concentrations of the 2mIM linker led to the formation of a framework with lower surface areas and particles aggregation, as evidenced by SEM analysis. Meanwhile, molar compositions of Zn^{2+}/2mIM in the range of 2 to 4 yielded the highest surface areas for ZIF-8, measuring around 1268 m^2 g^{-1} and 1943 m^2 g^{-1}, respectively. These results indicated that moderate molar compositions of Zn^{2+}/2mIM were capable of producing highly crystalline frameworks with a maximum surface area for ZIFs [3,54]. A higher content of imidazolate linkers in the reaction system accelerated the rate of the nucleation process and also influenced the particle sizes of the frameworks [44]. ZIF-8 was also synthesised in a microemulsion mixture of n-heptane and n-hexanol in TritonX-100, employing different molar compositions of Zn^{2+} to 2mIM (1:2, 1:5, 1:8, and 1:16) at room temperature (Figure 13) [55]. Uniform cubes of ZIF-8 nanoparticles were obtained with a molar ratio of 1:2/Zn^{2+}:2mIM and the particle size decreased from 110 nm to 40 nm as the concentration of the 2mIM linker increased [56,57].

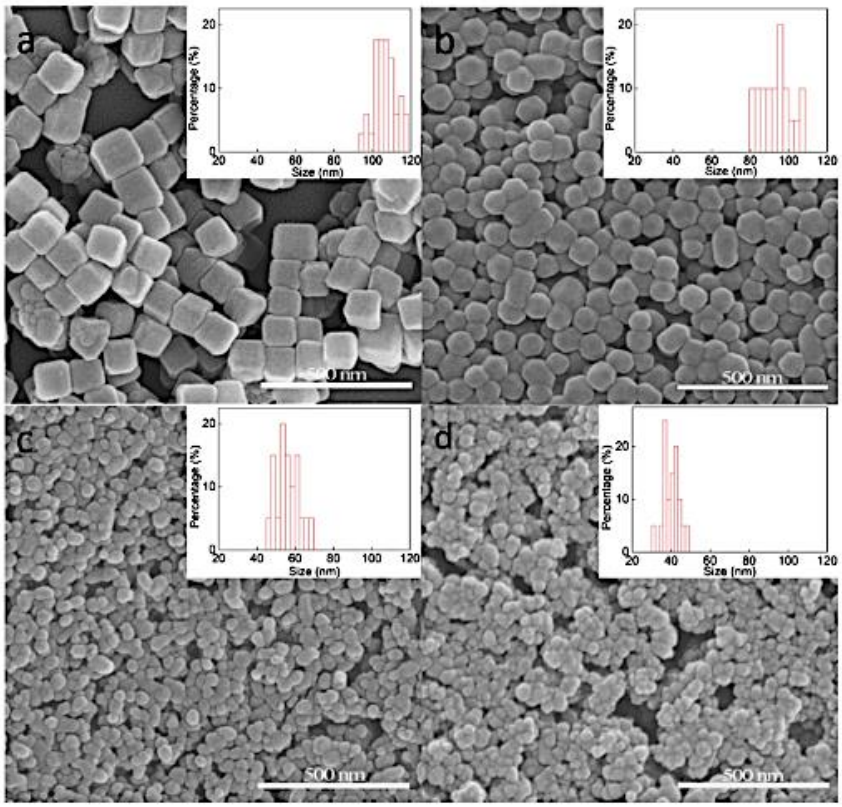


Figure 13. FESEM images of ZIF-8 nanoparticles obtained from different molar compositions of Zn^{2+}:2mIM (a) 1:2 (b) 1:5 (c) 1:8 (d) 1:16 [55]

The broader peak widths of the XRD patterns of ZIF-8-110 and ZIF-8-90 in Figure 14 was attributed to the formation of small crystal size in the samples, as a result from the high concentration of the imidazolate linker [55]. The construction of the framework is a meticulous process that necessitates the use of the appropriate molar composition of organic linkers and metal salts. This parameter is highly dependent on the specific synthesis method employed, as it significantly impacts the crystallinity and particle size of ZIFs.

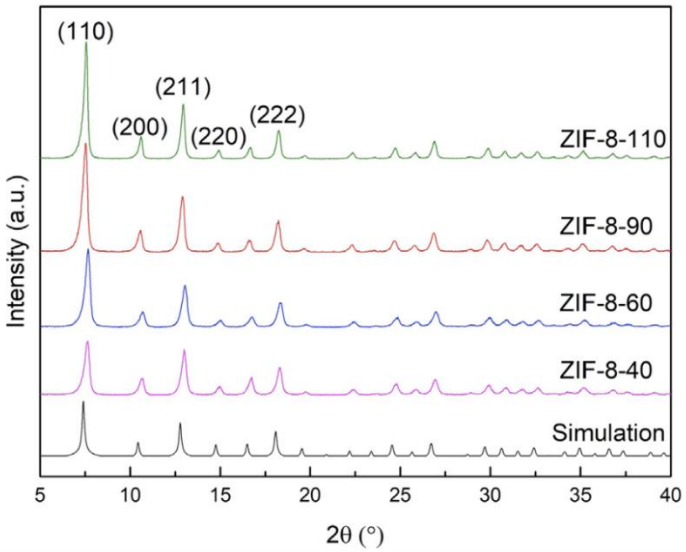


Figure 14. PXRD patterns of ZIF-8 nanoparticles with different sizes based on FESEM images (a) ZIF-8-110, (1:2) (b) ZIF-8-90, (1:5) (c) ZIF-8-60, (1:8) (d) ZIF-8-40, (1:16) [55]

Effect of temperature

The temperature of the chemical reaction is a critical factor in the formation of ZIF particles. Previous studies have reported that synthesis work conducted at different temperatures affects the crystallinity of ZIFs. ZIF-8 was prepared at varying temperatures (80, 100, 120, and 140 ^{o}C) under microwave-assisted solvothermal methods in methanol within 30 minutes [48]. A high crystallinity of ZIF-8 was observed based on PXRD patterns at 120 ^{o}C and 140 ^{o}C, which is close to the reference pattern of ZIF-8 synthesised under the solvothermal method. Meanwhile, the synthesis of ZIF-8 at a temperature lower than 100 ^{o}C was unable to generate a high crystallinity of ZIF-8 [48]. This is because the temperature is directly proportional to the pressure of the reaction under the microwave system and the solvothermal method [55]. In addition, the hydration of the metal salts decreases as the temperature of the reaction increased, as it is associated with entropic gains from the thermodynamic control leading to the release of water molecules from metal sites. This allowed more opportunity for deprotonated linkers to bond with metal sites [48].

Two ZIF-8 compounds were prepared at two different temperature, room temperature (ZIF-8-RT) and 140 ^{o}C (ZIF-8-HT) via hydrothermal synthesis [58]. Sharp peaks were observed in the PXRD patterns of ZIF-8-HT and ZIF-RT. Nonetheless, the full width half maximum values (FWHM) of the PXRD patterns of these ZIFs recorded different values at 7.3° [58]. The FWHM value of ZIF-8-HT was 0.14°, which is smaller than the FWHM value of ZIF-8-RT, which was around 0.20°. A larger FWHM value is responsible for peak broadening in PXRD patterns. The synthesis of ZIF-8 at higher temperature produced better crystallinity, due to the induction of high vapour pressure, as well as increased density, the vacant sites of metal centres, and the dimensionality of the frameworks [58]. The homogeneity in the heating process is a crucial nucleation rate for the construction of ZIFs [59, 60].

Effect of metal salt variations

The construction of ZIFs results from the formation of chemical bonds between the metal ions and the linkers. The types of metal salts used play a crucial role in determining the size of the ZIFs. According to Pearson's acid-base concept, metal salts with different counter ions possessing varying interaction strengths influence the nucleation rate. Higher concentration of metal salts and organic linkers in the reaction system contribute to an increased nucleation rate, leading to the formation of more nuclei which resulted in the production of large amounts of tiny crystals [61, 62]. Zinc acetate, $Zn(OAc)_2$ was the prominent metal salt for synthesising ZIF-8 in the hydrothermal method [47]. The morphology of the synthesised ZIF-8 particles using $ZnSO_4$ and $Zn(OAc)_2$ exhibited a rhombic dodecahedron for molar ratios of 2mIM/Zn^{2+} between 20 and 35. Furthermore, the formation of ZIF crystals is dependent on the molar composition of the precursors in the reactions. When ZIF-8 was synthesised using a high molar ratio of 2mIM/Zn^{2+}, truncated edges with bumpy surfaces were produced. In contrast, low molar ratios of 2mIM/Zn^{2+} resulted in rhombic dodecahedron morphology. As the molar ratio of 2mIM/Zn^{2+} decreased, the average size of ZIF-8 particles using $ZnSO_4$ also decreased from 1.64 μm to 231 nm.

Previous studies have highlighted that the size of ZIF particles depends on the molar ratio of precursors and the type of metal salts in the chemical reaction [47]. The morphology of ZIF-8 particles prepared from $Zn(NO_2)_3$ exhibited spherical and petal shapes, which is opposite to the findings obtained from the synthesis of ZIF-8 using $Zn(OAc)_2$ and $ZnSO_4$. ZIF-8 synthesised using $Zn(NO_2)_3$ showed better crystallinity framework than $Zn(OAc)_2$ and zinc sulphate, $ZnSO_4$, due to the high reaction temperature at 85 ^oC for 60 hours[46]. In terms of crystallinity, previous work has demonstrated that the high crystallinity of ZIF-8 could be obtained in the order of $Zn(NO_3)_2$ > $Zn(OAc)_2$ > $ZnSO_4$ > zinc chloride, $ZnCl_2$. Different metal salts will influence the synthesis process of ZIFs due to variations in pH resulting from the anionic ions of metal salts. Among these metal salts, ZIF-8 using $Zn(NO_3)_2$ recorded high catalytic activity in Knoevenagel condensation reactions due to the high external surface area of the ZIF-8 crystal. $Zn(NO_3)_2$ is composed of a soft acid metal ion, Zn^{2+}, with hard base anion, NO_3^-, which allows for more available vacant sites for the 2mIM linker to coordinate with Zn^{2+} ions, hence forming many nuclei and contributing to the framework's high surface area [46].

Effect of reaction time on synthesis

Another critical parameter to highlight for the formation of ZIFs is the effect of the reaction time on synthesis. It is widely accepted that a longer reaction time will yield higher quantity of products suitable for various promising applications. Previously, ZIF-8 was synthesised within 60 minutes using a sonochemical method under ultrasound irradiation, resulting in fine textural properties, however, with a low yield. The synthesis work conducted within three hours produced a high output of ZIF-8 crystal [25, 26]. Significant differences were observed between the short and long reaction periods of ZIF-8 from 5 minutes to 60 hours conducted in an aqueous medium. A longer reaction period resulted in ZIF-8 having a high surface area of 1027.20 $m^2 g^{-1}$ compared to the surface area obtained at five minutes, which was 878.59 m^2 g^{-1}. It is worth mentioning that reaction time is also interconnected with temperature; as they increase, better morphology is achieved and the product yield is enhanced [46].

ZIF-11 had different morphologies after being synthesised at 60 and 100 ^oC for 12 hours and six hours respectively. These shapes included rhombic dodecahedron shapes. The duration of the reactions strongly depends on the molar composition of the synthesis reaction, as demonstrated previously [40]. ZIF-11 was synthesised using three different molar compositions of Zn^{2+}/bzIM (1:4, 1:8, and 1:12). The same rhombic dodecahedral was formed at low and high concentrations, but the products were formed at different times. ZIF-11 crystals were fully formed after 48 hours for the molar composition of Zn^{2+}/bzIM at 1:4, which was attributed to the slow transition of pure phase crystalline of ZIF-11. In addition, pure crystalline ZIF-11 was achieved within 24 hours for high molar compositions of precursors. This demonstrates that the reaction duration depends on the molar composition and synthesis temperature, which influences the

recrystallisation and dissolution processes for the formation of ZIF [46].

Applications of ZIFs

ZIFs have been utilised in a wide range of applications owing to their exceptional chemical and thermal stability as well as flexible and adaptable frameworks that can be customised to suit specific applications. In this section, some of the significant applications of ZIFs are highlighted and discussed.

Adsorption of pollutants

Adsorption of pollutants is an alternative method for wastewater treatment due to its minimal production of secondary by-products and ease of operation. Activated carbon is a conventional adsorbent in wastewater treatment, and this adsorbent has a few limitations, including weak interactions with pollutants and problems related to regeneration in practical applications. Therefore, MOFs and ZIFs have emerged as promising adsorbents for wastewater treatment [40]. The structures of MOFs and ZIFs are flexible, which allow the incorporation of additional functionalities, such as unsaturated metal centres and active functional groups, to achieve targeted interactions with pollutants. For instance, ZIF-8 demonstrated rapid adsorption of methyl blue (MB), achieving a remarkable adsorption capacity of approximately 1917.5 mg g^{-1} within just 60 minutes. Despite the smaller pore diameter of ZIF-8 compared to the size of MB molecules, the high adsorption of MB is likely attributed to the formation of ionic bonds between the metal centre of ZIF-8, Zn^{2+}, and SO_3^- from MB. Figure 15 shows the proposed adsorption mechanism between SO_3^- from MB and Zn^{2+} from ZIF-8 [63].

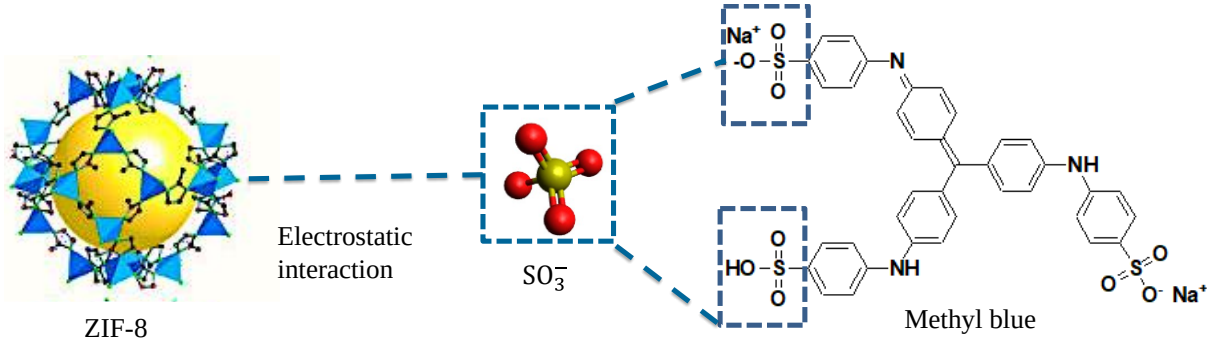


Figure 15. Adsorption mechanism between methyl blue and ZIF-8 [63]

Subsequent studies on the adsorption capabilities of Rhodamine B (RhB) and methylene blue (MB) have been previously explored. ZIF-8 was synthesised using ultrasound and a surfactant (Pluronic* P-123) at room temperature, resulting in ZIF-8 with a surface area of 745 $m^2 g^{-1}$ [64]. Organic dyes such as RhB and MB are classified as larger organic dyes (molecular sizes approximately 1.59 x 1.18 x 0.56 nm and 1.7 x 0.76 x 0.33 nm), preventing them from passing through the pore apertures of ZIF-8. Consequently, adsorption of RhB and MB occured on the external surface of ZIF-8 in between 2 to 48 hours, with adsorption capacities of 25.0 mg g^{-1} and 46.6 mg g^{-1}, respectively [65,66]. This highlights the significance of specific functional groups in organic dyes that enable better adsorption with ZIF, even when the molecule size exceeds the small micropores of ZIF-8.

ZIF-67 showed good adsorption performance with anionic dyes. The adsorption values obtained using ZIF-67 ranged from 37.7 mg g^{-1} for xylene cyanol (XCFF) to 800 mg g^{-1} for Cibacron Blue 3G-A (CB) and fluorescein angiography. High adsorption capacities with anionic dyes are due to the positive surface charges on the surface of ZIF-67, as confirmed zeta-potential values ranged from 2.88 mV to 14.37 mV in a basic solution (pH 7.00-10.00). It is noteworthy that water contact with the surface of ZIF-67 induces the

protonation of imidazolate linkers (2mIM-) and enhances the interaction between anionic dyes and ZIF-67, as depicted in Figure 16 [53, 64, 65]. It should be emphasised that the optimal pH of the aqueous solution for the adsorption studies depends on the types of pollutants.

ZIF-7 was used in the adsorption of trichloroethylene, and it was found that the optimal condition for conducting the adsorption study was low pH solution (pH < 6.00) within 120 minutes [67]. Under these conditions, ZIF-7 achieved a remarkable removal efficiency of approximately 96.29%, highlighting the enhanced removal efficiency of trichloroethylene in an acidic environment [68,69]. It is intriguing to note that the pH of the solution can influence the surface charge of the adsorbents and the degree of ionisation of the solution, which, in turn, affects the adsorption of pollutants. In a separate adsorption study, malachite green (MG) was adsorbed using ZIF-67. Several parameters, such as temperature, time, and concentration, were considered to determine the optimal condition for the adsorption study. Notably, as the temperature increased from 20 to 60 ^{o}C, a substantial increase in adsorption capacity was observed [70]. The maximum adsorption capacity was recorded at 60 ^{o}C with an adsorption capacity value of 2500 m^2 g^{-1}, making it one of the highest adsorption capacities among established adsorbents such as MIL-100 (Fe), chitosan beads, and commercial powder activated carbon [67]. A chemical interaction took place between the imidazole rings and MG via the π–π stacking interaction, which facilitated the adsorption of MG onto the surface of ZIF-67 [71, 72].

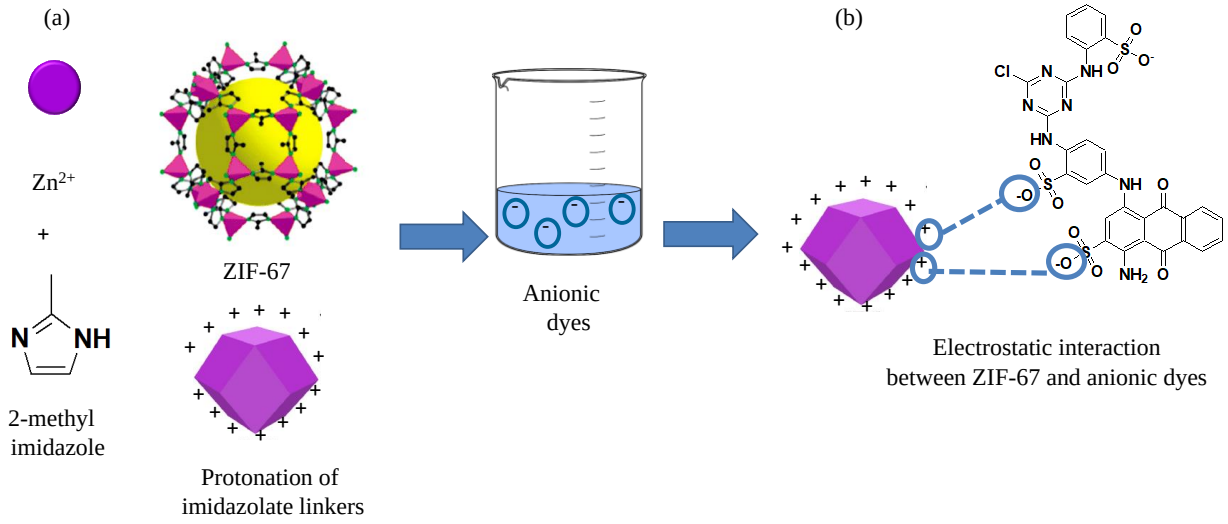


Figure 16. (a) Secondary building unit of ZIF-67 and (b) Interaction of ZIF-67 and anionic dyes [68, 69]

In another adsorption study, ZIF-67 showed excellent adsorption capacity towards Congo Red (CGR) from an aqueous solution, primarily attributed to electrostatic and π–π stacking interactions of the aromatic compound in CGR. Furthermore, the presence of Co^{2+} as the metal centre of ZIF-67 led to coordination between oxygen and nitrogen atoms from CGR, resulting in rapid adsorption in less than 40 minutes, even at a low concentration of 30 ppm. The impact of temperature was also evident, with an increase from 36.9 to 57.9 ^{o}C, leading to a significantly higher adsorption capacity of up to 714.3 mg g^{-1}, indicating the promise of ZIF-67 as an effective adsorbent for CGR [70, 73].

Antibiotics have been extensively used for the treatment of human and animal diseases, owing to their effectiveness. However, a substantial portion of antibiotics is not fully absorbed by humans and animals, resulting in the release of these compounds into the environment [69]. Ofloxacin is a third generation of quinolone that was used in humans, aquaculture, and animal husbandry to prevent livestock disease. ZIF-8 is

used as an adsorbent to remove ofloxacin with an adsorption capacity of approximately ~194.1 mg g^{-1} at pH 7 with an adsorbent amount of 20 mg and 100 ppm of initial concentration [74]. Ofloxacin exists as zwitterionic state within the pH of 8.44 to 9.2, resulting in a strong electric attraction with ZIF-8. Concurrently, ZIF-8 exhibits a positive charge in a basic pH [75,76] as shown in Figure 17. The adsorption of ofloxacin involves various interactions, including strong hydrogen bonding between the fluorine atom in ofloxacin with C-NH bonds in ZIF-8, as well as complexation with unsaturated metal sites that facilitates interaction with ofloxacin. Additionally, the presence of 2mIM rings within the ZIF-8 framework and the benzene ring of ofloxacin further enhance the adsorption capacity through π–π stacking interactions [74].

Similar chemical interactions were observed in the adsorption of norfloxacin using ZIF-8, a quinolone derivative [77]. ZIF-8 exhibited excellent adsorption efficiency at initial norfloxacin concentrations ranging from 10 mg L^{-1} to 30 mg L^{-1}. However, the efficiency of the adsorbent slightly diminished after 30 mg L^{-1} due to increased occupation of the available binding sites on ZIF-8 at higher norfloxacin concentrations. Furthermore, norfloxacin has a lower positive charge at pH levels between 4.5 to 6.2, allowing for enhanced adsorption with the positively charged ZIF-8 adsorbent due to lower electrostatic repulsion [78, 79].

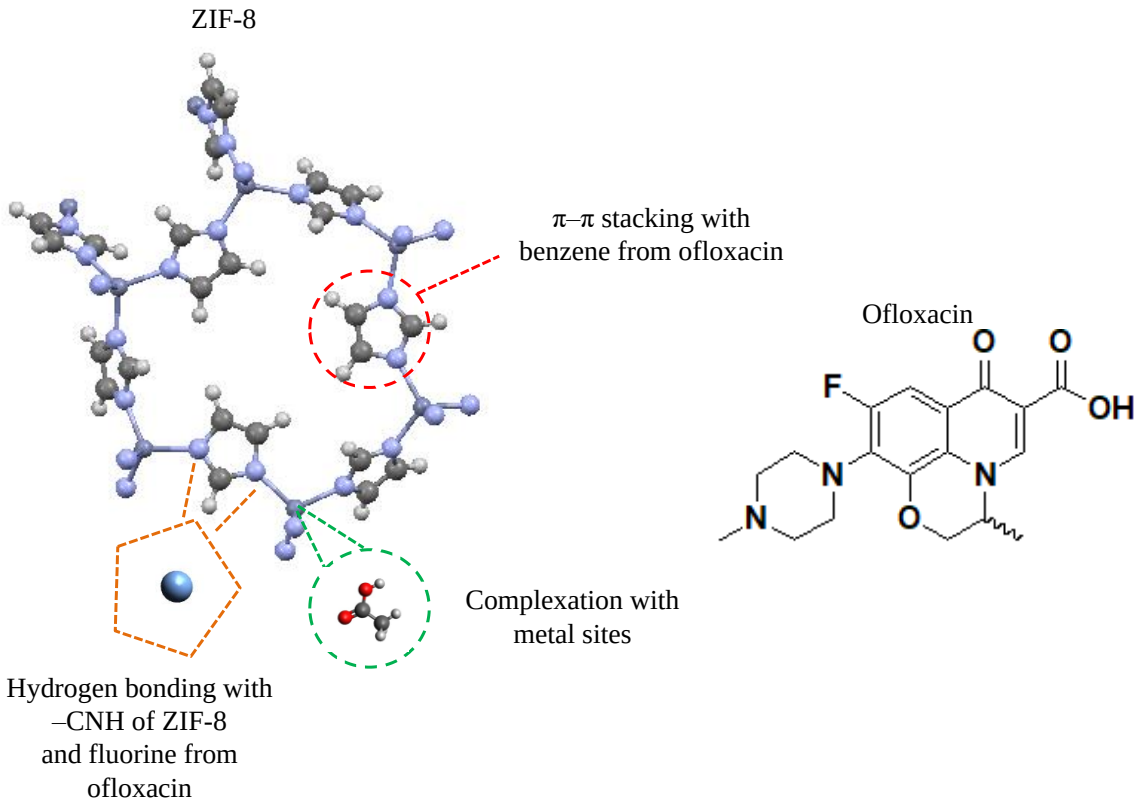


Figure 17. Mechanism of adsorption of ofloxacin with ZIF-8 [73-74]

Tetracycline (TC) and oxytetracycline hydrochloride (OTC) are commonly used antibiotics due to their effectiveness in treating bacterial infections and as veterinary drugs to promote animal growth [80]. However, only 30% of TC and OTC are absorbed by animals, while the remaining antibiotics are discharged into the environment in their original form, posing significant threats to the environment [81]. To address this issue, ZIF-8 was used for the removal of simultaneous mixtures of TC and OTC antibiotics from

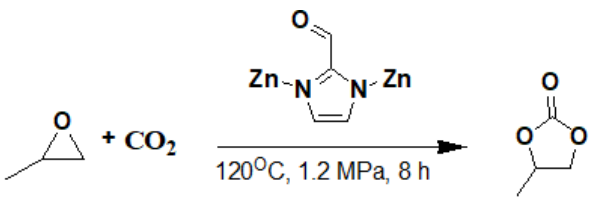
wastewater [80]. Fast adsorption was observed within 50 minutes at a pH 4 to 6, with an average simultaneous removal efficiency of 90.7% for TC and 82.5% for OTC at a concentration of 50 ppm. The impact of temperature on the simultaneous adsorption followed a pattern consistent with previous adsorption, indicating the reaction is an endothermic process. Higher temperatures led to rapid adsorption of TC and OTC due to molecular collision, which enabled greater interaction between adsorbates and the adsorbents [75,82].

In conclusion, several ZIFs, particularly ZIF-8, have proven their potential as adsorbents for the removal of organic pollutants such as dyes and antibiotics. The maximum adsorption capacity is determined by the types of organic pollutants and various parameters within the adsorption experiments. These characteristics help to facilitate the chemical interaction that occurs between ZIFs and organic contaminants.

Catalyst

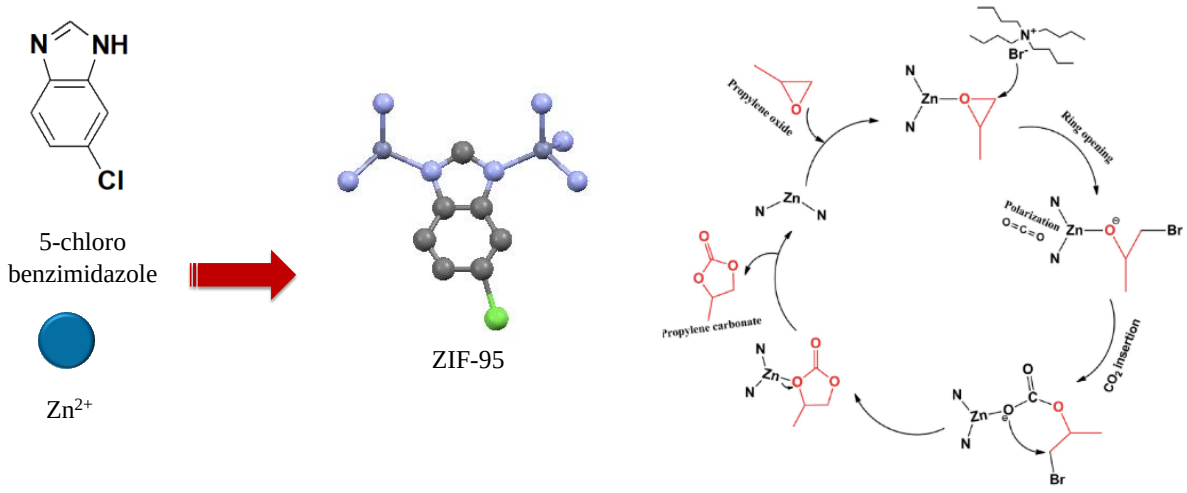
Aluminosilicate zeolites are well-established materials widely explored in many catalysis applications [83]. Owing to similar properties with aluminosilicate zeolites, ZIFs have emerged as efficient catalysts and co-catalysts for various catalytic reactions. Polylactide (PLA) is a widely recognised polymer derived from renewable resources that has received considerable attention due to its biocompatible properties [83–85]. Lactide (LA) is a monomer of PLA and undergoes ring-opening polymerisation (ROP) with a combination of a Lewis acid catalyst and organic bases to obtain cyclic PLA (cPLA) [86, 87]. ZIF-8 stands out as a promising Lewis acid catalyst for the polymerisation of $_L$-lactide. This framework demonstrates exceptional thermal stability up to 400 ^oC based on TGA analysis. In addition, it remains insoluble in many common organic solvents, signifying its chemical stability. In the previous study, ZIF-8 showed excellent catalytic activity at high concentrations ([M]/[I] = 100) and generated a high molecular weight of cyclic poly($_L$-lactide) (PLLA) of approximately 24.5 kg mol^{-1} with a conversion rate of 96%. This outcome aligns with MALDI-TOF mass spectrum results, where strong signals were observed for cyclic PLLA, indicating near complete conversion of L-lactide. The metal centre of ZIF-8, Zn^{2+}, functions as a Lewis acid site based on NMR spectroscopy, initiating chain growth by binding to the monomer. The Lewis acid sites are also working cooperatively with the active basic sites of ZIF-8, specifically the nitrogen atoms from the imidazolate ring, to catalyse the formation of cyclic PLLA via intramolecular transesterification. The PXRD pattern of the recycled ZIF-8 catalyst confirmed that the diffraction peaks remained unchanged after three runs of the ROP reaction, indicating no loss of catalytic activity during the recycling process [88].

The utilisation of CO_2 represents a promising strategy for reducing atmospheric levels of CO_2 caused by human activities. As such, the synthesis of cyclic carbonates using CO_2 offers a particularly alternative pathway to reduce the atmospheric level of CO_2 [89]. Scheme 1 illustrates the application of ZIF-90 as a promising catalyst for cycloaddition, eliminating the need for additional co-catalysts. This approach resulted in 88% of propylene carbonate (PC) with 90% selectivity at 120 ^oC within eight hours. The aldehyde group of ICA within ZIF-90 stimulates CO_2 insertions, leading to the formation of a ring-opening intermediate. ZIF-90 emerges as a truly promising material for catalysis, as it contains ICA linkers that can replace co-catalysts in the catalytic reaction [90].



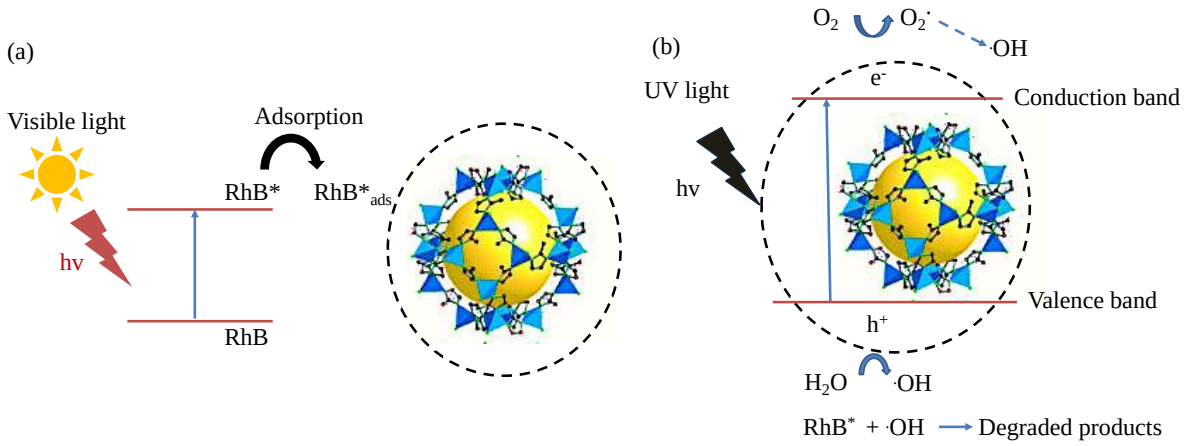
Scheme 1. Cycloaddition of propylene oxide (PO) and CO_2

In contrast with subsequent work in 2017, ZIF-95 showed excellent performance in the cycloaddition reaction, despite its structural similarity to ZIF-90. The maximum conversion of PO achieved with ZIF-95 was notably higher, reaching approximately 91%, with a selectivity exceeding 99% at the same temperature. Tetrabutylammonium bromide (TBAB) was used as a co-catalyst. The diffraction patterns for both frameworks remain unchanged even after they were reused for more than three cycles at higher temperatures, indicating their robust chemical and thermal stability. ZIF-95 featured an unsaturated tetrahedral Zn^{2+} centre that functioned as a Lewis acid site, initiating the reaction on the oxygen atom of PC as an electrophilic attack [91]. In Scheme 2, the bromide anion from TBAB attacks the carbon atom of PO, leading to ring-opening intermediates [92].



Scheme 2. The mechanism of cycloaddition of propylene oxide (PO) using ZIF-95 as a catalyst to form propylene carbonate (PC), [93]

In addition, ZIF-8 is also used for the epoxidation reaction of menadione with hydrogen peroxide, H_2O_2, achieving a remarkable 90% conversion to menadione-2,3-epoxide. This exceptional catalytic activity can be attributed to the presence of defects on the external surface of the framework, promoting interactions between H_2O_2 and the basic sites of ZIF-8. These basic sites include nitrogen atoms from the 2mIM ring and hydroxyl from methanol, facilitated via hydrogen bonding. The combination of the hydrophobic surface of ZIF-8 and the basic sites of the 2mIM ring contributes to a synergistic effect, resulting in nearly 100% selectivity [93]. Many ZIFs have been used as catalysts in the photodegradation of pollutants. Photodegradation is another alternative way to adsorb contaminants. The potential of ZIF-8 nanocrystals in the photodegradation of RhB was examined under various conditions, such as dark, UV, and visible irradiation conditions [94]. At 430 nm, ZIF-8 nanocrystals exhibited excellent degradation of RhB. This phenomenon is due to as the transition of the excited electrons in ZIF-8 nanocrystals to the conduction band to react with surface oxidants (O*) in order to form hydroxyl radicals (OH*), facilitating the photodegradation of RhB (Scheme 3). The photodegradation process under visible light irradiation was slightly slower due to the large bandgap energy (5.6 eV) of ZIF-8 and the relatively low photon energy of visible light (1.8-3.9 eV) for electron excitation to occur within the framework. Consequently, there is a limitation in the production of the required amount of oxygen radicals for the photodegradation process [94].



Scheme 3. Proposed mechanism of photodegradation of RhB using ZIF-8 under (a) visible light and (b) UV light [94]

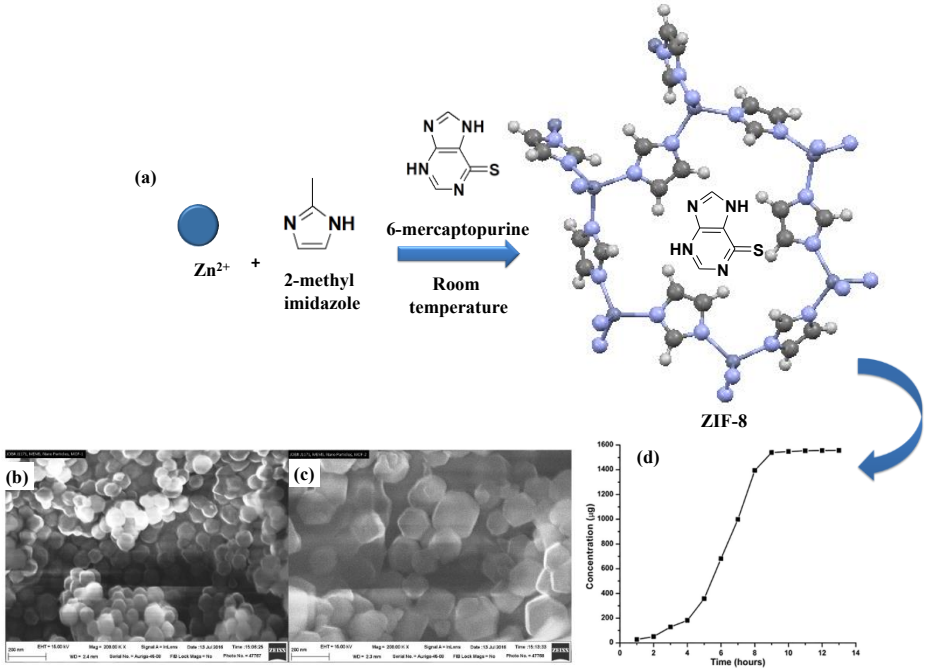
To address these challenges, ZIF-8 was successfully transformed by modifying its framework under thermal treatment to form a visible-light photocatalyst for decomposing gaseous formaldehyde [95]. This thermal treatment involved subjecting ZIF-8 nanocrystals to a temperature of 200 ºC in the presence of air to convert the -N=C of the 2mIM linker into -N=C=O functional groups, thereby expanding the light absorption range from UV to infrared. The presence of the -N=C=O functional group enabled the decomposition of formaldehyde, achieving nearly ~100% efficiency within one hour under simulated sunlight. This result was supported by the optical absorption band of ZIF-8 nanocrystals, which appeared at 715 nm. ZIF-67 also exhibited excellent performance in broadening the absorption spectrum for the photocatalytic activity of Cr^{6+} reduction. The UV-Vis absorption spectra revealed three distinct absorption maxima at 1009 nm, 589 nm, and 265 nm, corresponding to the near-infrared (NIR), visible and UV light regions, with C^{6+} reduction efficiencies of 71.22%, 69.52%, and 40.72% after two hours. It is suggested that the 2mIM ligand in the framework is responsible for absorbing light from different sources, facilitating charge transfer to the metal centre to produce excited electrons for reduction of $Cr^{5+}$to Cr^{3+} [96].

The photocatalytic reduction of CO_2 offers a promising approach for generating hydrocarbon fuels and renewable sources. The mechanism of CO_2 reduction using photocatalysts depends on electron-hole separation, their lifetime, and CO_2 adsorption capacity [96]. ZIF-67 was used as a co-catalyst for CO_2 reduction at room temperature in the presence of photosensitizer ($[Ru(bpy)_3]Cl_3.6H_2O$) and electron donor (Triethanolamine) [97]. This study revealed that two-dimensional ZIF-67 with a leaf-like morphology exhibited a high CO_2 transformation rate and showed high CO_2 adsorption capacity. No decomposition occurred during the photocatalytic activity involving ($[Ru(bpy)_3]Cl_3.6H_2O$) and ZIF-67 at 580 nm, indicating excellent electron transfer efficiency from the photosensitizer to the co-catalyst. Additionally, the strong interaction between CO_2 molecules and Co^{2+} reduced to Co^{+}, which facilitated the production of CO molecules for generating hydrogen gas, H_2 [98]. ZIF-8 was used as a catalyst to promote hydrogen production via sodium borohydride ($NaBH_4$) hydrolysis. ZIF-8 was synthesised using zinc hydroxide nitrate nanosheets ($Zn_5(OH)_8(NO_2)_2$) in the presence of NaCl salt and employed as a catalyst to accelerate hydrogen production. The hydrogen production rate using ZIF-8 (NaCl) was measured at around 3000 mL_{H2} min^{-1} g^{-1} [97,99], proving that ZIF-8 exhibited high catalytic activity without any reduction n performance after several cycles in the presence of $NaBH_4$. In conclusion, ZIFs are exceptional chemical and thermal stable materials to act as catalysts especially ZIF-67 due to its unsaturated metal centre, Co^{2+}.

Drug delivery system

Zinc is an essential micronutrient for human metabolism [100]. The imidazole group is the main functional group that constitutes the amino acid histidine. These combinations are significant for the development of ZIF as a promising biocompatible drug carrier [49]. 6-Mercaptopurine (6-MP) is a cytotoxic drug used to treat acute childhood leukaemia, provide maintenance therapy to prevent concurrent Crohn's disease, and as a treatment drug for autoimmune hepatitis (AH) [101]. ZIF-8 has been identified as a potential biocompatible drug carrier for 6-MP. An *in-situ* synthesis reaction was conducted to encapsulate 6-MP into ZIF-8 in DMSO at room temperature. Two peaks were observed in the UV-visible spectrum, representing ZIF-8 at 216 nm, and another peak at 320 nm which confirmed the presence of 6-MP on ZIF-8. The average particle size of ZIF-8 also increased the encapsulation of 6-MP, as confirmed by SEM analysis (Scheme 4). The drug release works efficiently at pH 5.0 within 10 hours, as the mildly acidic solution leads to the dissolution of ZIF-8 structure [102–104].

In a recent study, ZIF-8 showed potential in the drug delivery system for benznidazole (BNZ), a well-known drug to treat Chagas disease. The purpose of the encapsulation of BNZ into ZIF-8 is to overcome the low bioavailability and solubility of BNZ drugs in the human body [105]. The BNZ into ZIF-8 system was developed by the ex-situ method by mixing BNZ solution and ZIF-8 in acetone with the equimolar ratio of BNZ and ZIF-8. Encapsulation of benznidazole (BNZ) into the ZIF-8 framework exhibited a similar condition as 6-MP inZIF-8, achieving 80% of drug release at pH 4.8 after two days. The disintegration of the framework in an acidic solution allowed the movement of the imidazolate linker within the framework and promoted a greater opening of the pore windows of the framework [106]. Consequently, more diffusion of drug molecules can occur in weak acidic solutions (pH 5.0 – 6.0) [107, 108].



Scheme 4. (a) Encapsulation of 6-mercaptopurine (6MP) into the framework, (b) FESEM analysis of ZIF-8, (c) FESEM analysis of 6MP@ZIF-8 and (d) Drug release study at pH 5.0 [105]

The hydrophobic curcumin drug (CCM) was encapsulated in a ZIF-8 framework (CCM-ZIF-8), achieving a maximum efficiency of 83.3% within 15 minutes at room temperature [109]. CCM-ZIF-8 also exhibited superior stability in MeOH after 30 days, as evidently shown by the TEM image. A high average

drug release, reaching 88%, was recorded in an acidic pH solution within 72 hours, attributed from the protonation of 2mIM linkers. This protonation led to the disintegration of the coordination bond between the metal centre and 2mIM linkers, resulting in drug release. Cytotoxicity studies were conducted using a MTT assay to assess the activity of CCM-ZIF-8 and free CCM against HeLa cell lines. The study found a strong inhibitory effect on cell growth with CCM-ZIF-8 compared to free CCM at higher concentrations. At 62.5 μg mL^{-1}, CCM-ZIF-8 achieved a 57% cell death rate, irrespective to free CCM, which induced only 25% cell death [110].

D-α-Tocopherol succinate (α-TOS) has demonstrated significant cytotoxic effects on various cancer cells via the mitochondrial-dependent pathway while having no toxicity effect on other normal tissue. However, α-TOS has hydrophobic properties that limit its clinical applicability. Therefore, a drug delivery system (DDS) was developed by encapsulating α-TOS into a ZIF-8 framework (α-TOS@ZIF-8) through a one-pot synthesis reaction, followed by functionalisation with hyaluronic acid (HA) on the external surface of α-TOS@ZIF-8 [111]. The function of HA on DDS carriers is to enhance drug accumulation via the CD44 pathway towards the tumour region [109]. The formation of HA/α-TOS@ZIF-8 was confirmed by zeta potential measurements, where the value decreased from 22.1 mV (pure ZIF-8) to -17.9 mV. This negative value indicated that HA was successfully introduced onto the external layer of α-TOS@ZIF-8. The usage of HA/α-TOS@ZIF-8 has led to significant tumour growth inhibition, as demonstrated in *in vivo* and *in vitro* studies [112,113]. Similar studies were conducted with surface functionalisation using folic acid (FA) to reduce the cardiotoxicity of doxorubicin (DOX). DOX is an anticancer drug that was successfully modified using pH-sensitive linker cis-aconitic anhydride (CAA) to enhance drug loading into ZIF-8, forming CAD@ZIF-8. This was due to the lack of surface functional groups such as carbonyl and carboxyl groups on ZIF-8. CAD@ZIF-8 was functionalised with FA to enhance tumour targeting and avoid premature drug release. *In vivo* study revealed that mice treated with CAD@ZIF-8-FA showed a slight increase in weight and drug accumulation in the tumour region at 12 and 24 hours. In contrast, mice treated with DOX experienced a slight weight reduction, and the drug was distributed impartially to various body parts. This implies that CAD@ZIF-8-FA can deliver the drug and accumulate at the tumour region, as opposed to simply encapsulating DOX into ZIF-8. The modification of DOX enables specific targeting of tumour sites in the human body [114].

Conclusion

In conclusion, many new synthesised ZIFs have been successfully synthesised since 2008. It is challenging to incorporate more imidazolate linkers into the framework because different imidazolate linkers have varied reactivities in the synthesis reaction. The synthesis of mixed-linker ZIFs is considered as an interesting development of ZIFs because more functionalities can be added in the frameworks and the materials are suitable for a variety of applications. Instead of employing the solvothermal approach to generate known ZIFs, several methods were developed to produce existing ZIFs to reduce the amount of time needed for the reaction and consumption of hazardous solvents. Therefore, the synthesis strategies of ZIFs such as concentration of precursors, reaction time, the choices of metal salts and solvents must be taken into account as these factors will affect the crystallinity, crystal sizes and the stability of the frameworks. ZIFs have received considerable attention and researchers are currently exploring various applications for ZIF materials. The previous applications of ZIFs were summarised and discussed in detail. Imidazolate linkers provide abundant functionalities in the frameworks and the porous of ZIFs hold great potential across a wide range of applications. The functional properties of ZIFs can be tuneable by the incorporation of imidazolate derivatives linkers in a single framework to enhance the efficiency of ZIFs as catalysts, adsorbents and drug carriers.

Acknowledgement

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INHIBITION EFFECTS OF TRIDENTATE HYDRAZONE LIGANDS AS CORROSION INHIBITORS ON MILD STEEL IN SATURATED CO_2 ENVIRONMENT

(Kesan Perencatan Ligan Hidrazon Tridentat Sebagai Perencat Kakisan Pada Keluli Lembut Dalam Persekitaran CO_2 Tepu)

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Abstract

Two tridentate hydrazone ligands (AL01, AL02) were synthesized by condensation of an aldehyde and two hydrazides, benzohydrazide and 2-hydroxybenzohydrazide with OH substituent at the *ortho* position of the benzene ring. They were characterized using melting point, FTIR, NMR, elemental analysis, UV-Vis as well as mass spectroscopy. The formation of hydrazone ligands was confirmed by the appearance of *ν*(C=N) peak in the range of 1608 to 1648 cm^{-1}. The significant peak of N-H protons for AL01 and AL02 appeared at 8.82 and 8.41 ppm respectively, while the peak of phenolic proton (O-H) for AL01 was found at 12.28 ppm. Two peaks were observed in the wavelength range of 198-220 nm that can be attributed to π-$\pi^*_{(C=C)}$ and n-$\pi^*_{(C=C)}$ transitions of aromatic benzene, while the peaks for π -$\pi^*_{(C=N)}$ were observed in the range of 297–303 nm. The mass-to-charge (m/z) $[M+H]^+$ values of AL01 and AL02, which are 242.09 and 226.10, respectively, further confirmed their structures. The effectiveness of hydrazone ligands as corrosion inhibitors on mild steel in 3.5% NaCl solution saturated with CO_2 was examined using polarization and electrochemical impedance spectroscopy (EIS). The elemental constituents of the protective layer forms on mild steel immersed in the solution were further observed using scanning electron microscopy (SEM) coupled with energy-dispersive X-Ray (EDX)). Therefore, new corrosion inhibitors of tridentate hydrazone ligands, AL01 and AL02 were successfully synthesized and characterized using FTIR, NMR, elemental analysis, mass spectroscopy and UV-Vis. From polarization and EIS study, AL01 can be concluded to have higher inhibition efficiency, 84.30% at 500 ppm concentration compared to AL02, 74.90%. SEM-EDX analysis has confirmed the formation of the inhibitors' layer on mild steel as the surface of the mild steel immersed in solution with the presence of inhibitors have a smoother surface compared to that of untreated mild steel in 3.5% NaCl solution saturated with CO_2.

Keywords: CO_2, corrosion inhibitor, mild steel, protective layer, tridentate hydrazone

Abstrak

Dua ligan hidrazon tridentat (AL01, AL02) telah disintesis melalui pemeluwapan antara aldehid dan dua hidrazida, benzohidrazida dan 2-hidroksibenzohidrazida, dengan kumpulan pengganti OH pada kedudukan *orto* hidrazida. Mereka dicirikan menggunakan takat lebur, FTIR, NMR, analisis unsur, UV-Vis serta spektroskopi jisim. Pembentukan ligan hidrazon telah disahkan oleh kemunculan puncak *v*(C=N) dalam julat 1608 hingga 1648 $cm^{-1.}$ Puncak utama proton N-H untuk AL01 dan AL02 masing-masing muncul pada 8.82 dan 8.41 ppm, manakala puncak proton fenolik (O-H) untuk AL01 didapati pada 12.28 ppm. Dua puncak diperhatikan dalam julat 198-220 nm yang boleh dikaitkan kepada peralihan π-$\pi^*_{(C=C)}$ dan n-$\pi^*_{(C=C)}$ Kumpulan benzena aromatik manakala pucak bagi peralihan π-π*(C=N) telah diperhatikan dalam julat 297-303 nm. Nilai jisim-ke-cas (m/z) $[M+H]^+$ bagi AL01 dan AL02, iaitu 242.09 dan 226.10, masing-masing, memberikan pengesahan lanjut tentang strukturnya. Keberkesanan ligan hidrazon sebagai perencat kakisan pada keluli lembut dalam larutan NaCl 3.5% tepu dengan CO_2 telah diperiksa menggunakan polarisasi dan spektroskopi impedans elektrokimia (EIS). Kehadiran unsur pada lapisan pelindung yang terbentuk pada keluli lembut yang direndam dalam larutan diperhatikan selanjutnya menggunakan mikroskop elektron pengimbasan (SEM) ditambah dengan X-Ray penyebaran tenaga (EDX). Oleh itu, perencat kakisan baru, ligan hidrazon tridentat, AL01 dan AL02 telah berjaya disintesis dan dicirikan menggunakan FTIR, NMR, analisis unsur, spektroskopi jisim dan UV-Vis. Daripada kajian polarisasi dan EIS, dapat disimpulkan bahawa AL01 mempunyai kecekapan perencatan yang lebih tinggi iaitu, 84.30% pada 500 ppm berbanding AL02 iaitu, 74.90%. Analisis SEM-EDX telah mengesahkan pembentukan lapisan perencat pada keluli lembut kerana permukaan keluli lembut dengan kehadiran perencat mempunyai permukaan yang lebih licin berbanding keluli lembut yang tidak dirawat dengan perencat kakisan dalam larutan 3.5% NaCl tepu dengan CO_2.

Kata kunci: CO_2, perencat kakisan, keluli lembut, lapisan pelindung, hidrazon tridentat

Introduction

Corrosion is a common natural interaction between a pure metal and its surroundings where the metal tends to revert to its stable condition i.e., a compounded state, and the substance deteriorates because of energy being released in the process [1, 2]. It has a significant influence on a variety of machinery, infrastructures, industrial plants, and human life. Many things can suffer serious damage from corrosion, and the costs of the destruction are substantial [3]. A previous study by Tamalmani et al., stated that corrosion has been highlighted as one of the key causes of oil and gas infrastructure problems. Internal pipeline is one of the widely used means for transporting resources in the oil and gas sector, however, internal pipeline corrosion has seemed to represent a substantial threat [4]. It has been acknowledged that pipeline corrosion is primarily brought on by the presence of carbon dioxide (CO_2) as the major corrosive medium which commonly happens during transportation of hydrocarbons in the pipeline. Due to the solubility of CO_2 in water and brine (NaCl solution), the steel and the contacting aqueous phase undergo an electrochemical process resulted in carbonic acid (H_2CO_3) formation, that leads to severe corrosion in pipelines [4–6].

Mild steel is often utilized as a raw material for the construction of pipelines, nevertheless, due to its premature failure and degradation, a gross expenditure of billions of dollars has been incurred worldwide. These costs include replacement, maintenance, and lost productivity since mild steel is an essential component in an infrastructure, which includes pipelines, port facilities, and bridges [7]. This low-carbon steel is widely sought-after for high temperature applications, such as petrochemical processes and pipeline construction, due to its malleability and lack of brittleness. Mild steel is widely used in the oil and gas industries for transporting gases and liquids over long distances in pipelines from their sources to the consumers [8], despite having a low corrosion resistance because of its availability, affordability, and hardness [1]. Moreover, mild steel consists of porous regions within its structure that make it more prone to corrosion, especially in corrosion-aggressive environments such chemical industries, the ocean, and polluted environments [9, 10]. Transportation of crude oil in pipelines for instance, often comes naturally with impurities such CO_2, water, and oxygen, which are thought to be the main causes of corrosion in pipelines. Studies found that since CO_2 dissolves in aqueous solution to generate weak carbonic acid (H_2CO_3) [8, 11, 12], which is considered to be the most hazardous

type of acids to carbon steel, a combination of CO_2 with water indicates a serious corrosion problem. However, the condition gets worsen with the combination of CO_2 with brine solution (NaCl). A study reported by Almeida et al., found that the corrosion rate of steel in distilled water was 28.3 mm/y while in brine solution after being immersed for 30 minutes was 119 mm/y [13].

Therefore, investigations were conducted, and it was discovered that using corrosion inhibitors could be one of the strategies to resolve this problem since it is easy to put into application, affordable, and under control [10]. However, it was also discovered that the inorganic inhibitors used in passivation processes, such as nitrates, chromates, and derivatives of azole and thiourea, were hazardous to both humans and the environment. Additional research on effective, environment-friendly inhibitors that can provide a protective layer on metals is required to address these problems to mitigate the rate of corrosion [14]. Corrosion inhibitors need at least one polar group, such as heteroatoms that can facilitate the process of adsorption in an acidic environment to be able to bond to the metal surface. Additionally, the effect of corrosion inhibition may be enhanced by changing the molecule's size, orientation, and geometry. Aforementioned, organic compounds with unsaturated bonds or heteroatoms like O, N, and S as well as functional groups like -NH, NN, CN-, and CHO frequently work well as corrosion inhibitors [15].

Due to their unique chemical properties, hydrazones, which belong to a special family of organic molecules, have several biological and therapeutic uses. For example, hydrazone-based inhibitors derived from non-steroidal anti-inflammatory medicines (NSAIDs) such mefenamic acid, naproxen, and ibuprofen have been produced, which have shown to be effective against steel corrosion [16]. The most advantageous property of hydrazones is their high reactivity because of the azomethine group, which has two nitrogen atoms with nucleophilic properties and a carbon atom with both electrophilic and nucleophilic properties [17]. In addition, hydrazone-based inhibitors have also been identified as "green corrosion inhibitors" that prevent corrosion by adsorption on the metal surface since they are non-toxic, inexpensive, and biodegradable [18].

Hence, in this study, new corrosion inhibitors of tridentate hydrazone ligands without and with the presence of OH substituent at the *ortho* position of the benzene ring were synthesized, characterized, and evaluated as organic corrosion inhibitors. To the best of our knowledge, no reports on using AL01 and AL02 as corrosion inhibitors are available. Therefore, the relationship between the absence and presence of electron-donating group (-OH) [19] with the inhibition efficiency and corrosion rate of the mild steel is reported.

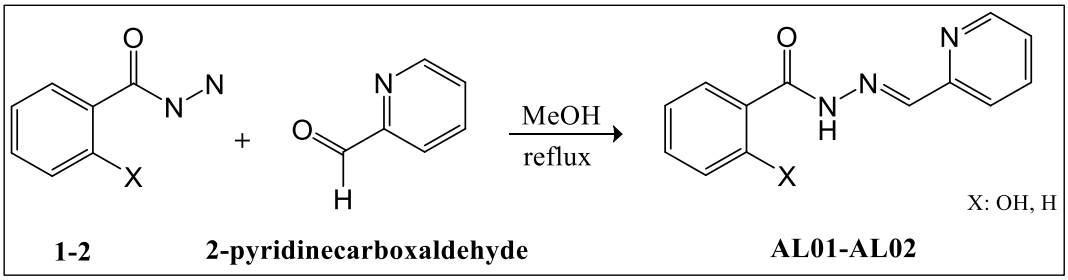
Materials and Methods

Materials

2-Pyridinecarboxaldehyde, 2-hydroxybenzohydrazide, benzohydrazide, and methanol purchased from commercial suppliers were used without further purification. Melting point determination in triplicate was carried out in glass capillary tubes using Buchii-B454 and the percentage compositions of the elements C, H and N of the compounds were determined by using Thermo Scientific Flash 2000 Elemental Analyzer with sulphanilamide as the standard. Fourier-transform infrared spectra (FTIR) were recorded using Perkin-Elmer FT-IR 1600 spectrometer by preparing samples as KBr discs. ^{1}H NMR spectra were recorded on Bruker Avance 600 MHz NMR spectrometer in deuterated solvents, DMSO and $CDCl_3$. UV-Vis spectra were obtained in acetonitrile ($5x10^{-5}$ M) in the 190-450 nm range Perkin Elmer Lambda 25 UV/Vis Spectrometer. Mass spectral data are measured in 1:1 ratio of acetonitrile: H_2O at concentration of 10 ppm using Thermo Scientific Vanquish Horizon UHPLC system fitted with Thermo Scientific TM Orbitrap Fusion Tribrid. The column used was Accucore TM Vanquish C18 (2.1 x 100 mm, 1.5 μm) at 37 ^{o}C.

Synthesis of tridentate hydrazone ligands

Scheme 1 shows the synthesis reaction for tridentate hydrazone ligands.



Scheme 1. Overall reaction between hydrazides and aldehyde

Synthesis of *(E)-2-hydroxy-N'-(pyridin-2-ylmethylene)benzohydrazide* (AL01)

2-Pyridinecarboxaldehyde (5 mmol, 0.5356 g) and 2-hydroxybenzohydrazide (5 mmol, 0.7608g) were dissolved in methanol (20 mL), and the mixture was heated under reflux for 30 minutes. The colorless solid obtained was filtered using vacuum pump and washed with ice cold methanol and air dried at room temperature. Yield: 83.5%. IR (KBr pellet, cm^{-1}): *v*(N-H) 3242 cm^{-1}, *v*(C=N) 1608 cm^{-1}, *v*(C=N) (pyridine) 1542 cm^{-1}, *v*(C=O) and 1630 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.28 (s, 1H), 8.82 (s, 1H), 7.97 (td, J = 7.8, 1.8 Hz, 1H), 7.69 (dd, J = 7.6 Hz, 1H), 7.64 – 7.54 (td, 1H), 7.58 – 7.54 (dd, 1H), 7.51 (dd, J = 1.6 Hz, 1H), 7.49 – 7.43 (td, 1H), 7.09 – 7.04 (td, 1H), 7.01 – 6.94 (dd, 1H), 2.19 (s, 1H). Anal. Calc. (%) For $C_{13}H_{11}N_3O_2$: C, 64.72 H, 4.60; N, 17.42. Found (%): C, 65.21; H, 4.66; N, 17.75.

Synthesis of *(E)-N'-(pyridin-2-ylmethylene) benzohydrazide* (AL02)

A mixture of 2-pyridinecarboxaldehyde (5 mmol, 0.5356 g) and bezohydrazide (5 mmol, 0.6808g) was prepared in 20 mL methanol. The reaction mixture was refluxed for 4 hours, and the solvent was concentrated using rotary evaporator. The concentrated product was cooled at room temperature until colorless precipitate formed. Yield: 66.7%. IR (KBr pellet, cm^{-1}): *v*(N-H) 3402 cm^{-1}, *v*(C=N) 1648 cm^{-1}, *v*(C=N) (pyridine) 1572 cm^{-1}, *v*(C=O) and 1651 cm^{-1}. ^{1}H NMR (500 MHz, DMSO) δ 8.57 (d, J = 75.9 Hz, 1H), 8.41 (s, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.88 (t, J = 6.5 Hz, 3H), 7.60 (t, J = 7.4 Hz, 1H), 7.52 (t, J = 7.6 Hz, 2H), 7.41 (dt, J = 31.4, 15.6 Hz, 1H), 2.06 (s, 1H). Anal. Calc. (%) For $C_{13}H_{11}N_3O$: C, 64.19; H, 5.39; N, 17.27. Found (%): C, 64.16; H, 5.33; N, 17.62.

Inhibition performance of corrosion inhibitors: Materials and electrolytes

Mild steel with surface area of 0.17 cm^2, 3.5% NaCl solution, CO_2 and N_2 to deoxygenate the solution [12]. Every experiment was carried out in unstirred, static solutions. Polishing Material, Potentiostat (Autolab, AUTO302N.FRA2 Netherlands), three electrode system glass cells with platinum electrode as counter electrode (CE), silver/silver chloride (Ag/AgCl) as reference electrode (RE) and mild steel as working electrode (WE). Tridentate hydrazone ligands (AL01 and AL02) were used as corrosion inhibitors.

Preparation of working electrode

The mild steel plate as working electrode was prepared by embedding the plate in epoxy resin, exposing a geometrical surface area of 0.17 cm^2 in 1000 mL of 3.5% NaCl solution saturated with CO_2 gas at pH 4-4.3. The metal plate was polished using various grade of SiC paper (300, 500 and 4000) prior to each experiment and degreased with ethanol and acetone.

Electrochemical impedance spectroscopy measurement (EIS)

Impedance measurements were performed after 15 min immersion of the metal plate in static condition at steady state open circuit potential at 25 °C [20] with slight modification. It was conducted in conventional three-electrode cell using platinum as the counter electrode and Ag/AgCl as the reference electrode. The AC current frequency range extended from 10 kHz to 0.1 Hz with 10 mV peak-to-peak amplitude. Electrochemical impedance spectroscopy generated Nyquist and Bode plots where the calculation of corrosion inhibition efficiency (IE %) of the inhibitors were calculated based on the R_p values using Equation 1,

$$IE\% = \left(\frac{R_{p(inh)} - R_{p(blank)}}{R_{p(inh)}}\right) x\ 100 \quad (1)$$

where $R_{p(blank)}$ and $R_{p(inh)}$ are the resistance charge transfer for bare mild steel and after the presence of inhibitor [1].

Polarization measurement

The corrosion behavior was further investigated through potentiodynamic polarization, immediately after the impedance spectra were recorded on the same metal plate without any additional surface treatment. The potentials were scanned at a scan rate of 0.5 mVs^{-1} from the corrosion potential (E_{corr}) in the cathodic direction and subsequently in the anodic direction. Potentiodynamic polarization gave the Tafel plot which was then extrapolated and corrosion inhibition efficiency (IE %) of the inhibitors was calculated based on the corrosion current density (i_{corr}) values using Equation 2,

$$IE\% = \left(\frac{i_{corr(0)} - i_{corr(i)}}{i_{corr(0)}}\right) x\ 100 \quad (2)$$

where $i_{corr(0)}$ and $i_{corr(i)}$ are the corrosion current densities for bare mild steel and after the presence of inhibitor [1].

Surface morphology analysis

The surface morphology and chemical composition were analyzed using scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) on the mild steel surfaces of samples that were immersed for 24 hours in 3.5% NaCl solution saturated with CO_2, with and without the presence of inhibitor at optimal concentration (500 ppm) [21].

Results and Discussion

Physical properties and elemental analysis

Table 1 displays physical characteristics of both ligands, and the experimental percentages of the elements C, H, and N of the compounds are closely corresponded to the theoretical values which proved their stoichiometry.

Table 1. Physical data for tridentate hydrazone ligands

Compound	Molecular Formula	Molecular Weight (g/mol)	Physical Properties	Melting Point (°C)	Elemental Analysis Found (Calculated) (%)		
					C	H	N
AL01	$C_{13}H_{11}N_3O_2$	241.25	White	225.8-227.3	**65.21** (64.72)	**4.66** (4.60)	**17.75** (17.42)
AL02	$C_{13}H_{11}N_3O$	225.25	Brown	111.0-113.6	**64.16** (64.19)	**5.33** (5.39)	**17.62** (17.27)

Infrared spectroscopy

The infrared spectra for AL01 shown in Figure 1, exhibit strong peak for *ν*(N-H), *ν*(C=N), *ν*(C=N) (pyridine), *ν*(C=O) at 3242 cm^{-1}, 1608 cm^{-1}, 1542 cm^{-1} and 1630 cm^{-1}, respectively. For AL02 spectra, the peaks for *ν*(N-H), *ν*(C=N), *ν*(C=N) (pyridine), *ν*(C=O) are at 3402 cm^{-1}, 1648 cm^{-1}, 1572 cm^{-1}, and 1651 cm^{-1}, respectively [22]. The stretching band for *ν*(C=N) of pyridine was at the lower frequency region compared to aliphatic *ν*(C=N) because of the N atom in the ring acts as an activating and electron-donating group which causes the absorption band to move to a lower frequency region by pushing the electron clouds towards the ring and increasing the electron density [21]. There was a broad absorption band of O-H for AL01 that appeared in the region of 3000 cm^{-1} [22]. Other prominent peaks for both ligands observed in Figure 1 are the C=C stretch (1466-1472 cm^{-1}), and the C-H sp^2 stretch (2925-2974 cm^{-1}), which indicate the existence of benzene rings in the structure [23].

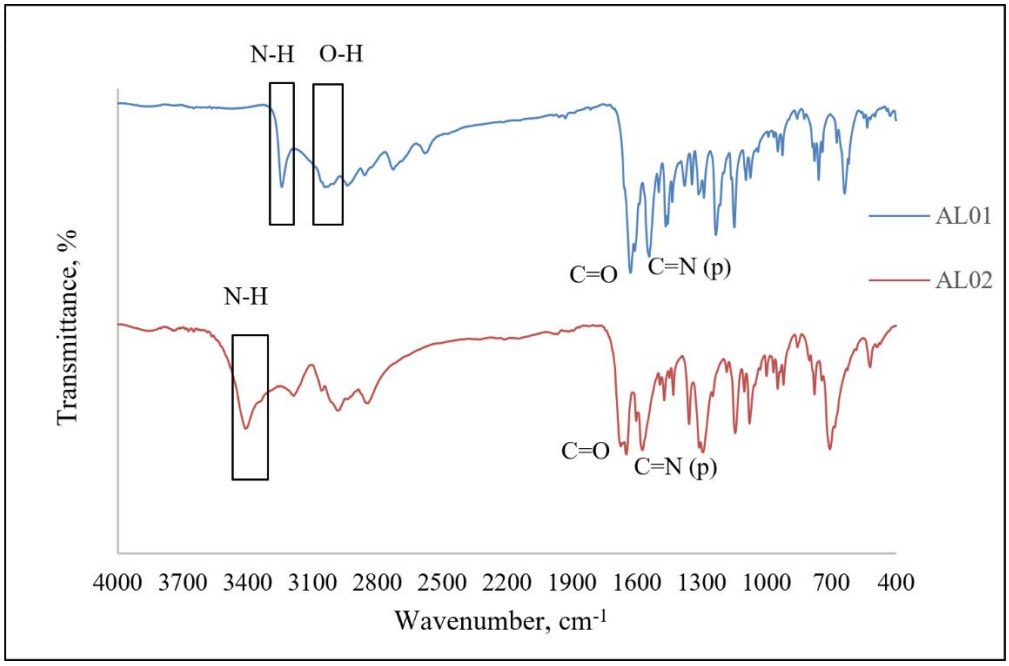


Figure 1. FTIR spectra of AL01 and AL02

Nuclear magnetic resonance spectroscopy

The ^{1}H NMR spectra for the ligand AL01 and AL02 were recorded in $CDCl_3$ and DMSO-d_6 respectively. Both ligands show a singlet peak at downfield region at 8.82 and 8.41 ppm, respectively, due to NH protons [24, 25] as shown in Figure 2(a) and Figure 2(b). For AL01 spectrum (Figure 2a), a singlet peak of phenolic proton appeared at 12.28 ppm strongly confirms the ligand structure [25]. The aromatic protons for benzene ring were observed in the range of 6.95-8.00 ppm for AL01 and in the range of 7.42-8.56 ppm for AL02 [26].

Ultraviolet-visible spectroscopy

The UV-Vis spectra of ligands AL01 and AL02 were measured at 5 x 10^{-5} M in acetonitrile between 190 to 450 nm. Two peaks in the absorption spectra of AL01 and AL02 in the range of 198-201 nm was due to the π-π*(C=C) transitions of aromatic rings [22]. However, for AL01 spectrum, there was a peak in the range of 205-210 nm which was assigned for n- π* transition as OH substituent at the *ortho* position was added to the benzene ring [27]. There was a weak band observed at 319 nm assigned for n-π* transition of C=N for AL01 while the peak for this transition was not found for AL02. There were also peaks of π-π* (C=N) transitions for AL01 at 302 nm and for AL02 at 299 nm. As shown in Figure 3, AL01 absorption is more bathochromic than AL02 because of the conjugated systems' effect from the OH functional group on the benzene ring. Hence, the absorption peaks appear at longer wavelengths compared to that of the benzene peak [27].

Liquid chromatography-mass spectrometry

The ligands AL01 and AL02 were characterized by LC-MS in the positive ion mode. When compared to the hypothesized structure, the mass spectrum of the ligand AL01 (Figure 4) exhibits a strong molecular ion peak at m/z = 242.09, which corresponds to the $[C_{13}H_{11}N_3O_2]+H^+$ (calculated RMM of AL01 = 241.25). Based on AL02 spectrum, an additional support for the structure was provided by the intense molecular ion peak at m/z = 226.10 corresponding to $[C_{13}H_{11}N_3O]+H^+$ which is in agreement with the molecular weight of the compound (225.25 g/mol) [28].

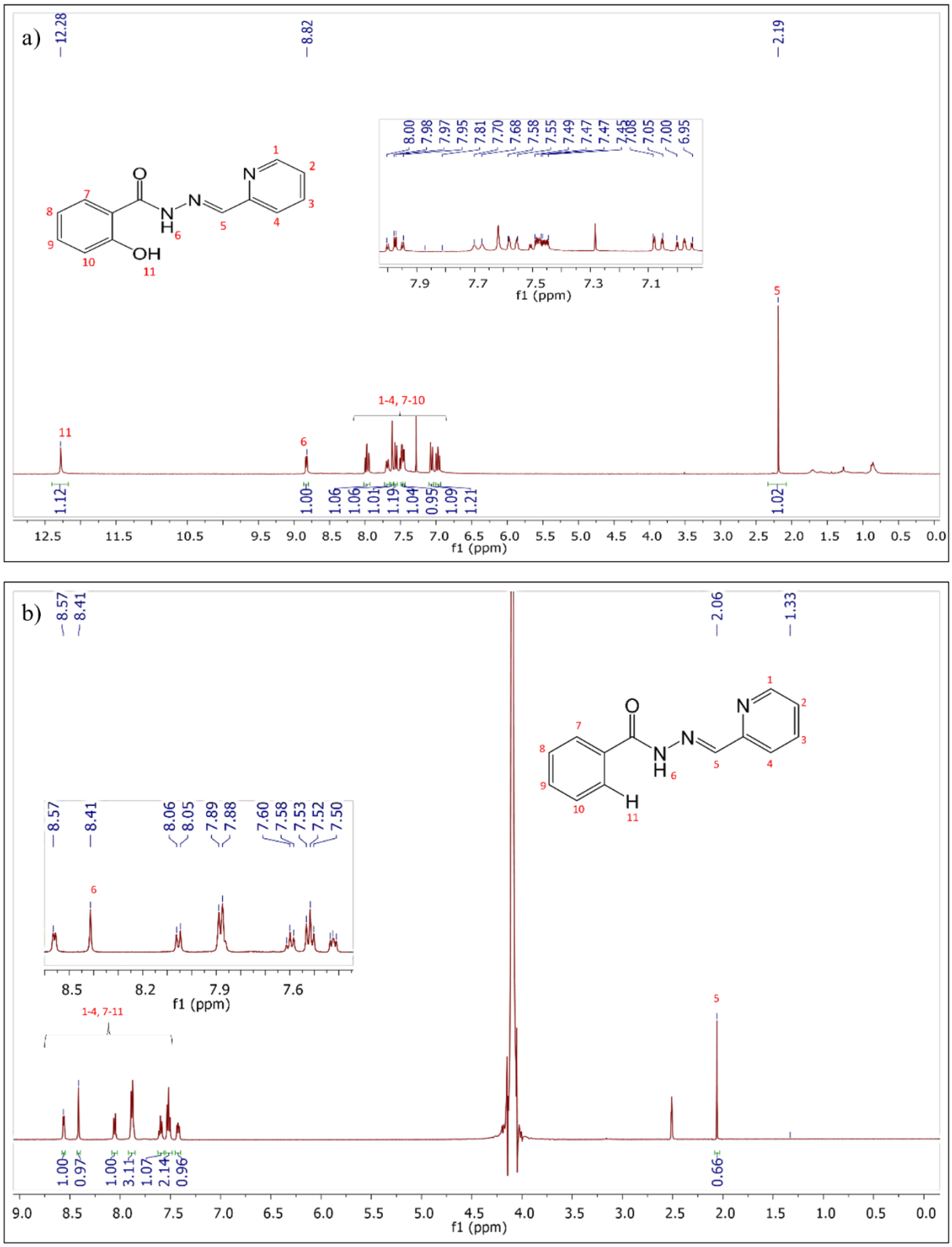


Figure 2. NMR spectra of a) AL01 and b) AL02

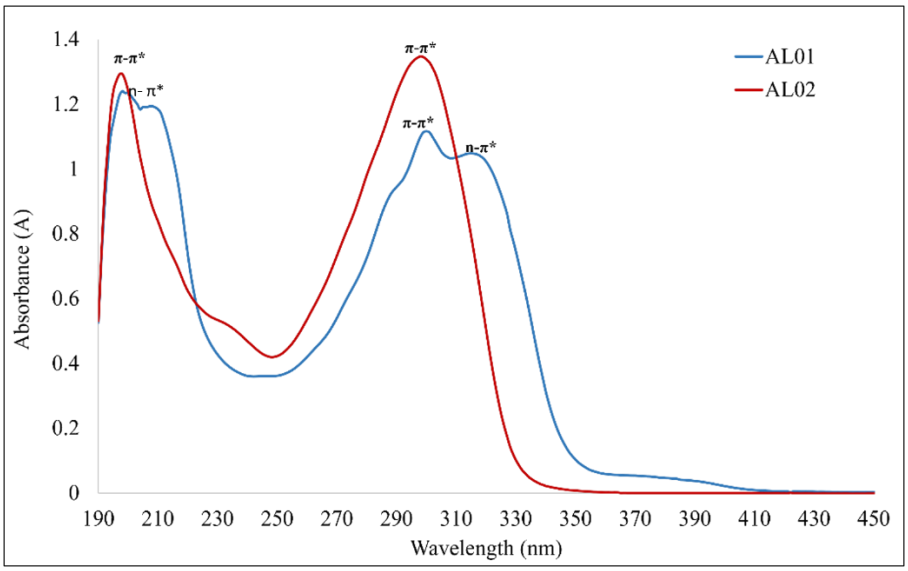


Figure 3. UV-Vis spectra of AL01 and AL02

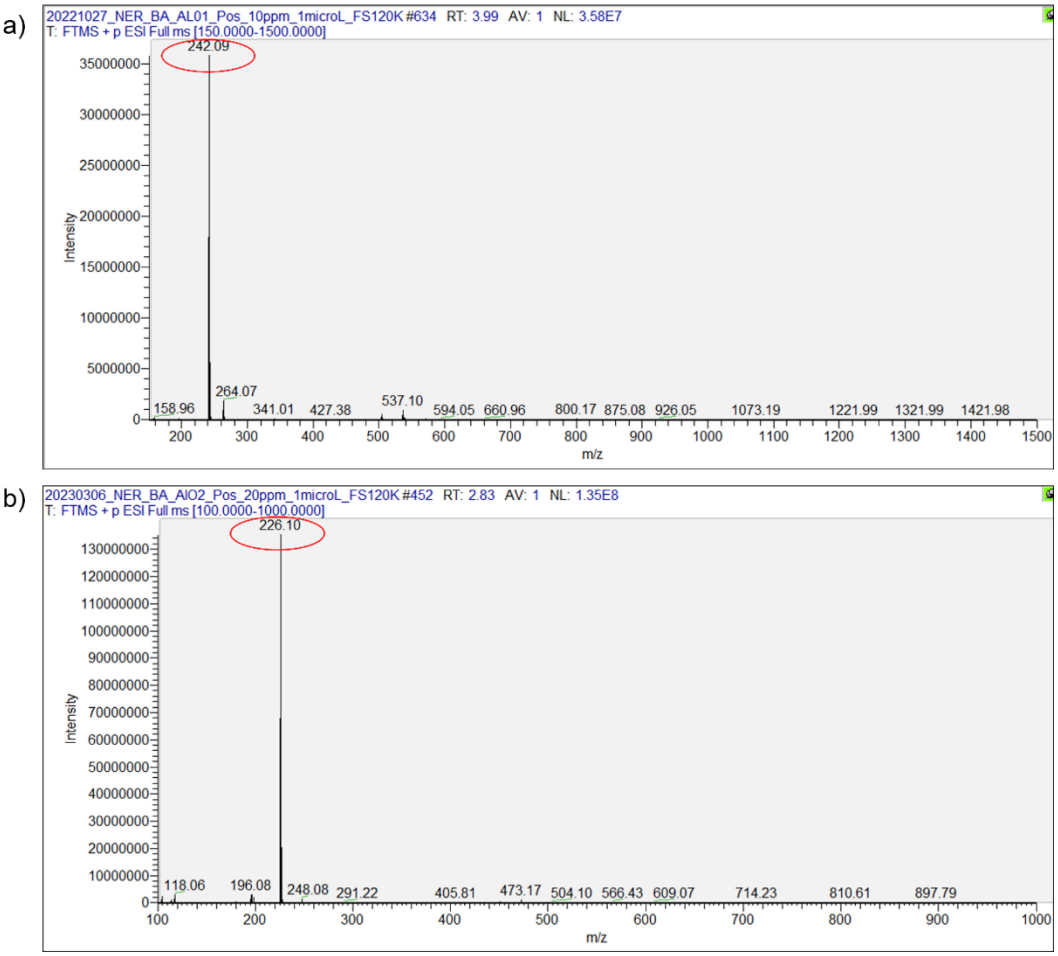


Figure 4. LC-MS spectra of a) AL01 and b) AL02

Inhibition performance of corrosion inhibitors: Potentiodynamic polarization (PDP)

Tafel extrapolation is a polarization technique to estimate corrosion rates that offers a faster alternative to traditional weight-loss method. The Tafel equation can be used to represent mixed potential theory using simple electrochemical kinetics, which can then be used to predict corrosion rate and potential based on the kinetics and thermodynamics of all reactions taking place on an electrode surface [29].

The potentiodynamic polarization parameters were computed using the Tafel extrapolation method. The corrosion rate values may be calculated using Equation 3, which is based on Faraday's equation [29]. Further on, the E_{corr} values were deducted from the intersection of the cathodic and anodic polarization curves [9].

$$\mathrm{CR\ (mpy)} = \frac{0.00327 \times i_{corr} \times EW}{\rho} \quad (3)$$

where i_{corr} is the corrosion current density in μA cm^{-2}, ρ is the density of the metal in g cm^{-3}, EW is the equivalent weight. Table 2 shows the potentiodynamic polarization parameters for the 3.5% NaCl solution, and the solutions with the presence of inhibitors.

Table 2. Potentiodynamic polarization parameters for blank, with presence of inhibitors AL01 and AL02

Compound	Concentration (ppm)	E_{corr} (V)	i_{corr} (μA/cm^2)	βa (V)	βc (V)	Corrosion Rate (mm/year)	Inhibition Efficiency (%)
Blank	3.5% NaCl	-0.6509	83.659	0.0502	-6.3902	0.9834	-
AL01	100	-0.6134	58.795	0.0356	0.1279	0.6912	30.00
	200	-0.5889	47.904	0.0300	0.1602	0.5631	42.74
	300	-0.6177	35.347	0.0618	0.2486	0.4155	57.75
	400	-0.6721	32.938	0.0409	0.3625	0.3872	60.63
	500	-0.6110	13.132	0.0463	0.2081	0.1544	84.30
AL02	100	-0.6583	43.790	0.0607	0.4808	0.5148	47.66
	200	-0.6989	38.570	0.0632	0.2813	0.4534	53.90
	300	-0.6733	34.502	0.0423	0.3106	0.4056	58.76
	400	-0.6729	34.025	0.0665	0.2524	0.4000	59.33
	500	-0.6426	21.002	0.0415	0.3638	0.2469	74.90

All data tabulated in Table 2 were originally obtained from Tafel plot as shown in Figure 5 below. It revealed that uncoated mild steel plate had the highest corrosion current density (i_{corr}), 83.659 μA/cm^2 and was therefore more prone to corrosion (active dissolution) [9]. Table 2 shows that there was a significant reduction in the corrosion current densities (i_{corr}) with increasing inhibitor concentration. In the presence of AL01 inhibitor, the value of i_{corr} decreased from 58.795 μA/cm^2 to 13.132 μA/cm^2, while in the presence of AL02, the value decreased from 43.790 μA/cm^2 to 21.002 μA/cm^2.

This was an obvious indication of how increasing inhibitor concentration resulted in an increasing number of hydrazone molecules to be adsorbed on the mild steel surface, inhibiting all forms of corrosion that were present at the metal-electrolyte interface [30]. The data proved that both inhibitors had an inhibitory impact on the aggressive mild steel solution, which effectively blocked the active site while the inhibitor was adsorbing to the mild steel surface [31]. Additionally, PDP data (Table 2) show that AL01 exhibits the highest level of inhibition, with an inhibition efficiency of 84.30%, followed by AL02 (74.90%).

This result was supported by the presence of OH functional group on the benzene ring in AL01 that could

enhance the adsorption process on the mild steel by increasing the molecule's electron charge density and molecule's binding affinity with the steel surface [12].

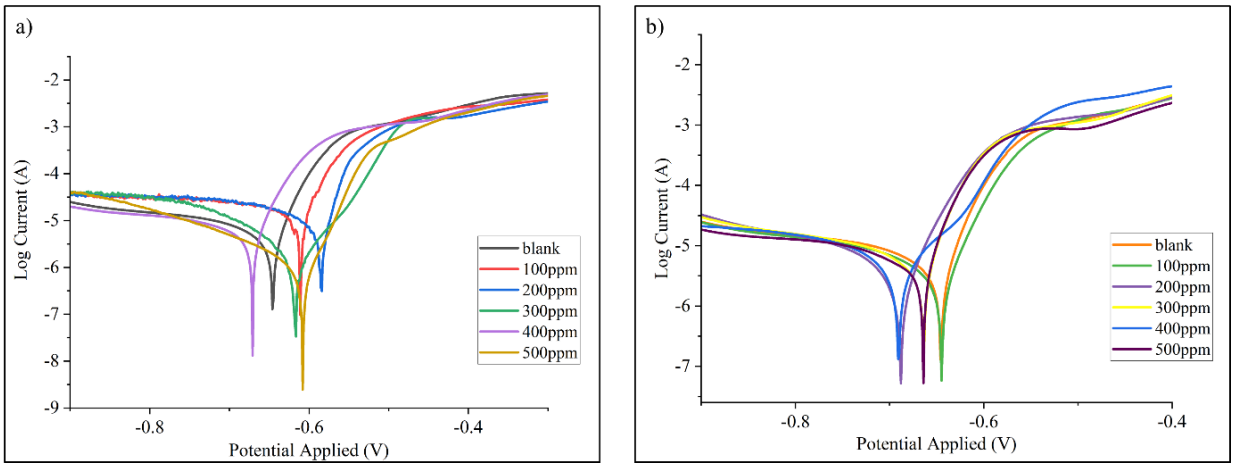


Figure 5. Tafel plots for ligands a) AL01 and b) AL02

The findings from Tafel plots (Figure 5) provided a helpful comparison of changes in potential corrosion values and the degree of corrosion current density reduction. This indicates that the corrosion reactions are inhibited [12]. When carbon dioxide gas dissolved in aqueous media, carbonic acid (H_2CO_3) was produced. Then, the bicarbonate ion (which could further dissociate to produce the carbonate ion) formed when the carbonic acid (H_2CO_3) partially dissociated. It has been widely recognized that, at the same pH, solutions containing H_2CO_3 would corrode mild steel more severely than solutions containing strong acids i.e., hydrochloric (HCl) or sulfuric (H_2SO_4) acids as they act as an additional source of hydrogen ions thus, lead to higher corrosion rates [11, 32].

Electrochemical impedance spectroscopy (EIS)

In addition, electrochemical impedance measurements were carried out to investigate the mechanisms of interaction between the electrode surface and the chemicals being studied, such as charge transfer, diffusion, and adsorption. Inhibitor adsorption on the metal substrate was corroborated by EIS studies, which also showed a rise in polarization resistance as the concentration of inhibitors increased [15]. Figure 6 shows Nyquist plots for mild steel at various concentrations of AL01 and AL02, in 3.5% NaCl solution saturated with CO_2.

Apparently, incomplete single depressed capacitive loops with nearly the same forms, both with and without inhibitors make up Nyquist plots, which show that the corrosion mechanism remains the same [17]. The non-homogeneity or roughness of the metal surface and the resistance to mass movement were the causes of the Nyquist plot's deviation from perfect semicircles, which was known as frequency dispersion. The semi-circle curve's diameter increased as the inhibitor concentration increased, demonstrating the inhibitor's ability to bind to mild steel forming a protective film over the metal surface [12, 31].

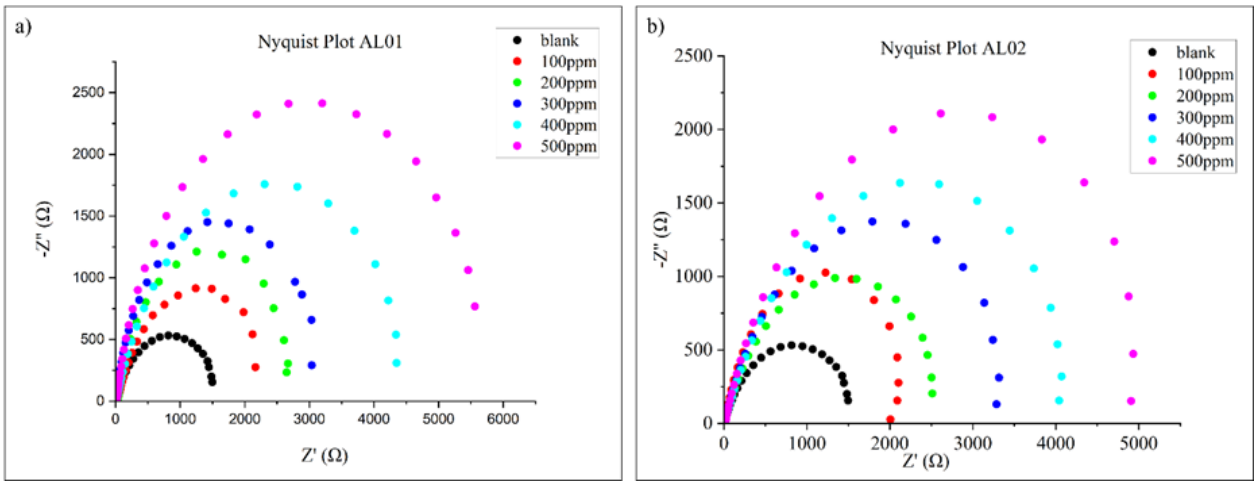


Figure 6. Nyquist plots for ligands a) AL01 and b) AL02

A basic Randle's circuit proposed in Figure 7 can be employed to represent the semi-circle Nyquist plots that were generated through the experiment. This circuit demonstrated that the charge transfer resistance (*Rp*) is parallel to the constant phase element (CPE) in series with the solution resistance (*Rs*) [31].

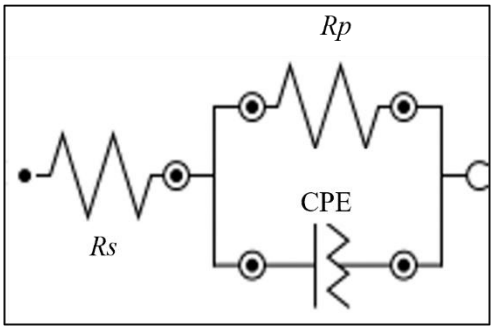


Figure 7. Simple Randle's circuit

Nyquist plots (Figure 6), indicate the samples' capacitive behavior, which consists of a single capacitive loop. The diameter of the Nyquist diagram represents the treated mild steel substrate's corrosion resistance (*Rp*, as indicated in EIS), which is correlated with the rate of corrosion. A greater semicircle diameter is indicative of a higher corrosion resistance, or a lower rate of corrosion. The resultant capacitive loop demonstrates that the charge transfer mechanism and the formation of a corrosion-inhibitory layer on the mild steel surface control the corrosion of mild steel in a 3.5% NaCl solution [9].

The impedance parameters obtained from Nyquist plots are presented in Table 3. The observed *Rp* values exhibited a significant increase in the presence of inhibitors, particularly at higher concentrations, in comparison to the *Rp* value obtained without the presence of an inhibitor. Consequently, the corrosion inhibition efficiency exhibited an increase. This behavior confirmed the formation of the protective film on mild steel. The observed trend in the diameter of the semi-circle curve indicates a positive correlation with the concentration of the inhibitor, hence, suggesting the occurrence of adsorption of the inhibitor on mild steel. This prevents the mild steel from corroding because the adsorbed layer formed hinders charge and mass transfers at the metal-inhibitor solution interface [31].

Table 2. Impedance parameter values for the corrosion of mild steel

Compound	Concentration (ppm)	Rs (Ω)	R_{ct} (Ω)	Inhibition Efficiency (%)
Blank	-	10.8	1596	-
AL01	100	29.8	2461	35.15
	200	35.84	2802	43.04
	300	16.9	3760	57.55
	400	13.1	4749	66.39
	500	19.4	5883	72.87
AL02	100	12.7	2185	26.96
	200	11.0	2781	42.61
	300	11.9	3700	56.86
	400	10.5	4545	64.88
	500	14.7	5529	71.13

The Nyquist plots and impedance data presented in Table 3 indicate that the corrosion resistance of both inhibitors was found to be improved by increasing the concentration to 500 ppm. The mild steel immersed in 3.5% NaCl solution with 500 ppm inhibitors produced the biggest semi-circle diameter, as shown in Figure 6. Furthermore, comparing AL01 and AL02, the diameter of the Nyquist plot was bigger for AL01 at 500 ppm suggesting a better corrosion resistance i.e., a more robust inhibitive layer [9].

SEM-EDX Analysis

Scanning electron microscopy (SEM) was employed to examine the morphology of the metal surface and the development of the protective film on the surface of mild steel. Following the inhibitors' binding, the steel surface was seen to be smoother [33]. Figure 8 shows the SEM micrographs for mild steel after 24 hours immersion in 3.5% NaCl solution with and without the presence of corrosion inhibitors.

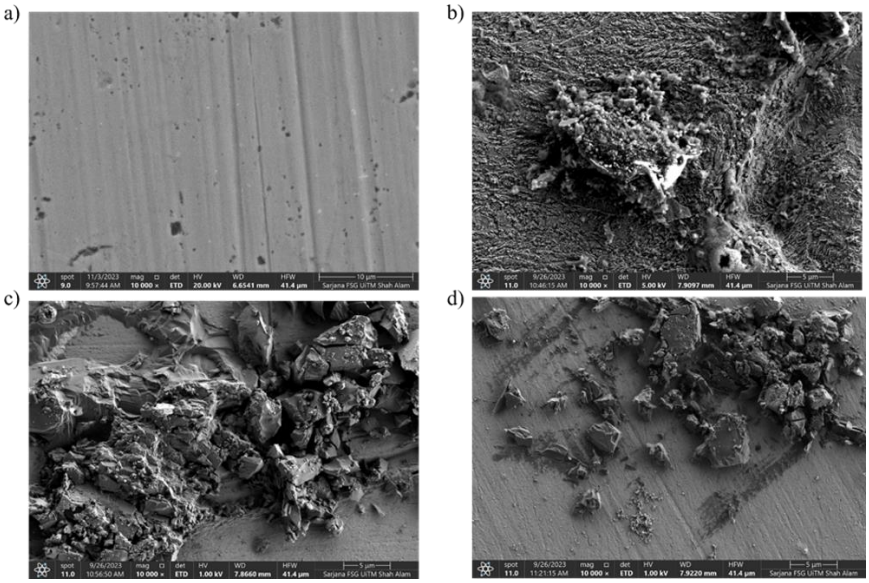


Figure 8. SEM micrographs of a) bare mild steel, (b) 3.5% NaCl solution, c) solution with 500 ppm of AL01 and d) solution with 500 ppm of AL02

Due to the manual abrasion process used during the polishing phase of the panels, scratch marks were apparent on the mild steel's exposed surface [9]. From observation, untreated mild steel plate in 3.5% NaCl solution (blank) (b) had a much rougher surface compared to surfaces with the presence of AL01 and AL02, which were substantially smoother. SEM examination confirmed that the presence of hydrazone ligands in the solution offered efficient corrosion retardation with the least amount of surface damage [9]. This is due to the formation of an adsorption layer that minimizes the effects of corrosion although the steel's surface treatment still left some residual scratches and polishing lines as in Figure 8(a) [34].

Energy dispersive X-ray spectroscopy (EDX) was used to evaluate the protective mechanism of inhibitors to find reductions in peaks related to iron, carbon, and chloride which are markers for metal corrosion [33]. According to the EDX study (Table 4), Fe, C and small traces of Si were detected as elements in bare mild steel. Noticeably, Fe content was decreased when treated with AL01 and AL02 from 97.25% to 82.19 wt.% and 95.35 wt.% respectively. This indicates the adsorption of inhibitors on the surface of the mild steel thus, reducing the Fe content [9]. For untreated mild steel there was a presence of Cl, signifying a corrosion attack from NaCl solution in aggressive environment [35]. However, when comparing the weight percentage (wt.%) (Table 4) of Cl element in the presence of inhibitors with that of the blank, there was no Cl detected when the inhibitors were present. This observation demonstrates the adsorption of inhibitors on the mild steel surface where elements such as C, N and O correspond to the inhibitor's composition, which were found on the mild steel immersed in the presence of AL01 and AL02 inhibitors. These are essential for stabilizing the molecule at the surface, which enhance the ability of the steel to resist corrosion [33, 35]. Hence, compared to AL02, which agrees with the data in Table 4, AL01 had a higher capacity to build a protective film corrosion because of its strong electron-donating ability due to the presence of OH functional group [19].

Table 3. Weight percentage of elements in mild steel specimen obtained from EDX spectra

Mild Steel Specimen	Fe (wt.%)	C (wt.%)	Si (wt.%)	Cl (wt.%)	Na (wt.%)	O (wt.%)	N (wt.%)
Bare mild steel	97.25	2.10	0.65	-	-	-	-
Untreated mild steel	75.51	1.89	-	6.28	15.37	0.95	-
Treated with AL01	82.19	13.60	-	-	-	2.47	1.74
Treated with AL02	95.35	3.23	-	-	-	1.07	0.35

Conclusion

New corrosion inhibitors of tridentate hydrazone ligands, AL01 and AL02 were successfully synthesized via condensation process derived from two different substituents of hydrazides. The ligands were characterized using FTIR, NMR, elemental analysis, mass spectroscopy and UV-Vis. From polarization and EIS study, it can be concluded that AL01 has the highest inhibition efficiency, 84.30% at 500 ppm compared to 74.90% of AL02. SEM-EDX analysis has confirmed the formation of inhibitors' layer on mild steel as the surface of the mild steel with the presence of inhibitors is smoother compared to that of the untreated mild steel in 3.5% NaCl solution saturated with CO_2. Hence, compared to AL02, AL01 has a higher inhibitory efficacy and the compound formed a stronger protective layer on the mild steel as the compound contains electron donating group (OH).

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IONIC TRANSPORT AND STRUCTURAL ANALYSIS OF BIOPOLYMER ELECTROLYTE BASED ON AGAROSE INTEGRATED WITH SODIUM NITRATE

(Kajian Pengangkutan Ion dan Struktur kepada Elektrolit Biopolimer Berdasarkan Penggabungan antara Agarose dan Natrium Nitrat)

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Abstract

The central focus of this paper revolves around investigating biopolymer electrolyte films characterized by exceptional ionic conductivity, a prerequisite for the practical implementation of sodium-ion batteries. This study successfully prepared the agarose-based biopolymer electrolyte using the solution casting method. The effects of adding various weight percentages (0, 10, 20, 30 and 40 wt.%) of sodium nitrate salt ($NaNO_3$) to agarose-based biopolymer electrolytes were characterized. Electrochemical Impedance Spectroscopy (EIS) was adapted to analyze the conductivity and dielectric relaxation phenomena of the agarose-$NaNO_3$ complex. The conductivity of agarose-based biopolymer electrolytes increases with the increasing salt concentration. The increase in ionic conductivity is due to the increase in the number of charge carriers and the mobility of the sodium ions. The highest room temperature conductivity was 3.44×10^{-5} S·cm^{-1} for the agarose-$NaNO_3$ biopolymer electrolytes containing 30 wt.% sodium nitrate. X-ray diffractometer (XRD) spectroscopy was employed to investigate the crystallinity of the agarose-based biopolymer electrolyte. It was confirmed that the agarose-based biopolymer with 30 wt.% of sodium nitrate is the most amorphous compared to the others, as it has the largest full width at half maximum (FWHM) and the smallest crystallite size. This indicated that the amorphousness of the biopolymer electrolyte boosts the Na^+ ions' mobility, increasing the ionic conductivity of the samples.

Keywords: biopolymer electrolyte, agarose, sodium nitrate, conductivity, dielectric constant, crystallite size

Abstrak

Tumpuan utama dalam artikel ini adalah berkisar tentang penyelidikan untuk filem electrolit biopolymer yang dicirikan oleh kekonduksian ionik yang luar biasa, prasyarat untuk pelaksanaan praktikal dalam bateri natrium-ion. Dalam kajian ini, elektrolit biopolimer berasaskan agarosa telah disediakan dengan jayanya menggunakan kaedah penuangan larutan. Kesan penambahan pelbagai peratusan berat (0, 10, 20, 30 dan 40 wt.%) garam natrium nitrat ($NaNO_3$) kepada elektrolit biopolimer berasaskan agarosa

dicirikan. Spektroskopi impedans elektrokimia (EIS) telah disesuaikan untuk menganalisis fenomena masa pengenduran kekonduksian dan dielektrik kompleks agarosa-$NaNO_3$. Kekonduksian elektrolit biopolimer berasaskan agarosa meningkat dengan kepekatan garam yang semakin meningkat. Peningkatan kekonduksian ionik adalah disebabkan oleh peningkatan bilangan pembawa cas dan mobiliti ion natrium. Kekonduksian suhu bilik tertinggi ialah 3.44×10^{-5} $S\cdot cm^{-1}$ untuk elektrolit biopolimer agarose-$NaNO_3$ yang mengandungi 30 wt.% natrium nitrat. Spektroskopi pembelauan sinar-X (XRD) digunakan untuk menyiasat kehabluran elektrolit biopolimer berasaskan agarosa. Telah disahkan bahawa biopolimer berasaskan agarosa dengan 30 wt.% natrium nitrat adalah yang paling amorf berbanding yang lain, kerana ia mempunyai lebar penuh terbesar pada separuh maksimum (FWHM) dan saiz hablur halus terkecil. Ini menunjukkan bahawa amorfus elektrolit biopolimer meningkatkan mobiliti ion-ion Na^+ sekali gus meningkatkan kekonduksian ionik sampel.

Kata kunci: elektrolit biopolimer, agarose, natrium nitrat, kekonduksian, pemalar dielektrik, saiz kristal

Introduction

Sodium-ion batteries offer promise as a feasible choice for large-scale energy storage owing to their advantages, such as the plentiful availability and cost-effectiveness of sodium-based components. The same organic liquid electrolytes used in lithium-ion batteries also contribute to continued safety issues, such as the risk of fire [1]. Polymer electrolytes provide an attractive alternative to liquid electrolytes, boosting sodium-ion batteries' safety. To ensure the success of this technique, all other battery components must be further developed. Electrolytes are essential to sodium batteries since they facilitate the transit of ions inside the cell. Polymers containing amorphous polymer-salt complexes are leading candidates to replace liquid electrolytes. This is because of its good temperature stability, large electrochemical window, and low electrolyte solution leakage.

Biopolymer electrolytes have gained significant attention in the field of sodium battery technology. These electrolytes are composed of natural biopolymers, such as agarose [2], chitosan [3], and alginate [4], which offer several advantages over traditional polymer or liquid electrolytes. Biopolymer electrolytes are environmentally friendly since they are derived from renewable sources. This makes them a sustainable alternative to conventional materials used in batteries. Additionally, these biopolymers possess good mechanical properties and high ionic conductivity, allowing for efficient ion transport within the battery system. A high concentration of functional groups allows for strong coordination interactions between ions and polymers. This leads to improved electrochemical performance, enhanced cycling stability, increased specific capacity retention rates, and low charge transfer resistance at electrode-electrolyte interfaces [5].

Agarose is a biopolymer from seaweed used as a host polymer to conduct ions. Biopolymer electrolytes based on agarose are an excellent potential replacement for synthetic-based polymer electrolytes in electrochemical devices [2]. Fossil fuels play a crucial role in producing synthetic polymers. However, substituting agarose for these synthetic materials can help reduce our reliance on fossil fuels, preserving them for future generations when their scarcity might pose a significant challenge. Fossil fuel formation is a time-consuming process, and our current consumption rate could eventually deplete this finite resource if we do not adopt more sustainable practices [6].

Adding sodium nitrate ($NaNO_3$) improves ionic conductivity in polymer electrolytes because it breaks down into sodium ions (Na^+) and nitrate ions (NO_3^-) when mixed with the polymer. These ions help electric charges move easily through the polymer's ion channels. This addition also boosts bulk and surface conductivities, as shown in X-ray studies revealing a stronger bond between $NaNO_3$ and the polymer, improving ion-polymer interactions and mobility [7]. Using sodium nitrate salts as dopant in polymer electrolytes has much potential to improve their performance in batteries. By interacting with polymers, these additives increase ionic conductivity, making it easier for charges to move while keeping the structure intact at normal temperatures.

This study is about cultivating a biopolymer electrolyte that combines agarose and sodium nitrate. The biopolymer electrolytes that will be utilized in this research are biopolymer electrolytes, which possess a greater conductivity when compared to solid polymer electrolytes. Thin films of biopolymer electrolytes will be produced and studied in this research. The thin films will be subjected to two methods of characterization of biopolymer electrolytes: Electrochemical Impedance Spectroscopy (EIS) to investigate ionic conduction analysis and X-ray Diffractometer (XRD) to determine the structural properties.

Materials and Methods

Preparation of biopolymer electrolyte

The biopolymer, agarose (molecular weight, Mw: 630.5 $g \cdot mol^{-1}$), was acquired from Next Gene in Puchong, Malaysia. Meanwhile, sodium nitrate ($NaNO_3$; Mw: 84.99 $g \cdot mol^{-1}$) as a dopant was attained from Chemiz (M) Sdn. Bhd. in Shah Alam, Selangor. Fisher Scientific, which is in Hampton, NH, USA, was where dimethyl sulfoxide (DMSO) (Mw: 256.41 $g.mol^{-1}$, ≥99.7%), which acts as a solvent, was obtained. The biopolymer electrolyte with a base of agarose and the integration of sodium nitrate was prepared using the method of solution casting. Firstly, 0.5 g of agarose was dissolved in 20 ml of the solvent dimethyl sulfoxide (DMSO). Next, sodium nitrate of different concentrations were added to the solution containing agarose and DMSO. The different concentrations of sodium nitrate are based on the weight percentages of 0, 10, 20, 30 and 40. The solution was stirred continuously at room temperature (300 K) using a magnetic stirrer at room temperature until a homogenous solution was obtained. The solution was cast on a petri dish and the sample was left to dry. The sample was dried in an oven to speed up the process of evaporation. The solvent that underwent evaporation was primarily composed of dimethyl sulfoxide, and upon its complete volatilization, autonomous biopolymer electrolyte films were effortlessly detached from the petri dishes, a visual representation of which is depicted in Figure 1.

Characterization of biopolymer electrolyte

The thin films of biopolymer electrolytes were characterized, and their ionic conductivity and electrical properties were analyzed based on impedance data from Electrochemical Impedance Spectroscopy. The apparatus that was used for this is the HIOKI 3532-50 LCR Hi-tester. The apparatus is consolidated with a computer of the frequency ranging between 100 Hz and 10 MHz at varying temperatures (303-373 K). The ionic conductivity, σ of an electrolyte, can be calculated using the formula in Equation 1. The distance between the electrodes in centimeters (cm) is t. At the same time, A is the contact area of the electrolyte and the electrodes in square centimeters (cm^2), whereas R_b is the bulk resistance of the sample electrolyte. Equation 1 can also be used to determine the ionic conductivity of a polymer electrolyte in the form of a thin film.

$$\sigma = \frac{t}{R_b A} \quad (1)$$

The thin films of the biopolymer electrolytes were characterized using X-ray diffractometer spectroscopy. Other than that, the structural behavior of the thin films was observed to identify the existence of amorphous and crystalline peaks in the thin films. The nature of the thin films, whether crystalline or amorphous, was determined. The apparatus in use for this is the PANalytical X'pert PRO diffractometer. The diffractometer has a copper K-alpha (Cu K-α) radiation of wavelength (λ) equal to 1.5418 Å. The angle of diffraction (2θ) of the diffractometer was set between 5° to 90° at room temperature.



Figure 1. The stand-alone thin film of agarose-$NaNO_3$ biopolymer electrolyte.

Results and Discussion

Conductivity analysis at room temperature

Impedance plot (Figure 2) of biopolymer electrolyte with 30 wt.% $NaNO_3$ shows a smaller depressed semicircle in the high-frequency region by comparing with 40 wt.%, and both samples containing an inclined spike in the low-frequency region. The semicircle indicates ionic conduction within the electrolyte, while the spike is linked to the impact of blocking electrodes. This impedance analysis employed blocking electrodes, creating a double-layer capacitance at the interface between the electrodes and the electrolyte.

The value of the bulk constant, $R_{b,}$ for each sample at different weight percentages was determined by finding the value on the real impedance, the Z_r axis at which the semi-circular arc converges with the spike. The value of the bulk constant is observed to be reduced as the weight percentage of the samples increases from 0 to 30 wt. %. The bulk constant, R_b, then increases as the weight percentage of the sample rises to 40 wt.%.

Table 1 and Figure 3 demonstrate the ionic conductivity of the samples with different weight percentages of $NaNO_3$ at room temperature. The ionic conductivity of the agarose-based biopolymer electrolyte starts at 1.21×10^{-9} $S\cdot cm^{-1}$ when devoid of $NaNO_3$ (0 wt.%), which shows that polymer agarose is the best choice for host polymer as it satisfies the conductivity ranges for pure polysaccharides biodegradable materials from $\sim 10^{-14}$ to 10^{-8} $S.cm^{-1}$ [8]. Steadily escalating to 3.69×10^{-7} $S\cdot cm^{-1}$ at 10 wt.% of $NaNO_3$, with a continuous ascent peaking at the highest conducting of 3.44×10^{-5} $S\cdot cm^{-1}$ at 30 wt. %. However, beyond this concentration, the ionic conductivity experiences a decline, diminishing to 6.84×10^{-7} $S\cdot cm^{-1}$ at 40 wt.%.

The enhancement of ionic conductivity is attributed to the higher number of ion dissociation events obtained because of the addition of $NaNO_3$ from 0 to 30 wt.% into the agarose-based polymer electrolyte system that results in the production of free ions (Na^+ cation and NO_3^- anion) that can be incorporated in the polymer host matrix [9]. Therefore, a high salt concentration promotes the degree of ion dissociation, thus increasing the ionic conductivity of the agarose-$NaNO_3$ biopolymer electrolyte system. On the other hand, the increase in the amorphous nature of the system also affects the enhancement of ionic conductivity. It leads to the flexibility of the agarose polymer chain and promotes segmental motion in this system. However, when ions are abundant in the samples, the viscosity increases, which leads to the formation of ion cluster carriers that cause a decline in both the number of ions and their mobility, which impedes ion movement. Therefore, the ionic conductivity of the samples is reduced to 40 wt.% of $NaNO_3$ because of the change in the flow of charge carriers [2].

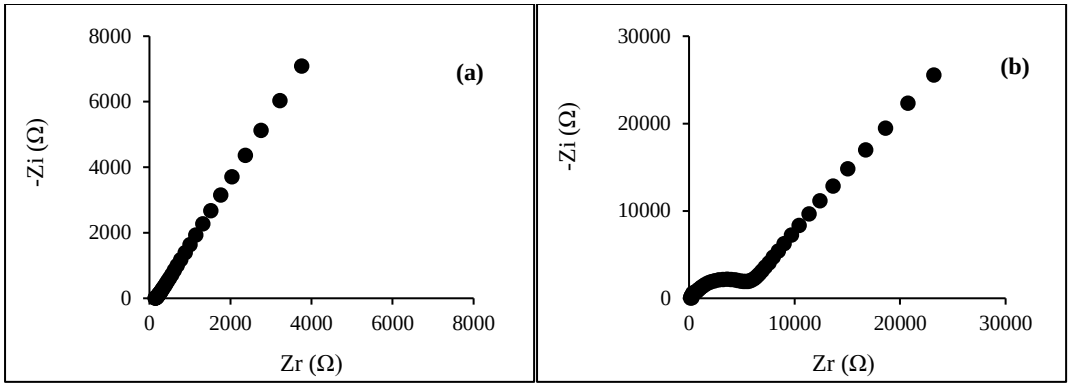


Figure 2. Impedance plot of agarose-based biopolymer electrolyte containing (a) 30 wt.% and (b) 40 wt.% $NaNO_3$ at room temperature.

Table 1. The bulk resistance and conductivity values of respected biopolymer electrolyte

$NaNO_3$ Content (wt. %) in Biopolymer Electrolyte	**Bulk resistance, R_b (Ω)**	**Conductivity, σ ($S \cdot cm^{-1}$)**
0	4.96×10^{7}	1.21×10^{-9}
10	8.33×10^{4}	3.69×10^{-7}
20	2.85×10^{3}	1.67×10^{-5}
30	9.57×10^{1}	3.44×10^{-5}
40	5.58×10^{3}	6.84×10^{-7}

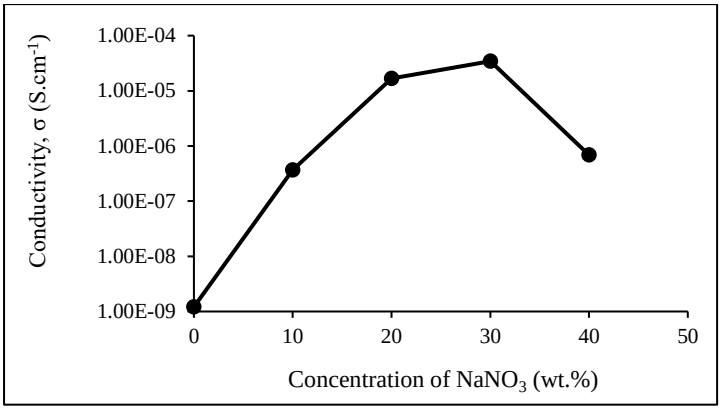


Figure 3. Conductivity plot of agarose-based biopolymer electrolyte containing $NaNO_3$ at room temperature

Temperature dependence conductivity analysis

One of the preliminary indicators for such electrolytes to have good thermal stability is that their ionic conductivity could be measured over a wide range of temperatures. In this work, the temperature dependence of the conductivity of agarose-$NaNO_3$ biopolymer electrolyte was carried out for the highest conducting sample containing 30 wt.% sodium nitrate salt within the temperature range between 303 K and 363 K (Figure 4). The conductivity of polymer electrolyte films is linearly dependent on the temperature, and this relation can be related to the Arrhenius rule as given in the following equation (2), where σ_0 is the pre-exponential factor, k is the Boltzmann constant, and T is the absolute temperature [10].

$$\sigma = \sigma_0 \exp\left(-E_a / k\,T\right) \quad (2)$$

The increase in ionic conductivity of biopolymer electrolytes with temperature can be attributed to the segmental motion of the agarose chain, which provides a higher free volume in the electrolyte upon an increase in temperature. The segmental motion in agarose allows Na^+ ions to hop from one site to another or provides a pathway for its movement. For that reason, it is inferred that the ionic motion in the agarose-$NaNO_3$ biopolymer electrolyte system is due to ion hoping facilitated by the dynamic segmental motion of the agarose. The conductivity amplifies with the temperature. This is due to the fact that the reintegration of ions during the step of solvent desiccation in the formation of thin films is weaker when heat energy is applied to the sample [2]. As a result, when the temperature rises and additional ions become accessible for conduction, it leads to the conductivity being significantly greater [2]. Therefore, at higher temperatures, the amorphous region progressively increases, and agarose chains obtain a faster internal mode in which bond rotations produce segmental motion to favor interchain and intrachain ion hopping. Fortunately, the degree of conductivity becomes appreciable.

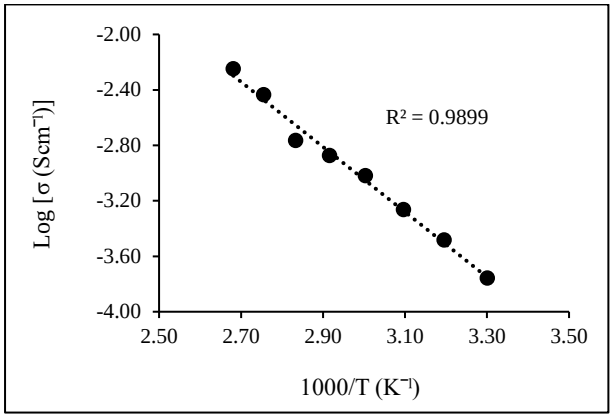


Figure 4. Arrhenius plots for the biopolymer electrolyte containing 30 wt.% of sodium nitrate

Dielectric properties analysis

Their dielectric behaviour is studied to better understand the ionic transport phenomenon in biopolymer electrolyte systems. The dielectric study in electrolyte systems helps us understand the polarization effect of the electrode–electrolyte interface and ionic conduction behaviour. In addition, the dielectric study is favourable due to the contribution of bulk material and interfacial effects at the electrode that are separable. The dielectric constant, ε_r, measures the ability of a material to polarise the amount of dipole alignment in a given volume and correlate with the capacity to store electric charge. Using the impedance data, the real component of complex permittivity is given by equation (3).

$$\varepsilon_r = \frac{Z_i}{\omega C_0 (Z_r^2 + Z_i^2)} \quad (3)$$

Figure 5(a) illustrates the temperature dependence dielectric constant at different frequencies for the highest conducting sample of Agarose-$NaNO_3$ biopolymer electrolyte with 30 wt.% $NaNO_3$ at various temperatures. At low frequencies, the plots rise sharply, indicating that the Na+ ions accumulate at the electrode–electrolyte interface and lead to the formation of an electrical double layer or space charge region in the electrolyte system. Therefore, it contributes to a high dielectric constant value by measuring stored charge directly related to charge carriers. It asserts the non-Debye dependence as the high reversal of the external electric fields [3]. No peak has been seen in the plots of the dielectric constant, which reaffirms that the increase in ionic conductivity is affected by an increase in Na^+ ions density. The increase in dielectric constant supposedly reflects the increase in the density of charge carriers.

A further analysis of the dielectric behavior would be more successfully achieved by using the formulation of

the dielectric modulus. A complex dielectric modulus formalism facilitates the identification and separation of the polarization effect from the bulk relaxation phenomenon in the electrolyte system. The electrical modulus is defined as the [11]:

$$M_r = \frac{\varepsilon_r}{\varepsilon_r{}^2 + \varepsilon_i{}^2} \quad (4)$$

Figure 5(b) demonstrates the real part of electric modulus against log f at different temperatures for the most favorable conductivity sample of Agarose-$NaNO_3$ biopolymer electrolyte containing 30 wt.% of $NaNO_3$. A decreased electric modulus is observed at lower frequencies, linked to the hidden electrode polarization impact. Conversely, at higher frequencies, both the real and imaginary electric modulus show an increase without a clear peak formation, and this rise in values can be attributed to the overall influence of the electrolyte samples. Furthermore, as the temperature rises, the M_r values incline, indicating an increased rate of movement for the charge carriers [2, 4].

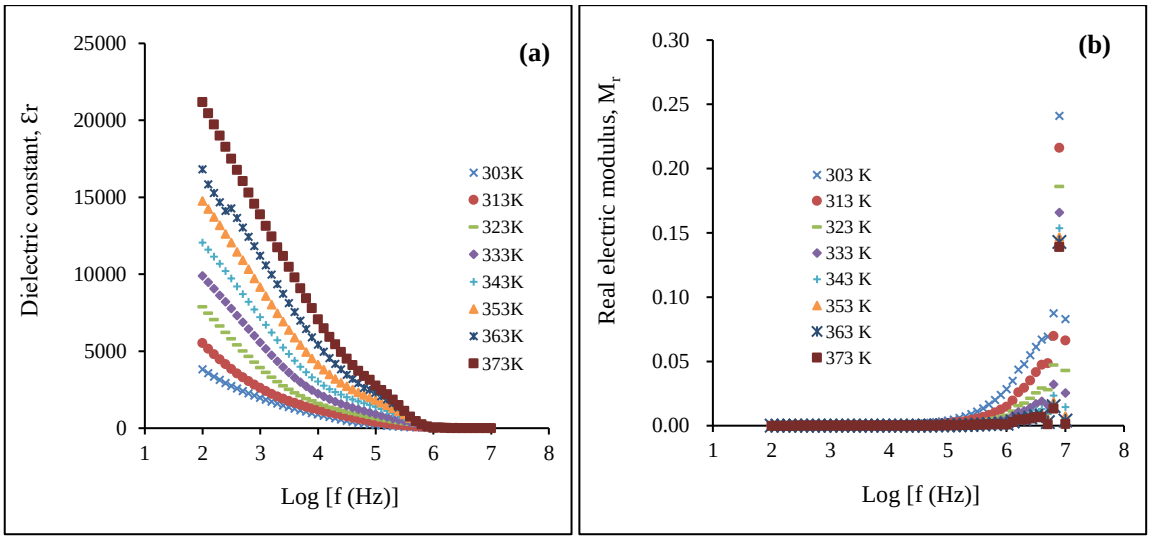


Figure 5. Variation of dielectric constant and real modulus component for optimized Agarose-30 wt.% $NaNO_3$ biopolymer electrolyte as a function of frequencies at various temperatures

The dielectric analysis is further parameterized by the loss tangent (tan $\delta = \varepsilon_i / \varepsilon_r$), and the relaxation time. Relaxation occurs when the loss tangent, tan δ, reaches its peak. The frequency at which the loss tangent, tan δ, reaches its peak is the maximum frequency (relaxation frequency), $f_{max,}$ which is the frequency used to calculate the relaxation time, τ. The relaxation time is calculated using the formula [12]:

$$2\pi f_{max} \tau = 1 \quad (5)$$

Figure 6 shows the plot of loss tangent, tan δ, against the natural logarithm of frequency for selected samples with 20 wt.% $NaNO_3$ best represents the time relaxation trend for the Agarose-$NaNO_3$ biopolymer electrolyte (313 K, 333 K, 353 K and 373 K). It is noticed that the calculated values of relaxation time, as tabulated in Table 2, decrease as the temperature increases. This is caused by the fact that when the temperature rises, ions transition between coordinate sites more quickly, which results in a shorter relaxation time. The results of the relaxation studies in this research correspond with the findings attained by Shetty et al. [13]. Temperature influences the graphs' frequency position and peak intensities, confirming the thermally activated dielectric relaxation process. The relaxation time describes how ionic charge carriers in materials align with the applied field's direction, and at higher temperatures, longer relaxation times are achieved. In high-temperature environments, more mobile ions involved in conduction are generated and undergo relaxation at higher frequencies.

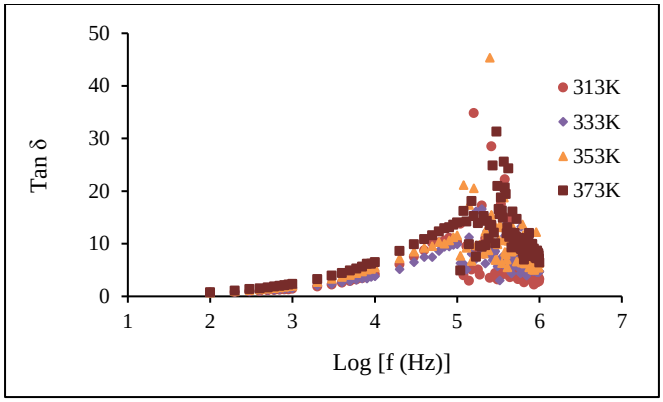


Figure 6. The plot of loss tangent as a function of the logarithm for biopolymer electrolyte with 20 wt.% of $NaNO_3$ at selected temperatures

Table 2. Relaxation time for agarose-$NaNO_3$ biopolymer electrolyte with 20 wt.% of $NaNO_3$

Temperature (K)	Maximum Loss Tangent, tan δ $_{max}$	Maximum Frequency, f $_{max}$ (Hz)	Relaxation Time, τ (s)
313	34.82	5.20	3.06×10^{-2}
333	16.57	5.30	3.00×10^{-2}
353	45.33	5.40	2.95×10^{-2}
373	31.32	5.48	2.91×10^{-2}

Structural properties analysis

The structural or semicrystalline properties of the agarose-$NaNO_3$ biopolymer electrolyte were investigated through XRD analysis. Figure 7(a-c) displays XRD spectra of agarose-$NaNO_3$ biopolymer electrolyte with varying $NaNO_3$ concentrations (0, 30 and 40 wt.%). The presence of amorphous peaks at approximately 2θ = 20° in the absence of $NaNO_3$ (0 wt.%) confirms that agarose exhibits a semicrystalline structure [2]. As more $NaNO_3$ is added to the agarose matrix, the peak broadens, where this broadening indicates an increased amorphous character in the biopolymer electrolyte system, leading to enhanced $NaNO_3$ dissociation into free ions and greater ion migration through the polymer chains. At a 40 wt.% of $NaNO_3$ concentration, the peak narrows compared to the electrolyte with the highest conductivity (30 wt.% $NaNO_3$), suggesting a reduction in the electrolyte's amorphous nature due to the reassociation of ions within the agarose matrix.

The peak was slightly absent in agarose-30 wt.% $NaNO_3$ biopolymer electrolyte indicating the complete dissociation of salts in the electrolytes [8]. The highest conducting sample confirms its characteristic as the most amorphous sample due to the broadest amorphous hump. Elevated amorphous eases the path for the Na^+ ions migration and mobility in the biopolymer electrolyte regions, thus facilitate superior ionic conductivity. A decrease in the degree of crystallinity is also implied by improved amorphousness, which serves as a boost to the ionic conductivity. The dominance of the amorphous region is mathematically confirmed by determining the degree of crystallinity, X_c and crystalline size, L of the electrolyte and evaluated by using equations (6) and (7), respectively [14, 15]:

$$X_c = \frac{A_c}{A_T} \times 100\% \quad (6)$$

$$L = \frac{0.9\,\lambda}{FWHM\,\cos\theta} \quad (7)$$

where A_c is area under the peak of crystalline region while A_T is the total area under the peak, λ is the X-ray wavelength (fixed at 1.5406Å), FWHM is the full width

at half maximum (a measure of the peak broadness) and θ is Bragg's diffraction angle.

In amorphous regions, the polymer chains are less organized, thus making it easier for salt to dissociate into free ions. The detail analysis of degree of crystallinity and crystallite size of 0 wt.%, 30 wt.% and 40 wt.% agarose-$NaNO_3$ biopolymer electrolyte are illustrated in Table 3 and Figure 8. The analysis supports the sample (agarose-30 wt.% $NaNO_3$) which exhibit the broadest XRD peak at 2θ = 20° which also demonstrate its amorphous nature by having the broadest FWHM, lowest degree of crystallinity (16%) and smallest crystallite size (0.0145 nm) among other samples.

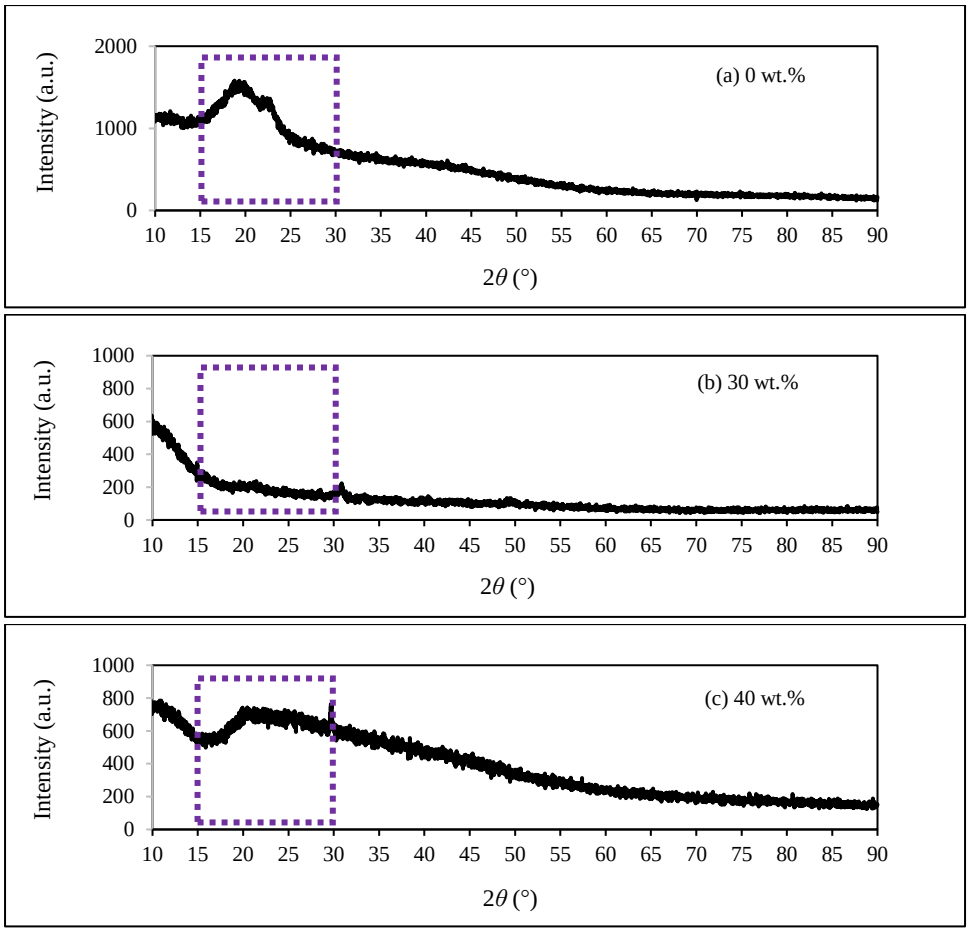


Figure 7. XRD pattern of agarose-$NaNO_3$ biopolymer electrolyte with different concentrations of sodium nitrate

Table 3. The analysis of agarose-$NaNO_3$ biopolymer electrolyte with different concentrations of sodium nitrate

$NaNO_3$ Content (wt.%)	**Full Width Half Maximum (FWHM)**	**Degree of Crystallinity, X_c (%)**	**Crystallite Size, L (nm)**
0	6.70	29	0.0219
30	10.44	16	0.0145
40	9.31	22	0.0161

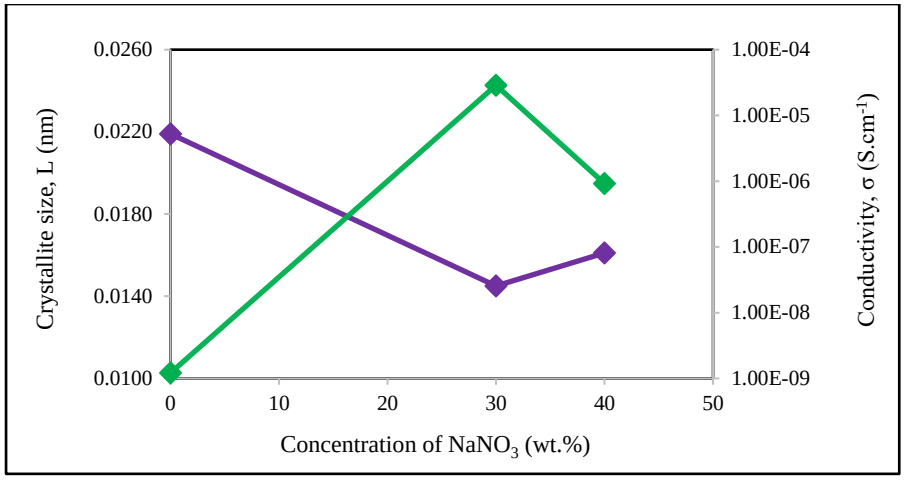


Figure 8. The size of crystallite of selected agarose-$NaNO_3$ biopolymer electrolyte with the respective room temperature conductivity values

Conclusion

In conclusion, Agarose-$NaNO_3$ biopolymer electrolyte was prepared in five different weight percentages of $NaNO_3$ by using the method of solution casting with the weight percentages of 0 wt.%, 10 wt.%, 20 wt.%, 30 wt.% and 40 wt.% $NaNO_3$. Electrochemical Impedance Spectroscopy (EIS) was employed to determine the biopolymer electrolyte's ionic conductivity and electrical properties. In contrast, X-ray diffractometer (XRD) spectroscopy was performed to observe the biopolymer electrolyte's structural behavior and amorphous nature. The conductivity studies at room temperature showed increased conductivity with the increasing sodium salt concentration. The increase in ionic conductivity is due to the increase in the number of charge carriers and the mobility of the sodium ions. The highest room temperature conductivity was 3.44×10^{-5} S·cm^{-1} for the agarose-30 wt.% $NaNO_3$ biopolymer electrolytes and beyond that, the ionic conductivity for agarose-40 wt.% $NaNO_3$ becomes reduced (6.84×10^{-7} S·cm^{-1}) as the aggregation of ions starts to occur. The dielectric properties analysis of the selected biopolymer electrolyte sample indicates a strong dependence on frequency and temperature. The structural properties analysis based on FWHM, degree of crystallinity and crystallite size for agarose-based biopolymer electrolytes confirm that the amorphous regions tend to facilitate higher ion mobility compared to crystalline regions, therefore contributing to higher conductivity, as ions can move more freely through the amorphous parts of the polymer.

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FABRICATION AND CHARACTERIZATION OF WATER-IN-PALM OIL NANOEMULSION AS A CARRIER FOR CATECHIN

(Fabrikasi dan Sifat Nanoemulsi Air dalam Minyak Kelapa Sawit sebagai Pembawa Katekin)

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Abstract

Catechins are known as potent antioxidants with numerous health benefits, including antimicrobial, antiviral, anti-inflammatory, anti-allergenic, and anti-cancer effects. However, there are some challenges to delivering catechins due to their low bioavailability, poor gastrointestinal absorption, and low stability. To overcome the limitations of catechin delivery, nanoemulsion technology with droplet sizes ranging from 10 to 1000 nm was used. In this study, water-in-oil (W/O) nanoemulsion was effectively prepared by employing palm oil (PO) as the oil phase, Span 80 as a lipophilic emulsifier, and Tween 80 as a hydrophilic emulsifier with high-energy approaches (high shear homogenizer). The optimum processing conditions for preparing water-in-palm oil nanoemulsion are as follows: the ratio of the oil phase to the water phase is 60:40, and the total concentrations of emulsifier mixtures are 11 w/w% with a hydrophilic-lipophilic balance (HLB) value of 7.1 using 15,500 rpm for 5 minutes of the high shear homogenizer. The best nanoemulsion showed an average of 185.6 nm particle size and a zeta potential of -38.4 mV. The optimized nanoemulsion containing catechin with a pH value of 6.82, showed a low conductivity value of this nanoemulsion (0.001mS/cm) which means that the continuous phase is oil. The nanoemulsion was stable at centrifugation of 4000rpm for 30 minutes and alongside 28 days of storage tests at 10˚C. Hence, this study successfully developed a stable water-in-palm oil nanoemulsion containing catechins (WPOC-NEs).

Keywords: catechin, nanoemulsion, emulsifiers, formulation, stability

Abstrak

Katekin dikenali sebagai antioksidan kuat yang menawarkan banyak manfaat kesihatan, termasuk kesan antimikrob, antivirus, anti-radang, anti-alergik, dan anti-barah. Walau bagaimanapun, terdapat beberapa cabaran dalam membuat penghantaran katekin disebabkan bioketersediaan mereka yang rendah, penyerapan gastrousus dan kestabilan yang lemah. Untuk mengatasi had penghantaran katekin, teknologi nanoemulsi dengan saiz zarah daripada 10 hingga 1000nm telah digunakan. Dalam kajian ini, nanoemulsi air-dalam-miyak telah disediakan dengan menggunakan minyak kelapa sawit sebagai fasa minyak, Span 80 sebagai pengemulsi lipofilik, dan Tween 80 sebagai pengemulsi hidrofilik dengan pendekatan tenaga tinggi (penghomogenatan ricih tinggi). Keadaan pemprosesan optimum bagi penyediaan nanoemulsi air-dalam-minyak mengandungi katekin adalah seperti

berikut: nisbah fasa minyak kepada fasa air adalah 60:40, dan jumlah kepekatan campuran pengemulsi adalah 11% dengan nilai keseimbangan hidrofilik-lipofilik 7.1 menggunakan penghomogenatan ricih tinggi. Nanoemulsi mengandungi katekin yang terbaik menunjukkan saiz zarah 185.6 nm, dan potensi zeta -38.4mV. Tambahan lagi, nanoemulsi dengan nilai pH 6.82 ini, menunjukkan nilai kondutiviti yang rendah iaitu 0.001mS/cm, yang bermaksud fasa berterusannya adalah minyak. Nanoemulsi ini juga stabil selepas diemparkan pada 4000rpm selama 30 minit dan juga sepanjang ujian simpanan selama 28 hari di suhu 10°C. Oleh itu, kajian ini telah berjaya membangunkan nanoemulsi air-dalam-minyak dengan mengandungi katekin yang stabil.

Kata kunci: katekin, nanoemulsi, pengemulsi, formulasi, kestabilan

Introduction

Catechin is a polyphenol compound found in various fruits, vegetables, and plant-based beverages. Catechins have sparked much interest because of their antiviral, anticancer, antioxidant, antimicrobial, anti-inflammatory, and possible medicinal applications in various viral infections [1]. Catechins have been studied extensively in terms of their biological functions. However, the bioavailability of catechins in vivo has remained limited due to their volatility. As a result of the creation of microemulsions (MEs) or nanoemulsions (NEs) for encapsulation of bioactive chemicals like catechin, it is feasible to improve stability, hence increasing catechin bioavailability and biological activity [2]. Bhushani et al. [3] reported that nanoemulsified catechin can increase their bioaccessibility compared to non-emulsified catechin.

A nanoemulsion is a kinetically stable system composed of two immiscible liquids (oil and water) that are stabilized by surfactants with droplet sizes ranging from 10 to 1000 nm [4]. The advantages of nanoemulsions (NEs) are numerous. Due to a higher surface area, they are suitable for drug administration. This could boost drug absorption through a variety of pathways [5]. A wide surface area also has an impact on the drug's transport characteristics, which is a critical component in ensuring long-term and targeted drug delivery. Drugs are protected from environmental changes, such as pH shifts and enzymatic breakdown, by being encapsulated within the nanoemulsion core [6].

Nanoemulsions consist of two types known as oil-in-water (O/W), where the continuous phase is water, and water-in-oil (W/O), where oil is the continuous phase. W/O nanoemulsion has great potential for pharmaceutical, cosmetic, agricultural, and food applications. This type of nanoemulsion can encapsulate the hydrophilic drugs in the inner water phase by improving the effect of sustained release, maintaining the retention time of the drug in the body, and lowering the metabolic rate [7]. This research applied high-energy emulsification approaches such as high-shear homogenizers to obtain nanodroplet emulsions. The emulsion will be forced into the narrow space between the rotor and stator, causing the droplets to break down and form smaller particles [8].

Overall, this research study aimed to encapsulate the catechin within the water in palm oil nanoemulsion to improve their bioavailability after oral administration or for the fabrication of topical application. Therefore, to form a stable W/O nanoemulsion, the choice of oil and surfactants is essential. Palm oil was chosen in this study since it can help to overcome the issue of low bioavailability, high toxicity, and poor solubility of several drugs, as well as improved drug permeability and shelf life [9]. Non-ionic surfactants were used as they can form a stable nanoemulsion and have a low toxicity potential that helps to reduce irritation [10]. The optimal hydrophilic-lipophilic balance (HLB) surfactant mixtures were first determined to obtain stable water-in-palm oil nanoemulsions containing catechin (WPOC-NEs). Then, the stable nanoemulsions obtained were characterized, including their droplet size, polydispersity index (PDI), zeta potential, and conductivity. To our knowledge, no work has been done on water-in-palm oil nanoemulsion containing catechin.

Materials and Methods

Materials

Catechin Hydrate was purchased from Sigma-Aldrich Co. (St. Louis, MO). Tween 80 and Span 80 were purchased from Chemiz (M) Sdn.Bhd. Palm oil was purchased from a local market. Distilled water was used

to prepare the water-in-palm oil nanoemulsion containing catechin.

Preparation of water-in-oil nanoemulsion

Water-in-palm oil (W/PO) nanoemulsion was prepared according to the method implied by Qonita et al. [11] and Baba Shekh et al. [12] with some modifications. The aqueous phase consisted of distilled water, Tween 80, Span 80, and catechin, which were mixed and stirred using a hotplate stirrer (Model HTS-1003, AB Labmart Malaysia) at 1200 rpm for 10 min. Then, the oil phase was added dropwise into the aqueous phase and homogenized at 15,500 rpm for 5 minutes using a high-shear homogenizer (T25 digital; IKA-Werk, GmbH & Co. KG, Staufen im Breisgau, Germany). All the procedures were standardized, that is (i) 36% water, 11% total surfactants mixture, 0.1% catechin, and 54% palm oil, and (ii) all procedures were performed at room temperature.

Selection of hydrophilic-lipophilic balance (HLB) of mixed surfactants

A range of 4.3-7.1 HLB values was used to prepare water in palm oil nanoemulsion for the HLB value determination. In all samples, the water-to-oil content ratio was maintained constant (40/60, w/w%). The surfactant consists of the mixtures Span 80 and Tween 80. The following calculation calculated calculation of HLB values of mixed surfactants:

$$HLB_{mix} = F_T\, HLB_T + F_S\, HLB_S, \quad (1)$$

where $HLB_{mix,}$ HLB_T, and HLB_S are the HLB values of mixed surfactants, Tween 80 (T80, HLB =15) and Span 80 (S80, HLB=4.3), while F_T and F_S are the weight fractions of Tween 80 and Span 80. All standard conditions were kept constant to prepare the nanoemulsions which were then centrifuged to evaluate their stability. Based on the calculation using Equation (1), a suitable HLB value was investigated until a stable W/PO nanoemulsion containing catechin was obtained for this study.

Fourier transform infrared spectroscopy (FTIR) – based attenuated total reflection (ATR)

Functional groups and chemical structures in the composition of the pure palm oil, span 80, tween 80, catechin, and water in palm oil nanoemulsion containing catechin were evaluated using a Perkin Elmer Fourier Transform Infrared, FTIR-based ATR (Spectrum 400 Perkin Elmer, Waltham, MA, USA) spectrometer in the range of 400 to 4000 cm^{-1}. All the samples were analyzed in their original forms without any kind of preparation.

Droplet size, zeta potential, polydispersity index, conductivity determination, and transmission electron microscopy (TEM) image

The most significant parameter for this study is the measurement of droplet size. The smaller the particle size, the bigger the surface area of the absorption of the drug. The droplet size, zeta potential, conductivity, and polydispersity index of WPOC-NEs were measured using dynamic light scattering, which scattered at an angle of 173˚ and a temperature of 25˚C. This procedure used a droplet-size analyzer (Zetasizer Nano ZS90; Malvern Instruments, Malvern, UK). To certify that the system has reached equilibrium, the droplet size was measured a day after the formulations were made [13].

The TEM image of the WPOC-NEs was obtained using Transmission Electron Microscopy (TEM) (Tecnai G2 Spirit BioTwin). Samples were diluted with deionized water and homogenized. A Formvar-coated copper grid was placed on top of a drop of diluted sample and left at room temperature (25 ± 0.5˚C) for 3 min. The samples on the filled copper grid were then negatively stained using 2% w/w phosphotungstic acid for 2 min and were air-dried before analysis [14].

pH determination

An S20 SevenEasy pH meter (Mettler Toledo, Schwerzenbach, Switzerland) was used to measure the pH of the optimized nanoemulsion containing catechin. The pH meter was calibrated with three pH standard buffer solutions (pH 4.00, 7.00, and 10.00) before taking pH measurements. The pH value was obtained by inserting the electrode directly into the sample.

Viscosity determination

A rotational Physica MCR 300 rheometer (Physica Messtechnic GmbH, Stuttgart, Germany) was used to

determine the viscosity of the optimized nanoemulsion containing catechin, with PP25 plate and plate geometries (gap of 1mm) at 25ºC. A controlled Peltier system (TEK150) with an accuracy of ± 0.1°C was used. The experimental data were fitted to the Power Law Equation:

$$\eta = k\dot{\gamma}^{n-1} \quad (2)$$

Based on the above equation, η is the viscosity (Pa.s), ý is the shear rate (s^{-1}), and k and n are the consistency index and flow behavior index, respectively.

Accelerated stability tests

The stability of nanoemulsions is characterized by their capacity to maintain their physical appearance (in a certain system) without phase separation or physical changes during a specific time of storage and use [15]. The optimized nanoemulsion containing catechin was submitted to two stability tests, namely, the centrifugation test and storage stability at different temperatures (10, 25, 50°C) for 28 days. If the visible appearance of creaming or phase separation occurred, the nanoemulsion containing catechin was said to be unstable.

The centrifugation test was performed using a SIGMA 2-16P centrifuge. The nanoemulsion containing catechin was placed into a centrifugation tube and then centrifuged at 4000 rpm for 30 min. The optimized nanoemulsion was then observed for the presence or absence of phase separation.

The storage stability test was performed at different temperatures and the optimized nanoemulsion was carried out by observing the changes in physical appearance on days 1,7,14, and 28.

Results and Discussion

Formulation and screening of water-in-oil nanoemulsion

Table 1 tabulates the W/PO nanoemulsion containing catechin. This study aimed to encapsulate catechin into water in palm oil nanoemulsion. Water-in-oil nanoemulsions comprise a dispersed aqueous phase with tiny droplets and a continuous oil phase. Oil-in-water nanoemulsions, on the other hand, are the dispersed phase disseminated into the continuous water phase [16]. As reported by Qonita et al. [11], the percentage of oil phase used was more than 50%, to form W/O nanoemulsions which was used in this study.

Tween 80 (T80) and Span 80 (S80), both non-ionic surfactants, were chosen in this study due to their strong biological compatibility, insensitivity to changes in electrolyte concentration or pH, and their established safety and approval for inclusion in specific pharmaceuticals, cosmetics, and food products [12].

Selection of hydrophilic-lipophilic balance (HLB) value of mixed surfactants

An acceptable HLB value of the surfactant utilized in the formulation is one of the primary driving forces for stable nanoemulsions. As it reflects the ideal mixture of surfactants that can produce stable nanoemulsions, it serves as a scale for choosing the suitable surfactants or mixtures of surfactants that are necessary for the oil and water phase [17]. The HLB system predicts the ideal emulsifier when the HLB values of the emulsifier and oil are matched. Well-known non-ionic and ethoxylated sorbitan alkanoates, which are Spans and Tweens, had a wide range of HLB values that helped adjust the HLB [18].

Table 1 shows the result surfactant mixtures (Tween 80/Span 80) of 26.2/73.8 (HLB 7.1) were suitable for producing W/PO nanoemulsion containing catechin, while others show separation. This HLB value did not show any physical separation during centrifugation, supporting the conclusion that the HLB 7.1 of the surfactant mixtures effectively stabilized the W/O nanoemulsion. The selected HLB range (4-9) covers water in oil and wettability agents in the classical HLB scale and was experimentally proven as an effective stabilizer for W/O systems [19].

Table 1. Composition of the water in palm oil nanoemulsion containing catechin for HLB screening

Composition						Observation
Surfactants(w/w%)		HLB	Palm Oil (w/w%)	Distilled Water (w/w%)	Catechin (w/w%)	Centrifugation
Tween 80	Span 80					
0.00	11.00	4.30	60.00	40.00	0.10	X
0.50	10.50	4.70	60.00	40.00	0.10	X
1.00	10.00	5.20	60.00	40.00	0.10	X
1.60	9.40	5.70	60.00	40.00	0.10	X
2.10	8.90	6.10	60.00	40.00	0.10	X
2.70	8.60	6.80	60.00	40.00	0.10	X
2.90	8.10	7.10	60.00	40.00	0.10	/

/ = stable or no physical change X = not stable and physical changes occur

ATR analysis based on FTIR

Figure 1 and Table 2 present the FTIR spectrophotometer and wavenumbers of palm oil (PO), Tween 80, Span 80, catechin, and water in palm oil nanoemulsion containing catechin (WPOC-NEs). FTIR analysis was performed to determine whether catechin was present in the water in palm oil nanoemulsion containing catechin. Figure 1(a), displayed a carbonyl (C=O) stretching peak at 1743.7 cm^{-1} and C–H stretching of alkyl chains at 2854.6 and 2922.9 cm^{-1}, which corresponds to the molecular structure of palm oil [20]. FTIR transmittance spectrum of surfactant Tween 80 was depicted in Figure 1(b) where a broad peak at 3493.3cm^{-1} indicates the presence of the O-H group. The aliphatic CH_2 symmetric and asymmetric stretching vibration is responsible for the 2920.1-2862.9 cm^{-1} band. In the domain 1735.8 cm^{-1}, the presence of C=O stretching methods may be seen. The band from 1349.7 to 1247.4 cm^{-1} is due to C-H bending, whereas the peak at 1097.5 cm^{-1} is attributed to C-O-C in $(CH_2CH_2O)n$.

Figure 1(c) indicates the spectrum of pure Span 80. The OH group is allocated to the peaks at 3410.7 cm-1. It should be noted that the absorption peaks at 1738.8 cm^{-1}, and 1458.8 cm^{-1} are due to C=O and C-H bending in CH_3 and CH_2 [21]. Characteristic IR peaks of catechin in Figure 1(d) were observed at 3355.3 (phenolic hydroxyl group), 1640.3, 1461.2 (phenyl ring), 1155.4, and 1095.8 (stretching vibration of C-O-C) cm^{-1} [22]. From Figure 1(e), the WPOC-NEs spectrum shows all of the distinctive peaks of catechin were attained by WPOC-NEs. The catechin distinctive peak within the WPOC-NEs was observed at 3356.3 (phenolic hydroxyl group), 1640.0, 1460.5 (phenyl ring), 1159.5, and 1096.8 (stretching vibration of C-O-C) cm^{-1}. Based on these findings, it was determined that catechin was present within the core of water in palm oil nanoemulsion containing catechin.

Table 2. Wavenumbers of IR of palm oil, tween 80, span 80, catechin, and W/PO NEs containing catechin

Sample	Wavenumbers (cm^{-1})				
	O-H Stretching	C-H Stretching	C=O Stretching	Phenyl Ring	C-O-C Stretching
Palm Oil	-	2854.6	1743.7	-	1115.3
Tween 80	3493.3	2920.1	1735.8	-	1097.5
Span 80	3410.7	2923.3	1738.8	-	1085.5
Catechin	3355.3	2925.6	-	1640.3	1095.8
WPOC-NEs	3356.3	2925.2	1741.7	1640.0	1159.5

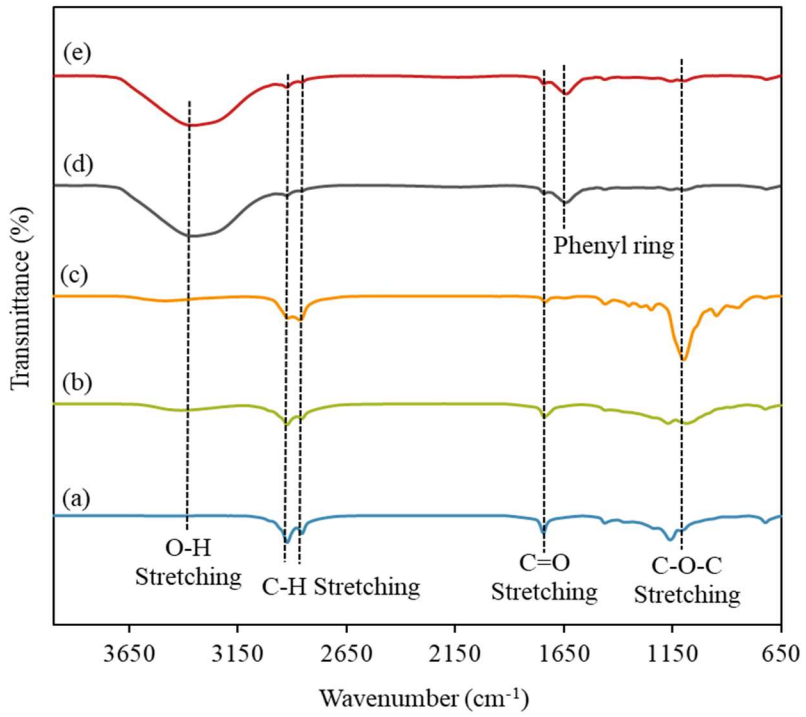


Figure 1. FTIR Spectra of (a)palm oil, (b)tween 80, (c)span 80, (d)catechin, and (e)WPOC-NEs

Droplet size, zeta potential, polydispersity index, conductivity determination, and transmission electron microscopy (TEM) image

The W/PO nanoemulsion containing catechin had mean droplet size, zeta potential, polydispersity index (PDI), and conductivity values of 185.60 nm, -38.4 mV, 0.251, and 0.001 mS/cm, respectively. The droplet size of a nanoemulsion ranging from 20 to 200 nm is substantially smaller than that of conventional colloidal systems. The small droplet size results in a larger colloidal surface area, which increases solubility and the rate of dissolution [23]. The zeta potential, which assesses a particle's electrokinetic potential, was utilized to determine the stability of the nanoemulsion. Readings higher than +30mV or lower than –30mV were said to be stable nanoemulsion [24]. The negatively charged zeta potential was caused by the polyoxyethylene chains in Tween 80, indicating sufficient Van der Waals electrostatic repulsions to stabilize and disperse the w/o system for an extended time [12].

The PDI value was used to examine the particle size distribution in the nanoemulsion. A PDI value close to zero implies a monodispersed system, whereas a PDI value close to one indicates a polydispersed system (a wide range of droplet sizes). Pongsumpun et al.[25] reported that a nanoemulsion system is termed monodispersed (with a restricted size distribution) if the PDI value is less than 0.3. Therefore, in this study, the optimized water in palm oil nanoemulsion containing catechin was found to be stable with a PDI value of 0.251, which conveys it is monodispersed (Figure 2) with a negative zeta potential value.

Conductivity is the measurement of the free amount of water and ions in a solution, as it will respond to any ions present. This parameter aids in determining the type of nanoemulsion produced [26]. The optimized nanoemulsion containing catechin had a low conductivity value of 0.001 mS/cm, indicating that the oil phase was a continuous phase and that the nanoemulsion created was a water-in-oil (W/O) nanoemulsion.

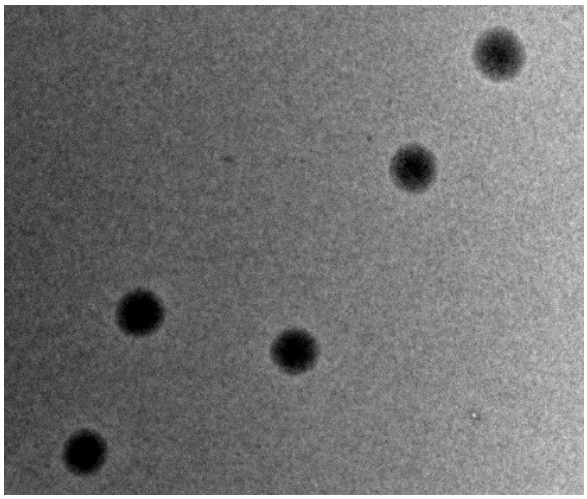


Figure 2. TEM image of WPOC-NEs

pH determination

The pH of the optimized W/PO nanoemulsion containing catechin was 6.82. These features are suitable for pharmaceutical applications, such as topical [27] and oral [28] applications.

Viscosity determination

A steady-state test was used to investigate the behavior of the W/PO nanoemulsion containing catechin. In the steady-state test, the W/PO nanoemulsion containing catechin displayed shear-thinning behavior (non-linear relationship), as shown in Figure 3. The flow behavior index (n) based on Equation (2) can be used to determine the rheological parameters of the W/PO nanoemulsion, as illustrated in Table 3. The value of n is 0.269 (n<1), indicating that the W/PO nanoemulsion was shear-thinning. The W/PO nanoemulsion's viscosity decreased with increasing shear rate, demonstrating that the W/PO nanoemulsion is a non-Newtonian pseudoplastic fluid (n<1). The non-Newtonian pseudo-plastic behavioral features of the W/PO nanoemulsion containing catechin are shown in Table 3. Notably, this type of behavior seen here matches the majority of over-the-counter pharmaceutical applications.

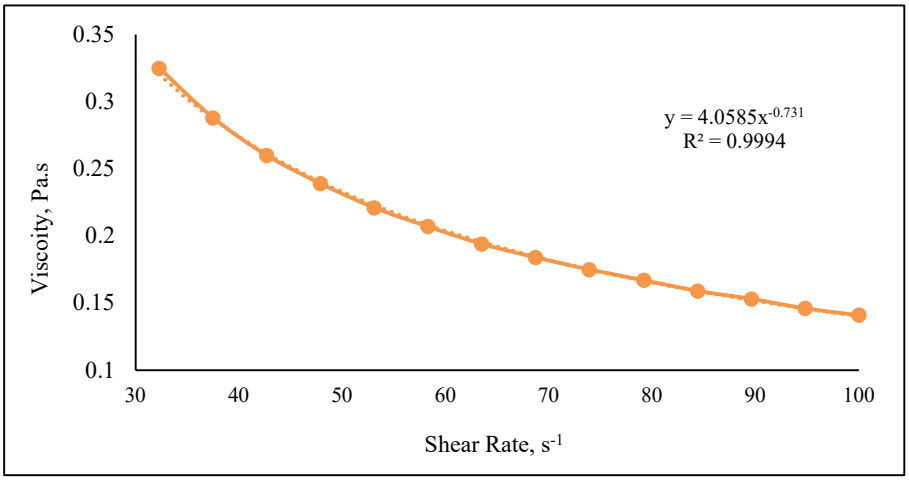


Figure 3. Graph of viscosity (Pa.s) versus shear rate (s^{-1})

Table 3. Flow behavior indices (n), consistency coefficients (k), and regression coefficients (R^2) of the water in palm oil nanoemulsion containing catechin

Sample	k	n	R^2
W/PO nanoemulsion containing catechin	4.0585	0.269	0.9994

Stability tests

For the centrifugation test, the W/PO nanoemulsion containing catechin showed no phase separation after centrifuging at 4000 rpm for 30 min. This indicates the formulation's remarkable stability against creaming or sedimentation and its expected long shelf life.

A storage stability study was conducted by exposing the W/PO nanoemulsion to three different temperatures (10, 25, 50 ℃) for 28 days. Table 4 denotes the physical stability of W/PO nanoemulsion containing catechin under three different storage temperatures. Based on the observations, the optimized nanoemulsion at 10 ℃ remained a homogeneous mixture and exhibited no physical or phase changes for 28 days. This shows that the W/PO nanoemulsion is stable at low temperatures.

However, at 25 and 50 ℃, the W/PO nanoemulsion was unstable after seven days and exhibited physical changes. This is due to the polyoxyethylene head groups of non-ionic surfactants of Tween 80 being known to be dehydrated when exposed to high storage temperatures. This alters the optimal curvature of the surfactant monolayer and reduces the range of steric repulsion, leading to instability [29].

Table 4. Physical stability of water in palm oil nanoemulsion containing catechin under different storage temperatures (10, 25, 50 ℃)

Storage Temperature (℃)	Storage Stability (days)				
	1	7	14	21	28
10	/	/	/	/	/
25	/	X	X	X	X
50	/	X	X	X	X

/ = stable or no physical change X = unstable and physical changes occur

Conclusion

This study successfully developed a stable water in palm oil nanoemulsion containing catechin with 54% palm oil, a ratio of Tween 80: Span 80 (26.2:73.8) with HLB 7.1 and 0.10% of catechin. The physicochemical characterization indicated that the W/PO nanoemulsion containing catechin was in the nanosize range, had a zeta potential of -38.4mV, and a PDI of 0.251, showing that the nanoemulsion produced was stable and monodispersed. The TEM image obtained showed that the monodispersity of the WPOC-NEs was correlated with the PDI value. The pH of the nanoemulsion was 6.82, which was within the acceptable pH range for pharmaceutical products and its conductivity was low, indicating the developed nanoemulsion was a water-in-oil nanoemulsion type. The nanoemulsion produced remained stable and had no phase separation under centrifuge test and during storage at 10℃ over 28 days. These results show that it will be helpful for rationalizing design of water-in-oil nanoemulsion-based delivery systems to encapsulate catechin in food, beverage, cosmetic, and pharmaceutical products.

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SCREENING OF PROMOTED CeO_2/Al_2O_3 CATALYSTS IN AQUEOUS PHASE GLYCEROL REFORMING AND HYDROGENOLYSIS INTO 1,2-PROPANEDIOL

(Penyaringan Mangkin Penggalak CeO_2/Al_2O_3 dalam Fasa Akueus Pembaharuan dan Hidrogenolisis Gliserol Kepada 1,2-Propanadiol)

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Abstract

A series of promoted CeO_2/Al_2O_3 catalysts (10Cu-90CeO_2/Al_2O_3, 10Ni-90CeO_2/Al_2O_3, 10Co-90CeO_2/Al_2O_3) were synthesized via method of impregnation and later calcined at 600 °C. Those catalysts were formerly tested for their physico-chemical properties by X-ray diffraction (XRD), H_2-temperature programmed reduction (H_2-TPR), and NH_3-temperature programmed desorption (NH_3–TPD). After characterized, it was then evaluated in the performance of catalytic glycerol conversion into 1,2-propanediol; propylene glycol (1,2-PDO; PG) via aqueous phase glycerol reforming and hydrogenolysis route under inert N_2 flow. Among the examined catalysts, CeO_2/Al_2O_3 with 10wt% Cu loading (10Cu-90CeO_2/Al_2O_3) showed optimum catalytic activity with 88.5% glycerol conversion (GC) and 35.5% PG selectivity at 300 °C reaction temperature, 2 h duration test, 30 cc/min of N_2 initial pressure, 0.1 g catalyst dosage and 10wt% glycerol concentration. The high catalytic performance of 10Cu-90CeO_2/Al_2O_3 was owing to the good copper-cerium-alumina interaction via its good metal reducibility at low temperature along with good acid capacity for the reaction.

Keywords: copper, cerium, alumina, acid capacity, glycerol conversion

Abstrak

Satu siri mangkin penggalak CeO_2/Al_2O_3 (10Cu-90CeO_2/Al_2O_3, 10Ni-90CeO_2/Al_2O_3, 10Co-90CeO_2/Al_2O_3) telah disintesis menggunakan kaedah pegisitepuan lalu dikalsinkan pada 600 °C. Kesemua mangkin telah dikaji sifat fiziko-kimia dengan menggunakan pembelauan sinar-X (XRD), penuruan suhu terprogram H_2 (H_2-TPR), dan nyahjerapan suhu terprogram NH_3 (NH_3-

TPD). Ia kemudian dinilai dalam penukaran pemangkinan gliserol kepada 1,2-propanadiol; propilena glikol (1,2-PDO; PG) melalui pendekatan pembaharuan dan hidrogenolisis fasa akueus dibawah aliran N_2. Diantara mangkin yang diuji, CeO_2/Al_2O_3 dengan 10wt% muatan Cu ($10Cu\text{-}90CeO_2/Al_2O_3$) telah menunjukkan aktiviti pemangkinan yang optimum dengan 88.5% penukaran gliserol (GC) dan 35.5% keterpilihan PG pada suhu tindak balas 300 °C, tempoh masa 2 jam, 30 cc/min tekanan awal N_2 dan 0.1 g dos mangkin dan 10wt% kepekatan gliserol. Prestasi pemangkinan yang tinggi oleh $10Cu\text{-}90CeO_2/Al_2O_3$ didorong oleh interaksi baik antara kuprum-ceria-alumina melalui penurunan logam yang baik pada suhu rendah dan kapasiti asid yang sesuai untuk tindak balas.

Kata kunci: kuprum, serium, alumina, kapasiti asid, penukaran gliserol

Introduction

The amount of glycerol produced as primary by-product of biodiesel worldwide is far exceeds the current market demand thus resulted in a surplus of crude glycerol, which then impacted the refined glycerol market. It was expected that the global crude glycerol market to provide approximately 680,000 tonnes by 2024 [1-2]. Therefore, one of the opportunities for valorising crude glycerol is by recycling and producing a demanded value-added product from it which is propylene glycol (PG) or 1,2-propanediol (1,2-PDO). This product is essentially used as an industrial solvent, intermediate chemical or multipurpose solvent, additive in food products, unsaturated polyester resins, pharmaceuticals, and cosmetics [3-4]. At present, PG is industrially produced by hydrating propylene oxide to 1,2-PDO from the oxidised propylene via petrochemical route [5-6]. With diminishing returns from petroleum and concerns over detrimental environmental effects on the rise, methods of synthesising PG from renewable sources would significantly desired. Therefore, to cope with uprising production and recycling glycerol from industry, catalytic aqueous phase hydrogenolysis (APH) intertwined with aqueous phase reforming (APR) of glycerol are among the routes that was opted as they can be operated at the same reaction conditions. By coupling both reactions, no external supply of hydrogen is required since in situ generated hydrogen obtained from glycerol reforming process is being utilised in same the reactor. This made the aqueous glycerol hydrogenolysis via reforming route as an alternative to the conventional hydrogenolysis since it can be run even at mild pressure with an inert gas environment without being equipped with external source of hydrogen to the reaction system [7]. However, it could not be denied that conventional hydrogenolysis could offer better product selectivity than the APR approach since the molecular hydrogen supplied could effectively react with the glycerol to give the desired product within low hydrogen pressure [4,6,10].

During aqueous hydrogenolysis process, glycerol is dehydrated and the primary C-OH bonds is cleaved over acid sites to form unstable intermediates such as acetol and it was further hydrogenated by which hydrogen atom was added to acetol over metal sites catalyst to give desired APH product which is propylene glycol [8]. However, formation of lower alcohol such as methanol as side reaction products could happen in the reaction due to the excess C-C cracking of the intermediate acetol [9]. Cerium oxide catalysts have been given more attention due to their effectiveness in various catalytic and hydrogenation reaction used as support, co-catalyst or promoter attributable to its good acid capacity as well as metal reducibility [7,10-11]. As well, a modification of cerium oxide as based-catalyst with promoter/co-catalyst such as Cu and Ni have been reported to improve and strengthen the metal-metal interactions and improved the catalytic performance via their chemical strength, acidity, metallic phase dispersion and reducibility (oxygen ion mobility) [12]. Therefore, the good features of a promoted CeO_2 catalyst would possibly enhance and promote efficiency towards primary C-OH bond cleavage of glycerol which eventually rearranged to activate/break the C=O bond of acetol before it undergoes hydrogenation in a second step to give PG product [13]. Furthermore, Al_2O_3 with high availability, unique acid properties and strong metal-support interaction has been selected as catalyst support to facilitate adequate surface area to allow active phase deposition, prevent sintering and therefore offering hydrogen spill medium for it to moves towards metal surface during reaction. Hence both metal and acid sites surface are the active sites for the reaction. The

performance of a series of promoted CeO_2/Al_2O_3 catalysts were tested in the glycerol conversion to produce PG under 300 ℃ reaction temperature, 2 h reaction time, 30 cc/min of N_2 pressure, 10wt% glycerol concentration, and 0.1 g catalyst dosage to relate the role of catalyst's metal reducibility and acid capacity in the reaction performance.

Materials and Methods

Chemicals

Aluminium oxide beads, Al_2O_3 (3mm) and cerium(II) nitrate hexahydrate ($Ce(NO_3)_2.6H_2O$) with 99% purity was supplied by Sigma-Aldrich. Copper(II) nitrate trihydrate, $Cu(NO_3)_2.3H_2O$ (≥99%) (MW: 241.60 g/mol), nickel(II) nitrate hexahydrate ($Ni(NO_3)_2.6H_2O$) (≥99%) (MW: 290.79 g/mol), and cobalt(II) nitrate hexahydrate ($Co(NO_3)_2.6H_2O$) (≥99%) (MW: 291.03 g/mol) were obtained from R&M Chemicals. Glycerol (≥99.5%) and N-methyl-N (trimethylsilyl)trifluoroacetamide (MSTFA) (≥98.5%) used as derivatizing agent GC derivatization was from Sigma Aldrich while pyridine (C_5H_5N) as binder solvent in derivitization process was from Merck. All chemicals used in this study were of either analytical or of reagent grade.

Preparation of catalyst

The catalysts were prepared by impregnation method. Prior to preparation, monometallic cerium was prepared first using 5g of precursor $Ce(NO_3)_2.6H_2O$ which was discretely dissolved and stirred homogeneously in 5 mL of distilled water. The solution was then added in 5g alumina beads (Al_2O_3) (referred as solution 1). The mixture was then stirred, left dried in drying oven for 24 hours at 90 °C, and calcined at 600 °C for 3 hours in a tube furnace with static air and a ramping rate of 10 ºC/min. The synthesised catalyst was denoted as CeO_2/Al_2O_3 catalyst. For preparing promoted CeO_2/Al_2O_3 catalyst, solution 1 was added with a proper amount of $Cu(NO_3)_2.3H_2O$ at 10wt% copper loading. After which, the similar method as indicated in preparation of Ce/Al_2O_3 was followed. The promoted catalyst was named as $10Cu\text{-}90CeO_2/Al_2O_3$ catalyst. Other promoted catalysts were prepared using similar method indicated by $10Cu\text{-}90CeO_2/Al_2O_3$.

Characterisation of catalyst

The phase composition structure and crystallite size of the catalysts were measured using X-ray diffraction (XRD) using Shimadzu diffractometer model XRD-6000. Hydrogen-temperature programmed reduction (H_2-TPR) was used to evaluate metal reducibility using a Thermo-Finnigan TPD/R/O 1100 SERIES equipped with a TCD (thermal conductivity detector). Typically, catalyst sample of 0.05g was placed in a U-shaped quartz reactor and pre-treated in flowing N_2 ($30cm^3$/min) for 0.5 h at 150 ºC to remove any moisture content followed by cooling it until room temperature. After pretreatment, H_2 chemisorption analysis was performed from temperature 50 ºC to 1000 ºC at a rate 10 ºC/min under $5\%H_2$/He flow ($30cm^3$/min). For determination acid sites of the catalyst, NH_3-temperature programmed desorption NH_3-TPD was used. The experiments were conducted on the same apparatus as the TPR experiments described above. The analysis that was conducted in this present study has already been explained in great detail in our previous study [14].

Set up for experiment

The catalytic reaction was carried out in a mechanically stirred 100 mL two-necked round bottom flask connected to N_2 gas flow (30 cc per min) of semi-batch reactor. In a typical run, 10 wt.% glycerol and 0.1g of catalyst was added into the reactor. Prior to reaction experiment, the reactor contained aqueous mixture was vacuumed and purged with N_2 for 10 min to remove the air inside. The reaction was then performed at 300 ºC and 2 h reaction time. Liquid product from the reaction was collected from the released of vapour species which then undergo condensation using external water-cooling circulator. Once reaction duration was completed, the reactor was cooled down to room temperature before the liquid product was collected and filtered from present catalyst upon analysis.

Product analysis

The liquid product of the experiment was analysed using a gas chromatography–mass spectrometry (GC-MS) coupled with an HP-5 capillary column after it had been extracted thrice using ethyl acetate. Prior for the sample preparation, the liquid product was derivatized with silyl

agent of N-Methyl-N-(trimethylsilyl)trifluroacetamide (MSTFA) and was then dissolved in pyridine (C_5H_5N) as binding solvent at 1:1:1 ratio followed by 30 min heating in oven at 70 ºC to allow complete bonding of silylation reaction. Next, 1 µL sample was injected into the GC inlet at 250 ºC and helium gas was served as the carrier gas. The initial temperature of the oven was set at 40 ºC and held for 6 min, then ramped up to 270 ºC with heating rate of 7ºC min^{-1}. The glycerol conversion and selectivity were determined by comparing the retention time of standard with experimental-based products.

Results and Discussion

Figure 1 shows the XRD analysis of CeO_2/Al_2O_3 and promoted CeO_2/Al_2O_3 catalysts (10Cu-90CeO_2/Al_2O_3, 10Ni-90CeO_2/Al_2O_3, 10Co-90CeO_2/Al_2O_3). As seen in Figure 1, for all catalysts, the intensity of each peak is hardly distinguished could possibly be due to the amorphous character of alumina present in the catalyst. Broad diffraction peaks of CeO_2 (2θ = 29.2º, 32.5º, 48.2º and 57.3º) and Al_2O_3 (2θ = 32º, 38.1º, 47º, 57º and 67.2º) phases were observed with low crystallinity in all catalysts. For promoted catalysts, formation of CuO, NiO and CoO peaks at was detected with the similar peak of CeO_2 and/or Al_2O_3 peak respectively as the oxides could be getting embedded inside the bulk of CeO_2/Al_2O_3 and hard to be distinguished. From Table 1, it was observed that the addition of metal species on CeO_2/Al_2O_3 increased the bulk metal's crystallite of CeO_2 at 2θ = 29.2º due to the metal species filled the interstitial spaces of bulk CeO_2.

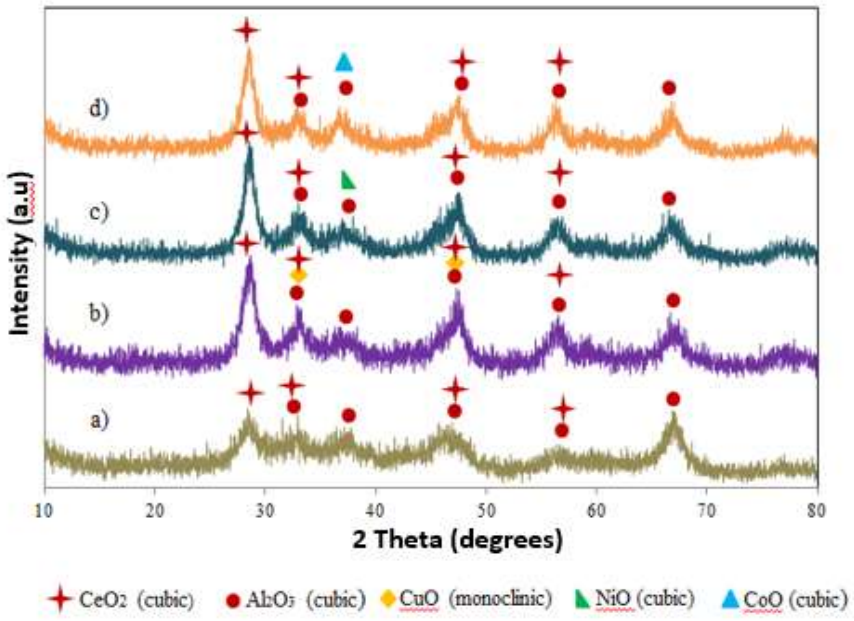


Figure 1. XRD diffractograms of calcined (a) CeO_2/Al_2O_3, (b) 10Cu-90CeO_2/Al_2O_3, (c) 10Ni-90CeO_2/Al_2O_3 and (d) 10Co-90CeO_2/Al_2O_3 catalysts

Figure 2 shows H_2-TPR profile of CeO_2/Al_2O_3 and promoted CeO_2/Al_2O_3 catalysts. From Figure 2, all catalysts gave reduction peak at higher temperature of ~800 ºC. The reduction peak at 800 ºC for all catalysts was indicated by the reduction of bulk ceria from CeO_2 to Ce_2O_3 since the reduction profile for CeO_2/Al_2O_3 was reduced at that temperature [15]. However, when copper was incorporated into CeO_2/Al_2O_3 matrix, a new reduction with high peak appeared at a much lower temperature ~180-330 ºC indicating reduction of the non-associated oxide of dispersed CuO species and the bulk reduction of CuO species, respectively since > 260 ºC was for reduction of CuO species interacted with support of either CeO_2 or Al_2O_3 [16-17]. The peaks could be attributed to the two steps reduction of CuO to Cu^+ and to metallic Cu^0 species [18]. Meanwhile, the

broader reduction peak of 10Ni-90CeO_2/Al_2O_3 than unpromoted CeO_2/Al_2O_3 was noticed at ~900 ºC. This could be due to the reduction of sintered oxide species or bulk nickel oxide interacted strongly with support. Accordingly, the broader reduction profile was caused by the slow diffusion or H_2 adsorption by the interphase due to the presence of clusters of well sintered metal species or alloy cluster [18]. For 10Co-90CeO_2/Al_2O_3, a reduction peak at ~1000 ºC was shifted to higher reduction temperature of CeO_2/Al_2O_3 attributing to the reduction of the metal oxide in complex species, where the interactions with the support were strong [19-20]. The findings in this study indicates that the addition of CuO with 10wt% Cu loading to the CeO_2/Al_2O_3 exhibited a significant promoting effect towards lower reduction temperature. Therefore, embedding Cu species into CeO_2/Al_2O_3 matrix resulted in good interactions between the Cu-Ce-alumina species that enhanced the reduction properties and thus metal reducibility of the catalysts.

Table 1. Physicochemical properties of the catalysts

	XRD	H_2-TPR			NH_3-TPD
Catalyst	**Crystallite Size[a] (nm)**	**H_2 Consumed at Different Temp (µmol/g)**		**Total Amount H_2 Consumed (µmol/g)**	**Total Amount NH_3 Adsorbed (µmol/g)**
		150-550 ºC	**> 550 ºC**		**> 550 ºC**
CeO_2/Al_2O_3	25.6	19	148	167	345
10Cu-90CeO_2/Al_2O_3	43.9	340	84	424	1009
10Co-90CeO_2/Al_2O_3	34.4	-	221	221	1007
10Ni-90CeO_2/Al_2O_3	32.1	-	978	978	1442

[a]The data were estimated according to the Debye Scherrer equation using the FWHM of the CeO_2 peak at $2\theta = 29.2^o$.

As seen from Table 1, amount of H_2 uptake for promoted CeO_2/Al_2O_3 catalysts was increased than the unpromoted catalyst indicating more H_2 was consumed since more oxides species was reducible at larger exposure area which increased H_2 adsorption. This was corroborated with the high and broader reduction peaks profile of the 10Cu-90CeO_2/Al_2O_3 and 10Ni-90CeO_2/Al_2O_3, respectively.

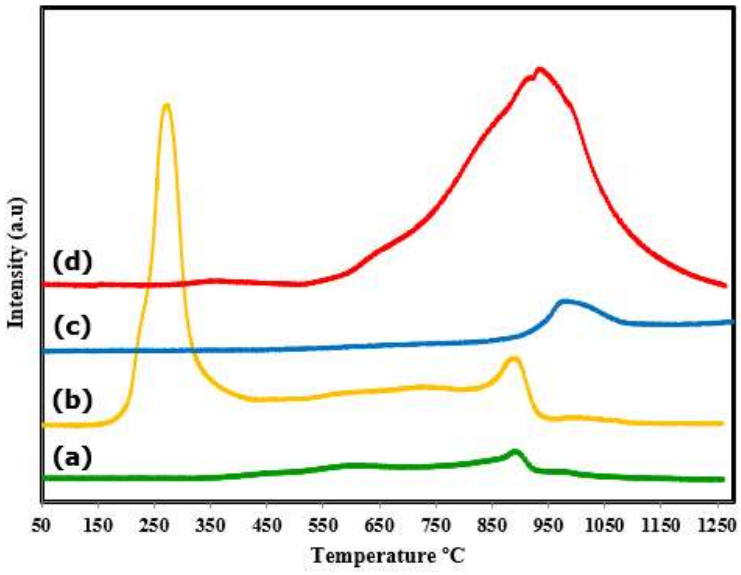


Figure 2. H_2-TPR reduction profile of calcined (a) CeO_2/Al_2O_3, (b) 10Cu-90CeO_2/Al_2O_3, (c) 10Co-90CeO_2/Al_2O_3 and (d) 10Ni-90CeO_2/Al_2O_3 catalysts

According to Zhu et al. [21], the acid sites of catalysts facilitated the activation of the C-OH bonds during dehydration step of glycerol to form unstable intermediate product before formation of PG. Figure 3 depicts the NH_3-TPD profiles of all catalysts while Table 1 lists their corresponding amount NH_3 desorption. The finding showed that the acidity was increased for promoted CeO_2/Al_2O_3 catalysts than the unpromoted CeO_2/Al_2O_3 with the order of CeO_2/Al_2O_3 < 10Co-90CeO_2/Al_2O_3 ≈ 10Cu-90CeO_2/Al_2O_3 < 10Ni-90CeO_2/Al_2O_3. This result demonstrated that the incorporation of copper, cobalt, and nickel oxide species into CeO_2/Al_2O_3 matrix promoted higher acid sites capacity since the presence more oxides species provide larger exposure area for NH_3 adsorption [8].

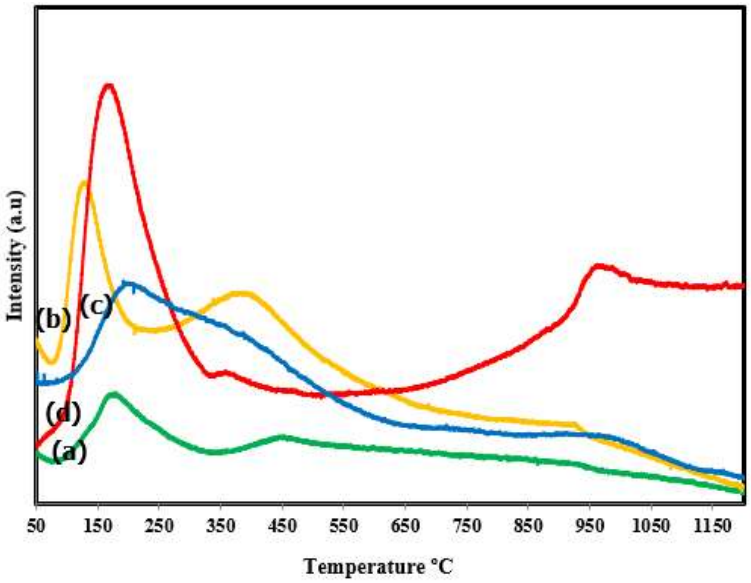


Figure 3. NH_3-TPD profile of calcined (a) CeO_2/Al_2O_3, (b) 10Cu-90CeO_2/Al_2O_3, (c) 10Co-90CeO_2/Al_2O_3 and (d) 10Ni-90CeO_2/Al_2O_3 catalysts

Table 2 shows the catalytic activity of CeO_2/Al_2O_3, and all promoted CeO_2/Al_2O_3 catalysts. All catalysts were evaluated based on their ability to convert glycerol and product selectivity to PG and methanol. From Table 2, the conversion of glycerol and PG selectivity were low with CeO_2/Al_2O_3 catalyst, however the activity was increased with the presence of promoted CeO_2/Al_2O_3 catalysts in the reaction. Among all tested catalysts, 10Cu-90CeO_2/Al_2O_3 contributed the highest glycerol conversion of 88% and 35.5% PG selectivity with the trend of CeO_2/Al_2O_3 < 10Ni-90CeO_2/Al_2O_3 < 10Co-90CeO_2/Al_2O_3 < 10Cu-90CeO_2/Al_2O_3. The high catalytic performance of 10Cu-90CeO_2/Al_2O_3 was attributed to its adequate acid sites (1009 µmol/g) that facilitated the dehydration of glycerol to form unstable intermediates which later converts into PG. The reduction profile of 10Cu-90CeO_2/Al_2O at significantly lower temperature with peak maxima of 260 ºC also contributed to the high activity performance as agreed with other studies suggesting that the reduction of copper oxide species was active at 200-300 ºC [16-17]. At that temperature, copper oxide species was reduced into metallic copper since that temperature was active for CuO reduction. In other words, reduction of copper oxide was occurred in situ by which the presence of metallic species would in turn hydrogenate the unstable intermediate on its surface into desired PG. Formation of of methanol in the reaction was due to the breaking of C-C bond [22]. In addition, when the activity of catalysts in this study was compared with the reported catalyst from literature [23] as shown in Table 2, the catalyst in this study exhibited good glycerol conversion and 1,2-PDO selectivity. This could be due to the catalyst's metal-metal interaction and acidity which contributed to the desired results during catalytic reaction.

Table 2. Performance by catalytic reaction of all catalysts

Sample	GC Conversion (%)	Product Selectivity (%) PG	Methanol	Ref
CeO_2/Al_2O_3	14.8	6.13	93.9	This work
$10Cu\text{-}90Ce/Al_2O_3$	88.0	35.5	64.5	This work
$10Co\text{-}90Ce/Al_2O_3$	84.5	12.3	87.7	This work
$10Ni\text{-}90Ce/Al_2O_3$	63.7	12.9	87.1	This work
$Ni/SiO_2–Al_2O_3$	40.0	7.9	1.21	[23]

Reaction conditions: 300ºC temperature, 2 h of reaction time, 30cc/min of N_2 initial pressure, 10wt% aqueous glycerol solution and 0.1 g of catalyst dosage

Conclusion

Among all the promoted CeO_2/Al_2O_3 catalysts tested, $10Cu\text{-}90CeO_2/Al_2O_3$ catalyst gave the highest conversion of glycerol (88%) and PG selectivity (35.5%) over reaction temperature of 300 °C, 2 hours reaction duration, 30 cc/min of N_2 initial pressure, 0.1g of catalyst dosage and 10wt% aqueous glycerol solution. This was attributed to the good $Cu\text{-}Ce\text{-}Al_2O_3$ interactions via its adequate acid capacity and the ability to be reduced at lower reduction temperature in the range that close to the experimental reaction temperature of 300 °C.

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CORROSION INHIBITION STUDY OF CARBOXYMETHYL CELLULOSE-IONIC LIQUID VIA ELECTROCHEMICAL AND MACHINE LEARNING TECHNIQUE

(Kajian Perencatan Kakisan Menggunakan Karboksimetil Selulosa-Cecairan Ionik Melalui Teknik Elektrokimia dan Pembelajaran Mesin)

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Abstract

Corrosion is a natural phenomenon defined as the deterioration of a substance or its properties due to interactions between the substance and the environment. Prolonged exposure to corrosive environment had negative consequences, including increased repair and maintenance costs, decreased structural integrity, and fatalities. An approach to address the issue is to use a corrosion inhibitor. Numerous inhibitors have lately been developed or made accessible on the market. However, some could be dangerous or contain of volatile organic compounds (VOCs). Our study introduces carboxymethyl cellulose (CMC) mixed with 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]) ionic liquid, also known as CIL, as a corrosion inhibitor on mild steel in seawater. The functional group of combined CIL was examined using Fourier transform infrared spectroscopy (FTIR). The study employed mild steel specimens immersed in varying concentrations of CIL, subject to electrochemical impedance spectroscopy (EIS) measurements at different temperatures. The obtained result of EIS measurement were analyzed to calculate corrosion inhibition efficiency (IE) of CIL. 46 of electrochemical data were fed into a machine learning technique to forecast the effectiveness of the inhibition. Results indicate when inhibition concentration rises, so does inhibition efficiency (IE). The inhibitory efficiency of CIL decreased as the temperature of the test solution rose. At an ambient temperature of 950 ppm, an IE result of 83% was recorded. Levenberg-Marquardt (LM), Bayesian Regularization (BR), and Scale Conjugate Gradient (SCG) training algorithms were compared via Neural Network Fitting Tool (NNTool). LM was found to be the best backpropagation training algorithm, providing the highest regression value (R) of 0.907 and the lowest mean square error (MSE) of 0.006 when compared to BR and SCG. With R value closer to 1 and MSE close to 0, the use of Artificial Neural Networks (ANN) appears to offer a new insight in predicting methods with the goal of easing the hassle and time-consuming of experimental work.

Keywords: carboxymethyl cellulose, corrosion inhibitor, electrochemical, ionic liquid, machine learning

Abstrak

Kakisan adalah fenomena semula jadi yang ditakrifkan sebagai kemerosotan bahan atau sifatnya akibat interaksi antara bahan dan persekitaran. Pendedahan yang berpanjangan kepada persekitaran yang mengkakis memberi kesan yang negatif, termasuk peningkatan kos pembaikan dan penyelenggaraan, penurunan integriti keatas struktur dan mengakibatkan kemusnahan. Salah satu penyelesaian kepada isu ini ialah menggunakan perencat kakisan. Banyak perencat kakisan telah dibangunkan atau boleh didapati di pasaran. Namun, sebahagian daripadanya mungkin berbahaya atau mengandungi banyak sebatian organik meruap (VOC). Kajian ini memperkenalkan karboksimetil selulosa (CMC) dalam 1-etil-3-metilimidazolium asetat ([EMIM][Ac]) cecair ionik, juga dikenali sebagai CIL, sebagai perencat kakisan pada keluli lembut dalam air laut untuk menangani isu alam sekitar. Kumpulan berfungsi kimia hasil dr campuran CIL ini telah diperiksa menggunakan spektroskopi inframerah transformasi Fourier (FTIR). Kajian ini menggunakan spesimen keluli lembut yang direndam dalam kepekatan CIL yang berbeza, diuji menggunakan spektroskopi impedans elektrokimia (EIS) pada suhu yang berbeza. data pengukuran EIS yang diperolehi dianalisis untuk mengira kecekapan perencatan kakisan (IE) CIL. 46 data elektrokimia digunakan di dalam teknik pembelajaran mesin (ML) untuk meramalkan keberkesanan perencatan. Keputusan menunjukkan bahawa apabila kepekatan perencatan meningkat, kecekapan perencatan juga menunjukkan peningkatan. Kecekapan perencatan CIL menurun apabila suhu larutan ujian meningkat. Pada kepekatan 950 ppm pada suhu ambang, keputusan kecekapan perencatan dengan peratusan 83% telah direkodkan. Algoritma latihan Levenberg-Marquardt (LM), Bayesian Regulisasi (BR), dan Gradien Konjugasi Skala (SCG) telah dibandingkan melalui aplikasi Ketepatan Jaringan Saraf (NNtool). LM didapati sebagai algoritma latihan rambatan belakang terbaik, memberikan nilai regresi (R) tertinggi 0.9072580 dan ralat min kuasa dua (MSE) terendah sebanyak 0.0054787 jika dibandingkan dengan BR dan SCG. Dengan nilai R yang menghampiri 1 dan MSE menghampiri 0, penggunaan Rangkaian Saraf Tiruan (ANN) memberi pandangan baharu dalam kaedah peramalam dengan matlamat untuk mengurangkan kerumitan dan penghambatan kerja eksperimen yang memakan masa.

Kata kunci: karboksimetil selulosa, penghambat pengaratan, elektrokimia, cairan ionik, pembelajaran mesin

Introduction

Mild steels are the most extensively used structural materials in a variety of industries due to their good material qualities such as mechanical durability, ease of processing, and low manufacturing cost. This material is frequently utilized in industries as a primary building material for the creation of structural shapes, automobile parts, buildings, plants, pipelines, bridges, and tin cans [1]. Mild steel, on the other hand, is prone to corrosion when exposed to conditions with high moisture levels and corrosive substances [2, 3]. Corrosion is defined as the degradation of a substance or its qualities because of interactions between the substance and its surroundings. It would be more feasible to avoid corrosion rather than strive to entirely eradicate it [4]. Hence, a corrosion inhibitor is utilized by adding it to a closed system to slow down, prevent, and lower the rate of corrosion [5]. Natural products are frequently being researched for corrosion inhibitors as an alternative to conventional corrosion inhibitors due to recent environmental concerns [6, 7, 8].

Carboxymethyl cellulose (CMC) is a cellulose derivative that has been identified as the most abundant and widely used cellulosed derivatives due to the presence of polar functional groups such as carboxyl and hydroxyl groups, which contribute significantly to CMC's strong adsorption ability to metal structures [9, 10]. Despite the fact that CMC is frequently used in the food and pharmaceutical industries [11], some research has indicated that this cellulose derivative is also effective as a corrosion inhibitor [9, 12]. In this project, 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]) ionic liquid was chosen as the CMC solvent due to its loosely bounded anions, which can strongly interact with the polar functional (-OH, -NH_2) groups of polysaccharides, causing them to solubilize and increasing the performance of CMC inhibitor [13]. The objective of this study is to investigate the stability and polarization resistance of carboxymethyl cellulose ionic liquids (CIL) as corrosion inhibitor for mild steel in seawater incorporate ANN for prediction study. Most researchers commonly utilize acidic and artificial NaCl electrolytes in their electrochemical studies involving CMC and CMC with additive [15-24]. In contrast, this research opts for natural seawater. The selection to employ seawater as the electrolyte offers the advantage of investigating the material's behavior in marine environments and offshore applications. This approach aids in gaining insights into the natural corrosion effects

in the environments and contributes to a better understanding, ultimately facilitating the development of strategies to mitigate corrosion in such settings. To reduce the time and effort required for laboratory work, a computational method based on machine learning was used to make predictions.

Artificial Neural Network (ANN) approaches were a viable choice for describing the corrosion inhibition behavior of carboxymethyl cellulose (CMC) in 1-ethyl-3-methylimidazolium acetate ionic liquid (CIL). The Levenberg-Marquardt (LM), Bayesian Regularization (BR), and Scaled Conjugate Gradient (SCG) training algorithms were used, and their performance indicators were analyzed. Furthermore, no machine-learning technique has been used to predict the corrosion inhibition of carboxymethyl cellulose in the ionic liquid ([EMIM][Ac]). By computing the corrosion inhibition efficiency prediction by using machine learning serves as an alternative to extrapolate the experimental data. The establishment of this model would be another new finding where it would beneficial to the research community. This model can be used to determine the corrosion inhibition behavior without the need for replicating the same experiment. This precise and robust model brought a new insight for the researchers parallel with the progression in Industrial Revolution 4.0 (IR 4.0).

Materials and Methods

Sample preparation

the experiment employed a square form of marine grade mild steel-chemical composition-carbon 0.051%, manganese 0.734%, silicon 0.126%, sulphur 0.004%, phosphorus 0.016% and iron remaining [14] with dimensions of 25mm x 25mm x 3mm. Prior to the EIS experiment, the coupons were polished with multiple grits (120, 600, 800, and 1200 grit) of emery paper to remove defects and attain the desired surface roughness. The coupon was then washed with acetone, rinsed with distilled water, quickly dried in air, and stored in desiccators before use. Carboxymethyl cellulose is constantly mixed with 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]) at 60°C for 30 minutes. Following that, the prepared inhibitor is subjected to Fourier transform infrared (FTIR) spectroscopy utilizing an INVENIO FT-IR Spectrometer with a wavelength range of 400 to 4000 cm^{-1} and a spectra resolution of 4 cm^{-1} to identify and analyze the functional group of a sample based on its interaction with infrared light. Immersion of mild steel in seawater is conducted for three hours to allow the adsorption of CIL onto the mild steel surface. The seawater was collected from Institute of Oceanography and Environment (INOS) Universiti Malaysia Terengganu.

Electrochemical impedance spectroscopy (EIS)

The impedance analysis of the mild steel interface in the presence and absence of CIL in seawater were characterized by using electrochemical impedance spectroscopy (EIS) Gamry 1010E. Three electrodes corrosion cell setup was used where the mild steel was employed as working electrode, Ag/AgCl as reference electrode, and platinum rod as counter electrode. Those electrodes were placed in a 200 mL beaker of seawater as electrolyte solution. For each experiment, volume of the test solutions was maintained to ensure the consistency of the parameter to all experiment. The testing parameters for the EIS are tabulated in Table 1. The ambient temperature was a foundational reference in standard laboratory conditions, establishing a baseline for electrochemical studies [25]. The elevated temperature of 50 °C was chosen to examine the moderate temperature effects on the corrosion inhibitor's behavior. At high temperature, 70 °C was utilized for higher temperature investigations. The decline in inhibition efficiency might happen as temperature rises was a characteristic observed in inhibitors physically adsorbed onto the metal surface resulted in desorption of some adsorbed corrosion inhibitor molecules from the metal surface[22, 26]. Electrochemical impedance spectroscopy (EIS) experiments were performed with a small amplitude perturbation 10 mV (peak-to-peak) for an AC signal where the surface area of the mild steel coupon was 3.125cm^2.

Table 1. Testing parameter of CIL for EIS experiment

Temperature (°C)	Concentration (ppm)	Frequency (Hz)
	0	
Room Temperature	350	
50	550	0.1 – 1000000
70	750	
	950	

The corresponding data then analyzed by using Gamry 1010E EChem Analyst. Determination of the corrosion respond parameters in the Nyquist plot was obtained via construction of equivalent circuit compatible with the Nyquist diagram. To fit the model using simplex method, theoretical transfer function Z(ω) was constructed parallel with the combination of a resistance R_p and a constant phase element (CPE), then, both in series with another solution resistance R_s as shown in Figure 1. To validate the suited equivalent circuit, the experimental data are fitted to the equivalent circuit and the circuit elements are obtained [27].

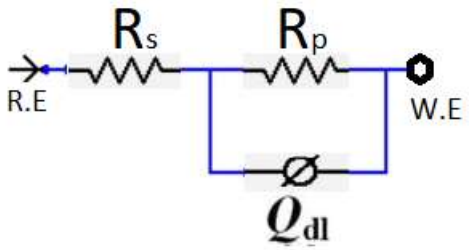


Figure 1. Equivalent circuit for the EIS Analysis

The inhibition efficiency (IE) was determined from Polarization Resistance, R_p according to Equation 1:

$$IE(\%) = \frac{R_{p(inhibited)} - R_{p(uninhibited)}}{R_{p(inhibited)}} \times 100\% \quad (1)$$

Artificial neural network

The Multilayer Feed Forward Neural Network (MFNN) was utilized to estimate the effectiveness of CIL's inhibition using MATLAB® software (R2021b, MathWorks®, Natick, MA, USA). The experimental data obtained from EIS characterization served as the source of the 46 datasets for the modelling investigation. Min-max technique of data normalization was adopted in this study which in the end, the values are between 0 and 1 for an accurate predictive model. The technique is based on the following Equation 2.

$$x = \frac{x_{input} - x_{min}}{x_{max} - x_{min}} \quad (2)$$

where x = (x1, . . ., xn) and x is the normalized data.

A satisfactory normalization is one of the most important aspects of the training process, which represents a direct influence on the model and offers benefits such as suitable results and a considerable decrease in calculation time [28].

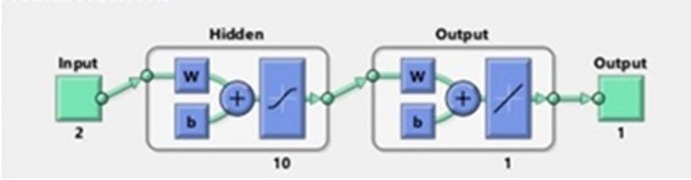


Figure 2. Artificial Neural Network Architecture

To forecast the inhibition efficiency (IE) behavior model of CIL, the Neural Network Toolbox in MATLAB was employed. The default settings of the NNTool were utilized, with 70% (32) of the data allocated for training, 15% (7) for validation, and 15% (7) for testing purposes, chosen randomly from a dataset of 46. The distribution of data between training, validation, and testing in a model of small data range to ensures the chosen subset accurately represents the entire dataset, reducing bias and minimizing the risk of overfitting [29]. As shown in Figure 2, architecture models are composed of three layers with a 2:10:1 structure. Temperature (°C) and Concentration (ppm) were the two parameters that made up the first layer, also known as the input layer. The model is built using 10 nodes in the hidden layer, and the output layer predicts inhibition as IE. As training algorithms, Levenberg-Marquardt (LM), Bayesian Regularization (BR), and Scaled Conjugate Gradient (SCG) were employed. Since there is no established standard procedure, a trial-and-error approach using 10 nodes with one concealed layer is used [30]. Performance Indicator (PI) used to measure the effectiveness and efficiency ANN developed. Here, 2 PI are used. Regression accuracy R is used to measure accuracy and mean square error (MSE) is used to measure error. The accuracy measures evaluate values from 0 to 1, whereby the best model is considered when the evaluated values are close to 1, while the best model for error measures are selected if the evaluated value is close to 0.

Results and Discussion

Fourier transform infrared (FTIR) spectroscopy

Figure 3 depicts the FTIR spectra of the cellulose ionic liquid (CIL). It displays a notable peak in the range of 3600-3100 cm^{-1}, corresponding to the stretching vibration of the O-H (hydroxyl) group [31]. This FTIR range revealed changes in the O-H stretching peak, signifying modifications in the hydrogen bonding pattern. The peak's increased broadening and intensity indicate the development of new formations of hydrogen bonds due to the disruption of the compact structure of the intramolecular hydrogen bonding in the glucose units of CMC [32]. The peak at the range of 1500-1620 cm^{-1} corresponds to the stretching vibration of the C=C group that can be found in the imidazole ring stretch [33]. Based on [34], it is suggested that around 1700 cm^{-1} corresponds to the C=O group and a peak at around 1200 cm^{-1} corresponds to C–N stretching. The peak at 1016 cm^{-1} in the FTIR range is typically associated with the symmetric stretching vibration of C–OC– in CMC [35]. The alkanes functional group exhibited spectral IR bands at around 750 cm^{-1} due to C-H rocking vibrations and at 600 cm^{-1} due to C-H bending vibrations [34]. The FTIR analysis revealed the presence of characteristic functional groups in the CMC mixed with the ([*EMIM*][Ac]) mixture, which was consistent with its expected molecular structure.

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Electrochemical impedance spectroscopy (EIS)

The corrosion inhibition efficiency of mild steel using CIL at Open Circuit Potential (OCP) was investigated by the area-normalized EIS tested in different concentration and solution temperature. With the different concentration used in the experiment, the result of polarization resistance, R_p shows an increasing trend of value as seen in Figure 4 and Table 2. The R_p for mild steel in the absence of CIL at ambient temperature was recorded with the value of 163.4 $\Omega.cm^2$. The R_p value of inhibited sample at ambient temperature for 350, 550, 750 and 950 ppm were recorded at 274.2, 482.6, 665.4, and 980.0 $\Omega.cm^2$ respectively. The calculated inhibition efficiency, IE were 40.30%, 65.98%, 75.12% and 83.09% for 350, 550, 750 and 950 ppm respectively. The increase of inhibition efficiency with increased concentration of the inhibitor may be attributed to increasing of the number of –O–,–OH, and –COOH groups, which increased the surface coverage of CMC molecules on the metal surface [9]. With the concentration variation, the electrochemical change is indicative of inhibition of corrosion process at increased concentration of the inhibitors by adsorbing on the surface of the metal thereby reducing the active surface area exposed to the aggressive corrosive electrolyte medium [1].

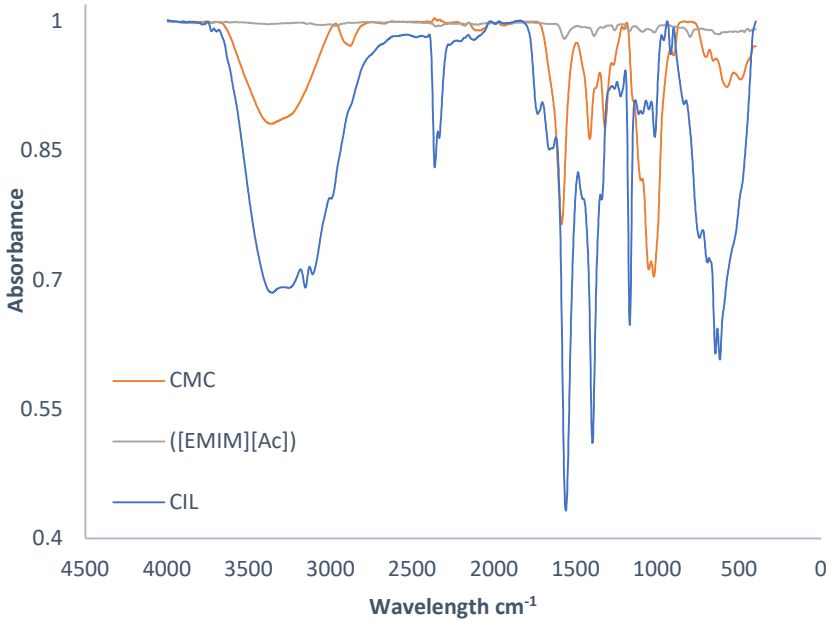


Figure 3. FTIR spectrum of CIL

Table 2. Impedance data for Mild steel in different concentration and temperature effect on CIL in seawater

Concentration (ppm)	Temperature (°C)	R_p (Ω.cm^2)	R_s (Ω.cm^2)	Y_0 (F)	IE %
blank	ambient temperature	163.400	3.129	0.014	0.00
350		274.200	4.739	0.004	40.30
550		482.600	6.856	0.005	65.98
750		665.400	3.914	0.020	75.12
950		980.300	4.231	0.014	83.09
blank	50	120.700	3.063	0.032	0.00
350		157.300	5.233	0.003	23.85
550		217.900	4.677	0.004	44.40
750		391.200	7.068	0.010	68.92
950		340.100	6.993	0.005	64.34
blank	70	85.270	3.111	0.031	0.00
350		155.400	5.465	0.008	45.06
550		201.600	3.239	0.004	56.85
750		295.600	4.876	0.002	70.59
950		249.200	4.876	0.002	65.21

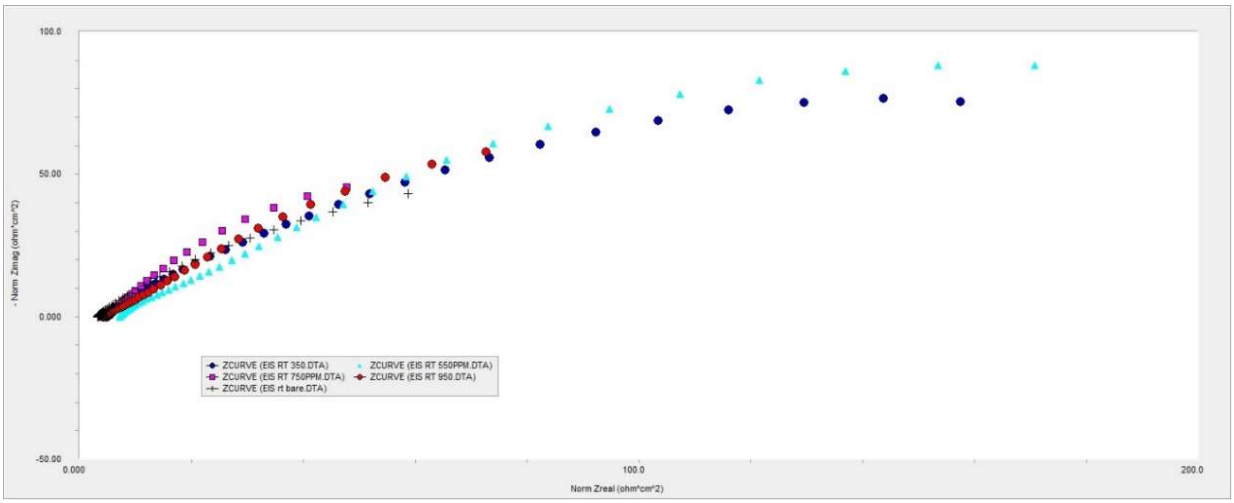


Figure 4. Nyquist Plot at ambient temperature

When testing the sample with a range of different temperatures, as shown in Table 2, the R_p for mild steel in both the presence and absence of CIL was recorded, with lower values obtained compared to the sample tested at ambient temperature. At 50 °C, the R_p recorded for mild steel in the absence of CIL was 120.7 Ω.cm^2. The R_p values of the inhibited sample at 50 °C for 350, 550, 750, and 950 ppm were recorded at 157.30 Ω.cm^2, 217.90 Ω.cm^2, 391.20 Ω.cm^2, and 340.10 Ω.cm^2, respectively. The inhibition efficiency was calculated as 23.85%, 44.40%, 68.92%, and 64.34% for 350, 550, 750, and 950 ppm at 50 °C, respectively, as shown in Table 2.

The Nyquist plot of inhibited and uninhibited samples tested at 70°C is shown in Figure 6. The plot shows a nearly perfect semicircle indicates that the electrochemical process is mainly contributed by the charge transfer process. Charge transfer is the difference in real impedance axis at low and high frequency regions. At 70 °C, the recorded R_p for mild steel in the absence of CIL was 83.27 Ω.cm^2. The inhibited samples record the R_p value at 155.40 Ω.cm^2, 201.00 Ω.cm^2, 295.60 Ω.cm^2, and 249.20 Ω.cm^2 for 350, 550, 750, and 950 ppm respectively. The calculated inhibition efficiency was 45.06%, 56.85%, 70.59% and 65.21% for 350ppm, 550ppm, 750ppm, and 950ppm at 70 °C respectively.

Temperature has a significant impact on corrosion rates and inhibiting efficiency. With the increase in the testing solution temperature from ambient to 70 °C, the inhibition efficiency decreased. At higher temperatures, the adsorption process may be hindered or reversed, leading to reduced inhibitor coverage and decreased corrosion protection due to reduced adsorption**,** as well as faster corrosion reactions because of the higher kinetic energy [36]. However, at temperatures of 50 °C and 70 °C, the inhibition efficiency decreased for both concentrations of 950 ppm. This might suggest a decrease in the effectiveness of the inhibition as the sample experienced the limitation of inhibitor adsorption. This occurs when inhibitor molecules do not have enough opportunities to adsorb onto a metal surface. According to [37], the decreased inhibition efficiency as the temperature increases is due to the physisorption mechanism, which might be attributed to an increase in the solubility of the precipitated inhibitor molecules on the mild steel surface. With the decrease in the number of molecules absorbed on the mild steel surface as the temperature rose, this resulted in a deficiency in the inhibitive performance.

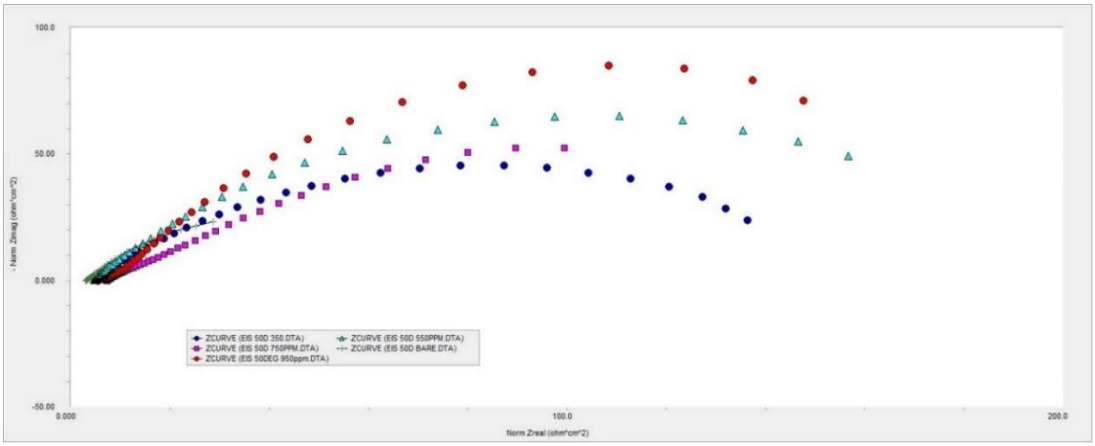


Figure 5. Nyquist Plot of CIL at 50°C testing solution temperature

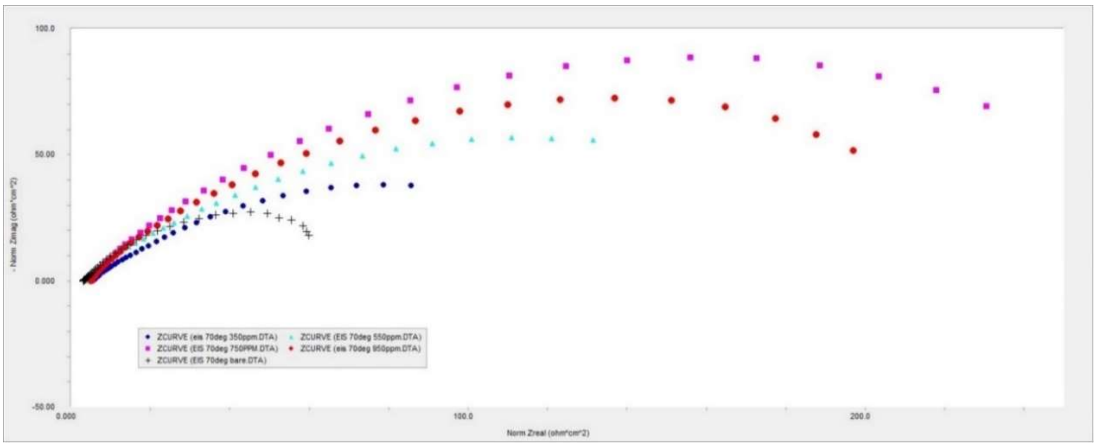


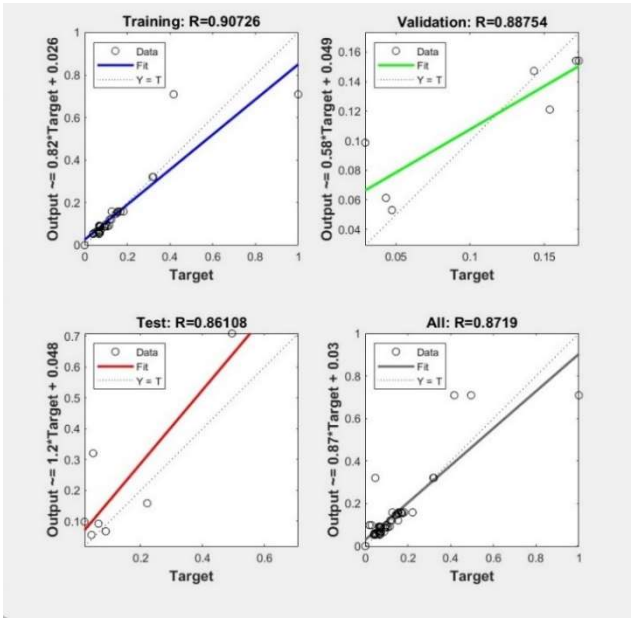
Figure 6. Nyquist Plot of CIL at 70°C testing solution temperature

Artificial neural network

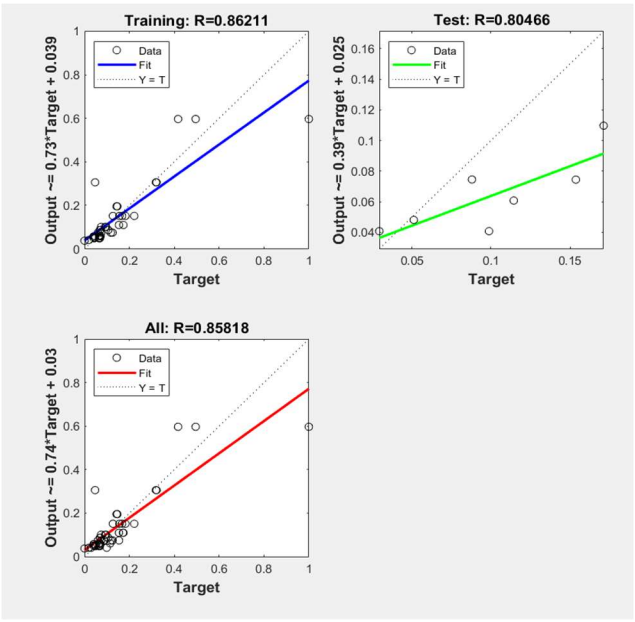
Table 3 shows the values of the performance indicator (PI) obtained from the modelling study via an artificial neural network. The correlation coefficients (R) for the Levenberg Marquardt (LM) training algorithm in the training, validation, and testing phases were 0.907258, 0.887539, and 0.861082, respectively. Based on the fitted line shown in Figure 7a, the fitted slope line falls within the expected range, showing the good relationship between the predicted and observed values obtained for the training, validation, and testing samples. The results from the Bayesian Regularisation (BR) training algorithm showed slightly lower precision as the R values for the training and testing phases were 0.862106 and 0.804661, in comparison to those obtained from LM, as shown in Figure 7b. The Scale Conjugated Gradient (SCG) training algorithm produced R values of 0.359149, 0.828020, and 0.333080 for training, validation, and testing, respectively. The SCG had the lowest precision of all the training algorithms.

Table 3. Performance indicator of training algorithm

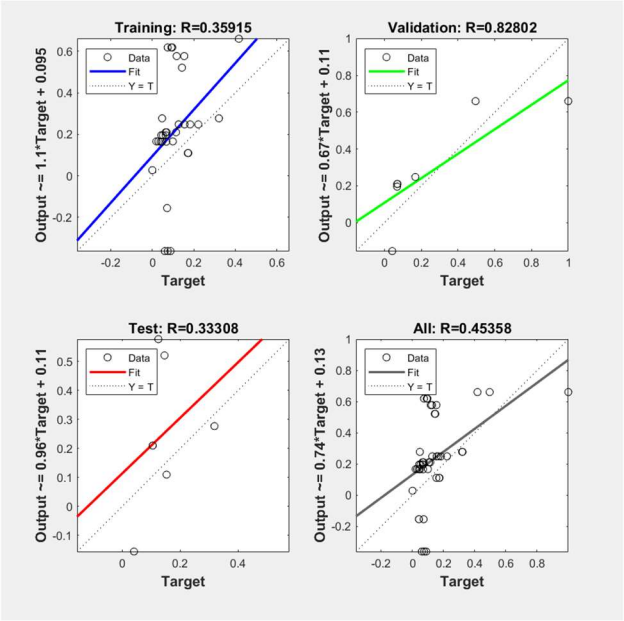
Training Algorithm	LM		BR		SCG	
PI	MSE	R	MSE	R	MSE	R
Training	0.005	0.907	0.008	0.862	0.073	0.359
Validation	0.001	0.888	-	-	0.035	0.828
Testing	0.019	0.861	0.002	0.805	0.058	0.333



(a)



(b)



(c)

Figure 7. Correlation of training, validation, testing and overall, between the actual and predicted corrosion rate for (a) Levenberg–Marquardt (LM), (b) Bayesian Regularization (BR) and (c) Scale Conjugate Gradient (SCG training algorithm for CIL inhibition efficiency prediction

Overall, the LM was found to be the best training algorithm. Other study also shows the use of LM gave better outcome [30]. This could be due to the LM backpropagation training algorithm is a modified version of Newton's method. The Levenberg-Marquardt algorithm also designed to approach second-order training speed without having to compute the Hessian matrix [38]. LM was selected as Backpropagation

Learning algorithm as it is known for its efficiency in minimizing the sum of squared errors and its ability to converge faster.

Conclusion

Corrosion inhibition performance of cellulose-ionic liquid (CIL) tested on marine grade mild steel with variation of temperature (°C) and concentration (ppm) using electrochemical analysis and further analyzed using Artificial Neural Network (ANN) has shown the promising result and observation. It was found that FTIR analysis shows the presence of functional groups in CMC mixed with ([EMIM][Ac]) mixture by formation of new hydrogen bonds as result of disrupting and breaking compact structure of intramolecular hydrogen bond of CMC which provides an adsorption site between the inhibitor and the metal. With increasing of CIL concentration, the result gives the higher inhibition efficiency and reducing corrosion rate. With the increase of testing solution temperature from ambient to 70°C, the result of inhibition efficiency is decreasing. LM was found to be the better training algorithm as the regression R is highest and MSE is lowest compared to BR and SCG. With the implication of the research result, CIL has the potential to be used as a natural and environmentally friendly corrosion inhibitor. To drive research forward with innovation and new techniques, the future prospects on enhancing the effectiveness of CIL as corrosion inhibitors should be implemented. This involves exploring various parameters including the immersion period, different CIL concentration or use of different inhibitor formulation. Assessing the corrosion condition under long period of time to simulate realistic conditions, testing CIL performance using different materials, comparing with different combination of ionic liquid, and conducting a thorough environmental impact assessment are possible to be implemented as well. Additionally, an approached of research on CIL interacts and influences marine biofilms on metal surfaces will give significance for the development of new technologies and implementation in marine industries. This feature acknowledges the intricate relationships that exist between corrosion inhibitors and the dynamic of marine environment, providing insights that lead to innovative solutions for effective corrosion protection in real-world marine applications

Acknowledgement

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ENRICHED SILICA FROM OIL PALM BIOMASS FOR MAGNETIC SOLID PHASE EXTRACTION OF ORGANOCHLORINE PESTICIDE: CHARACTERIZATION AND METHOD DEVELOPMENT

(Silika yang Diperkayakan daripada Biomas Kelapa Sawit untuk Pengekstrakan Fasa Pepejal Magnetik Organoklorin Racun Serangga: Ciri dan Pembangunan Kaedah)

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Abstract

This study focused on preparing magnetic silica bio-sorbent made from oil palm fronds and developing methods for analyzing trace amounts of organochlorine pesticides, including Aldrin, Dieldrin, and Heptachlor. The bio-sorbent, namely Fe_3O_4@SiO_2, was prepared through the sol-gel method by combining silica particles derived from oil palm fronds with commercial ferrous (II) oxide. The physical and chemical properties of the bio-sorbents were analyzed using ATR-FTIR, FESEM, EDX, and XRD. According to ATR-FTIR, the bio-sorbent contained absorption bands at 1100 cm^{-1} and 500-600 cm^{-1}, indicating the functional groups of Si-O and Fe-O. FESEM and EDX studies show the particles have a rough and irregular surface morphology that is composed of 63.9% oxygen, 16.97% silica, and 19.04% ferrous atomic weight. Meanwhile, XRD analysis confirmed that the bio-sorbent was composed of face-centered cubic Fe_3O_4 and amorphous SiO_2, with multiple peaks appearing at 2θ = 22.978 (101), 30.20 (220), 35.60 (311), and 57.50. A magnetic solid-phase extraction method using the synthesized bio-sorbent was developed prior to gas chromatography-mass spectrometry analysis of the selected OCPs. The optimal extraction conditions were determined via one variable at a time (OVAT) and resulted as follows: extraction time, 5 min; desorption solvent, ethyl acetate; desorption time, 5 min; mass of sorbent, 40 mg. The analytical performance of the method was evaluated under these conditions, with a detection limit of 0.1-0.53 mg/L and an extraction recovery of 80-92%. Several linear ranges were obtained for Heptachlor, Aldrin, and Dieldrin, including 0.6-1.8, 0.1-0.3, and 0.2-0.6 mg/L, respectively. The effectiveness of the bio-sorbent was proven by removing pesticides from actual paddy water samples, with calibration curves showing linearity over a wide range of values. The intra- and inter-day precisions were 0.50-1.86% and 0.69-2.54%, respectively.

Keywords: bio-sorbent magnetic silica, organochlorine, paddy water samples, magnetic solid phase extraction

Abstrak

Kajian ini bertujuan untuk menghasilkan biosorbent silika magnetik daripada pelepah kelapa sawit dan membangunkan kaedah untuk menganalisis jumlah jejak racun serangga organoklorin seperti Heptachlor, Aldrin, dan Dieldrin. Bio-sorben iaitu Fe_3O_4-SiO_2 telah disediakan melalui kaedah sol-gel dengan menggabungkan zarah silika yang diperolehi daripada pelepah kelapa sawit

dengan ferus (II) oksida komersial. Sifat fizikal dan kimia bio-sorben telah dianalisis menggunakan ATR-FTIR, FESEM, EDX, dan XRD. Menurut ATR-FTIR, bio-sorben mengandungi julat penyerapan pada 1100 cm^{-1} dan 500-600 cm^{-1}, yang menunjukkan kumpulan fungsi Si-O dan Fe-O. Kajian FESEM dan EDX menunjukkan bahawa partikel mempunyai morfologi permukaan yang kasar dan tidak teratur yang terdiri daripada 63.9% oksigen, 16.97% silika, dan 19.04% berat atom ferus. Sementara itu, analisis XRD mengesahkan bahawa bio-sorben terdiri daripada Fe_3O_4 kubik berpusat muka dan SiO_2 amorfus, dengan pelbagai puncak yang muncul pada 2θ = 22.978 (101), 30.20 (220), 35.60 (311), dan 57.50. Pembangunan kaedah pengekstrakan fasa pepejal magnet dengan menggunakan bio-sorben yang disintesis telah dijalankan diikuti analisis oleh kromatografi gas spektrometri jisim (GC-MS) daripada OCP yang terpilih. Keadaan pengekstrakan optimum ditentukan melalui satu parameter pada satu masa dan hasilnya seperti berikut: masa pengekstrakan 5 min; pelarut desorpsi, etil esitat; masa desorpsi, minit, dan sorbent jisim 40 mg. Prestasi analisis kaedah dinilai di bawah keadaan ini, dengan had pengesanan 0.1-0.53 mg/L dan pemulihan pengekstrakan 80-92%. Beberapa julat linear telah diperolehi untukHeptachlor, Aldrin dan Dieldrin, termasuk 0.6-1.8, 0.1-0.3, dan 0.2-0.6 mg/L masing-masing. Keberkesanan biosorben telah dibuktikan dengan mengeluarkan racun perosak daripada sampel air padi sebenar, dengan lengkung penentukuran menunjukkan kelinearan ke atas julat nilai yang luas.Ketepatan dalam dan antara hari ditemui dalam julat 0.50-1.86% dan 0.69-2.54%, masing-masing.

Kata kunci: bio-sorben silika magnet, organoklorin, sampel air padi, pengekstrakan fasa pepejal magnet

Introduction

In Malaysia, vast areas are devoted to cultivating oil palm, a significant agricultural product [1]. However, oil palm farming is associated with several environmental issues, such as deforestation and the accumulation of agricultural waste in the form of oil palm fronds (OPF). These fronds are often allowed to deteriorate or burn, resulting in greenhouse gas (GHG) emissions and air pollution [2]. Consequently, developing new uses for OPF can help reduce waste, generate economic benefits, and mitigate the negative environmental impact of oil palm farming. Researchers have devised various methods to convert palm oil biomass into valuable goods with added value, thereby decreasing the amount of palm oil biomass produced. One such valuable product is silica, which is found in the frond leaves of oil palms. Commercial silica is typically used as a precursor for other materials in industrial manufacturing that are costly and energy-intensive. Silica, on the other hand, is extracted from agricultural waste at moderate temperatures, resulting in an enormously cost-effective product [3]. Therefore, the production of silica from palm oil biomass can meet industrial demands because it can be used as a precursor in the manufacture of glass and cement, as well as in the biological sector as a catalyst carrier or adsorbent for metal removal. According to a study by Onoja et al. [4], acid-treated OPF ash contains up to 95.30% silica (SiO_2). Their controllable particle size, large surface area, great biocompatibility, and adsorptive properties make them useful in numerous technical applications, including low-loss, subwavelength-diameter single-mode optical waveguides, nanoscale optical sensors, atomic trapping and guiding [5, 6].

In agriculture, pesticides are utilized as plant protection products because they enhance crop quality and productivity. Despite their usefulness, pesticides are immature, environmentally stable, and easily dispersed. In 2016, the overall usage of pesticides, such as herbicides, fungicides, and insecticides, rose by 5.5 million tons per year, according to statistics from the Food and Agricultural Organisation (FAO) [7]. This increase was due to the growing demand for food, as it was anticipated that the world rice output would increase by 42% by 2050 [7]. Unfortunately, only 0.3% of the applied pesticides are necessary to affect the target insect, with the remaining 99.7% being leached into the ground, air, and water [9]. After being sprayed on crops, pesticides can leave the site through multiple mechanisms, including biodegradation, runoff, leaching, and volatilization. Pesticide runoff takes a long time to decompose, leading to soil contamination that could seriously impact human health if people are exposed to it.

Most pesticides have very low allowed residual limits, sometimes in the range of parts per trillion; therefore, enhanced sample collection and extraction methods are required to meet these low concentration levels. The presence of matrix molecules from complex matrices, such as soil, in the extract can interfere with the signal

response of the instrument in detecting the target compound [10]. Conventional sample preparation techniques for OCPs can be laborious and time-consuming because multiple stages are required [11]. A more recent method called Magnetic solid-phase extraction (MSPE) uses magnetic particles as adsorbents. This method is beneficial because it is simpler, more sensitive, and more selective than conventional solid-phase extraction (SPE) approaches [12]. It also allows for the rapid and efficient purification and concentration of pesticide samples. One of MSPE's key benefits is its ability to remove various pesticides from complex samples, owing to the large surface area and strong binding capacity of magnetic particles. The application of MSPE is eco-friendly because it does not utilize harsh chemicals, and the magnetic particles can be easily recycled, reducing the waste produced during analysis.

This study aimed to create and utilize sensitive extraction methods using Fe_3O_4-SiO_2-based magnetic solid phase extraction (MSPE) in combination with gas chromatography-mass spectrometry (GC-MS) for the determination of three organochlorinated (Aldrin, Dieldrin, and Heptachlor) in paddy water samples. The extraction parameters were optimized, including the extraction time, desorption solvent, desorption time, and sorbent mass, using the one-variable-at-a-time (OVAT) approach. The development of Fe_3O_4-SiO_2 from OPF was driven by an environmentally friendly concept, which was to adopt sustainable techniques by converting waste biomass into value-added silica-based adsorbent materials.

Materials and Methods

Chemical and reagents

All HPLC-grade solvents, including n-hexane, acetonitrile, acetone, ethanol, and ethyl acetate, were purchased from Merck (Germany). Additionally, reagents, such as ferrous oxide (Fe_3O_4), were acquired from Sigma-Aldrich. Three organochlorine pesticides, namely Aldrin, Dieldrin, and Heptachlor, with purity assay ≥ 99.9%, were purchased from Sigma-Aldrich. Standard stock solutions of aldrin, dieldrin, and heptachlor at concentrations of 1000 mg/L were prepared in acetonitrile. The prepared stock solutions were stored at 4°C until use. Working solutions of the standard mixtures were prepared by diluting standard stock solutions with acetonitrile.

Instrumentation

The FT-IR spectra were generated using an ATR-FTIR spectrometer (PerkinElmer Spectrum ONE FTIR with Universal ATR Polarization L1250064 ZnSe crystal) in transmittance mode over the wavenumber range of 4000-550 cm^{-1}. FESEM (Zeiss Supra 40VP FESEM) equipped with an EDX spectrometer was used for FESEM-EDX. XRD patterns and crystallinity behavior were obtained through XRD measurements using an X'Pert Pro X-Ray Diffractometer with Cu Kα radiation from 40 kV and 30 mA applied to the sample with a 2θ range of 5–100°.

Chromatographic analysis was conducted using a gas chromatography (Agilent 7890 B) system with a mass spectrometer detector (MS). Chromatographic separations were achieved on an HP-5 fused silica capillary (30 m x 0.32 mm, Agilent Technologies, USA). Helium was the carrier gas with a make-up gas flow rate of 1.1971 mL/min. The column temperature program began at 150 °C for 1 min, then increased to 200 °C at a rate of 30°C/min, held for 2 min, followed by an increase to 250 °C at a rate of 10°C/min, held for 2 min, and finally a ramp increase to 280 °C at a rate of 20°C/min. Splitless injection was maintained throughout the experiment to maintain the GC-MS interface at 250 °C.

Preparation of silica derived from oil palm frond

Oil palm fronds (OPF) were manually separated and washed with water to eliminate impurities. The sample was oven-dried (70 ˚C for 48h) and cut into smaller pieces before ground into fine powder. The following chemical treatment of OPF was adapted from the literature [13]. Briefly, OPF powder (50.0 g) was dispersed in HCl (1.0 M, 500Ml) and constantly stirred (400 rpm) for 2 h. The dispersion was cooled to room temperature and rinsed with distilled water until the washings reached a neutral phase. The neutralized OPF was then filtered and dried in an oven (80) for 24h. The OPF powder was heated in a preheated furnace at 600°C for 14h. The temperature gradually increased over 5 h,

then maintained for 9 h. The rate of temperature increase was 2°C/min. White ash from the treated OPF-Si was collected and stored in airtight containers at room temperature for further examination.

Preparation of sodium silicate (Na_2SiO_3)

Sodium silicate (Na_2SiO_3) was prepared using a modified procedure reported by Onoja et al. [14] by mixing OPF-SiO_2 (10.0 g) and 3.5M NaOH (82 mL) in a paraffin oil bath at 100 °C for 4 hours with constant stirring (200 rpm). The resulting sodium silicate solution was filtered using Whatman 41 filter paper at room temperature, and the filtrate was placed in a polypropylene vial with a screw-on cover for analysis.

Preparation of magnetic silica Fe_3O_4@SiO_2

The preparation of magnetic OPF-Silica was based on the developed method by Azmayawati et al. (2018) with minor modifications [14]. First, 1 g of Fe_3O_4 was diluted in 20 mL distilled water and ultrasonicated for 10 min. Subsequently, 5 mL of Na_2SiO_3 was diluted in 80 mL of distilled water and mixed with the Fe_3O_4 solution. The mixture was then ultrasonicated for an additional 20 min. Next, hydrochloric acid (HCl) was added dropwise while stirring the mixture at room temperature until the pH reached 7. The gel was aged and left at room temperature for 24 hours. The solution was then washed and vacuum-filtered to remove the gel. The sorbent was oven-dried for 24 h at 80°C and stored in a desiccator until needed.

Collection and pre-treatment of paddy water samples

Water samples from the paddy fields were collected in propylene bottles from Sawah Padi Bukit Gambir, Johor. The bottles were protected from UV by being covered with aluminum foil and kept at 4ºC. The samples were filtered twice using Whatman 125 mm filter papers to remove colloidal particles, and the filtered solutions were stored in glass bottles before analysis.

Magnetic solid phase extraction procedure

A 50 mg sample of Fe_3O_4@SiO_2 powder was added to a 10 mL aqueous solution containing 3 ppm of heptachlor, 0.5 ppm Aldrin, and 1 ppm of dieldrin. The mixture was vortexed in 5 min to allow the adsorption process. The solution was then separated by magnetic force and discarded. 1 mL of the desorption solvent acetonitrile (ACN) was added for the desorption of the targeted analytes and further sonicated for 5 minutes. The sorbent was separated by magnetic force, and the remaining ACN was concentrated to 0.5 mL via nitrogen before GC-MS analysis.

Results and Discussion

Characterization of the prepared adsorbent

The characteristics of the synthesized adsorbents were studied using ATR-FTIR, FESEM-EDX, and XRD. An ATR-FTIR study was conducted to compare the spectral data and discern any changes in the chemical characteristics of the prepared bio-sorbent. The origin of the Fe_3O_4 spectrum (Figure 1(a)) shows the vibrational mode at 586 cm^{-1} (stretching vibrations of the Fe−O bonds) and 1648 cm^{-1} (bending vibrations of Fe_3O_4 bound water), which is similar to a previous study reported by Lesiak et al. [1]. In contrast, the spectrum of SiO_2 -OPF (Figure 1 (b)) exhibits a wide peak at 3430 cm^{-1}, indicating the presence of silanol (Si-OH) groups and antisymmetric and symmetric stretching vibrations of siloxane groups at wavenumbers of 1073 cm^{-1} and 805 cm^{-1}, respectively [Onoja et al. (2017)]. The spectrum of Fe_3O_4@SiO_2 (Figure 1(c)) exhibits distinct peaks at 3366 cm^{-1}, 1629 cm^{-1}, 1088 cm^{-1}, 809 $cm^{-1,}$ and 577 cm^{-1}, which correspond to the stretching vibrations of the O-H, Fe-O, and siloxane groups, respectively, as reported by Zhang et al. [15]. In addition, the exhibited peaks were similar to those seen in the Fe_3O_4 and SiO_2-OPF spectra but with significantly reduced intensities. This suggests that SiO_2 was effectively integrated into Fe_3O_4, as supported by the corresponding EDX analysis as shown in Figure 2(b).

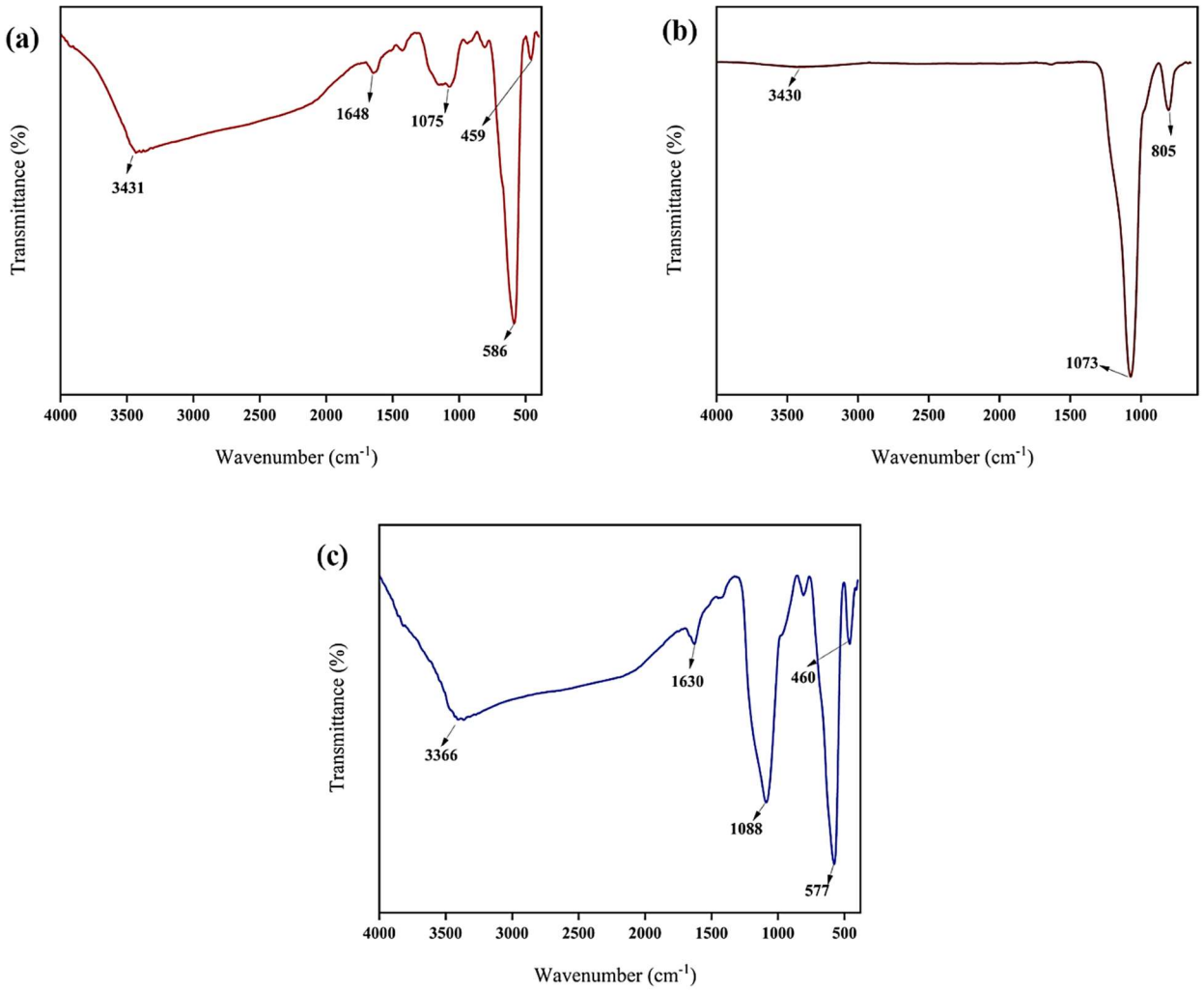


Figure 1. The ATR-FTIR spectra of the as-prepared (a) Fe_3O_4, (b) SiO_2 and (c) Fe_3O_4@SiO_2

Figure 2(a) depicts the FESEM micrographs of Fe_3O_4-SiO_2. As shown in Figure 2(a), the particles were obtained in agglomerated particles, rough surface structure, and the presence of cavities [14, 17]. Fe_3O_4 particles have a cubic shape and aggregate because of their high surface energy and adhesion properties. Nonetheless, the absence of a cubic structure in the FESEM images suggests that SiO2 has coated the magnetite and acted as a core to protect the magnetite from oxidation under acidic conditions, similar to a description by previous work [17].

The FESEM micrograph of Fe_3O_4-SiO_2 was further analyzed by mapping the elemental composition using energy-dispersive X-ray (EDX) analysis, as shown in Figure 2(b). Based on the result, the prepared bio-sorbent mainly consisted of 41.5 wt. % iron (Fe), 39.9 wt.% oxygen (O), and 18.6 wt.% silicon (Si), which correlated with the expected elements in Fe_3O_4-SiO_2. The high percentage of O could be from ferrous oxide and Si-OH in the silica layer, which acts as a shell for the core of the sorbent. The presence of Si can be interpreted as evidence of the successful synthesis of magnetic silica.

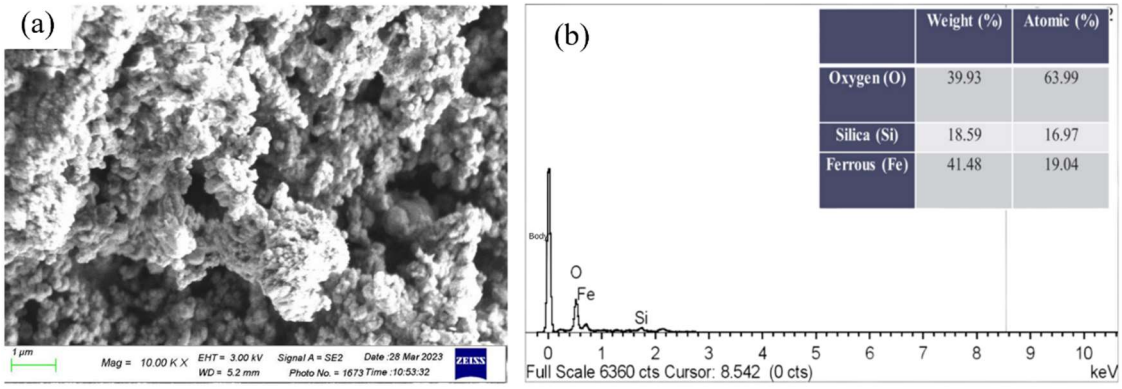


Figure 2. FESEM micrographs of the (a) $Fe3O_4$-SiO_2 and (b) EDX spectrum from specific regions of Fe_3O_4@SiO_2

The XRD diffractograms of each sample were analyzed to evaluate the impact of crystallinity on the sorbents. The diffractograms of Fe_3O_4@SiO_2, Fe_3O_4, and SiO_2 are shown in Figure. 3. The SiO_2 sample in Figure 3(a) displays a broad peak and a Bragg angle of $2\theta = 22.978°$ corresponding to the (101) Miller indices (ICDD No. 01-083-2300), indicating that it is in an amorphous form, which is consistent with previous studies [18]. While, the diffraction peaks seen at angles 2θ~ 30.20 (220), 35.60 (311), and 57.50 can be attributed to the face-centered cubic structure of Fe_3O_4 as documented in the JCPDS file number 85-1436 [19]. The diffraction pattern depicted in Figure 3c indicates a displacement of the peak, signifying that the presence of Fe_3O_4 has resulted in the shift. This alteration suggests that Fe_3O_4@SiO_2 particles have been formed successfully, with the magnetite phase being integrated into the silica surface [20]. With comparison, both peaks were present on the diffractogram of Fe_3O_4-SiO_2.

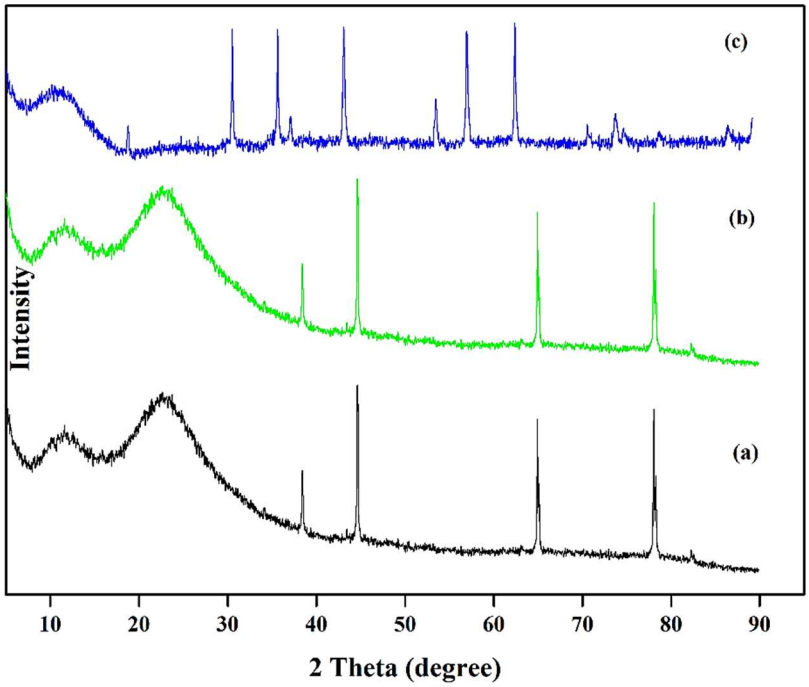


Figure 3. XRD patterns of (a) SiO_2, (b) Fe_3O_4 and (c) Fe_3O_4-SiO_2

Optimization of extraction conditions

The performance of the sorbent was assessed by MSPE for OCPs analysis in aqueous samples to determine the optimum extraction parameters. The one-variable-at-time (OVAT) approach was used to optimize the following MSPE parameters: extraction time, desorption solvent type, desorption time, and amount of sorbent. This approach reduces the use of chemicals, labor, and time. The optimization process involved utilizing distilled water spiked with heptachlor, aldrin, and dieldrin solutions. These solutions were prepared to achieve final concentrations of 3, 0.5, and 1 mg/L in 10 mL of water.

Effect of extraction time

The parameter of extraction time in analytical chemistry pertains to the period during which the extraction process isolates specific analytes from a sample matrix. The efficiency and effectiveness of the extraction procedure can be significantly influenced by critical factors. Precise optimization of the extraction time is crucial for obtaining reliable and accurate analytical results. This study focused on the impact of various extraction durations, ranging from 2.5 to 10 min, on the peak area of the analytes. Figure 4(a) illustrates the correlation between the peak area and extraction time, showing a significant increase in analyte extraction from 2.5 minutes to 5 min. However, the trend gradually decreases from 7.5 minutes to 10 minutes. This suggests that the most efficient extraction time for analyte adsorption onto the magnetic silica was 5 min. The decrease in trend may be because as the extraction time prolongs, there is an increased probability of analyte degradation or instability, leading to loss or alteration of the analyte and inaccurate, unreliable analytical outcomes [21]. Thus, 5 minutes of extraction time was selected for further experiments.

Effect of desorption solvent

The selection of the desorption solvent is crucial for facilitating the elution of the desired analytes from the sorbent material, which ultimately affects the overall efficiency and selectivity of the extraction process. As shown in Figure 4(b), ethyl acetate demonstrated the most favorable performance compared with other solvents such as n-hexane, acetonitrile, ethanol, and acetone. Ethyl acetate may have a greater selectivity towards chlorinated analytes than less polar solvents such as n-hexane, ethanol, acetonitrile, and acetone. This method enables the selective separation of heptachlor, aldrin, and dieldrin, reducing interference from other constituents in the sample matrix. Ethyl acetate is a solvent with moderate polarity (4.4) with favorable solubility characteristics for various organic molecules. Heptachlor, aldrin, and dieldrin are examples of chlorinated organic compounds. Owing to its moderate polarity (with dieldrin having a relative polarity of 5.40, heptachlor at 6.10, and aldrin at 6.75), ethyl acetate effectively dissolves these analytes from the sorbent material [22]. Therefore, ethyl acetate was selected as the desorption solvent for the subsequent analysis.

Effect of desorption time

Desorption time is a crucial parameter in extraction processes in analytical chemistry because it affects the efficiency and completeness of eluting target analytes from the extraction medium. The time range between 2.5 minutes and 10 min was examined, and the results showed that at 5 min, heptachlor and dieldrin were desorbed the most, while 7.5 minutes was required for aldrin. Proper control and optimization of desorption time are essential to achieve accurate and reproducible analytical results. The optimum desorption time was chosen as the one that was standardized for all three analytes. The differences in aldrin levels at 5 and 7.5 minutes were insignificant; therefore, the latter was accepted. A declining trend was observed as the sonication time was increased (from 7.5 to 10 minutes). This phenomenon may be attributed to the thermal effects caused by the sonicator, leading to the degradation of the analyte over time [23]. Therefore, 5 min was selected as the optimal desorption time.

Effect of mass sorbent

The adsorption capacity of the target analytes is influenced by the mass of the sorbent and its effect on both the adsorption capacity and the equilibrium between the sorbent and the sample matrix. The optimal sorbent mass for this extraction was 30 mg, as shown in Figure 4(d), with the highest extraction efficiency. This is because 30 mg of sorbent offers sufficient surface area

for the target analytes (heptachlor, aldrin, and dieldrin) to bind. Increasing the sorbent mass beyond this limit might not result in improved extraction effectiveness, as it could cause the sorbent particles to become overcrowded and limit mass transfer, thereby decreasing the extraction efficiency. Additionally, higher sorbent masses can saturate the active binding sites in the sorbent, leading to declining returns in extraction efficiency [24].

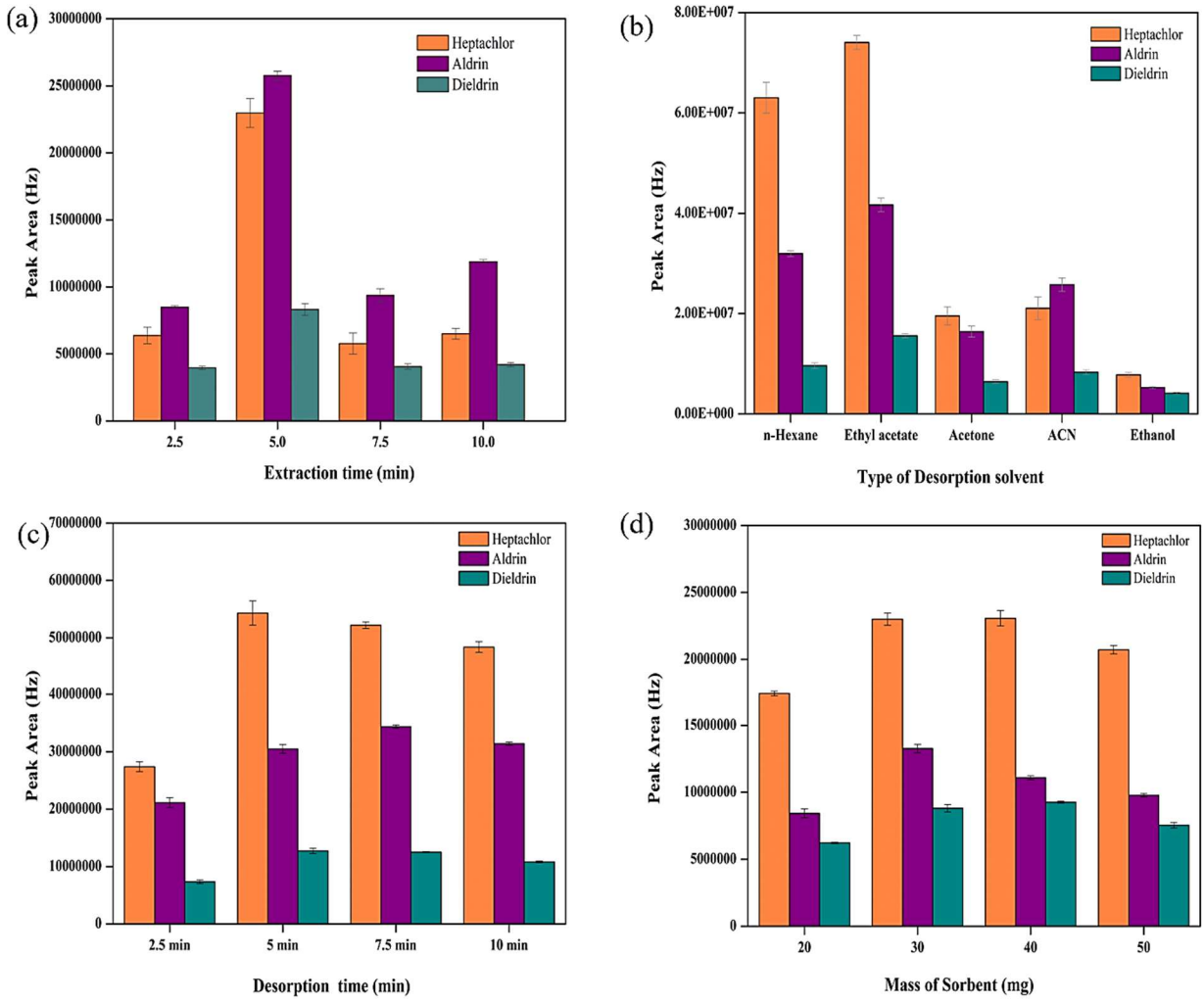


Figure 4. MSPE optimization conditions. (a) Extraction time (b) type of desorption solvent (c) desorption time (d) mass of sorbent

Method validation: Analytical performance

The accuracy of the proposed method for the magnetic solid-phase extraction of Fe_3O_4@SiO_2 was evaluated by analyzing certain organic pollutants in a water sample obtained from a paddy field. To determine the reliability of Fe_3O_4@SiO_2 for extracting three specific pollutants (heptachlor, aldrin, and dieldrin), a matrix-matched calibration approach was employed. The method was validated by calculating the limit of detection (LOD) and limit of quantification (LOQ) using the linear regression approach, standard deviation, and slope of the calibration curve. Calibration curves were generated using the least-squares approach, and peak areas were plotted against concentration. A strong linear relationship was observed between heptachlor, aldrin, and dieldrin, with R^2 values ranging from 0.98 to 0.99.

The limit of detection (LOD) was found to be 0.53-1.50 mg/L. The mean squared prediction error (MSPE) of Fe_3O_4-SiO_2 was evaluated in the context of pesticide analysis of paddy water samples.

Table 1. Performance of the proposed method under optimized conditions

Samples	Analytes	Linearity (mg/L)	Regression coefficient, R^2	LOD (mg/L)	LOQ (mg/L)
Paddy water	Heptachlor	0.6-2.4	0.99	0.53	1.62
	Aldrin	0.1-0.4	0.98	0.10	4.59
	Dieldrin	0.2-0.8	0.99	0.16	1.89

Analysis of real sample

The MSPE method, which utilizes Fe_3O_4@SiO_2, has been effectively used to analyze paddy water samples. The experiment involved adding spiked water samples to achieve final concentrations of 0.1 and 2.4 mg/L for heptachlor, 0.1 and 0.4 mg/L for aldrin, and 0.2 and 0.8 mg/L for dieldrin to conduct relative recovery tests. The results (Table 2) showed that the relative recoveries of the pesticides were within the standard range specified by AOAC International (2016), ranging from 80% to 95%. The intra- and inter-day precisions exhibited relative standard deviations within 0.50-1.86% and 0.69-2.54%, respectively. These results demonstrated the effectiveness of Fe_3O_4@SiO_2-MSPE as a technology for extracting pesticides from aqueous matrices. This study compared the new Fe_3O_4@SiO_2-MSPE method to conventional SPE methods that have already been published [25]. The results showed that the SPE method has recoveries ranging from 75.9 to 112.1 %, upon comparison with previous studies that used magnetic solid phase extraction to identify OCPs, the extraction time was shorter than that of conventional SPE. Figure 5 displays the gas chromatograms showing the presence of heptachlor, aldrin, and dieldrin in water samples from paddy fields.

Table 2. Method precision for intra-day and inter-day (%RSD) and percent recovery (%ER)

Analytes	Concentration (mg/L)	Inter-day RSD (%, n=9)	Intra-day RSD (%, n=3)	ER (%)
Heptachlor	0.6	1.86	2.54	80%
	1.8	0.93	1.93	90%
Aldrin	0.1	1.43	1.98	81%
	0.3	1.12	1.83	90%
Dieldrin	0.2	0.50	1.71	88%
	0.6	1.69	0.69	92%

Green assessment profile

New microextraction techniques are being developed to meet the demand for analytical sample preparation methods that are faster, less invasive, more selective, and require fewer resources. The AGREE calculator (shown in Figure 6) was used to evaluate the long-term feasibility of the proposed Fe_3O_4@SiO_2-MSPE procedure. The evaluation is considered eco-friendly because it meets a set of standards, such as reducing the number of extra sample treatments (4), avoiding derivatization during analysis and extraction (6), and using less energy (9), and fewer potentially harmful solvents (12). However, its primary limitation is that the target analytes must be determined offline, which means that Fe_3O_4@SiO_2 must be integrated into online or inline procedures.

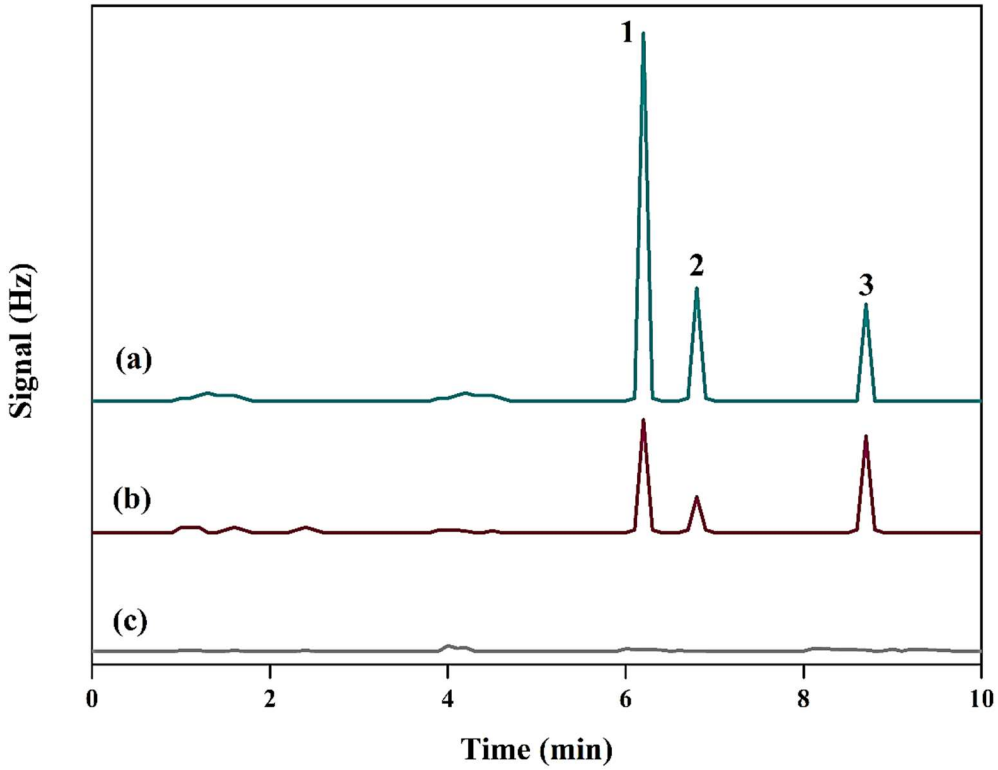


Figure 5. Chromatogram obtained from Fe_3O_4@SiO_2 MSPE-GC-MS of (a) blank of paddy water (b) and (c) paddy water spiked with at range 0.1 mg/L to 2.4 mg/L of analytes respectively. Chromatographic peaks: (1) Heptachlor, (2) Dieldrin, and (3) Aldrin.

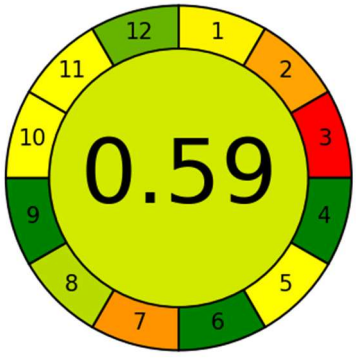


Figure 6. AGREE analysis of the method developed in this study

Proposed adsorption mechanism

Various interactions can occur between the sorbent and sorbate compounds, potentially affecting the extraction efficiency. These interactions, including electrostatic forces, hydrogen bonding, and hydrophobicity, can either attract or repel compounds involved. The properties of the sorbent materials, such as surface area, porosity, and sorption surface morphology, may also influence the extraction process of sorbate compounds [26]. The possible interactions between Fe_3O_4@SiO_2 and the OCPs include hydrogen bonding and van der Waals interactions, as schematically illustrated in Figure

7. Fe_3O_4@SiO_2 is sensitive to chlorinated groups of OCPs in its structure (aldrin and heptachlor) via van der Waals forces. Furthermore, silica has a polar functional group that interacts with the OCP molecule in a manner that makes it stick together, and the hydroxyl group of silica and the chlorine atom in the OCP compounds form strong hydrogen bonds. This interaction is facilitated by forming hydrogen bonds between the hydroxyl groups on silica and the anionic groups present in heptachlor molecules [27].

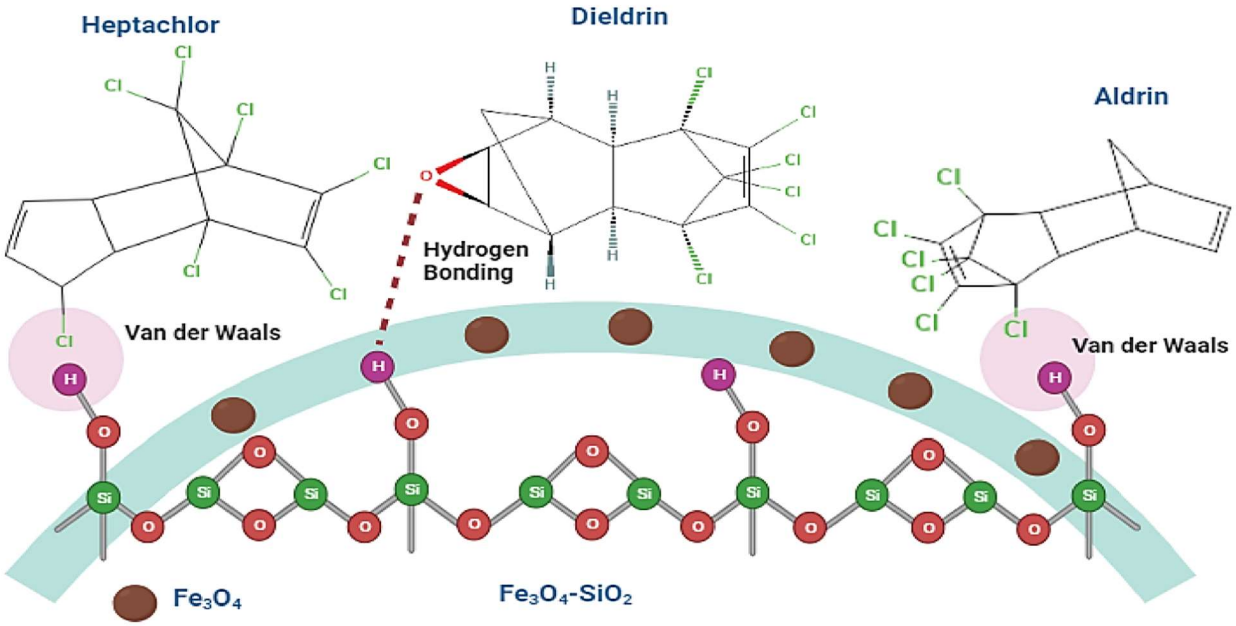


Figure 7. Schematic diagram of proposed interactions between sorbent and analytes

Conclusion

In summary, the present study included synthesizing a Fe_3O_4@SiO_2 composite material, which was then used as a magnetic sorbent in the magnetic solid-phase extraction (MSPE) method. This approach was coupled with gas chromatography-mass spectrometry (GC-MS) to quantify trace amounts of organochlorine pesticides (OCPs) in water samples collected from paddy fields. The main aim of this study is to develop a material that exhibits both environmentally friendly properties and magnetic sensitivity. The synthesizing and fabricating of a composite material consisting of Fe_3O_4@SiO_2 was successfully achieved via the sol-gel technique. The successful synthesis of Fe_3O_4 and its efficient extraction of organochlorine pesticides (OCPs) within a short timeframe was confirmed by the use of Fourier transform infrared spectroscopy (FTIR), field emission scanning electron microscopy (FESEM), and X-ray diffraction (XRD) methods. Significantly, the MSPE procedure offers efficient extraction within a short time frame (5 min) and only uses a small quantity of sorbent (40 mg). In addition, the suggested methodology demonstrated a low limit of detection (LOD), a broad range of linearity, and satisfactory accuracy. The use of the MSPE approach, combined with the GC-MS method, proved to be successful in analyzing organochlorine pesticide (OCP) residues in paddy samples. The findings obtained in this study exhibited good values for recoveries, enrichment factors, and relative standard deviations (RSDs), which significantly improved the sensitivity and simplicity of the sample preparation technique. Furthermore, the Fe_3O_4-SiO_2 adsorbent may be conveniently synthesized using abundant biomass sources, presenting supplementary benefits like cost-efficiency and ecological sustainability.

Acknowledgment

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A PORTABLE MICRO-VOLUME COLORIMETRIC ASSAY FOR ALUMINIUM DETERMINATION USING NATURAL REAGENT EXTRACTS FROM SAPPAN HEARTWOOD BY ULTRASOUND-ASSISTED EXTRACTION

(Satu Kaedah Uji Warna Isipadu-Mikro yang Mudah Alih untuk Penentuan Aluminium Menggunakan Ekstrak Bahan Semulajadi dari Teras Kayu Sappan Melalui Pengekstrakan Dibantu Ultrasonik)

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Abstract

An environmentally friendly small-scale ultrasound-assisted extraction and portable micro-volume colorimetry was proposed for the determination of aluminium in pharmaceutical formulations by extraction of Sappan heartwood in acetate buffer pH 5.5. Sonication parameters influencing the extraction of non-synthetic reagents from Sappan heartwood for quantification of aluminium including type of solvent, mass/volume ratio of solvent, extraction time and temperature were investigated. Efficiency of the ultrasound-assisted extraction procedure of aluminium from aluminium hydroxide gel was evaluated by comparing label values and the official method for pharmaceutical formulations. Under optimal conditions, a linear calibration graph ranging from 0.5 to 10 mg L^{-1} was obtained from micro-volume colorimetric determinations (at 530 nm) with limits of detection and quantification of 0.07 and 0.37 mg L^{-1}, respectively. Relative standard deviations were 2.3 and 1.3% for 1 and 3 mg L^{-1}, respectively. The developed system was successfully applied for the analysis of various forms of aluminium in pharmaceutical formulations (suspension and tablet). Good recoveries between 91 and 107% were obtained.

Keywords: Micro-volume colorimetry, ultrasound-assisted extraction, natural reagent, Sappan heartwood, aluminium

Abstrak

Satu kaedah pengekstrakan dibantu ultrasonik berskala kecil yang mesra alam dan uji warna isipadu-mikro mudah alih telah dicadangkan untuk penentuan aluminium dalam formulasi farmaseutikal dengan mengekstrak teras kayu Sappan dalam penimbal asetat pH 5.5. Parameter sonikasi yang mempengaruhi pengeluaran bahan kimia bukan sintetik dari teras kayu Sappan untuk pengukuran aluminium termasuk jenis pelarut, nisbah jisim/isipadu pelarut, masa pengekstrakan, dan suhu telah disiasat. Kecekapan prosedur pengekstrakan bantuan ultrasonik aluminium dari gel hidroksida aluminium di nilai dengan membandingkan

nilai label dan kaedah rasmi untuk formulasi farmaseutikal. Di bawah keadaan optimum, graf pemilihan linear dengan julat dari 0.5 hingga 10 mg L^{-1} diperolehi dari penentuan warna isipadu-mikro (pada 530 nm) dengan had pengesanan dan pengukuran masing-masing adalah 0.07 dan 0.37 mg L^{-1}. Sisihan piawai relatif adalah 2.3 dan 1.3% untuk 1 dan 3 mg L^{-1}, secara berturut-turut. Sistem yang dibangunkan telah berjaya digunakan untuk analisis pelbagai bentuk aluminium dalam formulasi farmaseutikal (ampaian dan tablet). Perolehan semula yang baik antara 91 dan 107% telah diperolehi.

Kata kunci: Uji warna isipadu-mikro, pengekstrakan dibantu ultrasonik, bahan semulajadi, teras kayu Sappan, aluminium

Introduction

Aluminium (Al), an abundant metal in the earth's crust, is a non-essential element commonly used to make aluminium-containing packaging for food/drink and cooking utensils [1]. It is also used for cosmetics production and pharmaceutical products [2]. Aluminium compounds are less toxic than heavy metals but excessive intake of Al has been associated with various diseases such as Alzheimer's, anaemia, osteomalacia and neurological syndrome [3-4]. Therefore, it is important to develop detection methodologies to monitor and quantify different matrices containing this metal.

Conventional methods for the determination of aluminium include flame atomic absorption spectrometry (FAAS) [5], graphite furnace atomic absorption spectrometry (GFAAS) [6], inductively coupled plasma-optical emission spectrometry (ICP-OES) [7], inductively coupled plasma-mass spectrometry (ICP-MS) [8], spectrophotometry [9-12], and fluorometry [13]. However, these methods require tedious pre-treatment as time-consuming processes that require professional operation with high instrumentation and maintenance costs. The development of green analytical techniques or procedures which provide economical and environmentally friendly advantages has now become an important aspect of sustainable analytical chemistry [14]. The use of a portable miniaturized system with green analytical methods involves stocking fewer samples, with reduced transport and analytical risks of contamination or analyte losses, together with positive environmental benefits [15].

Natural reagent extracts from various sources including vegetables, plant-based, animals, or bacteria require minimal treatment [16]. Natural reagents may be used instead of high purity chemical reagents when a reagent is only required in excess, or a crude extract contains the necessary active compound [17]. The use of crude plant extracts such as Indian mulberry (*Morinda citrifolia*) root extract [18], Indian almond (*Terminalia catappa* L.) leaf extract [19] and heartwood of Sappan wood (*Ceasalpinia sappan* L.) [20, 21] as chromogenic agents for analysis of Al in combination with flow-based analysis techniques [18-20] and paper-based analytical devices [21] have been reported as green analytical approaches. Contained plant compounds can be extracted by classical solvent extraction processes that include maceration with an organic solvent or boiling with water; however, these methods are time consuming and generate high waste production.

Sappan heartwood extracts are popular used for Al determination due to their high sensitivity and selectivity. The red homoisoflavonoid, brazilein is one of the main compounds found in the extracts of Sappan heartwood. In aqueous solution at pH 4.5, aluminium and pure brazilein extracted from Sappan heartwood form a 1:2 ratio complex via the ionized 10-hydroxyl group and 9-carbonyl oxygen of two molecules of brazilein bidentate ligands as shown in Figure 1 [22]. Traditional extraction methods of red dye from Sappan wood involve boiling the wood in water [23]. Various extraction methods have been proposed for the extraction of brazilein from Sappan heartwood such as maceration with ethanol [24], methanol [25], microwave-assisted extraction (MAE) [26-28] and ultrasound-assisted extraction (UAE) [29-30]. Among these, the ultrasound-extraction method is popular as a green analytical technique with clean protocol, safety, short operation time (less than 1 h) and moderate volume/concentration consumption of solvents and energy [31].

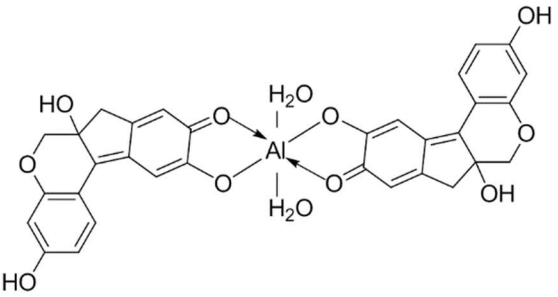


Figure 1. The proposed chemical structure of the Al(brazilein)$_2$ complex at pH 4.5

This study presented a new green analytical approach for Al determination based on simple small-scale ultrasound-assisted treatment procedures for (1) extraction of natural reagent from Sappan heartwood for complexation with Al, and (2) dissolution of Al in pharmaceutical products (suspension and tablet). A portable micro-volume colorimeter with an LED light was utilized to minimize the consumption of chemicals/reagents as a cost-effective device.

Materials and Methods

Chemicals and reagents

All chemicals used were of analytical reagent grade. Deionized water from Simplicity 185 (Millipore, Billerica, MA, USA) with resistivity 18.2 MΩ cm was used throughout the experiments. A 1000 mg L^{-1} aluminium (Al) standard solution (Merck, Darmstadt, Germany) was used in all the experiments. Working standard solutions of Al with different concentrations were prepared by appropriately diluting the stock solution. Hydrochloric acid (HCl, 37%) (Univar, Ingleburn, New South Wales, Australia) was used to digest the samples. Acetate buffer solutions at different pH were prepared from sodium acetate and acetic acid (Carlo Erba, Milano, Italy).

Instrumentation

A photo absorbance sensor, PiCOEXPLORER™ Model PAS-110 (Ushio Inc., Japan), controlled by a PAS-110 application via a smart phone/tablet mobile device was used for micro-scale colorimetric determination. A visible spectrophotometer, Genesys™ 20 (Thermo Fisher Scientific, USA) with Thermo Scientific VISIONlite 5 software was used for scanning the absorbance wavelength. Ultrasound-assisted extraction was carried out using a high power ultrasonic cleaning unit, Sonorex Digitec DT 100/H (Bandelin Electric GmbH & Co. KG, Berlin, Germany) with technical specifications: timer 0-30 min, 230 V, 50/60 Hz, frequency 35 kHz, and built-in heater 20 - 80 °C. All pH measurements were conducted using a pH meter (Model 713, Metrohm, Switzerland).

Preparation of natural reagent: Plant materials

Sappan heartwood powder (> 5 kg) was purchased from a local retail shop and dried in an oven at 50 °C for 24 h, storing in a dry place until use.

Ultrasound-assisted extraction method

Plant powder (0.5g) was accurately weighed into centrifuge tubes with caps (15 mL capacity) and 10 mL of 1 M acetate buffer solution pH 5.5 was added. The resulting solution were then mixed by a vortex and allowed to stand for 10 min at room temperature, termed as presonication time. The tubes were then immersed in an ultrasonic water bath and subjected to ultrasonic energy at 35 kHz for 30 min, termed as sonication time. The ultrasonic bath was set at 80 °C using a built-in heater. After sonification, the extract was centrifuged at 6000 rpm for 5 min, then passed through a filter paper (Whatman® No.1, UK) and made up to a volume of 10 mL with 1 M acetate buffer solution pH 5.5.

Conventional extraction method

Plant powder (5.0g) and deionized water (100 mL) were mixed in an extraction flask and heated on a hotplate until boiling. The extract was boiled for 10 min. After cooling, the extract was passed through a filter paper (Whatman® No.4, UK) and made up to a volume of 100 mL with deionized water.

Sample preparation: Pharmaceutical samples

Twelve aluminium hydroxide ($Al(OH)_3$) gel samples as suspensions (S1 – S6) and tablets (T1 – T6) were bought from local drug stores in Maha Sarakham Province, Thailand. Before analysis, 20 tablets were manually ground to powder and homogenized with an agate mortar and pestle.

Ultrasound-assisted extraction method

An accurately measured quantity of gel or fine powder (in case of tablet) as 0.024 to 0.030g of aluminium hydroxide (0.4-0.8 mL of aluminium hydroxide gel suspension, 0.010-0.020 g of aluminium hydroxide gel tablet) was transferred to a glass test tube. Then, 0.3 mL of hydrochloric acid was added and the mixture was immersed in an ultrasonic water bath for 10 min at 40 °C using a built-in heater. After sonification, the solution was cooled, filtered (in case of tablet) and diluted with 10 mL of deionized water.

Official method

A stock solution of the suspension was prepared following the pharmacopoeia procedures [32]. Briefly, the method involved the transfer of an accurately measured quantity of gel or fine powder (in case of tablet), equivalent to 1.2 to 1.5 g of aluminium hydroxide into a beaker. Then, 15 mL of hydrochloric acid was added and the mixture was heated gently until completely dissolved. The solution was then cooled, filtered (in case of tablet) and diluted with deionized water in a 500 mL volumetric flask.

Micro-volume colorimetric assay

This assay was performed in a micro-centrifuge tube (200 µL capacity), with reactive solutions measured by a portable colorimeter. For the construction of standard curves, 90 µL of natural reagent was added to each micro-centrifuge tube, followed by aliquots of eight different concentrations (0, 0.5, 1, 2, 4, 6, 8, and 10 mg/L) of aluminium standard solutions. Deionized water was used to make up the volume to 150 µL per tube. The contents were then mixed by shaking and kept at room temperature for 20 min, followed by reading at 530 nm (green LED) using PiCOEXPLORER™.

Results and Discussion

Ultrasound-assisted extraction of natural reagent from Sappan heartwood: Type of solvent

Normally, the extraction solvent has a strong impact on extraction yield by the solubility of the target analytes and also by physical parameters such as viscosity, vapor pressure and surface tension of the solvent [33]. From our previous study [20], the extraction solvent types (boiling with water, and maceration with different polar solvents, such as methanol, ethanol, and acetone) were optimized. An aqueous extract by boiling Sappan heartwood with water was chosen due to its simplicity and reduced chemical usage. After aqueous extraction, the extracts were mixed with sodium acetate and acetic acid to make a buffer solution of pH 5.5. This pH was chosen from its high sensitivity when comparing to other studied pH ranges from 3.0-6.0 [20]. However, the disadvantages of this method include tedious preparation steps and high consumption of water and chemicals (≈100 mL per batch per extraction). Therefore, ultrasound-assisted extraction (sonication time 30 min, temperature 80 °C) using 0.5 g of plant powder with water and acetate buffer pH 5.5 (10 mL) as extraction solvent was investigated for extraction efficiency compared to the conventional extraction method at the same mass ratio per extractant volume. Absorption spectra at 400-700 nm were recorded for the obtained solutions as shown in Figure 2. The conventional extraction method by boiling with water gave the highest absorbance of reagent extracts and reagent-Al complex. The decrease in absorption of the reagent and reagent-Al complex spectra obtained from ultrasound-assisted extraction was due to mass transfer limitation of soluble constituents from the material to the solvent by diffusion [34]. The absorption spectra obtained from ultrasonic energy with buffer solution were higher than with water. Therefore, acetate buffer pH 5.5 was chosen as the solvent for ultrasound-assisted extraction.

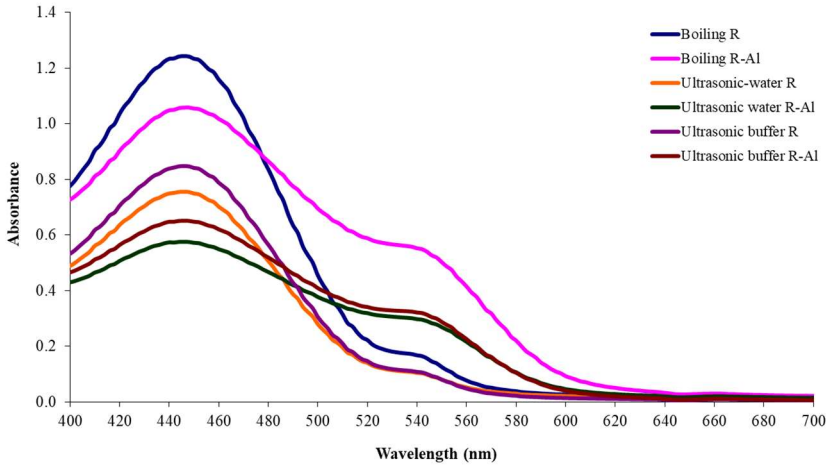


Figure 2. Absorption spectra of extracts from Sappan heartwood using different extraction procedures (boiling with water and ultrasonic-assisted extraction) and different extraction solvents (water and acetate buffer pH 5.5)

Effect of Sappan heartwood mass

In general, an increase in the solid sample mass improves sensitivity due to the transfer of higher amounts of analyte to the liquid phase. In this experiment, the volume of the extractant solvent was kept constant at 10 mL, while the mass of Sappan heartwood powder utilized in the extraction was varied from 0.1 to 1g. All other conditions were set according to the previous optimization experiments, with results shown in Figure 3. The absorbance of the reagent (at 446 nm) and reagent-Al complex (at 530 nm) increased with increasing amounts of plant powder from 0.1-0.5g, then slightly increased before remaining constant. A ratio of 0.5g of plant material per 10 mL of acetate buffer was selected as suitable for the extraction procedure from its high extraction reproducibility.

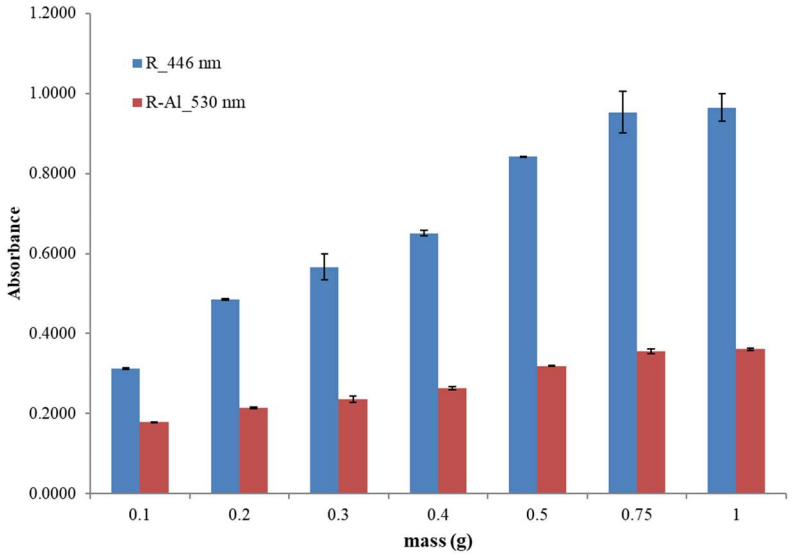


Figure 3. Effect of plant powder mass on the extraction of natural reagent performed in 10 mL of acetate buffer pH 5.5 with sonication time 30 min and temperature 80 °C

Extraction time

Long extraction time enhances extraction yields, but long extraction time may induce undesirable changes in the extracted compounds [35]. The influence of sonication time (10, 20, 30, 40, 50, and 60 min) on the yield of natural reagent extracts from Sappan heartwood using buffer pH 5.5 as extraction solvent was investigated with results shown in Figure 4. The yields of brazilein gradual increased from 10 to 30 min. After 30 min, there was a slight decrease in the yield, possibly due to degradation of the extracts at longer extraction times. This result indicated that ultrasonic irradiation played an important role in the extraction process. The increasing of the absorbance at 60 min might be due to the absorption of other species during degradation or the low reproducibility of the extraction process. An extraction time of 30 min was selected as suitable.

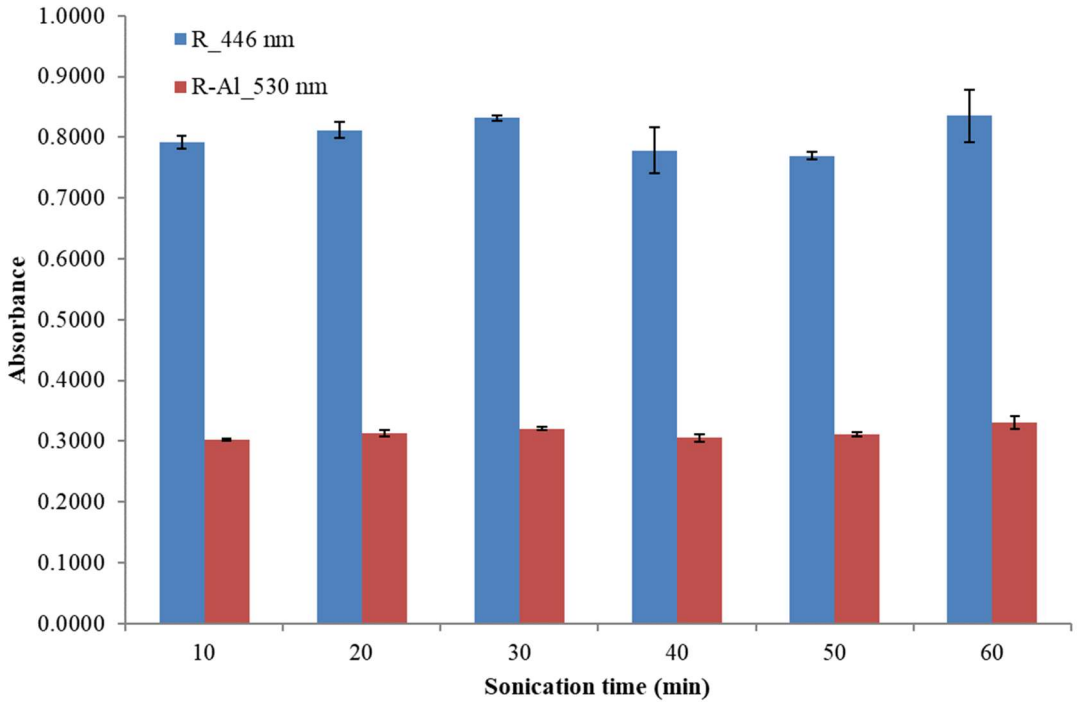


Figure 4. Effect of sonication time on the extraction of natural reagent performed with 0.5g of plant powder and 10 mL of acetate buffer pH 5.5 at 80 °C

Temperature

The extraction temperature is also important. Temperature contributes to efficiency of extraction. Usually, increase of temperature leads to an increase of extraction yield [33]. In this study, using an ultrasonic cleaning bath with built-in heater from 20-80 °C, the effect of different temperatures (30, 40, 50, 60, 70, and 80 °C) on the extraction yield was investigated. All other conditions were set according to the optimal parameters obtained above. Results are displayed in Figure 5. Extraction yield increased as temperature increased from 30 to 80 °C, showing that sonication temperature had a profound influence on the extraction of active ingredients from plant materials. Extraction temperature at 80 °C was selected as suitable.

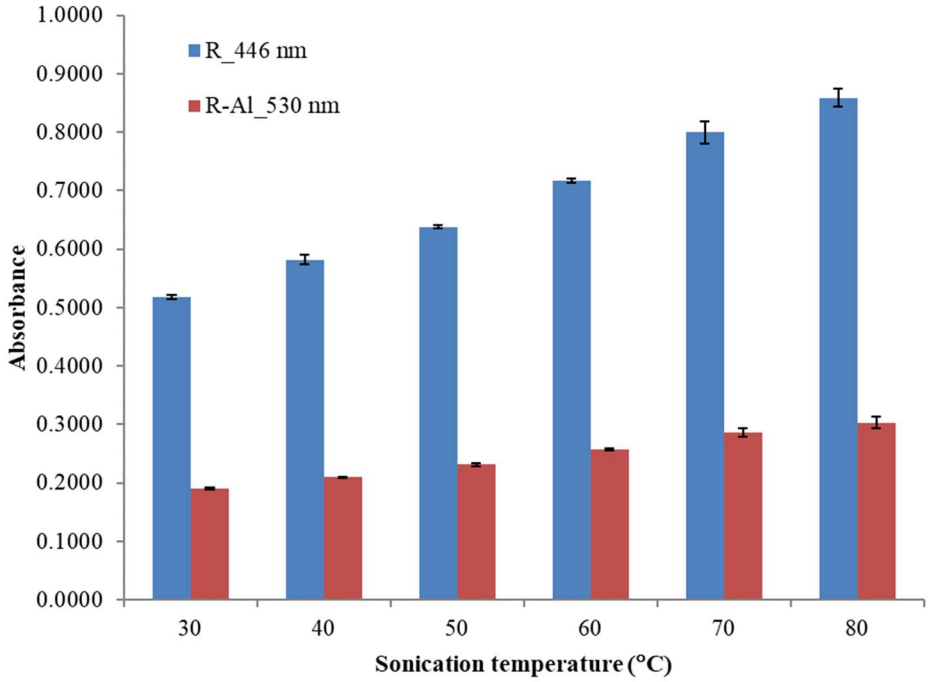


Figure 5. Effect of sonication temperature on the extraction of natural reagent performed with 0.5g of plant powder and 10 mL of acetate buffer pH 5.5 for 30 min

Extraction efficiency and reproducibility of crude plant extracts

After optimization, the extraction efficiency of the proposed small-scale ultrasonic-assisted extraction was lower than the conventional extraction by boiling with water (relative absorbance of 50%). However, the crude plant extracts contained sufficient active compounds to reacts with Al. To ensure the reproducibility of the various batches of natural reagent extracts for future use, the precision of absorbance of 10 batches of extracts prepared independently was evaluated. The relative standard deviation (%RSD) for the 10 batches was 3.8% and lower than the conventional extraction procedure [20]. The proposed small-scale ultrasonic-assisted extraction procedure had advantages over the conventional extraction procedure such as ease of operation, low chemical consumption, and high precision.

Determination of Al by micro-scale colorimetric procedure

Aluminium analysis was conducted using crude plant extracts containing brazilein. The complexation ratio of Al with brazilein in the crude plant extracts was 1:2 [22] with 530 nm the maximum complexation absorption wavelength. A marked change in color of the natural reagent with Al from yellow to reddish was observed.

Effect of mixing volume ratio of sample and reagent

The concentration of natural reagent extract used was 0.5g of heartwood /10 mL of buffer pH 5.5. The effects of sample and reagent volumes on the sensitivity of the system were studied at mixing volume ratios of Al:reagent 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:4, 8:2, and 9:1 for a total volume of 150 µL. As shown in Figure 6, the mixing volume ratio of Al:reagent at 4:6 (60:90 µL) gave the highest sensitivity and was selected as the optimal condition.

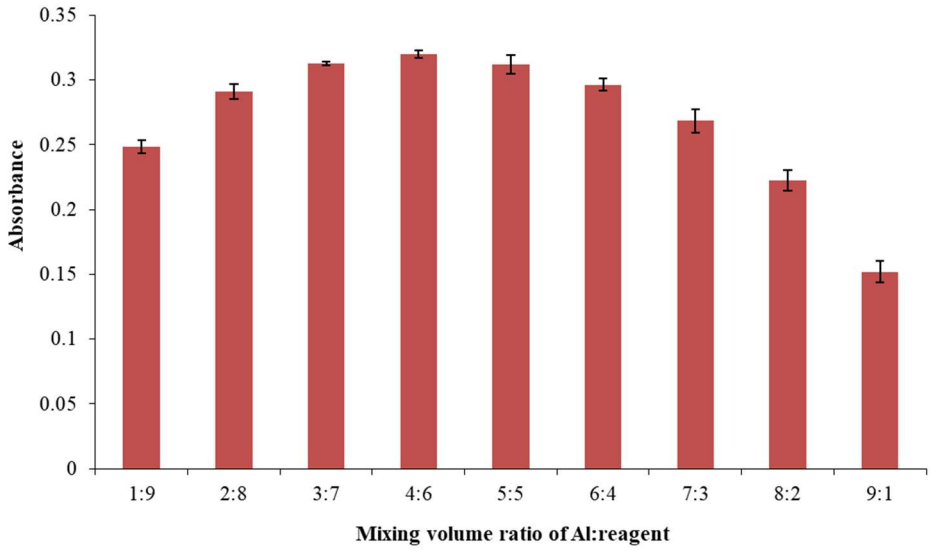


Figure 6. Effects of mixing volume ratios of sample (Al) and natural reagent extracts from Sappan heartwood at pH 5.5 on the sensitivity of the proposed micro-volume colorimetric measurement at 530 nm

Analytical features of the proposed system

The analytical characteristics of the proposed colorimetric assay were investigated. Using the optimal extraction conditions and the mixing volume ratio described above, standard calibration in the range 0.5-10 mg L^{-1} was constructed by plotting the absorbance against Al concentration. A linear graph was obtained with the calibration equation y = (0.0528±0.0024)x + (0.0111±0.0032) and R^2 = 0.9977, as shown in Figure 7. The limits of detection and quantification for Al were 0.07 and 0.37 mg L^{-1}, respectively with relative standard deviations for ten replicate determinations of 1 and 3 mg L^{-1} 2.3 and 1.3%.

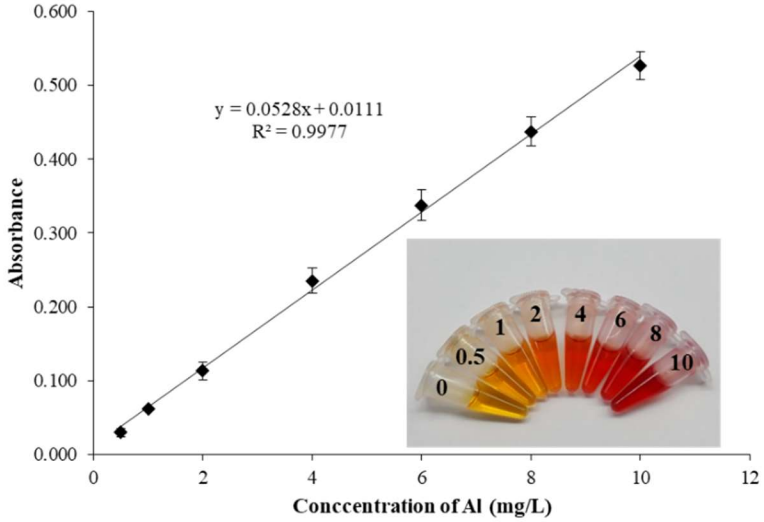


Figure 7. Visual detection and calibration curve of the aluminium standard with natural reagent extracts from Sappan heartwood at pH 5.5 obtained from micro-volume colorimetric measurement at 530 nm

The analytical performance of the proposed method was compared with previous methods using various natural reagents, as summarized in Table 1. The proposed method was not as sensitive as some other flow-based systems but the sensitivity was sufficient to measure Al in pharmaceutical samples (2.4-3.0 g/L). Our proposed micro-volume colorimetric assay consumed only 150 µL of each sample/standard and reagent solution per analysis. This assay also reduced contamination risk and analysis time and allowed portable on-field measurements.

Table 1. Analytical characteristics of the colorimetric determination method for Al using various natural reagents

Method	Natural Reagent	Extraction Method	Linear Range (mg L^{-1})	LOD (mg L^{-1})	Sample Volume (µL)	Ref.
Flow injection	Indian mulberry root extract	Maceration with 80%v/v acetone	0.1 – 1.0	0.05	75[a]	[18]
Flow injection	Indian almond leaf extract	Blending with acetate buffer pH 4.8	0 – 100	0.8	100[a]	[19]
Sequential injection	Sappan heartwood extract	Boiling with water	0.075 – 1.0	0.021	300[b]	[20]
Naked-eye detection	Cyanidin extracted from red cabbage	Extracted with MeOH and 2 M HCl (85:15 v/v)	1.35 – 2.70 (detection range)	1.35	1300[b]	[36]
Naked-eye and Vis-spectrophotometry	Pectin-rich apple extract-based gold nanoparticles	-	0 – 2.7	0.54	-	[37]
Naked-eye detection	Anthocyanin from black rice immobilized in carboxymethylcellulose/starch film	-	0 - 10	3.0	30 mL	[38]
Smartphone-based	Commercially fermented black carrot juice	-	1 – 50	0.2	2500[b]	[39]
Micro-volume colorimetry	Sappan heartwood extract	Small scale UAE	0.5 – 10.0	0.07	150[b]	This work

[a] sample volume only

[b] total analysis volume (sample+reagent+other solutions)

The effects of interfering species on the Al-natural reagent complex were investigated using the proposed method under optimal conditions. Various concentrations of foreign ions (Na^{+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, and Fe^{3+}) were spiked into a standard solution of 3 mg L^{-1} Al. An interfering concentration was considered as the value that caused signal variations higher than ±10%. Results showed that the system tolerated spiked cations up to at least a 1:1 concentration ratio of Al to interfering cations. Higher interference ratios were not studied since these minerals are normally

found in pharmaceutical formulations at lower amounts than Al compounds.

Ultrasound-assisted extraction of Al in pharmaceutical formulations

Aluminium hydroxide gel in suspension and tablet forms was used for optimization. The extraction efficiency or percentage label values (by comparing the amounts of aluminium found in aluminium hydroxide gel samples with their label values) of the results provided by ultrasonic variable parameters were evaluated under the optimal conditions. The mass of the sample, volume of the solvent, and type of solvent followed the standard method [32].

Sonication time

The influence of sonication time (1, 5, 10, 15, and 20 min) on the extraction efficiency of aluminium from aluminium hydroxide gel using hydrochloric acid as the extraction solvent and temperature 80 °C was investigated with results shown in Figure 8. Normally, long extraction time enhances extraction yields [35]. High percentage label of aluminium was obtained within 1 min for aluminium hydroxide gel suspension, while 10 min was required for the aluminium hydroxide gel tablet. Results indicated that sonication time played an important role in the extraction process of aluminium, especially in the form of aluminium hydroxide gel tablet. Therefore, an extraction time of 10 min was selected as suitable.

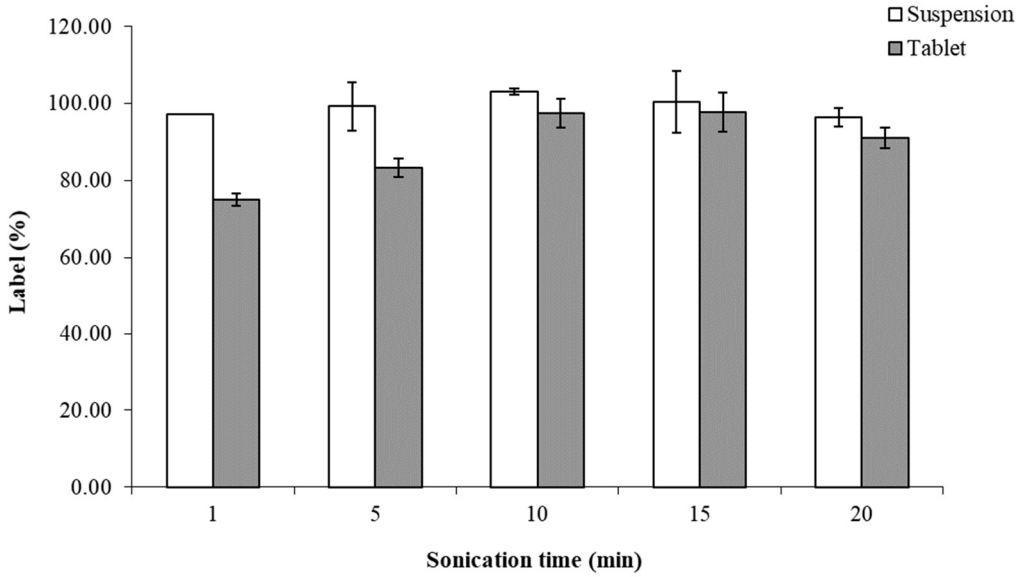


Figure 8. Effect of sonication time on the extraction of aluminium performed with 0.4 mL of $Al(OH)_3$ gel suspension or 11 mg of $Al(OH)_3$ gel tablet powder and 0.3 mL of concentration HCl at 80 °C

Temperature

The choice of extraction temperature is also important. The temperature strongly impacts the solvent's properties [33]. High temperature enhances solvent diffusion rates, while, low temperature enhances cavitation [35]. In this study, different temperatures (40, 50, 60, 70, and 80 °C) were investigated for extraction efficiency. All the other conditions were set according to the optimal parameters, with results displayed in Figure 9. High temperature during sonication did not influence the extraction of aluminium from aluminium hydroxide gel suspension and tablet. It might be due to aluminium ions were easily to dissolve from aluminium hydroxide gel under the acidic condition. Therefore, an extraction temperature of 40 °C was selected.

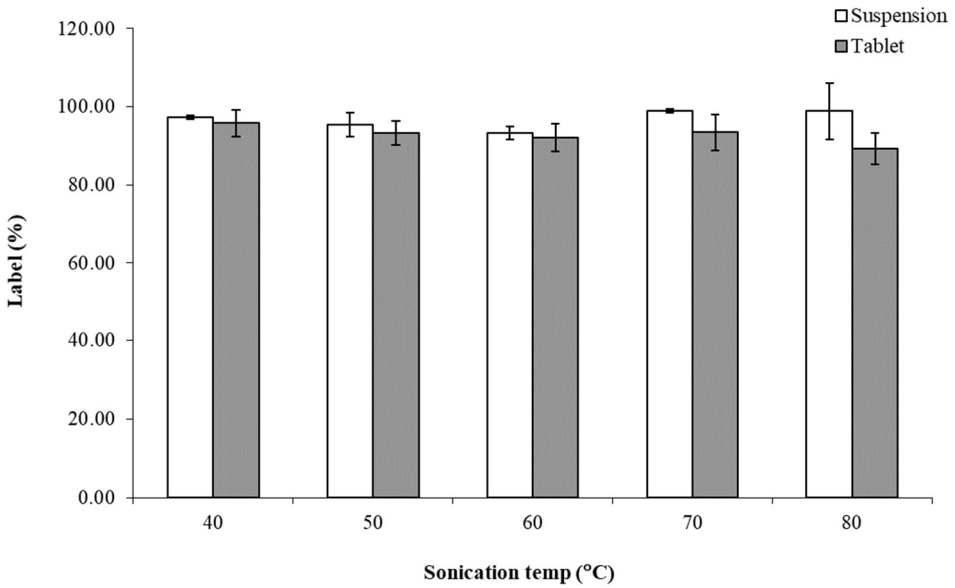


Figure 9. Effect of sonication temperature on the extraction of aluminium performed with 0.4 mL of $Al(OH)_3$ gel suspension or 11 mg of $Al(OH)_3$ gel tablet powder and 0.3 mL of conc. HCl for 10 min

Extraction efficiency

Small-scale ultrasound-assisted extraction efficiency was evaluated by comparing the results obtained from the official method for pharmaceutical formulations, as presented in Table 2. Satisfactory recoveries for both procedures were obtained at 103.3 and 96.8% for suspension and tablet forms, respectively. Results indicated that the extraction efficiency of the proposed small-scale ultrasound-assisted extraction procedure was comparable to the official procedure. Relative standard deviations of the aluminium concentration obtained after extraction by the proposed procedure were lower than for the official procedure.

Table 2. Extraction efficiency of the proposed ultrasound-assisted extraction procedure under optimal conditions

Pharmaceutical Form	Amount of Aluminium Hydroxide (mg) in 15 mL of Suspension or 1 Tablet		% Recovery[a]
	Reference Method (n = 10)	Proposed Method (n = 10)	
Suspension (960 mg Al / 15 mL)	960.5 ± 36.9 (3.8% RSD)	992.4 ± 12.9 (1.3% RSD)	103.3
Tablet (188.8 mg Al / tablet)	186.0 ± 9.4 (5.1% RSD)	180.0 ± 6.4 (3.6% RSD)	96.8

[a]Recovery (%) = [proposed method / reference method] x 100

Application to pharmaceutical samples

The proposed assay was used to determine aluminium content in pharmaceutical preparations, and the amounts of aluminium found in aluminium hydroxide gel samples were compared with their label values. Results are presented in Table 3. The aluminium contents in aluminium hydroxide gel suspension and tablet samples were acceptable at 90.0 to 110.0 % of the labeled amount of aluminium hydroxide [32]. Satisfactory recoveries ranging 91 to 107% for pharmaceutical samples were obtained.

Table 3. Determination of aluminium in pharmaceutical formulations (n = 3)

Sample[a]	Amount of aluminium hydroxide (mg) in 15 mL of suspension or 1 tablet		% Relative error[b]	%Recovery
	Label value	Found		
S1	960	986.3 ± 13.5	2.7	97.2 ± 3.4
S2	918	938.1 ± 6.9	2.2	101.2 ± 2.7
S3	900	910.7 ± 8.8	1.2	100.6 ± 3.5
S4	900	930.9 ± 7.4	3.4	104.0 ± 4.7
S5	600	603.4 ± 13.1	0.6	103.8 ± 3.6
S6	459	465.5 ± 6.6	1.4	106.6 ± 1.2
T1	188.8	183.8 ± 3.0	-2.7	90.9 ± 2.6
T2	191.3	188.7 ± 2.7	-1.4	91.2 ± 1.2
T3	248.6	235.2 ± 6.5	-5.4	95.2 ± 9.1
T4	191.3	179.8 ± 1.1	-6.0	101.0 ± 4.8
T5	188.8	184.6 ± 15.8	-2.2	104.4 ± 6.2
T6	153	147.6 ± 4.3	-3.5	94.6 ± 10.1

[a]S1-S6 : aluminium hydroxide gel suspension, T1-T6 : dried aluminium hydroxide gel tablet
[b]relative error = [(found - label value) / label value] x 100

Greenness assessment

The greenness of the proposed method was compared with the official method for Al in pharmaceutical sample [32] according to Analytical GREEnness (AGREE) metrics approach as seen in Figure 10. The calculation of the AGREE metrics software carried out by the application depends on 12 parameters that correspond to the 12 green analytical chemistry (GAC) principles [40]. Every principle or factor has a score range. According to the AGREE method, the proposed method scored 0.84 and stood out with portable miniaturized system, less amount of samples/reagent, less amount of waste, low energy consumption for sample pretreatment, high sample throughput and the use of bio-based reagents, while the reference method scored 0.42.

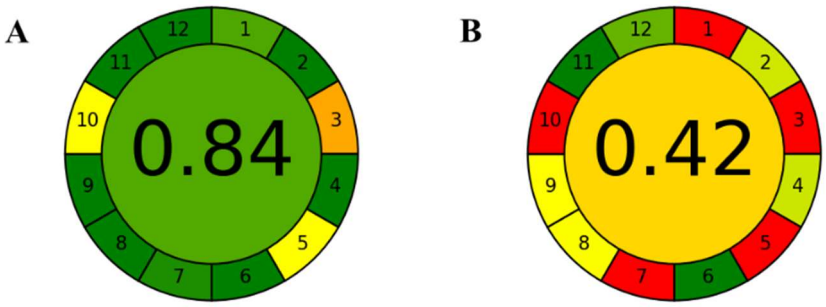


Figure 10. Results of AGREE analysis for the proposed micro-volume colorimetric assay (a) and the official method (b) for Al in pharmaceutical sample

Conclusions

A simple, reliable, cost-effective, and green analytical approach for the colorimetric determination of aluminium in pharmaceutical samples was presented. High efficiency sample preparation procedures based on ultrasound-assisted extraction were successfully applied

for small-scale extraction of natural reagent from Sappan heartwood, and also for aluminium from aluminium hydroxide gel samples. High throughput of more than 12 samples treated simultaneously was achieved using only a simple ultrasonic bath with a built-in temperature control. Our proposed micro-volume colorimetric assay significantly increased performance for the determination of aluminium in terms of cost-effectiveness, field-portability, and low chemical consumption. Satisfactory recoveries and high sample measurement frequency suggested that our proposed system showed high potential as a good alternative assay for the quality assurance of antacid suspensions and tablet products in the pharmaceutical industry. The "naked-eye" detection of this green approach can be used as a screening method for high aluminium content in wastewater samples from factories to improve environmental safety for the local residents.

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THE INFLUENCES OF SEASONAL MONSOONS UPON PHOSPHORUS, CHLOROPHYLL-A AND PHYSICAL CHARACTERISTIC OF THE KELANTAN WATERS

(Kesan Monsun Bermusim ke atas Fosforus, Klorofil-a dan Ciri-ciri Fizikal di Perairan Kelantan)

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Abstract

The influences of seasonal monsoons on the variability of phosphorus (phosphate (PO_4^{3-}), dissolved organic phosphorus (DOP), total particulate phosphorus (TPP)), chlorophyll-a, and physical characteristics in the water columns of Kelantan Waters were investigated based on the datasets collected by three cruises during the southwest monsoon (SWM), inter-monsoon (IM), and northeast monsoon (NEM) seasons. Concentrations of PO_4^{3-} were determined using a discrete analyzer and standard colorimetric techniques. DOP and TPP were measured using an indirect method that involved a UV digestion system. This system utilizes UV light to oxidize organic phosphorus and total phosphorus compounds into PO_4^{3-}, thereby making them measurable. Surface and vertical profiles were investigated and showed that almost all parameters measured showed significant differences ($p < 0.05$) among seasons. The seasonal trends in the surface and vertical distributions of PO_4^{3-}, DOP, and chlorophyll-a concentrations were found to have peaked during the NEM, followed by the SWM and IM periods. The combination factors of heavy rainfalls, large river discharges, and stronger wind-induced processes during the NEM created conditions conducive for the enhancement of PO_4^{3-}, DOP, and chlorophyll-a concentrations in the study area. Principal Component Analysis (PCA) demonstrated a strong correlation between chlorophyll-a and PO_4^{3-}, suggesting that PO_4^{3-} supply was the dominant source for phytoplankton biomass (chl-a) survival during our sampling period. This study has provided valuable information about phosphorus dynamics in the Kelantan Waters, establishing a baseline for future studies.

Keywords: intermonsoon, northeast monsoon, phosphate, dissolved organic phosphorus, South China Sea

Abstrak

Pengaruh musim monsun ke atas kepelbagaian fosforus (fosfat (PO_4^{3-}), organik terlarut fosforus (DOP), jumlah partikulat fosforus (TPP)), klorofil-a, dan ciri fizikal dalam turus air di perairan Kelantan telah dikaji berdasarkan dataset yang dikumpul oleh tiga pelayaran semasa monsun barat daya (SWM), peralihan-monsun (IM), dan monsun timur laut (NEM). Kepekatan PO_4^{3-} ditentukan menggunakan pengnalisis automatik diskret dan kaedah kolorimetrik piawai. DOP dan TPP diukur menggunakan kaedah tidak langsung yang melibatkan sistem pencernaan UV. Sistem ini menggunakan cahaya UV untuk mengoksida fosforus organik dan

sebatian jumlah fosforus menjadi PO_4^{3-}, menjadikannya boleh diukur. Profil permukaan dan menegak telah dikaji, dan menunjukkan hampir semua parameter yang diukur adalah berbeza secara signifikan ($p < 0.05$) di antara musim. Kami dapati trend musim dalam taburan permukaan air dan menegak kepekatan PO_4^{3-}, DOP, dan klorofil-a mencapai puncaknya semasa monsun timur laut, diikuti oleh monsun barat daya dan tempoh perlihan-monsun. Gabungan faktor hujan lebat, sungai besar, dan proses yang diperkuatkan oleh angin semasa monsun timur laut mencipta keadaan yang menyokong peningkatan konsentrasi PO_4^{3-}, DOP, dan klorofil-a di kawasan kajian. Analisis komponen utama (PCA) menunjukkan korelasi kuat antara klorofil-a dan PO_4^{3-}, menunjukkan bahawa bekalan PO_4^{3-} adalah sumber dominan untuk fitoplankton (chl-a) biojisim semasa tempoh penyempelan kami. Kajian ini memberikan maklumat berharga mengenai dinamik fosforus di perairan Kelantran and sebagai garis asas untuk kajian masa depan.

Kata kunci: monsun peralihan, monsun timur laut, fosfat, fosforus organik terlarut, Laut China Selatan

Introduction

Phosphorus (P) is long-recognized as an essential nutrient for life, required by all living organisms for growth, energy transport, and membrane synthesis [1, 2]. Phosphorus is present in oceans as inorganic phosphate (i.e., orthophosphate (PO_4^{3-}) and condensed phosphate) and organic phosphate in both dissolved and particulate forms (i.e., dissolved organic phosphorus (DOP) and particulate phosphorus (PP) [3, 4]. Microbial organisms can access a variety of P sources to satisfy nutritional P demands and preferentially utilize orthophosphate, as it can be directly consumed and assimilated, whereas DOP generally requires more energy for conversion into PO_4^{3-} before its metabolic assimilation [5, 6]. However, PO_4^{3-} is present in low concentrations, which is potentially limiting for microbial organisms in the oligotrophic oceans (tropical and subtropical) [1, 7]. These oligotrophic oceans are subject to smaller variations of seasonal temperature than temperate waters and are permanently underlain by a strong thermocline, which is considered the least variable and the least productive in the oceans' surface waters [8]. Microbes actively utilize DOP as an alternative P source when PO_4^{3-} is depleted in the ocean [7, 9-12]. DOP is a complex mixture of organic compounds in the ocean, and is predominantly mediated by phytoplankton through cell excretion, exudation, death, and autolysis [11], and can also be derived from organic compounds discharged from industrial, agricultural, and domestic sewage [12]. The recycling and utilization of DOP play a significant role in primary productivity, which may support approximately 90% of gross primary productivity [13, 14]. Meanwhile, most of the P is transported to the ocean by mainly being attached to the particles phase as total particulate phosphorus (TPP) through riverine input [15, 16], where TPP includes clay and silt-associated organic and inorganic P [9]. Additionally, living microorganisms attached in TPP (98%) are higher than that in particulate C (60%) and N (49%), and thereby result in low TPP in particular detrital sedimentary fractions as evidence of preferential utilization of P [7]. Thus, P availability in the ocean has significantly impacted primary productivity.

The Kelantan waters are located in the east coast of Peninsular Malaysia (ECPM), and lie in the southern region of the South China Sea, bordering the Gulf of Thailand. Kelantan Waters form a system that has a high variability of hydrographical factors predominantly based on its monsoon season. Four different monsoon seasons affect this region, i.e., the northeast (Nov–Feb), southwest (May–Aug), and two transitional shorter inter-monsoon (Mar–Apr and Sep-Oct) periods, which can be distinguished based on their wind flow [17, 18]. The northeast monsoon (NEM) is associated with the strong north-easterly winds that generate a strong southward current along the ECPM, and usually bring heavier rainy seasons and rough seas over this region [17, 19]. Meanwhile, the southwest monsoon (SWM) season generates weaker south-westerly winds and drives northward currents along ECPM, and typically receives minimum rainfall and drier weather regions [17, 19]. The prevailing SWM winds with the north-south orientation of the coastline induce offshore Ekman transport, thus being favourable for upwelling along the ECPM, and unfortunately, less favourable in Kelantan Waters [20,21]. The two inter-monsoons (IM) are referred to as the first IM period (March-April) and the second IM period (Sept-Oct), and have unique features of wind direction and surface current, which are characteristics similar to SWM and NEM accordingly,

but are relatively slow and driven by limited mixing [17, 22].

The selected parameters of phosphorus (PO_4^{3-}, DOP, and TPP), chlorophyll-a, and physical characteristics (salinity and temperature) are interlinked, forming a complex web of relationships that govern nutrient dynamics and ecosystem health. PO_4^{3-}, DOP, and TPP collectively provide a comprehensive view of phosphorus cycling, where PO_4^{3-} concentration in the water column directly affects the nutrient availability for phytoplankton biomass (Chl-a), establishing a fundamental link between phosphate levels and overall ecosystem productivity. Furthermore, DOP, represented as an organic form of phosphorus, is intricately linked to nutrient dynamics, and DOP measurement provides insights into the bioavailability of phosphorus, impacting microbial and algal communities. It is also important to measure TPP concentrations, which are associated with suspended particles and sediments, reflecting the interactions between nutrients and the physical environment. Moreover, chl-a serves as a proxy for primary productivity. The relationship between chl-a levels and nutrient concentrations, especially phosphate, is crucial for understanding the impact of nutrient availability on algal biomass and the overall ecosystem health in the study area. Meanwhile, salinity and temperature contribute to the physical context that influences nutrient distribution and biological processes in the water column. The existing knowledge regarding nutrient distribution in Kelantan Waters, particularly in terms of vertical distribution, remains notably scarce.

While previous studies have explored phosphorus (P) nutrient distribution in Peninsular Malaysia Waters during the southwest monsoon (SWM) [21, 23, 24], northeast monsoon (NEM) [23], and inter-monsoon (IM) [25], the focus on nutrient distribution within Kelantan Waters, especially in the vertical profiling in the water column, is still limited. Recognizing this critical gap, our study has aimed to comprehensively investigate the spatial distribution of P-based nutrients (PO_4^{3-}, DOP, and TPP), chlorophyll-a, and the physical characteristics (temperature and salinity) across both, the surface and vertical profiles in Kelantan Waters. In addition, this research has also sought to evaluate the temporal patterns of nutrient variations in response to the three monsoon seasons (IM, SWM, and NEM), and identify the key environmental factors governing these patterns. By addressing this research gap, our study contributes essential insights crucial for the effective and robust monitoring of nutrient dynamics in the Kelantan Waters. The interplay between all parameters measured would help researchers and environmental managers grasp the intricate relationships within the water column.

Materials and Methods

Study area and sampling activity

The study area of Kelantan Waters is located between 102° to 105° E longitude (Figure 1), which lies within the shallow continental shelf with less than 100 m in water depth. This study was part of the cruise expedition "HICOE 2017". Three cruises to the Kelantan Waters aboard the RV Discovery occurred from 1-2 April, 4-8 July, and 16-18 November 2017, respectively. A total of 7 sampling stations were sampled, which were in the transect that is oriented perpendicular to the coast of Kelantan and is about 260 km towards the offshore region (Figure 1). The sampling area is influenced by anthropogenic activities from its adjacent city of Kota Bharu, mainly discharged through the Kelantan River [21, 23]. Seawater samples were collected in Niskin bottles attached to a CTD (Conductivity-Temperature-Depth, Sea-Bird) probe frame. At each sampling station, surface water samples (~ 0.5 m) were collected for surface distribution, while water samples for vertical profiling were taken at 5, 10, 20, 30, 45, and 60 m depths, respectively. The seawater samples for PO_4^{3-}, DOP, and chlorophyll-a analyses were vacuum filtered onboard, through pre-combusted (5 hours at 450ºC) and pre-weighed GF/F filters (0.7 µm pore-size, Whatman). The filtrate samples were transferred into acid-washed polyethylene bottles and immediately kept frozen at –20 °C to reduce all the activities and metabolism of the organisms in the water. Meanwhile, unfiltered samples were used for TPP determination. Chlorophyll-a filters were folded in half twice, and placed in a 15 mL centrifuge tube wrapped in aluminium foil to protect the phytoplankton from light. Both the filtrate samples and the chlorophyll-a filters were immediately stored at –20 °C until laboratory analyses.

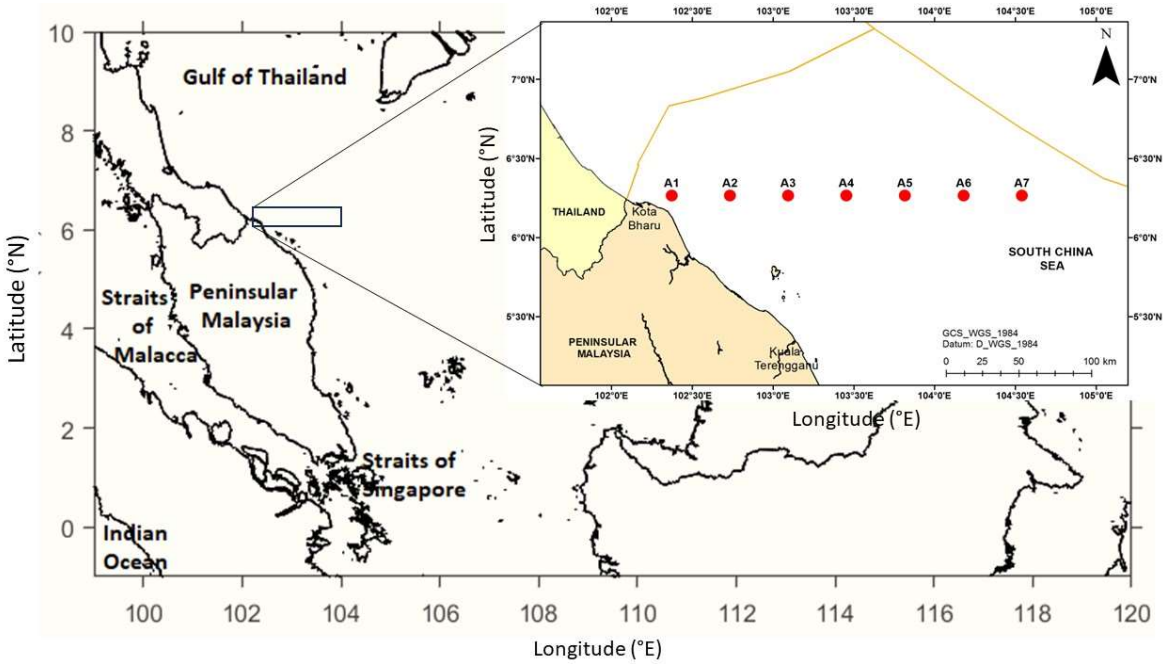


Figure 1. Map of the study area. Transect and stations are shown in the inset

Physical measurement

The physical data measurements began with temperature and salinity using a CTD sensor, and were displayed on a computer screen in the labs onboard. The sea surface current data were retrieved from the global ocean model, and the HYbrid Coordinate Ocean Model (HYCOM) global reanalysis (https://www.hycom.org/). The monthly means near-surface currents (u and v components of the current, unit: m/s) averaging over the top 10 m were used to investigate the influence of monsoons on the seasonal ocean circulation of the Kelantan Waters.

Laboratory analysis

In the laboratory, PO_4^{3-} samples were analysed based on standard colorimetric methods [26] using a SmartChem 200 discrete autoanalyzer (AMS Alliance), and were detected spectrophotometrically as a coloured complex. Basically, a water sample reacts with molybdate under acidic conditions (pH < 2) to form phosphomolybdic acid; the formed heteropolyacid is then reduced by ascorbic acid to produce a blue colour complex and detected at 880 nm using a SmartChem 200 discrete autoanalyzer. The determination of DOP relies upon the measurement of total dissolved phosphorus (TDP) and PO_4^{3-}, where DOP is calculated by subtracting PO_4^{3-} from TDP (DOP= TDP - PO_4^{3-}). TDP was first converted to measurable PO_4^{3-} by UV oxidation techniques using a Metrohm 909 UV digestion system, without the addition of any reaction initiators, catalysts, or oxidants. This process breaks down complex organic phosphorus forms, simplifying their quantification through analytical methods for inorganic phosphate PO_4^{3-}, which was subsequently measured using the same method as used for PO_4^{3-} determination above. This UV oxidation method is sufficient to oxidise 60–70% of DOP in the sample to PO_4^{3-} [27]. The unfiltered waters were used to determine the concentrations of TPP. The concentration of TPP measurement depends on the analyses of total phosphorus, TDP, and PO_4^{3-} (TPP = TP – TDP). TP was measured by converting all P forms to PO_4^{3-}, and the same method as used for TDP determination was applied. The Chl-a was determined using a UV-VIS spectrophotometer (Shimadzu 1201) after chlorophyll pigment was extracted from filters under subdued light in 90% (v/v) acetone and refrigerated at ~4 °C for 24 h [28].

Quality control (QC) measures were applied to ascertain the precision and accuracy of phosphorus determination

in this study, aligning with the EPA standard method [26]. These measures encompassed the analysis of duplicate samples, examination of blanks (utilizing deionized water treated as a sample), and the analysis of standard additions. Precision was evaluated through duplicate samples by estimating the standard deviation based on the results of a set of duplicate samples. The accuracy of measurements was assessed through the analysis of standards with known concentrations, reported as a percentage error. The percentage error was calculated using the following equation:

Percentage error = ((measured concentration – actual concentration) / actual concentration) x 100 (1)

Samples with error > 10% were reanalysed. The midrange standard concentrations and blanks were analysed periodically and simultaneously with the samples run at the beginning, intermediately and at the end of the run to confirm that instrument performance. The analyses yielded satisfactory results, with a percent measurement error staying consistently at less than 5%. Triplicates of every sample were analysed, and the concentrations of PO_4^{3-}, DOP, and TPP used here were the average of two measurements, and the overall analytical precision was within ± 2%. The detection limit was determined by calculating three times the standard deviations (DL=3SDb) of the blank measurement. The detection limit for phosphorus-based nutrients was found to be 0.05 μM P (n = 20).

Estimation of river nutrient input

Nutrient input from the Kelantan rivers, which were directly discharged to the study area, was estimated and calculated according to the following equation [23]

F = c x f (2)

where c is the nutrient concentration and f is the river flow. The information presented in this paper was calculated from data collected during the period of January-December 2017. The daily river flows in the Kelantan River are gauged and recorded by the Malaysian Department of Irrigation and Drainage (DID) and summed as monthly totals. Riverine nutrient concentrations were acquired from the Malaysian Department of Environment (DOE) for the Kelantan River estuary station, with sampling done at every two-three months of 2017. Unfortunately, load data is unavailable for the northeast monsoon season.

Statistical analysis

Statistical analyses were implemented by using the Xlstat 2019 (Addinsoft, France) software. We opted to apply a non-parametric test since most data distribution deviates from normality through the Shapiro-Wilk test (p<0.05, two-tailed). The Kruskal – Wallis with Bonferroni correction was employed as the post hoc analysis (at p<0.05), to test the significant differences of all parameters measured between stations (spatial pattern) and between monsoon seasons (temporal pattern). The degrees of potential correlations among the variables were identified through Spearman correlation analysis. The correlation was considered statistically significant when p-value < 0.05, and the strength of correlation was determined in three different classes (strong (r ≥ 0.60), moderate (r = 0.30 ≤, 0.59), weak r ≤ 0.29). Additionally, the compressed information of many correlated variables was further investigated through principal component analysis (PCA) to identify latent information generated from the spatiotemporal difference variations. In preparation for PCA, all variables were log-transformed to normalise their distributions, and eigenvalues >1 were considered significant principal components. Furthermore, to ensure the appropriateness of the data for PCA, Bartlett's test and Kaiser–Meyer–Olkin (KMO) was conducted. Bartlett's test should have a significance measure of less than 0.05 to indicate significant relationships among variables, and the minimum score of 0.60 was suggested for the KMO value. The high KMO values indicate a PCA with few errors [29].

Results and Discussion

Surface current

The surface current movement is largely controlled by wind, and Kelantan Waters' current circulation pattern is strongly influenced by monsoon winds. Figure 2 shows the sea surface current retrieved from the HYCOM in April, July, and November 2017, respectively, to represent current circulation during (A) IM, (B) SWM, and (C) NEM, respectively. In general,

the current direction during IM and NEM is similar, which moves towards the southward direction along Peninsular Malaysia; but both show different current patterns due to the stronger north-easterly winds occurring during NEM compared to IM. The variability of current circulation in the coastal area during IM due to winds is much weaker, which causes slow current movements, and which may be interrupted by tidal currents. Earlier research has proven that features of IM periods in East Coast Peninsular Malaysia include relatively weak winds as it is in prior monsoon seasons [17, 30], affected by local winds from the coast [17]. Strong north-easterly winds during NEM produce a stronger current that contributes to rough seas and brings colder water to Peninsular Malaysia [17, 31], with the current speed reaching up to 1.33 m/s during the sampling period. In contrast, surface currents during SWM showed a reverse direction, with the current circulation pattern flowing in the northward direction along the coast, with a maximum current speed recorded at 0.8 m/s. The prevailing winds during the SWM with the north-south orientation of the coastline are favourable for upwelling along the east coast of Peninsular Malaysia [20, 21]. However, so far, there has been no evidence of upwelling that can reach Kelantan Waters, because, as can be seen, the current movement diverts near 6˚N (Figure 2b) north-eastward towards the Vietnam coast [21, 32]. The variability of surface current circulation is an important factor to understand the oceanic environment. Strong water current causes water column mixing that may supply nutrients into the photic zone and vice versa.

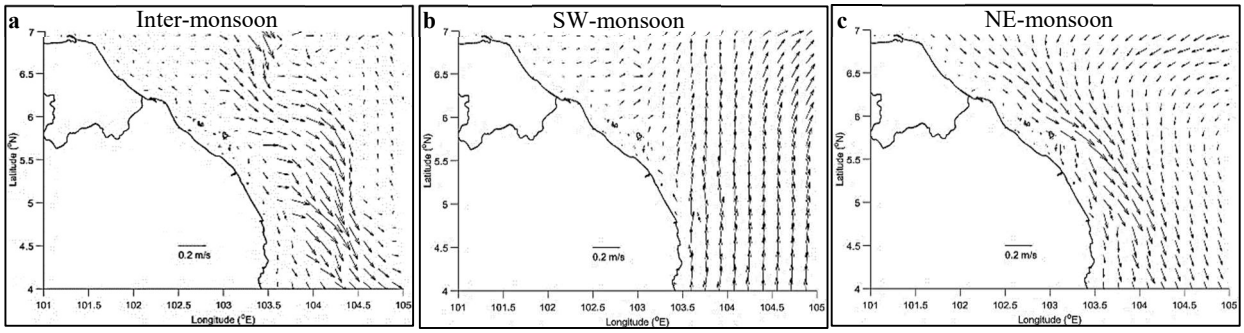


Figure 2. The seasonal variation of the mean surface current from HYCOM (unit: m/s) in Kelantan Waters

Kelantan river nutrient input

Kelantan River Nutrient Input in terms of PO_4^{3-} concentration was estimated based on the F = c x f equation. The results of Kelantan River Nutrient Input in terms of PO_4^{3-} concentration for April (IM) and July (SWM) were found to be 0.09 x 10^6 and 390 x 10^6 g per year, respectively. There were expectations of higher PO_4^{3-} inputs during November (NEM) based on higher rainfall and increased river discharge during NEM. Literature [23] recorded PO_4^{3-} riverine input from the river along the ECPM as 76% of its total annual input to ECPM waters in NEM, compared to SWM, where it was only 24%.

Surface distribution

The surface distribution of temperature, salinity, Chl-a, PO_4^{3-}, DOP, and TPP during three monsoon seasons for the seven stations area are illustrated in Figures 3 (a)-(r). The surface temperature in the study area exhibited relatively warm temperatures with a very slight variation, which was ubiquitous, with the surface temperatures reaching as high as 30 °C for all monsoon seasons. A clear decreasing gradient of surface temperatures toward the offshore region was recorded in the IM (28.26-30.4 °C; 29.13 ± 0.61 °C) (Figure 3a), while an opposite pattern was observed in SWM (29.56-30.7 °C; 30.08 ± 0.34 °C) (Figure 3b), with a few areas having a high temperature. Surface temperature measurements in NEM (Figure 3c) recorded low temperature values reaching 28 °C, especially in the coastal and middle areas (28-30.14 °C; 29.1 ± 0.52 °C). Figures 3d-f shows a surface salinity distribution pattern with low-salinity patches in the coastal area, which increase towards the open ocean. The lowest and the

highest salinity values of 28.18 psu and more than 34.16 psu were recorded during IM (31.98 ±2.0 psu), respectively. Meanwhile, the salinity pattern was slightly homogenous in the offshore areas during SWM and NEM, ranging from 29.69 – 33.38 psu (32.58 ± 1.3 psu) and 30.81 – 32.30 psu (31.81 ± 0.5 psu), respectively. The strong low-salinity distribution pattern observed in the coastal areas indicated the influence of freshwater from the adjacent river (Kelantan River) to our sampling area. This observation agrees well with existing studies in ECPM [21, 23, 25]. Overall, surface Kelantan Waters were characterised by relatively low salinity, and the warm temperature was variable between seasons. The Kruskal-Wallis test showed that salinity and temperature distributions were significantly different between seasons (p <0.05), but not significant among the stations (p>0.05).

The Chl-a gives an overview of phytoplankton biomass featuring monsoonal season influence. Surface Chl-a levels were significantly low with concentrations less than 0.2 µg/L, which varied from 0.03 to 0.17 µg/L (0.07 ±0.05 µg/L) during IM (Figure 3g), and 0.03-0.14 µg/L (0.07 ±0.04 µg/L) during SWM (Figure 3h), respectively. Both monsoon seasons were noticed with relatively uniform distribution, with a gradual decrease in Chl-a concentrations from coastal toward offshore stations. Meanwhile, high chlorophyll patches were observed in the coastal area during NEM, with concentrations ranging between 0.05 to 0.57 µg/L (0.14 ±0.2 µg/L). In NEM, particularly, the highest total rainfall (Figure 4) resulted in greater discharges from Kelantan rivers into nearshore areas. These discharges might have carried more domestic sewage from anthropogenic activities into the coastal seawater, leading to the higher nutrient concentrations and Chl-a observed in that season. Similarly, previous study [33] also found peak and extreme Chl-a concentrations (7.74 µg/L) along the coastal area of ECPM during NEM, and indicated that heavy rainfall and strong river discharge were the factors that affected the higher availability of Chl-a. Moreover, other previous studies also reported a similar pattern of Chl-a concentrations higher in coastal areas and lower towards offshore areas in Peninsular Malaysia Waters [21, 23, 34]. Statistically, the Kruskal–Wallis test shows that Chl-a concentrations were significantly different among the studied stations (p <0.05) and during monsoon season (p <0.05).

The concentrations of PO_4^{3-} (Figures 3 j-l) were measured to be low at less than 1 µM, and some were almost undetectable as the lowest concentration was below the detection limit (bdl) (bdl, <0.05 µM). Across the IM, SWM, and NEM, the following range of concentrations were recorded – bdl-0.22 µM (0.17 ± 0.07 µM), bdl-0.32 µM (0.14 ± 0.10µM), and 0.14-0.34 µM (0.29 ± 0.07 µM), respectively. The highest concentrations of DOP also have been estimated during NEM (Figure 3o), with concentrations ranging between 0.17-0.6 µM (0.38 ± 0.14 µM), followed by DOP concentrations during SWM (0.11-0.42 µM; 0.23 ± 0.10 µM) and IM (bdl-0.20 µM; 0.08 ± 0.07 µM). Generally, the DOP distribution patterns show large variations among monsoon seasons, with a uniform distributed pattern during IM (Figure 3m). TPP showed a similar trend with DOP, where a uniform distribution pattern was found in the IM (bdl-0.14 µM; 0.07 ± 0.05 µM) season, but irregular TPP distributions were found during SWM (0.23-0.59 µM;0.36 ± 0.16 µM) and NEM (0.12-0.44 µM; 0.29 ± 0.11 µM). Seasonally, the general trends of surface PO_4^{3-} and DOP show higher concentrations in NEM, probably attributed to the strong monsoonal winds that are associated with strong sea surface currents, resulting in increased nutrient input to the surface waters. The strong monsoon current was able to exchange Kelantan Waters and the adjacent bodies of water, since the surface current direction moves in the southward direction along Peninsular Malaysia, suggesting higher DOP and PO_4^{3-} during NEM, especially in the middle region, which could be transported from the Gulf of Thailand. Previous studies have found that the Gulf of Thailand-derived nutrients dominated the northern area of peninsular Malaysia [21, 23]. However, an unclear surface trend of TPP was observed in this study, with higher TPP during SWM. In terms of spatial distribution, PO_4^{3-}, DOP, and TPP showed a strong concentration onshore-offshore gradient, decreasing seaward. Except for TPP, the highest concentration was recorded at the nearshore station (A1) during NEM. The higher nutrients input during NEM at the nearshore station is in relation to the strong riverine input from Kelantan River due to higher

rainfall (900 mm) associated with greater river discharge (665 m^3/s), resulting in more PO_4^{3-} and DOP input to this station. Even so, there were still non-significant differences between stations (Kruskal Wallis $p > 0.05$), which were significant among the monsoons ($p < 0.05$).

The comparison of the data obtained in this study with previous studies in Peninsular Malaysia is useful in providing information on Chl-a and P distributions in Peninsular Malaysia (Table 1). Overall, the Chl-a concentration in this present study is much lower than in the previous study in ECPM [25] and coastal areas in Peninsular [6], but higher than the latest study in ECPM [23]. Surface PO_4^{3-} in this present study (0.14-0.34 µM) was recorded as slightly higher than other ECPM areas i.e., <0.10-0.11 µM [24] and <0.02-0.20 µM [23], but lower than coastal areas around Peninsular Malaysia (0.02-0.84 µM). Meanwhile, DOP concentrations (<0.11-0.60 µM) in this study were within the range previously reported for Peninsular Malaysia, by Lim et al. [6] (<0.01-0.61 µM) and Hee et al. [23] (<0.1-0.7 µM). However, as far as we know, no previous research has investigated TPP in Peninsular Malaysia Waters.

Depth-profile Distribution

The vertical profiles of temperature, salinity, and concentration of Chl-a, PO_4^{3-}, DOP, and TPP during all monsoon seasons are visualised in the contour plot, Figure 4. In IM, the temperature ranged from 26.32 - 30.04 °C, with an average of 28.24 ± 1.18 ºC, and salinity values were spread between 28.18-34.29 psu (33.11 ± 1.29 psu). The temperature (Figure 4a) and salinity (Figure 4d) profiles show similar stratification as observed at depths of 10-20 m, and there are significant differences between the depths ($p < 0.05$). The temperature and salinity profiles during SWM were already discussed in our previous work [21]. Briefly, the isotherm of 29.5 °C (Figure 4b) and isohaline of 32.5 psu (Figure 4e) was shoaled until 10 m in the region of 103 °E and bent downward towards the coast. This well-stratified water column was due to the Kelantan River plume and strong solar radiation during both IM and SWM. Both seasons of IM and SWM show a similar distribution pattern of warm temperatures and low salinities at ~10 m above the layer. This pattern indicated the typical features of riverine discharge that are influenced by the nearest adjacent river, i.e., the Kelantan River's direct input into the study area. The river outflow restricted the movement of water underneath from reaching the surface layer. Similar features were also observed in ECPM by [25], where these were studied during IM and SWM, and [20], during SWM. Moreover, weak water column mixing is mainly due to large surface water heating on the surface and weak surface current, which also explains why the underneath water did not reach the surface. In contrast, well-mixed water columns during NEM for temperature and slightly mixed for salinity, varied from 28.51- 30.14 °C (29.08 ± 0.26 ºC) and 30.81-33.10 psu (32.34 ± 0.5 psu), respectively. Temperature is vertically homogeneous with a mean of 29 °C occupied by all water columns (Fig. 4C), and offshore water of > 40 m depth showing a colder temperature (28.51°C). Thus, the temperature did not show any significant difference against the depth ($p > 0.05$). However, significant differences were observed in different depths ($p < 0.05$) for salinity, where salinity is more saline at the bottom layer, especially in offshore water (33.10 psu). The well-mixed water column was expected as a result of the large river discharge and strong water current, which increased the vertical mixing from the bottom to reach the surface layer. Although this study did not emphasise vertical water current, the river discharge and rainfall data obtained from the DID reveal that there was an increased rainfall amount associated with strong river discharge during November 2017 (NEM) as compared to April (IM) and July (SWM) 2017 (Figure 4). This is in agreement with previous studies in ECPM [17, 23], where NEM tends to have well-mixed water columns due to higher freshwater influence and strong wind speeds associated with the strong vertical current. The Kruskal-Wallis test results indicate significant differences among the monsoonal seasons regarding temperature and salinity ($p<0.05$). Seasonally, the vertical profile of Chl-a varied remarkably from the surface to the bottom, where Chl-a concentration showed a similar pattern with high concentration in the bottom and low concentration in the surface layers. Chl-a concentration varied from 0.02 to 0.73 µg/L (0.16±0.18 µg/L) during IM, with a weak trend of Chl-a movement towards the surface. Meanwhile, Chl-a concentration was recorded as 0.01 to 0.60 µg/L

(0.16±0.16 µg/L) during SWM, and 0.02 to 0.75 µg/L (0.22±0.17 µg/L) during NEM, respectively. The Chl-a variability shown in NEM slightly fluctuates and is higher in the middle of the water column. The strong vertical mixing mechanism induced by strong winds during NEM led to the re-suspension of bottom sediment and facilitates the release of nutrients. Consequently, this has supported increased phytoplankton biomass, reflected in higher Chl- a. concentration toward middle water. Previously, [35] revealed the occurrence of higher Chl-a concentration at the bottom water of the shallow region attributed to resuspension and turbulent water mixing, which modify the vertical dispersion rates of phytoplankton and the upward flux of nutrients and further promote the phytoplankton growth. Moreover, [36] also found higher Chl-a concentration at the bottom water due to the occurrence of turbulent vertical mixing that resulted in more efficient transport of nutrients from bottom to the water column and subsequently increased phytoplankton abundance and the chlorophyll-a content. Additionally, when there are low nutrients available in the surface water, phytoplankton tends to survive in bottom water without light limitations, especially in shallow water, due to the sediments releasing an abundant amount of nutrients [37]. Thus, in agreement with this study, where limited surface P availability in the surface water (except for PO_4^{3-} in NEM) and water depth was less than 60 m, phytoplankton (Chl-a) tended to grow in bottom layers. The Kruskal-Wallis test showed significantly different ($p < 0.05$) Chl-a levels among the monsoon seasons.

Table 1. The variability of the surface concentrations ranges of Chl-a, PO_4^{3-}, DOP, and TPP measured in different areas of Peninsular Malaysia

Location	Chl-a (µg/L)	PO_4^{3-} (µM)	DOP (µM)	TPP (µM)	Ref.
Current study	0.02-0.57	0.14-0.34	<0.11-0.60	0.01-0.69	
ECPM	0.068-7.744			-	[33]
ECPM	-	<0.10-0.11		-	[24]
ECPM	<0.01-0.06	<0.02-0.20	<0.1-0.7	-	[23]
Coastal around Peninsular Malaysia	2.77 ± 3.36	0.02-0.84	<0.01-0.61	-	[6]
ECPM	0.076-1.27	0.54-1.68			[25]

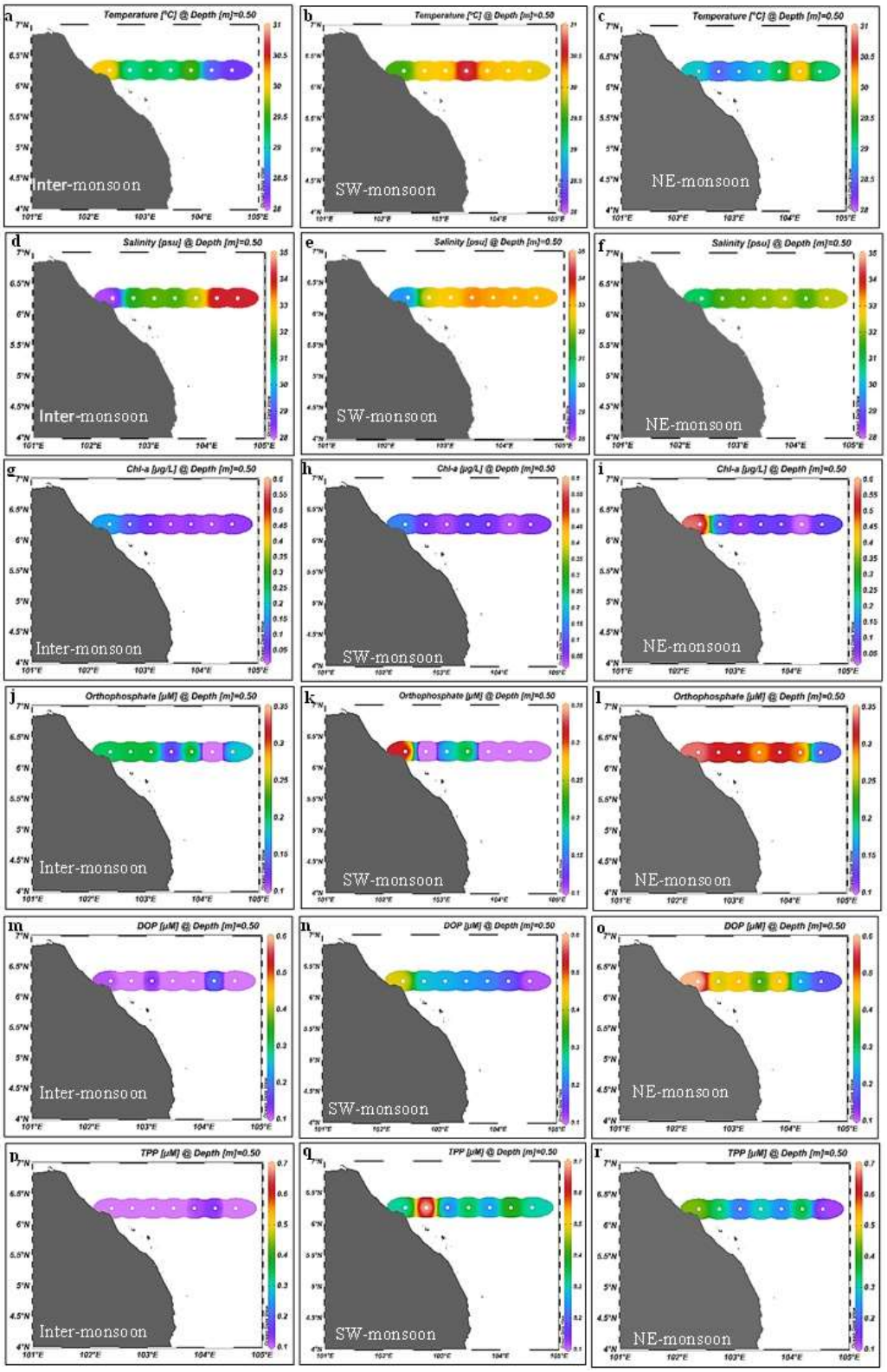


Figure 3. Seasonal variation of the surface physical characteristics, Chl-a, and P-based nutrients in Kelantan Waters

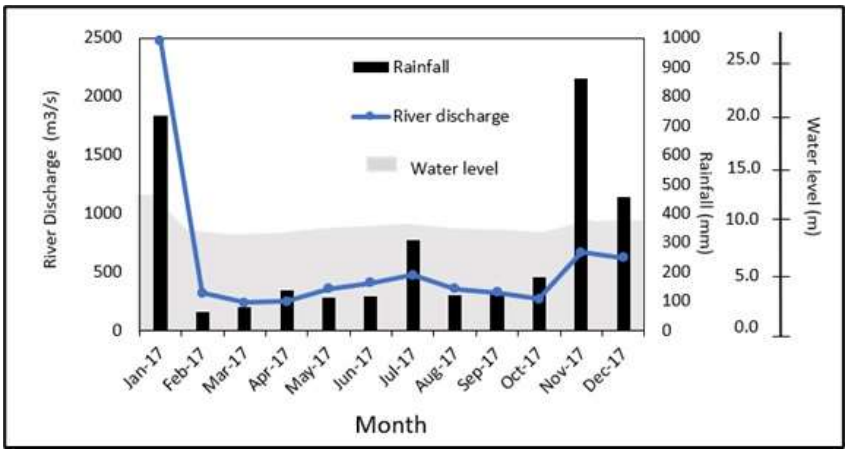


Figure 4. Monthly river discharge and rainfall in Kelantan River

The vertical profile of PO_4^{3-} during IM ranged between bdl to 0.44 µM (0.23 ± 0.09 µM), showing a large fluctuation, and an irregular variation for the entire water column (Figure 5d). Meanwhile, in SWM, PO_4^{3-} was exhausted or near the detection limit in most of the offshore area; nevertheless, higher concentrations were observed in the coastal area (bdl to 0.45 µM; 0.20 ± 0.13 µM). However, the Kruskal-Wallis test indicated that no significant depth change was noted (p >0.05) during both seasons. Conversely, the PO_4^{3-} distribution patterns showed a vertically uniform pattern in NEM (Figure 5F), and recorded concentrations of 0.20-0.45 µM (0.33 ± 0.05 µM), where a significant variability of PO_4^{3-} in the water columns was apparent (p <0.05). Uncommon PO_4^{3-} concentrations were notably higher in the offshore bottom water rather than in the coastal water, possibly due to stronger wind-induced vertical mixing during NEM; hence causing re-suspension of bottom sediments that brings up PO_4^{3-}-rich bottom water to the water column. PO_4^{3-}-rich bottom water is derived from remineralization processes of organic matter and sediment as a buffer to release nutrients to the water column when wind-induced currents disturb the water-sediment interface, especially in the shallow region [38]. However, this situation was not observed in the bottom water of the coastal area, which could be due to the lack of PO_4^{3-} resources in the bottom water, as a consequence of biological uptake or a strong river influence which transported PO_4^{3-} away to the offshore region. Earlier studies indicated that no matter how efficient the re-suspension event, the re-suspending of materials in the bottom also depends on the availability of the resources due to earlier re-suspension and/or benthic predation, which reduces the resources [35].

Overall, all monsoonal seasons have displayed a significant influence on PO_4^{3-} variation in the study area (p < 0.05). DOP was both seasonally and spatially significantly different (p <0.05). Vertically, the DOP distribution exhibited a general pattern of increased concentration with depth for all seasons ranging between 0.18 and 0.76 µM (0.39 ± 0.15 µM) in IM, 0.11 and 0.71 µM (0.41 ± 0.17 µM) in SWM, and 0.17 and 0.79 µM (0.51 ± 0.11 µM) in NEM, respectively. The DOP enrichment in the bottom depends on organic matter export processes, i.e., gravitational settling of living and dead cells or plankton biomass that can be remineralized into DOP. The DOP rapidly cycled and created a balance between local DOP production and utilisation processes occurring in the bottom water, leading to high DOP in the bottom water [39, 2]. However, DOP functioning in the bottom could not be clarified completely in this study, which can be clarified by further studying about P recycling in the future. Seasonally, the higher DOP concentrations have been estimated in the NEM, likely due to higher riverine run-offs induced by higher precipitation. According to [40], it was estimated that 61% of DOP in the Yellow Sea was mainly from riverine input, and [41] found peak DOP concentrations during wet seasons compared to dry seasons due to greater run-offs associated with higher precipitation. Thus, DOP distribution may vary depending on physical and chemical environmental conditions. Vertically, TPP distribution exhibited a

similar pattern with DOP, where concentration increased with depth. TPP was present in significant concentrations with depth ($p < 0.05$) for all seasons, and the concentrations ranged from 0.21-0.87 µM (0.54 ± 0.16 µM) during IM, 0.11-0.95 µM (0.46 ± 0.21 µM) during NEM, and 0.21-0.86 µM (0.54 ± 0.18 µM) during SWM, respectively. Monsoonal weather is an important factor that controls the distribution of Chl-a and nutrients measured in this present study, but plays a relatively insignificant ($p > 0.05$) role in TPP distribution.

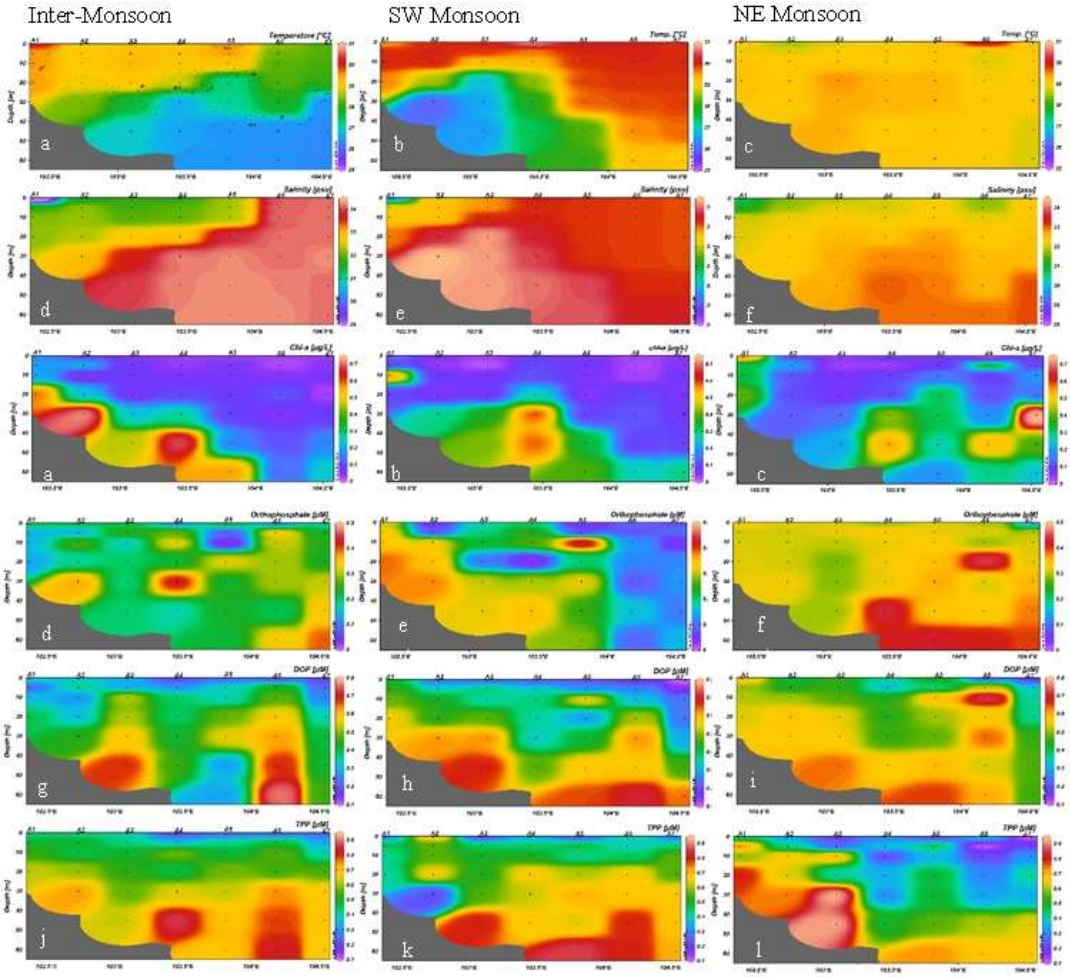


Figure 5. The seasonal cross-sectional profile of physical characteristics, Chl-a and P-based nutrients in Kelantan Waters

Principal component analysis (PCA)

The phosphorus species distribution within the Kelantan Waters is subject to influences not solely attributable to physical determinants but also governed by biological parameters, specifically chlorophyll-a (chl-a). This interplay is potentially modulated by the monsoonal fluctuations prevalent in the Southern South China Sea [21, 23]. The Kaiser-Meyer-Olkin (KMO) correlation coefficient, registering at 0.60, and the statistically significant Bartlett's sphericity test (p <0.001) collectively affirm the adequacy of the dataset for principal component analysis (PCA). The utilization of PCA proves instrumental in discerning the most salient variables in our investigation, facilitating a comprehensive understanding of the dataset while minimizing the loss of original information.

Consequently, PCA is applied to ascertain the potential influence of variables such as temperature, salinity, and Chl-a on the distribution of phosphorus variables throughout the study. The outcome of the PCA analysis reveals the acquisition of Principal Component 1 (PC1, denoted as F1 in Figure 6) for this study, possessing eigenvalues exceeding 1 (2.456) and accounting for 40.9% of the total variance in the dataset. This suggests a substantial explanatory power of the identified factors. The findings further suggest a strong correlation between salinity and Chl-a in determining the variability of phosphorus within the Kelantan Waters

The results of factor loading obtained from the PCA, as presented in Table 2, reveal distinctive contributions of various variables to the overall variability in phosphorus distribution within the Kelantan Waters. Specifically, phosphate exhibits the highest factor loading at 0.853, succeeded by DOP at 0.765, salinity at -0.698, and Chl-a at 0.687. These numerical values elucidate the respective influences of these variables on the observed fluctuations in phosphorus levels in the study area. Figure 6a visually elucidates the interrelationships between phosphorus species and other environmental factors in the Kelantan Waters. Notably, the concentration of phosphate demonstrates an inverse correlation with both salinity and temperature, while Chl-a stands as the singular variable deviating from this observed trend. The identified inverse relationships imply that regions characterized by lower salinity and temperature are prone to exhibit higher concentrations of phosphate. In contrast, the positive correlation between phosphate levels and Chl-a concentrations suggests that elevated phosphate availability contributes to increased phytoplankton biomass, subsequently augmenting Chl-a production. These findings align with prior research [21, 43], underscoring a significant correlation between Chl-a and phosphate, thereby emphasizing the essential role of phosphate in phytoplankton production. Consequently, the availability of phosphate emerges as a pivotal factor influencing Chl-a levels during the studied period.

Principal component analysis (PCA) was applied to delineate the distinctions in phosphorus distribution during the monsoon seasons, as elucidated in Figure 6b. This graphical representation underscores the statistically significant disparities ($p < 0.05$), previously ascertained through the Kruskal–Wallis test. The PCA results manifested as ellipses, characterizing the phosphorus distribution during the NEM, SWM, and IM seasons. Each ellipse encapsulated distinctive features corresponding to the respective monsoon seasons, except A1 during SWM. The discerned differences among the ellipses underscore the pivotal role played by monsoon seasons in shaping the observed variability in phosphorus distribution. Each monsoon season exhibited specific patterns and trends in phosphorus distribution, as delineated by the individual characteristics of the ellipses. Notably, the overlapping ellipses for SWM, IM, and NEM implied shared characteristics in phosphorus distribution during these seasons. This overlapping pattern suggests the persistence of certain factors and conditions, such as nutrient cycling, that influence phosphorus levels across SWM, IM, and NEM, highlighting the interconnected nature of phosphorus dynamics. The larger size of the ellipse for the SWM indicates its predominant influence on the observed phosphorus variability in the study area. This observation underscores the heightened impact of the SWM on shaping the phosphorus dynamics during the study period.

Table 2. Factor loading, eigenvalue, and percent of variance for 6 variables on the first two factor

Variables	Factor 1	Factor 2
Temperature	-0.173	**0.626**
Salinity	**-0.698**	0.396
Chlorophyll-a	**0.687**	-0.210
PO_4^{3-}	**0.853**	-0.169
DOP	**0.765**	0.441

Variables	Factor 1	Factor 2
TPP	0.392	**0.856**
Eigenvalue	2.456	1.526
Variability (%)	40.94	25.81
Cumulative	40.94	66.75

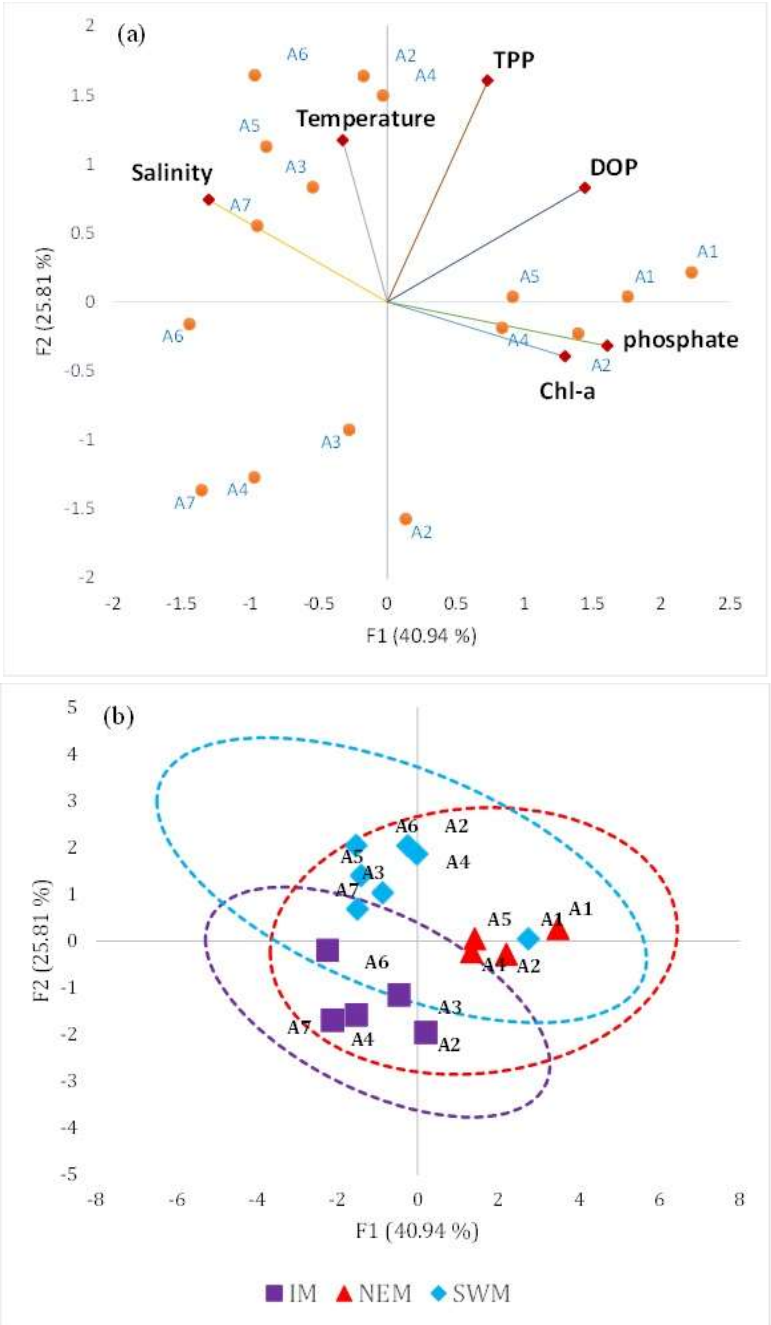


Figure 6. The correlation biplot using PCA of (a) phosphorus concentration and environmental factors and (b) phosphorus abundance

Conclusion

In this study, both surface and vertical distributions of PO_4^{3-}, DOP, TPP, Chl-a, and their physical characteristics in the Kelantan Waters were evaluated. Surface distributions of the most distinguished parameters demonstrated higher concentrations in the coastal waters compared to the offshore region, which indicates the influence of freshwater from the adjacent river of Kelantan River to our studied area. Supporting the warmer water with low salinity in the coastal area has proved the features of riverine discharge from the Kelantan River into the ocean. The influence of Kelantan riverine input is localized to the nearshore station (≤20 km), where further offshore areas may be influenced by the Gulf of Thailand-derived water. Offshore areas have their own ecological dynamics and biological processes, including nutrient cycling and phytoplankton activity, which can influence phosphorus levels. Vertical profiling showed higher concentrations of P-based nutrients and Chl-a in the deeper layer as compared to the surface layer, potentially derived from biological activities of remineralization processes and sediment as a buffer to release nutrients to the water column when wind-induced currents disturb the water-sediment interface. A direct link between the monsoon wind forcing with nutrients and Chl-a was noticed. Based on PCA, Chl-a strongly correlated with phosphate, which suggested that phosphate supply was the dominant source during our sampling. The monsoon weather pattern significantly acts as a key factor in controlling all the variables measured in Kelantan Waters, with SWM having a dominant influence on the variability observed in the study. Even though the obtained data are limited to one transect, the information provided an oceanographically meaningful spatial and temporal pattern of field data during all three monsoon seasons, which is vital for this study area, where a very limited amount of field data is available.

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ANALYSIS OF EFFLORESCENCE OF SPENT BLEACHING CLAY BASED GEOPOLYMER USING ALKALINE LEACHABILITY METHODS

(Analisa Peroian Geopolimer Berasaskan Tanah Liat Terluntur Menggunakan Kaedah Kelarutlesapan Alkali)

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Abstract

Geopolymers are a class of inorganic amorphous polymeric materials made up of silica, alumina and alkali- metal oxides. The presence of alkali in the geopolymer formulation aids in the dissolving of aluminosilicate precursors, resulting in the creation of microstructures and increases geopolymer strength. However, the alkali content is the most critical factor influencing efflorescence production. Therefore, this study evaluated the effects of curing parameters on efflorescence formation on spent bleaching clay based geopolymer. The efflorescence effects were analysed by alkaline leachability methods using atomic absorption spectroscopy (AAS) and electrochemical impedance spectroscopy (EIS). Neat geopolymer as well as cured geopolymer with different liquid/solid (L/S) ratio effects were studied. Alkaline leaching studies revealed water-cured geopolymers with liquid/solid (L/S) ratio of 2:1 shows that 974.9 ppm Na^+ ions leached from the geopolymer. This method helps to prevent efflorescence from reoccurring as most of the free Na^+ ions has already been leached from the geopolymer sample. The EIS analysis also showed that a 2:1 ratio had excellent resistance which reflects the mobility of the other ions such as silicon, aluminium and chloride ions. Functional group analysis using FTIR demonstrated the key peaks involved in development of geopolymer from spent bleaching clay (SBC) such as v(Si-O-Si / Si-O-Al) (bending & stretching), v(Al-O)(symmetrical), v(Si-O / Al-O) (asymmetrical) and v(O-H). Surface morphology analysis using SEM-EDX indicates that adequate silica-rich components were required to increase the mechanical strength of geopolymers by densifying their microstructure with more bulk and compact layers. From the examination of thermal stability of neat and treated geopolymer compositions decomposed at 700 °C confirming the thermal resistance of the material. Therefore, for more sustainable cements, alkali leachability can be performed on SBC-based geopolymer by water-curing as it revealed to have an exceptional performance on total Na leached, resistance and thermal stability as shown in this study.

Keywords: geopolymer, spent bleaching clay, efflorescence, leachability

Abstrak

Geopolimer adalah kelas bahan polimer amorfus tidak organik yang terdiri daripada silika, alumina dan logam-alkali oksida. Kehadiran alkali dalam formula geopolymer membantu dalam pelarutan bahan pemula aluminosilika, menghasilkan mikrostruktur serta menguatkan struktur geopolimer. Akan tetapi, kandungan alkali adalah faktor yang paling kritikal yang mempengaruhi pembentukan peroi. Oleh itu, kajian ini adalah untuk menilai sintesis geopolimer daripada tanah liat terluntur dan menganalisa peroian dengan kaedah kelarutanlesapan menggunakan AAS dan EIS. Geopolimer tulen serta geopolimer yang

dirawat dengan kesan nisbah cecair/pepejal (L/S) yang berbeza telah dikaji. Kajian kelarutlesapan alkali menunjukkan geopolimer yang diawet dengan air pada nisbah air/pepejal (L/S) 2:1 menghasilkan 974.9 ppm Na^+ ions melarutlesap daripada geopolimer tersebut. Cara ini mencegah pembentukkan peroi daripada berulang kerana kebanyakkan ion Na^+ bebas telah dilarutlesap daripada geopolimer. Analisa EIS menunjukkan bahawa nisbah 2:1 juga mempunyai rintangan yang baik yang mencerminkan pergerakan ion-ion yang lain seperti silicon, aluminium dan klorida. Analisa kumpulan fungsi menggunakan FTIR menunjukkan puncak-puncak utama yang terlibat dalam penghasilan geopolimer berasaskan tanah liat (SBC) yang terdiri daripada v(Si-O-Si / Si-O-Al) (bengkok & regangan), v(Al-O) (simetri), v(Si-O / Al-O) (tidak simetri) dan v(O-H). Analisis morfologi permukaan mengunakan SEM-EDX menunjukkan bahawa komponen kaya silika yang mencukupi diperlukan untuk meningkatkan kekuatan mekanikal geopolimer dengan memadatkan struktur mikronya dengan lebih banyak lapisan pukal dan padat. Daripada pemeriksaan kestabilan terma komposisi geopolimer yang asal dan dirawat itu terurai pada suhu 700°C menunjukkan kerintangan haba mereka. Oleh itu, untuk simen yang lebih mampan, kelarutlesapan alkali boleh dilakukan pada geopolimer berasaskan SBC dengan pengawetan air kerana ia menunjukkan prestasi yang luar biasa berdasarkan jumlah Na yang kelarutlesapan serta kestabilan termanya seperti yang dibuktikan dalam penyelidikan ini.

Kata kunci: geopolimer, tanah liat terluntur, peroian, kelarutlesapan

Introduction

Geopolymers or inorganic polymers, have excellent potential for industrial applications as they are both environmentally friendly and possess unique physico-chemical characteristics [1]. Geopolymers are alternative binder for fibre composites and cement for concrete that have outstanding physical, chemical, and mechanical prospects. They can also be employed in a variety of applications due to their outstanding physical properties, which include fire, heat, acid, and corrosion resistance, as well as high compressive strength. Fire-resistant and thermal-insulating materials, also low-tech and low- energy materials like cements, concretes and tiles are amongst them [2]. The appearance of efflorescence on geopolymer surfaces, independent of the precursor utilised, is a significant impediment to the application of geopolymer products. The formation of efflorescence might cause the geopolymer structure to deteriorate. The formation of efflorescence is linked to the leaching of sodium ion from geopolymers when the product encounters moisture or water. The material leaches alkali ions and transport them to the surface via capillary forces. These ions combine with carbon dioxide (CO_2) in the environment to generate hydrated sodium carbonate (Na_2CO_3) when the water evaporates. This takes effect notably when the material contains an excessive amount of alkaline substances which also has a high porosity [3]. Sodium hydroxide (NaOH) is extensively utilised during geopolymer synthesis as compared to potassium hydroxide (KOH) as NaOH optimises the dissolution procedure along with the combination of silicate solution [4]. This process is important since the solubility of aluminosilicate increases as the concentration of hydroxide increases. Geopolymer concrete with a greater NaOH concentration has a better compressive strength [5].

Numerous aluminosilicate substances are present in nature and some of them are also produced from industry wastes [6]. Spent bleaching clay (SBC) is a solid waste product from the refinement of palm oil where the disposal of bleaching clay as a byproduct from refining plants causes several environmental issues as well as financial losses to the country [7]. Spent bleaching clay (SBC) contains between 20-40% residual oil that may be a fire hazard [8]. Spent bleaching clay (SBC) is mostly made of SiO_2 followed by Al_2O_3, MgO, Fe_2O_3 and CaO, whilst its elemental components are C, Ca, O, Fe, Mg, Al, Si, P and K [9,10]. Besides, the main component utilised in this research is montmorillonite, which is a bentonite clay, with the potential to absorb several times its size, taking on a jelly-like consistency due to the hydration upon contact with aqueous solutions. Therefore, giving it a capacity for ion sorption, which can be improved by conducting an alkaline activation that results in the formation of a geopolymer or inorganic polymer.

As afore mentioned, the alkali concentration is vital to produce geopolymeric gels and to enhance the strength of the synthesised geopolymer [11]. Nevertheless, the alkali metals are weakly attached in the matrix and can readily be leached away in water. Thus, resulting in development of white efflorescence (Na_2CO_3) on the surface of the geopolymer, when it is in contact with water and moisture. This subsequently has a major

effect on degradation of geopolymer cements [12]. Furthermore, efflorescence on geopolymer surfaces, whether made of montmorillonite, metakaolin, fly ash, volcanic tuff, or other aluminosilicate precursors, is a significant hurdle for their utilisation [3]. Therefore, this work strategises towards the target of studying efflorescence formation by evaluating the amount of Na^+ leached using AAS and EIS are vital for future construction science.

It is hypothesised that the SBC-based geopolymer demonstrates greater physicochemical properties. However, excess efflorescence may disrupt the stability of the binder microstructure, which has been a persistent drawback in the development of geopolymers as reported in previous studies [3,12]. The characterisation techniques are used to support the formation of geopolymer from spent bleaching clay. This work develops the variation in curing conditions as the principal motive to study the development of efflorescence for the novel spent bleaching clay based geopolymer which has not been reported elsewhere.

Materials and Methods

Reagents and raw materials

Chemicals, reagents, and solvents employed in this investigation, specifically sodium hydroxide (NaOH), sodium silicate (Na_2SiO_3), sodium chloride (NaCl) were commercially purchased from local suppliers as analytical reagents such as Systerm, QReC and R&M Chemicals. They were utilised exactly as they are, with no further purification. Furthermore, all reactions were carried out in an ambient environment. While, spent bleaching clay was contributed by KL Kepong Oleomas Sdn Bhd, Selangor.

Geopolymer synthesis

Geopolymer was synthesised with starting materials, 15.6633g of NaOH and 54.8213g of Na_2SiO_3 mixed in 150 mL distilled water respectively. The ratio of the starting materials were NaOH : Na_2SiO_3 = 1 : 3.5, also known as the alkaline activator solution. The geopolymer paste and composites were attained by mixing the alkaline activator solution into 160.0g of SBC gradually. Empirically, the geopolymer composite complied to the ratio of alkaline activating solution (NaOH + Na_2SiO_3) : SBC of 1 : 2.27 [11,13]. Then, the composite was poured into molds and left in the oven at 70°C for 24 hours; conducive for curing process in to accelerate the gel formation [13,14]. Then, the geopolymer paste was left to undergo a hardening process for a week before proceeding with subsequent steps.

Characterisation of geopolymer

Perkin Elmer 100 FT-IR spectroscopy was utilised for FT-IR analysis by employing potassium bromide (KBr) pellets within spectral range 4000-400 cm^{-1} for determination of functional group in the synthesised geopolymer. In addition, for image analysis, Zeiss GeminiSEM 360 Field Emission Scanning Electron Microscope (FESEM) was utilised to examine the morphological phases. The characterisation techniques are important to showcase the formation of the geopolymer before the leaching study can be conducted.

Alkaline leaching test

Leaching test was conducted on the geopolymers to determine the alkaline leachability after 28 days of immersion test via AAS and EIS [15]. The leaching process was examined in two different conditions which were ambient curing and water curing, as per Table 1. Demolded geopolymers were placed in larger containers and sealed for ambient curing meanwhile, for water curing, the study progressed by immersing demolded geopolymer into distilled water in sealed containers for 28 days. The liquid/solid (L/S) for water curing varied from 2:1, 4:1, 10:1 and 20:1 to fully get rid of sodium ions from geopolymer sample. Ambient cured sample was immersed in water to collect the leachate for testing. The ratio of L/S was calculated based on previous study [11]. Thermogravimetric-differential thermal analysis (TGA-DTA) was explored to examine the mass loss as temperature rises. The test proceeded with a heating rate of 10°C/min from ambient temperature to 900°C. All untreated and treated geopolymer samples were evaluated for thermal stability.

Alkaline leachability study via AAS

After 28 days, the samples were analysed by Atomic Absorption Spectroscopy (AAS) for total amount of sodium ions (Na^+) leached. Initially, AAS instrument

was calibrated by using standard solution of 1, 2, 3, 4 and 5 ppm of NaCl, prepared from stock solution of 100 ppm. The standards were run, and a calibration graph was plotted. It is ensured that the $R^2 \geq 0.99$ prior to sample readings. Then, all the leachate of the water cured samples were examined and evaluated.

Table 1. Alkaline leaching sample details according to its curing condition

Curing Conditions	Liquid-to-Solid Ratio (L/S)	Sample
Water curing	2:1	A
	4:1	B
	10:1	C
	20:1	D
Ambient curing	N/A	E

Alkaline leachability study via AAS

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Alkaline leachability study via EIS

Impedance of the geopolymer samples from different curing conditions as per Table 1 were measured by the electrochemical impedance spectroscopy (EIS), Gamry Reference 600 electrochemical workstation with three electrode setups. First, the electrodes utilised were platinum as counter electrode (CE), graphite as working electrode (WE) and saturated calomel electrode (SCE) as reference electrode (RE). These electrodes were immersed in a beaker which contained a leached liquid sample of geopolymers from different curing conditions. Then, the Gamry Reference600 electrochemical workstation was opened to test the EIS with the frequency range set at 10^{-1} to 10^5 while AC amplitude of 10mV. The resulted Nyquist plots were analysed further.

Results and Discussion

Synthesis of geopolymer

Geopolymer was synthesized and characterized as an alternate new cement for concrete instead of ordinary Portland Cement (OPC). The preparation of geopolymer was accomplished via geopolymerisation process through aluminosilicate material dissolved in 0.39 mol of sodium hydroxide and 0.45 mol of sodium silicate to attain geopolymer and a by-product which is water molecules as shown in Figure 1.

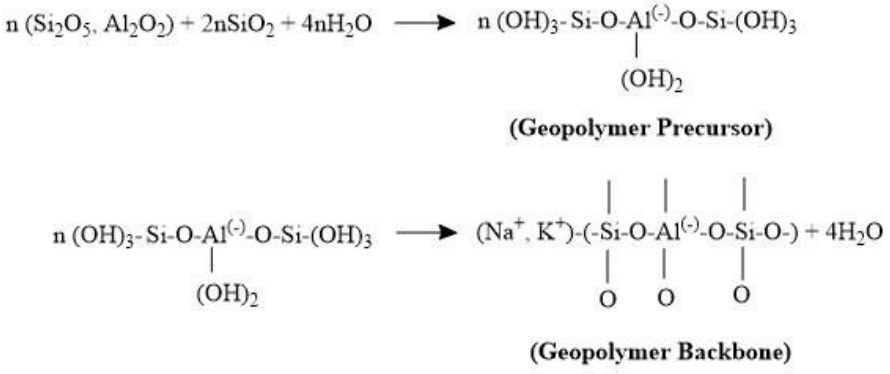


Figure 1. Mechanism of geopolymer synthesis [15]

Principally, the geopolymerisation technique consists of three main mechanisms [16]. Firstly, the dissolution of aluminosilicate materials in alkaline media by the breaking of covalent Si-O-Si and Al-O-Al bonds, resulting in the production of silicate and aluminate species. The hydroxyl (OH-) groups commence this process by attaching to the silicon atom, making the Si-O-Si bond more prone to breaking. At high pH, reactive aluminosilicates will dissolve quickly. Then, condensation of silicate and aluminate monomers will develop a gel until the solution approaches saturation, resulting in the release of NaOH, which reacts again. Finally, the gel undergoes polycondensation and structural rearrangement, resulting in a 3-D aluminosilicate structure of geopolymers. The final compound attained in this study is medium deep brown as shown in Figure 2. Physically, the cementitious geopolymer showed slight amount of efflorescence on its surface after aging for 28 days. There is no visible pore spotted on the surface of synthesised geopolymer.

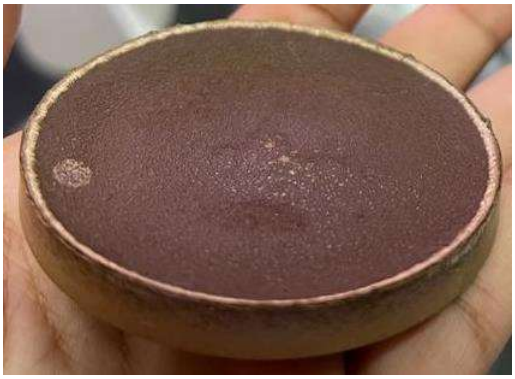


Figure 2. Geopolymer synthesized and aged for 28 days at room temperature

Functional group analysis

Functional group analysis of SBC and SBC-based geopolymer revealed all the expected bands of interest namely v(Si-O-Si / Si-O-Al) (bending), v(Si-O-Si / Si-O-Al) (stretching), v(Al-O) (symmetrical), v(Si-O / Al-O) (asymmetrical) and v(O-H) as seen in Figure 3. The spectrum ranging from strong intensity of SBC to moderate intensity of SBC-based geopolymer synthesised in this study showed a noticeable displacement. It is due to improvised geopolymer network organisation and there is more alkali cations present making the bonds to be less polar [18].

From the geopolymer spectra, the absorption band for v(Si-O-Si / Si-O-Al) strong bending vibration was observed at 523.94 cm^{-1} while a moderate IR band at 692.20 cm^{-1} was assigned as stretching vibrations of v (Si-O-Si/Si-O-Al) in geopolymer which is in close agreement with previous reported studies, as it is contributed to the stretching bands of tetrahedral aluminium [18]. Strong peaks from SBC around wavenumbers of 461.56 cm^{-1} and 531.33 cm^{-1} indicated montmorillonite structure which was then deconstructed and dissolved into Si-O and Al-O tetrahedral; characteristic of an amorphous aluminosilicate for geopolymer [20, 21].

Additionally, the presence of symmetrical v(Al-O) moderate vibration peak in AlO_4 with tetrahedral coordination can be observed at 789.88 cm^{-1} from the spectra of geopolymer [20]. Meanwhile, Li et al. highlighted that this modest peak can be attributed to Al-O-H bending vibrations or minor anionic ions such as silicate monomer, SiO^-. This is because the source material is rapidly dissolved forming small silicate monomers. The small peak seen in SBC spectra at 796.44 cm^{-1} disappeared in the geopolymer spectra, indicating that silicate monomers were consumed in favor of bulkier species [22].

The intense absorption band of asymmetrical Si-O/Al-O assigned at 1027.09 cm^{-1} is considered as the main absorption band. As the polycondensation process progresses, this band gets more concentrated, producing more aluminosilicates matrix. The band at 1027 cm^{-1} was likely due to an increase in the amount

of functional group attached to the molecular bond resulting in less non-bridging oxygen and Al being substituted in the silicate sites [18]. An absorption peak near 1600 cm^{-1} is attributed to bending vibration of (H-O-H) bonds, perhaps because the NaOH residue in the geopolymer which consequently produces Na_2CO_3 by interacting with CO_2 in the air [23].

Furthermore, the two absorption peaks in the 2800-3200 cm^{-1} range were the stretching vibrations of v(-Al-OH) and v(-Si-OH), indicating that tetrahedral structure was not damaged. This is mostly due to the evaporation of water in the paste that limited soluble ion movement, resulting in an inadequate reaction between clay precursor and the alkali activator [20]. It appeared that the band at approximately 3443.50 cm^{-1} is related to the stretching vibration of the O-H bond of geopolymer, whereas a comparable peak emerged at 3345.83 cm^{-1} for the SBC. The bands shifted likely because the usage of water during preparation of alkaline activator solution added with geopolymer slurry for geopolymerisation process. Considering water is a main byproduct of geopolymers, the results were comparable [24].

Carbon dioxide (CO_2) stretching vibration band at around 1300-1400 cm^{-1} was also discovered. These bands defined the geopolymer material [25]. These distinct peaks mostly indicate the presence of geopolymer compounds. The small-scale difference in intensity for the influence of carbonates was recorded at 1429.30 and 1387.02 cm^{-1}, corresponding to the stretching vibrations of O-C-O bonds in the carbonate group (CO_3^{2-}) caused by the carbonation reaction between carbon dioxide from the air and the geopolymer. The slight detected rise was caused by increased carbonation as a result of the high number of free ions. Carbonates contributed more to samples activated with sodium solution, which can react with atmospheric CO_2 to form Na_2CO_3 species [18].

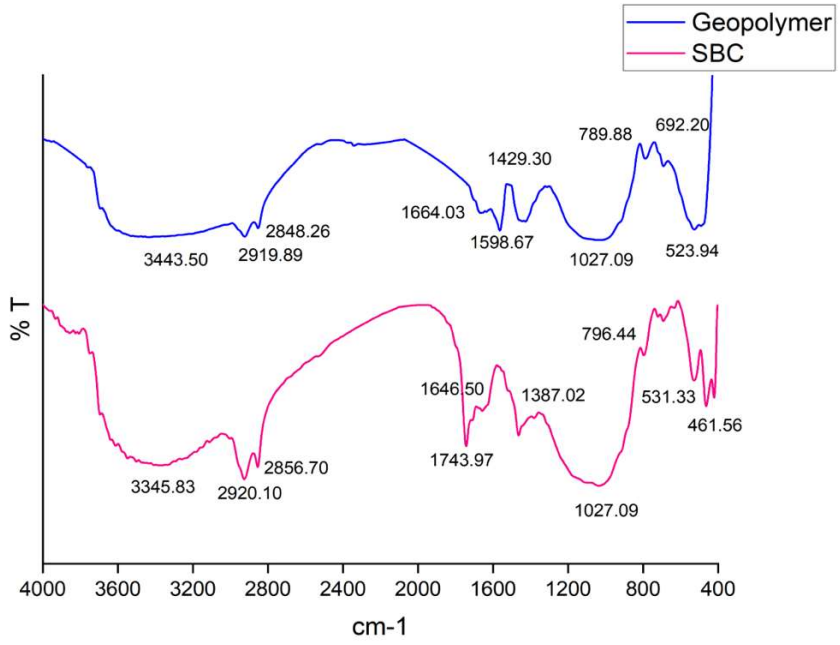


Figure 3. FTIR spectrum of geopolymer synthesized in this study and spent bleaching clay (SBC)

Surface morphology and elemental analysis

Figure 4 shows the SEM images of SBC and geopolymer. Figure 4 (a) shows that bulk, compact, and sponge-like structure are well formed for SBC. In contrast to other precursors, montmorillonite has a mainly fluffy and lumpy morphology, which may be characterised by the material's very amorphous structure caused by the dissolution of the multilayered plates structure [21,26]. This lumpy microstructure could be attributed to chemical modification in the clay galleries during palm oil refinement process. As a result, the composite is altered by an ionic liquid

(which is entrained oil and metallic contaminant) filled with aggregated montmorillonite particles that act as thickeners, and the ionic liquid completely form a thin film spread over the clay particles [27,28]. It also has a structure similar to illite, except that isomorphous substitution commonly occurs in the octahedral sheet, where every sixth aluminium is replaced by magnesium [21]. Figure 4 (b) shows an amorphous appearance with a rough, flaky, mild porous and less dense condition.

Additionally, the big lumps present in Figure 4 (b) exposes the homogenous part of sample. The aggregate seemed strongly bonded and a transition layer developed between SBC and the geopolymer matrix phase, indicating that a polymerisation process took place, generating a (-Al-O-Si-) chemical link. Thus, when a geopolymer substitutes the concrete, the alkali aggregated reaction does not induce product cracking [20]. Besides, the morphology of clay based geopolymer shifted to layer-like structure after geopolymerization process despite the curing temperature [19]. Meanwhile, the pores in geopolymers are mostly generated by water evaporation in the precursor. Pore structure parameters such as pore diameter, porosity, pore morphology and pore diameter uniformity have been shown to influence the microstructure and mechanical properties of geopolymers [29]. The rise of water absorption in geopolymer composition causing the microstructural deterioration, allowing water to exit the geopolymer network and be retained [30].

Spent bleaching clay (SBC) and geopolymer elemental composition were examined by using EDX analysis as per Table 2. The semi-quantitative elemental composition exposed the primary elements characterised were oxygen, carbon, silicon and aluminium with proportion values of 40.09%, 36.00%, 10.02% and 2.16% respectively, with traces of Ca, Fe, Mg, K and S. Based on these findings, the microstructure is primarily made up of amorphous sodium aluminosilicate binder (N- A-S-H) [31]. It is noteworthy that the composition of Na comprised almost 7% of the geopolymer's structure. Due to the alkali activator utilised during the geopolymerization reaction, the proportion of Na elements rose drastically [32,33]. Whilst, the Si molar ratios revealed the significance of binder phases in alkali activated geopolymers [31].

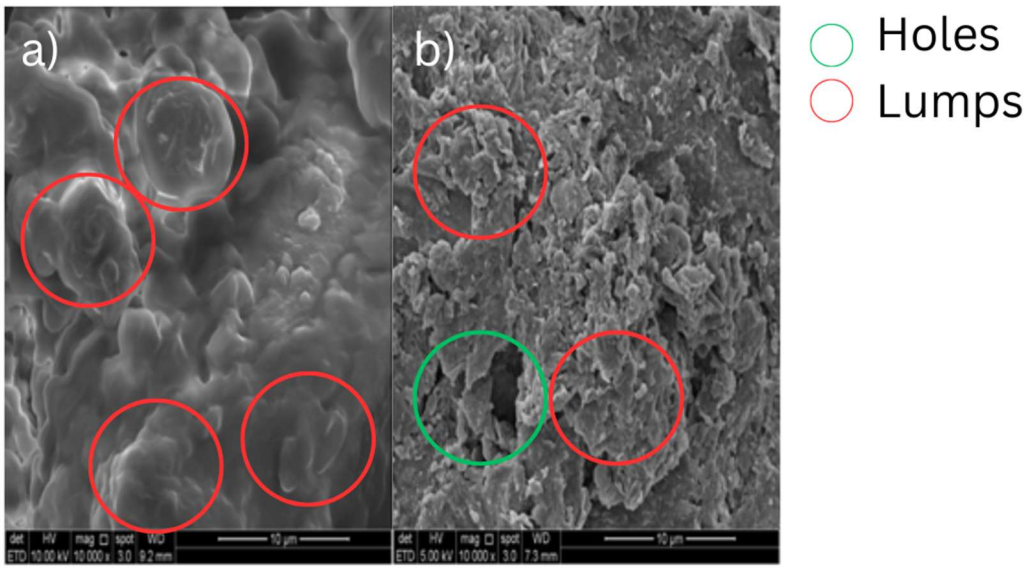


Figure 4. SEM image of (a) SBC and (b) geopolymer at 10,000x magnification

Table 2. EDX analysis of SBC and Geopolymer

Element	SBC (wt.%)	Geopolymer (wt.%)
O	22.95	40.09
C	64.40	36.00
Si	6.08	10.02
Na	NA	6.77
Al	1.57	2.16
Ca	0.81	1.85
Fe	3.57	1.74
Mg	0.62	0.64
K	NA	0.39
S	NA	0.34

Thermal analysis of neat and treated geopolymers

Figure 5 displays the thermogravimetric analysis and their differential curves for the neat and treated geopolymers developed in this work. From Figure 5, it was discovered that there is no discernible difference in the weight loss regardless of the curing method utilised. According to Gultekin and Ramyar, the geopolymers' free and weakly bonded-absorbed water caused the abrupt weight loss in the region of 0-200°C [34]. Another phenomenon, leading to the second weight loss, which occurs around 350°C, is the loss of hydroxyl from clay and organic residual oil, which makes up roughly 10-30% of the mass respective to the geopolymers. At 700 °C, this phase denotes the amorphization of the clay and the elimination of the montmorillonite precursor [1]. Dehydroxylation of the illite phase, the endothermic peak, begins at 600°C and concludes at 700°C, as can be shown in Figure 5 [35]. Besides, Marsh et al. stated that the continued curve is the formation of activated montmorillonite system [36].

Eventually, the presence of carbonates might also increase the compressive strength of geopolymer [37]. Besides, the unburnt organic carbon might be responsible for a mass loss of roughly < 1%, which were measured at the DTA peak of 170-180°C. Thermal analysis demonstrated that the clay-based geopolymers exhibited a thermally stable structure when heated up to 900°C. As a result of its improved thermal characteristics, SBC-based geopolymer may be more beneficial for manufacturing components that are stable at high temperatures than Portland cement systems. All in all, it can be summarised that all treated and untreated geopolymer samples possess good heat resistance, where it all decomposed only after 700°C.

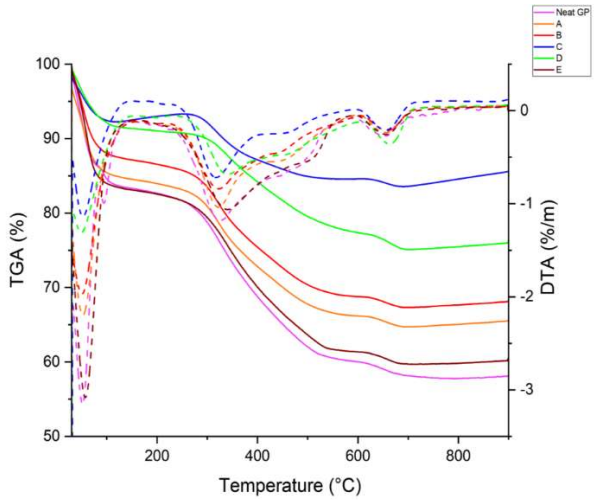


Figure 5. TGA-DTA of neat and treated geopolymer at different curing condition; water-cured (A-D) and ambient-cured (E)

Alkaline leachability via AAS

Geopolymer of same batch was tested for alkaline leachability from ambient curing and various liquid/solid (L/S) ratio as per Table 1 for water curing. Figure 6 depicts the concentration of sodium in the leachate after 28 days of leaching.

It is noticed that with increasing L/S ratio, there was a further drop in Na^+ leaching. This is because the increased of soluble silicate content in the geopolymer matrix probably results in less alkali leaching, which is consistent with water absorption values [38]. The less compact microstructure leads to fewer sodium associated in charge balancing sites. As a result of the porosity effects, the leaching of Na^+ is substantially enhanced [39]. This shows that resistance to efflorescence requires both decreased porosity and sodium incorporation in charge balancing sites [38].

As per Figure 6, alkaline leaching ability of geopolymer samples treated with L/S ratio of 2:1 is greater than for other samples with higher L/S ratios. Despite having a variable L/S ratio, samples C and D released nearly less Na^+ and at a slower rate in the leaching tests. Meanwhile, for all water-cured specimens, the leaching process significantly reduced the total alkali leaching after 28 days of immersion as compared to ambient-cured specimen. This is possibly due to the NaOH concentration in the sample and solution leveled off more slowly when pouring higher quantities of sample, in other words, more effective diffusion [3].

Besides, leaching behavior reduced for water-cured samples according to the L/S ratio from 2:1 to 20:1. Due to the high solution concentration of water content, the diffusion and mass transfer velocity were quick during the initial stage of leaching. The specimen surfaces begin to be progressively covered in residue as the reaction goes on and the concentration of the solution also starts to decrease. On account to this, the diffusion and mass transfer velocity slowed down, which given the lesser concentration that was leached out, indicated that the leachate solubility was also decreased [40]. Overall, water-cured specimen, sample A, with L/S ratio of 2:1 exhibited the most sodium ions leached from geopolymer as compared to other specimens.

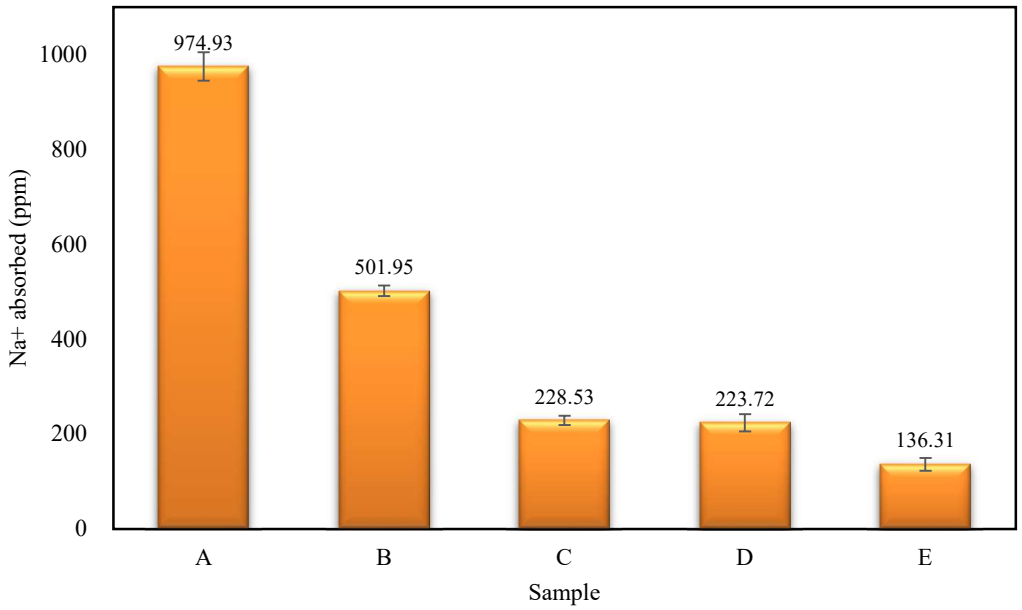


Figure 6. Sample A-D are water-cured specimens while E is ambient-cured specimen. Liquid/solid (L/S) ratio for water-cured specimens of A is 2:1, B is 4:1, C is 10:1 and D is 20:1

Alkaline leachability via EIS

The AC impedance spectra, also known as Nyquist plot, of treated geopolymers after 28 days of curing shown in Figure 7. It illustrates the oblique lines, which increases gradually due to ion diffusion effect. The resultant Nyquist plot shifts from left to right as curing age grows, and the value of resistance, which refers to the actual impedance of the junction, also rises. The elevation in resistance reflects the mobility of ions in the individual specimens, which in accordance with the L/S ratio [41]. On account to this, Biondi et al. reported that when immersed in water, Na^+ ions, as well as silicon and chloride ions, migrate in small amounts from the geopolymer [42]. According to Song et al., the parameter, θ linked to the surface condition of the montmorillonite particles and the applied voltage, E are the two key factors affecting the current of the geopolymer slurry. The θ represents the relationship between the dissolved surface area and the combined surface area of the montmorillonite particles. Consequently, an increase in θ will cause a rise in plasma current [43]. Therefore, the Nyquist plot from sample D with L/S ratio of 20:1 reflected highest impedance real part data to those of other ratios. This is because during the soaking phase, oxygen vacancies that have diffused from the solution interface to the geopolymer interface essentially promotes the formation of the leachates with high ion concentrations [44].

Charges in pore solution could be conveyed over the surface of the solid phase due to the differing resistance characteristics of solid and liquid phases [41]. Also, the concentration of ions in the geopolymer slurry rises as Si and Al dissolve, progressively increasing the resistance value of the sample. Furthermore, ambient-cured specimen, sample E, yield increased impedance response due to excellent electrolyte interfacial contact, probably anticipated to OH^- concentration solubility [45]. Potentially, the sample should have developed more aluminosilicate bonds during the curing phase. Therefore, sample A is considered to indicate excellent resistance in alkaline leaching since the other samples likely to have charge transferred in its solution.

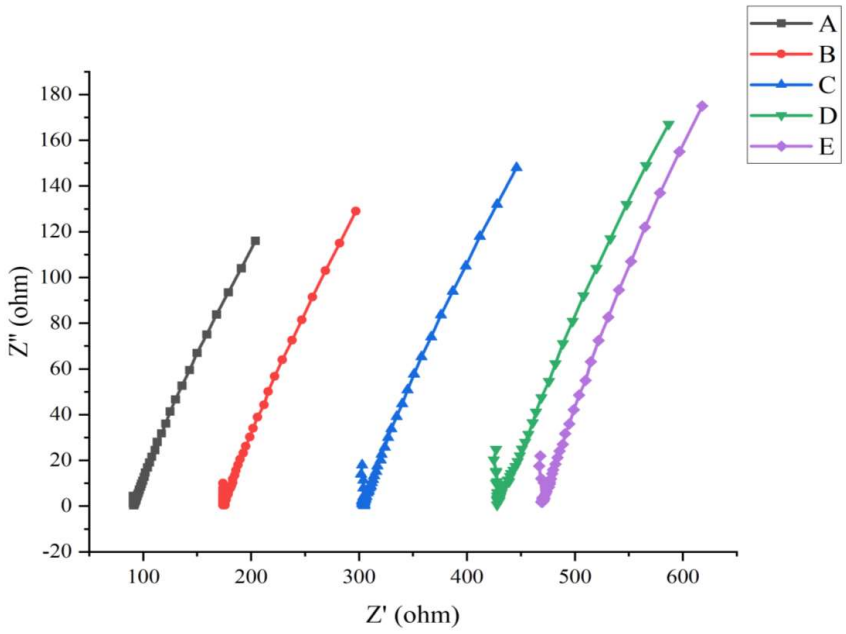


Figure 7. EIS responses in Nyquist curve at various curing condition; water-cured (A-D) and ambient-cured (E)

Conclusion

Aluminosilicates polymerised in an alkaline media at elevated temperature, produce a substance with cementitious characteristics. Spent bleaching clay-based geopolymer has been successfully designed by geopolymerisation, characterised and evaluated towards investigating the efflorescence development by using the leachability methods. This study details an

effort to investigate the alkaline leachability which causes the efflorescence phenomena on geopolymers using AAS and EIS. Mineralogically, the efflorescence still has impact on the main amorphous gels and residual crystalline phases formed from montmorillonite, which make up the majority of the clay-based geopolymers. According to this finding, free alkali concentration is significantly influenced by the alkalinity of the activating solution. Despite favouring the mechanical strength of the goods, higher alkalinities tend to enhance the leachability. Hence, sample A (L/S ratio 2:1) was analysed to have the most prominent curing condition to work towards efflorescence formation. In addition, the compound was analysed via several spectroscopic and analytical techniques, namely infrared (FT-IR), scanning electron microscopy (SEM-EDX), and thermogravimetric-differential thermal analysis (TGA-DTA). The montmorillonite based geopolymer in this study have significant thermal stability, as for both water and ambient-cured specimens yielded less weight loss as compared to neat geopolymer indeed. It is compatible with the outcome that high water and NaOH concentration reduces the strength of geopolymers to yield low compressive strength and a mild porous structure. On the other hand, studies on efflorescence propensity via AAS and EIS have demonstrated that curing geopolymers in water conditions is an efficient way to reduce efflorescence formed on their surface. This is due to the most Na^+ being leached away in sample A, liquid/solid (L/S) 2:1 ratio, with also an adequate thermal stability and impedance response. It is also demonstrated that ambient curing could not act preferably as a suggested curing condition with utmost exhibition.

Acknowledgement

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SYNTHESIS AND CHARACTERIZATION OF PALLADIUM(II) SCHIFF BASE COMPLEXES DERIVED FROM PHENYLAMINES AND THEIR CATALYTIC ACTIVITY FOR STILLE REACTION

(Sintesis dan Pencirian Palladium(II) Bes-Schiff Kompleks Dari Fenilamina dan Aktiviti Sebagai Pemangkin untuk Tindak Balas Stille)

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Abstract

Schiff bases with hydrogen and methyl at the *para*-position of phenylamines (B1H and B1Me) and their palladium(II) complexes, (PdB1H and PdB1Me) were synthesized and characterized by elemental analysis, FTIR, ^{1}H and ^{13}C NMR, magnetic susceptibility measurement and UV-Visible spectroscopy. The shifting of the *v*(C=N) and *v*(C-O) bands to lower frequencies in FTIR indicated that the complexation to palladium(II) occurred through these moieties. All complexes are diamagnetic and displayed low molar conductivity in acetonitrile, indicating non-electrolytic behaviour. B1H and B1Me are structurally characterized using single crystal X-ray diffraction. B1H and B1Me crystallize in orthorhombic space group $P2_12_12_1$, with a = 6.0879(2) Å, b = 9.1847(2) Å, and c = 21.0661(5) Å and a = 5.9051(9) Å, b = 9.2389(13) Å, and c = 23.297(3) Å, respectively. The palladium(II) complexes were screened for their catalytic activity in the Stille reaction. The reaction was monitored by measuring the % conversion of iodobenzene using GC-FID, where the reaction conditions used were 1.0 mmol% catalyst loading in the presence of triethylamine as the base and DMSO as the solvent at 80°C within 6 hours of the reaction time. It was observed that PdB1Me displayed the best catalytic performance for the Stille reaction, as indicated by the 80% conversion of iodobenzene.

Keywords: catalysis, palladium(II), Schiff base, Stille reaction, single crystal X-ray crystallography

Abstrak

Bes-Schiff dengan hidrogen dan metil pada fenilamina posisi *para* (B1H dan B1Me), dan kompleks paladium(II) (PdB1H dan PdB1Me), telah disintesis dan dicirikan oleh analisis unsur, FTIR, ^{1}H dan ^{13}C NMR, ukuran kerentanan magnet dan sinar ultraungu spektroskopi. Peralihan jalur *v*(C=N) dan *v*(C-O) kepada frekuensi yang lebih rendah dalam FTIR menunjukkan bahawa kompleksasi kepada paladium(II) berlaku melalui gugusan ini. Semua kompleks adalah diamagnet dan menunjukkan kekonduksian molar rendah dalam asetonitril yang menunjukkan tingkah laku bukan elektrolitik. B1H dan B1Me dicirikan secara struktur menggunakan pembelauan sinar-X kristal tunggal. B1H dan B1Me menghablur dalam ortorombik dengan kumpulan ruang $P2_12_12_1$, dengan a = 6.0879(2) Å, b = 9.1847(2) Å, dan c = 21.0661(5) Å dan a = 5.9051(9) Å, b = 9.2389(13) Å, dan c = 23.297(3) Å. Kompleks paladium(II) telah disaring untuk aktiviti pemangkinnya dalam tindak balas Stille. Tindak balas dipantau dengan mengukur % penukaran iodobenzena menggunakan GC-FID, di mana keadaan tindak balas yang digunakan ialah 1.0 mmol%

pemangkin dengan kehadiran trietilamina sebagai bes dan DMSO sebagai pelarut pada 80°C selama 6 jam. PdB1Me memaparkan prestasi pemangkin terbaik di kalangan kompleks untuk tindak balas Stille, ditunjukkan oleh penukaran 80% iodobenzena.

Kata kunci: pemangkinan, paladium(II), Bes-Schiff, tindak balas Stille, kristalografi sinar-X kristal tunggal

Introduction

The Schiff base complexes have received great attention in recent years because of their highly considered as stereochemical models in coordination chemistry, their easy mode of preparation and their structural diversity [1]. Schiff bases and their complexes have a wide range of applications such as bioactive agents [2], catalysts [3], medicines [4], and anti-corrosion agents [5]. Schiff base complexes play a key role in various homogeneous catalytic reactions and the activity of these complexes changes with the types of ligands, coordination sites and metal ions [6].

Schiff bases that are derived from amines and carbonyl compounds belong to a class of ligands involved in the coordination of metal ions via azomethine nitrogen [7]. Phenylamines, through the condensation reaction with various types of organic reagents such as aldehydes, ketones, thiosemicarbazides, carbazides, and similar compounds, are able to form flexible ligand systems that are able to coordinate with a variety of metals [8]. Schiff bases derived from phenylamines and their respective transition metal complexes exhibit potential catalytic activity [9].

Palladium is considered a very important metal from a coordination point of view and it plays a key role as a catalyst in a variety of catalytic activities by varying the ligand environment [10]. Palladium complexes are excellent and versatile catalysts in many important chemical reactions such as carbon-carbon coupling, alkylation, carbonylation, and oxidation [11]. Palladium(II) Schiff base complexes are mostly used to catalyse these couplings reactions as they offer high product yields, high regioselectivity, high stereoselectivity, and compatibility with many functional moieties.

The palladium-catalysed Stille cross-coupling reaction represents one of the most important transformations in constructing carbon-carbon bonds in organic synthesis [12, 13]. Generally, the combination of palladium catalysts with various phosphine ligands is commonly used in the Stille reaction [14]. However, phosphine ligands are air-sensitive and expensive, which places significant limits on their synthetic applications [15].

This paper aims to report the synthesis and characterization of bidentate Schiff base ligands with hydrogen and methyl at the *para*-position of phenylamines with *o*-vanillin and their palladium(II) complexes. Scheme 1 shows the synthesis of the Schiff base ligands and their palladium(II) complexes. The molecular structures of all compounds were elucidated through elemental analysis, magnetic susceptibility, molar conductivity, FTIR, ^{1}H NMR, ^{13}C NMR, UV−Visible analysis, and single crystal X-ray diffraction. The palladium(II) complexes were screened for their catalytic activity in the Stille reaction.

Materials and Methods

Materials and physical measurements

All chemicals and solvents received from commercial suppliers were used. Microanalyses for C, H and N were conducted on Thermo Finnigan Flash Elemental Analyzer 2000. Melting point determination was carried out in glass capillary tubes using Buchii-B454. The IR spectra were obtained using Perkin Elmer 1750X FTIR spectrophotometer (4000-400 cm^{-1}) with samples prepared as KBr discs. Proton (^{1}H) and carbon (^{13}C) NMR (600 MHz) spectra were recorded on Bruker Varian spectrometer in $CDCl_3$ and reported in ppm (δ) relative to TMS, used as the internal reference. The UV-Vis spectra were obtained on Perkin Lambda 35 in chloroform ($CHCl_3$) 1×10^{-4} M solution at room temperature, in the range of 200-800 nm, using a quartz cuvette. Magnetic measurements were carried out using the Guoy method with distilled water as a calibrant on the Sherwood Auto Magnetic Susceptibility Balance. A single crystal X-ray diffraction investigation was carried out on Bruker D8 QUEST CCD diffractometer with PHOTON III 7 detector, using graphite-monochromated Mo-Kα radiation (λ = 0.71073 Å) at 298 K. The collected frames were processed with the

software SAINT. Structures were solved by the direct methods and refined by full-matrix least-squares on F^2 using the SHELXTL software package. Mercury [16] software was utilized for the graphical representation of the X-ray results. Gas chromatographic analyses were performed on Agilent 7890A Network GC System using ethyl laurate as standard. The column used is HP-5 capillary columns with a temperature increment of 15°C per minute, and the final temperature was 300°C.

Synthesis of ligands

The Schiff base ligands were synthesized by condensation reactions between *o*-vanillin and phenylamine derivatives using a 1:1 molar ratio (Scheme 1) in hot ethanol, under reflux for 3 hours. The mixture was allowed to cool slowly to room temperature. Upon cooling, orange precipitate formed. The precipitate was filtered off, washed with ice-cold ethanol, and air-dried at room temperature. The crystals were obtained through slow evaporation of the filtrate solutions.

Synthesis of palladium(II) complexes

The ligands and palladium(II) acetate were dissolved in hot ethanol in a round-bottom flask using a 2:1 molar ratio. The mixture was stirred and refluxed for 6 hours. The mixture was allowed to cool slowly to room temperature. Upon cooling, brown precipitate formed. The precipitate was filtered off, washed with ice-cold ethanol, and air-dried at room temperature.

General procedure for the Stille reaction

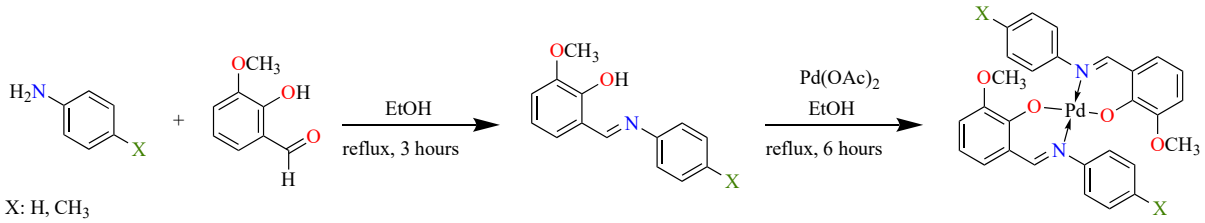
The general procedure (Scheme 2) is as follows: Iodobenzene (1 mmol, 0.2 g), tributylphenyltin (1.5 mmol, 0.55 g), triethylamine (Et_3N, 2.0 mmol, 0.20 g), palladium(II) complex (1 mmol%) (PdB1H, PdB1Me) and dimethylsulfoxide (DMSO, 7 mL) were mixed in Radley's 12-placed reaction carousel and refluxed at 80 °C for 6 hours. The reaction was monitored regularly using GC-FID.

Results and Discussion

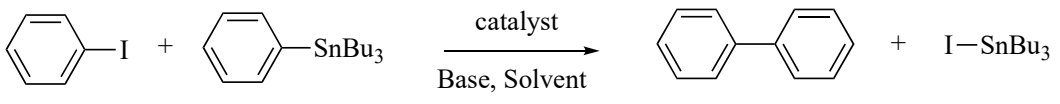
Elemental analysis

The physicochemical data of the compounds are presented in Table 1. According to the elemental analysis of C, H, and N for the compounds, the experimental values were in accordance with the calculated values of the proposed structure. The melting points of the parent ligands were typically lower than those of the complexes, which is likely due to the increased molecular sizes and the presence of stronger dative covalent bonds between C-N-Pd and ionic bonds between C-O-Pd [16].

Molar conductivity measurement for complexes revealed that the non-conductivity nature of the solution at room temperature indicating the absence of any electrolytes [17]. These values show that there are no ions existing in the outer-sphere coordination of the complexes. As expected, the magnetic susceptibility measurements showed diamagnetic behaviour reflected in the zero effective magnetic moment, $\mu_{eff} = 0$, in all complexes consistent with the 0 unpaired electrons of Pd(II) d^8 species having square planar geometry [18].



Scheme 1. Synthesis of the Schiff base ligands and their Pd(II) complexes



Scheme 2. General reaction of Stille reaction

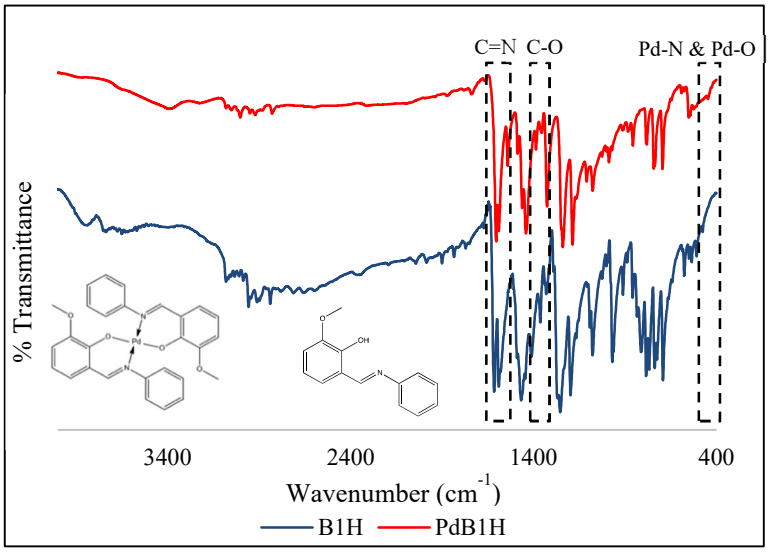
Table 1. Physicochemical data of ligands and their complexes

Compounds	Empirical Formula	Colour	Melting Point (°C)	Elemental Analysis (%)			Magnetism	Molar Conductivity ($\Omega^{-1}cm^2mol^{-1}$)
				C	H	N		
B1H	$C_{14}H_{13}NO_2$	Orange	227.26	73.99 (**73.29**)	5.77 (**5.68**)	6.16 (**6.05**)	-	-
PdB1H	$C_{28}H_{24}N_2O_4Pd$	Brown	558.93	60.17 (**59.39**)	4.33 (**4.19**)	5.01 (**4.84**)	Diamagnetic	1.275
B1Me	$C_{15}H_{15}NO_2$	Orange	241.29	74.67 (**73.79**)	6.27 (**6.24**)	5.81 (**5.71**)	-	-
PdB1Me	$C_{30}H_{28}N_2O_4Pd$	Brown	586.98	61.39 (**60.55**)	4.81 (**4.74**)	4.77 (**4.58**)	Diamagnetic	0.874

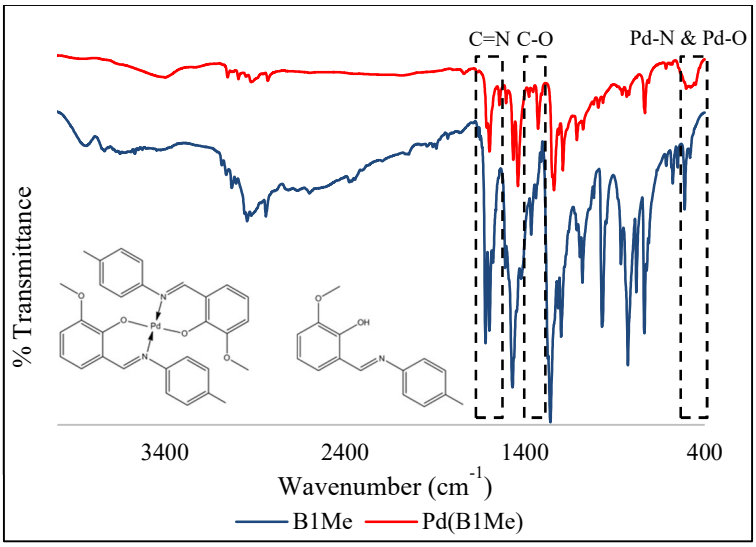
FTIR spectroscopy

The FTIR spectra of the ligands and palladium(II) complexes are shown in Figure 1. There is an absence of ν(OH) peak in the FTIR spectra of the ligands (Figure 1). This can be attributed to the formation of hydrogen bonding between the hydrogen and nitrogen atoms in the solid state. This is supported by the discovery of an intramolecular hydrogen bond in the X-ray crystallography analysis. The strong bands around 1615-1618 cm^{-1} are assignable to the azomethine ν(C=N) group for the ligands. This band was observed to be shifted slightly to lower frequencies of 1601– 1602 cm^{-1} in the complexes, likely due to the coordination of the imine nitrogen atom through the sharing of the N lone pair of electrons in a dative manner with the metal centre [19]. The involvement of deprotonated phenolic oxygen atoms in chelation was indicated by the shifting to lower frequencies of the $\nu(C\text{-}O)_{phenolic}$ band. The medium intensity $\nu(C\text{-}O)_{phenolic}$ band that was observed at 1253-1258 cm^{-1} in ligands shifted to lower wavenumbers of 1237-1240 cm^{-1} in complexes.

The coordination of the imine nitrogen and phenolic oxygen atoms to the metal centres was supported by the appearance of new peaks of ν(Pd-O) and ν(Pd-N) bands in palladium(II) complexes, which appeared in the regions of 507-572 cm^{-1} and 446-473 cm^{-1}, respectively [20].



(a)

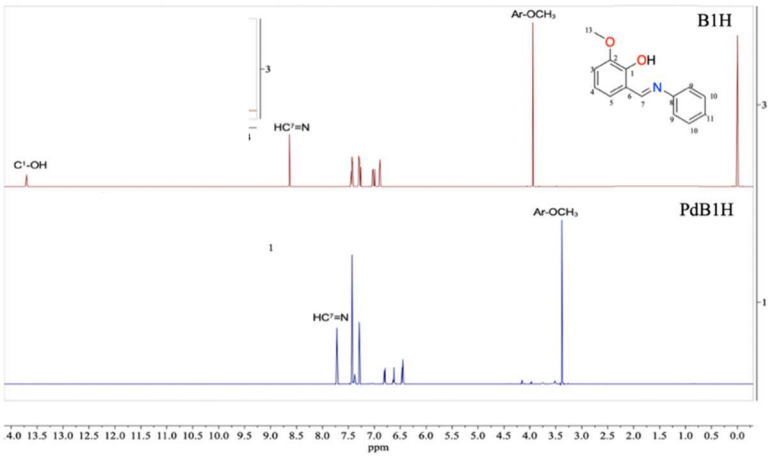


(b)

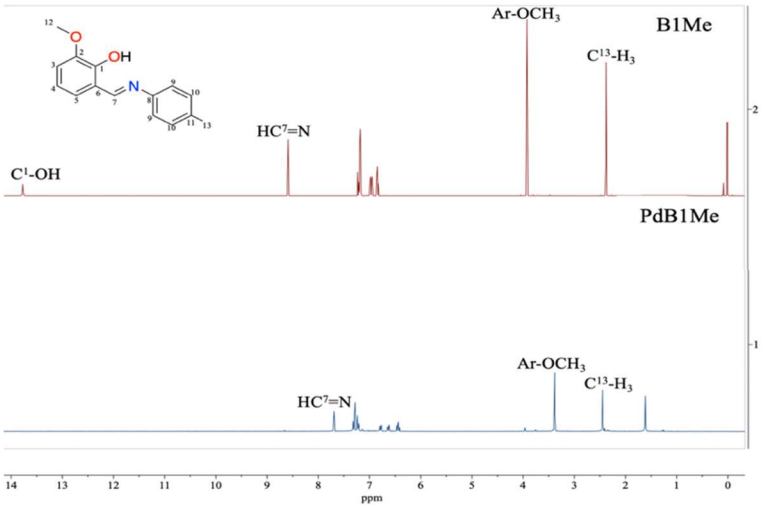
Figure 1. Infrared Spectra of (a) B1H and PdB1H and (b) B1Me and PdB1Me

Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H spectra of the ligands and palladium(II) complexes are presented in Figure 2. The ^{1}H NMR spectra showed integrations and multiplicities consistent with the proposed structures. The phenolic proton in ligands appears as a singlet in the downfield region in the range of 13.70 to 13.84 ppm [21]. Upon complexation, the peak of the phenolic proton was absent in both spectra of the complexes suggesting the deprotonation of the phenolic proton [22]. The chemical shift of the azomethine proton (-HC=N-) was shielded at the range of 7.69-7.72 ppm in the complexes compared to the ligands at 8.63 ppm, inferring coordination of the palladium(II) ion to the ligand through the azomethine nitrogen atom [23]. The metal donates the π electrons to the ligands π* orbital, adding the electron density to an antibonding molecular orbital, and this results in weakening of the C-O bond and C=N bond [24]. The spectra of the ligands and complexes show aromatic hydrogen appearing in the ranges of 6.88-7.44 and 6.41-7.32 ppm, respectively. The presence of the methyl groups attached to the structure of B1Me was confirmed by the appearance of singlet peaks at 2.38 ppm and 2.45 ppm at PdB1Me.



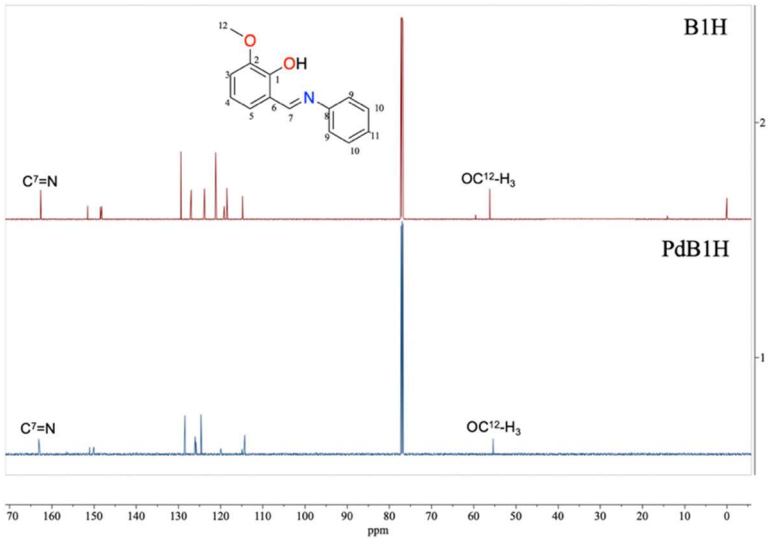
(a)



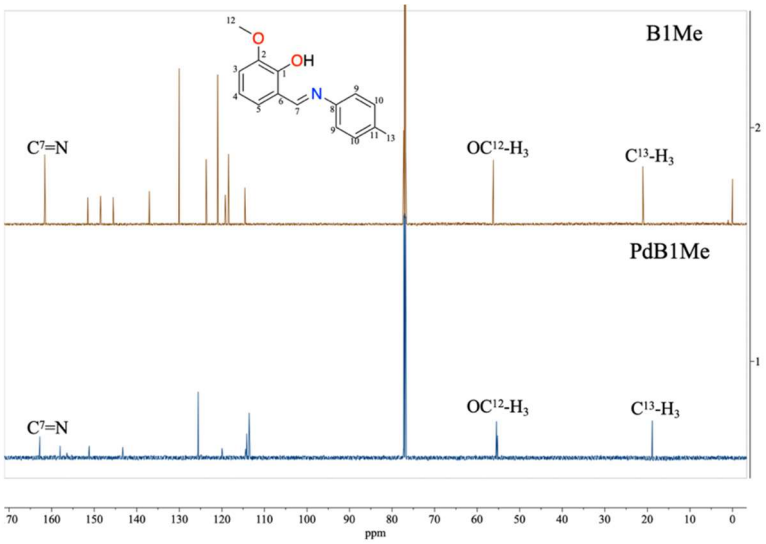
(b)

Figure 2. ^{1}H NMR spectra of (a) B1H and PdB1H and (b) B1Me and PdB1Me

The ^{13}C NMR spectra of the ligands and palladium(II) complexes in Figure 3, respectively recorded signals of sharp peaks representing the chemically non-equivalent carbon atoms in the compound. The phenolic carbon for the ligands was found in the range of 148.5-151.5 ppm and upon complexation, they appeared in the downfield region for complexes. The shifting indicates the establishment of new bond between metal centre and phenolic oxygen. The presence of the methyl group was detected at 22.7 and 19.6 ppm for B1Me and PdB1Me, respectively.



(a)



(b)

Figure 3. ^{13}C NMR spectra of (a) B1H and PdB1H and (b) B1Me and PdB1Me

UV-visible spectroscopy

The UV−vis spectroscopy spectra and data of the ligands and palladium(II) complexes are shown in Figure 4 and Table 2. The electronic transitions result in three main characteristic absorption peaks, i.e., π−π*(C=C), π−π*(C=N), and n−π*(C=N). The π−π*(C=C) peak of the ligand was observed at 210-229 nm, and the peak shifted to a bathochromic shift in the complexes, appearing at 220-253 nm for palladium(II) complexes. Besides, the π−π*(C=N) peak at 271-279 nm of the ligands underwent a bathochromic shift upon complexation, appearing at 301-305 nm. It arises due to the coordination of the lone pair of electrons with the metal ion [25]. The n−π* transitions of imine in the complexes were not found in the complexes, possibly due to the stabilization of the electron pair on the nitrogen atom upon the formation of the metal-nitrogen bond [26]. Upon complexation, there is the appearance of ligand to metal charge transfer at 420-421 nm, which suggests the square planar geometry around the Pd(II) centre [27]. Thus, it could be said that the magnetic susceptibility measurements for the PdB1H and PdB1Me, which show diamagnetic behaviour with square planar geometry, are complimentary to the UV-visible data obtained in Table 2.

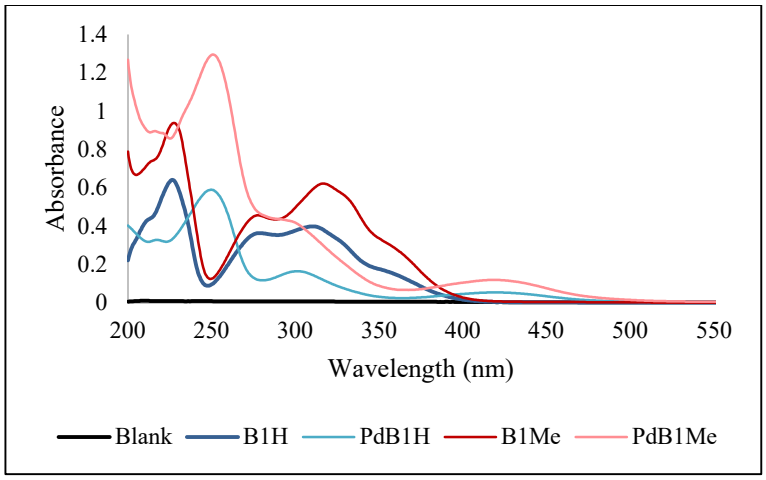


Figure 4. UV-visible spectra of ligands and complexes

Table 2. UV-Visible Data of Ligands and Their Pd(II) Complexes

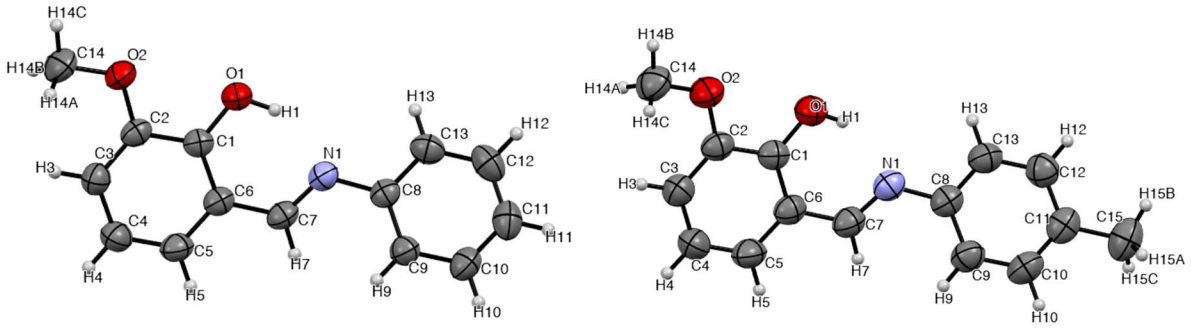
Compounds	π→π* (C=C)	π→π* (C=N)	n→π*	Ligand to Metal Charge Transfer
B1H	210 (1386), 225 (2108)	271 (1102)	310 (1331)	-
PdB1H	220 (1080), 253 (1918)	305 (539)	-	421 (183)
B1Me	213 (2441), 229 (3104)	279 (1517)	317 (2074)	-
PdB1Me	220 (2953), 253 (4274)	301 (1379)	-	420 (400)

Single X-ray crystallography

Suitable single crystals of B1H and B1Me were subjected to X-ray crystallography investigation to determine the structure. Both crystals were obtained through slow evaporation of their filtrate solutions. Crystallographic data collection parameters for the compounds are shown in Table 3. B1H and B1Me crystallize in orthorhombic space group $P2_12_12_1$, with a = 6.0879(2) Å, b = 9.1847(2) Å, and c = 21.0661(5) Å. and a = 5.9051(9) Å, b = 9.2389(13) Å, and c = 23.297(3) Å, respectively. The ORTEP diagrams of the compounds drawn at 50% probability ellipsoids are depicted in Figure 5.

Table 3. Crystallographic Data Collection Parameters for B1H and B1Me

Compounds	B1H	B1Me
CCDC number	2233459	2233467
Empirical formula	$C_{14}H_{13}NO_2$	$C_{15}H_{14}NO_2$
Formula weight	227.27	240.27
Temperature (K)	298.50	299.50
Crystal system	Orthorhombic	Orthorhombic
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions		
a (Å)	6.0879(2) Å	5.9101(14) Å
b (Å)	9.1847(2) Å	9.235(2) Å
c (Å)	21.0661(5) Å	23.303(6) Å
Volume (Å^3)	1177.92 (5) Å^3	1271.9(5) Å^3
Z	4	4
D_{calc} (Mg/m^3)	1.282 Mg/m^3	1.255 Mg/m^3
Absorption coefficient (mm^{-1})	0.086	0.084
F (000)	480.0	512.0
Crystal size (mm^3)	0.500 × 0.320 × 0.150 mm^3	0.39 × 0.27 × 0.13 mm^3
Theta range for data collection	6.96 to 56.6	6.854 to 56.582
Index ranges	$-7 \le h \le 8$, $-12 \le k \le 12$, $-27 \le l \le 28$	$-7 \le h \le 7$, $-10 \le k \le 12$, $-29 \le l \le 30$
Reflections collected	35437	22568
Independent reflections	2933 [R_{int}= 0.0603]	3145 [R_{int}= 0.0532]
Data/restraints/parameters	2933/0/159	3145/0/165
Goodness-of-fit on F^2	0.974	1.031
Final R indices [I > 2sigma(I)]	R1= 0.0400, wR2= 0.1189	R_1 = 0.0434, wR_2 = 0.1187
R indices (all data)	R1= 0.0524, wR2= 0.1314	R_1 = 0.0583, wR_2 = 0.1296
Largest diff. peak and hole	0.15/-0.17	0.29/-0.14



B1H B1Me

Figure 5. The ORTEP diagrams of compounds with the atom labelling scheme drawn at 50% probability ellipsoids

For B1H, the two benzene rings C1-C6 and C8-C13 are each non-planar with a maximum deviation of 0.010(2) and 0.015(2) Å for atoms C1 and C8, respectively, from their mean square planes. Both planes make a dihedral angle of 30.58°. The C-O bond length ranges from 1.352(2) to 1.419(3) Å in the B1H molecule. The C-N and C=N bond lengths of B1H are 1.279(2) and 1.396(3) Å, respectively (Table 4). The bond length values correspond to the previously reported values [28].

For B1Me, the two benzene rings C1-C6 and C8-C13 are each non-planar with maximum deviation of 0.018(2) and 0.012(2) Å for atom C1 and C8, respectively from their mean square planes. Both planes make a dihedral angle of 26.84°. The bond lengths and angles of B1Me are in normal ranges and the values in Table 4 are similar to those in a previous study [29].

Table 4. Selected Bond Lengths (Å) and Bond Angles (°) of B1H and B1Me

	B1H	**B1Me**
Bond Lengths (Å)		
O1—C1	1.352(2)	1.348(2)
O1—C2	1.368(2)	1.362(2)
O2—C14	1.419(3)	1.420(3)
N1—C7	1.279(2)	1.275(3)
N1—C8	1.396(3)	1.414(2)
Bond Angles (°)		
O2—O2—C14	117.12(16)	117.03(18)
C7—N1—C8	120.64(15)	121.31(16)

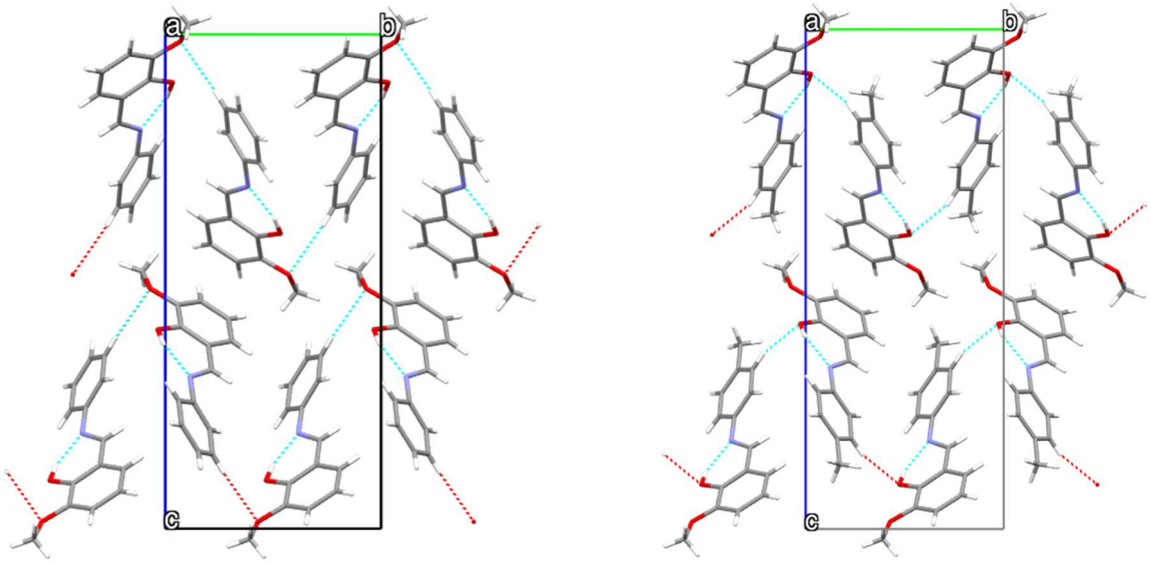
The packing diagram for B1H view down the crystallographic a-axis is shown in Figure 6. There is an intramolecular hydrogen bond O1—H1···N1 and the intermolecular hydrogen bond is drawn as dashed lines at C10—H10···O2 in the compound, as shown in Table 5. The packing diagram for the B1Me view along the a-axis is shown in Figure 6. There is an intramolecular hydrogen bond O1—H1···N1, and the intermolecular hydrogen bond at C10—H10···O1 is drawn as dashed lines in the compound (Table 5).

Table 5. Intramolecular and intermolecular hydrogen bonds of B1H and B1Me (Å, °)

D—H···A	**D—H**	**H···A**	**D ···A**	**D—H···A**
B1H				
O1—H1···N1	0.90(3)	1.83(3)	2.619(2)	146(2)
C10—H10···O2[(a)]	0.93	2.57	3.488(2)	167

D—H···A	D—H	H···A	D ···A	D—H···A
B1Me				
O1—H1···N1	0.82	1.90	2.621(2)	146
C10—H10···O1$^{(b)}$	0.93	2.47	3.365(3)	162

Symmetry code= (a): -x,1/2+y,3/2-z, (b): 1-x,-1/2+y,1/2-z



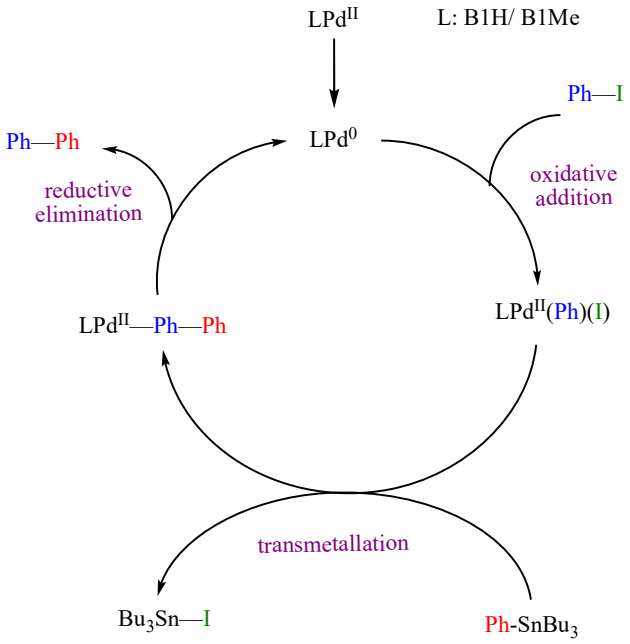
B1H B1Me

Figure 6. Packing B1H and B1Me view down crystallographic a-axis

Catalytic studies

The palladium(II) complexes in this study were screened for their catalytic activity in Stille reaction. The reaction was conducted by reacting iodobenzene with tributylphenyltin. The reaction conditions used were 1.0 mmol% catalyst loading in the presence of triethylamine as a base in dimethyl sulfoxide at 80 °C within 6 hours of the reaction time. Scheme 3 proposes the mechanism of the palladium-catalysed cross-coupling Stille reaction. The three key basic steps involved in the catalytic cycle are oxidative addition, transmetallation and reductive elimination. The mechanism begins with the oxidative addition of the organohalide to the Pd(0) in order to form a Pd(II) complex. Transmetallation with the organotin then proceeds where the R group of the organotin reagent replaces the halide anion on the palladium complex. Reductive elimination then gives the final coupled product, regenerates the palladium catalyst, and the catalytic cycle can recommence.

The reaction was monitored by measuring the % conversion of the aryl iodide starting material using gas chromatography [30]. The results are summarized in Figure 7. It was observed that PdB1Me displayed a better catalytic performance compared to PdB1H for the Stille reaction, as indicated by the 80% conversion of iodobenzene (PdB1Me) while for PdB1H, the % conversion is only 73%, this could be due to the presence of the electron donating group (-CH_3) [31, 32]. The presence of an electron-donating group in ligands will activate the benzene ring, thus as the π system in the benzene ring is activated, the electron density will increase [33]. By increasing the electron density, the formed metal-N bond will be shorter and stronger, thus this will improve the catalytic performance.



Scheme 3. Mechanism of palladium-catalyzed Stille reaction

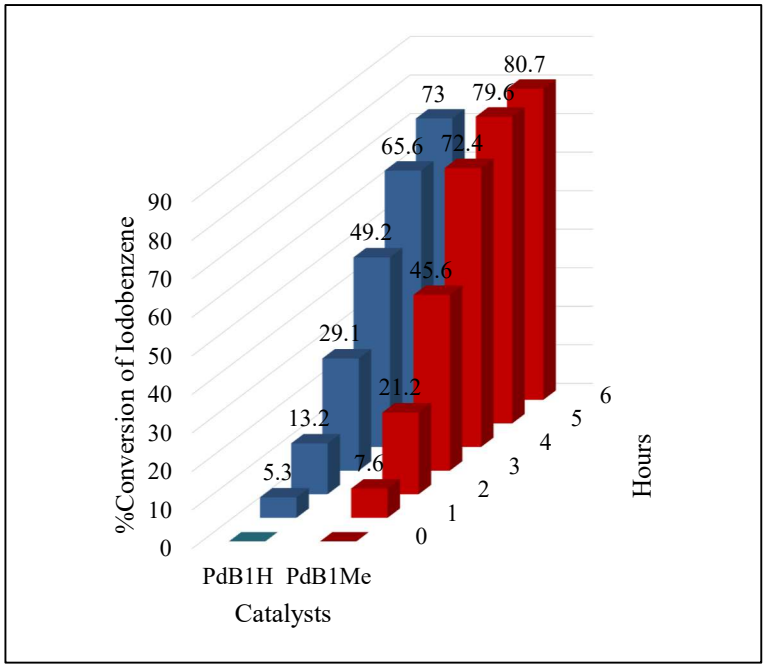


Figure 7. Activity of palladium catalysts on the Stille reaction between iodobenzene and tributylphenyltin

Conclusion

Two ligands and their palladium(II) complexes have been successfully synthesized and confirmed by characterization via various physico-spectral techniques. It can be concluded that the ligands act as bidentate chelators coordinated to the Pd(II) ion upon complexation based on the spectroscopic results and exhibited a square planar geometry. The good agreement between experimental and predicted spectral data of the Schiff bases strongly supported the elucidation of Pd(II) complexes. It was observed that both Pd(II) complexes displayed the properties of good catalysts for the reaction. PdB1Me was the most efficient catalyst for the

Stille reaction, which could be due to the presence of an electron donating group ($-CH_3$).

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FROM TRADITIONAL TO GREEN: EVOLUTION OF SHALE SWELLING INHIBITORS FOR SUSTAINABLE DRILLING

(Daripada Tradisional Ke Hijau: Evolusi Pencegahan Pembengkakan Syal Untuk Penggerudian Lestari)

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Abstract

The occurrence of shale swelling during water-based drilling operations poses substantial challenges to the stability of shale formations. Clay layer expansion is the primary cause of shale swelling, which results from interactions between clay minerals and drilling fluid components. The degree of expansion is determined by variables such as clay composition, ion exchange processes, osmotic pressure, ionic strength, temperature, and pressure. Therefore, this study explores various shale swelling inhibitors and meticulously investigates the underlying mechanisms. The effectiveness of conventional inhibitors, such as potassium chloride (KCl), ammonium chloride (NH_4Cl), and amine-based inhibitors, is well-established. However, it is important to note that these inhibitors do have certain limitations. Hence, the present work investigates a range of environmentally friendly inhibitors, including graphene oxide, ionic liquids, deep eutectic solvents, nanoparticles, nanocomposites, and biosurfactants. Graphene oxide exhibits notable efficacy in mitigating shale swelling and producing extensive, unbroken protective coatings. Ionic liquids, represented by 1-butyl-3-methylimidazolium chloride (BMIMCl), have demonstrated enhanced inhibitory characteristics in comparison to KCl, resulting in a reduction in bentonite swelling rates by 19.38%. Furthermore, it has been observed that deep eutectic solvents (DESs), such as NADES, exhibit remarkable inhibitory characteristics, resulting in a 49.1-62.8% reduction in the rate of swelling in clay samples. Nanocomposites, which involve the integration of single-walled carbon nanotubes (SWCNTs) and polyvinylpyrrolidone (PVP), have demonstrated successful mitigation of shale swelling and regulation of fluid loss. Moreover, biosurfactants such as chitosan-grafted l-arginine, flaxseed protein (FP), and flaxseed mucilage (FM), have exhibited potential as shale inhibitors that are both biodegradable and environmentally friendly. These findings contribute to ongoing efforts to improve the environmental sustainability of drilling operations and adhere to rigorous environmental protection standards. Nevertheless, more investigation, refinement, and practical application analysis are needed before they can be widely used.

Keywords: water-based drilling fluid, shale formation, shale swelling, inhibitors, environmentally friendly

Abstrak

Kejadian pembengkakan syal semasa operasi penggerudian berasaskan air menimbulkan cabaran besar kepada kestabilan pembentukan syal. Pengembangan lapisan tanah liat adalah punca utama pembengkakan syal, yang terhasil daripada interaksi antara mineral tanah liat dan komponen bendalir penggerudian. Tahap pengembangan ditentukan oleh pembolehubah seperti komposisi tanah liat, proses pertukaran ion, tekanan osmotik, kekuatan ion, suhu, dan tekanan. Oleh itu, kajian ini meneroka pelbagai perencat pembengkakan syal dan menyiasat dengan teliti mekanisme asas. Keberkesanan perencat konvensional, seperti kalium klorida (KCl), ammonium klorida (NH_4Cl), dan perencat berasaskan amina, adalah mantap. Walau bagaimanapun, adalah penting untuk ambil perhatian bahawa perencat ini mempunyai batasan tertentu. Oleh itu, kajian ini menyiasat pelbagai perencat mesra alam, termasuk graphene oksida, cecair ionik, pelarut eutektik dalam, nanopartikel, nanokomposit dan biosurfaktan. Grafin oksida mempamerkan keberkesanan yang ketara dalam mengurangkan pembengkakan syal dan menghasilkan salutan pelindung yang luas dan tidak pecah. Cecair ionik, yang diwakili oleh 1-butil-3-metilimidazolium klorida (BMIMCl), telah menunjukkan ciri-ciri perencatan yang dipertingkatkan berbanding dengan KCl, mengakibatkan pengurangan kadar pembengkakan bentonit sebanyak 19.38%. Tambahan pula, telah diperhatikan bahawa pelarut eutektik dalam (DES), seperti NADES, mempamerkan ciri-ciri perencatan yang luar biasa, mengakibatkan pengurangan 49.1-62.8% dalam kadar bengkak dalam sampel tanah liat. Nanokomposit, yang melibatkan penyepaduan tiub karbon berdinding tunggal (SWCNTs) dan polivinilpirolidon (PVP), telah menunjukkan kejayaan pengurangan bengkak syal dan pengawalan kehilangan bendalir. Selain itu, biosurfaktan seperti kitosan yang dicampurkan bersama l-arginin, protein biji rami (FP), dan lendir biji rami (FM), telah mempamerkan potensi sebagai perencat syal yang boleh terbiodegradasi dan mesra alam. Penemuan ini menyumbang kepada usaha berterusan untuk meningkatkan kelestarian alam sekitar operasi penggerudian dan mematuhi piawaian perlindungan alam sekitar yang ketat. Namun begitu, lebih banyak penyiasatan, penghalusan, dan analisis aplikasi praktikal diperlukan sebelum ia digunakan secara meluas.

Kata kunci: cecair penggerudian berasaskan air, batuan syal, pembengkakan syal, perencat, mesra alam sekitar

Introduction

Oil drilling is a challenging industrial process for finding and extracting hydrocarbon resources such as crude oil and natural gas. It entails drilling a borehole or wellbore through the earth's crust to reach reservoirs containing these valuable resources. The fundamental goal of oil drilling is to extract these resources safely and efficiently for use in a variety of applications such as energy production, transportation, and petrochemical manufacturing. To facilitate this process, drilling fluids plays a pivotal role. Drilling fluids, often known as drilling muds, are multipurpose fluids that are essential to the oil and gas drilling industry. These specialty fluids are painstakingly designed to accomplish a variety of critical duties during the drilling operation. Drilling fluids operate as cooling and lubricating agents while the drill bit bores into the earth's crust, minimizing the adverse effects of heat and friction on the drill bit. At the same time, they efficiently transport the rock cuttings generated during drilling, preventing obstructions, and keeping a clear wellbore. Drilling fluids also play an important role in preserving wellbore stability by exerting hydraulic pressure on the wellbore walls, preventing the entrance of formation fluids, which could cause well control concerns and blowouts. By preserving the integrity of cuttings for examination, they enable geologists and geophysicists to examine subsurface formations. Additionally, drilling fluid density is altered to give the drill string buoyancy, preventing it from getting stuck in the wellbore and promoting efficient drilling operations. In addition, these fluids contain corrosion inhibitors to protect drilling equipment and minimize formation damage, making them essential to the oil and gas industry's pursuit of safe, efficient, and environmentally responsible drilling practices.

Essential to the oil and gas drilling process, drilling fluids comprise a wide variety of formulations tailored to specific well conditions. These fluids are divided into three primary categories. First, water-based drilling fluids use water as their primary base fluid, which is frequently combined with additives. These compounds, which may include clays, polymers, and salts, serve to improve the viscosity, lubrication, and shale inhibition properties of the fluid. Second, oil-based drilling fluids use oil as their base fluid. These fluids are highly valued for their resistance to high-temperature and high-pressure drilling environments and their ability to provide superior lubrication for the drill bit. Lastly, gas-based drilling fluids, such as air or nitrogen, employ gases as the base fluid. They excel in specific drilling

scenarios, such as excavating coalbed methane. The selection of drilling fluid type is influenced by geological formations, well conditions, and environmental factors, and selecting the optimal formulation is essential for optimizing drilling performance and ensuring wellbore stability [1]. Each type provides a distinct combination of ingredients and functions to address the difficulties encountered during drilling operations.

In addition to the three main categories of drilling fluids, there is another important category known as synthetic drilling fluids. Synthetic drilling fluids, also known as synthetic-based muds (SBMs), are composed of synthetic or man-made base fluids as opposed to conventional oil or water. These base fluids can include esters, olefins, and other hydrocarbons that are synthetic. Drilling operations benefit from several advantages when synthetic drilling fluids are used. They are highly stable at elevated temperatures, making them ideal for excavating in high-temperature environments, such as offshore deep wells. Additionally, synthetic fluids have excellent lubricating properties, reducing equipment wear and extending their lifespan. Moreover, they have less of an impact on the environment than oil-based lubricants because they do not contain crude oil. When drilling through sensitive formations where contamination from conventional drilling fluids could cause significant damage, synthetic drilling fluids are particularly advantageous [2]. Synthetic drilling fluids are a valuable option for optimizing drilling performance while mitigating environmental impact in certain drilling scenarios due to their adaptability, stability, and environmental benefits. However, synthetic drilling fluids can be more expensive than their conventional equivalents, which can impact their selection based on the budget and drilling conditions of a project.

Particularly when considering the sustainability and environmental impact of drilling operations, the significance of water-based drilling fluids which consist predominantly of water as their base fluid, has risen in popularity. As industry endeavors to reduce its carbon footprint and minimize drilling's environmental impact, water-based fluids have emerged as a sustainable solution. However, water-based drilling fluids present their own set of challenges particularly when drilling through shale formations, where problems such as shale swelling can compromise wellbore stability [3]. Shale formations are characterized by their high concentration of clay minerals, notably smectite and montmorillonite clay minerals, which are recognized for their swelling properties. When drilling fluids containing water meet clay minerals, the water is absorbed, causing the clay to expand. This expansion, also known as shale swelling, can exert pressure on the wellbore walls and result in a few problems. Increased pore pressure within the formation can result in wellbore instability, which can lead to wellbore expansion and other problems such as blocked pipe incidents, lost circulation, and decreased drilling efficiency. Inefficient cuttings conveyance may also exacerbate drilling challenges. To combat these issues, shale inhibitors or shale stabilizers are added to water-based drilling fluids by the drilling industry. These additives are designed to minimize the interaction between the drilling fluid and shale minerals, thereby reducing swelling and sustaining the stability of the wellbore.

Shale swelling inhibitors are chemical additives used to combat the swell of clay minerals in shale formations during drilling operations. Potassium chloride (KCl) and amine-based inhibitors are two popular types of traditional shale swelling inhibitors. KCl prevents clay swelling by increasing the ionic strength of the drilling fluid, thereby decreasing the water absorption capacity of clay minerals. On the other hand, amine-based inhibitors function by adsorbing onto clay surfaces and modifying their chemical properties to render them less susceptible to hydration. Although these inhibitors have historically played a role in sustaining the stability of the wellbore, they have significant limitations related to conventional drilling methods. The release of KCl and certain amine-based inhibitors can contaminate soil and water, raising environmental concerns about the sustainability of these conventional approaches. In addition, these inhibitors may not function optimally under high-temperature or high-pressure drilling conditions, restricting their applicability for modern unconventional resources. There is also limited compatibility with some drilling fluid formulations

containing polysaccharides or polyanionic cellulose, further reducing the scope of usable formulations. The high cost of these traditional inhibitors additionally influences selection criteria, driving demand for more affordable shale swelling options.

To mitigate these drawbacks, the industry has shifted focus toward more sustainable and environmentally friendly approaches that align with stringent environmental regulations. The "green criteria" for new shale swelling inhibitors place priority on reduced toxicity, biodegradability, and minimizing environmental impact. This context encompasses not only environmental sustainability but also improved performance and adaptability under diverse drilling conditions. Alternatives like Graphene Oxide (GO) demonstrate promise for reducing shale swelling with less risk of ecological damage. Ionic Liquids (ILs) boast superior inhibition properties and a lower environmental footprint compared to conventional options. Emerging solvents like Deep Eutectic Solvents (DESs) effectively prevent swelling. Furthermore, biosurfactants, nanomaterials, and natural biomolecules offer environmentally conscious solutions to promote wellbore stability with minimal toxicity.

Shale swelling mechanism

The enlargement of shale during drilling with water-based drilling fluids is a complex process governed by several interrelated factors. Shale swelling is triggered when shale is exposed to drilling fluid with a different chemistry that causes clay minerals to absorb and adsorb water [4]. This typically occurs when shale formations that were confined in situ underground at high stresses are excavated and exposed to surface conditions or water infiltration. At the molecular level, as pore waters and moisture infiltrate into the shale, water molecules first get attracted to and bonded to the surfaces of clay mineral particles like montmorillonite or illite. These clays have an affinity for water and the additional mass intake causes them to expand their layered structures. These interactions between water and clay minerals are crucial to the phenomenon of shale expansion [5]. According to Alcázar-Vara and Cortés-Monroy, shale swelling occurs in three distinct stages: stage 1: surface hydration and inter-crystalline swelling; stage 2: intra-crystalline swelling from moisture ingress between mineral layers and stage 3: osmotic swelling from diffused double-layer expansion [6].

When shale comes into contact with water, the first stage of swelling occurs through capillary absorption of the water into the pores between the clay mineral particles (inter-crystalline pores) as shown in Figure 1. This stage corresponds to inter-crystalline swelling. As the water molecules enter the pores, they become attached to the surface of the clay mineral particles through hydrogen bonding and other surface forces. In sodium montmorillonite clay, the water dipoles readily attach to the siloxane cavities exposed on the edges of the clay mineral layers, as well as to cations like Na^+ adsorbed on the surface. The affinity for water absorption depends on the specific surface area available for bonding and the nature of the interchangeable cations present. Cations like Ca^{2+} attract fewer water molecules than cations like Na^+. The absorbed water forms a hydration shell around the particles, forcing them apart and leading to volumetric expansion and inter-crystalline swelling. The rate and amount of swelling depend on factors like the permeability of the shale, the availability of surface area for water bonding, shale mineralogy, and cation exchange capacity. Tight shales with low permeability and surface area tend to absorb less water and swell less. Highly reactive shales containing montmorillonite as the predominant clay mineral, with high cation exchange capacity, tend to imbibe larger amounts of water and exhibit greater inter-crystalline swelling.

The second stage of swelling occurs as water penetrates between the layered structural packets within the clay mineral particles themselves, known as intra-crystalline swelling. This process is most significant in smectite group clay minerals like montmorillonite, where the layered packets are held together only by relatively weak van der Waals forces. For instance, in montmorillonite clay, water dipoles can penetrate between the 2:1 layered silicate sheets that make up its structure. The extent of penetration and separation of the sheets depends heavily on the exchangeable cations present. Sodium montmorillonite will hydrate extensively, with unlimited swelling possible as water freely enters the interlayer spaces. On the other hand,

calcium montmorillonite has limited swelling due to the stronger binding of Ca^{2+} ions holding the sheets more tightly together. The intrusion of water leads to an increase in the basal spacing between the stacked sheets and a many-fold volumetric expansion - the intra-crystalline swelling. The presence of even small amounts of swelling clays like montmorillonite in shale can result in substantial overall swelling behavior.

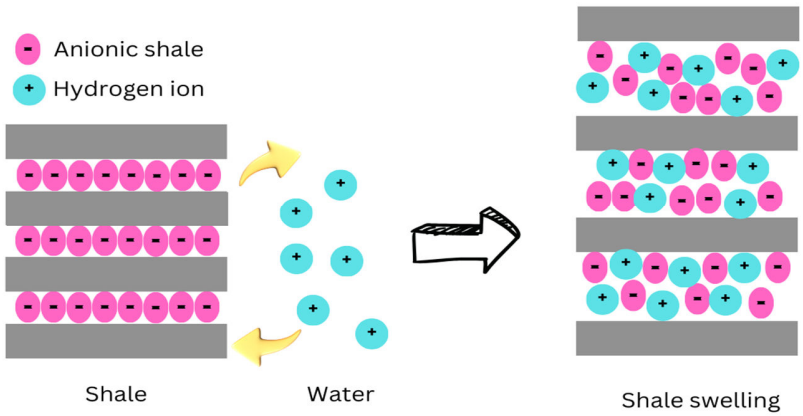


Figure 1. Surface hydration or inter-crystalline swelling

For the last stage, the introduction of water into the shale also induces osmotic pressure within the clay structure [7]. This osmotic pressure forces more water into the interlayer spaces, causing the clay layers to separate and swell. The degree of swelling increases as the osmotic pressure created by the drilling fluid rises, which is affected by variables including water content and salt concentration [8]. The ionic strength of the drilling fluid, which is determined by the concentration of salts such as potassium chloride (KCl) and calcium chloride ($CaCl_2$), can substantially influence the behavior of shale swelling [9]. The negatively charged surfaces of shale minerals attract positively charged ions or cations present in the drilling fluid. This exchange process results in the adsorption of cations onto the surfaces of clay minerals, which causes the clay strata to expand. Due to this, the distance between the strata grows, which contributes to the swelling effect [10]. Nevertheless, it was reported that higher salt concentrations in the drilling fluid somehow diminish the water's ability to permeate clay layers, thereby mitigating swelling to some extent [11]. Moreover, it was found that temperature and pressure also play a significant role in shale swelling. In a wellbore, elevated temperatures and pressures can intensify the absorption of water by shale minerals and the concurrent expansion of clay layers. These downhole conditions can have a significant effect on the amount of shale swelling encountered during drilling [12, 13]. Therefore, chemical inhibitors, such as surfactants or polymers, are frequently included in drilling fluid formulations to combat shale expansion issues. These shale inhibitors alter the surface properties of the shale, reduce water absorption, and prevent clay layers from expanding, as shown in Figure 2. By understanding the relevant factors, drilling engineers and fluid specialists can devise more effective strategies for combating shale swelling and preserving wellbore stability. It was concluded that the rate and magnitude of volume expansion depend on shale mineralogy, permeability to water, and pore pressures. This information guides the selection and formulation of drilling fluid additives intended to inhibit shale swelling. In addition, it permits the optimization of drilling parameters, such as temperature and pressure, to minimize the amount of edema encountered during operations. The ultimate objective is to improve drilling efficiency, reduce operational difficulties, and ensure the safety and integrity of wellbore systems. In addition, this understanding contributes to ongoing efforts in the oil and gas industry to develop environmentally sustainable drilling practices by minimizing the environmental impact of shale swelling and the use of drilling fluid

additives.

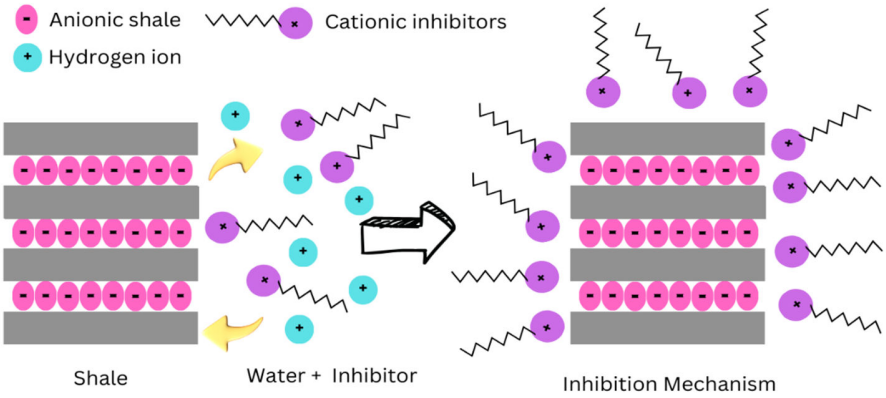


Figure 2. Shale inhibition mechanism by shale swelling inhibitors

Traditional shale swelling inhibitors

Potassium chloride (KCl) is the earliest and most extensively used shale swelling inhibitor for water-based drilling fluids. KCl inhibits the swelling of shale through an ion exchange mechanism. It does this by exchanging the Na^+ ions in the montmorillonite clay layers with K^+ ions that are weakly hydrated, as shown in Figure 3. This exchange of ions decreases the clay's affinity for water, preventing expansion and preserving the wellbore's integrity. Previously, it was found that by using KCl as the shale inhibitor, the activity of the drilling fluid increased, and the contact angles between the fluid and the shale increased, indicating a higher absorption rate [14]. According to the study, the ability of the drilling fluid to inhibit was inversely proportional to its ability to hydrate the surface. Osmotic hydration data showed that the shale had the lowest negative extreme swelling ratio with KCl, indicating its superior inhibitory effect, while the KCl drilling fluid displayed the highest surface adsorption rate. Moreover, another study found that KCl had an inhibitory effect on the mechanical properties of the samples, increasing their compressive strength and elastic modulus while decreasing their Poisson's ratio [15]. However, the results demonstrated that the clay content and presence of microcracks and bedding fissures in shale had a significant impact on the inhibitory effect of KCl on hydration. In addition, it was found that the relationship between KCl concentration and expansion rate is negatively exponential [16]. As the KCl concentration increased, the swelling rate decreased. When the concentration of KCl was 6%, the rate of swelling tended to be stable. The ultimate rate of swelling decreased from roughly 2% to 0.5% as shown in Figure 4. This high concentration increased the density of the drilling fluid, making it less suitable for use under specific drilling conditions.

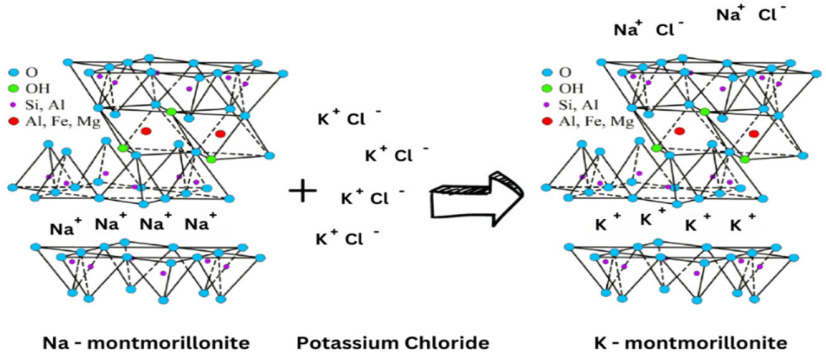


Figure 3. The replacement of Na^+ with K^+ within the montmorillonite structure

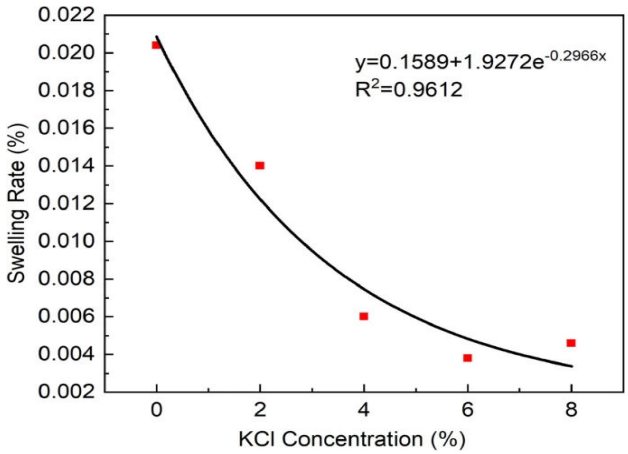


Figure 4. Final swelling rate of mudstone samples in relation to KCl concentration [16]

As a result of these limitations, various possible replacements for KCl have been investigated. Ammonium chloride (NH_4Cl), in contrast, functions as a shale inhibitor by facilitating the exchange of ammonium ions with the cations present on the surface of clays [17]. This ion exchange aids in preventing clay hydration and subsequent swelling. The NH_4^+ ion from NH_4Cl can replace Na^+ and other cations between the layers of the clay minerals. Since NH_4^+ has a lower hydration energy and tendency to attract water molecules compared to Na^+, the overall water uptake by the clay is reduced, thereby diminishing the swelling potential. According to a previous study, NH_4Cl is notably effective in stabilizing clays that are sensitive to potassium ions [18]. Moreover, NH_4Cl helps to establish an osmotic balance between the drilling fluid and the shale formation [19]. By controlling the water activity in the drilling fluid, the drive for water molecules to move into the shale and cause swelling is reduced. The presence of NH_4^+ ions also stabilized the clay structure by reducing the repulsive forces between the clay platelets [20]. This contributes to a reduction in the swelling and dispersion of the clays. Nevertheless, despite its benefits, the environmental impact of NH_4Cl, particularly its potential to contribute to eutrophication, and its corrosive nature are significant concerns. To surmount these issues, NH_4Cl is often used alongside corrosion inhibitors and advanced waste management techniques to minimize its environmental footprint.

The investigation of ammonium cations with similar hydration volumes to potassium ions (K^+) is a prominent strategy. This research led to the development of a variety of amine-based shale inhibitors, including polyetheramine, polyetherdiamine, polyvinylamine, and quaternary amine compounds. Amine-based shale inhibitors have emerged as a prospective alternative to potassium chloride (KCl) in water-based drilling fluids due to their ability to inhibit shale swelling effectively while mitigating some of the environmental concerns associated with KCl. Polyamines are particularly effective in inhibiting the swelling of smectite clays commonly found in shale formations. They work by adsorbing onto clay surfaces and neutralizing the charges that cause clay particles to repel each other and swell. In addition, amine-based inhibitors can inhibit shale at lower concentrations than KCl, which can aid in mitigating problems associated with drilling fluid density and disposal. Previously, the function of polyether amine as a shale inhibitor in the production of shale hydrocarbons has been studied [21]. The study demonstrated that the pH of polyether amine solution can influence its shale-inhibiting properties. Besides, it was found that polyether amine-derived bis-quaternary ammonium salt demonstrated superior shale inhibition performance compared to conventional stabilizers. The mechanism entails strong adsorption, an increase in hydrophobicity, and the reduction of clay particle repulsion by positive charges. This research provided an effective shale inhibitor, which is crucial for the efficient and sustainable development of shale gas and hydrocarbons. Moreover, the application of ammonium-

malic salts (AMS) as inhibitors for shale swelling has also been investigated [22]. The compound AMS-9, which was synthesized using malic acid and tetraethylenepentamine, demonstrated remarkable effectiveness in inhibiting the expansion of bentonite hydration. The addition of this substance resulted in a notable decrease in both the swelling and dispersion of bentonite within the water-based drilling fluid with the swelling rate dropping to 57.14% at a concentration of 0.5% AMS-9. According to this study, the inhibitory mechanism of AMS-9 on bentonite is likely attributed to the process of ion exchange between ammonium and Ca^{2+}, as well as the formation of hydrogen bonds and surface modifications. Furthermore, the potential of ammonium adipate solutions (AASs) as inhibitors for the phenomenon of shale swelling has also been studied [23]. The results showed that AAS-8 exhibited significant inhibitory effects on the hydration and swelling of clay. The substance exhibited the ability to regulate alterations in particle size inside bentonite and showed compatibility with additives such as carboxymethyl cellulose (CMC) and modified starch in water-based drilling fluids.

Nonetheless, the disposal of significant quantities of amine-containing drilling fluid can have negative environmental effects, including the disruption of ecosystems and the contamination of groundwater. In addition, certain amine compounds may have adverse effects on the rheological properties of the drilling fluid, which may affect drilling performance. Proper disposal procedures and monitoring are required to ensure that the use of amine-based inhibitors does not result in environmental contamination or damage to ecosystems. In response to the environmental and toxicity issues associated with amine-based inhibitors, there has been a growing interest in researching environmentally benign and low-toxicity shale inhibition alternatives over the past few years. As prospective shale inhibitors, researchers and industry are investigating various materials, including nanomaterials, ionic liquids, natural plant extracts, and other innovative compounds. These options seek to inhibit shale effectively while minimizing toxicity and environmental impact. The ongoing research in this area reflects a broader commitment to enhancing the environmental sustainability of drilling operations and conforming to more stringent environmental protection policies.

Green shale swelling inhibitors

Graphene oxide

Graphene oxide (GO) is a notable environmentally friendly shale swelling inhibitor that has garnered prominence in recent years. Graphene oxide is an oxidation of graphene that has demonstrated potential for mitigating shale expansion while minimizing environmental impact. In shale formations, GO particles readily adsorb onto shale clay mineral surfaces due to their hydrophilic groups and large area. Once adsorbed, the graphene oxide forms extensive hydrogen bonds and van der Waals forces, adhering tightly to the shale. This also allows the graphene oxide to act as a physical barrier that obstructs nano-sized pores and interlayer spacing between clay mineral platelets. By hindering water molecule intrusion, graphene oxide mitigates the swelling and dispersion of clay components. Additionally, graphene oxide imparts hydrophobicity to the shale surface, further diminishing the affinity of clays to hydrate and swell when contacting aqueous drilling fluids. Through this multipronged mechanism of physical obstruction of nanopores, hydrogen bonding, and hydrophobic modification, graphene oxide can effectively suppress shale swelling when used as an inhibitor additive. Significant reductions in shale swelling have been observed in various studies utilizing GO as a shale inhibitor, yielding encouraging results, as shown in Figure 5. The study demonstrated the ability to plug nano-sized pores, prevent water intrusion into shale, and prevent clay mineral swelling [24]. According to the findings of his study, GO exhibited superior performance to conventional inhibitors, forming a large, uninterrupted film to protect shale and adapting to various shale pore morphologies. Based on this foundation, glucopyranose-modified graphene (Glu-Gr) as a shale swelling inhibitor has also been studied [25]. The study revealed the exceptional dispersion recovery and inhibition durability of Glu-Gr, which surpasses conventional drilling mud. The mechanism of Glu-Gr, which is characterized by scanning electron microscopy (SEM) and X-ray diffraction spectroscopy (XRD), relies on establishing hydrogen bonds, physically obstructing clay nanopores, and preventing water penetration,

thereby diminishing shale swelling.

Moreover, *Janus amphiphilic* graphene oxide (JAGO) has also been used as a high-performance and dual-functionality shale swelling inhibitor [26]. It was found that the distinctive amphiphilic structure of JAGO, obtained by dodecylamine modification, has exceptional chemical inhibition and physical plugging properties. The effectiveness of JAGO derives from its capacity to adsorb onto clay surfaces, expel water molecules, and generate hydrophobic shields. In addition, choline chloride-modified graphene (Ch-G) has also been used as an eco-friendly and effective shale inhibitor [27]. The study demonstrated the inhibition mechanism, which includes hydrogen bonds, coordination, and electrostatic interactions with clay surfaces, thereby inhibiting shale expansion. Because of graphene's high performance, it has also been employed as a fluid additive [28, 29]. The use of silica nanoparticles (SiO_2-NPs) and graphene nanoplatelets (GNPs) as additives in drilling fluids exhibited synergistic effects, improving filtration properties, enhancing thermal stability, and increasing the fluid's ability to inhibit shale-related challenges. Collectively, these studies demonstrate the transformative potential of nanomaterial-based shale inhibitors, which offer innovative and environmentally friendly methods for ensuring wellbore stability in shale formations while minimizing environmental impact. While graphene oxide offers a novel approach to inhibiting shale swelling by physically blocking water absorption and chemically interacting with clay minerals, there remain significant economic and environmental challenges to its widespread adoption in the field. Research is ongoing to overcome the cost barriers, ensure environmental safety, and maintain operational stability and compatibility within drilling fluid systems. Through innovation and rigorous testing, the goal is to optimize the use of GO in drilling operations, harnessing its unique properties to enhance wellbore stability and efficiency.

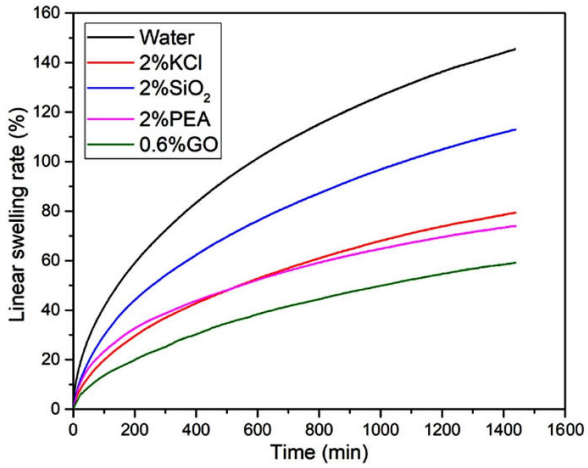


Figure 5. Linear swelling rate of Na-BT pellets in pure water, KCl, nano-SiO_2, PEA, and GO aqueous solutions [24]

Ionic liquids (ILs)

Aside from that, ionic liquids (ILs) have also emerged as an environmentally favorable option for reducing shale swelling in water-based drilling fluids. The mechanism by which ionic liquids inhibit shale swelling involves several interactions. They can replace water in the interlayer spaces of clay minerals through cation exchange, thus reducing the clay's ability to absorb water and swell. Additionally, the hygroscopic nature of ionic liquids can lower the activity of water in the drilling fluid, and their adsorption onto clay surfaces can alter the surface chemistry to further reduce swelling. In addition, ILs have garnered attention for their low toxicity and eco-friendly properties, making them a desirable option for sustainable drilling practices. Previously, the use of 1-butyl-3-methylimidazolium chloride (BMIMCl) as an inhibitor of clay expansion has been investigated [30]. It was found that BMIMCl exhibited superior inhibitory properties than the conventional stabilizer KCl, decreasing bentonite

swelling rates by 19.38%. FT-IR analysis confirms that this enhanced performance is due to the unique structure of BMIMCl, which features a hydrophilic head for adsorption on bentonite surfaces, hydrogen bonding, and a hydrophobic tail to create a water-repellent shield. Besides, the use of hydrophobic ILs in conjunction with gemini surfactants to prevent clay expansion has also been studied [31]. A novel combination of hydrophobic ionic liquid (trihexyltetradecyl phosphonium bis(2,4,4-trimethyl pentyl) phosphinate) and cationic gemini surfactant (GB) enhanced inhibition efficiency.

Contact angle measurements revealed a highly hydrophobic surface and linear swelling experiments indicate that the combined solution reduces bentonite swelling. This innovative technique improves wellbore integrity in drilling applications involving high temperatures. Besides, the used of multibranched ionic liquid (Trihexyltetradecyl phosphonium bis(2,4,4-trimethyl pentyl) phosphinate, Tpb-P) in water-based drilling fluid exhibited enhanced shale recovery and reduced linear swelling compared to conventional drilling fluids [32]. Contact angle measurements confirmed decreased shale surface hydrophilicity, and Tpb-P demonstrated the potential to maintain wellbore stability under high-temperature and high-pressure conditions. The results of these experiments demonstrated that the adsorption of ILs on clay minerals is dependent on their structure and concentration, leading to intercalation between clay layers. XRD analysis revealed increased basal spacing, implying IL intercalation, which was corroborated by FT-IR spectral data. The study provides insight into the inhibitory mechanisms and adsorption behavior of ILs on clay surfaces. Due to the unique properties of ILs, they have also been proposed as completion fluids to reduce formation damage. Moreover, experiments with core-flooding on confined sandstone formations with a high clay content demonstrated that completion fluids comprised of ionic liquids effectively stabilized clays, thereby preserving in-situ permeability [32]. These results indicated that completion fluids based on ionic liquids can be used for well completion without causing formation injury. This study was supported by using tetramethylammonium chloride (TMACl) and 1-ethyl-3-methylimidazolium chloride (EMIMCl). It was found that TMACl and EMIMCl ionic liquids outperformed the conventional inhibitor potassium chloride (KCl) [33]. TMACl exhibited superior inhibition due to its more positive charge, which facilitated adsorption and decreased clay enlargement. This study emphasized the importance of cationic and anionic components in ILs for shale inhibition.

SEM images revealed fewer cavities and a smoother clay surface when ILs were added, while XRD results indicated intercalation of ILs in clay layers. The type of anion also affected rheological properties, but clay swelling was not substantially affected. In comparison to a commercial clay inhibitor, ILs exhibited superior performance with fewer adverse effects on drilling fluid properties. These studies illuminate the diversity of ionic liquids and inhibitors, shedding light on their potential to revolutionize shale inhibition strategies, assure wellbore stability, and reduce environmental impact. Nevertheless, increasing the length of the alkyl chain increased the hydrophobicity of ILs, making it more challenging for ILs to minimize layer spacing and reduce water activity as shown in Figure 6.

Deep eutectic solvents (DESs)

Building upon the promising attributes of graphene and ionic liquids as environmentally benign swelling inhibitors for shale, numerous studies explore the effectiveness of deep eutectic solvents (DES) in a similar capacity. Deep Eutectic Solvents (DESs) are a class of fluids like ionic liquids but composed of a mixture of a hydrogen bond donor and a hydrogen bond acceptor, which can form a eutectic mixture with a melting point much lower than either of the individual components. DESs interact with clay minerals via solvation and ion exchange mechanisms, thereby decreasing the tendency of shale to swell. DESs can inhibit shale swelling effectively while exhibiting low toxicity and environmental impact. Previously, the novel deep eutectic solvent CU-DES as a potential shale inhibitor was studied [34]. From the study, it was seen that CU-DES exhibits remarkable inhibitory properties, outperforming conventional inhibitors such as KCl and PDA, especially at high temperatures (160°C). CU-DES substantially reduces the swelling rate of clay samples, increases the recovery of shale cuttings after hot-rolling,

and inhibits clay hydration and dispersion. Analytical techniques, such as particle size distribution measurement, Zeta potential measurement, SEM, FTIR, and XRD, demonstrated that CU-DES exerts its inhibitory effects via electrostatic attraction and hydrogen bonding with clay minerals. The findings of this study indicated that CU-DES is a prospective and cost-effective shale inhibitor for water-based drilling fluids, addressing wellbore instability issues. Recent research on DESs as shale swelling inhibitors has also been thoroughly studied [35].

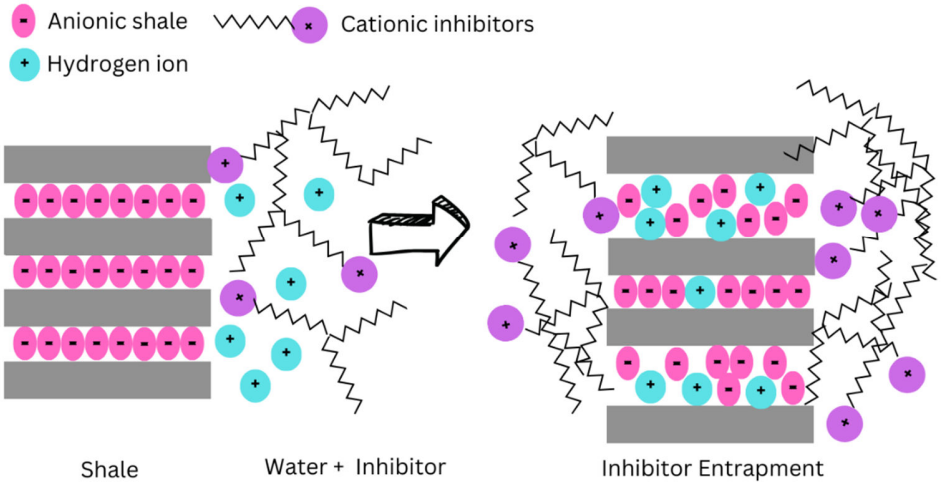


Figure 6. Inhibitor entrapment due to the length of the alkyl chain

Even at low concentrations, DESs exhibit extraordinary inhibitory performance, effectively reducing shale swelling. This study emphasizes the electrostatic attraction, hydrogen bonding mechanisms, and charge neutralization of clay surfaces. It highlighted the significance of comprehending DES-clay interactions for effective shale stabilization. As a drilling fluid adjuvant, a Natural Deep Eutectic Solvent (NADES) based on Epsom salt and glycerine was also introduced [36]. The results indicated that NADES significantly enhances the properties of drilling fluids. NADES reduces mudcake thickness by 26% and filtrate volume by 30.1% at a concentration of 1%. It inhibited the shale swelling by an impressive 49.14% due to hydrogen bonding and clay structure modification. The results of this study suggest that NADES could replace conventional inhibitors that are toxic and non-biodegradable. An extensive study using calcium chloride with NADES as a versatile drilling mud additive has also been done [37]. It was found that NADES improved mud rheology and inhibited hydrate formation by up to 80%, according to the findings. Moreover, it inhibited shale swelling by a remarkable 62.8% and enabled an 84.1% enhancement in shale recovery. These results are supported by surface tension, d-spacing, zeta potential, and FESEM analyses. This paper suggests that NADES is an environmentally favorable alternative to conventional additives, effectively addressing drilling operations' cost, biodegradability, and environmental impact issues. In comparison to conventional inhibitors, the results consistently demonstrate DES' superior inhibitory properties, cost-effectiveness, and environmental advantages. These results contribute to the advancement of environmentally sustainable drilling practices and highlight the need for additional research and optimization to realize their maximum potential in the industry.

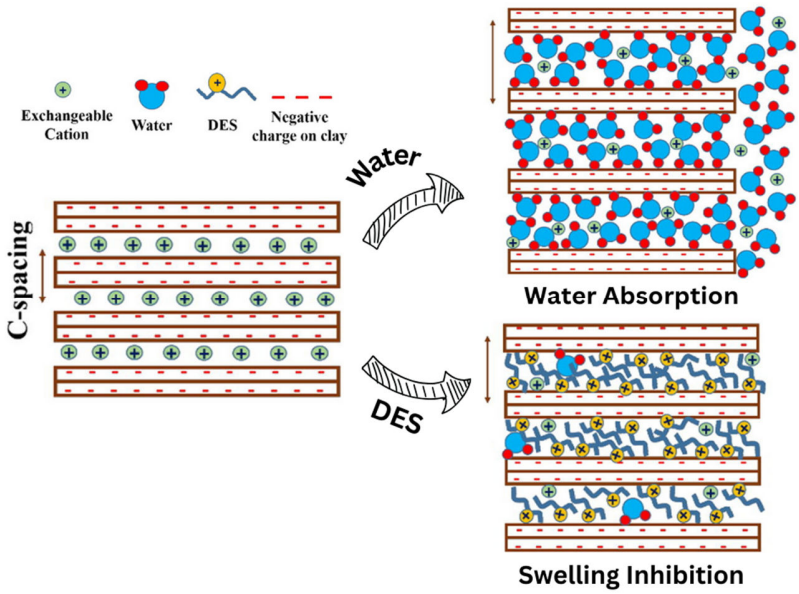


Figure 7. Shale swelling inhibition mechanism by using DES inhibitor [38]

Nanoparticles and nanocomposites

Similarly, numerous researchers have also conducted extensive studies on nanomaterials such as nanoparticles and nanocomposites. Nanoparticles and nanocomposites frequently perform their functions by adhering to clay surfaces and preventing water absorption. Figure 8 shows the surface of the shale sample using SEM. Their effectiveness in inhibiting shale swelling has been demonstrated, and their low environmental impact is encouraging. A novel method for enhancing the effectiveness of water-based drilling muds through the utilization of nanocomposites consisting of single-walled carbon nanotubes (SWCNTs) and polyvinylpyrrolidone (PVP) has been proposed [39]. The findings of their research demonstrated that the presence of 5-SWCNTs/PVP in the drilling fluid resulted in greater control of fluid loss and effectively reduced shale swelling. The combination of single-walled carbon nanotubes (SWCNTs) with polyvinylpyrrolidone (PVP) resulted in enhanced shale stability through the establishment of a protective barrier that effectively hindered the penetration of water. Additionally, the synergistic effect of ZnO nanoparticles and polymer additives in drilling fluids has also been investigated [40]. This study provided important insights into how the combination of these additives improves rheological properties, filtration control, and shale stability. The results indicated that this synergistic strategy significantly reduces shale swelling and enhances drilling fluid performance. Alternatively, the addition of two-dimensional nanoparticles, such as graphene and nanosheets, to water-based drilling fluids demonstrated that these nanomaterials significantly improve the rheological, viscoelastic, and filtration properties of drilling fluids while contributing to the stability of the wellbore [41]. In a recent study, the researchers examined the role of different nanoparticles in completion fluids [42]. The study specifically focused on the dual impact of these nanoparticles, which included both enhancing well stimulation and inhibiting clay swelling damage. The results of the study revealed that specific nanoparticles, specifically those possessing positive charges, could significantly enhance the mobility of crude oil and inhibit the swelling of clay. The study highlighted the importance of both the chemical composition and dosage of nanoparticles. Nevertheless, the necessity for further investigation to assess a broader range of nanoparticle categories and their performance in dynamic reservoir circumstances must be emphasized [43]. The study suggested delving deeper into the interactions between various nanoparticles and polymers to optimize their formulations for particular

downhole conditions. Therefore, to fully exploit the benefits of nanoparticles in drilling fluids, future research should strive to bridge the gap between laboratory experiments and actual drilling operations.

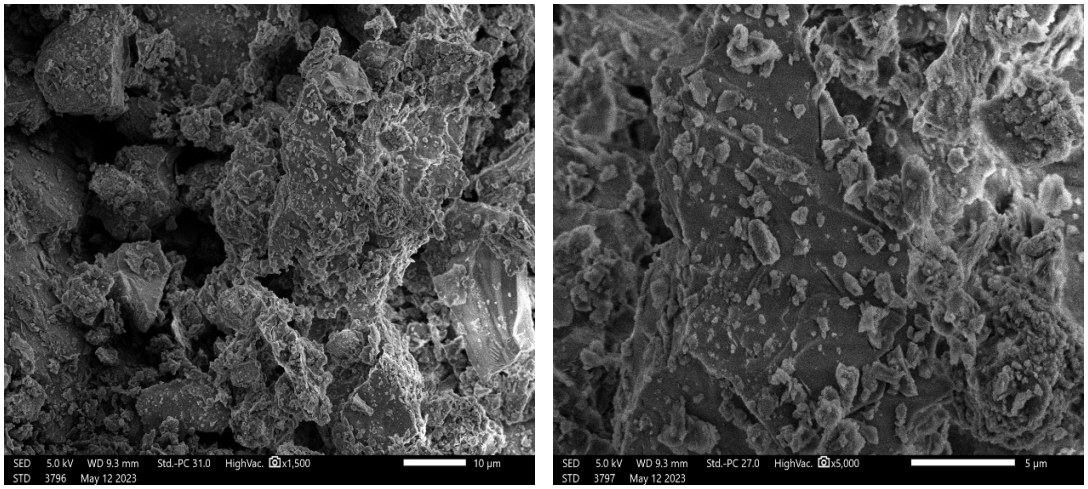


Figure 8. SEM images of the surface of shale taken at (a) 1500x resolution and (b) 5000x resolution

Biosurfactants

As eco-friendly shale inhibitors, biomolecules and biosurfactants derived from natural sources such as plants and microorganisms have been explored. Previously, a biodegradable and environmentally benign shale inhibitor based on chitosan-grafted l-arginine (CA) for shale formation was synthesized and used as a shale swelling inhibitor [44]. The results demonstrated that the inhibition of CA was superior to that of KCl, HPA, and SIAT, and that it can recover more than 90% of hot-rolled shale at 150 °C, indicating that CA has greater temperature resistance and prolonged durability. It was found that the positively charged CA effectively adhered to the surfaces of both MMT and shale, achieving multiple beneficial effects. It neutralized the negative charge associated with MMT, compacted the diffused electric double layer, and simultaneously enhanced the contact angle of both MMT and shale surfaces. This dual action resulted in an increased hydrophobicity of MMT and shale, effectively impeding their hydration enlargement and dispersion. Furthermore, compatibility experiments revealed that CA displayed compatibility with widely used treatment agents, demonstrating no impact on the rheology of water-based drilling fluids, and contributing to reduced fluid loss due to degradation.

Moreover, a fascinating investigation into the potential use of green and biodegradable surfactants, namely *Flaxseed Protein* (FP) and *Flaxseed Mucilage* (FM), as shale inhibitors in water-based drilling fluids has also been presented [45]. This research showed that these surfactants have the potential to reduce clay swelling and enhance drilling efficacy. The compatibility of these surfactants with salt (KCl) and their survivability under varying temperature conditions qualify them for further study. Nevertheless, the study suggests that future research should concentrate on optimizing the extraction methods to increase the yield and functionality of these surfactants, paving the way for more environmentally friendly drilling fluid additives. In addition, field-scale testing and compatibility evaluations with other drilling fluid components would shed light on the practical implementation of these surfactants in real-world drilling operations. Similarly, the feasibility of using Korean Red Ginseng root extract, rich in non-ionic surfactants, as a clay swelling inhibitor in water-based drilling muds has also been widely studied [46]. The research emphasized several distinctive properties of mud systems prepared with biosurfactants, including a low rheological profile, good sedimentation, and high recovery of shale cuttings. These characteristics demonstrated the efficacy of the biosurfactant in inhibiting clay swelling. The study also examined the

mechanisms underlying this inhibition, proposing that the biosurfactant produces a hydrophobic layer on clay surfaces, thereby decreasing water absorption and clay swelling. Optimizing the performance of the biosurfactant and investigating its potential as an environmentally friendly drilling fluid additive could be the focus of future research. Future research could entail scaling up the use of plant or food waste extract biosurfactants to assess their performance under field conditions. For practical applications, investigating its long-term stability and potential interactions with other drilling fluid additives would also be essential. Additionally, investigating cost-effective methods for obtaining the biosurfactant on a larger scale would make its industrial application more feasible.

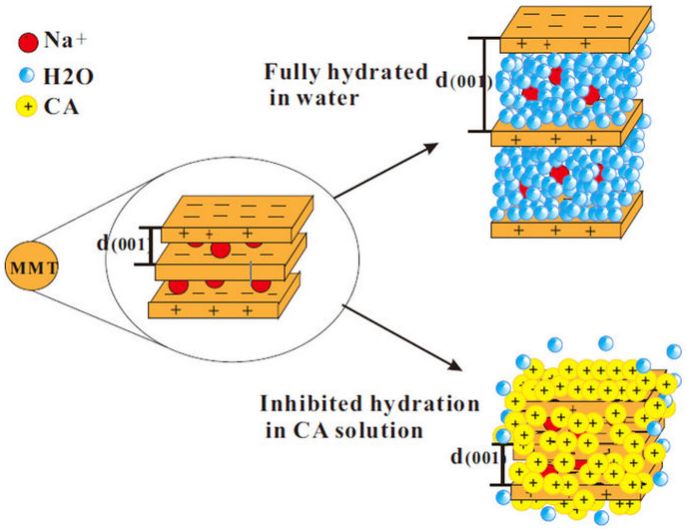


Figure 9. Inhibition mechanism of CA on shale surface [45]

Conclusion

In summary, the transition from conventional shale swelling inhibitors to environmentally friendly alternatives represent a significant paradigm shift within the drilling industry, highlighting the industry's commitment to conservation and environmental sustainability. Shale swelling, a common issue in drilling operations, is increasingly being handled using environmentally friendly inhibitors, which are replacing conventional options such as potassium chloride (KCl). The study has examined a range of environmentally friendly inhibitors, such as graphene oxide, ionic liquids, deep eutectic solvents, nanoparticles, nanocomposites, and biosurfactants. Graphene oxide, with its high surface area and functional groups, can interact with clay particles in shale to prevent water uptake. Ionic liquids and deep eutectic solvents (DESs) offer tunable properties that can be designed to reduce water activity and clay swelling without the environmental harm associated with inorganic salts like KCl. Nanoparticles and nanocomposites act at the nanoscale to block the pathways for water molecules or alter the surface chemistry of the clay minerals, preventing them from swelling. Biosurfactants, derived from biological processes, offer a biodegradable and less toxic alternative to traditional surfactants.

The transition to these greener inhibitors is not without challenges. There are concerns about cost, effectiveness under a range of geological conditions, potential health impacts, and long-term environmental effects. However, through ongoing research and development, improvements in these inhibitors are being made. Innovations in material science, chemistry, and engineering are leading to more effective and environmentally sustainable drilling practices. The use of environmentally friendly shale swelling inhibitors is a testament to the drilling industry's commitment to reducing its environmental footprint while still meeting global energy demand. By investing in these

technologies, industry not only adheres to stricter environmental regulations but also contributes to the broader goal of sustainable development. Attention may also turn to the life cycle analysis of these inhibitors to ensure their sustainability from production to disposal. Additionally, the integration of digital technologies like AI and machine learning could revolutionize the selection and application of swelling inhibitors by optimizing their performance in real time and predicting their behavior under various geological conditions. Furthermore, collaborative efforts between industry, academia, and regulatory bodies will likely be pivotal in establishing standardized protocols for the assessment and implementation of these green inhibitors.

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PHYSICOCHEMICAL CHARACTERIZATION OF ZnO/G-C_3N_4 FOR PHOTO-REMOVAL OF METHYL ORANGE UNDER LOW UV-LIGHT INTENSITY

(Pencirian Fizikokimia ZnO/g-C_3N_4 untuk Penyingkiran Metil Oren di Bawah Lampu UV Berkeamatan Rendah)

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Abstract

UV light-active photocatalyst of ZnO/g-C_3N_4 was synthesized by a facile mixing method with different ratio of g-C_3N_4 (0.01, 0.03, 0.05, 0.07, and 0.09 g). The photocatalytic activity of ZnO/g-C_3N_4 was tested towards methyl orange (MO) dye in aqueous solution using a low UV light intensity (7W). FTIR spectroscopic results obtained show the functional groups spectrum corresponding to the ZnO and g-C_3N_4. Besides, SEM results also show the formation of heterogenous photocatalysts as irregular shape particles of ZnO interlink with g-C_3N_4 surface. EDX analysis confirms the presence of Zn, C, O, and N elements correspond to ZnO/g-C_3N_4 photocatalyst. From the results obtained, ZG3 (0.1:0.03) photocatalysts exhibit almost complete removal of methyl orange under UV irradiation in a period of 60 min (~95%). The highest degradation rate constant of 5.26×10^{-2} min^{-1}, based on pseudo-first-order kinetics is estimated. The best photocatalyst (ZG3) showed high potential in degrading MO under low UV light intensity which can be used for photocatalytic water remediation application.

Keywords: g-C_3N_4, methyl orange, photocatalysis, UV light, ZnO

Abstrak

Fotomangkin cahaya UV-aktif ZnO/g-C_3N_4 telah disintesis dengan kaedah pencampuran mudah dengan nisbah g-C_3N_4 yang berbeza (0.01 g, 0.03 g, 0.05 g, 0.07 g dan 0.09g). Aktiviti fotomangkin ZnO/g-C_3N_4 telah diuji terhadap pewarna metil oren (MO) dengan menggunakan cahaya UV berkeamatan rendah (7W). Analisa FTIR spektroskopik yang diperoleh menunjukkan spektrum kumpulan berfungsi yang sepadan dengan ZnO dan g-C_3N_4. Selain itu, analisa SEM juga menunjukkan pembentukan fotomangkin heterogen ZnO sebagai zarah bentuk tidak sekata ZnO bersaling paut dengan permukaan g-C_3N_4. Analisa EDX mengesahkan kehadiran unsur Zn, C, O dan N sepadan dengan fotomangkin ZnO/g-C_3N_4. Melalui keputusan yang diperoleh, fotomangkin ZG3 (0.1:0.03) menunjukkan penyingkiran hampir lengkap pewarna metil oren di bawah penyinaran UV dalam tempoh 60 minit (~93%). Pemalar kadar degradasi yang tertinggi adalah 5.26×10^{-2} min^{-1} berdasarkan kinetik pseudo-tertib pertama telah dianggarkan. Fotomangkin terbaik (ZG3) menunjukkan potensi yang tinggi untuk degradasi MO dibawah keamatan cahaya UV

yang rendah yang boleh digunakan untuk aplikasi pemulihan air fotomangkin.

Kata kunci: g-C_3N_4, metil oren, fotokatalisis, cahaya UV, ZnO

Introduction

Since the last few decades, industrialization, technologies, and consumption of non-renewable resources have grown at a rapid pace, owing to the constant increase in demand for textile, dye, fertilizer, domestic, and plastic materials. As a result, environmental pollution and energy crises have already reached alarming proportions. Industrial wastes are more toxic and nonbiodegradable than municipal wastes because they contain fats, oil, grease, heavy metals, phenols, and ammonia, among other things [1]. The negative effects of these pollutants on human and wildlife reproductive health have become a major source of public concern [2]. Though since traditional wastewater treatment methods aren't designed to remove toxic organic pollutants, these toxins have ended up in aquatic life, where they can subsequently make their way into the human food chain [3].

In the fight against the accumulation of organic wastes in the environment and aquatic life, heterogeneous semiconductor photocatalysis has been proposed as an effective method of removing this pollution. Zinc oxide (ZnO) is a semiconductor metal oxide and widely regarded as the most potential photocatalytic materials for the abatement of pollutant in water. It has a wide bandgap of 3.37 eV, good mechanical, thermal, and chemical stability, nontoxic, cost-efficient, and can also absorb a large portion of the light spectrum (UV/solar) [4–6]. However, the photo-corrosion of pure ZnO caused by the photoinduced holes is found to be severe during the reaction, which could affect the stability and photocatalytic activity of ZnO [7]. Furthermore, the rapid recombination of photoinduced electrons and holes limits its practical application due to its weak photocatalytic activity. Several initiatives, such as metal doping, have been made to enhance its charge separation efficiency and prevent photo-corrosion [8], as well as non-metal doping [9], and heterojunction formation [10].

Recently, graphitic carbon nitride (g-C_3N_4) has proved to be a promising candidate to form heterojunctions with ZnO [11, 12]. The material in question is a metal-free, n-type polymeric semiconductor with a narrow bandgap of 2.7 eV that could provide a viable solution to increase charge separation [13]. Previous research has revealed that the heterostructure formation and synergistic interaction between ZnO and g-C_3N_4 continue to improve photocatalytic activity. Ismael, for example, synthesized ZnO/g-C_3N_4 using a simple calcination process, and the photocatalytic enhancement was attributed to an increase in the quantity of the surface hydroxyl group [11]. Alternately, Le et al. synthesized ZnO/mesoporous g-C_3N_4 using a hard-templating technique, which then discovered that those composite photocatalysts exhibited remarkable photocatalytic activity due to effective charge carrier separation at the heterojunction interfaces [14]. The charge recombination rate can be reduced with an appropriate band alignment, resulting in improved high electron transfer and increased photocatalytic activity. However, not many studies proposed the photocatalytic activity of ZnO through n-n heterojunction with g-C_3N_4 under low UV light intensity. Accordingly, an improved surface area and visible light absorption capacity of the g-C_3N_4-ZnO composite resulted in more active sites, photogenerated electron-hole pairs, and a lower frequency of photo-induced charge recombination, all of which improved the photoactivity potential [12]. On the other hand, the band adjustment at the interface was responsible for the type-II heterojunction formation and the subsequent charge transfer process from g-C_3N_4 to ZnO at the interface [15]. Direct addition of g-C_3N_4 to ZnO nanocomposite results in a decrease in crystalline size, an increase in optical bandgap, and a modification in nanocomposite morphology. When compared to pure ZnO, g-C_3N_4 significantly improves the photocatalytic performance of g-C_3N_4@ZnO nanocomposites under visible light irradiation [16].

In this work, ZnO/g-C_3N_4 composite photocatalysts were synthesized by a simple mixing approach with the use of commercially available ZnO and dicyanamide as the precursor for g-C_3N_4. By only changing the mass of g-C_3N_4, a variety of composites with various ratios were created. Utilizing X-ray diffraction (XRD), scanning

electron microscopy (SEM), energy dispersive spectroscopy (EDX), and Fourier transform infrared (FTIR), we looked at the structural, morphological, surface compositions, and functional groups of the ZnO/g-C_3N_4 photocatalysts. In order to assess the photocatalytic activity of ZnO/g-C_3N_4 under low UV light intensity (7 W), the probe molecule methyl orange (MO) dye was chosen. Grafting of non-metal materials like g-C_3N_4 onto a wide bandgap ZnO photocatalyst could be an alternative strategy to prepare a stable and efficient photocatalyst for water remediation process.

Materials and Methods

Materials

Commercial zinc oxide (ZnO, 99%) and dicyanamide ($C_2H_4N_4$, 99%) were purchased from Sigma-Aldrich (Selangor, Malaysia). Methyl orange dye was obtained from Bendosen Laboratory Chemicals (Selangor, Malaysia). All chemicals were used as received and deionized water was used throughout the experimental procedures.

Preparation of g-C_3N_4

Dicyandiamide (0.1 mol) was dissolved in a deionized water (50 mL) and magnetically stirred for 1 hour. Next, the solution was dried in the oven at 80 °C overnight, followed by calcination in a muffle furnace at 500 °C for 2 hours (heating rate: 5°C/min) in a close crucible. The obtained yellow powder of g-C_3N_4 was grounded and stored for later use.

Preparation of ZnO/g-C_3N_4 composite photocatalyst

ZnO/g-C_3N_4 composite photocatalyst was prepared by mixing the two materials through different mass ratios of g-C_3N_4. First, 0.01g of previously prepared g-C_3N_4 was mixed with 0.10g ZnO and dispersed in a deionized water (50 mL) with continuous stirring for 1 h. The collected light-yellow sample was dried in the oven at 80 °C overnight and labelled as ZG1. The same procedure was repeated with different masses of g-C_3N_4. The composition of the prepared photocatalysts is presented in Table 1.

Table 1. Compositions for the preparation of ZnO/g-C_3N_4

Photocatalyst	ZnO (g)	g-C_3N_4 (g)
ZG1	0.10	0.01
ZG3	0.10	0.03
ZG5	0.10	0.05
ZG7	0.10	0.07
ZG9	0.10	0.09

Physicochemical characterization of ZnO/g-C_3N_4 composite photocatalyst

The XRD patterns were recorded using a powder X-ray diffractometer (XRD6000, Shimadzu) with Cu Kα radiation (λ = 1.540 Å, 30 kV, and 30 mA) in the 2θ range of 10–70° with a scanning speed of 2°/min. The structural and elemental compositions were characterized using a scanning electron microscope equipped with an electron dispersive X-ray analyzer (SEM-EDX, TESCAN VEGA3). The presence of functional groups in the ZnO/g-C_3N_4 photocatalysts were analyzed by Fourier transform infrared (FT-IR) between 4000 to 500 cm^{-1} on a Perkin Elmer Infrared Spectrometer.

Photocatalytic evaluation of ZnO/g- C_3N_4 composite photocatalyst

The calibration plot of the selected pollutant methyl orange (MO) dye in aqueous solution under normal condition was carried out prior to the photocatalytic activity. The calibration curve was plotted based on different concentrations of the selected pollutant ranging from 5 to 25 mg/L. The linear equation was calculated from this calibration plot by measuring the absorbance of each sample at maximum wavelength (λ_{max} = 464 nm). Figure 1 shows the photodegradation of methyl orange in aqueous solution in a 250 mL glass photoreactor with a low intensity of UV-C light (7 W). A preliminary test which is photolysis was done on MO without the addition of photocatalyst to determine that no degradation of MO occurs when irradiated with low-intensity UV light. The system received a predetermined

amount of the powder photocatalyst. To assure the presence of dissolved oxygen in the solution, the photoreactor was then constantly supplied with air at a flow rate of 4 L/min. First, the working solution was agitated for 30 minutes in the absence of light to achieve adsorption-desorption equilibrium. The solution was then exposed to UV light for 60 minutes while being continuously stirred. The liquid (5-10 mL) was collected at predefined time intervals and filtered to remove the photocatalyst and the concentration left was monitored using a Perkin Elmer UV–vis spectroscopy (UV-2550, Shimadzu). In this study, the percentage of degradation was calculated using Equation (1) [17].

$$\text{Percentage of degradation (\%)} = \left[\frac{(C_t - C_0)}{C_0}\right] X\ 100\% \quad (1)$$

where C_o is the concentration of pollutant before irradiation and C_t is the concentration of pollutant at 't' min.

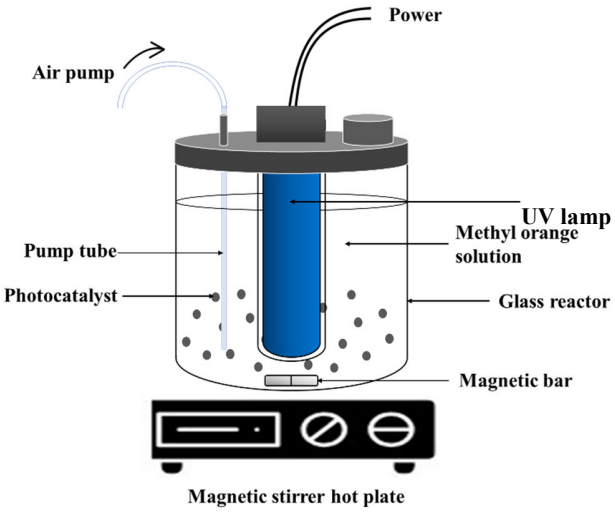


Figure 1. Schematic illustration of photoreactor set up for degradation of MO dye in aqueous solution using ZnO/g-C_3N_4 composite photocatalyst

The kinetics of the MO degradation by the prepared photocatalysts was studied based on the Langmuir–Hinshelwood (L–H) kinetic model using the following equation:

$$r = \left(\frac{dC}{dt}\right) = k_{obs}C \quad (2)$$

which can be rewritten as

$$ln\left(\frac{C_o}{C_t}\right) = k_{obs}t \quad (3)$$

where r is the reaction rate, k_{obs} is the rate constant (min^{-1}), and C_o and C_t are the concentration (mg/L) of MO initially and at different irradiation times, respectively.

Results and Discussion

Physicochemical characterization of ZnO/g-C_3N_4 composite photocatalyst: XRD analysis

The XRD patterns of pure g-C_3N_4, pure ZnO, and ZnO/g-C_3N_4 composite photocatalysts are shown in Figure 2. The pure g-C_3N_4 pattern (Figure 2a) shows some peaks as the (002) lattice planes' most strong peak, which corresponds to the interplanar stacking peak of conjugated aromatic rings, is identified at 28.16° [18–20]. The peak of 13.27° locates (100) lattice plane attributed to the in-plane structural repeating unit of N-bridged tri-s-triazine [18, 21]. In the meantime, ZnO's typical diffraction peaks are found at 31.78°, 34.40°, 36.25°, 47.54°, 56.58°, 62.82°, 67.94°, and 69.04°, with corresponding crystal indices of (100), (002), (101), (102), (110), (103), (200), (112), (201), and (202)

(Figure 2b), indicating that ZnO is the wurtzite as confirmed from the standard (JCPDS card 36–1451) [22, 23]. Next, the samples of ZG composite photocatalysts exhibit associated diffraction peaks for ZnO and g-C_3N_4 (Figure 2c-g). The presence of g-C_3N_4 diffraction peaks becomes more noticeable as the content of g-C_3N_4 was increased up to 0.09 g. It can be clearly observed that the ZG7 and ZG9 composite exhibits two-phase compositions of both ZnO and g-C_3N_4, as demonstrated by their diffraction peaks and the absence of any further peaks (Figure 2f and g). Comparing all composite photocatalysts to pure ZnO and pure g-C_3N_4, the positions of the distinctive peaks are often unchanged. This demonstrates that the ZnO/g-C_3N_4 combination has no effect on the stability of the lattice structures of individual ZnO and g-C_3N_4 [24].

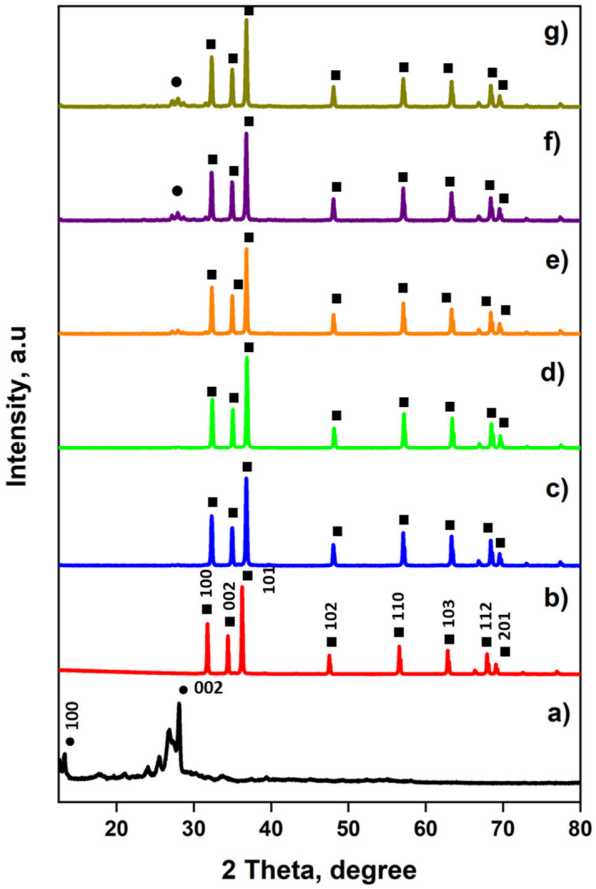


Figure 2. XRD patterns of a) pure g-C_3N_4, b) pure ZnO, c) ZG1, d) ZG3, e) ZG5, f) ZG7 and g) ZG9 photocatalysts

SEM-EDX analysis

Scanning electron microscopy (SEM) was used to examine the morphology of the prepared photocatalysts. At a magnification of 2000x, the photocatalyst images are noticed. The SEM morphology for pure ZnO, pure g-C_3N_4, and ZnO/g-C_3N_4 composite photocatalysts with various ratio compositions are shown in Figure 3. Pure ZnO reveals that it is made up of agglomerated and irregularly shaped particles (Figure 3a). It was previously reported that pure g-C_3N_4 depicted shape of irregular sheet-like meanwhile other researcher reported that pure g-C_3N_4 showed layered morphology and this is consistent with the results obtained in this study; pure g-C3N4 has a sheet-like surface and layered structure (Figure 3b) [25, 26]. Figure 3c)–d) depicts the morphology of ZnO/g-C_3N_4 composites with various g-C_3N_4 compositions. Figure 3c-g shows that ZnO are dispersed across the graphitic carbon nitride (g-C_3N_4)

surface, which helps the two phases form a heterojunction, improving charge transfer and separation as well as the photocatalytic activity of the composites [27]. This demonstrates the interactions between ZnO and g-C_3N_4 at the interface, which could help with internal electron migration to the photocatalyst surface and the effective separation of photoinduced excited photons [26].

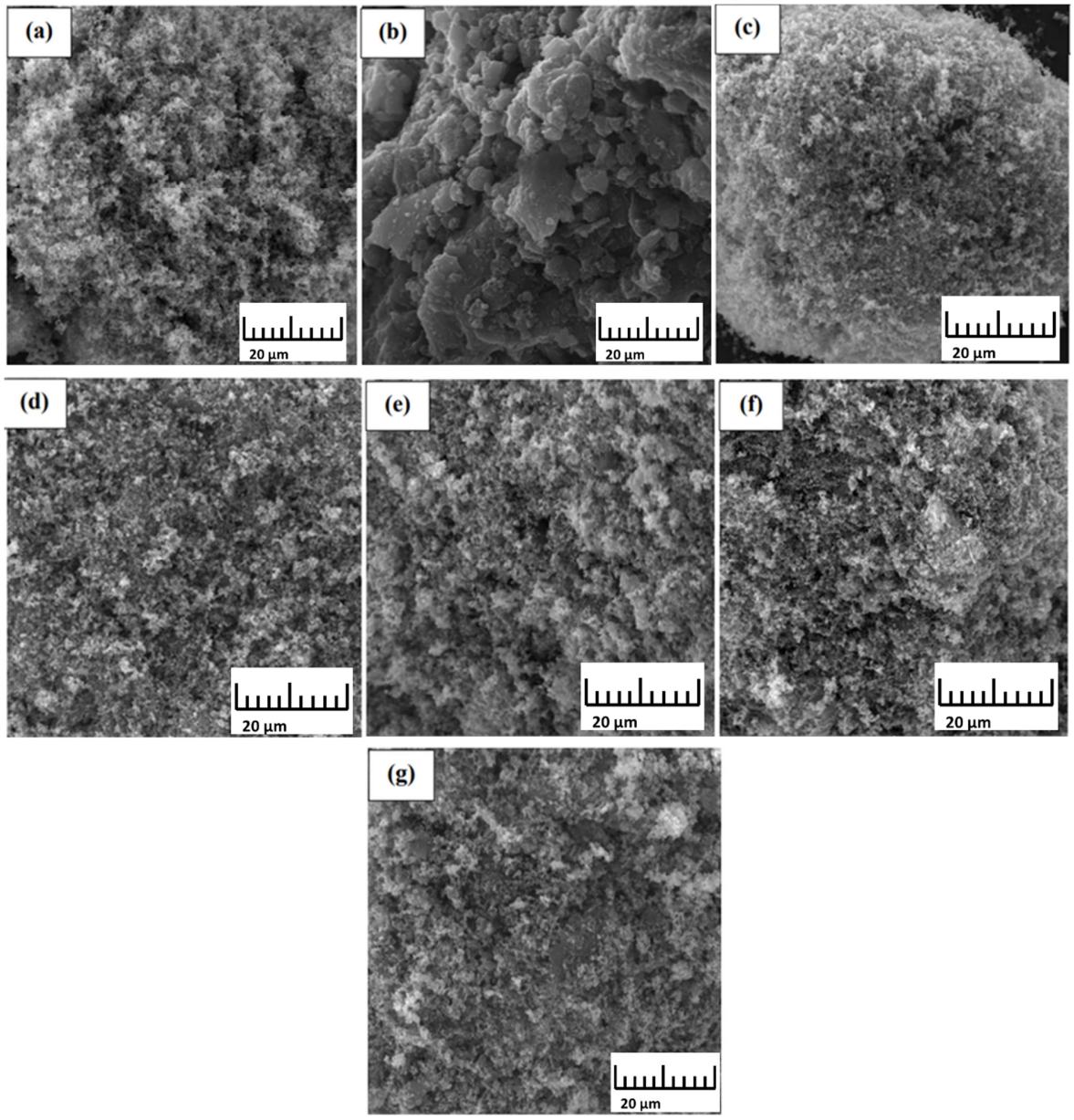


Figure 3. SEM images of (a) pure ZnO, (b) pure g-C_3N_4, (c) ZG1, (d) ZG3, (e) ZG5, (f) ZG7, and (g) ZG9

The EDX study was conducted to support the results from the SEM examination and to further confirm the presence of ZnO and g-C_3N_4 in the ZnO/g-C_3N_4 composite photocatalyst. The existence of zinc, carbon, oxygen, and nitrogen has been established for the ZG1 and ZG9 photocatalyst, confirming the composition of ZnO/g-C_3N_4, as illustrated in Figure 4a) and 4b) below.

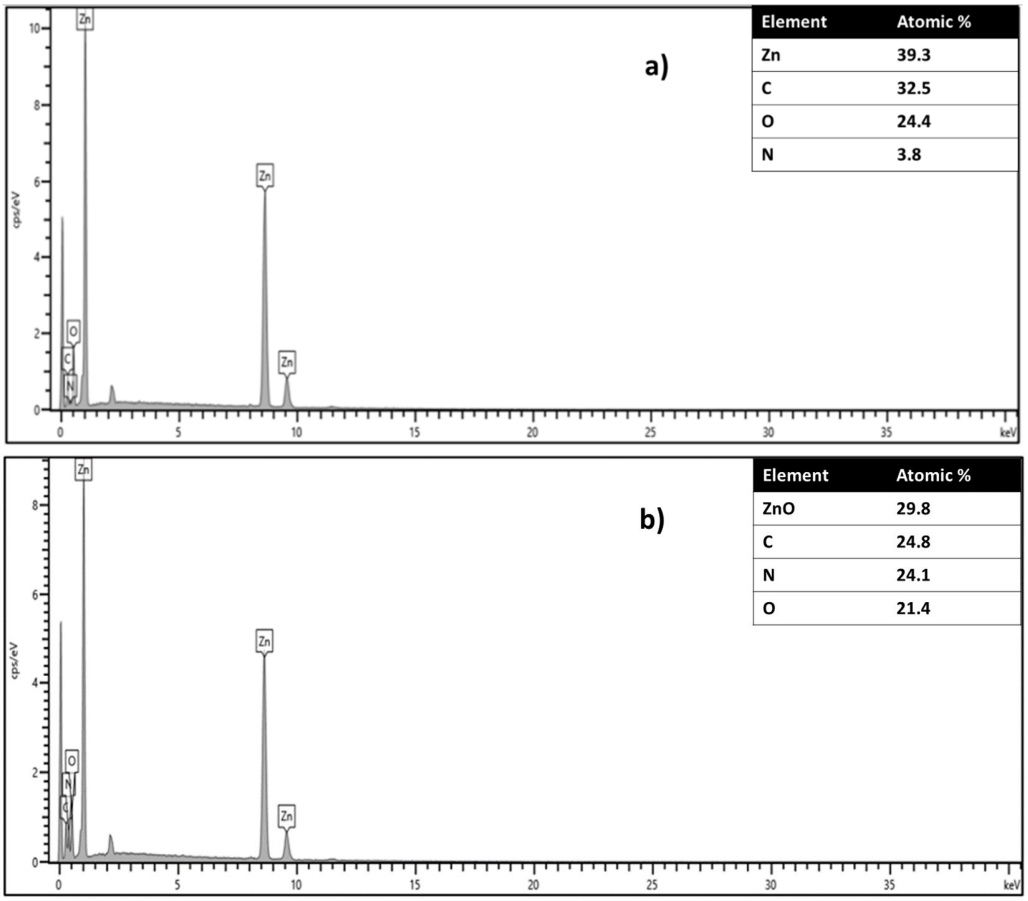


Figure 4. EDX analysis of a) ZG1, and b) ZG9

FTIR analysis

The distinct functional groups and chemical bonds present in the pure ZnO, pure g-C_3N_4, and ZnO/g-C_3N_4 composite photocatalysts were determined using the Fourier transform infrared spectroscopy (FTIR) analysis (Figure 5). From the spectrum, Zn-O bond vibrational modes can be detected in the band at the 560.80 cm^{-1} region. It is also possible to see a peak at 3348.90 cm^{-1}, which indicates the existence of a hydroxyl group in the ZnO component resulting from water molecules adhering to the oxide's surface [28]. The prepared g-C_3N_4 exhibits peaks between 1240 and 1640 cm^{-1} that indicates the presence of C_3N_4 heterocycle stretching modes. While aromatic C-N stretching vibration modes are responsible for the peak at 1463.47 cm^{-1}, C=N stretching is represented by the peak at 1613.81 cm^{-1} [29]. The triazine units of g-C_3N_4 have out-of-plane bending vibration modes that are associated to the peak at 797.32 cm^{-1} area [12]. Additionally, the N-H stretching modes are responsible for the absorption peak at 3098.56 cm^{-1}, which is between 2900 and 3400 cm^{-1} [15]. The spectrum of the ZnO/g-C_3N_4 composite photocatalyst are similar to the main peaks of g-C_3N_4, indicating that the component's structure was preserved even after combining with ZnO (subset a) and b) of Figure 5). Plus, the characteristics peaks of ZnO can also be seen in the spectrum because of its high content in the photocatalyst (subset c) of Figure 5). Moreover, the conjugated system of the g-C_3N_4 is stretched because the band red-shifted and the band strengths also diminished [30]. Thus, a broader conjugated system is generated between the ZnO and g-C_3N_4 [30]. The interfacial interaction between the ZnO and g-C_3N_4 will improve the photocatalytic performance as it will enable the transportation of photoinduced charges [26].

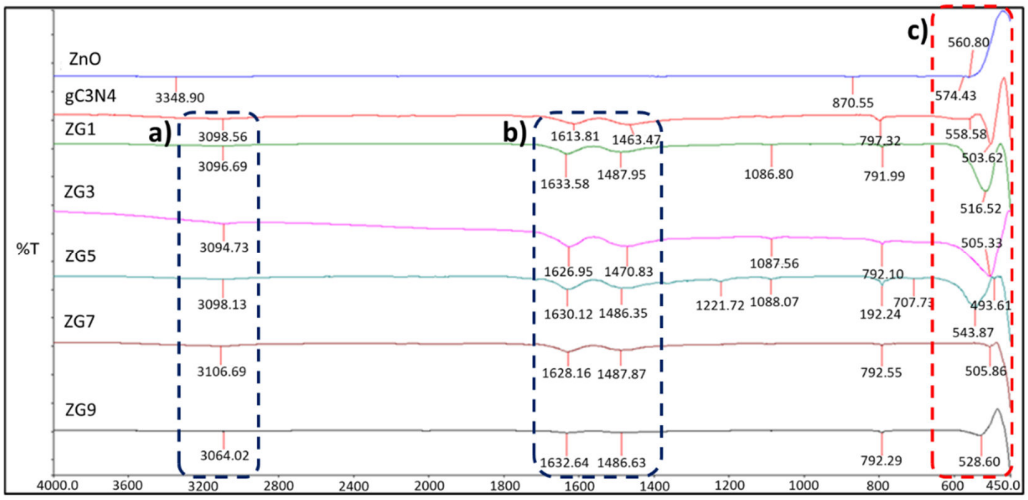


Figure 5. FTIR spectra of all prepared photocatalysts

Photocatalytic performance of ZnO/g-C_3N_4 photocatalyst

The photocatalytic degradation of MO dye by pure ZnO, pure g-C_3N_4, and ZnO/g-C_3N_4 composite photocatalysts with varied mass ratios is shown in Figure 6a). According to preliminary investigations, no degradation of MO was found throughout the photolysis process, indicating that MO was stable against UV light irradiation, with a negligible effect. In 60 minutes, pure ZnO shows 2.2 times the percentage of degradation greater than pure g-C_3N_4 (33%). In degrading MO, all generated ZnO/g-C_3N_4 composite photocatalysts demonstrate a higher percentage of degradation than pure ZnO (Figure 5a). Interestingly, with a maximum percentage of degradation of 95%, the ZG3 photocatalyst with a ratio of 0.1:0.03 is shown to be the best. It can be seen that the photocatalytic activity and degradation rate increase with an increasing amount of g-C_3N_4 up to 0.03g. As previously reported, the heterojunction that formed between ZnO and g-C3N4 is what led to the improved photocatalytic activity [11], [31–33]. However, as the g-C_3N_4 percentage increase (> 0.03), the photocatalytic activity and degradation rate falls dramatically due to the presence of aggregated particles, which lowers the surface area and light absorption capacity of the composite photocatalysts. When subjected to UV light, the photocatalyst may not function as well due to the reduced capacity of the photoinduced electrons to transfer electrons from ZnO to the g-C_3N_4 surface [34]. These results reveal the fact that there is a limitation to the amount of g-C_3N_4 that can be integrated into the ZnO surface without negatively impacting the percentage of degradation and the rate of deterioration. Therefore, ZG3 contains the appropriate ratio for the degradation of MO dye when exposed to low intensity of UV light irradiation. Given the strong synergistic interactions between ZnO and g-C_3N_4, it is clear that both elements are crucial for the enhanced photocatalytic activity.

As depicted in Figure 6b), the degradation of MO follows pseudo first-order kinetics. Likewise, the degradation percentage, the ZG3 has the greatest rate constant of 5.26 x 10^{-2} min^{-1} and, as a result, the highest rate of MO deterioration. Table 2 shows the degradation percentage and pseudo first-order kinetics for all produced photocatalysts.

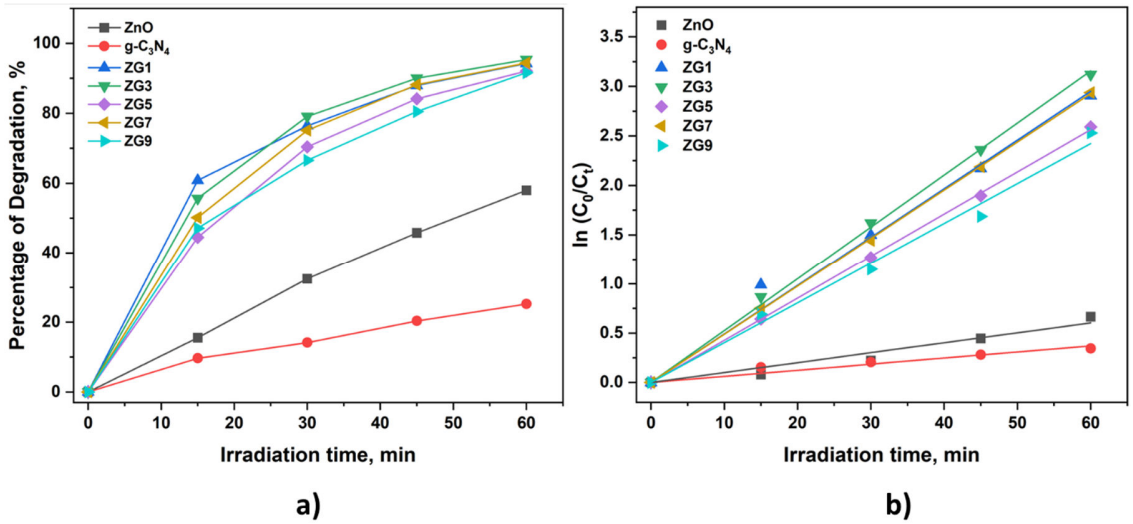


Figure 6. a) Degradation percentages and b) kinetics of the degradation of MO by all prepared photocatalysts

Table 2. Percentage of degradation and pseudo first-order kinetic for all prepared photocatalysts

Photocatalyst	Percentage of Degradation (%)	Rate Constant, K_{obs} (min^{-1}) × 10^{-2}	Rate (mg/L ·min)	Correlation Factor, R^2
Pure ZnO	57.97	1.01	0.1038	0.9782
Pure g-C_3N_4	25.20	0.62	0.0637	0.9809
ZG1	94.23	4.92	0.5057	0.9958
ZG3	95.35	5.26	0.5407	0.9995
ZG5	92.10	4.27	0.4389	0.9999
ZG7	94.41	4.88	0.5016	0.9999
ZG9	91.60	4.04	0.4153	0.9964

Conclusion

A simple mixing method was used to prepare the ZnO/g-C_3N_4 composite photocatalyst, resulting in an efficient photocatalytic removal of methyl orange (MO) dye under low UV light intensity. The findings demonstrate that the synthesized photocatalysts showed aggregated semi-spherical ZnO particles interlink with g-C_3N_4 surface. This can aid in achieving effective charge separation and lowering the rate of recombination of photoinduced charge carriers. Since ZG3 had the best photocatalytic performance which displayed ~95% photodegradation efficiency in a period of 60 min compared to other photocatalysts, it had the optimal ratio of ZnO and g-C_3N_4 contents. Overall, this research not only offers a different approach to construct ZnO/g-C_3N_4 photocatalysts through heterostructure synthesis, but also encourages simple and inexpensive processing methods.

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PHYSICOCHEMICAL STUDY ON ALKOXYLATED AZO-IMINE CHITOSAN-BASED BIOPOLYMER FOR ELECTROLYTE APPLICATIONS

(Kajian Fizikokimia ke atas Biopolimer Alkoksi Azo-Imina Berasaskan Kitosan bagi Aplikasi Elektrolit)

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Abstract

The performance of electrolytes in electronic applications are in decline due to low ionic conductivity, leakage, and inadequate thermal stability. Overcoming these challenges necessitates innovative approaches which includes electrolyte design, and materials selection, that should be taken into consideration. In this study, new additive alkoxylated azo-imine (**AZ**) material containing hybrid moieties of azo (-N=N-) and imine (-CH=N-) groups was successfully synthesized, characterized, and doped in biopolymer matrix. Chitosan-based biopolymer was formulated with **AZ** additive material as solid biopolymer electrolyte (SBE) through the concept of Donor (D) - π- Acceptor (A). The prepared **AZ** additive was characterized by selected spectroscopic and thermal analyses using FTIR, 1D NMR, and TGA before integration as SBE. The thermogram of **AZ** additive revealed good thermal stability up to 300 °C, making it suitable for application as electrolytes. Several weight percentages (2%-8%) of additive were doped with chitosan biopolymer as new SBEs (**F1-F4**). The conductivity of prepared SBE was measured via electrochemical impedance spectroscopy (EIS) which achieved the highest ionic conductivity of 8.22 x 10^{-3} S cm^{-1} at room temperature (303K). The preliminary findings suggest that this organic material has significant potential as an additive material in electrolyte application.

Keywords: additive, azo-imine, biopolymer electrolyte, conductivity, chitosan

Abstrak

Prestasi elektrolit dalam aplikasi elektronik semakin merosot kerana konduktiviti ion yang rendah, kebocoran, dan ketidakstabilan terma. Bagi mengatasi cabaran ini, pendekatan inovatif termasuk reka bentuk elektrolit dan pemilihan bahan perlu diberi perhatian. Dalam kajian ini, bahan tambah baharu azo-imina alkoksi (**AZ**) yang mengandungi moieti campuran azo (-N=N-) dan imina (-CH=N-) telah berjaya disintesis, dicirikan, dan dicampurkan ke dalam matriks biopolimer. Biopolimer berasaskan kitosan telah diformulasikan dengan bahan tambah **AZ** sebagai elektrolit pepejal biopolimer (SBE) melalui konsep Penderma (D) - π - Penerima (A). Bahan tambah **AZ** yang disediakan telah dicirikan menggunakan analisis spektroskopi dan terma yang terpilih menggunakan FTIR, 1D NMR, dan TGA sebelum digabungkan sebagai SBE. Termogram bahan tambah **AZ** menunjukkan kestabilan terma yang baik sehingga 300 °C, menjadikannya sesuai untuk digunakan sebagai elektrolit. Beberapa peratusan berat (2-8 wt. %) bahan

tambah telah dicampurkan dengan biopolimer kitosan sebagai SBE baharu (**F1-F4**). Kekonduksian SBE yang sediakan telah diukur melalui spekstroskopi impedans elektrokimia (EIS) dan mencapai konduktiviti 8.22 x 10^{-3} S cm^{-1} pada suhu bilik (303 K). Keputusan awal menunjukkan bahawa bahan organik ini mempunyai potensi yang baik sebagai bahan tambah dalam aplikasi elektrolit.

Kata kunci: bahan tambah, azo-imina, biopolimer elektrolit, kekonduksian, kitosan

Introduction

Electrolytes are a crucial component in energy storage devices, and these includes capacitors, batteries, and fuel cells [1]. The properties of the electrolyte, such as potential window, resistivity, conductivity, viscosity, stability, and flexibility, provides a huge impact on device performance [2]. A wide variety of electrolytes have been explored, including liquid electrolytes (aqueous, organic, and ionic liquid) [3] and solid-state electrolytes [4] as the potential ideal electrolyte. In this context, there is a high demand on liquid electrolytes-based alkali metals, lithium salts and organic solvent due to their performance in high ionic conductivity, capability to form stable contact with electrode, and the ability to reach high-rate performance in devices [5, 6]. Despite its superior performance, electrolytes have their own set of shortcomings as they are susceptible to corrosion and leakage issues, leading to device degradation and the possibility of self-discharge to electronic waste (e-waste) [7]. To mitigate the risks associated with liquid electrolytes and harmful material, solid-state electrolytes have been considered as a promising alternative.

Solid-state electrolytes offer various advantages properties including non-flammable, no flowing and corrosion after damage, and higher operation temperature [8]. The development of sustainable and eco-friendly materials for electrolyte applications has initiated a growing demand for new materials that can enhance performance. Thus, biopolymers have emerged as a promising candidate for polymer matrix due to their abundant availability, biodegradability, and excellent physicochemical properties [9]. Specifically, the preceding research employed biopolymers such as cellulose, alginate, and starch as solid-state electrolytes with the conductivity of these biopolymers is in the magnitude range of 10^{-5} - 10^{-10} S cm^{-1} [1, 10, 11]. Among various biopolymers materials, chitosan has garnered considerable interest in biopolymer-based electrolytes, primarily because of its unique characteristics including high mechanical strength, good thermal stability, and excellent film-forming ability [12]. Nonetheless, its ionic conductivity is often restricted, posing a challenge for its optimal use in high-performance applications [13].

In this context, researchers focus on effective methods to boost up the ionic conductivity of electrolytes by incorporating small portion of additives into the biopolymer matrix [14]. Some of the strategies to achieve an ideal ionic conductivity of at least 10^{-3} S/cm in electrochemical application includes utilizing ionic liquids [15], increasing the amounts of inorganic salts [16], and incorporate the organic liquid plasticizers [17] into biopolymer. Nevertheless, plasticization can result in deterioration of the mechanical integrity of the film and increased corrosive reactivity of polymer electrolyte as it requires large amounts of plasticizers [18]. Recently, the functional additives containing nitrogen (N), oxygen (O), carbon (C), and hydrogen (H) have been extensively explored to enhance the ionic conductivity of biopolymer electrolytes which consequently improve the electrolyte performance [19, 20]. Hence, this study aims to prepare new organic functional additives for polymer-based electrolyte application. The new hybrid moiety of azo (-N=N-) and imino (-C=N-) with alkoxy chain has been tested to act as an additive in the attempts to explore their suitability in chitosan biopolymer matrix. Individually, the moiety of (-N=N-) and (-C=N-) have soft and hard donor functionalities, offer π-system that highly desirable for various applications in electrolyte [20, 21], optoelectronic [22], and sensor [23]. These conjugated π-systems provide electron delocalization and π-π stacking interactions, resulting in improved charged transport properties [24]. Thus, in this study, azo-imine additive has been incorporated with chitosan-based biopolymer and in turn, the performance is tested on electrolyte application. The combination of the mixed

soft-hard donor character and the intriguing properties of these compounds should open a new avenue for electrolyte application. The mixing of prepared **AZ** additive in biopolymer matrix system is expected to support the ion dissociation; and as a result, a greater number of migrating ions may become available for charge transport.

Materials and Instrumentations

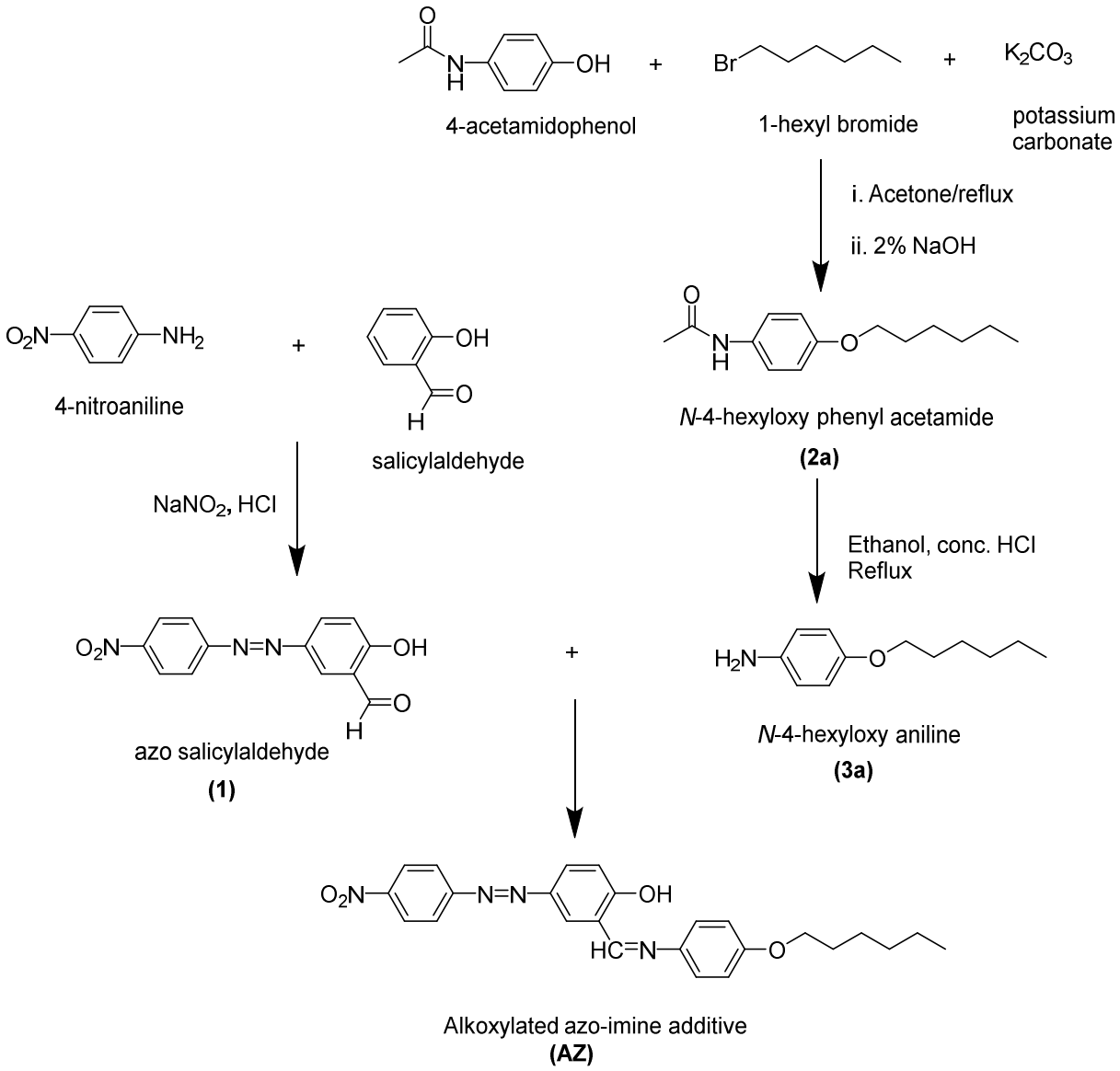
The reagents, 4-nitroaniline, 4-acetamidophenol, 1-bromohexane, chitosan powder (high molecular weight (M_w), 310,000-375,000, salicylaldehyde, glacial acetic acid, potassium carbonate and ethanol were purchased from commercial suppliers. It was used without further purification. Fourier transform infrared (FTIR) spectroscopy was performed in the wavenumber range of 4000 – 400 cm^{-1} using the Bruker Invenio-S instrument via the Attenuated total reflectance (ATR) method. 1D Nuclear magnetic resonances (NMR) of ^{1}H and ^{13}C were conducted using the Varian NMR System 500 MHz instrument using tetramethylsilane (TMS) as internal standard in deuterated chloroform ($CDCl_3$). Thermogravimetric Analysis (TGA) was conducted using the Perkin Elmer analyzer, with a heating temperature ranging from 30 to 600 ℃ and a flowrate of 10 ℃/min under nitrogen atmosphere. The conductivity of the prepared SBE were run using electrical impedance spectroscopy (EIS) within the frequency range of 1 MHz to 50 Hz, employing the HIOKI 3532-50 LCR Hi-Tester.

Synthesis of alkoxylated azo-imine additive (AZ)

Prior to obtain **AZ** additive, azo salicylaldehyde (**1**), *N*-4-hexyloxy phenyl acetamide (**2a**) and *N*-4-hexyloxy aniline (**3a**) acting as precursors were attained via different designated reactions. The experimental details on the precursors (**1**, **2a** & **3a**) have been reported in previous studies [24–26]. Several modifications in term of synthetic pathway and characterization were carried out and are discussed further in this contribution. The precursor **1** was synthesized via diazotization process, using 4-nitroaniline (0.015 mole, 2g) and salicylaldehyde (0.015 mole, 1.8g). The progress of the compound's reaction completion was monitored using Thin Layer Chromatography (TLC) performed under ethyl acetate: n-hexane (2:3) as a mobile phase. Then, the product obtained was filtered and washed with ethanol. The precipitate was then dried over silica gel until fully dried. For precursor **2a**, it was accomplished via Williamson ether reaction using 4-acetamidophenol (0.018 mole, 3g), 1-bromohexane (0.018 mole, 2.5g) and potassium carbonate (0.018 mole, 2.5g). The reaction resulted in a white precipitate and a colorless solution. TLC technique was used to determine the completion of the reaction. After cooling to room temperature and vacuum removal of the solvent, white solid was obtained. Then, the obtained white solid of **2a** was refluxed with constant stirring in a mixture of ethanol and concentrated acid for *ca.* 2 hours. This reaction has afforded **3a**, along with light brown solution. To obtain the **AZ** additive, the precursor of **1** (0.0018 mole, 0.5g) was fully dissolved in ethanol (100 mL) whilst, the precursor of **3a** (0.0018 mole, 0.35g) was completely dissolved in ethanol (30 mL) before being added dropwise to the precursor of **1**. Once both compounds were fully dissolved, reflux process was initiated. The reaction was refluxed for approximately 6 hours at 70 ℃, the boiling point of ethanol. When adjudged completion also via TLC, the orange precipitate obtained was filtered and collected. The overall chemical pathway for the synthesis of **AZ** additive is represented in Scheme 1.

Preparation of solid biopolymer electrolyte (SBE)

An amount of chitosan (2g) was dissolved in 2% acetic acid and stirred continously for 4 hours. The obtained **AZ** additive was dissolved in 1 mL acetic acid and added into chitosan solution until a clear solution was achieved. The composition of **AZ** additive (2 wt.% to 8 wt.%) were added into the chitosan solution to obtain new SBE (**F1** – **F4)** is presented in Table 1. The solution was then casted into glass petri dishes, followed by drying in an oven at 50℃ for 30 hours. In this work, a maximum amount of 8 wt. % was tested to form clear solution and film.



Scheme 1: Preparation of **AZ** additive

Table 1. Formulation of solid biopolymer electrolyte

Coding	Additive, wt.%	Weight percentage of additive, g	Chitosan, g
F0	0	0	2
F1	2	0.04	2
F2	4	0.08	2
F3	6	0.12	2
F4	8	0.16	2

Electrical impedance spectroscopy (EIS)

The prepared SBE was evaluated using EIS model HIOKI 3532-50 LCR Hi-Tester to determine its conductivity. For EIS measurement, 2 cm diameter of SBE was sandwiched between two stainless steel electrodes and tested in room temperature at a frequency range of 1 MHz to 50 Hz. The data was presented in the Cole-Cole plot form. The ionic conductivity, σ, of the electrolyte was calculated by using equation 1 [28] .

$$\sigma = \frac{t}{R_b \cdot A} \quad (1)$$

From eq. 1, t is the thickness of the electrolyte, R_b is the bulk resistivity and A is the (cm^2) electrode-electrolyte contact area. The value of R_b is retrieved from the Cole–Cole plot.

Results and Discussion

Spectroscopy technique

Figure 1 shows the IR spectra of precursors (**1**, **2a** & **3a**) and targeted compound of **AZ** additive. **AZ** additive revealed all the bands of interests namely ν(C-H), ν(C=N), ν(N=N), and ν(NO_2). The aliphatic C-H stretching vibrations arise at 2921 and 2856 cm^{-1} of the alkoxy substituent. The assignment of the stretching mode was made by comparison to their precursors **2a** and **3a** which indicates that the intensities of these bands increased. The absence of the stretching modes of residual amino, ν(NH_2) from precursor (**3a**) and aldehyde ν(CHO) groups from **1** together with the appearance of a typical band of imine bonds, ν(C=N) at 1614 cm^{-1} have provided strong evidence to the Schiff base formation reaction for **AZ** additive [29]. Two bands of ν(N=N) occur at 1500 cm^{-1} and 1440 cm^{-1} represent the asymmetric and symmetric N=N stretching vibrations of azo moiety [30, 31]. The shifting of ν(N=N) band to the higher wavenumber compared to **1** is due to the enhanced of mesomeric and inductive effect of the substituents. The characteristics of nitro group shifted to a slightly lower wavenumber at 1343 cm^{-1} due to stretching vibrations of ν(NO_2).

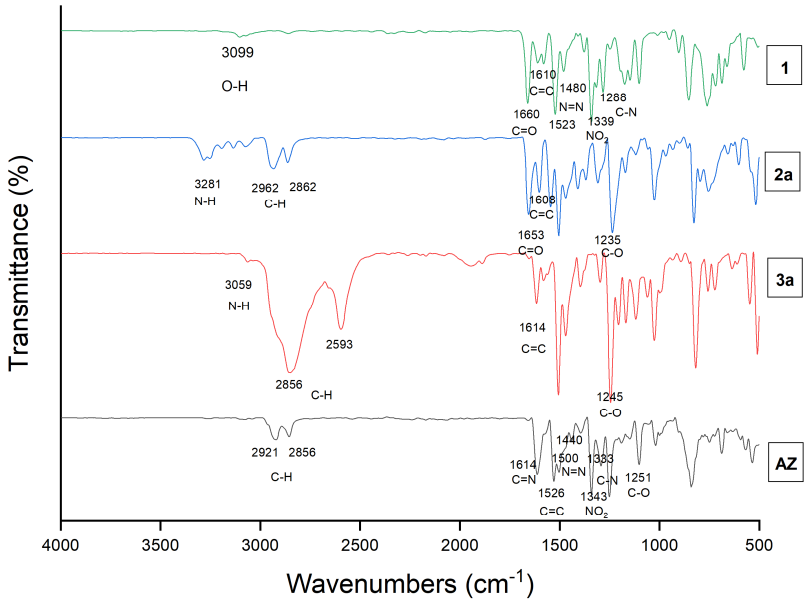


Figure 1. IR spectra of precursors and **AZ** additive

From Figure 2, the ^1H NMR spectrum for **AZ** additive reveals the methyl protons at δ_H 0.92 ppm while protons for methylene group were detected in the region of δ_H 1.35-1.82 ppm. The signal of R-O-CH_2 deshielded at δ_H 4.00 ppm due to the contribution of electronegativity of the oxygen atom. The closer the proton

approaches the electronegative oxygen atom, the greater the deshielding effect and therefore the resonance of R-O-CH_2 shifted towards downfield as compared to CH_3 which is located further from the oxygen atom. The values are in good agreement with the previous literature reported by Nakum and team [32]. The resonance of aromatic protons of *para*-substituted aromatic ring can be clearly observed as a pseudo-doublet resonance in the range of δ_H 6.82 ppm − 6.98 ppm and δ_H 7.66 ppm − 7.82 ppm and is considerably downfield due to large anistropic effect. The CH=N and hydroxyl protons found at around δ_H 8.06 ppm and δ_H 8.71 ppm respectively, are characterized by their unique singlet resonance at downfield [33], thus confirming the targeted compound.

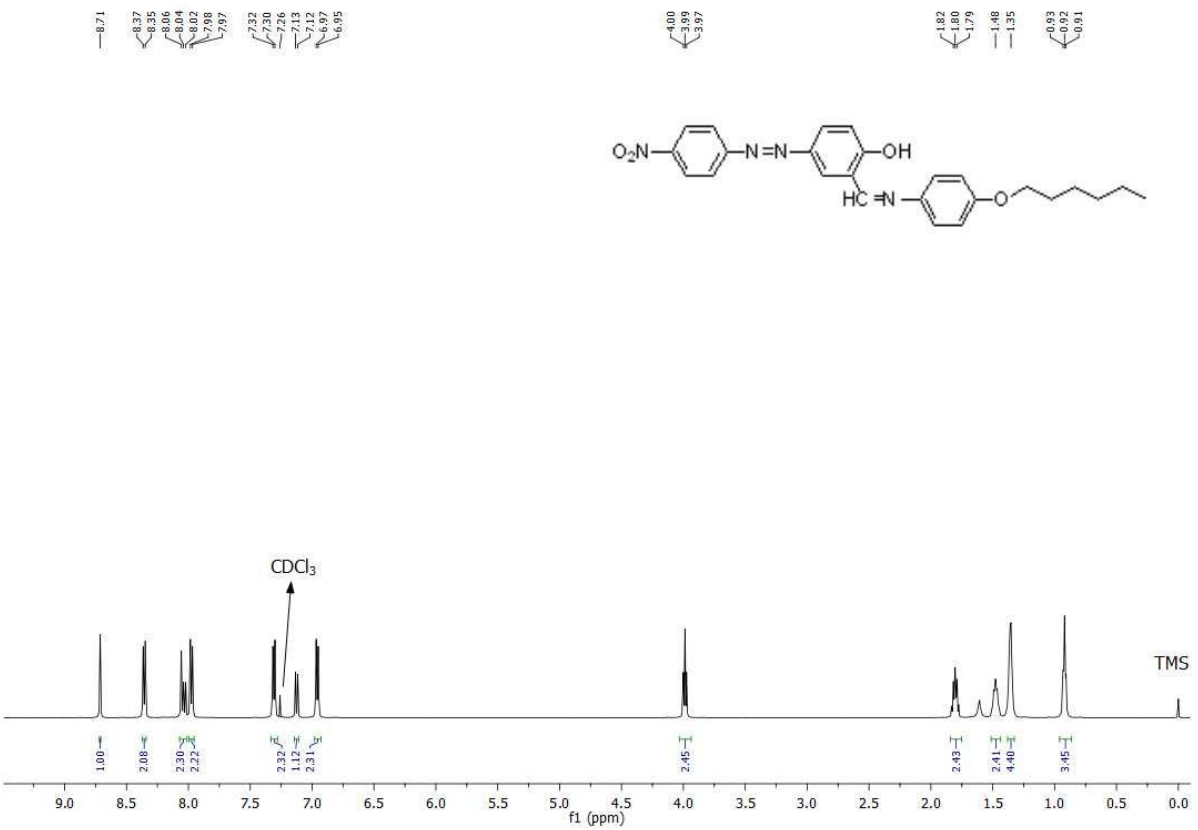


Figure 2. ^{1}H NMR for the **AZ** additive

Based on ^{13}C NMR spectrum of **AZ** additive, all expected resonances are at appropriate and expected chemical shifts. The carbon resonance of the terminal methyl group (CH_3) appears around δ_C 14.03 ppm, while for alkyl chain length, it appeared in the range of δ_C 22.60-35.57 ppm. The chemical shift for R–CH_2-O- can be found at δ_C 62.69 ppm due to the deshielding effect and is similar to Nakum et al. [34]. The resonances of aromatic ring carbon were assigned in the range of δ_C 115.29-155.85 ppm. The signal observed at δ_C 165.57 ppm is attributed to the C=N carbon atom which indicates the presence of imino linkage in the **AZ** additive.

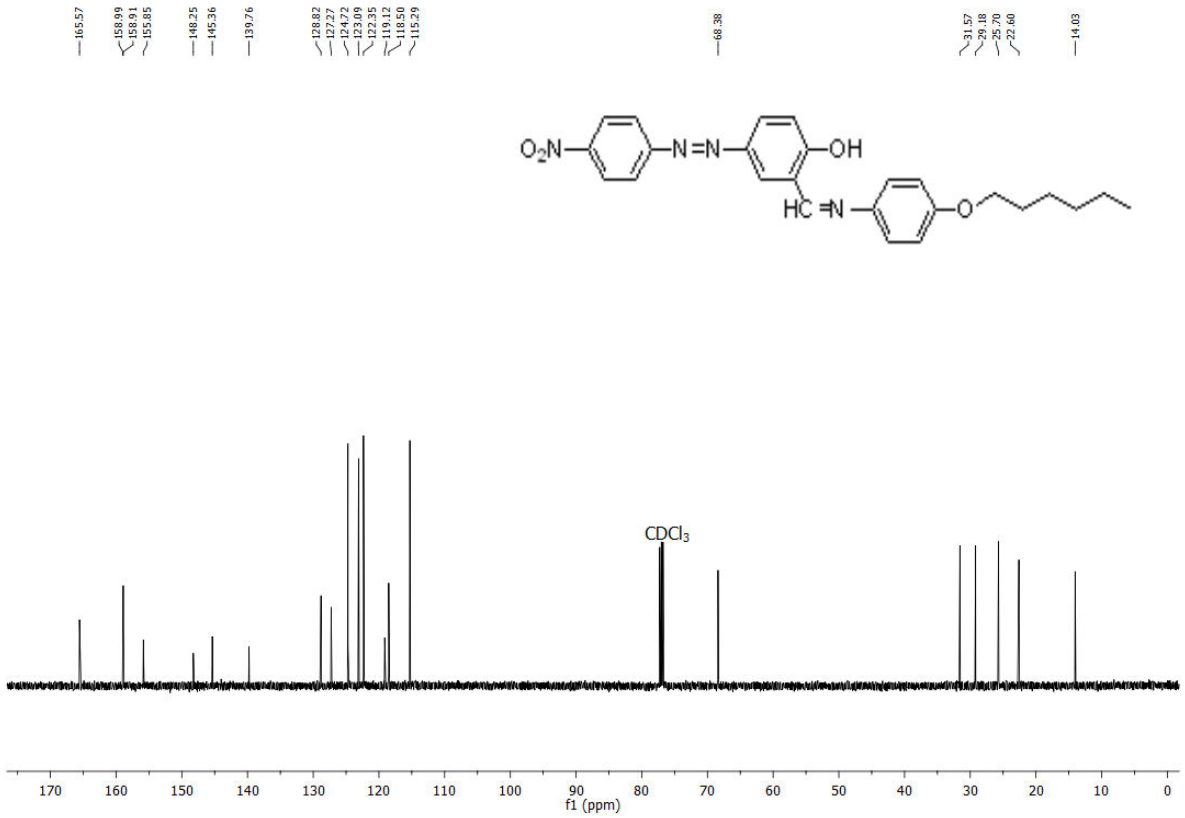


Figure 3: ^{13}C spectrum of the **AZ** additive

Thermogravimetric analysis (TGA)

TGA of precursors (**1**, **2a**, and **3a**) and **AZ** additive were carried out to investigate their thermal property. Based on the thermograms shown in Figure 4, no weight loss was observed at temperature below 120 °C. This indicated absence of water or solvent molecules that are left or trapped during the synthetic reaction and recrystallization. All synthesized compounds exhibited only one step of thermal degradation with different onset and offset values. By comparing the onset temperature of each compound, **AZ** additive was found to be stable up to 300 °C indicating that it has higher thermal stability compared to its precursor and therefore chosen as the additive to be tested in this study. The degradation of **AZ** additive was not complete at 600 °C, with 42.55% of residue left. This value coincides with the reported value by Ahmed et al. [35]. This may be due to the degradation of azo moiety which occurred through the cleavage of the azo bond by reductive process as suggested by previous studies [35, 36]. The trend of the thermal stability can be clearly observed by the presence of azo and alkoxy group in **AZ** additive. Ultimately, it can be concluded that the stability of additive increases as the degradation increases and it also depends on the substituent and molecular weight of the material. The thermal degradation parameters are given in Table 2.

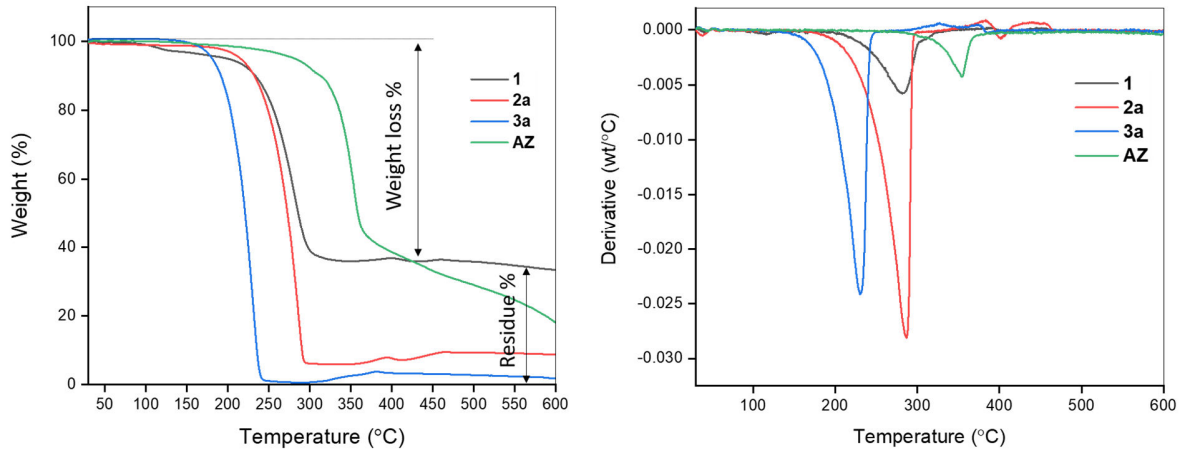


Figure 4. TGA and DTG of precursors and **AZ** additive

Table 2. Thermal degradation parameters of precursors and **AZ** additive

Compound	**T_{onset} (°C)**	**T_{max} (°C)**	**Weight Loss (%)**	**Residue (%)**
1	243	300	62.91	37.09
2a	254	289	92.6	7.4
3a	204	235	99.83	0.17
AZ additive	319	348	57.45	42.55

Ionic conductivity analysis

Impedance spectroscopy is typically used to study the electrical properties of the electrolytes. The Cole-Cole plot for the prepared SBE system is illustrated in Figure 5. From the plots, chitosan-based solid biopolymer electrolyte shows high-frequency region semicircle arc and low-frequency region inclination spike, where both are related to ionic conduction in the bulk of sample. Upon the addition of additives, the semicircle of chitosan disappeared and bulk resistance (R_b) decreases. The SBE with the incorporation of 4% additive (**F2**) gives the highest ionic conductivity value of 8.22 x 10^{-3} S/cm.

Figure 6 shows the ionic conductivity of the prepared SBE. The ionic conductivity value for chitosan SBE (without addition of additive) is 7.38 x 10^{-10} S/cm which is comparable with reported results by Rahman et al. [13]. The value shows the significant trends where it shows the increment of values upon the addition of **AZ** additive in the polymer host matrix of up to 8.22 x 10^{-3} S/cm for SBE containing 4 wt.% **AZ** additives. The increment in ionic conductivity can be attributed to the enhancement in the number density of charge carriers and efficiency of synthesized additives in dissociating chitosan matrix [37]. However, the value decreases after 6 wt.% because it has already reached the optimum concentration, thus leading towards sample aggregation [11]. From the preliminary result, **AZ** additive reveals good potential as an additive agent in SBEs as it can improve the ionic conductivity of the system with a potential in electrolyte application.

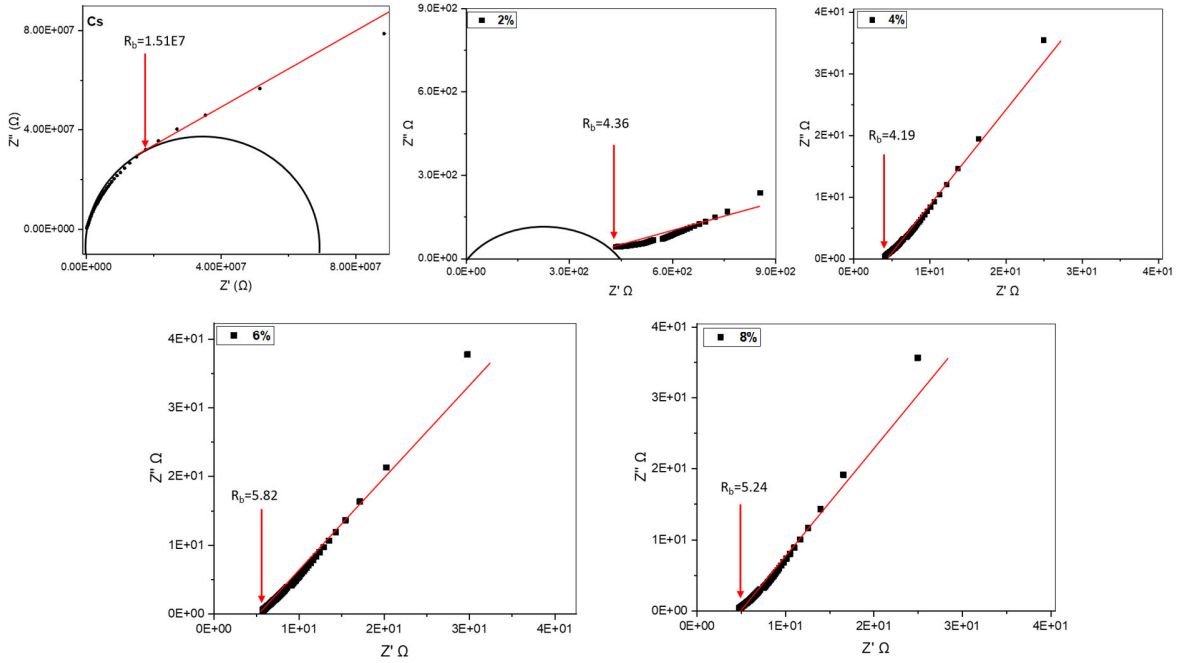


Figure 5. Cole-Cole plot of chitosan and variation weight percent of additive

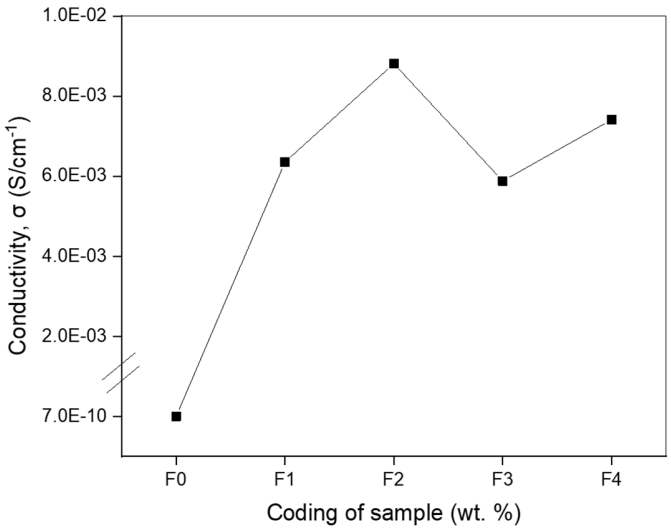


Figure 6. Conductivity of SBE system

Conclusion

This present work has successfully introduced a new **AZ** additive material integrated into chitosan biopolymer electrolyte to enhance charge carrier mobility and conductivity performance. All synthesized compounds including the precursors and **AZ** were analyzed via FTIR, 1D NMR, and TGA. TGA studies show that the effect of **AZ** influenced the thermal stability of the SBEs system by increasing the temperature decomposition (T_d) and is stable up to 300 °C. The ionic conductivity of the prepared SBEs system have been successfully evaluated using EIS and shows an increment in the order of 10^{-7} S/cm to 10^{-3} S/cm with the addition of **AZ** additive.

The maximum conductivity at ambient temperature (303 K) achieved up to 8.22 x 10^{-3} S/cm at 4% of additive. The preliminary finding reveals that the presence of active group of hydroxyl (-OH) and azo (N=N) can significantly affect the conductivity performance. Hence, the output from this work creates an alternative approach to initiate further investigation on new, efficient, stable additive materials in various optoelectronic application.

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PERFORMANCE OF MEMBRANE ELECTRODE ASSEMBLY USING Pt/C AND CoFe/N-C CATALYSTS IN PROTON EXCHANGE MEMBRANE FUEL CELLS

(Prestasi Pemasangan Elektrod Membran Menggunakan Pemangkin Pt/C dan CoFe/N-C dalam Sel Bahan Api Membran Pertukaran Proton)

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Abstract

The performance of membrane electrode assembly (MEA) hinders fuel cell commercialisation. MEA is greatly affected by humidification, temperature and hydrogen flow rate. In this study, the effects of operating conditions on MEA were determined using Pt/C and CoFe/N-C catalysts in proton exchange membrane fuel cells. Herein, two types of MEAs using the Nafion-212 membrane were prepared and tested. The anode and cathode of the first MEA were coated with Pt/C and CoFe/N-C catalysts, respectively, whereas the second MEA utilised a Pt/C catalyst on both electrodes. The electrode with Pt/C and CoFe/N-C catalysts was characterised using cyclic voltammetry and electrochemical impedance spectroscopy to obtain the electrochemical surface area (ECSA) and electrical conductivity of the electrode, respectively. The performance of two MEAs was tested under different operating conditions, such as various humidifier temperatures (40 °C, 60 °C, 80 °C and 100 °C) and hydrogen flow rates (100, 200, 300 and 400 mL/min). The electrode with a Pt/C catalyst exhibited higher ECSA (0.245 m^2/g) than the CoFe/N-C electrode (0.018 m^2/g). Similarly, the Pt/C electrode possessed higher conductivity (7.2×10^{-3} S/cm) than the CoFe/N-C electrode (4.4×10^{-3} S/cm). Consequently, the open-circuit voltage (OCV) of the second MEA with a Pt/C catalyst on both electrodes showed a higher value (0.890 V) than the OCV of the first MEA (0.790 V). Furthermore, the humidifier temperature was optimum at 80 °C, and it achieved power density levels as high as 10.14 and 3.43 mW/cm^2 for the second and the first MEA, respectively. In addition, the performance of MEA was affected by the hydrogen flow rate. At the optimum hydrogen flow rate of 400 mL/min for the first MEA, a power density of 4.93 mW/cm^2 was achieved. Meanwhile, the second MEA required a lower hydrogen flow rate (200 mL/min) to achieve a maximum power density of 10.14 mW/cm^2.

Keywords: proton exchange membrane fuel cells, MEA performance, Co-Fe/N-C, humidification temperature, hydrogen flow rate

Abstrak

Prestasi pemasangan elektrod membran (MEA) membawa kepada kesesakan pengkomersialan sel bahan api. MEA sangat dipengaruhi oleh pelembapan, suhu, dan kadar aliran hidrogen. Dalam kajian ini, menentukan kesan keadaan operasi pada MEA menggunakan pemangkin Pt/C dan CoFe/N-C dalam sel bahan api membran pertukaran proton (PEMFC). Di sini, dua jenis MEA yang menggunakan membran Nafion-212 telah disediakan dan diuji. Anod dan katod MEA pertama disalut dengan pemangkin Pt/C dan CoFe/N-C, manakala MEA kedua menggunakan pemangkin Pt/C pada kedua-dua elektrod. Elektrod dengan pemangkin Pt/C dan CoFe/N-C dicirikan menggunakan voltammetri kitaran dan spektroskopi impedans elektrokimia (EIS) untuk mendapatkan kawasan permukaan elektrokimia (ECSA) dan kekonduksian elektrik elektrod. Prestasi dua MEA diuji dengan keadaan operasi yang berbeza, seperti pelbagai suhu humidifier (40 °C, 60 °C, 80 °C, dan 100 °C) dan kadar aliran hidrogen (100, 200, 300, dan 400 mL/min). Elektrod dengan pemangkin Pt/C mempamerkan ECSA yang lebih tinggi (0.245 m^2/g) daripada elektrod CoFe/N-C (0.018 m^2/g). Begitu juga, elektrod Pt/C mempunyai kekonduksian yang lebih tinggi (7.2 x 10^{-3} S/cm) daripada elektrod CoFe/N-C (4.4 x 10^{-3} S/cm). Akibatnya, voltan litar terbuka (OCV) MEA kedua dengan pemangkin Pt/C pada kedua-dua elektrod menunjukkan nilai yang lebih tinggi (0.890 V) daripada OCV MEA pertama (0.790 V). Tambahan pula, suhu humidifier adalah optimum pada 80 °C dan mencapai tahap ketumpatan kuasa setinggi 10.14 dan 3.43 mW/cm^2 masing-masing untuk MEA kedua dan pertama. Di samping itu, prestasi MEA dipengaruhi oleh kadar aliran hidrogen. Dengan kadar aliran hidrogen optimum 400 mL/min untuk MEA pertama, ketumpatan kuasa 4.93 mW/cm^2 telah dicapai. Sementara itu, MEA kedua memerlukan kadar aliran hidrogen yang lebih rendah (200 mL/min) untuk mencapai ketumpatan kuasa maksimum 10.14 mW/cm^2.

Kata kunci: sel bahan api membran pertukaran proton, prestasi MEA, Co-Fe/N-C, suhu pelembapan, kadar aliran hidrogen

Introduction

Proton exchange membrane fuel cells (PEMFCs) have high efficiency, high power density, pollution-free properties because they use hydrogen as fuel [1] and flexible source of hydrogen [2]. PEMFCs can be used in electric vehicles [3] and maritime applications [4], as well as for portable and stationary applications [5]. The membrane electrode assembly (MEA) is the most important component in PEMFCs. The performance of MEA's is determined by the catalytic activity, the method of placing the catalyst at the catalyst layer (CL) [1], the effect of supporting a material for the CL [6] and the design structure of the flow field [7]. The ideal catalyst for PEMFCs has good conductivity and high stability [8], particularly the activity and stability of the catalyst at the cathode [9]. In general, the catalyst used in PEMFCs is Pt-based because it is optimum for the hydrogen oxidation reaction at the anode [10], [11]. However, the weakness of the Pt/C catalyst lies in its high cost and susceptibility to CO poisoning, which accelerates platinum particle growth, causing aggregation and structural changes. This phenomenon negatively impacts catalytic activity, which leads to gradual deactivation, thereby reducing fuel cell lifespan and efficiency [12].

Meanwhile, oxygen reduction at the cathode in PEMFCs is relatively slow [13]. Thus, a catalyst that can accelerate the reduction reaction at the cathode is necessary. Pt catalyst alloys with other transition metals is commonly used at the cathode to reduce the interatomic distance in the lattice and energy variations in the d-orbitals, which can facilitate the adsorption of oxygen on the cathode surface [9]. Moreover, the slow reaction at the cathode will lead to the improper occurrence of the stoichiometric equilibrium between the hydrogen and oxygen reactions that are supplied, thereby decreasing the performance of the PEMFC. The oxygen reduction reaction (ORR) of the CoFe/N-C catalyst makes it a good catalyst for the cathode because it increases the reduction reaction in rechargeable zinc–air batteries [14].

Meanwhile, the use of nitrogen graphene with iron fibre can increase the ORR on the membrane, whereas the N-doped cobalt carbon (Co/C) can reduce the oxygen evolution reaction (OER). Thus, the performance of the CoFe/N-C catalyst in PEMFC can be compared with that of the Pt/C catalyst that is often used in the PEMFC cathode. Using a non-platinum FeCo-based catalyst at the cathode has also been carried out using the printing method for catalyst synthesis [15]. Several studies have shown that the alloys of transition metals, nitrogen and carbon synergise with one another to obtain ORR electrocatalytic activity and high performance durability [16]. Based on the evaluation using the rotating disk electrode technique, a significant increase in ORR activity is observed in CoFe/N-C compared with Co/N-C and Fe/N-C (Table 1). This finding indicates a synergistic coupling effect between Co and Fe in alloy

metal nanoparticles supported on nitrogen-doped Vulcan carbon. The CoFe/N-C catalyst demonstrates higher current density at the same overpotential, indicating greater electrocatalytic activity [14].

Apart from the catalytic activity, the performance of MEA also depends on operating conditions. Humidification and gas flow rate are the operating conditions of MEA that can affect its performance [8, 17]. In MEA, humidification is important because it will affect the supply of water vapor to the membrane. If the water vapor supply is less, then the membrane will be dehydrated, and the evaporation of water in the membrane increases [18]. Meanwhile, increasing the temperature can increase the reaction kinetics [19]. Increasing the temperature of the humidifier keeps the water passing through the electrode in a vapor state to avoid condensation, which can create a flood condition on the electrode [20].

Table 1. Electrocatalytic data at a current density of 10 mA·cm^{-2} (E@10 mA·cm^{-2} for OER)

Materials	ORR		
	$E_{on\ set}$ (V vs RHE)	$E_{1/2}$ (V vs RHE)	Tafel slope (mV dec^{-1})
CoFe/N-C	1.03	0.821	86
Pt/C	1.01	0.817	93
Fe/N-C	0.95	0.764	80
Co/N-C	0.91	0.776	54

Source: [14]

Materials and Methods

Herein, we compared the performance of two MEAs. The electrodes of the first MEA used Pt/C 40 wt.% (Fuel Cell Store) and CoFe/N-C (self-synthesised, refer to paper [14]) catalysts at the anode and cathode, respectively, with catalyst loading of 1 mg/cm^2 of each electrode, whereas the second MEA used a Pt/C catalyst on both electrodes. The electrode size was 5 cm × 5 cm, and the electrolyte membrane used was Nafion-212 (Fuel Cell Store). A pin type flow field and gold-plated current collectors (Electrochem/Fuel Cell Store) were also used. The electrode structure consisted of a backing layer in the form of a carbon paper (Gashub, Singapore) and a microporous layer (MPL) comprising a mixture of carbon Vulcan XC-72R (Fuel Cell Store), ammonium bicarbonate (Sigma-Aldrich) and Teflon DISP 30 Fluoropolymer dispersion (Fuel Cell Store). The electrode structure also consisted a CL comprising a Pt/C catalyst (40 wt%, Fuel Cell Store) at the anode (for both MEA types) and Pt/C and Co-Fe/C-N catalysts (self-synthesised) for each cathode at two types of MEAs. Catalyst loading was 1 mg/cm^2, and the catalyst ink on the catalyst and MPL was stirred using an ultrasonic homogeniser for 15 min. The electrodes were characterised using an Autolab PGSTAT 204 by cyclic voltammetry and electrochemical impedance spectroscopy to determine electrochemically active surface area (ECSA) and electrical conductivity. ECSA and electrical conductivity are calculated using Equations (1) and (2) with modifications.

$$\text{ECSA (cm}^2\text{/g)} = \frac{q}{\Gamma . L} \quad (1)$$

where q is the charge density (C/cm^2), Γ is the equivalent constant (μC/cm^2), and L is the catalyst loading (mg/cm^2) [21, 22].

$$\text{EIS } (\sigma)\ \text{(S/cm)} = \frac{1}{Z_R} \times \frac{1}{A}, \quad (2)$$

where Z_R is the total resistance = Rp + Rs (Ω) (note: Rp, and Rs are obtained from Nyquist curve fitting), l is the thickness (cm), and A is the surface area (cm^2) [23, 24].

CV and EIS analyses were performed using Potentiostat/Galvanostat PGSTAT204 from Methrom with Nova 2.1.4 software to process the data. CV and EIS were performed by placing Pt/C or Co-FeN/C electrodes, Ag/AgCl and platinum rods as working, reference and supporting electrodes, respectively. In addition, NaOH solution with a concentration of 1 M served as the electrolyte with a potential range of −0.2 V to 0.6 V and a scan rate of 100 mV/s. The measurements were repeated for 5 cycles.

MEA was fabricated from two electrodes and Nafion 212 (NR 212) (Fuel Cell Store) as an electrolyte membrane with hot pressing at 135 °C of 2000 psi for 3 min. The performance of MEA (I-V and I-P's performance) was tested using the fuel cell test station SMART2 WonAtech (South Korea) with varying flow rates of hydrogen gas (UHP, Samator, 100, 200, 300 and 400 mL/min) and oxygen gas (UHP, Samator) at 100 mL/min. The humidifier temperature varied from 40 °C, 60 °C, 80 °C and 100 °C.

Results and Discussion

Electrochemical characterisation

MEAs consisting of Pt/C (anode) and Co-Fe/N-C (cathode), as well as Pt/C catalysts on both electrodes, were arranged in a stack. Before making the MEA, the electrodes were characterised using CV and EIS. These methods were used in electrode characterisation to determine catalytic activity based on the ECSA values from CV analysis and electrical conductivity from EIS analysis. Determining the ECSA value from the voltammogram is a common procedure, and the value depends on the area of the electrode as well as the kinds and the loading of the catalyst [25, 26].

EIS is a well-established diagnostic method in electrochemistry [27]. The voltammogram and Nyquist curve of the electrodes are shown in Figure 1. As shown in Figure 1, the performance of the electrode with a Pt/C catalyst was better than that with a CoFe/N-C catalyst. Figure 1(a) shows a voltammogram from CV analysis with a scan rate of 100 mV/s. The electric charge can be determined on the basis of the area of the peak curve of the voltammogram and the scan rate data. If the electric charge is observed from the voltammogram curve in Figure 1(a), then the area of the electrode with the CoFe/N-C catalyst is smaller than that with the Pt/C catalyst. This result was confirmed through a smaller ECSA value compared with the Pt/C catalyst.

Figure 1(b) represents the impedance characteristics of the electrode with Pt/C and CoFe/N-C catalysts. Electrodes with a Pt/C catalyst had an impedance value of 3.12 Ω, whereas CoFe/N-C electrodes had a real impedance value of 5.54 Ω. The electrical conductivity of the electrode can be determined with the known equation based on the impedance values [24]. The ability of an electrode to conduct electricity can be determined from the conductivity value. The electrical conductivity value in electrodes coated with Pt/C was 7.2×10^{-3} S/cm, whereas that in electrodes coated with CoFe/N-C catalysts was 4.4×10^{-3} S/cm (Table 2).

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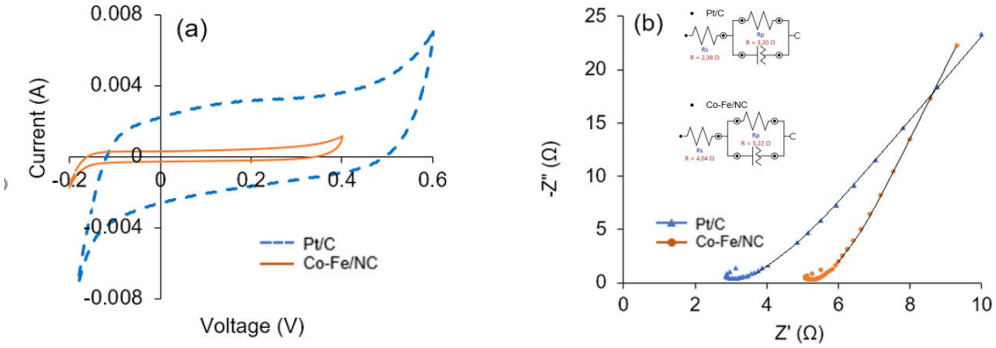


Figure 1. Electrode characterisation result using (a) CV and (b) EIS

Table 2. The electrical conductivity with Pt/C and CoFe/N-C catalysts

No.	Electrode	Impedance Parameters		Electrical Conductivity (S/cm)
		Rs (Ω)	Rp (Ω)	
1.	Pt/C	4.0357	5.2236	7.2×10^{-3}
2.	CoFe/N-C	2.3826	3.2046	4.4×10^{-3}

ECSA shows the active surface area of the catalyst, which is the response of the electrode in the form of current to the applied voltage. In turn, ECSA can be determined through the existing equations [28, 29] by considering the loading of the catalyst and the surface area of the electrode. Apart from the catalyst loading and surface area, ECSA also used a correction factor [30]. Moreover, based on the calculations, the ECSA value of the electrode with Pt/C and CoFe/N-C catalysts was 0.245 and 0.019 m^2/g, respectively. The distribution and surface area of the catalyst in the CL are the factors influencing the ECSA value of an electrode. Adding conductive carbon to the catalyst can lead to the even distribution of the catalyst [31, 32]. The even distribution of the catalyst in carbon may increase the active site of the catalyst. The carbon matrix in the catalyst is a factor affecting the ECSA value on a fuel cell electrode, which can make the catalyst spread more evenly. The more even the distribution of the catalyst in the carbon is, the larger the active site of the catalyst will be. A large surface area allows the distribution of Pt and CoFe metals amongst the carbon pores and even distribution on the surface of the CL [30].

The particle size of CoFe/N-C was more than 10 nm and uneven [14], whereas the particle size of Pt/C was approximately 3–4 nm [33]. The relatively smaller particle size of Pt/C led to a larger surface area of the Pt/C catalyst. Thus, it became a factor that led to a larger Pt/C ECSA value. Electrodes with Pt/C catalysts have an impedance value of 3.12 Ω, whereas CoFe/N-C electrodes have a real impedance value of 5.54 Ω. A high impedance value indicates the low conductivity of the electrodes. Moreover, EIS was used to analyse the electrochemical performance of an electrode, which will produce data from the sample response to a given frequency range in the form of real independence (Z′) and imaginary independence (Z″) values [34, 35]. The Nyquist curve can determine the impedance of electrodes through fitting to simulate the equivalent circuits.

Measurement of open-circuit voltage (OCV)

The OCV testing of the two MEAs showed their initial performance. The MEA with a Pt/C catalyst on both electrodes had a higher OCV of 0.890 V compared with that with a CoFe/C-N catalyst on one of the electrodes, which had an OCV of 0.720 V. Although the OCV value suggests an opportunity to use CoFe/C-N catalyst as a catalyst for PEMFC, the performance of the MEA is largely determined by its ability to maintain voltage with increasing load and not just under OCV conditions.

Performance testing of MEA at various humidifier temperatures

The performance of MEA was measured by testing the influence of humidifier temperature at 40 °C, 60 °C, 80 °C and 100 °C with a hydrogen flow rate of 200 mL/min. In addition, MEA treatment at high temperatures can increase the electrochemical kinetics, reduce the susceptibility to contamination and facilitate good water management [8]. Moreover, the performance of MEA at various humidifier temperatures is shown in Figure 2.

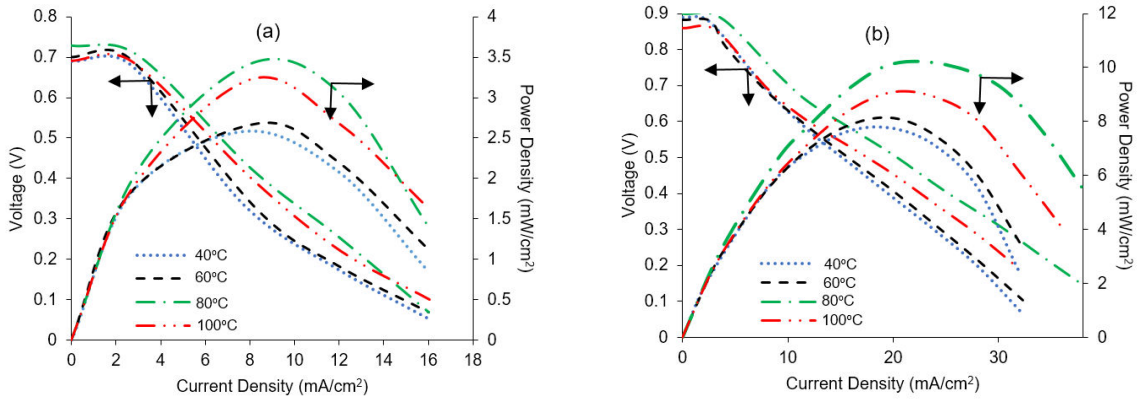


Figure 2. Effects of humidifier temperature on the performance of MEA with (a) CoFe/N-C and (b) Pt/C catalysts

The polarisation curve shows that the voltage decreases with the increase of load implemented as the electric current density [36]. Figure 2 shows that 80 °C is the optimum humidifier temperature because it can maintain voltage against an increase in current density, which is greater than those of other humidifier temperatures. The performance of MEA with CoFe/NC and Pt/C catalysts on the cathode and anode side (Figure 2[a]) was lower than that with a Pt/C catalyst on both sides (Figure 2[b]). This fact strengthened the OCV data. The MEA with a COFe/N-C catalyst in the cathode (Figure 2[a]) cannot support the performance of the Pt/C catalyst in the anode. However, in zinc–air batteries [14], the COFe/N-C catalyst is superior to Pt/C. The MEA shown in Figure 2(a) produced a maximum power density of 3.42 mW/cm^2 at a current density of 8.04 mA/cm^2, whereas the MEA with a Pt/C catalyst on both sides (Figure 2[b]) obtained a maximum power density of 10.14 mW/cm^2 at a current density of 20.04 mA/cm^2. Prior studies have investigated the impact of humidifier temperature on the performance of MEA in PEMFCs, using Pt catalysts on the anode and cathode. The humidifier temperature set at 80 °C delineates the boundary for high-temperature PEMFCs [37].

MEA performance test on various hydrogen flow rates

After obtaining the optimum humidifier temperature of 80 °C, the performance of MEA on PEMFCs was continuously tested on the basis of the effect of varying hydrogen flow rates (100, 200, 300 and 400 mL/min). The I-P and I-V performances are shown in Figure 3.

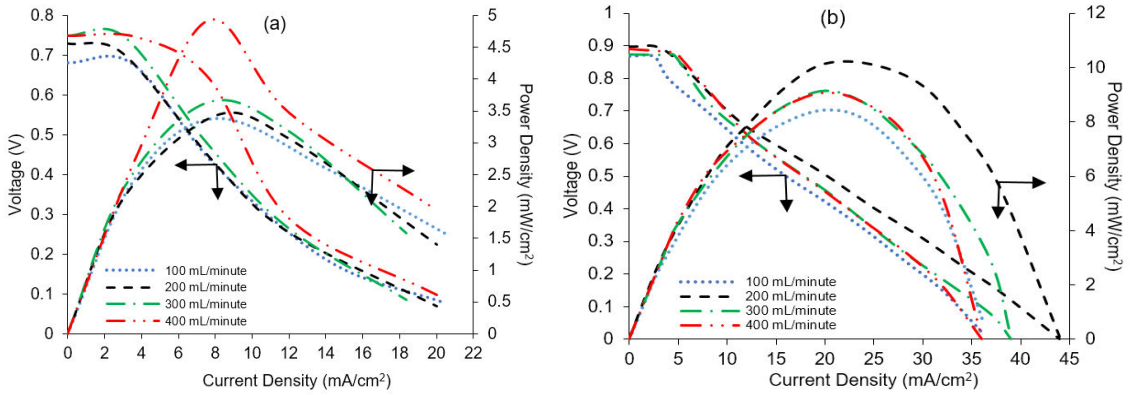


Figure 3. Effects of the hydrogen flow rate on the performance of MEA with (a) CoFe/N-C and (b) Pt/C catalysts

The ability of MEA to maintain a high voltage for a given current at a hydrogen flow rate of 400 mL/min compared with other hydrogen flow rates was shown on the basis of the I-V curve depicted in Figure 3(a) (MEA with CoFe/N-C and Pt/C catalysts). The hydrogen flow rate can well maintain the voltage during activation and ohmic polarisation; hence, the voltage does not decrease drastically with the increase of current density. However, MEA with a Pt/C catalyst on both sides (Figure 3[b]) at a hydrogen flow rate of 400 mL/min could not maintain the resulting voltage with increasing current density. This result was particularly true in the region of concentration polarisation because of an imbalance in the ratio between the supply of reactants and the supply of products. Meanwhile, at a hydrogen flow rate of 200 mL/min, MEA in PEMFC can better maintain the resulting voltage with increasing current density. The resulting I-V curve was gentler than the curves of other hydrogen flow rates.

Figure 3(a) shows that MEA with a CoFe/N-C catalyst on the cathode side had a fairly good performance with increasing hydrogen flow rate. This result was due to the maximally distributed hydrogen supply on the anode side, which increased the number of protons produced. The increase can be seen from the resulting power density of 4.93 mW/cm^2 at a current density of 7.72 mA/cm^2. However, it did not affect MEA with Pt/C catalysts on both sides (Figure 3[b]). Thus, this finding indicated that a hydrogen flow rate of 400 mL/min produced a lower power density than a hydrogen flow rate of 200 mL/min, that is, 10.14 mW/cm^2 at a current density of 20.04 mA/cm^2. MEA with a Pt/C catalyst on both sides of the electrode showed better performance than MEA with CoFe/N-C and Pt/C catalysts at the

cathode and anode side, respectively. Thus, Pt/C was the best catalyst for MEA of PEMFCs, and adequate hydrogen flow rate can lead to a good performance.

Conclusion

The characterisation of electrodes with a Pt/C catalyst showed a higher ECSA and electrical conductivity value compared with electrodes with a CoFe/N-C catalyst. Furthermore, a humidifier temperature of 80 °C produced the best performance on PEMFC for both MEAs with a Pt/C catalyst on both sides as well as Pt/C and CoFe/N-C catalysts on the anode and cathode sides, respectively. Meanwhile, increasing the hydrogen flow rate improved the performance of MEA. A hydrogen flow rate of 400 mL/min produced the highest performance for MEA with Pt/C and CoFe/N-C catalysts on the anode and cathode side, respectively. However, for MEA with a Pt/C catalyst on both sides (anode and cathode), the optimum hydrogen flow rate was 200 mL/min. Therefore, the CoFe/N-C catalyst can be an alternative for a Pt/C catalyst in fuel cells, especially PEMFCs.

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AN INSIGHT INTO PINEAPPLE PEEL WASTE ADSORBENT FOR IRON CONTAMINATED WATER THROUGH KINETIC AND ISOTHERM STUDY

(Tinjauan Terhadap Penjerap daripada Sisa Kulit Nanas bagi Air Tercemar dengan Ferum Melalui Kajian Kinetik dan Isoterma)

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Abstract

The swift growth of sectors like steel and coal mining leads to a higher release of heavy metals like iron into aquatic ecosystems, resulting in water pollution. In this study, the kinetics and isotherms studies were applied to pineapple peel waste based adsorbent for iron removal. An adsorbent derived from discarded pineapple peels was created through a chemical activation process, employing zinc chloride ($ZnCl_2$) as the activator. By examining the effects of various experimental parameters, including contact duration (30–240 min), concentration of iron (5–80 ppm), and the amount of adsorbent (2–24 g/L), the behaviour of the adsorbent for iron adsorption was carefully studied. Maximum iron removal of 99.24% efficiency with adsorbent dosage of 20 g/L was achieved through this study. The iron removal process was most accurately represented by the Freundlich isotherm model, exhibiting an R^2 value of 0.985. Furthermore, the kinetics investigation demonstrated an excellent fit with the pseudo-second-order model for iron adsorption, yielding an R^2 value of 0.999. These findings strongly indicate that pineapple peel waste holds promise as a viable adsorbent for eliminating iron from water bodies.

Keywords: Pineapple peel waste, iron removal, adsorption isotherm, adsorption kinetic

Abstrak

Pertumbuhan pesat sektor-sektor seperti keluli dan perlombongan arang menyebabkan peningkatan pelepasan logam berat seperti ferum ke dalam ekosistem akuatik, yang mengakibatkan pencemaran air. Dalam kajian ini, kajian kinetik dan isoterma telah digunakan bagi penyingkiran ferum menggunakan penjerap berasaskan sisa kulit nanas. Penjerap yang diperoleh daripada kulit nanas yang dibuang telah dihasilkan melalui proses pengaktifan kimia dengan menggunakan zink klorida ($ZnCl_2$) sebagai pengaktif. Dengan mengkaji kesan pelbagai parameter eksperimen, termasuk tempoh penjerapan (30–240 minit), kepekatan ferum (5–80 ppm), dan jumlah penjerap (2–24 g/L), tingkah laku penjerap untuk penjerapan ferum telah dikaji dengan teliti. Penyingkiran ferum maksimum sebanyak 99.24% dengan dos penjerap 20 g/L telah dicapai melalui kajian ini. Proses penyingkiran ferum paling tepat

diwakili oleh model isoterma Freundlich, dengan nilai R^2 sebanyak 0.985. Selain itu, penyiasatan kinetik menunjukkan kesesuaian yang sangat baik dengan model pseudo-tertib kedua untuk penjerapan ferum, menghasilkan nilai R^2 sebanyak 0.999. Penemuan ini menunjukkan bahawa sisa kulit nanas berpotensi sebagai penjerap yang berkesan untuk menyingkirkan ferum dari air.

Kata kunci: sisa kulit nenas, penyingkiran ferum, isoterma penjerapan, kinetik penjerapan

Introduction

Iron stands as one of the most plentiful elements in the earth's crust, occurring naturally in water in two primary forms: soluble ferrous iron, Fe(II) or $Fe(OH)^+$, and complexed ferric iron, Fe(III), which precipitates as $Fe(OH)_3$ [1]. Iron can also be found in water because of industrial activities like metal plating, mining, the iron and steel industry, and the corrosion of metal materials [1, 2]. As reported by Al-Anber, the safe level for Fe(III) ions for the human body is not more than 0.3 ppm and excessive iron ions may cause anorexia and oliguria. The high amount of iron can also give the water a metallic taste and odour [3]. To overcome these problems, special attention should be given to removing or reducing the amount of iron ions from industrial water effluents or water supply.

Liquid-phase adsorption is one of the most common methods used to remove heavy metals from wastewater due to the proper design of the adsorption process that will produce a high-quality treated effluent [2]. However, the adsorption of heavy metals such as iron by commercial adsorbent is expensive; therefore, research studies focus on developing adsorbents from agricultural waste to remove iron from the aqueous system [3]. Among these various adsorbents, including rice hull [2], rice husk ash [4], orange peel [5], olive stones [1, 6], wild jack, and jambul barks [7] have been employed for the purpose of eliminating iron ions from aqueous solutions. Based on recent works, adsorbent from pineapple peel waste can be considered an agricultural waste that shows remarkable potential towards the adsorption of toxic heavy metals from the aqueous solutions. For instance, Zn, Cr, Cd, Pb, Mn, and Cu ions have been removed from aqueous solutions [8–11].

Malaysia's pineapple (*Ananas comosus*) industry was ranked 18th world's largest pineapple producer with 334,400 metric tonnes produced by 13,733 hectares of planted area. As reported by Ahmad Zamri et al., about 335,488 tonnes of pineapple produced in Malaysia yielded 137,550 tonnes of pineapple peel [12]. Pineapple fruit typically consists of around 60% edible flesh and approximately 35% skin or peel, which is considered waste material. This peel is primarily composed of cellulose, hemicellulose, lignin, and pectin substances. With certain modifications, pineapple peel can be repurposed and utilised as an adsorbent material [10, 11]. Previous studies reported adsorbent from pineapple peel waste showed very high removal efficiencies of heavy metals such as Cd(II), Pb(II), Cu(II), Zn(II), and Cr(VI). For example, Turkmen et al. reported that the pineapple peel waste which was carbonised using microwave at 800 W yielded a maximum Zn(II) removal efficiency of 93% for both types of activating agent: $ZnCl_2$ and H_2SO_4 [13]. The similar result reported by Olowu et al. (2022) when pineapple peel adsorbent was used to remove Zn(II) and Cr(VI) from the aqueous solution with the maximum percentage removals of 89% and 91.2%, respectively [8]. Hence, considering both economic and environmental aspects, pineapple peel waste may serve as an effective adsorbent for the removal of iron from aqueous solutions.

Solid waste derived from processed pineapple peel waste has previously been employed as an adsorbent, demonstrating considerable potential for adsorbing harmful pollutants, including heavy metals. In this study, we assessed the capability of pineapple peel waste as an adsorbent for capturing Fe(III) ions from aqueous solutions. The investigation involved the assessment of various operational factors such as contact time, adsorbent quantity, and initial iron concentration through a batch adsorption study. Furthermore, we delved into the sorption mechanism by examining different kinetic models, including the pseudo-first- and pseudo-second-order models. To evaluate various isotherms and their capacity to correlate with experimental data, three isotherm models—Langmuir, Freundlich, and Temkin—were employed.

Materials and Methods

Materials

Iron standard solution, $Fe(NO_3)_3$, sulfuric acid, H_2SO_4, and zinc chloride, $ZnCl_2$, of analytical grade were procured from Merck chemicals. A stock iron solution

with a concentration of 1,000 mg/L was employed as the adsorbate, and different concentrations were prepared by diluting the stock solution with distilled water. pH measurements were conducted using the pH meter pH 150 from Eutech Instruments.

Preparation of adsorbent from pineapple peel waste

The adsorbent was prepared using a method reported by Rozaidi & Abu Samah with some modifications [14]. Pineapple peel waste collected from Pekan Pina Sdn. Bhd. was washed with distilled water. The peels were oven-dried at 80 °C for 24 h. Then, the dried peels were ground and sieved using a sieve with 300 µm size to obtain the particle size of less than 300 µm. After that, chemical activation was performed by mixing 30 g of sieved pineapple peels with 1.0 M of $ZnCl_2$. The procedure involved stirring the mixture for 24 h at room temperature using a magnetic stirrer, followed by filtration. The filtered peels were then dried in an oven at 80 °C overnight. Subsequently, the sample was placed in a sealed crucible and subjected to carbonisation at 800 °C for 1 h, with a heating rate of 10 °C per min. After cooling to room temperature, the sample was immersed in a 0.5 M H_2SO_4 solution for 30 min to remove the excess $ZnCl_2$. Following this, the sample was once again dried in an oven at 80 °C for 24 h after being filtered and washed with distilled water until the wash water reached a pH of 7. The samples were then transferred to a desiccator and stored in sealed plastic bags for future use [15].

Characterisation of adsorbent

To identify the primary functional groups, present in the adsorbent, Fourier transform infrared spectroscopy (FTIR) was employed, utilising a Nicolet AVATAR 370 DTGS, UK instrument. The analysis covered the functional groups within the samples across a range of 500–4500 cm^{-1}. FTIR analysis allows the samples to be absorbed at different wavelengths with characteristic beams produced at different wavelengths which will later be analysed and computerised in the form of wave numbers, expressed in cm^{-1}. All spectra included the wave numbers from 4000 to 500 cm^{-1} with 128 scans at a resolution of 2.0 cm^{-1}.

Furthermore, the adsorbents were pretreated by degassing at 200 °C for 2 h, in a vacuum condition, then the specific Brunauer–Emmett–Teller analysis (BET, Micromeritics ASAP 2010, USA) was used to determine the surface area (S_{BET}) of the samples.

Additionally, the morphology of the adsorbent was examined through scanning electron microscopy (SEM, JEOL JSM-7800F, Japan). The adsorbent was mounted using a carbon conductive pad on a metal stub and silver metals were placed at both sides of the samples. In a vacuum condition of an argon atmosphere, the samples were coated with gold for observation and analysis. When the scanning completed, the results were shown based on image formed at SEM display unit.

Batch adsorption study

Batch adsorption experiments were performed under standard conditions, where 2 g/L of the pineapple peel waste adsorbent was mixed with an iron-containing aqueous solution. The stirring process took place at ambient temperature and pressure. The mixture was continuously stirred magnetically for a duration of 240 min. To monitor the adsorption process over time, 10 mL of the mixture were withdrawn at 30-min intervals, and the amount of iron in these samples was analysed. The iron content was determined by using an atomic absorption spectrometer (AAS AAnalyst 400, Perkin Elmer). The experiment was repeated with different amounts of adsorbent (2–24 g/L) and different initial concentrations of iron (5–30 ppm). The removal of iron by the adsorbent was calculated using Equation 1.

$$Removal\ (\%) = \frac{C_i - C_t}{C_i} \times 100\ \% \quad (1)$$

C_i represents the initial iron concentration in the sample (measured in mg/L), while C_t denotes the concentration of iron at a particular time during the experiment (also measured in mg/L).

The amount of iron adsorbed on the adsorbent at certain time was calculated using Equation 2:

$$q_t = \frac{(C_i - C_t)V}{M} \quad (2)$$

Here, q_t represents the iron adsorption on the adsorbent at any given time (measured in mg/g), V indicates the volume of the solution in litres (L), and M signifies the mass of the adsorbent in grams (g).

Kinetic modeling

Pseudo-first- and pseudo-second-order kinetic models are commonly used to describe the adsorption kinetics [16]. The pseudo-first-order equation is given by Equation 3:

$$\frac{dq_t}{dt} = k_1 (q_e - q_t) \quad (3)$$

where q_t and q_e are the amount of solute adsorbed (mg/g) at time t and equilibrium, respectively and k_1 is the rate constant of the kinetic model (min^{-1}). The linearised equation of pseudo-first-order can by expressed by Equation 4:

$$\log(q_e - q_t) = \log\ q_e - \frac{k_1}{2.303} t \quad (4)$$

A plot of $\log(q_e - q_t)$ versus t can be used to obtain the rate constant.

The pseudo-second-order equation is expressed by Equation 5:

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \quad (5)$$

where k_2 is the pseudo-second-order rate constant (g/mg·min) and the linearised form is expressed in Equation 6:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e{}^2} + \frac{1}{q_e} t \quad (6)$$

A plot of t/q_t versus t gives the pseudo-second-order rate constant.

Adsorption Isotherms

Langmuir, Freundlich, and Temkin isotherms are commonly used to determine the adsorption isotherms. These equations explain the relationship between the solute concentrations in both liquid and adsorbent surface equilibrium. Equation 7 represents the Langmuir isotherms:

$$q_e = \frac{q_m K_L C_e}{1 + K_L C_e} \quad (7)$$

where q_e is the quantity of the adsorbed adsorbate bound per unit amount of adsorbent (mg/g), q_m is the maximum adsorption capacity, K_L is the affinity constant for Langmuir isotherm (L/mg), and C_e is the adsorbate equilibrium concentration (mg/L).

To determine the values of maximum adsorption capacity (q_m) and the Langmuir affinity constant (K_L), Equation 7 can be linearised into several form as shown in Equation 8.

$$\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{q_m K_L} \quad (8)$$

A plot of C_e/q_e versus C_e was plotted to determine the values of q_m and K_L.

Freundlich isotherm does not predict the saturation binding of the adsorption. It assumes a logarithmic relationship between the adsorption equilibrium and the solute concentration as shown in Equation 9.

$$q_e = K_F C_e{}^{1/n} \quad (9)$$

where K_F is known as the Freundlich constant (mg/g) and 1/n is a constant reflecting the solute affinity towards the adsorbent.

By taking logarithmic on both sides of the equation, a linearised form of Freundlich isotherm can be used as shown in Equation 10 to determine the adsorption constant (K_F) and its affinity (1/n) by plotting log q_e versus log C_e.

$$log\ q_e = log\ K_F + \frac{1}{n} \log C_e \quad (10)$$

The Temkin isotherm model infers that the adsorption energy decreases linearly with the surface coverage caused by adsorbent–adsorbate interactions [3]. The Temkin isotherm is described in Equation 11:

$$q_e = B\ lnA + B \ln C_e \quad (11)$$

where C_e is the adsorbate equilibrium concentration (mg/L) and q_e is the quantity of metal adsorbed per gram of the adsorbent (mg/g).

The Temkin isotherm equation can be linearised to Equation 12:

$$q_e = \frac{RT}{b}\ lnC_e + \frac{RT}{b} lnK_T \quad (12)$$

where RT/b = B (J/mol) and K_T = A (L/g) are the Temkin constants. R (8.314 J/mol·K) represents the universal gas constant at T (K) which is the absolute solution temperature.

A plot q_e versus ln C_e was plotted to determine the Temkin constant.

Results and Discussion

Characterisation of pineapple peel waste adsorbent: BET analysis

The Brunauer–Emmett–Teller (BET) surface area measurement was utilised to assess the adsorption capacity of the adsorbent. This measurement operates on the principle that a greater surface area of the adsorbent corresponds to a higher adsorption capacity. In other words, as the surface area of the adsorbent increases, its ability to adsorb substances, in this case, iron, also increases. This relationship underscores the importance of surface area in determining the effectiveness of the adsorbent in adsorbing the target substance [17]. After chemical treatment followed by carbonisation at 800 °C for 1 hour, the surface area of pineapple peel waste adsorbent obtained was 1080.91 m^2/g. A prior study documented that following the activation and carbonisation procedures applied to raw pineapple peel waste, there was a noteworthy enhancement in the surface area of the prepared adsorbent [18]. Besides, the surface area of raw pineapple peel waste was 7.9348 m^2/g [14]. The carbonisation process conducted at 800 °C had the effect of improving the elimination of volatile compounds. Additionally, the inclusion of $ZnCl_2$ as an impregnation agent heightened the heat energy within the process. This, in turn, led to the creation of new pores and an accelerated development of porosity in the adsorbent. Consequently, these factors collectively contributed to an increase in the surface area of the adsorbent. [19].

The N_2 adsorption isotherms for the adsorbent are depicted in Figure 1. The behaviour of the adsorbent corresponds to a Type I isotherm, indicating that the adsorbent possesses microporous characteristics. A Type I isotherm typically exhibits a convex curve, and its plateau is either horizontal or nearly horizontal. In this type of isotherm, the adsorption curve directly intersects with the line $P/P_0 = 1$ [18]. The amount of adsorption experienced a rapid increase in a region of low relative pressure ($P/P_0 < 0.2$) during the initial stages of adsorption. This observation suggests that the primary location for nitrogen molecule adsorption was within the microporous structure of the adsorbent [18].

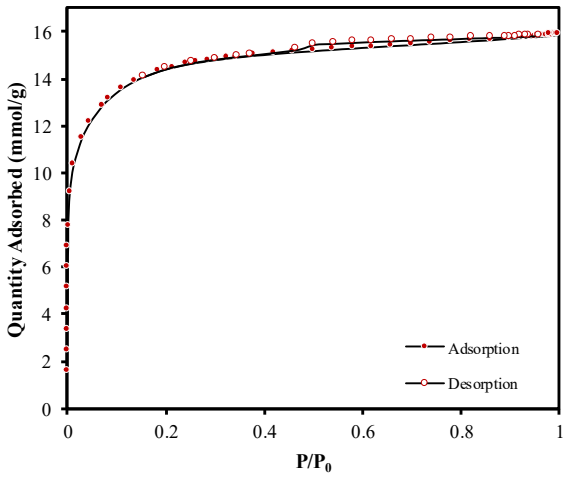


Figure 1. N_2 adsorption–desorption isotherms of adsorbent

Scanning electron microscopy (SEM) of adsorbent

Figure 2 shows the SEM images of raw pineapple peel waste, the prepared pineapple peel waste adsorbent, and the adsorbent after the adsorption process. SEM analysis was employed to observe the surface morphologies of the raw pineapple peel waste, the prepared adsorbent at 800 °C for 1 h, and the adsorbent after adsorption. The surface of raw pineapple peel waste is naturally uneven and features creases. However, the surface of the adsorbent exhibits a more intricate and porous structure after the modification process. This transformation can be attributed to the removal of volatile substances during carbonisation and activation, resulting in a stable carbon mass and the widening of pore networks within the

adsorbent sample, as documented in reference [20]. The porous nature of the adsorbent plays a significant role in facilitating the adsorption of heavy metals in wastewater. These pores serve as active sites for adsorption and increase the overall surface area of the adsorbent, as discussed in reference [21]. Furthermore, in this study, it was noted that the adsorbent's surface featured densely packed bundles of elongated cylindrical pores, signifying a strong adsorption capacity. This configuration provides an abundance of sites for capturing heavy metal ions, such as iron. This could be explained by the morphology of the adsorbent after adsorption where the surface morphology became rougher or more irregular due to the formation of metal ion complexes or precipitates on the surface of the adsorbent. It was observed that there are some cavities on the surface of the adsorbents [22].

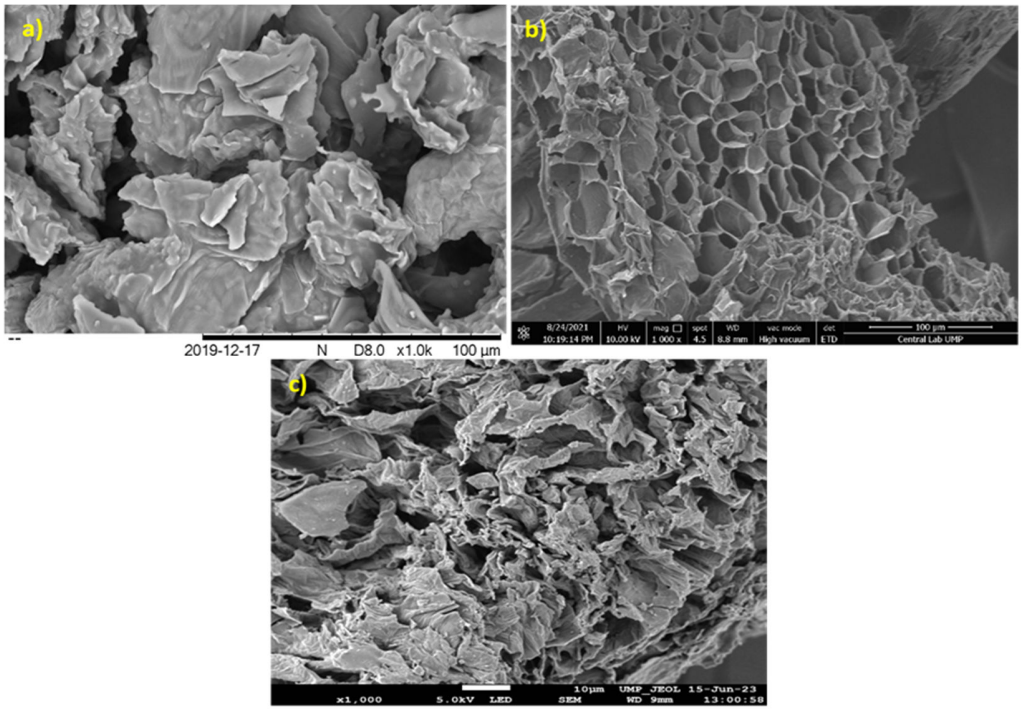


Figure 2. SEM images (1000× magnification) of (a) raw pineapple peel waste, (b) pineapple peel waste adsorbent and (c) adsorbent after adsorption

FTIR analysis

FTIR analysis was conducted to qualitatively identify the primary functional groups present on the surface of the adsorbent derived from pineapple fruit peel. Figure 3 displays the FTIR spectra of both the adsorbent and the raw pineapple peel. In the spectrum of the raw pineapple peel, a broad band appearing around 3,300 cm^{-1} is primarily associated with hydrogen bonds (O–H or NH_2) symmetrical stretching variations. Additionally, the peak at 2,917 cm^{-1} corresponds to aliphatic CH groups. However, after the modification process, several peaks within the range of 1,560–1,300 cm^{-1} can be attributed to CH_2 bending vibrations, O–H bending, and O–H stretching or C-– bending vibrations. Furthermore, a strong band at 1,089 cm^{-1} is attributed to the C–O of the $–OCH_3$ group, aligning with the lignin structure present in pineapple fruit peel [10] and [21]. The slight shift in absorption bands indicates the successful transformation of the raw pineapple peel into the adsorbent.

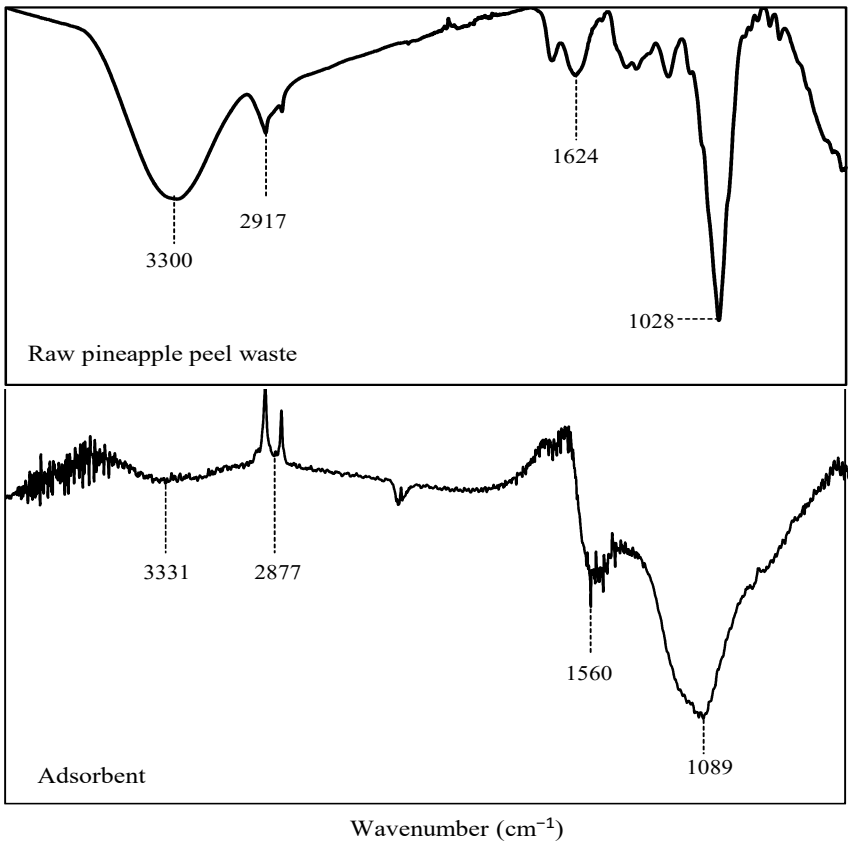


Figure 3. FTIR spectra for the raw pineapple peel waste and pineapple peel waste adsorbent

Batch adsorption studies: The effect of contact time

One of the most critical factors affecting adsorption performance is the contact time, as it illustrates the removal percentage of metal ions from the solution over time [24]. The effect of contact time on iron adsorption onto the pineapple peel waste adsorbent is shown in Figure 4(a). In the initial stages, the rate of adsorption exhibited a substantial increase and gradually reached equilibrium around the 150-min mark, achieving an efficiency of approximately 48.86% removal. Then, the final Fe(III) ions concentration became almost similar until 240 min. Therefore, the process was at steady state at 150 min. This situation happened when the metal ions rapidly adsorbed onto the adsorbent at the beginning caused by the availability of the empty active sites. In addition, the uptakes were gradually slower due to the decrease in vacant active sites [10]. It was reported that the properties of the adsorbent and its existing active sites are known to affect the time needed to reach equilibrium [23, 24].

The effect of adsorbent dosage

The impact of adsorbent dosage on the adsorption of iron onto the pineapple peel waste adsorbent was explored, as adsorbent dosage is recognised as another key factor influencing the adsorbent's capacity to adsorb metal ions at a given initial concentration [25–27]. In Figure 4(b), the removal percentage of Fe(III) ions and the adsorption capacity of the pineapple peel waste adsorbent are depicted across various dosages, all while maintaining a constant initial concentration of 5 ppm Fe solution. The graph demonstrates that 20 g/L gave the highest iron removal up to 99.24% and remained constant till 24 g/L. A higher amount of adsorbent dosage will increase the number of active sites since the adsorption depends on the availability of binding sites for iron [30].

The effect of initial iron concentration

In batch adsorption processes, the adsorption rate is a function of the initial concentration of heavy metal ions; hence it is a principal factor for a successful adsorption

process [31]. The percentage removal of iron at different initial concentrations from 5 to 80 ppm is shown in Figure 4(c). The removal efficiency increased drastically at 5 ppm before increasing gradually until 80 ppm with the highest removal of 80.13%. It shows that the pineapple peel waste adsorbent had a higher magnitude for Fe(III) ions adsorption. This may also be related to an increase in the concentration gradient driving force with the increase in metal ions concentration [4]. A comparable result was also reported where the adsorption of iron using rice husk ash gave higher iron adsorption capacity than manganese, which means that increasing the initial concentration of iron also improves the removal efficiency of iron [4].

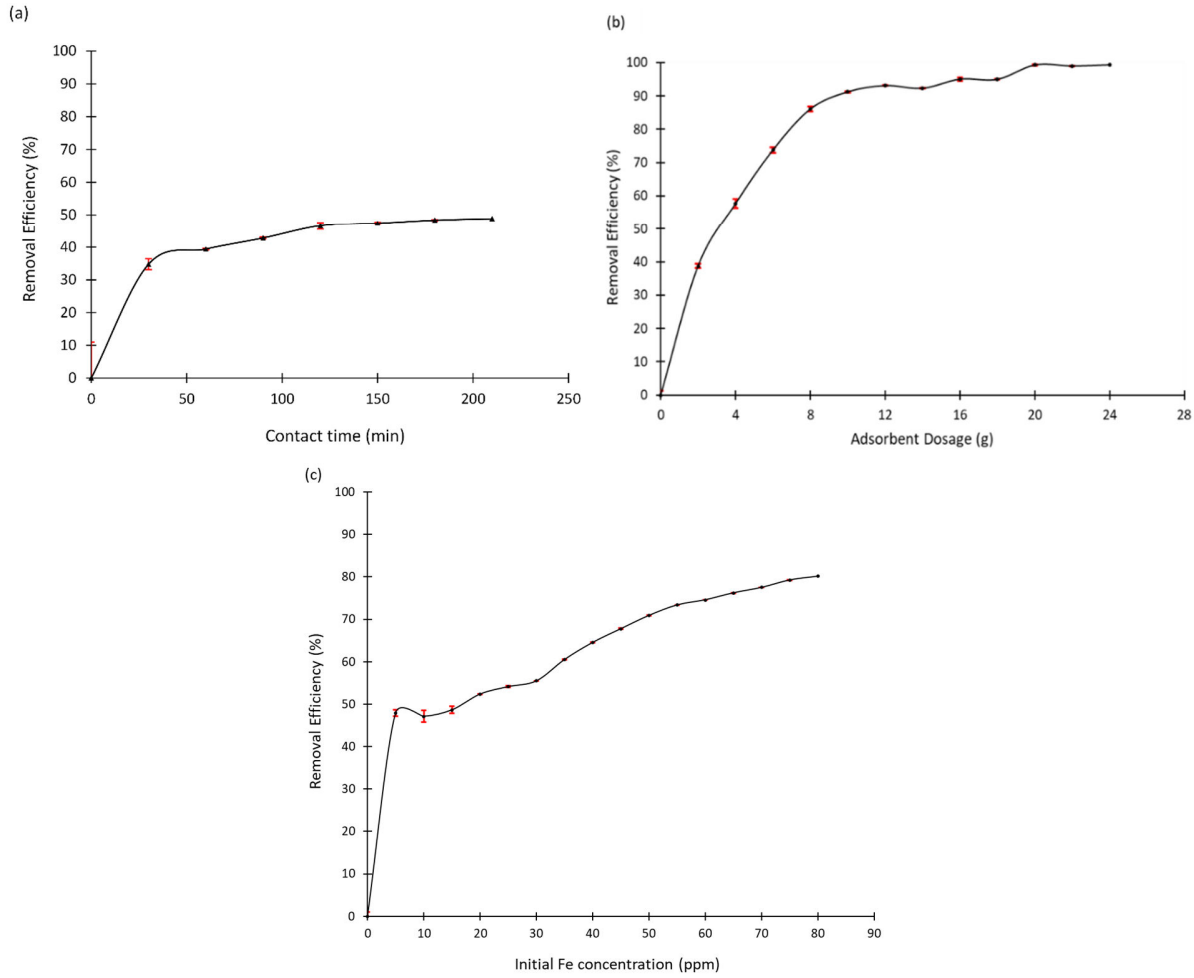


Figure 4. The effect of (a) contact time, (b) adsorbent dosage, and (c) initial iron concentration on the iron removal efficiency

Adsorption kinetic

Adsorption kinetics are used to explain the adsorption mechanism and characteristics. The pseudo-first- and pseudo-second-order kinetics models were applied to the adsorption data to specify the rate of adsorption of iron onto the pineapple peel waste adsorbent. Constants of pseudo-first- and pseudo-second-order were determined from the slope and intercept of the linear plots as shown in Figure 5(a) and 5(b), respectively.

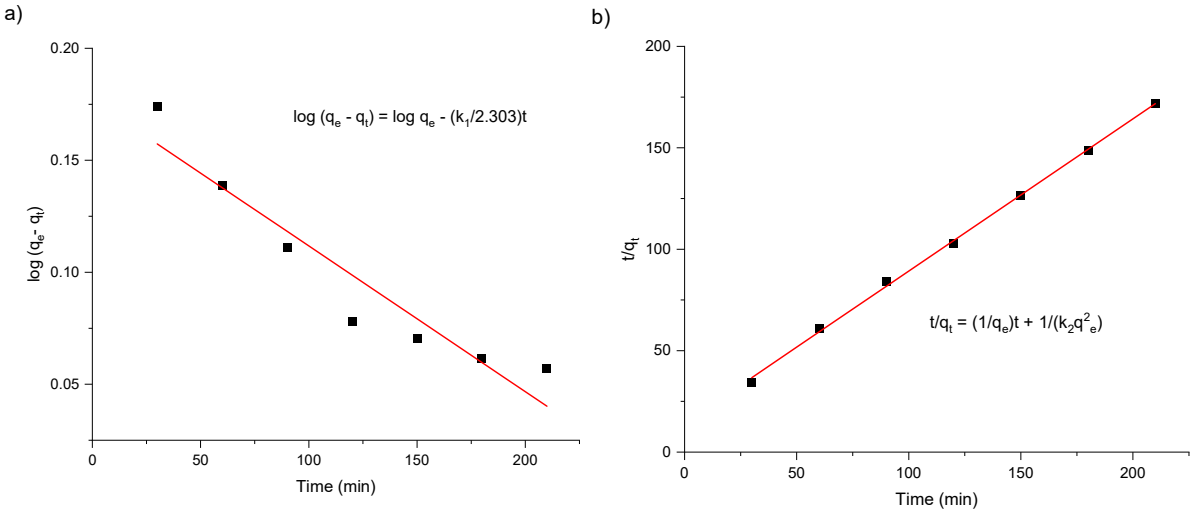


Figure 5. The plots of (a) pseudo-first- and (b) pseudo-second-order kinetic models for iron removal

Table 1. Pseudo-first- and pseudo-second-order parameters for iron removal

Model	Intercept	Slope	$q_{e\,(exp)}$ (mg/g)	$q_{e\,(cal)}$ (mg/g)	q^2_e	k	R^2
Pseudo-first-order	0.01535	−0.01228	1.198	−4.17664		0.028281	0.93689
Pseudo-second-order	14.19527	0.75037		1.332676	1.776025	0.039665	0.99872

Table 1 presents the parameters and regression coefficients (R^2) for both the pseudo-first-order and pseudo-second-order kinetic models. It is noteworthy that the pseudo-second-order model demonstrates a notably higher correlation coefficient ($R^2 > 0.9987$) in comparison to the pseudo-first-order model. This high R^2 value, close to unity, signifies a robust relationship between the parameters and underscores the suitability of the pseudo-second-order kinetic equation for describing the uptake of Fe(III) by the pineapple peel waste adsorbent [32]. Meanwhile, the equilibrium removal capacity calculated ($q_{e,\,cal}$) of the pseudo-second-order model was much closer to the equilibrium removal capacity experimental ($q_{e,\,exp}$) representing that the pseudo-second-order rate model gives a better summary of iron removal from solution to the pineapple peel waste adsorbent. This conveys that the pseudo-second-order model can assume a chemically rate-controlling removal process [33].

The comparison of kinetics results with values from other studies is listed in Table 2. Iron adsorption onto the pineapple peel waste adsorbent was inferred to follow pseudo-second-order kinetics. These findings suggest that the rate-limiting step in the adsorption process is chemisorption, which entails valency forces, including the sharing or exchange of electrons between the adsorbent and the adsorbate [31, 34]. These results are consistent with previous literature, which also indicated that the adsorption kinetics of Fe(III) ions by distinct types of adsorbents typically adhere to the pseudo-second-order model [4, 32].

Table 2. Kinetic parameters comparison of iron adsorption onto various adsorbents

Adsorbent	Pseudo-first-order model		Pseudo-second-order model			References
	k_1 (min^{-1})	R^2	k_2 (g/mg·min)	q_e	R^2	
Pineapple peel	0.0283	0.9369	0.0397	1.33	0.9987	This study
Natural Clay	0.056	0.986	0.02	7.42	0.998	[35]
Acid–activated clay	0.058	0.991	0.007	11.41	0.994	[35]
Natural zeolite	4.9	0.8558	0.22	8.70	0.9985	[36]
Orange peel	0.001	0.032	0.068	13.44	0.996	[5]

Adsorbent	Pseudo-first-order model		Pseudo-second-order model			References
	k_1 (min^{-1})	R^2	k_2 (g/mg·min)	q_e	R^2	
Limestone	0.069	0.919	5.913	0.019	0.997	[37]
Rice hull	0.017	0.90	0.000813	22.73	0.99	[2]
Natural feldspar	0.0283	0.83	0.0352	8.72	0.9987	[3]
Rice husk ash	-	-	0.033	1.340	0.995	[4]

Adsorption isotherms

An isotherm characterises the equilibrium association between the solute molecules bound to the adsorbent's surface and those remaining unbound in the liquid phase. In this study, Langmuir, Freundlich, and Temkin isotherms were employed to elucidate the equilibrium relationship between the concentration of solute in the liquid phase and its interaction with the adsorbent's surface. Batch adsorption isotherm experiments were conducted to investigate iron adsorption using the pineapple peel waste adsorbent at room temperature. The results of these adsorption isotherms are visually presented in Figure 6(a-c).

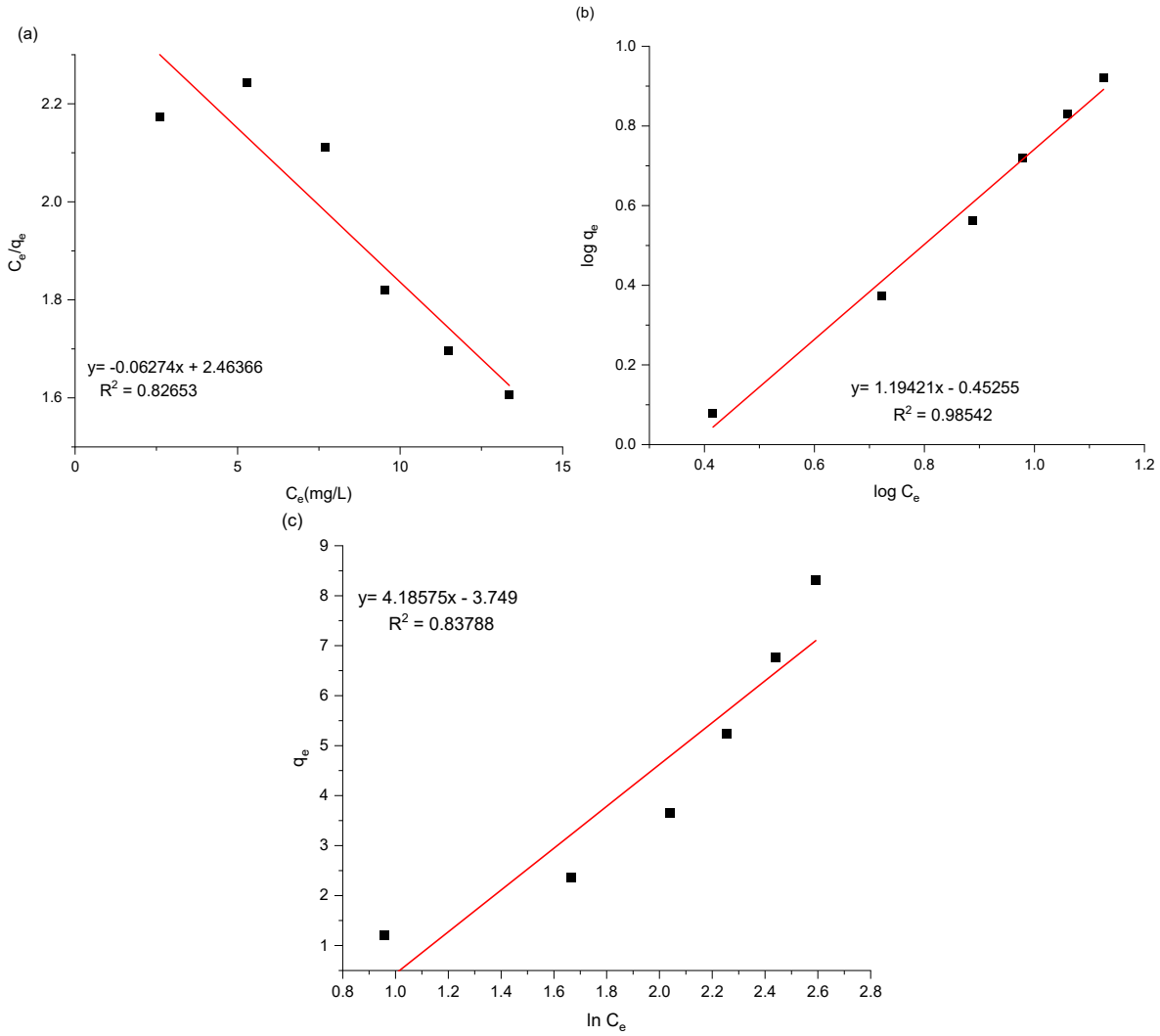


Figure 6. The plots of (a) Langmuir, (b) Freundlich, and (c) Temkin isotherms

Table 3. Langmuir, Freundlich, and Temkin isotherms parameters for iron removal

Isotherms	Constants	Value
Langmuir	$1/q_m$	0.0624
	q_m (mg/g)	15.9288
	K_L (L/mg)	0.0255
	R_L	1.1459
	R^2	0.8265
Freundlich	1/n	1.1942
	n	0.83737
	log K_F	0.4526
	K_F (L/mg)	2.8349
	R^2	0.9854
Temkin	Slope = RT/B	4.1858
	Intercept = RT/B (ln A)	−3.749
	B	597.8651
	A (L/mol)	0.3274
	R^2	0.8379

The results for all the parameters obtained from the Langmuir, Freundlich, and Temkin isotherms are summarised in Table 3. The Langmuir isotherm model was employed to fit the experimental data, yielding a correlation regression coefficient (R^2) of 0.8265. The maximum sorption capacity (q_m) was determined to be 15.94 mg/g using this model. However, it is evident that the data does not align well with the Langmuir model. This deviation may be attributed to interactions between the adsorbate or the fact that the adsorption process is not monolayer adsorption involving just one adsorbate molecule per adsorption site [38]. In this case, iron can be said to be adsorbed in inhomogeneous distribution to the adsorbent surface due to the irregular surface. So, there might be a 'favoured' point of strong adsorption side. The results indicate that the Freundlich isotherm best fitted the iron adsorption on pineapple peel waste adsorbent with R^2 of 0.9854. However, with $1/n > 1$, it is best described by the S-type isotherm. This experiment agrees with the hypothesis that at low concentrations, such compounds compete with water for the adsorption sites. It means that the surface has a low affinity for the adsorptive at low concentrations, which then increases at higher concentrations [39]. On the other hand, the Temkin isotherm model moderately fitted the experimental data for iron adsorption onto pineapple peel waste adsorbent as the R^2 value is 0.84. The comparison of the adsorption capacity of other adsorbents for iron removal from aqueous solutions is tabulated in Table 4.

Table 4. Comparison of adsorption isotherms for iron removal using other adsorbents

Adsorbents	Langmuir			Freundlich			Temkin			References
	q (mg/g)	K_L (L/mg)	R^2	K_f (L/mg)	1/n	R^2	A	B	R^2	
Pineapple peel	15.93	0.0255	0.827	2.835	1.194	0.985	0.327	597.87	0.838	This study
Natural Clay	18.86	0.044	0.990	0.805	0.543	0.985				[35]
Acid–activated clay	25.00	0.09	0.998	5.96	0.312	0.993	-	-	-	[35]

Adsorbents	Langmuir			Freundlich			Temkin			References
	q (mg/g)	K_L (L/mg)	R^2	K_f (L/mg)	1/n	R^2	A	B	R^2	
Natural zeolite	6.13	17.92	0.977	6.04	0.3497	0.945	-	-	-	[36]
Spent coffee grounds	0.615	2.803	0.999	0.530	0.6606	0.991	-	-	-	[28]
Chitosan	133.3	0.0643	0.93	3.807	0.388	0.979	-	-	-	[40]
Limestone	0.018	140.31	0.923	0.029	0.216	0.753	-	-		[37]
Rice hull	45.45	0.03	0.99	1.07	0.68	0.97	32.27	28.36	0.79	[2]
Natural feldspar	25.00	0.046	0.936	1.701	0.622	0.997	-	-	-	[3]
Orange peel	8.35	0.15	0.998	4.97	0.0937	0.900	-	-	-	[5]
Rice husk ash	66.66	0.026	0.927	1.0739	0.916	0.908	54.5	101.76	0.93	[4]
Palm oil mill effluent	1.116	0.25	0.998	0.376	0.281	0.966	-	-	-	[41]

Proposed mechanisms of iron adsorption

Adsorbent from pineapple peel waste consists of three main components: cellulose, hemicellulose, and lignin. These cellulosic components contain a majority of the oxygen functional groups, such as hydroxyl, ether, and carbonyl, that influence the adsorption properties of the iron onto the adsorbent [42]. The adsorbent after adsorption was examined using SEM analysis in Figure 2 and showed that the morphology of the adsorbent changed with the presence of some precipitation. The binding mechanism of the adsorbent and iron ions is proposed in Figure 7.

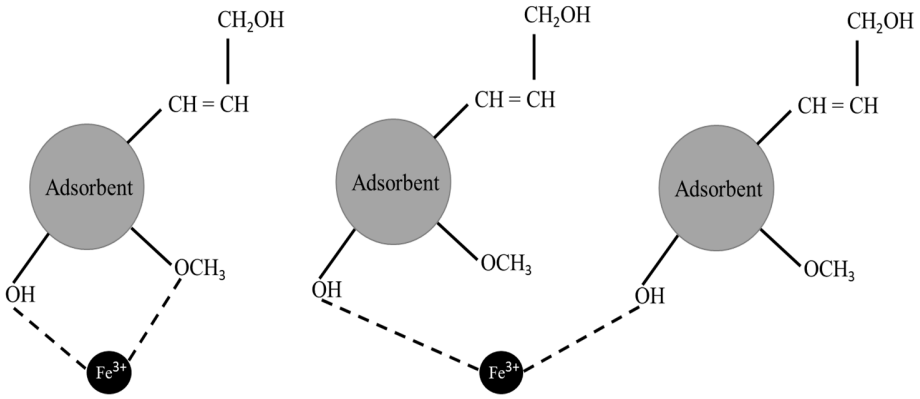


Figure 7. Binding mechanism of adsorbent and Fe(III) ions

Conclusion

In this study, the adsorbent from pineapple peel waste can potentially remove iron from wastewater. The results show that the adsorbent could remove 48.86% iron when 2 g/L of the adsorbent was used and increased to 99.24% when 20 g/L of the adsorbent was used. The adsorption isotherm study shows that iron adsorption fitted the experimental data of the Freundlich isotherm rather than Langmuir and Temkin isotherms due to the highest R^2 value of 0.985. From the Langmuir model, the maximum iron adsorption capacity of the adsorbent was 15.93 mg/g. The adsorption kinetics study shows that iron adsorption followed the pseudo-second-order kinetics model, indicating that chemisorption was the mechanism of adsorption that contributed to the iron removal from the aqueous solution. Based on the results

of this study, pineapple peel waste adsorbent was found to be effective in removing iron from an aqueous solution.

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DETERMINATION OF CELECOXIB IN HUMAN PLASMA BY EFFERVESCENCE-ASSISTED DISPERSIVE LIQUID–LIQUID MICROEXTRACTION AND HPLC/UV

(Penentuan Celecoxib dalam Plasma Manusia dengan Bantuan Buih Berbuak Pengekstrakan Mikro Penyebaran Cecair-ke-Cecair dan HPLC/UV)

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Abstract

In this study, effervescence-assisted dispersive liquid–liquid microextraction was utilized for rapid, simple, and inexpensive microextraction of celecoxib followed by HPLC/UV. In the proposed method, carbon dioxide microbubbles were produced by the reaction between oxalic acid and sodium bicarbonate. Various factors such as type and volume of extraction solvent, pH of the sample solution, content of the acidic and basic promoters, and type and volume of auxiliary solvent were optimized. Under optimum conditions, 60 µL chloroform (as an extraction solvent) was added to 2 mL methanol and 0.4g oxalic acid. The mixture was added to the test tube containing 0.8g sodium bicarbonate and 10 mL of sample solution (pH 6). The presented method offered wide linear dynamic range of 0.5-1000 µg L^{-1} with satisfactory extraction recovery of 94.4%, high pre-concentration factor of 206, and normal RSD% (2.15, n=3). Finally, the proposed method was successfully applied for the analysis of celecoxib in plasma samples.

Keywords: effervescence-assisted liquid-microextraction, celecoxib, plasma, acid-base reaction, carbon dioxide microbubbles

Abstrak

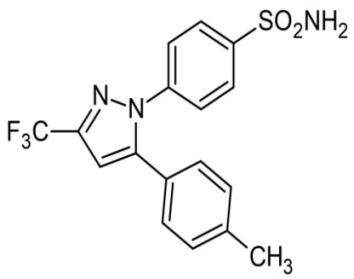
Dalam kajian ini, pengekstrakan mikro cecair-ke-cecair yang dibantu buih berbuak telah digunakan untuk pengekstrakan celecoxib yang cepat, mudah, dan murah diikuti oleh HPLC/UV. Dalam kaedah yang dicadangkan, buih mikro karbon dioksida dihasilkan oleh tindak balas antara asid oksalik dan natrium bikarbonat. Pelbagai faktor seperti jenis dan isipadu pelarut pengekstrakan, pH larutan sampel, kandungan penggalak asid dan bes, dan jenis dan isipadu pelarut bantu telah dioptimumkan. Di bawah keadaan optimum, 60 µL kloroform (sebagai pelarut pengekstrakan) ditambahkan ke dalam 2 mL metanol dan 0.4g asid oksalik. Campuran tersebut ditambahkan ke dalam tabung uji yang mengandungi 0.8g natrium bikarbonat dan 10 mL larutan sampel (pH 6). Kaedah yang dikemukakan menawarkan julat dinamik linear yang luas dari 0.5-1000 µg L^{-1} dengan pulihkan pengekstrakan yang memuaskan sebanyak 94.4%, faktor pra-pemekatan tinggi sebanyak 206, dan RSD% normal (2.15, n=3). Akhirnya, kaedah yang dicadangkan telah berjaya digunakan untuk analisis celecoxib dalam sampel plasma.

Kata kunci: pengekstrakan cecair-mikro yang dibantu buih berbuak, celecoxib, plasma, tindak balas asid-bes, buih mikro karbon dioksida

Introduction

Non-steroidal anti-inflammatory drug (NSAID), celecoxib (CEL) has been extensively prescribed for the treatment of inflammation and pain in osteoarthritis, rheumatoid arthritis, and painful menstruation [1]. Furthermore, the therapeutic effect of this drug on renal and pancreatic functions, as well as its chemo-preventive effect on various cancer types have been recently investigated and reported [2]. In spite of the excellent therapeutic effect of CEL, its high dosage can enhance the risk of heart attack. Some recent reports have also mentioned the distribution of CEL into human breast milk [3, 4]. In this context, the development of an efficient technique for the measurement of CEL concentration in biological matrices can contribute to optimizing the CEL dosage based on medical strategies and therapeutics for serious intoxication. The most common analytical method for measuring the CEL in bio-fluid samples is based on high-performance liquid chromatography (HPLC) [5-8]. The quality of analysis usually decreases due to the complexity of bio-fluids matrices and trace concentration of CEL. Hence, the development of a sample preparation technique to reduce the matrix interference and enrich the trace values of CEL is the main challenge.

The structure and corresponding log P value (octanol–water partition coefficient) and dissociation constant value (pKa value) of CEL are presented in Figure 1.



Log p= 3.5 pKa= 11.1

Figure 1. Chemical structure, Log p and pKa of Celecoxib

Extraction of trace contents of chemical compounds that can be analyzed by analytical instruments has found increasing popularity among the scientists. This extraction should be able to concentrate analytes and also sample cleanup contemporaneously. This process is called sample preparation [9, 10]. Liquid- liquid extraction (LLE) and Solid phase extraction (SPE) are among the earliest sample preparation methods [11, 12]. Scientists have also attempted to minimize the consumption of organic solvents and simplify this type of extraction. Liquid–liquid microextraction (LLME) and Solid phase microextraction (SPME) are really useful in the extraction of the analytes from various matrixes [13-15]. Liquid phase microextraction (LPME) is an interesting and applicable method capable of extracting analytes from an aqueous phase in mL to an organic solvent in μL [16, 17].

Among the conventional solvent-minimized extraction methods, dispersive liquid liquid microextraction (DLLME) is frequently applied due to its outstanding benefits such as fast microextraction process, high enrichment factor, and low consumption of organic reagents as extraction solvents [18-21]. In spite of the excellent performance of this method, there are several disadvantages in the initial version of DLLME method. The low partition coefficient of the target analytes due to use of the organic disperser solvents and inadequate contact surface between the organic extractor and sample solution are the shortcomings of the DLLME method. The use of hazardous chlorinated extractors and toxic disperser solvents increases the operational and environmental pollution risks which it is the other drawback of the mentioned method [22]. In recent years, effervescence-assisted dispersive liquid–liquid microextraction (EADLLME) method has been proposed as a new assisted technique with promising potential for pre-concentration of target analytes without using the disperser solvent [23-27].

The present study is focused on the development of EADLLME technique based on gas-generation reaction which could be an appropriate alternative for organic disperser solvent in DLLME method. In this method, CO_2 microbubbles are formed as disperser by the reaction between acid and base source in the sample solution. The produced CO_2 microbubbles enhance the dispersibility of organic extraction solvent which promotes the transfer of CEL to the organic phase. Also, the gas bubbles provide the large contact surface between CEL and extractant, a high extraction efficiency and rapid extraction equilibrium. The effect of several important factors on the extraction efficiency was investigated by utilizing one-at-a-time process to achieve the optimum micro-extraction conditions.

Finally, the developed EADLLME method was successfully applied to isolate and pre-concentration of CEL in the plasma samples. The analysis of target analyte was accomplished by high-performance liquid chromatography with ultraviolet detection (HPLC/UV). Precise and repeatable data were obtained to find further high pre-concentration factor and extraction recovery.

Materials and Methods

Materials and standard

Celecoxib was supplied from Arasto Pharmaceutical Chemicals Inc (Tehran, Iran). Oxalic acid, sodium bicarbonate, HPLC grade methanol (MeOH), and acetonitrile (ACN) were provided from Merck (Darmstadt, Germany). N-hexane, dichloromethane, carbon tetrachloride, chloroform, methanol and ethanol were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Plasma samples were obtained from Blood Transfusion Organization (Babol, Iran). Standard stock solution of CEL (500 ppm) was prepared by dissolving 50 mg of reference material into 100 mL of HPLC grade methanol which was refrigerated before use. Working solutions were prepared by diluting the stock solution in water.

Apparatus

HPLC analysis of CEL was carried out using an Alliance HPLC system from waters Company (Milford, USA). The HPLC instrument included a 2695 pump, an online degasser, a UV/vis detector (Model 2487), and a 6-port/3-channel injection valve encompassing a high-pressure manual injection valve (20 µL loop). The detector was adjusted at 252 nm. C_{18} (150 mm × 4.6 mm I.D., 5 µm) MZ-Analysentechnik GmbH HPLC column (Mainz, Germany) was also utilized for chromatographic separation. Empower software was employed for data processing. Isocratic elution was also conducted by a mixture of acetonitrile: water (75:25) at the flow rate of 1mL/min.

Effervescence-assisted dispersive liquid–liquid microextraction procedure

2 mL methanol (auxiliary solvent), 0.4g oxalic acid (acidic promoter), and 60 µL Chloroform (Extraction solvent) were mixed and then added to a conical test tube containing 0.8g sodium bicarbonate(basic promoter) in 10 mL water with the concentration of 1 µg mL^{-1} of CEL (pH 6). The acid-base reaction occurred rapidly upon adding the acidic promoter to the basic promoter solution (Eq. 1) and CO_2 (one of the reaction products) generated bubbles inside the test tube, resulting in a cloudy solution. After one minute, the mixture was centrifuged for 5 min at 5000 rpm and the dispersed droplets of organic phase were gathered at the conical bottom of the test tube. The extract was withdrawn using a micro-syringe and analyzed by HPLC/ UV.

$$2NaHCO_3 + (COOH)_2 \longrightarrow (COONa)_2 + 2CO_2 + 2H_2O \quad (1)$$

Results and Discussion

This work addressed the effects of various parameters such as type and volume of extraction solvent, content of acidic and basic promoters, pH of sample solution and type and volume of auxiliary solvent for the optimization of EADLLME procedure of CEL.

Extraction solvent

The following criteria should be considered in the selection of an extraction solvent for EADLLME: It should be immiscible with water and compatible with chromatographic systems and also have good efficiency for extraction of the analyte. Dichloromethane, chloroform, carbon tetrachloride (higher density than water), and n-hexane (lower density than water) were examined. Among these solvents, chloroform showed the highest Extraction recovery and was used for further studies (Figure 2).

Content of acidic and basic promoter

Four contents of oxalic acid (acidic promoter) and sodium bicarbonate (basic promoter) were examined for optimizing the promoter's content. The molar ratio of $NaHCO_3$: $C_2H_2O_4$ was set to 2:1. The Extraction recovery of celecoxib increased by raising the oxalic acid content to 0.4 g (Figure 3). So 0.4g of oxalic acid and 0.8g of sodium bicarbonate are considered as the optimized amount in the further studies.

Type and volume of auxiliary solvent

An auxiliary solvent dissolves the oxalic acid, promoting the dispersion of the extraction solvent in the aqueous phase. Methanol, ethanol, and acetonitrile were studied for this purpose. Extractions were conducted with oxalic acid (0.4g) and 1 mL methanol, ethanol and acetonitrile. Based on Figure 4, methanol

was the best auxiliary solvent. According to Figure 5, 2 mL methanol led to the highest extraction recovery in the extraction of CEL.

Volume of extracting solvent

Volume of extraction solvent is one of the main parameters that should be optimized. For this purpose, 60, 70, 80, 90, and 100 µL of chloroform were applied. Based on the results, extraction with 60 µL showed the greates extraction recovery, thus, this value was selected for further analyses (Figure 6). Under this volume, no organic solvent sedimented at the bottom of the test tube.

Effect of pH

The pH of the sample solution is another important parameter in the EADLLME method. The effect of this factor was explored on the extraction recoveries of CEL in the ranges of 3.0-8.0. Based on the obtained results (Figure 7), the extraction recovery of CEL decreased by decrementing the pH of sample to 3.0. The target analyte appeared into its ionic form at low pH value which reduced transferring into the organic phase and decreased extraction efficiency. At higher pH levels, the reaction between acid and base is likely to be disrupted, decreasing CO_2 production, followed by a reduction in the dispersion of the organic phase in the sample solution and extraction recovery. Therefore, pH of 6.0 was chosen as the optimum pH for the following experiments.

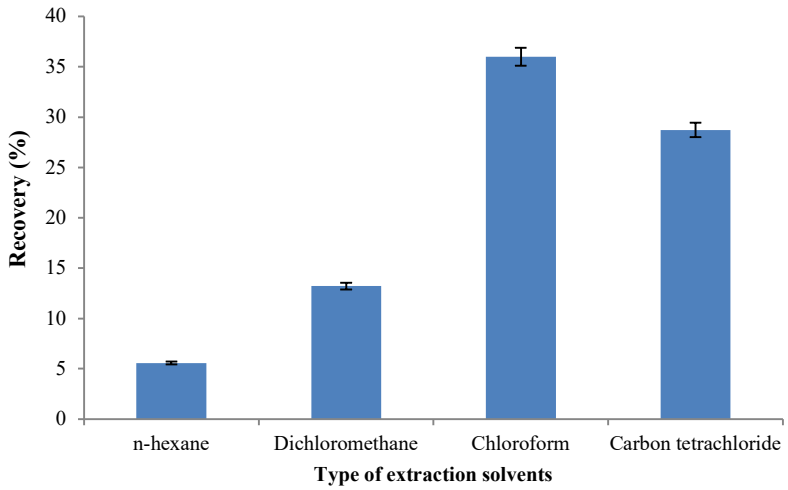


Figure 2. Effect of extraction solvent on the extraction recovery of CEL. Extraction condition: CEL 1µg mL^{-1}; sample volume 10 mL; extraction solvent volume: 100 µL; acidic promoter 0.3g; basic promoter 0.6g; auxiliary solvent; methanol, 1 mL; centrifuging time: 2 min at 4000 rpm. All experiments were done at triplicate and the mean value of the results is reported

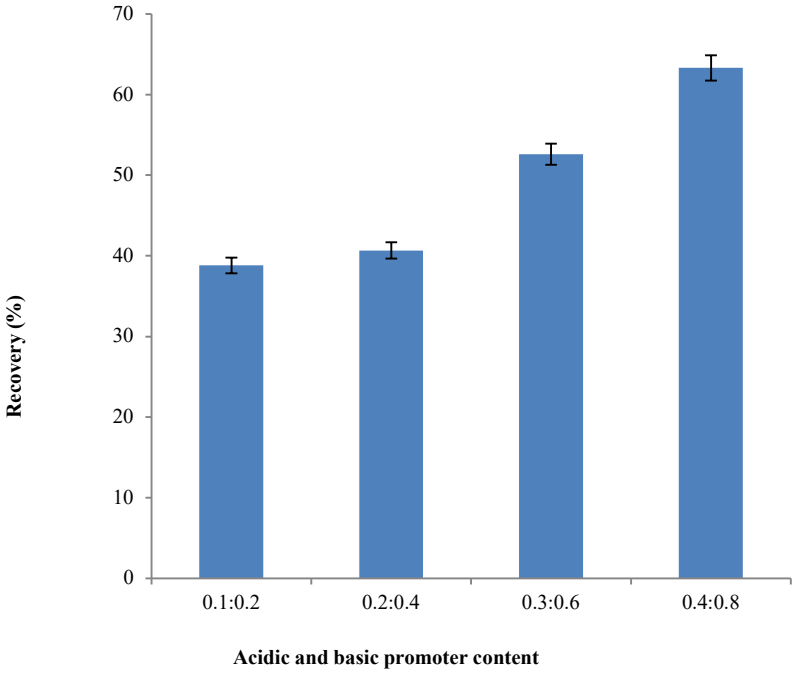


Figure 3. Effect of the content of acidic and basic promoter on extraction recovery. Extraction condition: CEL 1µg mL^{-1}; sample volume 10 mL; extraction solvent: $CHCl_3$, 100 µL; auxiliary solvent; methanol, 1 mL; centrifuging time: 2 min at 4000 rpm. All experiments were done at triplicate and the mean of results is reported

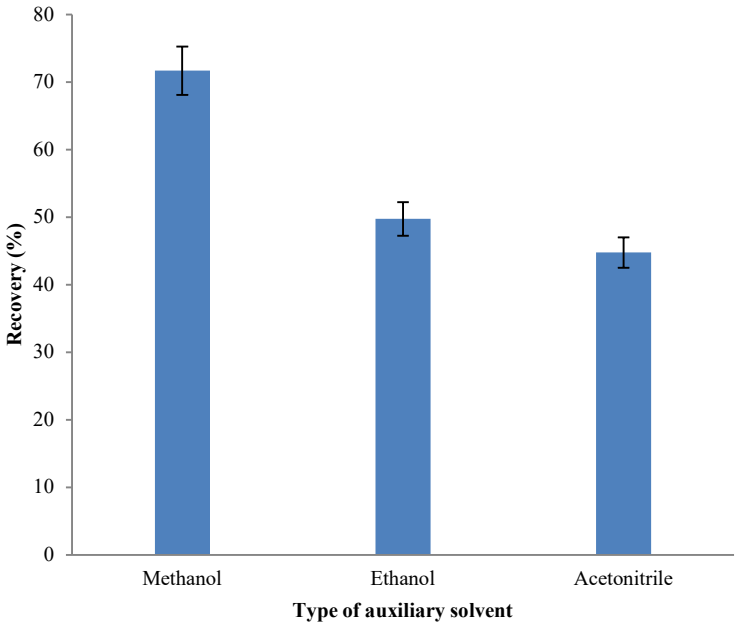


Figure 4. Effect of the type of auxiliary solvent on extraction recovery. Extraction condition: CEL 1 µg mL^{-1}; sample volume: 10 mL; extraction solvent: $CHCl_3$, 100 µL; acidic promoter: 0.4g; basic promoter 0.8 g; volume of auxiliary solvent: 1 mL; centrifuging time: 2 min at 4000 rpm. All experiments were performed in triplicate and the mean values of results are reported

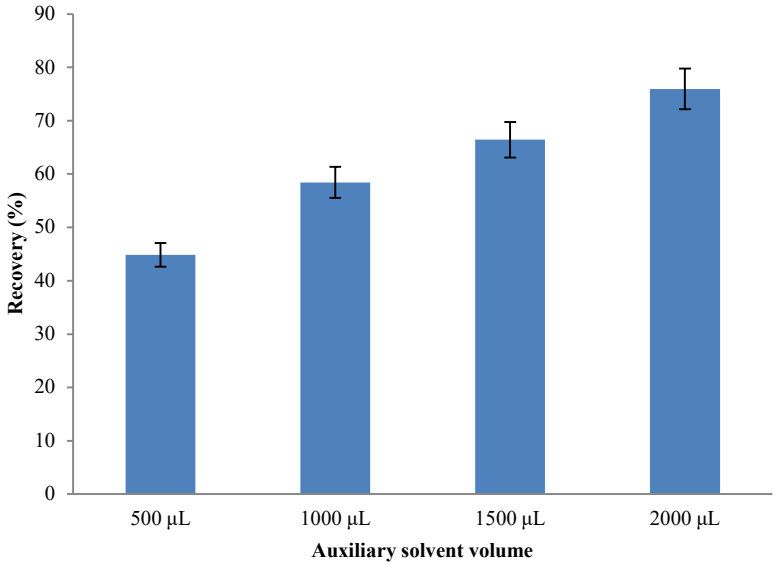


Figure 5. Effect of the volume of auxiliary solvent on extraction recovery. Extraction condition: CEL 1µg mL^{-1}; sample volume: 10 mL; extraction solvent: $CHCl_3$, 100 µL; acidic promoter: 0.4g; basic promoter: 0.8g; auxiliary solvent: methanol; centrifuging time: 2 min at 4000 rpm. All experiments were performed in triplicate and the mean values of results are reported

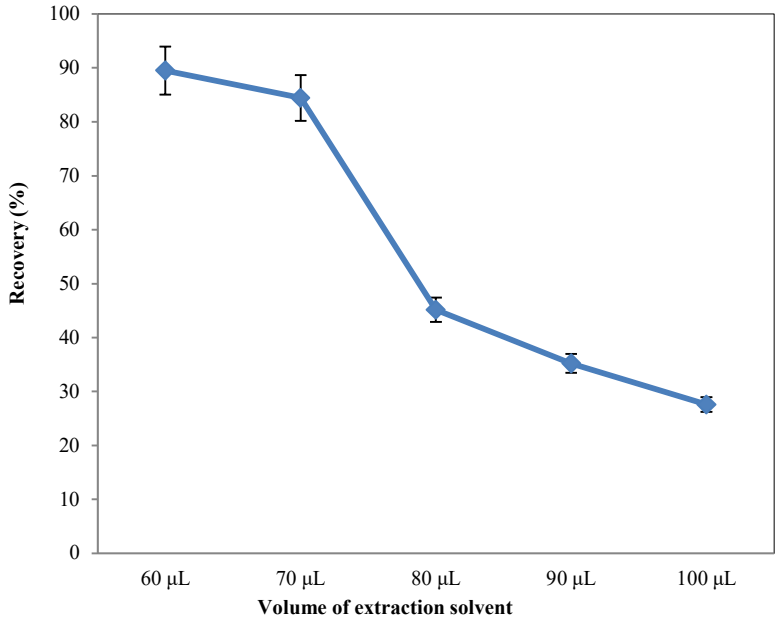


Figure 6. Effect of the volume of extraction solvent on extraction recovery. Extraction condition: CEL 1 µg mL^{-1}; sample volume: 10 mL; extraction solvent: $CHCl_3$; acidic promoter 0.4g; basic promoter 0.8g; auxiliary solvent: methanol, 2 mL; centrifuging time: 2 min at 4000 rpm, pH of sample solution, 6. All experiments were performed in triplicate and the mean value of the results are reported

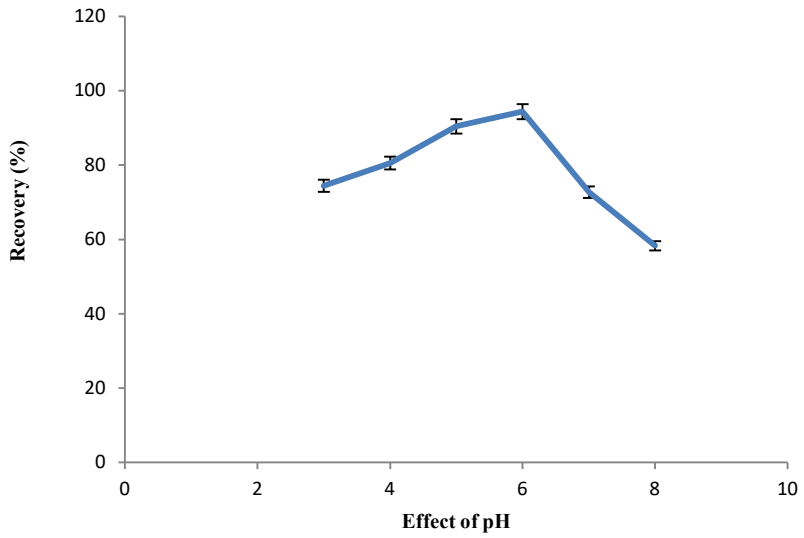


Figure 7. Effect of pH of sample solution on extraction recovery. Extraction condition: CEL 1 µg mL^{-1}; sample volume: 10 mL; acidic promoter: 0.4g; basic promoter: 0.8g; auxiliary solvent: methanol, 2 mL; extraction solvent: $CHCl_3$, 60 µL; centrifuging time: 2 min at 4000 rpm. All experiments were done in triplicate and the mean value of results is reported.

Method performance

The performance of EADLLME was investigated in optimal conditions. The limit of detection (LOD), linear dynamic range (LDR), correlation coefficient (r^2), Enrichment factors (EF), and extraction recovery (ER %) were evaluated which can be calculated using the following equations:

$$\text{LOD} = \frac{3\text{Sb}}{\text{m}} \quad (2)$$

Where S_b is the standard deviation of three replicates in the blank solution and m shows the slope of calibration curve.

Table 1 summarizes the performance of the EADLLME as an approach for extraction of celecoxib.

$$\text{EF} = \frac{Co}{Caq} \quad (3)$$

Where C_o denotes the concentration of the analyte in the organic phase (settled at the conical end of test tube) and C_{aq} represents the concentration of analyte in the aqueous phase.

$$\text{ER}=(\frac{Vo}{Vaq}) \times EF \times 100 \quad (4)$$

Where V_o and V_{aq} are the volume of the organic phase and aqueous phase, respectively [28].

The figures of merits of EADLLME method and other extractions of celecoxib are compared in Table 2.

Table 1. Figures of merit of the presented method

LDR (µg L^{-1})	Correlation Equation	R^2	PF[a]	LOD (µg L^{-1})	RSD %, n=3
0.5- 1000	$Y = 38.82C^{b} + 4221.1$	0.9912	206	0.1	2.15

[a] Preconcentration factor was calculated at 100 µg L^{-1}.
[b] Concentration in µg L^{-1}.

Table 2. Comparison of presented method with other extraction method applied for the extraction and quantification of Celecoxib

Methods	LOD (μg L^{-1})	LDR (μg L^{-1})	Recovery (%)	RSD	Ref.
EADLLME –HPLC/UV[a]	0.1	0.5-1000	94.4	2.15	The current method
LLE-HPLC/UV[b]	3.0	10- 2000	96.0	≤ 10	[6]
LLE-HPLC/UV	6.0	20-2000	85.0	≤ 10.7	[29]
LC-MS/MS[c]	-	5-4000	82.2	≤ 13.0	[30]
SPE-HPLC/UV[d]	7.5	25-2000	91.0	≤ 5.7	[31]
SPE-MIP-HPLC/UV[e]	8.0	50-100000	96.0	≤ 1.1	[5]

[a] Effervescence-Assisted Dispersive Liquid–Liquid Microextraction-high performance liquid chromatography with ultraviolet detection
[b] Liquid- liquid microextraction-high performance liquid chromatography with ultraviolet detection
[c] Liquid chromatography mass mass spectrometry
[d] Solid phase extraction- high performance liquid chromatography with ultraviolet detection
[d] Solid phase extraction- molecular imprinted polymer- high performance liquid chromatography with ultraviolet detection

Comparison of the extraction efficiency of presented method with ultrasonic/vortex/air- dispersive liquid-liquid microextraction methods

In this method, the use of CO_2 bubbles enhanced the dispersibility of organic phase into the sample solution. To confirm this claim, other DLLME techniques such as Air assisted-DLLME (ADDLME), Ultrasound assisted-DLLME (UADLLME), and vortex- assisted DLLME (VADLLME) were examined at optimum conditions. The obtained recoveries (Table 3) showed the high extraction recovery of the presented method with excellent repeatability.

Real sample analysis

The isolation and quantification of CEL in plasma sample was studied to investigate the applicability and accuracy of EADDLE method. The 3 mL of plasma sample were deproteinized by 5 mL of acetonitrile and diluted at the ratio of 1:3 by water and analyzed to assess the EADLLME method at optimal conditions (methanol as an auxiliary solvent; 2 mL, oxalic acid as an acidic promoter; 0.4g, sodium bicarbonate as basic promoter; 0.8g, pH of sample solution 6, chloroform as extraction solvent; 60 μL, and centrifugation time; 5 min). Table 4 lists the recoveries achieved for the plasma spiked at three different concentration levels (10, 50, and 100 μg L^{-1}). The obtained results demonstrated an admissible relationship between the obtained relative recoveries and spiked amount of CEL standards. Fig. 8 shows the data obtained from the proposed method of CEL existing in plasma samples.

Table 3. Comparison of the proposed method with other dispersive liquid-liquid microextraction methods applied for the extraction and quantification of CEL (50 μg L^{-1})

Methods	Recovery (%)	RSD
EADLLME-HPLC/UV[a]	94.4	2.15
UADLLME-HPLC/UV[b]	90.9	3.7
ADLLME-HPLC/UV[c]	96.2	3.1
VDLLME-HPLC/UV[d]	87.5	4.9

[a] Effervescence-Assisted Dispersive Liquid–Liquid Microextraction-high performance liquid chromatography with ultraviolet detection
[b] Ultrasound-assisted liquid-phase microextraction-high performance liquid chromatography with ultraviolet detection
[c] Air-assisted liquid-liquid microextraction- high performance liquid chromatography with ultraviolet detection
[d] Vortex-assisted liquid-liquid microextraction- high performance liquid chromatography with ultraviolet detection

Table 4. Analytical performance of presented method for plasma sample (n=3)

C_{added} (μg L^{-1})	$C_{founded}$ (μg L^{-1})	RR%[a]	RSD[b]	RSD[c]
10	9.8	98	1.87	2.13
50	50.3	100.6	2.27	1.84
100	101.6	101.6	2.05	2.46

[a] Relative recovery
[b] Relative standard deviation, interday (n=3)
[c] Relative standard deviation, intraday (n=3)

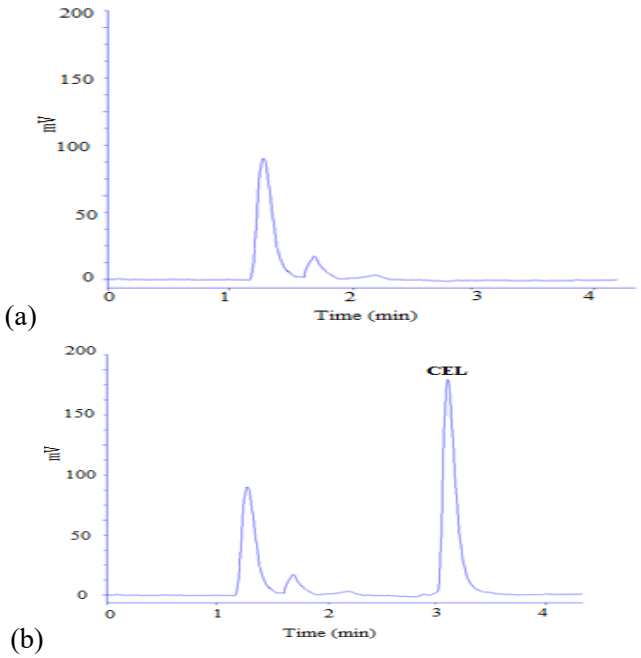


Figure 8. Chromatograms of blank plasma (a) and spiked plasma (b) at 100 μg L^{-1} of CEL

Conclusion

This study addressed the application of effervescence-assisted dispersive liquid–liquid microextraction (EADLLME) through acid-base reaction promotion for the extraction of CEL in the plasma sample and further determination through HPLC/UV. The carbon dioxide produced in the acid-base reaction distributes the extraction solvent throughout the aqueous phase with no complex apparatus. The method offered several advantages over other extraction methods. EADLLME is a simple, fast, accurate, and inexpensive approach with high extraction recovery.

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Fe_3O_4-DOPED POLYSULFONE MEMBRANE FOR ENHANCED ADSORPTION OF COPPER FROM AQUEOUS SOLUTION

(Membran Polisulfon Berdop Fe_3O_4 untuk Peningkatan Penjerapan Kuprum daripada Larutan Akues)

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Abstract

Water pollution, especially from industrial wastewater has become one of the major global environmental problems. As the result of rapid industrialization, the expansion of industries such as the electroplating industry has resulted in an increase in heavy metals effluent, especially copper, in the wastewater, and this poses detrimental effects on the biodiversity and environment. The abatement of copper pollution has received widespread attention, and continuous research advancement has been observed in adsorption and membrane technology. Nanofiltration membranes with nanopores recorded higher suitability to remove ions but at the expense of membrane fouling as a result of the formation of contaminants on the surface layer that blocks the diffusion of contaminants into the membrane substructure. This research highlights the incorporation of Fe_3O_4 nanoparticles into the polysulfone (PSf) membrane matrix as an adsorptive membrane and their possible adsorption mechanism towards Cu, which can manifest the combined characteristics of both removal techniques. Fe_3O_4 nanoparticles were synthesized using the co-precipitation method. Fe_3O_4-doped PSf membranes were then synthesized with various concentrations of Fe via the Non-solvent Induced Phase Separation (NIPS) technique. The physicochemical properties of the Fe nanoparticles and the membranes were evaluated using X-ray diffraction (XRD), Scanning Electron Microscope (SEM), water contact angle and porosity testing. Crystal phase analysis confirmed the formation of magnetite Fe_3O_4 in a cubic structure. Agglomerations of Fe NPs on the membrane surface were observed for membranes with lower Fe concentrations, suggesting the possibility of poor blending and this contributed to the lower adsorption capability of these membranes. Membranes with 2 wt.% Fe concentration (Fe-2.0) exhibited the highest Cu(II) ions adsorption capacity of 637 mg/g, which is trifold of those recorded for pristine PSF membrane (Fe-0.0). The adsorption data of Cu adsorption were best fitted into the Temkin isotherm and pseudo-second-order models, suggesting an adsorption mechanism involving an exothermic chemical interaction between Cu ions and the Fe_3O_4 NPs within the membrane. This research confirms the potential of incorporating Fe_3O_4 in the PSf membrane backbone to enhance Cu removal as an adsorptive membrane, even at lower NP concentrations.

Keywords: adsorptive membrane, copper removal, iron oxide, adsorption kinetics

Abstrak

Pencemaran air, terutamanya daripada air sisa industri telah menjadi salah satu masalah alam sekitar yang utama di seluruh dunia. Berikutan pengindustrian yang pesat, peluasan industri seperti industri penyaduran elektrik telah mengakibatkan peningkatan dalam efluen logam berat, terutamanya kuprum, dalam air buangan, dan ini menimbulkan kesan buruk kepada biodiversiti dan alam sekitar. Pengurangan pencemaran kuprum telah mendapat perhatian yang meluas, dan kemajuan penyelidikan berterusan telah diperhatikan dalam teknologi penjerapan dan membran. Membran penapisan nano dengan liang bersaiz nano mencatatkan kesesuaian yang lebih tinggi untuk menyingkirkan ion tetapi dengan mengorbankan penyisikan membran akibat pembentukan lapisan permukaan bahan cemar yang menghalang resapan bahan cemar ke dalam substruktur membran. Penyelidikan ini menekankan penggabungan nanopartikel Fe_3O_4 ke dalam matriks membran polisulfon (PSf) sebagai membran penjerap dan mekanisme penjerapan yang mungkin terhadap Cu, yang boleh menunjukkan ciri gabungan kedua-dua teknik penyingkiran tersebut. Nanozarah Fe_3O_4 disintesis menggunakan kaedah pemendakan bersama. Membran PSf terdop Fe kemudiannya disintesis dengan pelbagai kepekatan Fe melalui teknik pemisahan fasa teraruh bukan pelarut (NIPS). Sifat fizikokimia nanopartikel Fe dan membran dinilai menggunakan pembelauan sinar-X (XRD), mikroskop elektron pengimbasan (SEM), sudut sentuhan air dan ujian keliangan. Analisis fasa kristal mengesahkan pembentukan magnetit Fe_3O_4 dalam struktur kubus. Aglomerasi NP Fe pada permukaan membran diperhatikan untuk membran dengan kepekatan Fe yang lebih rendah, menunjukkan kemungkinan pengadunan yang lemah dan ini menyumbang kepada keupayaan penjerapan yang lebih rendah bagi membran ini. Membran dengan kepekatan Fe 2% berat (Fe-2.0) mempamerkan kapasiti penjerapan ion Cu(II) tertinggi sebanyak 637 mg/g, iaitu tiga kali ganda daripada yang direkodkan untuk membran PSF murni (Fe-0.0). Data penjerapan penjerapan Cu paling baik dipadankan ke dalam model isoterma Temkin dan pseudo-second-order, mencadangkan mekanisme penjerapan yang melibatkan interaksi kimia eksotermik antara ion Cu dan NP Fe_3O_4 dalam membran. Penyelidikan ini mengesahkan potensi menggabungkan Fe_3O_4 dalam tulang belakang membran PSf untuk meningkatkan penyingkiran Cu sebagai membran penjerap, walaupun pada kepekatan NP yang lebih rendah.

Kata kunci: membran penjerap, penyingkiran kuprum, ferum oksida, isoterma penjerapan, kinetik penjerapan

Introduction

The rapid growth of urban industrialization, including the chemical industries, uses enormous amounts of water and produces large amounts of waste effluent during the production processes. Copper (Cu), chromium (Cr), zinc (Zn), and cadmium (Cd) are the most common heavy metals present in these industrial effluents, making the release of heavy metals from such operations far more dangerous [1]. In fact, the electroplating industry alone produces a high amount of copper, in the range of 8.8 g/L [2]. These heavy metals are stable, non-biodegradable, toxic, and carcinogenic. They accumulate in living organisms and can cause severe problems for public health and the environment. The Environmental Protection Agency (EPA) has set the acceptable standard concentrations of Cu that can be discharged from industries to the environment, which falls within the range of 0.2 and 1.0 mg/L. Therefore, the remediation of water contaminated by Cu is critical to protect the environment as well as to promote water reuse.

Increased interest was observed towards membrane filtration, mainly ultrafiltration (UF) and nanofiltration (NF). UF is replacing the conventional method for water treatment and is becoming universally accepted due to the lower operating pressure and the simple membrane preparation, among others. However, small compounds and toxic ions cannot be removed using UF. Meanwhile, nanofiltration (NF) is used to remove heavy metals from water, but the performance of the NF membrane, is restricted by the species of the targeted heavy metal and the fact that it degrades as heavy metal concentrations increase up to 2000 mg/L [3]. In addition, NF membranes require higher mechanical strength, owing to the higher operating pressure and the functional surface layer that is critical to ensure the efficacy of the membrane separation. These often require complicated preparation of the functional surface layer such as interfacial polymerization and surface coating [4]. Polysulfone (PSf) membrane is often used in NF membrane formation due to its high chemical and mechanical resistance, but its hydrophobic nature often results in low flux and membrane fouling [5]. This has triggered the rapid development of efficient and cost-effective membranes for heavy metal removal, particularly Cu, which can overcome the limitations of NF and other common remediation techniques. The

invention of adsorptive membranes that combine the advantages of both adsorption and membrane filtration can also overcome the drawbacks of both techniques. The impregnation of prospective nano adsorbents into polymeric membranes can be achieved by characterizing them based on their affinity towards pollutants in the forms of ions and molecules [6].

Hydrophilic nanoparticles are often used to boost the hydrophilicity and flux of the PSf membrane, especially in removing Cu. The findings of Ayaz et al. [7] indicated that adding hydrophilic nanoparticles such as alumina into the PSf membrane matrix can increase the hydrophilicity of the membrane. This subsequently increases the water flux and Cu rejection from 40% for the nascent PSf membrane to 75% for PSf membrane with 1 wt.% alumina. Zeolite is also used in the PSf membrane to remove Cu. Hamid et al. [8] reported that the highest Cu adsorption capacity of 101 mg/g for PSf membrane doped with 15 wt.% of zeolite at an optimum pH of 5 as well as the anionic character of zeolite, attract the positive Cu ions for the adsorption process. Iron oxide nanoparticles have been amalgamated into membrane matrix due to their strong affinity towards Cu and hydrophilic nature, which can increase the hydrophilicity of PSf membranes in order to enhance the membrane performance towards not only Cu but other heavy metals. This includes lead [9], phosphate [10] and other contaminants such as protein. Said et al. [11] reported increased water flux and protein rejection with the addition of iron oxide into their PSf membrane, attributed to the higher porosity and hydrophilicity. Even though iron oxide has been reported as a popular nano adsorbent in many reports involving polymeric adsorptive membranes to remove heavy metals, the adsorption mechanism towards Cu (II) ions in highly concentrated copper aqueous solution has never been reported previously.

In this present work, the adsorption capability and mechanism of the Fe_3O_4-doped PSf membrane were evaluated. A set of adsorptive membranes was synthesized by incorporating various concentrations of Fe_3O_4 NPs into the PSf membrane matrix to remove Cu from the aqueous solution. The Fe_3O_4 NPs were prepared via the common co-precipitation method and the membranes were synthesized via the simple nonsolvent induced phase separation (NIPS) technique. The Fe_3O_4 NPs were characterized using XRD and the membranes were evaluated using FTIR, SEM, contact angle measurement and porosity analysis. The adsorption performance of the membranes was evaluated in terms of contact time and initial Cu concentrations. Subsequently, the adsorption mechanism of these membranes was also evaluated.

Materials and methods

Chemicals

Metal precursors such as $FeCl_2.6H_2O$, $(NH_4)_2Fe(SO_4)_2.6H_2O$ and $CuSO_4$ powders were procured from R&M chemicals. Polysulfone (PSf) polymer pellets were procured from Solvay Solexis (France). Dimethylacetamide (DMAc, 99.5%), used as the solvent, was purchased from Sigma-Aldrich (Germany). Ultrapure deionized water was used as the coagulant. All solvents and precursors were used as received.

Instrumentation

X-ray diffraction (XRD) pattern of the synthesized Fe nanoparticles was recorded using a diffractometer (Bruker AXS Inc., 30 kV, 10 mA), and equipped with a Cu-Kα radiation source (λ = 1.5412 Å). The samples were scanned from 15° to 70° (2θ). Surface morphological analysis of Fe_3O_4-doped PSf membranes was carried out using Hitachi SU8220 Scanning Electron Microscopy (Oxford Instrument, Oxford Shire, UK). FTIR spectra were recorded on Perkin Elmer FTIR spectrophotometer (Massachusetts, USA) with a crystal diamond universal ATR sampling accessory at room temperature. All spectra were recorded in the wavenumber range of 4000 to 500 cm^{-1}. The membrane porosity was measured using the gravimetric method. The membrane was immersed in ethanol for 2 hours. The wet membrane was then slightly patted to remove excess liquid on the surface and weighed to get the weight of the wet membrane, w_m. The membrane was then dried in an oven for an additional 2 hours and the weight of the dried membrane, w_d was weighed. The membrane porosity can be calculated using Equation 1:

$$\varepsilon = \frac{(w_m - w_d)/\rho_e}{((w_m - w_d)/\rho_e) + (w_d/\rho_p)} \quad (1)$$

where w_m is the weight of the wet membrane (g), w_d is the weight of the dry membrane (g), ρ_e is the density of ethanol and ρ_p is the density of the polymer, PSf. The water contact angle of the membrane surface was evaluated using the sessile drop method. A water droplet of 2 µL was dropped on the membrane surface and images of the droplet were taken after 5 s. The images were analysed using the ImageJ software to get the water contact angle of the membrane [12,13].

Synthesis of Fe_3O_4 nanoparticles

The Fe_3O_4 nanoparticles were synthesized via the co-precipitation method. High-purity precursors including $FeCl_2.6H_2O$ (3.9 g) and $Fe(SO_4)_2.6H_2O$ (5.4 g) were used in appropriate stoichiometric molar ratios. The precursors were dissolved in 200 mL of deionized water under vigorous stirring until a suspension solution was formed. A concentrated NaOH solution was then added dropwise until the pH of the suspension achieved 7 (Neutral). The suspension was vigorously stirred for 2 h before repeatedly washing with deionized water to remove the excess NaOH. The formed suspension was then filtered and dried in the oven at 110 °C for 5 h. The dried nanoparticle powders were kept in a desiccator prior to characterization and testing [3,14].

Synthesis of Fe_3O_4-doped PSf membranes

The Fe_3O_4-doped PSf membranes were prepared following the formulation shown in Table 1, via the phase separation method, particularly the Nonsolvent Induced Phase Separation (NIPS) method (Figure 1). Dope solutions with various wt.% of nanoparticles were prepared. DMAc was first heated to 60 °C, with constant stirring and pre-weighed PVP at 1 wt.% was added to the solvent as a pore former. Next, various wt.% of nanoparticles prepared in the previous subsection were added slowly to the solution [15]. Dried PSf granules at 18 wt.% were then gradually added and dissolved into the solution for 6 h. After mixing, the dope solution was degassed prior to casting, to remove any bubbles formed during the stirring process. The dope solution was then cast onto a glass plate using a casting machine, with a membrane thickness of 250 µm. The casted solution was then immediately immersed into a coagulation bath, containing deionized water for the phase inversion process to occur. The membrane formed was then washed to remove any excess solvent and dried at ambient temperature. The membrane was then placed in fresh DI water prior to characterization and performance tests.

Table 1. Composition of the synthesized Fe_3O_4-doped PSf membranes

Membrane	PSf, (wt.%)	PVP, (wt.%)	DMAc, (wt.%)	Fe NPs, (wt.%)
Fe-0.0	18	1	81.0	0.0
Fe-0.5	18	1	80.5	0.5
Fe-1.0	18	1	80.0	1.0
Fe-2.0	18	1	79.0	2.0

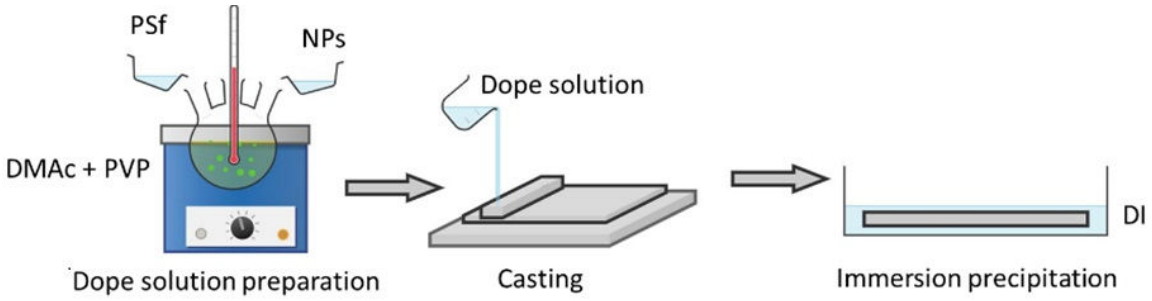


Figure 1. Fe_3O_4-doped PSf membrane preparation via NIPS

Batch adsorption studies

The batch adsorption study was performed to evaluate the adsorption characteristics of Fe_3O_4-doped PSf membranes towards copper. Effects of contact time and initial concentration on the adsorption capacity, q and copper rejection, R of the synthesized membranes were evaluated using Equation 2 and 3.

$$q_e = \frac{(C_o - C_e)V}{m} \quad (2)$$

$$R = \left[1 - \left(\frac{C_e}{C_o}\right)\right] \times 100\% \quad (3)$$

where q is the adsorption capacity (mg/g), Co and Ce are the initial and equilibrium concentration of metal ions in solution (mg/L), V is the volume of the solution (L) and m is the membrane dry mass (g).

The adsorption experiments were performed in batches and the effects of Cu initial concentration and contact time on Cu removal were evaluated. 12.5 mg of the membranes with various wt.% of Fe nanoparticles were cut into smaller pieces and placed into several centrifuge tubes containing 12.5 mL of various initial concentrations of Cu solution; from 0.05 to 1.2 M. The tubes were agitated at 160 rpm for 30 minutes. To study the effect of contact time, the membranes were placed into 0.05 M of Cu solution and were removed from the solution after 5, 10, 15, 20, 25 and 30 minutes, respectively. The final concentration of the Cu solution was measured using a UV-Vis spectrophotometer.

Adsorption Isotherm and Kinetic

The mechanism of Cu adsorption onto the Fe_3O_4-doped PSf membranes was further evaluated based on three isotherms models;1. Langmuir, 2. Freundlich and 3. Temkin. These isotherm models are best described based on the following linear equations as shown in Equations 4 to 6, respectively:

$$\frac{C_e}{q_e} = \frac{1}{q_{max}K_L} + \frac{C_e}{q_{max}} \quad (4)$$

$$lnq_e = \ln K_f + \frac{1}{n} lnC_e \quad (5)$$

$$q_e = \frac{RT}{b_T} lnK_T + \frac{RT}{b_T} lnC_e \quad (6)$$

where q_e is the equilibrium adsorption capacity (mg/g), q_{max} is the maximum adsorption capacity (mg/g), C_e is the equilibrium concentration of metal ions (mg/L) and K_L is the Langmuir adsorption constant (L/mg), n is the adsorption intensity constant, K_f is the Freundlich adsorption constant (L/g), R is the gas constant (8.314 J/mol), T is the absolute temperature (K), K_T is the Temkin equilibrium constant (L/g) and b_T (J/mol) is the constant associated with the adsorption heat. For the Langmuir model, an important parameter, known as the separation factor or equilibrium parameter, denoted as R_L, shown in Equation 7, is used to check the favourability of Cu adsorption onto the Fe_3O_4-doped PSf membranes.

$$R_L = \frac{1}{1+K_L C_0} \quad (7)$$

where K_L and C_o are the Langmuir constant and the highest initial concentration of Cu, respectively.

The kinetics of the adsorption were also evaluated based on these two common kinetic models; the pseudo-first-order and pseudo-second-order models. The pseudo-first-order and pseudo-second-order models are described in linear forms as Equation 8 and 9, respectively:

$$log\,(q_e - q_t) = logq_e - K_1 t \quad (8)$$

$$\frac{t}{q_t} = \frac{1}{K_2 \cdot q_e^2} + \frac{t}{q_e} \quad (9)$$

where K_1 (1/min) is defined as the pseudo-first-order adsorption rate constant, q_e (mg/g) and q_t (mg/g) are the adsorption capacities for Cu adsorption at equilibrium and at respective contact time and K_2 (g/mg min) is the pseudo-second-order rate constant.

Results and Discussion

Characterizations

The XRD pattern of the synthesized nanoparticles, shown in Figure 2 shows well-defined Bragg reflection characteristics of Fe_3O_4. The data shows diffraction peaks at 2θ = 30.2306°, 35.59°, 43.4167°, 57.3511° and 56.998° which can be indexed to the (220), (311), (400), (422) and (511) planes of Fe_3O_4 in a cubic phase, respectively which is consistent with the standard pattern for magnetite Fe_3O_4 (JCPDS no.- 96–900-7645) [16]. Based on the XRD pattern, the highest peak is approximately 35.59°, indicating that the (311) plane is the most ordered plane in the crystal lattice. The crystallite size was around 15 nm to 42 nm which agrees

with the findings by Upadhyay et al. [17], which reported a crystallite size ranging from 27 nm to 53 nm for Fe_3O_4 nanoparticles.

Figure 3a displays the FTIR spectra of the synthesized membranes. All membranes showed similar peaks at 1585 cm^{-1}, 1488 cm^{-1}, 1242 cm^{-1} and 1150 cm^{-1} [18]. These peaks confirm the presence of PSf as the backbone of these membranes although the reduced intensity of the absorption peaks for Fe_3O_4-doped PSf membranes suggested that the weight percentage of PSf was affected by the addition of Fe into the membrane matrix [19]. The presence of the carbonyl and sulfone group within the PSf polymer was confirmed by the characteristic peaks observed at approximately 558 cm^{-1}, 1242 cm^{-1}, 1150 cm^{-1} and 1585 cm^{-1} which correspond to the asymmetric stretching, symmetric stretching, and bending vibrations of the sulfone group, including the C-O-C, O=S=O and C=C aromatic, respectively [20, 21]. Peaks for the C-S stretching vibrations were also observed at 635 cm^{-1} for all membrane [22]. After the addition of Fe_3O_4 NPs, the intensity of the C-S peak increases (Figure 3b), due to the presence of Fe-O bond at around 635 cm^{-1}, confirming the succesful incorporation of the Fe_3O_4 nanoparticles on the PSf membrane [23].

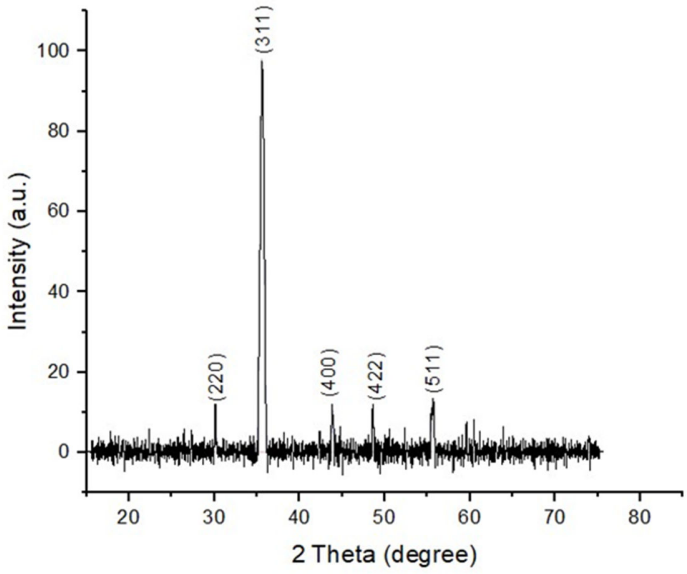


Figure 2. XRD pattern of the synthesized Fe nanoparticles

The surface morphology of Fe_3O_4-doped PSf membranes was appraised under SEM (Figure 4). Figure 4a indicates some pores on the surface of the Fe-0.0 membrane, with other visible pores, hidden under a very thin skin layer and uniformly distributed across the surface. As the concentration of Fe nanoparticles increased from 0.5 wt.% to 1.0 wt.% (Figure 4b and c), the dense skin layer thickens, evidenced by the less visible pores on the surface but at the expense of increasing Fe agglomeration on the surface layer. The blending of 2 wt.% of Fe into the PSf membrane (Figure 4d) also induced some nonsolvent penetration through the thick dense layer, forming pores on the surface and with less Fe agglomeration. These top surface morphologies also revealed that the blending of Fe nanoparticles in the membrane matrix was improved with increasing concentration of Fe. The top surfaces of the Fe-0.5 and Fe-1.0 membranes (Figure 4b and c) were covered by white particle agglomeration and this agglomeration was significantly reduced when the Fe concentration was increased to 2 wt.%. This observation can be explained by the miscibility of PSf with PVP and Fe nanoparticles. Since PVP is hydrophilic in nature and is not miscible with PSf, thermodynamically, it facilitated the penetration into the membrane during the solvent/ nonsolvent exchange. However, kinetically,

this can also increase the viscosity of the solution. Without the addition of the Fe nanoparticles (Fe-0.0), the thermodynamic effects are more dominant, resulting in a rapid demixing process, whereby the polymer precipitates very quickly, forming a very thin skin layer and presumably with a finger-like substructure [24, 25]. The addition of Fe NPs reduces the shear force of the dope solution due to the restricted shear flow of polymer chains by the NPs thus increasing the viscosity of the dope solution. This then potentially delayed the demixing process by slowing down the exchange between the solvent and nonsolvent in the sublayer, thus delaying the solvent polymer demixing, forming a denser skin layer before the demixing occurs [10, 26].

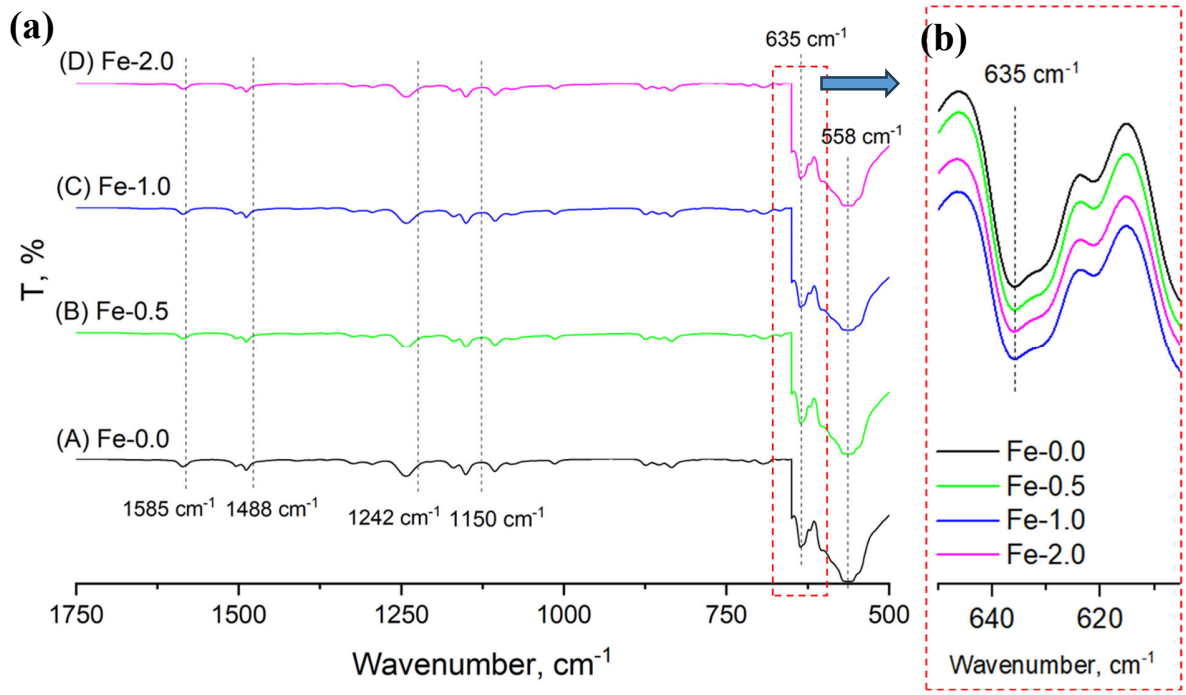


Figure 3. (a) FT-IR spectra of (A) Fe-0.0 (B) Fe-0.5, (C) Fe-1.0 and (D) Fe-2.0, and (b) FT-IR spectra of Fe-0.0, Fe-0.5, Fe-1.0 and Fe-2.0 at the Fe-O stretching band (635 cm^{-1})

The surface hydrophobicity or the wetting capability of the membrane is considered one of the important requirements for water filtration, specifically to improve the pure water flux and prevent membrane fouling. In addition, the surface hydrophilicity of a membrane also depends on the porosity, surface roughness, and chemical composition of the surface. Lower water contact angle measurements are a sign of higher hydrophilicity, which is preferable for wastewater treatment purposes because it can draw in water molecules and prevent foulants from adhering to or interacting with the surface of the membrane, improving its antifouling abilities and water flux [27]. Figure 5 shows the water contact angle (WCA), measured using the sessile drop method and the porosity of the membranes. As shown in Figure 5, the pristine PSf membrane exhibited a WCA of 77.40˚, showing a rather hydrophobic trait against water, while other membranes revealed a decreasing trend of WCA values than that of the pristine PSf membrane (Fe-0.0) with increasing Fe concentration. Fe-0.5, Fe-1.0 and Fe-2.0 recorded WCA values of 71.96˚, 53.99˚ and 50.93˚, respectively. The hydrophilicity of the membranes improved significantly due to the hydrophilicity nature of the Fe NPs, owing to the higher surface energy and the presence of the oxygen element in the Fe NPs [28], in the form of Fe_3O_4, as corroborated by the XRD result, albeit at lower concentration of Fe NPs into the membrane matrix. This subsequently promotes chemisorption and makes it attractive as an adsorptive membrane [29].

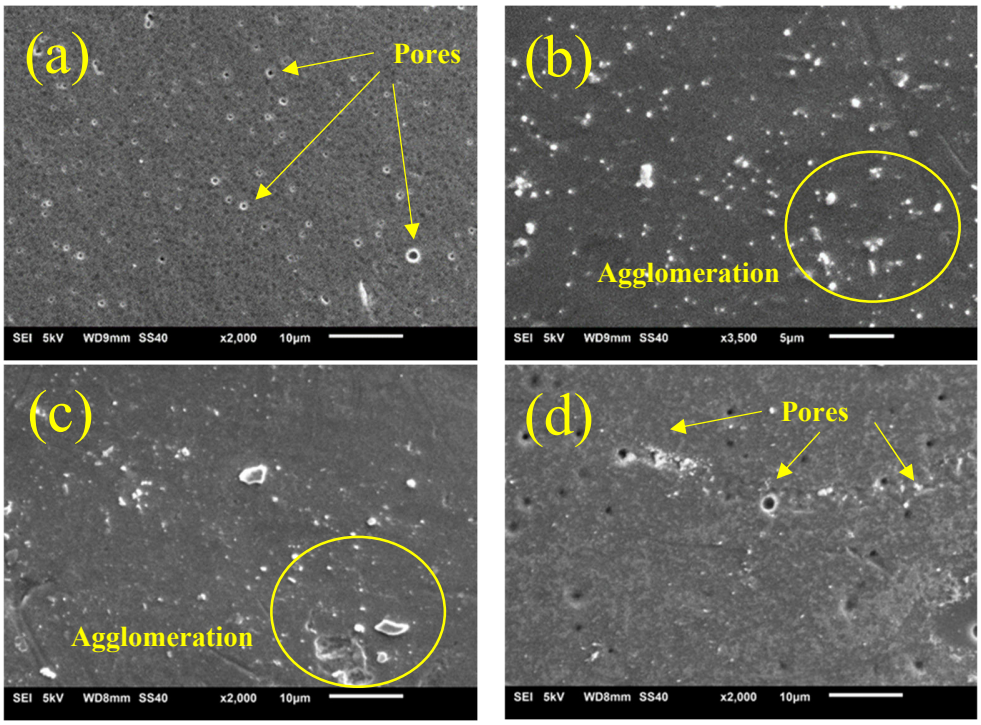


Figure 4. SEM images of the surface region of Fe_3O_4-doped PSf membranes at 2000x magnification: (a) Fe-0, (b) Fe-0.5, (c) Fe-1.0, and (d) Fe-2.0

In terms of the porosity of the membrane, a substantial decline was observed after the addition of Fe NPs, with Fe-0.5 membrane recorded the lowest porosity at 14%. This was more than half than the porosity recorded for the pristine PSf membrane (Fe-0.0) which was at 38%. Granting that the porosity of the Fe_3O_4-doped PSf membranes was lower compared to the pristine PSf membrane, the porosity immediately picks up the trend and shows a positive increase of porosity with increasing Fe concentrations. The formation of the denser skin layer at the top of the Fe-0.5, Fe-1.0 and Fe-2.0 membranes, resulting from the polymer-rich phase formation at the nonsolvent-film interface hindered the diffusion of solvent/nonsolvent of the membrane film after immersion into water. The closer porous substructure formed due to the delayed demixing subsequently increases the thickness of the top dense layer, reducing the porosity of the membrane [13]. The lower porosity of Fe-0.5 and Fe-1.0 membranes can also be attributed to the ineffective blending of Fe NPs in the membrane matrix causing the NPs to agglomerate. The formation of larger Fe agglomerates can reduce the free space of the membrane, thus reducing the porosity [26].

Batch adsorption study fo Cu

The synthesized Fe_3O_4-doped PSf membrane was evaluated as the adsorptive membrane for Cu removal. The adsorption capacity (mg/g) and the Cu removal percentage (%) of the membranes were established under two main adsorption parameters, such as the initial Cu concentrations and the contact time. The adsorption process was evaluated with a membrane mass of 12.5 mg, and 12.5 mL of Cu solutions at 27 ˚C and pH 7 except otherwise stated.

Effect of Fe_3O_4 doping

Figure 6 illustrates the effect of blending Fe_3O_4 into the PSf membrane matrix on the adsorption capacity. It is evident that the adsorption capacity was improved from below 267 mg/g to almost double (514 mg/g) when only 0.5 wt.% of Fe_3O_4 was blended into the membrane. The Cu removal increases almost trifold after 30 minutes of contact with the PSf membrane with 2 wt.% of Fe (Fe-

2.0) as compared to the pristine membrane (Fe-0.0). The data revealed a clear increasing trend of adsorption capacity with increasing Fe_3O_4 concentration with the Fe-2.0 membrane recording the highest Cu adsorption capacity of 670 mg/g. The root cause of this enhancement can probably be traced back to the higher Fe_3O_4 loading with less agglomeration, hence providing more available adsorption sites for Cu ions adsorption. Agglomerations observed for Fe-0.5 and Fe-1.0 might obstruct the potential adsorption sites for Cu ions, lowering the adsorption capacity of the membranes [3].

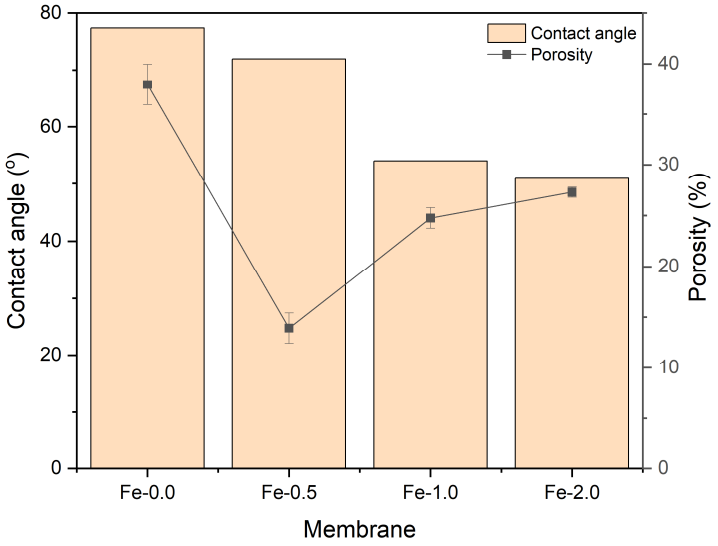


Figure 5. Water contact angle (˚) and porosity of the Fe_3O_4-doped PSf membranes

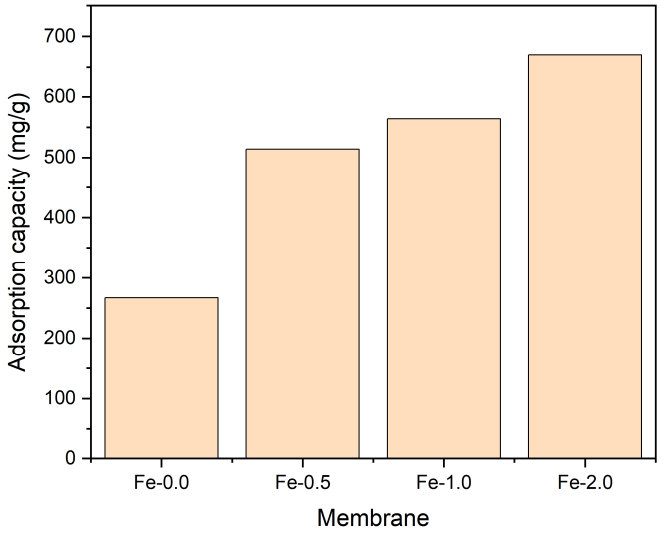


Figure 6 Effect of Fe_3O_4 doping into PSf membrane in terms of the adsorption capacity

Table 2 presents the comparison between the current work, Fe_3O_4-doped PSf membrane with previously reported adsorptive membrane for the adsorption of Cu ion from aqueous solution in terms of the adsorption capacity. The results revealed that the proposed Fe_3O_4-doped PSf membrane is a highly efficient adsorptive

membrane with the highest adsorption capacity for the treatment of Cu ions from aqueous solution. The Fe_3O_4-doped PSf membrane exhibited the highest adsorption capacity of Cu ions with a comparable NP concentration and contact time to other reported adsorbents.

Table 2. Comparison of the synthesized adsorptive membrane with other published adsorptive membranes for adsorption of Cu ions

Membrane	Adsorbent	Concentration of Adsorbent (wt.%)	Adsorption Capacity	References
PSf	$NiFe_2O_4$	3	42 mg/g	[30]
PES	PEG-coated $CoFe_2O_3$	2-6	-	[31]
PES	PANI/ Fe_3O_4	0.01-1	1.6 mg/g	[32]
PSf	Fe_3O_4	0.5-2	670 mg/g	This work

Effect of Cu initial concentration

Figure 7 depicts the removal of Cu(II) by Fe_3O_4-doped PSf membranes at different Cu initial concentrations (0.05, 0.06, 0.07 and 0.08 M). A steady increase in Cu adsorption can be seen for all membranes with respect to increasing Cu initial concentration ranging from 0.05 to 0.08 M. This occurred as a consequence of the increasing concentration gradient, which increased the driving force for the molecules to slit into the membrane pores and adhere to the surface of the NP [15]. Additionally, a higher number of Cu ions to be adsorbed onto the NPs can also facilitate the adsorption. At a lower Cu concentration of 0.05 M, the increasing Cu adsorption capacity was in the order of Fe-1.0 <Fe.0.5<Fe-2, while at higher Cu concentrations of 0.08 M, Fe-0.5 membrane recorded the lowest adsorption capacity of around 4613 mg/g. For membranes with lower adsorption capacity such as Fe-0.5 and Fe-1.0 membranes, the ineffective blending of the Fe NPs which results in particle agglomeration, observed in the SEM images might cause the uneven distribution of NPs in the membrane matrix subsequently reducing the number of readily available adsorption sites. These large agglomerates can also restrict the access of Cu(II) ions to the active sites on the surface of the membrane and in the membrane matrix.

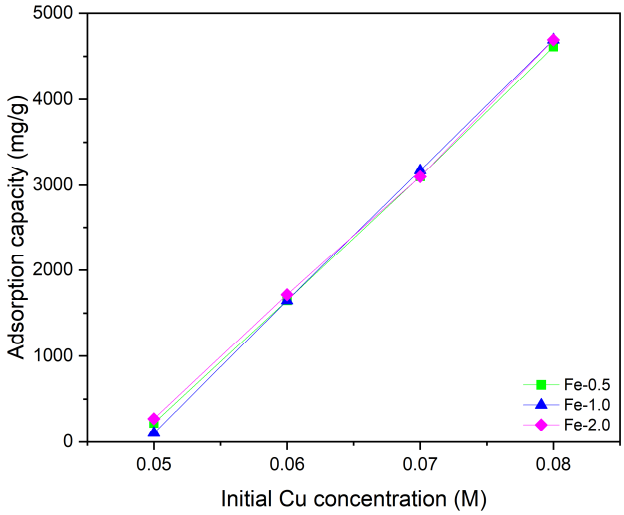


Figure 7. Effect of initial Cu concentration (M) on Cu adsorption capacity and rejection of the Fe_3O_4-doped PSf membranes (pH =7.0±0.1, membrane dosage = 1.0 g/L, contact time = 120 min, volume of feed solution = 12.5 mL)

Effect of contact time

The effects of contact time on the adsorption capability of the synthesized Fe_3O_4-doped PSf membranes are shown in Figure 8. In general, within 30 minutes, two distinct main stages were observed in the adsorption process for all three membranes. Following the common trend of any adsorption process, the adsorption was rapid in the first 5 minutes of contact time. The adsorption capacity of the membranes increases with increased concentration of Fe in the PSf membrane with 394 mg/g, 586 mg/g and 637 mg/g for Fe-0.5, Fe-1.0 and Fe-2.0, respectively. This rapid trend was due to the bulk diffusion of Cu ions into the membrane pores followed by the sorption of the ions onto the abundant vacant Fe nanoparticles in the pores. The higher number of adsorption sites at the beginning of the adsorption process was believed to be the driving force of this rapid Cu removal. No further increment of Cu removal was observed for the Fe-1.0 membrane as the vacant adsorption sites decreased and became saturated over time [33]. A slight difference can be observed in the adsorption process trends for Fe-0.5 and Fe-2.0 which can be attributed to the difference in morphologies of the membranes. Fe particle aggregates on the Fe-0.5 surface can also promote the adsorption of Cu on the surface particles which then blocks the penetration of more Cu into the membrane pores, slowing down the adsorption process altogether [9]. The adsorption activity of the Fe-2.0 membrane was further increased after 20 minutes, indicating that there are more available sites for Cu to adsorb hence improving the Cu removal even more.

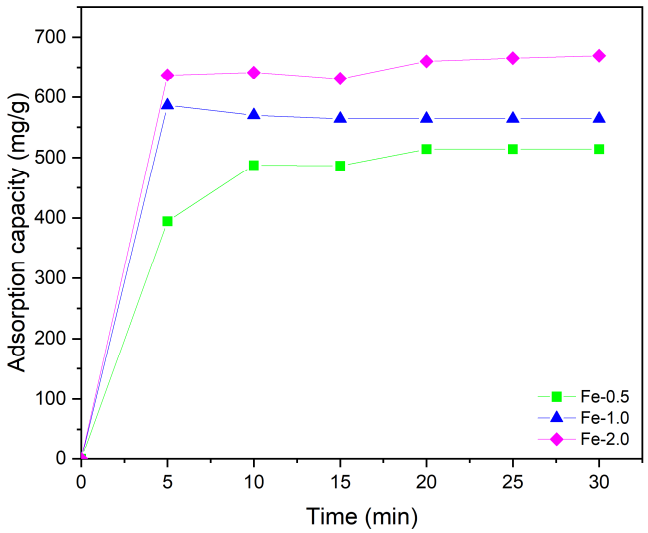


Figure 8. Effect of contact time (min) on Cu adsorption capacity and rejection of the Fe_3O_4-doped PSf membranes (pH =7.0±0.1, membrane dosage = 1.0 g/L, contact time = 60 min, volume of feed solution = 12.5 mL)

Evaluation of adsorption isotherm models and kinetics

In order to gain an understanding of the sorption mechanism of Cu ions onto these Fe_3O_4-doped PSf membranes, the experimental data were fitted into three main adsorption isotherms, specifically the Langmuir, Freundlich and Temkin models. The Langmuir isotherm model assumes that the adsorbate molecules are adsorbed onto a surface in a single layer, with each adsorption site having the same affinity for the adsorbate. The model also assumes that there is no transmigration of adsorbate molecules within the plane of the surface [34]. The Freudlich isotherm model describes the adsorption of a substance onto a heterogeneous surface with active sites having different binding energies. The adsorption favours the chemical adsorption process if the value of n is <1. However, if the value of n is >1, the adsorption then adopts the physical adsorption process [35]. The Temkin isotherm model focuses on the effect of adsorbate-adsorbent

interactions and the heat of adsorption on the adsorption process. It assumes a linear decrease in adsorption energy with increasing surface coverage, indicating that the adsorbate-adsorbent interactions weaken as the adsorption progresses [36].

Table 3 tabulates the isotherm parameters for all three models of all three membranes. In general, the equilibrium data of all membranes demonstrated stronger correlations with the Temkin isotherm model (Figure 9), evidenced by the higher correlation coefficients, R^2 of around 0.90-0.97, compared to those of the Langmuir and Freundlich isotherm models. The values obtained for the maximum adsorption capacity, q_{max} of Fe_3O_4-doped PSf membranes reveal that the adsorption of Cu onto these membranes does not follow the Langmuir isotherm. The corresponding R^2 for the Freundlich isotherm was higher than those of Langmuir showing a good linearity to the model. Still, the values of n did not fall within the range for favourable adsorption of between 1 to 10 [37]. Low values of n were observed for all membranes in the range of 0 to 1 indicating lower affinity between Cu ions and Fe NPs on the membrane [38], although this also suggests the adsorption involves chemisorption. The strength of the adsorption can also be described by the values of 1/n. All membranes demonstrated 1/n values above 1 indicative of cooperative adsorption whereby the adsorbed Cu ions can influence the adsorption of free Cu ions [39]. These results show that the linearized Langmuir and Freundlich models alone cannot properly describe the adsorption of Cu ions onto these membranes. As for Temkin, the variation of the adsorption energy, b_T was positive for all membranes, suggesting an exothermic sorption process between the Cu ions and the Fe_3O_4 NPs. This isotherm also validates the influence of Cu ions-Fe_3O_4 NPs interaction on the linear decrease of adsorption energy with increasing surface coverage [40].

It can also be inferred that these isotherms on their own might be underestimating the adsorption capability of these Fe_3O_4-doped PSf membranes, which contradicts the experimental data that suggests a higher performance of these membranes in removing Cu. However, the underestimation might also be due to the poor blending of the NPs resulting in huge agglomerates on the surface and in the membrane substructure. These agglomerates also occupied larger surface areas, reducing the available active sites for adsorption, hindering the proper adsorption of Cu ions onto the adsorption sites and consequently reducing the Cu removal and the misinterpretation of these isotherms [41].

Table 3. Isotherm parameters for Cu adsorption onto the Fe_3O_4-doped PSf membranes

Isotherm	**Parameter**	**Fe-0.5**	**Fe-1.0**	**Fe-2.0**
Langmuir	q_{max}	-12.3457	-8.3472	-16.1031
	b	-8119.07	-8039.9	-8077.29
	R^2	0.7814	0.8540	0.7686
Freundlich	K_f	2.53×10^{-19}	5.23×10^{-24}	3.43×10^{-18}
	1/n	5.4230	6.5660	5.1493
	n	0.1844	0.1523	0.1942
	R^2	0.8968	0.8484	0.8808
Temkin	K_T	0.00012	0.00015	0.00013
	b	0.0292	0.0218	0.0316
	R^2	0.9722	0.9441	0.9081

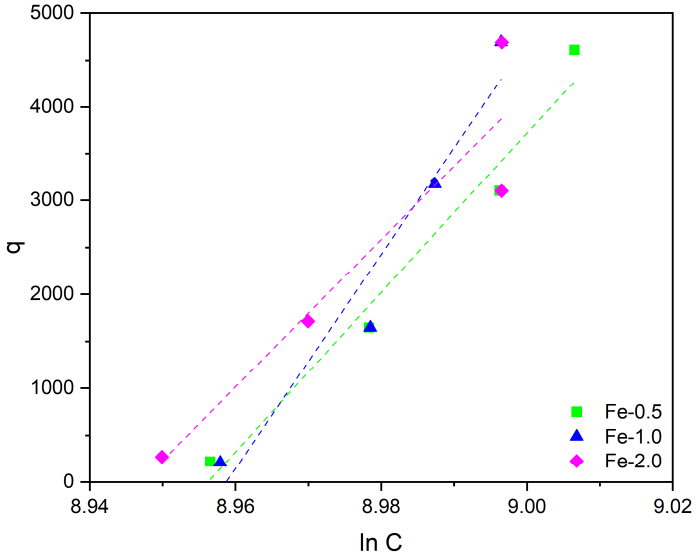


Figure 9. Temkin adsorption equilibrium of Cu onto Fe_3O_4-doped PSf membranes (pH =7.0±0.1, membrane dosage = 1.0 g/L, contact time = 60 min, volume of feed solution = 12.5 mL)

To investigate the controlling mechanism of the adsorption kinetics, two kinetic models were used; the Lagergren's pseudo-first-order and pseudo-second-order linear models. Table 4 revealed the kinetic parameters for both models and the linear regression of the Pseudo-second-order kinetics for the adsorption of Cu(II) onto all three membranes is shown in Figure 10, respectively. From the table, it can be seen that the correlation coefficient of the linear regression (R^2) for the pseudo-second-order model was higher for all membranes (0.98-1), suggesting that the adsorption kinetics of Cu ions onto these PSf membranes obeyed the Pseudo-Second order model. In this kinetic model, the adsorption of Cu ions onto the Fe_3O_4-doped PSf membranes adopts the chemisorption process and this chemical reaction is considered the rate-limiting step. It also depends on the concentration of the Cu ions and the NPs.

Table 4. Kinetic parameters for Cu adsorption onto the Fe_3O_4-doped PSf membranes

Membrane	Parameter	Fe-0.5	Fe-1.0	Fe-2.0
Pseudo 1st order	K_1	0.0320	0.0442	0.0574
	q_e	9.2452	6.6866	2.8115
	R^2	0.2980	0.7361	0.9912
Pseudo 2nd order	K_2	0.0013	0.0025	0.0274
	q_e	666.6670	666.6670	270.27
	R^2	0.9812	0.9991	1

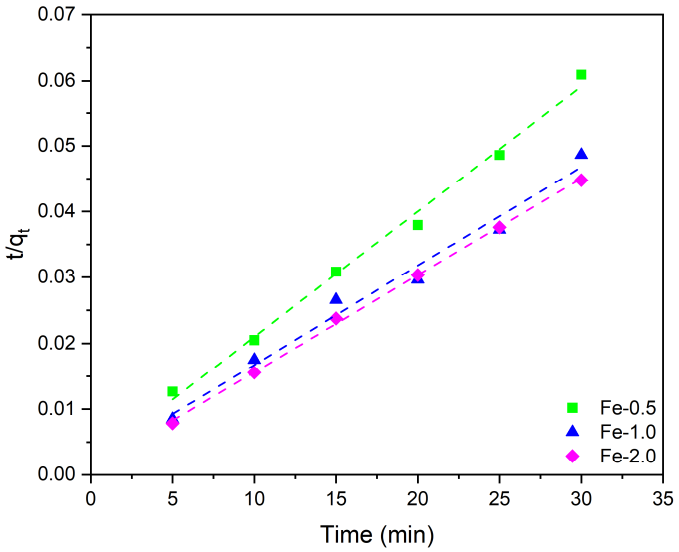


Figure 10. Linear regression of the Pseudo-second-order kinetics for the adsorption of Cu (II) onto Fe_3O_4-doped PSf membranes with various Fe_3O_4 NPs concentrations (pH =7.0±0.1, membrane dosage = 1.0 g/L, contact time = 60 min, volume of feed solution = 12.5 mL)

The adsorption of Cu ions onto Fe_3O_4-doped PSf membranes is believed to follow four general steps; (1) bulk diffusion of Cu ions into pores of PSf membrane; (2) sorption of Cu ions onto Fe_3O_4 NPs inside the pores; (3) chemical interaction between Cu ions and Fe_3O_4 NPs through electrostatic interaction; and (4) Adsorption equilibrium reached. Figure 11 illustrates the possible electrostatic interaction between the Cu ions and the NPs. The adsorption of Cu ions onto these Fe_3O_4 NPs that were incorporated into the PSf membrane was facilitated by the electrostatic interaction between the positive charges of Cu ions and negative charges on the Fe_3O_4 nanoparticle surface [30].

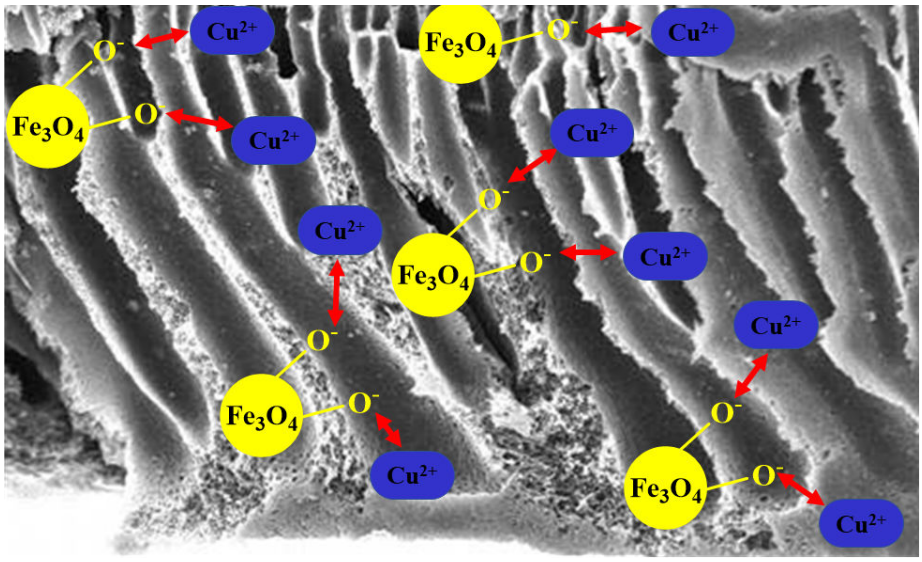


Figure 11. Schematic illustration of the possible mechanism for Cu adsorption on Fe_3O_4-doped PSf membrane

Conclusion

This work reported the synthesis of Fe_3O_4-doped PSf membranes for the removal of Cu(II) from wastewater, particularly from the electroplating industry. XRD analysis confirmed the successful synthesis of Fe_3O_4 NPs with crystallite sizes ranging from 15 to 42 nm. The Fe_3O_4-doped PSf membranes were successfully synthesized and the experimental results show a trifold increase in adsorption capability towards Cu, from 262 to 637 mg/g, proving that the Fe NPs help in improving the removal process of Cu. Membrane with 2 wt.% Fe_3O_4 showed the highest adsorption activity, credited to the easiness of Cu ions to travel to the adsorption sites, through the thicker dense layer with less agglomerations. The research also revealed that the adsorption process fitted well with the Temkin isotherm and the Pseudo-second order models with coefficient correlation, R^2 of around ~0.98, suggesting that the adsorption process adopts the chemisorption process and is exothermic in nature. This research demonstrated the potential of incorporating Fe_3O_4 NPs into the PSf membrane matrix to improve the adsorption of Cu ions and ultimately increase Cu removal as an adsorptive membrane in membrane filtration. Further research in this direction can contribute to the development of polymeric adsorptive membranes with embedded single and alloyed transition metals, including nickel and cobalt, for effective copper and any heavy metal removal.

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PRE-CONCENTRATION OF ORGANOCHLORINE PESTICIDES USING MICRO-SOLID PHASE EXTRACTION BASED ON BIO-SORBENT ALGINATE-SILICA BEADS DERIVED FROM OIL PALM FROND

(Pra-Kepekatan Racun Organoklorin Menggunakan Pengekstraksi Fasa Mikro-Pepejal Berasaskan Bio-sorben Alginat-Silika Komposit yang Diperoleh daripada Daun Pelepah Kelapa Sawit)

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Abstract

This study focuses on the extraction of organochlorine pesticides (OCPs) contained in environmental water samples by using encapsulated alginate-silica (Alg-SiO_2) composite beads. The composite beads were made by ionic crosslinking interaction between polymeric alginate and silica particles derived from oil palm fronds and were characterized by ATR-FTIR, FESEM, and EDX to study their chemical and physical properties. The Alg-SiO_2 composite beads were then used as the sorbent material for the micro-solid phase extraction (μ-SPE) to extract OCPs from aqueous samples. Optimization of method development for extraction of targeted analytes (heptachlor, aldrin, and dieldrin) was done by varying the extraction time, extraction solvent, desorption time, and sorbent mass before the gas chromatography-mass spectrometry analysis. Analytical figures of merit for μ-SPE were calculated under the optimized extraction conditions. Under the optimum conditions of 50 mg of sorbent extracted for 5 mins and desorbed using n-hexane for 7 mins, good linearity was achieved based on the data from method validation, linear ranges of 1–10 mg/L for heptachlor, 0.1–1.0 mg/L for aldrin, and 0.2–1.4 mg/L for dieldrin, with a limit of detection of 0.09–0.85 mg/L. The proposed method was successfully applied to determine organochlorine pesticides in paddy water samples. The wide range of the calibration curves was linear, and the coefficients of regression were all around 0.979-0.993. The recoveries were obtained in a satisfactory range of 82-98%, and the precision RSD value was in the range of 0.19-4.52%.

Keywords: alginate, silica sorbent, organochlorine, oil palm, micro solid phase extraction

Abstrak

Kajian ini memfokuskan kepada pengekstrakan racun perosak organoklorin (OCP) yang terkandung dalam sampel air persekitaran dengan menggunakan kapsulan manik komposit alginat-silika (Alg-SiO_2). Manik komposit dibuat melalui interaksi pertautan silang ion antara polimer alginat dan zarah silika yang diperoleh daripada pelepah kelapa sawit, dan dicirikan oleh ATR-FTIR, FESEM, dan EDX untuk mengkaji sifat kimia dan fizikalnya. Manik komposit Alg-SiO_2 kemudiannya digunakan sebagai bahan

penjerap untuk pengekstrakan fasa pepejal mikro (μ-SPE) untuk mengekstrak OCP daripada sampel akueus. Pengoptimuman pembangunan kaedah untuk pengekstrakan analit yang disasarkan (heptaklor, aldrin, dan dieldrin) dilakukan dengan mempelbagaikan masa pengekstrakan, pelarut pengekstrakan, masa nyahjerapan dan jisim penjerap sebelum analisis spektrometer jisim gas kromatografi. Angka analisis merit untuk μ-SPE dikira di bawah keadaan pengekstrakan yang dioptimumkan. Di bawah keadaan optimum 50 mg penjerap yang diekstrak selama 5 min dan nyahjerap menggunakan n-heksana selama 7 min telah menghasilkan julat linear yang baik. Berdasarkan data yang diperoleh daripada pengesahan kaedah, julat linear 1–10 mg/L untuk heptaklor, 0.1–1.0 mg/L untuk aldrin, dan 0.2–1.4 mg/L untuk dieldrin, dengan had pengesanan 0.09–0.85 mg/L. Kaedah yang dicadangkan telah berjaya digunakan untuk menentukan racun perosak organoklorin dalam sampel air padi. Julat luas keluk penentukuran adalah linear, dan pekali regresi adalah sekitar 0.979-0.993. Pemulihan diperolehi dalam julat yang memuaskan iaitu 82-98% dan nilai ketepatan RSD dalam julat 0.19-4.52%.

Kata kunci: alginat, silika sorben, organoklorin, kelapa sawit, pengekstraksi fasa mikro-pepejal

Introduction

Oil palm plants have contributed to Malaysia's gross domestic product by around 37.9% compared to other agriculture plants [1]. As stated in the statistic report, 77% of all residues come from oil palm production, which includes its frond, empty fruit bunch, trunks, kernel, and shells. As the number of oil palm plants increases, the waste undeniably increases. Dry oil palm fronds (OPF) only generated 44-47 million tons, which were burned as one of the disposal methods. This causes air pollution to the environment. In contrast, agricultural wastes are renewable resources that can be utilized to create energy, bio-based products, and items with additional value [2]. Consequently, numerous research and reviews exist on how these agricultural wastes can be utilized as useful resources rather than discarded [3, 4]. Oil palm fronds have numerous underutilized natural polymers, including cellulose, hemicellulose, and lignin. According to Roslan et al., OPF may contain siliceous material, attracting the attention of many researchers to the potential of silica extracted from this biomass [5]. Silica is an inorganic substance with excellent mechanical strength, high chemical stability, and thermal stability. Its internal connection is composed of siloxane, while the surface is abundant in silanol. This provides silica with excellent hydrophilicity. Furthermore, this silica is environmentally tolerant, which makes it widely used as an adsorbent carrier [6]. Thus, this study utilized silica extracted from oil palm fronds, which follows the government's announcement on "Zero Waste Initiatives" by reusing the oil palm fronds' wastes into silica powders. A study by Onoja et al. showed that ash from acid-treated OPF could produce about 95.30% silica (SiO_2), which is employed in many applications such as in diverse array applications, enzyme immobilization carriers, as well as functional biomaterial [7]. The hydroxyl groups on the surface of silica particles make it easy to chemically modify the surface and introduce functional groups [8] by controlling the pore volume, pore size, and tortuosity, and surface chemistry on the particle allows for tunable release profiles in controlled release applications involving silica particles [9]. However, the light and fine particles of obtained silica properties make it complicated to collect and reuse after extraction. Hence, encapsulating the silica with tensile polymeric alginate proves to be a favorable option for improving their recyclability without the loss of any of the fine particles [10].

Alginate (Alg) is a biomaterial with several uses in biomedical science and engineering due to its advantageous features, such as biocompatibility and ease of gelation [11], with the presence of hydrophilic and reactive functional groups providing superior adsorption [12]. This biopolymer is rich in D-mannuronic acid (M block) and L-guluronic acid (G block), which can be easily cross-linked utilizing bivalent ions such as calcium ions to create hydrogels during the gelation process [13]. Hence, it is frequently used to create a gel-phase adsorbent material that is easier to manipulate than the raw precursor material [14]. Nevertheless, this biomaterial has high rigidity, fragility, and weak elasticity, as well as weak mechanical properties [15]. Organic and inorganic alginate-based composites have been produced to improve the mechanical and thermal stability, as well as the swelling

properties of pure alginate polymers, which have been intensively studied for the removal of pollutants from aqueous solution over the past decade [15]. One of the materials studied was inorganic silica matrices that provided several benefits regarding chemical and mechanical stability [16]. These combined characteristics of silica and alginate composites have drawn interest since they may be superior compared to their individual compounds [17], which enhances their high biocompatibility and reasonable physical and chemical durability [18]. Thus, alginate-silica composites were proposed in the study as a new class of materials with promising applications in a variety of disciplines, including biomedicine, bio-catalysis, bio-separation, and bio-sensing [19]. The proposed synthesis mechanism of alginate-silica adsorbent is shown in Figure 1 [20].

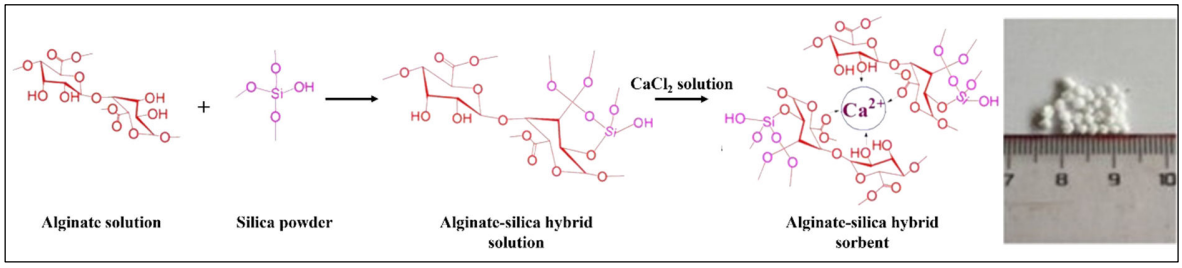


Figure 1. Synthesis mechanism of alginate-silica hybrid adsorbent

Amongst the aforementioned applications, very few studies of modification biowaste-silica for the pre-concentration of organochlorine pesticides have been investigated, as summarized in Table 1. Most of them applied different types of sorbents for OCPs or organophosphorus pesticides (OPPs). Organochlorine pesticides (OCPs) are one of the major pesticides that are used to manage insect pests on various types of crops. These OCPs are in the persistent organic pollutants (POPs) class, which are poisonous, bio-accumulative, and bio-magnified. The widespread adoption of these pesticides causes accumulation in the environment and food chains, and the incidence of OCP contamination in the human body can cause cancer, neurological system damage, generative diseases, and immune system disruption. The usage of OCPs is usually for pest and vector control in agricultural plantations such as oil palm, cocoa, and rubber in Malaysia [21]. However, the usage of OCPs has declined over the previous decade, as shown by various environmental monitoring studies [22, 23]. It is still possible to find OCPs and the products of their decomposition in the water, soil, sediment, and aquatic species in some regions of the world due to human activity. This is confirmed by several studies that have found the existence of OCPs in various samples in Malaysia. This includes the presence of OPCs (heptachlor and aldrin) in the surface water of Bertam and Terla River in Cameron Highland [24] and the detection of other types of OCPs such as endosulfan sulfate, 4-4'-DDT, and hexachlorocyclohexane isomers (α, β, γ, δ) in paddy soil situated in Machang, Kelantan [25] as well as detection of another group of OCPs, benzenehexachlorides (BHCs) in fish and sediment distributed in numerous countries including Malaysia [26].

These OCPs are present at the trace level, which needs sensitive, accurate, and efficient analytical procedures, which include sample preparation for their detection, identification, and quantification. Few sample preparations include liquid-liquid extraction (LLE) and solid phase extraction (SPE), which consume enormous volumes of toxic organic solvents and generate a substantial amount of waste that is unsafe for users and the environment [39]. Until today, SPE has mainly been used as sample preparation, especially on aqueous matrices, which mostly employ cylindrical cartridges packed with sorbent as SPE devices [40]. Countless researchers studied the miniaturization and solventless of SPE, such as dispersive SPE, or coined as μ-SPE. It is explained that this procedure implements loose

sorbent dispersed in the aqueous sample to allow the extraction of analytes and separated physically by magnetism, filtration, or centrifugation before the elution of analytes using solvent for the analysis [40]. This extraction method is favorable since it applies small amounts (microgram or milligram quantities) of sorbent, which allows the dispersion to occur and facilitates rapid and uniform interaction between sorbent and target analytes, resulting in faster equilibrium and enhancing the extraction efficiency [40]. Basheer et al. presented membrane-protected micro-solid-phase extraction (μ-SPE) as a replacement for classical multistep SPE, which allowed a reduction in the volumes of sorbent and desorption solvent as well as smaller sample volume and lower overall cost [41]. Sev eral studies implementing numerous types of μ-SPE techniques with results are summarized in Table 1. Therefore, the research aimed to investigate the use of the first-ever attempt of alginate-silica beads derived from oil palm frond as a promising bio-sorbent for the determination of OCPs in water samples with the aid of μ-SPE. Besides, this method works well for matrices that are moderately difficult to process by protecting the hydrophobic membrane and improving the sorbent as well as matrix influence to a greater extent.

Table 1. Summaries on analytical performance and extraction conditions of the developed procedure with other recently published works

Extraction Technique	**Sorbent Type**	**Sample Vol. (mL)**	**Desorption Solvent**	**Desorption Solvent Vol. (mL)**	**Total Extraction Time & Desorption Time (min)**	**LOQ (μg L^{-1})**	**RR (%)**	**Ref.**
d-μ-SPE	C18/Florisil/ Chromosorb G/AW-DCMS	100	EtAc	0.5	54	0.0017–0.0745	62.3-123	[27]
SBSE	SMNMa	60	ACN	1.50	57	0.23 – 2.94	82.6-104	[28]
d-μ-SPE	Zn-MOFb	20	ACN	0.20	~12	0.099 – 0.693	91.9-99.5	[29]
d-μ-SPE/ MSPE	Fe_3O_4@PDA-DES	100	ACN	1.50	~23	0.03 – 0.036	72.5-108	[30]
d-μ-SPE/ MSPE	Fe_3O_4@SiO_2 @KIT-6	100	ACN	2.00	~12	0.04 – 0.018	86.6-98.8	[31]
MIP-SPE	MIP	50	MeOH/HAc	1.50	-	0.23 – 0.41	79-104	[32]
d-μ-SPE	Fe_3O_4@TGA @TMU6	25	1-ButOH	0.10	35	1.65 – 3.30	88-107	[33]
SPE	PDMAT microbeads	100	DCM	10.0	-	0.006 – 1.99	69-139	[34]
SPE	PEG-CNT	70	ACN	0.80	-	0.033 – 0.099	95.2-99.8	[35]
d-μ-SPE/ MSPE	Fe_3O_4@APTES-GO /ZIF-8	30	MeOH	1.50	18	0.033 – 0.363	71.2-111	[36]
d-μ-SPE	MIL-101	10	EtAc	0.10	55	0.0083 – 0.053	87.6-98.6	[37]
d-μ-SPE/ MSPE	KHA/Fe_3O_4	30	MeOH	0.25	10	0.099 – 0.330	93.4-99.3	[38]
d-μ-SPE	Alginate – silica	10	n-hexane	1.00	22	0.27 – 2.55 (mg/L)	82 - 98	This work

Materials and Methods

Chemicals and materials

Three organochlorine pesticide standards: heptachlor ($C_5H_{10}Cl_7$, ≥ 98%), aldrin ($C_{12}H_8Cl_6$, ≥ 98%), dieldrin ($C_{12}H_{16}O$, ≥ 95%), Acetonitrile (C_2H_3N, 99.9%), and ethanol (C_2H_6, 99.9%, HPLC grade) were purchased from Sigma-Aldrich. Acetone (CH_3COCH_3, ≥ 99.5%) ACS reagent, ethyl acetate ($C_4H_8O_2$, 98%), n-hexane (C_6H_{12}, 99%), anhydrous calcium chloride ($CaCl_2$, ≥ 97%) and sodium alginate were all purchased from Sigma Aldrich (St. Louis, USA). Ultrapure water was collected from Barnstead Nanopure (Thermo Scientific).

Preparation of standard and sample solutions

A series of working solutions from stock solution, 1000 mg/L, were diluted in acetonitrile to prevent the decomposition of analytes and stored in amber glass at 4°C when not in use. Paddy water samples were collected from Bukit Gambir, Johor, and filtered through 0.45 µm nylon filter paper to remove any suspended particles. The samples were stored in a pre-cleaned bottle with acetone polyethylene at 4°C prior to the extraction. The sample was spiked with a standard mixture to give a final concentration of 5 mg/L of heptachlor, 0.5 mg/L of aldrin, and 1 mg/L of dieldrin common solution into a solution for a final volume of 10 mL for the precision and accuracy studies.

Preparation of silica derived from oil palm fronds

The method of synthesis was adapted from the literature [42]. Freshly harvested oil palm fronds (OPF) were then dried in the oven at 70 °C for 48 hours and ground into fine powder. The OPF powder was dispersed into 1 M HCl and constantly stirred for 2 hours at 400 rpm. The dispersion was allowed to cool at room temperature. The wet powder was washed with distilled water until the acid-treated OPF reached neutrality at pH 7. The neutralized OPF was filtered and dried in an oven at 80 °C for 24 hours. Then, the OPF powder was calcined in the furnace at 600 °C for 14 hours with a ramping time of 5 hours and a hold time of 9 hours at the rate of 2 °C/min. The white ash of silica was harvested and stored in capped vials at room temperature for further analysis.

Preparation of alginate-silica composite beads (Alg-Si)

Experimental methods for the preparation of alginate-silica were adapted and modified from the previous studies with minor modifications [43]. Alginate-silica (Alg-Si) composites were prepared by adding 2g of sodium alginate into 100 mL of distilled water and stirring using a magnetic stirrer at 60 °C to produce 2% (w/v) of sodium alginate solution until no longer coagulated. The heat was turned off, and the solution was left stirred. In the meantime, 10 mL of distilled water was added to 0.1g of silica powder and ultrasonicated with parafilm covered onto the beaker's mouth for approximately 10 mins. The silica solution was then added to the 2% (w/v) sodium alginate solution and stirred thoroughly. In the separated beaker, 4% (w/v) of $CaCl_2$ was prepared and diluted with distilled water. The Alg-Si mixture was then dropped wisely into the prepared $CaCl_2$ solution and left for 30 mins. The Alg-Si beads formed were filtered and rinsed with distilled water before being dried in an oven overnight at 80 °C to remove any excess cation solution. The Alg-Si was then stored and tested for the extraction procedure.

Characterization of alginate-silica composite beads

Several characterization methods were applied to study the physical and chemical properties of Alg-Si beads formedAttenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) with a resolution of 4 cm^{-1} and a total of 8 scans in the range of 500-4000 cm^{-1}. Scanning Electron Microscope (SEM) was used to determine the qualitative characteristics of the physical organization and morphology of pure alginate beads and alginate-silica beads as well as energy-dispersive X-ray spectroscopy (EDX) analyzer TEAM ™ EDS to measure the micro-area elements and the condition of element distribution in beads.

Micro-solid phase extraction (µ-SPE)

Several parameters were optimized by changing one variable at a time (OVAT), including extraction time, desorption solvent, desorption time, and mass of sorbent. OVAT was chosen because of the simplicity of the system and the limited number of parameters under consideration. With only four parameters to be examined, OVAT provides a practical and

straightforward approach, allowing for a focused exploration of individual factors while ensuring resource efficiency. This OVAT method was adapted from Othman et al. with minor modification, where 50 mg of the beads were added to 10 mL of spiked aqueous sample and agitated at 900 rpm for 15 mins to allow the desorption process to occur in 12-Radley carousel system. The supernatant was discarded, and 1 mL of desorption solvent (acetonitrile) was added to the beads before sonicated for 7 mins. Under a gentle stream of nitrogen gas, the collected solvent was pre-concentrated to 500 µL and transferred to the vial for GC-MS analysis [44]. The schematic flow diagram of µ-SPE is illustrated in Figure 3.

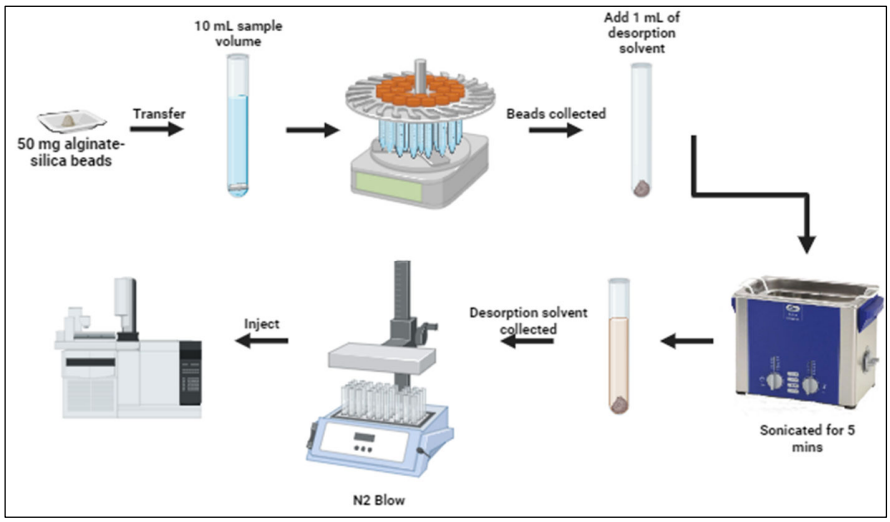


Figure 2. Micro-Solid Phase Extraction (µ-SPE)

Chromatographic conditions

The analysis was conducted using GC-MS, Shimadzu's (Kyoto, Japan) QP2010 GC-MS system, and a Shimadzu AOC-20i autosampler. The flow rate was set at 1.2 mL/min with pressure at 16 kPa at an average velocity of 41.633 cm/sec using helium as carrier gas. The injection was set without any intermediate splits. The separation was carried out on a silica capillary column (30 x 250 µm x 0.25 µm). The chromatographic conditions were optimized by varying the temperature. The temperature in the oven was increased from 150 °C (held for 1 min) to 200 °C at a rate of 30 °C per min (held for 2 mins), then to 250 °C at a rate of 10 °C per min, and finally to 280 °C at a rate of 20 °C per min. The data was collected in full scanning mode from 50 to 500 m/z to verify retention times. Selective ion monitoring (SIM) analysis was performed on all standards and sample extracts.

Method validation

Prior to sample analysis, the linearity (R^2), limit of detection (LOD), limit of quantification (LOQ), precision (RSD%), and accuracy (relative recovery%) of the method were evaluated.

Green analytical calculator (AGREE)

This analytical green calculator is a simple software applied in various research to evaluate the greenness of the analysis method. This calculator evaluated the greenness of the developed method when the AGREE metric values are near 1, correlated to a dark green. Meanwhile, a red color implies the least environmentally friendly, which is close to 0. This calculator has 12 scales, each of which corresponds to fundamental principles of green analytical chemistry, and the weight of each scale represents the importance of the principles of the analytical procedure [45]. The principle of the scale includes (1) the implementation of direct analytical techniques to avoid sample treatment, which can reduce the safety issues and health and environmental problems related to the methodology. However, direct analysis is usually inexecutable as the

appropriate form of the sample is required to increase its sensitivity; (2) the minimal usage of sample size and samples are recommended; (3) preferable *in situ* measurements whereby the location of analytical device should be close to the measurement location; (4) reduction usage of reagents and saves energy by the integration of analytical process and operations such as sampling with pre-concentration, simultaneous performance of derivatization with extraction or chromatographic determination; (5) automated and miniaturized methods; (6) no derivatization should be employed; (7) the proper management of analytical waste and a small volume of analytical waste should be implemented; (8) the multianalyte or multiparameter methods are preferred compared to methods using one analyte at a time; (9) the minimization of energy consumption in detection steps, sample preparation and analytical separation; (10) reagents obtained from renewable sources should be preferred; (11) toxic reagents should be eliminated or replaced by greener alternatives whenever possible; (12) the safety of the operator and environment whereby the several threats that are not avoidable should be selected [46].

Results and Discussion

Physical properties

Digital photographs were captured to examine the visual structure of the composite beads (Figure 4). Alg-Si beads, before drying, were obtained in asymmetrical white color and have a texture resembling hydrogel due to water content, as shown in (a). However, the size of Alg-Si shrunk to half after the drying process and turned to a yellowish color. In comparison, the pure alginate bead was prepared with a similar method and obtained as dark yellow beads.

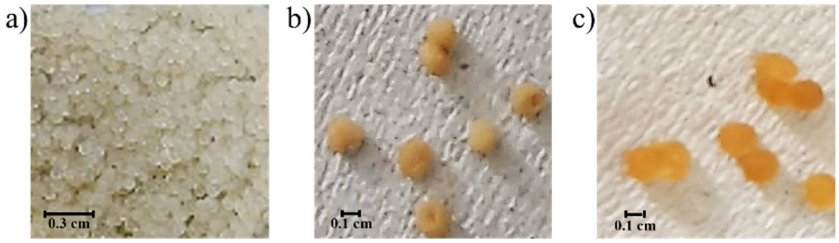


Figure 3. Digital photograph using phone camera with the scale of (a) Alg-Si beads before dried, (b) Alg-Si beads after dried overnight, (c) pure alginate beads after dried overnight

ATR-FTIR analysis

Figure 5 shows the spectra of OPF-silica powder, Alg-Si beads, and pure alginate beads. In the spectrum of OPF-silica powder, the existence of a broad band around 3407 cm^{-1} is attributed to the -OH stretching vibration of the silanol on the silica surface. The strong band at 805-1079 cm^{-1} corresponds to the asymmetric vibration of the Si-O-Si siloxane bond, which forms the silica matrix backbone [7]. The FTIR spectra of Alg-Si beads demonstrate that the absorption peaks at 3326 cm^{-1} correspond to the O-H absorption band, which is due to the stretching vibration of residual -OH groups and free hydrogen bonds [47]. The absorption peaks at 824 and 1029 cm^{-1} correspond to symmetric and asymmetrical stretching vibrations of Si-O-Si bonds, respectively. The bands at 1419 and 1598 cm^{-1} in the spectrum of the Alg-Si beads may be assigned to asymmetric and symmetric COO- deformation, respectively, confirming the interaction of carboxylate groups with silanol [48]. The FTIR spectra of pure alginate beads display characteristic bands at 3331 $cm^{-1,}$ which correspond to the OH stretching vibrations of hydrogen-bonded OH groups. Other distinctive peaks of pure alginate beads can be detected at 1424 cm^{-1} and 1598 cm^{-1}. They are caused by the asymmetric and symmetric stretching peaks of carboxylate (-COO), respectively. Table 2 summarizes the comparison of major absorption bands for OPF-silica powder, pure alginate beads, and alginate-silica beads with all the spectra in Figure 5.

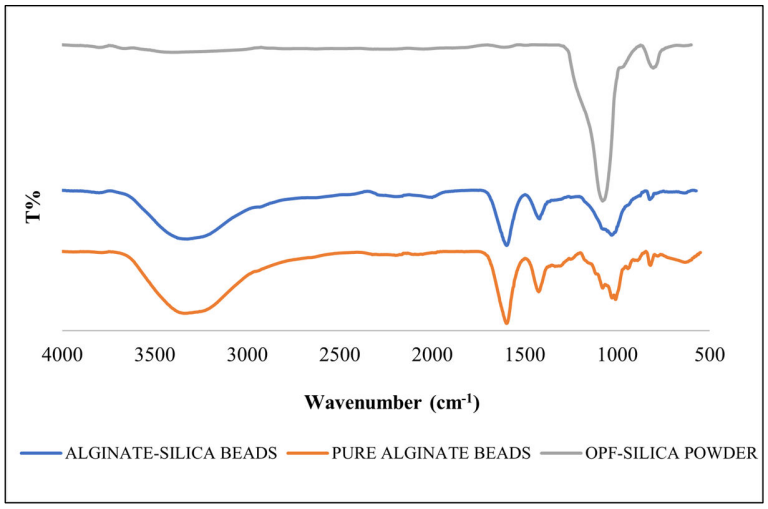


Figure 4. ATR-FTIR spectra of OPF-silica powder, Alg-Si beads, and pure alginate beads

Table 2. Comparison of major absorption bands for OPF-silica powder, Alg-Si beads, and pure alginate beads

Major Functional Groups	OPF-Si powder	Alg-Si beads	Pure alginate beads
	Wavenumber (cm^{-1})	Wavenumber (cm^{-1})	Wavenumber (cm^{-1})
Stretching of O-H	3407	3326	3331
Antisymmetric stretching of COO-groups	-	1598	1598
Symmetric stretching of COO-groups	-	1419	1424
Asymmetric stretching of Si-O-Si	1079	1029	-
Symmetric stretching of Si-O-Si	805	824	-

Field emission scanning electron microscopy

The morphology of Alg-Si beads and pure alginate beads was investigated by field emission scanning electron microscopy (FESEM). Under magnification 45X, 1000X, 3000X, and 5000X, micrographs of pure alginate and Alg-Si beads were viewed. Alginate and calcium chloride can form a rigid structure due to the sequential placement of G units on alginate, which generates a space for calcium ions to bind, with each ion able to link to four G units [49]. In accordance with Figure 4 (a-b), the surface of pure alginate beads was rough, irregular in shape, and possessed a deep and extensive fissure span. At the same time, the morphology study of Alg-Si beads in Figure 6 (c-d) found that the gully and folds on the surface of the hybrid material diminished after interaction with SiO2; the surface of the "wrinkles" ring shrunk and progressively became more regular, with no discernible fold ring appearing. Briefly, the surface structure of Alg-Si beads was more homogeneous and regular than that of pure alginate composite beads. In addition to having a rough surface, the Alg-Si beads have cavities created during the cross-linking process. The formation of cavities during sonication, in which alginate and silica combine to produce a homogenous web [50]. Moreover, these cavities facilitate pore diffusion during adsorption due to the significance of the inner specific surface with a low diffusion resistance in the composite beads (involving increased adsorption potential and rate). In conclusion, the composite particles of alginate and silica exhibited a porous network structure. A large number of hydroxyl and carboxyl groups in sodium alginate provided numerous chemical and physical cross-linking and reactive sites, which enhanced the deformation of resistance of cross-linked structures [51].

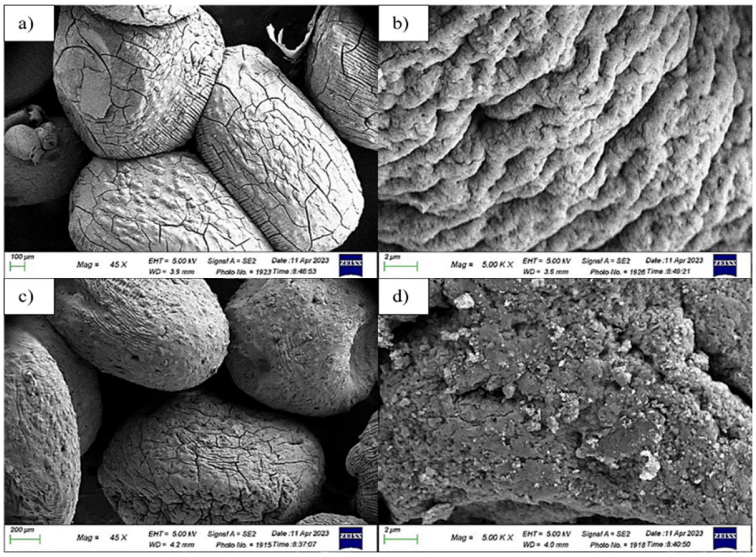


Figure 5. FESEM images for pure alginate beads at a) 45X, b) 1KX and Alg-Si beads at c) 45X, d) 5KX

Energy dispersive X-ray (EDX) analysis

In conjunction with energy dispersive X-ray (EDX), energy spectra of Alg-Si beads and pure alginate beads were measured to characterize the beads. It permits the quantification of the active molecules retained within the beads. Figures 7 a) and b) depict the EDX analysis of pure alginate and Alg-Si beads, respectively. EDX analysis revealed that pure alginate beads consisted primarily of oxygen (68.83%), calcium (28.55%), and a minor amount of sodium (2.62%) by weight. There were no additional impurity peaks in the EDX spectra, and this was consistent with the results obtained in the FTIR. Details of the chemical composition of pure alginate and Alg-Si beads were tabulated in Table 3.

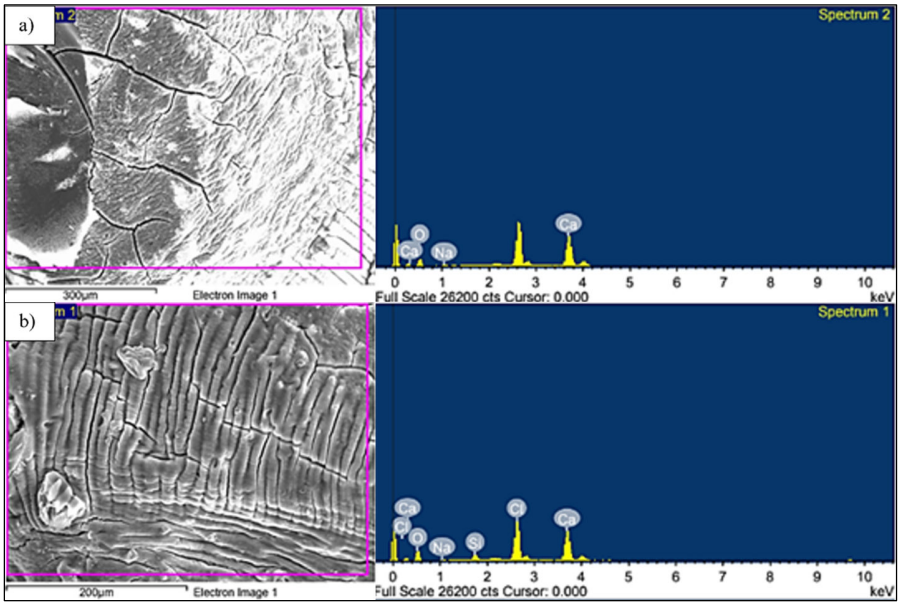


Figure 6. a) The EDX analysis of pure alginate beads and b) the EDX analysis of Alg-Si beads

Table 3. Chemical composition of pure alginate beads and alginate-silica beads based on EDX analysis

Element	Pure Alginate Beads		Alginate-Silica Beads	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
O	68.83	83.89	59.05	76.64
Na	2.62	2.23	0.71	0.64
Ca	28.55	13.89	18.16	9.41
Cl	-	-	19.55	11.45
Si	-	-	2.53	1.87

Optimization parameters of µ-SPE

Heptachlor, aldrin, and dieldrin were chosen as the target analytes for this analysis since they are the most frequently used OCPs in the agriculture industry due to their excellent efficacy in pest control and cost-effectiveness [52]. In order to find the optimal conditions for the extraction of OCPs performance from aqueous samples, several significant variables that can affect the extraction performance, including extraction time, desorption solvent, desorption time, and sorbent mass, were studied. Optimization was conducted by using OVAT (one variable at a time) in which the distilled water samples were spiked with each OPC at a concentration of 1 mg/L in a 10 mL solution. Triplicate extractions were performed for every parameter optimized in this analysis using gas chromatography-mass spectroscopy (GC-MS).

Effect of extraction time

In the µ-SPE principle, the sorbent is encapsulated in a polysaccharide alginate matrix; as a result, its sorption behavior is based on the equilibrium of mass transfer between the sample matrix and the sorbent, as opposed to the exhaustive extraction as in cartridge- or disc-based SPE. The sorption time was one of the most important parameters for enhancing the precision and sensitivity of the extraction procedure, as it is vital to allow enough time to ensure the equilibrium between the aqueous phase and sorbent [53]. Thus, various extraction times were evaluated in the range of 2.5 to 15 minutes to study the optimum time for the sorbent-analyte to achieve an equilibrium state. The impact of contact time between the sorbent and target analytes is depicted in Figure 6. It was discovered that the adsorption efficiency of OCPs was significantly increased up to 15 minutes, especially for heptachlor and aldrin. However, the adsorption of dieldrin was practically constant after 10 minutes. This could be due to the saturation of the surface area of the sorbent as time progressed and the competitive adsorption rate of the analytes [54]. Further extraction time after 15 minutes was not advisable as a few studies show that it will cause back extraction of analytes from the acceptor into the sample solution [55]. Thus, the optimal extraction time was determined to be 15 mins and was employed in subsequent experiments. The average was reported with associated error bars representing the standard deviation (n=3) in Figure 8.

Effect of desorption solvent

According to the principle of “like dissolves like”, compounds dissolve in a solvent when their chemical properties, particularly their polarities, are similar [56]. Polar and non-polar solvents are able to dissolve polar and non-polar compounds, respectively [57]. In this analysis, the desorption solvents n-hexane, ethyl acetate (EA), acetone, acetonitrile (ACN), and ethanol were assessed, with increasing polarity from n-hexane to ethanol to determine which one provided the maximum analyte recovery using µ-SPE technique. Figure 9 demonstrates that n-hexane performed better than the other solvents regarding desorption. N-hexane, which is non-polar with a polarity index of 0.1, provides a more favorable performance in desorbing the target analytes due to the hydrophobicity properties of the OCPs [58]. Meanwhile, due to its reduced compatibility with the hydrophobic OCPs in terms of polarity, ethanol exhibited the lowest desorption efficiency. Hence, the principle “like dissolves like” was applicable and produced the greatest results among various solvents used, and n-hexane was regarded as the optimal desorption solvent for further analysis.

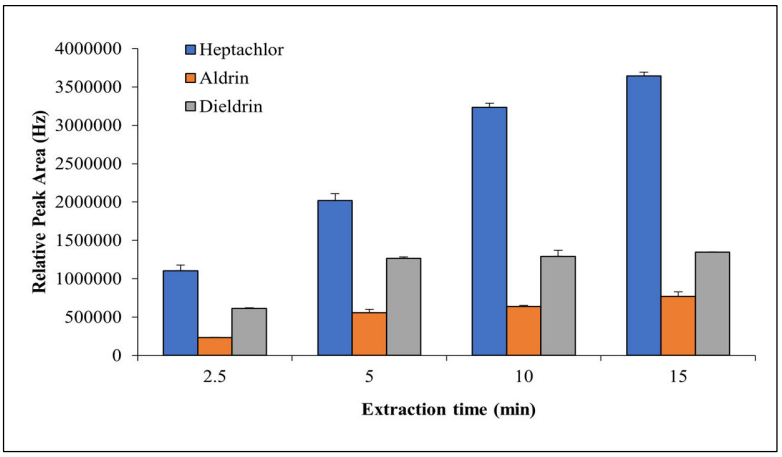


Figure 7. Graph of average relative peak height (Hz) against extraction time (min)

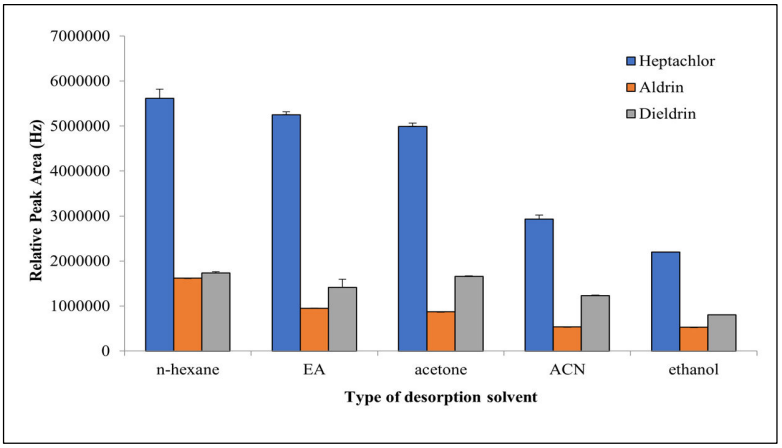


Figure 8. Graph of average relative peak height (Hz) against desorption solvent

Effect of desorption time

Desorption time was an additional significant element that could influence the peak area of the analytes. It has been determined that ultrasonication is an effective method for enhancing the desorption procedure [59]. The effectiveness of ultrasonic-assisted desorption is dependent on the parameters that influence the acoustic cavitation created during the process [60]. In addition to the ultrasonic power, the total amount of analytes eluted is also influenced by the time consumed. As depicted in Figure 10, there were only minor changes in desorption efficiency between 1,3,5,7 and 10 mins of ultrasonication. As the ultrasonic time increased from 3 to 7 minutes, a modest increase in the desorption of heptachlor, aldrin, and dieldrin was detected. Regardless of the result, at 1 and 10 mins, the trend of the graph appears to increase, although the data appears to be consistent at 3,5 and 7 mins, which are the span of time in the middle. Adding to this, extended ultrasonication decreased the efficacy of the OCP desorption. This result can be attributed to its comparatively high hydrophobicity among these analytes, indicating that the analyte prefers to diffuse into the hydrophobic pores of alginate-silica. It has been shown that ultrasonication can facilitate the desorption of adsorbed molecules and boost pore diffusion in the sorption process [60]. As a result of the re-adsorption of the target analytes, the peak area of each target OCP steadily decreased after a 7-minute desorption time [61]. Thus, the optimal desorption time for further analysis was determined to be 7 minutes.

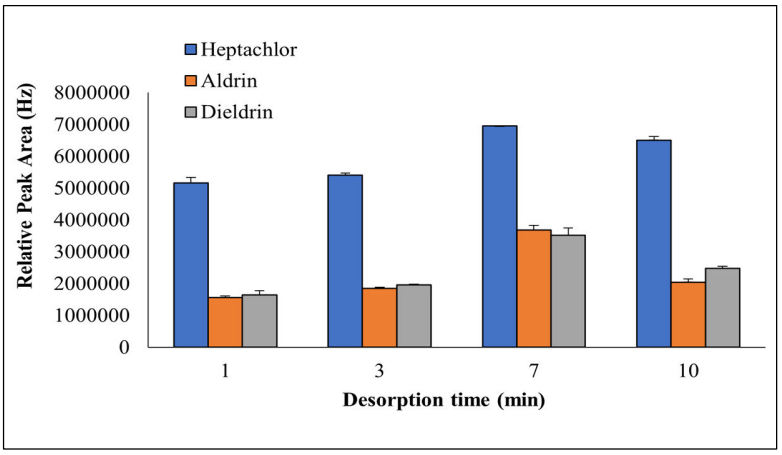


Figure 9. Graph of average relative peak height (Hz) against desorption time (min)

Effect of sorbent mass

In this study, 20, 30, 40, 50, and 60 mg of Alg-Si beads were used to optimize the sorbent mass. The results in Figure 11 demonstrate that the maximum peak area of analytes is attained with 50 mg of sorbents. With increasing sorbent mass, no discernible increase in peak area was seen; instead, the peak areas continued to diminish. This may be owing to the saturation of Alg-Si beads as the primary sorption site; hence, the use of an excessive amount of sorbent (more than 50 mg) made desorption more difficult and demanded a larger volume of desorption solvent [62]. Since the peak area for the analytes was at its maximum at 50 mg, 50 mg of sorbent was used for the following analysis.

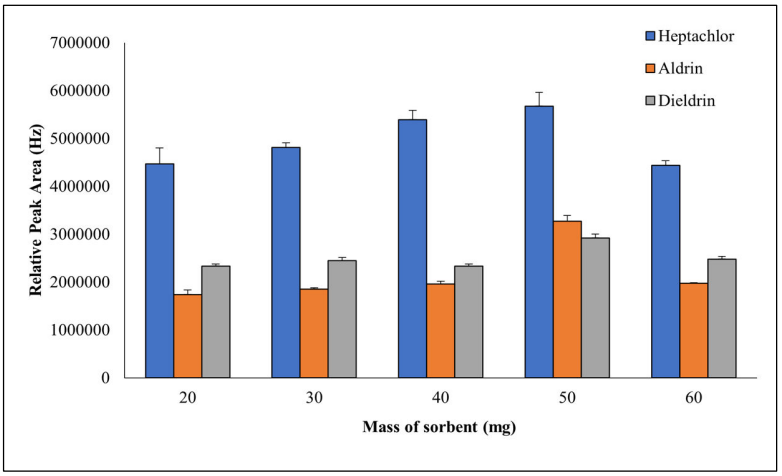


Figure 10. Graph of average relative peak height (Hz) against mas of sorbent (mg)

Optimum µ-SPE conditions.

The final adopted Alg-Si-µ-SPE conditions were: 15 min extraction time; n-hexane as desorption solvent; 7 min of desorption time; sorbent mass, 50 mg. Hence, these optimum extraction conditions were applied for subsequent studies.

Matrix-matched calibration

In analytical procedures, matrix effect (ME) can manifest as an undesired enhancement or suppression of the analytical response due to the eco-elution of interfering matrix components. Therefore, ME is a source of error in quantitative chromatography analyses of complex matrices such as pesticides, and it may be

compound or matrix-dependent. Commonly, ME is determined by comparing the slope of the calibration curve of analytes spiked into a blank paddy water sample to the slope of the calibration curve derived by spiking analytes into the solvent. In this study, matrix-matched calibration was performed on blank pesticides of varying concentrations of the OCPs.

Method validation

The proposed µ-SPE-GC-MS analytical method for the determination of three pesticides in paddy water samples under optimized experimental conditions was deemed valid. The analytical figures of merit, including the limit of detection (LOD), the limit of quantification (LOQ), the linear range, the relative recoveries (RR), the determination coefficient (R^2), and the precision (as relative standard deviation, RSD), were therefore determined. A calibration curve for each pesticide was constructed by adding six concentration levels of each analyte to a blank sample of paddy water. Heptachlor at 1, 4, 7, 10, and 13 ppm; aldrin at 0.3, 0.5, 0.7, 1, and 1.3 ppm; and dieldrin at 0.2, 0.6, 1, 1.4, 1.8 mg/L. Using least-squares linear analysis, the contours and corresponding regression equations were determined and subsequently validated by R^2 values. The spiked concentration differs because of the varying detection limits and anticipated ambient concentrations of the analytes [63], in which the calculated values indicate the expected levels of a particular substance or analyte in the surrounding environment. The optimized, developed µ-SPE procedure was validated to determine the adsorption capacity of Alg-Si particles as a functional bio-sorbent using paddy water as a sample, as shown in Table 4.

For each of the analytical curves, good R^2 values for each analyte were obtained in the range of 0.9796 to 0.9935. The LOD varied between 0.85 mg/L for heptachlor, 0.09 mg/L for aldrin, and 0.19 mg/L for dieldrin, as presented in Table 4, while the LOQ varied between 2.55 mg/L for heptachlor, 0.27 mg/L for aldrin, and 0.57 mg/L for dieldrin. Concerning precision, the examined OCPs exhibited intraday precision values of 10% or less. The intraday precision RSD% ranged from 2.52 – 2.78% for heptachlor, 2.34 – 3.29% for aldrin, and 3.52 – 4.52% for dieldrin. Meanwhile, the interday precision ranged from 1.82 – 2.31%, 0.30 – 1.91%, and 0.19 – 2.11% for heptachlor, aldrin, and dieldrin, respectively. Enrichment factor (EF) and extraction recovery (ER%) were also determined to evaluate the method's effectiveness. All the target pesticides provide RR (%) values above 80%, ranging from 82 – 84%, 90 – 91%, and 95 – 98%, for heptachlor, aldrin, and dieldrin, respectively. This result is comparable with another study conducted by Huang et al., which obtained a range of 87.6 – 98.6% for the same method with the application of different sorbents [64]. The EF values for the pesticides ranged between 6.74 – 16.87 for heptachlor, 9.64 – 34.73 for aldrin, and 4.38 – 20.25 for dieldrin. The results are summarized in Table 5. Meanwhile, Figure 12 shows the chromatogram of blank paddy water sample in three different analyses (blank, spiked at 1 mg/L and 10 mg/L, 0.1 mg/L and one mg/L, and 0.2 and 1.4 mg/L for heptachlor, aldrin, and dieldrin, respectively). All targeted analytes were detected in less than 10 minutes.

Table 4. Performance of the proposed method under optimized conditions

Sample	Analytes	Linearity (mg/L)	Correlation Coefficient	Regression Coefficient, R^2	LOD (mg/L)	LOQ (mg/L)
Paddy water	Heptachlor	1-10	y = 1246479x + 102580	0.9796	0.85	2.55
	Aldrin	0.1-1	y = 710263x + 166656	0.9908	0.09	0.27
	Dieldrin	0.2-1.4	y = 469676x + 62885	0.9935	0.19	0.57

Table 5. Method precision for intra- and inter-day (RSD), relative recovery (RR%), and enrichment factor (EF)

Analytes	Concentration (mg/L)	Intra-day RSD (%, n=3)	Inter-day RSD (%, n=9)	%RR	EF
Heptachlor	1	2.52	1.82	84	6.74
	10	2.78	2.31	82	16.87
Aldrin	0.1	2.34	0.30	90	9.64
	1	3.29	1.91	91	34.73
Dieldrin	0.2	3.52	0.19	95	4.38
	1.4	4.52	2.11	98	20.25

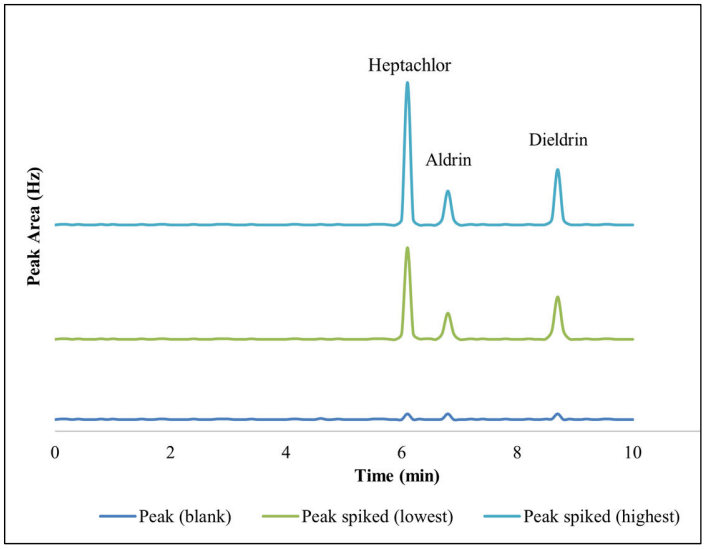


Figure 11. Chromatogram of paddy water sample at unspiked (blank), spiked (lowest concentration), and spiked (higher concentration)

Figure 12 shows the proposed interaction mechanism between the targeted analytes' OCPs and the obtained alginate-silica sorbent in this study. The extraction of OCP on alginate-silica sorbent is based on adsorption, whereby it possesses a porous structure comprising a substantial number of active sites accessible for binding with analytes [65]. It is explained that the binding type is contingent upon the nature of interactions between the adsorbent's surface and the molecular species of the analyte. For this interaction between OCP and alginate-silica composite, the interaction is physical, primarily governed by weak van der Waals forces, which is known as physisorption [65].

Green analytical calculator (AGREE)

The greenness of the developed method was evaluated using the AGREE metric calculator, which falls under a score (Figure 13) of 0.56. According to Rajendran et al., the determination of green classification lacks a predefined scale or metric; it hinges on the number of segments exhibiting a green color, with the value on the central pictogram approaching 1.0 [45]. The assessment implied an adequate green analytical methodology as it meets specific criteria, including (4) the integration of analytical processes for simultaneous steps, such as extraction and sonication (6), no derivatization agent was used during analysis and extraction, (10) no reagent used in the procedure, (12) the threats to the operator and environment were minimum due to minimum organic solvent consumption which approximately less than 1mL. The other two criteria represented by red are (3) the offline position of the analytical device and (9) the high consumption of energy-intensive techniques as the analysis was performed using GC-MS. The red colors of

the assessment will be improved for future work. Nevertheless, by concentrating solely on the advancement of sample preparation techniques, this study can be regarded as an environmentally friendly method in comparison to conventional SPE and LLE techniques.

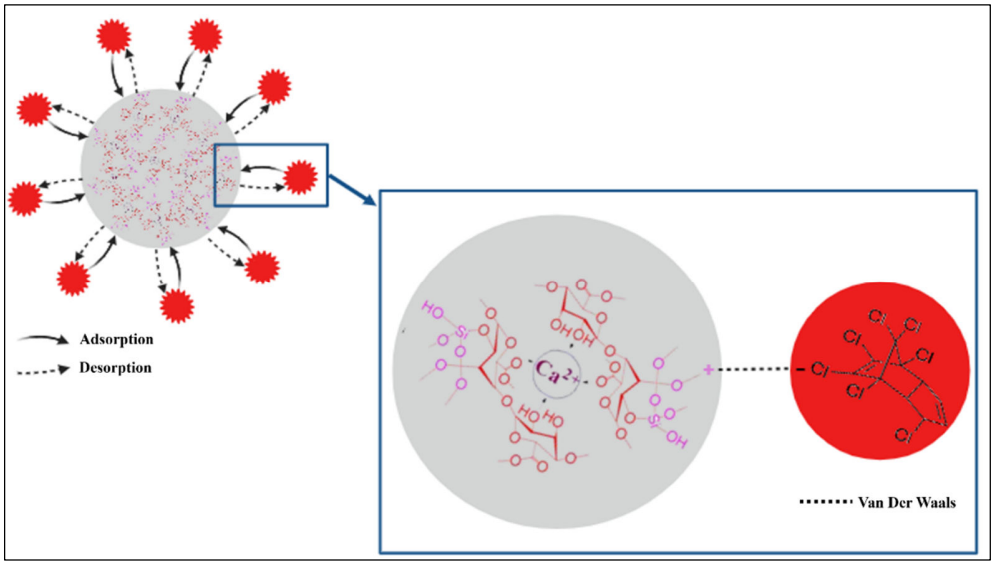


Figure 12. Interaction mechanism of extraction between alginate-silica sorbent with OCP (heptachlor) by van der Waals interaction

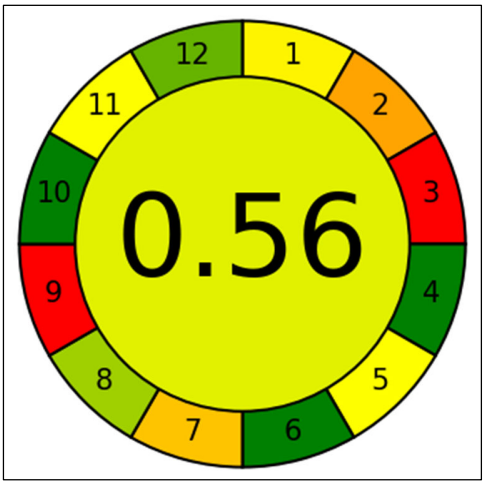


Figure 13. AGREE metric for greenness assessment of method developed

Conclusion

In conclusion, the chemical and physical properties of Alg-Si beads prepared by the encapsulation method were examined by characterization with ATR-FTIR and FESEM and showed that the functional groups of O-H, Si-O-Si, and COO^- groups contribute to the van der Waals, the interaction between prepared Alg-Si beads sorbent with targeted analytes. An uneven, rough surface area from the FESEM analysis of the sorbents could be attributed to the porosity of the beads obtained. The performance of Alg-Si-µ-SPE to extract OCPs from paddy water samples using alginate-silica beads was optimized, which included extraction time, desorption solvent, desorption time, and mass of sorbent.

Furthermore, the high adsorption capacity of the sorbent is demonstrated by the data obtained from method validation, which reveals linear ranges of 1-10 mg/L, 0.1-1.0 mg/L, 0.2-1.4 mg/L, for heptachlor, aldrin, and dieldrin, respectively with LOD and LOQ in between of 0.09-0.85 mg/L and 0.27-2.55 mg/L, respectively. The relative recoveries (RR) have been found in the range of 82-98% for all targeted analytes. OCPs were extracted from genuine paddy water samples, demonstrating the effectiveness of the developed sorbent. Most of the calibration curves were linear, and all regression coefficients were in the range of 0.97-0.99. The intra-day precision varied from 2.34-4.52% while the inter-day ranged from 0.30-2.31% by spiking the three analytes into a sample solution. The miniaturized solvents volume (μL) and sorbents mass (mg) utilized in this method could be considered a green method and safe for the environment. Furthermore, the silica produced from oil palm fronds (OPF) is likely to evolve considerably in various applications and not limited to analytical and material chemistry only.

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A REVIEW OF THE CURRENT TREND IN THE SYNTHESIS OF NITROGEN-RICH FUNCTIONAL POLYMERS FOR CARBON DIOXIDE ADSORPTION

(Sorotan Terhadap Trend Semasa Dalam Sintesis Polimer Fungsional Kaya Nitrogen Untuk Penjerapan Karbon Dioksida)

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Abstract

The consumption of fossil fuels (oil, coal, and natural gas) has increased due to the increasing demand for energy from various industries. The International Energy Agency, in its World Energy Outlook 2022, has noted a steady increase in global energy demand from fossil fuels from 350 EJ in 2000 to over 500 EJ in 2030. This increase has contributed significantly to the emission of greenhouse gases, mainly carbon dioxide (CO_2), leading to global warming and climate change. Carbon capture by adsorption on polymeric adsorbents is a strategic approach to mitigate climate change by reducing CO_2 concentration in the atmosphere. This review focuses on the preparation of various nitrogen-rich functional polymers, mainly polyamine, polyamide/polyimide, and covalent triazine framework. This article provides important information on improving the functionality of nitrogen-rich functional polymers for CO_2 adsorption. Brief insights into the CO_2 adsorption mechanism and molecular modeling using density functional theory are also included. The challenges and future recommendations are also addressed in this review.

Keywords: carbon capture, adsorbent, climate change, density functional theory

Abstrak

Penggunaan bahan api (minyak, arang, dan gas asli) telah meningkat kerana pertambahan permintaan untuk tenaga dalam pelbagai sektor industri. Agensi Tenaga Antarabangsa (IEA) dalam Tinjauan Tenaga Dunia 2022 telah mencatatkan pertambahan yang mantap dalam permintaan global tenaga daripada bahan api fosil dari 350 EJ pada tahun 2000 hingga melebihi 500 EJ pada tahun 2030. Peningkatan ini telah menyumbang kesan yang ketara pada pembebasan gas hijau terutamanya karbon dioksida (CO_2) yang

membawa kepada pemanasan global dan perubahan iklim. Penangkapan karbon menggunakan kaedah penjerapan ke atas bahan penjerap daripada polimer ialah satu strategi bagi mengatasi perubahan iklim dengan cara mengurangkan kepekatan CO_2 dalam atmosfera. Sorotan ini memfokuskan kepada penyediaan beberapa jenis polimer fungsional kaya nitrogen, antaranya poliamina, poliamida/poliimida, dan kerangka kovalen triazina. Artikel ini menyediakan maklumat penting bagi menambah baik kefungsian polimer kaya nitrogen bagi tujuan penjerapan CO_2. Pemahaman ringkas mengenai mekanisma penjerapan CO_2 serta pemodelan molekul menggunakan teori ketumpatan berfungsi turut disertakan. Cabaran dan cadangan masa hadapan turut disampaikan dalam sorotan ini.

Kata kunci: penangkapan karbon, bahan penjerap, perubahan iklim, teori ketumpatan berfungsi

Introduction

The increasing demand for energy due to population growth has led many countries to explore their natural resources to strengthen their economy. The increase in greenhouse gas emissions is mainly contributed by carbon dioxide (CO_2) generated from the combustion of fossil fuels and natural resources, impacting the environment, and causing global warming and climate change [1]. To address this issue, the Paris Agreement was adopted on 12th December 2015 by 196 parties at the United Nations Climate Change Conference 21 in Paris. It aims to reduce global warming to below 2 °C, if possible, to 1.5 °C compared to the pre-industrial level [2]. Carbon capture, utilization, and storage is considered one of the strategic climate mitigation options to meet this international goal. It refers to technologies that capture CO_2 emissions from main contributors, such as energy production and manufacturing facilities that utilize either non-renewable or renewable energy sources (e.g., biomass) [3]. If the captured CO_2 is not consumed, it is compressed and transported through pipelines, ships, rails, or trucks for use in various applications, or injected into deep geological formations, such as a gas reservoir or saline formation that traps CO_2 for permanent storage [4].

An increasing number of publications have reported on carbon capture using various methods, including absorption, adsorption, membrane separation, and cryogenic distillation [5]. However, the conventional CO_2 capture technology using liquid amine systems has some drawbacks. For instance, the regeneration and desorption of captured CO_2 from aqueous amine scrubbing systems can be energy-inefficient due to the high regeneration temperature. The significantly inefficient energy consumption requirements can lead to economic inefficiency. Other shortcomings, such as equipment corrosion and concerns about amine leakage into the environment, are frequently encountered in these conventional aqueous amine schemes [6]. If a system experiences amine leakage and is exposed to atmospheric oxidants, it can lead to a degradation reaction, producing toxic compounds (e.g., nitrosamines, nitramines, and amides) that pose a threat to human health and environmental safety [7]. Adsorption using solid materials is a practical technology that can be applied for CO_2 capture. Adsorption offers low energy requirements and low operating costs, including controlled waste generation, high CO_2 adsorption uptake in ambient conditions, high specific surface area, high mechanical durability, adequate pore size distribution, and accessibility for regeneration [8].

Adsorption is a sorption process of CO_2 molecules onto the surface of a solid adsorbent that has residual surface energy due to unbalanced forces. These unbalanced forces will attract CO_2 molecules colliding with the solid surface, causing them to remain on the solid surface. The attraction that forms between gaseous CO_2 and the adsorbent can be classified as a weak physical interaction (i.e., physical adsorption) and a stronger chemical bond (i.e., chemical adsorption) [9]. Several solid materials, such as zeolites and silica, have been reported for CO_2 capture. These materials possess high CO_2 capture capacity and high selectivity of CO_2 over nitrogen (N_2) under atmospheric conditions. Nevertheless, the adsorption capacity of zeolites can be affected by the presence of water. Hence, dehumidification would be necessary in such conditions prior to CO_2 capture. This additional step would significantly increase the investment cost, total energy consumption, and the difficulty of the operation [10].

Mesoporous carbon-based materials offer promising potential for CO_2 capture due to their widely available resources, high chemical and thermal stability, and inexpensive and tunable porous structure [11].

On the other hand, polymeric materials are great adsorbents for CO_2 adsorption due to the presence of different functional groups on their backbone. The adsorption capacity and selectivity of a polymeric adsorbent can be improved when these functional groups form specific interactions with CO_2. Surface morphology and tunable porosity can enhance the intraparticle transport of CO_2 toward the active sites of the adsorbent, thereby increasing its performance [12]. Recently, porous polymeric adsorbents have been receiving attention due to the increasing number of publications on the functionalization of polymers with various functional groups to improve the performance of adsorbents. A previous study reported the introduction of nitrogen- and oxygen-based functional groups that could enhance the CO_2 adsorption capacity of the adsorbent [13]. The functionalization of polymers has also led to the development of various combinations of porous support materials, such as zeolite and mesoporous silica, with functional polymeric materials. The functionalized polymeric solid sorbent can overcome the issue of low stability caused by the leaching of amines over repeated capture/regeneration cycles and the limited transportation of CO_2 to the active sites in an amine-impregnated CO_2 sorbent system [13]. Hassan et al. [14] also synthesized triptycene-based nitrogen-rich hyper-cross-linked polymer (HCP). Their results indicated a higher adsorption capacity of 160 mg/g at 273 K and 1 bar, and a lower specific surface area of 848 m^2/g. The specific surface area decreased as a result of the functionalization with the nitrogen functional group; however, this modification did not affect the performance of CO_2 adsorption. The inclusion of a nitrogen heteroatom in HCP has increased the affinity of the material toward CO_2 due to their dipole-quadrupole interaction [14].

Tremendous efforts have been dedicated to designing porous materials with different channel geometries, pore sizes, and chemical compositions to improve the effectiveness of sorbents. These design variations have considered several criteria, such as high adsorption capacity, cost-effectiveness, fast kinetics, energy efficiency, and long-term stability [15,16]. This review presents a comprehensive discussion of the current trends in the development of polymeric adsorbents for CO_2 adsorption. The introduction is presented in the first section, elaborating on the background study on CO_2 adsorption. This includes the main sources of CO_2 production, the current policies being applied, and carbon capture methods. In the second section, the preparation of nitrogen-functional polymers for CO_2 adsorption is discussed. The structure of CO_2 and the mechanisms of interaction with CO_2 are elaborated in the subsequent section. This review ends with conclusions and recommendations for future studies.

Preparation of nitrogen-rich functional polymer

Nitrogen-rich porous solid adsorbents have been widely explored for their CO_2 chemisorption ability under low CO_2 concentrations. Figure 1 shows nitrogen moieties like amine, amide, imide, triazine, melamine, imidazole, and hydrazine, which are often used to produce nitrogen-rich functional polymers in CO_2 capture applications [17–20]. This section presents a brief discussion on the preparation of nitrogen-rich polymers, mainly focusing on polyamine, polyamide/polyimide, and covalent triazine frameworks.

Preparation of amine-functionalized polymer

Polyethyleneimine (PEI) or polyaziridine is a polymer composed of an amino group and two carbon aliphatic CH_2CH_2 spacers, and it is widely applied as an adsorbent in CO_2 adsorption [21, 22]. This amine-bearing polymer is well known for its highly positive charge, and it can exist in a linear and branched chain with different properties. Linear PEIs are composed of secondary amines, whereas branched PEIs are composed of primary, secondary, and tertiary amino groups that can be found on the polymeric backbone [23]. For CO_2 adsorbent application, PEIs are commonly combined with different porous inorganic supports to achieve cost-effectiveness and high amine content through chemical grafting of amine reagent, physical impregnation, and impregnation-cross-linked method [24–26].

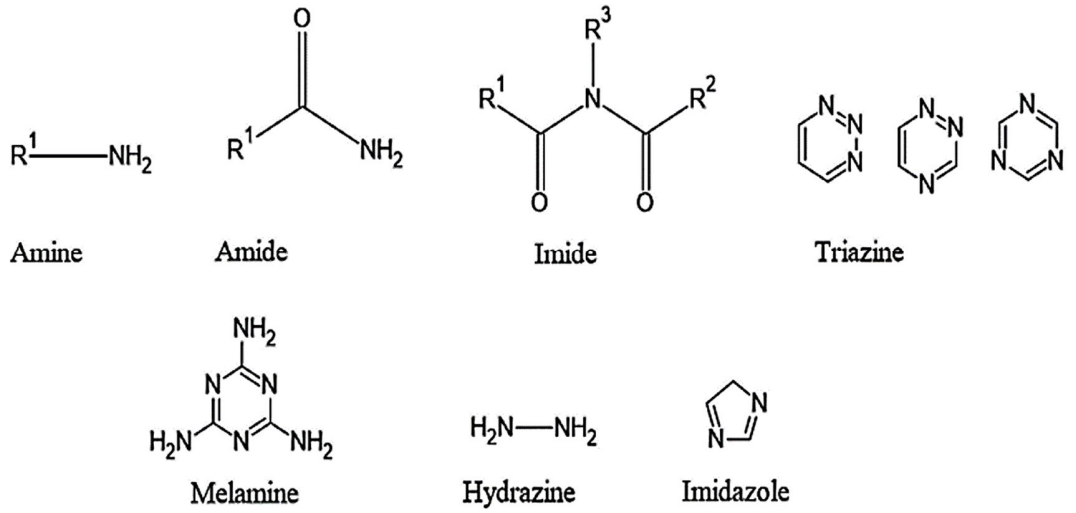


Figure 1. Nitrogen functional groups are commonly used in nitrogen-rich functional polymers

Physical impregnation onto porous materials

Physical impregnation of low vapor pressure amine polymers onto porous support materials is an easy and more scalable method [54, 60]. Li et al. [30] used the wet impregnation method by dissolving PEI in the solvent to obtain a homogeneous solution. Then, the porous material (Al_2O_3) was added to the solution, and the mixture was stirred until the solvent evaporated. The obtained material was dried in a vacuum oven overnight. The synthesized 50%PEI@Al_2O_3 adsorbent exhibited high adsorption stability for up to ten cycles with an adsorption capacity of 136 mg/g. The high operating temperature increased the kinetic effect, reduced the viscosity of PEI molecules, and increased the diffusion of CO_2 molecules, thereby increasing the likelihood of molecular collisions between CO_2 and PEI molecules. Nonetheless, this method also suffered from several drawbacks, such as amine leaching during the capture and regeneration cycles, and low active site accessibility after impregnation, which reduced the performance of the adsorbent [31]. Li et al. [32] reported a simplified and environmentally friendly method to prepare amine-modified amorphous silica to overcome the issue of particle agglomeration between PEI and silica by introducing a solventless mechanical grinding method. The CO_2 adsorption capacity of 70% PEI/SiO_2 was approximately 2.47 mmol/g at 70 °C and 1.5 bar. These results show that the absorbents have a remarkable CO_2 adsorption capacity at high temperatures.

In addition to infusion into porous solid supports, porous polymer supports are also used to enhance CO_2 performance. This approach will increase CO_2 adsorption performance with low mass transfer limitation caused by clogged pores by maximizing amine loading and improving the accessibility of gas through the sorbent [24]. Choi et al. [33] prepared metal-organic frameworks (MOFs) and immobilized polyacrylonitrile (PAN) fiber mats with PEI impregnation for CO_2 capture. The PEI was impregnated into the PAN-MOF electrospun mats using the wet impregnation method. They selected two types of MOFs, namely ZIF-8 and HKSUT-1, to prepare the PAN/MOF electrospun mats. The impregnation of PEI into the PAN/ZIF-8 electrospun mats enhanced their adsorption capacity by 45% and improved the CO_2/N_2 sorption selectivity due to the strong intermolecular interactions between the amine group in PEI and CO_2 molecules. However, a further increase in PEI loading reduced the adsorption capacity due to the reduction of surface area and pore volume.

Chemical grafting

A functional adsorbent with specific amine group and functional group densities suitable for investigating the

CO_2 adsorption mechanism can be obtained using the surface grafting method. However, this method is expensive and involves a complex procedure. Yin et al. [34] successfully synthesized PEI-modified poly(melamine)-polyacrylamide (MF/PAM-g-PEI), which demonstrated higher CO_2 adsorption of 2.8 mmol/g (10% CO_2 and 90% N_2; 30 mL/min) at 273 K compared to non-modified MF and PAM polymer networks. The polymer network was synthesized by interpenetrating PAM between the molecular chains of MF and functionalizing it with PEI to attain a solid amine adsorbent, as shown in Figure 2. The interpenetration of PAM into the porous amino resin improved its swelling capacity and provided amine grafting sites for the amine agents. Consequently, the adsorption capacity and the regeneration performance of the adsorbents increased.

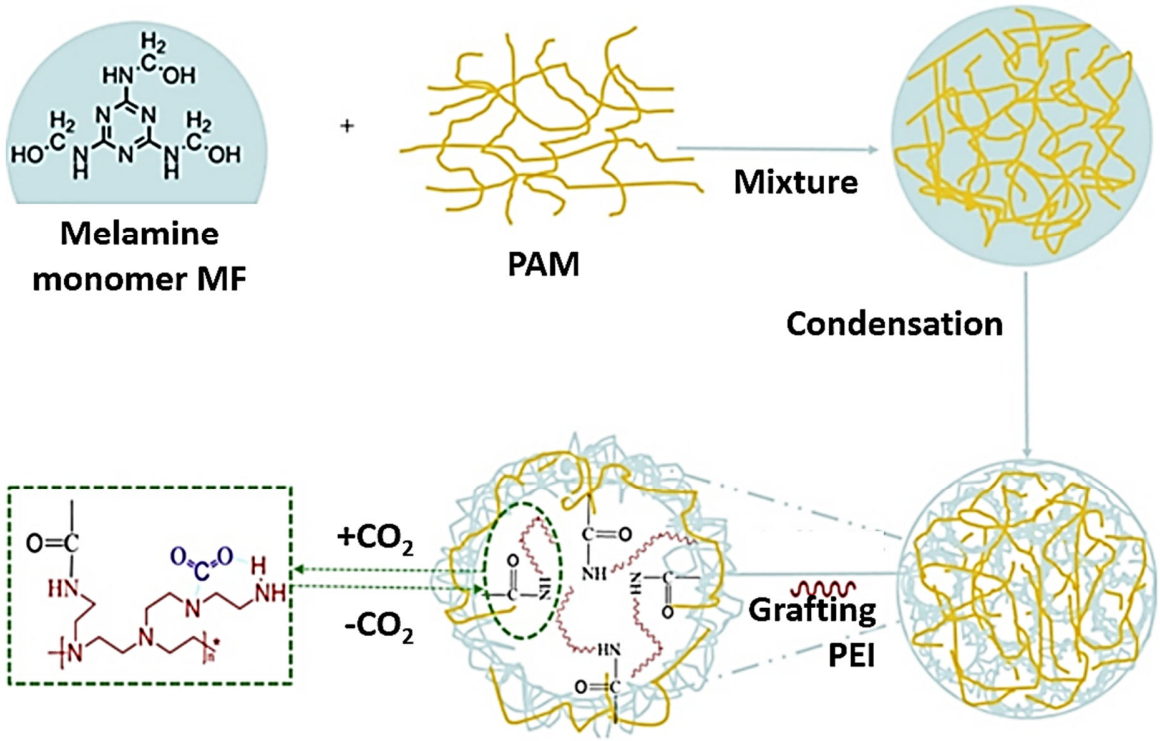


Figure 2. Schematic diagram of the synthesis of MF/PAM polymer network [34]

The grafting method was applied by Wang et al. [35] using nucleophilic substitution reaction without a catalyst to prepare the precursor for PEI-based polymers. The surface of the precursor was evenly coated with amine groups, while its internal side has a dual role of a pore-forming agent through self-decomposition and in creating nitrogen sites that can enhance surface polarity. The formation of narrowly distributed ultramicropores (d = ~0.6 nm) can attract CO_2 by relying on the van der Waals force as affinity attraction. This material achieved a CO_2 uptake of 4.92 mmol/g. The polymers exhibited the typical adsorption-desorption patterns of Type-I isotherms. Alongside the distribution of pore sizes, the polymers exhibited a recognizable microstructural feature, with ultramicropore sizes ranging from 0.6 to 0.9 nm [35]. Yang et al. [36] also synthesized an amine-functionalized polymer for CO_2 adsorption from flue gas under the pressure swing adsorption operating mode. Linear pentaethylenehexamine (PEHA) was functionalized on the open-cell foams of low-density and high-porosity polypropylene/polyolefin elastomer (PP/POE), which served as a support material.

Impregnation-cross-linking method

Porous poly(divinylbenzene) (PDVB) was synthesized using the high internal phase emulsion (HIPE) template. The porous structure was further tailored by adding a pore-forming agent and applying the post-cross-linking method. The adsorption capacity of meso-PDVB increased with the introduction of PEHA to 4.52 and 4.99 mmol/g at a 10% CO_2 concentration, with higher N efficiency observed under wet conditions [25]. The same group also reported the preparation of porous polyHIPE with silica hydroxyl groups that act as a silane coupling agent via the copolymerization of styrene, divinylbenzene, triethoxyvinylsilane (VTEO), and tetraethyl orthosilicate (TEOS), as illustrated in Figure 3. The surface morphology of polyHIPE in Figure 3 shows the formation of a thin layer of TEOS and VTEO compared to the surface morphology of polyHIPE without the presence of both silane groups. The material can be easily functionalized with aziridine via the Friedel-Crafts cross-linking reaction through the silanol group of polyHIPE, as shown in Figure 3, thus increasing the specific surface area and enriching the CO_2 chemical adsorption sites [37]. This group also synthesized a novel amine-functionalized adsorbent with an interconnected porous structure. The adsorbent was prepared using HIPE polymerization of styrene and divinylbenzene, and then functionalized with PEI in a one-step synthesis. The 4.0PEI70k@polyHIPE adsorbent showed a maximum adsorption capacity of 4.18 mmol CO_2 per gram of adsorbent at 20 °C (CO_2:N_2 = 1:9; volume ratio of 30 mL/min) under humid conditions. The monolithic structure produced from the HIPE template on a macroscopic scale of the materials offers a better advantage in industrial applications compared to powder adsorbent [38]. This is also supported by Haorui, who synthesized cross-linked polyHIPE with PEI, producing a sponge-like PEI-based solid amine adsorbent that exhibited a record high CO_2 adsorption capacity of 14.22 mmol/g with stable regeneration performance; hence, the adsorbent holds great potential in CO_2 adsorption and separation [39].

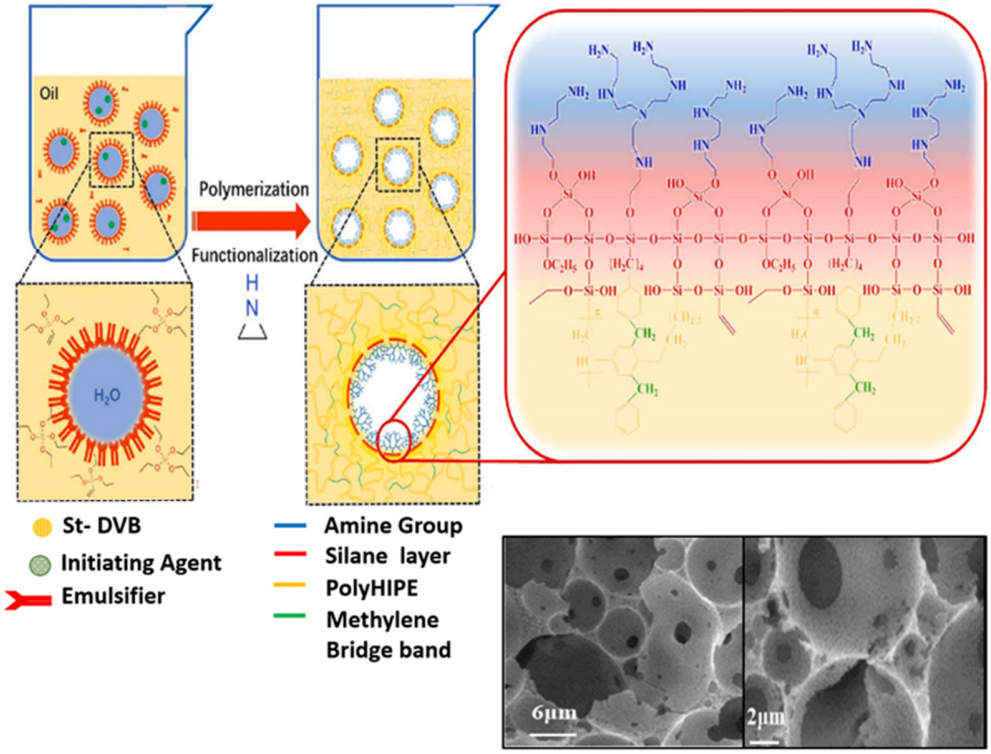


Figure 3. Synthesis of the hierarchically porous solid amine adsorbent and scanning electron microscopy images of cross-linked polyHIPE [37]

Table 1. Summary of different methods used for introducing amine group in polymer

No.	Polymer	Amine Introduction Method	Support	Adsorption Capacity	Advantages	Disadvantage	Ref
1	Polyethyleneimine/ tetraethylenepentamine (PEI/TEPA)	Physical impregnation	Macroporous silica	TEPA/SIO: 5.36 mmol/g PEI-1200: 3.75 mmol/g	• A simple, effective, and inexpensive method	• Low accessibility toward active sites after impregnation • The use of organic solvent during impregnation increases production cost • High amine loading can lead to agglomeration	[40]
2	PEI	Physical impregnation	Colloidal silica and ceramic fiber honeycomb monolith	PEI-SS (50%): 1.79 mmol/g			[41]
3	PEI	Physical impregnation	Silica support	2.3 mmol/g			[22]
4	TEPA grafted on polyethylene/ polypropylene (PE/PP) grafted with poly(glycidyl methacrylate) (PGMA)	Chemical grafting	PGMA-grafted fiber	117 mg/g	• Improved swelling capacity • Improved regeneration performance • The functionalization method can be tailored and the functional group on the surface porous material can be controlled	• Require complex sample preparation	[42]
5	Poly(aminoethyl methacrylate)	Chemical grafting	N/A	2.4 mmol/g			[43]
6	PEI	Chemical grafting	Poly(acrylic acid) foam	5.91 mmol/g			[44]
7	Molecular imprinted polymer (PEI and glycol diglycidyl ether)	Impregnation cross-linked	Molecular imprinted polymer	MIP: 6.58 mmol/g; NIP: 5.87 mmol/g.	• Improved regeneration stability • The monolithic structure of polyHIPE offers a greater advantage in industrial applications • Avoid high resistance to mass transfer during adsorption	• Complex synthesis processes	[45]
8	PolyHIPE-PEI	Impregnation-cross-linking method	PolyHIPE	6.22 mmol/g			[46]

Preparation of amide- and imide-functionalized polymers

Both amide- and imide-based polymers, which are commonly synthesized for CO_2 capture applications, consist of a carbonyl group and nitrogen atoms. These polymers can be prepared for CO_2 capture using several methods, such as polycondensation, polymerization, and radical copolymerization.

Radical copolymerization of polyacrylamide

Polyacrylamide is a polymer obtained from the radical copolymerization of acrylamide (AAm) or methacrylamide (MAAM), which demonstrated great potential in CO_2 adsorption and separation [45, 47, 48]. Yoo et al. [49] synthesized an MOF (MIL-101) loaded with polymethacrylate (PM). This polymer was synthesized using radical copolymerization in the presence of azobisisobutyronitrile as an initiator, while ethylene glycol dimethacrylate (EGDMA) and MAAM were used as monomers to produce PM@MOF, which was further reduced to R-PM@MOF to introduce free –NH_2 group. The CO_2 adsorption capacity and selectivity performance of this adsorbent increased under optimum PM loading and the reduction of PM to R-PM due to the introduction of an amine functional group [49].

In another work, Fayemiwo et al. [50] synthesized three different HCPs using radical bulk polymerization, whereby EGDMA was used as the monomer cross-linker, while MAAM, AAm, and triallylamine (TAAm) were used as the functional monomers. The results showed that the maximum CO_2 uptake was obtained by HCPMAAM-2 (1.45 mmol/g), followed by HCP-AAm and HCP-TAAm at 273 K. The highest CO_2/N_2 selectivity (15/85) was achieved by HCPMAAM. Meanwhile, HCP-TAAm showed low adsorption performance and selectivity, possibly attributed to the weak attraction between tertiary amine and CO_2. In comparison, HCP-MAAM-2 and HCP-Aam have NH_2 functional groups that can form strong interactions with CO_2 molecules. The same group also reported the life cycle assessment of HCP-MAAM as an adsorbent. The results indicated that this adsorbent has lower environmental impacts compared to molecularly imprinted polymers, as the latter used solvents sourced from fossil fuels [50]. Su et al. [51] reported an improved molecular imprinting polymerization method using the surface imprinting technique to produce CO_2 molecular imprinting polymer (CO_2-MIP). As shown in Figure 4, the porous polymer was prepared using surface radical polymerization with continuous stirring using acrylamide as a monomer, EGDMA as a cross-linker, and activated carbon (AC) synthesized from sunflower heads (biomass waste) acted as a carrier. The MIP was polymerized onto the surface of the AC. The results showed that the macropores of the carrier decreased after molecularly imprinted polymerization, whereas its micropores and mesopores increased, leading to a decrease in the average pore size. The results obtained indicated better performance than other CO_2-MIP samples prepared using suspension polymerization [51].

Polycondensation polymerization of polyimide

Polycondensation is a polymerization method that produces a long-chain polymer from single or multiple monomers by releasing water or any related by-products. Aromatic polyimide has gained attention in CO_2 capture applications as this microporous material has outstanding thermal stability, excellent chemical resistance, and abundant heteroatoms [52]. The polycondensation of an aromatic dianhydride and tridimensional geometry amine can produce microporous polyimide with a high specific surface area for excellent CO_2 adsorption.

Ning et al. [53] introduced a new composite adsorbent named PI-UiO/GO-1, which was synthesized using a chemical in situ knitting method involving the reaction among 4,4'-oxydiphthalic anhydride, 2,4,6-trimethyl-1,3-phenylenediamine, UiO-66-NH_2, and graphene oxide (GO). Compared to pristine UiO-66-NH_2, the PI-UiO/GO-1 adsorbent exhibited superior performance, including higher CO_2 capacity, higher CO_2/N_2 selectivity (64.71 compared to 15.43), and significantly improved acid-base resistance stability. Interestingly, grafting polyimide onto UiO-66-NH_2 had no impact on the microporous structure. However, the introduction of C-N covalent bonds and secondary amines in PI-UiO/GOs enhanced the selective adsorption of CO_2. This enhancement was achieved through the interaction between the negative charge of N and O atoms in polyimide and the electrophilic C in CO_2, thereby

enhancing the selectivity and CO_2 adsorption capacity of the adsorbent [53].

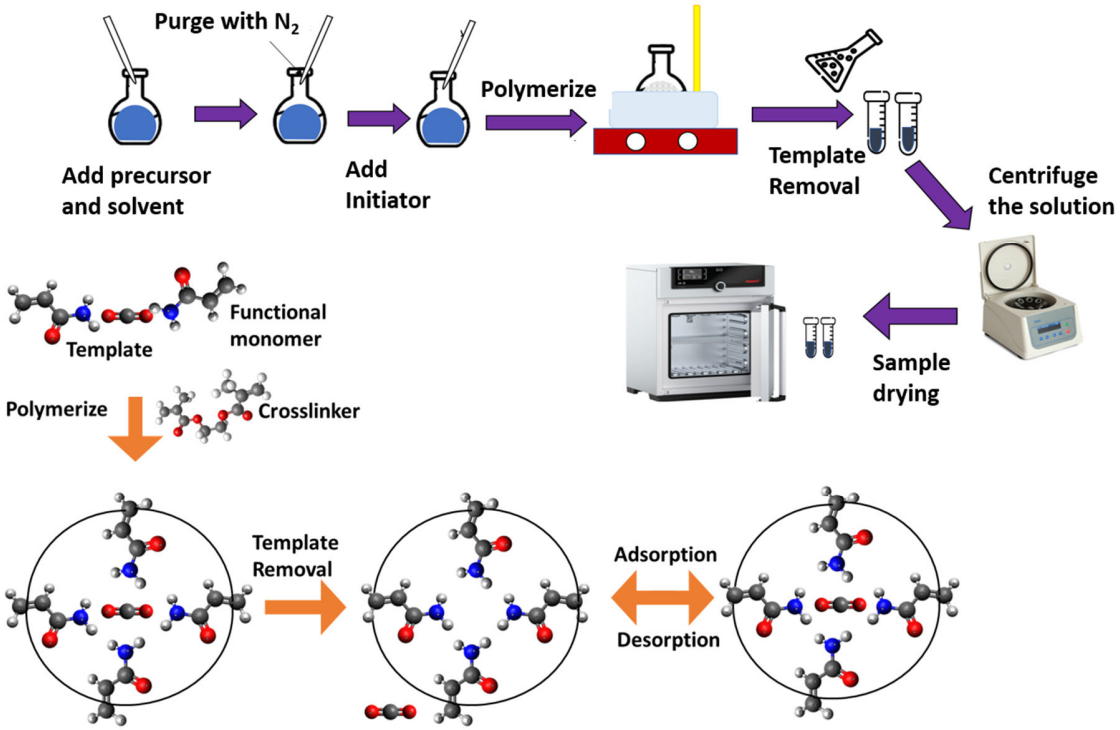


Figure 4. Schematic diagram of the steps in MIP preparation for CO_2 adsorption

Song et al. [54] reported a more simplified and economical method for preparing cross-linkable hyperbranched polyimide for CO_2 capture using polycondensation and thermal cross-linking. The cross-linked structure served as a scaffold that restricted the movement of polyimide chains, thus preventing the polymer skeleton from collapsing and facilitating the formation of micropores [54, 55].

Earlier research described the synthesis of two cross-linked microporous polyimides (i.e., TAPM-PEPA-CL and TAPA-PEPA-CL) using aromatic alkynyl monomers through thermal cross-linking polymerization in diphenyl sulfone [56]. The former exhibited a higher Brunauer-Emmett-Teller (BET) surface area and microporous volume (736 m^2/g and 0.175 cm^3/g, respectively) compared to the latter (351 m^2/g and 0.018 cm^3/g, respectively). Additionally, the adsorption capacity of TAPM-PEPA-CL was significantly enhanced. These results indicate that adjusting the topological structure of cross-linked microporous polyimides can influence their microporosity and CO_2 adsorption performance [56]. Wang et al. [57] also introduced cross-links to linear polyimide by synthesizing linear cross-linked polyimides with porous structures. This adsorbent showed higher porosity with improved CO_2 adsorption (5.7–9.3 wt.%) and good CO_2/N_2 selectivity (34.4–58.5) at 273 K compared to the other linear polyimide [57].

Preparation of covalent triazine framework

A covalent triazine framework (CTF) is often classified as a type of covalent organic framework (COF). It is synthesized through the classical ionothermal cyclotrimerization of a precursor containing carbonitrile groups. Covalent triazine frameworks possess distinctive attributes, including exceptional stability and robustness due to the presence of conjugated aromatic

C=N linkages, making them suitable for various adsorption applications [58, 59]. The design of CTFs allows for controlling the textural, structural, and physicochemical characteristics of the framework by manipulating the geometry, dimensions, and functionalities of the organic building units [60, 61]. Chen et al. [62] reported the preparation of four pyrene-based CTF materials using one-pot ionothermal polymerization. Pyrene units were chosen as the building blocks for forming CTFs due to their strong π–π interactions, which contribute to the dense structure of COFs. Additionally, the π-conjugated system derived from the pyrene group contributed to the overall structural rigidity of the CTFs. These newly developed materials exhibited remarkably high BET surface areas ranging from 1,415 to 1,753 m^2/g, along with notable CO_2 uptake capacities. The triazine-based frameworks have garnered significant interest in CO_2 adsorption research because their carbon backbones contain higher nitrogen content than other frameworks, leading to higher porosity characterized by both micropores and mesopores [62]. Liu et al. [17] prepared triazole-functionalized triazine-based porous organic polymer using copper-catalyzed azide-alkyne cycloaddition (Cu-AAC) polymerization, as shown in Figure 5. The presence of triazine and triazole rings in the CTF chain enriched the nitrogen content, thus enhancing the interaction between CO_2 and this porous material. A triazine ring with a pre-decorated azide functional group was used as the key building block for the synthesis of CTF. The Cu-AAC reaction led to the formation of 1,4-triazole, which showed a considerable CO_2 adsorption capacity of 52.0 cm^3/g at 273 K and 1 bar. They also reported a lower adsorption capacity due to the lower permanent porosity [17]. The CTF can also be prepared through the polycondensation of formamide and triazine-based amine to produce nitrogen-rich microporous polyaminals [63, 64]. The outcome of this reaction yielded various geometric configurations and functionalities, depending on the initial monomers used. As a result, it allowed for the tuning of BET surface areas and pore sizes within a range of 842–1,227 m^2/g and 0.53–3.82 nm, respectively [63]. The adsorption capacity of CO_2 can also be influenced by the pore size and BET surface area [63].

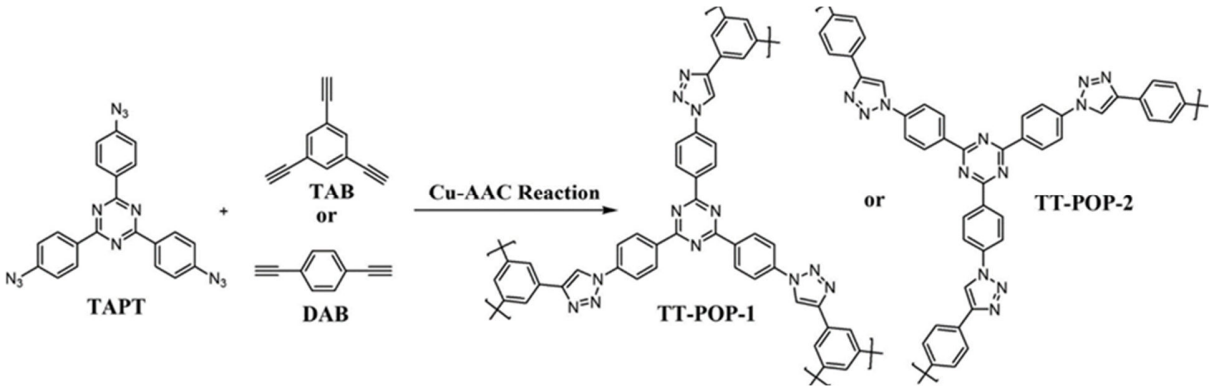


Figure 5. Schematic diagram of the synthesis of triazine-based porous organic polymer [17]

Carbon dioxide adsorption mechanism

Carbon dioxide has two oxygen atoms bonded to a central carbon atom through a covalent double bond. Its linear geometry causes the net molecular dipole moment to be zero, making it a non-polar molecule. Quadrupole interaction, or London dispersion interaction, can form between CO_2 molecules, where the middle carbon carries a partial positive charge and both terminal oxygens carry partial negative charges. The opposite charge that comes into contact forms the quadrupole interaction or electrostatic interaction (+/−). Figure 6 shows the electrostatic potential surfaces of CO_2, illustrating the distribution of charges on the molecular surface. The surfaces plotted in pink indicate the negative potential regions, while the surface in green indicate the most positive region. The surface potential site of CO_2 shows the carbon atom with a positive charge (poor electron) and the oxygen atoms at both ends with

a negative potential (rich electron). The potentials of the positive and negative regions can form electrostatic interactions with electrophilic and nucleophilic regions of other molecules [65, 66].

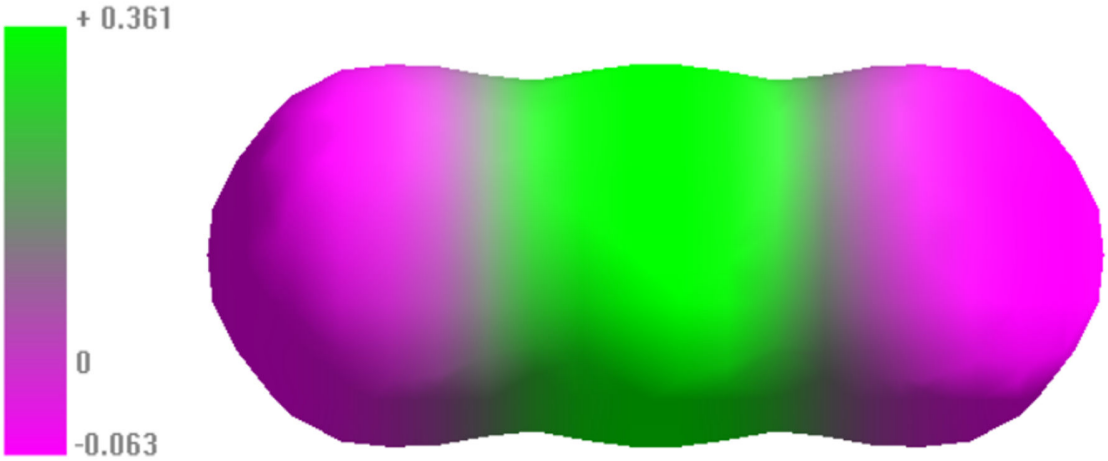


Figure 6. Electrostatic potential surfaces generated for two molecules.

The interaction between CO_2 and an amine (primary or secondary) functional group can lead to the formation of a zwitterion. Faisal et al. [67] reported the mechanism of the reaction between the primary amine of ethylenediamine (RNH_2), which was functionalized onto the surface of mesoporous carbon and reacted with CO_2 to form carbamate ions, as shown in Equation (1). Meanwhile, mesoporous carbon triethylenetetramine, which contains both primary and secondary amines (RNH_2 and R_2NH, respectively), reacted with CO_2, as illustrated in Equation (1) and (2) [67].

$$CO_2 + 2RNH_2 \rightarrow RNH_3^+ + RNHCOO^- \quad (1)$$

$$CO_2 + 2R_1R_2NH \rightarrow R_1R_2NH_2^+ + R_1R_2NCOO^- \quad (2)$$

$$CO_2 + RNH_2 + H_2O \rightarrow HCO_3^- + RNH_3^+ \quad (3)$$

$$RNH_3^+RNHCOO^- + H_2O \rightarrow RNH_2 + HCO_3^-RNH_3^+ \quad (4)$$

$$CO_2 + RN_3 + H_2O \rightarrow HCO_3^- + R_3NH^+ \quad (5)$$

This mechanism is supported by another study that used a different support-free cross-linked PEI sorbent [68]. They reported that primary and secondary amines reacted rapidly with CO_2 to form carbamates, as shown in Equations (1) and (2). This mechanism, which was proposed by Caplow et al. [69], is initiated by the nucleophilic attack of an amine that acts as a Lewis base to form a zwitterion. Later, this zwitterion is deprotonated by another amine acting as a Bronsted base, resulting in the formation of an ammonium carbamate ion [69]. The reaction between an amine and CO_2 in the presence of water can lead to the formation of ammonium bicarbonate, as shown in Equation (3). This reaction involves the interaction between zwitterion and water molecules, whereby only one amino group is needed to interact with one CO_2 molecule instead of two amino groups, as shown in Equations (1) and (2) [68]. The formation of ammonium carbonate can also occur through the hydrolysis of ammonium carbonate, as presented in Equation 4 [70]. In the case of tertiary amines, which possess no hydrogen atom or any weak amine nucleophiles, such as sterically hindered amines, this group of amines does not directly react with CO_2. Hence, water or other strong nucleophiles will attack the electrophilic CO_2 and generate bicarbonate/carbonate, as shown in Equation

(5) [68–71]. The reaction mechanism between CO_2 and amine, whether in a solution or solid adsorbent under dry or humid conditions, depends on the type of nucleophile, the Bronsted base, and the stabilizing component. The strong, stable interaction that forms between CO_2 and amine is the main reason why amine polymer is widely used. Numerous studies have reported the use of amine polymers combined with other porous materials, highlighting their remarkable potential as CO_2 adsorbents. However, extensive research must be done to modify the adsorbents so that they can fulfill this application. Suresh et al. [72] reported that based on an electron density difference map analysis, CO_2 was capable of interacting with oxygen molecules in amide carbonyl via Lewis acid-base reaction. They also reported weak H bonds with phenyl C−H and π–π interactions with the aromatic phenyl group [72]. An intermolecular interaction can form between CTF and CO_2 through the nitrogen-based functional group. Lee et al. [73] and Tan et al. [74] each reported a theoretical study of the intermolecular interaction between melamine and CO_2. The nitrogen atoms in the melamine molecule and the C in the CO_2 molecule (N-C) can interact, including the hydrogen bonds between the H-amine and the O-CO_2 molecule [73,74]. Tumnantong et al. [75] suggested that the adsorption mechanism of the synthesized modified natural rubber (MNR) composite was a combination of physisorption and chemisorption. This was further proven by the kinetic study conducted by the group, where Avrami's kinetic model fitted well with the adsorption kinetics data, showing a high R^2 value (>0.95). The Avrami exponent, n_a reflects the changes in the mechanism during adsorption. In the MNR foam composites, the values ranged from 1.0535 to 1.2469, indicating a complex reaction mechanism.

Application of molecular modeling in adsorption mechanisms

The Hohenberg-Kohn principle is the foundation of the density functional theory (DFT) approach. The electron density function is utilized in molecular simulation. This approach has low computing complexity and can determine the chemical bond energy, forecast the structures of compounds, and predict reaction processes. Table 2 presents a summary of DFT applications in studying the interaction between CO_2 and the polymer. Schukraft et al. [76] conducted a study on the interaction of adsorption sites in triazine-biphenyl HCP with CO_2 adsorption using DFT and in situ diffuse reflectance infrared Fourier transform spectroscopy. The study indicated that the strong intermolecular interaction between HCP and CO_2 was confirmed and compared using experimental infrared spectra and theoretical vibrational bands during CO_2 adsorption.

A study by Dash [66] involved the calculation of binding energy (BE) to study the interaction between a COF polymer containing a triazine group building block and CO_2 using DFT simulation. The results indicate that the BE between the triazine benzene and CO_2 complex is higher than the BE of the triazine benzene and N_2 complex, with approximately 18.1 kJ/mol and 13.47 kJ/mol, respectively. These differences are due to electrostatic interactions. These electrostatic interactions can be classified as dipole-quadrupole and dipole-induced dipole interactions. Moreover, the formation of hydrogen bonds can be explained by the interaction between the hydrogen atom of the aromatic ring and the oxygen atom of CO_2. The strength of the hydrogen bond increases with a greater number of aromatic rings [66]. Xu et al. [77] described the interaction between CO_2 and a polyimide matrix through molecular dynamic simulations. Their analysis through the radial distribution function revealed that CO_2 has greater intermolecular affinity with the polyimide chain; in addition, O and N atoms in the imide groups are identified as the preferred sorption sites for CO_2 molecules [77]. Zhang et al. [78] used four organic amines with different properties to prepare amine-functionalized X-5 adsorbents using the impregnation method. The study involved the strategy of matching mechanisms between different organic amines and porous resins for CO_2 adsorption using DFT calculations to investigate the interaction between the amine and CO_2.

Table 2 Summary of DFT applications in investigating CO_2 interaction mechanism.

Polymer	Purpose	Approach	Software	Ref
Amide-functionalized microporous organic polymer	To analyze CO_2 molecule interactions with amide functional groups via Lewis acid-base type interactions	DFT, geometry optimization by M06-2X/cc-pVTZ level of theory	Gaussian 09 program	[72]
Poly(1-vinylimidazole)	To study how CO_2 interacts with oligomers of n-VIm, where 'n' represents the number of monomers (from 1 to 4)	DFT, geometry optimization by B3LYP–D3/6–311++G* (d, p) level	Gaussian 03 program	[79]
α- and β-Polyvinylidene fluoride (PVDF)-supported imidazolium and pyridinium	To investigate the interaction of PVDF/ionic liquid (IL)-based system with binary mixtures of CO_2, CO, CH_4, and H_2 gases (the gas adsorption interactions involving PVDF tetramers, ILs, and the combinations of PVDF/IL complexes are elucidated by considering various quantum chemical descriptors)	DFT, geometry optimization by B3LYP, along with triple zeta basis set 6-311+G (d, p)	Gaussian 16 program and Gaussview 6.0	[80]
Polyvinyl chloride (PVC)/ polyvinyl imidazole (PVIm)	To analyze the interaction of CO_2 and methane gases with PVC and PVIm	DFT, geometry optimization by B3LYP–D3/6–311++G* (d, p) level	Gaussian 03 program	[81]
Graphene oxide/ polymer composite	To analyze the interaction of CO_2 and nitrogen gases with GO/polymer composite complex	Geometry optimization by DFT using the long-range corrected ωB97XD functional 39 together with the 6–31+G(d) basis set	Gaussian 16 package	[82]

Conclusion and future direction

This review discusses the preparation methods for three types of nitrogen-rich polymers, namely amine-functionalized polymer, amide- and imide-functionalized polymer, and covalent triazine framework. Several limitations, as well as advantages, have been identified in the implementation of these methods. Impregnating silica with high amine loading can cause agglomeration of the adsorbent, which may impact its adsorption capacity. This impregnation method can also suffer from leaching and degradation of amine during the regeneration cycle, which can affect the stability of the adsorbent. Despite these drawbacks, the sample preparation is easy and uses inexpensive chemicals (silica and PEI) compared to other methods. On the other hand, surface grafting is quite costly and requires complex preparation procedures. Nonetheless, this technique can overcome amine leaching, dissolution, and deactivation of the adsorbent. In-depth modification and cross-linking of amine can improve the stability of the adsorbent, even after ten regeneration cycles. Meanwhile, the use of HIPE template during polymerization can produce excellent, stable porous solid amine adsorbents with a three-dimensional interpenetrating network. The monolithic polymer produced from the HIPE template has a greater potential for industrial applications compared to powder adsorbent. This polymer also possesses a high pore

volume and specific surface area. However, the abundance of specific surface area will not benefit CO_2 adsorption as mesopore structures are favored for CO_2 adsorption. Free radical polymerization in the preparation of molecularly imprinted polymer can produce simple and highly selective tailor-made polymers. However, the use of solvents during the washing step can have an impact on the environment. This issue can be addressed by using a greener solvent or reducing the consumption of fresh solvent through recycling or using tools that can assist in the washing step. The use of imide-functionalized polymers in the polycondensation method can produce highly porous polymers with improved adsorption capacity and stability. The disadvantage of this method is that the chemical used is quite expensive and the preparation is complex. In the future, more research related to the formulation and design of materials on a large scale should be emphasized. In-depth analysis should also be considered to determine the cost of preparing the materials and the process involved during the adsorption of CO_2 onto adsorbents. The long-term stability and life-cycle assessment of adsorbents should be studied to determine their environmental impact before undergoing scalable production. Molecular modeling has proven to be a valuable approach for studying CO_2 adsorption and its interaction with various functional materials. The findings reveal that investigations at the molecular level can enhance the understanding of CO_2 interaction mechanisms. This study can greatly contribute to designing and optimizing functional polymers for carbon adsorption and storage purposes.

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THE CONVERSION OF CELLULOSE TO GLUCOSE USING NOBLE METAL PLATINUM SUPPORTED ON ZEOLITE

(Penukaran Selulosa kepada Glukosa Menggunakan Mangkin Logam Adi Platinum Disokong pada Zeolit)

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Abstract

Today, the chemical industry faces tremendous pressure to make breakthroughs in green materials, biofuels, and sustainable chemicals. In fact, the goal is to move industrial methods and technologies toward those that are cost-effective and environmentally friendly. Chemical transformation of renewable resources is essential to ensure the long-term sustainability of chemicals and to make sure that energy is sufficient for society in the future. The focus of this study was on the conversion of cellulose to glucose using platinum supported on zeolite as a catalyst. Wet impregnation method was employed to synthesize the catalyst, which was then characterized by thermogravimetric analysis (TGA), Fourier transform infrared (FTIR) spectroscopy, and Brunauer-Emmett-Teller (BET) analysis. According to the catalyst characterization performed using TGA, the nitrate precursors of the catalyst decomposed at temperatures between 200 and 280 °C. The finding was verified by FTIR, which showed a diminishing intensity of the peak around 1450–1300 cm^{-1}, indicating that the catalyst was free of precursors. Subsequently, the catalyst was calcined at 500 °C for 5 h. It is noteworthy that this catalyst has a large surface area of up to 648.13 m^2/g. A catalytic reaction study on the conversion of cellulose to glucose was carried out to investigate several factors, including substrate loading and catalyst loading. It was discovered that 0.4 g of cellulose and 0.1 g of platinum supported on zeolite contributed to a high yield of glucose and conversion of cellulose (22.07% and 54.9%, respectively). The study shows that the chemical transformation of cellulose to glucose is possible with the supported noble metal catalyst.

Keywords: cellulose, glucose, heterogeneous catalyst, platinum supported on zeolite, supported noble metal catalyst

Abstrak

Industri kimia pada masa kini menghadapi tekanan yang sangat besar untuk melakukan penemuan dalam bahan hijau, bahan bakar-bio, dan bahan kimia yang mapan. Malah, matlamatnya adalah untuk menggerakkan kaedah dan teknologi perindustrian ke arah yang kos efektif dan mesra alam. Transformasi kimia bagi bahan boleh diperbaharui adalah penting untuk memastikan keberlanjutan jangka panjang bahan kimia dan tenaga yang mencukupi untuk masyarakat pada masa hadapan. Oleh itu, fokus kajian ini adalah kepada penukaran selulosa kepada glukosa menggunakan platinum yang disokong pada zeolit sebagai mangkin. Kaedah pengisitepuan basah digunakan untuk mensintesis mangkin, dan ia kemudiannya dicirikan oleh analisis termogravimetri

(TGA), spektroskopi inframerah transformasi Fourier (FTIR), dan analisis Brunauer-Emmett-Teller (BET). Menurut pencirian mangkin yang dilakukan menggunakan TGA, prekursor nitrat daripada mangkin boleh diuraikan pada suhu antara 200 dan 280 °C. Penemuan ini telah disahkan oleh FTIR yang menunjukkan keamatan puncak yang semakin berkurangan sekitar 1450–1300 cm^{-1}, menunjukkan mangkin adalah bebas daripada prekursor. Seterusnya, mangkin dikalsin pada 500 °C selama 5 jam. Selain itu, mangkin ini didapati mempunyai luas permukaan yang besar sehingga 648.13 m^2/g. Kajian tindak balas mangkin mengenai penukaran selulosa kepada glukosa telah dijalankan untuk menyiasat beberapa faktor, termasuk pemuatan substrat dan pemuatan mangkin. 0.4 g selulosa dan 0.1 g platinum yang disokong pada zeolit didapati menyumbang kepada hasil glukosa dan penukaran selulosa yang tinggi (masing-masing 22.07% dan 54.9%). Kajian menunjukkan bahawa transformasi kimia selulosa kepada glukosa menunjukkan potensi dengan pemangkin logam adi yang disokong.

Kata kunci: selulosa. glukosa, mangkin heterogen, platinum disokong pada zeolit, mangkin logam adi sokongan

Introduction

The conversion of cellulose, the most abundant biomass on Earth, into valuable chemicals such as glucose, has gained significant attention in recent years due to its potential as a sustainable alternative to traditional fossil fuel-based processes [1]. The preference for utilizing biomass-derived resources is because these feedstocks do not compete with the food supply [2].

Glucose is utilized as a starting chemical in the chemical industry to produce various valuable compounds through biochemical and chemical processes. It serves as a renewable and abundant source for the synthesis of numerous products, including 5-hydroxymethylfurfural, levulinic acid, ethanol, organic acids (e.g., citric acid and gluconic acid), amino acids (e.g., lysine and glutamic acid), and polyols (e.g., sorbitol and xylitol) [3]. Glucose is also a key precursor in the synthesis of pharmaceuticals, polymers, and bio-based materials. Its versatility and availability make it an important starting material for diverse applications in the chemical industry, contributing to sustainable and environmentally friendly production processes.

Catalytic systems play a crucial role in facilitating this conversion, and researchers are continually exploring new catalysts to enhance the efficiency and selectivity of the reaction. One promising approach is the utilization of supported noble metal catalysts as heterogeneous catalysts, which have demonstrated remarkable performance in various biomass conversion reactions. Previously, homogeneous catalysts such as mineral acids, acidic ionic liquids, heteropolyacids, and Lewis acids (e.g., Al, Zr, and Cr salts) were utilized for cellulose degradation. Despite their high efficiency in catalyzing cellulose conversion, homogeneous catalysts pose several challenges, including equipment corrosion, high separation costs, and environmental pollution. Although homogeneous catalysts are extensively utilized in industry, they have inherent drawbacks that add complexity to the process. These drawbacks include low stability, difficulties in isolating and separating the final product from the reaction mixture, and the need for recycling expensive noble metals or ligands [4].

Zeolites are crystalline aluminosilicates with well-defined nanoporous structures, which possess both Brønsted and Lewis acid sites, provide a high surface area and shape-selective properties, and offer excellent support for catalytic species [5]. Due to their adjustable acidity, exceptional thermal stability, and impressive shape-selective properties, zeolites have proven effective in dehydrating cellulose in water. The porous nature of zeolites also allows for easy access to reactants and products, facilitating efficient mass transfer. The incorporation of noble metals, such as platinum (Pt), palladium (Pd), and gold (Au) onto zeolite, on the other hand, results in unique catalytic properties, such as high activity, selectivity, and resistance to deactivation, making them ideal candidates for biomass conversion [6].

Several recent journal articles have highlighted the effectiveness of noble metals supported on zeolite catalysts for cellulose conversion. For example, Negoi et al. investigated the catalytic performance of noble metal-supported zeolites in the hydrolysis of cellulose to sorbitol [7]. The authors demonstrated that the catalyst exhibited remarkable activity and selectivity (85%) toward sorbitol production, with a yield of over 50%

under mild reaction conditions. In another study, Ya'Aini et al. employed zeolite as the catalyst for converting glucose to levulinic acid (LA) [8]. The researchers observed that the catalyst exhibited excellent stability and selectivity, achieving a high LA yield of 65% under optimized reaction conditions. The catalyst also demonstrated good recyclability, making it a promising candidate for industrial-scale applications.

These recent findings underscore the potential of noble metals supported on zeolite as heterogeneous catalysts for the conversion of cellulose to glucose. The combination of noble metals' unique catalytic properties with the advantageous features of zeolite support holds great promise for the development of efficient and sustainable processes for biomass utilization. Hence, in this study, we investigated the impact of catalyst and cellulose loading on the conversion of cellulose to glucose, utilizing Pt supported on zeolite. Further research and optimization of these catalyst systems are expected to pave the way for the commercial production of valuable chemicals from cellulose, contributing to the advancement of green chemistry and the transition toward a more sustainable future.

Materials and Methods

Materials

The catalyst was synthesized using platinum (IV) nitrate ($Pt(NO_3)_2$) with 15% w/w of Pt and zeolite Y purchased from Sigma-Aldrich. For the reaction work, microcrystalline cellulose (extra pure, 100%) from Acros Organics and glucose (>99.9%) were employed as the substrate and for standard reference calibration purposes, respectively. All the chemicals were of analytical grade and used without further purification unless otherwise stated. Distilled water was utilized for all the experiments.

Catalyst synthesis

The catalyst was synthesized through the wet impregnation method, which aimed to bind the active sites to the support material. $Pt(NO_3)_2$ and zeolite Y were employed for catalyst preparation. The objective was to achieve a Pt content of 4 wt.% on the zeolite support. Initially, the zeolite was weighed using an analytical balance and evenly distributed on a Petri dish. Subsequently, 1.3g of $Pt(NO_3)_2$ was diluted with 2 mL of distilled water. The Pt solution was then dropped onto the zeolite sample in four separate batches using a syringe. During each application, the first 0.5 mL of the Pt solution was dropped in a circular motion, and the dispersion was carefully spread using a spatula. These steps were repeated until the entire Pt solution had been used. Following the impregnation process, the catalyst was dried in an oven at 120 °C overnight to eliminate any residual water content. Subsequently, the catalyst underwent calcination in a furnace for a duration of 5 h at a temperature of 500 °C. Finally, the catalyst was allowed to cool in a desiccator and then stored in a vial for future use.

Catalyst characterization

The catalyst underwent characterization through several analytical techniques, including thermogravimetric analysis (TGA), Fourier transform infrared (FTIR) spectroscopy, and Brunauer-Emmett-Teller (BET) analysis. A Mettler-Toledo STARe software system was used for TGA. Approximately 5–10 mg of the catalyst was weighed using an analytical balance and placed in an alumina crucible. The analysis was conducted over a temperature range from ambient (30 °C) to 600 °C, utilizing a heating rate of 15 °C/min and a continuous flow of high-purity air at 20 mL/min. The FTIR analysis was carried out using a Nicolet iS10 FTIR spectroscopy instrument, with a spectral range from 4000 cm^{-1} to 400 cm^{-1}. The spectral resolution was set at 8 cm^{-1}, and five scans were accumulated under an open-beam air background at room temperature. A Micromeritics 3Flex surface characterization analyzer was used to determine the surface area and pore volume of the catalyst. Prior to analysis, the catalyst was degassed for 12 h at 150 °C in nitrogen (N_2).

Reaction study

The pre-reduction of the catalyst was conducted in a 20 mL stainless steel batch reactor. Initially, 0.1g of catalyst and 10 mL of distilled water were loaded into the reactor. The pre-reduction process was carried out under conditions of 1 bar of hydrogen (H_2) at a temperature of 150 °C for a duration of 1 hr. Following the pre-reduction of the catalyst, 0.4g of cellulose was introduced into the reactor in preparation for the

subsequent reaction. The reactor was purged three times with N_2 to remove any residual air. Subsequently, the reactor was placed in an oil bath on a magnetic stirring hot plate. Once the desired temperature was reached, the stirrer and timer were set to initiate the catalytic reaction. The reactor was rapidly cooled by immersing it in a water bath to halt the reaction. The resulting product was collected and subjected to filtration. Liquid products were filtered using cotton wool, while solid products were filtered through filter paper. Following the filtration process, the liquid product was stored in a vial in preparation for high-performance liquid chromatography (HPLC) analysis. For the solid product, the filter paper was dried in an oven for 12 h at 80 °C, and the weights of the filter paper before and after drying were recorded to calculate cellulose conversion.

Product analysis

The liquid products obtained from the reaction were identified and quantified through HPLC with a refractive index detector. For this analysis, a mobile phase of 0.005 M sulfuric acid with a flow rate of 0.5 mL/min was utilized. The column temperature was maintained at 40 °C throughout the analysis.

Results and Discussion

Catalyst characterization: TGA

Based on the TGA data presented in Figure 1, two distinct weight losses were observed in the temperature ranges of 50–100 °C (I) and 210–280 °C (II) for the catalyst prior to calcination. The initial weight loss can be attributed to the removal of adsorbed water [9, 10], while the subsequent weight loss is associated with the decomposition of nitrates and the elimination of organic compounds [11, 12]. Notably, beyond 300 °C, no additional weight loss was noted, indicating the absence of further decomposition. Consequently, a calcination temperature of 500 °C was selected to ensure complete decomposition of nitrate precursors.

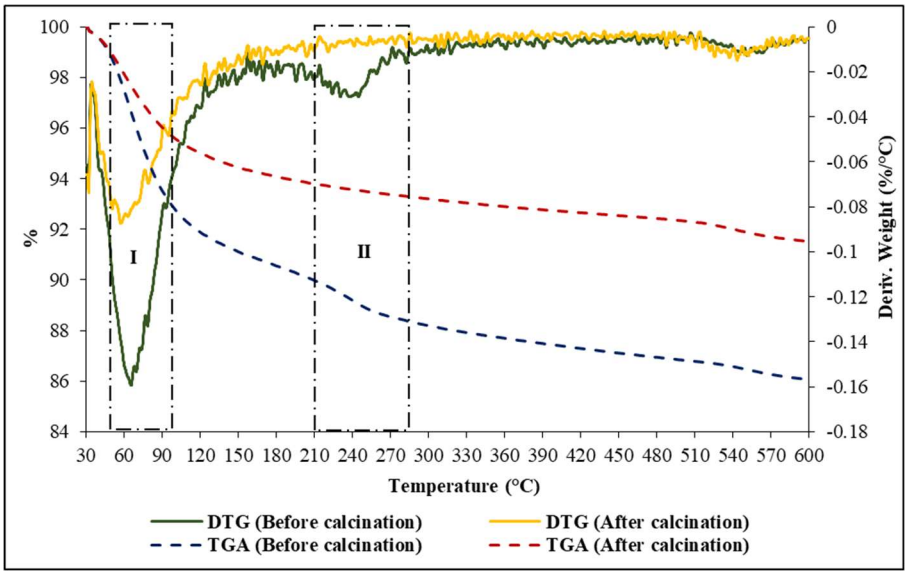


Figure 1. TGA and DTG of Pt/zeolite (before and after calcination)

Following the calcination process, the catalyst exhibited a single weight loss, which occurred within the temperature range of 50–100 °C, as indicated by the TGA pattern in Figure 1. Importantly, no weight loss was detected at temperatures exceeding 100 °C, signifying the complete decomposition of nitrates. Therefore, it is noteworthy that the optimal calcination temperature for achieving a precursor-free catalyst is 500 °C [13]. This observation aligns with the FTIR analysis results, which reveal the absence of nitrate peaks in the catalyst after calcination.

Catalyst characterization: FTIR

According to the FTIR spectra presented in Figure 2, the

spectral region from 3600 to 3200 cm^{-1} (I) is associated with the stretching and bending modes related to water molecules and their hydrogen bonding [14,15]. The O–H stretch bands observed at 3600–3200 cm^{-1} in the catalyst before calcination appear broader compared to those in the catalyst after calcination, owing to the expansion and contraction absorption of hydrogen bonds. This finding is supported by Jamal et al. [16], who also observed widened bands in their study. They examined various calcination temperatures and noted that lower temperatures resulted in broader bands at 3600–3200 cm^{-1}. This broadening effect is attributed to a decrease in the peak mode of O–H bond elongation and bending as the calcination temperature rises, which could reduce the absorption of water molecules on the catalyst surface.

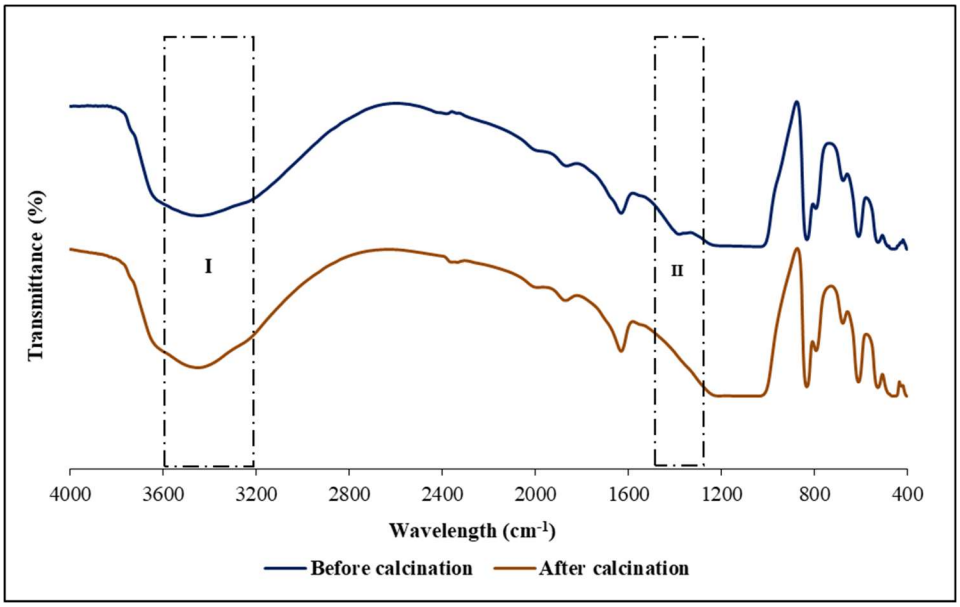


Figure 2. FTIR spectra of Pt/zeolite (before and after calcination)

In contrast, the spectral region between 1450 and 1300 cm^{-1} (II) signifies the presence of nitrate precursor ions (NO_3^-) within the catalyst prior to calcination [15,17]. Significantly, these NO_3^- bands were observed to disappear in the catalyst after undergoing calcination, providing clear evidence that nitrate decomposition was effectively accomplished at the calcination temperature of 500 °C. Consequently, it is important to highlight that the optimal temperature for eliminating the nitrate precursor is 500 °C.

Catalyst characterization: BET

Based on Table 1, the surface area and pore volume of the catalyst before calcination were notably high (665.40 m^2/g and 0.3575 cm^3/g, respectively). However, these values experienced a slight decrease after catalyst calcination. Nevertheless, the reduction observed in both surface area and pore volume amounted to only 17.21 m^2/g and 0.0128 cm^3/g, respectively. According to the findings by Baharudin et al., this decrease in surface area can be attributed to particle aggregation during calcination [18]. It is worth noting that despite the reduction in surface area and pore volume due to calcination, this process has the potential to maintain the structural stability of the catalyst [19]. Furthermore, it is important to highlight that even after calcination, the catalyst still retains a substantial surface area and pore volume, measuring up to 648.13 m^2/g and 0.3447 cm^3/g, respectively, in comparison to previous research [13].

Table 1. BET surface area and pore volume of catalysts

Sample	Surface Area[a] (m^2/g)	Pore Volume[b] (cm^3/g)
Pt/zeolite (Before calcination)	665.40	0.3575
Pd/zeolite (After calcination)	648.13	0.3447

[a]: Surface area was obtained from the BET method, [b]: Pore volume was obtained from the BET method.

Effect of cellulose loading on the yield of glucose and conversion of cellulose

As illustrated in Figure 3, it becomes evident that as the cellulose loading increases from 0.2g to 0.3g, both the yield of glucose and the conversion of cellulose exhibit a consistent increase. However, when cellulose loading further increased from 0.4g to 0.5g, there is a noticeable decrease in glucose yield and cellulose conversion. This observed trend aligns with the outcomes of prior studies conducted by Joshi et al. and Zhang et al. [12,20]. Several factors may contribute to this condition, including potential product feedback inhibition, a reduction in reactivity, or an insufficient dosage of catalyst for increased cellulose content. Moreover, higher cellulose loading might increase the likelihood of reactive compounds colliding and initiating cross-polymerization, leading to the formation of undesired products [21]. Consequently, the increase in cellulose loading ultimately results in the saturation of the catalyst's optimal surface area and active site availability.

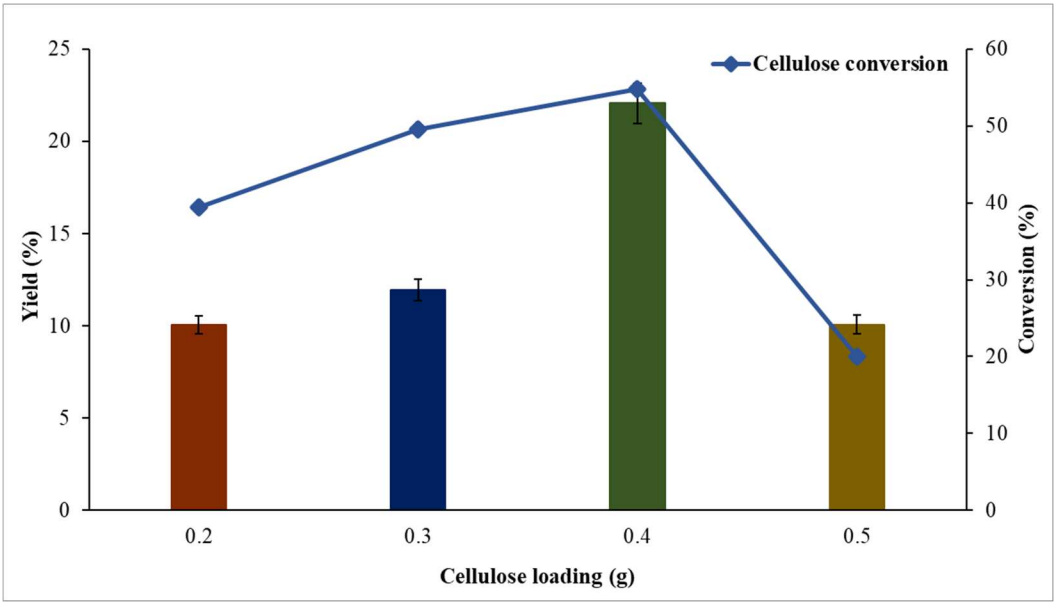


Figure 3. Effect of cellulose loading on the yield of glucose and cellulose conversion at 15 min

Note: a) Pre-reduction of catalyst conditions: 10 mL of distilled water, agitation speed of 1300 rpm, and 1 bar of H_2 for 1 h at 150 °C, b) Reaction conditions: 0.1 g of cellulose, reaction temperature of 210 °C, agitation speed of 1100 rpm, and reaction time of 15 min.

Effect of catalyst loading on the yield of glucose and conversion of cellulose

As shown in Figure 4, the highest cellulose conversion and glucose yield were 54.9% and 22.1%, respectively, when 0.1g of catalyst was employed in the reaction. It is worth noting that as the catalyst loading increased from 0.04g to 0.1g, both glucose yield and cellulose conversion also exhibited an increasing trend. This condition can be attributed to the increasing availability of active sites on the catalyst, which allows more

cellulose to enter and break down into the desired product at an accelerated rate. The lowest glucose production was observed when using 0.04g of the catalyst due to the limited number of active sites available to efficiently convert cellulose into glucose. This observation aligns with findings from other studies, such as those conducted by Wang et al. [21] and Zhang et al. [20], which demonstrated that increasing catalyst loading favored higher product yields. However, Kamal et al. found an opposite trend, where an increase in catalyst loading led to a decrease in glucose yield and cellulose conversion [22]. This might be attributed to unwanted side reactions occurring at higher catalyst loadings. Additionally, high catalyst loading could increase the viscosity of the reaction mixture, resulting in a slower reaction [23]. This leads to reduced mixing efficiency and increased mass transfer resistance within the mixture, ultimately limiting the availability of cellulose for the reaction. Consequently, it is important to refrain from adding additional catalyst once the required amount for the reaction has been reached.

The introduction of the catalyst accelerates the progress of the reaction. The use of the catalyst does not alter the equilibrium constant of the reaction; instead, it accelerates both the forward and reverse processes. This is achieved by providing an alternative pathway with a lower energy barrier, thus facilitating the production of the desired product [24]. As a point of reference, a control experiment without the use of a catalyst was conducted under the optimal conditions outlined in this paper. Without the catalyst, the glucose yield and cellulose conversion were at their lowest levels, which were 4.2% and 2.8%, respectively.

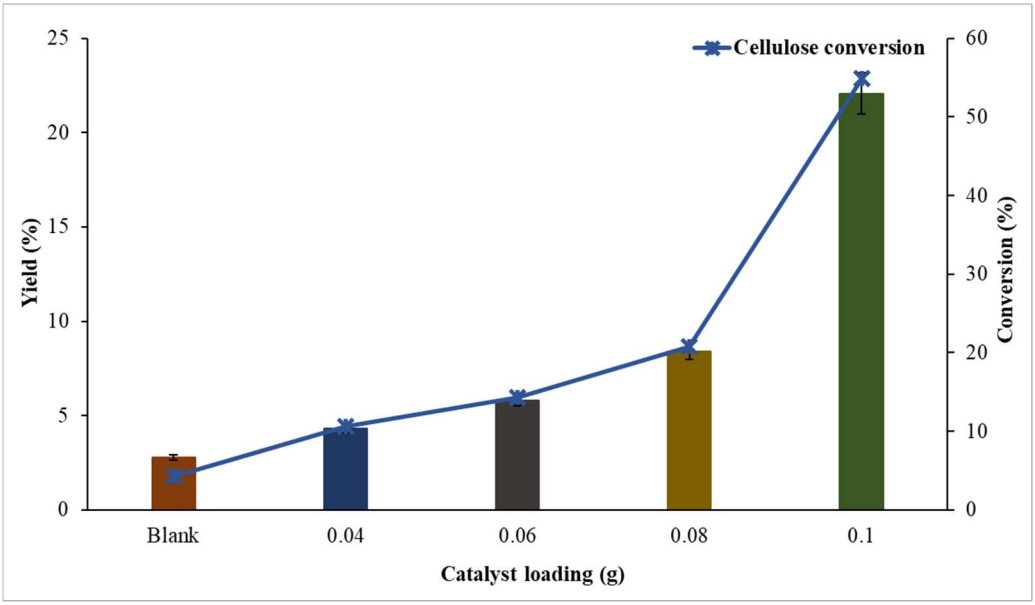


Figure 4. Effect of catalyst loading on the yield of glucose and cellulose conversion at 15 min

Note: a) Pre-reduction of catalyst conditions: 10 mL of distilled water, agitation speed of 1300 rpm, and 1 bar of H_2 for 1 h at 150 °C, b) Reaction conditions: 0.4 g of cellulose, reaction temperature of 210 °C, agitation speed of 1100 rpm, and reaction time of 15 min.

Conclusion

The catalyst synthesis involved $Pt(NO_3)_2$ and zeolite, aiming for a Pt content of 4 wt.% on the zeolite support prepared via the wet impregnation method. Catalyst characterization was performed using TGA, FTIR, and BET analysis. First, TGA was conducted from 30 °C to 600 °C, and the results revealed that the weight losses are attributable to water and nitrate decomposition. Subsequently, the FTIR analysis detected nitrate bands prior to calcination, which disappeared after calcination, confirming successful nitrate decomposition. Despite minor decreases, the catalyst maintained a substantial surface area and pore volume of up to 648.13 m^2/g and 0.3447 cm^3/g, respectively, affirming its stable structure.

In addition, the influence of cellulose and catalyst loading on glucose yield and cellulose conversion was explored. The results revealed that the optimal conditions for maximizing glucose yield and cellulose conversion were achieved when employing 0.4g of cellulose and 0.1g of catalyst, resulting in yield and conversion of 22.1% and 54.9%, respectively. These findings underscore the important role of catalyst selection and loading in achieving efficient biomass conversion processes, which contribute to the advancement of sustainable and eco-friendly chemical production.

Acknowledgement

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SYNTHESIS AND MODELLING OF FUNCTIONALIZED UiO-66 METAL-ORGANIC FRAMEWORKS FOR GAS ADSORPTION

(Sintesis dan Pemodelan Rangkaian Organik-Logam UiO-66 Terfungsi bagi Penjerapan Gas)

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Abstract

In this article, we present our experimental and computational findings on the adsorption of ethylene gas within variations of UiO-66 metal-organic frameworks (MOFs). UiO-66 MOF was functionalized with 2-vinylbenzoic acid (2VBA), 3-vinylbenzoic acid (3VBA), and 4-vinylbenzoic acid (4VBA) and characterized using powder X-ray diffraction techniques, FT-IR analysis and ^{1}H NMR. Based on structural analysis, the functionalized UiO-66 MOFs were found to possess the same structural stability as the 3D porous crystalline lattices of UiO-66, with hexanuclear zirconium oxyhydroxide clusters ($Zr_6O_4(OH)_4(RCO_2)_{12}$. Grand Canonical Monte Carlo simulation showed that the average loading of ethylene gas in UiO-66 and functionalized UiO-66 were relatively close. The adsorption was further investigated using radial distribution function analysis and it was found that the most favorable interactions were between the adsorbate and the carbon atom of the MOFs. These interactions were crucial for ethylene stabilization inside UiO-66 pore channels. The results presented here supported the viability of ethylene adsorption in UiO-66's modified terephthalate linker by demonstrating that functionalized UiO-66 not only retain structural integrity, but also have comparable ethylene adsorption at different pressures with stronger interactions with ethylene gas.
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Keywords: metal organic framework, gas adsorption, UiO-66, Grand Canonical Monte Carlo

Abstrak

Dalam artikel ini, kami melaporkan kerja eksperimen dan hasil pengiraan untuk penjerapan gas etilena dalam rangkaian logam organik (MOF) UiO-66. UiO-66 MOF difungsikan dengan asid 2-vinilbenzoik (2VBA), asid 3-vinilbenzoik (3VBA), dan asid 4-vinilbenzoik (4VBA) serta dicirikan menggunakan teknik pembelauan sinar-X serbuk, analisis FT-IR dan ^{1}H NMR. Berdasarkan analisis struktur, UiO-66 MOF yang difungsikan didapati mempunyai kestabilan struktur yang sama seperti kekisi kristal berliang 3D UiO-66, dengan gugusan zirkonium oksihidroksida heksanuklear ($Zr_6O_4(OH)_4(RCO_2)_{12}$. Grand Canonical Monte Carlo simulasi menunjukkan purata pemuatan gas etilena dalam UiO-66 dan UiO-66 yang difungsikan adalah agak hampir hasilnya.

Penjerapan disiasat lebih lanjut menggunakan analisis fungsi taburan jejari dan didapati bahawa interaksi yang paling baik adalah antara penjerap dan atom karbon MOFs. Interaksi ini adalah penting untuk penstabilan etilena di dalam saluran liang UiO-66. Keputusan yang dibentangkan di sini menyokong daya maju penjerapan etilena dalam penyambung tereftalat yang diubah suai UiO-66 dengan menunjukkan bahawa UiO-66 yang difungsikan bukan sahaja dapat mengekalkan integriti struktur, tetapi juga mempunyai penjerapan etilena yang setanding pada tekanan yang berbeza dengan interaksi yang lebih kuat dengan gas etilena.

Kata kunci: rangkaian logam organik, penjerapan gas, UiO-66, Grand Canonical Monte Carlo

Introduction

Fast ripening and pest attack are the two most common causes of food loss in climacteric fruits. Food losses can also occur as a result of insect pest attacks in stored agricultural products. Every season, the Muda Agricultural Development Authority (MADA) estimates that RM 980 million is lost in postharvest rice crop losses [1]. As a result, solutions for controlling the ripening process and securing product storage prior to consumption are in great demand. Ethylene gas usage causes fast ripening and promotes fungal growth during storage, reducing fruit quality and shelf life [2]. As a result, for effective long-distance transportation and subsequent storage, many fruits must be kept in a low ethylene concentration environment due to its adverse effect on postharvest fruit. This has led to a great deal of research on ways to enhance ethylene adsorption and storage.

Materials used for reducing postharvest losses must be applied carefully to the targeted climacteric fruits and staple foods. Furthermore, the material's biocompatibility must be considered and created from non-toxic materials. The environmental implications, including the synthesis technique and disposal features, must also be acknowledged. For the material to be employed in food storage applications, it should ideally be water soluble or biodegradable. Ethylene gas adsorption is frequently based on extremely porous adsorbents with large surface areas, such as clays and zeolites [3]. The first porous material known to be capable of encapsulating ethylene was zeolite [4]. However, the tiny pore size of zeolite creates a significant limitation in its application on gaseous molecule exchange and release [5].

Reticular materials, or metal-organic frameworks (MOFs), are a class of functional material with unique chemical and physical characteristics. It has long been known that MOFs, a kind of crystalline, porous material, are ideal host platforms for gas adsorption [6]. For example, copper-terephthalate (CuTPA) MOF has a total pore capacity of 0.39 cm^3/g and can encapsulate 654 L/L ethylene gas in a 50 mg section of the material. When stored in a 4 L container, CuTPA loaded with ethylene dramatically accelerated the ripening of bananas and avocados [7]. One of the most used MOFs for adsorption studies is UiO-66 (Figure 1a). Numerous studies have been conducted on the adsorption of CO_2 on UiO-66, and the results have been promising [8,9]. At 298 K and 100 kPa, Le et al. (2021) discovered that UiO-66 modified with 10% PABA had the greatest CO_2 absorption capacity of 2.47 mmol g^{-1} and ideal adsorbed solution theory (IAST)-based CO_2/N_2 selectivity, outperforming several other CO_2 benchmark adsorbents [10].

The UiO-66 metal-organic framework (MOF) has high surface area, variable pore size, and good chemical stability, making it a promising material for a wide range of applications. UiO-66 consists of 1,4-benzenedicarboxylic acid (BDC) as ligands connected by Zr_6O_8 as a metal cluster. Additionally, UiO-66 possesses easily accessible metal sites, which indicates that this material has potential applications in gas storage and separation. Due to its large specific surface area, which ranges from 1,000 to 1,500 m^2/g, UiO-66 is a great choice for gas adsorption such as ethylene gas [11]. Furthermore, UiO-66 has been shown to be resistant to high temperatures (up to 500°C) and a broad range of chemical conditions, making it perfect for the adsorption of gases. By altering the metal ions or organic linkers, UiO-66's pore size may also be changed, allowing for the selective adsorption of different gas molecules [11]. Additionally, the functionalized UiO-66 can also help to increase the gasses loading and adsorption capacity. Due to UiO-66 unique properties, a great deal of research has been conducted on UiO-66

and UiO-66 mixed linker, which have been used for a range of gas adsorption and separation techniques, such as the storage of gasses, and the capture of numerous gases including ethylene, carbon dioxide, and many others. For a variety of gas-related applications, UiO-66 is a good choice due to its versatility and tunable properties.

Here, to improve adsorption capability, the UiO-66 terephthalate linker was modified with 2-vinylbenzoic acid, 3-vinylbenzoic acid and 4-vinylbenzoic acid (Figure 1c-1e). UiO-66 and functionalized UiO-66 MOFs were synthesized by using solvothermal method. Powder X-ray diffraction techniques, FTIR analysis and ^{1}H NMR were used to elucidate the resulting structures. The adsorption capacity and host-guest interactions were studied using Grand Canonical Monte Carlo (GCMC) simulation. We were able to illustrate the characteristics and behaviour of ethylene adsorption within UiO-66 and functionalized UiO-66 MOFs using appropriate computational approaches to this system. Modifying the UiO-66 terephthalate linker should boost the ethylene adsorption while simultaneously increasing the number of interactions between host and guest molecules i.e., improved storage.

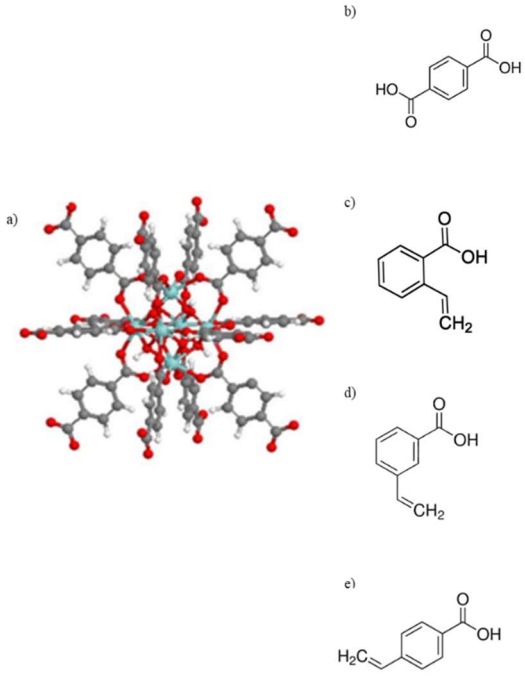


Figure 1. a) Unit cell of UiO-66, b) parent organic linker, c) 2-vinylbenzoic acid d) 3-vinylbenzoic acid, and e) 4-vinylbenzoic acid

Materials and Methods

Materials

All chemicals were obtained commercially (Aldrich) and used without further purification.

Preparation of UiO-66 and functionalized UiO-66

A standard synthesis of UiO-66 was performed by dissolving zirconium tetrachloride, $ZrCl_4$ (0.0625g, 6.4 mmol) in formic acid (2.5mL) and DMF (2.5mL) at room temperature in a volumetric flask. The mixture was stirred for 20 minutes and (0.05g, 0.05mmol) 1,4-dibenzenedicarboxylicacid was added into the mixture. The mixture was stirred again for another 20 minutes. The resulting mixture was placed in an oven at 120 ^{o}C for 24 h. After the solution cooled to room temperature in the air, the resulting solid was filtered and repeatedly washed with absolute ethanol for 3 days. Thus, the resulting white powder was filtered and dried under vacuum at ambient temperature. UiO-66 modified linker with 2-vinylbenzoic acid, 3-vinylbenzoic acid and 4-

vinylbenzoic acid were synthesized analogously by replacing 1,4-dibenzenedicarboxylicacid with ratio of 4:1 of 1,4-dibenzenedicarboxylicacid and modified linker, respectively.

Characterization

Approximately 3 mg of UiO-66 and modified samples were soaked in ethanol dispersed and dried as a thin layer on a glass plate before the PXRD measurements. PXRD data were collected at ambient atmosphere and temperature on a Bruker D5000 instrument with monochromatic Cu Kα radiation (λ=1.540 A˚) operated in Bragg-Bretano geometry. The IR experiments were performed in transmission mode on a FTIR Bruker VERTEX 80 spectrometer equipped with two detectors: a cryogenic MCT detector (spectral range 4000-600 cm^{-1}) for UiO-66 and modified UiO-66 MOFs. The MOFs under study was analyzed in the form of a thin film deposited on a silicon wafer. The thin film was prepared from an ethanol suspension of the samples. Solvent removal spectra were obtained by introducing the silicon wafer in a quartz cell that allowed *in situ* spectrum collection in a controlled atmosphere, and the sample was degassed at 373 K under dynamic vacuum (residual pressure). For the ^{1}H-NMR analysis, 20 mg of the sample were dissolved in a solution of natrium hydroxide, NaOH, and D_2O for a duration of 24 hours. The resultant solution was then utilized using a Bruker Advance DRX-300 NMR Spectrometer (300 MHz) to obtain the ^{1}H-NMR spectra.

Computational details

The UiO-66(Zr) crystal information file (.cif) with deposition number 733458 was obtained from the Cambridge Crystallographic Data Center [12]. The .cif file was utilised to carry out preliminary geometry-based analysis, such as pore sizes, using the BIOVIA Material Studio tool [13]. UiO-66 has the chemical formula [C39.36H23.52O34.80Zr6], and the metal centers, zirconium, Zr were completely coordinated. The unit cell lengths of a, b, and c UiO-66 were 20.7343, 20.7343, and 20.7343, respectively, and the atom arrangement in the unit cell produced a triclinic symmetry. Next, using Material Studio, 2-vinylbenzoic acid, 3-vinylbenzoic acid, and 4-vinylbenzoic acid were used to modify the terephthalate acid UiO-66 linker. Structures were modified by replacing one of the terephthalate linkers with 2-, 3-, or 4-vinylbenzoic acid using the Material Studio's "modify element" tool. The Forcite module was used to carry out energy minimization with a convergence tolerance of 10^{-4} kcal/mol. Furthermore, geometry optimization was performed using the Smart method with maximum iteration numbers of 1000 for all the MOFs series [14].

For geometry optimization, the convergence tolerances for displacement, energy, and force were set to 10^{-6}, 10^{-5}, and 10^{-4} kcal/mol, respectively. The cell parameters of the frameworks were also allowed to be relaxed at this stage. The Universal Force Field potential parameters were used to optimize UiO-66 and the functionalized UiO-66 MOFs [14]. In order to compute the electrostatic interactions between MOF atoms and the gas molecules, partial charge of the framework atoms was calculated using the Qeq (charge equilibration technique) and EQeq (extended charge equilibration method) methods, depending on the existing metal type [15]. GCMC simulations were used to examine the ethylene gas adsorption isotherms in UiO-66 and functionalized UiO-66 MOFs. All simulations were performed using the RASPA [16] package at 293 K [17] with pressures ranging from 0 to 100 kPa. The accessible pore volume and helium (He) void percentage of UiO-66, UiO-66-2VBA, UiO-66-3VBA, and UiO-66-4VBA were determined to be at 0.43, 0.44, 0.44 and 0.44 cm^3/g, respectively. Widom particle insertion was also used to calculate the He void fraction for UiO-66 and functionalized UiO-66, and the resulting values were set in the input file for adsorption simulations. A 12.0 Å cut-off was set for electrostatic and van der Waals interactions. The adsorption simulations used up to 2.5 x 10^5 Monte Carlo cycles, with 5 x 10^4 cycles for the initial equilibrium phase and 2 x 10^5 cycles for the production phase [18-20]. Each Monte Carlo (MC) move was set to having an equal chance of being translated, rotated, inserted, or deleted.

Results and Discussion

Characterizations of UiO-66 and functionalized UiO-66

The powder X-ray diffraction (PXRD) pattern of the synthesized UiO-66 and functionalized UiO-66 MOFs

are shown in Figure 2. The peaks are identical to the one previously reported research [21]. The identical XRD patterns of UiO-66 and functionalized UiO-66 show that these functionalized UiO-66 have the same 3D porous crystalline lattices as UiO-66, with hexanuclear zirconium oxyhydroxide clusters ($Zr_6O_4(OH)_4(RCO_2)_{12}$) and octahedral cages assembled with these modified terephthalate linkers. It was determined that the functionalization did not alter the framework topology of UiO-66(Zr) MOFs. The peak at $2\theta = 7.3°$ corresponds to the (111) crystal plane of the UiO-66 structure. This peak is one of the important peaks for UiO-66 and this peak was considered as fingerprint peak that indicates whether a sample has the UiO-66 framework. When compared to another UiO-66 sample, the peak for UiO-66-2VBA at 7.3° is sharper and more intense, indicating that the UiO-66-2VBA sample has good crystallinity properties. A second notable low-angle peak is seen at approximately $2\theta = 8.4°$, which is the crystal plane (200). Additionally, mid-angle peaks were noted at around $2\theta = 12.0°$, which is the peak associated with the (220). The (311) crystal plane is assigned a peak at about $2\theta = 14.0°$. The (222) crystal plane is thought to be responsible for the peak at about $2\theta = 15.8°$.

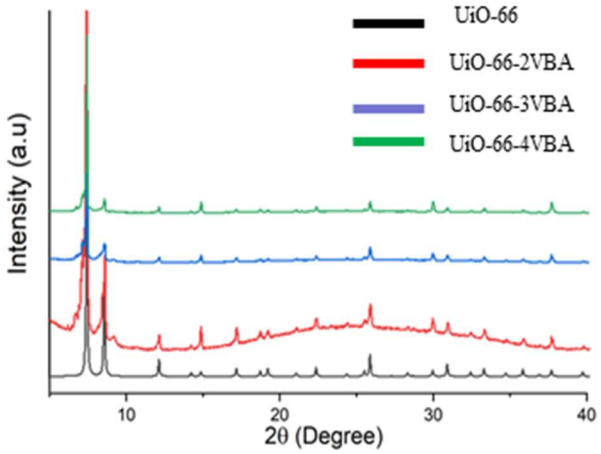


Figure 2. PXRD pattern for UiO-66 and functionalized UiO-66

The FT-IR spectra of UiO-66 and functionalized UiO-66 MOFs is shown in Figure 3. These four UiO-66 samples have IR spectra with comparable distinctive peaks. UiO-66 sample FTIR spectra show multiple major wavelengths. We can see a large peak around 3419 cm^{-1} that indicated -OH group present in the structure. Two highly linked C–O bonds in the carboxylate group of the ligand, BDC, result in two distinct peaks: an asymmetric C–O stretching band at 1586 cm^{-1} and a symmetric C–O stretching band at 1398 cm^{-1}. The C=C of the benzene ring of the ligand in the structure could be the source of the tiny bands at 1504 cm^{-1}. After synthesizing UiO-66 using a mixed linker, the peaks show that the ligand at 1586 cm^{-1} changed somewhat and widened. The existence of several functional groups with various vibrational frequencies, which cause overlapping bands and a broadening effect, is one potential explanation.

Solid state ^1H NMR of the three UiO-66-2VBA, UiO-66-3VBA and UiO-66-4VBA samples also exhibit similar characteristic peaks with the one previously reported [22]. According to Ky Vo et al. (2020) ^1H NMR spectra, UiO-66 had a single signal that was 7.84 ppm in strength and was attributed to the phenyl proton of the BDC ligand [23]. On the other hand, our research indicates that functionalized UiO-66 show three distinct peaks (Figure 4). The ^1H NMR spectra of functionalized UiO-66 show two notable peaks: one at 1.9 ppm, which is related to the vinyl chain, and another at 7.8 ppm, which is related to the aromatic protons in the ligand. The addition of vinyl groups to the UiO-66 framework is linked to the different peaks observed in the ^1H NMR spectra of the functionalized UiO-66. A noteworthy

peak was noted for the functionalized UiO-66. The result of bridging hydroxyl (OH) protons is a wide singlet at 5.5 ppm, which represents the four hydroxyl protons that bridge the zirconium clusters.

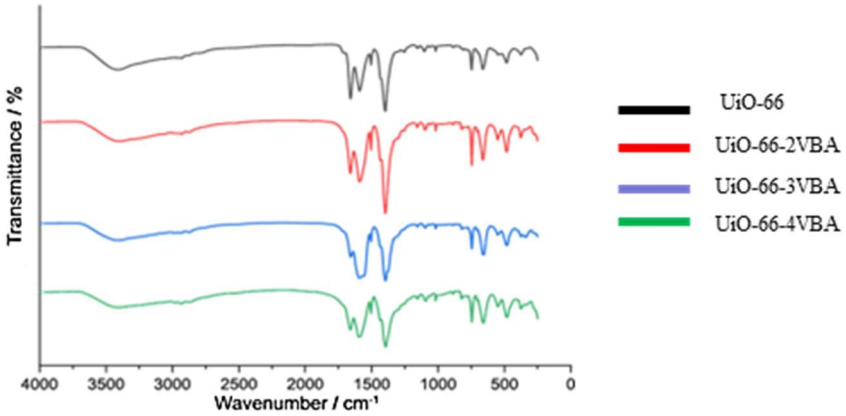


Figure 3. FT-IR spectra for UiO-66 and UiO-66 with mixed linker

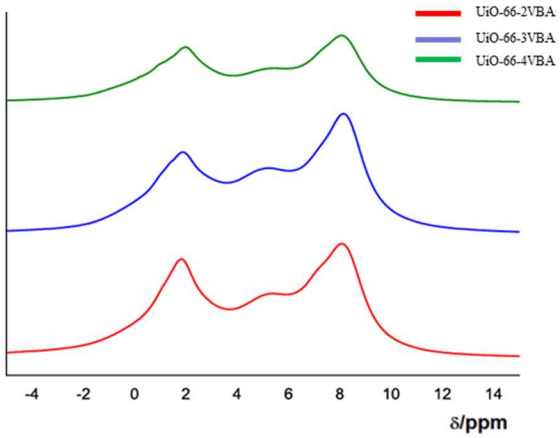


Figure 4. [1] H NMR Spectra of UiO-66 functionalized

Adsorption isotherm

Figures 5(a) and 5(b) show the ethylene gas adsorption isotherms in UiO-66 and functionalized UiO-66 at 293 K. Force fields mentioned in the method section were used and the ethylene uptake was simulated at pressures ranging from 0 to 100 kPa. The average loading for UiO-66 and functionalized UiO-66 rose as the pressure increased from 0 kPa to 100 kPa. When the MOFs are sorted according to the average loading for molecule per unit cell, the following order were produced; UiO-66-2VBA > UiO-66-4VBA > UiO-66-3VBA > UiO-66. At 100kPa, the maximum ethylene gas loading was 14.45 molecules per unit cell in UiO-66-2VBA, as compared to 14.05, 14.37, and 14.40 molecules per unit cell in UiO-66, UiO-66-3VBA, and UiO-66-4VBA, respectively. Although the highest loading was found for UiO-66-2VBA, the values between all MOFs were closely spaced. The present of the guest-guest interactions, which played a key role at higher pressures resulting in larger loadings, may explain the small variation in the average ethylene loading. The observations at higher pressures do not truly reflect the functionalized UiO-66's remarkable sensitivity to isotherms. Hence, the intermolecular interactions analysis was carried out to better understand the influence of functionalization on ethylene gas

adsorption.

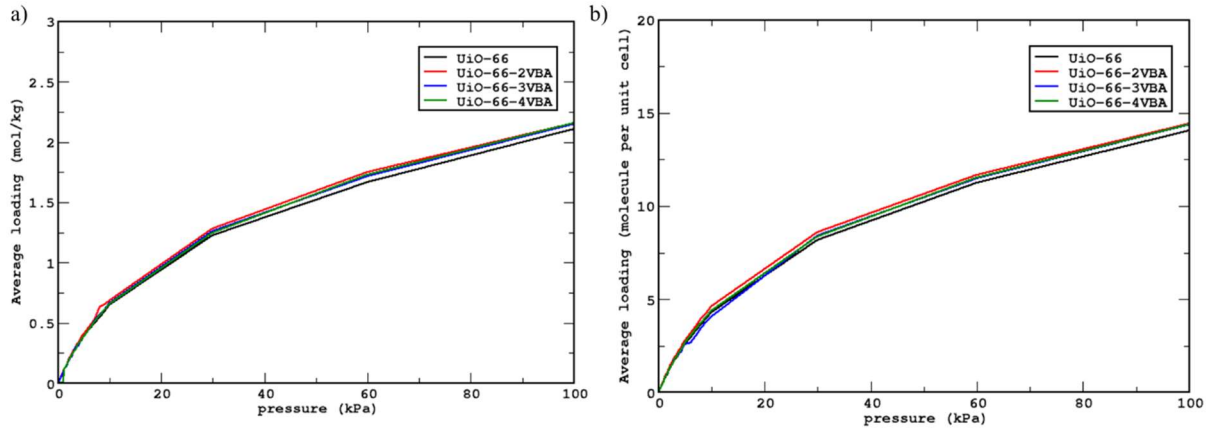


Figure 5. Simulated adsorption isotherms of ethylene molecule in UiO-66 and functionalized UiO-66: (a) pressure(kPa) versus average loading (mol/kg) and (b) pressure(kPa) versus average loading (molecule per unit cell)

Radial Distribution Function (RDF)

To study the interactions of the ethylene gas with UiO-66 and functionalized UiO-66 MOFs, the radial distribution function (RDF) was calculated using the following equation:

$$g_{ij}(r) = \frac{N_{ij}\,(\mathrm{r}, \mathrm{r} + \Delta\mathrm{r}).\ V}{4\pi r^2.\,\Delta r.N_i.N_j} \quad (1)$$

In this equation, N_{ij} (r, r + Δr) represents the number of particles *j* around particle *i* inside the area from r to r + Δr. *V* and *N* denote the volume of the system and the number of particles, respectively. Analyzing RDF allowed for assessing the distribution of ethylene gas molecules on the interaction sites of UiO-66 and functionalized UiO-66 MOFs. In general, the greatest distribution of gas molecules in the UiO-66 pores must follow the adsorbed quantity. The RDF plots for ethylene gas (Figure 6) showed an acceptable distribution of oxygen, hydrogen, carbon and zirconium atoms based on the adsorbed quantities and van der Waals surfaces of UiO-66 and functionalized UiO-66 MOFs. Although the ethylene gas adsorption quantity in UiO-66 and functionalized UiO-66 MOFs is not significantly different, the difference in terms of RDF values is noticeable. The level of distribution for the gas molecules was found in the order of UiO-66-2VBA < UiO-66-4VBA < UiO-66 < UiO-66-3VBA (Figure 6). Except for UiO-66, these results show an agreement with the adsorption isotherm results. Carbon, C and Zr atoms in the UiO-66-2VBA were discovered to be the most favorable sites for ethylene gas. C and Zr atoms have distribution *g*(*r*) values of 3.1 and 3.5 in UiO-66-2VBA, respectively. In comparison, the *g*(*r*) distributions for C and Zr atoms in UiO-66 are only approximately 2.0 and 3.1, respectively. These findings show that functionalizing UiO-66 with vinyl benzoic acid increased the interaction of ethylene gas with the C of the organic linker, while decreasing the interaction with H atoms.

Simulation snapshot analysis

The simulation snapshots for the adsorption of the ethylene gas in UiO-66, UiO-66-2VBA, UiO-66-3VBA and UiO-66-4VBA at 100 kPa along the y-axis is presented in Figure 7. As illustrated, all gas molecules were contained within pores of UiO-66. The simulated snapshots show molecules of ethylene gas adsorbed in UiO-66's porous structure. The ethylene molecules are located at the centre of the huge channels, where they interact with the framework via van der Waals forces. UiO-66's huge pore size enable effective ethylene adsorption, which results in a high adsorption capacity. The snapshots also indicate that ethylene gas molecules were adsorbed on the exposed zirconium metal sites. This Lewis's acid sites, which are made from coordinatively unsaturated zirconium atoms, facilitate robust interactions with the ethylene molecules. The

figure further shows that ethylene gas molecules are also dispersed throughout the framework, taking up the available pore space of UiO-66. It also can be observed that the functionalization of UiO-66 with vinyl benzoic acid helps to enhance the active sites for ethylene gas interactions. More ethylene gasses were adsorbed in the framework's pore in UiO-66-2VBA, UiO-66-3VBA, and UiO-66-4VBA. This might be owing to increased interactions between ethylene and the linker in the framework. It was clear that functionalizing UiO-66 had a positive effect on the adsorption of ethylene. As the pressure increased, the framework was gradually loaded with ethylene molecules at the metal sites. Hence, interactions between ethylene gas and Zr metal ions also increase. In comparison, the ethylene molecules' interaction with the Uio-66-2VBA framework is stronger than that of UiO-66, UiO-66-3VBA, and UiO-66-4VBA adsorption. This suggests that the ethylene gas adsorption inside UiO-66 MOF is enhanced by the mixed linker techniques.

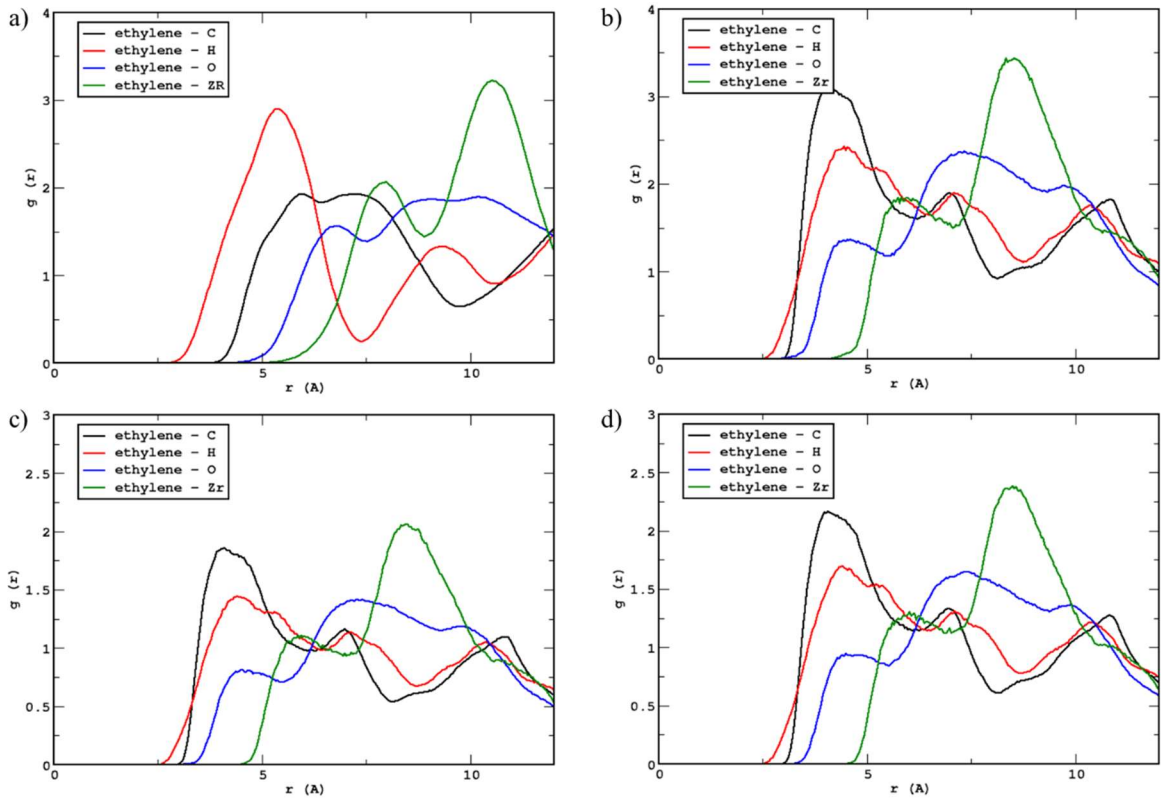


Figure 6. Radial distribution plots for (a) UiO-66, (b) UiO-66-2VBA, (c) UiO-66-3VBA, and (d) UiO-66-4VBA

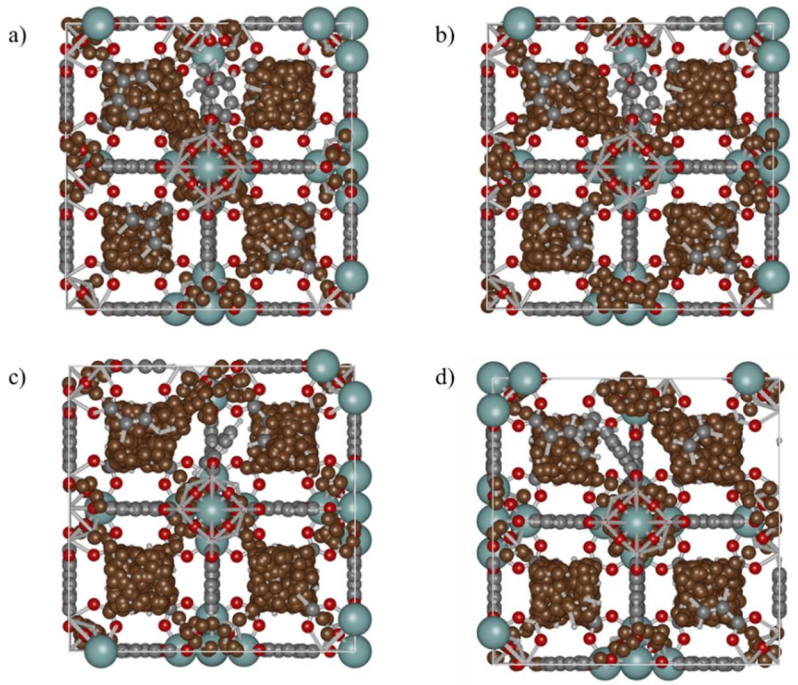


Figure 7. Simulation snapshot for (a) UiO-66, (b) UiO-66-2VBA, (c) UiO-66-3VBA, and (d) UiO-66-4VBA at 100kPa. The colors in the snapshot correspond to: linkers (grey), Zirconium atom (light blue), Oxygen atom (red) and ethylene gasses (brown)

Conclusion

We have successfully synthesized three functionalized, UiO-66 MOFs. The functionalization with different variations of vinyl benzoic acid did not disrupt the overall framework topology of the UiO-66(Zr) MOF. GCMC simulations provided an atomic-level understanding of ethylene gas adsorption in UiO-66, UiO-66-2VBA, UiO-66-3VBA, and UiO-66-4VBA at pressures ranging from 0 kPa to 100 kPa. The adsorption isotherm revealed the largest and most consistent ethylene loading in UiO-66-2VBA but did not differ significantly from the other MOFs studied. The carbon atoms in the functionalized linker and the Zr metal ion have the most interactions with ethylene gases. This was validated further by analyzing the simulation snapshots at 100kPa. Based on the findings, we determined that functionalization of UiO-66 MOF with vinyl benzoic acid will contribute towards more efficient ethylene gas adsorption and storage.

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NANOCRYSTALLINE CELLULOSE EXTRACTED FROM DISCARDED CIGARETTE BUTTS

(Selulosa Nanokristal yang Diekstrak daripada Sisa Puntung Rokok)

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Abstract

The increase in cigarette consumption has led to significant accumulation of non-degradable cigarette butt (CB) waste worldwide. CBs, though small, contain both cellulose acetate and toxic tobacco substances, posing environmental risks. This includes water pollution and harm to aquatic life by releasing toxins from leachate into waterways. This study addresses the problem by exploring the extraction and hydrolysis of cellulose acetate from discarded CBs, converting it into nanocrystalline cellulose (NCC). Several conditions were examined during this study such as the ratio of water and ethanol for the extraction (1:1, 1:2, 1:3 v/v%) and the efficiency of acid hydrolysis. Analysis techniques including FTIR, TEM, ICP-MS, and XRD were employed. TEM results showed that sample C has the smallest diameter of nanocrystal cellulose with needle-like shape that corresponded to the XRD results that show the highest Crystallinity Index (CI) of 64.8% with 3.66 nm crystallite size. The treatments successfully removed toxic chemicals from the CBs that were not detected in the retained NCC using ICP-MS. The preliminary results obtained open an avenue to reuse the CB to produce environmentally friendly NCC that can be used widely in many applications fields.

Keywords: cigarette butts, nanocrystal, cellulose pulp, extraction, cellulose acetate

Abstrak

Peningkatan penggunaan rokok telah membawa kepada pengumpulan sisa pepejal rokok yang sukar untuk terurai di seluruh dunia. Walaupun saiz puntung rokok (CB) kecil, tetapi ia mengandungi asetat selulosa dan toksin tembakau yang menyebabkan masalah pencemaran. Ini termasuk pencemaran air yang mengancam kehidupan akuatik kesan daripada pelepasan toksin ke dalam salur air. Kajian ini mengatasi masalah tersebut dengan mengkaji kesan pengekstrakan dan hidrolisis selulosa asetat daripada CB, mengubahnya menjadi selulosa nanokristal (NCC). Beberapa keadaan telah dikaji seperti nisbah air dan etanol untuk pengekstrakan (1:1, 1:2, 1:3 v/v%) dan efisiensi hidrolisis asid. Teknik analisis termasuk FTIR, TEM, ICP-MS, dan XRD digunakan. Hasil TEM menunjukkan sampel C mempunyai diameter selulosa nanokristal terkecil dengan bentuk jarum yang sepadan dengan hasil XRD yang menunjukkan Indeks Kristaliniti (CI) tertinggi iaitu 64.8% dengan saiz kristal 3.66 nm. Rawatan yang dilakukan berjaya mengeluarkan bahan kimia beracun daripada CB yang dikesan menggunakan NCC yang dihasilkan menggunakan ICP-MS. Keputusan awal ini membuka peluang untuk mengguna semula CB untuk menghasilkan NCC mesra alam yang boleh digunakan secara meluas dalam pelbagai bidang aplikasi.

Kata kunci: puntung rokok, nanokristal, pulpa selulosa, pengekstrakan, selulosa asetat

Introduction

A cigarette butt (CB) acts as a filter to a cigarette. It filters the toxic substances contained in cigarettes before going through humans. CB is made up of cellulose acetate and rolled with paper. Cellulose acetate is extensively used in textiles, cigarette filters for absorbing vapors and collecting particulate smoke components, surface coatings and inks as an additive, and as a carrier in photographic negatives, motion picture film (celluloid), microfilm, microfiche, and audio tape [1]. The global market for cellulose acetate is expected to reach 1.05 million metric tons by 2017, with an anticipated price of $1.80 per pound, according to a new analysis by Global Industry Analysis [2].

CB littering is ubiquitous in urban life and can be found abundantly in most public places especially streets, beaches, and food stalls. Studies have consistently found that a substantial majority of smokers litter CB (76.7%–84%) [3]. Even though it is small, the accumulation of CB may lead to environmental problems. Apart from being not easily degraded, the discarded CB may contain harmful chemicals such as nicotine as the major toxic substance. A smoker gets between 1.4 and 2.2 mg of the chemicals in each cigarette, with polycyclic aromatic hydrocarbons, N-nitrosamines, and aromatic amines being the most effective carcinogens [4]. The biodegradability of cellulose acetate is still questionable as some researchers reported that natural organisms could not degrade cellulose acetate with a degree of substitution of greater than 1.5. Therefore, it is not degraded by itself thus creating a big problem to dispose of the CB and recycle them.

The filter used for a cigarette is composed of cellulose acetate which is not easily degradable and may lead to the accumulation of CB on the ground and the toxins leaching out into the groundwater [5]. Recently, there have been several initiatives taken to overcome this problem such as recycling CBs to produce supercapacitors, bricks, insecticides, chemical inhibitors, sound absorbing material [6], or cellulose pulp [5]. Toxic chemicals trapped in the CB filters that leaching out and dissolving into the water can cause considerable damage to the environment and various organisms [3]. Incinerating or landfilling are not the best methods to dispose of cigarette butts. It incurs more costs and is challenging to dispose waste that contains a lot of organic and toxic materials in landfills. Since many toxic compounds are released into the air when the waste is burned, incinerating CBs is also an unsustainable approach [4]. Recycling CB is problematic due to the toxic substances contained in the filter. Any mechanism to dispose of the filter needs to consider its impact on the environment and the feasibility of the methods. The current method has initiated recycling CB to produce nano crystalline cellulose (NCC) by using pure ethanol in the extraction. However, the maximum yield of cellulose acetate and NCC produced was not high [7]. Therefore, to increase the yield, the extraction solvent needs to be studied. It is known that ethanol can play a role in particle size and morphology, where a decrease in the aqueous solution's dielectric constant due to the addition of ethanol causes supersaturation, which in turn leads to the development of many homogenous nuclei and reduced average particle sizes [8]. Furthermore, instead of pure ethanol, aqueous ethanol can be an eco-friendly method for the extraction of cellulose [9].

Thus, this study aimed to use aqueous ethanol for the extraction of cellulose acetate from discarded CBs and convert it into nano-crystalline cellulose to reduce the environmental impact of discarded CBs. Apart from that, the heavy metals content in the recovered cellulose pulp was investigated to ensure safe usage when reused as other materials such as in biomaterial, catalysis, or polymer coating.

Materials and Methods

Materials

CBs were collected in public areas in Kuantan, Pahang. Ethanol (99.8%), sodium hydroxide pellets (NaOH), sulfuric acid (H_2SO_4), and sodium hypochlorite (NaOCl) were obtained from Merck (Malaysia). All chemicals were of analytical grade. Double distilled water was used for all experiments.

Sample preparation

Excess paper and tobacco were manually removed from the collected CB to ensure the toxic substance from the tobacco does not leach out during the extraction. Then

about 10 g of cleaned CBs were weighted prior to soaking and later, they were washed with water for 24 h. Following that, the water was drained, and the cleaned CBs were gathered.

Cellulose acetate extraction and isolation of nano-crystalline cellulose

The extraction method followed previous research [7] with a slight modification. Approximately 10 g of the washed CBs (1:10 w/v%) were extracted using water and ethanol at the ratios of 1:1, 1:2, and 1:3 (v/v%), respectively, for 24 h at room temperature. After the extraction, the solid materials were collected as cellulose acetate (CA-CB). The obtained CA-CB was bleached with 1.25 w/v% sodium hypochlorite for 6 h at room temperature. Following the bleaching procedure, the samples were dried and deacetylated for 24 hours at room temperature using 1 w/v% ethanolic NaOH. After mixing the samples, nano-crystalline cellulose (NCC-CB) was then isolated using 55 wt% of sulfuric acid at 45 °C for 45 minutes to remove the amorphous areas from the cellulose that had been produced. The percentage yield of the extracted cellulose from CB (Cel-CB) was determined using Equation (1).

$$\%\, Yield\ of\ Cel-CB = \frac{Weight\ of\ extracted\ Cel-CB(g)}{Weight\ of\ CB(g)} \times 100\% \quad (1)$$

Subsequently, NCC-CB suspension was diluted 20 times its original volume at room temperature using double-distilled water. This process was repeated 5 times. Then, the NCC-CB suspension was freeze dried at −60ºC for 72 h. A pestle and mortar were used to grind the resulting film (NCC-CB) to a powder and stored in sample vial for further analysis. The yield of NCC-CB was calculated using Equation (2).

$$\%\, Yield\ of\ NCC-CB = \frac{Weigh\ \ of\ NCC-CB\ isolated(g)}{Weigh\ \ of\ Cel-CB\ used(g)} \times 100\% \quad (2)$$

Characterization

The obtained cellulose powder (NCC-CB) was characterized based on the particle size, surface, crystal shape, morphology and elemental analysis using Particle Size Distribution, FTIR, TEM, XRD and ICP-MS. These instruments were used to identify the physical properties, chemical properties, and microstructure of the NCC-CB. XRD with radiation at 45kV and 40mA provides information about crystallinity and phase composition. The samples were scanned with a step size of 0.05° and a scan rate of three seconds per step. The crystallinity index of NCC and crystallite size were calculated by using Equation (3) and (4), respectively. TEM provides the photomicrograph of the dimensions and microstructure of NCC-CB using high resolution transmission electron microscopy (HRTEM, JEM-2011/JEOL). ImageJ software was used to analyze the dimension of the NCC from TEM image. The functional groups of NCC-CB were confirmed using Fourier transform Infrared spectroscopy (FTIR) within a range of 4000 – 500 cm^{-1}. The elemental analysis was conducted using ICP-MS Elan 9000 (Perkin-Elmer).

$$CI(\%) = \frac{I_{crys} - I_{am}}{I_{002}} \times 100\% \quad (3)$$

Where I_{crys} is the maximum intensity of diffraction at the highest peak around 20° to 22° angle and I_{am} is the minimum intensity of amorphous material around 18° angle. The crystallite size of the sample was calculated using Scherrer equation as in Equation (4):

$$Crystallite\ size\ (nm) = \frac{0.94\lambda}{\beta\ cos\theta} \quad (4)$$

Where λ is the wavelength at 0.154, β is the full width half maximum (FWHM), and θ is the corresponding Bragg angle [10].

Results and Discussion

Cellulose and nano-crystalline yield

Nano-crystalline Cellulose-Cigarette Butt (NCC-CB) was isolated from CB after the extraction with ethanolic sodium hydroxide, bleaching, deacetylation, and sulfuric acid hydrolysis. NCC-CB differs to Cel-CB in terms of amorphous region, whereby, in NCC-CB, the amorphous region is lesser when compared to Cel-CB. This is due to acidic hydrolysis. According to Table 1, sample A with the extraction ratio of water to ethanol 1:1 (v/v) obtained 98.76% of Cel-CB yield, sample B with the extraction ratio of 1:2 (v/v) gives 97.76% yield of Cel-CB and sample C with the extraction ratio of 1:3 (v/v) yielded 83.98% of Cel-CB. As a comparison, the yield of Cel-CB obtained by previous research [7] was at 60% with the extraction ratio of water to ethanol of 1:1 (v/v) which was lower than the yield obtained in the current study. On the other hand, for the yield of NCC-CB isolated using 55% wt. of sulfuric acid at 45 °C for 45 minutes, sample C shows the highest yield at 33.7%, followed by sample B at 24.4% and sample A at 15.1%. The yield of NCC-CB obtained from the previous study [7] was a bit higher than the current study which was at 29.4% when using an extraction ratio of 1:1 (v/v). However, the yield of NCC-CB in this study was 33.7%, was improved with extraction ratio 1:3 (v/v) which is higher than previous studies that yielded 29.4% from discarded cigarette butt with the usage of 99.8% ethanol extraction [7], 23.5% from corncob [11], and 15-20% from olive fiber [12]. It shows that when ethanol in the extraction ratio is increased, the yield of NCC increases. Based on Table 1, it can be concluded that the extraction ratio of water to ethanol at 1:1 (v/v) gives the best yield of Cel-CB at 98.76% and the extraction ratio of 1:3 gives the best yield of NCC-CB at 33.7%. The trend of NCC increased with decreasing of the yield of Cel-CB. The yield from the treatment in Sample B and C was also higher than the yield obtained by other studies using pineapple leaves (32.1%) and sugarcane bagasse (28.6%) as a source of nanocellulose [10].

Table 1. Yield of Cellulose (Cel-CB) and Nano-crystalline cellulose (NCC-CB) from Cigarette Butt

Sample	Ethanol to Water Ratio (v/v)	Yield of Cel-CB (%)	Yield of NCC-CB (%)
A	1:1	98.76	15.1
B	1:2	94.76	24.4
C	1:3	83.98	33.7

Aqueous dispersion and morphology of nanocrystalline cellulose: Correlogram of particle size

Dynamic Light Scattering (DLS) determines how randomly particles move when they undergo Brownian motion, and it is not applicable when a particle's motion is not random. Figure 1 shows the DLS measurement. From Figure 1, sample A has increased correlation at higher delay times between 100000-1000000μs which indicates sedimentation, whereas for Sample B, there is no sudden increase which indicates that there is a lower level of sedimentation happening in Sample B. In Sample C, there is an increase in correlation that happened earlier than in Sample A.

Particle Size Distribution (PSD)

The particle size distribution of nanocrystalline cellulose (NCC) from cigarette butt is shown in Figure 2. The size distribution in Sample A shows the largest size distribution in a range 100–1000 nm with 655.4 nm indicated microcrystalline size (MCC). While in Sample B and Sample C, the largest size distribution was in the range of 10–100 nm with 41.75 nm and 30.47 nm, respectively, indicating nano cellulose crystal (NCC). The formation of size differs by the extraction ratio of water to ethanol which is conducted in Sample A, B, and C. It shows that the treatments had improved the cellulose crystal size. The Polydispersity index (PdI) for Sample A, B and C were 0.859, 0.400 and 0.410, respectively. High reading of PdI may result from a sample containing large particles reflected in Sample A. Samples B and C have the lowest PdI which corresponds to Sadeghifar [13], their NCC extracted with HBr of 1M gives PdI of 0.41 reflected small particles. In conclusion,

the extraction ratio of 1:2 and 1:3 (v/v) used for samples B and C improved the size distribution of NCC and this corresponds to the yield of NCC in Table 1.

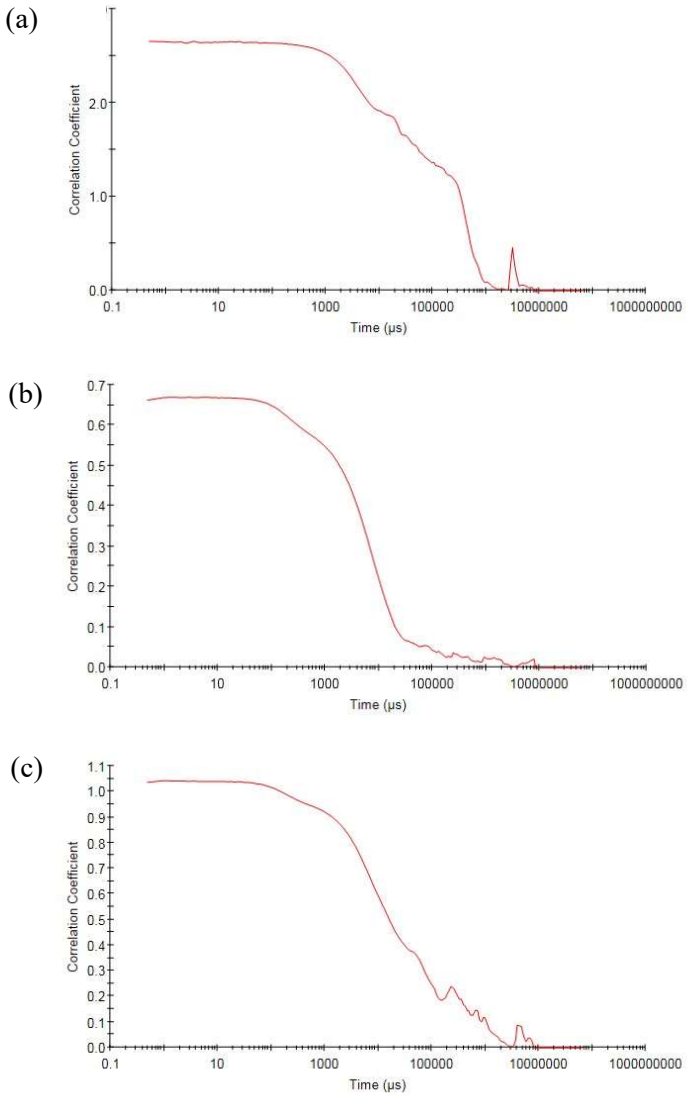


Figure 1. Dynamic light scattering (DLS) measurement of a) Sample A, b) Sample B and c) Sample C of NCC-DCB

Transmission electron microscope (TEM)

The crystallinity and morphology of the NCC-CB produced from this study were analyzed using TEM to analyze the nanometer size samples. Figure 3 shows the TEM micrograph for Sample A, B and C that varied the extraction ratio of water to ethanol 1:1, 1:2, and 1:3 (v/v), respectively. The TEM shows obtained nano-crystals cellulose resulting from sulfuric acid hydrolysis. The amorphous portion of cellulose microfibrils was predicted to be cleaved transversely by the acid hydrolysis treatment under regulated conditions, preserving the straight crystalline domains. Eventually, the treatment should cause the fibers' size to decrease from the micron to the nanometer scale [14]. Here it can be seen that the nanocrystals cellulose is rod in shape for Sample A and needle-like in shape for Sample B and C with a reduction in size due to the treatments, respectively. The observed behavior may be explained

by the fact that ethanol creates a layer and adsorbs more strongly than water on the surface, preventing agglomeration during crystallization [8]. The length and diameter range of Sample A is found to be in a micro size which corresponded to 50% of ethanol extraction, contrary to Sample B and C, where the size of the cellulose crystals is found to be in the nanometric range which corresponded to 66% and 75% of ethanol extraction. From Figure 3(b) and 3(c), the formation of nanocrystalline requires further extraction ratio to have complete shape and size of NCC as in other studies. However, other studies that used other sources of cellulose such as sugar palm fiber [15], pine wood and corncob [11] obtained smaller size than that was obtained in this study that due to higher exposure to more concentrated sulfuric acid (>60%) on the cellulose fiber. According to Yu [14], their nano-crystals diameter is in the range of 15–20 nm with different method of preparing NCC which only involves alkali treatment with NaOH, using aqueous chlorite to bleach, and acid hydrolysis with 10 $molL^{-1}$ of sulfuric acid. The size 170 nm in diameter nanocrystals were isolated from sugarcane bagasse which was extracted with 17.5% w/v of sodium hydroxide solution [16].

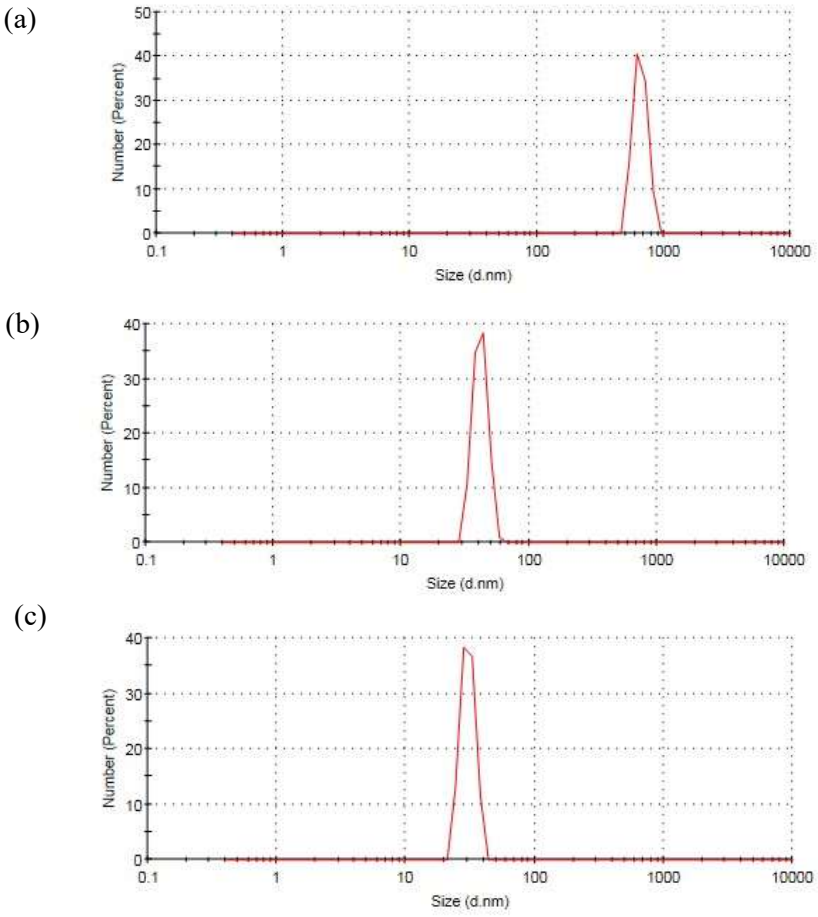


Figure 2. Particle size distribution of nano-crystalline from discarded cigarette butt for a) Sample A, b) Sample B and c) Sample C

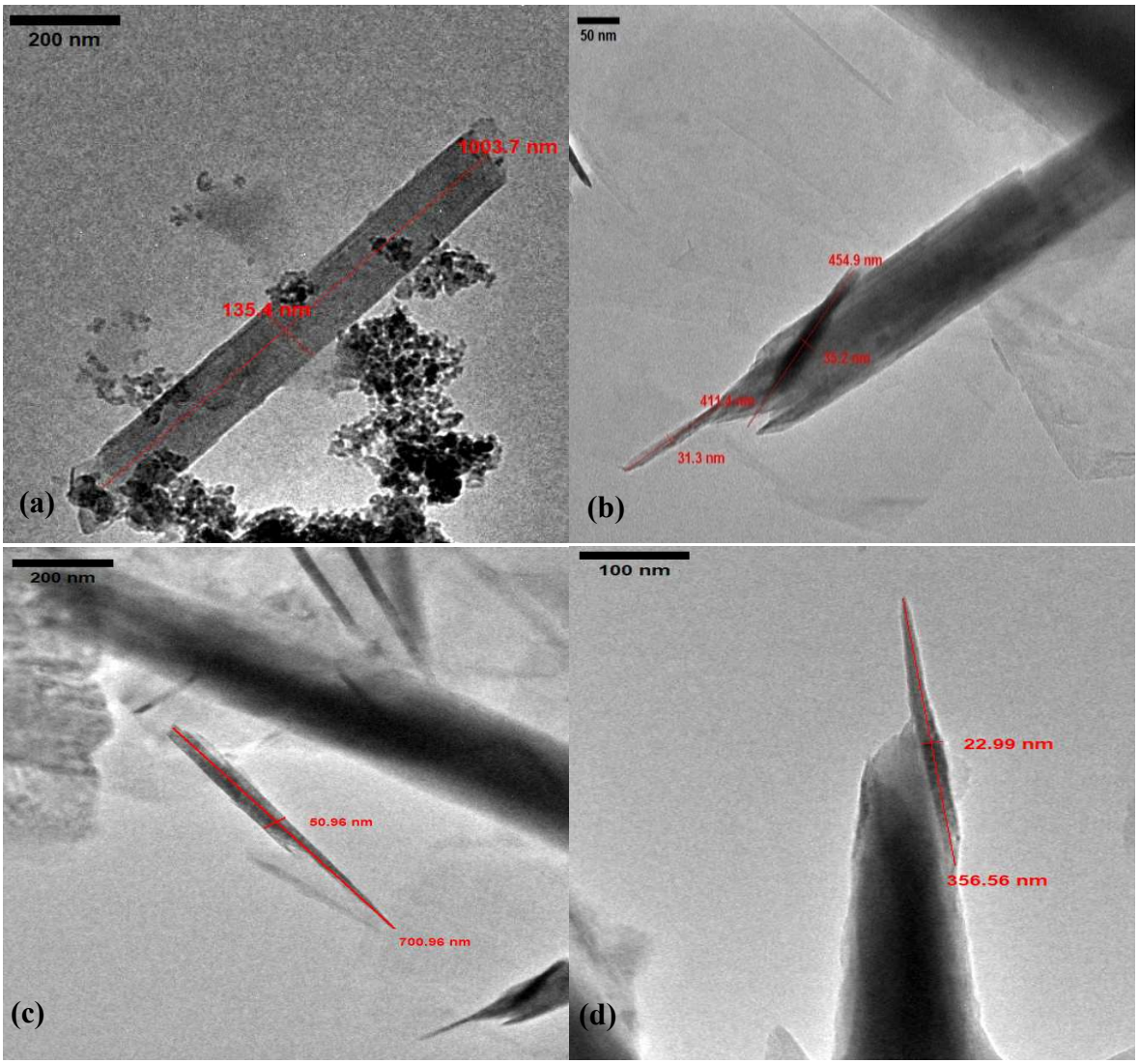


Figure 3. TEM image of nanocrystalline cellulose from a) Sample A; b and c) Sample B; and d) Sample C

The aspect ratio shown in Table 2 for this study indicated that the treatments did not interfere with the formation of the crystal. The aspect ratio is increasing as the ratio is increasing. The aspect ratio for treatment in Sample B and C exhibited more than ten (Table 2) which indicated that the isolated NCC has a mechanical stability for polymer composites synthesis corresponding to the minimal aspect ratio of more than ten to achieve optimal stress transmission during the fiber-matrix interaction[17].

Fourier-transform Infrared Spectroscopy (FTIR)

The successful isolation of the freeze-dried NCC–CB from cigarette butt was confirmed by FTIR. All three samples show a broad band at 3320 cm^{-1} in the absorbance regions of O–H with increasing intensities as the NCC yield increased with the treatment extraction ratio (Figure 4). The O-H stretching vibration of the O-H groups in cellulose molecules is shown by a broad band in the range of 3100 to 3600 cm^{-1} that is visible in the FTIR spectra of every sample. The spectra were compared with the cigarette butt extract without treatment that showed the two main absorbance regions at 3338 cm^{-1} and 1634 cm^{-1}, respectively, that indicate band for O–H and –NH group [18]. After the treatments for Sample A, B, and C there were no sign of C=O at

1743 cm^{-1} and –NH group as in untreated cigarette butt in the orange line in Figure 4. This shows that the treatments in this study can remove ketone and amine [7], [18]. The spectra of sample A, B and C show a C–H stretching band at 2890, 2880, 2894 cm^{-1}, respectively. Furthermore, a peak in band detected at 1363, 1368,1370 cm^{-1} is related to the bending vibration of the C–H and C–O bonds in the polysaccharide aromatic rings [19]. In addition, the absorption peak was linked to aromatic C–C in the plane symmetrical stretching vibration of aromatic rings seen in lignin at 1604 cm^{-1} in the untreated sample. These bands were no longer present in the FTIR spectra of the current NCC.

Table 2. The dimension and aspect ratio for sample with different extraction ratio treatment

Samples	*L*, length (nm)	*D*, diameter (nm)	Aspect ratio (*L*/*D*)
A	1003.7	135.4	7.413
B	454.9	35.2	12.923
	411.4	31.3	13.143
	700.3	50.9	13.731
C	356.6	23.0	15.504

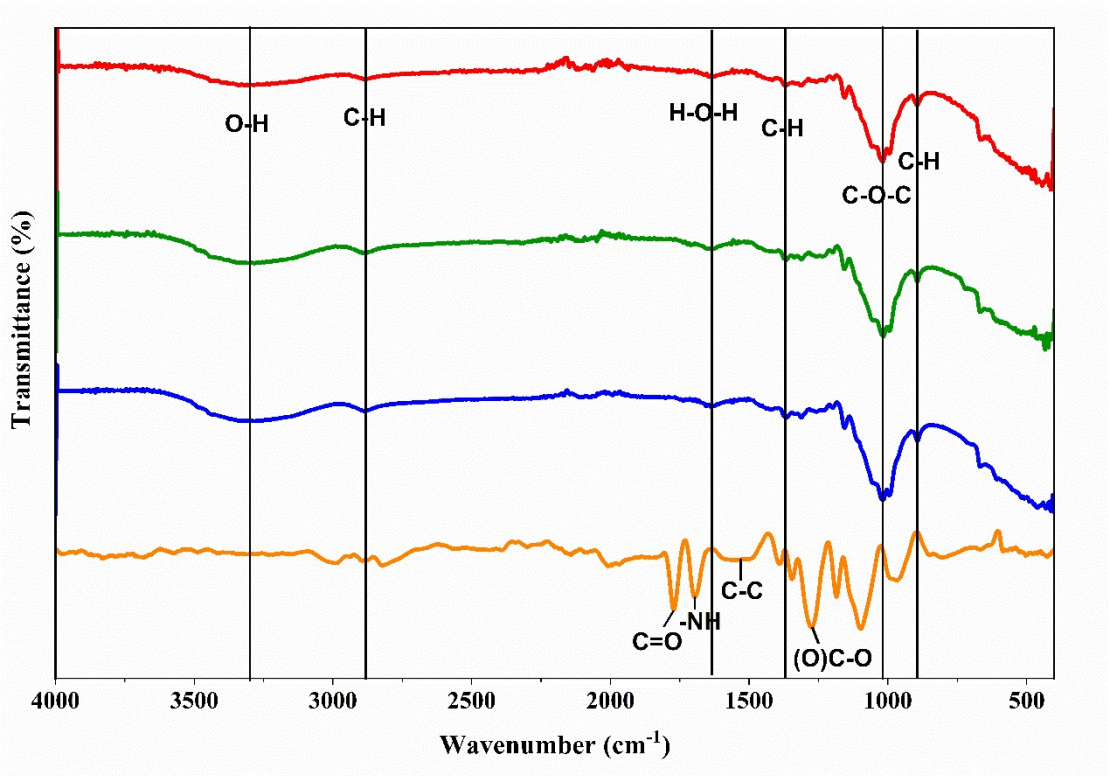


Figure 4. FTIR spectra of Sample A (red line), B (green line), C (blue line), and untreated cigarette butt (orange line)

Finally, the band peaks at 1016, 1018 and 1016 cm^{-1} for sample A, B and C respectively, due to the stretching vibration of the C–O–C pyranose ring, which is antisymmetric in phase. The most important absorption band is at 800–950 cm^{-1} (which is associated with the glycosidic linkages between glucose units in cellulose) [19] when comparing with the FTIR result obtained previously [7], 3320 cm^{-1} (O–H), 2900 cm^{-1} (C–H), 1646 cm^{-1} (H–O–H), 1437 cm^{-1} CH2, 1371 cm^{-1} (C–H), 1018 cm^{-1} (C–O–C) and 898 cm^{-1} (C–H) with the extraction of ethanol to water of 1:1 (v/v). This shows that the FTIR results for sample A, B and C have the

same absorption band to prove that it is NCC chemical structure, and the ratio of extraction did give little impact on FTIR results.

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X-ray powder diffraction (XRD)

The XRD patterns of the NCC-CB in Sample A, B and C show a characteristic of cellulose III structure with a wide peak around 2θ = 12° (101), 20° (10$\bar{1}$), 22° (002) and 25° (111) (Figure 5) similar shape with other NCC obtained from cigarette butt [7]. After acid hydrolysis, the isolated NCC-CB Crystallinity Index (CI) and crystallite size were calculated using Equation 3 and 4, respectively as shown in Table 3 for sample A, B and C. This data is lower than the data obtained previously [7] using CBs as the cellulose source, which is 94.5% for NCC. However, for this study, the CI was increasing as the treatment increased the extraction ratio. When referring to the untreated cigarette butt, the CI was 28.26% which was due to the amorphous region in the cellulose being removed during the acid hydrolysis [7]. There is no significant difference between all three samples. Sample A had the highest peak is highest at 22° whereas Sample B and C recorded the highest peak at 20°. This is due to the conversion of cellulose I to cellulose III as the extraction ratio increased [20]. Sample C shows the highest crystallinity Index (CI), 64.8% with the smallest crystallite size 3.66 nm. This suggests that the extraction ratio does not have a negative effect on crystallite of cellulose. Further, an increase in the extraction ratio can be extended to obtain more CI.

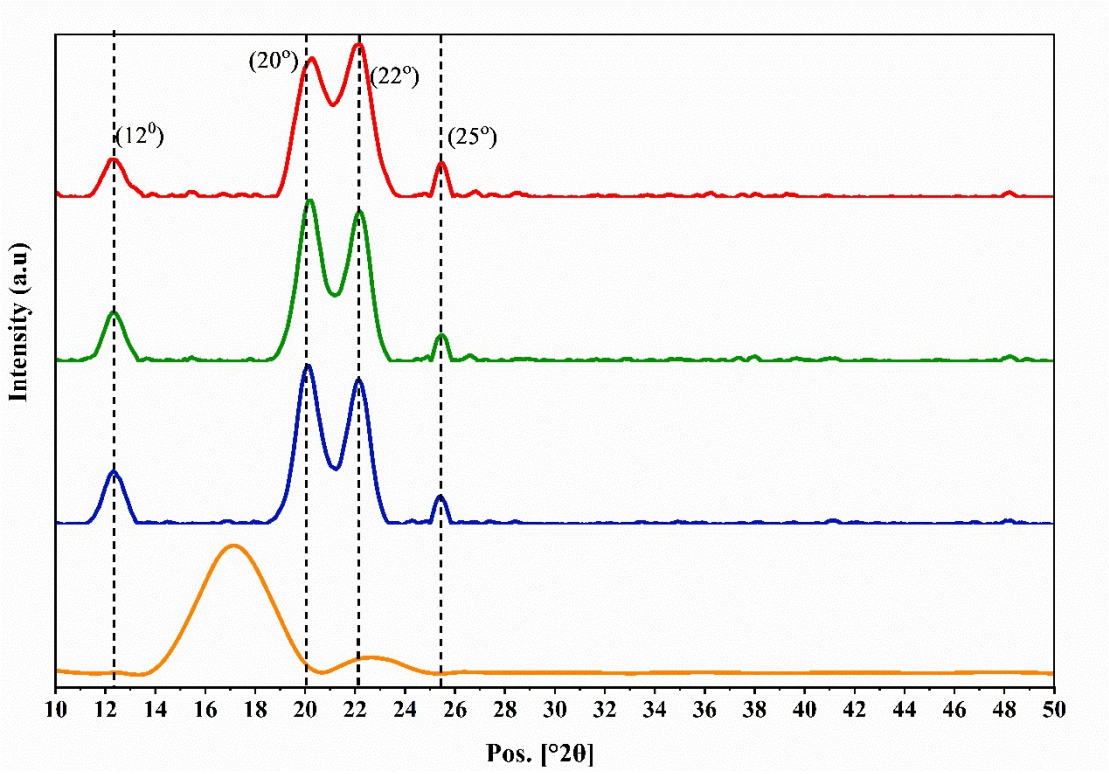


Figure 5. XRD results of Sample A (red line), B (green line), C (blue line), and untreated cigarette butt (orange line)

Table 3. Crystallinity index and crystallite size of nanocrystalline cellulose from cigarette butt

Sample	Crystallinity Index (%)	Crystallite Size (nm)
A	44.9	4.25
B	48.6	3.99
C	64.8	3.66

Inductively coupled plasma mass spectrometry (ICP-MS)

CB can contain toxic chemicals that come from cigarettes as it functions to filter the chemicals from getting into the human body. To reuse the CBs as a source of cellulose for NCC, the contents need to be examined. The metal contents in the powder NCC-CB were analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and compared with the previous study [18] as shown in Table 2. Based on Table 2, Sample A has most metal elements from the discarded CB due to the inappropriate extraction ratio during the isolation of the NCC-CB. Contrary to Sample B and Sample C, the metal contents were reduced and most of them were not traceable. The amount of heavy metal content reduced with increasing extraction ratio. This shows that the heavy metal contents in the cigarette butt can be removed, and this opens an avenue to recycle the waste in an environmentally safe way.

As the treatment ratio increased it improved the content in the CB and enhanced the CI of the isolated NCC from CB, and it is confirmed by the formation of the NCC-CB shown in Figure 3(b) and 3(c) for Sample B and Sample C. This shows that the samples contain high percentage of nano-crystals cellulose [21]. Apart from that, heavy metal contents in the discarded CB were removed successfully using all the extraction ratios, indicated by lead, nickel, copper, arsenic, cadmium, cobalt, manganese, and titanium were not traceable in all samples. However, further treatment needs to be done to remove or reduce other metals such as aluminum and iron [17].

Table 2. Elements analyzed by ICP-MS for Sample A, B, and C and compared with the metal leached from untreated cigarette butt [18]

	Concentration (ppm)			
Elements	**Sample A**	**Sample B**	**Sample C**	**Untreated Cigarette butt [18]**
Na	368	139	60.9	239
Mg	100	69.4	64.1	620
Al	1320	720	323	950
Pb	-	-	-	2.39
K	162	40.6	20.9	929
Ca	540	417	559	766
V	2.53	-	1.52	-
Cr	21.6	11.1	6.99	85
Fe	403	194	154	590
Ni	3.02	-	-	2.90
Cu	2.12	4.66	-	11
Zn	27.4	21.8	16	35.3
As	1.54	-	-	-
Ag	-	1.94	7.49	0.18
Cd	-	-	-	0.99
Co	-	-	-	0.04
Mn	-	-	-	178
Ti	-	-	-	106
Ba	-	-	-	48.7
Sr	-	-	-	39.6

Conclusion

In conclusion, reusing CB to isolate NCC can be a solution to the CB littering problem. The NCC can be used further in polymer industries. The NCC isolated in this study using water to ethanol of 1:3 (v/v) gives the best results of crystallinity index with 64.8%, polydispersity index of 0.40, and crystallite size 3.66 nm with needle-like shape showing the improvement in the treatment. The NCCs yield was considerably great between 24-34% and MCC yield was 15%, by only utilizing more aqueous ethanol (1:3 v/v), proving the preparation method was environmentally friendly. The

chemical structure of NCC in FTIR spectra of sample A, B and C present similar bands, indicating the same chemical structure for all the NCC. ICP-MS was conducted to determine the content of metal found in the samples and aluminum and iron were found to be the highest. Thus, further treatment needs to be done with aqueous ethanol (1:3 v/v) and sulfuric acid treatment to improve the NCC isolated from the CB to use in various application fields especially in polymer and medical. The results obtained here suggest that CB is capable of being one of the sources of cellulose to produce NCC with the advantage of being easily biodegradable and environmentally friendly.

Acknowledgement

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SYNTHESIS AND CHARACTERISATION OF REUSABLE PVA-SODIUM ALGINATE-Ni CATALYST IN HECK REACTION

(Sintesis dan Pencirian Pemangkin yang Boleh Digunakan Semula dalam Tindak Balas Heck)

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Abstract

Heck reaction is a carbon-carbon coupling reaction that essential for the development of natural products, agrochemicals, optical devices and drugs. During the synthesised of chemicals, the presence of catalysts is important. Normally, the reaction is carried out with the presence of palladium metal as catalyst. However, it has disadvantages such as high toxicity and considerably expensive. Compared to palladium, nickel represent a low cost and environmental friendly type metal. Therefore, nickel is effective to replace the palladium in their application as catalyst in Heck reaction. This study produced a Polyvinyl alcohol-sodium alginate-Nickel (PVA-SA-Ni) catalyst by using one pot synthesis method in the ultrasound irradiation system. The benefits in producing a catalyst not only is cost effective but also its reusability properties due to the presence of PVA-SA as a support. The PVA-SA-Ni was successfully synthesised and characterised by using FTIR, XRD, FESEM-EDX and AAS. The catalytic performance and reusability of PVA-SA-Ni was investigated in Heck reaction between 1-bromo-4-nitrobenzene and styrene. The percentage conversion rate of reactant to product was determined by using GC-FID. The PVA-SA-Ni showed the best performance in Heck reaction with 74 % conversion by using 1 mmol % in the presence of K_2CO_3 as a base at a temperature of 165 °C, and DMA as solvent within 4 h of reaction time.

Keywords: polyvinyl alcohol, sodium alginate, nickel(II) catalyst, Heck reaction, reusable catalyst

Abstrak

Tindak balas heck ialah tindak balas gandingan karbon-karbon adalah penting untuk pembangunan produk semula jadi, agrokimia, peranti optik dan ubat-ubatan. Semasa bahan kimia disintesis, kehadiran pemangkin adalah penting. Biasanya, tindak balas dilakukan dengan kehadiran logam paladium sebagai mangkin. Walau bagaimanapun, ia mempunyai kelemahan seperti kadar toksik tinggi dan harga yang agak mahal. Berbanding dengan paladium, nikel dilihat sebagai jenis logam kos rendah dan mesra alam. Oleh itu, nikel berkesan untuk menggantikan paladium dalam penggunaannya sebagai pemangkin dalam tindak balas Heck. Kajian ini menghasilkan mangkin Polivinil Alkohol-natrium alginat-Nikel (PVA-SA-Ni) menggunakan kaedah sintesis satu pot

dalam sistem penyinaran ultrabunyi. Terdapat banyak faedah dalam menghasilkan pemangkin ini bukan sahaja efektif dari segi kos tetapi dilengkapi dengan sifat kebolehgunaan semula kerana kehadiran PVA sebagai sokongan. PVA-SA-Ni yang telah berjaya disintesis dicirikan menggunakan FTIR, XRD, FESEM-EDX dan AAS. Prestasi pemangkin dan kebolehgunaan semula PVA-SA-Ni telah disiasat dalam tindak balas Heck antara 1-bromo-4-nitrobenzene dengan stirena. Kadar penukaran peratus bahan tindak balas kepada produk ditentukan dengan menggunakan GC-FID. PVA-SA-Ni menunjukkan prestasi yang baik dalam tindak balas Heck dengan penukaran 74 % dengan menggunakan 1 mmol % pemangkin, kehadiran K_2CO_3 sebagai bes pada suhu 165 °C, dan DMA sebagai pelarut dalam masa 4 jam tindak balas.

Kata kunci: polivinil alkohol, natrium alginat, pemangkin nikel(II); tindak balas Heck, pemangkin yang boleh digunakan semula

Introduction

The Heck reaction is a common practical reaction used in the synthesis of natural compounds, agrochemicals, and medicines. Palladium (Pd) catalysts are commonly used in Heck reaction [1] because of their high catalytic activity, high stability, and the reaction can occur at low temperatures [2]. Nevertheless, the price of Pd metal is expensive and toxic that is harmful to the environment leading to its limitations in industrial applications. An alternative to replace the Pd metal is nickel catalyst. It is cheaper, low toxicity, and good in catalytic activity [3].

The catalytic performance of Ni type catalyst in the Heck reaction is comparable with Pd type catalyst. However, the homogenous Ni catalyst has difficulty separating from the product of Heck. Ni-phosphine ligand also has its drawbacks, whereby phosphine is not cheaper, toxic and needs inert condition because it is sensitive to water and oxygen. Heterogeneous Ni type catalyst is more preferable due to easy recovery at the end of reaction, recyclable, reduce waste of chemical and environmentally friendly [4].

The heterogeneous type of catalyst is designed by the presence of solid polymer support in the structure of catalyst. Amongst the polymers, biodegradable polymers such as chitosan (CS) [5], PVA [6] and sodium alginate (SA) [7] are gaining more attention due to their great advantages. For instance CS is an expensive polymer from polysaccharides, non-toxic, biodegradable, renewable and not soluble in common solvents [5]. Interestingly, CS have OH and NH_2 groups that function as chelating ligand to Ni and other metals [8]. Tang et al. [8] synthesized CS together with carbon nanotube (CNT) to support Ni (CS-CNT-Ni). Excellent catalytic activity of CS-CNT-Ni is reported giving 100% acetylene conversion at 160°C. However, no study on recyclability was reported. Hajipour and Abolfathi [5] also synthesized CS with Ni and triazole (CS-triazole-Ni). The catalytic activity of CS-triazole-Ni was good at above 80% of conversion and the recyclability of catalyst is 5 times without any reduction in rate of conversion.

The PVA is also used as support for the synthesis of heterogeneous Ni type catalyst. PVA is non-toxic, biocompatibility, biodegradable and environmentally friendly [6]. Moreover, the presence of hydroxyl group in PVA structure cause it easy to dissolve in water [9] and also can function as ligand during the complexation [10]. Medjili, et al. [11] synthesized Ni supported catalyst with Cu, PVA and polyaniline (PANI). PVA-PANI-Ni-Cu catalyst was successfully used in removing methylene blue (94%) from 10^{-5} M initial concentration of dye. Lakhdari, et al. [6] also synthesis PVA-PANI-Ni-Cu. They applied the PVA-PANI-Ni-Cu in converting the toxic methanol to less harmful by-products, CO_2 and H_2O.

Sodium alginate (SA) is extracted from brown algae that contain polysaccharides. It is water soluble because it contains carboxyl (-COO^-) and hydroxyl (-OH) functional groups. The -COO^- and -OH functional group can coordinate with metal to form coordination complex. The SA will become a hydrogel when it is cross-link with $CaCl_2$ [12]. Qiao et al. [7] synthesized SA-Ni and used as catalyst for hydrogenation. SA-Ni catalyst has excellent catalytic activity in styrene hydrogenation where it successfully converts 99.9% styrene to ethylbenzene. It also can be reused up to 20 times without loss of initial activity.

Both PVA and SA have good interaction with metals. The properties of SA can be increased by blending with

PVA [13]. Knijnenburg, et al. [14] used PVA-SA to bind with Zn for fertilizer application. While Rahman et al. [15] synthesized PVA-SA-$PdCl_2$ for Heck reaction. The catalytic performance of PVA-SA-$PdCl_2$ for reaction of aryl halides with styrene gives an outstanding result of 100% conversion rate. In addition, the recyclability of PVA-SA-$PdCl_2$ is excellent which is over 11 cycles. PVA-SA as active metal site support has been found in many literatures. As noted, there are yet findings available that emphasising the applications of PVA-SA as polymer support for Ni catalysts. Hence, the current study aimed to synthesise polymer-supported Ni or PVA-SA-Ni catalysts employable in Heck reactions. Moreover, the recyclability and optimum reaction conditions of the catalyst in different parameters were determined. This is a fundamental study to determine the capability of synthesis polymer-supported Ni or PVA-SA-Ni as catalyst in Heck reaction.

Materials and Methods

Materials and instrumentation

Polyvinyl alcohol (PVA, 99+% hydrolysed) (Aldrich), sodium alginate (SA) (Sigma, medium viscosity), nickel(II) chloride ($NiCl_2$) (Aldrich, 99%), nitric acid (HNO_3)(R&M Chemicals, 65%), hydrogen peroxide (H_2O_2, 50%) (Bendosen), calcium chloride ($CaCl_2$) (Sigma Aldrich, 97%), distilled water, styrene (R&M Chemicals, 99%), 1-bromo-4-nitrobenzene (Aldrich, 99%), N,N-Dimethylacetamide (DMA) (R&M Chemicals, 99%), toluene (R&M Chemicals, 99.5%), potassium carbonate (K_2CO_3) (Fisher Scientific, 99.5%), methanol (Sigma-Aldrich, 99%) and sodium acetate (NaOAc) (Sigma-Aldrich) were purchased from standard commercial suppliers and used as received without further purification.

The characteristic of PVA-SA-Ni was characterized by using flame atomic absorption spectroscopy (FAAS), attenuated total reflection-Fourier transform infrared (ATR-FTIR), field emission scanning electron microscope-energy dispersive X-ray (FESEM-EDX), and X-ray diffraction (XRD). The Ni content in PVA-SA-Ni was measured by using FAAS (Perkin Elmer-AAnalyst 400) utilizing a flame approach. The spectra of samples were provided by ATR-FTIR (Perkin Elmer Spectrum 100) analysis and recorded in the range of 650 cm^{-1} to 4000 cm^{-1} by averaging 16 scans numbers at 4 cm^{-1} resolution.

The sample surface analysis was conducted utilizing FESEM-EDX (Hitachi, SU8220). The analysis using a Carl Zeiss electron microscope at 10 kV with 3 k magnification. Diffractogram of the samples by using XRD (Empyrean, PANanalytical). It was recorded over a 2-theta range of 2° to 50° by X-ray diffractometer with a angle of 0.02^o min^{-1}. The voltage and current were utilized at 40 kV and 20 mA respectively.

The percentages of conversion rate and turnover number (TON) of reactant determined the catalytic efficiency. The percentage conversion rate and percentage TON were calculated by using equation (1) and (2) below. The data was obtained from Gas Chromatography (GC) (Agilent Technologies, 7890A). The instrument was equipped with an HP-5 capillary column (15 m × 0.25 mm × 0.25 μm) with a flame ionization detector (GC-FID). The microliter samples were injected at 50 °C (held for 1 min) and the final temperature of 250 °C (held for 30 s). The temperature increased at a rate of 15 °C min^{-1}. The flow rate was set at 1.9162 mL min^{-1}.

$$\% \text{ conversion} = \frac{A_{initial} - A_{final}}{A_{final}} \times 100 \quad (1)$$

$$\% \text{ TON} = \frac{\% \text{ conversion}}{\% \text{ catalyst}} \quad (2)$$

Preparation of catalyst

In the preparation of $NiCl_2$ catalyst, the optimum parameter from previous study was used with 1 h ultrasonic synthesise time. The modification method was acquired from a study by Rahman et al. [15].

Catalytic testing

1-bromo-4-nitrobenzene reacts with styrene monomer to produce 1-bromo-4-styrylbenzene in Heck reaction. All chemicals: 1-bromo- 4-nitrobenzene (0.2000 g;1 mmol), styrene monomer (0.2083 g; 2 mmol), Na_2CO_3 (0.2500 g, 2.4 mmol), PVA-SA-Ni catalyst (1.0 mmol%) and DMA (10 mL) as solvents was mixed in a round bottle flask and refluxed at 165 ºC based on the boiling point of DMA solvent whilst being purge with the nitrogen gas

for 1 h. The conversion rate of crude products was analyzed by GC-FID. The Heck reactions were then studied with three parameters, including the effect of different reaction times, solvent, and bases.

Recyclability of catalyst

The catalyst was separated from the reaction mixture after reaction and washed with water and acetonitrile to remove salts and organic residual. The catalyst was then dried in a vacuum oven and subjected to use in a fresh new reaction.

Results and Discussion

Characterization of synthesized compound

This study successfully synthesized PVA-SA-Ni (Figure 1) by using a one-pot ultrasonic method. The color of PVA-SA-Ni obtained is pale green as shown in Figure 2 (transformed from white color of PVA-SA). The amount of Ni is 0.14615 % in 50 mg PVA-SA-Ni was determined by using AAS.

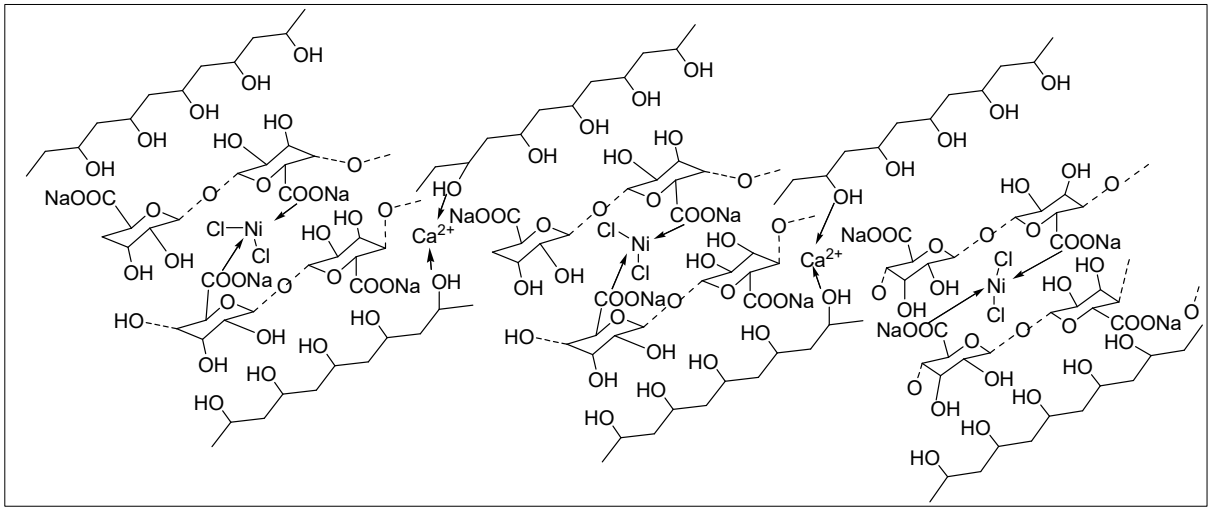


Figure 1. Structure of PVA-SA-Ni

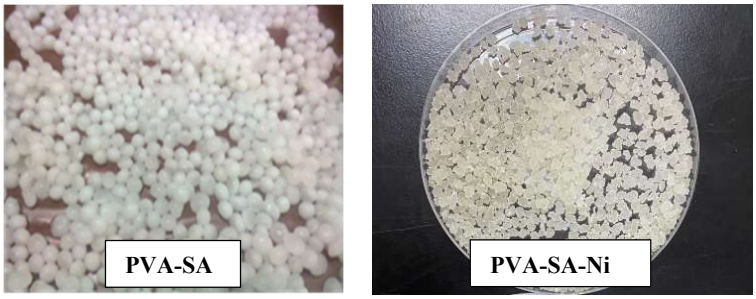


Figure 2. The images of PVA-SA and PVA-SA-Ni

The successful synthesis of PVA-SA-Ni catalyst was confirmed by using ATR-FTIR, FESEM-EDX and XRD. The functional group absorption bands of PVA-SA and PVA-SA-Ni using ATR-FTIR is summarized in Table 1 measured within 650 cm^{-1} to 4000 cm^{-1}. Based on Table 1, the functional group present in PVA-SA and PVA-SA-Ni catalyst is similar. This result showed that the PVA-SA structure was not altered during blending with $NiCl_2$ and only the position of functional group was affected [16].

Table 1 shows no significant changes for hydroxyl (-OH), alkyl (-CH_2 and -CH_3) and -CO groups. The -OH absorption bands appeared at 3345 cm^{-1} and 3355 cm^{-1} in both PVA-SA and PVA-SA-Ni catalyst due to presence of a large amount hydroxyl group in both

structure of PVA and SA [15]. The -CH_2 absorption bands of PVA-SA and PVA-SA-Ni appeared at 2920 cm^{-1} and 2924 cm^{-1}. The group of -CH_3 symmetry bending of PVA-SA and PVA-SA-Ni observed at 1320 cm^{-1} and 1333 cm^{-1}. While the absorption band of -CO visualized at 1085 cm^{-1} and 1086 cm^{-1}. Important absorption band is asymmetric and symmetric vibration of -C=O at 1636 cm^{-1} and 1429 cm^{-1} that moved to lower wavenumbers after complexation due to the interaction of $NiCl_2$ and -C=O from -COONa indicating the successful construction of PVA-SA-Ni catalyst [15, 17].

Table 1. The functional group of PVA-SA and PVA-SA-Ni

Functional Group	PVA-SA (cm^{-1})	PVA-SA-Ni (cm^{-1})
-OH stretching	3345	3355
-CH_2 stretching asymmetric	2920	2924
-C=O asymmetric stretching	1636	1616
-C=O symmetric stretching	1429	1423
-CH_3 symmetric bending	1320	1333
-C-O bending	1085	1086

The micrograph of outer surface and cross-section of PVA-SA and PVA-SA-Ni from FESEM (100x of magnification) is presented in Figure 3. The outer surface and cross section of PVA-SA beads in Figure 3 (A1) and Figure 3 (A2) had a rough and ridged surface. It has pores due to lumps stuck together. The presence of $NiCl_2$ in outer surface and cross section of PVA-SA-Ni Figure 3 (B1) and Figure (B2) caused modification on PVA-SA which resulted more pores on PVA-SA-Ni as compared to PVA-SA [16]. Based on Rahman et al. [15] study's every angle of pores allow catalytic reaction to occur by allowing diffusion of reactant and product utilised in the reaction that help in catalytic activity. The FESEM mapping of PVA-SA-Ni outer surface is shown in Figure 3 (**C1**) and cross-section in Figure 3 (**C2**) revealed that dispersion of Ni metal is homogenous over the whole mass of the beads through the binding process.

Meanwhile, the chemical composition of the PVA-SA and PVA-SA-Ni (Figure 4) was analysised by using an energy dispersive x-ray (EDX). The element present in PVA-SA was similar as reported by Rahman et al. [15] that contain element of carbon (C), oxygen (O), sodium (Na), calcium (Ca) and chlorine (Cl). In contrast, the PVA-SA-Ni detected contained 0.8 % mass of Ni atom. The crystalline phase in PVA-SA and PVA-SA-Ni was confirmed by using XRD as shown in Figure 5. Diffractogram of PVA-SA showed peaks appeared at 20° with shoulder similar to diffractogrom obtained by Sang Joon Lee, et al. [18]. Broad peaks obtained indicated the PVA-SA is in amorphous form similar to a study by Rahman, et al. [15]. In contrast with diffractogram of PVA-SA-Ni , the peaks at 20° is narrower and sharp, indicating that the presence of Ni promoted the crystallisation of PVA-SA [19]. While, peak of Ni at 44.4°, 51.7° and 76.4° [20] was not seen because the amount of Ni in PVA-SA-Ni is small based on data FAAS and EDX. From all the characterisation data of PVA-SA-Ni were sucessfully synthesised and the structure is illustrated in Figure 1.

Catalytic activity of PVA-SA-Ni in Heck reaction

The efficiency of PVA-SA-Ni as catalyst was tested in Heck reaction between styrene and 1-bromo-4-nitrobenzene. The study focused on optimizing the reaction condition and the results are summarized in Table 2. The initial test was conducted by using 1 mmol % of the PVA-SA-Ni catalyst, K_2CO_3 as base, DMA as solvent, reflux at 165 °C temperature based on DMA solvent boiling point with different times. The results are recorded at Entries 1-5.

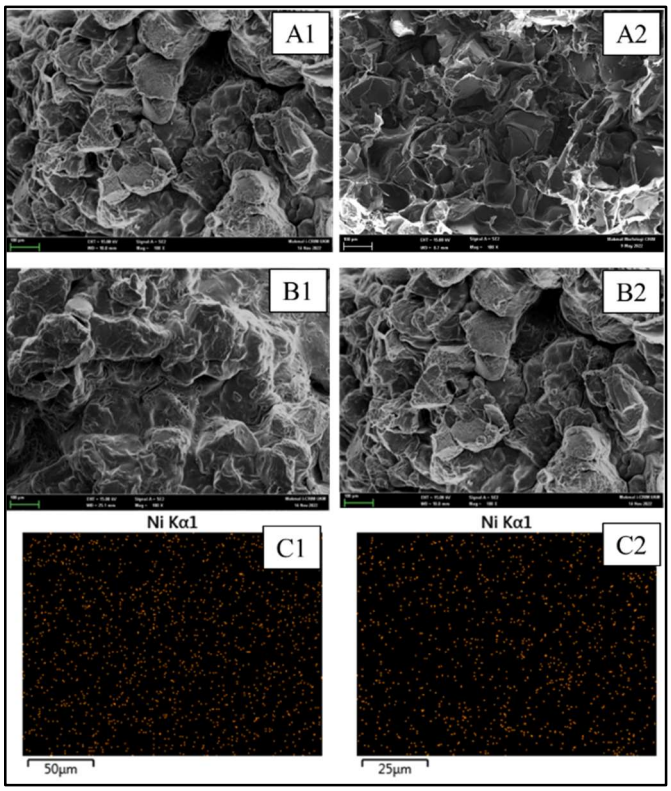
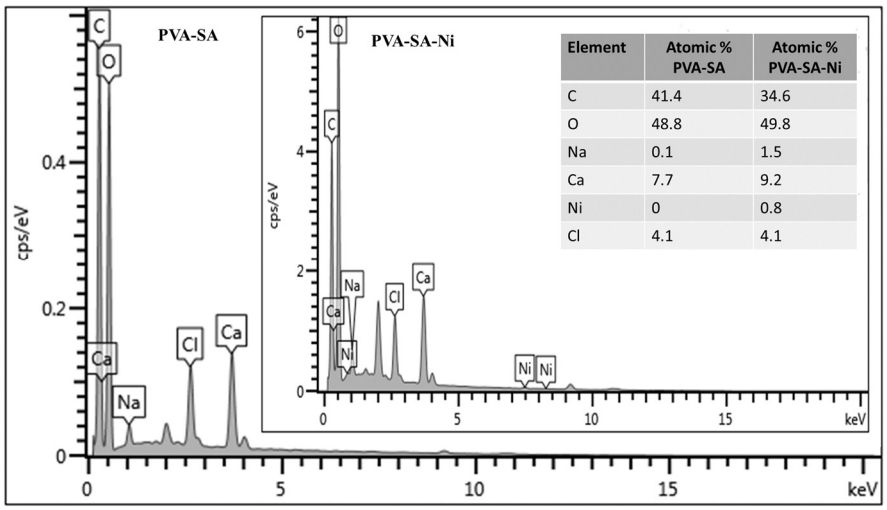


Figure 3. FESEM micrograph of PVA-SA (**A1** and **A2**), PVA-SA-Ni (**B1** and **B2**) and elemental mapping of Ni for PVA-SA-Ni (**C1** and **C2**)



Element	Atomic % PVA-SA	Atomic % PVA-SA-Ni
C	41.4	34.6
O	48.8	49.8
Na	0.1	1.5
Ca	7.7	9.2
Ni	0	0.8
Cl	4.1	4.1

Figure 4. The EDX images of PVA-SA and PVA-SA-Ni

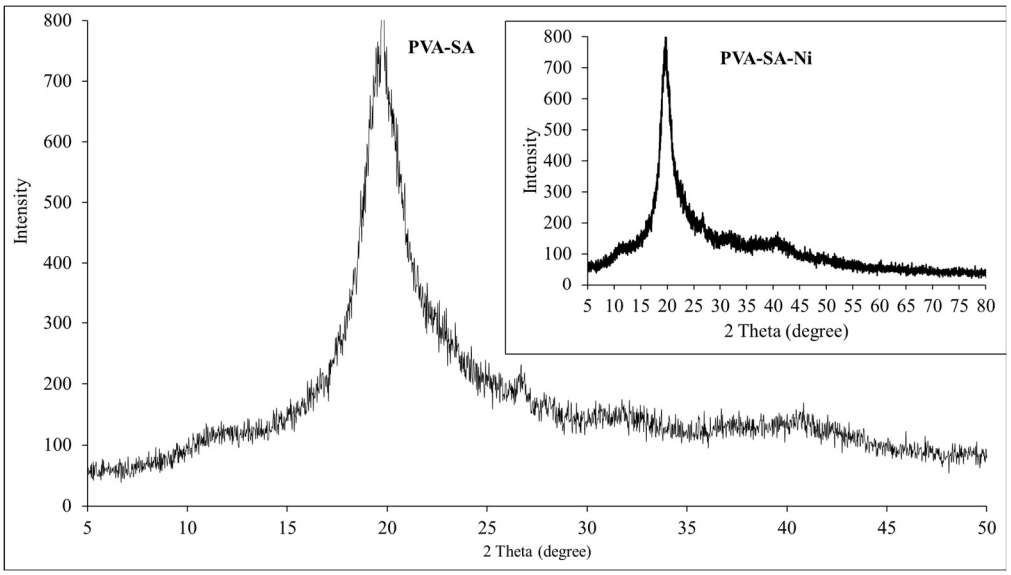


Figure 5. The diffractogram of PVA-SA and PVA-SA-Ni

The first 1 h of reaction, only 36% (Entry 1) of conversion rate was obtained. In order to confirm the substrates have sufficient time to convert to product, the reaction time was increased (Entries 2-5) [21]. Within 4 h of reaction times, 74% (Entry 4) of conversion rate was recorded. However, reaction time increase to 5 h showed no significant changes in conversion rate percentage because deactivation of catalyst caused by the reaction was too long [22]. Thus, 4 h was selected as optimum reaction time for future study in effect of bases, and solvents.

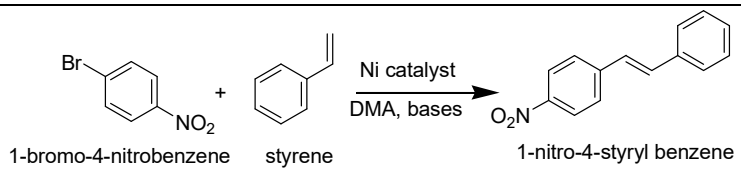
Catalytic activities also would increase by the presence of suitable bases. The function of base is to regenerate active complexes of catalyst by removing hydrogen halide from inactive catalyst complex [23]. In this study, K_2CO_3, Na_2CO_3 and NaOAc (Entries 4, 6-7) were used. The highest conversion rate percentage by using K_2CO_3 probably due to the solubility of base in solvent [21].

The effect of solvent in the catalytic activity has been studied by employing polar solvent DMA, 6.5 (Entry 4) and methanol, 6.1 (Entry 8) and non-polar solvent toluene (Entry 9). Based on Table 2, DMA was the most efficient solvent due to its higher basicity and nucleophilicity [24] as compared to methanol that is less polar. Toluene is not suitable in this catalytic activity due to its non-polar solvent.

From all the results obtained, used of 1 mmol% PVA-SA-Ni catalyst load gained the highest catalytic activity when K_2CO_3 acted as base, DMA as solvent, with temperature at 165 °C and time within 4 h were employed (Entry 4).

The parameter for entry 4 was used to pursue the recyclability study of PVA-SA-Ni catalyst. It is important to evaluate the ease separation of catalyst from reaction mixture. The catalyst was separated from reaction mixture by easy filtration step, wash, dried and used for next catalytic cycle. The reusability of PVA-SA-Ni was three times based on Entry 4, 10 and 11. In the 1st run, the conversion rate of the product was 74%. For the 2nd run, the conversion rate of product was 69%. However, for the 3rd run, the conversion rate obtained was 24% and the catalyst was denatured. The reusability of the heterogeneous catalyst was low maybe due to Ni leaching from FAAS analysis (59%). It is expected that this results in a loss of catalytic because in many cases showed low reusability and low catalytic activity due to the leaching [15].

Table 2. Optimisation of Heck cross-coupling reaction of PVA-SA-Ni (II) catalyst



Entry	Base	Solvent	Time (h)	%Conversion
1	K_2CO_3	DMA	1	36
2	K_2CO_3	DMA	2	40
3	K_2CO_3	DMA	3	51
4	K_2CO_3	DMA	4	74
5	K_2CO_3	DMA	5	69
6	Na_2CO_3	DMA	4	6
7	NaOAc	DMA	4	43
8	K_2CO_3	Methanol	4	2
9	K_2CO_3	Toluene	4	-
10	K_2CO_3	DMA	4	69
11	K_2CO_3	DMA	4	24

*The catalytic study was conducted by using 1 mmol % of PVA-SA-Ni at reflux condition

The plausible reaction mechanisms involved in Heck reaction between 1-bromo-4-nitrobenzene and styrene is shown in Figure 6. The first step is started with the reduction of catalyst, complex $NiCl_2$-A to the catalytic active species Ni(0)-A with the presence of K_2CO_3, where A is PVA-SA polymer. The active species of Ni(0)-catalysed coupling reaction in five steps; (2) oxidative addition of 1-bromo-4-nitrobenzene to generate (a), (3) styrene insertion to form a π-alkene complex (b), (4) migratory insertion to produce σ-alkyl Ni complex (c), (5) rearrangement of the complex by internal rotation to generate (d), β-hydride elimination to release targeted product (e), and the addition of base to regenerate the catalyst by removing hydrogen halide (HX) from the inactive complex.

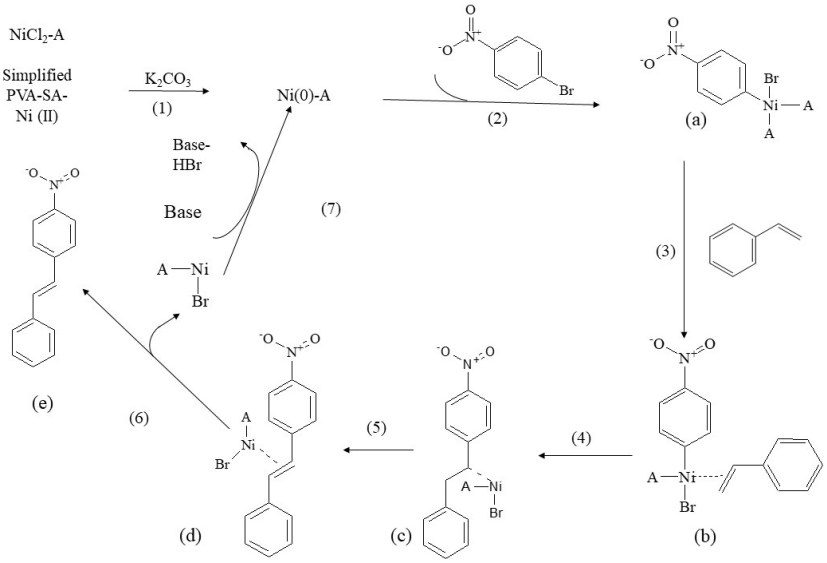


Figure 6. The proposed mechanism of Heck reaction in the presence of a PVA-SA-Ni as a catalyst

Conclusion

This study was successful in supporting $NiCl_2$ by using PVA-SA that form PVA-SA-Ni catalyst utilizing one pot ultrasonic method. The Ni loading in PVA-SA-Ni was 0.14615 % in 50 mg of catalyst. The successful synthesis PVA-SA-Ni was confirmed by using FAAS, ATR-FTIR, FESEM-EDX and XRD. The catalytic activity of catalyst in Heck reaction between 1-bromo-4-nitrobenzene and styrene was analyzed by using GC-FID gave 74% conversion rate at optimum condition. The optimum condition involved K_2CO_3 as base in DMA solvent at 165 °C and 4 h reaction time. The recyclability of catalyst is up to 3 cycles. Therefore, this study produced a novel green technique in preparing Ni catalyst that has potential for Heck reaction. However, study on the structure of catalysts improvement is needed by modification of parameter during synthesis to achieve high metal loading that influence conversion rate percentage and recyclability of catalyst. Further research on optimization of the synthesis parameters and exploring additional factors could potentially enhance the catalytic performance even more. Continuous improvement and refinement of the synthesis process are essential to maximize the efficiency and applicability of the catalyst.

Acknowledgement

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METHANOGEN INHIBITOR EFFECT ON ANAEROBIC DEGRADATION OF 1,2-DICHLOROETHANE BY SUNGAI ULAR SEDIMENT

(Kesan Perencat Metanogen Terhadap Degradasi Anaerobik 1,2-Dikloroetana oleh Sedimen Sungai Ular)

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Abstract

The toxicity of industrial organohalides such as 1,2-dichloroethane (1,2−DCA) and their longevity in the environment has piqued the public's interest. Anaerobic degradation by Organohalide-Respiring Bacteria (OHRB) has emerged as one of the effective techniques in the removal or degradation of toxic organohalides. Meanwhile, it is typical for methanogenesis to occur within OHRB-dechlorinating bacteria microcosms. These methanogens will compete with the OHRB in the cultures for nutrients and electron donors such as hydrogen, limiting the OHRB's development and dechlorination activity. Methanogen inhibitors are used to remove methanogens from enrichment cultures. The objective of this study is to investigate the effect of methanogen inhibitors on the degradation rate of 1,2-dichloroethane (1,2-DCA) by Sungai Ular sediments. The concentration of 2-Bromoethanesulfonate (BES) as a methanogen inhibitor was varied from 1−25 mM and its effects on the 1,2-DCA degradation were monitored using gas chromatography (GC). Methane which is the methanogenesis product, was also monitored to evaluate the effectiveness of the inhibitor. The results indicated the microcosm treated with 25 mM BES demonstrated the fastest 1,2-DCA degradation at 0.61 µM/day. The amount of methane produced showed a decline in all the BES concentrations, with 25 mM BES demonstrating the lowest methane production at 0.2 ppm. Metagenomic data after the BES treatment also revealed a decrease in the methanogen's population from 42% to 0.007% and an increase in OHRBs population 5% to 69%. This study showed that the addition of a methanogen inhibitor can significantly increase the degradation rate of the chlorinated compound by inhibiting the growth of the methanogens.

Keywords: organohalide-respiring bacteria, methanogen inhibitors, dechlorination, methanogen, 1,2−dichloroethane

Abstrak

Ketoksikan organohalida industri seperti 1,2-dikloroetana (1,2−DCA) dan jangka hayatnya dalam alam sekitar telah menarik minat orang ramai. Degradasi anaerobik oleh bakteria pernafasan organohalida (OHRB) telah muncul sebagai salah satu teknik yang berkesan dalam penyingkiran atau degradasi organohalida toksik. Sementara itu, adalah tipikal untuk metanogenesis berlaku dalam mikrokosmos bakteria penyahklorinan OHRB. Metanogen ini akan bersaing dengan OHRB dalam kultur untuk nutrien dan penderma elektron seperti hidrogen, mengehadkan aktiviti pembangunan dan penyahklorinan OHRB. Perencat metanogen digunakan untuk mengeluarkan metanogen daripada kultur pengayaan. Objektif kajian ini adalah untuk menyiasat kesan perencat metanogen terhadap kadar degradasi 1,2-dikloroetana (1,2-DCA) oleh sedimen Sungai Ular. Kepekatan 2-Bromoetanasulfonat

(BES) sebagai perencat metanogen telah diubah daripada 1−25 mM dan kesannya terhadap degradasi 1,2-DCA dipantau menggunakan kromatografi gas (GC). Metana yang merupakan produk metanogenesis, juga dipantau untuk menilai keberkesanan perencat. Keputusan menunjukkan mikrokosmos yang dirawat dengan 25 mM BES menunjukkan degradasi 1,2-DCA terpantas pada 0.61 µM/hari. Jumlah metana yang dihasilkan menunjukkan penurunan dalam semua kepekatan BES, dengan 25 mM BES menunjukkan pengeluaran metana terendah pada 0.2 ppm. Data metagenomik selepas rawatan BES juga mendedahkan penurunan populasi metanogen daripada 42% kepada 25% dan peningkatan populasi OHRB 5% hingga 30%. Kajian ini menunjukkan bahawa penambahan perencat metanogen boleh meningkatkan kadar degradasi sebatian berklorin dengan ketara dengan menghalang pertumbuhan metanogen.

Kata kunci: bakteria penafas organohalida, perencat metanogen, penyahklorinan, metanogen, 1,2−dikloroetana

Introduction

Anthropogenic organohalide pollution of land and water (e.g., through the manufacturing and use of pesticides, dry-cleaning solvents, ozone-depleting refrigerants, and industrial degreasers) is ubiquitous and constitutes a serious risk due to its negative effects on human health and ecosystems [1]. Anthropogenic organohalogens have significantly damaged the environment as a result of their widespread usage and frequently unregulated disposal. The Stockholm Convention lists polyhalogenated pollutants as the majority of persistent organic pollutants with a serious impact on human, animal, and environmental health [2,3]. Much research has been conducted in order to better understand the microbial detoxification of anthropogenic organohalogens and to create viable bioremediation techniques [4,5].

Halogenated alkane with the common name ethyl chloride (chloroethane) is commonly used in the production of tetraethyllead (TEL), which is an additive for gasoline. The 1,2-dichloroethane (1,2-DCA) compound belongs to a category of chloroethanes that have two chloro groups replaced at positions 1 and 2. Vinyl chloride, which is used to create a range of plastic and vinyl items such as polyvinyl chloride (PVC) pipes, furniture and car upholstery, wall coverings, household goods, and vehicle parts, is the most prevalent usage of 1,2-DCA. When toxicity occurs, higher concentrations of 1,2-DCA can circulate throughout the body and bind to glutathione instead of being detoxified and removed because the biotransformation systems are saturated [6].

Anaerobic degradation by using OHRBs has emerged as one of the effective methods in the degradation of toxic chlorinated compounds which include 1,2-DCA. The OHRBs are found in several phyla including the Proteobacteria, Firmicutes and Chloroflexi [7]. Reductive dehalogenase enzymes, which is a special characteristic of OHRB, allow them to employ various organohalides as terminal electron acceptors and live in a variety of terrestrial and aquatic habitats. The capacity of OHRB to utilize organohalides as terminal electron acceptors to provide energy for growth offers a possible remedy for cleaning up polluted locations [8].

One of the common problems in the OHRBs bacterial communities is the presence of methanogens. Since methanogens are usually the dominant anaerobic bacteria in most reduced aquifers, the electron donor and substrate consumption by methanogens could be a rate-limiting factor for OHRBs during anaerobic degradation of halogenated compounds [9]. These methanogens will compete with the OHRB in the cultures for nutrients and electron donors such as hydrogen, which will slow down the growth of the OHRB and its dechlorination activity [10]. The effectiveness of dechlorination is limited when methanogens are present because they can outcompete OHRB for available electron sources and acceptors. Methanogen inhibitors are compounds that selectively target and inhibit the activity of methanogens. The activity of OHRBs will be increased and methanogen competition will be decreased by the addition of the methanogen inhibitor to the consortia containing OHRBs [9]. The aim of this study is to investigate the relationship between the dechlorination process of the Sungai Ular sediments and the methanogen inhibitor, specifically 2-Bromoethanesulfonate (BES).

Materials and Methods

Sample collection

Inocula was obtained from sediments collected at Sungai Ular (4.046821°N, 103.395601°W) in Kuantan, Pahang. The sediments were taken at 15 cm below the riverbank surface using sterile 50 mL Falcon tubes. The sampling tubes is immersed beneath the sediment, filled with

sediment, and completely closed before being brought to the surface. Exposure to air was strictly limited and the samples were transferred into the serum bottle within 24 hours.

Preparation of anaerobic medium

The anaerobic medium contained 0.2% (w/v) KH_2PO_4, 0.27 % (w/v) NH_4Cl, 1% (w/v) NaCl, 0.41% (w/v) $MgCl_2.6H_2O$, 0.52% (w/v) KCl, 0.15 % (w/v) $CaCl_2.2H_2O$, 0.7% (w/v) $ZnCl_2$, 0.8% (w/v) $MnCl_2.4H_2O$, 0.06% (w/v) H_3BO_3, 0.19% (w/v) CoCl2.6H_2O, 0.02 % (w/v) $CuCl_2.2H_2O$, 0.24% (w/v) $NiCl_2.6H_2O$, 0.36% (w/v) $Na_2MoO_4.2H_2O$, 0.02% (w/v) $FeCl_2.4H_2O$ and 0.0128% (w/v) nitriloacetic acid [11]. The medium was flushed with nitrogen for approximately 2 minutes before being sealed with a rubber stopper and aluminum crimp cap. The medium was then autoclaved for 20 minutes at 121°C. Sterile $NaHCO_3$ solution (1g/L), $Na_2S.9H_2O$ + L-cysteine solution (25mg/L) and vitamin cyanocobalamin solution (40 mg/L) were then added to the serum bottles before the inoculation of sediments.

Degradation of 1,2-DCA using methanogen inhibitor

Six g of sediment (wet weight) was inoculated to each 120 mL glass serum bottle containing 65 mL medium prepared above. 20 µM 1,2−DCA as the electron acceptor and overpressure H_2 at 0.4 bar as the electron donor were added to the serum bottles. The methanogen inhibitor, 2-bromoethanesulfonate (BES) at 1, 10 and 25 mM were then added to the consortia. Microcosms were prepared in triplicates for each BES concentration. The serum bottles were then incubated at 25 °C in dark static conditions.

Gas chromatography analysis

1,2−DCA, methane and other volatile organic compounds were detected and quantified by analyzing headspace taken from the serum flasks using gas chromatography coupled to a flame ionized detector (GC-FID). The analysis was carried out using DB−624 column (30m × 0.32mm with 0.25µm film thickness; Agilent Technologies). The oven, injector and detector temperatures were fixed at 150 °C, 250 °C and 260 °C, respectively for 25 minutes. 1 mL of the sample was taken from the headspace and then manually injected into GC-FID. Chromatographic peak areas obtained from the GC-FID were converted to corresponding 1,2−DCA concentrations.

Results and Discussion

1,2−DCA degradation

Figure 1 shows the concentration of 1,2−DCA in the Sungai Ular anaerobic bacterial culture at different concentrations of BES as methanogen inhibitor. Microcosm without any BES addition labeled as "No BES" was included as control. 20 µM of 1,2−DCA was added at day 0 and an additional 20µM of 1,2−DCA was added at day 32, after all the 1,2−DCA fully degraded in all tested microcosms. During the first addition of 1,2−DCA (day 0-32), 25 mM BES recorded the fastest 1,2−DCA degradation when compared to other added concentrations of BES. However, the control culture (No BES) demonstrates faster 1,2−DCA degradation than the 25 mM BES culture. Nonetheless, following the second addition of 1,2−DCA at day 32, 25 mM BES started to exhibit more rapid 1,2−DCA degradation than the control culture and other BES concentrations. 1 mM BES displays the slowest 1,2−DCA degradation followed by the control culture and 10 mM BES, respectively.

Figure 2(a) depicts the chromatogram profiles for 1,2−DCA at day 1 after the transfer while Figures 2(b) and 2(c) depict the chromatogram profiles of No BES and 25mM BES consortia after several days. As can be seen from those figures, 1,2−DCA partially degraded in consortia with No BES while it was completely degraded in 25mM BES consortia. This indicates that the addition of BES accelerated the degradation rate of 1,2−DCA. Similar outcomes were seen in a study by Lin et al. [9], where the inclusion of BES led to the maximum trichloroethylene (TCE) removal efficiency of 99.8%, while BES-free cultures only achieved 77.4% TCE removal.

In the first cycle (day 0−32), lower 1,2−DCA degradations in the cultures with BES addition when compared to control culture can be caused by several factors. The first factor could be caused by slower build-up of the dehalogenating OHRB communities as compared to methanogen populations [12]. During this time, methanogens competed for hydrogen and substrates with the OHRBs and contributed to the lag of 1,2−DCA degradation [13]. Aside from that, numerous studies also pointed out that sometimes, instead of limiting the growth of OHRBs, methanogens synthesized cyanocobalamin (vitamin B_{12}) which is a

niche co-factor in OHRBs growth [3,4]. However, at the second cycle of 1,2−DCA addition (day 52-66), higher BES concentrations contributed to a more positive impact on the dechlorination of 1,2−DCA. It is possible that during this period, the populations of OHRBs began to expand more quickly than those of methanogens. Thus, high concentrations of BES favor the dechlorination by OHRB and limiting the methanogens' activity. Aside from that, the redox potentials and dechlorination levels of the polychlorinated compounds itself appeared to be involved in their capacity to inhibit methanogenesis and promote reductive dechlorination [14].

Methane production

Figure 3 depicts the production of methane, which is the product of methanogenesis in the BES-treated and untreated cultures. As depicted in Figure 3, all the cultures showed methane increment from day 1 to day 32. After that, the amount of methane started to decrease in all BES-treated cultures except the without BES culture. This is in line with the 1,2−DCA degradations displayed in Figure 1, where lower dechlorination activities were observed during day 0−32. An increase in methane production indicates higher methanogen activities and thus limiting the dechlorination activities. However, from day 32−66, the methane concentrations started to drop in all the BES treated cultures and an increase in 1,2−DCA dechlorination was also observed. Higher concentrations of BES were discovered to give favorable benefits in the reduction of methane production with 25 mM BES demonstrating the lowest methane production, followed by 10 mM and 1 mM BES. Meanwhile, the methane in treatment cultures without BES keeps increasing and thus causing slower 1,2−DCA degradation in it. Furthermore, chromatogram profiles in Figure 2 demonstrate that consortia without BES addition (Figure 2b) produced higher levels of methane but slower degradation of 1,2−DCA than consortia that had 25mM of BES added into it (Figure 2c). Consortia supplemented with 25 mM BES (Figure 2c) exhibit lower methane production and after a few days, it totally degraded 1,2-DCA.

A similar trend was also observed in other studies on the degradation of trichloroethylene (TCE) by *Dehalococcoides* sp. in which the addition of 0.5 mM BES was found to improve the dechlorination rate of TCE [9]. However, this study did not particularly study the effect of BES at different concentrations. In another study that investigates the effect of BES concentrations (10–30 mM) on pentachlorophenol degradation that was carried out by Zhu et al., methane was hardly detected at BES concentrations as low as 10 mM, indicating complete inhibition of methanogens in their study [15]. Nevertheless, these two previous studies agree with this study where there is a synergistic association between the increased rate of halogenated chemical degradation and the reduced methane production caused by the addition of BES.

Microbial community compositions

Figure 4 illustrates the metagenomic data by 16S rRNA gene sequencing before (Figure 4a) and after the BES treatment (Figure 4b). In this figure, the identified OHRB were *Desulfobaccia, Desulfobacteria, Desulfomonillia, Desulfuromonadia, Desulfovibrionia, Shewanella* and *Desulfitobacteriia*); while the identified methanogens are *Methanomicrobia, Methanococci, Methanosarcinia* and *Methanobacteria*. Blue color indicates the presence of methanogens in the microbial communities of Sungai Ular sediments. The findings show that the total bacterial diversity decreased in the microcosm treated with BES. The relative abundance of methanogens in the microcosm decreased from 42% to 0.007%. Meanwhile, the relative abundance of OHRBs population increased from 5% to 69%. These results suggest that methanogen proliferation may be considerably inhibited by high BES concentrations.

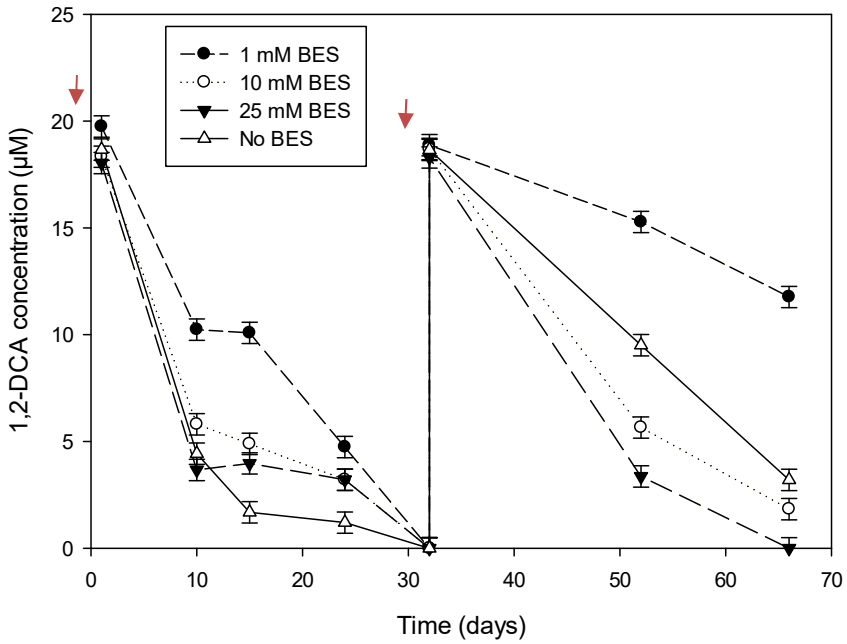


Figure 1. 1,2−DCA degradation at different BES concentrations by Sungai Ular sediments. Arrows (↓) indicate the addition of 1,2-DCA

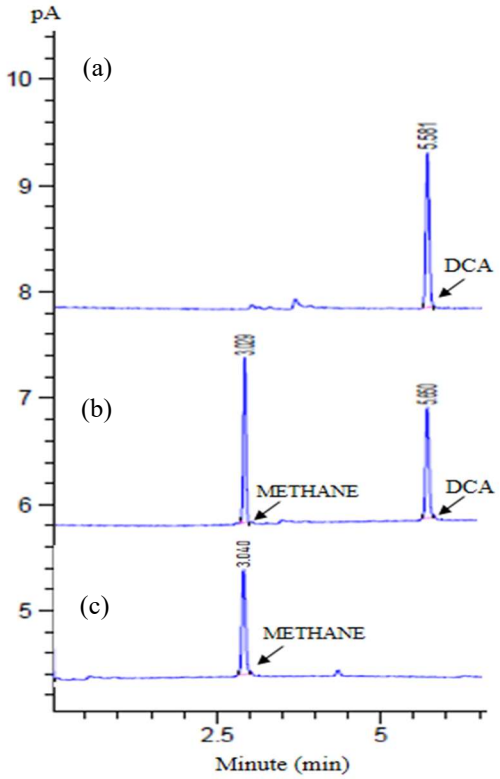


Figure 2. 1,2−DCA and methane chromatogram profile, (a) initial chromatogram of the consortia, (b) chromatogram of consortia with No BES treatment, (c) chromatogram of consortia with 25mM BES

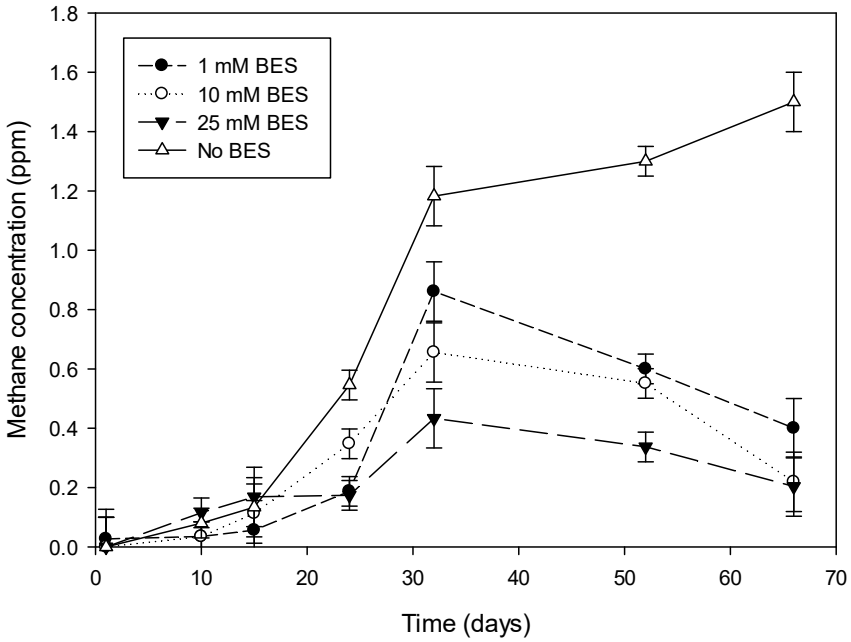
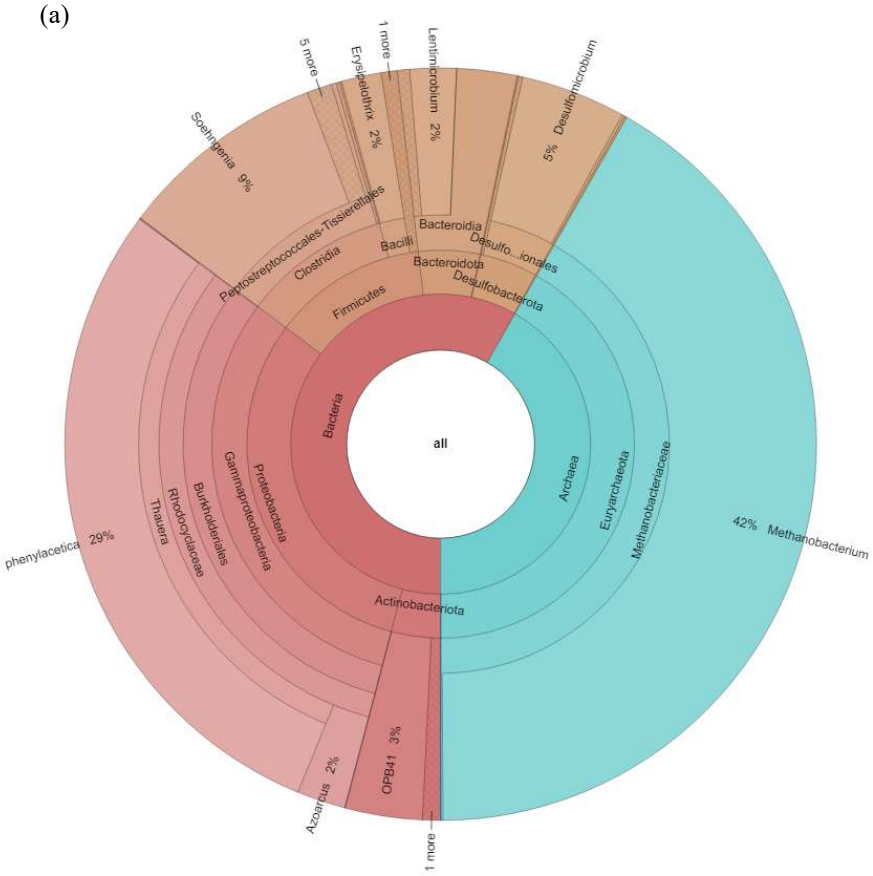


Figure 3. Methane concentration at different BES concentrations in Sungai Ular sediments

(a)



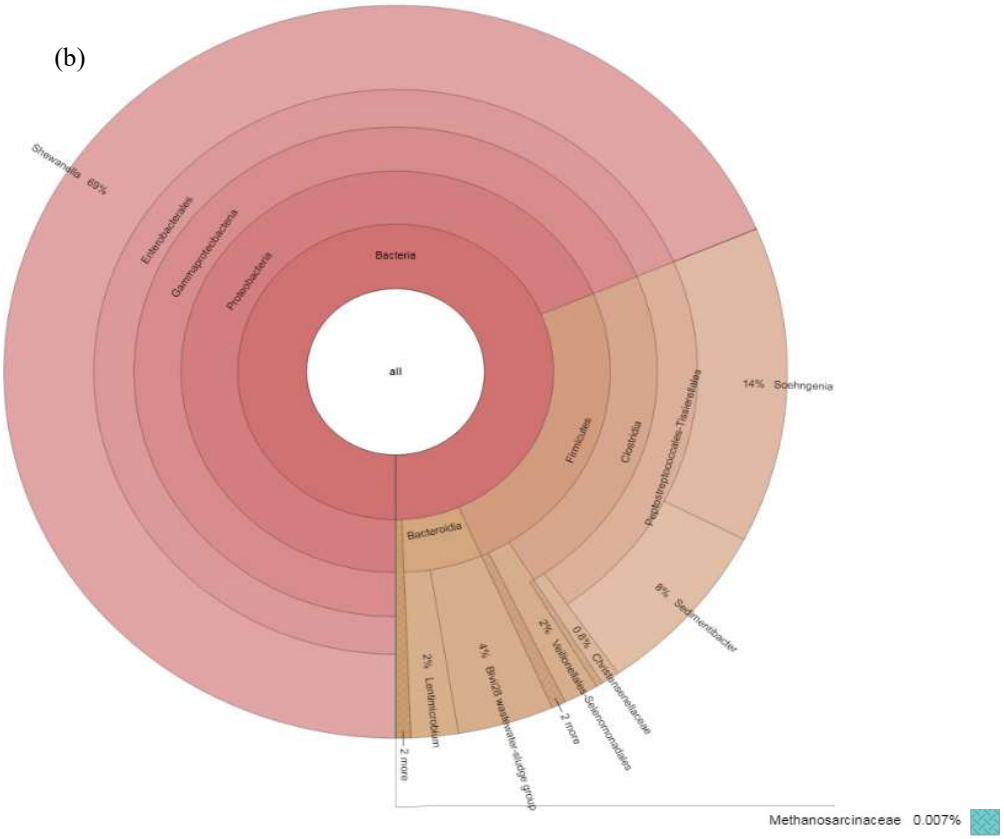


Figure 4. Bacterial communities in Sungai Ular sediments, (a) metagenomic data before the addition of BES, (b) metagenomic data after addition of 25 mM BES

Despite that, the synchronous relationship between methanogenesis and the anaerobic breakdown of halogenated substances remains incomprehensible [13,15]. Most studies on the effect of methanogen inhibitors on dechlorination were carried out in simple systems and its effect on largescale contaminated sites has never been tested. In addition, there are no definite studies concluding the negative impact of methanogens' presence on the dechlorinators population in the environment. The methanogens competition for nutrients and electron donors may hinder the OHRBs growth but simultaneously also provides a beneficial co-factor for OHRBs growth.

Conclusion

This study demonstrated that the degradation rate of 1,2–DCA was highly affected by different concentrations of BES. Higher 1,2–DCA degradation and lower methane production were found in the culture with higher BES concentration. The metagenomic data also supported the relationship between the BES concentrations with the methanogens and OHRBs population. Our results suggested a potential application for high concentrations of methanogen inhibitor in promoting faster degradation rates of chlorinated compounds.

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INVESTIGATIONS ON THE INCLUSION OF BETULINIC ACID INTO CD-MOF-1 PERFORMED BY QUANTUM MECHANICS CALCULATIONS AND MOLECULAR DOCKING SIMULATION

(Kajian Kemasukan Asid Betulinik ke dalam CD-MOF-1 Melalui Pengiraan Mekanik Kuantum dan Simulasi Dok Molekul)

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Abstract

In recent years, CycloDextrin Metal-Organic Frameworks (CD-MOFs) have been investigated to develop potential drug carriers and improve the solubility of some molecules by their encapsulation. Betulinic acid (BA) or 3β-hydroxy-lup-20(29)-en-28-oic acid is a pentacyclic triterpene of the lupane family which has a wide range of biological activities, and it is considered a promising candidate for clinical application but, its high hydrophobicity and limited aqueous solubility contribute to its poor bioavailability. In this work, we show through computational studies that CD-MOF-1 can strongly encapsulate betulinic acid molecules through hydrogen bond interactions. For this purpose, the optimized geometry of BA was implemented by the Density Functional Theory using the function of Becke, Lee, Yang, and Parr (DFT/B3LYP) method with a 6-31+G(d) basis set using the Gaussian09 program and GaussView 5.0 for visualization. We noticed that BA has a lower energy gap (Egap = 6.1615 eV) indicating that it is soft, less stable, more reactive, and polarizable. According to the map of electrostatic potential (MEP), the active site of betulinic acid is the carboxylic group and the latter molecule preferred electrophilic attacks. The molecular docking was performed using AutoDock Vina v1.1.2 program and Discovery Studio Visualizer 16.1. The best binding affinities of BA and CD-MOF-1 had the lowest values of -8.2 KCal/mol and -11.5 KCal/mol for the simple and the packed CDMOF-1 structure respectively. The docking revealed that BA bound in the hydrophobic cavities of CD-MOF-1 through hydrogen bonds interactions which are [CD-MOF-$1_{OH13\cdots O28}$BA (2.95 Å)], [CD-MOF-$1_{O13\cdots H30}$BA (2.54 Å)], and [CD-MOF-$1_{OH12\cdots H30}$BA (2.12 Å)] for the simplest structure and [CD-MOF-$1_{OH12A\cdots O28}$BA (3.00 Å)], [CD-MOF-$1_{H13A\cdots H30}$BA (2.72 Å)] and [CD-MOF-$1_{OH2A\cdots H30}$BA (2.66 Å)] for the packed structure 1×1×1. The packed CD-MOF-1 structure is the best with accurate results, this may be the factor of enhancement of betulinic acid solubility and bioavailability. The present theoretical results indicate the possibility of forming the host-guest inclusion complex between BA and CD-MOF-1 which may enhance the solubility of BA and then its efficiency for drug delivery.

Keywords: CD-MOF, betulinic acid, quantum mechanics, molecular docking, solubility

Abstrak

Kebelakangan ini, pembangunan kerangka kerja logam-organik siklodekstrin (CD-MOF) telah dikaji sebagai pembawa ubat yang berpotensi dan meningkatkan keterlarutan beberapa molekul melalui pengkapsulan mereka. Asid betulinik (BA) atau asid 3β-hidroksi-lup-20(29)-en-28-oik ialah triterpena pentasiklik daripada keluarga lupana yang mempunyai pelbagai aktiviti biologi, dan ia dianggap sebagai calon yang berpotensi untuk aplikasi klinikal, namun hidrofobisitinya yang tinggi dan keterlarutan dalam air yang terhad menyumbang kepada bioavailabilitinya yang lemah. Dalam kajian ini, kajian pengiraan menunjukkan bahawa CD-MOF-1 boleh memerangkap molekul asid betulinik dengan kuat melalui interaksi ikatan hidrogen. Untuk tujuan ini, geometri BA yang optimum telah dilaksanakan melalui kaedah Teori fungsi ketumpatan menggunakan fungsi Becke, Lee, Yang, dan Parr (DFT/B3LYP) dengan set asas 6-31+G(d) menggunakan program Gaussian09 dan GaussView 5.0 untuk visualisasi. Kami mendapati bahawa BA mempunyai jurang tenaga yang lebih rendah (Egap = 6.1615 eV) menunjukkan bahawa ia lembut, kurang stabil, lebih reaktif dan boleh dipolarisasi. Menurut peta potensi elektrostatik (MEP), tapak aktif asid betulinik ialah kumpulan karboksilik dan molekul terakhir lebih cenderung kepada serangan elektrofilik. Pendokkan molekul telah dilakukan menggunakan program AutoDock Vina v1.1.2 dan Discovery Studio Visualizer 16.1. Perkaitan pengikatan terbaik BA dan CD-MOF-1 mempunyai nilai terendah -8.2 KCal/mol dan -11.5 KCal/mol untuk struktur CDMOF-1 yang ringkas dan padat. Pendokkan mendedahkan bahawa BA terikat dalam rongga hidrofobik CD-MOF-1 melalui interaksi ikatan hidrogen iaitu [CD-MOF-$1_{OH13\cdots O28}$BA (2.95 Å)], [CD-MOF-$1_{O13\cdots H30}$BA (2.54 Å)], and [CD-MOF-$1_{OH12\cdots H30}$BA (2.12 Å)] untuk struktur paling ringkas dan [CD-MOF-$1_{OH12A\cdots O28}$BA (3.00 Å)], [CD-MOF-$1_{H13A\cdots H30}$BA (2.72 Å)] and [CD-MOF-$1_{OH2A\cdots H30}$BA (2.66 Å)] untuk struktur tersusun 1×1×1. Struktur CD-MOF-1 tersusun adalah yang terbaik dengan hasil yang tepat, yang berkemungkinan faktor peningkatan keterlarutan asid betulinik dan bioavailabiliti. Keputusan teori semasa menunjukkan kemungkinan pembentukan kompleks kemasukan hos antara BA dan CD-MOF-1 yang boleh meningkatkan keterlarutan BA dan kemudian kecekapannya untuk penghantaran ubat.

Kata kunci: CD-MOF, asid betulinik, mekanik kuantum, dok molekul, keterlarutan

Introduction

For millennia, natural products have been used as a source of natural bioactive compounds for the medication and treatment of various sicknesses [1]. Alkaloids, steroids, quinones, limonoids, flavonoids, as well as another significant component, terpenoids, are all being explored in medicine for their potential to improve health. Based on their chemical skeleton, terpenoids can be classified as monoterpenes, sesquiterpenes, diterpenes, triterpenoids, and steroids [2]. Triterpenes molecules, in particular, are the most abundant terpenoids and their study has increased dramatically during the last decade due to their different kinds of bioactivities [3], including liver protection, antioxidant, anti-cancer, immunomodulatory, anti-inflammatory, and anti-obesity [2, 4]. Betulinic acid is one of this family's most frequently studied compounds [3]. Indeed, betulinic acid (BA) or 3β-hydroxy-lup-20 (29)-en-28-oic acid is a pentacyclic triterpene of the lupane family isolated primally from the bark of white birch plants [5] (Figure 1). BA has a wide range of biological activities such as its cytotoxic, anti-inflammatory, antiviral (especially anti-HIV), antimalarial, anti-tumor, antibacterial, and antioxidant activities [6, 7]. Its first anticancer effect against melanoma was reported by Pisha et al. [8]. A main advantage of BA and its derivatives is that they are cytotoxic to different human tumor cells, while cytotoxicity is much lower in normal cells [9]. Many subsequent investigations have discovered that BA was demonstrated to be a very promising anticancer agent against various tumor cell lines such as breast, colon, lung, and brain [10, 11].

Therefore, betulinic acid is considered a promising candidate for clinical application [12, 13]. Despite its strong cytotoxic effect, BA exhibits low water solubility, characterized by high hydrophobicity leading to poor bioavailability [5, 10], it belongs to the Biopharmaceutics Classification System (BCS) class IV that is characterized by both low solubility and permeability [14]. To overcome these inconveniences, several approaches for improving the physicochemical and pharmacokinetic profile of the hydrophobic small drug molecules have been conducted such as cocrystallization, amorphous solid dispersions, liposomes, solubilization in co-solvents, complexation with β-cyclodextrin and nanoemulsions [6, 15, 16]. Although these approaches can enhance performance over pure drugs, they all have drawbacks, most of which

are linked to the drug's chemical stability and physical stability against crystallization [17].

A general approach to improve drug solubility can be demonstrated by incorporating such active molecules into appropriate Metal-Organic Frameworks (MOFs) [15]. Since 1999, when Yaghi et al. named materials connected by metal-ligand coordination as Metal-Organic Frameworks (MOFs), they have rapidly become a hot topic of research [18]. Indeed, MOFs are novel porous materials with ultrahigh surface areas, tunable pores, high crystallinity, designable crystal structures, permanent porosity, and abundant metal sites which have shown huge potential applications in various fields, including gas sorption and separation, catalysis, luminescence, biomedicine, environmental pollution, sensor, and so on [19-22]. In recent years, MOFs have been investigated for the encapsulation of organic or organometallic molecules as well as for the development of potential drug carriers [23]. To this end, they ensure the structural, chemical, and thermal stability of the active molecules through confinement in their pores and they experience rapid hydrolytic decomposition, resulting in the release of the drug molecule [15]. Recently, a concept of "edible" MOF named cyclodextrin metal-organic framework (CD-MOF) has been reported, it is synthesized by using γ-CD as an organic ligand, and low-toxic alkali metal potassium ions (K^+) as metal ions [24, 25]. CD-MOFs which have the empirical formula $[(C_{48}H_{80}O_{40})(KOH)_2]_n$ can be employed as a versatile host to encapsulate enough biological agents and convert crystal pharmaceuticals into molecular form, significantly increasing the solubility and bioavailability of poorly soluble drugs [26, 27].

One of the reasonable and helpful strategies in drug discovery is the quantitative structure-activity relationship (QSAR), which is important for the development and optimization of drug candidates as well as for improving their biological activities [28]. The development of a new medicine is a time-consuming, costly, difficult, and ineffective procedure that can take up to 15 years [29]. The concept of computational chemistry such as computer-aided drug design (CADD) has recently achieved a remarkable revolution in medicinal chemistry as well as the model for discovering pharmacologically interesting molecules [30]. This study aimed to investigate in silico, the inclusion of betulinic acid into CD-MOF-1 by quantum mechanics (QM) calculations, and molecular docking simulation, to indicate the possibility of forming the host-guest inclusion complex between BA and CD-MOF-1 which may enhance the solubility of BA and then its efficiency for drug delivery. To the best of our knowledge, this work has not yet been done in the literature, but it has been reported some similar work using protein as a receptor like myeloid differentiation 2 (MD2) [31], phospholipase A2 (PLA2) [32], human serum albumin (HSA) [33], and so on to show the biological properties of betulinic acid.

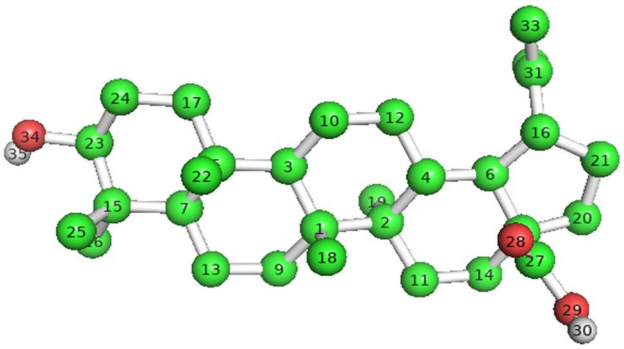


Figure 1. Molecular structure of betulinic acid retrieved after adding atomic partial charges based on B3LYP/6-31+G(d) level, (carbon atoms in green, oxygen atoms in red, and hydrogen atoms in grey)

Materials and Methods

Quantum mechanics (QM) calculations

Density functional theory (DFT) developed by Hohenberg and Kohn [34] and Kohn and Sham [35], is a method for calculating the electronic structures of molecules using the first principles of quantum

mechanics [29]. DFT calculations are a functional tool for determining the correct molecular structure corresponding to the lowest energy state at the potential energy surface. This approach is useful in exploring the structure-activity relationship since accurate molecular structure determination is required in this field. Researchers can utilize DFT calculations to obtain reliable information about molecules' electronic structure and properties, which can then predict their behavior in chemical reactions and interactions with other compounds [36]. In this study, all geometry optimizations were calculated by using the function of Becke, Lee, Yang, and Parr (B3LYP) with a basis set of 6-311+G(d). Atomic partial charges of CD-MOF-1 and betulinic acid were calculated by CHarges from ELectrostatic Potentials using a Grid-based (CHelpG) method based on B3LYP/6-31+G(d) level, the cluster structures used for those calculations are given in Figures 1 and 2 and their values in Tables 1 and 2 for betulinic acid and CDMOF-1 respectively. Thus, we were allowed to analyze some properties such as frontier molecular orbital (FMO) and molecular electrostatic map (MEP).

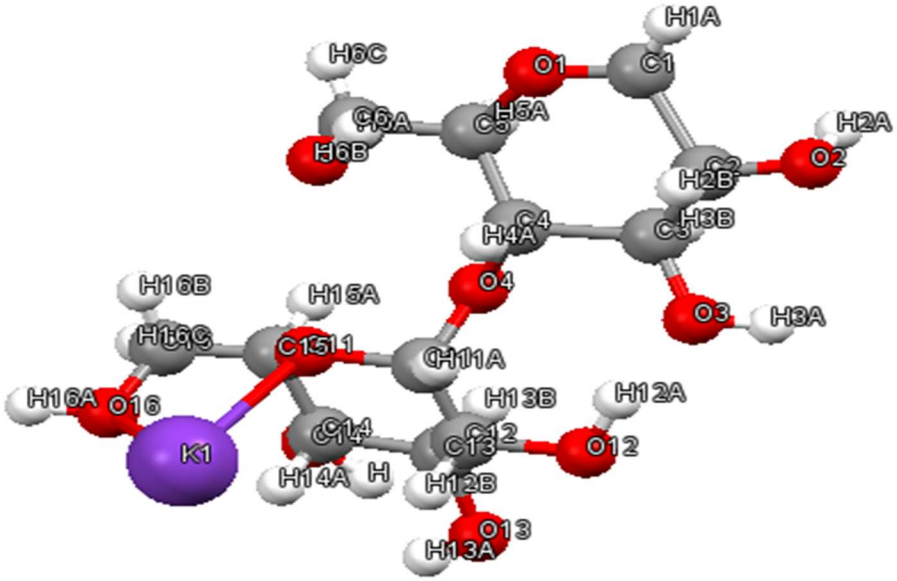


Figure 2. The cluster was created by cutting directly a piece of framework used for the calculation of atomic charges. One hydrogen (H) has been added to O14 to saturate the structure. (K, O, C, and H atoms are represented as light blue, red, grey, and white respectively)

Table 1. The partial atomic charge values of CD-MOF-1 as given by CHelpG calculation based on the B3LYP/6-31+G(d) method

Atoms	Charges	Atoms	Charges	Atoms	Charges
K1	0.070	C4	0.104	O13	-0.849
O1	-0.318	C5	0.434	H13A	0.480
C1	0.538	O6	-0.468	C13	0.254
O2	-0.701	H6A	0.375	O14	-0.616
H2A	0.492	C6	0.172	C14	0.341
C2	0.176	O11	-0.093	C15	-0.304
O3	-0.660	C11	-0.709	O16	-0.576
H3A	0.371	O12	-0.678	H16A	0.350
C3	0.472	H12A	0.333	C16	0.142
O4	-0.763	C12	0.605	H	0.418

Table 2. The partial atomic charge values of betulinic acid as given by CHelpG calculation based on the B3LYP/6-31+G(d) method

Atoms	Charges	Atoms	Charges	Atoms	Charges
C1	0.283	C13	-0.047	C25	- 0.177
C2	0.388	C14	-0.133	C26	-0.152
C3	0.181	C15	0.462	C27	0.728
C4	-0.249	C16	0.038	O28	-0.607
C5	0.098	C17	-0.191	O29	-0.603
C6	0.346	C18	-0.205	H30	0.409
C7	-0.058	C19	-0.191	C31	0.185
C8	-0.017	C20	0.081	C32	-0.052
C9	-0.072	C21	-0.118	C33	-0.182
C10	-0.030	C22	-0.134	O34	-0.759
C11	0.007	C23	0.305	H35	0.415
C12	-0.067	C24	0.054	Total	0.000

Molecular docking simulation: Receptor preparation

The CIF (crystallographic information files) of CD-MOF-1 was retrieved from the Cambridge Crystallographic Data Centre (CCDC) (https://www.ccdc.cam.ac.uk/structures/) using the reference code 773709 with the name catena-((μ8-γ-cyclodextrin)-di-potassium dihydroxyde) [37]. Atomic partial charges were added, then the AutoDock Tool was used to add hydrogen atoms and Kollman charges and the file has been saved in pdbqt format.

Ligand preparation

Betulinic acid molecule was obtained in Structure Data Format (sdf) from PubChem web platform (https://pubchem.ncbi.nlm.nih.gov/) in 3D conformation (CID 64971) and converted to the PDB file using OpenBabel software [38] of the Python Prescription (PyRx) suite, a virtual screening tool. Betulinic acid geometry was optimized, and atomic charges added, then, the preparation of the pdbqt file was done using the AutoDock Tool.

Molecular docking processes

Molecular docking simulations were carried out using the AutoDockTool 1.5.7 and AutoDock Vina v1.1.2 program. The number of points in the grid map of 42×46×40 Å was set to cover whole the framework spacing of 1.000 Å, which was centered at x = 12.971, y = 18.216, and z = 14.482. Grid-based energy evaluation methods combined with a Lamarckian Genetic Algorithm (LGA) were considered for pre-calculating grid maps according to the interatomic potentials of all-atom types present for each CD-MOF-1 pores and BA molecules. The docking has also been performed using the packed 1×1×1 CD-MOF-1 structure to have more accurate results. The exhaustiveness value was set as 8 to obtain an efficient binding conformation pose of the CD–MOF–drug complex. Using an AutoDock Vina, the docking analysis was performed, and the receptor-drug complex's binding affinity was observed as a negative score with a unit of kcal/mol and the same value for all 9 poses. Discovery Studio 16.1 software packages and Pymol were assisted to analyze and visualize the host-guest interactions.

Results and Discussion

Frontier molecular orbital (FMO) analysis

The most important orbitals in a molecule also called frontier molecular orbitals (FMOs) are the lowest unoccupied molecular orbital (LUMO), which can accept electrons, and the highest occupied molecular orbital (HOMO), which can donate electrons [36]. These orbitals are important for determining a compound's electrical properties, kinetic stability, optical polarizability, and chemical reactivity [29]. To understand the chemically reactive site of BA, the HOMO, and LUMO are obtained, where the negative region is presented in red and the positive one in green (Figure 3). As we can see from the given figure, HOMO is located over the alkene group, the cyclohexane and cyclopentane rings, which can be useful for donating electrons to the electrophilic sites of CD-MOF-1. On the other side, the formation of LUMO in the carboxylic group is important for accepting electrons given by

nucleophilic sites of CD-MOF-1.

The HOMO and LUMO energy values of betulinic acid were calculated as well as the global chemical parameters, like energy gap (Egap), ionization potential (I), electronic affinity (EA), chemical potential (μ), electronegativity (χ), chemical hardness (η), softness (S), electrophilicity (ω) and nucleophilicity (ε) according to equations (1 to 9) below (Table 3) [29].

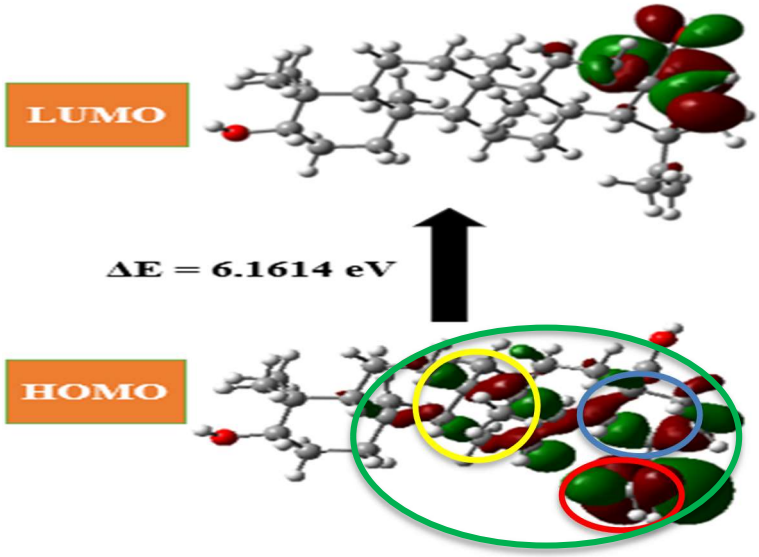


Figure 3. HOMO and LUMO orbitals of betulinic acid, the circles in green, yellow, blue, and red show the location of HOMO, cyclohexane, cyclopentane, and alkene groups respectively

Table 3. Electronic properties of betulinic acid based on B3LYP/6-31G+ (d)

Electronics properties	Values
E_{HOMO}	-6.5558 (eV)
E_{LUMO}	-0.3943 (eV)
Energy gap (E_{gap})	6.1615 (eV)
Ionization potential (I)	6.5558 (eV)
Electronic affinity (EA)	0.3943 (eV)
Electronegativity (χ)	3.4750 (eV)
Chemical potential (μ)	-3.4750 (eV)
Chemical hardness (η)	3.0807 (eV)
Chemical softness (S)	0.3246 (eV^{-1})
Electrophilicity (ω)	1.9599 (eV)
Nucleophilicity (ε)	0.5102 (eV^{-1})

The energy gap (Egap) was calculated by the difference of energy between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) shown in equation 1. For the molecular system containing π electrons, smaller Egap increases the mobility of π electrons to higher energy levels, with better energy distribution and higher reactivity [39]. According to Table 3, the value of Egap of betulinic acid is 6.1615 eV in the gas phase, which is less than 6.378 eV in water as reported by Khan et al. (2018). Khan et al. (2018) performed the DFT calculations of betulinic acid using the Solvation Model on Density (SMD) involving several solvents like water, dimethyl sulfoxide (DMSO), acetonitrile, n-octanol, chloroform [32]. That

model is called SMD, where the "D" stands for "density" to denote that the full solute electron density is used without defining partial atomic charges [40]. This means that betulinic acid is less stable and more reactive and its electrons can easily flow from the HOMO orbital to the empty LUMO orbital until reaching the equilibrium state.

The ionization potential (I) and electronic affinity (EA) are obtained from the reverse sign of the calculated energies of HOMO and LUMO as follows in equations 2 and 3 respectively.

$$Egap = ||E_{HOMO}|| - ||E_{LUMO}|| \quad (1)$$

$$I = -E_{HOMO} \quad (2)$$

$$EA = -E_{LUMO} \quad (3)$$

Electronegativity (χ) (equation 4) is a measure of an atom's ability to attract electrons towards itself in a chemical bond, it is a fundamental property of atoms that affects the nature of chemical bonding and the resulting properties of compounds. Electronic chemical potential (μ) (equation 5) which is the absolute reversed value of electronegativity means escaping the tendency of electron cloud from the ground state. Betulinic acid can be capable of forming polarized covalent bonds with other atoms having both relatively low electronegativity, such as alkali metals or alkaline earth metals, or strong electronegativity like the hydroxide group. The polarity of the bonds in the molecule can have effects on the physical properties of betulinic acid, such as solubility and polarizability, BA may tend to interact with other molecules with complementary electronic properties, which may affect its chemical reactivity.

$$\chi = \frac{I+EA}{2} \quad (4)$$

$$\mu = -\chi \quad (5)$$

Hardness (η) (equation 6) is a measure of the resistance of a molecule to electron transfer and is defined as the energy required to add or remove an electron from the molecule's HOMO or LUMO respectively. In order words, a molecule with a high hardness requires a large amount of energy to donate or accept an electron. On the other hand, softness (S) (equation 7) is the inverse of hardness and represents the ease with which a molecule can donate or accept electrons.

$$\eta = \frac{-Egap}{2} \quad (6)$$

$$S = \frac{1}{\eta} \quad (7)$$

The concept of hardness (η) and softness (S) is related to a compound's reactivity, so, to the energy gap of a molecule. As the energy gap increases, the molecule becomes harder (less soft), significantly less polarizable, and less reactive. The calculated values are 3.0807 eV and 0.3246 eV^{-1} for chemical hardness and softness respectively, as compared to 3.189 eV and 0.314 eV^{-1}. As shown above, betulinic acid has a smaller energy gap, so it is less soft, more reactive, and polarizable. The same behavior has been demonstrated by Muya and collaborators, they did the DFT calculations of betulinic acid using the continuum solvation model based on density (SMD) at B3LYP and M062X with 6-311 + G(d,p) basis sets, they conclude that betulinic acid appears soft and can interact with soft acceptor molecules [41].

Other important descriptors are the electrophilicity index (ω) and nucleophilicity (ε) used to describe the ability of a molecule or atom to accept or donate electrons in a chemical reaction. Electrophilicity refers to the tendency of a molecule to attract electrons, it is typically associated with electron-deficient species that have a partially positive charge and nucleophilicity refers to the tendency of a molecule to donate electrons, it is associated with electron-rich species that have a partially negative charge. Their values are defined as follows in equations 8 and 9:

$$\omega = \frac{\mu 2}{2\eta} \quad (8)$$

$$\varepsilon = \frac{1}{\omega} \quad (9)$$

Electrophilicity included both the ability of an electrophile to acquire additional electronic charge and a system's resistance to exchange electronic charge with the environment. Betulinic acid electrophilicity is 1.9599 eV at the B3LYP/6-31G+ (d), 1.48 eV at the B3LYP/6-31G(d) using SMD solvation model [32] and

1.15 eV at B3LYP/6-31G+ (d,p) and 1.19 eV at M062X/6-311 + G(d,p) using SMD model [41]. We notice that, with atomic partial charges, the electrophilicity value of BA increases at the B3LYP/6-31G+ (d). The electrophilicity character of betulinic acid proves that the active site of the latter molecule may be the carboxylic group.

Molecular electrostatic potential (MEP) and contours analyses

The MEP maps give an idea about the chemical reactivity of the compounds by determining electrophilic and nucleophilic attack regions in the molecule according to charge distributions [42]. The maps of electrostatic potential (MEPs) surfaces and contours were used to display the electron density on surfaces that surround the atoms within the molecules and sites for nucleophilic and electrophilic attacks. To predict the electrophilic and nucleophilic center of betulinic acid, MEP for optimized geometry was calculated at the DFT/B3LYP/6-31G+ (d). According to Figure 4a, potential decreases in the following order of colors red < orange < yellow < green < blue. Red and yellow regions indicate negative regions (rich electron) which promote nucleophilic site while the positive regions represented by the blue color promote electrophilic site. The green color represents a region of zero potential which indicates the neutral region [36]. In addition, MEP contours are more significant for illustrating the reactive nature of outermost orbital electrons (Figure 4b) [29]. The reactivity of betulinic acid is observed with red-colored contours and green lines show that electrons are not moving in the area. Figure 4 shows that the negative potentials are generated over the electronegative oxygen atom of the hydroxide group and the positive potentials are located in the oxygen atom of the carboxylic group. As shown above, for the molecular system containing π electrons, a smaller energy gap increases the mobility of π electrons to higher energy levels, which corroborates with MEPs showing that the carboxylic group of BA is preferred active site. This may be confirmed or not following this work by performing molecular docking simulation between betulinic acid and CD-MOF-1.

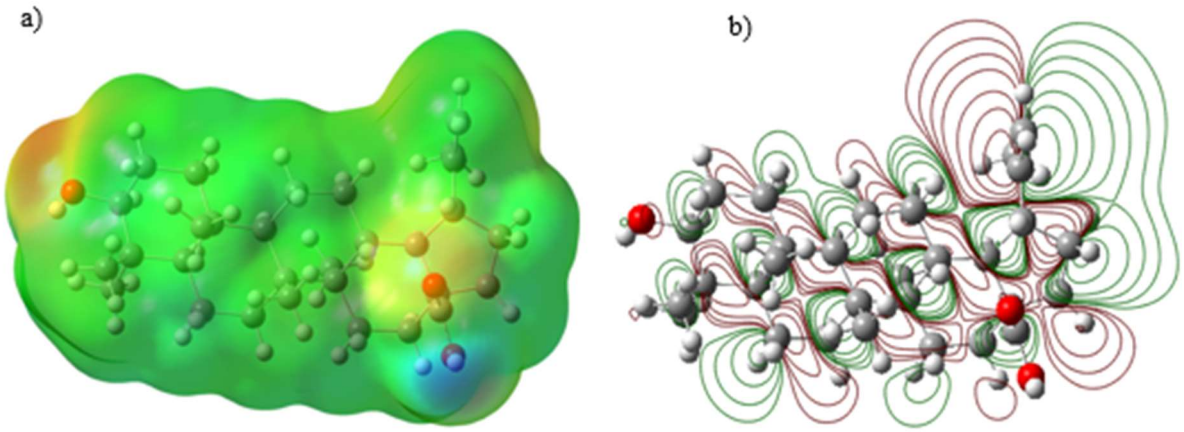


Figure 4. MEP of (a) surface and (b) contours of betulinic acid

Molecular docking analysis

CD-MOF has spherical voids with a diameter of 0.95 nm and pore size of 0.78 nm, which are body-centered cubic extended structures with surface areas of approximately 1200 m^2/g (Figure 5) [43]. The possible conformations of betulinic acid molecules were done by docking BA into CD-MOF-1, the hydrogen bond interactions are given in Table 4. The blind docking was done in triplicate and the binding affinity of all of the nine poses was -8.2 kcal/mol, the specific docking has also been performed thrice and the binding affinity varying between -8.2 and -8.1 kcal/mol, a very low difference. The best-docked conformation of betulinic acid, located in the center of the cyclodextrin pore has been analyzed by Discovery Studio as shown in Figure 6. We noticed that betulinic acid bound in the hydrophobic inner cavity of CD and its electrophilic side (carboxylic group) interacted with a hydrophilic outer surface of CD (constituted by D-glucopyranose units) and the nucleophilic side of BA (hydroxyl group) can just form

the unfavorable interaction. This confirms that the carboxylic group (COOH) of betulinic acid is its active site as predicted by the global descriptors and the MEPs. So, betulinic acid and CD-MOF-1 exhibit a stronger affinity examined by the hydrogen bond interactions, which means that CD-MOF-1 can stabilize BA against oxidation by tailoring its physical properties.

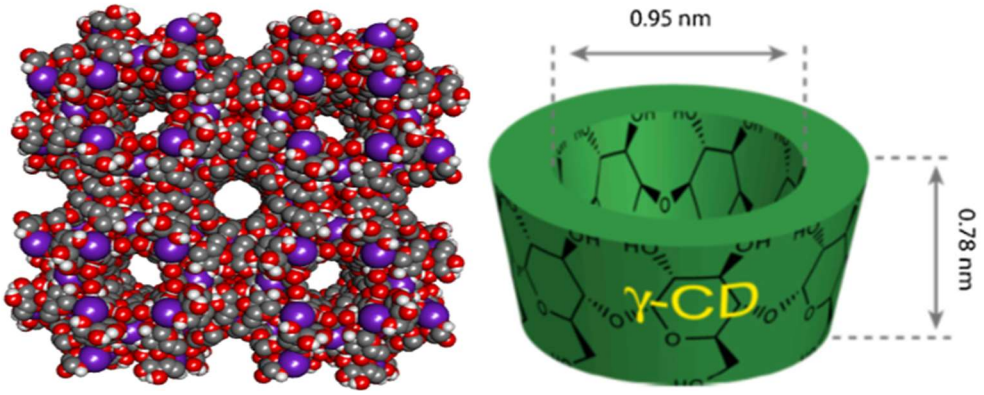


Figure 5. A space-filling representation of the extended solid-state structure, showing the $(\gamma CD)_6$ repeating motifs adopting a body-centered cubic packing arrangement (C gray, O red, K purple)

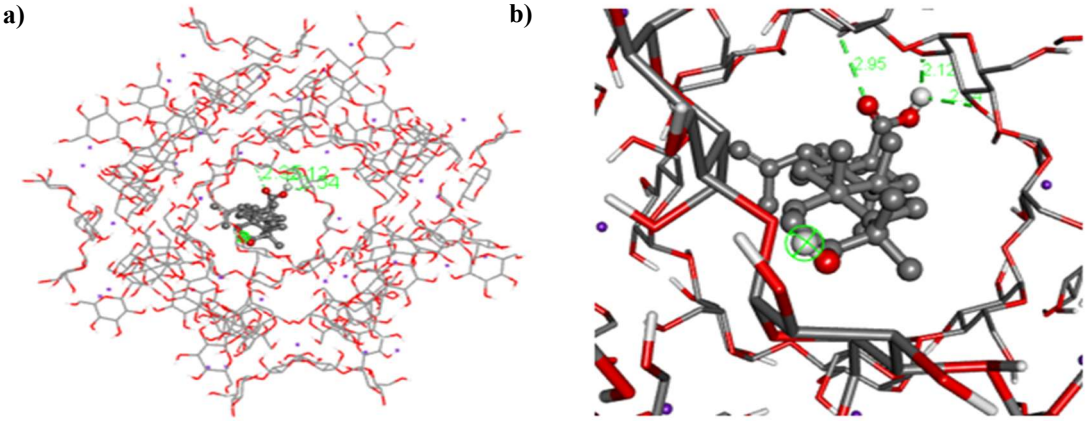


Figure 6. (a) The best binding affinity of BA into CD-MOF-1, (b) the zoom of the cyclodextrin pore where we have hydrogen bonds interactions, the green lines are hydrogen bonds between CD-MOF-1 and BA

Many researchers noticed that the drug candidate may be most effective when the binding energy is lowest and the number of H-bond donors with the receptor is largest [30]. Figure 6 shows three hydrogen bonds between BA and CD-MOF-1, which are [CD-MOF-$1_{OH13}\cdots_{O28}$BA (2.95 Å)], [CD-MOF-$1_{O13}\cdots_{H30}$BA (2.54 Å)], and [CD-MOF-$1_{OH12}\cdots_{H30}$BA (2.12 Å)]. So, CD-MOF-1 may be a good carrier of betulinic acid in drug delivery and can enhance its bioavailability and solubility, by improving its biological efficiency.

To have more accurateness, molecular docking simulation has also been done with the packed 1×1×1 of CD-MOF-1 structure, the binding affinities of the blind and specific docking of all of the nine poses were -11.3 kcal/mol more significant than -8.2 kcal/mol. All the poses are located in the cavities of the cyclodextrin, Figure 7 shows hydrogen interactions between BA and CD-MOF-1 which are [CD-MOF-$1_{OH12A}\cdots_{O28}$BA (3.00 Å)], [CD-MOF-$1_{H13A}\cdots_{H30}$BA (2.72 Å)] and [CD-MOF-$1_{OH2A}\cdots_{H30}$BA (2.66 Å)]. These interactions are not similar to the ones above, but they are longer and more significant in terms of energy and distance, so the CD-MOF-1 packed structure is the best to have accurate results.

The observed binding affinities in molecular docking studies provide valuable insights into the strength of interaction between betulinic acid and CDMOF-1, these binding affinities can have several implications for stabilizing betulinic acid against oxidation:

- Identification of stable binding interactions: High binding affinity often indicates strong and stable interactions between the ligand and receptor. In the context of stabilizing betulinic acid molecules against oxidation, this suggests that betulinic acid is tightly bound to CDMOF-1, which may shield it from interacting with oxidizing agents in the surrounding environment.
- Prevention of oxidation-induced conformational changes: Binding to the CDMOF-1 molecule in a specific conformation may prevent betulinic acid from adopting alternative conformations that are more susceptible to oxidation. By stabilizing betulinic acid in a particular orientation, CDMOF-1 may help shield vulnerable functional groups from oxidation.
- Design of antioxidant compounds: Insights gained from molecular docking studies can aid in the design of small molecules with enhanced antioxidant properties. By understanding the structural features that contribute to high binding affinity and stability against oxidation, we can modify betulinic acid or design novel molecules that better fulfill the desired criteria.

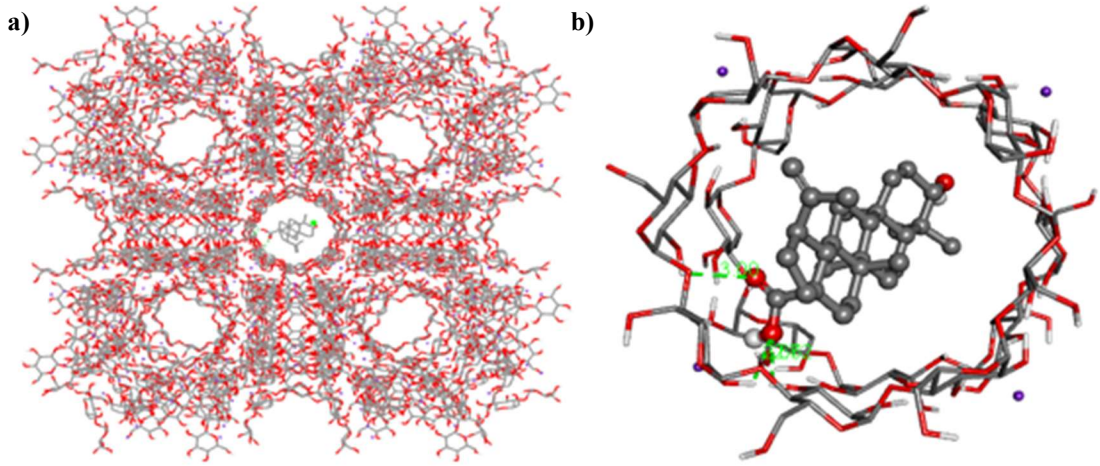


Figure 7. The best binding affinity of BA into CD-MOF-1 using the packed 1×1×1 structure of CD-MOF-1, the green lines are hydrogen bonds between CD-MOF-1 and BA

Table 4. Hydrogen bond interactions between CD-MOF-1 and betulinic acid

CD-MOF-1 Structure	**Hydrogen bond interactions between CD-MOF-1 and BA (Interaction distance)**		
Simple	CD-MOF-1_{OH13}··· $_{O28}$BA (2.95 Å)	CD-MOF-1_{O13} ···$_{H30}$BA (2.54 Å)	CD-MOF-1_{OH12} ··· $_{H30}$BA (2.12 Å)
Packed 1×1×1	CD-MOF-1_{OH12A} ···$_{O28}$BA (3.00 Å)	CD-MOF-1_{H13A} ··· $_{H30}$BA (2.72 Å)	CD-MOF-1_{OH2A} ··· $_{H30}$BA (2.66 Å)

Conclusion

CD-MOF-1 is a porous coordination compound, that exhibits many unique characteristics such as high surface area, high porosity, and tunability leading to numerous applications in many fields including drug delivery. In the present work, we have theoretically obtained the host-guest interactions between betulinic acid and CD-MOF-1 by performing QM calculation to understand the behavior of BA inside the CD-MOF-1, and molecular docking simulation to determine the best

binding affinity of BA into CD-MOF-1. The global descriptors such as energy gap, electronegativity, chemical hardness and softness, and electrophilicity show that betulinic acid has a smaller energy gap which involves less stability, more reactivity, and more polarizability. The MEPs map shows that the active site of betulinic acid is the carboxylic group and the latter molecule preferred electrophilic attacks. Molecular docking revealed that BA bound in the hydrophobic cavities of CD-MOF-1 through hydrogen bonds [CD-MOF-$1_{OH13\cdots O28}$BA (2.95 Å)], [CD-MOF-$1_{O13\cdots H30}$BA (2.54 Å)], and [CD-MOF-$1_{OH12\cdots H30}$BA (2.12 Å)] for the simplest structure and [CD-MOF-$1_{OH12A\cdots O28}$BA (3.00 Å)], [CD-MOF-$1_{H13A\cdots H30}$BA (2.72 Å)] and [CD-MOF-$1_{OH2A\cdots H30}$BA (2.66 Å)] for the packed structure 1×1×1. The packed CD-MOF-1 structure is the best with accurate results, this may be the factor of enhancement of betulinic acid solubility and bioavailability. So, CD-MOF-1 may be investigated and validated as an efficient carrier to enhance the solubility and bioavailability of betulinic acid. However, translating theoretical findings from molecular docking studies into practical applications can face several challenges and limitations such as accuracy of predictions, validation, flexibility and dynamics, solvent effects, chemical accessibility, and synthesis. In forthcoming research, the integration of molecular docking and molecular dynamic simulations unveils potential mechanisms governing binding affinities, enabling virtual observation. This integrated approach guides experimental synthesis and characterization validation from in vitro to preclinical studies, offering cost-effective methods to advance drug discovery, particularly in nanomedicine for cancer treatment, ensuring sustainable research practices.

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UNVEILING THE IMPACT OF BACTERIAL CELLULOSE DERIVED FROM NATA DE COCO AND SORBITOL ON BIOCOMPOSITE FILM PROPERTIES FOR POTENTIAL FOOD PACKAGING

(Menyingkap Kesan Selulosa Bakteria Diperoleh daripada Nata De Coco dan Sorbitol Terhadap Sifat-Sifat Filem Biokomposit Sebagai Bahan Pembungkus Makanan Alternatif)

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Abstract

The surge in plastic production in recent years has demonstrated the urgent need for eco-friendly alternatives. Bacterial cellulose (BC) has emerged as a promising candidate for replacing petroleum-based materials. However, the inherent rigidity, fragility, and brittleness of BC have posed formidable challenges, particularly in food packaging. This study investigates the impact of combining BC (1.0g, 2.5g, 5.0g, 7.5g, 10.0g) derived from nata de coco and sorbitol (0.5g, 1.0g, 1.5g, 2.0g, 2.5g) on the physical, mechanical, chemical, and thermal properties, as well as the surface morphology of the biocomposite films. Films were produced by varying BC and sorbitol quantities via the solvent casting method. Evaluations encompassed film thickness, moisture content, water solubility, mechanical characteristics, FTIR, TGA, and FE-SEM. Tensile strength increased with BC content but decreased with added sorbitol. FTIR confirmed the consistency of functional groups in the BC/Chitosan/Sorbitol biocomposite film with those in BC. TGA indicated improved thermal stability with increased BC and decreased sorbitol content. FE-SEM images revealed a smoother biocomposite film surface with sorbitol. The optimal composition was determined to be 10.0BC and 0.5S due to its low moisture content, low water solubility, high tensile strength, and minimal TGA weight loss. Overall, this study highlights the potential of BC/Chitosan/Sorbitol films as environmentally friendly and sustainable alternatives to traditional synthetic food packaging materials.

Keywords: bacterial cellulose, nata de coco, chitosan, sorbitol, biocomposite film

Abstrak

Peningkatan pengeluaran plastik dalam beberapa tahun kebelakangan ini telah menghasilkan keperluan mendesak untuk pembangunan pengganti plastik yang mesra alam. Dalam konteks ini, selulosa bakteria (SB) mempunyai potensi yang memberangsangkan sebagai pengganti bahan berasaskan petroleum. Walau bagaimanapun, ketegaran dan kerapuhan SB telah menimbulkan pelbagai cabaran, terutamanya dalam konteks kegunaannya sebagai pembungkus makanan. Dalam kajian ini, kami mengkaji kesan gabungan BC dan sorbitol terhadap sifat fizikal, mekanikal, kimia, termal, dan morfologi permukaan filem biokomposit berdasarkan BC. Filem-filem ini dihasilkan dengan mengubah kuantiti BC (1.0g, 2.5g, 5.0g, 7.5g, 10.0g) dan sorbitol (0.5g, 1.0g, 1.5g, 2.0g, 2.5g) menggunakan kaedah pelarut. Sampel filem yang terhasil dinilai berdasarkan ketebalan filem, kandungan lembapan, kelarutan dalam air, sifat-sifat mekanik, FTIR, TGA, dan FE-SEM. Hasil kajian menunjukkan bahawa nilai kekuatan regangan meningkat seiring dengan peningkatan BC, tetapi berkurangan apabila kepekatan sorbitol bertambah. FTIR

menunjukkan bahawa kumpulan berfungsi yang wujud dalam filem biokomposit BC/Chitosan/Sorbitol sepadan dengan kumpulan berfungsi BC. TGA pula mendedahkan bahawa kestabilan termal filem komposit meningkat dengan peningkatan BC dan penurunan sorbitol. Peningkatan kuantiti BC menyebabkan filem mengalami kehilangan berat yang minimum, tetapi apabila tahap sorbitol meningkat, filem mengalami kehilangan berat yang lebih besar FE-SEM menggambarkan bahawa permukaan filem komposit menjadi lebih licin dengan penambahan sorbitol. Komposisi optimum ditentukan sebagai 10.0BC dan 0.5S, disebabkan oleh kandungan lembapan yang rendah, kelarutan dalam air yang rendah, kekuatan regangan yang tinggi, dan kehilangan berat yang minima dalam TGA. Keseluruhannya, kajian ini membuktikan bahawa filem komposit berdasarkan BC mempunyai potensi untuk digunakan sebagai alternatif yang boleh dikompos secara biodegradasi dan mampan kepada bahan pembungkusan makanan sintetik tradisional.

Kata kunci: selulosa bakteria, nata de coco, kitosan, sorbitol, filem biokomposit

Introduction

The packaging industry plays a crucial role in the global economy, particularly in the food and beverage segment, which holds the majority market share of around 85% [1]. However, the reliance on conventional plastic packaging materials has led to significant environmental concerns due to their non-biodegradable nature [2]. The immense volume of plastic waste generated worldwide is expected to triple by 2060, surpassing one billion metric tons. Alarmingly, only a minimal fraction (9%) of this plastic waste is recycled, leading to exacerbated resource depletion, contributing to global warming, acidification, eutrophication, and posing risks to human health [3]. Consequently, there is an increasing global demand for environmentally friendly alternatives to traditional packaging materials. Traditional approaches to biodegradable food packaging, which use proteins, polysaccharides, and lipids from agricultural byproducts or synthetic biopolymers such as PLA and PHA, encounter significant challenges because of their reduced tensile strength and insufficient moisture and oxygen barriers. These properties are essential for preserving food safety and quality. Despite their eco-friendly nature, these methods struggle with scalability and cost-effectiveness, primarily due to the elevated price of raw materials, energy-intensive processing, and advanced equipment requirements [4]. Moreover, the environmental advantages of polymers, such as PLA, are mitigated by their dependence on industrial composting facilities, which are scarcely available. On the other hand, naturally degradable materials such as chitosan show promise but are plagued by sourcing and quality consistency issues [5]. These limitations highlight the urgent necessity for new, sustainable, and economically viable food packaging solutions that effectively balance environmental benefits with performance requirements for widespread commercial adoption.

In this context, Nata de coco (NDC) emerges as an environmentally sustainable and promising alternative to conventional plastic packaging materials, primarily due to its role as a BC source. BC, derived from Nata de coco, is a natural and renewable biopolymer primarily produced by *Acetobacter xylinum* through the fermentation of coconut water [6]. This unique production process involves the metabolic conversion of glucose found in coconut water, enriched with carbon and nitrogen, by *Acetobacter xylinum*, resulting in the extracellular production of cellulose as a valuable metabolite [7]. BC shares a chemical composition identical to plant cellulose but distinguishes itself by the absence of lignin or hemicelluloses, leading to superior physical characteristics compared to conventional plant cellulose [8]. Its high tensile strength and water-holding capacity make BC stronger, more resistant to breaking, and capable of absorbing more water. Furthermore, BC exhibits high purity and crystallinity, contributing to excellent transparency and barrier properties [9]. These properties make BC an attractive potential replacement for synthetic food packaging materials [10]. Nevertheless, despite its potential, using BC alone for food packaging applications is not ideal due to its natural brittleness when dried. Dried BC has a rigid and less pliable structure, resulting in a rough appearance and reduced flexibility. To overcome this limitation, BC is often combined with other biopolymers or plasticizers, such as chitosan or glycerol, to enhance its pliability, achieve a more plastic-like appearance, and improve overall properties while minimizing brittleness [11,12].

Chitosan, the second most abundant natural biopolymer after cellulose, is primarily sourced from the byproducts

of the shellfish industry [5]. It has demonstrated remarkable versatility in various applications, notably in food packaging, due to its non-toxicity, biodegradability, film-forming attributes, solubility, and inherent antimicrobial properties. Furthermore, chitosan and bacterial cellulose exhibit similar chemical structures and mutually complementary characteristics, fostering enhanced molecular interactions among their polysaccharide chains [13]. Consequently, chitosan emerges as a highly promising candidate for utilization as a filler in the production of BC-based films, offering the prospect of enhanced mechanical properties and film-forming capability [14]. In contrast to films composed solely of chitosan, BC/chitosan composite films present distinct advantages in terms of mechanical resilience, including improved thermal stability and reduced oxygen permeability, making chitosan a highly viable candidate for augmenting BC-based films [15].

Moreover, incorporating plasticizers such as sorbitol, recognized for its low molecular weight and non-volatile nature, into film formulations can significantly enhance their flexibility, elongation, and toughness, thereby effectively addressing the inherent brittleness of BC [16]. The materials can also reduce film stiffness and brittleness, enhance transparency, and decrease water vapor transmission rate. One commonly used plasticizer is sorbitol, which has a low molecular weight, non-volatile nature, and high boiling point. Sorbitol exhibits significant intermolecular interaction with polymers, reducing polymer strength while increasing flexibility and extensibility. It is a polyhydric alcohol monosaccharide molecule, also known as hexitol or glucitol, with a chemical formula of $C_6H_{14}O_6$ [17]. Incorporating sorbitol as a plasticizer into film formulations can influence film thickness, flexibility, density, and water content [15], offering a means to enhance the properties of BC and make it more suitable for food packaging applications.

In the past few years, numerous studies have explored the incorporation of BC and chitosan for food packaging applications [18–20]. BC showed a reinforcing effect and can improve the physical properties of whey protein concentrate films [21]. However, despite these advancements, comprehensive studies considering film formulations and interactions between BC from NDC, chitosan, and sorbitol are still lacking in the literature. Building on this gap of knowledge, this study aimed to investigate the impact of varying BC and sorbitol concentrations on the physical, mechanical, and thermal properties of BC-based biocomposite films for potential use in sustainable food packaging. The study aims to determine the ideal composition of BC and sorbitol that ensures low moisture content, high tensile strength, and improved thermal stability. This research is expected to contribute to the advancement of an environmentally friendly substitute for traditional plastic packaging materials.

Materials and Methods

Materials

1.5 kg of food-grade nata de coco (Brand: Desa Alpha, Manufactured by Rico Food Ind. Sdn. Bhd.) in the form of cubes was purchased from the local supermarket. Chitosan of medium molecular weight and sodium hydroxide were obtained from Sigma-Aldrich. Sorbitol and acetic acid were purchased from R&M Chemicals. All other chemicals used were of the analytical grade.

Purification of bacterial cellulose (BC) from nata de coco

The nata de coco cubes were thoroughly washed and soaked in distilled water twice daily for 10 days to ensure the pH reached 7. This step was done to neutralize the acidity and remove any impurities from the nata de coco cubes. After that, an alkaline treatment was performed by stirring the nata de coco cubes in 0.01 M NaOH at 80 °C for 90 min to remove any remaining bacterial cell debris, microorganisms, and other dissolved substances. Subsequently, the nata de coco cubes were washed multiple times with distilled water at room temperature until the pH reached 7 [22]. The purified nata de coco was then ground and mechanically blended for 1 hour using a kitchen blender (ProMix hand blender, Philips). The resulting BC slurry was collected and stored at room temperature for future use.

Preparation of BC/Chitosan/Sorbitol biocomposite films

The biocomposite films were prepared using a solvent casting method, following the procedure by Yanti et al. [23]. The film-forming solution consisted of BC suspension, chitosan, and sorbitol as plasticizers, as shown in Table 1. The chosen ranges for BC and sorbitol were established through preliminary experiments

aimed at identifying levels that yield films with flexibility and without brittleness, suitable for further testing and application. Chitosan solution was first prepared by dissolving 0.25g of chitosan powders in 25 mL of 1 %v/v acetic acid. All film formulations were homogenized using an ultrasonic homogenizer for 5 min with a pulse of 5 sec and amplitude of 60%. The resulting film solutions were then cast onto an acrylic mold (8 cm × 12 cm) and dried at 50 °C for 12 hours in an oven. The dried films were peeled off from the petri dishes and stored in zip-lock bags for further analysis. The biocomposite films were previously conditioned for 48 h at 25 °C and 54% RH before evaluating their physicochemical properties.

Table 1. Composition of BC and sorbitol in film formulations.

Sample Name	Bacterial Cellulose (g)	Sorbitol (g)
0.5S	10.00	0.50
1.0S	10.00	1.00
1.5S	10.00	1.50
2.0S	10.00	2.00
2.5S	10.00	2.50
1.0BC	1.00	0.50
2.5BC	2.50	0.50
5.0BC	5.00	0.50
7.5BC	7.50	0.50
10.0BC	10.00	0.50

Characterization of BC/Chitosan/Sorbitol films:

Film thickness

A digital micrometer (3109-25A, InSize, China) was used to measure the thickness of the formed film with an accuracy of 0.001 mm. The thickness of the film was measured at five different spots, and the average thickness was calculated and recorded.

Moisture content (MC)

Test specimens (2 cm × 2 cm) at three random positions of each film type were uniformly cut and placed on glass Petri dishes. The moisture content of the films was determined by drying test specimens in an oven at 105 °C for 24 h until a constant dry weight was obtained according to the method briefly explained by Azaza et al. [24]. The dry weight of the film was measured after drying, and the MC was calculated using Equation (1) below:

$$\text{Moisture Content (\%)} = \frac{\text{Initial sample weight (g)} - \text{Dried sample weight (g)}}{\text{Initial sample weight (g)}} \times 100 \quad (1)$$

Water solubility (WS)

The water solubility of the film was measured according to the method described by Lee et al. [25]. Briefly, to determine the initial dry weight, samples were cut to sizes (2 cm × 2 cm) in triplicate and dried at 105 °C for 24 hours. The dried films were then immersed in 50 mL of distilled water and stirred at room temperature for 24 hours at 100 rpm. After 24 hours, any remaining films were removed and dried at 105 °C for 24 hours to obtain the final dry weight. The percentage of water solubility in samples was calculated using the following Equation (2):

$$\text{Water Solubility (\%)} = \frac{\text{Dried sample weight (g)} - \text{Final dried sample weight (g)}}{\text{Dried sample weight (g)}} \times 100 \quad (2)$$

Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of the film samples were measured according to the ASTM D882-00 method (ASTM., 2000) using a texture analyzer (Stable Micro Systems TA-XT plus, Surrey, UK). Each BC and BC/Chitosan/Sorbitol film was cut into a rectangular shape (10 cm × 1 cm). All the tests were carried out at least twice with different strips. TS was calculated using the following Equation (3):

$$TS = \frac{\text{Force Maximum (Fmax)}}{\text{Area (A)}} \quad (3)$$

where Fmax is the maximum load, and A is the cross-sectional area.

EAB was calculated using the following Equation (4):

$$EAB = \frac{\Delta L}{L_0} \times 100 \quad (4)$$

where ΔL is the film extension, and L_0 is the initial length of the film sample.

Structure by Fourier-transform infrared (FTIR)

The film samples were cut into small pieces (2 cm × 2 cm) and analyzed using an FTIR Spectrophotometer (Nicolet iS5, Thermo Fisher Scientific, USA). For each sample, 32 scans with a resolution of 4 cm^{-1} were collected over the wavenumber ranging from 600 – 4000 cm^{-1} in transmittance mode.

Thermogravimetric analysis (TGA)

TGA was carried out on selected film samples to evaluate their respective mass losses when it is subjected to continuous heating. TGA was performed using TGA Instrument Q500 V 6.7 (TA Instruments, USA) with a 10 °C/min heating rate from 25 to 600 °C under nitrogen gas purging with a flow rate of 40 ml/min.

Surface morphology by field emission scanning electron microscopy (FE-SEM)

The surface morphology of the pure BC and selected BC/Chitosan/Sorbitol films were visualized using a Field Emission Scanning Electron Microscope (FE-SEM) JEOL JSM 7800 F with an accelerating voltage of 15 kV.

Results and Discussion

Physical appearances of BC/Chitosan/Sorbitol films

The physical appearance of the films was evaluated by analyzing their opacity and transparency, as shown in Figure 1. The results indicated that the opacity of the pure BC film was nearly 100% due to the high concentration of BC fibers. The higher value of opacity signified smaller transparency. BC is a polymer composed of ultrafine nanofibrils that form a three-dimensional web-shaped structure, held together by intermolecular and intramolecular hydrogen bonding [26]. As the BC content increased, the film became rougher and less homogenous. Conversely, the addition of sorbitol improved homogenization and gave the film a smoother appearance due to its high hydrophilicity. These findings align with previous research by Wang et al. [27], which also observed a significant increase in film opacity with increasing BC concentrations from 0% to 10%.

Film thickness, moisture content, and water solubility

Table 2 presents valuable insights into the characteristics of the film formulations with varying proportions of bacterial cellulose and sorbitol. The thickness of these films ranged from 0.0384 to 0.2507 mm and can be adjusted by modifying the amounts of BC and sorbitol. The results suggest that adding BC and sorbitol can improve film thickness. The film with a greater sorbitol concentration (2.5S) formed a thicker (0.2526 mm) film than the film with a lower amount of BC and sorbitol (1.0BC), which produced a thinner (0.0718mm). These findings are comparable to similar reports on the effects of sorbitol on the characteristics of carrageenan edible film. Edible film features and basic material composition are two aspects that impact the thickness of edible film. As the sorbitol concentration increased, the edible coating showed a corresponding thickness increment. This is because when the total solids in the edible film solution increased, the formation of the edible film occurred. Therefore, the higher the concentration of sorbitol, the higher the thickness of the film due to the solid content and characteristics of sorbitol [28].

In contrast, the moisture content exhibited less significant variations upon the introduction of BC but demonstrated a substantial increase when sorbitol concentration was augmented, ranging from 4.82% to

89.51%. This phenomenon mirrors findings from a study by Ghamari et al. [29], which investigated active edible film based on milk proteins infused with Nigella sativa essential oil. The study emphasized the crucial role of sorbitol concentration in moisture content, highlighting that its increment can affect the hydrophilic nature of sorbitol. This is because sorbitol has hydroxyl groups in its structure that can actively form hydrogen bonding with water.

Furthermore, the water solubility of the films exhibited a notable decline, decreasing from 32.88% to 5.29%, as BC and sorbitol were incorporated in greater quantities. The reduction in water solubility can be attributed to the enlarged surface area resulting from BC and the formation of intermolecular interactions between the agar matrix and BC. These interactions enhance the cohesion of the biopolymer matrix, leading to decreased water solubility [27]. Therefore, it is evident that as the proportions of BC and sorbitol increased, the water solubility of the films decreased significantly, an essential property for their potential applications

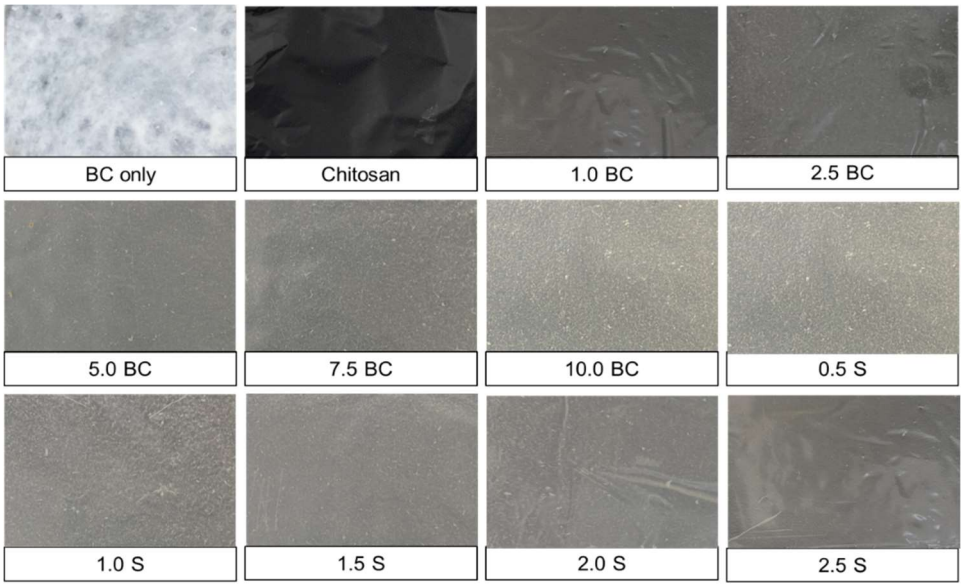


Figure 1. Visual appearances of BC/Chitosan/Sorbitol films. Digital photographs are taken under natural light to show color, transparency, and texture differences. No magnification was applied

Table 2. Thickness, moisture content, and water solubility of the BC/Chitosan/Sorbitol films

Sample Name	Thickness (mm)	Moisture Content (%)	Water Solubility (%)
BC	0.0384	8.31	64.88
0.5S	0.0822	58.57	8.87
1.0S	0.1620	66.76	6.93
1.5S	0.1580	74.72	5.29
2.0S	0.1896	89.51	18.60
2.5S	0.2526	78.42	19.21
1.0BC	0.0718	4.82	27.95
2.5BC	0.0774	11.32	26.98
5.0BC	0.0774	4.86	16.23
7.5BC	0.0760	8.37	32.63
10.0BC	0.0886	16.67	12.46

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was employed to investigate the intricate chemical interactions occurring between BC and sorbitol. This analytical approach allowed for the observation of shifts and alterations in the intensity of characteristic peaks within the spectra, providing valuable insights into both the physical and chemical interaction between these substances and shedding light on their compatibility [30].

Figure 2 compares FTIR spectra between BC and BC/Chitosan/Sorbitol films, varying in the amounts of BC and sorbitol. Figure 2(a) notably highlights the characteristic peaks of BC present in all the samples. The broad absorption band in the 3200-3500 cm^{-1} range was attributed to the stretching vibrations of hydroxyl groups (-OH) of BC and sorbitol [31]. The existence of hydroxyl groups within BC facilitates the formation of different intermolecular and intramolecular hydrogen bonds. The establishment of these hydrogen bonds, both between molecules and within individual molecules, plays a fundamental role in shaping the physical characteristics of cellulosic materials [12]. Meanwhile, the peak around 2925 cm^{-1} corresponds to C-H stretching vibrations, while the peak at 1637 cm^{-1} can be attributed to the –CO group. The peaks at 1411 and 1314 cm^{-1} signify the –CH asymmetric deformation and –CH bending vibrations, which serve as critical signatures for samples with chitosan [32]. Furthermore, the peak around 1056 cm^{-1} corresponds to C–O symmetric stretching. In contrast, the presence of the hydroxyethyl group is indicated by the shift of the C-O-C stretching vibration peak to 888 cm^{-1} [15].

The introduction of sorbitol as a plasticizer induced a noticeable shift in the peak positions, transitioning from 3500 cm^{-1} (as observed in pure BC in Figure 2(b)) to lower wavenumbers, typically around 3300 cm^{-1}. This shift is primarily attributable to the formation of hydrogen bonding interactions involving BC, chitosan, and sorbitol [27]. Interestingly, the FTIR spectra of plasticized films (Figure 2(b)) displayed only marginal reductions in intensity. This observation suggests the presence of intermolecular interactions facilitated by the formation of hydrogen bonds. Consequently, it can be inferred that the plasticizer did not induce significant structural alterations in BC; instead, it engaged in cooperative interactions with the material.

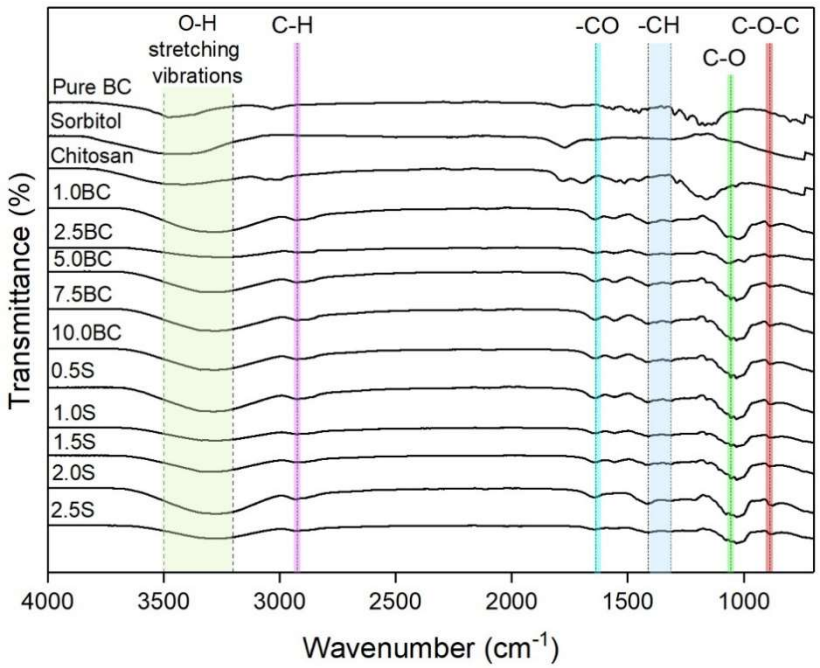


Figure 2. FTIR spectra for BC/Chitosan/Sorbitol films containing various amounts of BC and sorbitol

Mechanical properties

The evaluation of mechanical properties (Table 3), including tensile strength (TS), Young's modulus (YM), and elongation at break (EAB), is fundamental in assessing the potential of BC/sorbitol biocomposite films for food packaging applications. The control sample, which consisted solely of BC, served as the baseline for subsequent comparisons, recording a TS of 10.60 MPa, a YM of 629.91 MPa, and an EAB of 2.36%. As the BC concentration incrementally increased, these mechanical properties were affected. The findings align with previous analysis suggesting that a BC reinforcing effect within composite matrices can ultimately enhance the TS [33,34]. However, the phenomenon of agglomeration at higher BC concentrations observed in the 10.0BC formulation resulted in diminished TS, possibly due to impaired stress distribution [35]. On the other hand, adding sorbitol reduced the sample's YM due to its function as a plasticizer. Notably, the 2.5S formulation showed the lowest YM at 44.55 MPa, suggesting a significant improvement in film flexibility. This reduction aligns with theoretical expectations, where plasticizers weaken intermolecular hydrogen bonds in cellulose, thereby enhancing chain mobility and reducing stiffness [18].

Moreover, the YM analysis delineated a decrease with incremental BC and sorbitol additions. Initially, the BC-only films displayed considerable stiffness, a characteristic trait of materials with high crystallinity [23]. As BC concentrations were increased, stiffness initially rose due to the additional cellulose fibers reinforcing the structure of the films. However, similar to TS findings, excessive BC countered stiffness due to ineffective stress distribution within the matrix [35]. In contrast, the systematic addition of sorbitol to the film formulations markedly decreased the YM, reflecting its function as a plasticizer. For instance, the 2.5S formulation showed the lowest YM at 44.55 MPa, indicating substantial increases in film flexibility. This reduction in stiffness with added sorbitol supports the anticipated outcome where plasticizers disrupt hydrogen bonding within cellulose, thereby increasing chain mobility and decreasing material rigidity [36].

EAB further elucidates the ductility and adaptability of the films under strain. Initially, BC-only films displayed low EAB values, signifying limited elasticity, which is typical for rigid polymer matrices. As BC content was increased, there was a moderate increase in EAB, suggesting that higher levels of BC might slightly enhance the flexibility due to the distribution of stress across a denser network of cellulose fibers. On the other hand, increasing amounts of sorbitol had a more pronounced effect on EAB, substantially improving the stretchability of the films. Particularly, films with higher sorbitol content, such as the 2.5S sample, demonstrated significant increases in elongation capabilities. This behavior, indicative of enhanced film plasticity, highlights the efficacy of sorbitol in facilitating polymer chain slippage that induces more deformable film structures [36]. Such properties are desirable in packaging applications where material flexibility and resistance to breakage under load are essential.

Comparatively, the range of TS in this study, between 5.02 and 17.47 MPa, aligns closely with that of the modified BC films derived from sago liquid waste. However, it is noteworthy that the YM and EAB parameters observed in this study were slightly lower compared to those reported for modified BC derived from sago liquid waste. The modified BC displayed a YM range of 2.93-6.54 GPa and an EAB range of 10.27-29.67%, suggesting opportunities for further refinement of materials and tailored adjustments for specific applications.

Thermogravimetric analysis (TGA)

The thermal behavior of selected BC/Chitosan/Sorbitol films was investigated using TGA to unravel their thermal stability and degradation profiles. The obtained TGA curves and their first derivative (DTG) are depicted in Figure 3. DTG was the first-order differential of the TG curve; that is, the upward peaks in the DTG curve were consistent with the maximum weight loss of the TG curve. Notably, the findings unveiled an intriguing similarity in the thermal degradation behavior among all BC/Chitosan/Sorbitol films, aligning closely with the patterns observed in BC and sorbitol. The TGA curves revealed a multi-stage thermal degradation process, shedding light on the intricate transformations that occur within these materials when subjected to high temperatures [27]. Initially, a subtle weight loss, occurring around 100 °C, was linked to the evaporation

of water and the presence of residual acetic acid in the polymer matrix [33]. Subsequently, a distinct and substantial weight loss phase unfolded within the temperature range of 200 °C to 350 °C, marking the second thermal event that is closely associated with polymer decomposition [30]. It is worth noting that cellulose typically undergoes thermal degradation within the 300-450 °C temperature range. Incorporating chitosan and sorbitol into the film composition influenced the second stage of thermal degradation, with the primary thermal degradation step for chitosan and sorbitol initiating at approximately 300 °C [37]. Overall, the results regarding thermal stability suggest that the BC/Chitosan/Sorbitol biocomposite films remain stable when subjected to temperatures below 200 °C. As such, this biodegradable composite material is better suited for use with food products following sterilization or pasteurization processes.

Table 3. Tensile strength (TS), Young's modulus (YM), and elongation at break (EAB) of the BC/Chitosan/Sorbitol films

Sample Name	Tensile (MPa)	Young Modulus (MPa)	Elongation at Break (%)
BC	10.60±1.00	629.91	2.36±0.04
0.5S	41.08±2.58	1054.43	4.14±0.05
1.0S	14.95±1.09	426.03	3.71±0.64
1.5S	25.32±2.33	396.34	6.76±0.20
2.0S	15.32±0.76	248.37	6.47±0.04
2.5S	3.83±0.20	44.55	9.21±0.94
1.0BC	6.10±0.14	104.42	6.45 ± 0.05
2.5BC	19.20±0.38	255.99	8.60 ± 0.27
5.0BC	7.90±0.24	54.26	12.91 ± 0.09
7.5BC	20.65±0.85	318.89	11.29 ± 0.45
10.0BC	29.59 ±0.26	403.46	7.78 ± 0.63

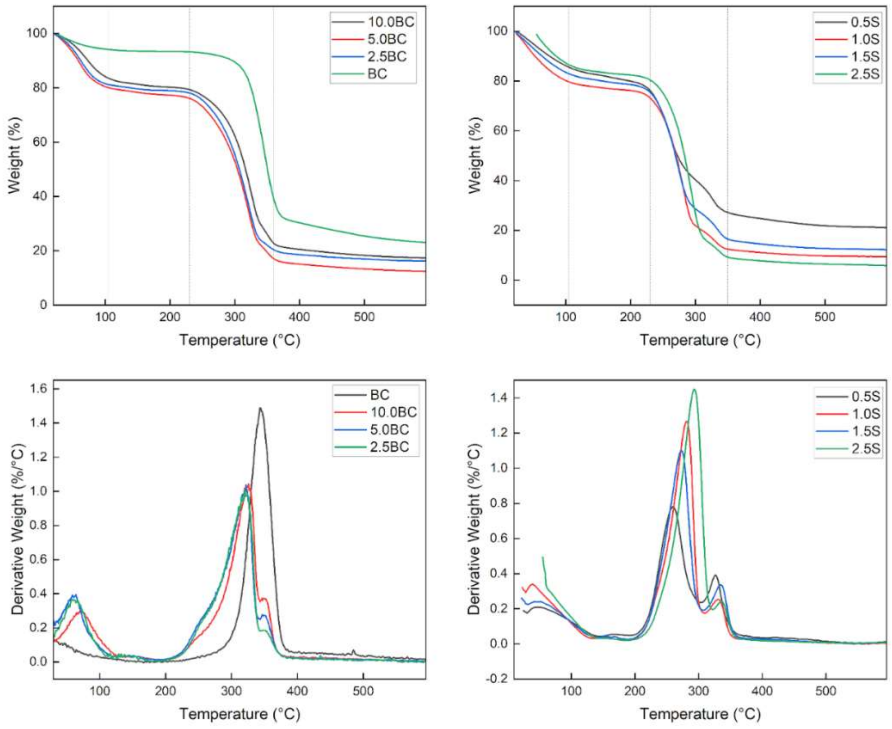


Figure 3. TG and DTG curves of selected BC/Chitosan/Sorbitol films

Morphology by FE-SEM

Figure 4 presents the surface morphology of pure BC film and selected BC/Chitosan/Sorbitol film samples (pure BC, 1.0BC, 10.0BC, 0.5S, 2.5S). The FE-SEM image of the pure BC film reveals the fibrous 3-D ultrafine networks of well-arranged nanofibers stabilized by hydrogen bonds existing in cellulose units [12,22]. However, the addition of sorbitol to the film formulation resulted in slightly smooth and homogeneous surfaces because of an even distribution of the material in the BC-based film [27]. Due to the hydrophilicity properties of sorbitol, an increase in the amount of plasticizer (from 0.5S to 2.5S) resulted in the least appearance of BC fibers and a wetter texture. In contrast, the 1.0BC film containing 0.5g of sorbitol had a dry texture but the same surface morphology.

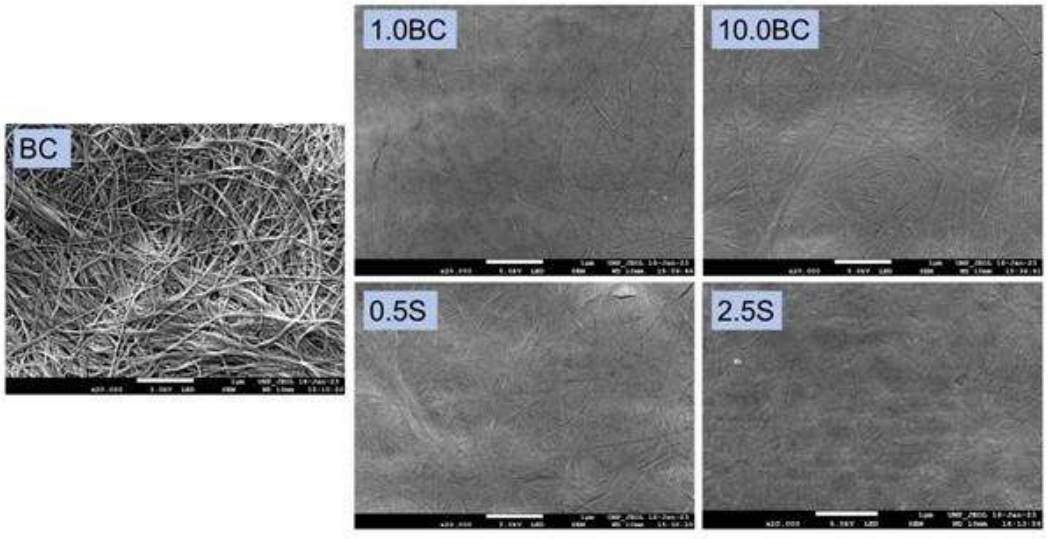


Figure 4. FE-SEM images of selected BC/Chitosan/Sorbitol films at 20000x magnifications

This study presents a comprehensive exploration of BC/Chitosan/Sorbitol films, which were developed using nata de coco as a key resource. Various amounts of BC and sorbitol were added to the films to improve their properties and compare their characterization. The results showed that an increase in BC content led to a significant improvement in the tensile strength, elongation at break, and thermal stability of the films due to hydrogen bonding interactions between the macromolecule chains of BC and the plasticizers. Conversely, the addition of sorbitol influenced moisture content and TGA weight loss. The optimum film composition emerged as 10.0BC and 0.5S, characterized by low moisture content, low water solubility, high tensile strength, and minimal TGA weight loss. These properties position this film as an excellent candidate for extended food packaging, offering a shield against physical, chemical, and biological contaminants while facilitating long-term content preservation. This research not only advances our understanding of biocomposite films but also underscores their potential in diverse applications, particularly in the realms of sustainability and food preservation.

The promising results of this study are anticipated to be the benchmark for future investigations into the incorporation of antimicrobial agents, natural dyes, and other natural plasticizers into the BC/Sorbitol matrix. Additionally, scale-up processes and lifecycle analyses will be crucial for assessing the commercial viability and environmental impact of these biocomposite films. Exploring these avenues will significantly contribute to the advancement of sustainable food packaging technologies.

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GLYCEROL ACETYLATION INTO ACETINS OVER SnO_2-BASED BIMETALLIC OXIDE CATALYST

(Pengasetilan Gliserol kepada Asetin Menggunakan Mangkin Dwilogam Oksida Berasaskan SnO_2)

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Abstract

Tin oxide-based bimetallic oxide catalysts, MO_x (MO_x = Al_2O_3, TiO_2, Y_2O_3) with a molar ratio of MO_x:SnO_2 of 1:1 was prepared by the solid-state method. X-ray diffraction (XRD) analysis showed that tin oxide, SnO_2, was present in all samples as a cassiterite phase with a tetragonal structure. The presence of other oxides, aluminum oxide (Al_2O_3), titanium oxide (TiO_2), yttrium oxide (Y_2O_3) in the respective Al_2O_3/SnO_2, TiO_2/SnO_2, Y_2O_3/SnO_2 were also identified and confirmed by X-ray fluorescence (XRF) analysis. A small exothermic peak at 480 °C and 750 °C in the differential thermal gravimetric (DTG) analysis revealed the oxidation of sub-oxide defects, took place which are very common in SnO_2-based samples. Further Fourier transform infrared spectroscopy (FTIR) analysis indicated the presence of Sn-O bonds, reflecting the abundance of tin-oxygen bonds in the materials. Acidity measurements by titration showed that both TiO_2/ SnO_2 and Y_2O_3/SnO_2 have the same acidity value of 0.23 mmol/g whereas Al_2O_3/SnO_2 and monoxide SnO_2 at 0.20 mmol/g. These catalysts were tested for the acetylation of glycerol to monoacetin (MA), diacetin (DA) and triacetin (TA) in a batch reactor at a molar ratio of glycerol: acetic acid of 1:6 w/v, a temperature of 100 °C, a reaction time of 2 h and a catalyst loading of 0.5 g. The tests showed complete conversion of glycerol by all catalysts, with the selective product being mainly MA, which was 64%, 62%, 57% and 43% for Al_2O_3/SnO_2, Y_2O_3/SnO_2, SnO_2 and TiO_2/SnO_2, respectively. The percentage selectivity of the more valuable products, DA + TA, was produced in the following order, TiO_2/SnO_2 > SnO_2 > Y_2O_3/SnO_2 > Al_2O_3/SnO_2 with 57%, 43%, 37%, and 35%, respectively. Further analysis of TiO_2/SnO_2 revealed that the catalyst not only had a high acidity, but also an increased BET surface area of 9.8 m^2/g compared to 8.6 m^2/g for SnO_2 when TiO_2 was incorporated into SnO_2. Analysis of the surface elements by energy dispersive X-ray spectroscopy (EDX) revealed a high surface oxygen content, which is consistent with the high acidity of the sample.

Keywords: glycerol acetylation, tin oxide, SnO_2-based bimetallic oxide, monoacetin, diacetin

Abstrak

Mangkin dwilogam oksida berasaskan timah oksida, MO_x (MO_x = Al_2O_3, TiO_2, Y_2O_3) dengan nisbah molar MO_x:SnO_2 pada 1:1 telah disediakan melalui kaedah keadaan pepejal. Analisis pembelauan sinar-X (XRD) menunjukkan timah oksida, SnO_2, hadir di dalam semua sampel sebagai fasa kasiterit dengan struktur tetragonal. Kehadiran oksida lain iaitu aluminum oksida (Al_2O_3), titanium oksida (TiO_2), itrium oksida (Y_2O_3) dalam Al_2O_3/SnO_2, TiO_2/SnO_2, Y_2O_3/SnO_2 masing-masing juga dikenal pasti dan disahkan oleh analisis pendarflor sinar-X (XRF). Puncak eksotermik kecil pada 480 °C dan 750 °C dalam analisis pembezaan gravimetrik terma (DTG) mendedahkan pengoksidaan kecacatan sub-oksida telah berlaku, iaitu kejadian yang biasa terjadi bagi sampel berasaskan SnO_2. Selanjutnya, analisis spektroskopi inframerah transformasi Fourier (FTIR) menunjukkan kehadiran ikatan Sn-O yang menterjemahkan limpahan ikatan timah-oksigen dalam bahan. Pengukuran keasidan secara penitratan menunjukkan kedua-dua TiO_2/SnO_2 dan Y_2O_3/SnO_2 mempunyai nilai keasidan yang sama iaitu 0.23 mmol/g, manakala Al_2O_3/SnO_2 dan monoksida SnO_2 pada 0.20 mmol/g. Semua mangkin telah diuji untuk pengasetilan gliserol kepada monoasetin (MA), diasetin (DA) dan triasetin (TA) dalam reaktor kelompok pada nisbah molar gliserol:asid asetik 1:6 b/i, suhu 100 °C, masa tindak balas 2 j dan muatan mangkin 0.5 g. Hasil ujian menunjukkan penukaran lengkap gliserol oleh semua mangkin, dengan pemilihan produk rata-ratanya MA iaitu 64%, 62%, 57% dan 43% bagi masing-masing Al_2O_3/SnO_2, Y_2O_3/SnO_2, SnO_2 dan TiO_2/SnO_2. Manakala penghasilan produk yang lebih bernilai iaitu DA + TA, adalah mengikut urutan $TiO_2/SnO_2 > SnO_2 > Y_2O_3/SnO_2 > Al_2O_3/SnO_2$ dengan masing-masing 57%, 43%, 37%, dan 35%. Analisis lanjutan terhadap TiO_2/SnO_2 mendedahkan bahawa mangkin ini bukan hanya mempunyai keasidan yang tinggi tetapi juga mempunyai peningkatan luas permukaan sebanyak 9.8 m^2/g berbanding 8.6 m^2/g bagi SnO_2, apabila TiO_2 digabungkan pada SnO_2. Analisis unsur permukaan oleh spektroskopi penyebaran tenaga sinar-X (EDX) menunjukkan kandungan oksigen permukaan yang tinggi, selaras dengan keasidan tinggi sampel berkenaan.

Kata kunci: pengasetilan gliserol, timah oksida, dwilogam oksida berasaskan SnO_2, monoasetin, diasetin

Introduction

Energy is a vital resource in the modern world and plays a role in a country's economic growth. With the world's population growing at an unprecedented rate, the demand for energy continues to rise. The transportation sector is the largest consumer of energy, with a growth rate of 1.5% per year [1]. Fossil fuels, which are non-renewable energy sources, account for about 90% of the transportation sector's energy consumption. The excessive use of fossil fuels has a significant impact on the environment, particularly in the form of greenhouse gas emissions and climate change. To reduce these impacts, the world is moving towards renewable energy sources such as wind, solar, hydro, geothermal and biodiesel, with the latter being the fastest growing industry.

In Malaysia, the 18 biodiesel plants in operation produced a total of 2.33 million tonnes of biodiesel in 2021 [2,3] and produced a total of 233,000 tonnes of glycerol as a by-product, i.e. about 10% for every tonne of biodiesel produced [3]. The biodiesel industry will continue to grow due to its renewability and reduction of CO_2 emissions compared to fossil fuels. Unfortunately, this also produces a considerable amount of glycerol, the limited use of which in the domestic industry will lead to waste disposal problems. Therefore, the catalytic conversion of glycerol into value-added products is an important issue for the sustainable production of biodiesel [4]. Currently, the acetylation of glycerol to acetins is the focus of interest, as acetins have multiple industrial applications, such as in cryogenics, cosmetics, as a fuel additive and as a raw material for biodegradable polyesters [5]. When glycerol reacts with an acetylating agent such as acetic acid or acetic anhydride, the reaction goes through a consecutive step. First, monoacetin (MA) is formed, which is later converted to diacetin (DA) and then to triacetin (TA), leading to a decrease in MA and an increase in DA and TA. This behavior was attributed to the protonation of the carbonyl oxygen atom of acetic acid and nucleophilic attack by glycerol to form an acylium ion intermediate on the surface of the catalyst, which favors subsequent nucleophilic attacks on the hydroxyl groups of glycerol. This requires a high density of acidic sites on the catalyst surface. Therefore, strongly acidic catalysts such as sulfuric acid, hydrofluoric acid and p-toluene sulfonic acid are used and have been shown to improve glycerol acetylation performance [6].

However, these acids are homogeneous catalysts, which leads to a difficult separation process and environmental problems in wastewater production [7]. Therefore, the development of an acidic solid catalyst for the acetylation of glycerol to acetins with excellent selectivity and high activity is highly desirable. Solid catalysts are attractive for this reaction because they can be easily separated from the reaction mixture, are reusable, and have the potential for higher catalytic

activity and selectivity. Solid catalysts are attractive for this reaction because they can be easily separated from the reaction mixture, are reusable, and have the potential for higher catalytic activity and selectivity. In addition, a solid catalyst can facilitate a chemical reaction by providing a surface for the reaction molecules to attach to and chemically convert into the desired product. Some common examples of solid catalysts are basic zeolites, alkaline earth metal oxides, hydrotalcites and carbon-based materials [25, 26].

The combination of metal oxides as acidic solid catalysts has shown great potential for the formation of bimetallic oxides, which have been reported for various reactions, such as Co_3O_4/SnO_2 [8] and TiO_2-ZrO_2 [9] in the acetalization of glycerol, Al_2O_3-ZrO_2 in the carboxylation of glycerol [10] and CaO-SnO_2 in the transesterification of soybean [12]. These metal oxides are cheap, abundant, effective and of great interest for heterogeneous catalysis due to their unique properties. As one of the leading tin producing countries in the world, Malaysia has diversified its tin industry away from mining and primary industries to manufacturing, services, and technology sectors. This diversification has reduced the country's reliance on tin exports. This work is therefore concerned with the research of tin oxide-based catalysts for the acetylation of glycerol, with the aim of improving their efficiency and selectivity while reducing their environmental impact. As mentioned earlier, the rationale behind this study is rooted in addressing the limitations of current strong acid catalysts which are effective but pose challenges in terms of separation processes and environmental concerns. At the same time, proper disposal of crude glycerol is imperative due to its salt, free fatty acids, and methanol content, which can contaminate soil and contribute to environmental problems. This underscores the significance of finding value-added uses for crude glycerol, particularly through the glycerol acetylation process. Several studies have shown that tin oxide is desirable as a stable and durable active site in heterogeneous catalysts [11, 12]. According to Xie and Zhao [12], the use of CaO-SnO_2 in the transesterification of soybean oil resulted in high generation of fatty acid methyl esters (FAME) with a yield of 83.9%, contributed by the acidic SnO_2, which improved the stability of the bimetallic oxide catalyst. Bewana et al. [8] concluded that the highest activity of Co_3O_4/SnO_2 (75:25) gave above 90% conversion of glycerol with 100% solketal selectivity. The catalyst was very stable and can be recycled for up to 6 cycles with little catalyst loss. Inspired by these works, a bimetallic oxide based on SnO_2 is synthesized by the solid-state method. Tin oxide is combined with metal oxide, MO_x = TiO_2, Al_2O_3, and Y_2O_3, in a molar ratio of MO_x:SnO_2 of 1:1. It has been reported that the MO_x used in this work exhibit high oxygen adsorption due to several oxygen vacancies. The acidic properties of these materials possess are utilized and employed in the acetylation of glycerol with acetic acid, as shown in Figure 1.

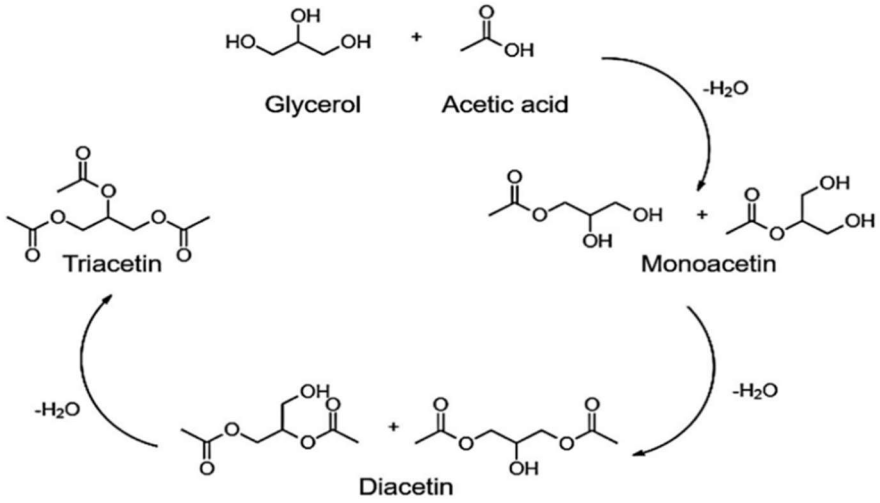


Figure 1. Acetylation of glycerol with acetic acid to produce monoacetin, diacetin, and triacetin

Materials and Methods

Materials

Anhydrous glycerol (99%), titanium dioxide, TiO_2 (98%), hydrochloric acid, HCl (99%), sodium hydroxide, NaOH (99%) and sodium chloride, NaCl (99.5%) were purchased from R&M Chemicals. Tin(IV) oxide, SnO_2 (99%), aluminum oxide, Al_2O_3 (99%), acetic acid, CH_3COOH (99.5%) and phenolphthalein (99%) were purchased from Systerm Chemicals. Yttrium oxide, Y_2O_3 (99.99%) was purchased from Sigma-Aldrich. All chemicals obtained were used without further purification.

Catalyst preparation

The SnO_2-based bimetallic oxide catalysts were synthesized through solid-state method [27]. A stoichiometric amount of TiO_2, SnO_2 in a 1:1 ratio was used. The measured chemical components were ground and well homogenized using a laboratory grade mortar and pestle. The grinding was maintained for a period of 30 minutes to allow proper intercalation of the Ti^{4+} and Sn^{4+} precursors. The precursor mixture was then calcined in a tubular furnace with a carbon dioxide flow at 400 °C for a duration of 2 hours at a heating rate of 5 °C /min. The calcined sample was named as TiO_2/SnO_2. The procedures for the solid-state synthesis of Al_2O_3 and Y_2O_3 with SnO_2 in a 1:1 ratio, respectively were repeated and the calcined samples were named Al_2O_3/SnO_2 and Y_2O_3/SnO_2. A monoxide SnO_2 was also prepared following the same procedures.

Catalyst characterisation

Thermal gravimetry analysis (TGA) and derivative thermogravimetry (DTG) were undertaken using Mettler Toledo 851e to determine the thermal stability of the synthesized catalysts. The sample was loaded into the sample pan and was heated from 50 to 900 ºC at a rate of 10 ºC min^{-1} under a continuous flow of nitrogen at 50 mL min^{-1}. The TGA provided information on absolute mass changes while the DTG focused on the rate of mass change as a function of temperature.

Fourier transform infrared spectroscopy (FTIR) spectra were obtained using a Perkin Elmer 1650 spectrometer. The sample was placed in the sample holder and inserted into the instrument with the spectroscopic range of 400 cm^{-1} to 4000 cm^{-1}. XRD measurements were obtained using a Shimadzu X-ray diffractometer model XRD 6000 using CuK_α radiation with a wavelength (λ) of 1.54 Å, scanning speed of 4º per minute, and operated at 30 kV and 15 mA. The patterns produced were compared to reference patterns supplied by the International Centre for Diffraction Data (ICDD).

Elemental composition and chemical compound analysis of the calcined SnO_2 and mixed oxide samples were conducted using Energy Dispersive X-ray Spectroscopy (EDX) attached to Scanning Electron Microscope (SEM) model JEOL, JSM6400 and using X-ray fluorescent (XRF), Shimadzu EDX-720, respectively. The surface morphology was delivered by the former analysis. Brunauer-Emmet-Teller (BET) surface areas were determined by N_2 absorption at -196 ºC using Thermo-Finnigan Sorptomatic 1990 series analyzer. Prior to the analysis, the sample was degassed at 150 ºC for 2 hours. The analysis was conducted at 5 different pressures along the isotherm before the results were converted to surface area measurements. While the Barret-Joyner-Halenda (BJH) method was used to calculate the pore volume and pore diameter of the samples from the desorption branch isotherm.

Acid titration

The necessary solutions were prepared in advance, with 0.1 M HCl prepared by diluting concentrated hydrochloric acid, HCl (99%) and 0.1 M NaOH prepared by diluting sodium hydroxide, NaOH (99 %). A phenolphthalein indicator solution was obtained by dissolving phenolphthalein powder in isopropanol. The catalyst powders, 0.1 g, were dispersed in a 20 ml NaOH solution, stirred for an hour, filtered, and the filtrate was added with phenolphthalein. The filtrate solution was then titrated with a HCl solution. Color change from pink to colorless was noted which signified the endpoint. These steps were repeated for three times for each catalyst to obtain average data.

Glycerol acetylation

The catalytic performance of the synthesized catalysts was carried out for the acetylation of glycerol to acetins in a laboratory batch reactor, as illustrated in Figure 2. The catalyst in powder form, 0.5g in weight, was introduced into a 250 mL round bottom flask. Glycerol and acetic acid (glycerol: acetic acid = 1:6 w/v) was carefully added and a magnetic bar was put into the mixture. A reflux condenser was fitted to the flask and

the flask was immersed in a silicon oil bath. The reaction was conducted at temperature of 100 °C for a duration of 2 hours under reflux conditions. The mixture was continuously stirred under a constant rate of 450 rpm. Upon completion of the reaction, the product was cooled down and subjected to centrifugation at 3000 rpm for 10 minutes. The catalyst was separated from the mixture using filter paper. To account for error bars and evaluate the statistical significance of the results, these procedures were repeated three times. The reaction products obtained were analyzed using gas chromatography coupled with a flame ionization detector (GC-FID) (Agilent 7890A), equipped with a DB Wax column (30 m × 0.25 mm ID × 0.25 µm). For internal standard and solvent, 1,3-butanediol and acetonitrile were utilized, respectively. A volume of approximately 1 µL of the final sample was injected into the column initially set at 80 °C for 3 minutes. Subsequently, the temperature was raised to 260 °C with a heating rate of 10 °C min^{-1}, and then at 300 °C, with a heating rate of 30 °C min^{-1}. The area of the peak was utilized for constructing the calibration curve and conducting quantification analysis. The total time required for GC-FID analysis was 22.3 minutes. Helium was employed as the carrier gas with a flow rate of 1.3 mL min^{-1}. The glycerol conversion and selectivity of MA, DA, and TA were calculated using the following equation.

$$\text{Glycerol conversion, \%} = \frac{Amount\ of\ glycerol\ converted}{Amount\ of\ glycerol\ fed}\ x\ 100 \quad (1)$$

$$\text{Selectivity, \%} = \frac{Amount\ of\ specific\ product\ formed}{Total\ amount\ of\ product\ formed}\ x\ 100 \quad (2)$$

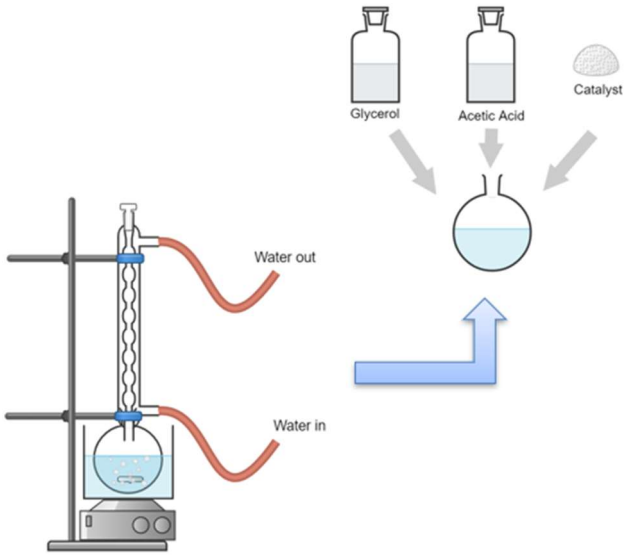


Figure 2. Experimental set up for glycerol acetylation process

Results and Discussion

The thermal properties of the mixed oxide catalysts, Al_2O_3/SnO_2, TiO_2/SnO_2, Y_2O_3/SnO_2 catalysts were investigated by thermogravimetric analysis and differential thermogravimetric analysis (TGA-DTG). The DTG is the derivative of the TGA curve and is displayed together with the TGA curve, Figure 3. The TGA weight loss from room temperature to 120 °C is likely due to the loss of adsorbed water molecules. A further loss of up to 400 °C could be due to the removal of hydroxyl groups (dehydroxylation) from the lattice [28]. These processes are accompanied by a weak endothermic peak on DTG profiles, with a maximum at 100 °C and 225 °C, respectively. It should be noted that the total loss due to bound and unbound water molecules was not recorded for all samples, except for Y_2O_3/SnO_2 and this was only for 0.25% [29]. The weight gain was observed in the TGA plot from 400 °C to 900 °C, with the differential thermogravimetric (DTG) curve revealing a small exothermic peak at about 480 °C and

750 °C. This phenomenon appears to be associated with the oxidation of sub-oxide defects. Oxygen defects are very common in SnO_2 due to oxygen vacancies where oxygen atoms are missing in the crystal lattice [13]. These vacancies can be caused by thermal treatment or by defects introduced during solid-state synthesis. These small exothermic peaks are accompanied by a small loss, 1%, corresponding to the removal of a certain amount of oxygen from the lattices, which leads to crystallization of the material [14].

The FTIR spectra of the SnO_2-based bimetallic oxides are displayed in Figure 4. The spectra of the mixed oxide showed no absorption band in the region of OH vibration (3000-4000 cm^{-1}), suggesting that the OH group could be removed during calcination at high temperature. A similar observation was made by Deshmukh and Kadam [15] who did not observe OH vibration in tin oxide treated with microwaves at high power. The SnO_2-based bimetallic oxides are typically a metal oxide compound consisting of tin (Sn), aluminum (Al), titanium (Ti), yttrium (Y) and oxygen (O) atoms in their respective mixtures. This observation confirms that these mixed oxides do not contain OH functional groups in their chemical structure, so there are no OH bonds to give rise to OH stretching or bending vibrations in the FTIR spectrum of SnO_2-based bimetallic oxides. An enlarged view of the FTIR spectra in the range of 400 to 700 cm^{-1} shows that the peaks at 610-630 cm^{-1} are due to the vibrations of the Sn-O terminal bonds and the peaks at 410-430 cm^{-1} are due to the vibrations of the O-Sn-O bridging bonds [14]. The well-defined absorption band indicates the presence of Sn-O bonds in the sample and reflects the abundance of tin-oxygen bonds in the materials. Nevertheless, the vibrations associated with Al-O, Ti-O and Y-O bonds should be observed in the FTIR spectra in the 400-700 cm^{-1} range, which are coincided with the vibrations of Sn-O.

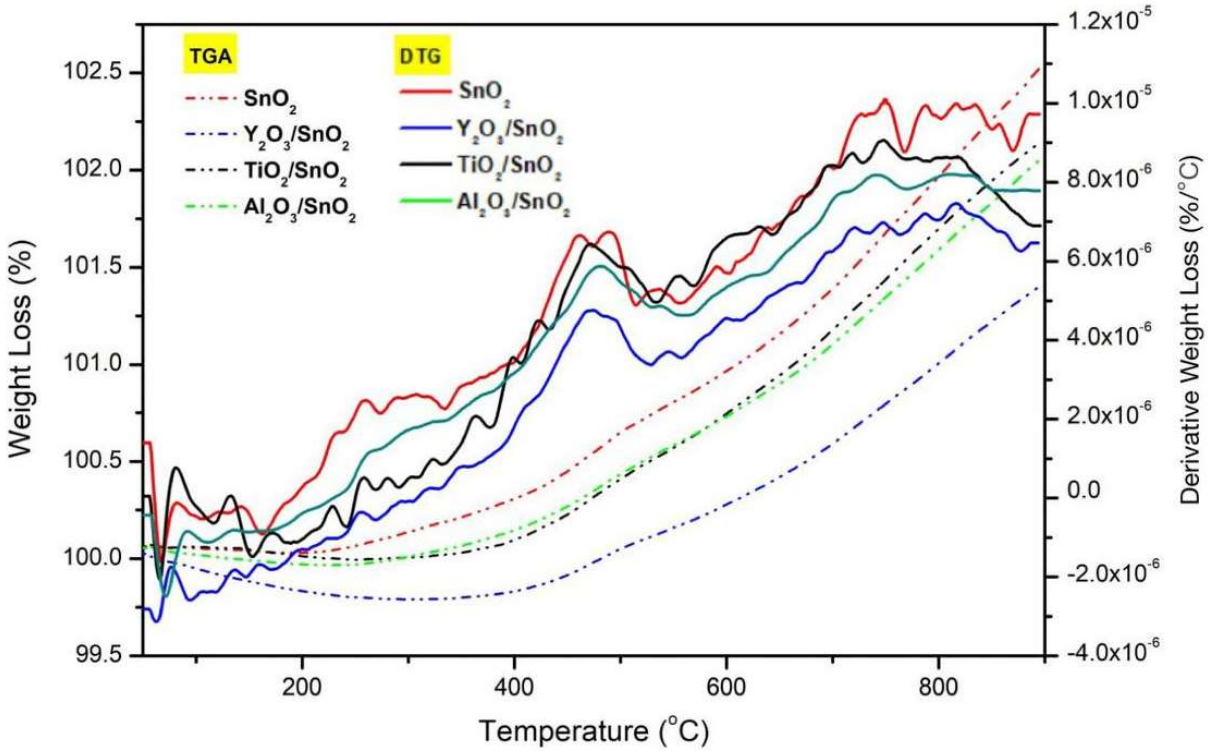


Figure 3. TGA-DTG curves for SnO_2-based bimetallic oxide catalysts

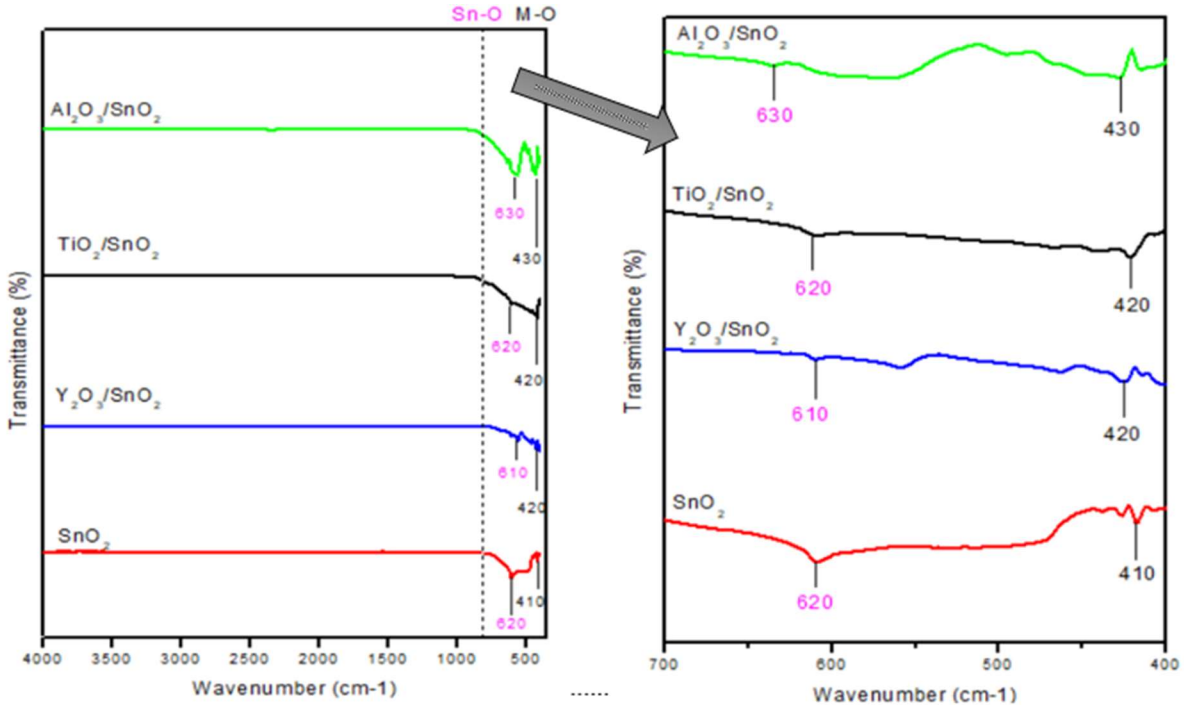


Figure 4. FTIR spectra of SnO_2-based bimetallic oxide catalysts.

The X-ray diffraction (XRD) patterns of the calcined catalysts are presented in Figure 5. For all materials, the main phase of tin oxide (SnO_2) cassiterite with a tetragonal structure was identified using ICDD file no. 98-010-5764, revealing distinct diffraction peaks at 2θ = 26.7°, 34.0°, 38.1°, 39.1°, 51.9°, 54.9°, 58.0°, 62.1°, 64.9°, 66.1°, 71.4°, and 78.9°. This indicates that the tin oxide species are present in all calcined catalysts and are in the form of a tetragonal phase, which is consistent with Bowles' report in 2021 [16]. The presence of other metal oxides, MO_x phases, Al_2O_3 (ICDD: 98-008-4608), TiO_2 anatase (ICDD: 98-004-0606), Y_2O_3 (ICDD: 98-003-5889), in the respective SnO_2-based bimetallic oxides was also detected. This is perhaps not surprising as the mixed oxide contains a stoichiometric amount of MOx and SnO_2 (MOx: SnO2 = 1:1). This is based on the preparation of the mixed oxide in the solid-state method, where one mole of MOx was mixed with one mole of SnO_2.

The apparent emergence of the sharp and intense peaks in all samples suggested that the obtained SnO_2-based mixed oxide was formed with a well-ordered crystal structure. A third phase of aluminium tin, $Al_{0.975}Sn_{0.025}$ (ICDD: 98-009-731), titanium tin oxide, $(Ti_{0.1}Sn_{0.9})O_2$ (ICDD: 98-005-7620) and yttrium stannide, YSn_2 (ICDD: 98-011-0094) was observed for all mixed oxide samples as Al_2O_3, TiO_2 and Y_2O_3 react with SnO_2 to form agglomerates of precipitates. Interestingly, a fourth phase, diyttrium distannate, $Y_2(Sn_2O_7)$ (ICDD: 98-003-9694) was observed in Y_2O_3/SnO_2. This is because the ionic radius of the ion Y^{3+} (93 pm) is larger than the ionic radius of the ion Sn^{4+} (71 pm). The ion Y^{3+} does not occupy the ion Sn^{4+} in the crystal lattice, instead Sn^{4+} tends to occupy the Y^{3+} site. As a result, the crystalline quality of the tin oxide was reduced. The opposite was true for TiO_2/SnO_2 and Al_2O_3/SnO_2, where both Al^{3+} (50 pm) and Ti^{4+} (61 pm) have a relatively smaller ionic radius than Sn^{4+}, which can be easily replaced the Sn^{4+} in the SnO_2 lattice. This substitution would disrupt the regular tin dioxide lattice. However, the composition of the resulting mixture depends on the extent of substitution and the ratio of TiO_2 and Al_2O_3 in the mixture. Since the substitution is limited (at a molar ratio of MOx: SnO_2 = 1:1), the regions of pure TiO_2 and Al_2O_3 are still present in the mixture, while the crystalline nature of SnO_2 has been preserved.

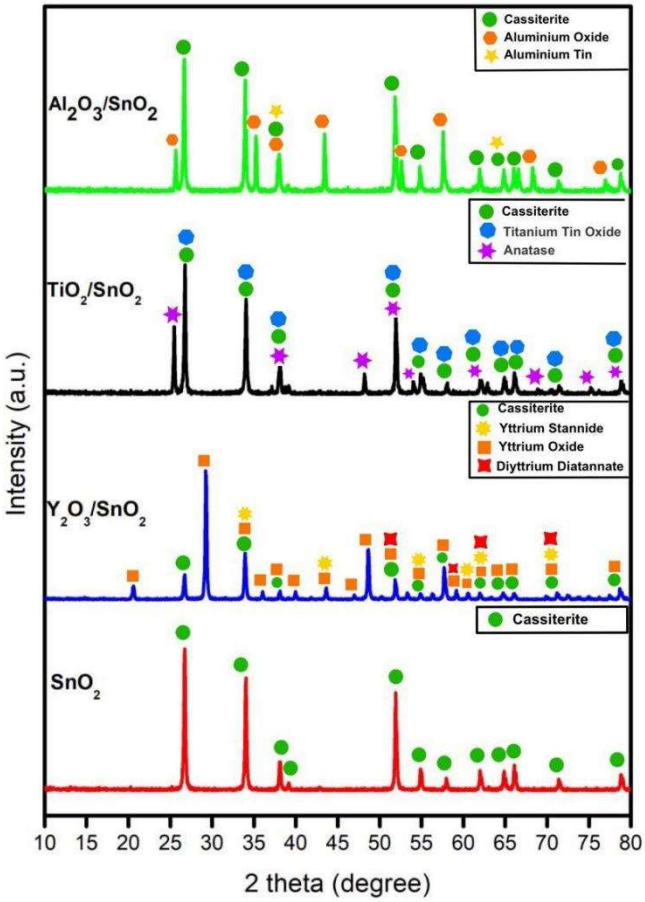


Figure 5. XRD profiles of SnO_2-based bimetallic oxide catalysts

XRF analysis was performed to determine the chemical composition of the catalysts. Table 1 shows the amount of metal oxide (MO_x) present in the catalyst samples in weight percent (wt.%). TiO_2/SnO_2 exhibits the chemical components of 47.8% TiO_2 and 51.4% SnO_2, which is closest to the 1:1 molar ratio. The Y_2O_3/SnO_2 has the lowest SnO_2 content (25.9 %) due to low occupation of the Y^{3+} crystal lattice by Sn^{4+}, which is consistent with the XRD analysis. The highest SnO_2 content (71.1%) is delivered by Al_2O_3/SnO_2, which is due to a larger SnO_2 occupation in the bimetallic oxide sample. The low weight percentage of Al_2O_3 in the Al_2O_3/SnO_2 mixture indicates that the substitution of Al_2O_3 into the SnO_2 lattice at a ratio of 1:1 may not be very efficient despite the smaller atomic radius of Al^{3+} compared to Sn^{4+}. This could be due to the different crystal structures of Al_2O_3 (hexagonal) and SnO_2 (rutile), which hinder the incorporation of Al_2O_3 into the SnO_2 lattice.

The acidity of the surface of the catalyst is a crucial factor affecting its activity. This acidity is attributed to the presence of Lewis acid sites on the surface of the metal oxide. The acid sites coordinate with electron-rich species of the carbonyl group of acetic acid and thereby activate it for the subsequent reaction with glycerol [34]. In this work, the acidic surface properties of the catalyst were investigated by titration after a displacement reaction. The attached free OH ions on the solid catalyst, previously dispersed in sodium hydroxide solution, were treated with acid to neutralize, and displace them from the surface by protonation. Table 1 shows the acidity measurements using the titration technique for SnO_2-based bimetallic oxide catalysts. The acidic sites are

associated with the surface tin cations [17] and the acidic character increases with the addition of MO_x = TiO_2 and Y_2O_3 (i.e. TiO_2/SnO_2 and Y_2O_3/SnO_2).

The SnO_2-based bimetallic oxide catalysts were investigated for the acetylation of glycerol to acetins. Figure 6 shows the catalytic performances of the prepared SnO_2-based bimetallic oxide catalysts expressed as glycerol conversion (GL) and selectivity to different acetins, namely monoacetin (MA), diacetin (DA), and triacetin (TA). As can be observed, after only 2 h of the process, all the glycerol present in the system was transformed to acetins, as 100% GL was achieved. This is not surprising, as it is known that the reaction of glycerol with acetic acid is an autocatalytic process that can convert glycerol to acetins even in the absence of a catalyst [18,19]. However, in the non-catalyzed process, the conversion rate of glycerol is low, about 9 % after 1 h of the reaction carried out at 80 °C [20]. Thus, in the presence of a catalyst, even a monoxide (SnO_2) showed a sufficient catalytic activity for glycerol conversion. The most important performance is the selectivity towards acetins, whereby during the 2 h reaction time at a temperature of 100 °C mainly a monosubstituted product, i.e. monoacetin (MA), was formed. The differences between the MA selectivity of SnO_2, Al_2O_3/SnO_2 and Y_2O_3/SnO_2 were not that significant. All these catalysts produced mainly MA (over 50%), and the selectivity for MA obtained after 2h of reaction time were 57%, 64% and 62%, respectively. For TiO_2/SnO_2, the selectivity to MA was lower compared to the other catalysts and was 43 %. While for the trisubstituted product (triacetin, TA) was formed in small amounts. SnO_2, Al_2O_3/SnO_2 and Y_2O_3/SnO_2 gave only about 1-2% TA selectivity. A study investigating the selectivity for substituted products at different reaction temperatures (80 °C and 110 °C) concluded that the higher reaction temperature (110 °C) promoted the formation of a mixture of products, with the conversion of MA to higher esters being significantly faster [21].

Table 1. XRF analysis and acidity measurements of SnO_2-based bimetallic oxide catalysts

Catalyst	Metal Oxide	Weight (wt.%)	Acidity (mmol/g)
TiO_2/ SnO_2	SnO_2	51.4	0.23
	TiO_2	47.8	
	Total	99.2	
Y_2O_3/SnO_2	SnO_2	25.9	0.23
	Y_2O_3	60.6	
	Total	86.5	
Al_2O_3/SnO_2	SnO_2	71.1	0.20
	Al_2O_3	27.8	
	Total	98.9	
SnO_2	SnO_2	98.0	0.20

In this work, TiO_2/SnO_2 showed the highest selectivity for TA at 8%, while the combined selectivity's for DA+TA showed that TiO_2/SnO_2 was the most active catalyst at 57%. TiO_2/SnO_2 provides an acidic site for activation of the carbonyl group of acetic acid, resulting in an acylium ion that is more susceptible to nucleophilic attack by the oxygen atom of the hydroxyl of glycerol [6, 18, 19]. Upon release of a water molecule, monoacetin (MA) is formed, which further reacts with acetic acid by a similar mechanism to form diacetin (DA) and triacetin (TA). The observed difference in selectivity for DA+TA between Y_2O_3/SnO_2 and TiO_2/SnO_2 catalysts, despite similar acidity (Table 1), could be due to differences in their acidic sites. Y_2O_3/SnO_2 may offer unique acidic sites that could influence the reaction pathways, exact mechanism, and effects on the formation of diacetin and triacetin compared to TiO_2/SnO_2.

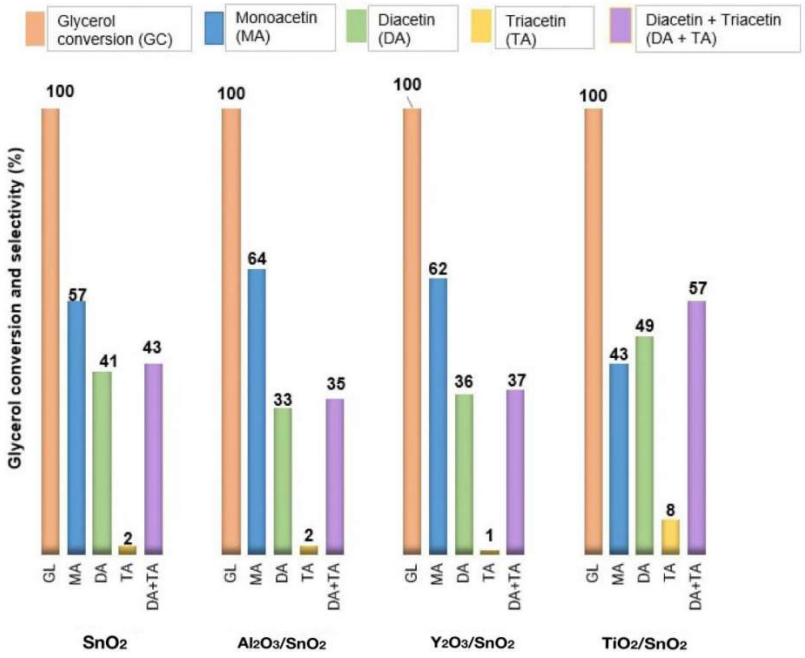


Figure 6. Glycerol conversion and product selectivity. Reaction conditions: glycerol to acetic acid molar ratio of 1:6, catalyst load 0.5 g, temperature 100 °C, time 2 h, and stirring rate 450 rpm. GL: Glycerol conversion, MA: monoacetin, DA: diacetin, TA: triacetin

The TiO_2/SnO_2 catalyst was further analyzed due to its promising catalytic activity in glycerol acetylation. The monometallic SnO_2 was also analyzed for comparison. Textural analysis of TiO_2/SnO_2 and SnO_2 by nitrogen adsorption-desorption showed a type IV isotherm (Figure 7 (a)), a typical feature of mesoporous materials, i.e. materials with a pore width between 2-50 nm [22]. The pore size distribution of both TiO_2/SnO_2 and SnO_2 is shown in Figure 7 (b). It is obvious that the pore size distribution of TiO_2/SnO_2 catalysts has a larger pore width compared to SnO_2 catalysts, while the pore volume of SnO_2 increases (Table 2). At the same time, TiO_2/SnO_2 shows a slightly higher BET surface area of 9.8 m^2/g compared to 8.6 m^2/g for SnO_2. Although the value of the changes is not significant, it shows that the addition of TiO_2 to SnO_2 affects its textural properties. Such a potent combination of suitable pore width and high acidity inherent in TiO_2/SnO_2 could enable more efficient selectivity of DA+TA in the glycerol acetylation process [35].

The morphology of TiO_2/SnO_2 was analysed with the SEM (see Figure 8). The catalyst exhibited a non-uniform particle shape similar to the morphology of the SnO_2 monoxide, indicating that the addition of TiO_2 did not affect the morphology of the tin oxide. Elemental analysis of the surface was performed using energy dispersive X-ray spectroscopy (EDX) and the analytical values are listed in Table 2. As can be seen in Figure 8, both TiO_2/SnO_2 and SnO_2 emit characteristic L-shell X-ray emission lines of tin, SnL. The atomic percentage of tin, calculated from the intensity of the SnL peaks relative to the other peaks, gave a value of 42.5 % Sn in SnO_2. This value decreased drastically for the mixed oxide TiO_2/SnO_2, with the heteroatom Sn accounting for 19.1 % and Ti for 12.7 %. The dominant atom in the bimetallic oxide was oxygen, based on the K-shell X-ray emission lines of oxygen, with a proportion of 66.3 %, which is higher than that of the monoxide SnO_2 at 56.1 %. These data demonstrate the efficiency of the solid-state mixture in increasing the surface oxygen in the bimetallic oxide. The immediate consequence of the high surface oxygen content was an increase in the surface acidity of the catalyst, which is consistent with the acidity measurement in Table 1. Therefore, TiO_2/SnO_2 provides a higher selectivity of DA + TA as it has a higher acidity that allows for a consecutive reaction of glycerol with acetic acid.

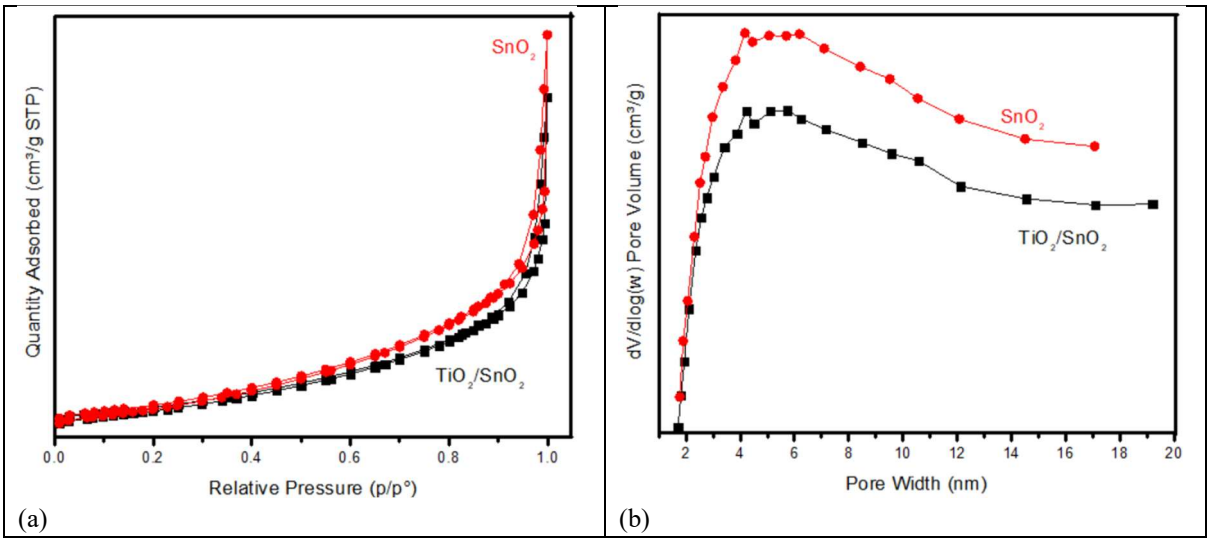


Figure 7. (a) N_2 adsorption–desorption isotherm and (b) pore size distributions of the synthesized TiO_2/SnO_2 and SnO_2 catalysts

Table 2. Textural and elemental analysis of SnO_2 and TiO_2/SnO_2 catalysts.

Catalyst	BET Surface Area ($m^2\ g^{-1}$)	Pore Volume (nm)	Pore Width (nm)	Element	Weight (%)	Atomic (%)
SnO_2	8.6	0.001829	4.9693	SnL	83.69	42.5
				OK	14.88	56.1
				Total	98.57	98.6
TiO_2/SnO_2	9.8	0.001783	5.0286	SnL	56.51	19.1
				OK	26.43	66.3
				TiK	15.14	12.7
				Total	98.08	98.1

SnL = Sn (Tin), OK = O (Oxygen), TiK = Ti (Titanium)

The catalytic activity of the TiO_2/SnO_2 catalyst was compared with a biomass-based catalyst (Biosolid) [5], (1Ag–10Cu-doped rice husk silica) [23], a mixed oxide-supported catalyst (MoO/TiO_2-ZrO_2) [24] and a zeolite catalyst (H-USY) [25] (Table 3). The summary shows that the reaction catalysed with TiO_2/SnO_2 gave better combined DA+TA production than other catalysts. In addition, the TiO_2/SnO_2 catalyst also yielded acetins at a lower reaction temperature (100 °C) than other catalysts, proving that the tin oxide-based catalyst promotes glycerol conversion and acetin selectivity. This is a very important result that should be highlighted as tin oxide is one of the most important minerals in Malaysia that has a high potential for use in the chemical industry

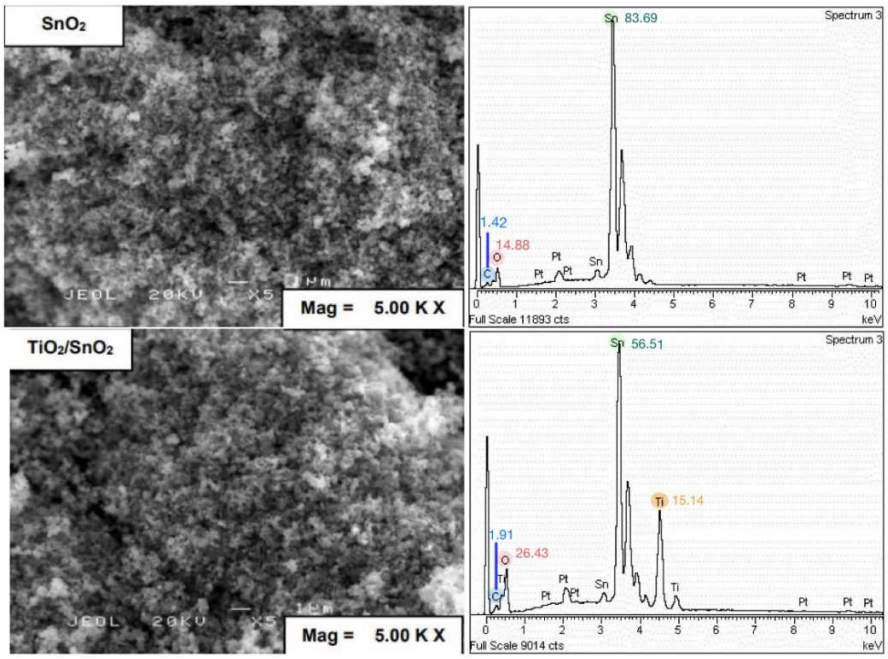


Figure 8. SEM image and EDX analysis of SnO_2 and TiO_2/SnO_2 catalysts. Carbon and platinum signals are attributed to the SEM carbon grid and platinum coating

Table 3. Comparison on glycerol acetylation into acetin

Catalyst	Reaction Parameters				GL (%)	Selectivity (%)				Reference
	T (ºC)	MR	CL	t (h)		MA	DA	TA	DA+TA	
TiO_2/SnO_2	100	1:6	10 wt.%	2	100	43	49	8	57	Present study
Biosolid	120	1:6	16 wt.%	2	100	47	48	6	54	[5]
1Ag–10Cu-doped rice husk silica	108	1:10	0.08 g	5	83.4	68.3	18.5	13.2	31.7	[23]
Zirconia-based: MoO/TiO_2-ZrO_2	120	1:6	5 wt.%	3	100	52	40	7	47	[24]
Zeolite-based: H-USY	110	1:9	-	4.5	78	74	21	6	27	[25]

GL = glycerol conversion, T = reaction temperature, MR = molar ratio glycerol: cetin, t = time, MA = monoacetin, DA = diacetin, TA = triacetin

Conclusion

Heterogeneous catalysts based on tin oxide, SnO_2, were investigated for the acetylation of glycerol acetylation with acetic acid to monoacetin (MA), diacetin (DA) and triacetin (TA). The catalyst was prepared by a solid-state method by adding metal oxide MO_x (MO_x = Al_2O_3, TiO_2, Y_2O_3) in a molar ratio of 1:1 to tin oxide. Monoxide SnO_2 was also prepared as a comparison. Remarkably, all catalysts used achieved complete conversion of glycerol, indicating their potential for acetylation reactions. However, glycerol was mainly converted to MA, as all catalysts showed. The selectivity towards DA and TA was rather low, except for TiO_2/ SnO_2, which showed a higher selectivity towards DA + TA (57 %) than other catalysts. Thus, TiO_2/ SnO_2 proves to be a compelling option for use as a heterogeneous

catalyst in glycerol acetylation. This innovative approach could effectively integrate tin oxide into the catalyst industry, increasing its versatility and value.

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MICROPLASTIC POLLUTION IN MALAYSIA: STATUS AND CHALLENGES – A BRIEF OVERVIEW

(Pencemaran Mikroplastik di Malaysia: Status dan Cabaran – Suatu Tinjauan Ringkas)

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Abstract

Microplastics (MPs) are widespread and infiltrate the environment through unregulated use in diverse industrial processes such as the manufacturing of cleaning products, cosmetics, fertilizers, and pharmaceuticals, as well as from the degradation of larger plastic items. Due to this ubiquitous nature, combined with the fact that MPs possess the capacity for biomagnification within the food chain, MPs may constitute a major threat to human health. Hence, the scientific community and regulatory authorities are increasingly focusing their attention on this issue, leading to continuous developments in both legislation and the scientific literature concerning MPs. The review aims to provide an overview of the state-of-the-art occurrences of MPs, identification, detection, and quantification of MPs, degradation of MPs, and health effects of MPs from the perspective of Malaysia. A brief overview of the latest scientific research and recent developments on MPs (until the year 2024) will provide insights into the current understanding of MPs and their health implications. Furthermore, this review will serve as a valuable reference for further studies related to MPs in Malaysia and could help in informing future policy decisions.

Keywords: Microplastic, emerging contaminants, occurrence, analytical methods, degradation

Abstrak

Mikroplastik (MPs) adalah sebaran meluas dan meresap ke dalam alam sekitar melalui penggunaan yang tidak terkawal dalam pelbagai proses industri seperti pengilangan produk pembersihan, kosmetik, baja, dan farmaseutikal, serta daripada peluruhan bahan plastik yang lebih besar. Disebabkan sifatnya yang meluas ini, serta hakikat bahawa MPs mempunyai kapasiti untuk biomagnifikasi dalam rantaian makanan, MPs boleh menjadi ancaman besar kepada kesihatan manusia. Oleh itu, komuniti saintifik dan pihak berkuasa bagi pengawalan semakin memberi tumpuan kepada isu ini, yang membawa kepada perkembangan berterusan dalam perundangan dan literatur saintifik mengenai MPs. Tinjauan ini bertujuan untuk memberikan gambaran keseluruhan tentang kejadian MPs, pengenalpastian, pengesanan, dan pengukuran MPs, degradasi MPs, dan kesan kesihatan MPs dari perspektif Malaysia. Gambaran ringkas tentang penyelidikan saintifik terkini dan perkembangan terkini berkaitan dengan MPs (sehingga

tahun 2024) akan memberikan pemahaman semasa tentang MPs dan implikasinya kepada kesihatan. Selain itu, tinjauan ini boleh dijadikan sebagai rujukan yang berharga untuk kajian lanjutan berkaitan dengan MPs di Malaysia dan boleh membantu dalam membentuk keputusan dasar pada masa akan datang.

Kata kunci: mikroplastik, pencemar baharu, kemunculan, kaedah analisis, degradasi

Introduction

Microplastics (MPs) are scientifically defined as plastic fragments with sizes of less than 5 mm and are of different shapes, sizes, and polymer compositions [1]. MPs are further classified into two, which are primary and secondary MPs [2]. Primary MPs, including polyethylene (PE), polypropylene (PP), and polystyrene (PS) particles, are derived from many different sources [3], while secondary MPs originate through physical, chemical, and biological processes that lead to the breakdown of plastic debris [4, 5]. Researchers estimate that ten million metric tons of plastic waste and MPs enter the oceans every year and project that the total amount of plastic waste circulating the world's oceans will be 150 million tons by 2025 [6]. Malaysia has one of the largest plastic production industries globally comprising more than 1,300 plastic manufacturers. In 2016, Malaysia exported resin products with a total value of RM30 billion Malaysian Ringgits (RM) to plastic manufacturers worldwide [7]. The plastic industry in Malaysia is segmented into seven main sectors including agriculture, household products, packaging, construction, electronics, automotive, and others such as furniture and medical devices [7]. In Asia, the percentage of plastic in solid wastes generated in Malaysia ranks second after the Philippines [8]. In 2020, roughly 148,000 tons of plastic were used for food packaging in Malaysia [9]. Based on the Worldwide Fund (WWF) report, Malaysians are among the biggest per-capita plastic packaging users in South-East Asia and the region is responsible for more than half of the plastic litter in the world's oceans. It is reported too, that Malaysia produced 0.94 million tons of improperly disposed of plastic waste, of which 0.14 to 0.37 million tonnes may have potentially leaked into the ocean [10]. The country was thus, ranked the eighth-worst country responsible for mismanagement of plastic waste and third in the list of countries contributing most to marine plastic pollution [11].

MPs are ubiquitous, highly stable, have a long endurance, break up readily and have the potential to leach toxic plastic chemical additives. MPs can adsorb a variety of persistent organic environmental pollutants, thereby enhancing their bioavailability, toxicity, and dispersion [12, 13]. Then, it will eventually penetrate the food chain and might cause health problems such as endocrine disruption and gut inflammation when ingested by humans and other organisms [10, 14, 15]. Given the significant threat to posed by MPs pollution in Malaysia, this review aims to examine the updated status and challenges of MPs in Malaysia by discussing aspects such as MPs occurrence and distribution, uptake by aquatic organisms, degradation, effects on human health, and analytical methodologies for detection and analysis up to the year 2024. It is hoped that readers and researchers will benefit from this review by gaining a better understanding of MPs and by developing appropriate strategies for the management, mitigation, removal, and analysis of MPs.

Understanding MPs: Properties and Distribution

To effectively tackle this issue, a comprehensive understanding of MPs is crucial. This section will delve into the physical, chemical, and biological properties of MPs, exploring factors such as size, shape, polymer composition, and their potential for interaction with environmental pollutants. Following this, we will examine the abundance and distribution of MPs in Malaysia, focusing on data from various environmental compartments (e.g., rivers, coastal waters, and sediments).

Physical, chemical, and biological properties of MPs

Due to the degradation of MPs through processes such as oxidation, solar exposure, biofilm growth, and thermal aging [2], different physical properties of MPs have been reported [16, 17]. The degradation process breaks the particles into micro and nano-sized particles [17, 18], and facilitates their entry into living systems [19]. The shape of these polymeric particles plays a significant role in their interactions with biological systems in the aquatic environment. A study reported by Au et al. in 2015 [20] revealed that the toxic effect of polypropylene fibers on the amphipod *Hyalella azteca* was influenced by the particle shape. Additionally, the surface area of the MPs can be correlated to particle size. The alterations in the surface area of MPs particles are closely linked to their size, with surface ablation attributed to particle fragmentation [21]. Examples of shapes of MPs include microfibers, pellets, and fragments. The size can be whittled down by repeated filtration to provide the finest particles, the density can be from high to low density, colour can be varied, and

the fragmentation increases the surface area gradually.

In addition, MPs can exist in amorphous or semi-crystalline form [22]. The most common MPs polymer types found in the aquatic environment are polyethylene (25%), polyethylene terephthalate (16.5%), polyamide (12%), polypropylene (14%), polystyrene (8.5%), polyvinyl alcohol (6%), and polyvinyl chloride (2%) [15], [23], [24]. Polyethylene and polypropylene are semi-crystalline polymers, while polyvinyl chloride, polystyrene, and polyethylene terephthalate are amorphous polymers [21]. In general, plastic particles are resistant to microbial attack due to their high crystalline nature, thus posing challenges for the biodegradation process [25]. However, recent research has shown that various microorganisms, including bacteria and fungi, can contribute to the degradation of plastics [26, 27]. Other factors such as the soil conditions, the type of organisms involved, pre-treatment methods, polymer characteristics (e.g., mobility, toxicity, crystallinity, molecular weight, functional groups, substituents, and the presence of plasticizers or additives), as well as both biotic and abiotic conditions, are also vital factors in the biodegradation process [28].

Abundance and distribution of MPs in Malaysia

Examining the prevalence of microplastic pollution requires two-part approach where understanding both its abundance, which reflects the quantity of particles present, and its distribution, revealing the spatial extent and variability across various ecosystems. Learning the abundance and distribution of MPs is especially crucial for researchers to understand the sources and transportation pathways of these pollutants. Data of MPs abundance have yet to cover all waterway and terrestrial environments in Malaysia (Figure 1). However, the growing research is providing valuable insights. It is also important to note that the reported data are resulted from different methods and some studies reported the data with different unit.

As presented in Table 1, MPs are detected in estuarine, beaches, marine and freshwaters, coastal and offshore, agricultural soil, and air in various states of Malaysia. Sediments act as a sink for MPs, leading to higher concentrations compared to water. Therefore, many studies reported MPs abundance is higher in sediment than in surface water for Seberang Prai, Pulau Pinang and Setia Wetland, Terengganu (4000 pcs/kg and 5.97 items/g respectively). While in surface water, Seberang Prai was reported to have the highest abundance of MPs (1407 pcs/L) in Malaysia. MPs are detected higher in abundance in Seberang Prai than in Kuala Muda and Balik Pulau because of industrialization and urbanization intensity of the area. MPs have also been detected in agricultural soil in the Klang Valley, with concentrations ranging from 1.5-6.0 particles/kg. This suggests agricultural activities as a potential source of MPs pollution in soil [29]. Compared to other Asian countries, Malaysia has a lower MPs abundance in soil than China but higher than Bangladesh. Finally, only a few studies have measured MPs in Malaysian air, covering locations like Kuala Lumpur, Bangi, and Terengganu. Perlis was reported to have higher airborne MPs concentrations than Kuala Lumpur, possibly due to fewer buildings [30].

The scarcity of data, particularly regarding geographic coverage, restricts our complete understanding of the national picture of MPs pollution in Malaysia. While existing studies provide valuable insights, further research employing standardized methodologies is crucial. This research should encompass diverse environmental compartments (e.g., rivers, coastlines, remote areas) to obtain a more comprehensive understanding of MPs abundance and distribution. This information is essential for assessing the potential ecological and human health risks associated with MPs pollution in Malaysia.

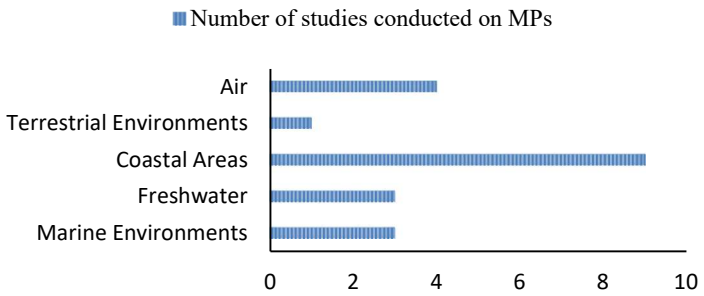


Figure 1. Number of studies conducted for abundance of MPs in Malaysia

Table 1. Abundance of MPs in Environmental Compartment in Malaysia

State	Location	Sample Type	Microplastics Concentration	Reference
Perlis	Timah Tasoh, Perlis	Air	983 ± 146 particles/m²/d	[30]
Pulau Pinang	Seberang Perai	Surface water	1407 ± 124.265 pcs/L	[31]
		Coastal bottom sediment	350 ± 25.892 pcs/kg	
		Estuarine sediment	4000 ± 29.174 pcs/kg	
	Penaga	Surface water	358 ± 50.991 pcs/L	
		Coastal bottom sediment	18.070 pcs/kg	
		Estuarine sediment	940 ± 15.773 pcs/kg	
	Balik Pulau	Surface water	273 ± 24.943 pcs/L	
		Coastal bottom sediment	255 ± 22.368 pcs/kg	
	Kuala Muda	Surface water	201 ± 21.214 pcs/L	
		Coastal bottom sediment	290 ± 24.505 pcs/kg	
		Estuarine sediment	430 ± 7.234 pcs/kg	
Selangor/Kuala Lumpur	Klang River estuary	Surface water	2.47 particles/L	[32]
	Langat River	Surface water	1464.8 items/L	[33]
	Sementa Man grove Area, Kapar	Sediment	-	[34]
	Bangi	Air	340 ± 30 MPs/m²/day	[35]
	Klang Valley	Soil	1.5–6.0 particles/kg	[29]
	Univerisit Teknologi Malaysia (Kuala Lumpur)	Air	97 - 775 particles/m² /day	[36]
Negeri Sembilan	Port Dickson	Surface water	4.65 particles/L	[37]
Johor	Skudai River	Surface water	360±60 particles/ per kg	[38]
	Tebrau River	Surface water	640±80 particles/ per kg	[38]
Pahang	Kuantan Port	Surface water	0.14–0.15 pcs/L	[39]
Kelantan	Kelantan Bay	Sediment	-	[40]
	Kelantan River	Surface water	179.6 items/L	[33]
Terengganu	Kuala Nerus	Surface water	0.13–0.69 pcs/L	[39]
	Terengganu estuary	Surface water	1687 particles/ m^{-3} and 1900 particles/ m^{-3} in estuary and offshore, respectively	[41]
	Sungai Dungun	Surface water	102.8 items/m^3	[42]
	Pulau Perhentian	Surface water	588.33 ± 111.77 items/L	[43]
	Pulau Redang	Surface water	314.67 ± 58.08 items/L	[43]
	Pulau Kapas	Surface water	359.8 ± 87.70 items/L	[43]

	Pulau Tenggol	Surface water	294.33 ± 101.64 items/L	[43]
	Setiu Wetland	Surface water	0.36 items/L	[44]
		Sediment	5.97 items/g	[44]
	Chagar Hutang	Air	274 ± 95 MPs/m^2/day	[35]
	Coastal area	Air	5476 ± 3796 MPs/m^2/ day	[45]
Sarawak	Miri River Estuary	Surface water	10.7 - 14.3 particles /L	[46]
		Sediment	283.7 - 456.2 particles/kg	[46]
	Miri Coast, NW Borneo	Sediment	83 - 327 particles/90g	[47]
	Pasir Pandak Beach	Sediment	60 - 328 items/m^2	
Sabah	Tanjung Aru Beach, Kota Kinabalu	Sediment	857 MPs/kg	[48]
	UMS ODEC Beach, Kota Kinabalu	Sediment	160 MPs/kg	[48]

Impacts of MPs Pollution

The presence of microplastics (MPs) in the environment poses significant threats to both aquatic ecosystems and human health. This section will explore the potential consequences of MPs pollution, focusing on two key areas, which are the uptake of aquatic organism and potential human health risks.

Uptake by aquatic organisms

Studies have shown that MPs have made their way into a range of organisms living in Malaysia's aquatic environments, including seafood. These minute plastic particles are ingested by organisms through both direct consumption and indirect trophic transfer mechanisms [1]. Planktonic creatures, larvae, invertebrates, and fish have all been found to have ingested MPs in Malaysian waters [1]. This alarming trend extends to commercial fish species in Malaysia, raising potential health risks for consumers [10].

The contamination of seafood with MPs poses significant threats to both food security and human health. Studies have revealed that commercially valuable fish and shellfish species are affected by MPs contamination [49]. For instance, an investigation discovered that 80% of sampled *Decapterus muroadsi* fish had ingested MPs resembling their natural prey, indicating a pathway for these particles to enter the human food chain. The presence of MPs in seafood is therefore a cause for concern regarding its potential health implications for consumers.

Beyond seafood, MPs have also been identified in other organisms inhabiting Malaysia's aquatic environments. A study in the Terengganu estuary and offshore waters of Malaysia detected MPs in seawater and zooplankton [41]. This widespread presence underscores the extensive contamination of MPs within Malaysia's marine ecosystem. Another study reported that marine species such as *Scapharca cornea*, cage cultured and wild *Lates calcarifer*, *Namalycastis sp*., and zooplankton have ingested MPs [50]. This suggests that MPs are found in Malaysia's aquatic environment and are being ingested by various wildlife species.

A study delved into the presence of MPs within commercially harvested marine fish off the northwestern coast of Malaysia's Peninsular region [51]. The study unveiled the presence of MPs in the digestive tracts of four distinct species of commercial marine fish: *Atule mate, Crenimugil seheli, Sardinella fimbriata*, and *Rastrelliger brachysoma*. This observation underscores

the potential risk of MPs infiltrating the human food chain through the consumption of contaminated fish. Another specific example of MPs ingestion by animals in Malaysia is the study conducted by Ratnam and Mohd Zanuri [52]. They found that blood cockles (*Tegillarca granosa*), a commonly consumed seafood in Malaysia, were contaminated with MPs in Kuala Juru, Pulau Pinang. This contamination poses a threat to seafood safety and potentially jeopardizes human health due to the potential toxicity of MPs.

Another study focused on the contamination of water bodies in Malaysia and its impact on marine organisms intended for human consumption [54]. The study found that commercial fish in seafood markets and abiotic sea products such as salts were contaminated with MPs. Marine organisms, including zooplankton, mussels, oysters, shrimp, and fish, were found to ingest MPs, further contributing to their presence in the human food chain. Efforts are underway to address the issue of MPs pollution in Malaysia. One study explored the possibility of polypropylene MPs degradation by bacteria isolated from Malaysian mangrove ecosystems [54]. Encouragingly, the study identified specific bacterial strains capable of breaking down polypropylene MPs, suggesting biodegradation as a potential remediation strategy.

The existence of MPs in seafood items presents challenges when assessing their health effects. The proportion of fiber-type MPs varies among different food items, and there are disparities between MPs levels in food and the adverse outcomes observed in animal studies [55]. Therefore, further research is imperative to gain a more comprehensive understanding of the potential health risks associated with consuming MPs in seafood [51, 59].

Effects of MPs exposure to human health

The potential health risks associated with MPs exposure in human is still under investigations. However, existing research suggested several possible pathways of exposure and potential health effects relevant to Malaysia. The primary route suggested is ingestion, often through the consumption of seafood as suggested earlier. Inadequate wastewater treatment facilities may also contribute to MPs contamination in drinking water (ingestion route) [56]. Exposure through inhalation route can occur when airborne MPs are respired, and dermal exposure is possible through personal care products and clothing containing MPs. The findings from cellular and animal experiments have demonstrated that MPs can impact various systems within the human body, including the digestive, respiratory, endocrine, reproductive, immune, and nervous systems.

Initially, when MPs are ingested, they can affect the digestive system, which may result in inflammation and subsequent gastrointestinal symptoms. These symptoms include abdominal pain, bloating, and alterations in bowel habits. MPs can also disrupt the balance of beneficial and harmful bacteria in the intestinal microbiome, contributing to gastrointestinal symptoms [57, 58]. Furthermore, MPs can serve as carriers for environmental toxic substances, such as bisphenol A (BPA), which can be absorbed into the body and cause health issues related to the endocrine and reproductive systems. Recent research even detected MPs in the placentas of pregnant women, emphasizing the need for further study on potential adverse effects [59].

In addition to their physical effects on the digestive system, MPs can also cause chemical toxicity by absorbing and accumulating environmental toxins such as heavy metals and polycyclic aromatic hydrocarbons [60]. These toxins can enter the body when MPs are ingested, leading to various gastrointestinal symptoms like nausea, vomiting, and abdominal pain. Both in vitro experiments with human cells and *in vivo* data from mice suggest that MPs can elicit adverse health effects, primarily through mechanisms involving inflammation, oxidative stress (resulting in increased production of reactive oxygen species), disturbances in lipid metabolism, gut microbiota imbalances, and neurotoxicity. For instance, exposure to certain MPs has been shown to increase the expression of inflammatory genes like Interleukin 6 (IL-6) and interleukin 8 (IL-8) in human cells [61]. Animal experiments have shown that MPs can disturb lipid metabolism in the liver, increase oxidative stress, affect acetylcholine esterase activity, and finally lead to intestinal microbiota

imbalances. Furthermore, exposure to MPs can exacerbate lipid-metabolism disturbances and inflammatory responses, especially in diabetic mice [62].

Concerning the immune system, accumulated exposure to MPs has been observed to induce chronic inflammation and homeostasis changes in animal experiments [63]. In addition, studies involving human lung cells have shown that MPs can activate innate immunity by regulating the expression of genes and proteins involved in the immune response [61]. Further research is required to fully understand the negative effects of MPs on the human immune system. Regarding the respiratory system, inhalation of MPs can lead to oxidative stress in the airways and lungs, resulting in respiratory symptoms such as coughing, sneezing, shortness of breath, and fatigue. Recent research has even linked nano-sized plastics to mitochondrial damage in human respiratory cells [64]. Finally, there is neurotoxicity induced by MPs has been reported in animal experiments, with findings showing that exposure to MPs can accumulate in the central nervous system, causing microglia activation and neuron damage [65]. This can lead to cognitive dysfunction, locomotor function changes and altered anticholinesterase activity in animals.

In conclusion, MPs have the potential to affect numerous systems in the human body. Even though many of the studies have been conducted on animals, further research is required to completely understand the degree of their effects and the mechanisms involved in humans. A complete understanding of these hazards and their long-term implications is still developing.

Analytical methodologies for determination of MPs

Effectively addressing MPs pollution requires reliable methods for their detection and analysis. This section will explore the various analytical methodologies currently employed to determine the presence and abundance of MPs in the environmental compartment, aquatic animal, and consumer products in Malaysia. Generally, the experimental design of MPs detection usually comprises of sample collection, sample preparation, sorting (physical characterization) and chemical characterization (Figure 2).

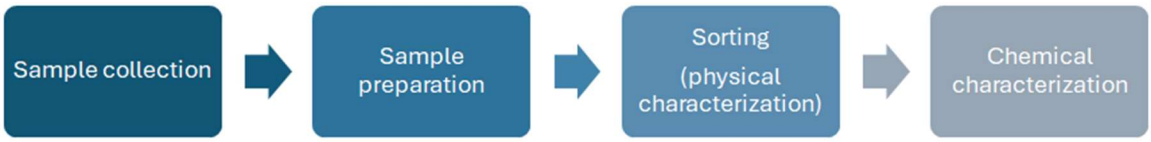


Figure 2. The general flow of MPs particles analysis

Sample collection

The technique to collect sample for MPs analysis in Malaysia depends on the type of sample itself and the purpose of the analysis (Table 2). For environment sample like marine water and sediment, the purpose was mostly to characterize and quantify the abundance of MPs particles in the environment or area selected. For instance, to quantify the extent of MPs in mangrove forest in Selangor, triplicate samples of sediment were collected from the surface of soil until 5 cm depth based on 50 cm x 50 cm quadrant [34]. Sampling quadrant was also adapted for sediment sample collection in Kota Kinabalu [48] and Pasir Pandak Beach [66]. For atmospheric MPs deposition, sample collection applied passive sampling technique[30, 45].

MPs are prevalent in marine environments and have been reported in commercial marine fish and other aquatic organism worldwide. For aquatic organism sample, like, bivalve or fishes, the purpose of the study is to investigate the digestion of those animals of the MPs particles polluted in the sampling area. Sample collection will either from the local area/market [67, 68, 69] or random collection by hand [70]. Processing of those animals will be dissection to obtain the guts, gills, and tissue. Sometimes, the whole organism was processed for the analysis of MPs particles itself. To analyse MPs particles in items like bottled water, fish meal products and personal products, these products were sourced from local market [71-74].

Table 2. Sample collection for different matrices in MPs particle analysis in Malaysia

Matrix	Sample Collection and Process
Surface water	Collection using stainless steel bucket and sampler [39, 46]
	Collection using water grab sampler [40]
Sediment	Collection using shovel [34]
	Collection using box corer [38]
	Collection using Ekman Grab sampler [40, 46]
	Collection using scoop [48]
Fishes	Caught at coordinated location [75]
	Purchases from local market [68]
Zooplankton	Collection by water pump [41]
Bivalve	Handpicked during low tide [70]
Sea cucumber	Random collection [76]
Products (dried fish, salt, canned sardine, bottled water, and fish meal product)	Purchased from Malaysian market [73, 74, 77, 78]
Human colectomy	Collection from patients who are scheduled to do colectomy. Specimen harvested after surgery [79]
Polychaete worm	Collection by hand [80]
Atmospheric deposition (air)	Collection through passive sampling in 24 h using a glass petri dish containing a glass microfibre filter paper [30, 45]

Sample preparation

After collection, the preparation of samples involves extraction of MPs from the sample matrices. Extraction of MPs includes manual sorting, density separation, filtration, and digestion method. Digestion process using acid or enzyme is needed for organic materials to dissolve. Digestion process is applied for matrices like fish, polychaete worm, and canned and dried fish. After digestion, some environment matrices apply density separation in sorting out the MPs particles. Density separation is applied through salt addition like sodium chloride (NaCl) or sodium iodide (NaI) or zinc chloride ($ZnCl_2$) [81]. Then, particles will be filtered by sieving through size exclusion or membrane filter. Filtered and separated MPs particles will be then characterized by physical and chemical characteristics.

Sorting (physical characterization)

After extraction, the isolated MPs are set for characterization to determine the particle size, shape, colour, surface texture and other characteristics to discriminate MPs from other particles (physical characterization). Then, this process is usually followed by identification of chemical composition such as the functional groups, molecular weight and structure, and degree of polymerization of polymers in MPs [82].

The goal of physical characterization is to do preliminary analysis to determine shape, size, and morphology of MPs collected [83]. In other words, the filtered MPs particles will be screened and distinguished from non-plastic before chemical characterization. Some main physical characterization techniques are visual inspection, laser diffraction particle size analysis and dynamic light scattering. For MPs analysis in Malaysia in this paper, the methods employed for physical characterization are visual analysis (Table 3). Visual analysis technique in physical characterization is applied for most type of samples such as environment (sediment and water), animals, (fishes and bivalve) and products (canned food, packed food, and bottled water) and personal products. The process of visual analysis consists of observing the MPs particles with naked eyes or with microscopes. Then, the particles will be classified and counted based on the shape, colour, and size [83]. Analysis with naked eyes only applies for greater size range of MPs particles (1-5 mm). On the other hand, microscope images that can provide the shape and the quantity of MPs particles. This technique

was seen used on MPs analysis 1-5 μm in size for all samples in Table 2. On the other hand, those MPs particles that are lacking distinct colours and shapes are sorted using SEM. In studies analysing MPs in dried fish, canned sardines, marine fishes, and human colectomy specimens [79], SEM was combined with EDX. This common approach provides elemental analysis, enabling the differentiation of inorganic MPs particles.

Chemical characterization

Determination of the composition of the detected and extracted MPs is crucial to assist the establishment of the methods for treating MPs pollution [84]. For MPs analyses in Malaysia, Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, and thermal analysis (Table 3).

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) provides information about the functional groups present for MPs that are more than 20 μm in size [81]. It obtains chemical information of chemical bonds and functional group of samples through vibrational spectroscopy technique. FTIR is a non-invasive analysis that requires simple sample pre-treatment process. Also, it is also less vulnerable to interference from autofluorescence originating from plastic additives components [82]. Currently, FTIR is widely used for chemical characteristics of MPs analysis in Malaysia.

There are three analysis techniques of FTIR which are specular reflection, transmission and attenuated total reflection (ATR). The selection of the right techniques depends on the properties of the sample. ATR mode, for example, will be selected for samples that are thick or strong infra-red (IR) absorbent. MPs have been identified and characterized by ATR- FTIR for marine water [39], sediment samples [34, 39, 46, 66, 85] and personal products collected in Selangor [71]. Micro-FTIR is also growing technique in Malaysia for analysing MPs as small as 10 μm. Researchers have used it to study MPs not only in deposited atmospheric dusts, but also in human tissue samples (from colon surgery) and in zooplankton samples.

Raman spectroscopy

Raman spectroscopy is one of the widely used methods for identifying chemical composition of MPs. It works by analysing the frequency shift of light scattered from a sample due to the Raman effect. The frequency shift can determine the sample's vibrational modes and identify the chemical substances present. Raman spectroscopy is widely accepted for its many advantages including minimal sample damage, requirement of small sample sizes, potential for high throughput screening, and environmental friendliness (as reported by Huang et al. [82]).

In contrast to FTIR, Raman spectroscopy faces limitations when it comes to detecting for samples with fluorescence [82] . Besides, the Raman spectra generated by plastic additive within MPs and contaminants adhering to the particle surfaces may overlap with the Raman spectra of the plastic polymers itself. This interferes the function of Raman spectroscopy in identification of the MPs. A. Karami et al. [67] developed a time effective and low-cost protocol for sample preparation of MPs analysis in fish while protecting the integrity of the plastic polymers. Raman spectroscopy has been shown to be a reliable technique to identify MPs in dried fish, salt, and tropical shark species [68, 77, 86]. Raman spectroscopy can detect MPs greater than 10 μm in size, whereas micro-Raman spectroscopy offers higher resolutions, enabling detection up to 1 μm [83]. A. Karami et al. has successfully used micro-Raman to detect MPs in canned sardine and sprat products with size ranging from 190 to 3800 μm. Karbalei et al. detected MPs in commercial fish meal with particle size from 149-7800 μm [73].

Thermal analysis

Thermal analysis is a technique to study the functional change of temperature and time of a material [82]. Pyrolysis-GCMS (Py-GC/MS) is one of the classical methods for thermal analysis that was used for analysis of MPs in Polychaete worms in Setiu Wetlands [80]. Analysis with Py-GC/MS using a selective ion monitoring mode detected pyrolytic products and fragment ions associated with various polymers, including polyvinyl chloride, polypropylene, polyethylene, polyethylene terephthalate, polyamide,

and polymethylmethacrylate. The study demonstrated the valuable application of Py-GC/MS techniques for monitoring MPs, especially when dealing with analytical sample in large quantities. Usage of Py-GCMS must be complemented with optical techniques due to its inability to determine the number and other physical characteristic of the MPs extracted.

While the current analytical methods offer valuable insights, advancements in MPs analysis are constantly evolving. These advancements hold great promise for improving our understanding of MPs pollution in Malaysia. Currently, visual counting is a common method for quantifying MPs. However, this technique can be time-consuming and prone to human error. Emerging techniques, such as flow cytometry and microfluidics, offer the potential for automated, high-throughput analysis, allowing for more accurate quantification of MPs abundance in environmental samples [87, 88]. At the same time, there is a crucial need to identify the source of the MPs pollution for understanding their pathways into the environment and to develop effective mitigation plan. Advanced spectroscopic techniques, such as Raman spectroscopy coupled with multivariate analysis, can potentially be used to fingerprint the specific types of plastic present in MPs [89]. This information can help to identify the sources of plastic pollution, such as specific industries or consumer products.

As MPs research in Malaysia continues to grow, it is essential to establish standardized protocols for sample collection, preparation, and analysis across different research groups, as previously emphasized. This standardization will ensure data consistency and facilitate comparisons between studies conducted in various locations and by different researchers.

In conclusion, the analytical methods employed in Malaysia provide valuable insights into the presence and abundance of MPs in the environment. However, continuous advancements in MPs analysis offer exciting possibilities for the future. Techniques like flow cytometry and microfluidics hold promise for more accurate and automated MPs quantification, while advanced spectroscopy with chemometrics can help pinpoint the sources of plastic pollution. Moving forward, efforts to standardize sampling and analysis protocols across research groups in Malaysia are crucial for strengthening the nation's expertise in MPs analysis. By embracing these advancements and fostering a collaborative research environment, Malaysia can become a leader in tackling the challenge of MPs pollution.

Current challenges and data gaps

A report published by Minister of Science, Technology and Innovation (MOSTI) has revealed a roadmap plan to achieve towards zero single-use plastics in 2030 [7]. This plan highlighted the significant challenges hindering Malaysia’s efforts to achieve zero single-plastic use by 2030. These challenges include:

a) Limited public awareness: Many Malaysians lack sufficient knowledge about the environmental impact of single-use plastics and the benefits of alternatives.
b) Low recycling rate: The current recycling infrastructure struggles to handle the volume of plastic waste generated, leading to improper disposal and environmental pollution.
c) Cost factor: Biodegradable alternatives to single-use plastics are often more expensive, making it difficult for consumers and businesses to adopt them readily.
d) Enforcement difficulties: Effectively enforcing regulations against single-use plastics can be challenging due to limited resources and monitoring capabilities.
e) Waste management needs: Improvements are needed throughout the plastic waste management system, from collection and sorting to recycling and treatment facilities.

While these challenges are well-defined, several critical data gaps hinder the development of the targeted solutions. Table 4 described some of the data gaps that need to be addressed.

Table 3. Summary of common methods used in extraction, physical and chemical characterization of MPs

Matrix	Extraction Method	Physical Characterization Method	Analytical Instrument	References
Sediment	Density separation	SEM, spectroscopy, microscopy	FTIR, ATR-FTIR, μ-FTIR	[38, 46, 48]
Water	Filtration	SEM, microscopy	FTIR, ATR- FTIR, μ-FTIR	[33, 39, 42, 43]
Soil	Density separation followed by filtration	Microscope	FTIR	[29]
Air	Filtration	Stereomicroscope, selective fluorescent staining using Nile Red	μ-FTIR, ATR-FTIR	[35, 45]
Biota (tissues)	Digestion (chemical or enzymatic) followed by filtration	SEM, Stereomicroscope, FESEM-EDX	FTIR, μ-Raman spectroscopy, μ-FTIR, ATR-FTIR	[68, 69, 70]

Abbreviations: *FTIR=Fourier Transform Infrared Spectroscopy; μ-FTIR=Micro-FTIR; ATR-FTIR= Attenuated total reflectance-FTIR; FESEM-EDX=Field Emission Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy; SEM=Scanning Electron Microscope*

Table 4. Data gap in MPs pollution in Malaysia

Area of Data Need	Specific Data Need	Importance for Addressing MPs Pollution
Sources of MPs pollution	Types and quantities of MPs from various industries	To identify major contributors of MPs to the environment
Distribution of MPs	Research on MPs abundance in rivers, coastal waters, and offshore environments throughout Malaysia should continue to cover the rest of the area which are not covered	To understand the spread and accumulation of MPs in different environments.
	Research on atmospheric MPs deposition should be further conducted	To understand the spread and accumulation of MPs in different environments
Impacts of MPs pollution	Potential pathways of microplastic ingestion (food, water)	To develop strategies to mitigate these risks
	Epidemiological studies on health effects of microplastic exposure.	To evaluate potential human health risks from microplastic contamination
	The presence of MPs in seafood and other products should be further examined covering all areas and types	To develop strategies to mitigate these risks

Conclusion

Microplastic pollution has emerged as a complex environmental challenge in Malaysia, with significant implications for both ecological health and human well-being. This review has examined the current understanding of MPs pollution in Malaysia, encompassing its abundance, potential impacts on biota and human health, and the analytical methods employed for its detection. The research conducted so far highlights the widespread presence of MPs in various environmental matrices in Malaysia, raising concerns about their potential ecological and human health risks. The potential for MPs to accumulate in the food chain and their ability to adsorb harmful pollutants necessitates further investigation t fully understand their long-term effects. Moving forward, addressing MPs pollution in Malaysia requires a comprehensive multi-pronged approach. This includes promoting the development and adoption of sustainable plastic production practices, coupled with effective waste management strategies to minimize plastic waste generation and improper disposal. Additionally, ongoing research efforts are crucial to develop efficient technologies for MPs detection, removal, and potential biodegradation. Public awareness campaigns are also essential to promote responsible plastic consumption and waste disposal practices among Malaysians. By implementing a combination of these strategies, Malaysia can play a leading role in combating MPs pollution and preserving its rich natural environment for future generations.

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SYNTHESIS AND MOLECULAR DOCKING SIMULATION OF CINNAMIC ACIDS DERIVATIVES AGAINSTS DENV2 NS2B/NS3

(Sintesis dan Simulasi Pengedokan Terbitan Asid Sinamik Terhadap DENV2 NS2B/NS3)

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Abstract

Dengue virus (DENV) is known as one of the serious global threats to human health since its emergence in the past few decades. However, clinically approved antiviral drugs are still not available for DENV treatment. The increased number of cases reported yearly had led to the on-going research in finding an effective drug for dengue. Cinnamic acid is a naturally occurring compound which consists of α, β-unsaturated carboxylic acid and is known for its wide pharmacological properties. Cinnamic acid derivatives such as caffeic acid are one of constituents in *Carica papaya* leaf which are used as a folk remedy for dengue. Recently, cinnamic acid was reported to show a potential against dengue virus. In this study, eight cinnamic acid derivatives were synthesized by incorporating cinnamic acid and amide moieties through the reaction of cinnamic acid with corresponding amines. All compounds were characterized by using ^{1}H and ^{13}C nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR), mass spectrometry (MS) and CHN elemental analysis. Then, the synthesized compounds were simulated for molecular docking to investigate their binding affinity with the Wichapong homology protein model crystal structure, DENV-2 NS2B/NS3pro. The lower the value of FEB of a compound, the higher its binding affinity towards protein. The in-silico study revealed that compounds **6b1-6b4**, with *tert*-butyl substituents had the highest binding affinity and good interaction with DENV-2 NS2B/NS3 serine protease. This might be due to the presence of a *tert*-butyl moiety in the structure, which could interact with amino acid residue of protease, thereby increasing the binding interaction between the ligand and the protein. Compound **6b2** showed the lowest FEB value despite having less H-bond interactions as compared to compound **6b3**. This was probably due to the presence of more non-covalent interaction and closer bond distance which helped to enhance its binding stability. In summary, this study showed that cinnamic acid could be a promising candidate for the development of antiviral drugs to target DENV.

Keywords: cinnamic acid, synthesis, anti-dengue virus, docking

Abstrak

Virus denggi (DENV) telah dikenali sebagai salah satu ancaman global yang serius terhadap kesihatan manusia setelah

kemunculannya beberapa dekad yang lalu. Walau bagaimanapun, masih belum ada ubat antiviral yang diluluskan secara klinikal bagi rawatan DENV. Peningkatan jumlah kes yang dilaporkan setiap tahun telah membawa kepada penyelidikan yang berterusan dalam pencarian ubat yang berkesan untuk rawatan denggi. Asid sinamik adalah bahan semulajadi yang mempunyai kumpulan asid karbosilik α, β-tak tepu dan dikenali dengan pelbagai sifat farmakologinya. Asid sinamik seperti asid kafeik merupakan antara komposisi yang terkandung di dalam daun *Carica papaya* yang digunakan secara tradisional untuk rawatan denggi. Kebelakangan ini, asid sinamik telah dilapor menunjukkan potensi terhadap DENV. Di dalam kajian ini, lapan terbitan asid sinamik telah disintesis dengan menggabungkan asid sinamik dan amida melalui tindak balas asid sinamik dengan amina yang bersesuaian. Semua sebatian dicirikan dengan menggunakan 1H and ^{13}C Resonans Magnetik Nuklear (RMN), Inframerah Transformasi Fourier (FTIR), spektrometri jisim (MS) dan analisis unsur CHN. Sebatian yang disintesis kemudiannya disimulasikan menerusi pengedokan molekul bagi mengkaji keafinan pengikatan mereka bersama struktur kristal homologi protein Wichapong, DENV-2 NS2B/NS3pro. Kajian *in siliko* menunjukkan bahawa sebatian **6b1-6b4**, yang mengandungi kumpulan penukargantian *tert*-butil menunjukkan tenaga pengikatan yang tinggi dan interaksi yang baik dengan DENV-2 NS2B/NS3 serine protease. Ini mungkin disebabkan oleh kehadiran *tert*-butil dalam strukturnya, yang boleh berinteraksi dengan asid amino protein, dengan itu meningkatkan interaksi pengikatan antara ligan dan protein. Semakin rendah nilai FEB sebatian, semakin kuat ikatannya dengan enzim. Sebatian **6b2** menunjukkan nilai FEB terendah walaupun ia mempunyai kurang interaksi ikatan H-bond berbanding dengan sebatian **6b3,** dan ini mungkin disebabkan oleh kehadiran interaksi non-kovalen yang banyak dan jarak ikatan yang berdekatan telah membantu meningkatkan kestabilan ikatannya. Secara keseluruhannya, kajian ini menunjukkan bahawa sebatian asid sinamik berpotensi untuk pembangunan ubat-ubatan antiviral bagi merawat DENV.

Kata kunci: asid sinamik, sintesis, anti-denggi, pengedokan

Introduction

Dengue virus (DENV) is the most significant virus in the *Flaviviridae* family with the highest morbidity and mortality rates and has been a serious global threat in the past few decades [1]. The latest statistics from the World Health Organization (WHO) have shown an increasing trend of DENV infection throughout Malaysia with 127,407 reported cases in 2019 [2]. Despite this alarming issue, no current effective vaccine or antiviral treatment is available for treating dengue patients. Prevention against DENV infection by large depends on mosquito vectors control as well as the use of a natural remedy [3, 4, 5]. This underscores an urgent need to discover and develop a novel antiviral agent that is safe and effective in controlling dengue infection.

Cinnamic acid has gained a lot of interest due to its unique structure with great potential for pharmacological properties. It is a group of aromatic carboxylic acids which appear naturally in the plant kingdom. Cinnamic acids occur in all green plants as well as the reproductive organs of flowering plants [6]. Cinnamic acids with varied substitutions on the aryl ring and their esters have been identified in natural bee products, including honey and propolis [7]. It is also readily available from coffee beans, tea, cocoa, apples, tomatoes and cereals [8]. It is a safe and extensive source of material due to its natural and low toxicity properties.

Cinnamic acid and its related molecules have a variety of biological activities, such as anticancer, antimalarial, and antimicrobial [9, 10, 11]. Additionally, cinnamic acid was also reported to be an effective antiviral drug against Zika virus (ZIKV) by inhibiting RNA-dependent RNA polymerase (RdRp) activity at the post-entry stage of ZIKV replication cycle [12]. Cinnamic acid was also reported to have excellent activity against *Tobacco mosaic virus* (TMV) and Hepatitis C virus (HCV) [13,14]. Despite their rich medicinal tradition and remarkable biological activities, the antiviral activity of cinnamic acid related molecules against DENV infection remained underutilised for several decades. To our knowledge, cinnamic acid derivatives were first evaluated for their anti-dengue activity and were reported by Rees and co-workers in 2008. Zosteric acid (*para*-sulfoxy-cinnamic acid), derived from Zostera Marina and CF238, was found to exhibit the process of virus entry. It was suggested to entrap the virus on the surface of the cell in a non-permissive binding mode and prevent the fusion process of the virus into the cell [15]. Recently, a new cinnamic acid, 3-(2-chlrophenyl) acrylic acid was synthesised based on the structural scaffold found in *Carica papaya*, in which the extract was used as a folk remedy for dengue [16]. It was

reported that the compound showed higher inhibition activities against DENV2 NS2B-NS3 protease as compared to quercetin [17]. Arylcyanoacrylamide, aminobenzamide and α-ketomide were also reported to have a significant inhibitory effect on dengue protease NS2b/NS3 [18, 19, 20]. Due to this, this study aimed to develop a dengue inhibitor by incorporating cinnamic acid with amide, which has been reported to show potential towards DENV.

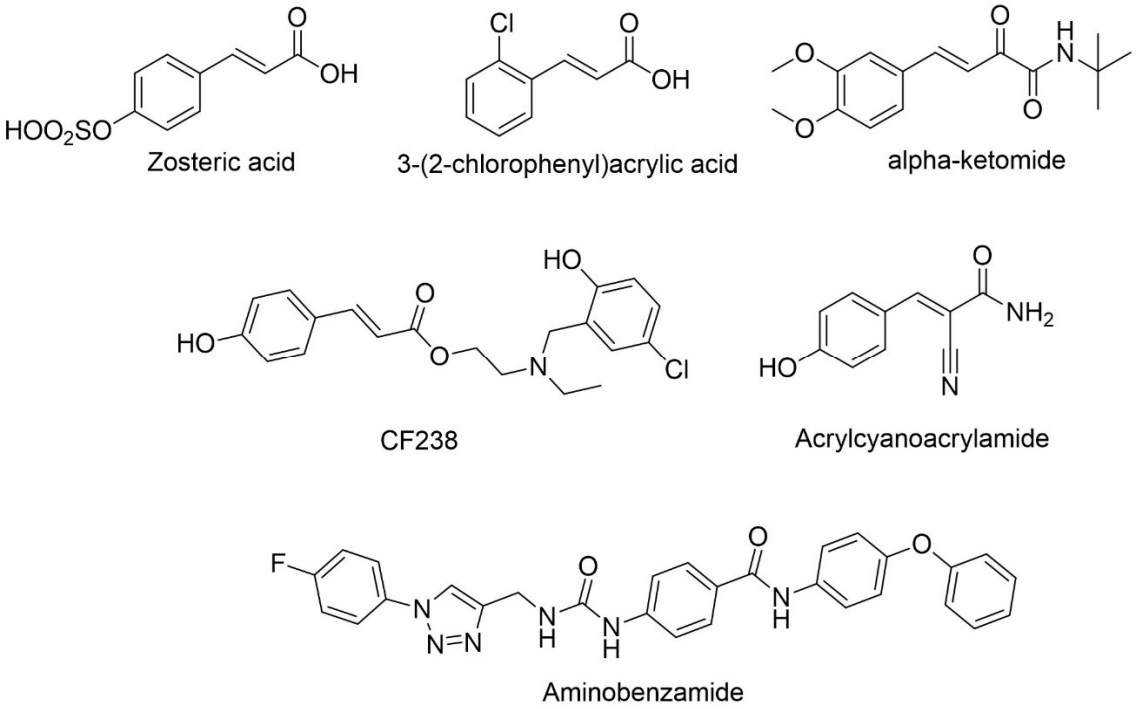


Figure 1. Structure of zosteric acid, CF238, 3-(2-chlorophenyl) acrylic acid, arylcyanoacrylamide, aminobenzamide and α-ketomide.

DENV is a single-stranded RNA genome which encodes a single open reading frame (5-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3) with three structural and seven non-structural proteins [21]. The N-terminal of NS3 is a trypsin-like serine protease, in which when combined with its co-factor (NS2B) it can cleave the viral polyprotein. Disruption of NS2B-NS3 protease inhibits viral replication [22]. This poses NS2B-NS3 protease of DENV-2 as a promising target for antiviral drug design [23]. The three catalytic triad amino acid residues present in NS3 serine proteases were His51, Asp75 and Ser135. The NS2B protease acted as an NS3 serine protease co-factor for an optimal catalytic activity [24]. The homology protein crystal structure generated by Wichapong et al. (2010) was used due to lack of DENV-2 NS2B/NS3 serine protease inhibitor-bound 3D structures. Moreover, some protease database structures were found to have missing amino acid residues [25].

In the selection of an effective medicine for patient, in addition to having a high biological activity, the targeted molecule must exhibit low toxicity and adhere to drug-like properties as outlined in Lipinski's Rule of Five. These properties, such as molecule size, hydrogen bonding characteristic and lipophilicity, play a crucial role in determine the permeability of molecule throughout the body [26]. Evaluation for these ADME (for absorption, distribution, metabolism, and excretion) parameter can be evaluated using SwissADME web tool [27].

In this study, eight cinnamic acid derivatives were synthesised, characterised, and subjected to molecular

docking simulations to investigate their binding interactions with the protein target of DENV-2 NS2B/NS3 protease. The drug-likeness parameter of compounds was calculated by using SwissADME.

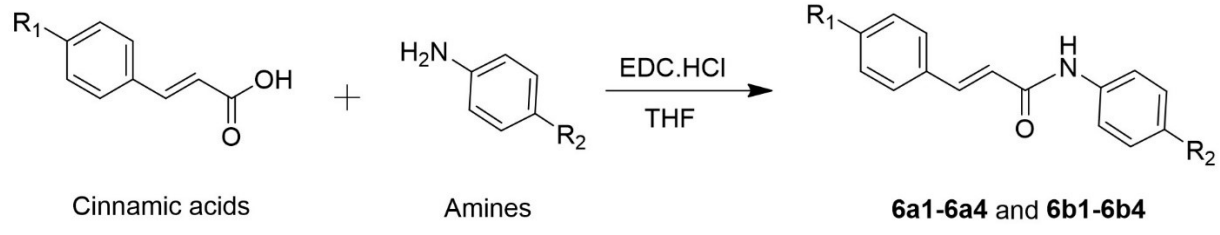
Materials and Methods

Chemicals and instrumentations

The chemicals and solvents used were purchased from Sigma-Aldrich Co., Acros Organics, QRec and Merck Chemical Co. (Reagent Grade) and were used without further purification. Thin-layer chromatography (TLC) was performed on a plastic-backed pre-coated with silica gel plates (Merck silica gel, 60 F254), in the solvent system of ratio Hexane:ethylacetate (7:3). Completion of the reaction was determined by the appearance of product and disappearance of reactant spots under UV-fluorescence (λ_{max}= 254 nm), respectively. All reactions were carried out in heat-dried glassware under dry nitrogen atmosphere by using a ballon, unless otherwise stated. All liquids transferred were conducted by using standard cannula techniques. All spectral data were obtained on the following instruments: Attenuated total reflected-infrared (ATR-IR) spectra were recorded by using Perkin Elmer 100 spectrometer model at a spectral range of 4000—400 cm^{-1}. ^{1}H (400 MHz) and ^{13}C (100 MHz) Nuclear magnetic resonance spectra were obtained on Bruker Avance II 400 spectrometer and were reported in unit ppm on the δ scale. NMR analyses used DMSO-d_6 and TMS solvents as the internal standards. The data were analyzed by using Bruker TopSpin software package. The chemical shift was internally referenced to solvent DMSO-d_6 (^{1}H δ 2.50; ^{13}C δ 39.5). The mass spectrometer experiments were performed by using LCMS Orbitrap Discovery (Thermo Scientific), electrospray (ES) negative mode and the samples were dissolved in methanol.

Preparation of cinnamic acid derivatives

The cinnamate-amide derivatives (**6a1-6a4** and **6b1-6b4**) were synthesized according to the reported literature procedure with slight modifications [28]. A substituted cinnamic acid (2.0 mmol) was added to 15 mL tetrahydrofuran (THF) and treated with 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC.HCl) (3.0 mmol, 0.575g) under a nitrogen atmosphere. The solution was stirred at 0 ˚C for 30 min, and 2.0 mmol of amine was slowly added within 15 min. Then, the solution was stirred overnight at room temperature under nitrogen atmosphere. The completion of the reaction was monitored using TLC (Hexane: ethyl acetate, 7:3). The solvent was removed under reduced pressure, and the residue was treated with water and extracted with ethyl acetate. The organic extract was washed with 5% HCl solution, 5% $NaHCO_3$ solution and brine. The organic layer was dried over $MgSO_4$, filtered, and then evaporated under reduced pressure. The reactions are illustrated in Scheme 1.



Scheme 1. Preparation of cinnamic acid amide derivatives (**6a1-6a4** and **6b1-6b4**)

3-(4-hydroxyphenyl)-N-4-phenylacrylamide (6a1)

Pale brownish powder (0.272g, 56.8%), m.p.156 ˚C (lit. 156 ˚C, [29]), compound **6a1** was prepared from the reaction of 4-hydroxycinnamic acid (0.33g, 2.0 mmol) with aniline (0.19g, 2.0 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (400 MHz, DMSO-d_6): δ_H ppm, 10.12 (1H, s, NH), 9.98 (1H, s, OH), 7.67-7.75 (2H, m, H-ar), 7.48-7.56 (3H, m, H-ar and CH), 7.29-7.38 (2H, m, H-ar), 7.06 (1H, t, *J*= 8.0 Hz, H-ar), 6.86 (2H, d, *J*= 12.0 Hz,), 6.66 (1H, d, *J*= 16.0 Hz, CH); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C ppm, 116.3 (2×CH-ar), 119.1 (=CH), 119.6 (2×CH-ar), 123.6 (CH-ar), 126.2 (Cq), 129.2 (2×CH-ar), 130.0 (2×CH-ar), 140.0 (Cq), 140.8 (=CH), 159.7 (C-O), 164.5 (C=O); FTIR (ATR, cm^{-1}): *v* 3317 (N-H, secondary amide), 3022 (O-H, phenol), 2922 (C-H, aromatic), 1651 (C=O), 1599 (N-H bend, secondary amide), 821 (C-H oop, aromatic); Found MS (ES, negative mode) $[M-H]^+$ 238.09 $C_{15}H_{12}NO_2$, requires 238.27. Anal. Calculated for $C_{15}H_{13}NO_2$: C, 75.30; H, 5.48; N, 5.85; Found: C, 73.55; H, 5.51; N, 5.24.

3-(4-hydroxyphenyl)-N-(4-methoxyphenyl) acrylamide (6a2)

Pale greenish-yellow powder (0.383g, 71.2%), m.p.167 ˚C, compound **6a2** was prepared from the reaction of 4-hydroxycinnamic acid (0.33g, 2.0 mmol) with 4-methoxyaniline (0.25g, 2 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (400 MHz. DMSO-d_6): δ_H ppm, 10.00 (1H, s, NH), 9.97 (1H, s, OH), 7.65 (2H, d, *J*= 8.0 Hz, H-ar), 7.53 (1H, s, H-ar), 7.46 (2H, d, *J*= 8.0 Hz, H-ar), 6.92 (2H, d, *J*= 8.0 Hz, H-ar), 6.85 (2H, d, *J*= 8.0 Hz, H-ar), 6.63 (1H, d, *J*= 16.0 Hz, CH), 3.74 (3H, s, OCH3); ^{13}C NMR (100 MHz. DMSO-d_6): δ_C ppm, 55.6 (OCH_3), 114.4 (2×CH-ar), 116.3 (2×CH-ar), 119.2 (=CH), 121.1 (2×CH-ar), 126.3 (Cq), 129.9 (2×CH-ar), 133.1 (Cq), 140.3 (=CH), 155.6 (C-O), 159.6 (C-O), 164.1 (C=O); FTIR (ATR, cm^{-1}): *v* 3328 (N-H, secondary amide), 3066 (OH, phenol), 2946 (C-H, aromatic), 1679 (C=O), 1598 (N-H bend, secondary amide), 821 (C-H oop, aromatic); Found MS (ES, negative mode) $[M-H]^+$ 268.10 $C_{16}H_{13}NO_3$, requires 268.30. Anal. Calculated for $C_{16}H_{15}NO_3$: C, 71.36; H, 5.61; N, 5.20; Found: C, 68.45.15; H, 5.79; N, 4.79.

3-(4-hydroxyphenyl)-N-(4-methylphenyl) acrylamide (6a3)

Yellowish brown powder (0.306g, 60.5%), m.p. 167 ˚C [29], compound **6a3** was prepared from the reaction of 4-hydroxycinnamic acid (0.33g, 2.0 mmol) with 4-methylaniline (0.21g, 2 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (400 MHz. DMSO-d6): δ_H ppm, 10.01 (1H, s, NH), 9.95 (1H, s, OH), 7.59 (2H, d, *J*= 8.0 Hz, H-ar), 7.48 (3H, t, *J*= 12.0 Hz, H-ar and CH), 7.13 (2H, d, *J*= 8.0 Hz, H-ar), 6.84 (2H, d, *J*= 8.0 Hz, H-ar), 6.62 (1H, d, *J*= 16.0 Hz, CH), 2.26 (3H, s, CH_3); ^{13}C NMR (100 MHz. DMSO-d_6): δ_C ppm, 20.9 (CH_3), 116.3 (2×CH-ar), 119.2 (=CH), 119.6 (2×CH-ar), 126.3 (Cq), 129.6 (2×CH-ar), 129.9 (2×CH-ar), 132.5 (Cq), 137.5 (Cq), 140.5 (=CH), 159.6 (C-O), 164.3 (C=O); FTIR (ATR, cm^{-1}): *v* 3250 (N-H, secondary amide), 3012 (O-H, phenol), 2920 (C-H, aromatic), 1653 (C=O), 1594 (N-H bend, secondary amide), 816 (C-H oop, aromatic); Found MS (ES, negative mode) $[M-H]^+$ 252.10 $C_{16}H_{14}NO_2$, requires 252.30. Anal. Calculated for $C_{16}H_{15}NO_2$: C, 75.87; H, 5.97; N, 5.53; Found: C, 68.45; H, 5.79; N, 4.79.

3-(4-hydroxyphenyl)-N-(4-chlorophenyl) acrylamide (6a4)

Yellow powder (0.352g, 64.0%), m.p. 187 ˚C [30], compound **6a4** was prepared from the reaction of 4-hydroxycinnamic acid (0.33g, 2.0 mmol) with 4-chloroaniline (0.26g, 2 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (400 MHz. DMSO-d6): δ_H ppm, 10.12 (1H, s, NH), 9.98 (1H, s, OH), 7.72 (2H, d, *J*= 7.9 Hz, H-ar), 7.48-7.56 (2H, m, H-ar), 7.33 (2H, t, *J*= 7.7, 8.1 Hz, H-ar), 7.05 (1H, t, *J*= 7.4, 7.3 Hz, H-ar), 6.85 (2H, d, *J*= 8.4 Hz, H-ar), 6.66 (1H, d, *J*= 16.0 Hz, CH), 3.47 (1H, s, OH); ^{13}C NMR (100 MHz. DMSO-d_6): δ_C ppm, 116.3 (2×CH-ar), 118.7 (2×CH-ar), 121.1 (2×CH-ar), 127.1 (Cq), 129.1 (2×CH-ar), 130.1 (2×CH-ar), 138.9 (Cq), 141.2 (=CH), 159.8 (C-O), 164.6 (C=O); FTIR (ATR, cm^{-1}): *v* 3367 (N-H, secondary amide), 3112 (O-H, phenol), 2925 (C-H, aromatic), 1662 (C=O), 1594 (N-H bend, secondary amide), 826 (C-H oop, aromatic); Found MS (ES, negative mode) $[M-H]^+$ 272.05 $C_{15}H_{11}ClNO_2$, requires 272.72. Anal. Calculated for $C_{15}H_{12}ClNO_2$: C, 65.82; H, 4.42; N, 5.12; Found: C, 65.72; H, 4.83; N, 4.75.

3-(4-*tert*-butylphenyl)-N-4-phenylacrylamide (6b1)

Pale yellow powder (0.515g, 92%), m.p.142 ˚C [31], compound **6b1** was prepared from the reaction of 4-*tert*-butylcinnamic acid (0.41g, 2.0 mmol) with aniline (0.19g, 2.0 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (400 MHz. DMSO-d_6): δ_H ppm, 10.23 (1H, s, NH), 7.74 (2H, d, *J*= 8.0 Hz, H-ar), 7.57 (3H, m, H-ar and CH), 7.46 (2H, d, *J*= 12.0 Hz), 7.34 (2H, t, *J*= 8.0 Hz, H-ar), 7.07 (1H, t, *J*= 8.0 Hz, H-ar), 6.84 (1H, d, *J*= 16.0 Hz, CH), 1.30 (9H, s, 3×CH_3), ^{13}C NMR (100 MHz. DMSO-d_6): δ_C ppm, 31.4 (3×CH_3), 35.0 (Cq), 119.7 (2×CH-ar), 121.9 (=CH), 123.7 (CH-ar), 126.3 (2×CH-ar), 128.0 (2×CH-ar), 129.2 (2×CH-ar), 132.5 (Cq), 139.8 (Cq), 140.5 (=CH), 153.1 (Cq), 164.2 (C=O); FTIR (ATR, cm^{-1}): *v* 3314 (N-H, secondary amide), 2953 (C-H, aromatic), 1662 (C=O), 1597 (N-H bend, secondary amide), 825 (C-H oop, aromatic); Found MS (ES, negative mode) [M−H]+ 278.06 $C_{19}H_{20}NO$, requires 278.38. Anal. Calculated for $C_{19}H_{21}NO$: C, 81.68; H, 7.58; N, 5.01; Found: C, 79.87; H, 7.17; N, 5.96.

3-(4-*tert*-butylphenyl)-N-(4-methoxyphenyl) acrylamide (6b2)

Brown solid (0.357g, 96.2%), m.p.175 ˚C [31], compound **6b2** was prepared from the reaction of 4-*tert*-butylcinnamic acid (0.41g, 2.0 mmol) with 4-methoxyaniline (0.25g, 2 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (DMSO-d_6, 400 MHz): δ_H ppm, 10.09 (1H, s, NH), 7.62-7.65 (2H, d, *J*= 12.00 Hz, H-ar), 7.52-7.56 (3H, t, *J*= 8.00, 8.00 Hz, H-ar), 7.45-7.47 (2H, d, *J*= 8.00 Hz, H-ar), 6.91-6.93 (2H, d, *J*= 8.00 Hz, H-ar), 6.76-6.80 (1H, d, *J*= 16.00 Hz, CH), 3.74 (3H, s, CH_3), 1.28 (9H, s, 3×CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ_C ppm, 31.4 (3×CH_3), 35.0 (Cq), 55.6 (OCH_3), 114.4 (2×CH-ar), 121.1 (2×CH-ar), 122.0 (=CH), 126.3 (2×CH-ar), 127.9 (2×CH-ar), 132.5 (Cq), 133.0 (Cq), 139.9 (=CH), 152.9 (Cq), 155.7 (C-O), 163.7 (C=O); FTIR (ATR, cm^{-1}): *v* 3311 (N-H, secondary amide), 2954 (C-H, aromatic), 1660 (C=O), 1597 (N-H bend, secondary amide), 825 (C-H oop, aromatic); Found MS (ES, negative mode) [M−H]+ 308.17 $C_{20}H_{22}NO_2$, required 308.41. Anal. Calculated for $C_{20}H_{23}NO_2$: C, 77.64; H, 7.49; N, 4.53; Found: C, 78.85; H, 7.32; N, 5.31.

3-(4-*tert*-butylphenyl)-N-(4-methylphenyl) acrylamide (6b3)

Yellow solid powder (0.541g, 92.3%), m.p.135 ˚C, compound **6b3** was prepared from the reaction of 4-*tert*-butylcinnamic acid (0.41g, 2.0 mmol) with 4-methylaniline (0.21g, 2.0 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (400 MHz. DMSO-d_6): δ_H ppm, 10.15 (1H, s, NH), 7.57-7.63 (5H, m, H-ar), 7.46 (2H, d, *J*= 8.0 Hz, H-ar), 7.14 (2H, d, *J*= 8.0 Hz, H-ar), 6.82 (1H, d, *J*= 16.0 Hz, CH), 2.27 (3H, s, CH_3), 1.28 (9H, s, 3×CH_3); ^{13}C NMR (100 MHz. DMSO-d_6): δ_C ppm 21.0 (CH_3), 31.4 (3×CH_3), 35.0 (Cq), 119.7 (2×CH-ar), 122.0 (=CH), 126.3 (2×CH-ar), 128.0 (2×CH-ar), 129.6 (2×CH-ar), 132.5 (Cq), 132.7 (Cq), 153.0 (Cq), 140.2 (=CH), 163.9 (C=O); FTIR (ATR, cm^{-1}): *v* 3245 (N-H, secondary amide), 2954 (C-H, aromatic), 1654 (C=O), 1605 (N-H bend, secondary amide), 816 (C-H oop, aromatic); Found MS (ES, negative mode) $[M-H]^+$ 292.17 $C_{20}H_{22}NO$, requires 292.41. Anal. Calculated for $C_{20}H_{23}NO$: C, 81.87; H, 7.90; N, 4.77; Found: C, 83.18; H, 7.64; N, 5.47.

3-(4-*tert*-butylphenyl)-N-(4-chlorophenyl) acrylamide (6b4)

Pale yellow solid powder (0.439g, 69.9%), m.p.135 ˚C, 6b4 was prepared from the reaction of 4-*tert*-butylcinnamic acid (0.41g, 2.0 mmol) with 4-chloroaniline (0.26g, 2.0 mmol) in the same manner as described in Scheme 1. Data analysis: ^{1}H NMR (400 MHz. DMSO-d_6): δ_H ppm, 10.37 (1H, s, NH), 7.75 (2H, d, *J*= 8.9 Hz, H-ar), 7.56-7.61 (3H, m, H-ar), 7.47 (2H, d, *J*= 8.4 Hz, H-ar), 7.39 (2H, d, *J*= 8.9 Hz, H-ar), 6.79 (1H, d, *J*= 15.7 Hz, CH), 1.30 (9H, s, 3×CH_3); ^{13}C NMR (100 MHz. DMSO-d_6): δ_C ppm 31.4 (3×CH_3), 35.0(Cq), 121.2 (2×CH-ar), 126.3 (2×CH-ar), 127.3 (=CH), 128.1 (2×CH-ar), 129.2 (2×CH-ar), 132.3 (2×Cq), 138.8 (Cq), 140.9 (=CH), 153.2 (Cq), 164.3 (C=O); FTIR (ATR, cm^{-1}): *v* 3318 (N-H, secondary amide), 2958 (C-H, aromatic), 1662 (C=O), 1594 (N-H bend, secondary amide), 824 (C-H oop, aromatic); Found MS (ES, negative mode) $[M-H]^+$ 312.12 $C_{19}H_{19}ClNO$, required 312.83. Anal. Calculated for $C_{19}H_{20}ClNO$: C, 72.72; H, 6.42; N, 4.46; Found: C, 72.02; H, 6.26; N, 5.23.

Molecular docking

The in-silico studies (molecular docking) were conducted to investigate the binding interactions of the synthesized compounds towards DENV-2 NS2B/NS3 protease and in search for the best orientation of ligand-protease complex with the lowest free energy of binding (FEB). This step served as a good tool to predict and match the desired binding site, understand possible conformation of the compounds and further clarify the binding interactions in the binding pocket [32].

Preparation of protein

The DENV non-structural protein homology model, NS2B/NS3pro with tetrapeptide inhibitor (Bz-Nle-Lys-Arg-Arg-H) developed by Wichapong and co-worker [33] was used as the protein target. This model was constructed based on DENV2 complex cofactor-protease and by using the crystal structure of NS2B/NS3pro West Nile Virus (WNV) as the template. Meanwhile, the coordinate was taken from DENV2 NS2b/NS3 crystal structure (PDBID: 2FOM). The NS2B/NS3pro was prepared as macromolecule prior to docking by using AutoDock 4.2 (www.autodock.scrips.edu). The protein was initially prepared by removing its inhibitor complex (Bz-Nle-Lys-Arg-Arg-H) by using Discovery Studio Client 2020 (www.accelrys.com). Both protein and ligand were prepared by using AutoDockTools 1.5.7. Kollmann charges were added to the protein structure while Gasteiger charge was added to the ligand. Both prepared protein and ligand were saved in pdbqt format file prior to docking simulation.

Preparation of ligand

The cinnamic acid derivatives were constructed in two-dimensional (2D) and three-dimensional (3D) structures and optimized by using ChemDraw Professional 16.0. The structures were saved in sdf format before being converted to pdb format.

Docking analysis

The docking simulation was performed by using AutoDock 4.2. In the positive control docking, extracted native ligand was re-docked into the binding sites of the protein. Coordinates of the redocked Bz-Nle-Lys-Arg-Arg-H were used as an active site of docking. The grid box size of dimension 60 × 60 × 60 Å in x, y, z coordinates and grid spacing of 0.375 Å were employed to cover the protein active site. The docking of ligands was applied with Lamarckian Genetics Algorithm (GA) search program to generate 100 runs. The re-docked results with the low root mean square deviation (RMSD) value of less than 2.0 Å were applied as the fix parameter for ligands simulation.

ADME properties

Lipinski's rule of five, also known as Pfizer rule is used in the prediction of a new compound to act as a drug molecule based on the membrane permeability and absorbance in the organism. The drug-likeness parameter of compounds was calculated by using a free access online server, SwissADME (http://swissadme.ch/)[27].

Results and Discussion

In this study, eight cinnamic acid amide derivatives were synthesized by the reaction of cinnamic acid with corresponding amine derivatives. EDC.HCl was used as a catalyst to produce cinnamic acid amide derivatives (**6a1-6a4** and **6b1-6b4**) in moderate to excellent yield (56.8-96.2%). Based on the literature, a common approach used to improve the reactivity of carboxyl group towards nucleophilic reagent is by employed a carbodiimide reaction [34, 35, 36]. The proposed plausible mechanism for the formation of cinnamic acid amide derivatives is shown in Figure 2.

The reaction starts with deprotonation of carboxylic group by EDC.HCl to form an active *O*-acylisourea intermediate. The highly electrophilic *O*-acylisourea intermediate is unstable and a good leaving group [35]. This intermediate was resonance stabilized, which then underwent a nucleophilic attack from amine to form an amide bond and removed urea by-product [36]. ^{1}H and ^{13}C NMR spectra of synthesized compounds were available in Supplementary material. The chemical shift for ^{1}H and ^{13}C NMR for **6a1-6a4** and **6b1-6b4** are tabulated in Table 1 and Table 2, respectively. The proton signal for N-H of all synthesized compounds was observed as a broad singlet at the downfield region of δ_H 10.00-10.23 ppm. Compared with the literature, the proton of N-H appeared at more downfield region [29,

30, 31]. This may be due to the temperature, concentration and solvent affecting the chemical shift of amide proton [37]. High intensity singlets of methyl and methoxy moieties in **6a1-6a4** were observed at δ_H 2.26 and 3.74 ppm, respectively. The electronegativity of oxygen attached to CH_3 in the methoxy group caused it to be more deshielded and their chemical shifted downfield, as compared to methyl moieties. A similar pattern was observed in ^{13}C NMR, whereby the signal for methoxy carbonyl appeared at a more upfield region (δ_C 20.9 ppm) than methyl carbonyl (δ_C 55.6 ppm). The chemical shift for C=O of amide appeared at the downfield region, 163.7-164.6 ppm. The position of C-O of methoxy (δ_C 155.6-155.7 ppm) than C-O of hydroxyl was observed at slightly upfield (δ_C 159.6 ppm—159.8 ppm) [37].

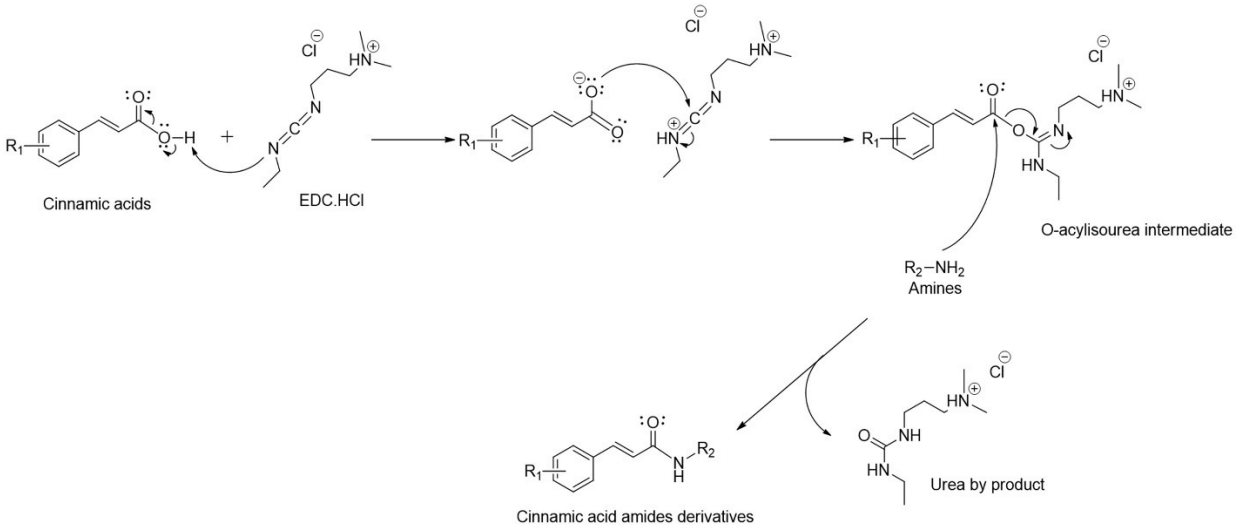


Figure 2. Plausible mechanism for the formation of cinnamic acid amide [35]

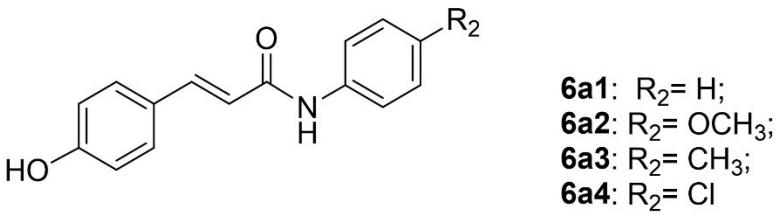


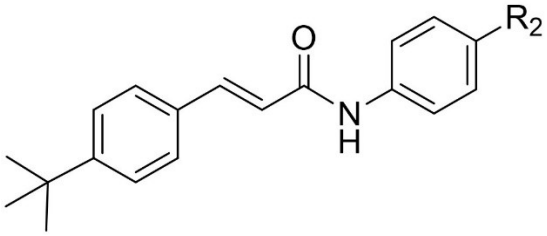
Figure 3. Structure of compounds **6a1-6a4**

Table 1. Chemical shift ^1H and ^{13}C NMR for compounds **6a1-6a4**

Comp.	Chemical Shift ^1H NMR (δ, ppm)				Chemical Shift ^{13}C NMR (δ, ppm)				
	OH	N-H	CH_3	OCH_3	C=O of amide	C-OH	C-OCH_3	CH_3	OCH_3
6a1	9.98 (s)	10.12 (s)	-	-	164.5	159.7	-	-	-
6a2	9.97 (s)	10.00 (s)	-	3.74 (s)	164.1	159.6	155.6	-	55.6
6a3	9.95 (s)	10.01 (s)	2.26 (s)	-	164.3	159.6	-	20.9	-
6a4	10.02 (s)	10.12 (s)	-	-	159.8	159.8	-	-	-

The proton signal for *tert*-butyl moieties in **6b1-6b4** appeared as singlet at δ_H 1.28 to 1.30 ppm, corresponding with literature [31]. The presence of three methyl groups in the moieties caused it to be more shielded and upfield compared to methoxy (δ_H 3.74 ppm) and single methyl moieties (δ_H 2.27 ppm) [37]. The same trend for the peak of moieties position was observed in ^{13}C NMR. The presence of carbon signal

between δ_C 34.9 to 35.0 ppm observed in ^{13}C NMR for **6b1-6b4**, and it is absence for **6a1-6a4**. This peak indicated the presence of quaternary carbon (Cq) of *tert*-butyl moieties presence in **6b1-6b4**. The chemical shift of proton and carbon for all compounds is very small, except for **6a4** and **6b4**. The proton signal for NH and carbon signal for carbonyl of amide in both **6a4** and **6b4** slightly shifted more downfield compared to other compounds. This might be due to the presence of an electronegative Cl at the para position of the aromatic ring, which caused an anisotropic effect on the ring and thus deshielded the proton of amide attached to the ring. Though the anistropic field diminishes with distance, the presence of the oxygen in the amide may also cause the NH proton to be more deshielded [37].



6b1: R_2= H;
6b2: R_2= OCH_3;
6b3: R_2= CH_3;
6b4: R_2= Cl

Figure 4. Structure of compounds **6b1-6b4**

Table 2. Chemical shift ^1H and ^{13}C NMR for compounds **6b1-6b4**

Comp.	Chemical Shift ^1H NMR (δ, ppm)				Chemical Shift ^{13}C NMR (δ, ppm)					
	$C(CH_3)_3$	N-H	CH_3	OCH_3	C=O of amide	$C(CH_3)_3$	Cq of $C(CH_3)_3$	C-OCH_3	CH_3	OCH_3
6b1	1.30 (s)	10.23 (s)	-	-	164.2	31.4	35.0	-	-	-
6b2	1.28 (s)	10.09 (s)	-	3.74 (s)	163.7	31.4	35.0	155.7	-	55.6
6b3	1.28 (s)	10.15 (s)	2.27 (s)	-	163.9	31.4	34.9	-	20.9	-
6b4	1.30 (s)	10.37 (s)	-	-	164.3	31.4	35.0	-	-	-

The FTIR spectrum of major functional groups for **6a1-6a4** and **6b1-6b4** are portrayed in Table 3. The absorption band of N-H bending vibration stretching for secondary amide was reported to generally appear at the range of 3370 to 3170 cm^{-1} [38]. The FTIR spectrum of synthesized compounds featured the absorption band of N-H stretching vibration at 3367 to 3245 cm^{-1} and the absorption band of N-H bending appears around 1594 to 1605 cm^{-1}, which implying the presence of secondary amine in the structure [37]. The high intensity of bands was due to the combination of N-H bending band with C-N stretching band. The presence of O-H in **6a1-6a4** could be observed by the presence of a broad O-H band within the range of 3022 to 3112 cm^{-1} [29]. A strong band of carbonyl C=O of amide stretching was observed around 1654 to 1662 cm^{-1}, corresponding with literature [29]. A prominent out-of-plane C-H bending vibrations band appeared at 808 to 869 cm^{-1} for all compounds attributed to a para-disubstituted aromatic compound, which were in rapport with its structures [37]. The spectral of compounds obtained agreed with the proposed structure.

Both theoretical values of synthesized compounds for MS and CHN analysis were calculated using ChemDraw Ultra 12.0. An electron spray ionization mass spectrometer (ESI) in negative mode was used to measure the mass-to-charge ratio (m/z) of the synthesized compounds in this study. The experimental m/z for all compounds agreed with the theoretical value. The elemental composition of a synthesized compound, including carbon, hydrogen, and nitrogen (CHN), provides essential information for chemical identification [39]. This study aimed to determine the molecular formula of **6a1-6a4** and **6b1-6b4** in the respect of theoretical versus the experimental results. However, the experimental results obtained show slightly different from the theoretical value. The percentage weight of carbon for **6a1**, **6a2**, and **6b1-6b4** varied slightly from the theoretical value by -1.75 to 2.91%. While the difference in nitrogen percentage from the theoretical value for **6a1** and **6b1-6b4** ranged

from -0.96 to 0.61%. These discrepancies may be attributed to the presence of solvents, which contribute to the calculation of weight percentage of experimental value. The theoretical values of oxygen showed good agreement with the experimental results for all compounds. However, the slight difference observed in the experimental nitrogen value compared to the theoretical value was maybe due to the presence of oxygen, leading to the generation of nitrogen oxide and resulting in a lower yield of nitrogen. It has also been suggested that the degree of variability in elemental result may be due to difference in the instrumental methods used for quantifying each element [40]. The data of CHN analysis are tabulated in Table 4.

Table 3. The IR absorption peaks for the synthesized compounds (**6a1-6a4** and **6b1-6b4**)

Group	Wavenumber (ν, cm^{-1})					
	N-H (secondary amide)	**OH (phenol)**	**C-H (aromatic)**	**C=O**	**N-H bend (secondary amide)**	**Out-of-plane C-H (aromatic)**
6a1	3317	3022	2922	1651	1599	821
6a2	3328	3066	2946	1654	1598	820
6a3	3250	3012	2920	1653	1598	816
6a4	3367	3112	2925	1662	1594	826
6b1	3314	-	2953	1662	1594	825
6b2	3311	-	2954	1660	1597	825
6b3	3245	-	2954	1654	1605	816
6b4	3318	-	2958	1662	1594	824

Table 4. The CHN data for the synthesized compound (**6a1-6a4** and **6b1-6b4**)

Element	Carbon		Hydrogen		Nitrogen	
	Theory (%)	**Experimental (%)**	**Theory (%)**	**Experimental (%)**	**Theory (%)**	**Experimental (%)**
6a1	75.30	73.55	5.48	5.51	5.85	5.24
6a2	71.36	68.45	5.61	5.79	5.20	4.79
6a3	75.87	76.03	5.97	5.59	5.53	5.31
6a4	65.82	65.72	4.42	4.83	5.12	4.75
6b1	81.68	79.87	7.58	7.17	5.01	5.96
6b2	77.64	78.85	7.49	7.32	4.53	5.31
6b3	81.87	83.18	7.90	7.64	4.77	5.47
6b4	72.72	72.02	6.42	6.23	4.46	5.23

In the positive control docking, the center of the grid box was determined by the center of Bz-Nle-Lys-Arg-Arg-H at 23.038, 3.372, -0.316 in the x, y and z coordinates, respectively. The Lamarckian genetic algorithm was employed with the covalent map parameter to constrain the molecular geometry of peptide inhibitor [41, 42].

The internal validation was done by re-docking tetrapeptide inhibitor (Bz-Nle-Lys-Arg-Arg-H) with RMSD value in the range of 1.5-2.0 Å, depending on ligand size, which indicating that the docking was able to reproduce native conformation [43]. The redocked tetrapeptide inhibitor to DENV2 NS2B/NS3pro was observed to have a similar conformation with an initial pose of the inhibitor with RMSD value 1.98 Å, as illustrated in Figure 5. The docking parameter obtained was utilized in docking simulations of ligand-DENV2-NS2b/NS3 protease study by using the synthesized compounds as ligand.

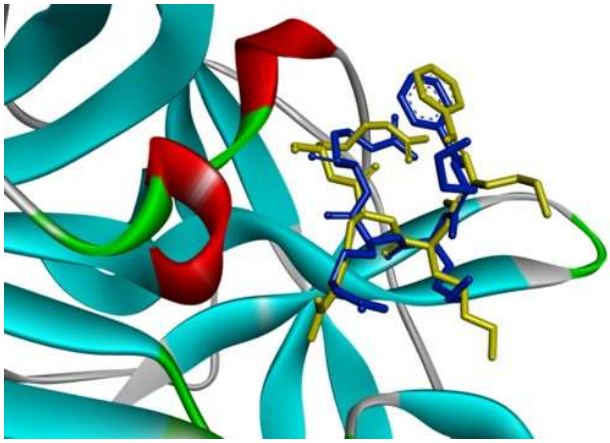


Figure 5. The overlay of the redocked Bz-Nle-Lys-Arg-Arg-H to DENV2 NS2B/NS3pro with RMSD of 1.61 Å Yellow: original; blue docked ligand conformation

The ligand-protein docking results based on free energy of binding (FEB) values and detailed type of interactions that occurred between the ligands and protease are shown in Table 5. Compounds with the lowest FEB were considered to have the strongest binding affinity towards DENV2 NS2b/NS3pro. All docked compounds displayed positive interaction with NS2b/NS3pro, with a binding energy of less than the peptide inhibitor. Generally, the difference between compounds **6a1-6a4** and **6b1-6b4** was the substitutes at the *para* position in the aromatic ring. The aromatic system with *para*-substituent with high electron density was reported to exhibit good activity against DENV2 NS2b/NS3 [18]. The *para*-substituted compounds possess an advantage in molecular interaction due to their optimal conformation, favorable electrostatic interactions, lower steric hindrance, π-π stacking and hydrophobic complementary [44].

Among the eight docked compounds, ligands with *tert*-butyl substituents had the lowest free binding energies, ranging from -6.69 kcal/mol to -7.72 kcal/mol. Compounds **6b2** and **6b3** were the top two hit compounds which showed the best result with minimum FEB values of -7.72 kcal/mol and -7.26 kcal/mol, respectively. The greater negative score binding free energy indicated a stronger binding affinity between the compound and enzyme, suggesting that the compounds have a higher probability of biological activity [45, 46]. The detailed ligand-binding site interaction in 2D structural views of compounds **6b2** and **6b3** are shown in Figure 6.

Table 5. The free energy of binding (FEB) of synthesized compounds

Compound	Free Binding Energy (kcal/mol)	Interaction	Type of Interaction	Bond Distance (Å)
6a1	-6.54	HIS B:51	H-Bond	2.07
		VAL B:52	π-Alkyl	5.35
		ASP B:129	H-Bond	1.92
		PHE B:130	H-Bond	2.47
		PRO B:132	H-Bond	4.17
		TYR B:161	π-π Stacked	1.79
6a2	-6.65	ILE B:36	π-Alkyl	3.89
		HIS B:51	H-Bond	2.03
		VAL B:52	π-Alkyl	5.36
		ASP B:129	H-Bond	1.88
		PHE B:130	H-Bond	2.56
		PRO B:132	H-Bond	1.91

		TYR B:161	π-π Stacked	4.13
6a3	-6.90	ILE B:36	Alkyl	5.37
			π-Alkyl	3.98
		VAL B:52	π-Alkyl	5.25
		ASP B:129	H-Bond	2.61
		PHE B:130	H-Bond	2.05
		PRO B:132	H-Bond	1.78
		TYR B:161	π-π Stacked	4.69
6a4	-7.03	ILE B:36	Alkyl	4.42
		HIS B:51	H-Bond	2.09
		VAL B:52	π-Alkyl	5.39
		ASP B:129	H-Bond	1.96
		PHE B:130	H-Bond	2.53
		PRO B:132	H-Bond	1.77
		TYR B:161	π-π Stacked	4.09
6b1	-6.69	HIS B:51	π-Cation	4.41
			π-π Stacked	3.73
		GLY B:151	H-Bond	2.06
6b2	-7.72	TRP B:50	π-Alkyl	5.08
		VAL B:72	Alkyl	4.82
		ILE A:86	Alkyl	4.03
		GLY B:153	H-Bond	2.06
		VAL B:154	π-Sigma	3.70
			Alkyl	4.21
			H-Bond	1.89
		VAL B:155	π-Alkyl	5.04
			Alkyl	5.13
6b3	7.26	TRP B:50	π-Alkyl	5.18
		HIS B:51	π- π Stacked	4.36
		ASP B:75	π-Anion	4.00
		GLY B:153	H-Bond	2.13
		TYR B:161	π- π Stacked	4.03
			π-Alkyl	4.00
6b4	-7.16	TRP B:50	π-Alkyl	5.19
		HIS B:51	π- π Stacked	4.38
		ASP B:75	π-Anion	4.00
		GLY B:153	H-Bond	2.14
		TYR B:161	π-Alkyl	4.14
			π- π Stacked	4.07
Positive control (Bz-Nle-Lys-Arg-Arg-H)	-5.21	-	-	-

Aside from the catalytic residue, involvement of amino acid residues, such as Val52, Val72, Pro132 and Tyr150 (in the NS3 domain) and Ile86, Gly151, Gly153 (in the NS2B cofactor domain) in ligand-protein interaction helped to contribute to the binding [47, 48]. The *tert*-butyl moiety of **6b2** and **6b3** formed an alkyl and π-alkyl interaction with Trp50 and Val72. Compound **6b2** and DENV2 NS2B/NS3pro complex showed H-bond interaction between O of amide with Gly153 and O of methoxy group with Val155. The methoxy group and *tert*-butyl substituents showed non-bonding interaction of alkyl and π-alkyl with Val72, Ile86 and Trp50. Although no interaction occurred with the catalytic triad residues (His51, Ser135 and Asp75), the binding of 6b2 to the active site of protease was stabilized through one H-bond with Gly153 and the support of non-covalent interaction with Trp50, Val72, Ile86, Val154, and Val155. [47].

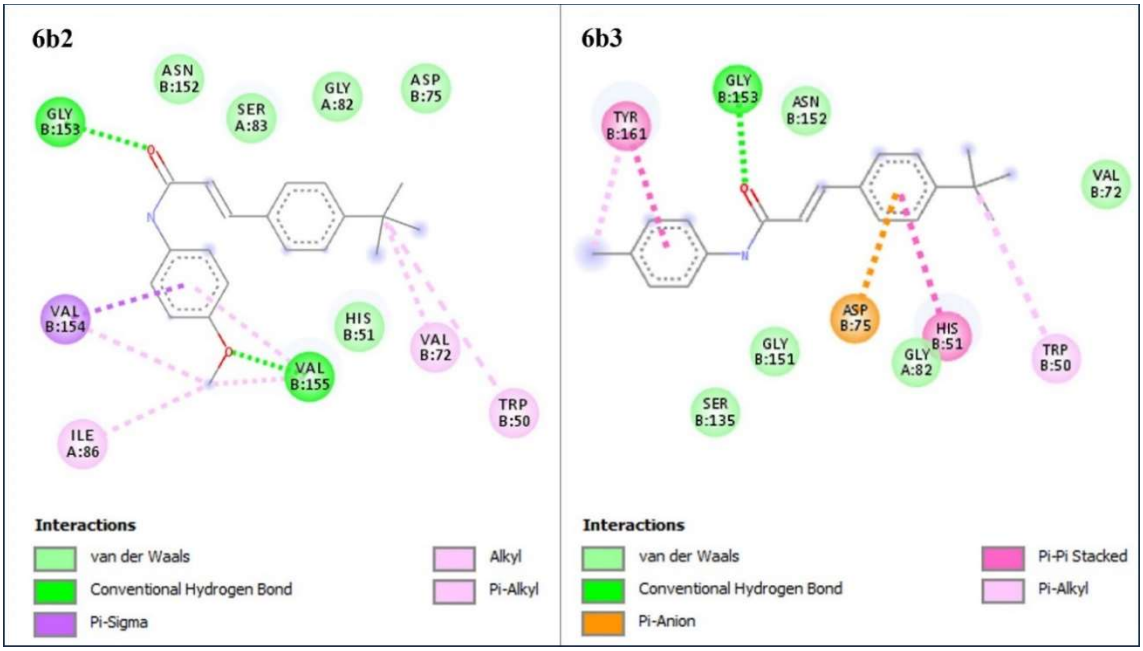


Figure 6. 2D protein-ligand interaction of **6b2** and **6b3** against NS2B/NS3pro

The docked pose showed that compound **6b3** formed one hydrogen bond interaction with Gly153 of NS3. The phenyl ring forms π-π stacking and π-anion with catalytic triad His51 and Asp75, respectively. The ligand also formed an interaction with Tyr161 and Trp50. Even though 6b3 interacted with two catalytic triad residues, its binding free energy was slightly higher than **6b2**. This might be due to a lack of non-covalent interaction to improve binding affinity for **6b3** as compared to **6b2**. The strength of hydrogen bonding increases as it gets closer to the geometry [49]. The distance of H-bond interaction between ligand-protein for **6b2** was 2.06 Å and 1.89 Å, which is less than in **6b3**. This made 6b2 bind more firmly to the protein, and thus its FEB value was more negative. ADME (absorption, distribution, metabolism, and excretion) describes the internal process of drug movement throughout the body. The ADME results for physiochemical properties of compounds **6b2** and **6b3** are tabulated in Table 6. The lipophilicity of drug candidate molecules was determined by the ratio of partition coefficient of n-octanol and water (log $P_{o/w}$), and its value should be less than 5 for it to have good absorption [50]. The number of H-Bond donors (HBD) needed to be less than 5 and H-Bond acceptors (HBA) should be less than 10 [51, 52].

Table 6. Physiochemical properties of compounds **6b2** and **6b3**

Properties	**The Top Two Hit Compounds**	
	6b2	**6b3**
Log S	-5.06	-5.29
Solubility	Moderately soluble	Moderately soluble
Heavy atoms	23	22
Number of rotatable bonds	6	5
Number of H-bond acceptors (HBA)	2	1
Number of H-bond donors (HBD)	1	1
Log $P_{o/w}$	3.51	3.45
TPSA (Å^2)	38.33	29.10

According to Kenny, elimination of HBD could lead to small increases in lipophilicity and improved permeability of the molecule [53]. Topological polar surface area (TPSA) score for a drug should be less than 130 $Å^2$, the solubility of the molecule log S, should not exceed more than 6 [54]. Both compounds were found to be within the acceptable range of drug-likeness parameter of compounds. An excessively high lipophilicity can contribute to the immobilization of the compound within a given layer [55]. Based on the ADME results, **6b2** and **6b3** were suggested to have moderate permeability against the cell barrier to be distributed to molecular targets.

Conclusion

In conclusion, the present work has demonstrated that eight cinnamic acid derivatives were successfully synthesised through the reaction of cinnamic acid with various amines. The molecular studies revealed a significant molecular interaction between the synthesised compounds and DENV2 NS2B/NS3 protease, which indicated their potential activity as a protease inhibitor. It was observed that both compounds with a hydroxyl group and *tert*-butyl substituents displayed a high binding affinity and good interaction with DENV-2 NS2B/NS3 serine protease as compared to the tetrapeptide inhibitor (Bz-Nle-Lys-Arg-Arg-H).

Amongst the eight compounds, **6b2** and **6b3** showed the best results with the minimum value of FEB. Compound **6b2** (-7.72 kcal/mol) exhibited the lowest FEB as compared to compound **6b3** (-7.26 kcal/mol). The number of ligand-protein interactions and their distance have an important influence on the FEB value. Although hydrogen bond is deemed as important in enhancing the ligand-protein binding affinity, the bond distance, and another types of interaction, such as non-bonding interaction could also help to compensate for enhancing the binding stability and binding affinity of the ligand. The ADME results showed that compounds **6b2** and **6b3** have the potential to serve as lead molecules and be developed as inhibitors for DENV2 NS2B/NS3pro to treat dengue. However, further validation through in-vitro and in-vivo analysis is required.

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FORMULATION AND EVALUATION OF BODY SCRUB USING PINEAPPLE WASTE AS NATURAL EXFOLIATOR

(Formulasi dan Penilaian Skrub Badan Menggunakan Sisa Nanas sebagai Pengelupas Semulajadi)

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Abstract

The study explores potential of repurposing pineapple waste which known to contain antioxidants and bromelain as a body scrub to combat increasing amount of pineapple waste generated during pineapple production. The cream body scrub was formulated using varying concentrations of pineapple powder namely S0 (0%), S1 (3.5%), S2 (4.5%), and S3 (5.5%). The resulting formulations were evaluated for their physical and sensorial characteristics, including color, consistency, pH, viscosity, spreadability, foamability, odor, washability, grittiness, irritability, and exfoliation capability. Additionally, an online market survey was also conducted. Analysis showed that S0 had the highest pH value (7.97), viscosity (8120 cP), and spreadability (21411.71g). S1 with the lowest pineapple concentration exhibited the lowest irritancy (6.68) and the highest acceptance of pineapple body scrub with fragrance (6.48) and without fragrance (6.48), while S2 showed the highest acceptance for grittiness (6.45) and washability (7.18). S3 exhibited the highest foamability (2.1 mL within 30 minutes) and was the darkest (L=51.59±2.39) among other samples. The presence of pineapple powder is related to the exfoliation capacity of the body scrub (with a significant correlation, p <0.05). Body scrubs with 3.5% and 4.5% pineapple powder have the highest acceptance among respondents and are recommended for future research development.

Keywords: pineapple, waste, natural exfoliator, formulation, evaluation

Abstrak

Kajian ini meneroka potensi penggunaan semula sisa nanas yang diketahui untuk mengandungi antioksidan dan bromelain sebagai skrub badan untuk menangani peningkatan jumlah sisa nanas yang dihasilkan semasa pengeluaran nanas. Skrub badan krim diformulasikan menggunakan kepekatan serbuk nanas yang berbeza-beza iaitu S0 (0%), S1 (3.5%), S2 (4.5%), dan S3 (5.5%). Formulasi yang terhasil dinilai untuk ciri fizikal dan derianya, termasuk warna, ketekalan, pH, kelikatan, kebolehtebaran, kebolehbuih, bau, kebolehbasuh, kekasaran, kerengsaan, dan keupayaan pengelupasan. Selain itu, tinjauan pasaran dalam talian juga telah dilaksanakan. Analisis menunjukkan bahawa S0 mempunyai nilai pH tertinggi (7.97), kelikatan (8120 cP), dan kebolehtebaran (21411.71g). S1 dengan kepekatan nanas yang paling rendah menunjukkan kerengsaan yang paling rendah (6.68) dan penerimaan skrub badan nanas yang paling tinggi dengan pewangi (6.48) dan tanpa pewangi (6.48), manakala S2 menunjukkan penerimaan yang paling tinggi untuk kekasaran (6.45) dan kebolehbasuh (7.18). S3 mempamerkan kebolehbuih tertinggi (2.1 mL

dalam masa 30 minit) dan merupakan yang paling gelap (L=51.59±2.39) antara sampel lain. Kehadiran serbuk nanas adalah berkaitan dengan keupayaan pengelupasan skrub badan (dengan korelasi yang ketara, p <0.05). Skrub badan dengan serbuk nanas 3.5% dan 4.5% mempunyai penerimaan tertinggi dalam kalangan responden dan disyorkan untuk perkembangan kajian masa hadapan.

Kata kunci: nanas, sisa, pengelupas semula jadi, formulasi, penilaian

Introduction

According to Malaysian Pineapple Industry Board (MPIB), Malaysia has annually produced 400,000 to 600,000 metric tons of pineapple [1], accumulating 137, 550 tons of peel waste [2]. The waste has caused vast amount of pollution due to poor agro-waste management. The common way to manage the agro-waste is by discarding the waste via open burning, which releases harmful pollutants into the atmosphere, aggravating soil pollution, water pollution, and food contamination. Besides, waste is also dumped into the landfills, creating a breeding environment for disease outbreaks due to the presence of pests such as rats and flies. Due to the problems, alternative ways to reduce agro-waste from pineapple and its environmental impact are at urge. Pineapple waste has shown to contain high quantities of bioactive chemicals with antioxidant qualities and serves as a significant reservoir of bromelain protein [3]. The antioxidative nature of bromelain additionally aids in promoting skin health by combating oxidative stress [4]. Therefore, utilizing pineapple waste for a natural exfoliating body scrub is an excellent way to repurpose waste and reduce landfill contributions.

Body scrub is a type of physical exfoliator used to exfoliate dead skin and keep the pores of the skin clean from oil and grime [5]. Exfoliator helps boost blood circulation, thus providing revitalized and healthy-looking skin. Physical exfoliators can be sourced from natural exfoliants or synthetic exfoliants. Natural exfoliants refer to a list of non-exhaustive products such as seeds, nut shells, and grains, whereas synthetic exfoliators consist of particles such as polyethylene or polypropylene beads. However, synthetic exfoliators have caused microplastic pollution as they enter streams of waste water [6] and rise concern to marine ecosystems [7]. Hence, natural exfoliants made from pineapple peel and core would be more sustainable for the environment. The objectives of the study were to conduct market survey for development of pineapple body scrub, to formulate body scrub from the waste parts of pineapple which are peel and core and to evaluate its parameters, such as color, consistency, pH, viscosity, spreadability, foamability, odor, washability, grittiness, and irritability along with its exfoliation capability.

Materials and Methods

Formulation of pineapple body scrub

Pineapple wastes (peel and core) were collected from restaurants and fruit stalls nearby Muar, Johor. The pineapple wastes were washed and cut into smaller pieces [8]. The cut pineapples were dried using oven for 20 hours at 70 ℃. After that, the samples were ground and then sieved using sieved shaker (500 microns) to obtain uniform size of pineapple powder. The pineapple powder was weight to produce different concentrations which are 3.5g in 100g base cream (3.5%), 4.5g in 100g base cream (4.5%), and 5.5g in 100g base cream (5.5%). Base cream of pineapple body scrub was formulated with references to a previous study conducted by Hendrawati et al. [9]. Table 1 shows the formulation ingredients with percentage (%),

Table 1. Formulation ingredients for base scrub

Material	Weight (%)
Glycerin	15
Stearic acid	12
Cetyl alcohol	4
Trietanolamine	3
Isopropyl	2
Propylparabene	0.02
Distilled water, up to	100

The oil phase of the ingredients which are stearic acid and cetyl alcohol were mixed and melted in a beaker until it reached 70 ℃ in water bath. Then, as it was completely melted, the temperature was lowered to 65 ℃. As the temperature dropped, isopropyl and propyl

paraben were added to the oil phase mixture while stirring until it was homogenous. Then, the water phase ingredients such as glycerin and water were mixed and heated at 80 ℃ in a different beaker. It was then being cooled to 65 ℃ while triethanolamine was being slowly added. Both of the oil and water phase were mixed using hand mixer until a fine cream emulsion was produced. After that, the base cream was left to cool until the temperature dropped to 40 ℃ [9]. Different concentrations of pineapple powder which are 3.5%, 4.5%, and 5.5% were then added to the 100g base cream respectively with continuous stirring. The body scrubs were kept in airtight container. The body scrubs were labeled based on their concentrations as S0 (0%), S1 (3.5%), S2 (4.5%) and S3 (5.5%).

Evaluation of pineapple body scrub

The performance of the body scrubs was evaluated in two categories which were physical and sensorial characterization. The color, consistency, pH, viscosity, spreadability, and foamability for physical characterization, while odor, washability, grittiness, irritability, and exfoliation capability for sensorial characterization. The parameters of body scrubs were compared to the standard, previous study or commercialized product for a particular evaluation. In sensorial characterization, the acceptance level of body scrubs for odor with and without fragrance, grittiness, washability, irritability and overall acceptability were evaluated using 9-point hedonic scale, where 1 (extremely dislike), 2 (dislike very much), 3 (moderately dislike), 4 (slightly dislike), 5 (neither like nor dislike), 6 (slightly like), 7 (moderately like), 8 (like very much), and 9 (extremely like). The results were expressed in mean.

Color

In physical characterization, the color of the body scrubs was observed in petri dish at room temperature using MiniScan EZ portable spectrophotometer for eight days with two days interval. The results were displayed in L*a*b* reading, where L*, expressed lightness (100-0), a*, show the red-green field, where positive values for red, while negative values for green, (-128, +127), while *b, shows the blue-yellow field, where positive values for yellow, while negative values for green (-128, +127) [10]. The change of color was calculated using equation below.

$$\Delta E_{ab} = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2} \quad (1)$$

where, 0< ΔE < 1 = colour difference cannot be noticed, 1< ΔE < 2 = colour difference can be noticed by experienced observer, 2< ΔE < 3.5 = colour difference can be noticed by unexperienced observer, 3.5< ΔE < 5 = clear difference in colour can be noticed, and 5< ΔE = two different colours can be noticed

Consistency

The consistency was tested under microscope with 40x magnification for three parts of the body scrub which were the top, middle and bottom.

pH

The pH of the scrub was measured by utilizing Eutech Instruments pH 700 pH meter using a 1:9 ratio between sample and distilled water.

Viscosity

The viscosity was measured using Brookfield DV3T Rheometer, where the result would be displayed as centipoise (cP).

Spreadability

Spreadability was measured using a texture analyzer with stable micro systems, where a smaller force indicates higher spreadability [11].

Foamability

The foamability was measured by mixing one gram (g) of body scrub with five milliliters of water and observed for 30 minutes.

Irritability

The scrubs were applied to the skin and left on for 10 minutes. The irritability of the body scrub assessed using a 9-point hedonic scale, where a greater number signifies lesser irritability.

Exfoliation capability

The exfoliation capability of body scrubs was also

evaluated by calculating the colour changes before and after applying the body scrubs [12] using the Equation 1 above. The exfoliation capability of body scrub S0, S1, S2 and S3 was observed using the SPSS application one-way ANOVA test, where the factor observed was the concentration of pineapple powder. Meanwhile, other parameters were observed using descriptive analysis, where the results were expressed in mean or mean ± standard deviation.

Market survey of pineapple body scrub

An online market survey was conducted to gain information about market trends [13]. The online survey was performed through a Google Form questionnaires and distributed randomly through the WhatsApp application, which was expected to receive 100 to 200 responses, and the results obtained were analyzed and tabulated. The questionnaires consisted of three parts, Part 1: Demographic information, Part 2: Feedback on existing product, and Part 3: Interest and preferability in body scrub.

Results and Discussion

Pineapple body scrub product

The fresh pineapple that was obtained weighed about 2.86 kilograms (kg) while the dried and grinded pineapple was 336.78g. The moisture content of the dried pineapple was 11%. The moisture content of dried fruits should be around 10% to 15% to ensure safe storage [14]. The cream form of base cream was formulated and the concentration was mixed resulting to four concentrations, S0 (0%), S1 (3.5%), S2 (4.5%) and S3 (5.5%). Figure 1 shows the samples of body scrub.



Figure 1. Samples of body scrub

Analysis of body scrub product

In physical characterization, the color of body scrubs was determined using MiniScan EZ portable spectrophotometer, and S3 has the darkest color (L*= 51.59 ± 2.39) among all the other body scrubs, and it also has the highest color difference from the start and the end of the experiments with 5.28 differences. This is because, color formed usually affected by color of its constituent, therefore as S3 has the highest concentration of pineapple powder, it has the darkest color, while the lower pineapple concentration, the lighter the color. S0 has the lightest color (L= 68.49 ± 5.30) as it has no pineapple powder, and the base cream is quite white in color. Next, the consistency of body scrubs was observed after being left for a week, the result shows that all body scrubs were homogenous and consistent, as no separation of formulation was observed. The pH of body scrubs were examined in previous study to be around 6.29 to 7.61 [15]. The pH of body scrubs in this experiment ranges from 7.16 to 7.97. However, pH levels of all formulations still fell within the acceptable range for cosmetic products as outlined in accordance with SNI 16-4339-1996 which is from 4.5 to 8.0.

Next, the viscosity of body scrubs is in line ranges from 4660cP (S1) to 8120cP (S0), which is slightly lower than previous study [16], however still in line with SNI 16-4399-1996 standard for skin care which is about 2000-50000 cP [17]. The viscosity of body scrubs increases with pineapple powder concentration for S1, S2 and S3. As for spreadability, commercialized product has 23451.37g, making S2, having the most similar values to the standard commercialized product (Brand N),

though, the spreadability among other sample do not have huge differences, S0 has the highest spreadability with 21411.74g force, while S1 has the lowest spreadability as it has more force (24438.57g). Meanwhile, S3 has the highest foamability which is 2.1mL for 30 minutes and is equal to the commercialized product. In foamability, a trend can be observed where, the higher the pineapple powder concentration, the more foam is formed. Table 2, shows the result for parameters tested for physical characterization.

Table 2. Parameters of body scrub for physical characterizations

Attributes	Color	ΔEcolour	pH	Viscosity (cP)	Spreadability (g)	Foamability (mL/30mins)
Brand N	L= 64.09 ± 0.63 a = 3.78 ± 0.37 b = 9.49 ± 0.77	1.67	6.64	-	23451.37	2.1
S0	L= 68.49 ± 5.30 a = -0.06 ± 0.11 b = -0.86 ± 0.84	4.31	7.97	8120	21411.74	1.6
S1	L= 56.06 ± 2.40 a = 3.57 ± 0.11 b = 15.94 ± 1.69	3.63	7.48	4660	24438.57	1.8
S2	L= 54.64 ± 1.82 a = 4.28 ± 0.95 b = 17.01 ± 3.28	5.25	7.38	4760	23225.68	2.0
S3	L= 51.59 ± 2.39 a = 4.32 ± 0.74 b = 15.56 ± 3.07	5.58	7.16	5120	22509.14	2.1

According to 40 respondents, S0 has the lowest acceptance on both odor with fragrance (4.48) and odor without fragrance (4.55). This is most likely because, S0 lacks natural ingredient, which is the pineapple powder, allowing the respondents to directly smell the chemical ingredients in the base scrub. Meanwhile, S1 has the highest acceptance for both odor with fragrance (6.48) and odor without fragrance (5.50). There is no observable trend on odor, which is supported by previous study, where no significant difference was observed in acceptance of odor [9]. Next, S0 has the lowest acceptance on grittiness (4.60) as it has no pineapple powder. The body scrub with the highest acceptance on grittiness is S2 (6.45), which has 4.5% pineapple powder. This result is precisely the same as previous study, where body scrub with 4.5% concentration was the most preferable. This is because, scrubs that have higher than 5% of concentration may cause skin tissue to damage and cause dry skin [9]. Therefore, this shows that the respondents preferred mild grittiness as S3 with highest concentration of pineapple powder has the lowest acceptance (5.85) among body scrubs with pineapple powder, while S1 has 6.25 acceptance level. Next, S0 has the lowest washability acceptance (5.40) while S2 has the highest washability acceptance (7.18). S0 has the lowest washability as base cream has thick consistency [18]. Next, S1 with lowest pineapple concentrations (3.5%) has the highest acceptance for reaction of irritability, which indicates that the body scrub is non-irritant or has the least irritation (6.68). As the level of acceptance gradually decreases with increase of pineapple powder concentrations, thus S3 has the lowest acceptance on irritability (6.15). This indicates that the concentration 5.5% might be too harsh for respondents' skin. As the results, S1 and S2 has the highest overall acceptance (6.98), while S0 has the lowest overall acceptance (5.20). In Table 3, the results for sensorial characterization were shown.

Table 3. Level of acceptance for body scrubs in sensorial characterization

Sample	Odor With Fragrance	Odor Without Fragrance	Grittiness	Washability	Non-Irritant	Overall Acceptance
S0	4.48	4.55	4.60	5.40	6.40	5.20
S1	6.48	5.50	6.25	6.88	6.68	6.98
S2	6.30	4.93	6.45	6.63	6.63	6.98
S3	6.13	4.90	5.85	6.15	6.15	6.27

Next, the exfoliation capability of body scrubs of S0, S1, S2 and S3 were evaluated on 40 respondents. The capability was accessed by using SPSS one-way ANOVA, in which the factor that was being studied was the presence of pineapple powder, the null hypothesis was "presence of pineapple powder has no effect on exfoliation capability" and alternative hypothesis was "presence of pineapple powder has effect on exfoliation capability", where if the p >0.05, the null hypothesis is accepted. However, the exfoliation capacity shows that the significance value ($F_{3,116}$=6.612, p <0.05), thus indicating that the alternative hypothesis was accepted. As a result, adding an exfoliant, such as pineapple powder, contributes to the exfoliating capability of the body scrub as physical exfoliator should contain gritty particles. Table 4 shows the result of ANOVA exfoliation capability.

Table 4. ANOVA exfoliation capability of pineapple body scrub

Sample	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21.865	3	7.288	6.612	.000
Within Groups	127.863	116	1.102		
Total	149.728	119			

Market survey of pineapple body scrub

Results of 196 respondents were obtained from market survey, where 128 (65%) of the respondents are female and the remaining 68 (35%) are male. Most of the respondents used body care products, especially shower gel or body wash (84%) while about 80 respondents use body scrub. Respondents tend to choose their product based on brand (61%), especially a well-known brand, as they are more familiar with it [19]. In addition, respondents also purchased products by looking at its ingredients (54%). Given that, respondents wish to have more products with natural ingredients (25%) from existing body care products. Next, 108 respondents (55%) might be willing to try new products from new company especially if the price is within their budget. The future development of pineapple body scrub may be risky due to the lack of a brand. It is also not the essential body care product among respondents, but with appealing features such as natural ingredients and a fair price, there could potential for the product. Respondents preferred pump packaging (46%) compared to tube (39%) and jar packaging (14%). Pump packaging allows easier access to product and has good hygiene as buyers do not need to make direct contact with the product while using it. As for formulation of body scrub, half of the respondents preferred cream form (50%) compared to gel form (46%). Cream has higher physical stability which increases effectiveness of pineapple powder [20]. Cream form also has more moisturizing effect and lots of previous study has been done involving cream body scrub. Meanwhile, majority of respondents (60%) might be interested in pineapple body scrub, while 61 respondents (31%) were interested in pineapple body scrub products, where 77 respondents (39%) are willing to pay for RM10 - RM15 for 100g of pineapple body scrub. Hence, after conducting total cost spent for the experiment, it was observed that the pineapple body scrub can range at RM 5/100g without profit using ingredients and packaging preferred by the respondents. In Table 5, the results of the market survey are shown.

Table 5. Result for market survey based on majority of the respondents

Attributes	Result Based on Majority	Number of Respondents (%)
Gender	Female	128 (65)
Type of body care product used	Shower gel/Body wash	165(84)
Way of choosing body care product	Brand	119(61)
Suggestion of improvement for existing body care product	Natural ingredients	48(25)
Willingness to try new product	Maybe	108(55)
Preferred packaging for body scrub	Pump	85(46)
Preferred formulation for body scrub	Cream	98(50)
Interest in pineapple body scrub	Maybe	117(60)
Willingness to pay for pineapple body scrub	RM10-15	77(39)

Conclusion

The body scrub was formulated in cream form with pineapple waste as the natural exfoliator in pump packaging in accordance with the market survey. In the physical characterization test, S3 has the darkest color (L=51.59±2.39) and shows highest foamability (2.1mL within 30 minutes) among the body scrubs. The consistency is equal for all body scrubs. S0 has the highest pH value (7.97) among other samples, it also has highest viscosity (8120 cP) and highest spreadability (21411.74g). In sensorial characterization, the highest acceptance of pineapple body scrub for the odor with and without fragrance was S1, which is with fragrance (6.48) and without fragrance (5.50). S1 also showed the highest acceptance for nonirritant (6.68). S2 showed the highest acceptance for grittiness (6.45) and washability (7.18). S1 and S2 received the highest overall acceptance. The exfoliation capacity shows the significance value ($F_{3,116}=6.612$, $p<0.05$) between S0, S1, S2 and S3. In conclusion, the body scrub with pineapple waste as natural exfoliator was successfully developed and accepted among the respondents. Body scrubs with 3.5% and 4.5% pineapple powder concentrations are recommended for future development. Besides, it is recommended to observe the shelf-life of body scrub at different temperature for future consideration.

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A REVIEW ON CONVERSION OF MICROPLASTICS INTO VALUE-ADDED PRODUCTS: CHALLENGES AND PERSPECTIVES

(Ulasan Tentang Penukaran Mikroplastik Kepada Produk Nilai Tambah: Cabaran dan Perspektif)

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Abstract

Microplastics have emerged as a pressing environmental concern, exerting profound impacts on ecosystems, water bodies, terrestrial landscapes, and human food sources. In light of the global plastic waste crisis, innovative strategies are being explored to manage and recycle plastic waste, with an emphasis on microplastics. Research endeavours aimed at transforming waste microplastics into valuable resources align seamlessly with circular economy principles. Microplastics can be collected using surface water sampling, air sampling, sediment sampling, soil sampling, shoreline sampling, as well as wastewater and effluent sampling. Microplastics can be chemically and physically characterised for composition selection and then converted using biological, chemical, and mechanical approaches. Biological conversion involves microbial activity and enzyme utilisation, chemical conversion involves chemically breaking down polymers into smaller molecules that can be used as feedstock for valuable materials, while mechanical conversion applies physical force to reduce polymer size. Both conventional and biodegradable plastics can undergo biological, chemical, and mechanical recycling to an extent to maintain their value and prevent the waste of non-renewable resources. However, there are challenges to overcome in the conversion of microplastics, including cost-effectiveness, scalability, environmental friendliness, and regulatory considerations. Appropriate macroplastic management and life cycle assessment analyses are still crucial for transitioning to a sustainable and circular economy.

Keywords: microplastics, conversion techniques, value-added products

Abstrak

Mikroplastik telah muncul sebagai kebimbangan alam sekitar yang mendesak, memberikan impak yang mendalam terhadap ekosistem, badan air, landskap terestrial, dan sumber makanan manusia. Dalam konteks krisis sisa plastik global, strategi inovatif sedang dikaji untuk mengurus dan mengitar semula sisa plastik, dengan penekanan pada mikroplastik. Usaha penyelidikan yang bertujuan untuk mengubah mikroplastik sisa menjadi sumber daya bernilai selari dengan prinsip ekonomi bulat. Mikroplastik boleh dikumpulkan melalui pengambilan sampel air permukaan, pengambilan sampel udara, pengambilan sampel sedimen, pengambilan sampel tanah, pengambilan sampel garis pantai, serta pengambilan sampel air sisa dan air buangan. Mikroplastik

boleh dicirikan secara kimia dan fizikal untuk pemilihan komposisi, dan kemudian diubah menggunakan pendekatan biologi, kimia, dan mekanikal. Penukaran biologi melibatkan aktiviti mikrob dan penggunaan enzim, penukaran kimia melibatkan pemecahan kimia polimer menjadi molekul yang lebih kecil yang boleh digunakan sebagai bahan mentah untuk bahan bernilai, manakala penukaran mekanikal menggunakan daya fizikal untuk mengurangkan saiz polimer. Plastik konvensional dan plastik terbiodegradasi boleh mengalami kitar semula biologi, kimia, dan mekanikal untuk mengekalkan nilai mereka dan mengelakkan pembaziran sumber tidak boleh diperbaharui. Walau bagaimanapun, terdapat cabaran yang perlu diatasi dalam penukaran mikroplastik, termasuk kos-efektif, skalabiliti, kemesraan alam sekitar, dan pertimbangan peraturan. Pengurusan makroplastik yang sesuai dan analisis penilaian kitar hidup masih penting untuk bergerak ke arah ekonomi lestari dan bulat.

Kata kunci: mikroplastik, teknik penukaran, produk bernilai tambah

Introduction

Microplastics have ubiquitously permeated the ecosystems of the planet, penetrating the atmosphere, water bodies, terrestrial regions, and even human food sources, signifying a critical predicament of global contamination [1-4]. Microplastics, small plastic particles less than 5 millimetres in size, are at the forefront of significant environmental concerns and thus have been intensively studied to understand their abundance, distribution, and sources [5-7]. The various sources of microplastics include fragmentation of larger plastic items, personal care products, textiles, industrial processes, and plastic resins used in manufacturing [8, 9]. In addition, the potential impacts of microplastics on aquatic organisms include feeding, growth, reproduction, as well as bioaccumulation of environmental pollutants from microplastic-pollutant complexes due to the vector ability of microplastics [10-13].

The persistence of microplastics in the environment and their potential to cause adverse ecological and health effects have escalated the search for sustainable solutions to address their accumulation. Research into the waste utilization of microplastics for value-added products is a new cutting-edge field being presently considered and expected to further intensify, staying abreast with the presence of multiple driving forces from international, national, environmental, economic, and social aspects [14]. The global plastic waste crisis has reached an annual microplastic waste generation of 3 million tonnes [15], leading to increased international and national interests, such as UNEP, in finding innovative ways to manage and recycle plastic waste, including microplastics [16]. Resource scarcity and the circular economy are also at the leading edge of the environmental drives propelling research in utilising waste plastics [17]. While the extraction of virgin materials and fossil fuels for manufacturing is resource-intensive and can contribute to environmental degradation, research into converting waste microplastics into value-added products can help to reduce resource consumption and thus align with the principles of the circular economy [18]. From an economic viewpoint, industrial opportunities arise from transforming microplastics into valuable materials, generating revenue from value-added products, reducing costs associated with waste disposal, and developing new markets for recycled materials [19, 20]. Consumer and industry demand for environmentally friendly and sustainable products is growing due to the plastic-free movement and public awareness of plastic pollution and environmental issues [21]. Additionally, the conversion of microplastics can occur through biological, chemical, and mechanical conversion techniques but research is lacking compared to macroplastics and conventional plastics [22].

Therefore, in the pressing quest to address global plastic pollution, the efficacy of converting conventional microplastics into value-added products presents a pivotal state-of-the-art juncture in sustainable resource management and environmental mitigation. This article reviews the collection, physical and chemical characterisation, biological, chemical, and mechanical conversion methods, potential value-added product applications, and environmental considerations associated with conventional microplastics.

Collection and characterisation of conventional microplastics

Supply chain management is essential for successful waste material manufacturing by coordinating the flow of materials from waste collection to final product manufacturing [23]. In other words, microplastic collection methods are vital for adequate material

supply in manufacturing and vary depending on the environment where microplastics are collected. In microplastic research, microplastics are commonly collected using manta trawl, grab sampling, and bulk water filtration targeting marine and freshwater [24, 25]. In general, major collection methods include surface water sampling, air sampling, sediment sampling, soil sampling, beach and shoreline sampling, wastewater and effluent sampling, and biota sampling (Table 1) [26-29]. Surface water sampling involves collecting water samples from rivers, lakes, and oceans, and filtering them to separate microplastics [27, 29]. Sediment sampling isolates microplastics from water bodies, while air sampling collects airborne particles [26, 30]. Biota sampling for microplastics has been used to examine organisms like fish and shellfish to determine ingestion occurrence or their ingestion rates on microplastics [29, 31]. Soil sampling extracts microplastics from sources like plastic mulch in agriculture or atmospheric deposition [28], while wastewater and effluent sampling at wastewater treatment plants provides insights into microplastic presence and fate in waste streams [32]. Additionally, beach and shoreline samplings involve manually picking up debris or using specialised devices to collect microplastics [33].

Table 1. The method types, advantages, and limitations of common collection approaches for microplastics

Methods	Advantages	Limitations	References
Surface water sampling	• Provides information on plastic distribution. • Comparatively simple and widely used.	• Restricted to surface-bound microplastics. • May underestimate the subsurface microplastic amount.	[24] [25] [29]
Air sampling	• Helps assess the microplastic amount in the atmosphere. • Non-invasive and can cover large areas.	• Difficult to distinguish microplastics from other particles. • Limited information on microplastic sources.	[26]
Sediment sampling	• Gives information on the accumulated history of plastic. • Allows study of buried microplastics.	• Time- and effort-consuming. • Sediment and benthos disturbance during sampling.	[30]
Soil sampling	• Gives information on plastic contamination of terrestrial ecosystems. • Can evaluate the uptake potential of plants.	• Microplastics might be mixed with natural particles. • Separating microplastics from organic matter is challenging. • Limited standardized protocols.	[28]
Beach and shoreline sampling	• Direct observation of shoreline plastic pollution. • Aids in understanding the ocean-to-land plastic transfer.	• Only a small portion of all marine microplastics are captured. • Limited to accessible shorelines. Microplastics may not be as noticeable if macroplastics are present.	[33]
Wastewater and effluent sampling	• Perspectives on plastic input from different sources. • Allows targeting of pollution sources.	• Variation in microplastic loads and types in different effluents. • Small particle detection in complicated matrices is difficult. • Limited data on microplastic fate after release.	[32]
Biota sampling	• Identifies potential harm that microplastics could cause to ecosystems. • Links plastic pollution to higher trophic levels.	• Invasive on living organisms. • Requires specialized techniques for extraction and analysis. • Limited species-specific data. Distinguishing environmental particles from ingested microplastics is challenging.	[31] [29]

Among the microplastic collection methods listed, air sampling and surface water sampling can be considered relatively clean and hygienic with less time and effort consumption, since air and water samplings mainly

involve collecting particles that are airborne, in marine, or freshwater environments, reducing the risk of direct contact with potentially contaminated materials. However, the process faces numerous challenges, such as microplastics from wastewater and effluent for conversion into food packaging or clinical applications may require additional treatment for consumer safety, the polymers making up microplastics contain chemical additives such as plasticizers, flame retardants, and antimicrobial agents. These additives need to be discarded priory the conversion process. The wide range and intricate nature of microplastics in terms of their types, shapes, sizes, colours, compositions, and additives pose challenges in their separation, classification, and characterization. In real environment, the low quality and purity of microplastic waste, potentially harboring contaminants like organic matter, metals, pathogens, and other pollutants, pose risks to the functionality and safety of the resultant products. Biota sampling involves not only contact with biological samples that may have been infected but also the dissection of living organisms [34, 35]. On the other hand, sediment sampling, soil sampling, beach and shoreline sampling, may have varying levels of cleanliness, hygienic conditions, and time and effort consumption (e.g., separation of sediment and microplastics) depending on the specific circumstances and precautions taken during the sampling process. Sample processing includes collection, transportation, storage, filtration, separation, and extraction techniques (Figure 1).

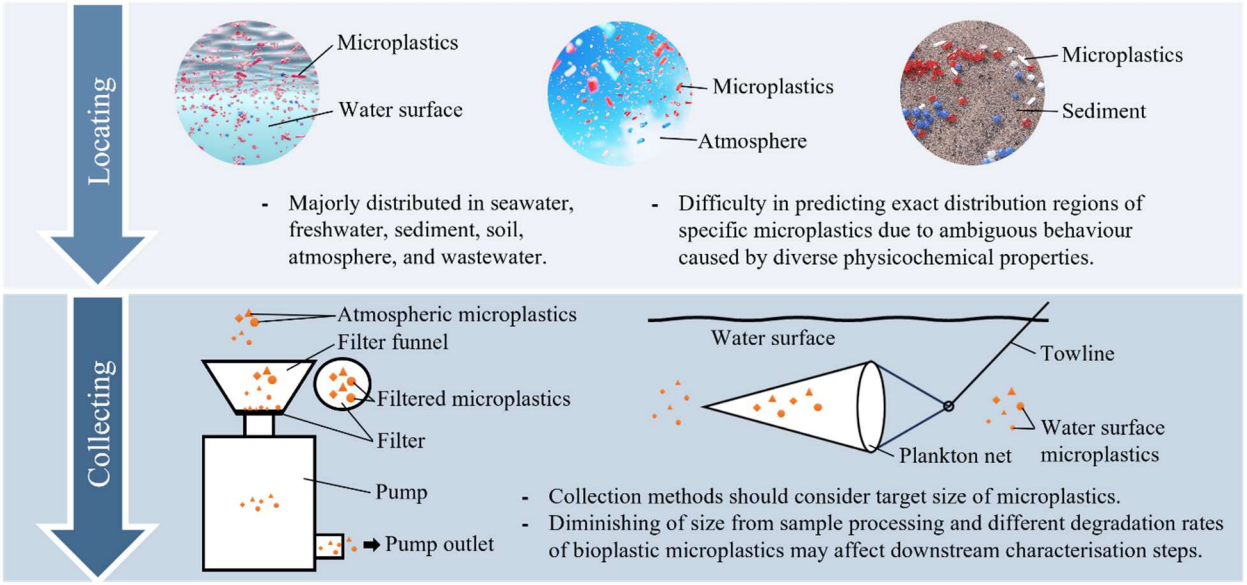


Figure 1. General conceptual illustration of collecting microplastics [26-29, 36-40]

In microplastic conversion to value-added products, additional microplastic characterisation steps are required to categorise or selectively collect microplastics according to their composition, using fluorescent spectroscopy, Fourier-transform infrared spectroscopy (FTIR), micro-Raman spectroscopy, mass spectrometry, chromatography, pressurized fluid extraction (PFE), density separation, and fluorescence microscopy via hydrophobic fluorochrome dye staining (e.g., Nile red staining) to verify the composition or properties of the collected microplastics. Presently, a blend of physical methods such as microscopy and chemical methods like spectroscopy is extensively employed for microplastic examination. This strategy reduces the occurrence of inaccurate positive and negative results. [41-44]. In plastics manufacturing, identical resin compositions are crucial for homogenous products as variations in these compositions can lead to defects, inconsistencies, or undesirable characteristics, affecting the appearance and performance of the final product [45, 46]. For industrial conversion of microplastics to value-added products, rapid collection and characterisation methods of high capacity are required to channel the microplastics to the appropriate manufacturing techniques and product types.

Physical characterisation

Physical characterization of plastic plays a significant role in determining and better understanding the properties of a plastic polymer including to determine the optimum surrounding condition to catalyse high-efficiency biodegradation of the plastic polymer and to ensure a higher quality and reliability of the plastic product that can adapt to a variety of applications. Due to the complicated nature and properties of the polymer, specific tests are required for each unique characteristic of each polymer and among the physical properties that are commonly tested and measured are comprising of mechanical properties such as flexural rigidity, tensile strength, hardness and wear and tear resistance [47]. Tensile strength of a plastic polymer is described and tested through the application of the maximum tensile stress before the polymer is fractured into fragments which are commonly utilized in load-bearing applications. According to Sola et al. [48], the tensile test is one of the commonly used mechanical tests in the industrial sector due to multiple factors such as relatively short, simple procedures and lack of in-depth requirements of research and development allowing industrial players to test for tensile strength without spending an extra cost for external services.

As the world has reached a point of a global boiling era as mentioned by the UN secretary general, studying the effect of heating of plastic to the polymer and its degradation properties have never been more relevant as the temperature of the global surface increasing at an alarming rate since the past decade [49] (Niranjan, 2023). Thermal-based analysis of polymers comprises a few analyses that are thermogravimetric analysis (TGA), differential thermal analysis (DTA), differential scanning calorimetry (DSC) and evolved gas analysis (EGA). As summarized by Ng et al. [50], TGA functions to analyse mainly the thermal stability, properties of composites and oxidative stabilities of polymer samples. The above-mentioned thermal analysis: DMA are reported to study the viscoelastic properties of polymeric samples that would further complement the TGA analysis. Another similar analysis that applies the thermal-based analysis is Melt Flow Index Measurement (MFI), which studies the process of thermoplastic polymers by measuring of flow of molten plastic [51]. This process is crucial for the application of moulding injection as well as extrusion processes.

Chemical characterisation

Polymers regardless of size have their respective monomers, linkages, reactive chains, or end-groups that determine their characteristics that could enable the creation of polymer networks reliably and repeatedly with desired features. Through chemical characterization, researchers can unveil surface functionalities, including oxidation, hydrophobicity, and adsorbed substances. Moreover, they can understand changes in polymer structure resulting from UV exposure, mechanical forces, and microbial activity. [52, 53]. There are many methods for identifying objects and determining their chemical composition as shown in Figure 2 [54]. SEM-EDS aids in quick differentiation between non-plastic and plastic pellets and can find microscopic particles that are missed by optical inspection. EDS was used to determine the qualitative elemental composition of the microplastic surface. In addition, tool plays a critical role in the identification of pollutants linked to microplastics, such as toxic metals. [55, 56].

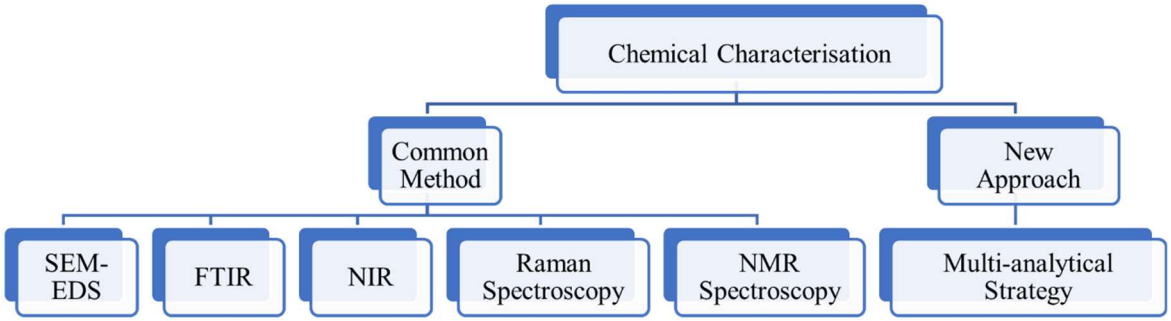


Figure 2. Methods in chemical characterisation

The most popular method for locating microplastics is Fourier-transform infrared (FTIR) which is typically used to determine the polymeric composition of the microparticles, while Raman spectroscopy since it is straightforward, reliable, and non-destructive [52, 57-59]. However, the detection of domain differences within a sub-micrometre region with traditional Raman or FTIR spectroscopy offers average chemical information at a bulk area with very limited spatial resolution, which may restrict the analysis of nanoplastics that are smaller than the spatial resolution [60, 61]. The use of hybrid approaches, namely atomic force microscopy-Raman (AFM-Raman) and AFM-IR techniques, have been considered to map and identify composites at the nanoscale on sample surfaces. AFM has the ability to function in various modes, such as contact mode, tapping mode, or phase mode, which are determined by the force feedback type and the probe's oscillation frequency. [62, 63]. Apart from that, NIR spectroscopy also can be used to characterize and differentiate microplastics and is capable of seeing deeper into the plastic compared to other spectroscopy methods [54, 64]. Quantitative ^{1}H NMR spectroscopy gives a way to identify microplastics both qualitatively and quantitatively which is also a cost-efficient, quick, and easy technology that provides a significant advantage over other detection techniques. In fact, this technique is non-destructive and non-invasive, eliminating the need for sample preparation or solvents. [65-67]. Thermal degradation of MPs has been discovered to be advantageous since it can simultaneously identify the type of polymer and detect chemical components, additives, and gases in the intricate formulation [68]. Thermal degradation includes pyrolysis as well as GC-MS where the product of pyrolysis (heating and melting of the microplastics) will undergo GC-MS for separation and detection of compounds present. This method has the capability to address certain drawbacks of spectroscopy approaches, including low detection limits, matrix interferences, and sample heterogeneity. [69, 70].

Analysis of MPs in environmental samples is hampered by microplastic contamination from the sampling and laboratory environments, which can affect both qualitative and quantitative determination that includes the most popular methods [71]. A series of newly created analytical protocols for the precise and selective detection of the polymers, along with specialized methods that include extraction of polymer using different solvents, hydrolytic depolymerization, purification, and quantification procedures that are tailored for condensation polymers, pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS), and followed by physical characterization process [72]. ^{1}H NMR spectroscopy and the thermal desorption gas chromatography-mass spectrometry (TD-GC-MS) technology were used in a multi-analytical strategy to characterize the microplastics in different degradation times. Utilizing multiple strategies can surpass the limitations of each individual method, offering a more thorough and dependable insight into the characteristics of microplastics, including particle number, size, shape, mass, composition, and additives. [73, 74].

Conversion of conventional microplastics

As the development and applications of plastic have revolutionized the modern-day world, the constant increase in the usage of plastic in daily life has raised environmental concerns as plastic waste persists in the environment longer than intended. The valorisation of plastic waste, conventional plastic, shows a prospect in supporting the circular economy model [75]. Conversion of conventional microplastic to added value products takes the recycling principle to another level which is not just the recycling of the product itself but down to the monomer recycling of the said products through depolymerization of microplastic. Depolymerization of the polymer allows the monomeric products to be recycled to produce different yet environmentally friendly and economically valuable products closing the loop of recycling [76].

Research regarding the conversion of waste products to added value has been done extensively in the past decade as the valorisation of waste offers a prospective solution supporting the circular economy [77]. The waste-to-energy conversion technology involves the utilization of waste products to produce a variety of fuels that can be further used to generate other products and maintain a sustainable energy supply (Figure 3) [4]. Among the pathways discovered to increase the conversion of waste products into added value are biological, chemical, and mechanical pathways. Biological conversion utilizes the natural ability of microorganisms to further recycle the end-of-use plastic to produce simpler compounds that can be

recovered [78]. In addition, pyrolysis has gained popularity recently as the chemical pathway of conversion of plastic waste toward the production of gasoline, diesel and oils via heating of waste materials in the absence of oxygen [79]. Mechanical conversion of waste to value added products on the other hand, involves the change in the physical state of the waste products via the shredding and grinding process without altering the chemical or biological structure of the waste products [80].

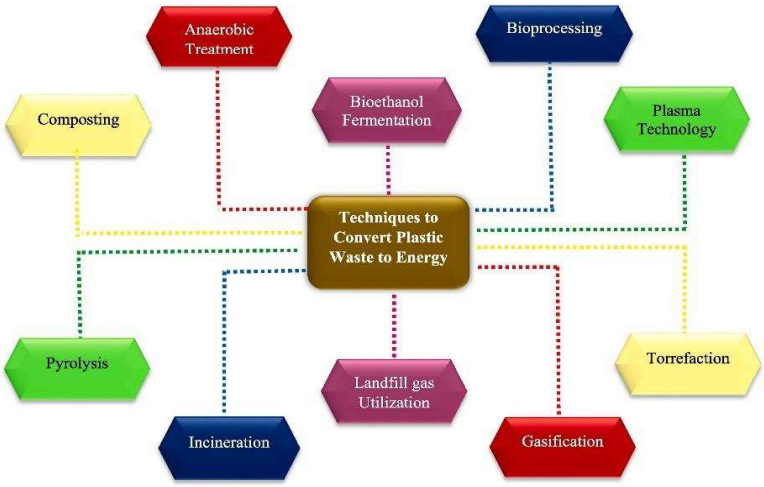


Figure 3. Techniques to convert plastic or microplastics waste to energy

Biological conversion

In general, the bioconversion of traditional biowaste such as agricultural, domestic, and industrial waste to value-added products comprises four primary conversion pathways, microbial, enzymatic, fermentation, and composting conversion pathways [81]. In this review, the valorisation of plastic waste through bioconversion pathways will be further explored. Biological conversion of plastic waste refers to the recent advances of innovative strategies for adding value to end-of-life plastic-based products by exploiting the fundamental abilities of the microbial factory [82]. Conventional petrol-based polymers are categorized into two groups depending on their chemical structure. The two categories of petrol-polymer are polymers that possess hydrolysable ester bonds and functional groups of esters and amides (PET and PUR) that have higher biodegradability compared to non-hydrolysable (PE, PS, PP, and PVC) which possess a stable carbon chain backbone [23, 78]. Recent advances in biological plastic conversion comprise two main elements: plastic-degrading microorganisms and enhancing enzymes for degradation. Both elements play a crucial part in the process of bioconversion of plastic to obtain the desired end-products. The efficiency and productivity of biological conversion of plastic waste into substrate depends on multiple factors such as the type of plastic-degrading microorganism used, depolymerization through enzymatic reactions and end-product value [23, 83].

Plastic-degrading microorganisms

Studies on the utilization of microorganisms in the degradation of plastic waste have been extensively done in the pursuit of a sustainable approach to plastic waste solutions. In a study conducted by Yoshida et al. [84], reported that *Ideonella sakaiensis* 201-F6 can produce enzymes with the ability to degrade PET into terephthalic acid (TPA) and ethylene glycol (EG) which can be assimilated for metabolic activity. It has been found that *I. sakaiensis* is capable of surviving on PET alone as a carbon source whilst utilizing two main enzymes that are PETase and MHETase [85, 86].

The genus *Pseudomonas* sp. is also among the most researched bacteria due to its versatility in degrading a wide range of polymers. A study conducted by Lee et al. [87] showed that *Pseudomonas aeruginosa* isolated from the guts of superworms exhibit biodegradability properties towards four synthetic plastics, PE, PS, PPS and PP with the fastest degradation on the PE type plastic. The terrestrial bacterium *Bacillus subtilis* possesses synthetic degrading abilities and has been

reported to efficiently degrade conventional plastic type PET with the rate of 9.26% in 30 days which supports biodegradation of conventional plastic through microbial factory [88]. To date, there are currently 949 microorganisms associated with plastic according to the plastic microbial biodegradation database (PMBD) [89].

Enzymatic depolymerization

Degradation of synthetic oligomers and polymers by enzymatic activity commonly involves two categories of enzymes that are hydrolases (e.g., esterases, lipases, cutinases, proteases) and oxidases (e.g., dehydrogenases, laccases) [90]. Depolymerization functions to chemically breakdown polymers from macromolecules to lower compounds of oligomers and monomers through the process of enzymatic activities such as hydrolysis, oxidation, or hydroxylation that will allow the reuse of these monomers through repolymerization process [78, 91]. For example, conventional plastic such as PP, PE, and PVC poses similar structure of C-C as the main chain that can be further turned into various type of fatty acids through the act of enzyme activity [75, 92, 93]. In a different study by Zhang et al. [94], conventional plastic PS can be converted into aromatic monomers, which leads to the oxidation of styrene epoxide catalyzed by the enzyme styrene monooxygenase (SMO) or styrene dioxygenase (SDO).

The biodegradation of PET employs a two-enzyme system which utilizes the PETase to hydrolyze PET into TPA, EG and MHET and MHETase enzyme to further hydrolyze MHET into TPA and EG [84, 86, 95]. To date, most PET-degrading enzymes have been found in two main genera of bacteria, *Pseudomonas sp.* and *Bacillus sp*. While other bacterial strains produce PET degrading enzymes such as *Brevibacillus parabrevis, Staphylococcus aureus, Streptococcus pyogenes* and *Clostridium thermocellum,* research studies have shown that the usage of microbial consortia comprising *Pseudomonas sp*. and *Bacillus sp.* have proven to have increased degradation efficiency of PET polymer [96, 97].

Chemical conversion

Chemical conversion or chemical upcycling approaches for converting conventional microplastics into value-added goods are gaining popularity as a potential solution to the world's plastic pollution problem. It involves a process that breaks down the polymers into smaller molecules which could then be used as a feedstock for a variety of valuable chemicals and materials. Microplastics are microscopic plastic particles, frequently less than five millimetres in size, found in a variety of environmental compartments such as oceans, rivers, and soil. Converting these microplastics into valuable materials has the potential to minimize their environmental impact while also providing economic rewards. This section briefly discusses the two techniques of chemical upcycling widely studied over the years.

Depolymerization

Depolymerization is a process where long polymer chains are broken down into small monomer units allowing new products to be synthesised using these monomers. Research was done to investigate the techniques of depolymerization such as using transition metals [98], enzymes [99], catalysts [100], electrocatalysis [101] and more. In 2012, a team of researchers used a PNP-Ru pincer complex (Ru-1, 0.1 mol %) as a catalyst to perform the first hydrogenative depolymerization of waste poly(propylene carbonate) (PPC) which yielded 1,2-propylenediol and methanol [102]. Besides, Mao et al. [101] reported the utilization of electrocatalytic upcycling of PET microplastics to formate, terephthalic acid and potassium sulphate using a novel catalyst Mn0.1Ni0.9Co2O4-δ rod-shaped fibre (RSFs) catalyst. Recently, enzymes are being used to facilitate the hydrolysis of amides, esters, or urethane bonds within microplastics, such as polyamides, polyesters, or polyurethanes, for the generation of monomers or oligomers, which can subsequently undergo analysis using techniques includes chromatography or spectroscopy. In addition, fungi are introduced to secrete various digestive enzymes and proteins that attach to the polymer surface, penetrate it, and degrade macromolecules into small organic compounds, such as carbon dioxide, water and methane [103, 104].

Pyrolysis

Pyrolysis is a thermochemical technique that decomposes waste materials into energy products such as liquid oil and gases in hypoxic conditions and at high operating temperatures ranging from 400 to 800°C [105, 106]. A myriad of studies has been

conducted on the conversion of microplastics to value-added products via the pyrolysis technique. Nabgan and coworkers [107] investigated the potential of utilizing microplastic wastes to produce value-added products using the pyrolysis-catalytic steam reforming technique. They synthesized the nano-catalyst, Ni-Pt/Ti-Al, and loaded it into the pyrolysis reactor before introducing the phenol-dissolved microplastics into the fixed-bed continuous flow quartz reactor at 500 – 700°C at standard atmospheric pressure. Several liquid oils were generated such as 1,2-bis(trimethylsilyl)benzene, bis(2-ethylhexyl) phthalate, cyclohexane-1,3-dione, 2-allylaminomethylene-5,5-dimethyl- and more. Agriculture is one of the largest plastic waste producers. The generated waste plastic mulching films are normally disposed of conventionally (incineration and landfilling) which introduces greenhouse gases and microplastics in the ecosystem [108]. Because of this Jung and coworkers [108] investigated the disposal of the mulching films through a CO_2-assisted catalytic pyrolysis. It was reported that hydrogen gas, short-chained and long-chained hydrocarbons were formed through thermal degradation of the mulching films. When a Ni/SiO_2 catalyst was used the hydrogen gas production skyrocketed to 100 times more. Furthermore, when CO_2 was introduced as a flow gas CO production was observed.

Gasification

Gasification is a technological process that converts any carbonaceous (carbon-based) raw material into synthesis gas (syngas). Gasification takes place in a gasifier, which is typically a high temperature/pressure vessel in which oxygen (or air) and steam come into direct contact with the coal or other feed material, resulting in a sequence of chemical processes that convert the feed to syngas and ash/slag (mineral wastes) [109]. The gasification of polypropylene was studied by Wang et al. [110]. They compared the effects of the presence of a catalyst with none. Expectedly, the catalyst (Ni/Al_2O_3) set-up has vastly improved the yield of H_2, CO, hydrocarbons and total syngas by 137%, 38.2%, 98.2%, and 44.1%, respectively. Not only that, Moghadam et al. [111] conducted a study on syngas production from palm kernel shells and polyethylene wastes via the gasification process. The paper reported that a co-gasification process under optimized conditions was able to yield 422.4 g/kg feedstock and 135.27 g/kg feedstock of syngas and H_2, respectively.

Mechanical conversion

Mechanical conversion is the mechanical force applied as a catalyst to reduce the molecular weight of the polymer [112]. Properties of plastics can be classified in terms of weight, hardness, tensile strength, thermal properties, electrical properties, and chemical properties [113] (Alauddin et al., 1995). Thus, breaking the plastic with external mechanical force could break and change those properties to some level. A study highlighted that mechanical conversion affects the structure of the polymer and reduces the quality of the product in terms of mechanical properties regardless of the type of the polymer [114]. Yet, mechanical recycling is a practical method for maintaining plastic's inherent worth, preventing the waste of non-renewable resources [115].

Grinding and milling techniques

Milling in general is a technique or process of cutting or grinding material using a rotary cutter and incorporates the conversion of mechanical energy into thermal energy and was the first technique to produce microplastic particles [116, 117]. The milling and grinding process is a crucial step before thermal conduction [118]. Meanwhile, a study by Eitzen et al [116] shows that longer cryogenic milling and pre-cooling duration could produce small particles of polystyrene (PS).

Cryogenic grinding with a wet-sieving technique could enable maximum yield of the polyvinyl chloride (PVC) and smaller microparticles could be obtained if the microparticles undergo 2 cycles of the grinding process [119]. There is also a new grinding technique that has been revealed which is the melt grinding technique that has the advantage of not having drying steps in the usual grinding technique where the time consumption was high [120]. Cryogenic milling and grinding are the processes that incorporate low temperatures to overcome the issue mentioned before such as heating despite milling or grinding conventional microplastic as each type of microplastic has respective characteristics that differ by physical and chemical properties. In conclusion, the grinding and milling technique is the early stage before the plastics are further proceeded for extrusion and injection moulding

as smaller plastics can be melted in large quantities in a shorter time compared to large plastics.

Extrusion and injection moulding

Injection moulding is a technique where injecting material into a mould with high temperature and pressure in a closed system [121], meanwhile, extrusion is a technique of pushing out a material through an opening with the help of an extruder [122]. The extrusion and injection moulding of conventional plastic can cause issues such as degradation of their features and usability arise when a combination of heat and mechanical shear affects the recycling of both post-industry and post-consumer plastics [123]. Changes in chain length and mechanical characteristics are a constant problem, even though the methods of degradation vary among polymers, especially upon thermal conduction [124]. For instance, the thermomechanical process results in a drastic reduction in poly (ethylene terephthalate) (PET) characteristics [125]. Also, polyolefins that include HDPE, LDPE, and PP shows varied mechanism; HDPE and LDPE have a high probability in chain branching, but PP has a high probability in chain scission upon extrusion [124, 126]. Extrusion is best for long, uniform products and is cost-effective for mass production. Injection moulding suits complex, irregular items with precision for custom orders. Unlike extrusion, it handles both thermoplastics and thermosets [127]. Figure 4 shows a combination of mechanical and chemical technique to convert plastic waste into value added products such as fuels.

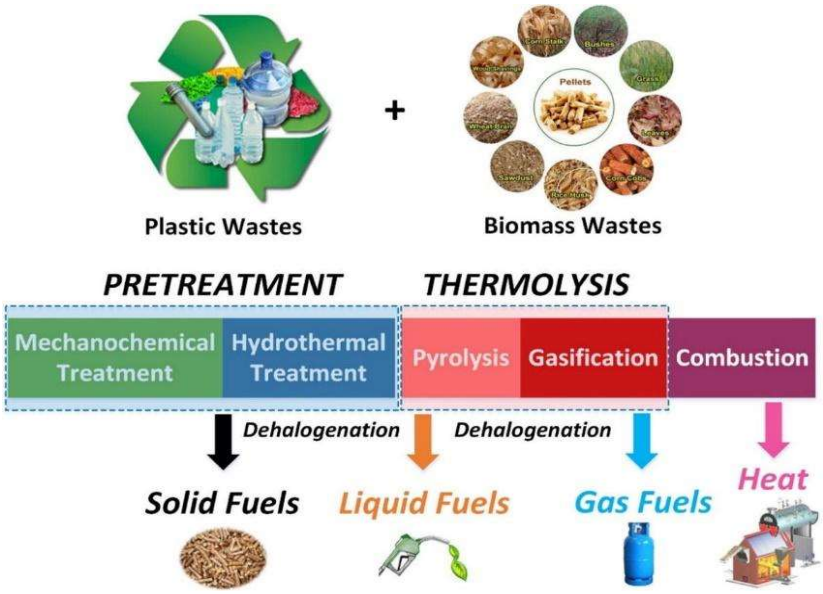


Figure 4. Mechanical and chemical treatment of valorisation of plastic waste

Potential applications of value-added products

The transformation of microplastics into value-added products requires consideration of material properties, process selection, waste utilisation, and environmental concerns. Material properties, such as composition, size, and shape, play a significant role in determining the suitability of waste materials for specific manufacturing processes [128], as well as the compatibility between waste materials and other components or additives for proper integration and performance since the properties of manufactured products should meet the required standards and specifications for their intended applications [129]. The selection of conversion and manufacturing processes is dependent on available resources and technologies, where process optimization and innovation are required to improve efficiency and sustainability. In microplastic research, the state-of-the-art focus has presently pivoted towards the waste utilisation, material properties, conversion process selection, and environmental implications of all types of microplastics [20, 106, 129]. These leading-edge endeavours underscore the imminent potential to transform microplastics into a range of valuable products, emphasizing the timeliness and urgency of such innovative pursuits.

Utilizing nature's process of breaking down complex compounds into simpler compounds mechanism in the innovative approach of recycling plastic waste has opened a new door towards plastic waste management. The biological approach to converting plastic waste through the coupled act of plastic-degrading bacteria and enzymatic degradation has shown promising results. For example, the intermediate produced from enzymatic reactions of PETase and MHETas can be used for further metabolic reactions, in PHA polymer production through microbial fermentation [78, 97, 130]. Enzymatic hydrolysis of plastic waste, including impurities from PLA-PET, is being researched as an eco-friendly method of bio-recycling plastic trash. Recent studies suggest an innovative hybrid approach merging thermal and electrochemical techniques to convert both plastic waste and biomass into exceptionally pure hydrogen and carbon nanotubes. Moreover, the method introduces a carbon capturing process using a molten salt, which can subsequently be transformed into valuable carbon nanotubes through electrochemical means powered by renewable energy sources. [131, 132].

Challenges associated with the conversion of microplastics to value-added products

Regulatory and policy considerations

Plastic is the main material used for packaging and textiles in various sectors. Excessive plastic usage has led to tremendous plastic waste generation. According to Tiwari et al. [133], the top three countries that produce the most plastic are in Asia within the Indo-Pacific region, namely India, China and the Philippines. Many countries have established regulations, action plans and policies to regulate and control plastic waste generation (Table 2) Despite many regulations that have been enforced, the accumulation of plastic usage at the rate of 25 million tonnes per year continues due to their persistence in the environment [134]. In addition, in certain countries, the mitigation plans and regulations are mostly focusing on plastics but not microplastics. Today's microplastic is a global issue since it can be found everywhere. Vectors for drugs in medical sectors and microbeads in beauty care products are known as primary microplastics, while secondary microplastics are tiny plastics deteriorated from primary microplastics [135, 136].

As a result of growing concern towards these tiny plastic particles, few countries have imposed national legislation to combat microplastic specifically (Table 3). All these plastic grains eventually end up in the ocean, posing major threats to the ecosystem, where they have been reported to be found in all levels of aquatic environments [137, 138]. Microplastics that are consumed by fish will eventually contaminate the human body through seafood consumption [139]. Consequently, this sparks a growing number of research and publications on the effects of microplastic towards fish and human beings. Kutralam-Muniasamy et al. [140] indicated that the actual consequences of microplastics are merely understood scientifically. In a recent study, Carrington [141] reported that microplastic has been detected in human blood for the first time. Although the impacts of microplastic in the human body are still being explored, a solution to substitute conventional plastic with an eco-friendlier alternative is urgently needed.

Fundamentally, reducing macroplastic usage through prohibition and restriction use of plastics will reduce the presence of microplastic in the ecosystem. However, Mitrano et al. [142] stated that it is unclear whether technical bans can help in the reduction of microplastic. They opined that proper macroplastic management is the ideal way to reduce microplastic as a transition towards a circular economy. An amendment to the government framework is suggested to focus on waste utilisation by repurposing and converting the waste into other products. Although many regulations were in place, microplastic pollution is hard to solve, so the focus should be concentrated on the utilisation of microplastic into value-added products, to address the circular economy concept and to comply with the Sustainable Development Goals 6, 7, 12, 13, 14, 15. We need specific guidelines, regulations and policies to regulate and control microplastic pollution, by regulating microplastic production, supporting microplastic conversion into value-added products, and encouraging a circular economy that reduces the usage of raw resources.

Table 2. Policies and regulations on plastic waste established by countries

Year	Country	Regulations
2016	India	Plastic Waste Management Rules (2016)
2008	China	China State Council had issued a Notice restricting the Production and Sale of Plastic Shopping Bags
2020	China	National Development and Reform Commission (NDRC) and the Ministry of Ecology and Environment (MEE) released an "Opinions on Further Strengthening the Control of Plastic Pollution
2022	Philippines	Extended Producer Responsibility Act (EPRA) 2022
2022	Philippines	Department of Environment and Natural Resources (2020) has banned the usage of unnecessary single-use plastics in offices under the National Solid Waste Management Commission.
2011	Italy	Banned traditional plastic bags and substituted them with biodegradable bags
2012	Sweden	Started to focus on the replacement of fossil-based raw materials with bio-based raw materials in their research and development needs
2013	Canada	Banned the usage of traditional plastic bags in Toronto
2023	United Arab Emirates (UAE)	Banned shopping bags that are not oxo-photodegradable.
2021	Australia	Published the National Plastics Plan 2021 to address and reduce plastic waste generation
2019	Scotland	Banned plastic cotton buds in 2019 and 2021, a ban was imposed on single-use plastic items
2018	Malaysia	Malaysia's Roadmap Towards Zero Single-use Plastics 2018-2030
2021	Malaysia	Ministry of Environment & Water (2021) published the Malaysia Plastic Sustainability Roadmap 2021-2030

References [143-150]

Table 3. Policies and regulations on microplastic established by countries

Year	Country	Regulations
2014	Netherlands	Ban the usage of microbeads in cosmetic products.
2015	United States (US)	Microbeads-Free Waters Act of 2015
2017	Canada	Microbeads in Toiletries Regulations 2017
2016	France	Law n° 2016-1087 for the recovery of biodiversity, nature and landscapes
2018	Italy	General Budget Law 2018 no. 205 of 27
2017	New Zealand	Waste Minimisation (Microbeads) Regulations 2017
1998	Sweden	Ordinance (1998: 944) on prohibitions, etc. in certain cases in connection with the handling, importation and export of chemical products
2017	England	Environmental Protection (Microbeads) Regulations 2017
2018	Scotland	Environmental Protection (Microbeads) Regulations 2018
2018	Wales	Environmental Protection (Microbeads) Regulations 2018
2018	Northern Ireland	Environmental Protection (Microbeads) Regulations 2018
2023	United States (US)	National Strategy to Prevent Plastic Pollution proposed by the United States Environmental Protection Agency

References [151-158]

Future directions and emerging trends

In the future, there is a need for standardised methodologies for microplastic analysis, quantification, and classification. The accurate characterisation of microplastics is crucial for appropriate categorisation and isolation of microplastics before downstream conversion processing, hence escalating future research is expected on the development of advanced techniques that allow for rapid and precise analysis of microplastics. For example, method improvisations on mechanical recycling and biodegradation optimisation through specific designs of microbial consortia or enzymes could recover high-quality conventional microplastics and accelerate the breakdown process of

biodegradable microplastics. As innovative conversion technologies emerge, future microplastic research could include the development and integration of circular economy models into broader frameworks that incorporate microplastic recovery, conversion, and reuse, thus reducing the dependence on virgin plastics. While future research expansion is expected on the conversion of conventional microplastics to biofuels, the simultaneous production of biodegradable microplastics and biofuels from renewable resources might also be considered. In addition, future regulatory frameworks are also expected to encompass both conventional and biodegradable microplastics, ensuring that products claiming to be biodegradable undergo rigorous testing to validate their claims before "green" or eco-friendly labels are permitted. Policymakers must collaborate with researchers and industries to develop effective waste management strategies that prevent microplastics from entering the environment. By embracing a multidisciplinary and holistic approach, international and national collaboration among researchers, industries, policymakers, governments, and non-governmental organizations is paramount for driving meaningful progress in microplastic research via the exchange of knowledge, resources, and expertise.

Conclusion

This study explores the potential of converting conventional microplastics into value-added products by comprehensively exploring the collection, characterisation (i.e., physical and chemical), conversion (i.e., biological, chemical, and mechanical) methods, providing valuable insights into the potential of mitigating microplastic pollution while deriving value from these materials. The physical and chemical characterisation of microplastics highlights the distinctive attributes of conventional microplastics to tailor suitable conversion approaches based on the various compositions of microplastics. Biological conversion is a promising avenue, as it shows the ability of microorganisms to degrade both types of microplastics, suggesting the potential for bioremediation of microplastic pollution. Chemical and mechanical conversion pathways also offer routes to synthesize value-added products such as biofuels from microplastic materials. Furthermore, the exploration of potential applications of value-added products derived from microplastics underlines their potential to contribute to the transition towards a more sustainable and circular economy. Regulatory and policy considerations are also crucial, as the conversion processes may be subject to varying regulations, requiring coordination and adaptive governance structures. Moreover, challenges in the conversion processes, such as technical constraints and economic feasibility, emphasise the need for continued research and development efforts. Collaboration between academia, industry, and policymakers is essential in mitigating plastic pollution and fostering circular economies.

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INNOVATOVE ECO-FRIENDLY WOOD ADHESIVE FORMULATIONS: EXPLORING THE EFFICACY OF GELATINIZED CASSAVA STARCH AND POLYVINYL ALCOHOL CROSSLINKED WITH CITRIC ACID

(Formulasi Pelekat Kayu Mesra Alam: Inovatif Meneroka Keberkesanan Pati Ubi Kayu Bergelatin dan Alkohol Polivinil Berpaut Silang dengan Asid Sitrik)

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Abstract

In an effort to develop eco-friendly bio-adhesives for wood applications, this study explores the effect of citric acid as a new cross-linking agent on the properties of a cassava starch/polyvinyl alcohol (PVA)-based wood bio-adhesive. The bio-adhesive was synthesized in a three-step process: first, the hydrolysis of cassava starch using hydrochloric acid, followed by the oxidation of cassava starch with ammonium persulfate, and finally, the polymerization of starch and PVA with citric acid. The reaction was conducted for 100 min in a three-neck flask, using a mercury stirrer at 70°C under atmospheric pressure. The amount of citric acid added to the adhesive was calculated based on the dry weight of the cassava starch, with percentages ranging from 0%, 0.5%, 1%, 1.5%, to 2%. The adhesive characteristics were then evaluated in line with the ASTM D906 wood adhesive standard. Tests were conducted to measure adhesive viscosity, solid content, dry and wet shear strengths, thermal stability, and surface morphology. Molecular changes resulting from the addition of citric acid were analyzed using Fourier transform infrared spectroscopy. This research demonstrated that the addition of citric acid improved the adhesive properties. Specifically, the addition of 2% citric acid (AD-CA2.0) produced optimal results across several analyses. These included a high viscosity reading of 2910 cP, dry and wet shear strengths of 1.21 MPa and 0.89 MPa, respectively, and high solid content of 36%.

Keywords: bio-adhesive, cassava starch, polyvinyl alcohol, citric acid, polymerization

Abstrak

Sebagai pengiktirafan mendapatkan bio-pelekat mesra alam untuk aplikasi kayu, kajian ini meneroka kesan asid sitrik sebagai agen penghubung silang baharu ke atas sifat-sifat bio-pelekat kayu berasaskan kanji ubi kayu/polivinil alkohol (PVA). Bio-pelekat disintesis melalui tiga langkah: hidrolisis kanji ubi kayu dengan asid hidroklorik (HCl), pengoksidaan kanji ubi kayu dengan ammonium persulfat ($(NH_4)_2S_2O_8$), dan pempolimeran kanji dan PVA dengan asid sitrik. Tindak balas dijalankan selama 100 minit dalam kelalang leher tiga dan pengacau merkuri pada suhu 70 °C dan tekanan atmosfera. Berdasarkan berat kering kanji ubi kayu, peratusan asid sitrik yang ditambahkan pada pelekat berbeza antara 0%, 0.5%, 1%, 1.5%, dan 2%. Selaras dengan piawaian pelekat kayu ASTM D906, kelikatan pelekat, kandungan pepejal, kekuatan ricih kering dan basah, kestabilan terma dan morfologi permukaan telah diuji. Perubahan molekul telah dianalisis menggunakan spektroskopi Fourier Transform Infrared (FTIR). Penyelidikan ini menunjukkan bahawa penambahan asid sitrik meningkatkan ciri pelekat. Penambahan 2% asid sitrik

(AD-CA2.0) mempunyai hasil yang optimum untuk beberapa analisis seperti kelikatan tinggi pada 2910 cP, kekuatan ricih kering dan basah pada 1.21 MPa dan 0.89 MPa, dan kandungan pepejal tinggi pada 36%.

Kata kunci: bio-pelekat, kanji ubi kayu, polivinil alkohol, asid sitrik, pempolimeran

Introduction

The global rise in human population has led to an escalating demand for wood furniture, which in turn has increased the consumption of wood adhesives, a pivotal component in the wood industry [1]. Numerous studies have underscored this trend, highlighting a significant annual increase in the demand for wood adhesives [2]. In industry, the capacity of an adhesive to combine two materials in a short amount of time is preferred because it is practical and simple [3, 4].

Wood adhesives are commonly produced by chemical reactions [5], with two main types being utilized in the industry: conventional and natural adhesives [6]. Conventional adhesives are typically derived from petroleum, a nonrenewable resource. Formaldehyde was one of the earliest standard adhesives used to bind wood pairs [7]. It continues to dominate the production of wood-based adhesives owing to its effectiveness, stability, and cost-efficiency [8]. For instance, the urea-formaldehyde adhesive, known for its superior water resistance, is commonly used for interior products, while the phenol-formaldehyde adhesive is preferred for exterior applications [9].

Empirical evidence supports the health risks associated with the use of conventional adhesives. Formaldehyde, a known carcinogen that is used to treat human organ systems [10], poses potential threats not only during the manufacturing process but also in the final products. This is because formaldehyde naturally occurs in wood products and its emissions are generated by the residual formaldehyde content of the resin. Exposure to formaldehyde can induce skin and lung irritation, genotoxicity, and cancer [11]. Additionally, formaldehyde emits volatile organic chemicals that cause significant risks to both humans and the environment [12].

Various investigations on bio-adhesives have been conducted as potential substitutes for petroleum-based adhesives in wood bonding. The objective is to mitigate the adverse effects of synthetic adhesives on both human health and the environment [13]. The raw ingredients for bio-adhesives are abundant and inexpensive resources found in nature. These included materials such as soy, tannins, lignin, gelatin, wood fibers, plant polymers, and starch [14, 15].

Cassava starch, a material rich in amylose and amylopectin, has been utilized as a raw ingredient for bioplastic and paper adhesives [16]. This suggests that cassava starch could potentially serve as a raw material for bio-adhesive production. However, wood panels constructed entirely of starch are currently too fragile for practical use [17]. The hydroxyl groups in starch chains are hydrophilic, causing the starch to quickly interact with water, resulting in poor moisture tolerance. Moreover, when exposed to sufficient moisture and heat, the starch undergoes gelatinization, absorbing water and swelling [18].

A solution to this issue is the addition of PVA to the bio-adhesive, which reduces the rate of water absorption and decreases starch swelling. In the meantime, to ensure effective binding between starch and PVA, a cross-linking agent is required. Previous studies have investigated the influence of cross-linking agents like PTSA and bio-oil on the quality of bio-adhesive materials [19], [20]. However, these agents have been found to reduce adhesive quality, highlighting the need for the development of improved cross-linking agents.

Citric acid, with its three functional carboxyl groups, holds promise as it can potentially form bonds with the hydroxyl groups in starch and PVA. Its incorporation as a cross-linking agent in cassava starch bio-adhesives has demonstrated improved water resistance and mechanical qualities [21]. Despite these promising results, no studies have been conducted to analyze the effects of citric acid on the adhesive properties of starch. This investigation, therefore, sought to fill this research gap by focusing on the performance of cassava starch and polyvinyl alcohol (PVA) using citric acid as the cross-linking agent. A range of tests were

carried out to assess the physical properties and structural changes of the bio-adhesive, including viscosity, dry and wet shear strengths, solid content, thermogravimetry, and Fourier transform infrared (FTIR) analyses.

Materials and Methods

Materials

This study utilized cassava starch purchased from PT. Budi Acid Jaya. PVA and sodium dodecyl sulfate (SDS) procured from CV. Gudang Kimia, Yogyakarta. Hydrochloric acid (HCl, 37%) and citric acid were obtained from the Chemical Reaction and Catalyst Laboratory, UGM, Yogyakarta. Ammonium persulfate ($(NH_4)_2S_2O_8$) was provided by the LPPT UGM (Yogyakarta). Wooden boards (*Tectona grandis*) were purchased from UD. Makmur Jaya, Yogyakarta.

Bio-adhesive synthesis

The bio-adhesive was prepared by dissolving 50 g of cassava starch in 100 mL of 0.5 M HCl. The solution was stirred for 15 min to ensure homogeneity, before being placed in a batch reactor for gelatinization. In this study, the reactor, designed using a three-necked flask and a mercury stirrer, was maintained at a temperature of 70 °C and 1 atm pressure. The stirrer was set to a speed of 500 rpm. After 10 min of gelatinization, 0.4 grams of $(NH_4)_2S_2O_8$ were added to the solution. In addition, 1 gram of SDS was added to the solution after the process had run for 30 minutes. Approximately 15 g of PVA dissolved in 50 mL of distilled water was added to the reactor. The process continued with adding citric acid at a certain concentration, and the reaction was carried out for 1 h. All procedures were repeated with citric acid concentrations of 0%, 1%, 1.5%, and 2% based on the weight of cassava starch. The schematic of bio-adhesive is provided in Figure 1.

Viscosity analysis

The viscosity of the adhesive was analyzed using the ASTM D1084 standard [22]. Approximately 200 mL of the adhesive solution was poured into a glass beaker for this experiment. The adhesive viscosity was measured using a DV-E Brookfield Viscometer. The solution viscosity results were displayed on the monitor.

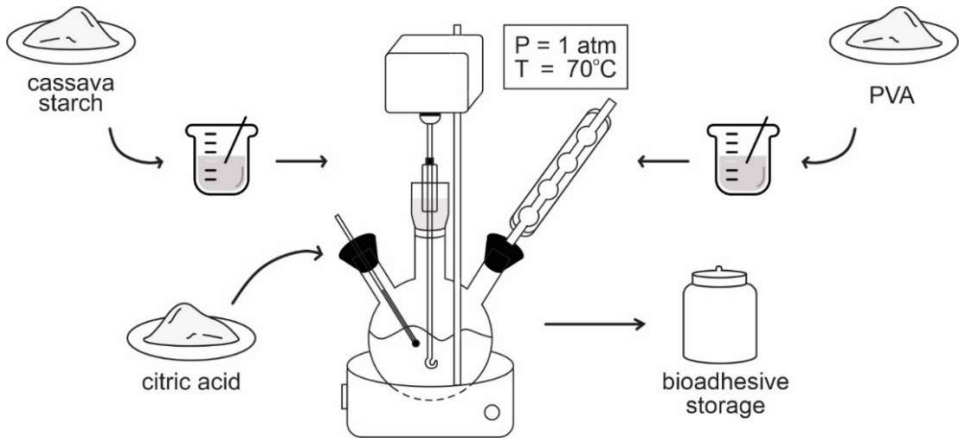


Figure 1. Schematic diagram of cassava starch bio-adhesive synthesis

Dry shear strength analysis

The dry shear strength was measured according to ASTM D906 [23] guidelines. Wood pieces measuring $30 \times 25 \times 2$ mm^3 were attached to the bio-adhesive and pressed under 3.27×10^{-2} N/mm^2 for 24 h before being stored at room temperature for seven days. The maximum bonding strength of the sample was determined at a speed of 2 mm/min using a universal testing machine in the Department of Chemical Engineering UGM. The shear strength of the adhesive was estimated using Equation (1).

$$\sigma_t = \frac{W}{A \times 1000} \quad (1)$$

Where σ_t is the shear strength of the adhesive (MPa),

W is the maximum force load (N), and A is the bonding area of the wood (mm^2). Dry strength analysis was conducted five times.

Wet shear strength analysis

The wet shear strength of the adhesive was calculated using the ASTM D1183 standard [24]. Identically sized wood pairs, already analyzed for dry shear strength, were bonded, and compressed under a pressure of 3.27×10^{-2} N/mm^2. After a day, the sample was submerged in distilled water for 24 h. Following this, the sample was dried and stored at room temperature at a relative humidity of 50%. Using a universal testing machine, we measured the maximum load of the sample at a testing speed of 2 mm/min. We used Equation 1 to calculate the wet shear strength. To ensure accuracy, we repeated this analysis five times and calculated the average of the results.

Solid content analysis

The solid content of the adhesive was determined according to ASTM-D2369 [25]. One gram of bio-adhesive was initially placed in a petri dish and heated at 110 °C ± 2.5 °C for an hour. After stabilizing the sample temperature using a desiccator for 10 min, we weighed the samples. We then used Equation 2 to determine the solid content of the adhesive.

$$S = \frac{w_2 - w_0}{w_1 - w_0} \times 100\% \quad (2)$$

Where S denotes the solid content of adhesive (%), w_0 signifies the mass of the petri dish (gram), w_1 is the mass of petri dish and sample before heating (gram), and w_2 is the mass of petri dish and sample after heating (gram). The solid content analysis was repeated three times.

Fourier transform infrared spectroscopy

The change in the functional groups in the adhesive because of the reaction process was investigated using a spectrophotometer FTIR Nicolet Is10 Mb 3000 from LPPT UGM in Yogyakarta, Indonesia. The spectral range of this study was between 650 and 4000 cm^{-1}, and 32 scans were recorded.

Thermogravimetry Analysis

Thermogravimetric analysis was performed at the Chemical Instrument Analysis Laboratory using a thermogravimetric analyzer (TA Instruments), housed in the DSC-60 Plus Shimadzu, Department of Chemical Engineering, Gadjah Mada University. The sample was dried in an oven at 60 °C for 3 h to obtain an adhesive powder with a size of 200 mesh or finer. The sample was placed in an alumina crucible, and another empty alumina crucible was used as a control. In an N_2 environment, the sample was gradually heated to 600 °C at a rate of 10 °C per min.

Adhesive morphology analysis

To analyze the morphology of the newly prepared adhesive, we used a Dino-Lite microscope AM8917 from the Department of Chemical Engineering, UGM and a scanning electron microscope (SEM) JSM-6510LA from LPPT, UGM. For the Dino-Lite microscope analysis, we placed the sample on a clean transparent glass. The light of the Dino-Lite microscope was turned on, and the magnification was adjusted to 500×. An image of the adhesive morphology was then captured for further analysis.

Results and Discussion

This study investigated the effects of citric acid addition on the quality of bio-adhesives made from cassava starch and PVA. The experiment varied the addition of citric acid between 0%, 0.5%, 1%, 1.5%, and 2%, based on the mass of cassava starch. The samples were labeled as AD-CA0 (blank sample), AD-CA0.5, AD-CA1.0, AD-CA1.5, and AD-CA2.0, according to their respective citric acid content. The experiment was conducted in a batch reactor system at 500 rpm.

In general, the synthesis of cassava starch bio-adhesives using citric acid involves a three-step process. The initial step involves hydrolysis and gelatinization of cassava starch using HCl. This process, carried out at 70 °C and 1 atm of pressure, breaks the glycosidic linkages in the starch to create an acid-thinned starch. The starch was then oxidized with ammonium persulfate $(NH_4)_2S_2O_8$) to produce carboxyl groups (–COOH). hydrolysis and oxidation mechanisms are shown in Figure 2.

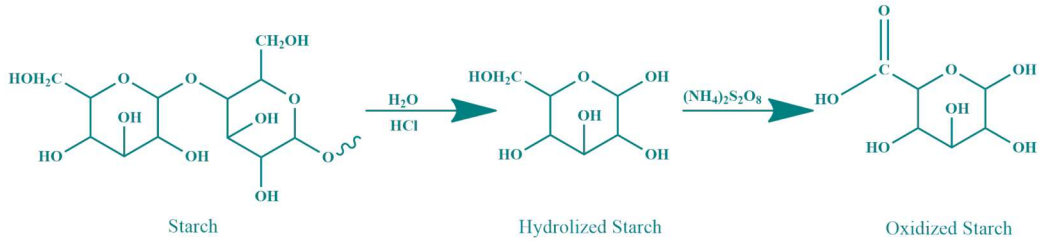


Figure 2. Reaction mechanism of cassava starch hydrolysis and oxidation

The carboxyl group of oxidized starch can form a bond with citric acid, which acts as a cross-linking agent. This bond formation gives rise to ester-starch. In addition, this oxidized molecule can induce retrogradation following gelatinization, a process where gelatinized starch reverts to its original form. Figure 3 illustrates the mechanism by which starch, and citric acid generate ester-starch.

Moreover, starch and PVA have the potential to form a bonding molecule known as ester starch/PVA. Figure 4 depicts the formation of bonding molecules between the starch and PVA using citric acid.

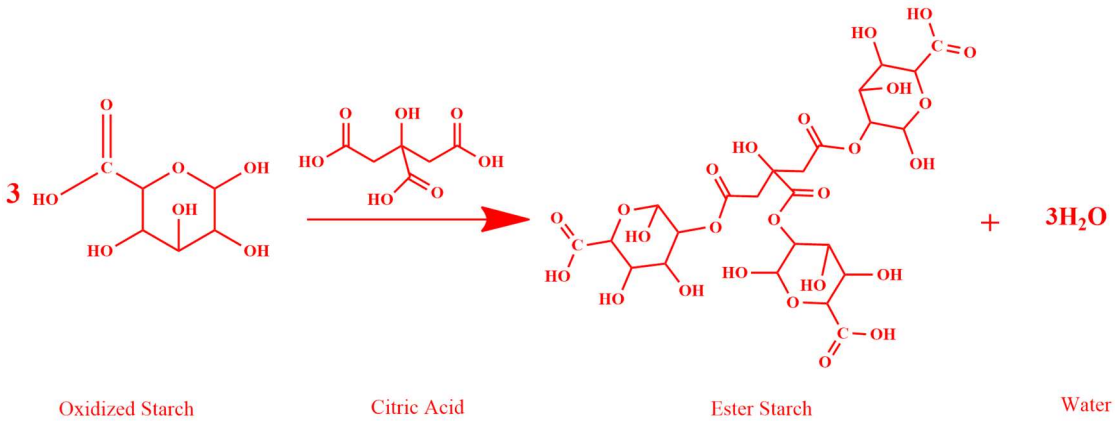


Figure 3. Reaction mechanism of ester starch synthesis

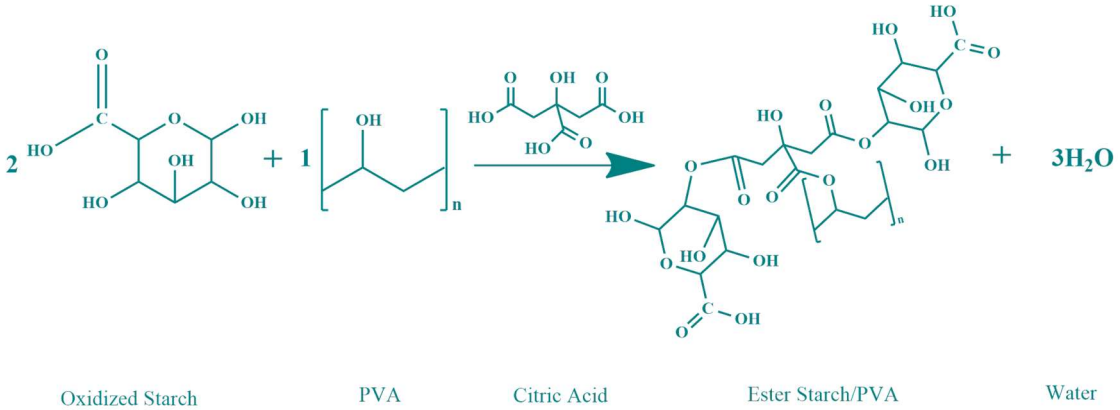


Figure 4. Reaction mechanism of ester starch/PVA synthesis

Viscosity analysis

The viscosity of each adhesive was measured to determine its physical properties. This characteristic is necessary as it greatly affects the adhesive capacity to coat wood surfaces. If the adhesive is too thick, it struggles to penetrate the wood pores, resulting in poor bonding. Conversely, a liquid adhesive fails to bond two pieces of wood together because it is rapidly absorbed by the wood pores. Figure 5 provides a visual example of this explanation.

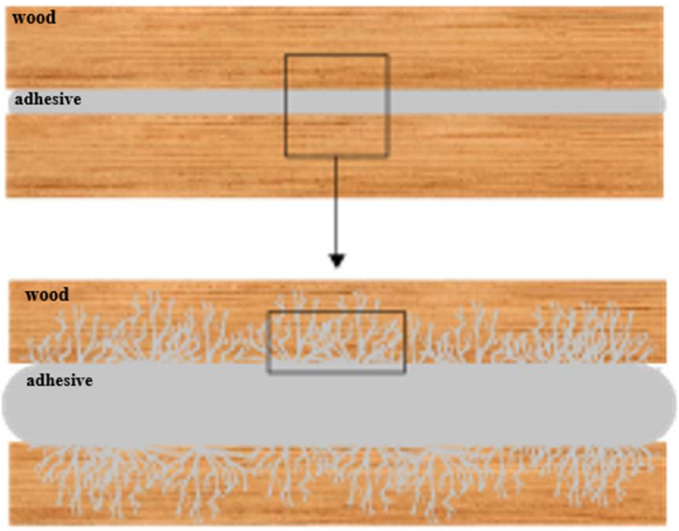


Figure 5. An illustration of bio-adhesive adsorption into the wood pores

In this experiment, viscosity was examined using a Brookfield Viscometer spindle. Given the nature of the adhesive as a non-Newtonian fluid, whose thickness fluctuates over time, the spindle was adjusted from its smallest to largest size and from its slowest to the fastest speed [26].

As shown in Figure 6, the adhesive viscosity increased with the addition of citric acid. The viscosity of AD-CA0, the reference sample without citric acid, was recorded at 1137 cP. Upon inclusion of 0.5% citric acid, the viscosity of AD-CA0.5 steadily increased to 1222 cP. The adhesive viscosity dramatically increased to 2312 cP and 2882 cP at concentrations of 1% (AD-CA1.0) and 1.5% (AD-CA1.5), respectively. Finally, adding 2% citric acid resulted in an adhesive viscosity of 2910 cP (AD-CA2.0).

This progressive increase in viscosity with the addition of citric acid underlines its role as a potent cross-linking agent that significantly influences adhesive properties. This is because the addition of citric acid to the adhesive greatly enhances the interaction of starch, PVA, and the cross-linker forming a polymer [27]. Citric acid, endowed with three carboxyl groups, functions as a bridging agent to enhance the number of hydrogen and covalent bonds between molecules. When citric acid is added to the mixture, its carboxyl groups react with the hydroxyl groups on starch and PVA to form a stable ester and reduce the mobility of the polymer chains. This polymerization process contributes to a denser solution, making the adhesive thicker and heavier. Therefore, higher concentrations of citric acid result in a more viscous adhesive.

Our results align with those of Kang et al. [28] and Wang et al. [29], who found that the addition of a cross-linking agent significantly increased polymer viscosity and enhanced adhesive mechanical properties. A comparison of our findings showed the consistency of cross-linker addition to the viscosity of the polymer. This finding also indicates that the concentration of citric acid as the cross-linker significantly contributes to the viscosity properties of the adhesive.

Dry and wet shear strength analysis

The shear strength of an adhesive is a crucial component that significantly influences its overall performance. The objective of this assessment was to determine the adhesive capacity to join two wood pieces. Additionally, we sought to determine how environmental factors affect the adhesive quality. Each sample was analyzed five times to confirm the accuracy of the data collected during this study. According to Figure 8, the adhesive strength was greater under dry conditions than that under wet conditions because water and starch share the same similar polarity properties. Therefore, the adhesive tends to dissolve in water easily, thereby reducing its bonding strength. The illustration of adhesive, wood, and water is shown in Figure 7.

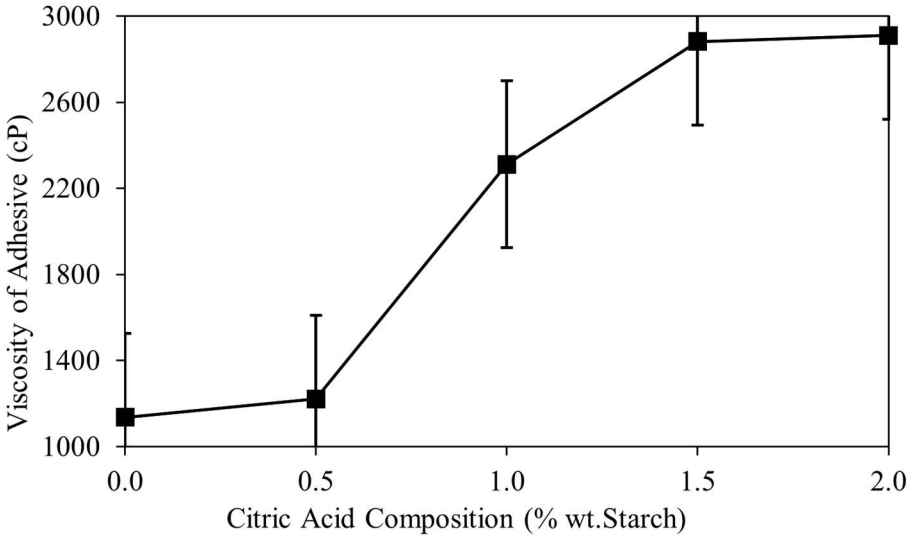


Figure 6. The viscosity of adhesive in various percentages of citric acid concentration

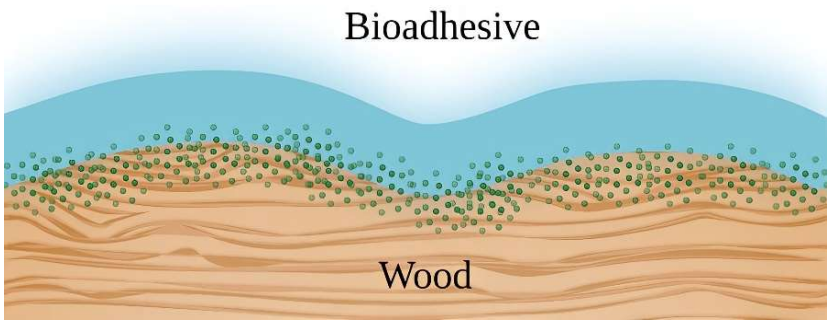


Figure 7. Illustration of the interaction between adhesive and water

An analysis of the shear stress demonstrated that the addition of citric acid significantly enhanced the adhesive's strength. In dry conditions, the reference sample (AD-CA0) exhibited a shear strength of 0.66 MPa. With citric acid addition, the shear strengths of samples AD-CA0.5, AD-CA1.0, and AD-CA1.5 increased progressively to 0.86 MPa, 1.02 MPa, and 1.16 MPa, respectively. AD-CA2.0 reached the highest dry shear strength with a value of 1.21 MPa. By contrast, under wet conditions, the reference sample (AD-CA0) recorded the lowest shear strength at 0.54 MPa. The shear strengths of samples AD-CA0.5, AD-CA1.0, and AD-CA1.5 reached 0.68 MPa, 0.70 MPa, and 0.84 MPa, respectively. The sample AD-CA2.0 demonstrated a maximum shear strength at 0.89 MPa.

Our findings echo those of Zhao [30] and Widyorini [31], who demonstrated that the addition of citric acid enhances polymer strength. The cross-linking reaction between citric acid and starch/PVA lowers the number of hydroxyl groups in the starch, thereby reducing the adhesive affinity for water and enhancing its resistance to moisture. However, when the adhesive is immersed in water, its bonding capability weakens owing to its similar polarity with water, making it more soluble and less resistant in wet conditions.

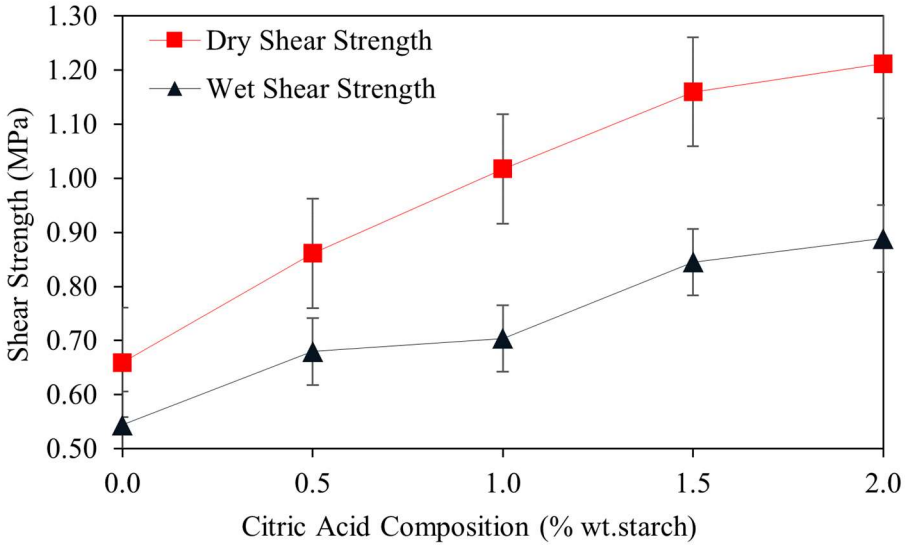


Figure 8. Dry and wet shear strength of adhesive in various percentages of citric acid concentration

Solid content analysis

The measurement of the adhesive's solid concentration is a critical step in determining the proportion of solid components. This analysis aids in evaluating the adhesive homogeneity and its ability to coat the substrate during the gluing process. Furthermore, determining the solid content provides insight into the adhesive's volatility level. This is crucial because high volatility can adversely affect the health of users. A well-balanced mixture of solid materials in the adhesive results in superior performance in terms of homogeneity and volatility [32].

In this study, we examined the solid content of the adhesive according to the ASTM D2369 standard. As illustrated in Figure 9, the addition of citric acid increased the adhesive's solid content. The solids made up 29% of the reference sample (AD-CA0). Upon adding citric acid, the percentage of solids rose to 33%, 34%, and 35% for samples AD-CA0.5, AD-CA1.0, and AD-CA1.5, respectively. Sample AD-CA2.0, with a 36% proportion of solids, included the highest percentage of solids.

This increase in solid content can be attributed to the bonding formed between cassava starch and polyvinyl alcohol owing to the addition of citric acid, thereby reducing the number of volatile components. Consequently, the reaction between citric acid and cassava starch/PVA increases the number of solid components. This observation aligns with a study conducted by Marquez [33], who discussed the potential of citric acid to modify adhesive properties and enhance the molecular solid weight of the adhesive. However, a high percentage of solids can hinder the adhesive performance owing to difficulties in application on wood. To ensure robust wood bonding, the adhesive needs to quickly coat the surface of the wood pores.

Fourier transform infrared spectroscopy

FTIR spectroscopy was performed to understand the structural modifications of the chemical molecules during the reaction. The molecular structures of

cassava starch, the 0% citric acid sample (AD-CA0), and the 2% citric acid sample (AD-CA2.0) were analyzed for comparison. Figure 10 illustrates the results of the analysis.

Figure 10 shows the alteration peak at 994 cm^{-1}. This implies that the strength of the C–O bonds in cassava starch was lower than that in the reference sample (AD-CA0). Additionally, the strength of the peak at 3262 cm^{-1} increased, indicating that the number of hydroxyl groups in each molecule of hydrolyzed starch increased. These modifications explain why cassava starch underwent hydrolysis during the procedure. Furthermore, the peak intensity at 1639 cm^{-1} for sample AD-CA0 increases, indicative of the creation of carboxyl functional groups (C=O) owing to oxidation. At 2942 cm^{-1}, the peak intensity increased as the fraction of carboxyl groups in AD-CA2.0 increases. This indicates that the sample underwent greater oxidation during polymerization.

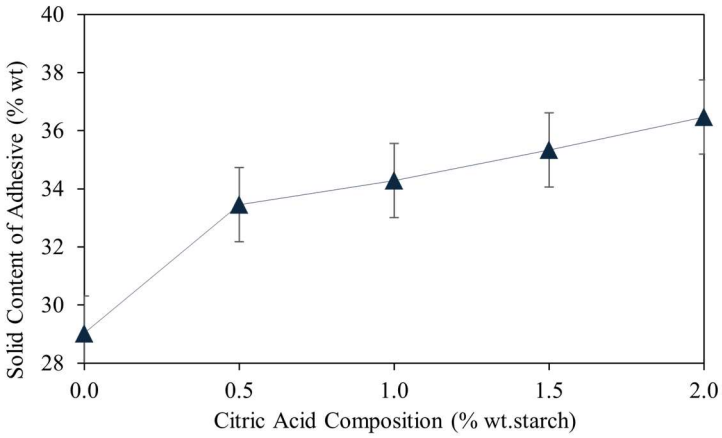


Figure 9. The solid content of adhesive in the various percentages of citric acid addition

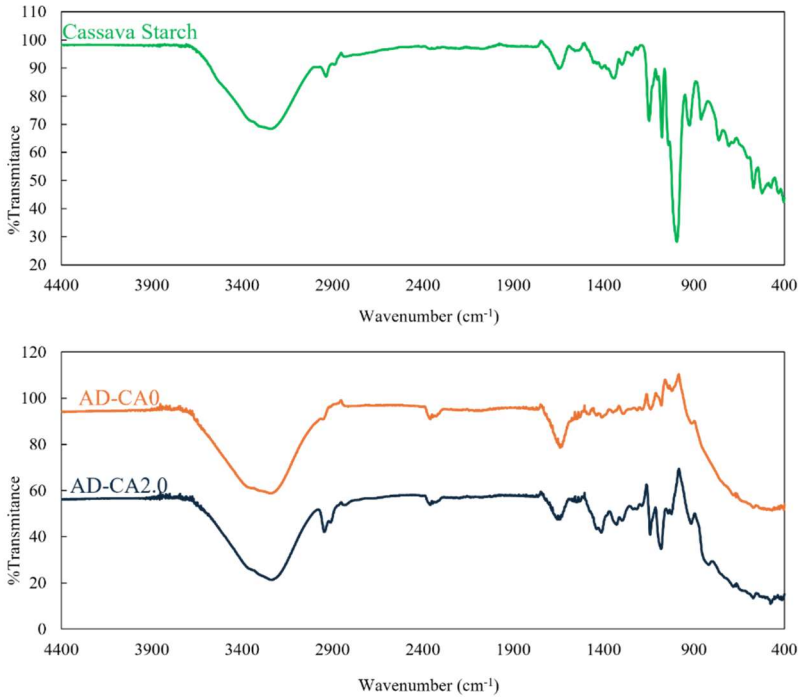


Figure 10. Infrared spectra of cassava starch, AD-CA0, and AD-CA2.0

Thermogravimetry analysis

Thermogravimetric analysis was performed to determine the thermal stability of the material. The thermal stabilities of both the reference sample (AD-CA0) and the sample with citric acid addition (AD-CA2.0) were examined across a temperature range between 0 °C and 600 °C. Figure 11 shows the results of thermogravimetric analysis. The mass loss of the reference sample (AD-CA0) and citric acid sample (AD-CA2.0) was separated into two stages based on this curve.

For the AD-CA0, the initial temperature degradation stage ranges from 0 °C to 100 °C, whereas the second stage spans from 100 °C to 600 °C. In the first stage, the sample experienced a weight loss of approximately 5%. This is attributed to the evaporation of water and monomers present in the adhesive. During the second stage, owing to the breakdown of hydrogen and covalent bonds in the adhesive, the weight progressively decreases until it reaches 600°C.

For the sample containing citric acid (AD-CA2.0), the first step of degradation occurred between 0 °C and 142 °C, and the second stage occurred between 142 °C and 600 °C. The initial stage saw a weight loss of about 7%, consistent with the evaporation of water and monomers as temperature increased. Owing to the dissolution of covalent bonds in the adhesive and the degradation of the adhesive leftovers, the mass loss rate decreased steadily during the second stage. This data suggests that the addition of citric acid can indeed enhance thermal stability of the adhesives.

Morphology analysis

A Dino-Lite microscope and a SEM were used to analyze adhesive surface morphology. This experiment was conducted to investigate the homogeneity of the adhesives. The adhesive morphology before (AD-CA0) and after (AD-CA2.0) citric acid addition was compared. As illustrated in Figure 12, the adhesive's surface became smoother with citric acid introduction. SEM analysis confirmed an increase in the adhesive's homogeneity following citric acid addition (Figure 13). This can be attributed to citric acid enhancing the likelihood of a reaction between cassava starch and PVA which made the homogeneity of adhesive increase.

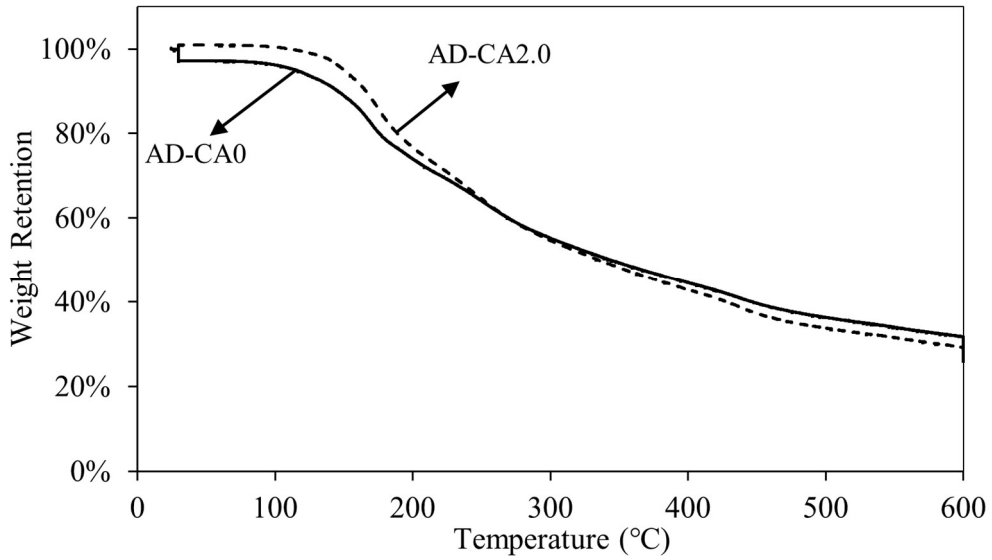
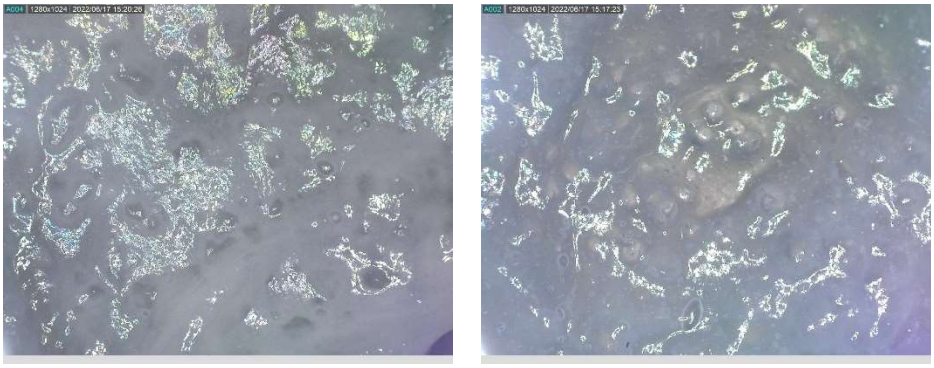
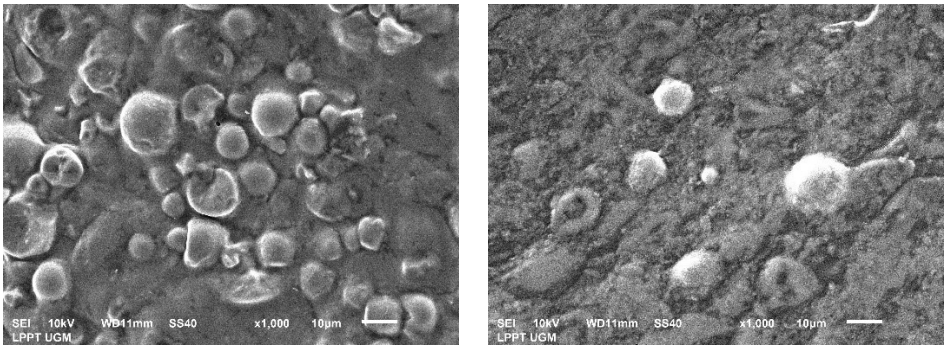


Figure 11. Thermogravimetric curves of citric AD-CA0 and AD-CA2.0



a b

Figure 12. The morphology of cassava starch/PVA adhesive before (a) and after (b) citric acid addition using dino-lite microscope



a b

Figure 13. The morphology of cassava starch/PVA adhesive before (a) and after (b) citric acid addition using scanning electron micrograph

Conclusion

Incorporating citric acid as a cross-linking agent considerably improved the quality of the cassava starch bio-adhesive. The physical attributes of the adhesive, such as viscosity, solid content, and dry and wet shear strengths, were improved with citric acid addition. Sample AD-CA2.0, with 2% citric acid, demonstrated the highest bond quality in this experiment, recording a viscosity of 2910 cP, and dry and wet shear strengths of 1.21 MPa and 0.89 MPa, respectively. Furthermore, the solid content of this sample was 36%. The adhesive underwent hydrolysis, oxidation, and polymerization according to FTIR measurements. Furthermore, the morphological study revealed that citric acid addition improved adhesive uniformity. These findings have opened up new avenues for further research to explore the long-term durability and environmental sustainability of cassava starch bio-adhesives modified with citric acid. Future studies could investigate the aging behavior of adhesive bonds under various environmental conditions, such as temperature and humidity fluctuations, to assess their performance over extended periods.

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EXPLORING DEEP EUTECTIC SOLVENTS AS A POTENTIAL SOLVENT FOR THE EXTRACTION OF FOOD ADDITIVE ALLURA RED IN FOOD TECHNOLOGY APPLICATION

(Mengeksplorasi Pelarut Eutektik Mendalam sebagai Pelarut Berpotensi untuk Pengekstrakan Pewarna Makanan Merah Allura dalam Aplikasi Teknologi Makanan)

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Abstract

In recent years, synthetic food dyes have gained significant popularity as additives, replacing natural dyes, with azo dyes comprising approximately 65% of the commercial dye market. However, concerns regarding the toxic effects of azo dyes used in food colouring have prompted the development of advanced analytical methods to detect them in various food matrices. These methods typically involve intricate pre-concentration processes, time-consuming steps, and the use of expensive instruments, thus emphasising the need for highly sensitive and selective approaches. Hence, this study aims to explore the application of deep eutectic solvent (DES) as a potential solvent for the extraction of Allura Red AC (ARAC). First, the synthesised magnetic nanoparticles functionalised with DES (silicone surfactant: dodecanoic acid) (SS: DoAc@Fe_3O_4) adsorbent was successfully characterised using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) for the identification of functional bonds. This adsorbent was subsequently applied in the magnetic solid phase extraction (MSPE) technique to extract ARAC in soft drink samples. In contrast to pure Fe_3O_4 particles, the targeted ARAC can be easily adsorbed onto the SS: DoAc@Fe_3O_4 surface due to the formation of strong hydrophobic interaction between ARAC and the adsorbents, thus amplifying the extraction efficiency. Further optimisation was done via one-variable-at-a-time (OVAT) and Taguchi experimental design to determine the optimised condition for ARAC extraction. The OVAT analysis identified that using methanol: acetonitrile (1:1) as the desorption solvent, incorporating Na_2SO_4 to boost extraction efficiency, employing salt volume of 200 µL, and utilising a sample volume of 20 mL produced the most favourable outcomes. Meanwhile, the Taguchi experiments pinpointed the optimum conditions as pH 2, an adsorbent dosage of 25 mg, an extraction duration of 2 minutes, a desorption period of 3 minutes, and a desorption solvent volume of 400 µL. Under the optimised conditions, the R^2 value obtained was 0.999 while the limit of detection (LOD) and limit of quantification (LOQ) values were 0.1 mgL^{-1} and 0.4 mgL^{-1}, respectively. The studied ARAC also obtained relative recovery values between 80% to 111%. Therefore, it is safe to conclude that the newly proposed SS: DoAc@Fe_3O_4-based MSPE is an environmentally friendly, simple, affordable, and effective technique for the extraction of ARAC in drink samples.

Keywords: Allura red AC, deep eutectic solvents, dodecanoic acid, magnetic solid phase extraction, silicone surfactant

Abstrak

Kebelakangan ini, pewarna makanan sintetik semakin popular dan kerap digunakan sebagai bahan tambahan menggantikan pewarna semula jadi, dengan pewarna azo merangkumi kira-kira 65% daripada pasaran pewarna komersial. Walau bagaimanapun, kebimbangan mengenai kesan toksik pewarna azo yang digunakan dalam pewarna makanan telah mendorong pembangunan kaedah analisis lanjutan untuk mengesannya dalam pelbagai matriks makanan. Kaedah tersebut biasanya melibatkan proses pra-kepekatan yang rumit, prosedur yang memakan masa, dan penggunaan instrumen yang mahal, sekaligus menekankan keperluan untuk pendekatan yang sensitif dan terpilih. Oleh itu, kajian ini bertujuan untuk meneroka aplikasi pelarut eutektik mendalam (DES) untuk mengekstrak pewarna merah allura AC (ARAC). Pertama, penjerap zarah nano magnetik difungsikan dengan DES (surfaktan silikon:asid dodekanoik) (SS:DoAc@Fe_3O_4) telah berjaya disintesis dan dianalisis menggunakan spektroskopi transformasian Fourier inframerah dengan pantulan keseluruhan dikecilkan (ATR-FTIR) untuk mengenal pasti ikatan berfungsi. Bahan penjerap ini kemudiannya digunakan dalam teknik pengekstrakan fasa pepejal magnetik (MSPE) untuk mengekstrak ARAC dalam sampel minuman ringan. Berbeza dengan zarah Fe_3O_4 tulen, ARAC lebih mudah diserap ke permukaan SS:DoAc@Fe_3O_4 disebabkan oleh pembentukan interaksi hidrofobik yang kuat antara ARAC dan penjerap, sekaligus menguatkan kecekapan pengekstrakan. Pengoptimuman lanjut kemudiannya dilakukan melalui satu-pembolehubah-pada-satu-masa (OVAT) dan reka bentuk eksperimen Taguchi bagi menentukan keadaan optimum untuk pengekstrakan ARAC. Analisis OVAT mendapati bahawa penggunaan metanol:acetonitrile (1:1) sebagai pelarut penyahjerapan, penambahan garam untuk meningkatkan kecekapan pengekstrakan, penggunaan isipadu garam sebanyak 200 µL, dan penggunaan isipadu sampel 20 mL memberikan hasil yang paling baik. Sementara itu, eksperimen Taguchi menunjukkan keadaan optimum sebagai pH 2, dos penjerap sebanyak 25 mg, tempoh pengekstrakan selama 2 minit, tempoh penyahjerapan selama 3 minit, dan isipadu pelarut penyahjerapan sebanyak 400 µL. Dibawah keadaan yang dioptimumkan, nilai R^2 yang diperolehi adalah 0.999 manakala nilai had pengesanan (LOD) dan had kuantifikasi (LOQ) masing-masing adalah 0.1 mgL^{-1} dan 0.4 mgL^{-1}. ARAC yang dikaji juga memperoleh nilai pemulihan relatif antara 80% hingga 111%. Oleh itu, dapat disimpulkan bahawa MSPE berasaskan SS:DoAc@Fe_3O_4 yang dicadangkan adalah satu teknik mesra alam, mudah, berpatutan, dan berkesan untuk pengekstrakan ARAC dalam sampel minuman.

Kata kunci: Pewarna merah allura AC, pelarut eutektik mendalam, asid dodekanoik, pengekstrakan fasa pepejal magnetik, surfaktan silikon

Introduction

Food colourants, also known as pigments, encompass dyes or substances that impart colour to food and beverages. They are broadly utilised both in commercial food production and home cooking settings [1]. These colourants consist of diverse chemical substances added to food matrices, serving several purposes. They are employed to enrich or safeguard the sensory attributes of food items, which could otherwise be compromised or destroyed throughout the processes of manufacturing or storage [2]. Additionally, food colourants are employed in food technology applications to achieve the desired appearance and colour of the final product. The visual aspects are considered a vital role in the modern consumer's food choices, with colour being an integral part of food and beverages [3]. Over the past few years, a multitude of artificial food dyes have been introduced as an alternative to natural colourants. They are predominantly utilised to attain distinct attributes, such as enhanced visual appeal, intensified hues, increased colour stability, and uniformity [4, 5].

Synthetic food colourants offer several commercial benefits, such as affordability, resilience to light and pH changes, and excellent colour stability in comparison to natural colours [6]. These colourants are chemical compounds derived from coal tar derivatives, with many of them containing azo-group [7]. However, there are concerns in the market as some foods and beverages may contain unapproved synthetic colourants or excessive amounts of approved synthetic colourants [8]. Extensive research has revealed synthetic colourants as a significant contributor to food poisoning and can lead to severe health issues. It is also not safe to use approved synthetic colourants for the long term [9]. The use of unapproved colourants or the indiscriminate use of approved colourants is known to have adverse effects on animal and human health. There is a growing demand for the development of precise, accurate, sensitive, and selective analytical methods for the qualification and quantification of food colourants, owing to their extensive use in food manufacturing processes [10]. Among the synthetic dyes utilised in the food industry, azo dyes dominate the commercial dye market, accounting for about 65% of the share [11].

Azo dyes are synthetic organic colourants known for their vibrant and intense colours, making them popular additives in various food products. However, these dyes possess certain characteristics that warrant attention. They are typically resistant to aerobic conditions but can be metabolised by intestinal flora, leading to the formation of aromatic amines [12]. Such compounds have been associated with adverse effects on health, including headaches in adults, neurotoxicity, genotoxicity, and carcinogenic properties [13]. Most countries worldwide have taken measures to prohibit the usage of a significant number of azo dyes in food products as a response to the associated health concerns. Over the past few decades, strict regulations have been put in place to govern the use of azo dyes in both domestic food supplies and exports of various nations [14]. However, despite the presence of diverse regulatory frameworks, the overall approach is grounded in similar stages that demand well-established protocols for risk assessment.

Magnetic solid phase extraction (MSPE) has been developed based on traditional SPE [15]. In recent years, nano-magnetic materials have attracted much interest due to their unique size and physical properties. They possess a high surface area, resulting in greater adsorption capacity for analytes [16]. Because of their super-paramagnetic property, nanoparticles can be attracted by a magnet, but do not retain magnetism after the field is removed. Additionally, they do not agglomerate after the magnetic field is removed, thus not affecting sorption ability [17]. Meanwhile, the magnetic sorbent can be used repeatedly to enrich and separate the target analyte [18]. However, the naked Fe_3O_4 adsorbent is limited for selective analyte adsorption. Therefore, surface modification is necessary to design highly selective adsorbents to improve selectivity or affinity towards analytes [19]. Compared with traditional sample pre-treatment, MSPE offers many advantages, such as simplicity, low cost, and environmental friendliness, making it highly suitable for isolating and enriching target analytes from food samples [20]. The demand for environmentally friendly solvents has led to the adoption of ionic liquids (ILs) as replacements for traditional organic solvents, which are volatile and toxic. However, the green aspects of ILs have come under scrutiny due to their poor biocompatibility and biodegradability [21].

As a solution to this concern, deep eutectic solvents (DESs) have been introduced as a green alternative to ILs since 2004. DESs can be tailored to exhibit specific properties, such as hydrophobicity or hydrophilicity, providing the advantage of selectively extracting target compounds. This selectivity leads to higher purity and concentration of the emerging contaminants (ECs) obtained from the extraction process [22]. DESs can be easily modified by adjusting the composition of their components, making them adaptable to meet the specific demands of the extraction process. This versatility makes DESs well-suited as adsorbents for extracting or removing ECs in various matrices [23]. They offer numerous benefits, including high solvency, low toxicity, low environmental impact, biodegradability, and cost-effectiveness, as they are derived from renewable resources. Furthermore, DESs are easy to recycle without imposing negative effects on the environment.

Food analysis has become an exceptionally crucial procedure in ensuring the safety and quality of food products [24]. Furthermore, the adverse toxic effects linked to azo dyes employed for food colouring have spurred the advancement of exceptionally sensitive and selective analytical methods for their detection in various food matrices [25]. Despite the existence of many analytical methods previously studied, the majority of conventional methods, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE), regrettably entail intricate pre-concentration, lengthy procedures, and the use of expensive equipment [26, 27]. An appropriate approach for preparing samples is imperative before conducting instrumental analysis, aiming to eliminate interferences arising from intricate food matrices that could potentially obstruct the chromatographic system. Hence, the development of green solvents has attracted significant interest in the demand for sustainable and green processes [28].

Based on our previous laboratory findings, a newly synthesised magnetic adsorbent named SS:DoAc@Fe_3O_4 using non-ionic silicone surfactant and dodecanoic acid as a DES in magnetic solid-phase extraction (MSPE) was reported as a reliable approach for the extraction of organophosphorus in honey and

vegetable samples. In this study, similar magnetic adsorbent is employed to explore the extraction performance towards Allura Red AC (ARAC) using the MSPE technique. The focus on ARAC is prompted by its extensive application as food colorant in the food industry. ARAC (E129), also known as Red 40, is an azo dye originally derived from petroleum with a dark red colour and water solubility. It serves as a food colouring substitute for amaranth and is commonly added to sodas, baby medicines, and cotton candy. In the United States, ARAC is the most frequently used food colouring in beverages, candy, popsicles, deli meats, cheeses, salmon, over-the-counter (OTC) medications, and liquid suspensions. This study explored the potential application of DES-based magnetic adsorbent, a novel adsorbent synthesised by Elencovan et al. in solid phase extraction methods for the determination of ARAC in food matrices [29]. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) analysis was conducted to confirm the successfully re-synthesised material. Then, the newly synthesised SS:DoAc@Fe_3O_4 adsorbent was applied in the MSPE technique. All the methods were optimised and validated for the analysis of ARAC in soft drink samples.

Materials and Methods

Chemicals and reagents

The food dye (ARAC) was purchased from R&M Chemicals (Essex, UK). Ultrapure water (18.2 MΩ/cm) supplied by the Sartorius Milli-Q system (Aubagne, Germany) was used to prepare the standard stock solution for ARAC at the concentration of 1000 mgL^{-1}. Daily preparation of the standard working solutions involved dilution of a sufficient quantity of the stock solutions with ultrapure water for the extraction analysis. Additionally, the stock solution for salt was created by dissolving the requisite amount of salt in ultrapure water to achieve a concentration of 25% (w/v). Miracle S240, a non-ionic silicone surfactant, was obtained from G-Planter (Johor, Malaysia) with the IUPAC designation of 3-[methyl-bis(trimethylsilyloxy) silyl]propan-1-ol;2-methyloxirane; oxirane. Sodium sulphate (Na_2SO_4) was purchased from Qrec (Chonburi, Thailand) while methanol and acetonitrile of HPLC grade were obtained from Fisher Scientific (Seoul, Korea). R&M Chemicals (Essex, UK), Friedemann Schmidt Chemicals (Parkwood, Australia), Sigma-Aldrich (Darmstadt, Germany), and Chemiz (Shah Alam, Malaysia) supplied the iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), ammonium hydroxide (25%), sodium hydroxide (NaOH), and hydrochloric acid (HCl), respectively. Absolute ethanol was purchased from Fisher Scientific (Seoul, Korea).

Instrumentation

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) was employed to identify the functional bonds present in the newly synthesised SS:DoAc-based DES. The spectra were examined using Fortier from Perkin Elmer USA in the 4000 to 600 cm^{-1} range. The Perkin Elmer Lambda 25 UV-Vis spectrophotometer was used to analyse the analytical response of ARAC. Meanwhile, the Minitab 2022 (v21.3.1) software was used to investigate the signal-to-noise ratios of the experimental parameters that were optimised for the MSPE technique via the Taguchi design of experiments.

Synthesis of SS:DoAc-based DES

The SS:DoAc-based DES used in this study was synthesised according to the procedure mentioned in Elencovan et al. [29]. The co-precipitation method was used to synthesise the SS:DoAc@Fe_3O_4 adsorbent. The DES was made by combining silicone surfactant (SS) as a hydrogen bond acceptor (HBA) and dodecanoic acid (DoAc) as a hydrogen bond donor (HBD) at a molar ratio composition of 1:5, respectively. A clear, homogeneous liquid of SS:DoAc-based DES was produced by heating the mixture at 70 °C for 15 min with continuous stirring. Then, 150 µL of DES was added to the mixture of 1.4576 g of $FeCl_2.4H_2O$ and 3.7306 g of $FeCl_3.6H_2O$. Subsequently, 75 mL of ultrapure water was incorporated into the mixture. The mixture underwent vigorous stirring using a magnetic stirrer for 30 minutes at 85 °C under nitrogen gas stream protection. Next, 10 mL of ammonium hydroxide (25%) was added dropwise to the mixture and agitated constantly for 60 minutes. The synthesised SS:DoAc@Fe_3O_4 adsorbent was later collected using an external magnet and rinsed repeatedly, alternating between ethanol and ultrapure water to ensure complete removal of any residual unreacted chemicals. Lastly, the isolated SS:DoAc@Fe_3O_4 adsorbent was placed to dry

in an oven for 24 hours at 80 °C.

Proposed SS:DoAc-based DES in MSPE technique

The magnetic solid-phase extraction (MSPE) method was conducted as described by Elencovan et al. with some modifications [29]. Firstly, 25 mg of SS:DoAc@Fe_3O_4 adsorbent was weighed precisely and incorporated into 10 ml of sample solution that was spiked with targeted analyte, followed by the addition of 800 µL of 25% (w/v) sodium sulfate (Na_2SO_4) to increase the ionic strength. The sample vial was vortexed for 2 minutes at 2500 rpm using a vortex mixer to promote the adsorption of the targeted analyte onto the SS:DoAc@Fe_3O_4 adsorbent. An external magnet was later used to isolate the SS:DoAc@Fe_3O_4 adsorbent and supernatant was discarded. Next, 700 µL of methanol:acetonitrile (1:1) acting as a desorption solvent was added to the isolated SS:DoAc@Fe_3O_4 adsorbent. The mixture underwent sonication for 2 minutes to facilitate the elution of the target dye analyte from the SS:DoAc@Fe_3O_4 adsorbent into the desorption solvent. Subsequently, the SS:DoAc@Fe_3O_4 adsorbent was separated using an external magnet and the desorption solvent was carefully retrieved with the aid of a micropipette tip and filtered using a 13 mm nylon syringe filter with a pore size of 45 µm. The final solution was then transferred into a 20 mL vial. Lastly, the desorption solvent containing the targeted analyte was subjected to UV-Vis spectrophotometry analysis for further identification and quantification.

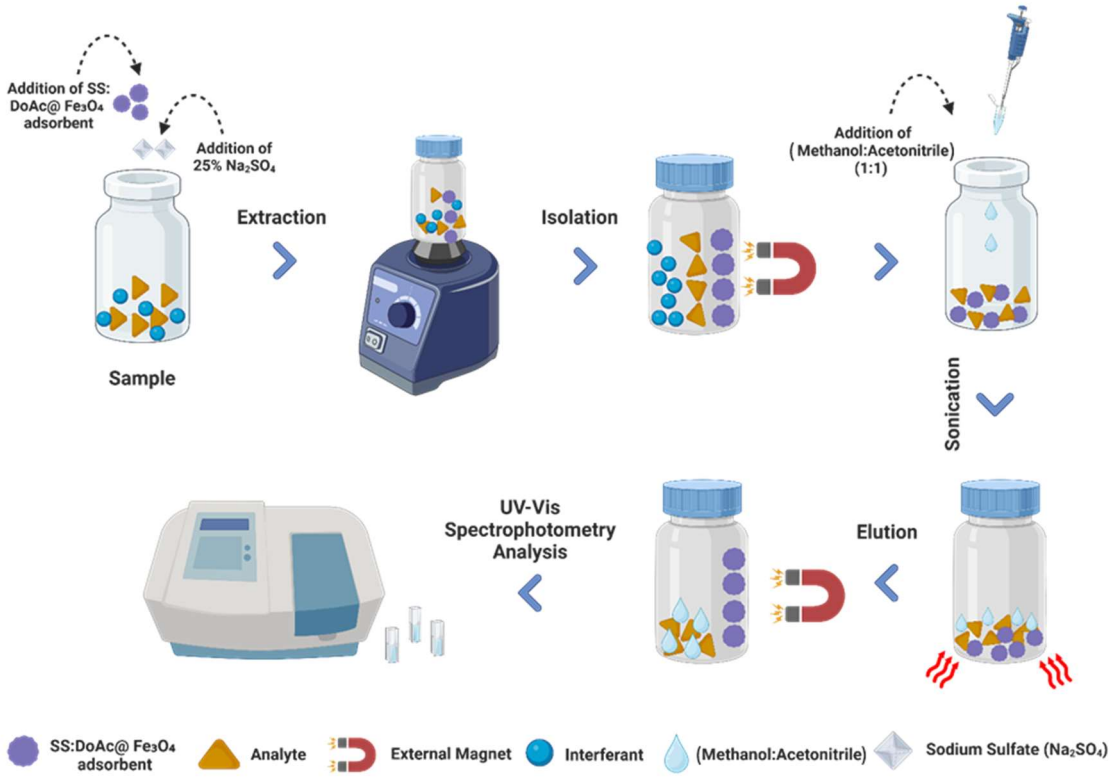


Figure 1. Schematic diagram of MSPE technique

Preliminary screening

A preliminary study was conducted via the MSPE approach to compare the performance of newly synthesised SS:DoAc@Fe_3O_4 adsorbent with control (Fe_3O_4 particles). Other extraction parameters included 25 mg of adsorbent, 10 mL of sample solution, 2 minutes of extraction time, 800 µL of 25% (w/v) Na_2SO_4, 700 mL of methanol:acetonitrile as desorption solvent, and 2 minutes of sonication. The adsorbent's performance in extracting ARAC was analysed using UV-Vis spectrophotometry at λ_{max}=501 nm.

Optimisation of MSPE technique via One-Variable-at-A-Time (OVAT) design of experiments

The OVAT (one-variable-at-a-time) design of experiments is a simple and straightforward approach in experimental design where one independent variable is varied while keeping all other variables constant. This method involves changing one factor at a time to observe its individual effect on the dependent variable. It is often used in the preliminary stages of research and allows researchers to quickly evaluate the impact of individual factors on a system without the complexity of considering interactions between multiple variables. In this study, in-depth analysis was conducted on the types of desorption solvent, addition of salt, volume of salt, volume of sample solution, and point zero charge as well as how they affect the extraction performance of the SS:DoAc-MSPE method. All experiments were conducted in triplicates (n=3). Four different types of desorption solvents, including methanol, acetonitrile, a 1:1 mixture of methanol and acetonitrile, and isopropanol, were evaluated in this study. To assess the effectiveness of target analyte extraction and recovery with and without the addition of salt, 25% (w/v) Na_2SO_4 was used to study the effect of salt. The effect of salt volume was investigated using volumes of 25% (w/v) Na_2SO_4 in the range of 200 to 800 µL. Different volumes of sample solution were investigated in this procedure ranging between 10 to 25 mL.

Point of zero charge

The points of zero charges (pzc) of the adsorbent were determined using this approach [18] where 25 mg of newly synthesised SS:DoAc@Fe_3O_4 adsorbent and control (Fe_3O_4 particles) were added to 10 mL solutions with pH levels of pH 2, 4, 6, 8, and 10, respectively. An orbital shaker was used to agitate the mixture for 24 hours. Subsequently, the final pH of the mixtures was determined using a pH meter and the graph of ΔpH versus the initial pH was plotted.

Optimisation of MSPE technique via Taguchi design of experiments

A process can be optimised using the Taguchi design of experiments method by identifying the crucial parameters that influence the output and determining the ideal levels of these parameters. This strategy uses the multi-variable at a time method, which is comparable to the conventional approach of OVAT. Five different parameters (pH of sample solution, mass of adsorbent (mg), extraction time (min), desorption time (min), and volume of desorption solvent (µL)) were analysed simultaneously where only one parameter was manipulated at a time whereas the rest were held constant. The extraction efficiency was assessed using the average of three replicate measurements (n=3), which were conducted for each parameter. Deionised water with 10 mgL^{-1} of ARAC was used to conduct the optimisation analysis. Spectrophotometric peak regions corresponding to the target dye wavelength were employed to analyse the effects of various factors on the effectiveness of the targeted dye's extraction. The effect of sample pH was studied at the range of pH 2, 4, 6, and 8. The SS:DoAc@Fe_3O_4 adsorbent ranging from 5 to 25 mg was also investigated. Additionally, the effect of extraction time was analysed for durations between 0.5 to 3 mins; the effect of desorption time was analysed for durations between 1 to 5 mins; and the effect of desorption solvent volume was analysed at the range of 400 to 700 µL. In this study, the extraction efficiency is the target property and should be maximised; thus, the favourable optimisation criterion is the larger-the-better (LTB) type of signal of noise.

Method validation

The linearity range, precision, limit of detection (LOD), limit of quantification (LOQ), recovery values, and matrix effect of the proposed SS:DoAc@Fe_3O_4-based MSPE approach was evaluated by conducting a range of tests on real matrix samples according to the International Council for Harmonisation of Technical Requirements of Pharmaceuticals for Human Use (ICH) guidelines [31].

Linearity

The linearity of the proposed DES in the MSPE method was examined by employing the optimised parameter conditions. Samples of soft drinks were spiked with various amounts of the standard dye analyte, ranging from 0.5 to 10 mgL^{-1}. A peak area following sample pre-treatment was applied to create a linear calibration curve with a coefficient of determination (R^2) close to 1.

Limit of detection (LOD) and limit of quantification (LOQ)

To assess the sensitivity of the proposed technique, the LOD and LOQ were calculated using the formulas in Eq. 1 and Eq. 2, respectively, where s is the standard deviation of ten replicate readings of the blank sample and b is the gradient of the calibration line [32].

$$\text{LOD} = \frac{3.3s}{b} \quad (1)$$

$$\text{LOQ} = \frac{10s}{b} \quad (2)$$

Precision analysis

The intra-day and inter-day precision at dye concentrations of 10 mgL^{-1} were measured to determine the precision of the proposed method. Both intra-day and inter-day precision analyses were conducted using three replicates (n=3). Prior to establishing the precision value, the standard deviation (SD) of the data was determined and provided in the form of percentage of relative standard deviation (%RSD). Subsequently, the SD and %RSD values were calculated using Eq. 3 and Eq. 4, respectively.

$$\text{SD} = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}} \quad (3)$$

$$\text{RSD} = \frac{\text{SD}}{\bar{x}} \times 100 \quad (4)$$

where x is the outcomes of each run, n is the number of measurements, and $\bar{x}$ is the mean.

Recovery study

Three different dye concentrations were incorporated into the sample matrix to evaluate the accuracy of the suggested technique. The relative recovery (RR%) of dye was determined for each concentration level using three replicates (n=3) via Eq. (5).

$$\text{RR (\%)} = \frac{\text{Cs-Cu}}{\text{Co}} \text{ x } 100 \quad (5)$$

Where C_s is the dye concentration that has been spiked into the sample matrix, C_u is the dye concentration that has not been spiked into the sample matrix, and C_0 is the initial standard solution spiked into the sample matrix. In this study, the standard dye solution was spiked with dye at low, medium, and high concentrations (1, 3, and 10 mgL^{-1}) to analyse the drink samples.

Matrix effect

The process of co-extracting and co-eluting chemicals from actual matrix samples can have a significant impact on the spectrophotometry signals of dye. Therefore, Eq. (6) was used to study the matrix effect [33].

$$\text{Matrix effect (\%)} = \frac{(A_{M+S} - A_M)}{A_O} \text{ -1 x } 100 \quad (6)$$

Where A_{M+S} denotes the spectrophotometric response of the dye in a sample matrix that has been spiked, A_M denotes the spectrophotometric response of the dye in a sample matrix that has not been spiked, and A_O denotes the spectrophotometric response of the dye in a standard solution. A dye concentration of 10 mgL^{-1} was used to assess the matrix effect.

Real sample analysis

Commercial soft drinks were purchased from the local supermarket. First, an adequate amount of liquid drink sample was degassed for 10 minutes via sonication [34]. The liquid sample was later filtered through 0.45-µm nylon syringe filters, and 1.0 mL of the sample solution was taken and transferred into a 100-mL volumetric flask and diluted with ultrapure water [35]. Afterwards, the pH of the liquid samples was adjusted to 2 using 1 M hydrochloric acid. The MSPE technique was then applied to analyse the liquid samples.

Results and Discussion

Characterisation of DES

Figure 2 displays the FT-IR spectra of Fe_3O_4, SS:DoAc-based DES, and SS:DoAc@Fe_3O_4. The existence of the Fe-O stretching vibration at a frequency between 580 and 620 cm^{-1} serves as evidence for the successful formation of the Fe_3O_4 particles in spectra (a) and (b). The large absorption peak of approximately 3000 cm^{-1} in the Fe_3O_4 and SS:DoAc@Fe_3O_4 adsorbents indicated the existence of hydroxyl group (OH stretching). The Si−O−Si functional group of silicone surfactant (SS) is responsible for the stretching vibration in SS:DoAc@Fe_3O_4 materials at a wavenumber of around

1402 cm^{-1}. When Fe_3O_4 was coated with SS:DoAc-based DES, the C=O stretching band associated with the dodecanoic acid (DoAc) of SS:DoAc DES produced a wavenumber of 1622 cm^{-1}. These results are in line with the findings reported by previous studies [36, 37]. When compared to pure Fe_3O_4, the distinctive peaks of Si−O−Si stretch at 1402 cm^{-1} and C=O stretch at 1622 cm^{-1} established the success of the coating of SS:DoAc on Fe_3O_4. The attribution of the FT-IR spectra is summarised in Table 1. The FT-IR results denote the successful synthesis of the unique SS:DoAc@Fe_3O_4 material.

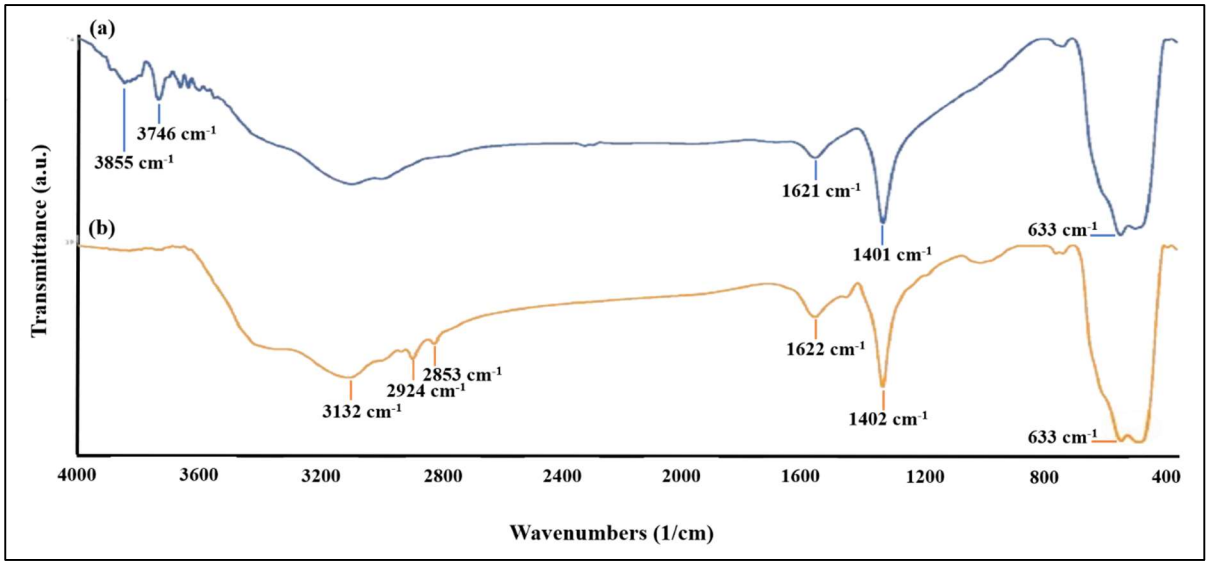


Figure 2. FT-IR spectra of (a) pure Fe_3O_4, (b) SS:DoAc@Fe_3O_4

Table 1. Main IR frequencies with assignments

	Wavenumber (cm^{-1})	
Assignment	**Fe_3O_4**	**SS:DoAc@Fe_3O_4**
OH stretching	3855	3132
Fe−O stretching vibration	633	633
Si−O−Si stretching	-	1402
C=O stretching	-	1622
C−H	3746	2924

Preliminary screening

An initial study was conducted using the MSPE technique with pure Fe_3O_4 particles and SS:DoAc@Fe_3O_4 adsorbent. As illustrated in Figure 3, the SS:DoAc@Fe_3O_4 adsorbent demonstrated a greater analytical response to dye extraction compared to pure Fe_3O_4 particles. This demonstrates that the coating of DES-based SS:DoAc on Fe_3O_4 particles enhanced the adsorbent's capacity for extraction. The extraction of the non-polar dye investigated in this study is facilitated by the hydrophobic SS:DoAc-based DES. Due to the strong hydrophobic interaction that developed between the target dye and the adsorbents, the target dye could be readily adsorbed onto the SS:DoAc@Fe_3O_4 surface. In the MSPE extraction technique, hydrophobic deep eutectic solvents (HDES) have been reported as an alternative to traditional extraction medium. HDES has been proven to be efficient absorbents with excellent CO_2 affinity and selectivity [38]. They are also efficient in removing hydrophobic compounds from aqueous samples [39]. Moreover, HDES has been utilised as an effective modification of inexpensive adsorbent for the extraction of pharmaceuticals and dyes [40]. Hence, the SS:DoAc@Fe_3O_4 adsorbent was preferred for further optimisation because the SS:DoAc-based DES coating could significantly enhance the extraction performance.

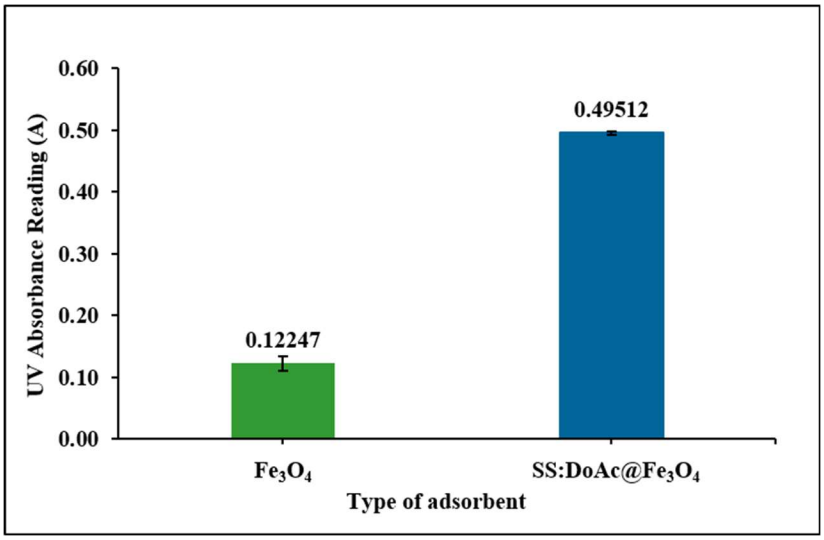


Figure 3. Type of adsorbent. (Condition: 10 mL of sample solution; 25 mg of adsorbent; 2 mins of extraction time; 800 µL of 25% (w/v) Na_2SO_4; 700 µL of methanol and 2 mins for desorption time), n=3

Optimisation of MSPE via OVAT design of experiments: Type of desorption solvent

The three frequently used organic solvents (methanol, acetonitrile, and methanol:acetonitrile (1:1) and isopropanol) with varied polarity were evaluated to determine their ability to elute the target dye to ensure the maximum recovery feasible. The results in Figure 4 show that the application of methanol:acetonitrile (1:1) as eluent resulted in the highest analytical signal, which was subsequently followed by methanol, acetonitrile, and isopropanol. The polarity and solvent characteristics of methanol and acetonitrile contribute to their efficacy as desorption solvents. Methanol is a polar-protic solvent, whereas acetonitrile is a polar-aprotic solvent and has a greater dipole moment. These two solvents are compatible with standard mobile phase additives and buffers and are completely miscible with water [41]. Methanol and acetonitrile, however, have been discovered to complement each other in terms of extraction efficiency. Both solvents can interact with non-polar compounds like ARAC due to their mid-polarity, making it easier to extract these compounds from food samples [42]. As a result, the target ARAC is more easily eluted from the SS:DoAc@Fe_3O_4 adsorbent into methanol:acetonitrile. Therefore, methanol: acetonitrile was chosen as a potential candidate for the desorption solvent in the forthcoming optimisation study.

Salt addition

The addition of salt was found beneficial in the extraction of ARAC because it enhances extraction efficiency by effectively separating dye molecules from the aqueous phase [43]. The recovery of the target analyte was favoured when salt was added to the sample solution due to the salting-out effect where it spiked the ionic strength of the aqueous matrix without causing additional reactions or chemical changes. This condition selectively reduced the analyte's solubility in the aqueous phase and increased the amount of analyte that could be adsorbed onto the SS:DoAc@Fe_3O_4 adsorbent [44]. Sodium sulfate (Na_2SO_4) is often used for salting out processes due to its properties where it is highly soluble in water and does not react with most compounds, making it an inert substance in this type of extraction. The effect of salt addition on the extraction performance was studied where sample solution added with salt was compared to sample solution without the addition of salt. The type of salt employed in this procedure is Na_2SO_4 with a concentration of 25% (w/v). In contrast to the standard sample solution, adding salt drastically increased the extraction yield, as shown in Figure 5. Therefore, 25% (w/v) of Na_2SO_4 was chosen for further investigation.

Volume of salt

The effect of salt volume was investigated by adding 25% (w/v) Na_2SO_4 at the range of 200 to 800 µL. As

depicted in Figure 6, the highest analytical response was the extraction yield obtained in 200 µL of 25% (w/v) Na_2SO_4. However, the extraction efficiency decreased with the addition of 800 µL of 25% (w/v) Na_2SO_4. This is due to the possibility of a salt-rich layer forming at the interface between the aqueous and organic phases, which potentially reduces the efficacy of the extraction method [45]. Thus, 200 µL of 25% (w/v) Na_2SO_4 was preferred for further investigation.

Volume of sample solution

The effect of sample solution volume was investigated with the range of 10 to 25 mL. As depicted in Figure 7, the highest analytical response was the extraction yield obtained in 20 mL of sample solution. A considerable amount of sample solution is necessary to obtain a higher pre-concentration factor [46]. The adsorption rate exhibited little impact for sample volumes between 10 and 20 mL but it drastically dropped between 20 and 25 mL (see Figure 7). This resulted from the use of high volume of sample solution, which may dilute the target analyte and minimise the efficacy of extraction and the analytical response of the method.

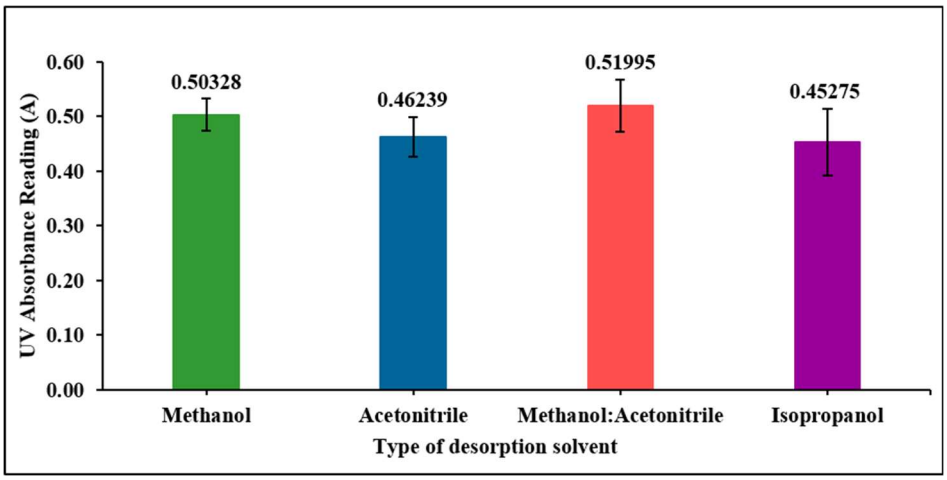


Figure 4. Effect of desorption solvent. (Condition: 10 mL of sample solution; 25 mg of adsorbent; 2 mins of extraction time; 800 µL of 25% (w/v) Na_2SO_4; and 2 mins for desorption time), n=3

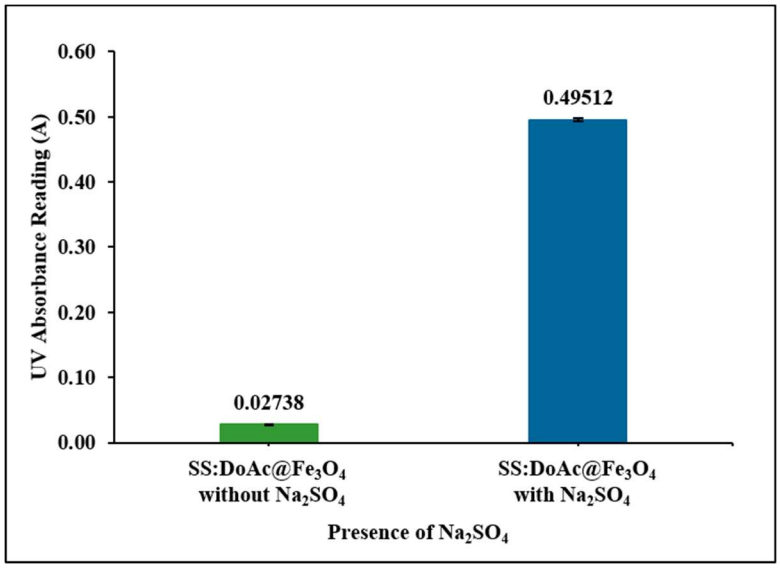


Figure 1. Effect of presence of Na_2SO_4. (Condition: 10 mL of sample solution, 25 mg of adsorbent; 2 mins of extraction time; 800 µL of 25% (w/v) Na_2SO_4; and 2 mins for desorption time), n=3

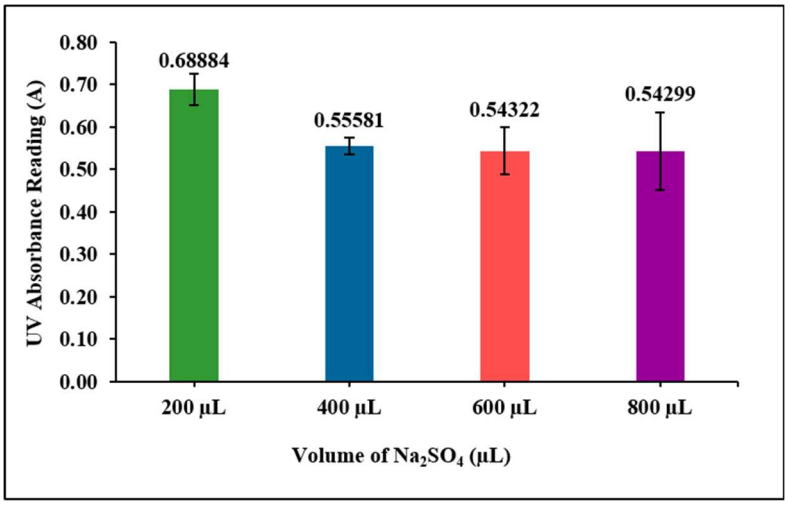


Figure 2. Effect of volume of Na_2SO_4. (Condition: 10 mL of sample solution, 25 mg of adsorbent; 2 mins of extraction time; and 2 mins for desorption time), n=3

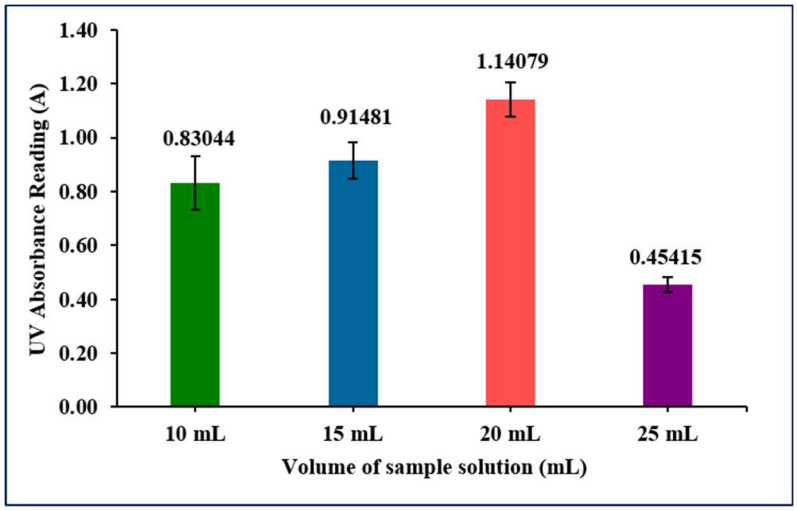


Figure 3. Effect of volume of sample solution. (Condition: 800 µL of 25% (w/v) Na_2SO_4, 25 mg of adsorbent; 2 mins of extraction time; and 2 mins for desorption time), n=3

Point of zero charge

The pH at which the net surface charge of the particles reaches zero is known as the point zero charge (pzc) of an adsorbent [47]. Determining the point of zero charge (pzc) of an adsorbent is crucial in adsorption studies. The surface of the adsorbent is positively charged whenever the solution's pH is lower than pzc. The surface may attract anions in this condition. However, if the pH of the solution is greater than pzc, the surface is negatively charged and may attract cations [48]. Figure 8 shows the Δ pH plotted against the initial pH graph. The pH value for zero-point charge of the SS:DoAc@Fe_3O_4 adsorbent was found to be at pH 2. Whereas the surface of SS:DoAc@Fe_3O_4 adsorbent became positively charged at pH level less than pH 2, making it feasible for anionic species like Allura Red AC to be adsorbed.

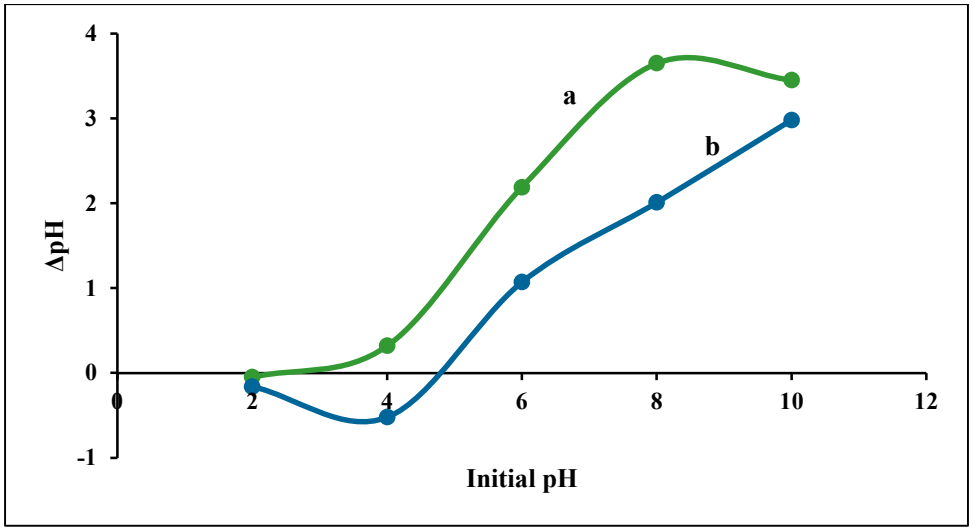


Figure 8. Point of zero charge of a) Fe_3O_4 and b) SS:DoAc@Fe_3O_4, (n=1)

Optimisation of MSPE technique via Taguchi design of experiments: pH of sample solution

The efficacy of the extraction is significantly affected by the pH of the sample solution by establishing the ionic state of any possible adsorbents, adsorbates, and other species in the sample [49]. The effect of sample pH was investigated with pH values ranging from 2 to 8. As shown in Figure 9, the extraction recovery of ARAC significantly increased in the pH range of 2 to 4 and decreased from 6 to 8. Given that the pKa of the target analyte, ARAC, is 11.4 and that 50% of this dye is protonated at the pH value of 11.4, the partial deprotonation of the phenol group for ARAC may be the cause of the decreased extraction of the analyte [50]. The results align with those reported by Yu and Fan who found that the adsorption rate of ARAC was higher at pH 2 or 3 but decreased as the pH increased from 3 to 7 [46]. Another study also mentioned that in an acidic solution (pH 2), the ARAC dye functions as an anionic dye molecule and two of its sulfonate (SO^{3-}) groups may be electrostatically attracted by protonated amino (NH^{2+}) groups. The maximum dye adsorption was seen at solution pH 2, which may be due to the electrostatic interaction between dye molecules and adsorption sites [51]. The target ARAC could be present as a stable acidic molecule with a greater affinity for adsorbents at pH 2, which resulted in the maximum extraction yield. As a result, pH 2 was selected as the appropriate pH for subsequent tests.

Mass of adsorbent

The quantity of adsorbent was optimised to achieve the highest extraction yield where different amounts of SS:DoAc@Fe_3O_4 in the range of 5 to 25 mg were investigated. As shown in Figure 9, the extraction recovery for the investigated ARAC rose exponentially with adsorbent mass up to 25 mg. The dosage of the adsorbent determines the amount of target dyes being adsorbed since it offers the sites needed to load the dyes. In this regard, more accessible sites are deemed advantageous for maximising extraction [49]. This agrees with Saha et al. who stated that surface area and the number of accessible adsorption sites increased as the mass of adsorbents increased in the extraction of Allura Red [51]. Therefore, 25 mg of the SS:DoAc@Fe_3O_4 adsorbent was established for the subsequent experiments.

Extraction time

According to Yu and Fan, the extraction method used in MSPE is based on a time-dependent equilibrium in which target molecule mass transfer rises with time until adsorption equilibrium is attained [46]. The period of time needed to establish extraction equilibrium is crucial for improving both the mass transfer and the interaction between the sample solution and the solvent [50]. The extraction time was examined between 0.5 and 3 mins in order to maximise the extraction yield. As seen in Figure 9, the analytical response elevated significantly in the first 2 mins. This could be prompted by the

powerful attraction forces between the dye molecules and the adsorbents. To achieve equilibrium, dye molecules quickly adsorb onto the adsorbents' exterior surfaces, followed by pore diffusion into their matrix [51]. Further prolongation of the extraction process notably after 2 mins demonstrates that the adsorption of ARAC dye onto the adsorbent did not show a significant progress, which could be related to back extraction [52]. Hence, 2 mins of extraction time was selected for forthcoming investigations.

Desorption time

The impact of desorption time was investigated between 1 and 5 mins. The results in Figure 9 show that 3 mins of sonication was sufficient to elute all the target ARAC from the adsorbent into methanol:acetonitrile (1:1). The analytical response increased gradually and declined after 3 mins of elution. According to Naing et al., prolonged sonication may have caused the target analytes to re-adsorb onto the adsorbent, thus decreasing the extraction yield [52]. Conversely, the adsorbents' magnetic properties allowed them to be quickly isolated from the sample solution in less than 2 mins using a powerful magnet as opposed to filtration or centrifugation [46]. Therefore, the optimal desorption time was established at 3 mins of sonication.

Desorption solvent volume

The impact of desorption solvent volume on the elution of target ARAC was also investigated to obtain the highest possible extraction yield. As shown in Figure 9, the analytical signal quickly decreased following the increasing volume of elution solvent. Methanol:acetonitrile (1:1) beyond 400 µL caused the target ARAC to be diluted in the solvent, which gradually reduced the spectrophotometric response. Desorption solvent volumes below 400 µL were not employed in this study because the remaining solvent obtained after the desorption process was insufficient to be subjected to UV-Vis spectrophotometry analysis. Subsequently, 400 µL of methanol:acetonitrile (1:1) was fixed to prepare for future optimisation.

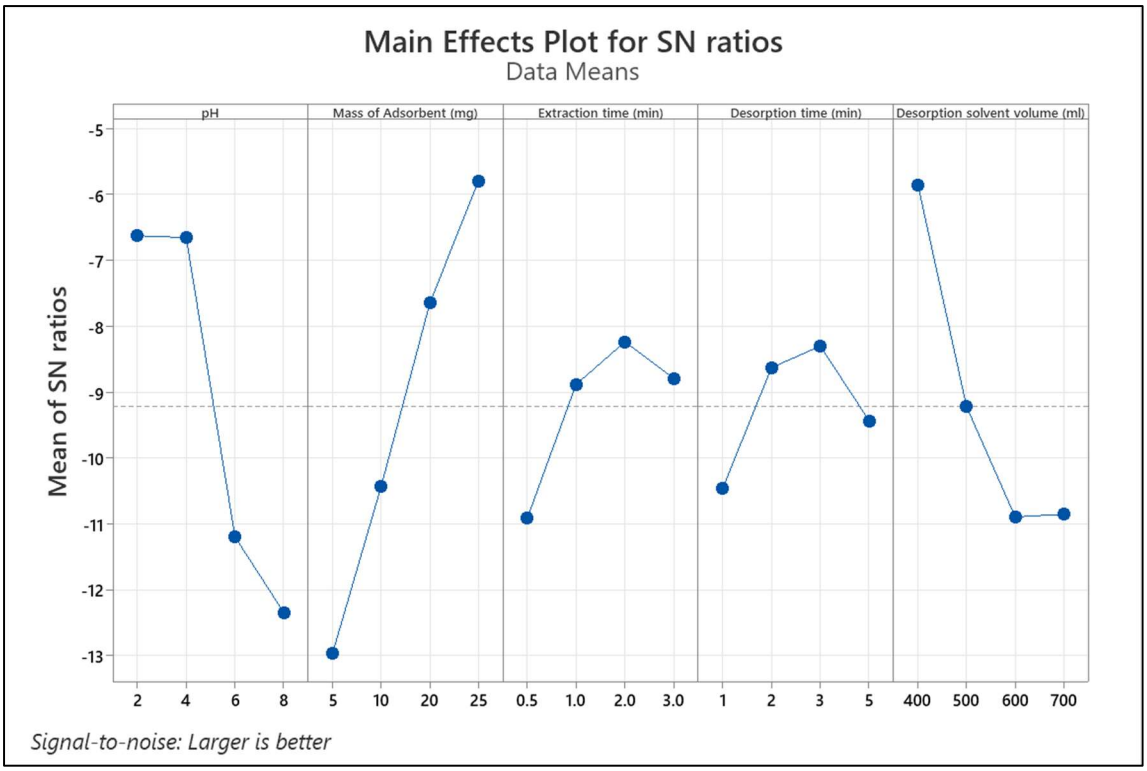


Figure 9. Signal-to-noise ratios involving sample pH; mass of adsorbent, extraction time, desorption time, desorption solvent volume

Method Validation

The proposed approach was implemented to monitor ARAC in soft drink samples to evaluate its feasibility. The calibration curve was plotted within the range of 0.3 to 10 mgL^{-1} for soft drinks samples under optimised experimental parameters. The coefficient of determination (R^2) derived from the linearity curve was 0.999. The LOD obtained was 0.1 mgL-1 and the LOQ was 0.4 mgL-1, and both values are lower compared to the maximum permitted level of ARAC in soft drinks. In accordance with Directive 94/36/EC from the European Parliament and Council, the highest acceptable concentration of ARAC in soft drinks is 100 mgL^{-1} [53]. The intra- and inter-precision values for the developed technique ranged between 5.05% and 5.37%, respectively. The matrix effect obtained is 4% across all investigated ARAC, indicating that the analysis of the soft drink samples had minimal effect on the recovery of ARAC. Table 2 provides a summary of the figure of merits.

Table 2. Analytical performance data of MSPE technique using SS:DoAc@Fe_3O_4 adsorbent

Assay	Allura Red AC
Linearity (mgL^{-1})	0.3 - 10
Coefficients of determination (R^2)	0.999
Intraprecision (%) (n=3)	5.05
Interprecision (%) (n=3)	5.37
LOD (mgL^{-1})	0.1
LOQ (mgL^{-1})	0.4
Matrix effect (%)	4

Real sample analysis

Samples of commercial soft drinks were subjected to the validated MSPE method using the SS:DoAc@Fe_3O_4 adsorbent. The samples were spiked with 1, 3, and 10 mgL^{-1} of the target ARAC to examine how the sample matrix affected the results. The recoveries obtained from soft drinks samples ranged from 80% to 111%. Table 3 lists the recovery values for the real sample.

Table 3. Relative recovery study of SS:DoAc@Fe_3O_4 based MSPE of ARAC in soft drink samples.

Analyte	Spiked (mgL^{-1})	% Recovery (% RSD, n = 3)		
		Sample A	Sample B	Sample C
Allura Red AC	0	nd	nd	nd
	1	111 (11.6)	101 (7.4)	93 (4.6)
	3	88 (9.2)	84 (6.2)	85 (1.1)
	10	80 (10.7)	83 (2.6)	90 (3.9)

Linear range: 0.3-10 mgL^{-1}

[a]nd-not detected.

[b]Mean of triplicate analysis.

[c]RSD-Relative standard deviation (n = 3)

Comparison with previous studies

As shown in Table 4, the developed method obtained lower LOD compared to those reported by dos Reis et al. [54]. Compared to other methods, the LOD obtained was slightly lower due to the instrument used in this study. UV-Vis has lower sensitivity compared to other instruments, such as HPLC. However, the result is comparable to other studies as the extraction time needed in this study was only one minute.

Table 4. Comparison of performance of ARAC extraction using various extraction techniques

Extraction Technique	Matrix	Instrument	LOD (mg L^{-1})	LOQ (mg L^{-1})	Extraction Time (min)	Reference
VA-SS-LPME	Confectioneries, beverages, drugs and syrups	UV-Vis spectrometer	0.051	0.172	2	[55]
LLE	Milk, meat and fish products, confections, wines, juices and beverages	HPLC-DAD	0.029	0.088	2 – 5 (based on samples)	[56]
MSPE	Paediatric syrup, colored sugar-coated chickpeas, and mineral water	HPLC-UV	0.05	0.1	5	[48]
LLE	Guava pulp	HPLC-DAD	15	30	0.5	[54]
MSPE	Soft drink	UV-Vis spectrometer	0.1	0.4	2	This study

Abbreviation: VA-SS-LPME: vortex assisted sequential-simultaneous liquid phase micro-extraction; LLE: liquid-liquid extraction; MSPE: magnetic solid phase extraction; HPLC-DAD: high performance liquid chromatography with diode array detector; HPLC-UV: high performance liquid chromatography with ultraviolet detector.

Conclusion

This study aimed to explore the application of SS:DoAc-based DES functionalised Fe_3O_4 (SS:DoAc@Fe_3O_4) adsorbents, which are inexpensive and environmentally benign solvents, to increase the effectiveness and sustainability of the extraction technique. The work was successful in demonstrating the feasibility and efficacy of SS:DoAc@Fe_3O_4 for the extraction of ARAC through an extensive experimental method and analysis. The results showed that the proposed adsorbent has higher extraction efficiency, high selectivity, and great reusability. Compared to unmodified Fe_3O_4 particles, the SS:DoAc-based DES functionalised Fe_3O_4 allowed for simple and quick separation of the adsorbent from the sample matrix. This was made possible by the strong hydrophobic interaction between ARAC and the SS:DoAc@Fe_3O_4 adsorbent.

Through a combination of OVAT and Taguchi design methodologies, we successfully optimised the extraction conditions for ARAC using magnetic solid phase extraction. The OVAT analysis revealed that having a methanol:acetonitrile (1:1) mixture as the desorption solvent, salt addition for enhanced extraction performance, salt volume of 200 µL, and a sample volume of 20 mL yielded optimal results. Meanwhile, Taguchi experiments determined the ideal conditions as pH 2, 25 mg of adsorbent dosage, 2 minutes of extraction time, 3 minutes of desorption time, and a desorption solvent volume of 400 µL. These findings enhance the current understanding of the extraction process and offer practical guidelines for maximising ARAC extraction efficiency. Under optimised experimental parameters, the studied ARAC derived the R^2 value of 0.999 for linearity curve while the LOD and LOQ were 0.1 mgL^{-1} and 0.4 mgL^{-1}, respectively. This infers that the developed MSPE approach capitalising on the SS:DoAc@Fe_3O_4 adsorbent is indeed an excellent tool for the analytical separation and identification of ARAC. The findings of this work significantly support the notion that DES-based MSPE is an extraction technique with high potential for the extraction of azo food dyes, hence providing new opportunities for efficient and sustainable dye analysis in the food industry. This research represents a significant advancement in the direction of more environmentally friendly and effective extraction techniques, making a substantial contribution to the overall development of green techniques for the analysis of food dyes.

Several suggestions for further research can be made based on the findings and conclusion deduced from this study. First, it is important to work on optimising the DES composition to find the best solvent system for a variety of azo food dyes. This can be accomplished by

experimenting with various molar ratios and DES component combinations. Moreover, examining the applicability of the suggested approach using other analytical tools, such as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS), can expand our knowledge in this specific field of study and improve its credibility. Investigating the application of DES-based MSPE to complex food matrices, like processed foods and different types of beverages, will also offer beneficial practical insights. For industrial implementation, it is essential to evaluate the scalability, viability, and compatibility of the process. Furthermore, it would be desirable to conduct an environmental impact assessment (EIA) to compare the sustainability and energy efficiency of DES-based MSPE to traditional approaches. These recommendations for future research must be taken into consideration to ensure consumer safety and environmental protection. This will help to promote effective and sustainable methods for food dye analysis in food technology applications.

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AUTHENTICATION OF CLOVE LEAF OIL IN PRODUCTS (*Syzygium aromaticum* (L.) Merr. & L. M. Perry) USING GC-MS AND FTIR METHODS COMBINED WITH CHEMOMETRIC

Pengesahan Minyak Daun Cengkih (*Syzygium aromaticum* (L.) Merr. & L. M. Perry) dalam Produk Menggunakan Kaedah GC-MS dan FTIR Yang Digabungkan dengan Kemometri

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Abstract

The production cost and market demand for clove oil are significantly high due to its great commercial value and benefits. However, the extraction yield of clove oil is very low at around 1%, causing the act of adulteration of essential oils to reduce production costs. Therefore, this study aims to determine the addition of adulterants in essential oils using the authentication technique. The samples used are clove oil from dry leaf distillation, products A, B, and C on the market. The GC-MS and FTIR methods combined with PCA (Principal Component Analysis) and PLS (Partial Least Square) multivariate calibration analysis were selected in the authentication to detect clove oil adulteration. The constituent's presence in distilled clove oil from GC-MS analysis was eugenol (49.63%), β-Caryophyllene (28.25%), alpha-Humulene (8.92%), alpha-Copaene (2.15%), delta-Cadinene (1.61%), and Caryophyllene oxide (1.50%). Six concentrations were prepared for FT-IR analysis of clove leaf oil and turpentine oil mixture, which was estimated by PLS and PCA chemometrics. Turpentine oil was used as counterfeit, which was usually added to clove oil products. The PLS analysis of FTIR obtained optimized wavenumbers, 2960-2860 cm^{-1}. The equation y=0.9998x+0.0096 had an R^2 value of 0.9998, as well as RMSEC, RMSECV, and RMSEP values of 0.22%, 0.76%, and 1.20%, respectively. The PCA analysis can categorize the oil based on the main component types of distilled clove leaf oil, turpentine oil, and market oil, namely products A, B, and C. The results showed that product A oil was in the same quadrant as distilled clove leaf oil. Moreover, no clove leaf oil product had similar physical and chemical characteristics to turpentine oil.

Keywords: authentication, clove leaf oil, Fourier transform infrared, gas chromatography-mass spectrometry, turpentine oil

Abstrak

Minyak cengkih mempunyai nilai dan faedah komersial yang besar, pada masa ini permintaan untuk ketersediaan minyak cengkih di pasaran adalah sangat tinggi namun, hasil perahan minyak cengkih adalah sangat rendah, iaitu sekitar 1% dan mengakibatkan kos pengeluaran minyak cengkih yang agak tinggi. Ini menyebabkan pemalsuan minyak pati untuk mengurangkan kos pengeluaran

dan mengekalkan jumlah minyak pati. Satu teknik yang boleh digunakan untuk mengesan kehadiran bahan tiruan dalam minyak pati adalah melalui pengesahan. Kaedah GC-MS dan FTIR digabungkan dengan analisis penentukuran multivariat PCA (analisis prinsip utama) dan PLS (*partial least square*) telah dipilih dengan tujuan untuk mengesan tindakan pemalsuan pada minyak cengkih. Kandungan minyak daun cengkih suling daripada hasil analisis GC-MS iaitu eugenol 49.63% β-Caryophyllene 28.25%, alpha-Humulene 8.92%, alpha-Copaene 2.15%, delta-Cadiene 1.61% dan Caryophyllene oksida 1.50%. Analisis FT-IR dijalankan dengan membuat 6 siri kepekatan campuran minyak daun cengkih dan minyak turpentin dan kemudiannya dianalisis dengan kemometrik PLS dan PCA. Keputusan analisis PLS daripada FTIR menunjukkan bahawa nombor gelombang yang dioptimumkan ialah 2960-2860 cm^{-1}. Persamaan yang terhasil ialah y = 0.9998x + 0.0096 dengan nilai R^2 0.9998; Nilai RMSEC (0.22%; nilai RMSECV 0.76%; dan nilai RMSEP 1.20%. Hasil analisis PCA mampu mengklasifikasikan minyak berdasarkan jenis komponen utama minyak suling daun cengkih, minyak turpentin, dan minyak daun di pasaran (A, B, dan C), di mana minyak produk A berada dalam kuadran yang sama dengan minyak daun cengkih suling dan tiada produk minyak daun cengkih yang mempunyai ciri fizikal dan kimia yang serupa dengan minyak turpentin.

Kata kunci: pengesahan, inframerah transformasi Fourier, kromatografi gas-spektometri jisim, minyak daun cengkih, minyak terpentin

Introduction

Clove (*Syzygium aromaticum*) is a spices that has long been used in medicine and as a food preservative [1]. This plant is a natural source that contains many secondary metabolite compounds, namely phenolics such as eugenol, eugenol acetate, and gallic acid. Therefore, its leaf oil, namely clove oil, has great potential for applications in the pharmaceutical, cosmetic, food, and agricultural fields [2]. The main constituents in clove flower buds, stalks, and leaves are eugenol and β-caryophyllene [3]. Meanwhile, the essential oil content in clove leaves is 58.6% eugenol, 21.4% β-caryophyllene, 5.6% α-humulene, and 2.9% caryophyllene oxide [4].

Clove oil can be produced from flowers and stems, including leaves that have the highest economic value. The price range from various parts is not significantly different, making it a widely traded product. This makes the leaves more economically valued for their potential to be processed into oil [5].Currently, the market demand for clove is very high due to its great commercial value and benefits. However, the extraction yield is significantly low at around 1%, leading to a high cost of production. This has consequently caused the adulteration act of essential oils to reduce production costs [6]. According to Hong et al. [7], essential oil adulteration is a major problem in today's global market by mixing cheaper components of lower quality in high-value essential oils.

Detection of clove oil adulteration is carried out to ensure that the oil sold in the market meets the established quality standards. Meanwhile, a frequently used type of counterfeiting material is the addition of turpentine oil with the main component which can cause an increase in the α-pinene content. This will reduce the specific gravity and solubility value of the oil in alcohol and affect the odor [8]. Turpentine oil is widely selected as an adulterant in essential oils because it is relatively cheap, with a lighter and less polar fraction is lighter [9]. The technique that can be performed to detect the addition of counterfeiters in essential oils is authentication. This technique is conducted using GC-MS and FTIR methods, which will be analyzed with PCA (principal component analysis) and PLS (partial least square) multivariate calibration to detect counterfeiting of clove oil [10].

The technique commonly used for essential oil quality control is gas chromatography (GC) coupled with mass spectrometry (MS). This is because the detection of volatile compounds in essential oils can quickly, easily, and accurately be identified using the technique. In recent years, spectroscopic techniques with a PCA chemometrics approach have often been proposed as an alternative to chromatographic methods in detecting the adulteration of essential oils [11].

Another method commonly used is Fourier transforms infrared (FTIR) combined with multivariate calibration chemometrics PLS and PCA to detect specific functional group absorption [12]. The authentication of essential oils using the FTIR is carried out in the fingerprint area,

which is the characteristic of a compound with the spectrum and sample concentration entered in a one-step model [13]. In this study, the profile analysis of the chemical components of clove oil using GC-MS and FTIR methods was will be analyzed by multivariate calibration of PCA and PLS to authenticate clove oil products in the market.

Materials and Methods

Materials

The material used was dry clove leaves harvested in June 2022 from Green Herbal Yogyakarta Company. Fresh clove leaves were determined at the Ahmad Dahlan University Biology Laboratory by sending fresh whole samples, including leaves and stem, for accurate identification. Subsequently, the clove oil brands A, B, and C as samples were obtained from e-commerce (Shopee). Turpentine oil was obtained from PT. Syailendra Bumi Investama.

Tools

The tools used were a set of distillation tools, beakers, measuring pipettes, micropipettes, GC-MS Agilent 7890A instrument with specifications, capillary column type HP-5DB MSDB 5DB-5 (60 m x 250µm x 0.25µm), carrier gas used helium, injector temperature 250 °C with an injection volume of 0.2 µL, split ratio 1:200 with a split speed of 50 mL/minute and the initial temperature of 50 °C held for 2 minutes. The rate of increase is as follows 10 °C/minute to 99 °C, 2 °C/minute to 225 °C held for 20 minutes, and 5 °C/minute to 250 °C. The final temperature reached 250 °C. The detector used was Agilent 5975C MS with a mass range of 35-550.

The FTIR instrument used Thermo Scientific Nicolet iS10 with beam splitter using KBr and deuterated triglycine sulfate (DTGS) detector. The readings were taken at wavenumbers: 4000-650 cm^{-1}, resolution 8, and 32 scans.

Procedure: Clove leaf preparation

A 3 kg dried clove leaves obtained from Green Herbal Yogyakarta Company were dry-sorted, and the sizes were reduced by 2-3 cm.

Authentication method: Analysis using GC-MS with PCA chemometrics

Samples of clove oil, turpentine oil, and market products were put into 2 mL vials and placed sequentially in the sample container of the GC-MS device. The n-hexane solvent was also placed in a 2 mL vial in the last container. A total of 0.2 µL oil samples were automatically injected into the GC-MS using a syringe. After the injecting one sample, the syringe automatically washes and perform column cleaning. At the column cleaning stage, the GC-MS was run without inserting any sample. This process was repeated three times, and the samples were identified using MS. Subsequently, data were analyzed on GC-MS in the form of the relative area obtained from chromatogram results and m/z fragmentation of compounds.

Sample calibration and validation for FTIR with PCA and PLS chemometrics

A total of 7 concentrations was prepared for each oil in the form of distilled clove leaf oil (MC) 100%v/v, turpentine oil (MT) 100%v/v. Further concentration series (2 mL) were also prepared, namely MC:MT (90:10), (80:20), (70:30), (60:40), and (50:50) %v/v for calibration and validation. This was followed by the analysis of clove leaf oil, turpentine oil, as well as samples A, B, and C using an FTIR [12].

Data analysis: GC-MS data analysis of PCA chemometrics combination

GC-MS data in the relative area was collected by grouping the essential oil components with clove oil products on the market brands A, B, and C using PCA followed by biplot with MINITAB 20 software. This grouping was based on data diversity, which produces scree, score, loading plots, and biplot graphs [14].

FTIR data analysis of combined PLS and PCA chemometrics

The FTIR data were analyzed by multivariate calibration in MINITAB 20 program using PCA and PLS techniques for quantification. The original spectrum absorbance data, the spectrum with a preliminary process involving all absorption features, and part of the absorption data were processed according to the spectrum segmentation area. Subsequently, the

calculation of the RMSE value was obtained by squaring the subtraction of the actual value with the predicted value summed and divided by the number of data [15].

Results and Discussion

Component analysis of clove leaf oil

The analysis of clove leaf oil using the GC-MS method was applied to determine the oil component and the percentage of essential oil compound content. The method had a high sensitivity to quantitatively and qualitatively volatile compounds. Furthermore, it was a fast and accurate method for separating complex mixtures and producing data on the structure as well as the identity of organic compounds. In GC-MS, the AUC produced by the chromatogram was directly proportional to the concentration of the components contained in the sample. The number of essential oil components and their percentage levels were obtained from analysis with GC. This was followed by using MS to determine the type of compounds contained in clove leaf oil using spectra derived from NIST and WILEY libraries. Meanwhile, the chromatogram of GC separation results of clove leaf essential oil is presented in Figure 1.

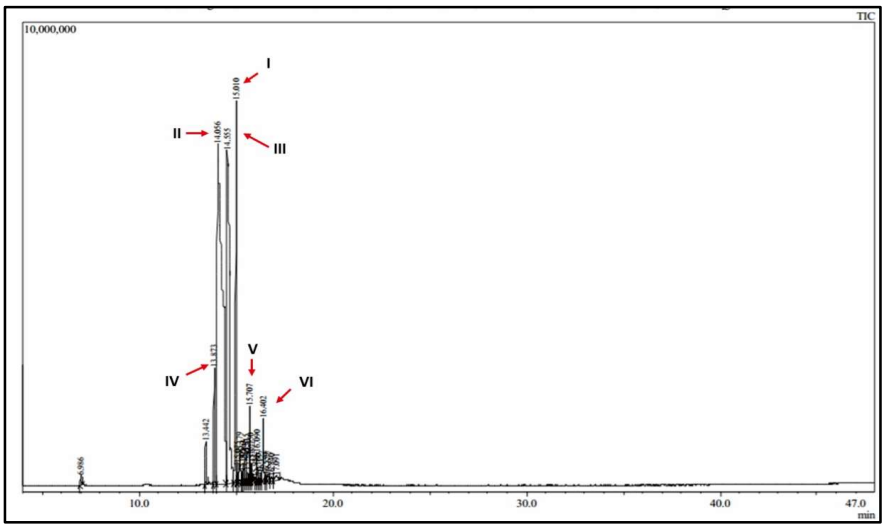


Figure 1. GC Chromatogram for clove leaf oil

Based on the chromatogram results of clove leaf oil in Figure 1, 25 chromatogram peaks were obtained, which showed the profile of the oil components. Subsequently, 6 main peaks were selected, each of which was estimated to contain eugenol (3-Allyl-6-methoxyphenol), trans-caryophyllene, α-humulene, α-copaene, δ-cadinene, and caryophyllene oxide. The component analysis of clove leaf essential oil was also replicated 3 times, and the results are presented in Table 1.

The results of the analysis using GC-MS method presented in Table 2 obtained the constituent components percentage of clove leaf oil, including eugenol (3-Allyl-6-methoxyphenol) (49.63±0.20) > trans-caryophyllene (28.25±0.22) > α-humulene (8.92±0.09) > α-copaene (2.15±0.04) > δ-cadinene (1.61±0.27) > caryophyllene oxide (1.50±0.05). There were also varying CV (Coefficient of Variation) values, with the smallest value of 0.40 in the eugenol compound. Meanwhile, the largest CV value of 16.92 was found in the δ-cadinene compound, which was caused by the large deviation between replications.

Analysis of clove leaf oil using GC-MS produced a retention time (tR) based on the separation results from GC. The mass spectra analysis was carried out using the base peak with the highest frequency in the spectrum, namely 100%, and Similarity Index (SI). Furthermore, to determine the closeness of the chemical structure of the essential oil components, the standard target was compared with the compound components in the spectra

by comparing the spectra of the NIST and WILEY 9.LIB libraries in the GC-MS software [16]. The detected compound was similar to the comparison data when the SI value approached 100% or above 90%. The analysis results in Table 2 showed that the detected clove leaf oil components had an SI value of >90%. Therefore, it can be concluded that the components of clove leaves were appropriate to the comparator spectra.

Table 1. The analysis results on the essential oil components of clove leave with GC-MS

No.	tR (minutes)	% Area	% Area±SD	CV (%)	SI	BM	Compound
I	14.056	49.49	49.63±0.20	0.40	97	164	eugenol
	14.058	49.54					
	14.080	49.86					
II	14.555	28.08	28.25±0.22	0.78	97	204	trans-caryophyllene
	14.555	28.17					
	14.556	28.50					
III	15.011	9.01	8.92±0.09	1.01	97	204	α-humulene
	15.011	8.91					
	15.011	8.83					
IV	13.873	2.19	2.15±0.04	1.68	95	204	α-copaene
	13.874	2.14					
	13.872	2.12					
V	15.707	1.30	1.61±0.27	16.92	95	204	δ-cadinene
	15.706	1.80					
	15.706	1.74					
VI	16.402	1.55	1.50±0.05	3.00	96	220	caryophyllene oxide
	16.404	1.50					
	16.403	1.46					

Note: SI = Similarity Index, MW = Molecular Weight

Table 2. The analysis results on the main components of clove leaf oil, turpentine oil, product A, B, and C

Oil Components	Percentage(%)				
	Clove Leaf Oil	Product A	Product B	Product C	Turpentine Oil
Eugenol	49.63±0.20	49.81±2.79	21.92±0.20	28.32±0.12	N/A
β-caryophyllene	28.5±0.12	32.49±0.14	19.97±0.12	12.83±0.02	N/A
α-humulene	8.92±0.09	10.43±0.03	10.03±0.05	3.13±0.45	N/A
α-copaene	2.16±0.04	1.58±0.03	1.71±0.07	0.57±0.01	N/A
δ-cadinene	1.61±0.27	0.53±0.02	3.30±0.02	1.21±0.26	N/A
Caryophyllene oxide	1.50±0.05	2.16±0.03	3.89±0.03	1.31±0.08	N/A
α-pinene	N/A	N/A	0.53±0.06	7.32±0.06	55.30±0.61
δ-carene	N/A	N/A	N/A	23.19±2.47	19.49±0.22
β-pinene	N/A	N/A	0.33±0.13	1.79±0.12	3.56±0.06
Camphene	N/A	N/A	N/A	N/A	2.533±0.17

Description; N/A= Not detected

Component profiles comparison of clove leaf oil, turpentine oil, and other clove leaf oil products in the market (A, B, and C)

This study used three types of clove leaf oil products from e-commerce (online shops) because the manufacturers were more varied. The selection of clove leaf oil products was based on predetermined criteria, namely clove leaf oil that was 100% pure from leaves and without mixture, within a price range of IDR 20,000-35,000, purchased by at least 100 buyers, and having a good store rating.

One type of clove leaf oil product had a certificate of analysis (CoA), namely product A. The CoA will show that the product has good quality and meets predetermined quality standards. This guaranteed the purity of the product and ensured no adulterants such as turpentine oil was contained. Meanwhile, products B and C do not have CoA and were suspected of adding adulterated oil because the price was very cheap with a large enough volume.

In this study, adulterated turpentine oil was obtained from Ddistillers (Syailendra Bumi Investama Company). a Central Java which has a CoA. Generally, turpentine oil is usually used as an adulterated oil because the price is relatively cheap, with a lighter and less polar fraction. However, the addition of turpentine oil can cause changes in the physicochemical properties of clove leaf oil, such as refractive index and specific gravity. This can also reduce the number of chemical components in the oil, thereby influencing the aroma of the oil. No derivatization was carried out in the analysis stage using GC-MS because the sample was a volatile compound.

Table 3. Functional group prediction results and vibration model of clove leaf oil and turpentine oil

Tape	Wavenumber (cm^{-1})			Functional Groups	Vibration Models	Intensity
	Clove Oil	Turpentine Oil	Reference [17, 18]			
1	3522	-	3000-3600	O-H (alcohol, acid, H bonding)	Hold out	Medium
2	2925	2953	3000-2840	C-H	Hold out	Strong
3	1637	1640	1667	C=C (para aromatic)	Buckling	Weak
4	1511	-	1600 and 1475; 1511 (eugenol acetate)	C=C (aromatic)	Hold out	Medium
5	1461	1462	1469	Methylene ($-CH_2-$)	Buckling	Medium
6	1451	1449	1446	Methyl ($-CH_3$)	Buckling	Medium
7	1265, 1231, 1204, 1181, 1147, and 1121	-	1300-1000	C-O (ether)	Hold out	Strong

The results presented in Table 3 showed that the largest component of clove leaf oil was eugenol. In the distilled clove leaf oil, eugenol was 49.63±0.20 > Product A (49.81±2.79) > Product C (28.32±0.12) > Product B

(21.92±0.20). Meanwhile, in the β-Caryophyllene compound, the content was Product A (32.48±0.14) > clove leaf oil (28.253±0.22) > Product B (19.97±0.12) > Product C (12.83±0.02). Eugenol is a phenolic compound and the largest component in clove oil, with a distinctive color and aroma in clove leaf oil, which can turn brown when in contact with air due to oxidation events. The previous study by Hasim et al. [4] related to the isolation of compound content in clove leaves showed that the eugenol component (58.6%) and β-Caryophyllene (21.4%) were obtained. Meanwhile, according to the Indonesian National Standard 06-2387-2006, the requirement for eugenol content in clove leaf oil is at least 78%, and β-Caryophyllene is a maximum of 17%. The concentration of eugenol and β-Caryophyllene levels can be different due to several factors, namely variations between seasons, soil conditions, growing areas, cultivation and harvesting processes of clove leaves, as well as distillation processes [19]. The component analysis of clove leaf oil, turpentine oil, as well as products A, B, and C, are shown in Table 2.

The alpha-pinene compound was turpentine oil's largest component (55.30±0.61), detected in products B and C with values of 0.53±0.06 and 7.32±0.06, respectively. The result of the component analysis in product B detected beta-pinene compound of 0.33±0.13, while C found delta-carene 23.19±2.47 and beta-pinene 1.793±0.12. Meanwhile, products B and C may contain adulterants, namely turpentine oil. Bhuiyan [20] analyzed compound components in clove leaf oil *Syzygium caryophyllatum* (L.) Alston species by GC-MS method and detected alpha-pinene compounds of 0.33% and beta-pinene of 0.45%. According to Oboh et al. [21], in a study related to the components in clove oil, the GC-MS method found alpha-pinene compounds and beta-pinene with values of 13.09% and 45.44% in *Eugenia aromatica Kuntze* species.

PCA of clove leaf oil with turpentine oil and market products

The PCA analysis was carried out by entering the AUC of pure clove oil components, the AUC of turpentine oil, pure clove leaf oil, as well as the AUC of products A, B, and C. This process was replicated three times to determine whether there were affecting factors. The location of the quadrants showed the similarity between the physicochemical properties of the constituent components of clove leaf oil.

PCA analysis was conducted using MINITAB 20 software, the latest version of MINITAB. The result of the analysis was a principal component (PC) that represented a large amount of variation in the initial data, where PC1 contained the most variance. The output of the PCA analysis of the component profiles of clove leaf oil, turpentine oil, as well as products A, B, and C are shown in Figure 2.

PCA analysis showed that each PC produced eigenvalue, proportion, and cumulative. The eigenvalue describes the variation explained by the data for each PC and the magnitude of the how much the variables on the formation of the matrix properties [22]. Proportion described the data variation for each influential PC with the number of original data variables. Meanwhile, the cumulative value describes the cumulative amount of proportion.

Figure 2 shows that the 16 PC generated corresponded to the number of variable data entered. The analysis results indicated that PCA can reduce the initial data with 16 PC variables and explained by 2 new variables (PC1 and PC2) to provide data information by 97%. This is revealed from the eigenvalue >1, close to zero, without effect, and was not correlated [23, 24]. Meanwhile, the score plot of the chemical components of clove oil, turpentine oil, and product oil is shown in Figure 3.

The score plot in PCA described a plot of two PC that provided information regarding the patterns present in the sample. The plot of the initial PC usually had the most variation in the data. The score plot displayed in this study was divided into 4 quadrants, where the plots of turpentine oil and products B and C oil were in different quadrants. Meanwhile, clove leaf oil and product A were in the same quadrant. This indicated that the closer the distance between plots, the closer the relationship among the samples analyzed [25].

Based on Figure 2, the main component of turpentine oil was in a different quadrant, located separately from the other oils analyzed. The product oil showed that the main components are not similar to the adulterated sample's. Meanwhile, product A and clove leaf oil are in the same quadrant, indicating that both samples had similar physical and chemical properties. Products B and C were in different quadrants from clove leaf oil but not with turpentine oil due to the presence of impurities or other oil mixtures. The results showed that the PCA chemometric method can be used to analyze or classify clove leaf oil with adulterants and products in the market.

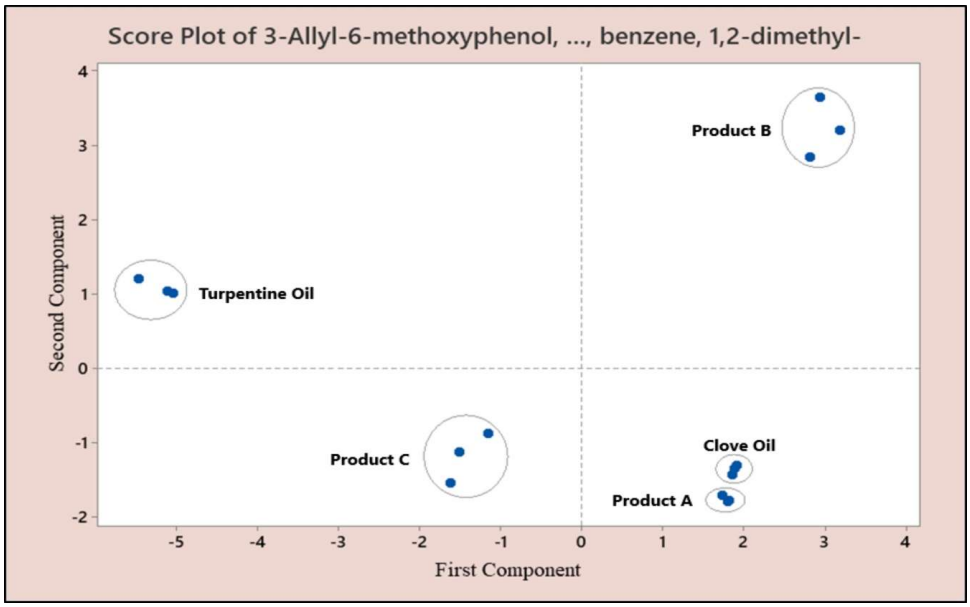


Figure 2. PCA plot scores from GC-MS of clove leaf oil, turpentine oil, product A, product B, and product C

Profile analysis of clove leaf essential oil by FTIR spectroscopy

The stages of analyzing the essential oil profile of clove leaves with the FTIR spectroscopy method were conducted using the functional groups. This was followed by the quantitative analysis with PLS and PCA.

Analysis of clove leaf essential oil by FTIR spectroscopy

FTIR spectroscopy is a widely used technique for essential oil authentication. This is because FTIR spectrophotometry can measure vibrations of functional groups as a consequence of electromagnetic radiation with the sample to produce a spectrum with fingerprinting properties [26]. Essential oils are volatile compounds consisting of a complex system of homogeneous mixtures of various compounds, and each species contains more than 100 constituents. Furthermore, essential oil fingerprint analysis can be used to distinguish species and chemotypes (common components) of different plants, representing a qualitative approach to the authentication and quality evaluation of essential oils [27, 28].

FTIR spectra can be distinguished from other compounds or samples based on wavenumber position, intensity, and the number of peaks [28]. The spectral analysis results of clove leaf oil and turpentine oil at wavenumbers between 4000 and 400 cm^{-1} are shown in Figure 3. The axis (x) is the wavenumber indicating the functional group, and the axis (y) is the absorption intensity [29]. The spectra analysis results showed that the infrared absorption peaks of both samples were at various wavenumbers. Further, Table 3 presented the predicted functional groups and vibrational models of clove leaf oil and turpentine oil.

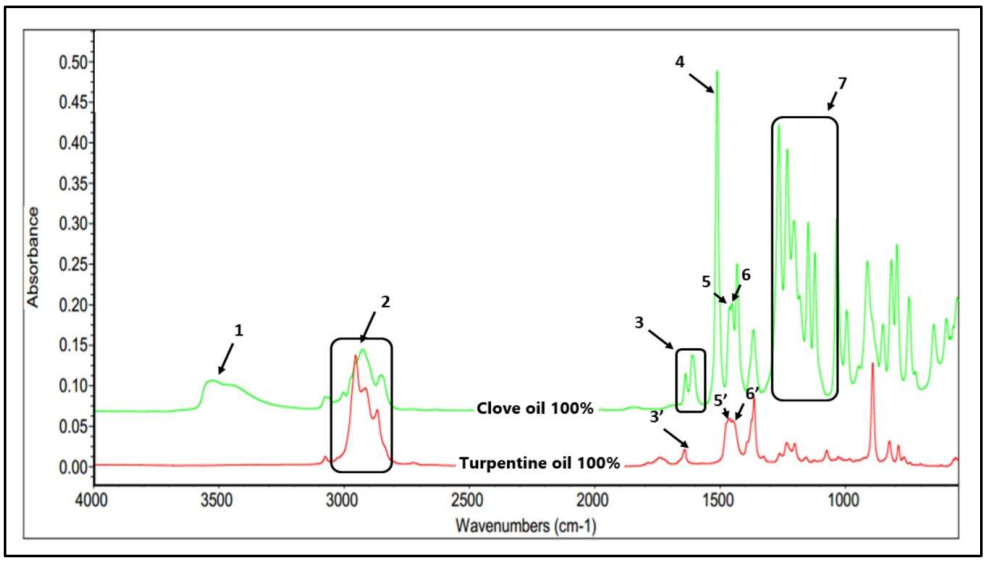


Figure 3. FTIR spectra of clove leaf oil and turpentine oil at wavenumbers 4000-600 cm^{-1}

The analysis results of the clove leaf oil functional groups in Table 3 showed a peak at wavenumber 3522 cm^{-1}, which was the O-H Phenol group with stretching vibrations. O-H groups typically at wavenumbers 3600-3000 cm^{-1}, as stated by Yang et al. [30] related to the analysis of eugenol compounds using FTIR instruments at wavenumbers obtained 3417 cm^{-1} with stretching vibrations. At wavenumber 2925 cm^{-1}, there was a C-H alkane functional group with stretching vibrations. Meanwhile, in turpentine oil at wavenumber 2953 cm^{-1}, the C-H alkane group will appear at 3000-2850 cm^{-1} [17]. At wavenumber 1511 cm^{-1}, an aromatic C=C functional group with the usual stretching vibrations. This indicated the presence of aromatic groups such as benzene in eugenol compounds, which were the main components of clove oil. The absorbance results in this study are 1637 cm^{-1} for the clove oil and 1640 cm^{-1} for turpentine oil, which is the C=C stretching vibration of the aromatic group [17]. Eugenol showed distinctive sharp peaks between 1640-1500 cm^{-1} of C=C aromatic because the functional groups of eugenols included double bonds. Therefore, these wavenumbers can provide unique spectral information with significant contributions to substance identification [31, 32, 33]. The FTIR spectra of clove oil and turpentine oil are shown in Figure 3.

In turpentine oil, the wavenumber 1640 cm^{-1} appeared, indicating the C=C alkene functional group with stretching vibrations, which showed the alkene group in the -pinene compound [34]. At 1461 cm^{-1} in clove oil and 1462 cm^{-1} in turpentine oil, there was a functional group (-CH_2-) with bending vibrations that usually appeared around the wavenumber 1465 cm^{-1} [17]. Furthermore, at wavenumbers 1451 cm^{-1} from clove, as well as 1449 cm^{-1} and 1363 cm^{-1} from turpentine oil, there was a bending vibration of the methyl group (-CH_3) [17]. The wavenumber of 1363 cm^{-1} in turpentine oil can indicate that α-pinena contains a gem-dimethyl group (C-(CH_3)$_2$) [34]. In clove leaf oil, wavenumbers 1265, 1231, 1204, 1181, 1147, and 1121 cm^{-1} contain C-O ether groups with stretching vibrations, which often appear at 1300-1000 cm^{-1} [17]. This wavenumber also indicated the presence of an ether group in the eugenol compound, such as C-O ether that appears at 1098 cm^{-1} [30]. The wavenumber 1300-1000 cm^{-1} not only described the C-O functional group but also explained the presence of CH_2 deformation vibrations in eugenol and eugenol acetate, besides C–C strain and C–H bending vibrations [18].

Based on the spectrum produced from clove leaf oil, the functional groups of the constituent components of clove leaf oil are eugenol and turpentine oil which contain α-pinene compounds. The wavenumber areas 1560-1480 cm^{-1}, 1814-1700 cm^{-1}, and 2954-2780 cm^{-1}

in clove leaf oil can be used in the PLS model developed for quantifying total eugenol [33]. The concentration series between clove oil and turpentine oil is presented in Figure 4. The peaks showed a variation in peak intensity in the difference in the concentration of clove oil and turpentine oil. Meanwhile, the variation in peak intensity during FTIR analysis indicates an effect that occurs when mixing clove leaf oil and turpentine oil.

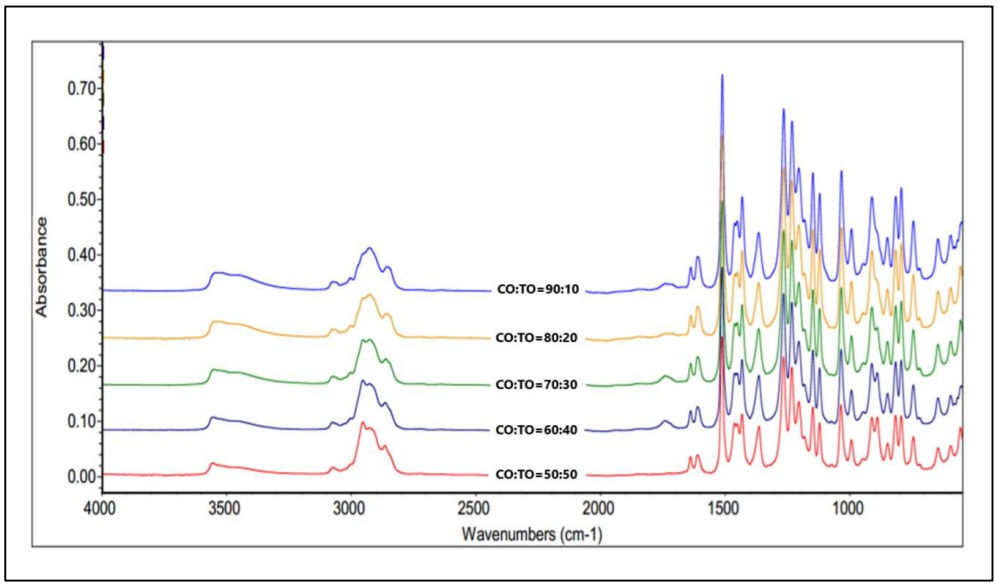


Figure 4. FTIR spectra of concentration series on clove leaf oil and turpentine oil at wavenumbers 4000-600 cm^{-1}

The absorbance results at each concentration ratio were used in the PLS and PCA chemometrics analysis. The FTIR spectra of clove leaf oil products A, B, and C circulating in the market are presented in Figure 5. Oil products were directly analyzed in the form of absorbance data using FTIR at wavenumbers 4000-400 cm^{-1}. The peak number of clove oil waves on the market is similar to that of the refined clove oil. However, there were differences in peaks indicating the OH functional group, namely products B and C, as shown in Table 4.

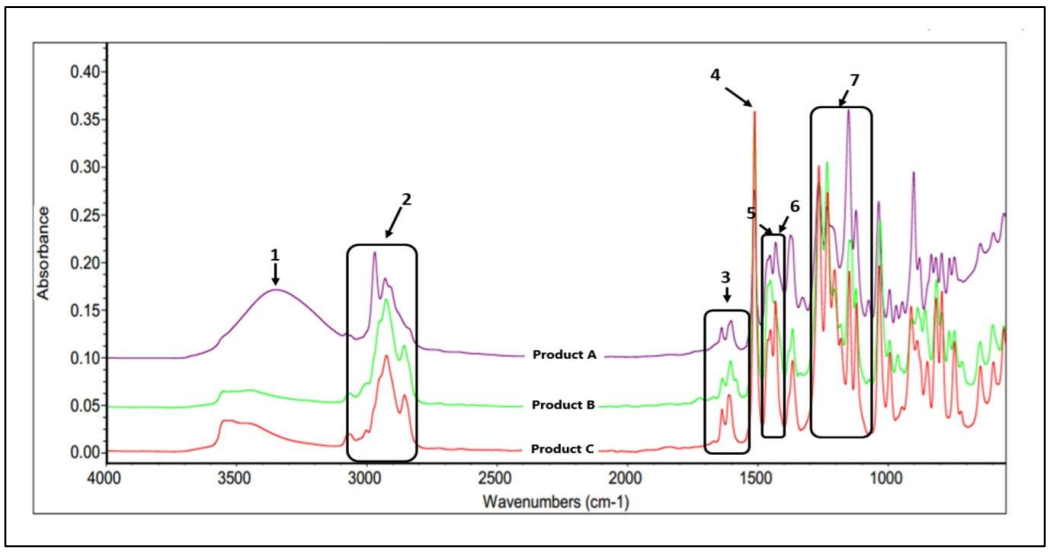


Figure 5. FTIR spectra of clove oil products in the market at wavenumbers 4000-600 cm^{-1}

Table 4. Prediction results on functional groups and vibration models of clove leaf oil products on the market

Tape	Wavenumber (cm^{-1})				Functional Groups	Vibration Models	Intensity
	Product A	Product B	Product C	Reference [17, 18]			
1	3350	-	-	3000-3600	O-H (alcohol, acid, H bonding)	hold out	Medium
2	2969	2925	2924	3000-2840	C-H	Hold out	Strong
3	1638	1636	1637	1667	C=C (para aromatic)	buckling	Weak
4	1513	1511	1511	1600 dan 1475; 1511 (eugenol acetate)	C=C (aromatic)	Hold out	Medium
5	1453	1451	1451	1469	Methylene ($-CH_2-$)	Buckling	Medium
6	1431	1433	1431	1446	Methyl ($-CH_3$)	Buckling	Medium
7	1267, 1233, 1151, 1122, and 1036	1264, 1233, 1182, 1248, 1141, and 1122	1265, 1232, 1205, 1148, 1121, and 1034	1300-1000	C-O (ether)	Hold out	Strong

Quantitative analysis with PLS

PLS regression is an analytical method that uses a linear combination of the predictor variables, not the original ones. In PLS, variables that show a high correlation with the response variable are given extra treatment because they will be more effective in the prediction process. In this method, a linear combination of predictor variables that were highly correlated with the response variable was selected to explain the existing variation. It was expected that only a few linear combinations of the predictor variables are needed to describe the majority of the variation [22].

PLS regression can be formed from a component that describes the correlation between x and y variables. Each component in the PLS regression was obtained by maximizing the covariance between the y variable and every possible linear function of the x variable. In an FTIR spectroscopy analysis, PLS is often used to extract information from complex spectra with overlapping peaks, impurities, and noise from FTIR spectroscopy instruments [35].

Before performing a PLS analysis, the first step was determining the wavenumber to be used for modelling calibration and validation by optimizing it. The wavenumber to be selected for optimization contains functional groups or fingerprint regions to determine the performance of the calibration model. It was also evaluated to provide a good correlation between the actual values of clove leaf oil, products A, B, and C oil, and turpentine oil to the predicted value using FTIR spectroscopy, expressed by the high coefficient of determination (R^2) close to 1. The R^2 value can be used to validate the accuracy parameter because R^2 describes the closeness of the predicted value to the actual value. Furthermore, the wavelength used during the analysis was selected based on the lowest error value in the calibration model, which was expressed by the Root Mean Standard Error of Calibration (RMSEC) [36, 29].

PLS connects two data matrices, x (actual value) and y (predicted value), with a linear multivariate model. The regression equation is used to find an expression that fits the data, where the linear regression equation model y=bx+a, y describes levels predicted with an FTIR spectrophotometer. The value of b is the slope of the straight line, x is the actual sample content, and a is the straight line intercept of the y-axis [37]. The optimization of wavenumber in clove oil is presented in Table 5.

Table 5. Wavenumber optimization for PLS multivariate calibration shows the relationship between the actual value (x-axis) and the predicted value (y-axis)

Wavenumber (cm^{-1})	Coefficient of Determination (R^2)	Regression Equation	RMSEC (%)
900-800	0.9991	y=0.9991x+0.0680	0.57
1300-1100	0.9908	y=0.9908x+0.7185	1.87
1400-1320	0.9955	y=0.9955x+0.3476	1.29
2900-2800	0.9993	y=0.9993x+0.0484	0.48
2960-2860	0.9998	y=0.9998x+0.0096	0.22

Multivariate calibration with the PLS method will provide relevant and specific spectral information based on the chemical properties of the calibration model at certain wavenumbers. Therefore, the selection of wavenumbers is based on optimization results which provide a linear relationship between the actual value and the predicted FTIR value [38]. As shown in Table 5, the wavenumbers used for calibration and validation of PLS modelling are 2960-2860 cm^{-1}. The relationship curve between the actual value (x-axis) and the predicted value (y-axis) of the calibration model is shown in Figure 7a.

The next process is calibration modelling at wavenumbers 2960-2860 cm^{-1}. The results, as shown in Figure 14 obtained the regression equation y=0.9998x+0.0096 and a coefficient of determination (R^2) of 0.9998. This indicated that the independent variable (x-axis) can explain 99.98% of the dependent variable (y-axis), with an RMSEC value of 0.22%. Meanwhile, the large value of the R^2 and the small RMSEC illustrate that the accuracy and precision of the developed model are acceptable.

The prediction model that has been made is evaluated using the cross-validation method by applying the leave-one-out technique. In this technique, one piece of data is deleted, and a new model is created with the remaining data. This validation is known as internal validation, where the parameter is the Root Mean Square Error of Cross Validation (RMSECV) value. Meanwhile, the validation result is said to be good when it produces a low RMSECV value, and the R^2 is close to 1. The curve of the relationship between the actual value (x-axis) and the predicted value (y-axis) using internal validation is shown in Figure 7b. Based on this curve, R^2 is 0.9992, and the RMSECV value is 0.76%, with a regression equation of y=0.9992x+1.5617. A small RMSECV value indicates that the error is getting smaller. Hence, the model built has better capabilities.

The next step is to carry out external validation to determine whether the validation model is applied to the new sample. Evaluation can be conducted by calculating the RMSEP value and the R^2. A low RMSEP value and an R^2 value close to 1 illustrate a successful creation of a regression model. The relationship curve between the actual value (x-axis) and the predicted value (y-axis) using external validation is shown in Figure 7c.

The R^2 obtained from the above curve is 0.9945, and the RMSEP value is 1.20%, with a regression equation of y=0.9945x+0.4918. The results of the external validation relationship curve, where the RMSEP value is getting smaller and the R^2 is close to 1, indicate that the validation model created can be applied to new samples.

Accuracy is expressed from R^2, where a value close to 1 illustrates that the relationship between the actual and

the predicted values is well formed, and the precision is improving. Precision was indicated by the smaller value of RMSEC, RMSECV, and RMSEP, showing that the model error was minimal. Based on several parameters, namely calibration, internal validation, and external validation, the value of $R^{2,}$ which was close to 1, showed better results. Meanwhile, smaller RMSEC, RMSECV, and RMSEP values described greater accuracy and higher precision in both models were made.

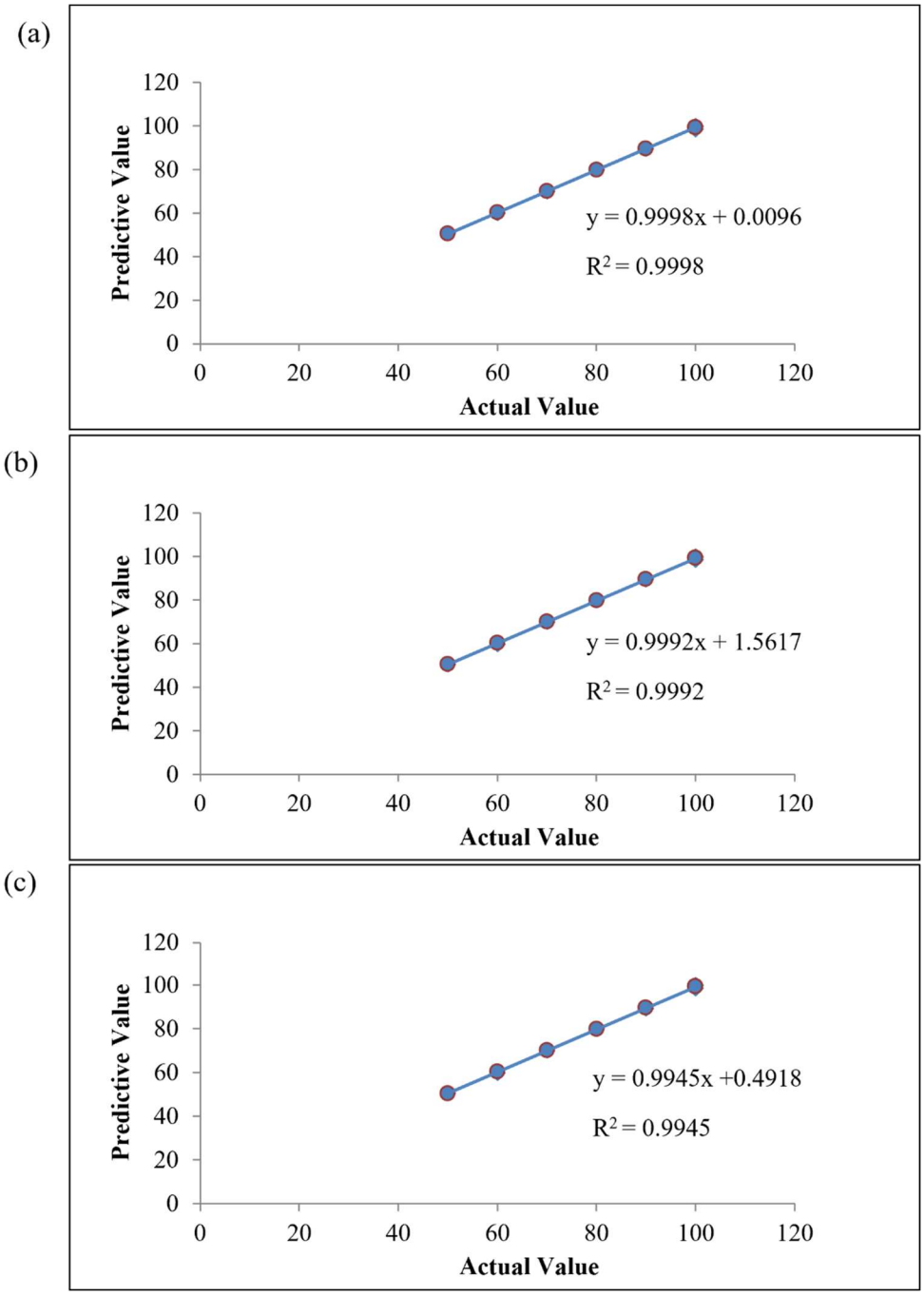


Figure 2. Relationship curves between actual values (x-axis) and predicted values (y-axis) of (a) calibration model, (b) internal validation model, and (c) external validation model at wavenumbers 2960-2860 cm^{-1}

Analysis with PCA

The FTIR spectra were analyzed using PCA, which is a data interpretation method that is performed by predicting data. This method reduces the number of variables in the matrix to generate new variables while retaining data information. The new variable is in the form of a score or main component [22]. PCA can reduce multivariate data when there is a correlation between variables. It also can be used for grouping data because samples with similar PC have similar Physicochemical properties [23].

The wavenumber that had been previously optimized was selected for PCA and used for quantitative analysis, namely 2960-2860 cm^{-1}. This wavenumber was selected for PCA modelling because of its ability to provide good separation of the samples to be evaluated, namely clove leaf oil, products A, B, and C oil, as well as turpentine oil as a forgery. The output of PCA analysis from FTIR is shown in Table 6. The results showed that analysis with PCA at wavenumbers 2960-2860 cm^{-1} reduced the initial data, namely 97 PC. This can be explained with three new variables, namely PC1, PC2, and PC3, capable of providing 100% data information. This can be seen from the eigenvalue>1, indicating that the data affect and correlate [24].

Table 6. Output PCA analysis of component profiles on the clove leaf essential oil, turpentine oil, product A, product B, and product C at wavenumbers of 2960-2860 cm^{-1}

Eigen analysis of the correction matrix											
Eigenvalue	82.235	17.151	4.552	0.054	0.008	0.001	0.000	0.000	0.000	0.000	0.000
Proportion	0.791	0.165	0.044	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Cumulative	0.791	0.956	0.999	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
Eigenvalue	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Proportion	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Cumulative	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

A score plot comparison of clove oil and turpentine oil using PCA is shown in Figure 8a. The analysis with PCA showed that both samples are well separated, as indicated by the grouping in different quadrants, with a latent variable, namely the PCA score plot. Samples with the same PC values are similar in their physicochemical properties [39]. The grouping of pure clove leaf oil, products A, B, C, and turpentine oil was carried out. Three oil samples on the market claim to contain 100% pure clove leaf oil. The results of the PCA score plots in Figure 9b showed that 4 quadrants distinguish between clove leaf oil, turpentine oil, as well as products A, B, and C.

Based on the results of the score plot, product A oil was in the same quadrant as pure clove leaf oil. This is shown in the clove leaf oil profile analysis by GC-MS and using PCA chemometrics. These results indicated that product A had the same physical and chemical characteristics as clove leaf oil, while products B and C were in different quadrants. Based on this analysis results, PCA was successfully used to classify clove leaf oil, products A, B, and C, as well as turpentine oil. Meanwhile, future investigation regarding the main components in products A and B required further analysis such as using PCR.

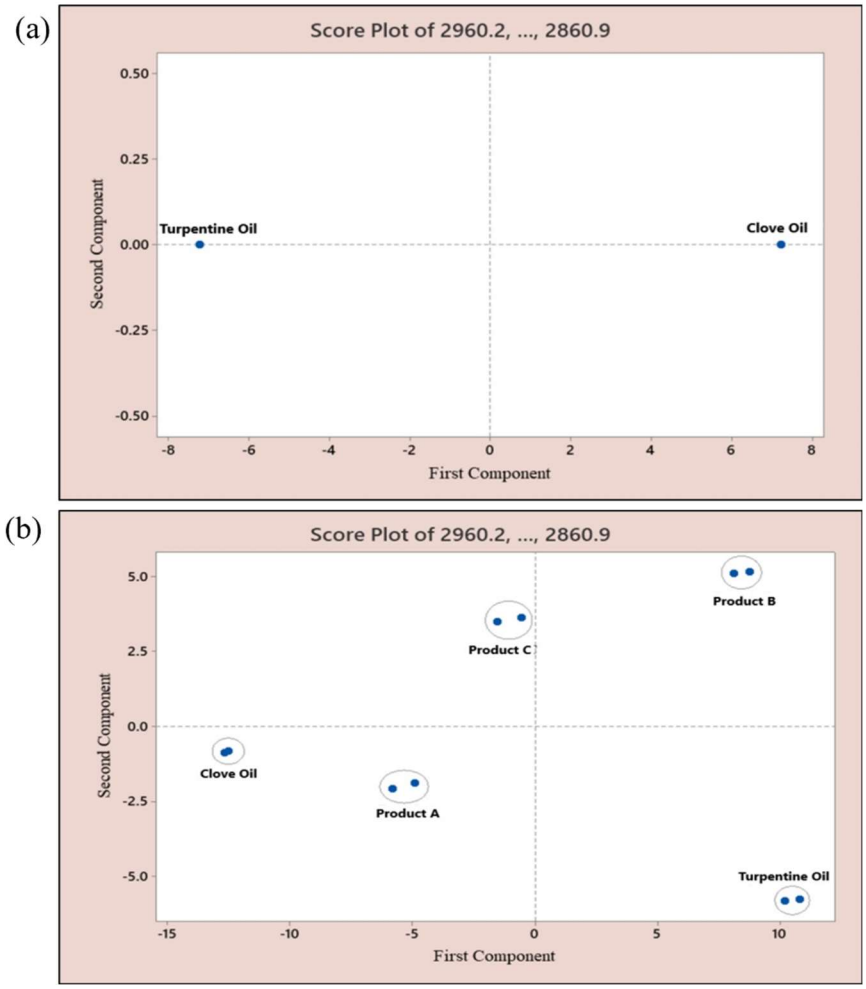


Figure 8. PCA Score Plot of FTIR (a) clove leaf oil and turpentine oil (b) clove leaf oil, turpentine oil and products A, B, C

Conclusion

The chemical components in the clove leaf oil were eugenol (3-Allyl-6-methoxyphenol), trans-Caryophyllene, alpha-Humulene, alpha-Copaene, delta-Cadinene, and Caryophyllene oxide. The results of grouping clove leaf oil using PCA based on the type of oil component showed that product A and distillate clove leaf oil were in the same quadrant. Therefore, both samples had similar physical and chemical properties. Clove leaf oil profile analysis using FTIR at wavenumbers 2960-2860 cm^{-1} with combined PLS yielded method validity results as indicated by R^2, RMSEC, RMSECV, and RMSEP values of 0.9998, 0.22%, 0.76%, and 1.20%, respectively. The results of PCA multivariate analysis showed that PCA can classify leaf oil, turpentine oil, and products A, B, and C. The authentication of oil products in the market, namely A, B, and C shows that no clove leaf oil products contain adulterated turpentine oil.

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SYNTHESIS OF TIN DOPED ZINC OXIDE (TZO) NANOWIRES: EFFECT ON DYE ADSORPTION

(Synthesis Zink Oksida Nanodawai Terdop Timah: Kesan Kepada Penjerapan Pewarna)

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Abstract

In this study, pristine zinc oxide (ZnO) and tin doped zinc oxide (TZO) nanowires (NWs) had been synthesised on a fluorine doped tin oxide glass substrate via a microwave-assisted ultrasonic irradiation technique. This is a facile and rapid technique that combined ultrasonic irradiation technique during preparation of precursor solution with microwave assisted hydrothermal technique during synthesisation of nanowires. The ultrasonic irradiation technique helps to breakdown the agglomerated and large molecules inside the precursor solution mixture into smaller molecules. The microwave assisted technique helps to increase the temperature of the precursor solution from internal to external in shorter time through the increasing of interaction between molecules. It is important to produce a wide surface area of this material in the photoanode of dye-sensitive solar cells (DSSCs) configuration. Specifically, dye adsorption was an important aspect to grasp, as it can be reflected in the performance of the DSSC device. The concentrations of tin, Sn dopant were varied between 0.3 and 1.8 at.%. As-prepared ZnO and TZO NWs were analysed using field emission scanning electron microscopy and X-ray diffraction. Then, the samples that were soaked in N719 were analysed with ultraviolet-visible-near infrared spectroscopy. The (002) peak of pristine ZnO was regressed, and the identified (100) and (101) peaks of ZnO were slightly shifted towards higher degrees after the addition of Sn dopants. The surface morphology of TZO NWs had displayed a more uniform transformation, with the smallest crystallite size at 51 ± 11 nm (0.9 at.%) due to the enhanced surface area. Unfortunately, it continued to degenerate for concentrations ranging from 1.2 to 1.8 at.%. Urbach energy analysis also suggested that the lowest defect at 0.9 at.% TZO (76 meV) produced the highest crystallinity with a less disorderly thin film. These properties had upheld the dye adsorption and trapping in the ZnO-based thin film. Thus, TZO NWs were the most promising candidates as photoanodes in DSSCs.

Keywords: crystallinity, dye adsorption, microwave, zinc oxide, tin doped

Abstrak

Dalam kajian ini, zink oksida tulen dan zink oksida terdop timah nanodawai telah disintesis ke atas substrat kaca timah oksida terdop florin menggunakan teknik penyiaran ultrasonik bantuan mikrogelombang. Teknik mudah dan pantas ini merupakan hasil gabungan teknik penyiaran ultrasonik yang digunakan semasa proses penyediaan larutan prekursor dengan teknik hidroterma bantuan mikrogelombang yang digunakan semasa proses pensintesisan nanodawai. Teknik penyiaran ultrasonik dapat membantu penguraian molekul yang beraglomerat dan besar dalam campuran larutan prekursor menjadi molekul yang lebih kecil. Teknik bantuan mikrogelombang pula dapat meningkatkan suhu larutan prekursor dari dalaman ke luaran dalam masa yang singkat melalui peningkatan interaksi antara molekul. Penghasilan kawasan permukaan yang luas merupakan aspek yang amat penting bagi bahan fotoanod dalam konfigurasi sel suria pekaan pewarna. Secara khusus, penjerapan pewarna merupakan aspek penting kerana ia akan mencerminkan prestasi peranti sel suria pekaan pewarna. Kepekatan dopan timah telah divariasi dalam lingkungan 0.3 hingga 1.8 peratusan atom. Zink oksida tulen dan zink oksida terdop timah nanodawai yang tersedia telah dianalisis menggunakan mikroskopi elektron pengimbasan pancaran medan dan pembelauan sinar-X. Kemudian, sampel yang telah direndam dalam pewarna N719 dianalisis dengan spektroskopi ultralembayung-cahaya nampak-inframerah dekat. Puncak utama (002) pada zink oksida tulen telah susut dan puncak-puncak identiti melibatkan puncak (100), (002) dan (101) milik zink oksida telah beranjak ke arah yang tinggi setelah penambahan dopan timah. Morfologi permukaan zink oksida terdop timah nanodawai memperlihatkan perubahan ke arah keseragaman dengan saiz kristal paling kecil pada 51 ± 11 nm (0.9 peratusan atom) disebabkan kawasan permukaan dipertingkat, namun terus merosot pada julat kepekatan 1.2 – 1.8 peratusan atom. Analisis tenaga Urbach juga mencadangkan terdapat kecacatan terendah pada 0.9 peratusan atom zink oksida terdop timah (76 meV) dengan menghasilkan saput tipis yang berhabluran tertinggi dan kurang ketaktertiban. Sifat-sifat tersebut telah menyumbang kepada penjerapan pewarna dan pemerangkapan dalam saput nipis berasaskan zink oksida. Oleh itu, zink oksida terdop timah nanodawai adalah calon berpotensi menjadi fotoanod dalam sel suria pekaan pewarna.

Kata kunci: habluran, penjerapan pewarna, gelombang mikro, zink oksida, terdop timah

Introduction

Dye-sensitized solar cell (DSSC) is an emerging third-generation technology in the photovoltaic field. This alternative may provide cleaner and replenishable energy sources for future generations by reducing carbon footprints. The basic structure of DSSC consists of a substrate with a conductive layer, a photoanode, a dye, an electrolyte, and a cathode. Photoaode and cathode are also known as working electrodes and counter electrodes, respectively. According to Green et al.'s 61st version of the solar cell efficiency table, Ecole Polytechnique Fédérale de Lausanne (EPFL) recorded the most recent DSSC efficiency at 12.25% [1]. Compared to other types of third-generation emerging solar cells, DSSCs' efficiencies progressed in small steps. We identified the photoanode layer as one of the major factors that limited the efficiency of DSSCs. The most commonly used photoanode was titanium dioxide (TiO_2). The irregular electric channels in TiO_2 trigger issues, leading to recombination that significantly impacts solar cell performance [2, 3].

The other alternative to TiO_2 was an II-VI semiconductor combination of zinc and oxide from metal and chalcogen elements that can create vast application materials. Zinc oxide (ZnO) is a benign, abundantly available, and thermally stable material. Zinc oxide nanostructure can be found in zero dimensions as nanodots [4] or nanoparticles [5], one dimensions as nanowires (NWs) [6] or nanorods [7], two dimensions as nanosheets [8] or nanoplates [9] and three dimensions as nanoflakes [10] or nanoflowers [11]. Based on previous studies, there are a few approaches to grow one-dimension (1D) ZnO nanostructures that include chemical bath deposition [12], hydrothermal [13], wet chemistry [14], solvothermal [15] and water bath [16]. However, these methods require longer reaction times, complex processes, and high-end instruments. For instance, the study conducted by Dloo et al. have found that using the hydrothermal technique required more than 2 hours to synthesis ZnO NWs [17]. Syrrokostas and his team have utilised chemical bath deposition process for 7 hours to form ZnO NWs that achieved the desired morphology [18]. Other than that, Guo et al. has managed to grow ZnO NWs within 1.5 hours using microwave assisted hydrothermal process [19]. However, the synthesisation process was utilising modified commercial oven that could inject the precursor solution continuously during the reaction. Thus, a rapid and straight-forward technique would be beneficial to the current technology of ZnO NWs synthesis.

The porosity of the photoanode compound directly influences dye adsorption, conductivity, and charge

transport across the DSSC [20]. A branched ZnO photoanode has the advantage of increasing dye adsorption properties by filling the space between the NW structures [21]. Previous studies have found that tin, Sn has a compatible structure as it has a lower ionic radius (Sn^{4+} ion: 0.69 Å) compared to Zn^{2+} ion with an ionic radius of 0.74 Å [22]. Therefore, at low doping concentrations, the oxide lattice remains unaffected [23]. Besides, Sn helps to improve the surface properties by increasing the surface area of ZnO thin film via the formation of smaller crystallite sizes than pure ZnO [24]. The doping of Sn must be controlled (below 2 at.%) to prevent clumping undesired truncated cone-shaped nanostructure that can affect the quality of ZnO thin film [25]. Sn also has the ability to improve electrical conductivity in a ZnO photoanode by enhancing the carrier concentration by introducing double-charge carriers [26]. This situation is favourable to boost the efficiency of DSSC [22, 23]. This study had combined the environmental-friendly ultrasonic irradiation with rapid microwave assisted technique to synthesis pristine ZnO and tin doped ZnO (TZO) NWs. These 1D TZO NWs were synthesised by varying the dopant concentrations (atomic percentage, at.%) to identify the optimum properties for DSSC photoanode applications.

Materials and Methods

The procedures are categorised based on step-by-step processes involved to synthesis and analyse the properties of pristine ZnO and TZO thin film that grown on FTO glass substrate.

TZO NWs thin film growth

The FTO glass was specified into 0.11 cm thickness and 2 × 2 cm^2 sized substrates. Then, aluminium doped ZnO seed layer had deposited on oxide-free substrate using spin coating technique [27]. Followed by that, tin doped ZnO (TZO) NWs were grown via microwave-assisted ultrasonic irradiation method was carried out. An equimolar of 12.5 mM of zinc acetate dihydrate, $Zn(CH_3COO)_2 \cdot 2H_2O$; hexamethylenetetramine, $C_6H_{12}N_4$ (HMTA) and tin (IV) chloride pentahydrate, $SnCl_4 \cdot 5H_2O$ powder were used as precursor, stabiliser and dopant, respectively. The amount of Sn dopant was varied to 0.3 – 1.8 at.%. All the reagents were dissolved in a beaker filled with 1000 mL distilled water that acted as solvent or medium for the reaction. Then, the mixture solution was stirred at 80 ºC for 40 minutes to yield clear and homogeneous solution. Next, the mixture solution was sonicated at 50 ºC for 30 minutes using an ultrasonic water bath (Hwasin Technology Powersonic 405, 40kHz) and aged for 60 minutes at room temperature. After that, the mixture solution is continued to age at room temperature with stirring at speed of 3. The solution was poured into a Schott bottle. Then, the optimized seed layer-coated glass substrates were placed at the bottom of the Schott bottle with one substrate each. Afterward, the container was placed inside the microwave oven (SHARP 25 L Microwave Oven with Grill R754AST) which was set to power of 600 W and microwaved for 60 minutes. The excess nanostructures deposited on the substrates were rinsed with distilled water thoroughly. After that, the samples were annealed (hard-baked) in a preheated furnace at 500 ºC for 60 minutes. Finally, the samples were removed from furnace for fast cooling process and proceeded with further analyses. The as-prepared pristine ZnO and TZO NWs grown on FTO were characterised with X-ray diffraction (XRD) to investigate the crystal orientations and crystallinity of the samples. The XRD model used was PANalytical X'Pert PRO with Cu K-alpha radiation wavelength of 1.5419 Å. The crystallite size was calculated based on the Scherrer formula provided in Equation 1. The k in this equation was the Scherrer constant with the value of 0.94. The λ represented the X-ray wavelength (CuK_α = 1.5419 Å), β denoted as the line broadening at full width half maximum while θ was the Bragg's angle. β and θ were both measured in radians.

$$D = \frac{k\lambda}{\beta \cos\theta} \quad (1)$$

Additionally, dislocation line density (DLD) of the thin films represented the length of dislocation lines per unit volume of the crystal which can be determined using Equation 2.

$$\delta = \frac{1}{D^2} \quad (2)$$

Furthermore, the interplanar distance or known as d-spacing can be figured out by substituting θ, n and λ

which were Bragg's angle, order of diffraction and wavelength of X-rays, respectively. The order of diffraction usually was 1 for first order. Value of d-spacing can be obtained through the Bragg's law formula in Equation 3.

$$2d \sin\theta = n\lambda \quad (3)$$

Besides that, Field Emission Scanning Electron Microscope (FESEM) to identify the morphology and particle distributions. The FESEM model utilised was Analytical High Resolution Field Emission Scanning Electron Microscope Apreo 2S (Thermo Fischer Scientific). The surface morphology images of the samples were taken at 10 kV and 60 000x magnification. Further analyses were done using ultraviolet-visible-near infrared (UV-vis-NIR) spectroscopy to analyse the defects in the samples. The UV-vis-NIR model used was Carry 5000 Varian with the fixed spectral range of 300 – 1200 nm. The data from UV-vis-NIR were analysed to obtain the Urbach energy value. The absorption coefficient was fitted to exponential function of energy. The derived formula of Urbach energy involved in this study was listed in Equation 4 and 5. The most linear gradient of plotted ln α against photon energy (hv) was enabled the gaining of Urbach energy value.

$$\alpha = \alpha_0 \, exp\left(\frac{hv}{E_u}\right) \quad (4)$$

$$\ln\alpha = \left(\frac{1}{E_u}\right) hv + \ln\alpha_0 \quad (5)$$

Dye adsorption and desorption process

Dye loading process was performed on pristine ZnO and TZO NWs thin film in order to analyse the adsorption and desorption ability of the photoanode. Dye that has been chosen as the light sensitizer in this study was a ruthenium-based dye namely Di-tetrabutylammonium cis-bis (isothiocyanato) bis (2,2′-bipyridyl-4,4′-dicarboxylato) ruthenium (II) or also known as N719. 0.5 mM of ethanolic N719 dye solution was allowed to be stirred homogenously at room temperature before ready to be used. FTO glass substrate with each type of NWs photoanode were then soaked into the prepared solution for 24 hours. The soaked sample was picked up and dried from excess dye solution. Then, dye loading process was carried out to analyse the adsorb and desorb action of photoanode thin film. Equation 1 was used to measure the dye loading in photoanode. Dye loading of photoanode thin film was determined by the multiply of dye concentration and volume of solution which then divided by the electrode area. The unit for dye loading in Equation 6 was mol/cm^2 while M denoted as dye concentration in mol/L, volume, V in unit of L and A was the electrode area in cm^2. The size of area covered with electrode was controlled at 3.6 cm^2. The dye loading value was consistent for all pristine ZnO and TZO thin films which was 0.833 μmol/cm^2. The dye loading value was smaller compared to other studies due to the large dye exposed surface area.

$$Dye\ loading = \frac{M \times V}{A} \quad (6)$$

The FTO glass/ZnO SL/pristine ZnO or TZO NWs/N719 dye configuration was illustrated as in Figure 1. Next step was the samples were washed with ethanol during unloading process to study the desorption of the photoanode. The transmittance of dye adsorbed and desorbed for all samples were plotted and discussed further in Results and Discussion section.

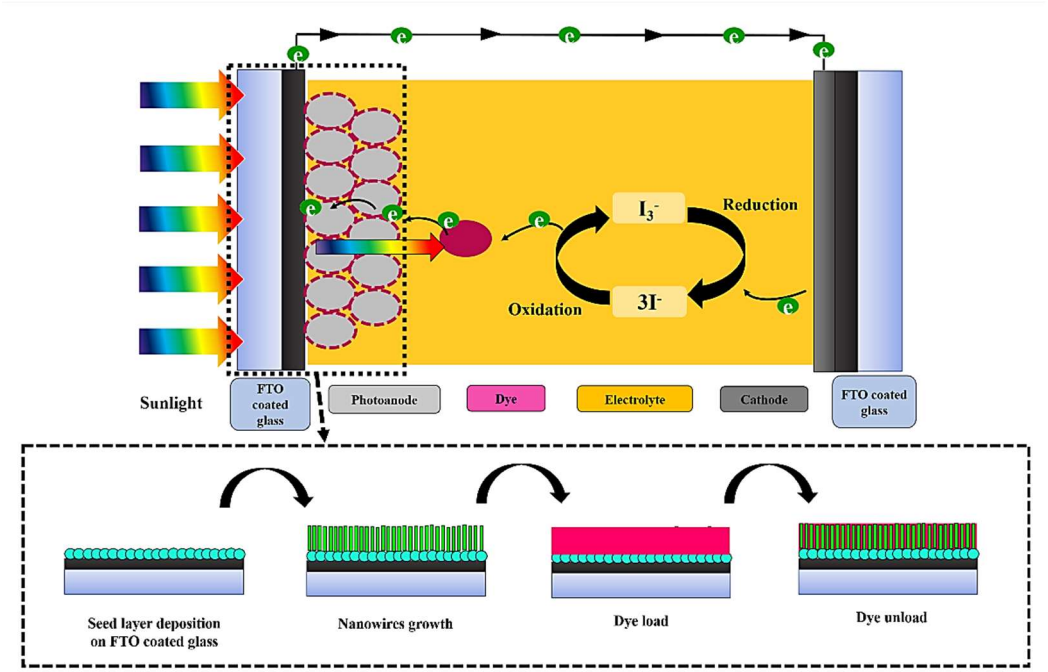


Figure 1. Stepwise processes that involved NWs photoanode formation and dye adsorb-desorb actions that build up DSSC configurations

Results and Discussion

This section presented the results and some elaboration based on physical and optical properties analysis that had been investigated on ZnO-based thin film. The analyses done were discussed in detailed based on each subtopic.

Crystal orientation and crystallinity analysis

The XRD pattern shown in Figure 2 (a) and Figure 2 (b) had indicated the hexagonal wurtzite structure with existence of peaks (100), (002), (101), (102), (110), (103) and (112) at 2θ of 32.29°, 34.96°, 38.35°, 52.11°, 57.06°, 62.17° and 66.26°. These peaks were the blueprints of pristine ZnO material according to JCPDS 00-036-1451. The small peak at 34.5° was belonged to FTO [28]. After the addition of Sn as dopants (0.3 – 1.8 at.%), the same peaks remained without any appearance of additional peak. After doped, the major peaks within the range of 30 – 40 degrees had slightly shifted to higher degree positions. Previous work on studying the effect of dopant concentration also had identified comparable tendency such as in this work [11]. This increment had suggested that there was shrinkage in d-spacing due to the consequence of compressive stress [29]. The slightly difference in atomic radii of Zn^{2+} ion with Sn^{4+} ion at 0.69 Å had shifted the 2θ crystal positions to relatively higher value [22]. The most dominant (002) peak that observable in pristine ZnO sample was suppressed and left with (101) as the most significant major peak after Sn dopant was added. This trend also can be noticed from earlier study by varying the Sn dopants in 2 – 8 wt.% [22]. The crystallite size and interplanar distance of pristine and all TZO samples had shown similar trends which indicated that both factors were closely related to each other. On the other hand, the FWHM and dislocation line density had the opposite relationship with the other two factors which mentioned before. These patterns can be seen from previous studies done by other researchers [29, 30].

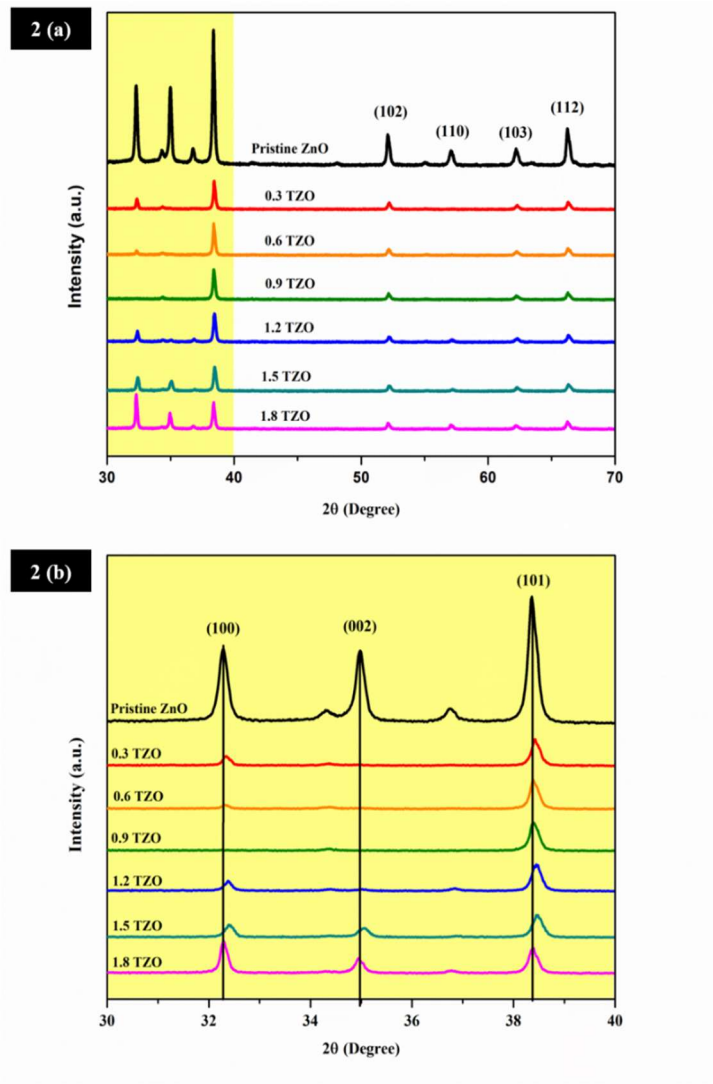


Figure 2. XRD patterns of pristine ZnO and various TZO samples within the range of (a) 30 – 70 degrees and (b) 30 – 40 degrees

Table 1. Crystallite size, dislocation line density and interplanar distance of pristine and varied TZO samples at (101) plane

Sample	FWHM (Degree)	Crystallite Size (nm)	Dislocation Line Density (E14 m^{-2})	Interplanar Distance (Å)
Pristine ZnO	0.214	41.09	5.92	2.347
0.3 TZO	0.223	39.44	6.43	2.340
0.6 TZO	0.203	43.32	5.33	2.344
0.9 TZO	0.214	41.09	5.92	2.344
1.2 TZO	0.232	37.91	6.96	2.341
1.5 TZO	0.244	36.05	7.70	2.340
1.8 TZO	0.223	39.43	6.43	2.345

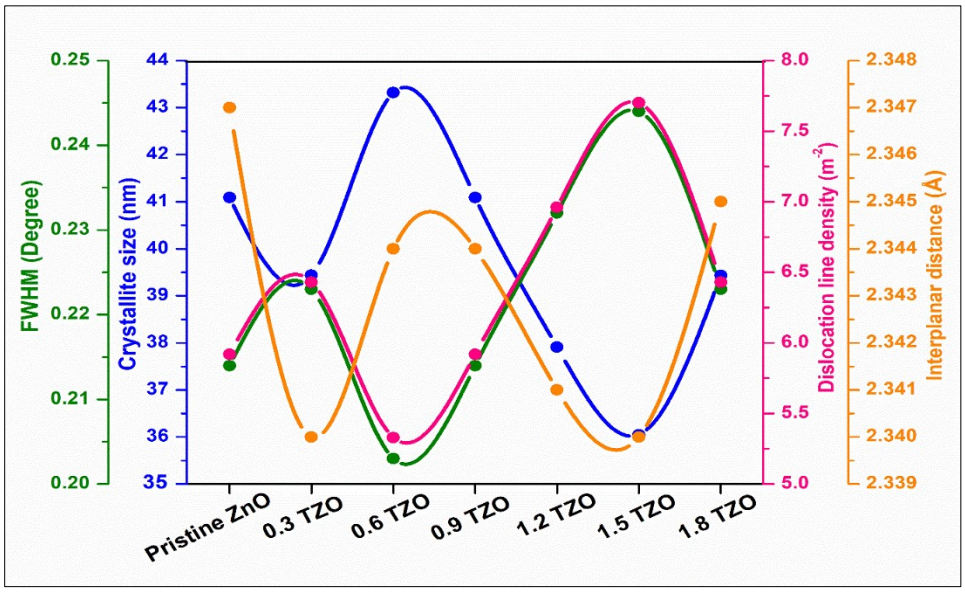


Figure 3. The crystallite size, dislocation line density and interplanar distance of pristine ZnO and varied TZO samples at (101) plane

Surface morphology and uniformity analysis

FESEM images had displayed pristine ZnO and TZO (0.3 – 1.8 at. %) NWs in Figure 4 (a – g). The particle size decreased and uniformity increased with the increment in Sn dopant concentration in ZnO to form nanowires. High in uniformity is one of the crucial aspects to produce decent morphological, optical and electrical properties. The smallest and most uniform TZO nanowire structures had displayed at 0.9 TZO (51 ± 11 nm). The introduction of Sn ions had changed the lattice of ZnO due to difference in ionic radius between Sn4+ ions (0.69 Å) and Zn^{2+} ions (0.74 Å) [22]. In contrary, after this point (1.2 at.% onwards), the nanowire structures started to show non-uniformity trend and become agglomerated and deformed. This phenomenon occurred due to Ostwald ripening effect which small crystals pick up the excess solute and amalgamate into large nanostructures [31]. This defect can be observed via FESEM surface images after excess Sn was doped (1.5 and 1.8 TZO). Previous researchers had suggested that the doping of tin must be restrained under 2 at.% to prevent clumping undesired truncated-cone shaped nanostructure which may disturb the quality of ZnO thin film [25]. Thus, this agglomerated nanostructures are expected to affect the resistivity and worsen the electrical properties of the photoanode thin film [25]. The summarised data on average particle size for all samples were tabulated in Table 2.

Defect analysis

Urbach energy indicates the degree of structural disorder, defects, and impurities in the material. High in Urbach energy translate into high defects density, more disorder and low in crystallinity. The defects can generate localised states that can traps carriers (electron and hole) and caused recombination. 0.9 TZO in Figure 5 had shown the lowest Urbach energy (76 meV) which reflected through the most uniform average particle size obtained in FESEM analysis and had achieved near lowest FWHM (0.214°) and dislocation line density (5.92 E14 m^{-2}). The Urbach energy values for all samples were tabulated in Table 3.

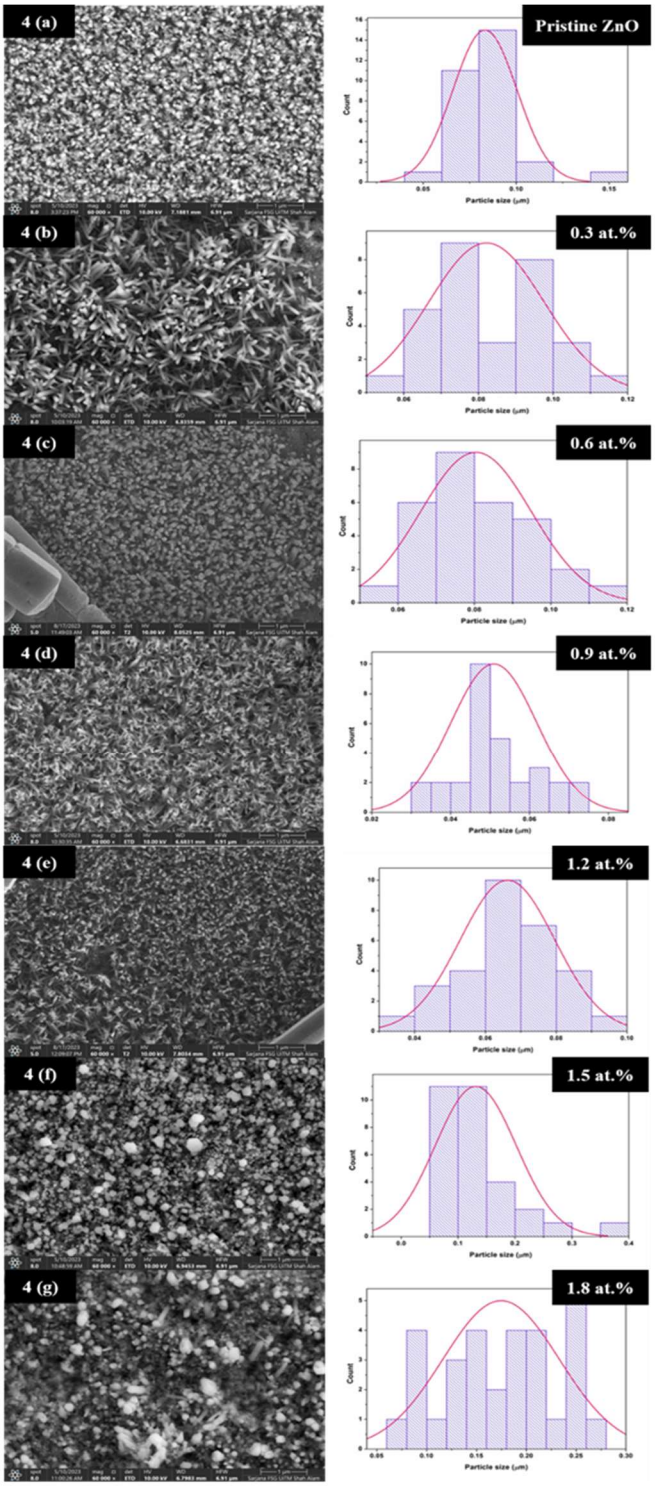


Figure 4. FESEM images and particle distributions of (a) pristine ZnO and (b – g) 0.3 – 1.8 at.% of TZO NWs

Table 2. The average particle size from particles distribution analysis in FESEM images for each sample

Label in Figure 4	Sample	Average Particle Size (nm)
(a)	Pristine ZnO	83 ± 17
(b)	0.3 TZO	82 ± 15
(c)	0.6 TZO	80 ± 14
(d)	0.9 TZO	51 ± 11
(e)	1.2 TZO	66 ± 13
(f)	1.5 TZO	131 ± 71
(g)	1.8 TZO	175 ± 58

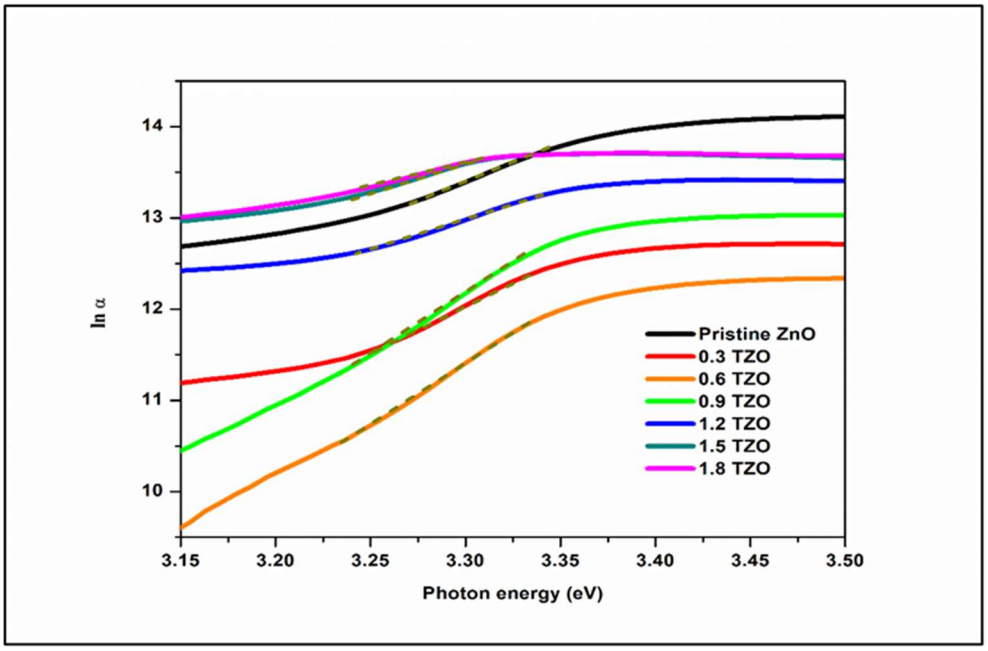


Figure 5. Plot of ln α against photon energy of pristine ZnO and 0.3 – 1.8 at.% of TZO NWs

Table 3. Urbach energy of pristine ZnO and 0.3 – 1.8 at.% of TZO NWs

Sample	Urbach Energy (meV)
Pristine ZnO	111
0.3 TZO	98
0.6 TZO	77
0.9 TZO	76
1.2 TZO	161
1.5 TZO	175
1.8 TZO	176

Dye adsorption and desorption analysis

From the plotted UV-vis-NIR graphs in Figure 6 (a – g) and the summarised data in Table 4 had found that the transmittance patterns of pristine ZnO was boosted with the helped of Sn dopants. This was due to the fact that Sn had successfully reduced the particle size as shown in FESEM images. However, the pattern had started to deteriorate after 1.2 TZO samples as the transmittance started to drop in respond to inhomogeneity of the NWs formation. For the dye adsorption ability, the transmittance of the window layer is reduced at the specific wavelengths corresponding to the dye's absorption peaks. The transmittance recorded by N719 dye was 13.50%. Numerous previous studies had discovered that this type of dye was one of the most promising and highly potential candidates for DSSC sensitiser application [32, 33]. The increased in the incident light absorbed by the dye molecules which resulted in a higher probability of generating electron-hole pairs. In this case, adsorbed dye molecules on ZnO nanostructures had maintained its adsorption even after desorption process (based on Table 4) was favourable for DSSC photoanode application. This strong bonding between dye molecules and metal oxide nanostructures were helpful for charge injection movement, dye functioning and generating the stability of solar cell performance [34]. There was not much difference in transmittance level during adsorption and desorption actions for pristine ZnO (1.25%), 0.3 TZO (2.71%), 1.2 TZO (2.21%), 1.5 TZO (5.28%) and 1.8 TZO (0.91%). This situation had established the dependability of better dye trapping character on the multiplane crystal orientation. The same pattern also displayed through less efficient dye trapping effect on 0.6 (7.79%) and 0.9 TZO (9.95%) due to much larger difference in trasnmittance before and after desorption process. This might due to the crystal growth leaned towards single plane (101). Less efficient dye trapping properties can be improved via application of buffer layer on top of the structure.

Table 4. The average transmittance within visible region (400 – 800 nm) of pristine ZnO and various TZO samples

Label in Figure 4	Sample	Average Transmittance (%)		
		As-prepared Sample	Dye Adsorbed (S1)	Dye Desorbed (S2)
(a)	Pristine ZnO	71.49	18.13	19.38
(b)	0.3 TZO	73.31	15.64	18.35
(c)	0.6 TZO	78.68	46.32	54.11
(d)	0.9 TZO	78.03	40.80	50.75
(e)	1.2 TZO	56.48	33.12	35.33
(f)	1.5 TZO	50.22	44.99	50.27
(g)	1.8 TZO	47.56	41.08	41.99

Conclusion

TZO nanowires with the desired properties were successfully synthesized using facile and ultrarapid microwave assisted ultrasonic irradiation technique. High uniformity, more crystalline, low defect, high transmittance and acceptable dye adsorption properties are the qualities can be found in 0.9 TZO thin film. Crystallinity can greatly influence the electrochemical stability of the thin film. The recombination will be suppressed if the defect level is low. Thus, this optimised TZO photoanode is expected to boost the efficiency and performance of DSSCs. Therefore, the fabrication of DSSCs is one of the ways to pave the pathway towards refining the future of renewable and clean energy generation.

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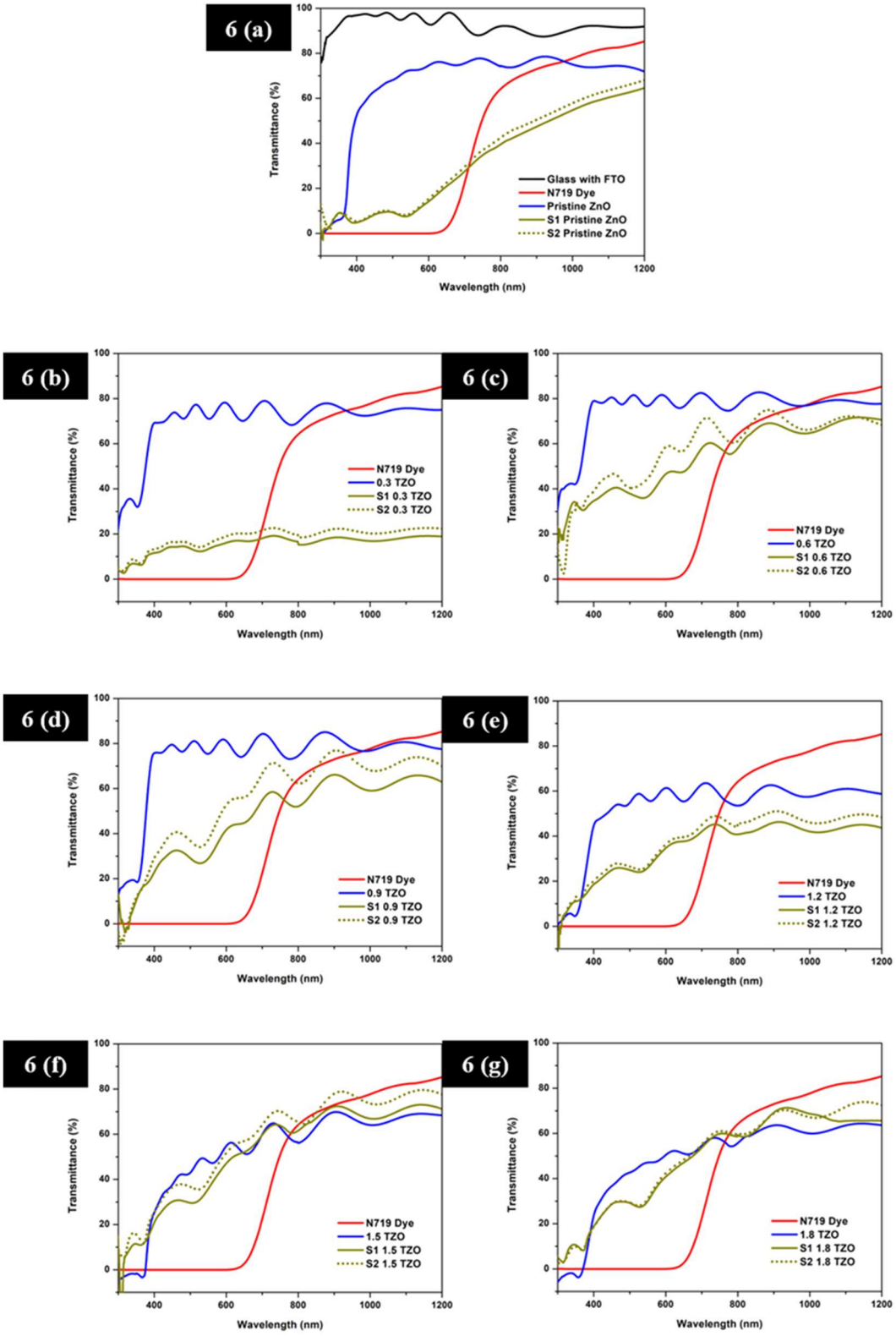


Figure 6. Transmittance of (a) pristine ZnO and (b – g) 0.3 – 1.8 at.% TZO NWs with dye adsorbed and desorbed plot

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MEMBRANE-PROTECTED MICRO-SOLID PHASE EXTRACTION OF CARCINOGENIC AROMATIC AMINES IN WATER SAMPLES PRIOR TO GC-FID ANALYSIS

(Pengekstrakan Fasa Pepejal Mikro Yang Dilindungi oleh Membran untuk Amin Aromatik Karsinogenik dalam Sampel Air dengan Analisis GC-FID)

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Abstract

A simple method to extract aromatic amines in environmental water samples was developed using β-cyclodextrin-toluene diisocyanate (β-CD-TDI) polymer enclosed with tea bag filter paper fashioned as a micro-solid phase extraction (μ-SPE) device combined with GC-FID. In this study, an efficient sorbent (β-CD-TDI polymer) was successfully synthesised and characterizaed for the microextraction of aromatic amines such as *p*-Toluidine, 4-chloroaniline, 2-naphthylamine, and 4-aminobiphenyl using FTIR, FESEM and TGA. Under optimized conditions (40 mg of β-CD-TDI polymer in 5 mL sample volume with 75 min extraction time and desorption with 300 μL of acetonitrile under 30s desorption time), β-CD-TDI polymer was proven to be an effective adsorbent for the extraction of aromatic amines in environmental water samples with a satisfactory percentage recovery achieved from 80 to 99%. All the studied analytes showed good linearity in the range of 5 to 500 μg L^{-1} and the correlation of determination (R^2) was from 0.9980 to 0.9990. The LODs obtained were from 1.67 to 3.18 μg L^{-1}, and LOQs were from 5.05 to 9.63 μg L^{-1}. This method gives precision values for both intra- and inter-day within accepted variable limits (<15% of RSD). The self-made cellulose-based μ-SPE device revealed that it can be simply prepared, easy to operate, reusable, cost-effective, reduce chemical consumption during extraction, and additionally speed up the extraction process, making it more efficient.

Keywords: μ-SPE, β-cyclodextrin-toluene diisocyanate, aromatic amines, GC-FID, environmental water

Abstrak

Satu kaedah mudah untuk mengekstrak amin aromatik dalam sampel air alam sekitar telah dibangunkan menggunakan polimer β-siklodextrin-toluen diisosiyanat (β-CD-TDI) yang dilingkungi dengan kertas penapis beg teh yang dihasilkan sebagai peranti pengekstrakan fasa pepejal mikro (μ-SPE) yang digabungkan dengan GC-FID. Dalam kajian ini, penyerap yang berkesan (polimer β-CD-TDI) berjaya disintesis dan dicirikan untuk pengeluaran mikro amin aromatik seperti p-Toluidin, 4-kloroanilin, 2-naftilamina, dan 4-aminobifenil menggunakan FTIR, FESEM, dan TGA. Di bawah syarat yang dioptimumkan (40 mg polimer β-CD-TDI dalam 5 mL isipadu sampel dengan masa pengekstrakan 75 minit dan desorpsi dengan 300 μL asetonitril dalam masa desorpsi 30 saat), polimer β-CD-TDI terbukti menjadi penjerap yang berkesan untuk pengekstrakan amin aromatik dalam sampel air alam sekitar dengan perolehan peratusan yang memuaskan mencapai dari 80 hingga 99 %. Semua analit yang dikaji menunjukkan lineariti yang baik dalam julat 5 hingga 500 μg L^{-1} dan korelasi penentuan (R^2) adalah dari 0.9980 hingga 0.9990. LOD yang diperolehi adalah dari 1.67 hingga 3.18 μg L^{-1}, dan LOQ adalah dari 5.05 hingga 9.63 μg L^{-1}. Kaedah ini memberikan nilai ketepatan untuk kedua-dua intraday dan inter-day dalam had batas variabel yang diterima (<15 % daripada RSD). Peranti μ-SPE berbahan selulosa buatan sendiri menunjukkan bahawa ia boleh disiapkan dengan mudah, mudah beroperasi, boleh digunakan semula, berkos rendah, mengurangkan penggunaan bahan kimia semasa pengekstrakan, dan tambahan pula mempercepatkan proses pengekstrakan, menjadikannya lebih cekap.

Kata Kunci: μ-SPE, β-siklodekstrin-toluena diisosiyanat, amin aromatik, GC-FID, air persekitaran

Introduction

Environmental pollution is the main concern today due to numerous fears caused by chemical contaminants to humans and other living beings with their high toxicity and widespread usage [1]. Among the number of organic pollutants found in the environment, aromatic amines (AAs) mainly gained the significant interest of researchers. Even in low concentrations, AAs negatively impact living organisms [2]. In recent years, aromatic amines have been used widely in high-in-demand developing sectors such as pharmaceuticals, pesticides, dyestuffs, and rubber industries. The production process, usage, and disposal of these products are the preliminary pathways aromatic amines enter into the environment. For instance, the hydrolysis of azo dyes and pesticides releases AAs directly into the water bodies.

Until now, more than 30 aromatic amines have been identified in the environment due to their thermodynamic stability, high polarity, and water solubility. Significant reactivity of amino groups enables them to be quite easily transported in and out of aquifers [3]. The United States Environmental Protection Agency (USEPA) and European Environmental Agency (EEA) have classified AAs as refractory organic materials as they are difficult to decompose by natural biological and chemical processes [4,5,6]. Therefore, reliable risk assessment of AAs is highly important, and the demand for trace level easy, efficient, and accurate analysis for various aromatic amines.

In addressing this matter, our current study focuses on a simple and cost-effective extraction technique to analyse these pollutants in water. To date, pollutant extraction using adsorbents is a highly favoured analytical technique and new sorbents are being widely studied using materials such as activated carbon powders and fibres, nanomaterials, paper mill sludge and cyclodextrin polymers [2]. Numerous cyclodextrin-based polymeric materials have been developed as sorbents for extracting environmental pollutants due to their eco-friendly nature and high regeneration value [4]. Cyclodextrins (CDs) are natural products obtained from the enzymatic degradation of starch. They are cyclic oligosaccharides built from (α-1,4)- linked D-glucopyranose units. CDs contain a hydrophobic interior and hydrophilic exterior in their torus structure. They have a structurally related series of macrocyclic oligosaccharides called α-, β-, and γ-CD [7]. Compared to other cyclodextrins, β-CD can form inclusion complexes with an extensive range of molecules through various interactions [8]. Besides, β-CD polymers are easy to synthesise, stable, water-insoluble, environment-friendly, and economical. Therefore, it is a good option for environmental-related applications.

The complexity of matrices, low concentrations of analytes in samples, and insufficient selectivity and

sensitivity in conventional methods commonly challenge the direct determination of aromatic amines in environmental samples. Therefore, the necessity for a simple and efficient sample preparation procedure arises constantly. Sample preparation is the conversion of complex sample matrices into an efficient analysable form via the elimination of interferences using separation and pre-concentration techniques [9]. Therefore, in analytical procedures, sample preparation can be expressed as critical and the most time-consuming step, which potentially could generate the highest error percentage on the work floor because it directly contributes to important analytical data such as the limit of detection (LOD), accuracy and precision [10]. Among the various sample preparation techniques, solid phase extraction (SPE) and liquid-liquid extraction (LLE) are the classical approaches for AAs. However, many disadvantages are associated with these classical methods, such as using a large volume of solvents, multi-step methodology, time-consuming extraction procedures, and difficulty in phase separation [11].

Recently, sample preparation has progressed towards a miniaturisation approach. Basheer and co-workers introduced an extraction technique using a small amount of sorbent packed inside a porous tea bag filter shaped like an envelope [12]. This technique is termed micro-solid phase extraction (μ-SPE) and has proven to be highly beneficial over classical extraction techniques. The method simultaneously provides better reproducibility, high accuracy, rapidity, high pre-concentration factor, and low consumption of organic solvents [13]. This miniaturised extraction technique has a growing interest in separation science due to its low cost, simplicity, and environmental compatibility compared to other commercial extraction techniques [14]. Hence in this research, the extraction of AAs from environmental water was studied using a similar μ-SPE technique. To our knowledge, β-cyclodextrin-toluene diisocyanate (β-CD-TDI), sorbent-based μ-SPE, is yet to be explored.

Typically, this study focuses on developing the μ-SPE method for four aromatic amines, namely 4-chloroaniline, 2-naphthylamine, 4-aminobiphenyl, and *o*-toluidine which are frequently found in environmental water samples and classified as carcinogens [15]. The study on the μ-SPE of AAs was conducted using polymeric sorbent (β-CD-TDI). β-CD-TDI was synthesised, characterised, and packed into a sealed porous cellulose-based envelope (teabag) prior to its application in the μ-SPE of AAs.

Materials and Methods

Materials and reagents

Commercially available β-CD [$(C_6H_{10}O_5)_7.3H_2O$] (molecular weight:1134.987 g mol^{-1}) was purchased from Calbiochem® (Calbiochem®, U.S.A.), and the linker 2,4-Toluene diisocyanate (TDI) (98%, v/v) was supplied by Sigma Aldrich (Aldrich, Buches SG, Switzerland). *N, N*-Dimethylformamide (DMF) (99.9%) was purchased from SeccoSolv® (Merck KGaA, 64271 Darmstadt, Germany). Analytes, *o*-toluidine, 4-chloroaniline, 2-naphthylamine, and 4-aminobiphenyl were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade, 99.9%), methanol (MeOH, HPLC grade, 99.9%), and n-hexane (HPLC grade, 95%) were acquired from Fisher Scientific, USA.

The individual standard stock solutions (200 mg L^{-1}) of four aromatic amines were prepared in 25 mL of MeOH and stored in the refrigerator at 4℃ to prevent degradation. The working standard mix was freshly prepared by diluting stock solutions with ultra-pure de-ionized water from the in-house Merk Milli-Q system. Cellulose-based heat sealable tea bag filter paper (Glatfelter brand) purchased from PCL Solutions (Pvt) Ltd, Sri Lanka.

Instrumentation analysis

Fourier transform infrared (FTIR) spectra were recorded on Perkin Elmer Spectrum 400 (Perkin Elmer, Waltham, MA, USA) in the range of 4000 to 400 cm^{-1} and resolution of 1 cm^{-1} using attenuated total reflectance (ATR) mode. Field emission scanning electron microscopy (FESEM) analyses were obtained using Hitachi SU8220 (Hitachi, Japan) with 1, 2, 10, and 50 μm magnifications under 1 and 2 kV voltages. Thermogravimetric analysis, TGA4000 (Perkin Elmer, USA) was used for thermal analysis of the synthesised polymer material where weight loss was measured for 10.0 mg of polymer using a linear temperature program at 10℃ min^{-1} within the temperature range of 50℃ to 900℃ under inert nitrogen atmosphere.

Chromatographic conditions

Separation and quantification of the selected AAs were performed on an Agilent 7890A gas chromatography

(GC) system equipped with an Agilent 5975C series flame ionization detector (Agilent, USA). The column used was the Agilent HP-5MS fused-silica capillary column (30 m x 0.32 mm, internal diameter, and film thickness: 0.25 µm). The following temperature program was used for the analysis of aromatic amines. Initially, the temperature was set to 60℃ for 1 min, then it was increased to 250℃ at the rate of 20℃ min^{-1} and held for 2 min. The total run time for GC-FID was 12.5 min. Nitrogen was used as the make-up gas (flow rate: 26.121 mL min^{-1}) and He as the carrier gas with a flow rate of 30 mL min^{-1} and pressure of 4.0 psi. The injector temperature was held at 300 °C with split-less mode, and the FID temperature was set to 300 °C. Quantitative calculations were made based on GC peak areas.

Preparation of materials: Synthesis of β-CD-TDI

The polymer was prepared according to the method referred to in the literature [16]. Firstly, 2.0 g of β-CD monomer (1.76 mmol) was added into 40 mL of anhydrous *N, N*-Dimethylformamide (DMF) solvent in a round bottom flask and flushed with nitrogen gas. Then 2.54 mL (17.6 mmol) of 2,4- toluene diisocyanate (TDI) crosslinking reagent was added dropwise into the solvent-β-CD system, with constant stirring at 700 rpm and heating at 80℃. The ratio of β-CD: TDI linker was 1: 10. The system was stirred and heated continuously for 24 hrs at the pre-set parameters. After completion, the reaction mixture was precipitated with excess acetone and allowed to settle for 10 min. This was done to remove excess solvent present in the reaction mixture. The mixture was then rinsed several times with acetone and de-ionized (DI) water to remove any unreacted starting materials. After rinsing, filtration was done using suction filtration, and the final product was dried in a vacuum desiccator for 48 hours prior to use [20].

Development of the µ-SPE device

The µ-SPE device consists of a porous membrane envelope filled with sorbent materials. In this study, cellulose-based tea bag filter paper was used as the porous membrane due to its high stability in the organic solvents and easy wettability. To prepare the µ-SPE device, the tea bag filter paper was cut into 1.5 cm x 1.8 cm rectangles, and 3 edges were heat-sealed. A glass pipette tip cut-off was used to insert 40 mg of β-CD-TDI polymer into pre-cleaned miniature tea bags (the variation of weights was less than 5%). The open ends were then heat-sealed to secure the sorbent content within the envelope. Each device was carefully weighed to ensure consistency in the preparation. The µ-SPE devices were soaked in acetone for 2 hrs to remove any organic impurities, followed by washing with DI water for another 2 hrs to remove any organic or inorganic impurities. For each washing step, 2 min vortex was used to achieve a better cleaning effect. Finally, the prepared device was stored in methanol until further use. Devices were pre-dried in the air before the extraction process. This method is highly cost-effective as 9 miniature devices could be prepared from one tea bag [14].

Micro-solid phase extraction procedure

A standard mixture of targeted AAs was extracted using the prepared µ-SPE device. For each extraction in a 20 mL sample vial, the µ-SPE device was placed into a 5.0 mL sample solution (pH 7.0) and agitated (100 rpm) for 75 min followed by a 1 min vortex (500 rpm) at room temperature. The device was removed with a pair of tweezers after extraction, transferred into a 5 mL sample vial, and dried in an oven at 80℃ for 15 min to remove trapped water. The analytes were eluted with acetonitrile (300.0 µL) using vortex for 30 s at 800 rpm. The eluent was then transferred into GC vials using 45 µm syringe filters. The extraction efficiency was subsequently analysed using GC-FID. The whole procedure is briefly illustrated in Figure 1.

Method validation

Method validation is an important process in analytical method development that provides evidence of whether the analytical process measures what is intended and is acceptable for its desired purpose. In the present study, optimised µ-SPE conditions for selected AAs were studied in terms of linearity, limits of detection (LOD), limits of quantification (LOQ), inter and intraday precision, and recovery.

Linearity

Linearity can be defined as the ability of the method to obtain signals proportional to the concentration of the analyte. Eight standards of different concentrations of aromatic amine mixtures were obtained by serial dilution from the stock solution. µ-SPE using optimised parameters was carried out for the prepared series of standards. The eluent was analysed using GC-FID, and the matrix match calibration plot for all four analytes

was prepared. The coefficient of determination for each plot was then calculated.

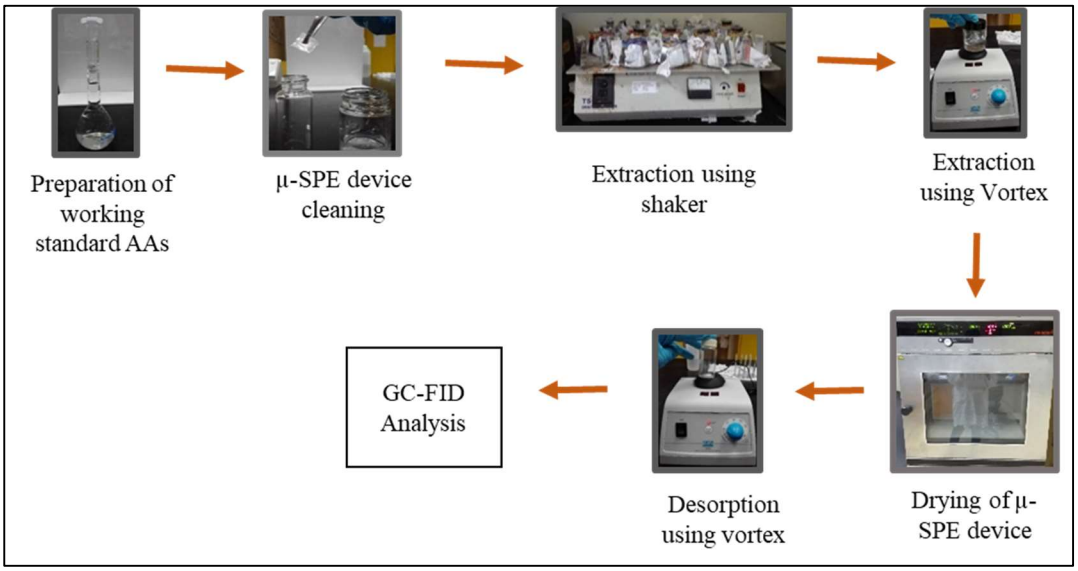


Figure 1. Schematic illustration of µ-SPE procedure for aromatic amines analysis

Limit of detection (LOD) and Limit of quantification (LOQ)

The limit of detection (LOD) can be defined as the lowest concentration of the target analyte, which could be detected by GC-FID with a certain confidence level and ought to be lower than the maximum residual limits (MRLs) in real samples. Meanwhile, the limit of quantification (LOQ) is the lowest concentration of an analyte that can be quantified with some degree of certainty. LOD and LOQ were calculated from equations 1 and 2**,** respectively.

$$LOD = 3.3 \times \frac{SD}{m} \quad (1)$$

$$LOQ = 10 \times \frac{SD}{m} \quad (2)$$

where m is the slope of the matrix match calibration plot and SD is the standard deviation of the blank residuals [17].

Pre-concentration factor (PF)

The pre-concentration factor is defined as the concentration ratio of the analyte in the enriched phase after extraction and the initial solution. This was calculated for each targeted analyte for the sample spike concentration of 200 µg L^{-1} using Equation 3.

$$PF = \frac{P_f}{P_i} \times \frac{V_i}{V_f} \quad (3)$$

P_f is the peak area of the analyte in the enriched extract after extraction, P_i is the peak area of the analyte in standard solution, V_i is the initial sample volume, and V_f is the desorption volume [18].

Matrix effect (ME)

The effect of interferences due to the co-extracting and co-eluting matrix substances can significantly influence the chromatographic signal of target analytes. The matrix effect can be calculated as the ratio of slopes of the standard and spiked calibration plots (equation 4).

$$ME = \frac{Slope\ of\ matrix\ matched\ calibration\ plot}{Slope\ of\ standard\ calibration\ plot} \quad (4)$$

Recovery

The recovery study was done by spiking a known concentration of analyte into the real samples. The spiking concentrations were chosen based on low, medium, and high concentrations for recoveries, i.e., 50.0, 75.0, and 100.0 µg L^{-1}, and the RSDs were determined using three different vials as triplicates ($n=3$). The percentage of recoveries (R %) for the real sample was calculated using Equation 5.

$$R\ \% = \frac{C_{observed} - C_{unfortified}}{C_{fortified}} \times 100\ \% \quad (5)$$

$C_{observed}$ is the concentration of AAs in the spiked sample, while $C_{unfortified}$ is the concentration of AAs in the unspiked sample. $C_{fortified}$ represents the initial spiking concentration [19].

Precision

Inter-day and intraday precisions of the developed technique were assessed by conducting extractions of 100.0 µg L^{-1} standard AAs mixture. Ten samples were tested for intraday precision, and inter-day precision was carried out on five consecutive days with five extractions per day. The results are presented as relative standard deviation (RSD %). Standard deviation (SD) and relative standard deviation (RSD %) were calculated using equations **6** and **7,** respectively.

$$SD = \sqrt{\frac{\sum(x - \tilde{x})^2}{n - 1}} \quad (6)$$

$$RSD\ \% = \frac{SD}{\tilde{x}} \times 100\ \% \quad (7)$$

where x is the observed result for the run, $\tilde{x}$ represents the mean value, and n is the number of measurements (repeatability) [19].

Reusability

The same µ-SPE devices were repeatedly used to determine the efficiency of reused polymers. Three of the 40 mg of β-CD-TDI polymer-packed tea bags were used for the extraction of 5.0 mL samples of AAs mixture (100.0 µg L^{-1}, pH 7.0). Extraction was done according to the optimised method. The same experiment was repeated for 8 additional cycles to study the reusability of the µ-SPE devices.

Real sample pre-treatment

To evaluate the applicability of the developed method, four different environmental water samples were analysed under optimized conditions. Tap water samples were collected from the working laboratory. Meanwhile, river water and lake water samples were collected in pre-cleaned amber glass bottles from the Pantai River, Malaysia, and Universiti Malaya (UM), Malaysia, respectively. Wastewater samples were collected from a cannel near the UM library. The collected water samples were filtered trice using 0.45 µm Millipore cellulose membrane filters right after sampling, and the pH was recorded. Finally, the filtered samples were covered with aluminium foil and stored in the refrigerator at 4℃ prior to analysis [9].

Results and Discussion

Synthesis and characterization of β-CD-TDI polymer: Mechanism of reaction

In the synthesis of cyclodextrin polymers, the use of diisocyanate as a cross-linking agent is a conventional direct reaction method. The reactions between β-CD and TDI were carried out at 80 °C for 24 hours. The polymerization mechanism follows the exothermic nucleophilic addition reaction as shown in Figure 2, where two electrons from OH- groups in β-CD donate to the C atom in the NCO- group of diisocyanate (Ar-aromatic ring structure). Thus, the double bond between N and C atoms breaks and forms 'urethane linkage' (-NH-C(=O)-O-) [13]. This polymerization reaction is popular among researchers working in this field due to its simplicity and high yields [16].

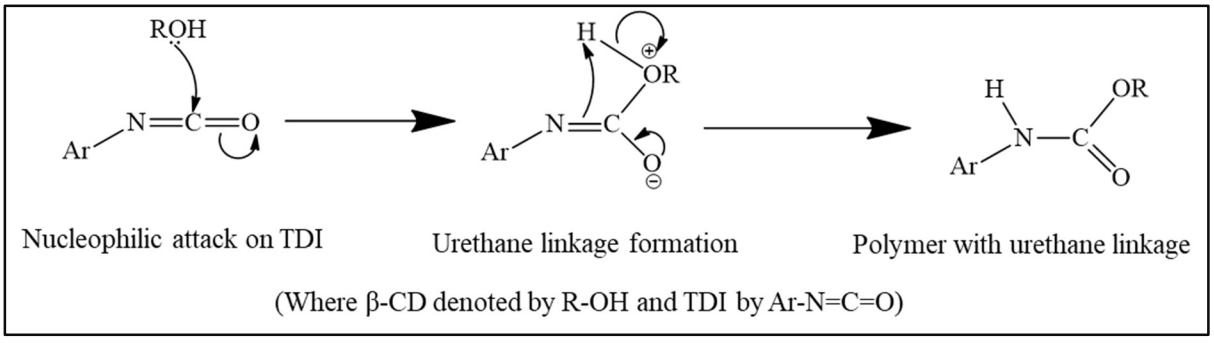


Figure 2. Proposed mechanism for the urethane linkage formation between β-CD and TDI

FTIR analysis

The spectrum of the β-CD-TDI polymer was compared with native β-CD and TDI spectra, as shown in Figure 3. In the spectrum of β-CD, the peaks at 1024 and 1080 cm^{-1} corresponded to C-OH stretching vibrations, while those at 3300-3400 cm^{-1} are attributed to -OH groups, the common functionalities in both native β-CD and TDI. However, in the β-CD-TDI spectrum, the absence of a peak around 2270 cm^{-1} (corresponding to the isocyanate group) and the appearance of stretching vibrations of carbamate linkage -NHCO at 1534 cm^{-1} indicates the completion of polymerization reaction [20]. Furthermore, the newly formed polymeric material (β-CD-TDI) exhibited peaks at 1600-1585 cm^{-1} and 1500-1400 cm^{-1} due to carbon-carbon stretching vibrations in the aromatic ring attached to TDI. The absorption band at 1221 cm^{-1} is attributed to the C-O-C characteristics of urethane linkage in the β-CD-TDI polymer [16]. These results confirm the successful polymerization of β-CD-TDI polymer.

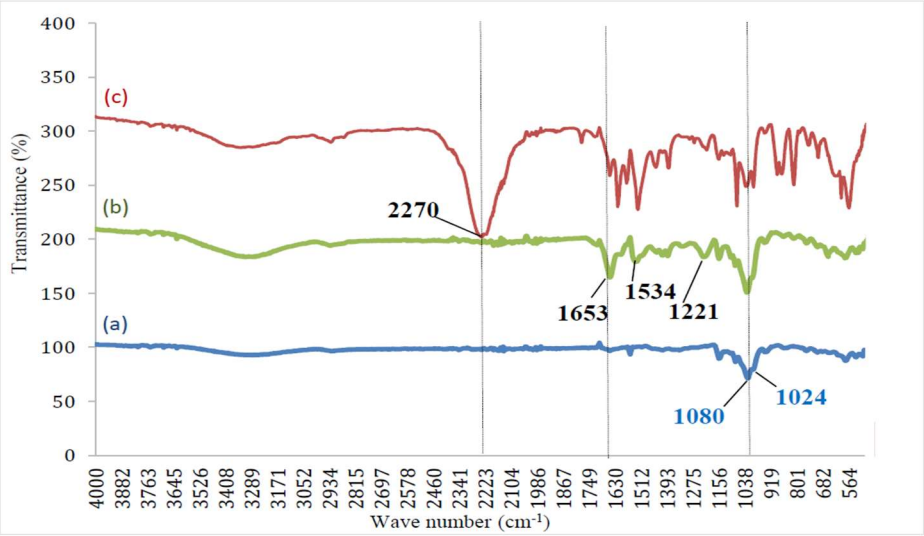


Figure 3. FTIR spectra of (a)β-CD, (b)β-CD-TDI polymer and (c)TDI

Morphology analysis

The polymer morphology analysis was carried out by FESEM analysis, and the results are given in Figure 4, illustrating both β-CD and β-CD-TDI images at different magnifications. FESEM images allow facile identification of irregularities on the surface of co-polymer materials [21]. The FESEM images of the β-cyclodextrin monomer shown in Figure 4 (a) have a smooth surface structure with deep cracks. The surface morphology of the β-CD-TDI polymer in Figure 4 (b) differs from the parent β-CD monomer. It records a non-smooth irregular surface with visible pore openings. Therefore, the results show that introducing the TDI linker to the β-CD has modified the surface characteristics of the polymer. Thus, it can be concluded that the synthesised polymer likely contains a more porous nature.

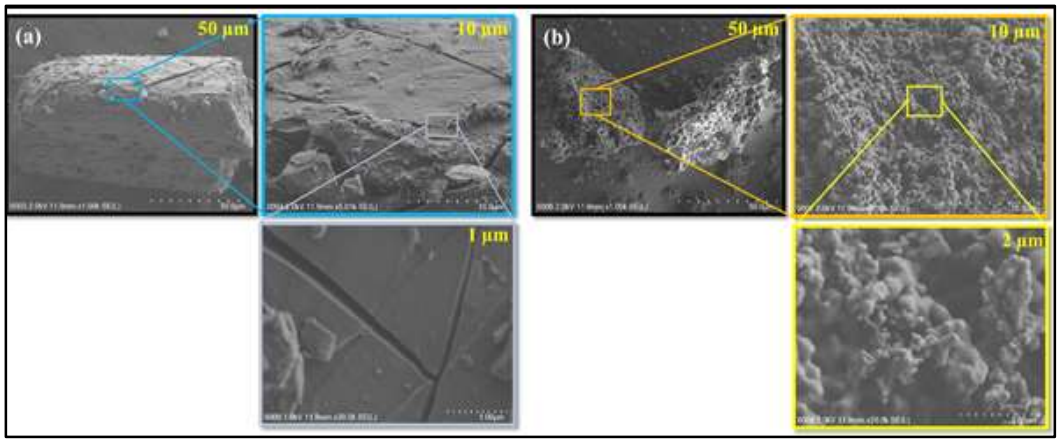


Figure 4. FESEM images 30k x magnification of (a) β-CD and (b)β-CD-TDI polymer

Thermal analysis

Thermal analysis is important in understanding the stability or function of the material at room temperature as well as in very high temperatures for extended periods. Besides, it is necessary to pre-determine the stability of the polymer during high-temperature applications. Polymer stability can be affected by various complex, interrelated phenomena, such as changes in the crystallinity of the bulk phase and polymer structure. Based on the TGA spectra of β-CD-TDI (Figure 5), the weight percentage reduction before 100 °C is around 8%. The evaporation of adsorbed moisture onto the polymer may cause a weight reduction [20, 22]. Further increment in temperature leads the polymer to lose weight as it degrades under the oxygenated environment. The polymer starts to degrade around 310 °C which is seen in the TGA curve. This higher weight loss is due to the cleavage of the urethane linkage and volatilization of the resultant product of decomposition [16]. It can be concluded from the thermal analysis data that the β-CD-TDI polymer is stable below 310℃ and can be used for high-temperature applications.

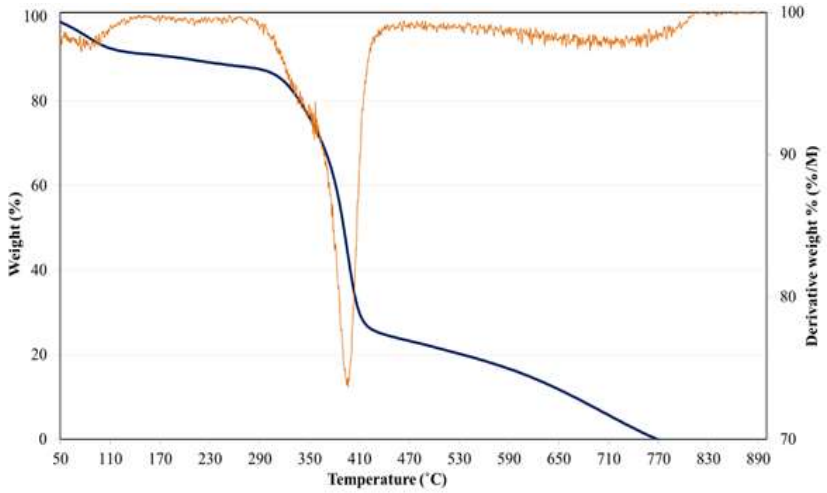


Figure 5. TGA analysis on weight (%) and derivative curves of β-CD-TDI polymer between 50 - 900℃

Optimization of micro-solid phase extraction (µ-SPE) parameters

Seven extraction parameters were selected to improve the performance of β-CD-TDI sorbent-based µ-SPE of aromatic amines. This includes optimising the type of desorption solvent, amount of adsorbent in the µ-SPE device, volume of desorption solvent, desorption time, sample volume, sample pH, and extraction time. The optimization procedure was started using an analyte concentration of 5.0 mg L^{-1}. All the optimizations were carried out in triplicates using GC-FID analysis.

Effect of types of desorption solvent

In the µ-SPE technique, the desorption step plays a vital role in desorbing maximum amounts of AAs from the β-CD-TDI polymer. Hence it is important to choose an appropriate desorption solvent that can provide optimum desorption efficiency. In this study, several organic solvents that were compatible with the GC system, namely methanol (MeOH), acetonitrile (ACN), and hexane, were explored. These solvents were chosen because the boiling point is lower than the analyte and for the GC system solvents that are easy to volatile are preferred [23]. Also, the wettability of the tea bag membrane, solubility of the analyte, and polarity of the solvents were considered. Due to the high porosity and fast water absorption of the cellulose-based tea bag filter paper, analytes could easily diffuse through and enhance the interactions with sorbent. Thus, the effect of tea bag material was neglected. Referring to Figure 6 (a), ACN was proven to yield the highest peak areas for all four analytes studied, whereas hexane showed the poorest elution capability. AAs are moderately polar compounds that form inclusion complexes with the β-CD-TDI during the extraction process [22]. Therefore, it may not be easily desorbed if the incorrect solvent is used. Hence, compared to non-polar solvents like hexane, polar solvents like methanol and acetonitrile were more suitable for the desorption of AAs. Among them, AAs were shown a strong affinity towards ACN, a moderately polar solvent, rather than β-CD-TDI achieving higher extraction efficiency. Therefore, ACN was chosen as the desorption solvent for further studies [14].

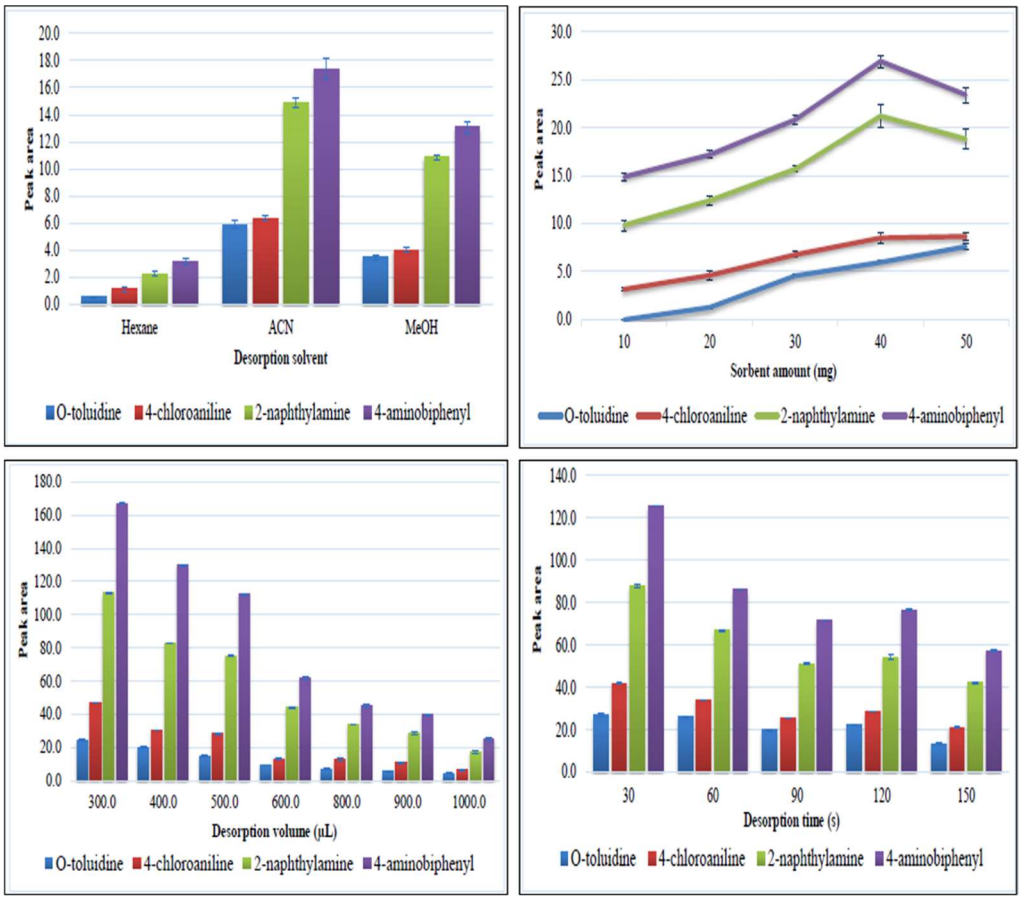


Figure 6. The effects of different types of desorption solvents (a), amount of adsorbent (b), volumes of desorption solvents (c) and different desorption times (d). (Extraction conditions: concentration of each AAs, 5 mg L-1; type of desorption solvent, hexane, MeOH and ACN; mass of β-CD-TDI, 10-50 mg; sample volume, 10 mL; extraction time, 30 min; desorption volume, 300 - 1000 µL and desorption time, 30- 150 s)

Effect of adsorbent amount

The effect of sorbent amount was observed using 10, 20, 30, 40, and 50 mg of polymer. According to Figure 6 (b), 40 mg of the β-CD-TDI polymer gave the largest peak area compared to other variables. Theoretically, the higher the amount of adsorbent used, the higher the concentration of analytes that can interact with polymer because of the increase in the surface area of the polymeric material. As the mass of the polymer increased from 10 - 40 mg, the peak area also increased, but beyond this point, there was no significant enhancement of the peak area for most of the analytes. This was due to the extraction of the maximum amount of analyte concentration from the solution; hence further increase in the mass of the adsorbent does not affect the extraction efficiency [16]. After 40 mg, a decrease in peak areas of some analytes may be due to steric hindrance or mobility disturbances occurring during analyte migration [14]. Thus, 40 mg of the β-CD-TDI polymer was chosen as the optimum amount for the extractions.

Effect of desorption volume

The effect of desorption solvent volume was studied in the range of 300.0 - 1000.0 µL. As observed in Figure 6 (c), 300.0 µL gave the highest peak area, and this micro-volume justifies the microextraction technique used in this study. This is probably due to the increasing dilution factor and the solubility of analytes in the aqueous phase the extraction step was increased which will lead to a decrease in the extraction efficiency. Thus, the smaller the volume is, the more concentrated the solvent becomes.100.0 and 200.0 µL were insufficient for desorption because those volumes were insufficient to make better contact with the prepared µ-SPE device. Therefore, 300 µL was selected as the optimum desorption volume for further studies.

Effect of desorption time

The effect of desorption time towards the extraction efficiency of AAs was investigated in the range of 30-150 s using a vortex (800 rpm). The observed results showed the best extraction efficiency was obtained at 30 s, as shown in Figure 6 (d). Beyond 30 s, when the time was increased, the peak area decreased as the equilibrium reaction of mass transfer moved to the opposite direction leading to the re-adsorption of analytes to the sorbent [14,24]. Thus, the 30 s desorption time was used in this study.

Effect of extraction volume

Sample volume also plays an important role in the extraction of AAs. Based on Figure 7 (a), when sample volume increases, the peak area also increases drastically, and 5.0 mL records the greatest value. When the volume of the sample increases up to 5.0 mL, proper dispersion of analytes occurs, leading to higher extraction efficiencies [25]. After reaching the maximum extraction efficiency, the binding sites of the sorbent are saturated with the targeted analytes; thus, further increase of volume will show a plateau. In contrast, the efficiency was decreasing, which may be due to migration difficulties between the sample solution and the surface of the sorbent [14]. Therefore, 5.0 mL was chosen as the optimal sample volume for this experiment.

Effect of sample pH

Sample pH plays a vital role in the extraction process because the degree of ionization and speciation of AAs depends on the pH of the solution. The effects of pH ranging from 2.0 - 9.0 were studied for the extraction of the selected AAs using β-CD-TDI as sorbent. It could be seen from Figure 7 (b) that the total amount of extractions improved significantly with the increase of pH from 2.0 to 7.0. The maximum peak area was found for pH 7.0, which was the natural pH of the aromatic amine mixture. Thereafter, the peak area decreased with an increase in pH until pH 9.0. The obtained results could be due to the sorption mechanism, which was probably dominated by the Van Der Waals forces, inclusion complex formation, л-л interactions, and hydrogen bonding between the β-CD-TDI and the aromatic amines [26]. Among these, inclusion complex formation plays a main role in the extraction [14]. When AAs were in the acidic medium, they were in ionic form, and due to that, the inclusion complex formation was low, whereas л-л interactions and hydrogen bonding may dominate. A higher pH (pH >7) also records the low capability of inclusion complex formation. However, higher chances for inclusion complex formation occur at pH 7, probably due to higher extraction efficiency. This is due to the neutral behaviour of AAs at the neutral pH [27]. Hence, pH 7.0 was selected as the optimum pH for the extractions.

Effect of extraction time

The effect of extraction time was another key parameter affecting the efficiency of extraction. In the µ-SPE technique, the mass transfer of AAs from the sample solution towards the polymeric sorbent is a time-dependent and equilibrium-based process rather than an exhaustive extraction process. The optimization for this parameter was carried out in the time range of 15 to 90 min using the shaker at 100 rpm and followed by a 1 min vortex at 500 rpm to provide better contact between the analyte solution and the polymer. The optimum time for the extraction was observed at 75 min (as shown in Figure 7 (c)), and the extraction efficiency was reduced after that point. Prolonged extraction decreases extraction performance due to a reverse equilibrium reaction [14,28]. Thus 75 min was selected as the optimum extraction time.

Reusability of µ-SPE device

The reusability of the prepared µ-SPE device was assessed through eight consecutive extraction cycles to check its extraction efficiency. Three µ-SPE devices were continuously reused to extract 100.0 µg L^{-1} standard AA solutions and then analyzed by GC-FID. According to the results, it was found that after five extractions, the response reduced significantly (> 25%), and the decreasing pattern continued, as shown in Figure 7 (d). Thus, this indicates that the µ-SPE device can be used only five times with no significant drop in performance. The non-reusability may be caused by the diffusion of oxygen and water into the polymer structure [21] and exposure to high agitation in a vortex where some amount of polymer might leach out from the teabag membrane after a few repetitions. Although the µ-SPE device cannot be reused more than five times, it still is low cost and records good reproducibility.

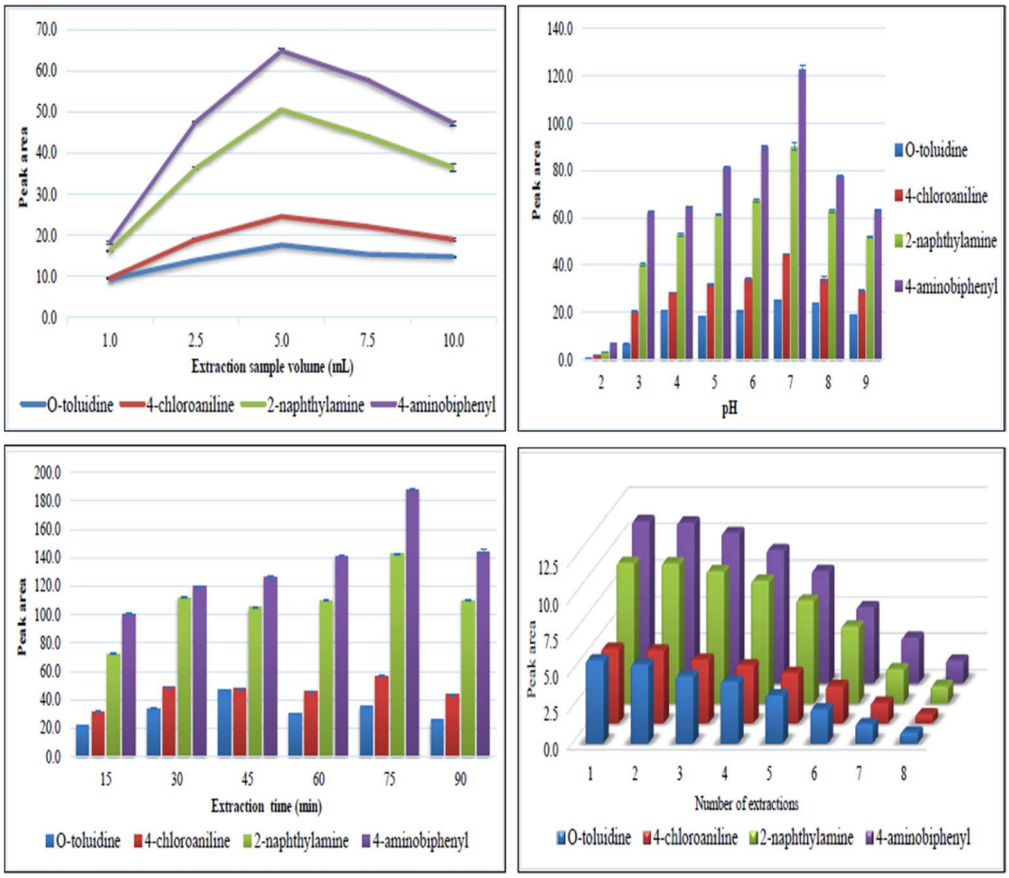


Figure 7. (a)The effects of different volumes of sample, (b)pH of sample, (c)extraction times and (d)reusability analysis of the µ-SPE device. (Extraction conditions: concentration of each AAs, 5 mg L-1; type of desorption solvent used :ACN; mass of β-CD-TDI, 40 mg; sample volume, 1 - 10 mL; extraction time, 15 - 90 min; desorption volume, 300 µL;desorption time, 30s and samples pH, 2-9)

Analytical performance of the proposed method

Method performance was analysed using the optimised parameters for μ-SPE of aromatic amines using β-CD-TDI. Under the optimised μ-SPE conditions, four selected aromatic amines were separated using GC-FID. A series of tests have been done to obtain the linear range, limits of detection, limits of quantification, and precision of the developed method. A series of spiked samples were extracted using the optimised μ-SPE method. The chromatographic data from GC-FID was used to prepare the calibration plots for each analyte (n=3). *o*-toluidine, 4-chloroaniline, and 2-naphthyl amine gave linear graphs between the ranges of 25.0 - 500.0 μg $L^{-1,}$ while 4-aminobiphenyl records the linear range of 5.0 - 500.0 μg L^{-1} for matrix match calibration. Based on matrix match calibration plots shown in Figure S2, the four aromatic amines gave linearity with a satisfactory coefficient of determination (R^2) ranging from 0.9980 to 0.9990. The results are tabulated in Table 1.

The lowest detection limits and limits of quantification for the developed method were calculated using the blank residue method. LOD values of the selected AAs for the proposed method as within the range of 1.67 - 3.18 μg L^{-1} and LOQs from 5.05 μg L^{-1} to 9.63 μg L^{-1}. The results of instrumental detection limits were found to be higher than method detection limits (*o*-toluidine: 215.70 μg L^{-1}, 4-chloroaniline: 289.54 μg L^{-1}, 2-naphthylamine: 219.21 μg L^{-1}, and 4-aminobiphenyl: 193.93 μg L^{-1}). Therefore, the developed method is sensitive enough to determine lower AA concentrations that cannot be detected by direct injection of AAs into the GC-FID. Furthermore, the obtained LOD values for the analytes (< 3.18 μg L^{-1}) were below the permissible levels suggested in the water. As mentioned earlier, the lowest possible concentration level for *o*-toluidine is 20 μg L^{-1} in ambient water (as indicated by USEPA), and the limit for 4-chloroaniline is 0.2 mg L^{-1} for domestic water and 28 μg L^{-1} according to the state drinking water guidelines of Florida [29,30]. According to USEPA, regional screening levels for chemical contaminants, 2-naphthylamine, is permitted up to 3.9 μg L^{-1} and 4-aminobiphenyl up to 3.0 μg L^{-1} in tap water [29].

The reproducibility of the optimised method was determined by intraday and inter-day precision studies. The samples were spiked with 100.0 μg L^{-1} standard AA mixture. The inter-day study was done on five consecutive days with 5 replicates per day, whereas 10 replicates were done on the same day to analyse intraday precision. Relative standard deviation (RSD) values for intraday ranged between 1.75% - 2.84%, and inter-day was 1.93% - 2.47%. The assessed values on precision were found to be within the variable limits of < 5% (different μ-SPE devices were able to produce reproducible results). Hence, the obtained results indicate that the developed μ-SPE method can be applied to determine trace levels of AAs with reproducible results. Moreover, pre-concentration factors for the target analytes were calculated from the ratios of the peak areas before and after the extraction of analytes. This was investigated to evaluate the extraction efficiency of the proposed μ-SPE method. As shown in Table 1, the extraction method provided moderately high pre-concentration factors ranging from 38 to 152. This may be due to the better mass transfer between the sorbent, β-CD-TDI, and sample solution facilitated by the continuous flow of sample solution via the cellulose teabag membrane [12].

Real samples analysis

The developed μ-SPE method was applied to real samples to determine the AA. The extractions were done under optimised conditions. Four water samples were collected inside UM premises: lake water from UM lake, water from Pantai River, tap water from the laboratory, and wastewater from the wastewater cannel. Samples were filtered twice using 0.45 μm Whatman filter papers to remove any possible particulates prior to extraction. The pH of the samples was tested prior to the analysis and adjusted accordingly. To study the matrix effect and recovery of the real samples, all four water samples were spiked with the analyte concentrations of 50.0, 75.0, and 100.0 μg L^{-1}. Simultaneously, the analysis of the blank samples was conducted for comparison purposes. The slopes of the standard and spiked calibration plots were compared through statistical testing to evaluate the effect of possible interferences from dissolved organic matter in real samples. As per the results tabulated in Table 1, a significant difference can be seen between the two slopes (ME > 0.05), which indicates the presence of interference from the sample matrix [24]. Therefore, the matrix match calibration plots were used for real sample analysis.

As illustrated in Table 2, recoveries of the aromatic amines for all the water samples were between 80 – 99% with % RSDs of 0.80 – 5.82%. The extraction recoveries for tap water ranged from 80 – 97% with % RSD values between 1.15 – 3.39%, for river water 80 – 95% (% RSD; 1.01 – 5.82%), lake water 81 – 99% (% RSD; 0.80 – 4.52%) and wastewater 80 – 99% (% RSD; 1.06 – 5.80%). The obtained results were within a satisfactory level. Typical chromatograms of lake water samples with and without spiking with aromatic amine standards (blank, 50.0, 75.0, and 100.0 μg L^{-1}) are demonstrated in Figure 8.

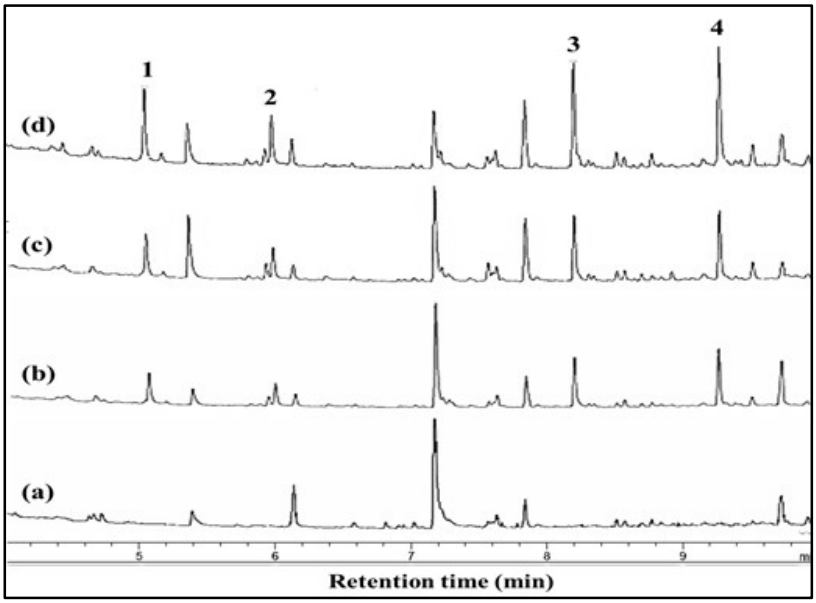


Figure 8. GC-FID chromatograms of unspiked lake water sample (a), lake water sample spiked with 50 μg L^{-1} AAs standard (b), lake water sample spiked with 75 μg L^{-1} AAs standard (c), and lake water sample spiked with 100 μg L^{-1} AAs standard (d). Analytes peak: o-toluidine (1), 4-chloroaniline (2), 2-naphthylamine (3), and 4-aminobiphenyl (4)

Table 1. Analytical performance data for the β-CD-TDI based μ-SPE method

Analytes	Regression Equation	Linear Range (μg L^{-1})	Coefficient of Determination (R^2)	RSD (%)		LOD (μg L^{-1})	LOQ (μg L^{-1})	Pre-Conc. Factor (PF)	Matrix Effect (ME)
				Inter-day (n=5)	Intra-day (n=10)				
***o*-toluidine**	Y = 0.0657x-0.4198		0.9990	2.18[a]	2.69[b]	2.66	8.07	38[d]	0.38
4-chloroaniline	Y = 0.0569x+0.3852	25- 500	0.9980	2.47[c]	2.68[c]	2.77	8.41	152	0.27
2-naphthylamine	Y = 0.1069x-0.3159		0.9983	2.06	2.84	3.18	9.63	102	0.24
4-aminobiphenyl	Y = 0.0882x+1.7317	5 - 500	0.9980	1.93	1.75	1.67	5.05	93	0.30

[a] Average of RSD % of five consecutive days

[b] Average of RSD % of ten different samples within a day

[c] Spiked with 100 μg L^{-1} standard AAs mixture

[d] Calculated for the concentration of 200 μg L^{-1}

Table 2. The relative recoveries and RSD of AAs in real water samples with spike concentrations of each analyte

Analyte	Average recovery (%) (% RSD, n = 3)				
	Spiked concentration (µg L^{-1})	Tap Water	River Water	Lake Water	Wastewater
***o*-toluidine**	0	N/D	N/D	N/D	N/D
	50	[a]90 [b](2.47)	87 (2.06)	82 (3.51)	83 (4.27)
	75	92 (2.29)	83 (3.03)	89 (3.95)	80 (5.80)
	100	96 (3.71)	93 (1.26)	99 (1.40)	99 (2.23)
4-chloroaniline	0	N/D	N/D	N/D	N/D
	50	84 (2.72)	82 (2.33)	88 (1.59)	83 (1.78)
	75	87 (3.39)	82 (5.08)	88 (4.52)	81 (4.45)
	100	97 (2.61)	85 (2.02)	87 (4.39)	94 (1.90)
2-naphthylamine	0	N/D	N/D	N/D	N/D
	50	90 (1.52)	86 (0.96)	84 (2.41)	80 (3.52)
	75	81 (1.15)	91 (1.01)	87 (2.20)	83 (3.32)
	100	96 (2.71)	95 (2.50)	97 (1.71)	96 (1.59)
4-aminobiphenyl	0	N/D	N/D	N/D	N/D
	50	80 (2.62)	80 (1.62)	81 (0.80)	(80 (1.62)
	75	82 (3.18)	83 (5.82)	81 (0.99)	81 (1.06)
	100	90 (1.79)	87 (5.18)	94 (2.15)	94 (1.77)

N/D – Not detected
[a] Average value from triplicate individual vial
[b] % RSD – Relative standard deviation (n=3)

Comparison study with other extraction methods

The analytical performances of the developed β-CD-TDI based µ-SPE method were compared with previously reported methods in Table 3. The obtained detection limits for the present work (1.67 – 3.18 µg L^{-1}) were lower than previous work that used LPME coupled with GC-FID [31] but were higher than the other methods. However, this method still provides a lower LOD value than the legally accepted lower limits proving its applicability for environmental water samples. The reproducibility results (< 5%) and percentage recoveries of the aromatic amines (80 – 99%) were within the acceptable range compared to other studies. Moreover, the current µ-SPE method is a cost-effective, simple, and green technique that consumed a lower sample volume (300 µL). Therefore, this method is beneficial over the other methods.

Table 3. Comparison of the proposed method with previous methods applied for the extraction of AAs in environmental water samples

Extraction Method	Sorbent	Analytical Method	Linear Range (µg L^{-1})	LOD (µg L^{-1})	Solvent Volume (µl)	Precision (% RSD)	Recovery (%)	Ref.
LPME	-	GC-FID	50 - 7000 90 - 10000	12 - 61	-	3.5 - 14.5	92 - 104	[32]
MSPE	PpPD/Fe_3O_4	GC-FID	0.05 - 100	0.007 - 0.01	250	< 5.9	93.0 - 99.8	[12]
SPE	β-CD-HMDI	HPLC-DAD	-	0.002 - 0.008	2000	3.5 - 9.2	38 - 108	[33]
SPE	C18 cartridge-SDS	HPLC-UV	3.1-125	1-4.5	3000	< 5.8	84.8 - 93.5	[34]
HS-SPME	PDMS-IL	GC-FID	0.005 - 0.5	0.001 - 0.1	-	3.5 - 8.5	83.3 - 95.0	[35]
Mixed Matrix Membrane Microextraction (MMMM)	MIP	HPLC-DAD	1 - 500	0.06 - 0.17	250	< 10	80 - 96	[9]
DLLME-SAP	DES-UA	HPLC-UV	-	0.07 - 0.11	40	2.9 - 6.2	85.1 - 99.9	[36]
µ-SPE	β-CD-TDI	GC-FID	25 - 500 5 - 500	1.67 - 3.18	300	< 5	80 - 99	This Method

BS-DLLME-binary solvent dispersive liquid-liquid microextraction; AALLME- Air assisted liquid-liquid microextraction; HS-SPME- Head-space solid-phase micro extraction; PDMS-IL-Poly(dimethylsiloxane)-ionic liquid; PpPD/Fe_3O_4-Poly(para-phenylenediamine) modified magnetic nanoparticles.

Conclusion

The synthesised β-CD-TDI polymer was packed inside the cellulose tea bag, successfully characterised by several analytical techniques, and applied as sorbent for the micro-solid phase extraction of aromatic amines combined with GC-FID analysis. Moreover, the self-made cellulose-based μ-SPE device revealed that it can be simply prepared, easy to operate, reusable, and cost-effective. This approach is environmentally friendly because this has reduced the consumption of organic solvents and sorbent. The method offers good repeatability and simple, rapid, and robust characteristics. The developed method was effectively applied to real water samples, including the lake, river, tap, and wastewater, with good sensitivity to analyse aromatic amines in aqueous samples and good reproducible results and better recoveries.

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SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL EVALUATION OF NOVEL THIAZOLIDINEDIONE DERIVATIVES AS EMERGING ANTIDIABETIC AGENTS

(Sintesis, Pencirian dan Penilaian Biologi Terbitan Novel Thiazolidinedion Sebagai Agen Antidiabetik yang Baru Muncul)

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Abstract

In the present research, derivatives of thiazolidinedione were synthesized and established as potent antidiabetic activity through *in vitro* alpha-amylase inhibition activity and *in vivo* animal experimentation. A total of 15 molecules were synthesized namely, SMI-IV-2, SMI-IV-4, SMI-IV-7, SMI-IV-9, SMI-IV-11, SMI-IV-15, SMI-IV-19, SMI-IV-22, SMI-IV-23, SMI-IV-28, SMI-IV-31, SMI-IV-32, SMI-IV-34, SMI-IV-41and SMI-IV-47. Thereafter, *in vitro* alpha amylase activity was carried out followed by *in vivo* animal study performed in STZ induced animal. Metformin was used as standard for result comparison. Furthermore, various biochemical parameters were analyzed. Initially, the novel thiazolidinedione derivatives were synthesized by the reaction between thiourea and chloroacetic acid and resulted first compound as 1,3-thiazolidine-2,4-dione which was further treated with pyridine-4-carbaldehydeand resulted (5*Z*)-5-[(pyridin-4-yl)methylidene]-1,3-thiazolidine-2,4-dione which was further reacted with acid chloride and aromatic aldehydes and resulted the derivatives of compound (5*Z*)-3-[(2*E*)-3-phenylprop-2-enoyl]-5-[(pyridin-4-yl) methylidene]-1,3-thiazolidine-2,4-dione which was named as SMI-IV. A total of five derivatives were selected based on *in vitro* and *in vivo* results and further characterized by melting point, thin layer chromatography, IR spectroscopy, ^{1}HNMR, ^{13}CNMR and mass spectra. *In vitro* alpha amylase study showed potent inhibition of alpha amylase activity by all the derivatives with dose dependent manner. Among the all derivatives, SMI-IV-4 showed IC50 value less than others (39.56%) and nearer to the standard (24.36%). *In vivo* animal experimentation was also revealed that derivative SMI-IV-4 significantly reduced the blood glucose level at 24 hours to 166 mg/dl when compared to the standard metformin (115 mg/dl). Overall results concluded that thiazolidinedione derivatives showed potent antidiabetic activity especially the derivatives viz. SMI-IV-4, SMI-IV-11, SMI-IV-22, SMI-IV-31, and SMI-IV-41 among them SMI-IV-4 showed more significance when compared to standard metformin.

Keywords: antidiabetic, *in vitro* assay, *in vivo* experiment, streptozotocin, spectroscopy, thiazolidinedione

Abstrak

Dalam penyelidikan ini, derivatif thiazolidinedion telah disintesis dan ditubuhkan sebagai aktiviti antidiabetik yang kuat melalui aktiviti perencatan alfa-amilase *in vitro* dan eksperimen haiwan *in vivo*. Sebanyak 15 molekul telah disintesis iaitu, SMI-IV-2, SMI-IV-4, SMI-IV-7, SMI-IV-9, SMI-IV-11, SMI-IV-15, SMI-IV-19, SMI-IV-22, SMI-IV-23, SMI-IV-28, SMI-IV-31, SMI-IV-32, SMI-IV-34, SMI-IV-41dan SMI-IV-47. Selepas itu, aktiviti amilase alfa *in vitro* telah dijalankan diikuti dengan kajian haiwan *in vivo* dilakukan dalam haiwan yang disebabkan oleh STZ. Metformin digunakan sebagai standard untuk perbandingan hasil. Tambahan pula, pelbagai parameter biokimia telah dianalisis.Pada mulanya, derivatif thiazolidinedion novel telah disintesis melalui tindak balas antara tiourea dan asid kloroasettik dan menghasilkan sebatian pertama sebagai 1,3-thiazolidine-2,4-dion yang kemudian dirawat dengan piridin-4-karbaldehid dan menghasilkan (5Z) -5- [(piridin-4-yl) metilliden]-1,3-thiazolidine-2,4-dion yang kemudiannya bertindak balas dengan asid klorida dan aldehid aromatik dan menghasilkan terbitan sebatian (5Z)-3-[(2E)-3-fenilprop-2-enoil]-5-[(piridin-4-il) metilliden]-1,3-thiazolidine-2,4-dion yang dinamakan sebagai SMI-IV. Sebanyak lima derivatif telah dipilih berdasarkan keputusan *in vitro* dan *in vivo* dan selanjutnya dicirikan oleh takat lebur, kromatografi lapisan nipis, spektroskopi IR, ^{1}HNMR, ^{13}CNMR dan spektrum jisim. Kajian *in vitro* alfa amilase menunjukkan perencatan kuat aktiviti alfa amilase oleh semua derivatif dengan cara bergantung kepada dos. Di antara semua derivatif, SMI-IV-4 menunjukkan nilai IC50 kurang daripada yang lain (39.56%) dan juga lebih hampir kepada standard (24.36%). Eksperimen haiwan *in vivo* juga mendedahkan bahawa terbitan SMI-IV-4 dengan ketara mengurangkan paras glukosa darah pada 24 jam kepada 166 mg/dl jika dibandingkan dengan metformin standard (115 mg/dl). Keputusan keseluruhan menyimpulkan bahawa derivatif thiazolidinedion menunjukkan aktiviti antidiabetik yang kuat terutamanya derivatif iaitu SMI-IV-4, SMI-IV-11, SMI-IV-22, SMI-IV-31, dan SMI-IV-41 antaranya SMI-IV-4 menunjukkan lebih ketara jika dibandingkan dengan larutan piawai metformin.

Kata kunci: antidiabetik, ujian *in vitro*, eksperimen *in vivo*, streptozotocin, spektroskopi, thiazolidinedion

Introduction

Diabetes mellitus (DM) is characterized by hyperglycemia, a complicated chronic disease that can develop when insulin production or action is impaired. Patients with this condition are at increased risk for macro- and microvascular consequences due to the chronic metabolic imbalance that characterizes it [1]. These difficulties, in turn, increase the likelihood of cardiovascular diseases (CVDs) and other hospitalizations that patients may have if they do not receive adequate care [2]. One of four plasma glucose (PG) criteria must be met for a clinical diagnosis of diabetes: (i) an elevated fasting plasma glucose (FPG) of more than 126 mg/dL; (ii) a 2-hour PG of more than 200 mg/dL during a 75-g oral glucose tolerance test (OGTT); (iii) an uncontrolled PG of more than 200 mg/dL accompanied by classic hyperglycemia symptoms; or (iv) a hemoglobin A1C level greater than 6.5% [3]. An American Diabetes Association report recently suggests that there might not be a suggested test for diagnosis. All people, regardless of weight, should start getting tested at 45 years of age [4]. People who have at least one extra risk factor for developing diabetes, are overweight or obese, and have diagnostic symptoms but are asymptomatic should still get tested [5].

Heterocyclic compounds with two heteroatoms incorporated into five-membered ring structures have significant biological efficacy. Thiazole is a fundamental structure found in the thiazolidine-2,4-dione, which is one of the physiologically active heterocyclic molecules [6]. Thiazolidine-4-one and thiazolidine-2-one is compounds that exhibit various functions and contain a carbonyl group at the 4th and 2nd positions, respectively. The presence of two carbonyl groups at the 2nd and 4th positions in the thiazole ring results in the formation of thiazolidine-2,4-dione. The substituents at positions 3 and 5 of the thiazolidine-2,4-dione structure are modifiable [7]. Peroxisome proliferator activated receptor-gamma is a nuclear receptor that plays a critical role in controlling gene expression linked to energy balance and lipid metabolism [8]. In the compound nucleus, PPARγ can limit the incidence of problems, increase insulin sensitivity, and boost lipocyte distinction. The glucose metabolism linked to insulin resistance can be controlled using thiazolidinediones (TZDs), which are PPARγ agonists with high affinity but no risk of

hypoglycemia [9]. There are now several medications that can be used to lower blood sugar levels in people with diabetes, including sulfonylureas and biguanides. Because of the serious adverse effects these medications caused, it is critical to find a new class of chemicals to address these issues [10].

Several TZDs like troglitazone, pioglitazone, and rosiglitazone are available in the market, but they are having severe side effects like idiosyncratic hepatotoxicity, fluid retention and weight gain. Furthermore, troglitazone and rosiglitazone were prohibited, and pioglitazone's label was revised due to risk of bladder cancer. Emerging strategies, guided by structural considerations of ligands, receptors and their interactions, offer the potential to create thiazolidinedione derivatives with diminished adverse effects [11]. Thereafter, many literatures were also reported that thiazolidine-2,4-dione derivatives are well known for diabetic management [12,13]. Therefore, detail structural characterization and synthesis of many thiazolidinedione derivatives are required for the development of small molecules as physiologically active agents as potent antidiabetic drugs.

Materials and Methods

Scheme and procedure: Below scheme-1 was highlighted the overall scheme of synthesis of compound-IV.

Step-I

A solution of 56.4g (0.6M) chloroacetic acid and 45.6g (0.6M) thiourea was dissolved in 60 mL of water in a 250 mL three-necked flask. After 15 minutes of stirring, white precipitates should have formed in the mixture. Once the contents of the flask were mixed, to dissolve the precipitates, 60 mL of strong hydrochloric acid was gradually added using the dropping funnel. The mixture was then refluxed for 10-12 hours at 100-110˚C while being stirred. White needle clusters formed from the flask's contents solidifying as they cooled. After removing any remaining hydrochloric acid, the product was filtered and rinsed with water before being dried. From ethanol, it was recrystallized.

Step-II preparation of compound-II:

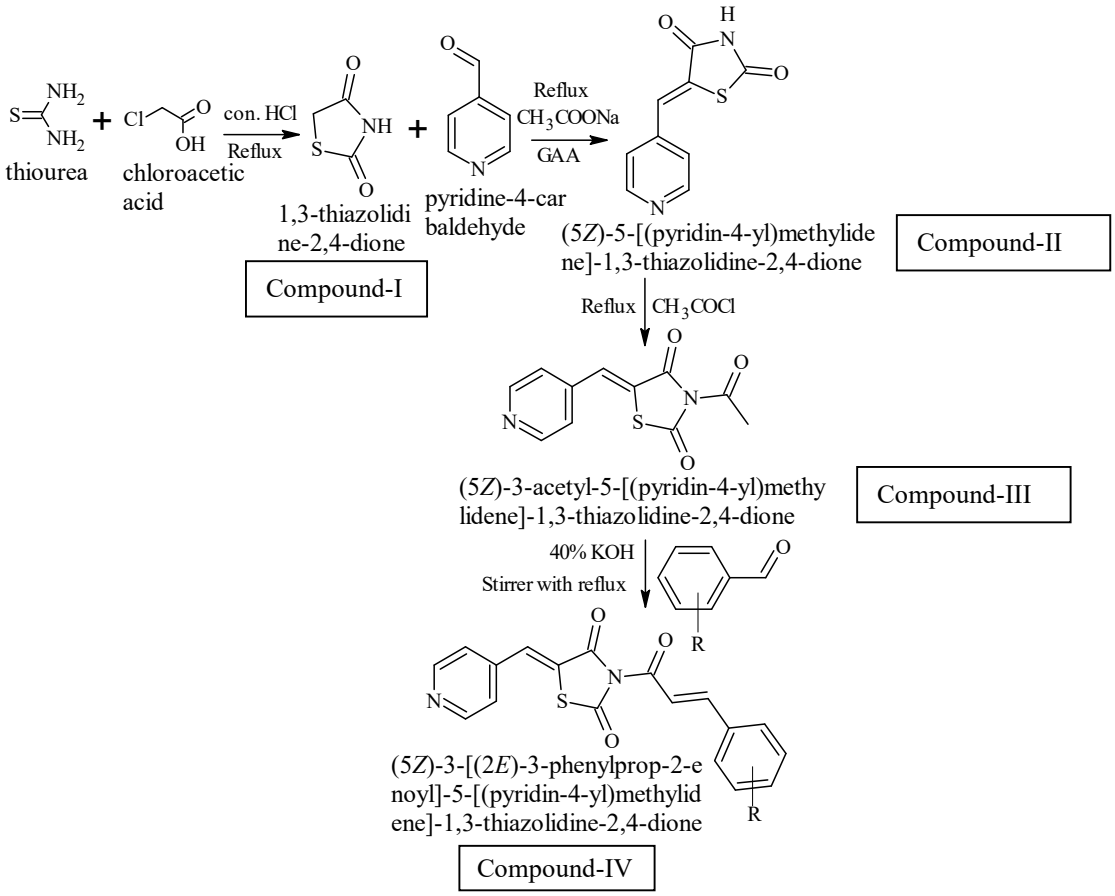
After being refluxed for 6 hours and cooled, a solution containing 1,3-thiazolidine-2,4-dione molecule (I) (1.66 g, 0.005 mol), (pyridine-4-carbaldehyde 0.005 mol) in ethanol (15 mL), and 5 drops of glutathione (GAA) was prepared. The resulting solid was extracted from the methanol by filtering, drying, and recrystallization.

Step-III preparation of compound-III

A solution of brine, consisting of 36% aqueous sodium chloride, was mixed with 5g (0.038 mol) of sodium acetate trihydrate in 50 mL. On top of it, 0.025 mol of compound-II was added. Next, while the mixture was still at room temperature, 2 mL of acetyl chloride (0.028 mol) in 3mL of acetone was added drop wise with stirring. An additional hour was spent stirring the reaction mixture. The effervescence was stopped by adding saturated $NaHCO_3$ solution. A solution was acidified with concentrated hydrochloric acid. Compound III, which is sparingly soluble, washed out as a solid. It crystallized from a solvent solution consisting of MeOH, MeOH, and water after being filtered under suction.

Step-IV preparation of derivatives of compound-IV:

In a round bottom flask equimolar mixture of compound-III and substituted aromatic aldehydes were dissolved in ethanol and was subjected for reflux on a water bath for 3.30 min in the presence of few drops of acetic acid. The reaction mixture was later poured into ice-cold water and the solid formed was filtered out and dried. The derivatives of compound-IV were obtained then recrystallized from ethanol (Scheme-I). Thereafter, many derivatives were synthesized from compound-IV and their physicochemical properties were depicted in below Tables 1 to 6.



Scheme-1. Synthesis steps for compound-IV

Biological evaluation: *In vitro* α-amylase inhibitory assay

Minor adjustments were made to the standard procedure before the experiment was conducted. A 0.2 mL solution containing 0.01M $CaCl_2$ and 0.5M Tris-HCl buffer (pH 6.9) was used to suspend 2 mg of starch azure. The tubes with the substrate solution were pre-incubated at 37.5 °C for 15 minutes after 5 min of boiling. All the derivatives (SMI-IV) were dissolved in dimethyl sulfoxide (DMSO) and prepared the various concentrations (10, 20, 40, 60, 80, and 100 µg/mL) by added 0.2 mL of thiazolidinedione derivatives (SMI-IV) to the substrate solution in the tube, with a specific concentration. To the tube with the thiazolidinedione derivatives (SMI-IV) and additionally, 0.1 mL of porcine pancreatic amylase in Tris-HCl buffer (2 units/mL) was added to the substrate solution. We ran the reaction for 10 minutes at 37 °C. To halt the process, 0.5 mL of 50% acetic acid was added to each tube. Centrifuged at 3000 rpm for 5 minutes at 4 °C, the reaction mixture was strained. A spectrophotometer was used to measure the absorbance of the supernatant at 590 nm. The α-amylase inhibitory effects of the fifteen derivatives that were chosen were tested using the same approach. The standard medicine utilized was acarbose, which is known to inhibit α-amylase [12, 13].

***In vivo* anti-diabetic activity: Streptozotocin (STZ)-induced diabetes**

Male albino Wistar rats weighing 160±20 g and around 3-4 weeks old were obtained (Ethical Ref No: IAEC/Sangli/2023/74). Unless otherwise specified, all rats were housed in an animal housing facility under

typical circumstances, including an ideal temperature of 22 °C, relative humidity, and a 12-hour light/dark cycle. Before the trial began, all the rats spent four days becoming used to the lab environment. At a dosage of 60 mg/kg body weight, 80 albino Wistar rats that had fasted overnight and weighed 160 ± 20 g were injected with a solution of STZ that had been dissolved in Citrate buffer (0.1 M). A glucometer (Accu-Chek) was used to assess the animals' fasting blood glucose levels from the vein of their severed tails 48 hours after injection. Diabetic animals were defined as those with a fasting blood glucose level between 300 and 450 mg/dL, and 8 animals were randomly assigned to each experimental group (total 8 groups). Oral dosages of 250 mg/kg of thiazolidinedione derivatives (SMI-IV) were administered to each group using a fine-mesh solution of test samples made in 1% gum acacia. The typical dose of metformin used as an antidiabetic medication was 250 mg/kg of body weight. Blood samples were taken after 24 hrs from the animal's tail, 300 to 1440 minutes after they were treated with the test sample, reference medication. Body weights were determined followed by the blood serum was separated from the blood using centrifugation, which ran for 20 minutes at 3000 rpm. Total cholesterol, triglycerides, low-density lipoprotein (LDL), and low-density lipoprotein (HDL) were all estimated using commercially available kits. Serum insulin was determined using an enzyme-linked immunosorbent test (ELISA) kit. Whether it's a conventional medicine or a test chemical, that percentage of blood glucose reduction Graph Pad Prism was used to plot the blood glucose level (mg/dL) against time (min). To demonstrate the improvement in hyperglycemia, the area under the curve (AUC) between 0-300 and 0-1440 minutes was determined by comparing the AUC of the groups treated with the test substance and the standard treatment [14, 15].

Statistical analysis

Results were tabulated as mean ± SEM. Data was evaluated using one-way ANOVA followed by Dunnett's comparison. The differences were considered significant at $p \leq 0.05$.

Table 1. Physicochemical properties of compound-I, II, III

Sl. No	Parameter	Compound-I	Compound-II	Compound-III
1	Molecular formula	$C_3H_3NO_2S$	$C_9H_6N_2O_2S$	$C_{11}H_8N_2O_3S$
2	Molecular weight	117.22	206.21	248.25
3	Theoretical yield	8.65gm	8.32gm	8.14gm
4	Practical yield	8.32gm	8.14gm	7.88gm
5	% yield	96.18%	97.83%	96.80%
6	Melting point	120-122^0 C	126-128^0 C	130-132^0 C
7	Recrystallization solvent	Ethanol	Methanol	Methanol
8	Solvent for TLC	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)
9	R_f value	0.90	0.84	0.96

Table 2. Physicochemical properties of derivatives of compound-IV

Sl. No	Parameter	SMI-IV-2	SMI-IV-4	SMI-IV-7
1	Molecular formula	$C_{18}H_{12}N_2O_4S$	$C_{18}H_{13}N_3O_4S$	$C_{18}H_{11}FN_2O_4S$
2	Molecular weight	352.36	367.39	370.35
3	Theoretical yield	1.10gm	1.10gm	1.10gm
4	Practical yield	0.98gm	0.87gm	0.91gm
5	% yield	89.09%	79.09%	82.72%
6	Melting point	128-130^0 C	130-132^0 C	132-134^0 C
7	Recrystallization solvent	Ethanol	Ethanol	Ethanol
8	Solvent for TLC	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)
9	R_f Value	0.85	0.79	0.87

Table 3. Physicochemical properties of derivatives of compound-IV

Sl. No	Parameter	SMI-IV-9	SMI-IV-11	SMI-IV-15
1	Molecular formula	$C_{18}H_{12}N_2O_7S_2$	$C_{19}H_{14}N_2O_3S$	$C_{19}H_{13}N_3O_5S$
2	Molecular weight	432.42	350.39	395.39
3	Theoretical yield	1.10gm	1.10gm	1.10gm
4	Practical yield	1.02gm	0.97gm	1.01gm
5	% yield	92.72%	88.18%	91.81%
6	Melting point	124-126^{0}C	130-132^{0}C	136-138^{0}C
7	Recrystallization solvent	Ethanol	Ethanol	Ethanol
8	Solvent for TLC	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)
9	R_f Value	0.82	0.90	0.88

Table 4. Physicochemical properties of derivatives of compound-IV

Sl. No	Parameter	SMI-IV-19	SMI-IV-22	SMI-IV-23
1	Molecular formula	$C_{20}H_{16}N_2O_3S$	$C_{19}H_{15}N_3O_3S$	$C_{18}H_{13}N_3O_4S$
2	Molecular weight	364.41	365.40	367.37
3	Theoretical yield	1.10gm	1.10gm	1.10gm
4	Practical yield	0.98gm	1.04gm	1.00gm
5	% yield	89.09%	94.54%	90.90%
6	Melting point	120-122^{0}C	122-124^{0}C	130-132^{0}C
7	Recrystallization solvent	Ethanol	Ethanol	Ethanol
8	Solvent for TLC	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)
9	R_f Value	0.88	0.94	0.86

Table 5. Physicochemical properties of derivatives of compound-IV

Sl. No	Parameter	SMI-IV-28	SMI-IV-31	SMI-IV-32
1	Molecular formula	$C_{18}H_{12}N_4O_5S$	$C_{18}H_{11}ClN_2O_3S$	$C_{18}H_{11}ClN_2O_4S$
2	Molecular weight	396.37	370.80	386.80
3	Theoretical yield	1.10gm	1.10gm	1.10gm
4	Practical yield	0.99gm	1.02gm	0.99gm
5	% yield	90.00%	92.72%	90.00%
6	Melting point	118-120^{0}C	124-126^{0}C	122-124^{0}C
7	Recrystallization solvent	Ethanol	Ethanol	Ethanol
8	Solvent for TLC	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)
9	R_f Value	0.91	0.89	0.72

Table 6. Physicochemical properties of derivatives of compound-IV

Sl. No	Parameter	SMI-IV-34	SMI-IV-41	SMI-IV-47
1	Molecular formula	$C_{18}H_{10}Cl_2N_2O_3S$	$C_{18}H_{10}F_2N_2O_3S$	$C_{18}H_{11}BrN_4O_5S$
2	Molecular weight	405.24	372.34	475.27
3	Theoretical yield	1.10gm	1.10gm	1.10gm
4	Practical yield	0.98gm	0.86gm	0.90gm
5	% yield	89.09%	78.18%	81.81%
6	Melting point	131-133^{0}C	125-127^{0}C	129-131^{0}C
7	Recrystallization solvent	Ethanol	Ethanol	Ethanol
8	Solvent for TLC	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)	Chloroform: Methanol (9:1)
9	R_f Value	0.80	0.90	0.78

Results and Discussion

All the derivatives were experimented on potential antidiabetic activity and among them 5 derivatives were selected based on the result. Spectral Characterization of 5 selected derivatives of SMI-IV-4, 11, 22, 31 and 41 were characterized using spectroscopic techniques such as IR, ^{1}HNMR, ^{13}CNMR and mass spectral studies (data shown in supplementary file). All the data shown in Tables 7 to 10.

Table 7. FT-IR spectrum data of derivativesSMI-IV-4, 11, 22, 31, and 41

SMI-IV-4		SMI-IV-11		SMI-IV-22		SMI-IV-31		SMI-IV-41	
Wave number (cm^{-1})	**Functional Group Assigned**	**Wave number (cm^{-1})**	**Functional Group Assigned**	**Wave number (cm^{-1})**	**Functional Group Assigned**	**Wave number (cm^{-1})**	**Functional Group Assigned**	**Wave number (cm^{-1})**	**Functional Group Assigned**
3060-3150	-OH stretching	3000-3050	-N-H stretching	3000-3060	-N-H stretching	3000-3090	-N-H stretching	3040-3000	-N-H stretching
3010-3050	N-H stretching of 1^{0}amine	2850-2990	Aromatic C-H stretching	2850-2995	Aromatic C-H stretching	2900-2800	Aromatic - C-H stretching	2970-2850	Aromatic C-H stretching
3000-2950	N-H stretch 2° or 3° amine	2300-2410	Aliphatic C-H stretching	2300-2450	Aliphatic C-H stretching	2350-2500	Aliphatic C-H stretching	2390-2510	Aliphatic C-H stretching
2900-2850	Aromatic C-H stretching	1430 1450 1490	C = O stretching	1410 1420 1430	C = O stretching	1435 1440 1450	C = O stretching	1450 1460 1470	C = O stretching
2380	Aliphatic C-H stretching	700	CH_3 stretching	820	CH_3 stretching	690	-CH stretching	705	-CH stretching
1490	C = O stretching	1110	C-S stretching	1105	C-S stretching	1100	C-S stretching	1110	C-S stretching
800	-CH stretching					845	-C-Cl stretching	790	-C-F stretching
1090	C-S stretching								

Table 8. H^1 NMR spectrum data of derivativesSMI-IV-4, 11, 22, 31, and 41

SMI-IV-4		SMI-IV-11		SMI-IV-22		SMI-IV-31		SMI-IV-41	
δ Value in ppm	**Peak Assigned (Multiplicity)**	**δ Value in ppm**	**Peak Assigned (Multiplicity)**	**δ Value in ppm**	**Peak Assigned (Multiplicity)**	**δ Value in ppm**	**Peak Assigned (Multiplicity)**	**δ Value in ppm**	**Peak Assigned (Multiplicity)**
4.315	$-NH_2$ (2H)	4.0-4.2	$-NH_2$(2H)	3.8-4.5	$-NH_2$(2H)	2.0-2.3	-CH(3H)	2.4-3.2	-CH(3H)
2.60-3.35	-CH(3H)	1.95-3.7	$-CH_3$, 3CH (6H)	2.0-3.4	$-CH_3$, 3CH (6H)	6.6-8.0	Ar-H (multiplate)	6.7-8.9	Ar-H (multiplate)
6.5-9.5	Ar-H (multiplate) (7H)	6.6-8.3	Ar-H (multiplate)	6.6-8.8	Ar-H (multiplate)				
10.96	-OH (1H)								

Table 9. ^{13}CNMR spectrum data of derivativesSMI-IV-4, 11, 22, 31, and 41

SMI-IV-4		SMI-IV-11		SMI-IV-22		SMI-IV-31		SMI-IV-41	
Carbon's	**δ Value in ppm**	**Carbon's**	**δ Value in ppm**	**Carbon's**	**δ Value in ppm**	**Carbon's**	**δ Value in ppm**	**Carbon's**	**δ Value in ppm**
3C CH	32.35 40.10 74.25	1C $-CH_3$	33.45	1C $-CH_3$	33.50	3C CH	59.80 66.78 80.45	3C –CH	59.80 66.78 80.45
12C from Aromatic ring	110-150	3C CH	40.09 74.70 79.45	3C CH	40.09 74.70 79.75	12C from Aromatic ring	110-160	12C from Aromatic ring	115-145
3C from C=O	147.60 154.10 167.05	12C from Aromatic ring	110-150	12C from Aromatic ring	110-150	3C from C=O	166.96 177.40 179.45	3C from -C=O	148.25 162.33 167.05
		3C from C=O	153.10 167.05 168.02	3C from C=O	153.10 167.05 168.02				

Table 10. Mass spectrum of synthesized compounds

Parameters	SMI-IV-4	SMI-IV-11	SMI-IV-22	SMI-IV-31	SMI-IV-41
M^+ Peaks (m/z)	367.2	365.3	365.3	369.2	372.2
Base Peak	267.2	337.1	305.2	287.1	289.2
Molecular weight	367.2	365.3	365.3	369.2	372.2

***In vitro* alpha-amylase inhibition activity**

Alpha-amylase inhibition activity was carried out for all the derivatives of compound-IV but the selected 5 derivatives showed significant results when compared to standard acarbose. The IC_{50} value was less in standard (24.36) with greater inhibition of enzyme. The same trend followed with the derivatives. SMI-IV-4 showed less IC_{50} value among the derivatives and showed higher inhibition in 100 µg/mL than other derivatives. Table 11 showed the tabulated result where per cent inhibition was recorded in different concentrations.

Table 11. Alpha-amylase inhibitory effects of selected derivatives of compound-IV

Compounds	% of Inhibition at Different Concentration (µg/mL) of Various Derivatives						IC_{50} value (µg/mL)
	10	20	40	60	80	100	
SMI-IV-4	2.41	5.10	11.85	19.46	25.34	32.46	39.56±1.85
SMI-IV-11	2.98	6.18	14.10	20.97	24.75	29.34	66.94±1.94
SMI-IV-22	3.97	7.86	15.76	21.78	25.13	30.94	62.42±1.59
SMI-IV-31	3.64	7.10	15.40	22.55	26.71	31.70	55.40±1.65
SMI-IV-41	3.20	6.51	13.89	20.37	24.74	31.48	57.46±1.64
Standard Acarbose	4.58	9.12	20.34	29.74	36.74	42.58	24.36±1.52

Data replicated three times (n=3)

***In-vivo* anti-diabetic activity: Streptozotocin (STZ)-induced diabetes**

Male albino Wistar rats were used for the antidiabetic activity experimentation in STZ induced animals. In STZ-induced diabetic rats, a loss of β-cells leads to a significant shortage of insulin and, later, a fasting blood glucose level that is higher than usual. A thiazolidinedione derivative (SMI-IV) was administered to each animal. In case of body weight of the animals after 24 hours, control animals did not show much variation than initial (160.52g) but STZ induced animals showed elevated body weight (168.21 g) whereas STZ induced standard showed decreased in weight (154.10g) and the derivative SMI-IV-4 significant decreased in body weight (156.01g) (Table 12). Similar way, various other biochemical parameters viz. total cholesterol, triglycerides, LDL and HDL contents were estimated where similar results were revealed as earlier. Total cholesterol content was significantly lower by the SMI-IV-4, 101.12 mg/dL than STZ induced animals (203.44 mg/dL) which was near value of STZ induced standard drug (90.32 mg/dL). Similarly, the results were the same as triglycerides, LDL and HDL content (Table 13). Except for a notable decrease with thiazolidinedione derivatives (SMI-IV), results demonstrate moderate to low antihyperglycemic efficacy in blood glucose AUC in single dose animal trials. The thiazolidinedione derivative SMI-IV-4 significantly lowered the blood glucose level at 24 hours to 166 mg/dL when compared to the standard metformin (Table 14, 15).

Table 12. Determination of body weight of animals

	Initial (g)	After 24 hours (g)
Control	160.45±1.43	160.52± 1.23
STZ	160.36± 2.21	168.21± 2.21
STZ+ SMI-IV-4	161.12±2.20	156.01±1.04$^{\#*}$
STZ+ SMI-IV-11	160.38±1.36	158.22± 2.21$^{\#}$
STZ+ SMI-IV-22	159.30±1.41	157.67 ± 0.45$^{\#}$
STZ+ SMI-IV-31	161.04±0.05	159.66 ± 0.11
STZ+ SMI-IV-41	160.21±2.05	159.32 ± 1.03
STZ+ Metformin	159.36 ±1.28	154.10 ± 0.37$^{\#*}$

Values, mean ± SE; (n=8); *$p \leq 0.05$, **$p \leq 0.01$, compared with corresponding control values; $^{\#}$ when compared with standard, $^{\#\#}$when compared with STZ

Table 13. Determination of biochemical parameters after 24 hours

Parameters	Concentration (mg/dL)			
	Total Cholesterol	**Triglycerides**	**LDL**	**HDL**
Control	87.41± 0.43	76.22± 1.01	37.32± 2.41	33.41± 1.11
STZ	203.44±0.11**	168.34± 0.41**	148.32±0.32**	21.26± 0.21**
STZ+ SMI-IV-4	101.12± 0.20$^{\#\#}$	92.07± 1.06	56.82±1.46$^{\#\#}$	31.30± 1.03$^{\#\#}$
STZ+ SMI-IV-11	129.10± 1.36*	102.22± 0.22*	62.43± 2.56$^{\#\#}$	30.18± 2.01
STZ+ SMI-IV-22	120.39± 1.41*	111.01 ± 0.75*	74.40±2.41$^{*\#\#}$	29.12± 1.42
STZ+ SMI-IV-31	113.10± 1.57$^{*\#\#}$	99.06 ± 0.17$^{*\#\#}$	76.30±1.07$^{*\#\#}$	27.40± 0.48
STZ+ SMI-IV-41	116.03± 0.42$^{*\#\#}$	109.31 ± 0.36*	78.21± 0.65$^{\#\#}$	25.36± 0.71
STZ+ Metformin	90.32 ± 1.03$^{*\#\#}$	85.12 ± 1.33$^{*\#\#}$	48.20±0.11$^{*\#\#}$	35.61± 0.10$^{\#\#}$

Values, mean ± SE; (n=8); $*p \leq 0.05$, $*p \leq 0.01$; compared with corresponding control values, $^{\#}$ when compared with standard, $^{\#\#}$when compared with STZ.

In the present study, many derivatives were synthesized from thiazolidinedione compound-IV and evaluated for antidiabetic activity. There are many derivatives synthesized in which the best 15 derivatives were selected based on the properties and activity. *In vitro* alpha amylase enzyme inhibition activity was carried out. For glucose-stimulated insulin release in pancreatic beta cells, glucose metabolism is critical. Additionally, it has been suggested that stimulating enhanced glucose metabolism could enhance ATP synthesis in pancreatic β cells, which in turn could contribute to an increase in insulin release [16]. Many literatures were revealed earlier about *in vitro* alpha amylase inhibition activity as one of the mechanisms in controlling diabetics [17, 18]. The same method was followed in this investigation and revealed significant inhibition of alpha-amylase by the derivatives of thiazolidinedione. STZ was used as diabetic inducer in Wistar animals. One of the animal models of diabetes mellitus is diabetic rats generated by streptozotocin [19]. It has been widely utilized to cause diabetes in experimental rat models and is well known for its specific cytotoxicity to pancreatic islet beta cells. In STZ-induced diabetes, Metformin is frequently used as a standard antidiabetic medication to assess the effectiveness of various hypoglycemic medications [20]. In STZ-induced diabetic rats, a loss of β-cells leads to a significant shortage of insulin and, later, a fasting blood glucose level that is higher than usual. A thiazolidinedione derivative (SMI-IV) was administered to each animal. Except for a notable decrease with thiazolidinedione derivatives (SMI-IV), results demonstrate moderate to low antihyperglycemic efficacy in blood glucose AUC in single dose animal trials.

Table 14. Anti hyperglycemic activity of 5 best derivatives on STZ-induced diabetic rats at the dose of 250 mg/kg)

Compounds	Glucose Value in mg/dL					
	0 min	5 hours	10 hours	15 hours	20 hours	24 hours
Control	435±2.0	422±1.5	415±1.0	410±0.6	410±0.5	408±0.5
STZ	435.43± 1.2	442.22 ±0.32	457.09± 1.80	476.54 ±0.47	489.23 ±1.33	523.11± 0.51
STZ+ SMI-IV-4	433.2±1.2	390±1.0	345±1.2	290.32 ±0.8$^{\#\#,**}$	212±1.0$^{**\#\#}$	166.21±0.5$^{\#\#**\#}$
STZ+ SMI-IV-11	435.01±1.5	395±1.2	324±1.3	276±1.2	234±1.0$^{**\#}$	200±1.0$^{*\#}$
STZ+ SMI-IV-22	435.03±1.3	375±1.2	330±1.2	268±1.5	236±1.2$^{**\#}$	190±1.3$^{*\#}$
STZ+ SMI-IV-31	435±1.5	385±1.3	320±1.2	274±1.0	234±1.0$^{**\#}$	170±1.0$^{**\#}$
STZ+ SMI-IV-41	435±1.0	380±1.5	310±1.0	260.3±1.2$^{\#**\#\#}$	223.2±1.0**	180±1.0$^{\#\#**\#}$
STZ+ Metformin	433±1.2	210.3±1.0$^{*\#\#}$	160.1±1.0$^{**\#\#}$	146.7±1.0$^{**\#\#}$	140.3±1.0$^{**\#\#}$	115.4±1.0$^{**\#\#}$

Values, mean ± SE; *$p \leq 0.05$, **$p \leq 0.01$; compared with corresponding control values; $^{\#}$when compared with standard, $^{\#\#}$when compared to standard.

Table 15. Area under the curve (AUC) values

Compounds	Blood Glucose AUC	
	0 min	5 hours
Control	1.5462	1.5123
SMI-IV-4	0.7854	0.6524
SMI-IV-11	0.8913	0.8457
SMI-IV-22	0.8861	0.8261
SMI-IV-31	0.8758	0.7942
SMI-IV-41	0.8729	0.8134
Metformin	0.7828	0.4562

Conclusion

The study revealed that the synthesized novel derivatives of thiazolidinedione (SMI-IV) exhibited significant lowering of blood sugar in STZ induced albino Wistar rats. 15 derivates were synthesized from the compound IV and based on the practical yield, 5 derivatives were selected viz. SMI-IV-4, SMI-IV-11, SMI-IV-22, SMI-IV-31, and SMI-IV-41. All derivatives showed potent antidiabetic activity but among them, derivative SMI-IV-4 showed significant antidiabetic activity.

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CATALYTIC REACTION STUDY OF LEVULINIC ACID ESTERIFICATION TO ETHYL LEVULINATE USING MODIFIED CARBON CATALYST AND ACIDIC DES

(Kajian Tindak Balas Mangkinan Pengesteran Asid Levulinik kepada Etil Levulinat Menggunakan Mangkin Karbon Terubah Suai dan DES Berasid)

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Abstract

Ethyl levulinate can be synthesized by the esterification reaction of levulinic acid with ethanol in the presence of a solid acid catalyst. In this research, the catalyst was prepared from lignin as a carbon precursor at 400 °C for 2 h and modified via sulfonation in a hydrothermal reactor to produce a modified lignin-based carbon catalyst. Acidic DES, which acted as a co-catalyst, was prepared from a choline chloride-sulfanilic acid mixture. The catalyst and acidic DES were characterized to study their chemical and physical characteristics. The catalytic activity was evaluated in the levulinic acid esterification by the effect of parameters such as ethanol to levulinic acid molar ratio (3–15), catalyst loading (5–25 wt%), and reaction time (1–5 h). The reaction performance was further investigated over the catalyst and acidic DES at optimum reaction conditions. The results demonstrated that the catalyst has good thermal stability up to 400 °C with a large surface area of 368.92 m^2/g to facilitate the reaction. The optimum conditions of levulinic acid esterification obtained over a modified lignin-based carbon catalyst were a 6 molar ratio of ethanol to levulinic acid, 10 wt% catalyst loading, and 3 h at 80 °C, to give 62.51 mol% of ethyl levulinate yield. The reaction performance was further improved when acidic DES was introduced with 81.65 mol% of ethyl levulinate yield. The catalyst and acidic DES have shown their potential for levulinic acid esterification and further catalyst modifications must consider obtaining better product yield.

Keywords: carbon catalyst, acidic DES, ethyl levulinate, levulinic acid, lignin

Abstrak

Etil levulinat boleh dihasilkan melalui tindak balas pengesteran asid levulinik dengan etanol dengan kehadiran pemangkin pepejal asid. Dalam penyelidikan ini, pemangkin disediakan daripada lignin sebagai pelopor karbon pada 400 °C selama 2 jam dan diubah suai melalui proses sulfonasi dalam reaktor hidroterma untuk menghasilkan mangkin karbon berasaskan lignin terubah suai. DES

berasid yang bertindak sebagai pemangkin bersama disediakan daripada campuran asid kolina klorida-sulfanilik. Pemangkin dan DES berasid dianalisa untuk mengkaji ciri kimia dan fizikal. Aktiviti mangkinan oleh pemangkin dinilai dalam tindak balas pengesteran asid levulinic melalui kesan parameter seperti nisbah molar etanol kepada asid levulinik (3–5), pemuatan mangkin (5–25 wt%), dan masa tindak balas (1–5 jam). Prestasi tindak balas telah disiasat lebih lanjut terhadap pemangkin dan DES berasid pada keadaan tindak balas optimum. Dapatan kajian menunjukkan bahawa pemangkin mempunyai kestabilan terma yang baik sehingga 400 °C dengan luas permukaan 368.92 m^2/g bagi memangkin tindak balas. Kondisi optimum pengesteran asid levulinik yang diperolehi daripada mangkin karbon berasaskan lignin terubah suai adalah 6 nisbah molar etanol kepada asid levulinik, 10 wt% pemuatan mangkin, dan 3 jam masa tindak balas pada 80 °C, untuk menghasilkan 62.51 mol% hasil etil levulinat. Tindak balas mangkinan telah ditambah baik menggunakan DES berasid yang bertindak sebagai pemangkin bersama dengan 81.65 mol% hasil etil levulinat. Pemangkin dan DES berasid telah terbukti berkesan dan berpotensi untuk pengesteran asid levulinik dan pengubahsuaian mangkin selanjutnya harus diambil kira untuk mendapatkan hasil produk yang baik.

Kata kunci: mangkin karbon, DES berasid, etil levulinate, asid levulinik, lignin

Introduction

The overabundance of lignocellulosic biomass (LB) generated from agriculture waste and forest scrap daily has urged researchers to convert them into valuable biochemical and biofuels. Levulinic acid (LA) is one of the most prominent LB-derived products which comprises two major functional groups (e.g., carboxylic acid and ketone), making it a top-notch building block precursor to synthesize various biochemical, including ethyl levulinate [1]. The catalytic esterification of LA with ethanol to produce ethyl levulinate (EL) has been an attractive approach. The short-chain fatty ester of EL is considered a potential biochemical to be utilized in fast-growing industries, such as pharmaceutical, food additive, agriculture, biofuel, and fuel additive [2]. Focusing on its implementation for liquid transportation fuel additive, EL possesses unique characteristics of high lubricity, stable flash point, and low toxicity [3]. Owing to high miscibility with conventional fuels, EL can be easily blended with gasoline, diesel, or biodiesel-diesel blend. The presence of EL in these energy-rich fuels can alter major fuel properties, such as flash point, viscosity, and lubricity [4]. Moreover, the addition of EL specifically into diesel can produce better and cleaner combustion in the engine cylinder [5]. EL is also known as an oxygenated fuel additive due to its high oxygen content, which can minimize soot formation and unburned hydrocarbon by blending 10% of EL into the fuel [6].

Traditionally, the esterification of LA employed inorganic acid (i.e., H_2SO_4 and HCl) to catalyze the reaction. However, using these acids to catalyze esterification reactions could harm the environment due to waste disposal as well as a higher tendency for equipment corrosion [7]. Therefore, the development of a solid acid catalyst for LA esterification is among the key engineers to initiate an environmentally sustainable approach for the reaction. There are many types of solid acid catalysts available to facilitate LA esterification, such as acidic resins, acidic zeolites, metal oxides, acidulated metal oxides, functionalized silica, functionalized clays, heteropolyacid salts, metal phosphates, and carbonaceous acid [2]. These catalysts can encourage a better catalytic system because they can be easily handled and have high recyclability to minimize catalyst disposal [8]. However, the performance of various heterogeneous catalysts has become an issue in providing high product yield. Subsequently, exploration for the development of new or modified solid acid catalysts continues to improve the catalytic reaction of LA esterification.

Currently, carbon-based catalysts are continuously highlighted as solid acid catalysts for LA

esterification. The catalysts can be produced from different types of carbon precursors such as carbon nanotubes [9], lignin-furfural carbon cryogel [10], ionic liquid-furfural based carbon [11], functional carbon material [12], and carbon derived from pine needle [13], corn stalk [14], and sugarcane bagasse [15]. Other than these materials, lignin, a rich carbon aromatic material that can be derived from LB, shows its potential as a carbon precursor for catalyst preparation. Lignin is the second abundance of natural organic biopolymer; yet, it remains underutilized. It is classified as a low-value material or waste from paper and pulp industries [16]. Lignin is considered a good candidate to be used as a carbon precursor for catalyst production, along with giving added value to it. Carbon-based catalysts offer the advantages of inexpensive and sustainable raw material plus versatile surface modification to improvise the catalyst's active sites [17]. The modification of carbon material via sulfonation of concentrated H_2SO_4 is usually selected to provide sulfonate functional group (SO_3H) on the carbon structure. According to Zainol et al. (2019) [10], SO_3H can possess Brønsted acidic sites, which are highly important factors for the mechanism of LA esterification to take place and enhance the EL yield. The SO_3H placement on the catalyst surface has demonstrates the critical role in catalyzing the reaction.

Another strategy to further promote catalytic activity is the application of deep eutectic solvents (DES). Such method has gained attention to facilitate many biomass conversion processes (e.g., pretreatment of biomass, cellulose dissolution, and catalysis) [18]. DES is a eutectic mixture, a combination of hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD), characterized as low volatility, low toxicity, and biodegradability [19]. A number of DESs are currently being investigated for LA esterification to EL where choline chloride performed as HBA and mixed with different types of HBD, such as acetic acid, malonic acid, oxalic acid, citric acid, formic acid, and p-toluene sulfonic acid [4]. Among available HBD, the use of sulfanilic acid in DES production is still limited, especially to catalyze this particular reaction. Sulfanilic acid has two specific active sites (NH_2 and SO_3H) in one molecule [20]. The production of DES from choline chloride and sulfanilic acid could provide more availability of active sites for LA esterification reaction. Moreover, less finding is known about the impact of the synergy between carbon catalyst and DES on the desired yield, which offers a fresh perspective on the potential of DES in the EL synthesis.

Hence, this work employed lignin as a carbon precursor to produce a modified lignin-based carbon catalyst (MCC) for LA esterification. The carbon was later modified with concentrated H_2SO_4 via hydrothermal sulfonation in a Teflon autoclave reactor, which is a self-generated pressure vessel that can assist in the strong attachment of SO_3H onto the catalyst surface. The DES was prepared using a choline chloride-sulfanilic acid mixture and employed as a co-catalyst. Both MCC and DES were characterized to study their physiochemical properties. In the LA esterification to EL, the effects of reaction parameters over MCC were evaluated such as the molar ratio of ethanol to LA, catalyst loading, and reaction time. Additionally, the catalytic reaction with DES in optimized reaction conditions was tested to determine the improvement of EL yield.

Materials and Methods

Materials

Levulinic acid (98%), $C_5H_8O_5$ was purchased from Merck KGaA. Ethanol (95%), C_2H_6O was obtained from Chemiz. Lignin and sulfanilic acid (99%), $C_6H_7NO_3S$ were bought from Sigma-Aldrich Company. Sulfuric acid (97%), H_2SO_4 was

supplied by Supelco. Finally, choline chloride $[(CH_3)_3NCH_2CH_2OH]Cl$ (99%) was obtained from Acros Organics. All chemicals were used immediately as received without any further purification.

Preparation of modified lignin-based carbon catalyst and DES

Lignin was calcined to lignin-based carbon (LC) in the furnace box at 400 °C for 2 h with the ramped temperature of 10 °C/min. The LC was washed with distilled water and dried in the oven at 90 °C overnight. The sulfonation process was conducted in the hydrothermal autoclave reactor at 180 °C for 6 h by mixing the LC with concentrated H_2SO_4 at a 1:10 carbon (g) to acid (mL) ratio. After sulfonation was completed, the sample mixture was diluted with distilled water before being continuously washed and filtered until the pH of the filtrate was around 6.5–7. The sample was dried overnight in the oven at 80 °C before being utilized for the reaction. Meanwhile, DES was prepared from choline chloride to sulfanilic acid with a molar ratio of 2. The choline chloride and sulfanilic acid mixture was continuously stirred at 140 °C for 8 h or until a clear homogeneous liquid was obtained.

Catalyst and DES characterization

The existence of related functional groups and thermal stability of LC, MCC, and DES were evaluated by Fourier transform infrared (FTIR) and thermogravimetric analysis (TGA). An FTIR spectroscopy from PerkinElmer One Spectrum was used at the infrared (IR) range of 400–4,000 cm^{-1}. TGA was conducted using the TGA/DTC 1 STAR System (MATTLER TOLEDO) through heating from 30–850 °C under nitrogen N_2 ambient at the heating rate of 10 °C/min. The physical morphology and surface area of the LC and MCC were determined using scanning electron microscopy images (SEM) and N_2 sorption analysis. The SEM images were captured at magnification of 5000X and 1000X in sizing of 10 μm and 50 μm. The surface area analysis started with degassed at 200 °C for 6 h in a vacuum, followed by N_2 sorption at -196 °C by Micrometritics 3-Flex equipment. Then, the Brunauer-Emmett-Teller (BET) and t-plot were used to calculate the surface area and pore structures.

The esterification reaction of LA and product analysis

The reaction catalyzed by MCC was done in a three-way round-bottom flask equipped with a reflux condenser at a constant temperature of 80 °C. The effect of reaction parameters was studied at the molar ratios of ethanol to LA (3–15), catalyst loading (5–25 wt%), and reaction time (1–5 h). The liquid product was collected and filtered to separate the catalyst from the desired product. After the optimum reaction was obtained, another consecutive reaction was conducted using DES as a co-catalyst. MCC with DES (10 wt.%) and DES with 40 and 60 wt.% (without the presence of MCC) were performed for the esterification reaction of LA to EL. All the liquid products were quantified by Gas chromatography-mass spectrometry (GC-MS) using an Agilent Technology model 7890 GC system. The GC-MS was equipped with an HP-5MS column of 60 m length, 0.25 mm diameter, and 0.25 μm film with the ramped temperature at 60 °C, which later increased to 180 °C (20 °C/min) until 280 °C (320 °C/min). The coolant was set at 50 °C.

Results and Discussion

Figure 1 shows the FTIR spectroscopy which demonstrates the changes in chemical bonding and functional groups of all the samples that were impacted by their preparations. The SO_3H was observed through the band spectra between 1,250–1,000 cm^{-1} for S–O and S=O. The S–O stretching

was observed at a wavenumber of 1,050 cm^{-1} and S=O stretching at 1,250–1,150 cm^{-1} [21]. The peak intensity of MCC was higher compared to LC which can be related to the improvement of SO_3H due to sulfonation. The results agreed with those reported by Li et al. [22]. The detection of SO_3H is crucial since this functional group mainly acts as an acid site on the catalyst surface. Besides, the detection showed that sulfonation was successfully conducted on the LC. The C=C and C=O functional groups were observed at wavenumber around 1,750–1,600 cm^{-1}. The peak intensity of MCC at 1,600 cm^{-1} was due to the stretching of the C=C group by the aromatic ring structure of carbon material [15]. The C=O of MCC can be observed at 1,700 cm^{-1}, which was related to the carboxylic acid group (COOH) resulting from the oxidation on the LC surface by strong acid [23]. A broad peak at wavenumber around 3,400–3,000 cm^{-1} reflects the O–H stretch of the samples [24]. Additionally, MCC showed a broader peak than LC due to the presence of SO_3H and absorbed moisture in the sample.

For DES, the S–O and S=O stretching correlated to SO_3H were also detected between 1,250–1,000 cm^{-1}. Similar to MCC, the detection of SO_3H is important since it was responsible for providing additional acid sites for the reaction to take place. The C–C peak of the aromatic ring can be seen around 1,500 cm^{-1} and C=C group at 1,600 cm^{-1} represents the aromaticity of the compound. The C=O peak stretching was undetected since COOH was absent in the DES compound. The observable S–O, S=O, C–C aromatic, and C=C spectra were mostly due to the use of sulfanilic acid as HBD in DES preparation. Moreover, the N-based component of DES, including N–H stretching vibration around 3,300–3,250 cm^{-1}, was identified [25]. The combination of choline chloride and sulfanilic acid resulted in the detection of a wide peak of the O–H stretch around 3,400–3,000 cm^{-1} for SO_3H and the hydroxyl groups of DES.

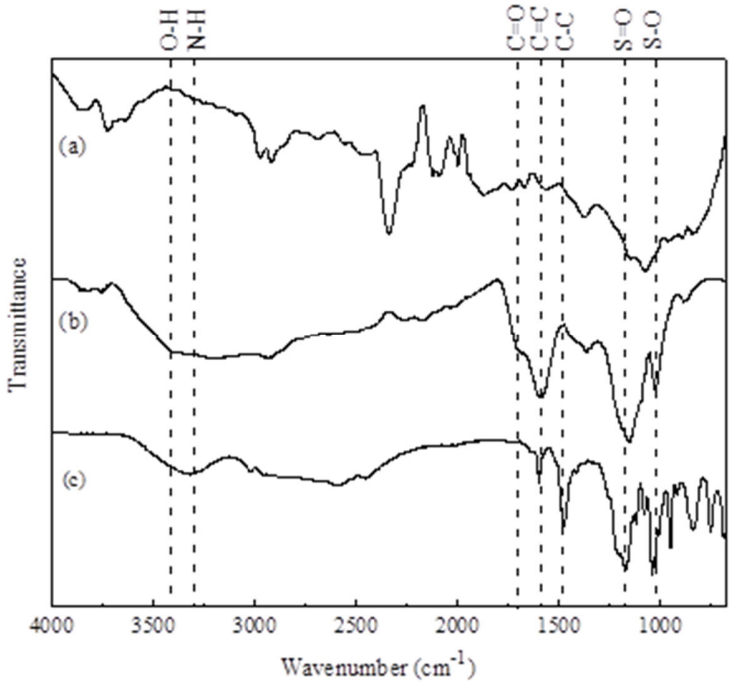


Figure 1. FTIR spectra for (a) LC, (b) MCC, and (c) DES

TGA was used to study thermal stability and the decomposition of the samples through the differential thermal gravimetric (TGA/DTG) curves. The results are presented in Figure 2. The appearance of DTG peaks below 200 °C for all the samples was caused by the existence of highly volatile (light organic-based) components and humidity in the samples. The degradation of these components caused weight loss of approximately 8–15 wt%, which is comparable to the findings reported by Hassan et al. [26]. The degradation of remaining light components in the LC structure occurred at heating temperatures of 200–500 °C. According to DTG, the low and broad peak of LC demonstrates that the sample was less affected by thermal degradation up to 500 °C, and there was approximately 88 wt% of residual weight. The application of thermal conditions (400 °C, 2 h) during LC preparation explains its good thermal stability. Above 500 °C, the linear decrease of the TGA curve at a slower rate corresponds to the continuous degradation of stable heavier organic components of LC.

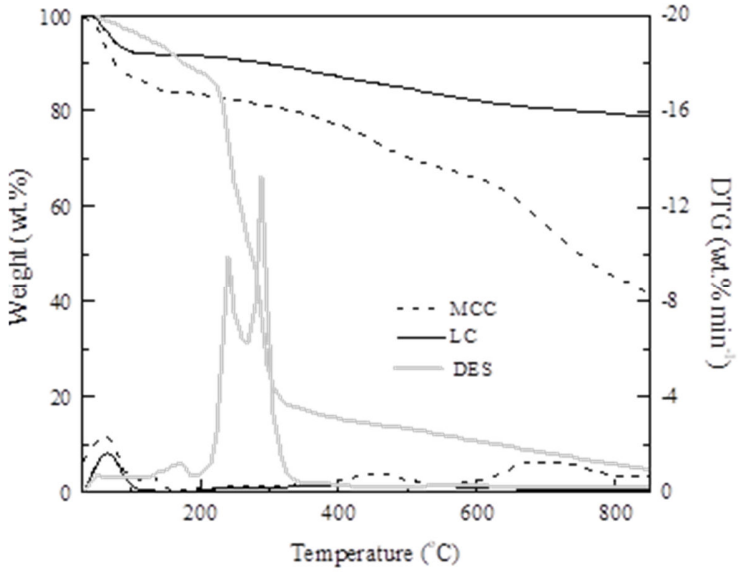


Figure 2. TGA-DTG curves for LC, MCC, and DES

A comparable DTG curve was observed for MCC at the heating temperatures of 200–400 °C. The weight loss of approximately 20 wt% was due to the decomposition of the weak acid site, such as the COOH group, in the MCC sample. As the heating temperature increased to 500 °C, the DTG peak appeared to represent the degradation of SO_3H strong acid site and 70 wt% residual weight was recorded. Thus, MCC can be suggested to have good thermal stability up to 400 °C to maintain its catalytic activity. A major decomposition of MCC was noticeable above 600 °C with the gradual decrease of the TGA curve. This pattern can be explained by the decomposition of the heavy components including the aromatic ring structure. Overall, the thermal stability of MCC was reduced compared to LC because of the acid modification and removal of ash during sulfonation.

The results of the thermal stability of DES were different with LC and MCC where a high DTG peak was observed from 200–400 °C. Here, a

significant weight loss major of about 80 wt% occurred due to the major degradation of the DES components including SO_3H and NH_2. The results imply that DES was thermally stable up to 200 °C. Above 400 °C, the TGA curve of DES showed low degradation rate, reflecting the continuous decomposition of the DES components. Based on the TGA of MCC and DES, a reaction below 200 °C was suggested for the reaction performance teting.

Figure 3 shows the SEM images of the morphological structure of lignin-based carbon and modified lignin-based carbon catalyst. The particles for both samples demonstrated irregular shapes and agglomerate particle structures. The SEM images of the catalyst showed a smooth and porous structure compared to lignin-based carbon. In the study by Sangsiri et al. (2022) [27], the SEM images of carbon catalyst after the sulfonation step with H_2SO_4 comprised of agglomerated particles. The surface was impacted by concentration acid treatment in a high-pressure condition, manifesting this observable surface morphology. The acid treatment has caused an unknown component present on the surface of the carbon structure to be removed or degraded during this process. These occurrences resulted in non-uniform porous including mesopores and micropores of the catalyst.

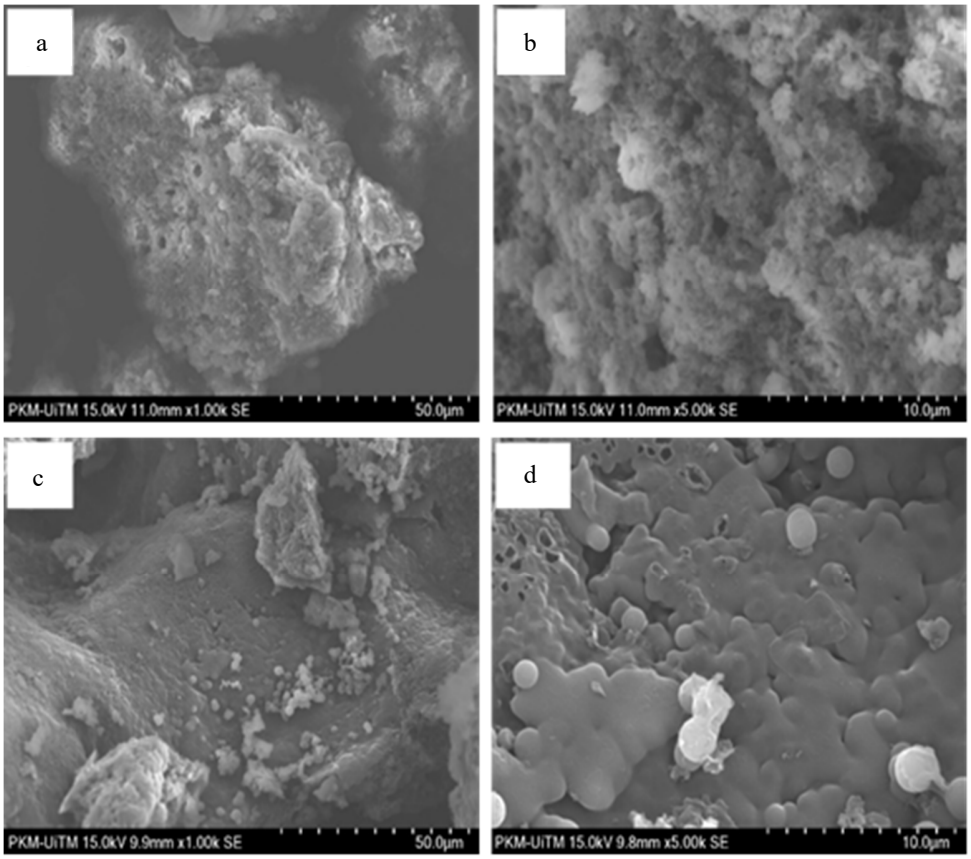


Figure 3. SEM images for (a, b) LC, and (c, d) MCC at 1000X and 5000X, respectively

The results of the N_2 sorption analysis of lignin-based carbon and modified lignin-based carbon catalyst are tabulated in Table 1. The mesopore and micropore areas for carbon material are 17.92 m^2/g and 0.05 m^2/g, respectively, making the sample's BET surface area of 17.98 m^2/g. After sulfonation, the catalyst's BET surface area was greatly improved to 368.92 m^2/g, with 126.7 m^2/g and 242.22 m^2/g of mesopore and micropore area, respectively. Sulfonation by using concentrated H_2SO_4 in a high-pressure condition significantly impacts the catalyst surface area. The degradation of organic components that exist on the carbon surface aided with high-pressure conditions during sulfonation leads to larger open pores. The high surface area is excellent for assisting the physical adsorption of LA on the surface of the catalyst.

Table 1. Surface area and pores distribution

Catalyst	S_{BET} (m^2/g)	S_{meso} (m^2/g)	S_{micro} (m^2/g)	V_{meso} (cm^3/g)	V_{micro} (cm^3/g)
Lignin-based carbon	17.98	17.93	0.05	0.092	0.001
Modified lignin-based carbon catalyst	368.92	126.70	242.22	0.119	0.100

The influence of reaction parameters such as the molar ratio of ethanol to LA, catalyst loading, and reaction time were also explored in this study. Figure 4(a) shows the effect of the molar ratio of ethanol to LA on the EL yield. The result demonstrated that the amount of LA and ethanol affected the yield of EL where the increment can be observed from 32.78 to 52.67 mol% as the molar ratio of ethanol to LA increased from 3 to 6. Then, the yield of EL was reduced to 16.94 mol% when the molar ratio of ethanol to LA was increased to 15. Further increasing of ethanol causes a reduction in the yield of EL due to a diluted mixture by high ethanol loading, reducing the tendency adsorption of LA on the catalyst acid sites. A similar trend was reported in previous work where high amount of ethanol diluted the LA concentration and reduced the reaction efficiency [28]. Hence, the molar ratio of ethanol to LA of 6 is the optimum condition for the reaction of LA to EL in the presence of a modified lignin-based carbon catalyst.

In Figure 4(b), the effect of MCC loading on the EL yield was observed from 5 to 25 wt%. The yield of EL showed an improvement from 51.46 to 54.73 mol% when the catalyst loading was increased from 5 to 10 wt%. A reduction of EL yield can be seen when the reaction was conducted with higher loading of MCC up to 25 wt%. This phenomenon is expected due to the high quantity of the catalyst concentration, which could also promote the high viscosity of the reaction mixture and limitations of mass transfer rate [29]. The increased viscosity up to a certain extent would affect the mass transport limitations and impede the catalytic activity [30]. Luan et al. (2018) [31] studied the catalyst loading ranging between 1 to 6 mol% and found that the catalyst loading of 4 mol% was sufficient enough to achieve the maximum conversion of LA. The EL yield does not amend as the amount of catalyst loading increases. Thus, for this work, the catalyst loading of 10 wt% was adequate to provide a reasonable EL yield.

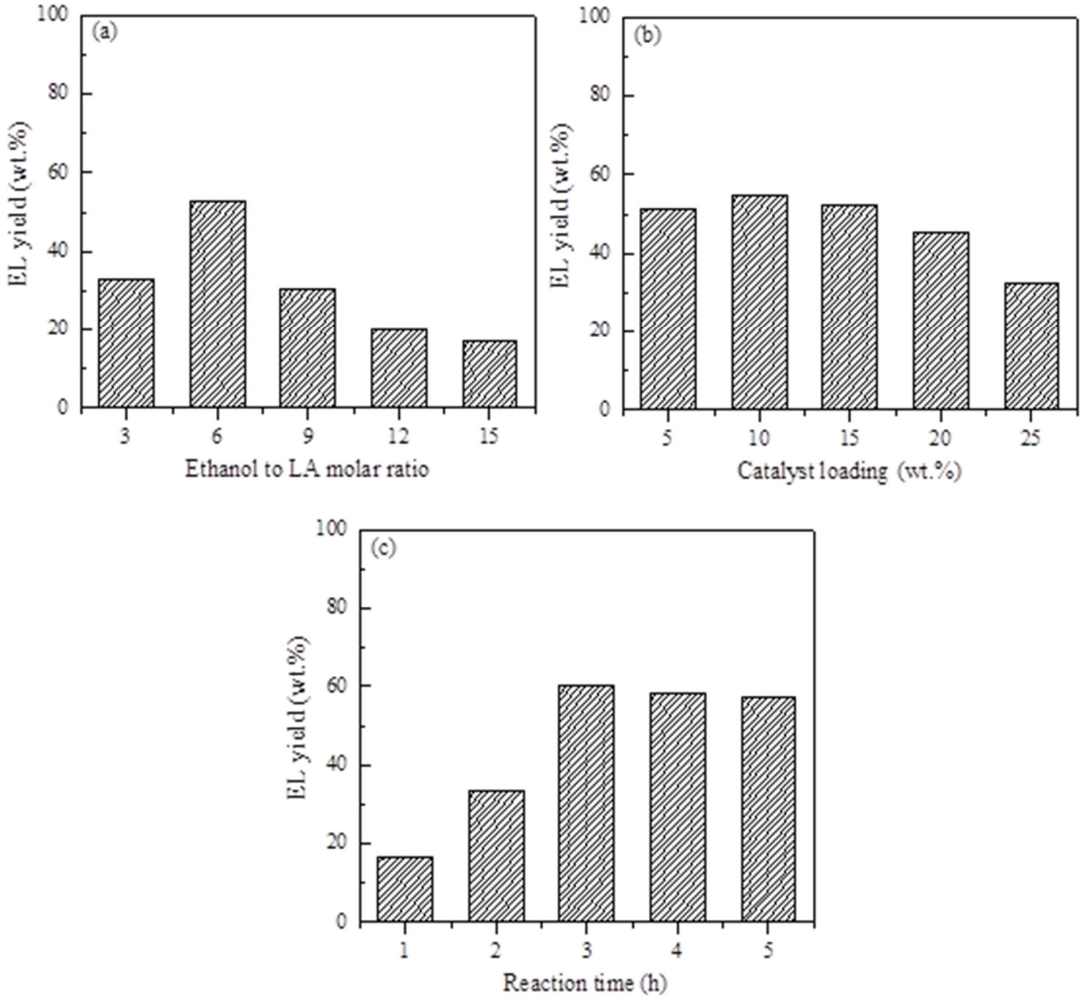


Figure 4. Parameter study of LA esterification (a) LA to ethanol molar ratio, (b) catalyst loading, (c) reaction time over MCC catalyst

The result of the reaction time for esterification of LA to EL is illustrated in Figure 4(c). The EL yield showed increment with the increasing reaction time up to the highest value of EL yield (62.51 mol%) at 3 h. As the reaction time was further increased to 5 h, the yield of EL displayed only small changes, indicating that the reaction had reached the equilibrium. Only a small reduction is observed in the EL yield at a prolonged reaction time and a comparable trend was obtained and reported in previous work [10]. This small reduction of yield is most probably due to the reversible reaction by hydrolysis after the reaction reaches equilibrium. The breaking of the ester bond into their respective functional groups may happen when the process of hydrolysis and reversible occur throughout the esterification reaction [32]. As for this work, the reaction parameter study provides the selected condition of a 6 molar ratio of ethanol to LA, 10 wt.% of catalyst, and 3 h of reaction time for LA esterification with ethanol in the presence of the MCC.

Figure 5 presents the reaction performances of LA esterification reactions to EL over various carbon catalyst and DES in the obtained optimum reaction conditions. The use of LC to catalyze the reaction acted as a benchmark, where the reaction provided 3.55 mol% EL yield. LC lacks of active sites with limited availability for reaction, which eventually leads to poor yield. As discussed, MCC provided 62.51 mol% EL yield and its performance demonstrated the function of sulfonation to provide acid sites as compared to LC. These findings have confirmed the importance of LC sulfonation to anchor the SO_3H on the catalyst surface and improve the catalytic activity of MCC. In the reaction catalyzed by MCC and 10 wt% DES, the EL yield was enhanced to 81.65 mol% compared to the use of MCC catalyst alone. The synergetic effects between MCC and DES provide more acid sites in the reaction mixture and eventually promote high EL yield. The increase of acid sites most likely induced more adsorption of reactants on acid sites before subsequent reaction mechanism steps to EL. The result of MCC with 10 wt% DES was notably comparable to DES only at 40 wt% and 60 wt% without the presence of MCC, where the EL yields recorded are 78.5 mol% and 80.1 mol%. The potential of the combination of 10 wt.% of MCC with lower DES loading (10 wt%) can be used for the reaction performance which has similar performance with 60 wt.% of DES used in the reaction. Thus, MCC has a major impact on the reaction and the combination with DES can further improve the reaction performance with high EL yield.

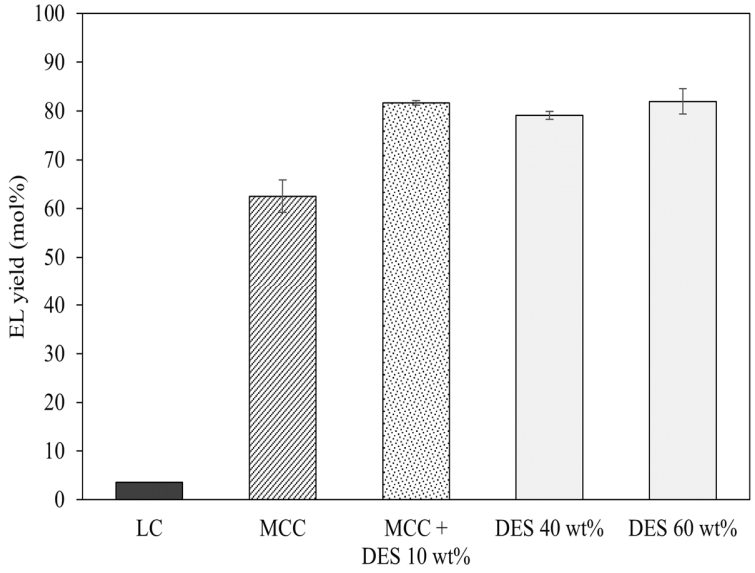


Figure 5. The performance of LA esterification to EL over LC, MCC, MCC with 10 wt% and 40 wt%, and 60 wt% of DES only at optimum reaction conditions

Conclusion

In this research, the esterification of LA with ethanol using MCC was conducted successfully. Sulfonation in high-pressure conditions greatly improves the surface area of the catalyst. Furthermore, the catalyst demonstrated good thermal stability of acid sites up to 400 °C with a large surface area. A reasonable EL yield of 62.51 wt.% at optimum conditions of 6 molar ratio of ethanol to LA, 10 wt% of catalyst loading, and 3 h

reaction time at 80 °C over MCC. Lignin has the potential to be used as carbon material for solid acid preparation, yet requires further improvement via any possible catalyst modification to enhance the reaction performance. The introduction of DES as a co-catalyst, which comprised the choline chloride-sulfanilic acid mixture, was proven effective in inducing a higher reaction performance of 81.65 mol% EL yield.

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DEVELOPMENT OF WATER-BASED DRILLING MUD BY USING LIGNIN FROM NATURAL RESOURCES AS THINNING AGENT

(Pembangunan Cecair Penggerudi Berasaskan Air Menggunakan Lignin Sebagai Agen Penipisan)

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Abstract

Drilling in a geological formation at high temperatures and pressures during offshore oil and gas exploration is often the cause of water-based mud damage. This study extracts lignin from coconut husk, oil palm fibre, and sawdust through the organosolv process. This lignin was a thinning agent in a water-based drilling mud formulation. The lignin was characterised by using Fourier Transform Infrared (FTIR) spectroscopy. The IR spectra of both lignin and lignosulfonic acid sodium salt showed bands at 3695.52 cm^{-1} and 2972.38 cm^{-1}, indicating hydroxyl groups in phenolic and aliphatic structures, the stretching of C-H bonds in aromatic methoxyl groups and methyl and methylene groups of side chains, respectively. When analysing mud properties, lignin's rheological and fluid loss properties were compared to lignosulfonic acid sodium salt, a commercial thinning agent. The dosage determination tests indicated that 0.6% was the optimum choice of lignin and lignosulfonic acid sodium salt. After thermal ageing, the rheological properties and fluid loss characteristics showed that lignin might be utilised as a thinning agent for drilling operations at temperatures as high as 90°C.

Keywords: lignin, lignosulfonic acid sodium salt, thinning agent, formic acid, organosolv process

Abstrak

Penggerudian dalam formasi geologi pada suhu dan tekanan tinggi semasa penerokaan minyak dan gas luar pesisir sering menjadi punca yang merosakkan cecair penggerudi berasaskan air. Dalam kajian ini, lignin daripada sabut kelapa, gentian kelapa sawit, dan habuk papan diekstrak melalui proses organosolv. Lignin ini digunakan sebagai agen penipisan dalam rumusan cecair penggerudian berasaskan air. Lignin dikenalpasti menggunakan spektroskopi inframerah transformasi Fourier (FTIR). Spektrum IR kedua-dua sampel lignin dan asid lignosulfonat menunjukkan jalur pada 3695.52 cm^{-1} dan 2972.38 cm^{-1}, menunjukkan kumpulan hidroksil dalam struktur fenolik dan alifatik, regangan ikatan C-H dalam kumpulan metoksil aromatik dan kumpulan metil dan metilena rantai sampingan, masing-masing. Apabila menganalisis sifat cecair penggerudi, sifat reologi lignin dan sifat kehilangan bendalir dibandingkan dengan asid lignosulfonat, yang merupakan agen penipisan komersial. Ujian penentuan dos menunjukkan bahawa 0.6% adalah pilihan optimum garam natrium lignin dan asid lignosulfonat. Selepas penuaan terma, sifat reologi dan ciri kehilangan bendalir menunjukkan bahawa lignin mungkin digunakan sebagai agen penipisan untuk operasi penggerudian pada suhu setinggi 90°C.

Kata kunci: lignin, asid lignosulfonat, agen penipisan, asid formic, proses organosolv

Introduction

Drilling fluid is an essential component of drilling operations, used to aid in creating a stable and gauged borehole to the desired depth with minimal damage to surrounding formations. Water-based drilling fluids (WBM) are becoming more popular with increasing environmental and economic concerns, particularly in high-
temperature, high-pressure (HTHP) environments. Dispersed WBM is one of the most widely used and cost-effective drilling fluids. However, drilling at high temperatures and pressures can cause filtration loss and damage the flow properties of drilling fluids.

Therefore, thinning agents are often added to drilling fluids to reduce viscosity and prevent gelation and thickening. High-viscosity fluids tend to increase filtration loss and can damage flow caused by low efficiency in flowing through rock formations in oil reservoirs. By reducing viscosity, thinning agents will help drill fluids flow more easily through these formations, thus reducing filtration loss and minimizing damage to the flow channels [1]. Drilling fluids used in HTHP environments tend to undergo gelation or thickening, which can further reduce flow and filtration loss. Thinning agents can prevent this gelation process by interacting with the fluid molecular structure, keeping it more fluidized. By maintaining the drilling fluids in these conditions, thinning agents can help to prevent the formation of gels and subsequent thickening of the drilling fluids, thus ensuring a smoother flow through the reservoir and reducing filtration loss [2]. Currently, most commercial thinning agents use chrome lignosulfonate, the most common thinner in water-based muds. Lignosulfonate is a natural polymer found in wood and plant materials. However, it was chemically modified to enhance its thinning properties. Adding chromium ions produces chrome lignosulfonate, which can be toxic to aquatic organisms and ecosystems if released into the environment [3].

Therefore, in this study, lignin from coconut husk (LCH), lignin oil palm biomass fibre (LOPBF) and lignin sawdust (LS) will be prepared as a thinning agent for water-based drilling muds using the organosolv method, with formic acid as the solvent for extraction. The materials used are non-toxic, abundant, and inexpensive renewable biomass with high lignocellulosic content. Lignin from coconut husk, oil palm biomass fibre and sawdust have the potential as a thinning agent for water-based drilling muds due to its high thermal stability and chemical resistance, as well as its ability to prevent damage to aquatic and terrestrial environments.

This study aims to develop lignin from coconut husk, oil palm biomass fibre and sawdust as a more environmentally friendly alternative to chrome lignosulfonate as a thinning agent for water-based drilling muds. The rheological properties of lignin were analyzed and characterized using FTIR.

Materials and Methods

Raw materials

This experiment's primary materials are sawdust, coconut husk and oil palm biomass fibers (OPBF). It is also known as renewable resources. Raw materials were collected from millworks and a local mill near University Malaysia Pahang Al-Sultan Abdullah and were received in ambient-dry conditions. The raw materials were inserted into the oven at 100°C for 24 hours to minimize the moisture content. Next, the raw materials were mechanically prepared using a blander and were sieved using 250-micrometre sieves. The organosolv medium uses formic acid as a solvent [4]. All chemical substances, such as formic acid, bentonite, and sodium carbonate, were purchased from Sigma-Aldrich (UK).

Organosolv process

The pulping process is the first step of the extraction process. At a solid to-solvent of 1:10, formic acid will be added to the raw materials in the flask and let to boil on a hot plate for 3 hours. 400ml of formic acid was added to the 40g raw material and was heated and stirred for 400 rpm at 105°C for 3 hours. After 3 hours, the flask and its contents can cool to room temperature. The content is then filtered through a Filter Press [4]. The

black liquid is obtained during the filtration procedure, and the remaining liquid may be classed as solution hemicellulose, and cellulose will not be utilized.

Evaporation process

The black liquor was evaporated using a Rotary Evaporator at 80°C and 90 rpm to separate the acid from the raw lignin-containing hemicellulose. The process continued until the black liquor was 60% dry [5]. 100 ml distilled water was then added to the balance of the liquor, and the raw lignin was manually stirred to rinse before going to the filtration process again.

Isolation of lignin

This step was used to isolate the lignin. The residue that got after the filtration was put in an oven at 100°C, atmospheric pressure, for 24 hours. The lignin was successfully obtained.

FTIR analysis

The infrared bands and absorbance were investigated using a Perkin Elmer 2000 FTIR spectrometer. Samples were scanned in the range of 4000 to 1000 cm^{-1} at a resolution of 4% using the KBr pellet technique [3].

Mud preparation

Five sets of lignin composition were used in mud formulation, as tabulated in Table 1. In addition to lignin, bentonite, sodium carbonate, and distilled water were incorporated in the mud and weighed precisely using a balancer. The mixing was done at 3000 rpm for 15 minutes.

Table 1. Composition of Mud

Materials	MUD 1	MUD 2	MUD 3	MUD 4	MUD 5
Lignin (g)	0	0.55	0.82	1.37	1.6
Lignin (w/w%)	0	0.2	0.3	0.5	0.6
Bentonite (g)	20	20	20	20	20
Sodium Carbonate (g)	1	1	1	1	1
Distilled Water (ml)	250	250	250	250	250

Mud property test

This experiment completed four main sets of tests: viscosity, fluid loss, density, and pH. Three equations will be used to compute apparent viscosity, μ_a (a), plastic viscosity, μ_p (p), and yield point, Γ_y (y) that is:

$$\mu_a = \theta 600/2 \quad (1)$$

$$\mu_p = \theta 600 - \theta 300 \quad (2)$$

$$\Gamma_y = 0.511(\theta 300 - \mu_p) \quad (3)$$

The filtrate loss (FL) volume is the amount of fluid that can pass through a porous filter medium after it has been exposed to a differential pressure for a set period. The mud's rheological features were investigated using a Fann model 286 viscometer, with mud viscosity measurements at two different rotation rates of 300 rpm (θ300) and 600 rpm (θ600). The viscometer's shear stress readings at 300 and 600 rpm are θ300 and θ600, respectively. Using a Fann model roller oven series 2500 and 500 mL Fann stainless steel ageing cell at 90°C for 24 hours, the ageing temperature (T) effects on water-based mud rheological and filtration loss qualities were studied [6]. The summary of the mud property test is tabulated in Table 2.

Table 2. Mud Property Test

Mud Property Test	Focus on Test	Machinery
Viscosity	1. Investigate the effects of various dosages of lignin in WBM at room temperature and under vigorous agitation (90°C).	Fann Model Viscometer
Fluid Loss	2. Mud doses of 0%, 0.2%, 0.3%, 0.5%, and 0.6% w/w were utilized.	Fann Model Filter Press
Density		Fann Model Mud Balance
pH		pH Meter

Results and Discussion

An FTIR analysis was performed to confirm the presence of lignin following the organosolv process. Figure 1 illustrates the spectra of LOPBF, LS, LCH, and LASS. In the infrared spectra of lignin OPBF, the alcohol is identified by a broad band with wavelengths ranging from 3695.53 cm^{-1} to 3663.42 cm^{-1}. Bands 2972 cm^{-1} and 2843.55 cm^{-1} reflect the stretching of C-H bonds in alkane groups. The stretching N=C=S was shown at bands 2077.72 cm^{-1}. The stretching of conjugated aldehyde yields a narrow broadband at 1691.72 cm^{-1}. Medium bands at 1512.51 cm^{-1} demonstrate the presence of nitro chemicals. The alcohol component is identified once again at 1345.74 cm^{-1} to 1331.20 cm^{-1}. The lignin OPBF structure grew robust, producing aromatic amines at 1268.33 cm^{-1} bands. C-H deformations create the band at 1164.06 cm^{-1} to C-O stretching. Finally, bands ranging from 1054.55 cm^{-1} to 1032.58 cm^{-1} show C-O bond stretching in the primary alcohol group.

A wide band at 3695.55 cm^{-1} to 3680.17 cm^{-1} in the LS spectrum may be attributed to the alcohol group. Alkane bands range from 2980.63 cm^{-1} to 2843.55 cm^{-1} at C-H stretching. The stretching of an isothiocyanate functional group results in a prominent band at 2077.84 cm^{-1}, indicating the strength of N=C=S stretching. A stretching O-H characterises the phenol functional group at bands at 1345.76 cm^{-1}. The medium peak indicates the C-O stretching vibration in the LS major alcohol group at 1054.67 cm^{-1}. The relative strength of absorption bands 1032.61 cm^{-1} is given to an out-of-plane S=O bond, which identifies as a sulfoxide functional group.

The alcohol group is represented by the broad, sharp bands in the LCH spectra ranging from 3695.47 cm^{-1} to 3662.92 cm^{-1}. The bands at 2972.39 cm^{-1} to 2843.57 cm^{-1} show an alkane functional group. A stretching C=C also happens at a wavelength of 1658.10 cm^{-1}. These peaks represent the alkene functional group of disubstituted (cis). N-O stretching occurs at 1535.79 cm^{-1} and O-H bending at 1391.87 cm^{-1}, indicative of the alcohol functional group. The structure of LCH at bands 1136.47 cm^{-1} displays a strong C-O stretching bond at the tertiary alcohol functional group. At band 1032.62 cm^{-1}, the structure exhibits C-O stretching and corresponds to an out-of-plane S=O functional group at band 1012.29 cm^{-1}.

The alcohol functional group is identified in the LASS infrared spectrum by a broad band with wavelengths ranging from 3695.52 cm^{-1} to 3680.218 cm^{-1}. Bands 2972.38 cm^{-1} and 2843.55 cm^{-1} demonstrate the stretching of C-H bonds in alkane groups. An influential band at 2077.84 cm^{-1} is due to the stretching of an isothiocyanate functional group, indicating the strength of N=C=S stretching. A stretching C=C distinguishes the conjugate of the alkane functional group at bands at 1641.08 cm^{-1}. LASS structure at bands 1345.91 cm^{-1}, revealing alcohol functional group with medium O-H stretching. The structure of LASS becomes strong between 1136.33 cm^{-1} and 1032.56 cm^{-1}, where the structure shows C-O stretching, indicating a tertiary alcohol functional group, and corresponds to an out-of-plane S=O stretching, indicating a sulfoxide functional group.

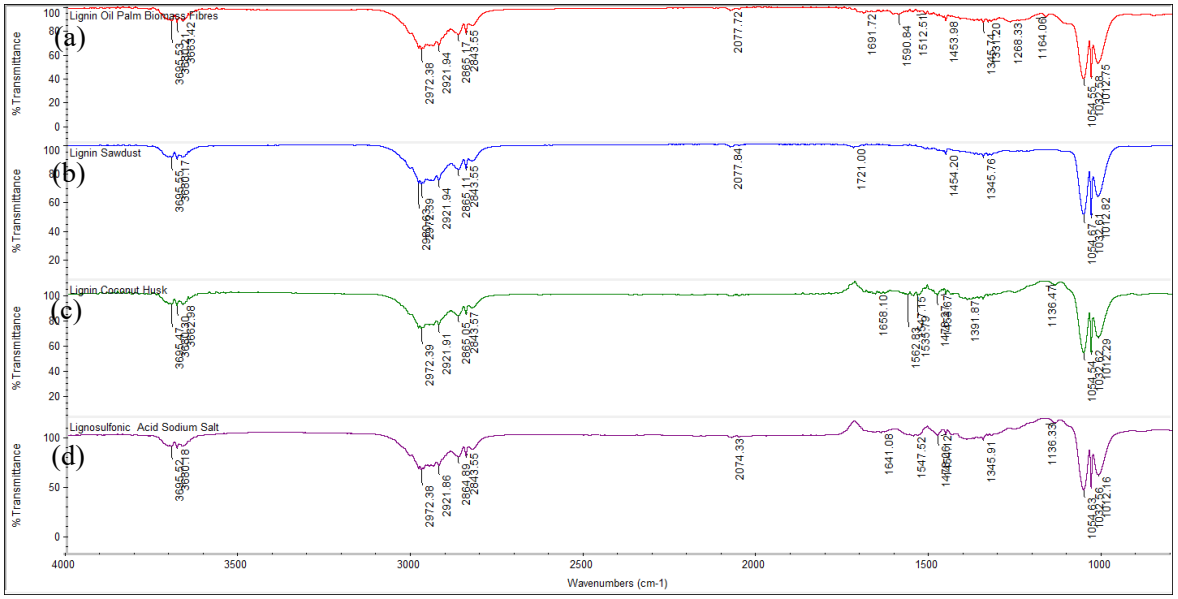


Figure 1. FTIR spectra of (a) lignin oil palm biomass fibre (LOPBF); (b) lignin sawdust (LS); (c) lignin coconut husk (LCH); (d) lignosulfonic acid sodium salt (LASS)

Base mud rheological properties

The goal of the present study was to investigate the feasibility of using lignin from oil palm biomass fibre (LOPBF), sawdust (LS) and coconut husk (LCH) as thinning agents in WBM. An attempt was made to compare the rheological properties of WBM containing LOPBF, LS and LCH with those of commercial thinning agents known as lignosulfonate acid sodium salt (LASS). Results of the experiment are tabulated in Figure 2(a)-(f), where the optimum composition of lignin in this experiment is 0.6 w/w %. Referring to Figure 2(a), as the composition of lignin in a WBM increased, the apparent viscosity decreased. This is due to the polymer chains in lignin interacting, making the mud less viscous [7]. In addition, lignin can create aggregates, which aids in lowering mud viscosity. These aggregates may develop due to the interaction of lignin with other mud components such as clay minerals or salt. The aggregates can alter the rheological characteristics of the mud, making it less viscous [8].

The plastic viscosity of WBM containing LCH shows the lowest value compared to LOPBF, LS and LASS at 0.2, 0.3 and 0.5 w/w/ % of lignin. It is apparent from Figure 2(b) that the plastic viscosity decreases as the lignin composition in WBM increases. The polymer chains in lignin interact with each other in a way that causes the mud to become less viscous [9]. Plastic viscosity is a measure of a fluid's resistance, which is WBM, to flow when it is being sheared or when forces are applied to it. It is an essential property of WBM since it affects the ability of the mud to lubricate and cool the drilling bit and to suspend and transport cuttings to the surface [10]. This is because as the concentration of the polymer increases, the intermolecular forces between the polymer chains become stronger, causing the mud to become more viscous. The higher the concentration of lignin, the lower the value of plastic viscosity. Usually, a lower plastic viscosity is preferred to minimize the potential for formation damage. This type of mud is suitable for drilling soft and porous formations. LOPBF and LS exhibit greater plastic viscosity when compared to LCH and commercial LASS. It is, therefore, ideal for use in harder formations for better hole cleaning.

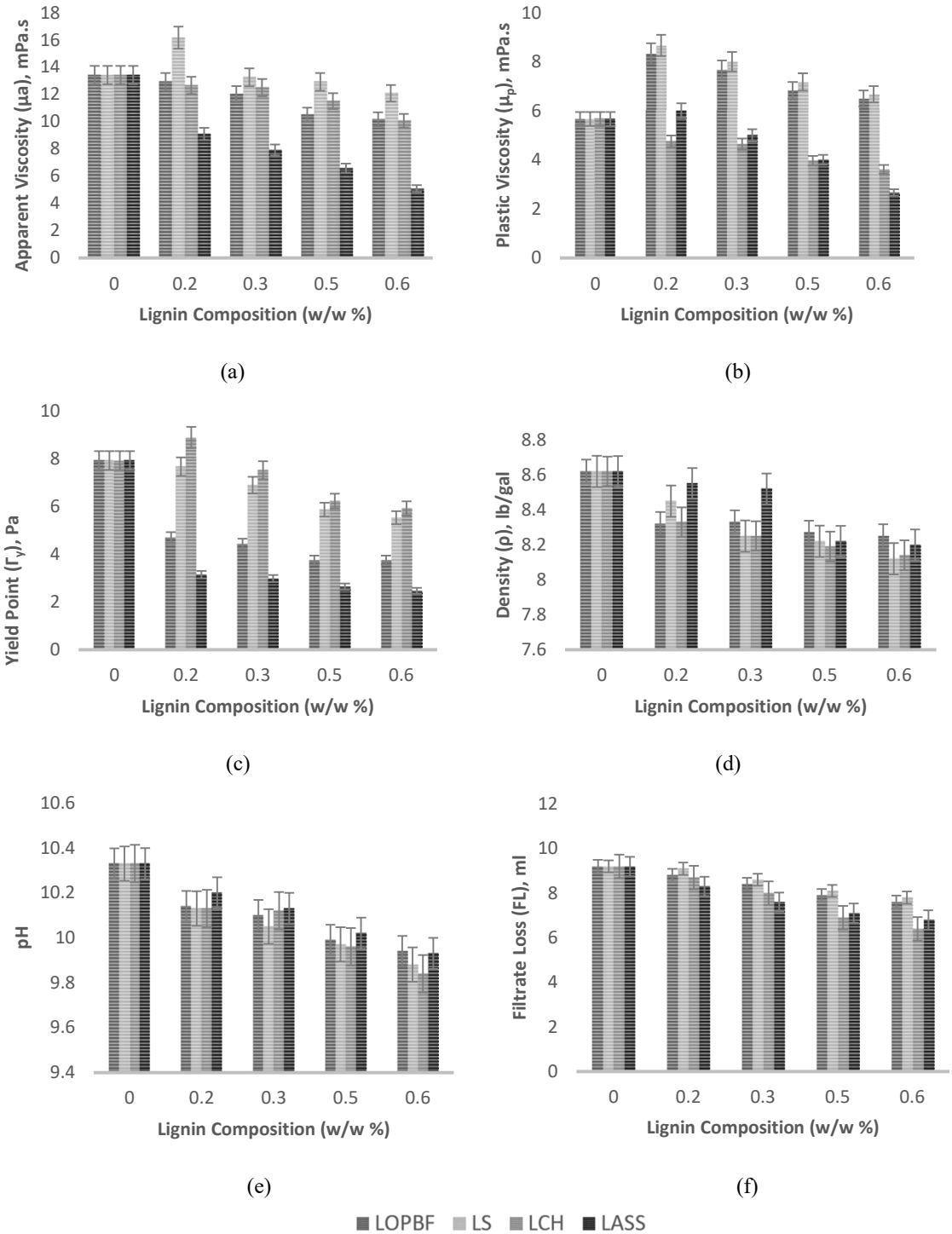


Figure 2. Base mud rheological properties at different lignin composition. The experiment was conducted at 27 °C. (a) apparent viscosity (μ_a); (b) plastic viscosity (μ_p); (c) yield point (Γ_y); (d) density (ρ); (e) pH; (f) filtrate loss (FL)

As the lignin content increased, the fluid's viscosity decreased, resulting in a lower yield point. As tabulated in Figure 2(c), the lowest yield point results from WBM containing LOPBF and LASS at a 0.2 - 0.6 w/w % lignin composition. The development of lignin-water complexes causes the yield point of a WBM to fall as the concentration of lignin increases. These complexes can develop when lignin molecules encounter water molecules in the mud, causing the lignin molecules to become more scattered and less able to resist movement. This lignin molecule dispersion reduces the mud's overall viscosity and lowers the yield point [11].

Overall, the density and pH trend also decreased when lignin composition was increased, as presented in Figures 2(d) and 2(e), respectively. The optimal lignin content is 0.6 w/w %, resulting in the lowest pH of LCH and density of LS. This is due to lignin's acidity, which can reduce the pH of the mud. Some clay minerals may flocculate or cluster together as the mud's acidity rises. As a result of the flocculated clay particles taking up less space, the mud density may drop. Measuring the density and pH of a water-based mud is an essential aspect of mud engineering as it helps to ensure that the mud properties are within the desired range for a particular drilling operation. Fluid loss (FL) in a WBM refers to the amount of FL in the formation while drilling. Based on the result in Figure 2(f), the lowest fluid loss value is represented by LCH with a lignin composition of 0.6 w/w %. This is because the lignin can form a thin filter cake on the wall of the wellbore, which acts as a barrier to prevent fluid loss. As the concentration of lignin increases, the filter cake becomes thicker and more effective at avoiding fluid loss [6].

Thermal aging test

In this work, the experiment was designed to simulate a high agitation process at 90°C. This temperature was used to confirm the consistency of rheological properties, including fluid loss, density, and pH of WBM containing LOPBF, LS, and LCH. The filtrate volume and rheological data were recorded before and after the hot-rolling process. The results obtained are tabulated in Figure 3, showing that the apparent viscosity (Figure 3-a), plastic viscosity (Figure 3-b), yield point (Figure 3-c) and filtrate loss (Figure 3-d) of lignin increased swiftly after the thermal rolling process. This finding suggests that LCH has better rheology than other mud formulations at high temperatures (90°C) after a hot-rolling process for 24 hours. This outcome may be due to the lignin's ability to form a stable filter cake at high temperatures. The chemical structure of lignin allows it to be more thermally stable than other fluid loss control additives, such as bentonite. When the mud's temperature increases, the mud's viscosity decreases, making it more challenging to maintain the integrity of the filter cake. LS, however, have higher thermal stability and can keep their viscosity at higher temperatures, allowing them to form a more effective filter cake and reduce fluid loss [10].

The density of WBM also smoothly increases when the lignin undergoes the thermal rolling process. The highest rise is on the optimum 0.2 w/w % concentration of lignin added, which gave LCH the highest increasing density value. This could be due to the chemical and physical properties of LCH. The density rises because the solubility of dissolved salts such as calcium and magnesium can increase dissolved ions in the mud, which can also increase mud density [10, 12]. Temperature can also impact the pH of the mud. The lower is on the optimum 0.6% concentration of lignin added, which gave LS the lowest pH value, which tends to be acidic. At warmer temperatures, the activity of microorganisms in the mud can increase, resulting in the creation of organic acids that can reduce the pH of the mud. Furthermore, heat can produce chemical processes in the mud, such as acid-base reactions and oxidation-reduction reactions, which can modify the pH of the mud.

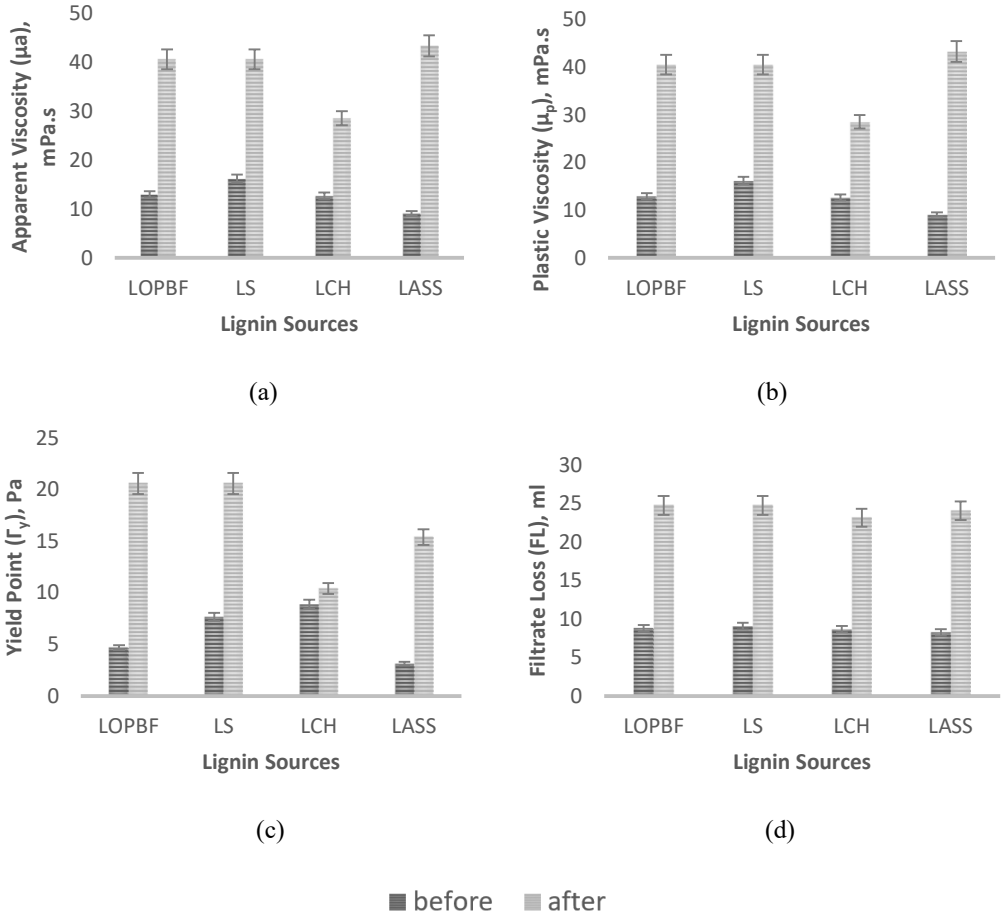


Figure 3. Base mud rheological properties of different lignin sources showing before and after the thermal rolling process at 90 °C. (a) apparent viscosity (μ_a); (b) plastic viscosity (μ_p); (c) yield point (γ_y); (d) filtrate loss (FL)

Conclusion

This study was conducted to observe the effectiveness of LS, LOPBF, and LCH as a thinning agent for water-based drilling mud rheological properties. Formic acid was used as a solvent during the organosolv process to extract lignin from LS, LOPBF, and LCH fibers. Various w/w% of the lignin were examined, including ageing temperature at 90°C and room temperature. Organosolv process was used in this experiment because it is a friendly method for the environment. Research has been done on the six criteria: apparent viscosity, plastic viscosity, yield point, fluid loss, density, and pH. The study showed that as lignin concentration increases, it could effectively function as a thinning agent to lower the viscosity. The viscosity will decline until the concentration of lignin in the mud becomes sufficiently high, causing the WBM to elevate the viscosity instead of reducing it.

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EVALUATION OF *Caulerpa lentilifera* (SEAGRAPE) EXTRACT AS A POTENTIAL ANTI-CORROSION ADDITIVE IN EPOXY COATING FOR MILD STEEL PROTECTION IN THE MARINE ENVIRONMENT

(Penilaian Ekstrak *Caulerpa lentilifera* (Anggur Laut) sebagai Bahan Tambahan Berpotensi Anti-Kakisan dalam Salutan Epoksi untuk Perlindungan Keluli Lembut dalam Persekitaran Marin)

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Abstract

Corroded metals have gained considerable attention due to the significant economic losses they cause, both financially and in terms of their impact on the marine environment. Numerous attempts have been made to mitigate the effects of corrosion using different preventive techniques, including the incorporation of corrosion additives into protective coatings. One such additive, *Caulerpa lentilifera* (CL), commonly known as seagrape, was studied for its potential as a green corrosion additive in epoxy coatings. The anticorrosive activity of various amounts of CL extract (1-5 wt%) added to epoxy coatings on mild steel in a 3.5 wt% NaCl solution was examined using the potentiodynamic polarization (PdP) technique. Fourier Transform Infrared (FTIR) analysis was also conducted to identify the primary phenolic components present in the CL extract. Electrochemical analysis showed that the addition of CL extracts significantly decreased the corrosion rate compared to bare mild steel. According to the findings, epoxy coatings containing 3 wt% CL extracts exhibited 24% more anticorrosive performance compared to the bare sample. Therefore, CL extract shows promising potential as an anti-corrosion additive in epoxy coatings for marine applications.

Keywords: *Caulerpa lentilifera*, epoxy coating, anticorrosion properties, corrosion additive, eco-friendly

Abstrak

Logam berkarat telah mendapat perhatian yang cukup besar kerana kerugian ekonomi yang ketara, baik dari segi kesan kewangan mahupun kesannya terhadap alam sekitar marin. Pelbagai usaha telah dilakukan untuk mengurangkan kesan kakisan dengan menggunakan teknik pencegahan yang berbeza termasuk penggabungan bahan tambahan kakisan dalam salutan pelindung. *Caulerpa lentilifera* (CL), biasanya dikenali sebagai anggur laut, dikaji untuk bahan tambahan kakisan hijau yang berpotensi dalam salutan epoksi. Aktiviti anti-menghakis dari pelbagai komposisi ekstrak CL (1-5% berat) yang ditambahkan ke dalam salutan epoksi pada keluli lembut dan diletakkan ke dalam larutan 3.5% garam natrium klorida telah diperiksa menggunakan teknik polarisasi potensiodinamik (PdP). Analisis inframerah transformasi Fourier (FTIR) juga telah dilakukan untuk mengesahkan komponen fenolik utama yang terdapat dalam ekstrak CL. Analisis elektrokimia menunjukkan bahawa penambahan ekstrak CL

kadar kakisan telah menurun dengan ketara berbanding keluli lembut yang kosong. Menurut penemuan, salutan epoksi yang mengandungi 3 % berat ekstrak CL menunjukkan prestasi anti-menghakis 24% lebih banyak berbanding dengan sampel kosong. Oleh itu, ekstrak CL mempamerkan potensi yang menjanjikan sebagai bahan tambahan anti-kakisan dalam salutan epoksi untuk aplikasi marin.

Kata kunci: *Caulerpa lentilifera*, salutan epoksi, sifat antikakisan, bahan tambahan kakisan, mesra alam

Introduction

Metals and alloys are crucial materials for numerous industries, including the marine industry. Most marine structures, such as ship hulls, upper decks, anchor chains, bulk carriers and cargo containers, are composed of metals, especially mild steel. Mild steel, recognized as low-carbon steel, is more malleable and ductile due to its lower carbon content compared to standard carbon steel. This makes it considerably easier to mould into shapes or bends without fracturing or cracking. In addition, mild steel offers other beneficial properties, such as being inexpensive, readily available, excellent thermal and electrical conductivity, and good tensile strength [1, 2, 3]. Unfortunately, mild steel is prone to corrosion and tends to experience uniform corrosion when exposed to chloride-containing mediums or corrosive environments for extended periods [4, 5, 6].

Corrosion is a spontaneous phenomenon that promotes metal deterioration in corrosive environments through chemical and electrochemical reactions between metallic materials [7]. Metal damage due to corrosion activities has received much attention in recent years because it has a significant financial impact on maintenance, replacement, material losses, and safety across many industries, including the marine sector [8, 9]. Additionally, corroded products can lead to pollution that poses a risk to human health. As a result, corrosion studies are vital for prolonging the lifespan of metals in various situations since the issue affects both structural integrity and human safety [10, 11].

Even though corrosion cannot be completely prevented, the rate of the process can be efficiently reduced. Modern technology has made it possible to control the onset and long-term consequences of corrosion through several methods. The application of a protective coating to metal surfaces is the most efficient approach to prevent corrosion, as it acts as a physical barrier [3, 11, 12]. Organic coatings have attracted a great deal of interest because they are composed of environmentally friendly organic compounds such as polymers or resins. In this regard, epoxy resin is versatile and commonly used as an organic coating in several industries due to its unique properties. Literature has reported that epoxy resin possesses excellent physical and mechanical properties, thermal and chemical resistance, good adhesion, high cross-linking density, and ease of application [4, 13, 14, 15]. Although epoxy resin offers multiple benefits, its characteristics can be further strengthened by incorporating a corrosion inhibitor.

Corrosion inhibitors used as coating additives can strengthen the barrier properties of epoxy coatings and slow the rate of corrosion through their inhibition mechanisms[10, 16]. Synthetic and inorganic inhibitors like phosphates and chromates are frequently and successfully utilized in small amounts; however, they are hazardous, carcinogenic, costly and non-renewable [1]. Moreover, they have negative impacts on the environment, marine ecosystems and human health [11, 17, 18]. As alternatives to conventional inhibitors, researchers are focusing on organic or natural inhibitors. Plant and seaweed extracts are

examples of natural inhibitors that are non-toxic, environmentally safe, easily obtainable, inexpensive, renewable, and exhibit good corrosion resistance. Currently, increased public awareness of health and environmental issues motivates the development of non-toxic and eco-friendly coatings [2, 10, 13, 19, 20].

According to the literature, plant and marine algae extracts are rich sources of phytochemicals, which are chemical compounds found in all parts of plants. These phytochemicals, such as polyphenols, flavonoids, tannins, saponins, carboxylic acids, amines, and other bioactive components, exhibit various beneficial properties, including self-healing, anticorrosion, antifouling, antimicrobial, and UV shielding effects [13, 19, 21, 22, 23]. Nowadays, industries in the coating sector are increasingly utilizing plant extracts as natural inhibitors due to their anticorrosion effects, which are comparable to synthetic inhibitors [11, 24]. Furthermore, the use of plant extracts in industrial applications aligns with the principles of green chemistry and sustainable practices [25]. As a contribution to this research field, *Caulerpa lentilifera* (CL) has been selected as a novel resource for corrosion additives in epoxy coatings.

Caulerpa lentilifera, a green seaweed species belonging to the *Caulerpa* genus, is famously known as seagrapes or green caviar due to its resemblance to tiny grapes [26, 27]. This genus is usually found in the coastal waters of countries in East and Southeast Asia, such as Thailand, Vietnam, Indonesia, Malaysia, the Philippines and Japan. In recent years, it has gained popularity as a culinary ingredient, often used in salads, snacks or sushi. In terms of texture and flavour, it has been described as succulent and salty taste [19, 28, 29]. The plant is commercially grown in ponds because of its ease of propagation, rapid growth rate, and potential medical benefits [30]. Previous studies have investigated various marine algae extracts as eco-friendly corrosion inhibitors and found that they significantly reduce corrosion. For instance, a study by Kamaland Sethuraman [31] showed that *Caulerpa racemosa* exhibited good corrosion performance on mild steel in a 1 M HCl solution. Similarly, based on a study in 2014, *Kappaphycus alvarezii* was found to efficiently protect mild steel from corrosion in both 1 M HCl and H_2SO_4 solutions [1]. Ramdani et al. [32] suggested that *Caulerpa prolifera* acts as a mixed-type inhibitor to successfully inhibit corrosion on carbon steel in a 1 M HCl solution. Nikitasari et al. [2] confirmed that *Euchema* extract acts as a mixed-type inhibitor on API 5L carbon steel in a 0.1 M HCl solution. Meanwhile, Kokilaramani et al. [7] discovered that both *Padina pavonica* and *Sargassum tenerrim*um seaweed extracts function as mixed-type inhibitors, where both can restrict biofilm development on the surface of mild steel to slow down the corrosion process. Despite these promising findings, no research has been conducted on epoxy-based coatings incorporated with CL extract for anticorrosion properties on mild steel substrates in a 3.5 wt% NaCl medium. Therefore, this study aims to investigate the anticorrosive activities of CL-epoxy coatings for marine applications.

Materials and Methods

Materials and chemicals

Dry seagrape (*Caulerpa lentilifera*-CL*)* was purchased from a local supplier in Sabah and used as a natural additive in the epoxy-based coating. The chemical used for the extraction procedure was 95% ethanol, while sodium chloride (NaCl) CP grade was used for corrosion solution testing; both chemicals were supplied by Bendosen Chemicals. For the coating formulation, epoxy resin (Bisphenol A and butyl glycidyl ether) and a hardener (diethylenetriamine) were purchased from Pace Technologies. All chemicals were used

without further purification. The sample materials employed in this research were mild steel coupons.

Ethanolic extract preparation

CL was thoroughly cleaned multiple times to remove all dirt and foreign substances before being rinsed with distilled water. Next, the sample was dried in an oven for 24 hours and ground until it was crushed into a powdered form. Then, 25 g of crushed CL was soaked in a 250 ml flask filled with 95% ethanol for three days. The solution was filtered, and the crude extract of CL was collected using a rotary evaporator. The extracts were refrigerated until further use. The extraction process is summarized in Figure 1.

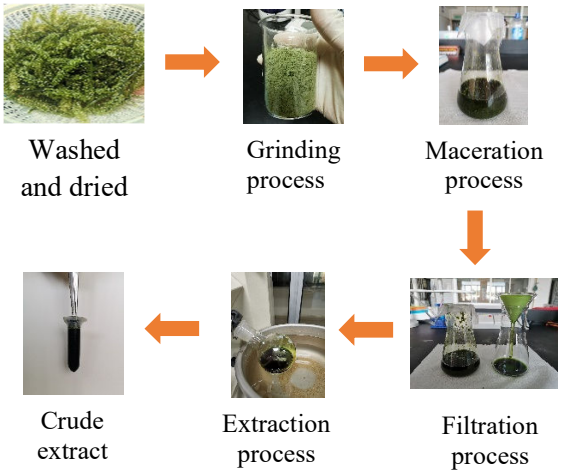


Figure 1. The ethanolic extraction process of *Caulerpa lentilifera*

Specimen preparation

Mild steel SS400 coupons measuring 2.5 cm x 2.5 cm x 1.5 cm were selected for this study, and their chemical compositions are presented in Table 1. The coupons were polished with different grit sizes of silicon carbide paper (240-1200), followed by cleaning with acetone and deionized water. The substrates were air-dried and immediately used afterwards for corrosion analysis.

Table 1. Chemical composition percentage of mild steel SS400

Composition	Percentage Composition (%)
C	0.14
Cr	0.015
Mn	0.705
Si	0.255
P	0.009
S	0.007
Cu	0.006
Fe	Bal.

Coating preparation

Different amounts of CL extracts (0-5 wt%) were mixed with epoxy matrix and stirred continuously for 2 hours at a speed of 500 rpm. The homogenous mixture was then sonicated for 15 minutes before the addition of the hardener based on the manufacturer's instructions. The formulated solution was drop-cast onto the mild steel surface. Subsequently, the samples were dried in an oven for 24 hours. The coating formulations are detailed in Table 2.

Table 2. Coating formulations of epoxy-CL solutions

Samples	CL Extract (wt.%)	Epoxy (wt.%)
BARE MS	-	-
EP	0	100
EP-CL1	1	99
EP-CL2	2	98
EP-CL3	3	97
EP-CL4	4	96
EP-CL5	5	95

Characterizations: Fourier transform infrared (FTIR) spectroscopy

The presence of phytochemical substances in the CL extract was examined using a Fourier Transform Infrared (FTIR) spectrometer, specifically the Thermo Nicolet iS20 FTIR Spectrometer. The characterization is used to determine the functional groups or other structural information of CL extract within the frequency range of 500–4000 cm^{-1} [5].

Ultraviolet-visible (UV-Vis) spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy is a commonly used analytical method for accessing the absorption properties of CL crude extract. In this study, a UV-1800 Shimadzu UV spectrophotometer was employed, operating within the typical range of UV and visible light wavelengths from 200 to 800 nm.

Potentiodynamic Polarization (PdP) analysis

The corrosion behaviour of the CL crude extract incorporated with epoxy matrix and bare samples was investigated using potentiodynamic polarization (PdP) methods. The experiment was conducted using a Gamry Instrument Interface 1010E, a common approach to determine the performance of protective coatings in inhibiting or slowing the corrosion rate. The measurements were performed using a three-electrode electrochemical cell, with samples having 1 cm^2 exposed area serving as the working electrode, a platinum rod as the counter electrode, and a saturated calomel electrode (SCE) as the reference electrode. All samples were analyzed at room temperature in a 3.5 wt% NaCl aqueous solution. The polarization curves were obtained by scanning the open circuit potential (OCP) between -250 and 250 mV at a scan rate of 1 mVs^{-1}.

Results and Discussion

Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the CL extract captured during this experiment are displayed in Figure 2 and matched using an IR absorption table. The findings were also compared with the results of other researchers as a reference, and the outcomes are

summarized in Table 3. In the spectra, several comparable bands were observed, which are associated with specific functional groups. The FTIR analysis of the CL extract revealed the presence of phenol, alcohol, alkanes, alkynes, amines, carbonyl, aromatic amines, and carboxylic acids [33, 34].

Table 3. FTIR peak values of the *Caulerpa lentilifera* crude extract

No.	Peak values	Bonds	Functional Groups
1	3319.02	O-H stretch, N-H stretch	Alcohol, phenol, amines
2	2976.88	C-H stretch	Alkanes
3	2901.88	C-H stretch	Alkanes
4	1644.27	C=O stretch	Carbonyl
5	1449.95	C-H bend	Alkanes
6	1402.84	C-H bend	Alkanes
7	1273.69	C-N stretch	Aromatic amines
8	1084.57	C-O stretch	Alcohol, phenol, carboxylic acid
9	1042.20	C-O stretch	Alcohol, phenol, carboxylic acid
10	877.51	C-H bend	Aromatics
11	536.69	=C-H	Alkynes

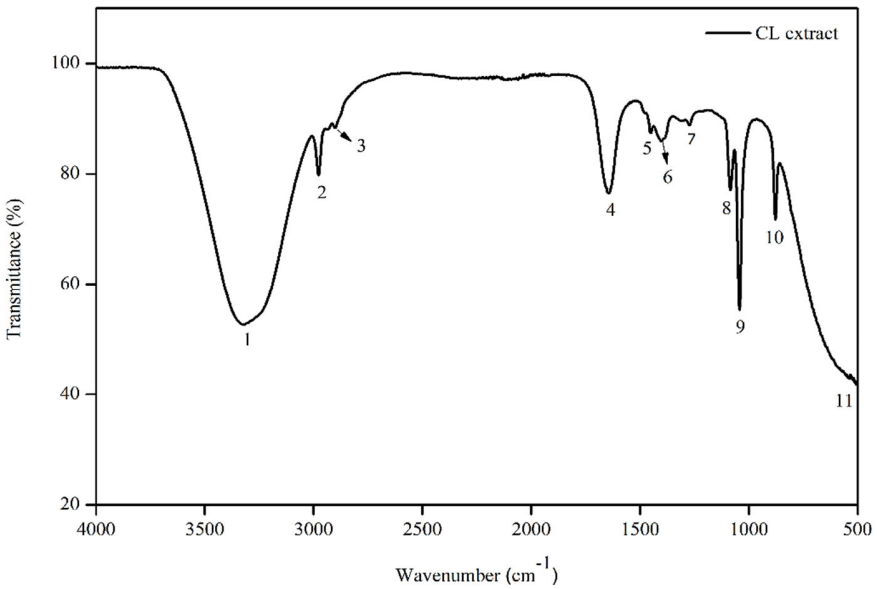


Figure 2. FTIR spectrum of the *Caulerpa lentilifera* crude extract

The weak absorption bands observed at 536.69 cm^{-1} in the CL extract signify the C-H bending vibration that confirms the presence of carbohydrates. The peaks detected at 877.51 cm^{-1} are ascribed to the aromatic C-H out-of-plane vibration, indicating the presence of an aromatic ring pigment compound. A stretching vibration of C-O was exhibited by the strong absorption peak at

1042 - 1085 cm^{-1}. Additionally, the presence of chlorophyll groups is demonstrated by the presence of C-H stretching vibrations at 2976.88 cm^{-1} and 2901.88 cm^{-1}. A broad band observed at 3319.02 cm^{-1} corresponds to the hydroxyl (O-H) and amines (N-H) groups [35, 36]. According to Khoukhi et al. [37], amine compounds are frequently employed as corrosion inhibitors for ferrous metals in natural aqueous environments. In brief, the chemical analysis of CL extract reveals the presence of elements like O, H, N and C that are capable of acting as corrosion inhibitors and contributing to the reduction of corrosion rate [2].

Ultraviolet-visible (UV-Vis) spectroscopy

The absorbance spectra of the CL extract were recorded and are shown in Figure 3. The findings were also compared with those of other researchers for reference, and the results are summarized in Table 4.

Table 4. UV-Vis results of the *Caulerpa lentilifera* crude extract

Peak no.	Wavelength (nm)
1	202
2	270
3	399
4	502
5	660

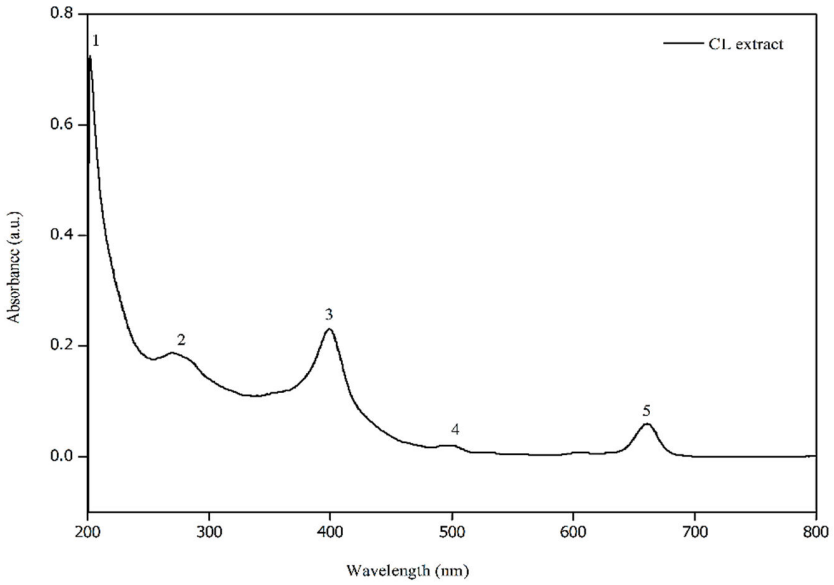


Figure 3. UV-Vis spectrum of the *Caulerpa lentilifera* crude extract

According to the data in Table 4, the UV-Vis spectra of the CL extract exhibit similar peaks within the wavelength range of 202-660 nm, indicating the existence of chlorophylls, phenolics, alkaloids, flavonoids and their derivatives [7, 36, 38]. Additionally, Kamaland Sethuraman [31] reported a broad peak visible at 269-315 nm, which reflects the properties of the carbonyl function conjugated to an aromatic ring system. This finding was confirmed by the IR absorption band in the

range of 1630-1687 cm^{-1} in the IR spectra (Table 3). Our results are consistent with those of earlier studies, with peak values in extracts ranging from 200 nm to 680 m.

Potentiodynamic polarization (PdP) analysis

The results of the polarization analysis were presented as Tafel plots. Figure 4 depicts the Tafel plots of uncoated and epoxy-coated mild steel with different concentrations of CL extract (0-5 wt%) immersed in a 3.5 wt% NaCl solution for 1 hour. The parameters derived from the curves, such as corrosion potential (E_{corr}), corrosion current density (I_{corr}) and corrosion rate (CR), are tabulated in Table 5. The corrosion protection efficiency (*% P.E.*) of coated mild steel samples was calculated using equation 1 [4]:

$$P.E = \frac{I_{ucorr} - I_{ccorr}}{I_{ucorr}} \, x \, 100\,\% \qquad (1)$$

where I_{ucorr} and I_{ccorr} represent the corrosion current density of uncoated and coated samples in saline solution, respectively.

Table 5. Parameter polarization for uncoated and coated epoxy-CL extracts

Samples	Corrosion Potential, E_{corr} (mV)	Corrosion Current Density, I_{corr} ($nAcm^{-2}$)	Corrosion Rate, CR (mpy)	Protection Efficiency, P.E. (%)
BARE MS	244	174	0.0793	0
EP	244	167	0.0763	4
EP-CL1	246	161	0.0734	7
EP-CL2	286	141	0.0646	19
EP-CL3	264	133	0.0607	24
EP-CL4	270	143	0.0651	18
EP-CL5	257	180	0.0822	-3

Based on Table 5 and Figure 4, it is evident that the presence of CL extract in the epoxy coating shifted the E_{corr} value to a more positive region compared to the uncoated and EP samples. Specifically, the E_{corr} value of both uncoated and EP samples was 244 mV. With the addition of CL extract, the E_{corr} values increased from 244 mV to 246 mV (EP-CL1), to 286 mV (EP-CL2), 264 mV (EP-CL3), 270 mV (EP-CL4) and 257 mV (EP-CL5). In addition, the I_{corr} values of the coated samples exhibited a decreasing trend when CL extract was added to the epoxy coating, except for 5 wt%, compared to the uncoated sample. The I_{corr} value of bare mild steel was 174 $nAcm^{-2}$, whereas the I_{corr} reading for the EP sample was 167 $nAcm^{-2}$, which was lower than the value of the uncoated sample. This indicates that the epoxy polymer itself can resist the corrosion of mild steel in aggressive environments [4, 11]. However, it was observed that the incorporation of CL extracts into epoxy coating at 1-3 wt% significantly reduced the I_{corr} values to 161 $nAcm^{-2}$ (EP-CL1), 141 $nAcm^{-2}$ (EP-CL2) and 133 $nAcm^{-2}$ (EP-CL3). This reduction is likely due to the coating layer acting as a protective barrier that prevents corrosion agents from affecting the substrate and thereby limiting both anodic and cathodic processes [14]. Despite this, increases in extract loading of 4-5 wt% in the epoxy coating reversed the trend, with I_{corr} readings rising to 143 $nAcm^{-2}$ (EP-CL4) and 180 $nAcm^{-2}$ (EP-CL5), respectively. Nonetheless, the excessive incorporation of CL beyond its optimal range increases the I_{corr} of EP-CL4 and EP-CL5, with possible weakening effects on the barrier

characteristics of the coating and triggering a corrosion attack. Furthermore, as displayed in Table 5, the values of CR were determined using the Gamry Echem Analyst software. The CR readings for EP-CL1 to EP-CL4 coating samples were found to be lower than those for the epoxy coating alone, which indicates that adding CL extract to epoxy coatings strengthens the barrier characteristics of the coating [10, 16]. Hence, the % P.E. for samples EP to EP-CL5 was determined to be 4%, 7%, 19%, 24%, 18% and -3%, respectively, as shown in Table 5. Therefore, PdP proves that CL extract functions as a mixed-type inhibitor with protection effectiveness ranking as follows: 3 > 2 > 4 > 1 > 0 > 5 wt%.

From these findings, it is proposed that the incorporation of CL extract in epoxy coating suppresses both the anodic and cathodic reactivity of mild steel in saline media. This suppression is attributed to the existence of bioactive compounds in the extract, which is consistent with the FTIR and UV-Vis findings. The functional groups of phenol, hydroxyl, carbonyl and amine contain heteroatoms, π-bonds and aromatic rings that are tightly bound to epoxide groups via a cross-linking reaction between the two phases. As a result, corrosion is slowed down by a maze effect, which restricts corrosive substances from penetrating the coating and lengthens their penetration path [2, 4, 5, 39, 40]. This behaviour explains the observed decrease in the *CR* of mild steel substrate in a 3.5 wt% NaCl solution when CL extract is used as a green inhibitor. Therefore, EP-CL3 with 3 wt% CL extract demonstrated the best corrosion protection for mild steel.

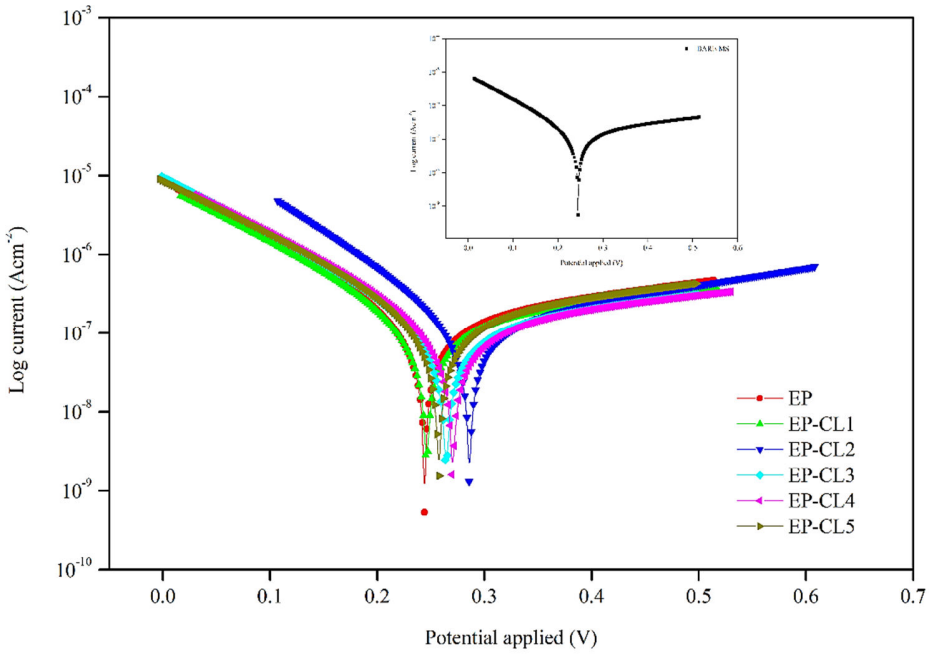


Figure 4. Tafel plots of uncoated (inset image) and coated mild steel in 3.5 wt% NaCl solution

Conclusion

Caulerpa lentilifera extract was successfully utilized as a green coating additive in epoxy coatings at different concentrations (1-5 wt%) for mild steel in a 3.5 wt% NaCl solution for the first time. The FTIR and UV-Vis analyses of the CL extract indicate the existence of phenol, alcohol, alkanes, alkynes, amines, carbonyl, aromatic

amines, and carboxylic acids. The anticorrosive activity of the coatings on mild steel exposed to the saline solution was evaluated using the potentiodynamic polarization technique. The results showed that the incorporation of CL extract in epoxy coatings improved the corrosion resistance of mild steel in the saline solution by reducing the I_{corr} and corrosion rate values. The outcomes of this study revealed that EP-CL3 offered excellent corrosion protection compared with other samples.

Acknowledgement

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EFFECT OF EXTRACTION METHODS ON YIELD, TOTAL PHENOLIC CONTENT, ANTIOXIDANT, AND ANTI-TYROSINASE PROPERTIES OF *Combretum indicum* LEAVES

(Kesan Kaedah Pengekstrakan Terhadap Hasil, Jumlah Kandungan Fenolik, Sifat Antioksida, dan Anti-Tirosinase Daun *Combretum indicum*)

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Abstract

Plants form a natural source of key bioactive chemicals equipped with excellent antioxidant and anti-tyrosinase properties. The restorative plant *Combretum indicum* (*C. indicum*), which belongs to the *Combretaceae* family, was widely used in customary treatment in the past. The target of this study is to assess the effects of water as a solvent, traditional extraction strategies (maceration and Soxhlet extraction), and extraction time on *C. indicum* yield. The total phenolic content (TPC), as well as antioxidant and tyrosinase inhibitory activities, were observed by utilising an ultraviolet-visible spectrophotometer. DPPH radical scavenging was used to determine the antioxidant property, while a mushroom tyrosinase inhibition assay was utilised to evaluate the anti-tyrosinase activity. Maceration carried out for 72 h showed good outcomes for TPC, antioxidant, and anti-tyrosinase activities. The results showed that TPC, antioxidant assay, and anti-tyrosinase activities had a significant linear correlation (p <0.05). It is evident from the study that *C. indicum* leaves, which contain numerous phytochemicals, are strong antioxidants and tyrosinase inhibitors that can potentially play a significant role in the pharmaceutical and cosmetic industries.

Keywords: antioxidant, anti-tyrosinase, *Combretum indicum*, maceration, Soxhlet

Abstrak

Tumbuh-tumbuhan menghasilkan sumber semula jadi bahan kimia bioaktif utama yang dilengkapi dengan sifat antioksida dan anti-tirosinase yang sangat baik. *Combretum indicum* (*C. indicum*) ialah tumbuhan pemulihan dan ia digunakan secara meluas sebagai rawatan adat pada zaman dahulu. Sasaran kajian ini ialah untuk menilai kesan air sebagai pelarut, strategi pengekstrakan tradisional (maserasi dan Soxhlet), dan masa pengekstrakan ke atas hasil *C. indicum*. Jumlah kandungan fenolik (TPC), aktiviti antioksida, dan aktiviti perencatan tirosinase telah diperhatikan dengan menggunakan spektrofotometer ultraungu tampak. Perencatan radikal DPPH digunakan untuk menentukan sifat antioksida, manakala ujian perencatan tirosinase cendawan telah digunakan untuk mengkaji aktiviti anti-tirosinase. Kaedah maserasi selama 72 jam telah meningkatkan hasil TPC, aktiviti antioksida, dan aktiviti anti-tirosinase. Keputusan menunjukkan bahawa TPC, aktiviti antioksida, dan aktiviti anti-tirosinase mempunyai korelasi linear yang ketara (p <0.05). Ia terbukti daripada kajian bahawa daun *C. indicum* yang mengandungi banyak fitokimia ialah antioksida yang kuat dan perencat tirosinase yang berpotensi memainkan peranan penting dalam industri

farmaseutikal dan kosmetik.

Kata kunci: antioksida, anti-tirosinase, *Combretum indicum*, maserasi, Soxhlet

Introduction

With the developing interest of the public in skin brightening, lightening agents are widely used in cosmetic formulas under dermatological supervision. The dynamic substances that lighten the skin complexion are either natural or synthetic and can act at various degrees of melanogenesis. They are utilised to treat different skin pigmentation issues, or basically, to obtain a lighter complexion because a fairer skin complexion in various societies can be defined as beauty. However, recent studies have shown that some of the synthetic antioxidants and anti-tyrosinases contribute to antagonistic impacts, prompting their prohibition or restricted use under the Food and Drug Administration and other global guidelines. There are concerns about utilising mercury and hydroquinone in skin-brightening products due to their reported adverse effects on human skin wellbeing. The adverse impact of inorganic mercury present in skin cleansers and other skin care products includes damage to the kidney, dermatological skin issues, and reduced skin protection from microbial infections. Other adverse impacts, including anxiety, misery, mental illness, and peripheral neuropathy, can also occur [1].

Consequently, there is a continuous quest for a natural antioxidant and plant-based anti-tyrosinase to replace these synthetic ingredients. Antioxidant activity refers to the ability of a molecule to scavenge free radicals and avert oxidative damage resulting from oxidative stress [2]. Meanwhile, tyrosinase is an enzyme containing copper that is crucial for the formation of melanin [3]. Anti-tyrosinase activity involves the inhibition of tyrosinase function, one of the frequently used techniques to deal with skin pigmentation issues [4]. The phenolic compounds found in plants exhibit strong antioxidant and anti-tyrosinase properties. For instance, extracts from plants like *Ginkgo biloba* have demonstrated antioxidant activity along with an anti-tyrosinase effect [5]. Hence, antioxidant and anti-tyrosinase activities are closely interconnected, and phenolic compounds possess both the ability to scavenge free radicals and inhibit tyrosinase.

Phytochemical extraction is the process of separating bioactive compounds from plant materials using an appropriate solvent and extraction technique [6]. It plays a crucial role in natural product research and drug discovery [7]. The most common extraction methods used are maceration and Soxhlet extraction. The effectiveness of the extraction process is influenced by multiple factors, such as the choice of solvent, extraction time, and temperature [8]. Through the optimisation of these factors, phytochemical-rich extracts can be produced at their highest potential yield and quality, thereby enhancing their applicability in pharmaceutical and cosmetic research.

Combretum indicum (*C. indicum*), a member of the *Combretaceae* family, is a fast-growing woody vine found in various regions, including Malaysia, the Philippines, Thailand, Bangladesh, and India [9]. Other names for the plant include *Quisqualis indica* and Rangoon creeper [10]. A previous study by Barik et al. [11] stated that it possesses various medicinal properties and has therefore been thoroughly used in traditional and folk medicine. This plant possesses a strong potential in the commercial landscape; however, there is minimal research on the benefits and potential of this plant to be used as the base in modern skin care products. This plant is non-seasonal, does not rely on the growing seasons, and grows quicker compared to other plants. Rather than simply overlooking its potential, it would be advantageous to assess this plant as much as possible due to its easy availability for the community and economic purposes. Plants are the prime sources of natural antioxidants. *C. indicum* is a great source of phenols and flavonoids, which play an important role in free radical scavenging [12]. Pharmacological studies have shown various capacities of the plant in antimicrobial, anti-inflammatory, antioxidant, antipyretic, antidiarrheal, and antihyperglycemic activities [13].

The purpose of this research is to identify the best extraction method that gives a high amount of yield and

total phenolic content (TPC), as well as better antioxidant and anti-tyrosinase activities from the leaf extract of *C. indicum*. The study applied maceration and Soxhlet extraction and used ultrapure water as the solvent. Water stands out as the safest and most cost-effective and environmentally friendly solvent that is highly capable of extracting polar compounds, such as polyphenols, flavonoids, and other hydrophilic constituents present in plant materials [14]. The ability of water to form hydrogen bonds enhances the solubilisation and extraction of bioactive molecules.

Materials and Methods

Sample collection

Samples of *C. indicum* leaves were gathered from the Blok W area, Universiti Malaysia Pahang Al-Sultan Abdullah, Gambang, Pahang, Malaysia. The leaves were then cleaned using distilled water and dried in an oven at 70 °C for a day. Later, a blender was used to pulverise the *C. indicum* leaves into powder, which was then stored in a zip-lock bag until further use [15].

Preparation of *C. indicum* leaf extract

The extract was prepared using maceration and Soxhlet extraction with some modifications. First, 40 g of the milled powder was soaked in 400 mL of ultrapure water. The sample was vigorously stirred in an incubator shaker for 12, 24, 48, and 72 h for maceration and 12 h for Soxhlet extraction. Then, Whatman No. 1 filter paper was used to filter the extracts. The filtered extract was centrifuged for 10 min at 2,500 rpm to remove the fine particles of the leaf powder. The extract was then freeze-dried and stored in a chiller until further use [15].

Extract Yield Percentage

The percentage of yield (%) of *C. indicum* extract was calculated using Equation 1:

$$\text{Percentage Yield} = \frac{\text{Weight of } C.\ indicum \text{ extract gained (g)}}{\text{Initial weight of powder extract (g)}} \times 100\% \quad (1)$$

Total phenolic content

For each extract, the TPC was determined using the Folin-Ciocalteu (FC) method [16] with minor modifications. The sample was diluted in 1:10 mg/mL, and gallic acid calibration solutions (50–300 µg/mL) were prepared. Then, 1 mL of extract or a standard solution of gallic acid was added to a 25 mL volumetric flask containing 10 mL of distilled water. Subsequently, 1 mL of FC reagent was added to the mixture. After 5 min, 10 mL of 7.5% w/v sodium carbonate was added. To prepare a precise 25 mL solution, 3 mL of distilled water was added, and the mixture was incubated for 45 min at 25 °C. The absorbance of the sample was determined using an ultraviolet-visible (UV-Vis) spectrophotometer at 765 nm. Following that, a gallic acid calibration curve was constructed. Using the regression equation, the concentration of gallic acid in each extract was estimated. Finally, the results were evaluated as TPC in milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g) using Equation 2:

$$\text{TPC} = \frac{\text{Concentration of sample (mg/mL)} \times \text{Volume used during the assay (mL)}}{\text{Mass used during the assay (g)}} \times 100\% \quad (2)$$

DPPH assay

The free radical scavenging activity of the prepared extracts was quantified in vitro by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals using the method applied by [17]. The samples were diluted with water (1 mg/mL), and ascorbic acid (1–0.062 µg/mL) was used as a standard. The DPPH stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol. Then, 6 mL of DPPH was mixed with 0.2 mL of plant extract or the standard solution in a test tube, followed by incubation for 30 min. The absorbance was measured using a UV-Vis spectrophotometer at 517 nm. The control sample was prepared by substituting 0.2 mL of the test sample with 0.2 mL of methanol. The experiment was conducted in triplicate, and the radical scavenging activity was calculated using Equation 3:

$$\text{DPPH Radical Scavenging Activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (3)$$

Where $A_{control}$ is the absorbance of the control sample and A_{sample} is the absorbance of the test sample.

Mushroom tyrosinase inhibition assay

As previously reported by [18], inhibition tests with certain modifications were carried out in this study, where L-3,4-dihydroxyphenylalanine (L-DOPA) was used as a substrate to evaluate the inhibitory effect of *C. indicum* leaf extract on tyrosinase activity. The response combination consisted of 6.85 mL of phosphate buffer (0.05 M, pH 6.5), 0.15 mL of mushroom tyrosinase (2,500 units/mL), 2 mL of plant extract solution, and 1 mL of 5 Mm L-DOPA. After L-DOPA was added, the dopachrome formation of the reaction mixture was monitored at 492 nm instantaneously. Kojic acid (200, 250, 300, 350, and 400 µg/mL) was used as a standard. The control sample was prepared by substituting 2 mL of the test sample with 2 mL of phosphate buffer (0.05 M, pH 6.5). The experiment was conducted in triplicate, and the inhibition activity of tyrosinase was calculated using Equation 4:

$$\text{Anti-Tyrosinase Activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (4)$$

Where $A_{control}$ is the absorbance of the control sample and A_{sample} is the absorbance of the test sample.

Results and Discussion

Extraction Yield

Figure 1 shows the impact of extraction time on the yield of *C. indicum* leaf extract. The extract yield of the plant was observed to gradually increase from 4.19% at 12 h to 6.96% at 24 h, 8.88% at 48 h, and 9.28% at 72 h using maceration. The increasing trend in the extract yield over the extraction period shows a time-dependent extraction process. The highest extract yield of 9.28% obtained at 72 h using maceration implies that the increase in retention time extends the interaction between the solvent and the matrix and, therefore, improves the extraction efficiency. The swelling of the cell wall increases the solvent's penetration into the plant material, thus increasing the leaf extract. Moreover, the maceration technique naturally swells plant matrices over an extended period of time by achieving solute-solvent diffusivity through osmotic pressure [19]. Apart from maceration, Soxhlet extraction is an alternative for herb extraction in a shorter time. The Soxhlet extraction technique showed better recovery of 10.08% at 12 h compared to maceration. This could be due to the heat used by the Soxhlet device to continuously vaporise and condense the solvent. The heating involved in the extraction process enhances the efficiency of the plant cell wall disruption and increases the solubility of the compounds in the solvent, leading to higher yields. Research studies have indicated that Soxhlet extraction is capable of breaking down cell walls more effectively compared to other extraction techniques, including maceration and ultrasound [20]. However, it should be noted that exposing plant materials that are thermolabile to high temperatures may cause herbal degradation [21]. Besides, Soxhlet extraction involves higher solvent consumption and energy requirements [22]. On the other hand, maceration is a gentle process and applies a milder temperature, which does not destroy bioactive compounds. Furthermore, it is simple, more convenient, and cost-effective [23]. Factors such as the preservation of compounds, cost, sustainability, and equipment complexity are important to consider when selecting the most suitable extraction method. Although the yield of Soxhlet extraction was slightly higher (10.08%) at 12 h compared to maceration at 72 h (9.28%), the difference in yield is relatively small. Hence, maceration at 72 h emerges as the optimal extraction method to yield *C. indicum* extract due to its efficiency, cost-effectiveness, sustainability, and preservation of compounds.

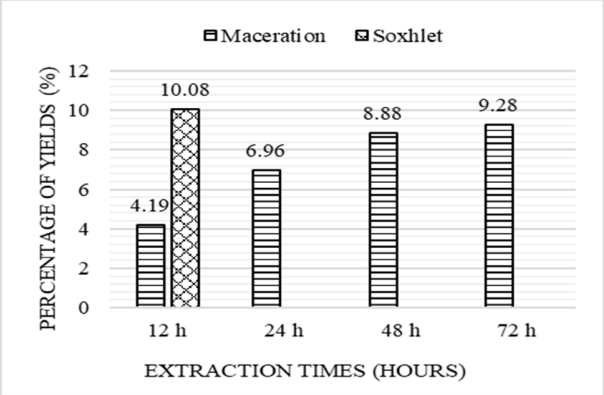


Figure 1. The extract yield of *C. indicum* leaves obtained using maceration and Soxhlet extraction

Total phenolic content

The TPC of each extract with a concentration of 0.1 mg/mL was determined by the FC method using gallic acid as the standard. A calibration curve with the regression equation of $y = 0.0027x + 0.0987$ and $R^2 = 0.9923$ was constructed using the absorbance values obtained at different concentrations of gallic acid. Figure 2 shows the TPC for different extraction times with the use of maceration and Soxhlet techniques. The lowest TPC of 64 ± 0.1275 mg GAE g^{-1} was observed at 12 h, and the highest TPC of 136 ± 0.3003 mg GAE g^{-1} was observed at 72 h using maceration. The results show that the TPC of the extract increases as the extraction time increases from 12 to 72 h. This is because more phenolic compounds are gradually released from the *C. indicum* leaves into the solvent over time. Increasing the extraction duration enhances the extraction of polyphenolic compounds [24].

The TPC of the *C. indicum* leaves extracted at 12 h using Soxhlet extraction was 135 ± 0.1342 mg GAE g^{-1}. The result obtained shows that Soxhlet extraction is able to give a comparable result to maceration at 72 h in a shorter time. Similar to the extracts derived from the parts of *Calophyllum incrassatum*, the barks of *Calophyllum rubiginosum*, and the leaves and barks of *Calophyllum canum*, the technique of Soxhlet extraction showed a high phenol extraction rate in a short time [25]. However, the high temperature used in Soxhlet extraction might contribute to the degradation of the heat-sensitive properties of some phenolic compounds [26]. A study observed the degradation of catechins in tea due to the high temperature used in Soxhlet extraction. The total polyphenol concentrations from Soxhlet extraction at 70 °C were lower than those from maceration at 40 °C [27]. Although Soxhlet extraction has a time-efficient nature, the increased temperature implied during extraction may impair the integrity of certain phenolic compounds. It is important to consider both extraction efficiency and the preservation of phenolic compounds when selecting the optimal extraction method [28]. Thus, maceration at 72 h is the optimal method for the extraction of *C. indicum* leaves due to its ability to extract phenolic compounds while maintaining its integrity.

Antioxidant activity

The DPPH test is a reliable and widely used method to assess the ability of an extract to scavenge free radicals [29]. The results of DPPH inhibition for different extraction times and methods are shown in Figure 3. The antioxidant activity of the *C. indicum* leaves extracted using maceration was reported to be 11.098% at 12 h, 29.016% at 24 h, 39.306% at 48 h, and 83.398% at 72 h. The results indicate a clear trend of increasing antioxidant activity with prolonged extraction time. An increased extraction time allows the extraction of a wider range of bioactive compounds, including phenolic compounds, which are well-known for their antioxidant capability [24]. Overall, the maceration technique for 72 h exhibited the highest antioxidant activity at 83.398%, surpassing other extraction times, including Soxhlet extraction, which showed 79.809% of antioxidant activity. Hence, maceration demonstrated 4% greater antioxidant efficacy compared to Soxhlet extraction. The elevated TPC values observed at longer extraction

times signify a higher concentration of antioxidant compounds within the extract. From the past outcome, it can be derived that the highest number of antioxidants corresponds to the highest TPC in the extracts, which reveals the positive correlation between TPC and antioxidant activity [30].

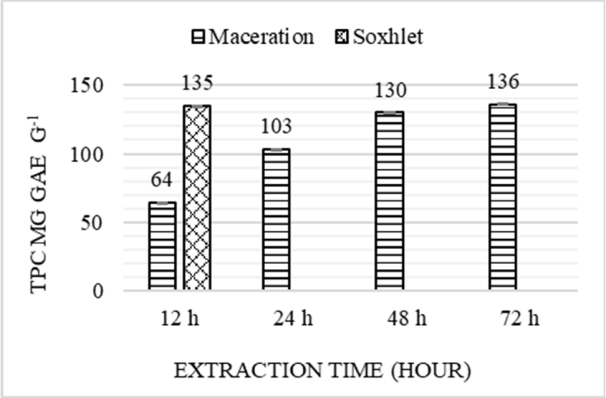


Figure 2. The TPC of *C. indicum* leaves extracted using maceration and Soxhlet extraction. The values are the means ± standard deviation of triplicate analyses. Columns labelled with different extraction times are significantly different (p <0.05) by the ANOVA single factor test

A significant contribution to antioxidant ability is made by phenolic and flavonoid molecules [31]. A study by [32] deduced that all parts of *C. indicum* possess strong antioxidant potential. Recent evidence indicates that diets high in polyphenolic compounds play an important role in combating oxidative stress disorder due to their antioxidant behaviour [33]. Hence, the polyphenolic compounds found in *C. indicum* leaves may potentially prevent oxidative diseases related to stress.

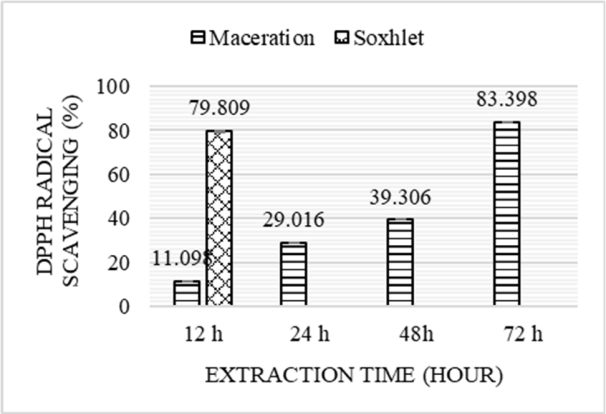


Figure 3. Antioxidant activity of *C. indicum* leaves extracted using maceration and Soxhlet extraction. The values are means ± standard deviation of triplicate analyses. Columns labelled with different extraction times are significantly different (p <0.05) by the ANOVA single factor test

Anti-tyrosinase activity

The relationship between the inhibitory action of tyrosinase and the antioxidant activity of plant extracts is closely linked to their phenolic content [34]. In this study, a direct correlation between the anti-tyrosinase activity and the TPC was observed from 12 to 72 h extraction periods (20.16 ± 0.0040% to 46.37 ± 0.0029%, respectively), as depicted in Figure 4. The

anti-tyrosinase activity of the extract demonstrates a progressive increase over time. Meanwhile, the 12 h extraction using Soxhlet extraction exhibits the lowest tyrosinase inhibition activity, registering at 13.31 ± 0.0037%. This observation is expected to be the aftereffect of bioactive material deterioration at high temperatures during Soxhlet extraction. Besides, high temperatures can also accelerate chemical reactions, including oxidation and hydrolysis, which may contribute further to the degradation of bioactive compounds present in the plant material [35]. Notably, the 72 h maceration technique demonstrated 33.06% greater tyrosinase inhibition activity compared to the 12 h Soxhlet method, highlighting the better efficacy of prolonged maceration in extracting phenolic compounds with anti-tyrosinase properties.

Tyrosinase is a pivotal enzyme in melanin synthesis [36]. The inhibition of its activity garners considerable attention in the skin care and cosmetic industries for its role in treating hyperpigmentation [37]. Additionally, the anti-tyrosinase activity of the *C. indicum* leaf extract obtained through maceration at 72 h further underscores its potential relevance in addressing hyperpigmentation concerns.

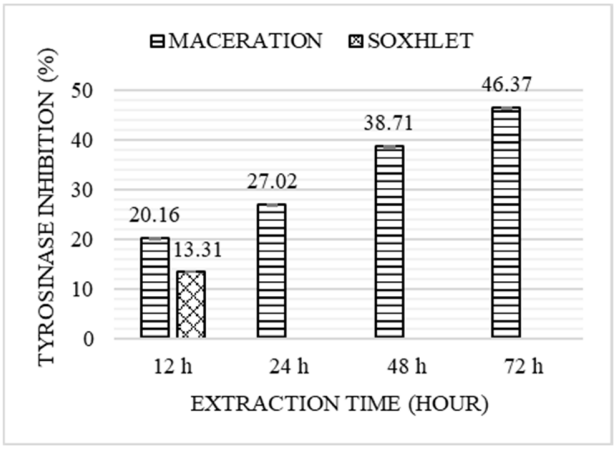


Figure 4. Anti-tyrosinase activity of *C. indicum* leaves extracted using maceration and Soxhlet extraction. The values are means ± standard deviation of triplicate analyses. Columns labelled with different extraction times are significantly different (p <0.05) by the ANOVA single factor test

Conclusions

In conclusion, this study revealed that extraction time and extraction methods have a significant impact on the yield, TPC, antioxidant, and anti-tyrosinase properties of the extracts obtained. The findings show that 72 h of maceration is the most effective method to extract *C. indicum* leaves for an optimum yield. As Soxhlet extraction requires a shorter time and negligible energy consumption to finish the extraction cycle of the plant, it remains viable for use. However, in order to obtain extracts with good antioxidant and anti-tyrosinase properties without any possible thermal degradation of the compounds, maceration for 72 h was determined to be the best technique to extract *C. indicum* leaves. Furthermore, the maceration strategy used in this study showed significant results, where the antioxidant activity was 29.02% and the anti-tyrosinase activity was 20.16%. This shows that the target of this study is effectively accomplished. Therefore, the extraction method of *C. indicum* leaves is a success. Overall, it can be said that *C. indicum* leaves could act as a potential source of natural antioxidants to be utilised for nutraceuticals and utilitarian food applications, as well as in the pharmaceutical and cosmetic industries concerning their viability as skin-brightening or lightening agents for their excellent anti-tyrosinase properties.

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PHYTOCHEMICAL PRODUCTION ENHANCEMENT ON *IN VITRO* CULTURE OF *Clinacathus nutans*

(Peningkatan Pengeluaran Fitokimia Pada Kultur *In Vitro Clinacathus nutans*)

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Abstract

This study assessed the effects of 6-Benzylaminopurine and wood vinegar on *in vitro* shoot multiplication, and as elicitors in enhancing the phytochemical content of *Clinacanthus nutans* extract. A nodal explant of *C. nutans* was cultured *in vitro* with single or combination treatments in MS medium supplemented with 6-Benzylaminopurine (BAP) or wood vinegar (WV). The growth performance of regenerated shoots was documented after eight weeks of culture. The total phenolic content, total flavonoid content, and antioxidant activities of the leaf extracts were also studied. The results demonstrated that all single treatments with BAP successfully regenerated and multiplied shoots and leaves. While in combination treatments, the data revealed that 2 mg/L BAP with 1% WV (B2WV1) medium treatment led to the highest number of shoots and leaves per explant and the highest total phenolic content and antioxidant activities in the leaf extract. This concludes that combining wood vinegar and BAP treatments in the culture medium caused significant shoot and leaf growth and enhanced the production of leaf's secondary metabolites. These findings highlight the potential of 6-Benzylaminopurine and wood vinegar as elicitors to enhance the production of secondary metabolites in *Clinacanthus nutans*, providing valuable insights for further research in plant biotechnology.

Keywords: *Clinacanthus nutans*, 6-Benzylaminopurine, wood vinegar, *in vitro*, elicitor, phytochemicals

Abstrak

Kajian ini menilai potensi 6-Benzilaminopurin dan cuka kayu untuk penambahan jumlah pucuk *in vitro* dan sebagai elisitor dalam meningkatkan kandungan fitokimia ekstrak *Clinacanthus nutans*. Eksplan nod *C. nutans* dibiakkan secara *in vitro* dengan rawatan tunggal atau gabungan dalam medium MS ditambah dengan 6-Benzilaminopurin (BAP) atau cuka kayu (WV). Prestasi pertumbuhan pucuk yang dijana semula telah didokumenkan selepas lapan minggu kultur. Jumlah kandungan fenolik, jumlah kandungan flavonoid, dan aktiviti antioksidan ekstrak daun turut dikaji. Keputusan menunjukkan bahawa semua rawatan tunggal dengan BAP berjaya menjana semula dan membiak pucuk dan daun. Manakala rawatan gabungan menunjukkan bahawa 2 mg/L BAP dengan 1% WV (B2WV1) membawa kepada bilangan pucuk dan daun tertinggi bagi setiap eksplan, serta jumlah kandungan fenolik dan aktiviti antioksidan tertinggi dalam daun. ekstrak. Ini menyimpulkan bahawa menggabungkan cuka kayu dan rawatan BAP dalam medium kultur menyebabkan pertumbuhan pucuk dan daun yang ketara serta meningkatkan pengeluaran metabolit sekunder daun. Penemuan ini menyerlahkan potensi 6-Benzilaminopurin dan cuka kayu sebagai elisitor untuk meningkatkan pengeluaran metabolit sekunder dalam *Clinacanthus nutans*, memberikan pandangan berharga untuk penyelidikan lanjut dalam bioteknologi tumbuhan.

Kata kunci: *Clinacanthus nutans,* 6-Benzilaminopurin, cuka kayu, *in vitro,* elisitor, fitokimia

Introduction

Clinacanthus nutans, a medicinal plant widely distributed in Malaysia, Thailand, Indonesia, and the Philippines, has traditionally been valued for its potential antioxidant properties [1]. This plant species contains antioxidant phytochemicals crucial in neutralizing free radicals and preventing cellular damage [2]. Antioxidants are indispensable in the body as they neutralize harmful free radicals, safeguard cells against oxidative damage, and reduce the risk of chronic diseases. Free radicals are associated with various severe conditions such as Parkinson's disease, Alzheimer's disease, atherosclerosis, heart attacks, and chronic fatigue syndrome [3]. External antioxidants can aid the body in efficiently scavenging free radicals. Currently, there is significant interest in antioxidants, particularly those that can counteract the anticipated harmful effects of free radicals in the human body and prevent the degradation of lipids and other food components [4]. Synthetic antioxidants like butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), tertiary butylated hydroquinone (TBHQ), and gallic acid esters were commonly used in the past century. However, their usage has been restricted due to concerns about potential severe health effects [5]. Given the health risks associated with synthetic antioxidants, there is a growing interest in developing natural-based antioxidants from plant sources. Overall, antioxidants derived from natural sources are preferred over synthetic ones [6, 7].

Secondary metabolites are compounds that do not directly contribute to plant growth, development, or reproduction. However, they play crucial roles in environmental interactions, particularly in defense against herbivores and viruses. Examples of these bioactive molecules include alkaloids, flavonoids, terpenoids, and other chemical compounds [8]. Phenolics, as secondary metabolites, are typically present in limited quantities within plant organisms, and their biosynthesis is influenced by various stress factors, both biotic (such as pathogen-induced damage) and abiotic (such as drought, extreme temperatures, and nutrient deficiencies) [9]. Consequently, plant *in vitro* culture provides a reliable system for inducing phenolic accumulation under controlled stress conditions [10]. *In vitro* culture, also known as micropropagation, involves growing and maintaining plant cells, tissues, or organs under aseptic conditions in a controlled environment. This technique facilitates the rapid multiplication of plantlets, allowing for the mass production of genetically identical plants in a short timeframe [11].

The tissue culture technique significantly enhances shoot regeneration and plant growth rates compared to traditional soil-based methods. However, some plants cultivated in culture media still exhibit low shoot regeneration rates due to inadequate nutrient absorption [12]. By employing *in vitro* selection and manipulating growth regulators, enhancing specific desirable traits in propagated plants becomes feasible. Tissue culture methodologies have been extensively utilized to augment the production of phytochemicals in plant species. The manipulation of culture conditions and specific growth regulators can stimulate the production of desired phytochemicals [13]. Nevertheless, the outcomes of tissue culture can vary based on the specific culture conditions employed. The biosynthesis of target phytochemicals can be prompted by adjusting the type and concentration of growth regulators and incorporating elicitors and other additives [14]. 6-Benzylaminopurine (BAP) is a cytokinin, a plant hormone crucial for regulating various physiological processes such as cell division, differentiation, and development. BAP fosters cell division and proliferation in *in vitro* cultivated plant tissues, leading to increased biomass. Additionally, BAP has been shown to influence the biosynthesis of secondary metabolites in plants [15].

Elicitation involves the application of specific chemical or physical agents to tissue cultures to stimulate the production of secondary metabolites. Elicitors may include substances like jasmonic acid, salicylic acid, or chitosan. Treatment with elicitors mimics the plant's natural defense mechanisms, promoting increased synthesis of phytochemicals [16]. Plants respond to various stressors, such as elicitors, by eliciting an immune response, triggering stress signaling pathways to produce a range of defense compounds collectively known as secondary metabolites [17]. These plant defense metabolites, such as phenolic acids, flavonoids,

and alkaloids, hold medicinal and commercial significance as health-promoting compounds [18].

Wood vinegar, or pyroligneous acid or liquid smoke, is a liquid produced from the destructive distillation of wood or biomass. It contains various compounds, such as acetic acid, butyric acid, catechol, and phenol [19]. Wood vinegar (WV) has been used for various purposes, including as a natural pesticide, fertilizer, and soil conditioner [20]. Wood vinegar contains organic compounds that can influence plant growth and development positively. These compounds act as natural plant growth regulators, promoting root growth, enhancing soil microbial activity, and improving plant nutrient uptake [21, 22]. These properties make wood vinegar an attractive alternative to synthetic growth promoters or elicitors. The use of wood vinegar as an elicitor in plant tissue culture and micropropagation offers several advantages and aligns with current trends in sustainable agriculture and plant biotechnology. Wood vinegar is a natural byproduct of wood processing and biomass decomposition, making it a sustainable and eco-friendly option for agricultural applications [23]. Previous studies have indicated that wood vinegar can stimulate callus formation, shoot proliferation, and root initiation in various plant species [24, 25]. WV contains natural plant growth regulators that can influence plant growth and development positively [19]. Unlike synthetic growth regulators or chemical elicitors, wood vinegar is derived from renewable resources and does not pose environmental risks associated with synthetic compounds.

Additionally, wood vinegar's ability to enhance nutrient availability and microbial activity in the soil can indirectly contribute to improved plant health and secondary metabolite production [26, 27]. In addition, wood vinegar has natural pesticidal properties, and its application can deter certain pests and diseases that may otherwise hinder plant growth [19]. Its phenolic compounds can stimulate the plant's own defense mechanisms by triggering the production of phytoalexins, which are antimicrobial substances produced by plants in response to pathogen attack. This strengthens the plant's ability to resist infections and enhances its overall resilience by increasing the secondary metabolites production [28]. Utilizing wood vinegar as an elicitor in tissue culture and micropropagation can be cost-effective compared to synthetic growth regulators or specialized elicitors [29]. Agricultural waste or biomass-derived products like wood vinegar offer a sustainable and economically viable alternative for enhancing plant growth [19] and phytochemical production [30, 31]. However, WV's complex composition requires careful dosage optimization for each application to maximize its effectiveness due to potential synergistic or antagonistic interactions among its constituents [32]. Therefore, optimizing WV administration is crucial to understanding its mechanism as an elicitor of plant bioactive compounds, as demonstrated here for an economically important crop like *C. nutans*.

As reported in previous research by Hashim et al. [7], an evaluation of phytochemical constituents across different parts of *C. nutans* revealed higher values of Total Phenolic Content (TPC) at 228.06 mg GAE/g DW, Total Flavonoid Content (TFC) at 29.76 mg QE/g DW, and quercetin at 0.021 g/g in intact leaf extracts. These findings also highlighted the superior antioxidant properties of intact *C. nutans*, with DPPH scavenging activity recorded at 15.50% and FRAP values at 15.68 $FeSO_4$/g. Notably, previous successful studies employing elicitors were conducted exclusively on intact *C. nutans* plants, such as the work by Mohan [33] using short-term heat stress as an elicitor, which significantly increased rutin, quercetin, and kaempferol production. However, there has been limited exploration of phytochemical values in *C. nutans in vitro* cultured plants, a little less on evaluating the effect of elicitor towards enhancement of plant secondary metabolites within the *in vitro* cultured plant. Therefore, there is a need for a preliminary study to *in vitro* culture this plant and screen its phytochemical constituents.

Combining BAP with other additional supplements will synergistically enhance the propagation process, as demonstrated in a previous study where kinetin, a cytokinin plant hormone, was used alongside BAP to achieve high rates of multiple shoot induction [34]. It is important to note that the impact of wood vinegar and BAP hormone on plant micropropagation can vary based on factors, specifically its application in the growth medium. Hence, optimization is crucial to determine the ideal application and concentration levels for promoting plant growth and maximizing the production of phenolic and flavonoid compounds. The present study aimed to develop an easy and efficient protocol for direct shoot

regeneration of *C. nutans* in medium with the addition of BAP or WV in single or by combination. Subsequently, the regenerated plants were subjected to different biochemical analytical assays through antioxidant enzyme activities, total phenolic and flavonoid content (TPC and TFC) determination, and free radical scavenging activity.

Materials and Methods

Wood vinegar preparation

The wood vinegar used in this agricultural study was sourced from a reputable supplier in Kuala Sepetang, Taiping, Perak, Malaysia. It was certified to be at least 95% pure, with an acetic acid concentration between 1% and 5%. The solution underwent filtration using Whatman No. 1 filter paper to remove debris, oily phases, or suspended particles, followed by autoclaving and storage at 4°C until further use.

Media preparation for shoot regeneration

Murashige and Skoog (MS) medium were used for shoot regeneration. MS powder at 0.44 % (w/v) was dissolved in distilled water with 3% (w/v) of sucrose. The 6-Benzylaminopurine (BAP) or WV was added in a single or combination medium at different concentrations in preparation for nine different medium treatments. Gelrite (7 g) was dissolved in the solution for 1 liter of medium final volume. The culture medium was autoclaved for 15 minutes at 121 °C and 15 psi. Single treatments were prepared individually by adding 6-Benzylaminopurine (BAP) or wood vinegar (WV) to the culture medium. Different concentrations of BAP (0.5, 1, and 2 mg/L) and WV (1, 2, and 4 %) were used in separate batches to assess their effects on plant tissue cultures. On the other hand, the combination treatments were prepared by adding a fixed concentration of 2 mg/L BAP to the culture medium. Various concentrations of wood vinegar (WV) ranging from 1, 2, and 4% were combined with the BAP-containing medium. The 2 mg/L of BAP hormone was used based on optimization studies conducted during preliminary testing that yielded the most favorable outcomes in promoting plant growth and development. Control groups were included in the experimental setup and served as baseline references for comparison against the single and combination treatments.

Establishment of aseptic culture

The sterilization step was according to Hashim et al. [1]. The nodal explants were excised from the plant and cleaned adequately under running tap water for five minutes. Explants were prewashed for one minute with 70% (v/v) ethanol and then rinsed thrice with sterile filtered water. Next, explants were dipped inside 0.1% $HgCl_2$ for 30 minutes and rinsed thrice for 5 minutes each. The sterile explant was then air-dried.

Shoot regeneration

The explants were inoculated vertically on MS medium for culture initiation. The cultures were incubated at 25 ± 2 °C under light (40 µmol m^{-2} s^{-1} Photosynthetic Photon Flux Density (PPFD) for 25–30 days. The growth performances of successfully regenerated shoots and leaves were measured in week eight by calculating the number of shoots per explant, shoot length, number of leaves per explant, and leaf length. The shoot and leaf length were measured by the unit of cm. The regenerated shoots were then sub cultured once every four weeks in a new medium.

Extraction

The regenerated plant was oven-dried (60°C, 24 h), and the extraction was performed according to the protocol described by Ali et al. [35] with minor modifications. Each finely ground sample (100 mg) was mixed with methanol (10 ml). The mixtures were sonicated (10 min) 3 times with a resting period of 30 min in between and centrifuge (8000 rpm, 10 min). The supernatants were collected and stored at 4° C.

Total phenolics and flavonoids determination

The total phenolic content of samples was determined using Folin-Ciocalteu reagent and gallic acid as standard, according to Singleton and Rossi [36], with minor modifications. Briefly, 1 mg of dried crude extract was dissolved in 1 mL methanol. Next, 0.2 ml of the mixture was added with 0.6 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent. After 5 min, 1 mL of saturated sodium carbonate solution (8% w/v) was added to the mixture, and the volume was made up to 3 mL with distilled water. The reaction was kept in the dark for 30 min. After centrifuging, the absorbance of extracts was measured using a visible spectrophotometer at 765 nm against a reagent blank without the extract. A stock solution of gallic acid (500 mg/L) was prepared in

distilled water and diluted to appropriate concentrations (5–500 mg/L) to construct the calibration curve. The total phenol concentration in samples was measured using the calibration equation ($y = 0.0024x$, $r^2 = 0.9896$) and expressed as µg of gallic acid equivalent (GAE) per g dry weight. All assays were carried out in triplicate.

The aluminum chloride colorimetric method was used to determine the total flavonoid content of the sample. Quercetin was used to make the standard calibration curve for total flavonoid determination. The standard solutions of quercetin were prepared by serial dilutions using methanol (5–200 mg/L). An amount of 0.6 mL diluted standard quercetin solutions or extracts was separately mixed with 0.6 mL of 2% aluminum chloride. After mixing, the solution was incubated for 60 min at room temperature. The absorbance of the reaction mixtures was measured against a blank at 420 nm wavelength with a Varian UV-Vis spectrophotometer. The concentration of total flavonoid content in the test samples was calculated from the standard curve equation ($y = 0.0153x$, $r^2 = 0.9615$), and results were expressed as mg quercetin equivalent (QE)/g of dried plant material. All the determinations were carried out in triplicate.

Antioxidant analyses

The DPPH free radical scavenging assay described by Gabr et al. [21] was used for antioxidant activity determination. The stock reagent solution (1 mM) was prepared by dissolving 22 mg of DPPH in 50 ml of methanol and stored at 20 °C until use. The working solution (0.06 mM) was prepared by mixing 6 ml of stock solution with 100 ml of methanol. Extract samples (1mg/ml) and synthetic antioxidant (BHA) solutions (0.1 ml of each) were vortexed for 30 s with 3.9 ml of DPPH working solution. After a 30-minute incubation period at room temperature in the dark, absorbance was recorded at 515 nm using a spectrophotometer. The DPPH solution without extract served as a control. The scavenging activity was calculated as follows:

$$\% \text{ inhibition} = [(\text{absorbance of control} - \text{absorbance of sample})/\text{absorbance of control})] \times 100 \quad (1)$$

Ferric Reducing Antioxidant Power (FRAP) assay was carried out according to the method of Wee [22]. FRAP reagent was freshly prepared by mixing 300 mM sodium acetate (pH 3.6), 10 mM 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) dissolved in 40 mM of HCl, and 10 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) in a ratio of 10:1:1. A total of 200 µl of extracts was gently mixed with 3 ml of FRAP reagent and incubated at 37°C in water bath for 30 minutes. The increase in absorbance at 593 nm was measured after 30 minutes against the FRAP reagent blank. Standards of known FeSO4 (0-500 µmol) were used to generate a calibration curve ($y = 0.0025x + 0.05$, $R^2 = 0.9972$), and the results were expressed as mg $FeSO_4$ equivalent per g dry weight samples (DW).

Statistical analysis

Each sample for the analytical test was carried out in triplicate, and the date was presented as average ± SE of the three-absorbance reading. The data was analyzed by the Analysis of variance (ANOVA) followed by Duncan's multiple range test. The data analysis was performed using SPSS version 20.

Results and Discussion

Shoot growth performance

A comparative analysis was conducted between single and combined treatments involving 6-Benzylaminopurine (BAP) and wood vinegar (WV) to assess their efficacy in establishing cultures. The study discerned that including BAP hormone in the single treatment medium notably stimulated shoot growth, with the optimum promotion observed at 2 mg/L BAP, resulting in 2.77 shoots per explant (refer to Table 1). Conversely, when using the MS medium with a sole addition of wood vinegar (WV), successful initiation and significant promotion of shoots were achieved. Specifically, the WV1 medium (1% WV) successfully initiated 2.64 shoots per explant (refer to Table 1). Moreover, the combination treatment involving 2 mg/L BAP and 1% WV (B2WV1) yielded higher plant shoot numbers and lengths than single treatments. The MS medium supplemented with B2WV1 exhibited the maximum number of shoots per explant (10.60), showcasing its superior efficacy in promoting shoot proliferation. Additionally, this supplemented medium demonstrated notable shoot length, measuring 2.51 cm, as indicated in Table 1.

Table 1. Regenerated plant growth performance after 8 weeks of culture

Code	Number of Shoots Per Explant	Length of Shoots (cm)	Number of Leaves Per Explant	Length of Leaves (cm)
CS	1.33 ± 0.03 [h]	0.72 ± 0.01 [e]	2.70 ± 0.02 [i]	0.64 ± 0.02 [d]
Single				
B0.5	1.30 ± 0.02 [i]	0.63 ± 0.02 [f]	2.77 ± 0.01 [h]	0.60 ± 0.04 [e]
B1	1.63 ± 0.02 [g]	0.89 ± 0.02 [d]	3.53 ± 0.02 [f]	0.90 ± 0.02 [c]
B2	2.77 ± 0.01 [c]	1.90 ± 0.06 [b]	4.17 ± 0.02 [c]	1.59 ± 0.02 [a]
WV1	2.64 0.01 [d]	0.46 0.04 [h]	3.91 0.02 [k]	0.36 0.02 [f]
WV2	0.00 [j]	0.00 [j]	0.00[k]	0.00 [h]
WV4	0.00 [j]	0.00 [j]	0.00[k]	0.00 [h]
Combination				
B2WV1	10.60 ± 0.03 [a]	2.51 ± 0.05 [a]	9.30 ± 0.01 [a]	0.56 ± 0.01 [e]
B2WV2	2.17 ± 0.03 [f]	0.24 ± 0.02 [i]	1.33 ± 0.01 [j]	0.39 ± 0.03 [f]
B2WV4	0.00[j]	0.00 [j]	0.00 [k]	0.00 [h]

(CS: control; B0.5: 0.5 mg/L BAP; B1; 1 mg/L BAP; B2: 2 mg/L BAP; WV1: 1% WV; WV2: 2% WV; WV4: 4% WV; B2WV1: 2 mg/L BAP + 1 % WV; B2WV2: 2 mg/L BAP + 2 % WV; B2WV4: 2 mg/L BAP + 4 % WV). Values (separate significance for each parameter) with the same superscripts were not found to be significantly different from each other.

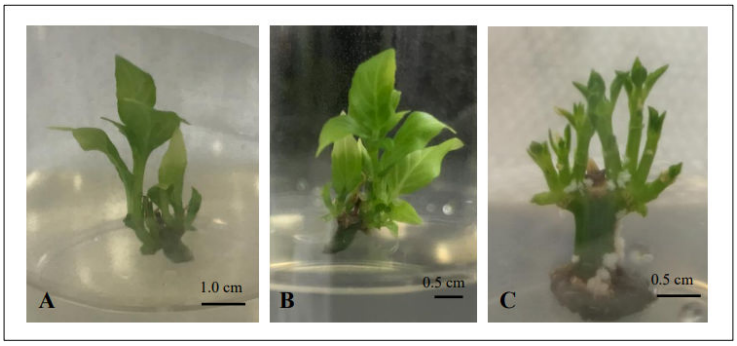


Figure 1. Development of regenerated plant from nodal explant culture in medium supplemented with (A-B) 2mg/L BAP (B2) (C) 2 mg/L BAP + 1 % WV (B2WV1)

Leaf numbers serve as a critical parameter for evaluating the *in vitro* growth of explants, particularly concerning their developmental progression. Data revealed a significant impact of the WV and BAP addition on leaf production in regenerated shoots. An optimal leaf count was observed in the combination treatment. Specifically, the MS medium supplemented with 2 mg/L BAP and 1% WV (B2WV1) demonstrated the highest leaf production, averaging 9.30 leaves per explant. However, increasing the WV concentration to 2% (B2WV2) resulted in a decline in leaf number to 1.33 leaves per explant. Moreover, although the leaf number peaked in the B2WV1 treatment, the leaf size was relatively small, averaging 0.56 cm per leaf. In contrast, a sole treatment of 2 mg/L BAP in the MS medium yielded the longest leaves, averaging 1.59 cm in length. Figure 1 visually depicts the development of adventitious shoots from nodal explants of *C. nutans* under single (B2) and combination (B2WV1) medium treatments. Notably, the B2 treatment showed robust growth of leafy shoots, while B2WV1 exhibited distinctive adventitious shoots with smaller leaf sizes.

This study demonstrates the substantial positive impact of synergistically combining wood vinegar (WV) and the BAP hormone to enhance plant growth. It highlights their potential as advantageous supplements in tissue culture applications. Previous studies have identified acids and phenols as crucial wood vinegar constituents, exhibited significant biological activity and promoted plant growth [37]. WV is acknowledged for its capacity to improve crop yield, bolster plant health, and elevate harvest quality [19]. The organic acids, phenols, and other organic compounds found in wood vinegar (WV)

act as typical allelochemicals that regulate plant growth by influencing ion absorption, respiratory metabolism, hormone equilibrium, and protein synthesis [27]. The exogenous application of phenolic compounds in plants plays a crucial role in enhancing plant growth and mitigating the effects of abiotic and biotic stress through mechanisms such as promoting the lignification of plant cell walls, which enhances shoot length [38]. This observation aligns with the findings of Ofoe et al. [39], who highlighted that the synergistic effect of the combined foliar application of WV and soil fertilizer contributed to increasing tomato leaves' growth performance.

In addition, the growth of regenerated shoots and leaves in the combination treatment is significantly influenced by the concentrations of wood vinegar (WV). High concentrations of WV exert an inhibitory effect on plant growth, leading to a decline in shoot production with further increases in WV concentration (2% and 4%) in the combination treatment. The detrimental effects of high WV concentrations can be attributed to elevated medium acidity levels, which are unfavorable for plant development and can induce phytotoxicity, acting as stressors and potentially functioning as herbicides [31]. Acidic conditions can also adversely affect plant growth by damaging cell structures and suppressing growth responses [40]. Furthermore, specific components in wood vinegar may interfere with nutrient uptake or disrupt physiological processes within plants, potentially leading to reduced leaf size or altered growth patterns. Therefore, adding WV at an appropriate concentration is crucial to optimize shoot growth [19].

This study suggests that moderate concentrations of WV, when used in conjunction with BAP, create a conducive environment for plant growth by enhancing the activity of key enzymes involved in metabolic pathways related to stress response and secondary metabolite production. This study highlights the potential of combining WV and BAP hormone as a synergistic treatment to enhance plant growth and resilience. The organic compounds in WV, particularly when used at optimal concentrations, can significantly improve plant health and productivity. Future research should focus on elucidating the precise molecular mechanisms underlying these synergistic effects and exploring the long-term implications of such treatments. Additionally, further studies could investigate the potential application of this combination in different plant species and under various environmental conditions to generalize the findings and develop comprehensive guidelines for practical use in agriculture and tissue culture. By understanding and optimizing the concentrations and conditions for applying WV and BAP, their full potential could be harnessed to improve plant growth and yield, paving the way for more sustainable and efficient agricultural practices.

Effect of hormonal and WV treatment on polyphenols content of *in vitro* cultured leaf extract

Figure 2 illustrates the varying phenolic compound concentrations in different treatments. Applying BAP and WV has significantly elevated Total Phenolic Content (TPC) in *C. nutans*. Specifically, 1% WV (WV1) increased TPC from 23.68 to 77.45 mg GAE/g DW, representing a 3.27 times increment. Meanwhile, 2 mg/L BAP (B2) raised TPC from 23.68 mg GAE/g DW to 121.95 mg GAE/g DW, marking a more increment given 5.15 times. Furthermore, the combined treatment of 2 mg/L BAP and 1% WV (B2WV1) exhibited the highest phenolic increment, reaching 219.75 mg GAE/g DW from the initial 23.68 mg/g (control) given 9.27 times increase.

A slightly different trend was observed in the total flavonoid content of the extract. The highest total flavonoid content (56.60 mg QE/g DW) was noted with the application of 2 mg/L BAP (B2), representing 5.25 times increase. Following this, B2WV1 displayed 35.95 mg QE/g DW, 3.27 times increase compared to the control's 10.99 mg QE/g DW (Figure 2). These results indicated that supplementation of both BAP and WV in MS medium has a stimulatory effect on the accumulation of phenolics and flavonoids in the regenerated plant of *C. nutans*. This phenomenon can be attributed to the stimulating effects of BAP on secondary metabolism and the bioactive attributes of WV components.

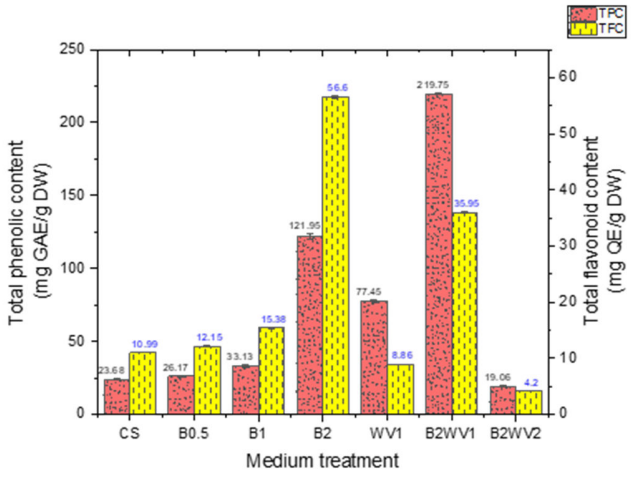


Figure 2. Total phenolic and flavonoid compounds of regenerated shoots of *C. nutans* from different medium treatments

Exogenous application of BAP hormone at high concentration has been shown to enhance the yield and medicinal quality of *C. nutans* by promoting the accumulation of phenolics and flavonoids. Due to oxidative stress in target cells, the elicitor stimulates the plant to increase immune responses and PAL gene expression, leading to an increase in secondary metabolites, especially phenolic compounds, which are part of the systemic acquired resistance process [41]. Zhang et al. [42] demonstrated that BAP can upregulate gene expression levels, particularly PAL, 4CL, CHS, and F3GT, crucial enzymes in the flavonoid synthesis pathway. Baskaran et al. [43] reported that the application of BAP in media resulted in elevated levels of phenolic and flavonoid content in the shoot cultures of *Coleonema pulchellum*. Enhancement in the PAL activity in response to exogenously applied TDZ and BA is linked to the stimulating effects of these PGRs on the regulation of the PAL gene at the transcriptional level [44].

Furthermore, the elevated levels of phenolics and flavonoids in *C. nutans* following BAP treatment can be attributed to the inclusion of Wood vinegar (WV). In this investigation, WV acts as an elicitor by effectively enhancing the production of secondary metabolites, mainly phenolic compounds and organic acids. The phenols derived from Wood vinegar (WV) are pivotal in mitigating plants' abiotic and biotic stress effects through various mechanisms. Phenols prevent pathogens from infiltrating the host plant and influence the activity of specific enzymes like antioxidant enzymes and the synthesis of compounds such as proline and phenolic compounds [45]. Additionally, acetic acid in WV may activate plant defense gene regulators. This aligns with prior research indicating that acetic acid in the medium successfully boosted the production of plant secondary metabolites [46, 47, 48, 49].

The current study reveals that combining 6-Benzylaminopurine (BAP) hormone and wood vinegar generates a synergistic effect, enhancing stress response and triggering increased production of secondary metabolites. The medium treatment influences the activity of enzymes involved in flavonoid and phenol biosynthesis. Earlier research has shown that Karrikin present in WV function similarly to other phytohormones, interacting with gibberellins, ethylene, and cytokinin to regulate plant growth and productivity [50, 39]. This suggests that WV may have induced and collaborated with enzymes and BAP hormones to increase plant yield. Integrating BAP with WV not only enhances the accumulation of beneficial secondary metabolites but also suggests a practical approach for improving plant resilience and productivity.

The DPPH and FRAP assay (Figure 3) indicate significant differences in the ability to scavenge free radicals among all medium treatments. The plant grown in medium with 2 mg/L BAP and 1% WV (B2WV1)

extract exhibited a more potent antioxidant activity (DPPH = 5.59 % and FRAP = 25.46 g $FeSO_4$/g DW) than 2 mg/L BAP (B2) given DPPH = 2.42 % and FRAP = 12.37 g $FeSO_4$/g DW. All extracts could directly react and quench the DPPH and Fe^{3+} radicals. The combination treatment of BAP and WV (B2WV1) exhibited the highest activity when compared with a single treatment of BAP or WV individually.

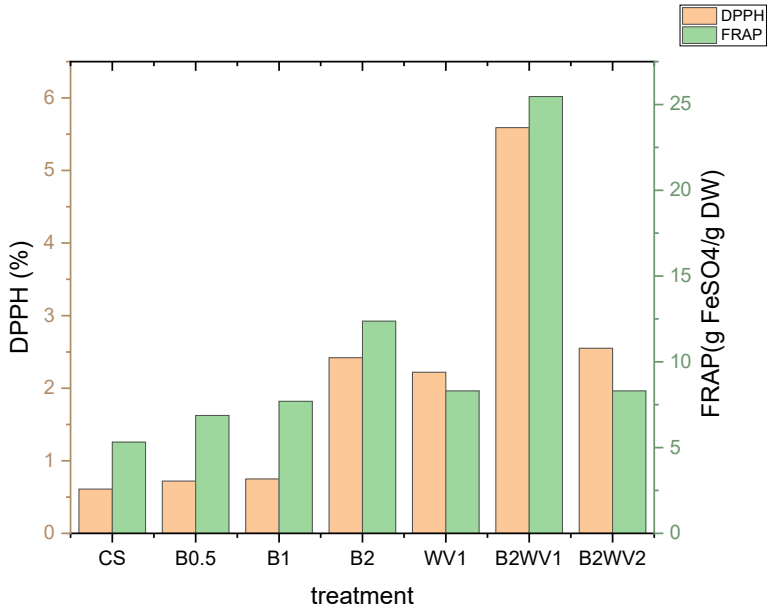


Figure 3. DPPH and FRAP activity of regenerated plant of *C. nutans* that cultured on MS medium comprising BAP and WV

Correlation coefficient of TPC, TFC, and DPPH and FRAP assay is given in Table 2. Results showed that TPC is highly correlated with DPPH and FRAP assay (R2 = 0.991 and R2 = 0.929). This means that TPC is highly contributed to the antioxidant capacities of *C. nutans* extracts more than the correlation coefficient with TFC towards DPPH and FRAP assay (R2 = 0.258 and R2=0.312), which might partially be due to their lower antioxidant activity (Fig. 2c).

Table 2. Correlation between measured parameters of the regenerated plant of *C. nutans*

	TFC	**DPPH**	**FRAP**
TPC	0.307*	0.991*	0.929*
TFC		0.258*	0.312*
DPPH			0.890*

Correlation is significant at the 0.05 level with the label (*).

The positive correlations between phenolic and flavonoid levels elucidate their role in enhancing the antioxidant potential of the extract. This study reinforces the findings of Ghasemzadeh et al. [51], Khoo et al. [52], and Baharuddin et al. [53], where the robust antioxidant activity of *C. nutans* extract was attributed to its phenolic and flavonoid content. Phenolics and flavonoids are recognized for their antioxidant properties, facilitated by their hydroxyl groups that contribute to radical scavenging capabilities [54]. Kapoor et al. [55] also reported a positive correlation analysis between Total Phenolic Content (TPC) and DPPH free radical scavenging activity in *R. imbricata* callus cultures exposed to various light spectra. Similar positive correlations have been observed between phenolics,

flavonoids, and antioxidant activity in diverse medicinal plants like *Cichorium pumilum* [56].

Incorporation of BAP and WV into MS basal media not only regulated the *in vitro* morphogenesis processes, leading to direct shoot regeneration in *C. nutans*, but also influenced the production of phenolic and flavonoid compounds. The *in vitro* synthesis of these phytochemical constituents is intricately governed by direct shoot induction from the cultured explants. The varying effects of BAP and WV treatments on the production of these beneficial compounds can be ascribed to several factors that impact *in vitro* shoot regeneration in *C. nutans*, such as the maintenance of the regenerated shoot phase *in vitro* and the defense mechanisms of plant tissue against supplemented growth regulators [57]. By understanding and optimizing the concentrations and conditions for applying WV and BAP, their full potential could be harnessed to improve plant growth and yield, paving the way for more sustainable and efficient agricultural practices. Future research should focus on elucidating the precise molecular mechanisms underlying these synergistic effects and exploring the long-term implications of such treatments. Additionally, further studies could investigate the potential application of this combination in different plant species and under various environmental conditions to generalize the findings and develop comprehensive guidelines for practical use in agriculture and tissue culture.

Conclusion

In conclusion, it was determined that *C. nutans* has good plant regeneration potential from nodal explant in MS medium supplemented with BAP hormone and wood vinegar. The highest number of shoots and leaves per explant was found on MS media supplemented with 2 mg/L BAP and 1% WV. Also, the present study showed that adding BAP and WV gives the highest elicitation effect on promoting the production of total phenolic and flavonoids. The antioxidant activities in *C. nutans* regenerated plant extract are also highly correlated with the total phenolic content. This study showed that adding BAP and WV, especially in combination treatment for promoting the *C. nutans* plant regeneration, also enhances the content of pharmacologically valuable metabolites in *C. nutans*.

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EFFECTS OF *Pandanus amaryllifolius* LEAF EXTRACT CONCENTRATIONS ON THE MICROSTRUCTURAL PROPERTIES OF ZnO NANORODS VIA BIOLOGICALLY SYNTHESIZED ZnO SEED LAYER FORMATION

(Kesan Kepekatan Ekstrak *Pandanus amaryllifolius* Terhadap Ciri-Ciri Mikro dan Struktur Nanorod ZnO Melalui Pembentukan Lapisan Benih ZnO Bersifat Biologi)

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Abstract

An integrated strategy of spin-coating and solution immersion methods was carried out to produce well-aligned zinc oxide nanorods (ZnO NRs). First, a biologically synthesized ZnO seed layer (B-ZnO SL) solution was prepared using zinc nitrate hexahydrate as a precursor added with *Pandanus amaryllifolius* (PA) leaf extract at different concentrations (3% w/v, 6% w/v, and 9% w/v) as a stabilizer. This B-ZnO SL solution was deposited on a glass substrate using the spin-coating method. Second, ZnO-HMTA solution was prepared using zinc nitrate hexahydrate and hexamethylenetetramine (HMTA) as a precursor and stabilizer, respectively. This ZnO-HMTA solution was deposited onto the previously prepared B-ZnO SL. The optimum PA leaf extract concentration for enhanced ZnO NRs was determined by measuring the absorbance of B-ZnO SL via ultraviolet–visible analysis. The highest absorbance corresponded to B-ZnO SL that consisted of 6% w/v of PA leaf extract with an absorption peak of 375 nm. Using X-ray diffraction, three primary peaks for ZnO NRs were detected at $2\theta = 31.77°$, 34.42°, and 36.24°, corresponding to (100), (002), and (101) ZnO crystallographic planes, respectively. By using field emission scanning electron microscopy, the smallest diameter of 96 nm was measured for the ZnO NRs synthesized on the B-ZnO SL with 6% w/v PA leaf extract. This study is the first to report on the efficacy of PA leaf extract at different concentrations to investigate the development of a highly crystalline and dense ZnO NRs structure.

Keywords: zinc oxide, nanorods, plant extract, spin-coating, solution immersion

Abstrak

Satu strategi bersepadu kaedah lapis-putar dan perendaman larutan telah dilakukan untuk menghasilkan nanorod zink oksida yang

teratur (ZnO NRs). Pertama, larutan lapisan benih ZnO yang dihasilkan secara biologi (B-ZnO SL) disediakan menggunakan zink nitrat heksahidrat sebagai perintis dan ditambah ekstrak daun *Pandanus amaryllifolius* (PA) pada kepekatan berbeza (3% w/v, 6% w/v, dan 9% w/v) sebagai penstabil. Larutan B-ZnO SL ini telah dimendapkan pada substrat kaca menggunakan kaedah lapis-putar. Kedua, larutan ZnO-HMTA disediakan menggunakan zink nitrat heksahidrat dan heksametilenatetramina (HMTA) masing-masing sebagai perintis dan penstabil. Larutan ZnO-HMTA ini dimendapkan ke atas B-ZnO SL yang disediakan sebelumnya. Kepekatan ekstrak daun PA yang optimum bagi peningkatan ciri-ciri ZnO NRs ditentukan dengan mengukur serapan B-ZnO SL melalui analisis ultralembayung–tampak. Serapan tertinggi yang diperoleh selaras dengan B-ZnO SL yang mengandungi 6% w/v ekstrak daun PA pada puncak serapan 375 nm. Dengan menggunakan belauan sinar-X, tiga puncak utama ZnO NRs dikesan pada 2θ = 31.78°, 34.47°, dan 36.26°, masing-masing sejajar dengan satah kristalografi ZnO (100), (002), dan (101). Dengan menggunakan mikroskopi elektron pengimbasan pancaran medan, diameter terkecil 96 nm diukur bagi ZnO NRs yang dihasilkan di atas B-ZnO SL dengan 6% w/v ekstrak daun PA. Kajian ini adalah yang pertama melaporkan keberkesanan ekstrak daun PA pada kepekatan yang berbeza untuk mengkaji perkembangan struktur ZnO NRs yang sangat berhablur dan padat.

Kata kunci: zink oksida, nanorod, ekstrak tumbuhan, lapis-putar, perendaman larutan

Introduction

Oriented zinc oxide nanorods (ZnO NRs) are particularly remarkable among diverse nanostructures due to their elevated levels of crystallinity, well-defined density of states, efficient one-dimensional charge transport, and substantial specific surface area. These attributes render them highly applicable for implementation in semiconductor devices and various other optoelectronic applications. While nanostructured ZnO is typically nontoxic, the synthesis process utilizes solvents or chemicals with moderate to high toxicity [1-3]. Hence, researchers are using a more eco-sustainable approach, such as solution immersion technique [4-5]. This method mainly uses monoethanolamine (MEA) or hexamethylenetetramine (HMTA) as a stabilizer to produce nanostructured ZnO such as ZnO NRs. Using this approach prevents the aggregation of the precursor solution [6-8]. As the eco-sustainable approach has evolved toward plant-mediated synthesis, the achievements to date and prospects of a plant-mediated method to produce nanostructured ZnO have been summarized in several reports [9-11]. In recent years, comparable rod-shaped nanostructured ZnO has been synthesized using leaf extract [12-16]. However, it is still challenging to produce consistent ZnO NRs using this strategy, especially via single-step synthesis as reported in earlier research [17]. The primary objective of this study is to synthesize oriented, well-aligned ZnO NRs with improved crystallinity via biologically synthesized ZnO seed layer (B-ZnO SL) formation. This was accomplished via an integrated strategy of spin-coating and solution immersion methods. *Pandanus amaryllifolius* (PA) leaf extract at different concentrations were used to prepare B-ZnO SL via spin-coating method. The optimum B-ZnO SL was determined by UV–vis analysis. The ideal microstructure of B-ZnO SL was also investigated using FESEM to correlate the efficacy of PA leaf extract concentrations during B-ZnO SL preparation with the development of oriented and well-aligned ZnO NRs. Our study anticipates that the incorporation of B-ZnO SL will substantially enhance the structural and morphological characteristics of ZnO NRs.

Materials and Methods

To achieve the study objectives, the methodology was separated into several stages: (1) Preparation of glass substrate, (2) i) Preparation of B-ZnO SL solution, ii) Deposition of B-ZnO SL solution onto a cleaned glass substrate via spin-coating method, (3) i) Preparation of ZnO-HMTA solution, ii) Deposition of ZnO-HMTA solution onto B-ZnO SL via solution immersion method. The resulting ZnO NRs on B-ZnO SL (top view) is shown in Figure 1 (a) while Figure 1 (b) illustrates the schematic diagram of the cross-sectional area of sample.

The glass preparation stage is critical for successfully synthesizing refined crystalline and well-aligned ZnO NRs. The B-ZnO SL solution was prepared using 0.1 M zinc nitrate hexahydrate [$Zn(NO_3)_2{\cdot}6H_2O$] as a precursor with PA leaf extract at concentrations of 3% w/v, 6% w/v, and 9% w/v as a stabilizer. The solution was mixed based on the standard procedure as described elsewhere [17,18-19]. By using the spin-coating method, 10 drops of the prepared solution were deposited on the glass substrate at 3000 rpm for 30 s. Then, the deposited ZnO seed layer was preheated at 150 °C for 10 min. The procedure was repeated five times to produce 5 seed

layers of ZnO. After the spin-coating process, the deposited ZnO seed layer was annealed at 450 °C for 1 h. Next, equimolar ZnO-HMTA solution was prepared using $Zn(NO_3)_2{\cdot}6H_2O$ and HMTA at 0.1 M as a precursor and stabilizer, respectively. The solution mixing process was performed as described elsewhere [17-19]. The optical properties of B-ZnO SL synthesized using different PA leaf extract concentrations were determined using a UV–vis spectrophotometer (Cary 60 UV-Vis). The morphologies of optimum B-ZnO SL and ZnO NRs were observed using field emission electron scanning microscopy (FESEM; JEOL JSM 7600F), while the phase and crystal structure of ZnO NRs were assessed by using X-ray diffraction (XRD; PANalytical X'Pert Powder). Finally, energy-dispersive X-ray (EDX) analysis was done to confirm the presence of zinc and oxygen.

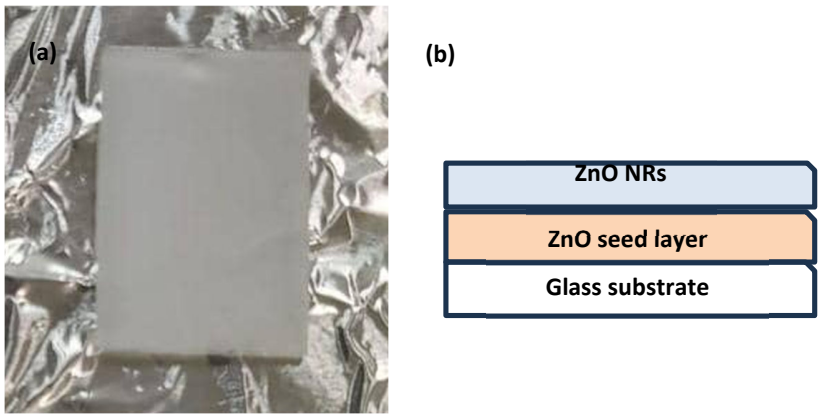


Figure 1. (a) Top view, (b) schematic diagram of cross section view of ZnO NRs deposited onto B-ZnO SL

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Results and Discussion

Optical properties of B-ZnO SL

The optical properties of B-ZnO SL synthesized with different PA leaf extract concentrations as a stabilizer are shown in Figure 2. The absorption peak centered at *375 nm is the characteristic peak for hexagonal wurtzite ZnO, which is the same pattern obtained from previous research [20-22]. The absorption peak shows a red shift compared to that of bulk ZnO (365 nm) [23]. This red shift can be attributed to the development of shallow levels inside the band gap due to the presence of foreign atoms in the lattice [24,25]. The absorbance of the ZnO seed layer increased as PA leaf extract concentration was increased to 6%. The use of PA leaf extract as a stabilizer in the ZnO seed layer might have provided more flavonoid compounds, as evidenced by the increase in UV absorption compared to the control sample (0% PA). The optimum PA leaf extract concentration as a stabilizer was determined to be 6% w/v based on the UV–vis finding.

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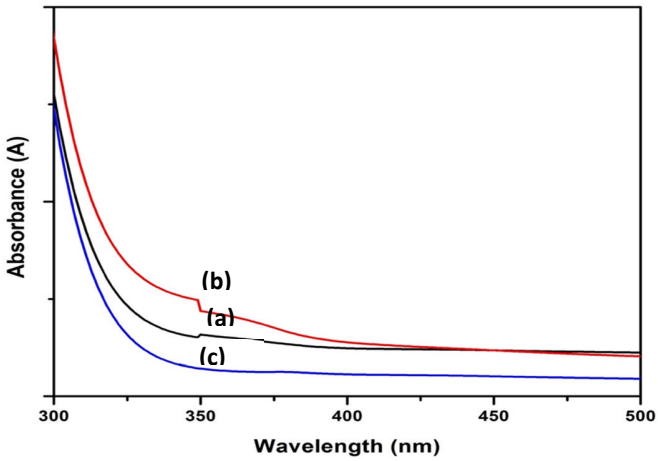


Figure 2. Absorbance of B-ZnO SL synthesized using PA leaf extract with concentrations of (a) 3% w/v, (b) 6% w/v, and (c) 9% w/v

Morphological properties of B-ZnO SL

The morphology of B-ZnO SL synthesized with 6% w/v PA leaf extract was studied and compared with that of the ZnO seed layer at which 0% w/v of PA was utilized (control sample). The B-ZnO SL thin film prepared with 6% PA leaf extract has a spongelike, porous morphology, as shown in Figure 3 (b). Laurenti and Cauda described this morphology as a porous nanobranched structure with a high specific surface area [26]. With a high specific surface area, the porous B-ZnO SL provides high energy for the formation of well-aligned nanorod structures. The particles had an average size in the range of 8–10 nm. In comparison, the ZnO seed layer thin film prepared using 0% PA leaf extract (Figure 2 (a)) has a slightly bigger particle size (12.38 nm). The 0% PA sample also consisted of many aggregates. Poor colloidal stability of nanoparticles in fluid may cause aggregation [27,28]. ZnO nanoparticles also have a high tendency to aggregate which may lower the surface energy. As aggregates become larger and denser with strong bonds, the surface reactivity decreases. The utilization of 6% PA as a stabilizer in B-ZnO SL provided more biomolecules such as flavonoid compounds. These compounds act as a stabilizing agent that resulted in homogeneous ZnO nanoparticles compared to the control sample.

Microstructural properties of ZnO NRs

The orientation of ZnO NRs significantly changed when PA leaf extract concentration was varied when preparing the B-ZnO SL growth solution. This is evidenced by the increase in UV–vis absorbance as PA leaf extract concentration was increased (Figure 2). UV–vis absorbance was related with the flavonoid compounds in the PA leaf extract, which were responsible in coordinating and stabilizing the ZnO nanoparticles within B-ZnO SL. This directly impacted the overall microstructural properties of ZnO NRs (Figures 4 & 5). The samples exhibit significant diffraction peaks (Figure 4), indicating a comparable enhanced crystallinity to ZnO NRs synthesized via the integrated approach used in this study. Furthermore, the absence of other peaks suggests no contaminants in the sample. This XRD pattern is known as the hexagonal wurtzite structure (JCPDS No. 36-1451). The three peaks at $2\theta = 31.77°$, 34.42°, and 36.24° correlate to the (100), (002), and (101) crystalline planes of ZnO, respectively. These three dominant peaks are consistent with those found in earlier research using hydrothermal [29] and sol–gel immersion processes [30]. As PA leaf extract concentration was increased during the preparation of B-ZnO SL, the peak intensity of ZnO NRs in XRD data also increased. The intensity or the count numbers can be assigned as the relative crystallinity of the sample. The decrease in ZnO NRs crystallinity when 9% w/v of PA leaf extract was used can be explained by the excessive amount of biomolecule compounds present in the B-ZnO SL solution. Instead of stabilizing the Zn and

O atoms, this excessive stabilizing agent was no longer efficient, producing lower ZnO purity [31]. In addition, the ZnO NRs synthesized in this study showed a superior purity as only three prominent peaks appeared in XRD, compared with the rod-shaped ZnO synthesized in a previous study [32].

The synthesized ZnO NRs (Figure 5 (a)) had a rodlike structure with irregular orientation as shown by the FESEM images in Figure 5 (a). This structure caused a bent or tilted configuration when the NRs were deposited onto the B-ZnO SL at 0% PA leaf extract (control sample). Utilizing different PA leaf extract concentrations as stabilizers during the preparation of the B-ZnO SL yielded distinct properties in terms of rod structure orientation and the diameter of the final deposited ZnO NRs. The smallest diameter of ZnO NRs measured was approximately 96 nm. The smaller diameter size and the uniformity of ZnO NRs when deposited onto B-ZnO SL (6% w/v) can be correlated with a synergistic effect between the increase in crystallinity as evident in Figure 4 (b). This is also further supported by the prominent (002) peak observed in the XRD data. This is due to the presence of dense ZnO nanoparticles with lower number of aggregates as shown in Figure 3 (b). With lower number of aggregates, more energy was directed to coordinate the Zn and O efficiently in the ZnO NRs. This facilitates the vertically aligned ZnO NRs perpendicular to the substrate as evident in Figure 5 (c). These findings show a remarkable microstructural improvement compared to previous rod-shaped ZnO fabricated via other biosynthesis routes [33]. EDX was conducted to further evidence the presence of elemental zinc and oxygen in ZnO NRs, and the result is shown in Figure 6. Only Zn and O atoms were detected, confirming a high purity of the prepared ZnO.

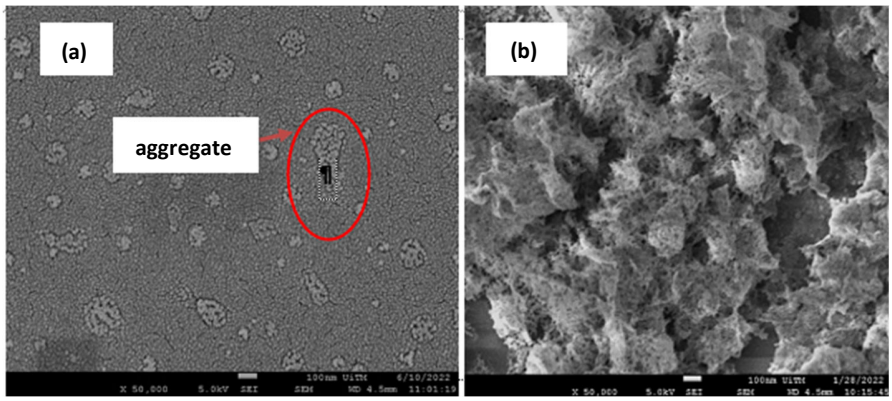


Figure 3. FESEM images of B-ZnO SL synthesized with (a) 0% and (b) 6% w/v PA leaf extract

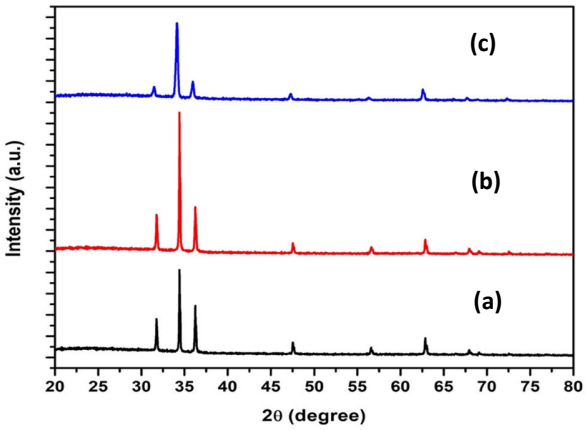


Figure 4. XRD pattern of ZnO NRs deposited onto B-ZnO SL synthesized with PA leaf extract concentrations of (a) 3% w/v, (b) 6% w/v, and (c) 9% w/v

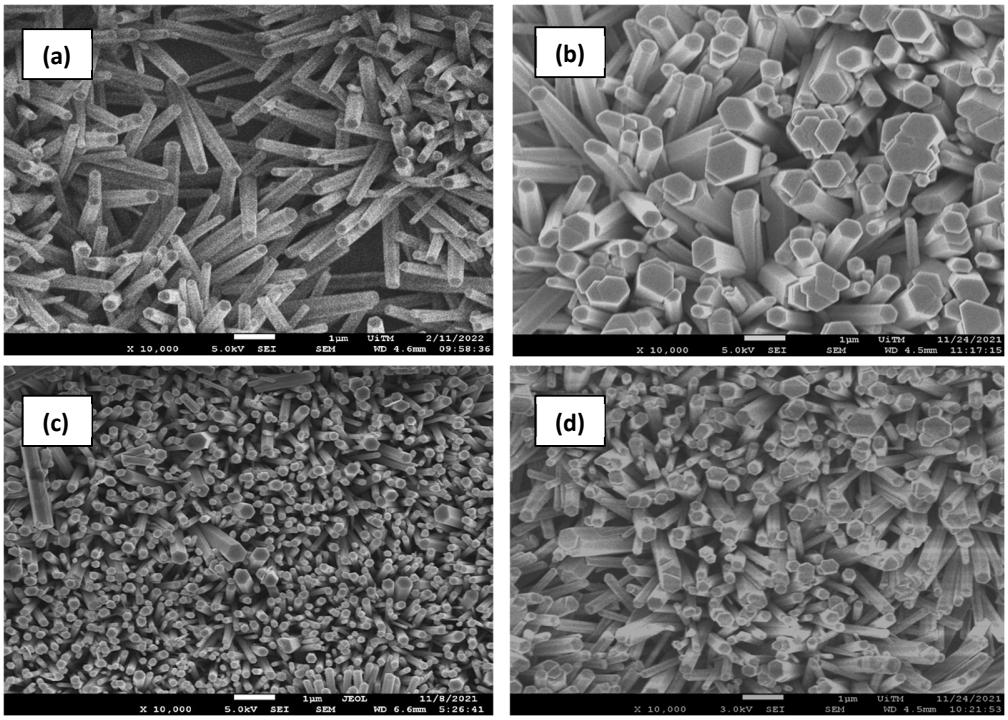


Figure 5. FESEM images of ZnO NRs deposited onto B-ZnO SL synthesized with PA leaf extract concentrations of (a) 0% w/v, (b) 3% w/v, (c) 6% w/v, and (d) 9% w/v

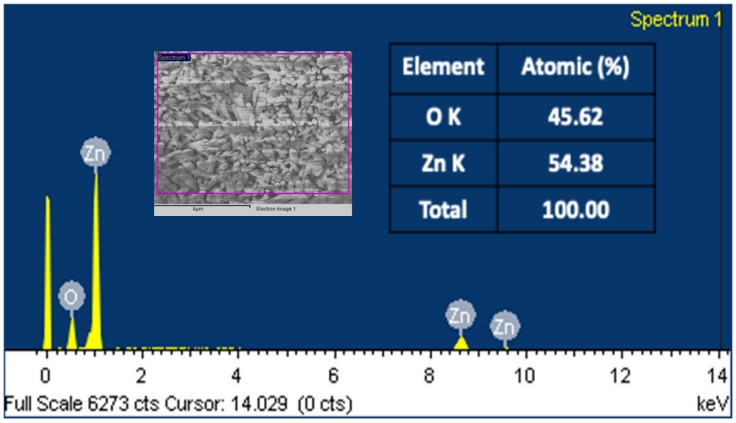


Figure 6. EDX data of ZnO NRs deposited onto B-ZnO SL synthesized with 6% w/v PA leaf extract

Conclusion

ZnO NRs were successfully prepared on a B-ZnO SL synthesized with PA leaf extract. The optimum PA leaf extract concentration was 6% w/v, at which the highest absorbance of the corresponding B-ZnO SL was obtained. The improved absorbance was attributed to the relatively higher amount of flavonoid compounds present in the corresponding sample. The crystallinity of the ZnO NRs improved for the sample deposited on B-ZnO SL prepared with 6% w/v PA leaf extract, with a dominant peak of the (002) orientation compared to the samples deposited on seed layers prepared with other concentrations. The average diameter of the ZnO NRs was 96 nm as shown through FESEM analysis, which is a 70% reduction from the control sample. The significant diameter reduction together with improved

vertical alignment of ZnO NRs may benefit their application for conductive electrodes and UV or humidity sensors. Subsequent studies should include a detailed analysis of other synergistic parameters to arrive at a comprehensive conclusion.

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A COMPARATIVE STUDY OF STRUCTURAL AND MORPHOLOGICAL CHARACTERISTICS OF POLYPYRROLE WITH CHEMICAL OXIDATION BY IRON(III) CHLORIDE, MICROEMULSION POLYMERISATION BY AMMONIUM PERSULFATE AND MICROEMULSION POLYMERISATION BY $FeCl_3$

(Satu Kajian Perbandingan Ciri-Ciri Struktur dan Morfologi Polipirol dengan Pengoksidaan Kimia oleh Besi (III) Klorida, Pempolimeran Mikroemulsi oleh Ammonium Persulfat dan Pempolimeran Mikroemulsi oleh $FeCl_3$)

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Abstract

Polypyrrole polymers are special polymers in which it has good conductivity of electricity for use in various fields due to its biocompatibility, thermal stability, easy to produce, etc. By use of various polymerisation methods like electro-chemical polymerisation, chemical polymerisation, microemulsion polymerisation, ultra-sonification polymerisation, vapour phase polymerisation, etc., pyrrole monomer is synthesised with various oxidants, dopants, surfactants, etc., to produce polypyrrole polymers. Two methods are widely used to produce polypyrrole one is chemical oxidation, and another is microemulsion polymerisation. There are few research papers on comparative studies of two methods and also between the two-oxidising agent used in the microemulsion method. The main objective is to compare both chemical oxidation by $FeCl_3$ polymerisation with microemulsion by iron(III) chloride and recompare the microemulsion by ammonium persulfate (APS) with microemulsion by iron(III) chloride. Therefore, three samples were first created: 0.1 M chemical oxidation, where iron(III) chloride was used as oxidising agent and sodium dodecyl sulfate as dopant. The second sample was 0.1 M microemulsion where ammonium persulfate was used as oxidant, sodium dodecyl sulfate as dopant, amyl alcohol as stabiliser. The three samples were 0.1 M microemulsion, where the oxidant was iron(III) chloride, sodium dodecyl sulfate acted as dopant, and amyl alcohol as the co-dopant. The three samples were characterised by Fourier-transform infrared spectroscopy (FTIR), field emission scanning electron microscopy (FESEM), and energy-dispersive X-ray spectroscopy (EDX), X-ray diffraction (XRD), and electrochemical impedance spectroscopy (EIS). The test result from EIS shows that the conductivity of 0.1 M chemical oxidation is 0.2211 S/cm, and that of

0.1 M microemulsion polymerisation is 0.9958 S/cm, and 0.1 M microemulsion by $FeCl_3$ is 1.1619 S/cm. The result from characterisation shows the successful development of polypyrrole, which can be used in fuel cell, supercapacitors, electrodes, biosensors, etc.

Keywords: polypyrrole, polymerisation, microemulsion

Abstrak

Polimer polipirol adalah polimer khas yang mempunyai kekonduksian elektrik yang baik untuk digunakan dalam pelbagai bidang kerana biokompatibilitinya, kestabilan haba, kemudahan penghasilan, dan sebagainya. Dengan menggunakan pelbagai kaedah pempolimeran seperti pempolimeran elektro-kimia, pempolimeran kimia, pempolimeran mikroemulsi, pempolimeran ultra-sonifikasi, pempolimeran fasa wap, dan lain-lain, monomer pirol disintesiskan dengan pelbagai oksidan, pendop, surfaktan, dan lain-lain untuk menghasilkan polimer polipirol. Dua kaedah yang digunakan secara meluas untuk menghasilkan polipirol ialah pengoksidaan kimia dan pempolimeran mikroemulsi. Terdapat beberapa kertas penyelidikan mengenai kajian perbandingan dua kaedah ini dan juga antara agen pengoksidaan yang digunakan dalam kaedah mikroemulsi. Objektif utama adalah untuk membandingkan kedua-dua pengoksidaan kimia oleh pempolimeran $FeCl_3$ dengan mikroemulsi oleh besi (III) klorida serta membandingkan mikroemulsi oleh APS dengan mikroemulsi oleh besi (III) klorida. Oleh itu, tiga sampel pertama kali dicipta: pengoksidaan kimia 0.1 M, di mana besi (III) klorida digunakan sebagai agen pengoksidaan dan natrium dodekil sulfat sebagai pendop. Sampel kedua ialah 0.1 M mikroemulsi di mana ammonium persulfat digunakan sebagai oksidan, natrium dodekil sulfat sebagai dopant, dan alkohol amil sebagai penstabil. Sampel ketiga ialah 0.1 M mikroemulsi, di mana oksidan adalah besi (III) klorida, natrium dodekil sulfat bertindak sebagai pendop, dan alkohol amil sebagai pendop bersama. Ketiga-tiga sampel itu dikaji menggunakan spektroskopi inframerah transformasi Fourier (FTIR), mikroskopi elektron pengimbasan pancaran medan (FESEM), spesifikasi sinar-X penyebaran tenaga (XRD), dan spektroskopi impedans elektrokimia (EIS). Keputusan ujian daripada EIS menunjukkan bahawa kekonduksian pengoksidaan kimia 0.1 M ialah 0.2211 S/cm, pempolimeran mikroemulsi 0.1 M ialah 0.9958 S/cm, dan mikroemulsi 0.1 M oleh $FeCl_3$ ialah 1.1619 S/cm. Hasil daripada pencirian menunjukkan kejayaan pembangunan polipirol, yang boleh digunakan dalam sel bahan api, superkapasitor, elektrod, biosensor, dan lain-lain.

Kata kunci: polipirol, pempolimeran, mikroemulsi

Introduction

Polypyrrole is formed when the pyrrole monomer is synthesised with various oxidants and dopants in an aqueous or non-aqueous medium with proper time and temperature; this process is also known as polymerisation. The factors that affect conductive are oxidative agents, molar ratio, dopants, and temperature. In one of the research projects by Varga and co-workers, $FeCl_3.H_2O$, iron (III) sulfate hydrate and ammonium persulfate (APS) were used as oxidative in a nanotube polymerisation. Shows, $FeCl_3.H_2O$ oxidising agent had shown the maximum conductivity 66 S/cm as compare to other oxidising agent shows less than 10 S/cm of conductivity [1]. If oxidant concentration is high, the rate of polymerisation will increase but the conductivity will be decreased. Hence, a ratio of initial oxidation to pyrrole monomer is important, it requires high oxidising for electron removal at a particular degree so that over-oxidising is prevented [2,3]. Dopant plays an important role in increasing the conductivity of a polymer. The mechanism is to produce polarons or bi-polarons to carry the charge. To increase conductivity, various dopants are used, for instance, fumaric acid, BSna, itaconic acid, HCl, etc. For surfactant, SDS is mostly used as a dopant in polypyrrole [4]. Meanwhile, the solvent utilised in the process is also important for good conductivity. To achieve high conductivity, the donor number and nucleophilicity should be less in the solvent [5].

Temperature plays a vital role in polymerisation, where a liner polypyrrole that is produced due to polymerisation kinetic becomes low at low temperature because of charge carrier migration and due to conjugation regulation. Which shows that polypyrrole conductivity can be achieve in low temperature [6]. To produce polypyrrole, there are various methods, such as chemical oxidation, electrochemical polymerisation, microemulsion, ultra-sonication polymerisation, vapour-phase polymerisation, electro-spinning, photo-polymerisation, and mechanochemical polymerisation; however, chemical oxidation and microemulsion are more widely used. Chemical oxidative polymerisation

method is the oldest and simplest method for the bulk production of polypyrrole. This is done by preparing two solutions: a monomer solution and an oxidation solution. Then, they are combined to produce a polymer. This can be carried out in an aqueous or non-aqueous medium, where the powder is then produced [7]. Many researchers had changed dopant, oxidising agent and conducted the experiments in one of the research shown that, the use of iron(III) chloride in chemical oxidation increased the conductivity as compare to ammonium persulfate [8]. In microemulsion polymerisation method two components are used a surfactant and two immiscible liquids to stabilise the immiscible liquid surfactant is used. The use of right surfactant particles is crucial in this polymerisation, which results in polymeric particles [9].

Numerous studies have been conducted on APS and $FeCl_3$ as oxidising agent, SDS as dopant. In one of the study shown that the increase of dopant SDS concentration from 0.01 M to 0.2 M the conductivity resistance was decreased [10]. In another research $FeCl_3$ was used but instead of dopant, capping agent hexadecyltrimethylammonium bromide and moringa oleifera for production of Polypyrrole nanowires and nanofibers [11]. Meanwhile, potassium peroxydisulfate was used as oxidising agent, SDS and Cetrimonium bromide as surfactant and hydrochloric acid as dopant in microemulsion polymerisation of polypyrrole [12]. There are very limited research papers on comparation study of chemical oxidation and microemulsion, second microemulsion by SDS as dopant, amyl-alcohol as stabiliser and $FeCl_3$ as oxidising agent. Hence, to fulfil the research gap this research is conducted.

Materials and Methods

Sodium dodecyl-sulfate (molecular weight is 288.38%, reagent grade is 99%), pyrrole (molecular weight is 67.09 g/mol, reagent grade is 98%), amyl-alcohol (molecular weight is 88.15 g/mol, reagent grade is 98.5%), ammonium persulfate (APS) (molecular weight is 228.18 g/mol, reagent grade is 98%), methanol (molecular weight is 32.04 g/mol, reagent grade 99%), and $FeCl_3$ (molecular weight is 162.21 g/mol, reagent grade is 99%).

Preparation of polypyrrole

In the preparation of polypyrrole, two methods are used chemical oxidation and microemulsion polymerisation is used as in Table 1, where the pyrrole is treated with different dopant to material ratio, time, temperature, and molarity.

Table 1. Sample preparation by various polymerisation methods

SL No.	Method of Polymerisation	Molarity (M)	Dopant	Oxidising Agent	Time (hour)	Temperature (°C)
1	Chemical oxidation	0.1	Sodium dodecyl-sulfate	$FeCl_3$	4	25
2	Microemulsion polymerisation	0.1	Sodium dodecyl-sulfate	Ammonium persulfate	24	0–4
3	Microemulsion polymerisation	0.1	Sodium dodecyl-sulfate	$FeCl_3$	24	0–4

Preparation of polypyrrole by using a chemical oxidation

In 100 mL of distilled water, 0.1 M of SDS was added and stirred using a magnetic stirrer for 5 min prior to adding 0.1 M of $FeCl_3$ in the solution and stirring it for another 5 min at a temperature of 25°C. Then, 0.1 M of pyrrole was added dropwise in the solution of $FeCl_3$ and SDS solution. The solution was kept under continuous stirring around 500 rpm (revolution per minute) at a temperature of 25°C for 4 hours, since the polymerisation of pyrrole is an exothermic reaction. When the solution of oxidation and dopant react with pyrrole, the solution will slowly turn into black colour and during polymerisation a black ring is observed around the beaker which is formed just above the beaker. After 4 hours the solution is kept in storage for 12 hours, so that polymerisation takes place and precipitation is also observed. The solution is filtered and washed

thoroughly by use of ethanol and distilled water. Then remaining substance from the filter paper is transferred to clay container for drying at 60°C–70°C on a hot plate. Finally, the dried sample is transformed into powder form by use of mortar and pestle and stored in a tight container surrounded with silica gel to prevent moisture.

Preparation of polypyrrole by using a microemulsion polymerisation (APS)

In 100 mL of distilled water 0.1 M of SDS is added and stirred for 20 min at an RPM of 500 at temperature of 0°C to 4°C. Then 0.1 M of pyrrole monomer is added in SDS solution and stirred for 30 min. After 30 min 0.1 M of amyl alcohol is added to the solution and stirred for another 10 min before adding 0.1 M of ammonium persulfate (APS) and allowed the mixture to stirred for 24 hours at temperature of 0°C to 4°C. Black ring is observed around the beaker above the solution, where methanol is subsequently added to the solution to dismiss the polymerisation reaction. The solution is than centrifuged at 10000 rpm for 25 min to produce precipitation. Then, this precipitation is passed through filtration process along with washing thoroughly by methanol and distilled water. Dry the sample at 60°C–70°C at hot plate for 4 hours and by the help of mortar and pestle it is converted into powder form and stored in tight container surrounded with silica gel to prevent moisture.

Preparation of polypyrrole by use of microemulsion polymerisation ($FeCl_3$) process

In 100 mL of distilled water 0.1 M of SDS is added and stirred for 20 min at an rpm of 500 at temperature of 0°C to 4°C. Then 0.1 M of pyrrole monomer is added in SDS solution and stirred for 30 min. After 30 min 0.1 M of amyl alcohol is added to the solution and stirred for another 10 min before adding 0.1 M of iron (III) chloride and allowed the mixture to stirred for 24 hours at temperature of 0°C to 4°C. Black ring is observed around the beaker above the solution. The solution is then centrifuged at 10000 rpm for 25 min to produce precipitation. Next, this precipitation is passed through filtration process along with washing thoroughly by methanol and distilled water. The sample is dried at a temperature ranging from 60°C–70°C on a hot plate for 4 hours and by the help of mortar and pestle to convert it in into powder form and stored in a tight container surrounded with silica gel to prevent moisture.

Results and Discussion

The FTIR spectra found from polypyrrole samples as in Table 1 and by the help of Table 2. [13] radical, wavelength and bond formation were taken as references. The bands 3400, 3400, and 3394 are present in 0.1 M chem, 0.1 M emulsion, and 0.1 M emulsion $FeCl_3$, which show the presence of N-H bonds in the polypyrrole due to N-H stretching vibration. The presence of bands 1552, 1542, and 1542 in 0.1 M chem, 0.1 M emulsion, and 0.1 M emulsion $FeCl_3$ shows the presence of C=C bonds, C=C stretching vibration indicates the π bond, which is responsible for conductivity due to delocalised π that allows electron movement. The bands 1174 and 1038, 1174 and 1038, 1152 and 1038, in 0.1 M chem, 0.1 M emulsion and 0.1 M emulsion $FeCl_3$ show the presence of C-N bond due to C-N stretching shaking of oxidised. The bands 830 and 772, 848 and 786, 846 and 774 in 0.1 M chem, 0.1 M emulsion and 0.1 M emulsion $FeCl_3$ show the presence of C-H bond, where C-H is responsible for plan deformation. The observation of band in current study is justified and available in journal, thereby confirming the polymerisation of pyrrole [13–16].

Table 2. Reference for FTIR spectra for polypyrrole [13]

Radical	Wavelength Range (cm^{-1})	Bond Formation
C=C	1700–1500	C=C bond formation in the molecule
C-H	860–680	C-H bond in molecule
C-N	1200–1025	C-N bond in the molecule
N-H	3500–3300	N-H bond in the molecule

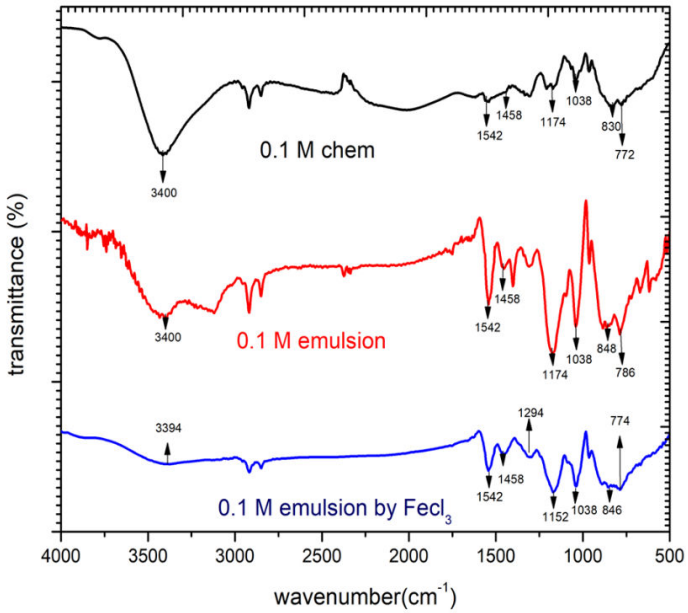


Figure 1. FTIR spectra of polypyrrole of different oxidation and same molarity (abbreviations: 0.1 M chem is 0.1 M chemical oxidation, 0.1 M emulsion is 0.1 M microemulsion by APS and 0.1 M emulsion by $FeCl_3$ is 0.1 M microemulsion by $FeCl_3$)

For surface characterisation of the three samples, Figure 2. shows the images of FE-SEM of (a) 0.1 M of chemical oxidation, (b) 0.1 M of microemulsion by APS and (c) 0.1 M of microemulsion by $FeCl_3$ of polypyrrole. The surface of three samples has rock like shape and distinctive rough surface and small granules [17,18]. By comparing Figure 2. FESEM images of samples (a) and (c), it is observed that polypyrrole produced from microemulsion by $FeCl_3$ has a rougher surface than chemical oxidation, since $FeCl_3$ used as oxidising agent, SDS as dopant in both methods with the same molarity.

To confirm that three samples have the necessary elements, mainly nitrogen and carbon, in polypyrrole, the EDX analysis was conducted. The tests results of EDX shows equivalent peaks as in Figure 3. (a), (b) and (c). Both carbon and nitrogen are observed in the three samples in sample (a) the atomic % of carbon and nitrogen are 80.16% and 19.84%, respectively. The details of the three samples values are measured in atomic and weight% are listed in Table 3. The following results are reported in previous work [19,20].

Table 3. EDX weight % and atomic % of polypyrrole of three samples

Number of samples	Carbon		Nitrogen	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
Sample (a) 0.1 chemical oxidation	77.60	80.16	22.40	19.84
Sample (b) 0.1 microemulsion by APS	64.20	67.65	35.80	32.35
Sample (c) 0.1 microemulsion by $FeCl_3$	59.36	63.01	40.64	36.99

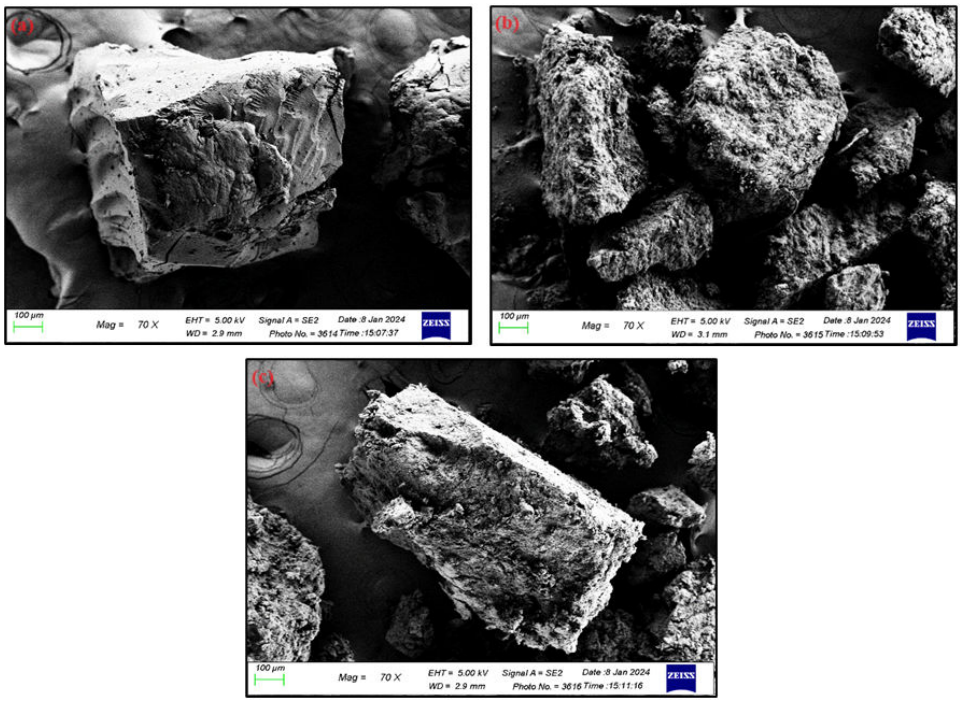


Figure 2. FESEM images of sample (a) chemical oxidation, (b) microemulsion (APS), and (c) microemulsion by $FeCl_3$

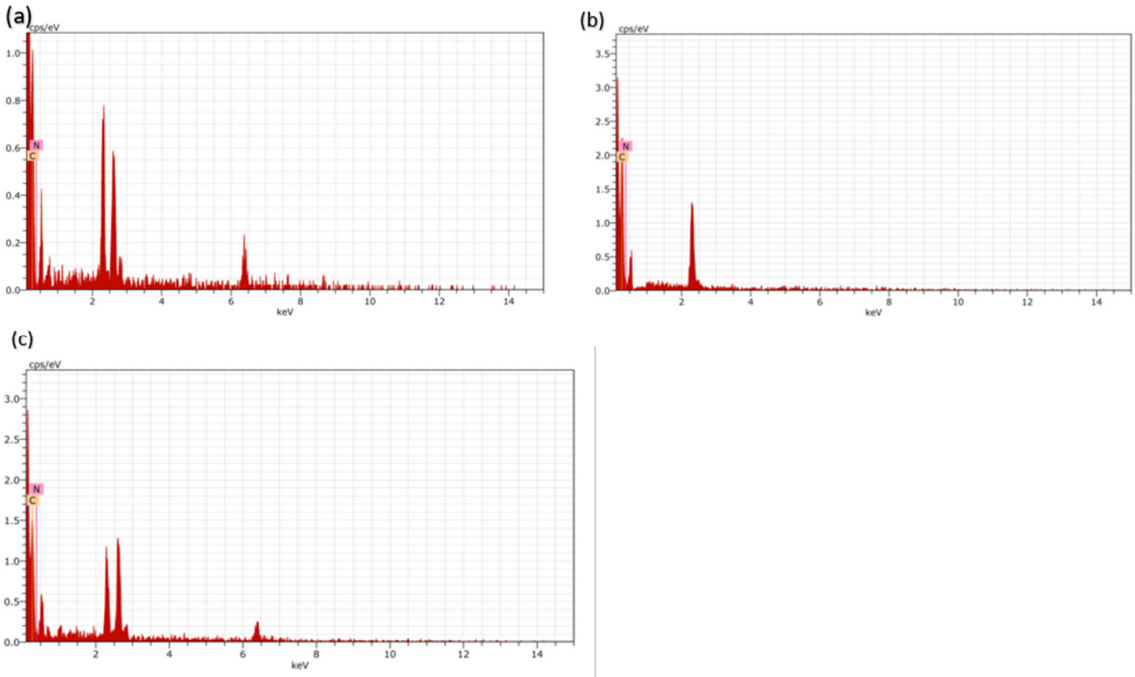


Figure 3. EDX result of sample (a) 0.1 M chemical oxidation, (b) 0.1 M microemulsion by APS (c) 0.1 M microemulsion by $FeCl_3$

The XRD patterns shows the broad peaks that are registered at 2θ equal to 20° and 21° of 0.1 M chemical oxidation, 0.1 M microemulsion APS as oxidant and 0.1 M microemulsion were $FeCl_3$ as oxidant as in Figure 4, the results detect amorphous region present in polypyrrole in three samples [21]. For the average crystallite size is taken from sharp peak at 20° and 21° by help of Scherrer's formula [22].

$$D = \frac{K \times \lambda}{\beta \times \cos\theta} \quad (1)$$

Where D is the crystallite size, K is the shape factor if the shape is unknown the value can be 0.89. θ is the maximum peck intensity at diffraction angle and β is full width at half maximum of diffraction angle in radian (FWHM). In the study sample 0.1 M microemulsion by $FeCl_3$ the cos θ value was 20.42875, β value was 0.03542 by converting FWHM radian value to degree from origin software. The K value was 0.89 and λ value is 1.5418 by putting all this values in (1) the average crystalline size was about 39 nm. The details values and results are mentioned in Table 4. The equation and calculation are taken from the research paper [23].

Table 4. Crystalline size by help of XRD analysis of sample 0.1 M chemical oxidation, Sample 0.1 M microemulsion by APS and Sample 0.1 M microemulsion by $FeCl_3$

	K	**Λ**	**β**	**θ**	**Crystalline size (nm)**
Sample 0.1 M chemical oxidation	0.89	1.5418	0.0673	20.95817	20.82
Sample 0.1 M microemulsion by APS	0.89	1.5418	0.05537	20.64522	25
Sample 0.1 M microemulsion by $FeCl_3$	0.89	1.5418	0.03542	20.42875	39

To determine the crystallinity and amorphous percentage of sample 0.1 M chemical oxidation, Sample 0.1 M microemulsion by APS and Sample 0.1 M microemulsion by $FeCl_3$. Crystallinity index in percentage [24] is used by the help of XRD data, the sharp peaks at 20° to 21° as in Figure 4.

$$Crystallinity\ index\ in\ \% = \frac{\text{area of crystalline peaks}}{\text{area of all peaks(crystalline+amorphous)}} \times 100 \quad (2)$$

In 0.1 M chemical oxidation the area of crystallinity peaks is 2060.739 and area of all peaks is 5888, then by putting the value in Equation (2) the crystallinity index percentage is 35% which means 65% of the material is amorphous. The details of all the three samples are in Table 5. The results of crystalline size from Table 4., crystallinity index % from Table 5. and broad peaks at 20° and 21° in Figure 4. it is seen that the samples are semi-crystalline in nature [25–27].

Table 5. Crystallinity index percentage of three samples

	Area of Crystalline Peaks	**Area of All Peaks**	**Crystallinity Index Percentage**	**Amorphous Percentage**
Sample 0.1 M chemical oxidation	2060.739	5888	≈35%	≈65%
Sample 0.1 M microemulsion by APS	839.5607	5744	≈15%	≈85%
Sample 0.1 M microemulsion by $FeCl_3$	396.8462	2736	≈15%	≈85%

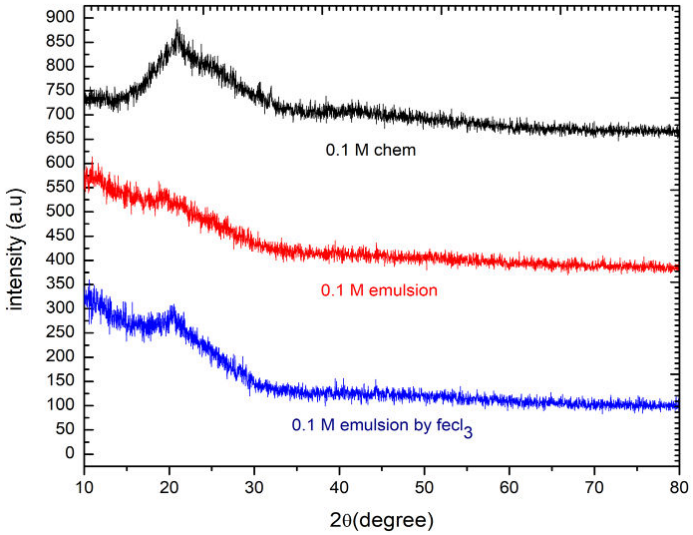


Figure 4. XRD of polypyrrole with different molarity and polymerisation method

For conductivity test Electrochemical Impedance Spectroscopy (EIS) is performed as in Figure 5 Cole-Cole plot of three samples (a), (b) and (c) the aim is to determine the conductivity of the samples. the values are obtained from Nyquist plot or Cole-Cole plot where ($Z^{/}$) is the real imaginary component and ($Z^{//}$) is the imaginary impedance component. The semi-circle formation in the plot denotes high frequency region and straight sloping line denotes low frequency region [28]

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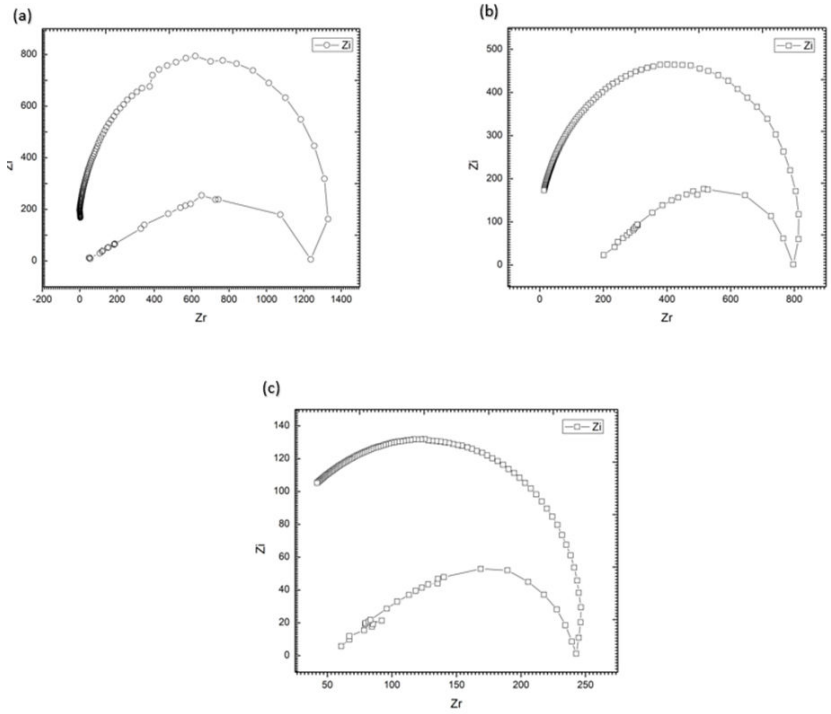


Figure 5. Cole-Cole plot of three samples (a) 0.1 M chemical oxidation, (b) 0.1 M microemulsion by APS and (c) 0.1 M microemulsion by $FeCl_3$

To confirm the conductivity of three samples as polypyrrole which is conductive in nature EIS test is executed. The conductivity is taken out by help of the conductivity formula:

$$\sigma = \frac{t}{R_b \times A} \quad (3)$$

where, σ is the ionic conductivity in $S.cm^{-1}$, t is the thickness of the sample in cm, R_b is the bulk resistance taken from the Cole-Cole plot, and A is the area of the contact between electrode and electrolyte in cm^2 [29]. In sample (a), t value is 0.272 cm, R_b value is 6.263017Ω and area is 0.1964 cm^2 putting the value in (3) gives $0.2211 \Omega^{-1} cm^{-1}$ of conductivity. The details of the three samples, namely, 0.1 M chemical oxidation, 0.1 M microemulsion by APS, and 0.1 M microemulsion by $FeCl_3$ are shown in Table 6.

Table 6. conductivity measurement by EIS of samples (a) 0.1 M chemical oxidation, (b) 0.1 M microemulsion by APS and (c) 0.1 M microemulsion by $FeCl_3$

Sample	Thickness (T) (cm)	Resistance Bulk (R_b) (Ω)	Area of the Electrode and Electrolyte (A) (cm^2)	Conductivity (Ω^{-1} cm^{-1})
Sample (a)	0.272	6.263017	0.1964	0.2211
Sample (b)	0.272	1.340834	0.1964	0.9958
Sample (c)	0.272	1.228653	0.1964	1.1619

Conclusion

By the test results and references it is concluded that by use of chemical oxidation and microemulsion the three samples are successfully polymerised from pyrrole monomer to polypyrrole. For the comparison study between chemical oxidation by $FeCl_3$ and microemulsion by $FeCl_3$, also comparison between microemulsion by APS and microemulsion by $FeCl_3$. Various characterisation tests were conducted, FE-SEM shows the rough, rocky surface in all three samples but by comparing Figure 2. FESEM images of sample (a) and (c) it is observed that polypyrrole produce from microemulsion by $FeCl_3$ has rougher surface than chemical oxidation since $FeCl_3$ used as oxidising agent, SDS as dopant in both method with same molarity. However, in case of microemulsion by APS and microemulsion by $FeCl_3$ same rough surface structure can be seen. Moreover, to confirm the chemical properties EDX and FTIR is performed where carbon and nitrogen are found in large number and by the FTIR the peaks show the bond formation in polypyrrole (i.e., C=C, C-H, C-N and N-H). Further to confirm XRD is performed to find the nature of the samples which are semi-crystalline in nature and the crystalline size are investigated. For conductivity measurement EIS test is performed where 0.1 M microemulsion by $FeCl_3$ have the highest conductivity of 1.1619 Ω^{-1} cm^{-1}. Thus, it illustrates that microemulsion by $FeCl_3$ is a much better option than the other two samples. Therefore, it is concluded that microemulsion by $FeCl_3$ is superior to APS oxidant for better conductivity, which can be used for future applications like in textile fields, medical fields, anti-corrosion, etc., by combining with other metals or non-metals.

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MULTIPLE REACTION MONITORING (MRM) OF PORCINE-SPECIFIC PEPTIDE MARKERS FROM LACTATE DEHYDROGENASE AND SERUM ALBUMIN IN PROCESSED MEAT FOR HALAL AUTHENTICATION

(Pemantauan Tindak Balas Pelbagai (MRM) Penanda Peptida Khusus bagi Khinzir dari *Lactate Dehydrogenase* dan *Serum Albumin* dalam Daging yang Diproses untuk Pengesahan Halal)

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Abstract

Multiple Reaction Monitoring (MRM) of four peptides is presented to detect the presence of pork in meat products using a triple quadrupole system (LC-QQQ-MS). Commercial samples were digested using the tryptic digestion method. MRM acquisition was optimized using Skyline software. Then, the presence of peptides was tested and verified on processed meat products, each with a different type of processing. The result reveals that peptides EVTEFAK (m/z = 450.2873), LVVITAGAR (m/z = 412.2134), FVIER (m/z = 388.7369), and TVLGNFAAFVQK (m/z = 647.8613) were consistently detected in the processed meat and displayed porcine-specific properties. Peptide LVVITAGAR, the most intense peptide, was eluted at 7 min while FVIEIR, as the second highest peak, was eluted at 9.5 min. On the other hand, peptide EVTEFAK was eluted at 4.5 min, and TVLGNFAAFVQK, a peptide with the lowest intensity, appeared between 12.5 to 13 min. Specifically, one peptide is derived from lactate dehydrogenase, and three from serum albumin protein. It demonstrated that porcine-specific peptides could be simultaneously targeted by the MRM method with the help of Skyline software that helps optimize the analytes' specific precursor ion, product ion, and fragmentation behaviour under collision energy. The present result reveals that MRM will enable rapid halal detection of contaminants occurring during the manufacturing or supply chain and ensure food integrity is preserved along the food supply chain.

Keywords: triple quadrupole, halal supply chain, peptide marker, species authentication

Abstrak

Pemantauan tindak balas berganda (MRM) empat peptida untuk mengesan kehadiran daging khinzir dalam produk daging menggunakan sistem caturkutub ganda tiga (LC-QQQ-MS) adalah dilaporkan. Sampel komersil dicerna dengan kaedah

penghadaman tryptic. Parameter pemerolehan MRM telah dioptimumkan menggunakan perisian Skyline. Seterusnya, kehadiran peptida telah diuji dan disahkan pada produk daging yang diproses dimana setiap satunya melalui jenis pemprosesan yang berbeza. Keputusan menunjukkan bahawa peptida EVTEFAK (m/z = 450.2873), LVVITAGAR (m/z = 412.2134), FVIER (m/z = 388.7369) dan TVLGNFAAFVQK (m/z = 647.8613) dikesan secara konsisten dan mempunyai sifat khusus daging khinzir. Peptida EVTEFAK, LVVITAGAR, FVIER dan TVLGNFAAFVQK dikesan secara konsisten dalam daging yang diproses dan menunjukkan sifat khusus khinzir. Satu peptida diperoleh khusus daripada laktat dehidrogenase dan tiga daripadanya adalah protein serum albumin. Ia menunjukkan bahawa peptida khusus untuk mengesan khinzir boleh disasarkan secara serentak oleh kaedah MRM dengan syarat pengetahuan tentang ion prekursor khusus analit, ion produk dan tingkah laku pemecahan di bawah tenaga perlanggaran dioptimumkan. Keputusan kajian menunjukkan bahawa MRM akan membolehkan pengesanan cepat bahan halal tercemar yang berlaku semasa proses pembuatan atau rantaian bekalan dan memastikan integriti makanan terpelihara di sepanjang rantaian bekalan makanan.

Kata kunci: caturkutub ganda tiga, rantaian bekalan halal, penanda peptida, pengesahan spesies

Introduction

Halal authentication methods have been growing tremendously with the aid of state-of-the-art technology. A DNA-based method such as Polymerase Chain Reaction (PCR) [1] and real-time PCR [2] were consistently reported in the literature, followed by a protein-based approach using enzyme linked immunosorbent assay (ELISA) [3], 2-Dimensional – Polyacrylamide Gel Electrophoresis (2D-PAGE) [4] and until recently, Mass Spectrometry (MS)-based authentication [5] has been proposed as a halal detection method. The analysis of pork adulteration in commercial meat products is challenging due to their composition, complexity, and, very often, inhomogeneity upon processing [6]. Moreover, food matrices that consist of fat, spices, and various salts, including commonly used sodium chloride and sodium nitrite, antioxidants, vegetable additives, or milk proteins, could affect detection sensitivity [7-8]. Therefore, researchers have continuously developed new authentication methods to overcome these challenges.

MS-based analysis has gained increasing consideration in medical, agriculture, animal science, and pharmaceutical fields. It offers excellent sensitivity and selectivity in which the mass-to-charge ratio m/z or structural information of a compound can be obtained in a shorter time. A considerable amount of literature has been published on the MS-based meat speciation technique, which explains the potential of peptides as molecular markers in meat species detection. For instance, [9] developed an Ultra-Performance Liquid Chromatography (UPLC) method that can be used to simultaneously separate myoglobin from water buffalo, bovine, pig, horse, chicken, and ostrich. The biomarker was subsequently used as a fast procedure to detect pork and beef meat in pre-mixed raw beef burgers. In addition, [10] mentioned that quantitative detection of chicken meat using stable isotope peptides was able to detect the presence of chicken in a mixture of meat. Moreover, [11] successfully identified 20 heat-stable peptide biomarkers unique to chicken duck and goose, and a detection method has been successfully developed using nano-LC-QTOF-MS/MS. Meanwhile, species-specific markers for detection in Bolognese sauce [12] and a combination of peptide detection and chemometrics have been demonstrated as well [13].

Targeted MS approaches such as Single Reaction Monitoring (SRM) and Multiple Reaction Monitoring (MRM) have tremendous potential for specific and reproducible qualitative and quantitative measurement of changes in the levels of peptides of interest to biologists and biomedical researchers [14]. The most common application of targeted MS could be observed in medical research, such as the detection of biomarkers for breast cancer [15], neurodegenerative disease [16], and hepatocellular carcinoma or cancer [17]. To this point, there is a current shortage of MRM-based assays in the meat science field, particularly on meat authentication. Previously, [18] developed an MRM method from myoglobin protein for QTRAP triple-quadrupole mass spectrometer to discriminate between beef, lamb, horse, and pork. Meanwhile, research conducted by [19-20] established a peptide-based detection method for the Quadrupole ion Trap Mass Spectrometry (QTRAP-MS) system whereby contaminations of pork with the most sensitive biomarker peptide were detected by MRM3. A previous study [19] reported a peptide-based detection method for

a QTRAP-MS system, in which contamination of pork with the most sensitive biomarker peptide was detected down to 0.13% in the beef matrix without pre-enrichment of analytes.

In contrast, the present study was conducted to verify the presence of biomarkers in detecting pork from commercially available meat products using MRM of triple quadrupole (QQQ) MS system. Previously published work on seven peptide biomarkers has successfully been identified using the LC-QTOF platform in heat-treated meat [21]. The present paper is a continuation of previous work whereby the specificity of the peptide was tested on commercial samples. Notably, the MRM platform was pursued as it reduced the time spent screening the total and extracted ion chromatogram. The work of examining the full spectrum in searching the marker peptides is no longer required, and analysis can be conducted in a single run. Moreover, all the listed peptides have been carefully analysed by checking their full MS/MS spectrum scan and species specificity, illustrated in the shotgun proteomics profiling. Once developed and optimized for selectivity and species specificity, the peptide biomarkers were used in the MRM assay to demonstrate proof of concept for detecting adulterated pork meat products in commercial meat products. The development of an MRM assay to verify candidate porcine-specific peptide biomarkers was established in this study. Briefly, it involved the development of MRM transitions and protocol, which was facilitated by the Skyline MRM method development application. Then, verification or refinement of potential biomarkers through MRM into some credible peptides that are, in fact, specific indicators for pork contamination proved that the listed biomarkers are qualified to be used for the species authentication method.

Materials and Methods

Sample preparation for adulterated and commercial samples

One boiled pork and beef meat and seven commercial samples were used in the study (N = 8). Initially, protein extract from boiled pork meat (100°C) was used for peptide screening and selection. Next, seven processed meat samples were purchased from local and international markets (Table 1). Species identity was listed according to the label. All samples were immediately stored in a 50 ml falcon tube at -80°C. Next, the meat and processed meat products were pulverized in liquid nitrogen prior to proceeding with extraction.

Table 1. List of samples used for species authentication using identified peptides

Type of Product	Species Declared (as indicated on the label)	Country of Origin or Manufactured (as indicated on the label)
Pork	pork	Malaysia
Corned beef	beef	Russia
Sausage	pork and chicken	Malaysia
Meatball	pork and chicken	Malaysia
Meatball	beef	Malaysia
Hamburger	pork, chicken and fish	Japan
Smoked Chicken Frankfurter	chicken	Malaysia
Chicken Luncheon	chicken	Malaysia

Extraction of proteins

Sample preparation and in-solution trypsin digestion were performed according to the previously described procedure [21, 22]. Approximately one gram of heat-treated pork and processed meat products were homogenized using handheld homogenizer (Wiggen Hauser D500, USA) in 5 ml of cold extraction buffer containing 7 M Urea, 2 M thiourea, 50 mM DTT, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.4% (v/v) carrier ampholytes (pH 3-10, Bio-Rad, USA) and 50 µL of protease inhibitor cocktail (Calbiochem, USA). Then, the solution was centrifuged at 12,000 g for 10 min at 4°C. The resulting supernatant was subjected to an acetone precipitation procedure in which the total volume of protein was mixed with four volumes of cold acetone (pre-chilled at -20ºC overnight). The mixture was then vortexed and incubated at -20ºC overnight.

Subsequently, the samples were centrifuged at 12,000g at 4ºC for 10 min. The resulting pellet was air-dried and resolubilized in 50 µL urea extraction buffer, spun down before the supernatant was collected, and stored at -20ºC. Total extractable protein concentration was determined using a Bradford protein assay kit according to the manufacturer's protocol (Bio-Rad, Hercules, USA). The protein assay was done in triplicates for each sample. The same urea buffer was used as the background reference for the assay. All samples were purchased once and extracted in triplicates, resulting in a total of 24 extracts.

In-solution trypsin digestion

Approximately 100 µg of protein was taken from the protein extracts. Briefly, the protein was incubated in 50 µL of 10 mM DTT/100 mM ammonium bicarbonate for 30 min at 60°C. The mixture was initially allowed to cool prior to proceeding with the alkylation process in 50 µL of 55 mM IAA/100 mM ammonium bicarbonate for 20 min at room temperature (25°C, in a dark place). In-solution tryptic digestion was performed with 1 µg trypsin digestion buffer (Promega trypsin gold, Madison, WI, USA) overnight at 37°C. Tryptic peptides were then dried in a rotational vacuum centrifuge (CHRiST RVC 2-18, Germany) and stored at -20ºC. The desalting procedure using ZipTip C18 (Millipore, USA) was performed according to protocols described by the manufacturer to remove any interfering agents. Next, eluted peptides were re-suspended in milli-Q water containing 0.1% formic acid. Then, chromatographic separation was carried out.

Targeted porcine-specific biomarker in meat products

The initial peptide list for MRM was adopted from the LC-QTOF-MS analysis of cooked pork by Sarah et al. [21]. The list is composed of seven peptides of high-to-moderate abundance in which the targeted peptides were derived from three different proteins: lactate dehydrogenase, creatine kinase, and serum albumin. All the information from the peptides was exported to method optimization tools, namely Skyline Targeted Proteomics Environment Optimization Software, which was downloaded from https://skyline.ms/project/home/software/Skyline/begin.view. Briefly, the full-length protein sequences were imported into Skyline in FASTA format. The peptide filtering condition has been set as follows: maximum length of peptide of 20 and at least eight amino acids. Later, a list of all possible y-series fragment ions, for both z = 2 and z = 3 precursor ion-charge states, spanning a m/z range from 300 to 1,400, was generated. A transition list that detailed the possible collision energy for each precursor ion and product ion was created by the software, and the information was further used to optimize the actual MRM acquisition parameters. During the acquisition, the instrument will be directed to monitor each precursor and product ion transition at various CE voltages during a specific retention time, as suggested by the transition list.

LC-QQQ-MS analysis

In brief, trypsin-digested peptides were separated by the Agilent 1290 Infinity UHPLC system, while MS analysis was conducted using an Agilent 6490 QQQ mass spectrometer (LC-ESI-QQQ-MS), which was interfaced with a standard-flow ESI (Jet Stream) source. About 5 µl of each sample was injected using the autosampler, with a total of 24 injects altogether. All the samples were maintained at 4°C using a thermostat autosampler. UHPLC separation was performed with AdvanceBio Peptide Map, C18 capillary columns (C18, 2.1 x 100 mm, 2.7 µm particles, cat no. 655750-902) in 95% mobile phase A (100% water, 0.1% formic acid), 5% mobile phase B (90% acetonitrile, 10% water, 0.1% formic acid) at a flow rate of 0.3 ml/min. The total time of analysis was set at 23 min. Initial conditions were maintained for 1 min, and peptide elution occurred at linear gradients of 1-40% mobile phase B for 15 min. This is followed by 5 min at 95% mobile phase B prior to returning to initial conditions in 2 min. Each sample was analyzed in triplicates. Each run was separated by two blank runs to wash and re-equilibrate the column prior to the next run to minimize sample carryover.

The MS conditions were as follows: drying gas temperature, 300°C; drying gas flow, 8 mL/min; nebulizer pressure, 35 psi; corona current, 10 nA; and capillary voltage, 4,000 V. MRM mode was applied for quantitative analysis using precursor/product ion information. All the data acquisition was processed, evaluated, and visualized using MassHunter Workstation (version B.03.01), which was processed using MassHunter Quantitative analysis software (B.04.00) (Agilent Technologies, USA). Ion

chromatograms were extracted with a mass tolerance of 10 ppm for MRM data.

Results and Discussion

Optimization of MRM precursor and product ion pairs using Skyline software

Table 2 indicates the final parameter of MRM acquisition suggested by Skyline software in which several parameters such as parent ion, product ion, and collision energy have been assessed to suit the targeted search. Initially, for each parent ion, approximately five to seven product ions were proposed by the software as having a high potential to be detected by LC-ESI-QQQ-MS. Moreover, at least seven collision energies were predicted to suit each product ion, which resulted in a total of 273 possible runs. The information obtained from this analysis is imperative to ensure that the most intense precursor and fragment ions for MRM method development were selected. All the parameters given by Skyline software were assessed to identify the presence of pork. Out of seven peptides with 273 possible runs, only four peptides were consistently detected with a clean and specific signal, whilst chromatograms with considerably high amounts of noise, interfering peaks, and non-specific signals were eliminated. Using Skyline as an optimization tool provides specificity of detection without detecting unnecessary transitions and reduces the time taken by instrument parameters to optimize each peptide [23]. In QQQ MS, three quadrupole (Q) rods were used, in which Q1 is used for precursor ion isolation, Q2 works as a collision cell containing a collision gas (usually argon), and Q3 analyses product ions produced from the precursor ion isolated in Q1. Therefore, only ions with a particular m/z value will be able to escape the quadrupole, while the rest will collide with the quadrupole walls [24, 14].

There are four peptides that gave positive spectra, which were then further analysed to ensure their specificity (Figure 1). Remarkably, two of the peptides, LVVITAGAR and FVIEIR, exhibit product ion with the highest intensity, while the other 2, EVTEFAK and TVLGNFAAFVQK, were present in lower intensity. Peptide LVVITAGAR, the most intense peptide, was eluted at 7 min while FVIEIR, as the second highest peak, was eluted at 9.5 min. On the other hand, peptide EVTEFAK was eluted at 4.5 min, and TVLGNFAAFVQK, a peptide with the lowest intensity, appeared between 12.5 to 13 min. A closer visualization of the extracted MRM chromatogram revealed a good detection of peptide biomarkers despite having the lowest peak. Such chromatogram reflects the relative abundances of distinct proteins in commercial products. The most intense product ions produced for each precursor ion were combined with all detected peptides to develop a final MRM method.

To achieve a true positive identification, all the product ions were confirmed to be eluted at the same point with the corresponding precursor ion, where the elution condition and retention time of all transitions were carefully observed. All the product ions were confirmed to be successfully eluted at the same retention time as the precursor ion with varying collision energy. It was, therefore, necessary to select the optimum pairs of product ion and collision energy that could produce the most intense peak.

Assessment of products ion in processed meat products

The porcine-specific peptide biomarker was evaluated by LC-ESI-QQQ-MS on seven different commercial meat products. Total ion chromatograms of seven products tested (Figure 1) display that the porcine-specific peptides were successfully detected in the tryptic fragmentation of three commercial product samples, which were declared to contain pork as labelled on the packaging. Three samples, Japanese hamburger, pork meatball, and pork sausage, were detected as positive. The result illustrated the overview of the MRM signals detected from all four porcine-specific peptides without any non-specific signal observed in the chromatogram. Meanwhile, the other products, namely, beef ball, chicken luncheon, corned beef, and smoked chicken frankfurter, exhibited a flat spectrum, indicating no traces of pork in the products. The four peaks were consistently absent, which led to the conclusion that they were free from any pork protein component.

Table 2. Parameter of MRM acquisition method for porcine-specific peptides from lactate dehydrogenase and serum albumin tested in processed meat products (Reproduce from [21])

No	Peptide Sequence (Protein)	Accession Number	RT (min)	Precursor Ion (charge state)	Product Ion	Collision Energy	Dwell
-1	LVVITAGAR (Lactate dehydrogenase)	GI:288860136 (*Sus scrofa*)	7	450.2873 (z=2)	786.4932 (y8)	18	20
					687.4048 (y7)	12	20
					588.3364 (y6)	12	20
					475.2523 (y5)	12	20
2	EVTEFAK (Serum albumin)	GI:52353352 (*Sus scrofa*)	4.5	412.2134 (z=2)	595.3286 (y5)	19.8	20
					494.2509 (y4)	10.8	20
					365.2083 (y4)	10.8	20
3	FVIEIR (Serum albumin)	GI:52353352 (*Sus scrofa*)	9.5	388.7369 (z=2)	629.3881 (y5)	10.1	20
					530.3197 (y4)	10.1	20
					417.2356 (y3)	10.1	20
					288.213 (y3)	16	20
4	TVLGNFAAFVQK (Serum albumin)	GI:52353352 (*Sus scrofa*)	12.5	647.8613 (z=2)	592.3553 (y5)	24.1	20
					521.3382 (y4)	30.1	20

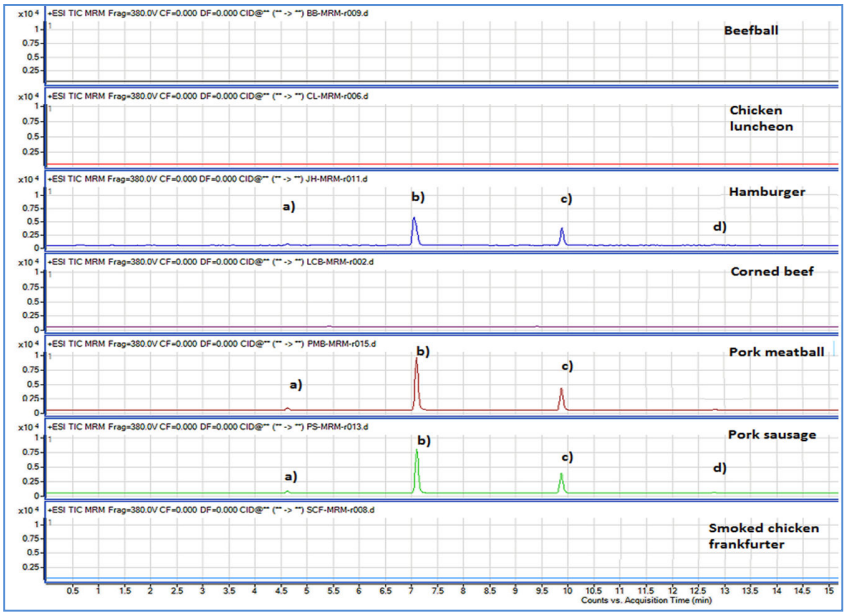


Figure 1. MRM chromatogram of seven different commercial meat products detecting peptides: a) EVTEFAK, b) LVVITAGAR, c) FVIEIR, and d) TVLGNFAAFVQK

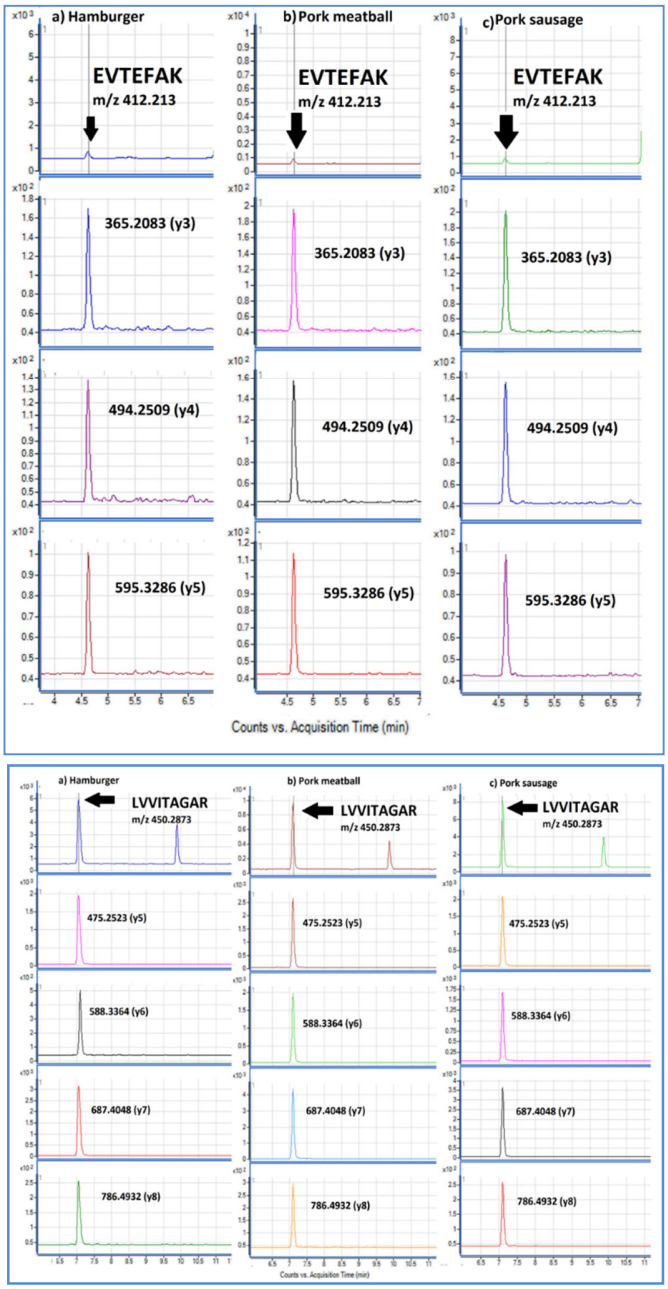


Figure 2. MRM chromatogram of peptide EVTEFAK and LVVITAGAR detected in hamburger, pork meatball, and sausage with the specific signal produced by product ion 365.2083, 494.2509 and 595.3286 and 475.2523, 588.3364, 687.4048 and 786.4932, respectively. All product ions were eluted at similar retention

MRM chromatograms from three commercial samples (sausage, meatball, and hamburger) that were positively identified to contain pork were examined in detail. The extracted MRM transitions of porcine specific-peptides marker within 23 min is presented (Figure 2). The first peak, peptide EVTEFAK, was eluted at 4.5 min, while peptide LVVITAGAR, the highest peak detected from lactate dehydrogenase protein, was eluted at 7 min. Peptide FVIEIR, as the second highest peak observed in the chromatogram, was eluted at 9.5 min while peptide TVLGNFAAFVQK, which was detected as the lowest chromatogram peak, was observed at 12.5 min (Table 2).

After examining the spectra, retention time, and abundance of parent ion, the spectra of product ions were further inspected using several parameters, such as the intensity of peptide and the quality of MS/MS spectra. Note that product ions must be eluted within the same retention time as parent ions, indicating the identity of the peptides. This suggests that a highly specified transition that monitors multiple specific ion transitions at high sensitivity from both parent ion and product ion has been performed simultaneously and scanned for a specific mass of interest in Q1 and Q3, respectively [25-26].

The peptide LVVITAGAR was set on finding the specific precursor mass (m/z = 450.2873) on Q1, with the collision energy being optimized to produce a diagnostic charged fragment of that parent ion. Meanwhile, Q3 was set to detect the specific mass of those product ions (786.4932, 687.4048, 588.3364, and 475.2523) produced by their respective parent ions. However, only ions with specified transition were detected in all commercial samples.

The peptide EVTEFAK with precursor ion 412.2134 has been set to detect product ions 595.3286, 494.2509, and 365.2083. Specific signals of precursor and product ions have been observed in three out of four samples, indicating the presence of pork in the products as declared on the label. Similarly, peptide FVIEIR (m/z = 388.7369) produced a good transition signal in both precursor and product ions (629.3881, 530.3197, 417.2356, and 288.213) upon detection in hamburger, pork meatball, and pork sausage. Meanwhile, peptide TVLGNFAAFVQK (m/z = 647.8613) demonstrates signal specificity despite having a slightly low chromatogram with only two product ions, 592.3553 and 521.3382, detected.

All the results above indicate that only analytes with specific precursor/product ion combinations will be detected. In a complex biological sample, there might be other protein components that could produce a 450.2873 m/z precursor ion during ESI. However, there is a low probability that they will also fragment to a 786.4932, 687.4048, 588.3364, and 475.2523 m/z product ion. This technique is termed MRM, and fragmentation is denoted as 450.2873 > 786.4932, 450.2873 > 687.4048.

Targeted MS has become the method of choice for the analysis of complex protein samples. Previous related studies that have documented the importance of targeted MS were mostly from a medical-related background, whereby the technique has been used to quantify and monitor the progress of drug therapy [27] and disease [28], [15]. In relation to the Agilent 6490 QQQ mass spectrometer, an MRM-based assay for the determination of cardiovascular disease and disease classification has been developed using the same instrument [28]. Since the instrument produces high sensitivity and selectivity of peptides, the present study utilizes its capacity to develop a powerful porcine detection method for halal authentication. Research by [29] used LC-ESI-QQQ-MS/MS 6460 from Agilent Technologies, a similar mass detector to this study. They reported positive MRM detection of duck, goose, and chicken peptide markers in various processed types of meat such as duck terrine, semi-dried sausage, and kabanos-type sausages.

As far as the application of MRM for pork detection is concerned, several research articles reported the work on identifying peptide biomarkers for the detection of pork in meat matrices. In a recently published study by [19], a total number of 12 biomarkers were detected. Among the reported peptides, they described peptide YDIINLR from troponin T protein, which was known to provide species-specificity for detecting pork and horses. Moreover, it has been tested against a mixture of raw meat. However, in the present study, no chromatogram of YDIINLR was observed from the cooked meat samples following multiple runs of MRM analysis, which suggested that peptide YDIINLR from troponin T was discovered not to be suitable for detection of thermally processed meat. Meanwhile, MRM of myoglobin peptides was also tested in meat against mixtures of several species of proposed peptide pairs, which is called the Corresponding Protein Corresponding Peptide (CPCP) strategy as a rote to relative quantitation [18].

Commercial meat products produced through technological processes possess several challenges for MS analysis due to protein denaturation following the process. Processes like cooking [30], freezing, and thawing methods [31] could influence the protein quantity and extractability of the samples. The porcine-

specific peptides obtained [21] were tested on processed products, and it was verified whether the peptide application was suitable for such products or only limited to meat detection. Here, proteins extracted from the meat products with various technological processes and purchased in the local and international supermarkets (Table 1) demonstrated a good match, with three exhibiting positive results. Hence, it indicates that the proposed peptides are suitable for processed products. The present study has successfully validated that a species-specific fragment was identifiable in all complexes of meat products, including sausages, corned beef, or canned meat, by applying MRM.

Previous studies conducted by [19-20] reported that species-specific marker peptides for horse and pork were identified in commercial samples such as frikadeller, meatloaf, and spaghetti sauce, which has been subjected to complex and harsh processing like mixing, cooking, and frying using MRM^3 experiment. Identified marker peptides were demonstrated to resist the thermal processing of different meat products. Furthermore, the sensitive and specific detection of pork or horses in processed food has been demonstrated to be down to 0.24% in a beef matrix system. Contrary to this study, peptides TLAFLFAER and SALAHAVQSSR are from myosin-4 and myosin-1 and -4, respectively, with no marker from serum albumin and lactate dehydrogenase reported [20]. Another research utilized raw or semi-dry ham as study material and employed peptides from Myosin Light Chain (MLC) isoforms, namely KPAAAAAPAPAPAPAPAPAPPK and EAFTVIDQNRDGIIDKEDLRDTFAAMGR, which indicated that the fragments of sequence are most likely to be the most resistant to trypsin, proteolytic enzymes as well as to technological processes [32].

Notably, the protein reported in this study agrees with [33], who reported the significance of lactate dehydrogenase as a marker of beef quality between *in vitro*-digested aged and cooked meat. Meanwhile, three peptides, ADFTDVTK, DVFLGSFLYEYSR, and EDPHACYATVFDK, from serum albumin have been demonstrated in MRM experiments as potential peptide biomarkers for the detection of sheep in processed products [34]. Moreover, serum albumin has been reported as the precursor of functional bio-peptide for developing commercial functional foods and nutraceuticals [35] and was also discovered in protein exudates obtained from pig meat injected in different levels of brine [36].

To make this research more meaningful, several recommendations shall be considered for future work. Future work should identify the remaining potential biomarker candidates, which could be present in trace amounts at the low abundance proteins group. Hence, sample fractionation and purification could improve the extraction of biomarker candidates, which are too low for a reliable assay. Moreover, it is recommended that internal standards or labelling of candidate peptides using stable isotopes be introduced. Known labelled standards, such as a stable isotope of the targeted peptide, would need to be introduced into the sample prior to sample injection to ensure a more confident result during routine analysis.

In general, the objectives of the present study were satisfactorily achieved. Notably, this research provided several important findings that may contribute to the advancement of halal authentication procedures. This study also highlighted a better understanding and insight into species specificity provided by precursor and product ion selection. All these findings shall be extended for the detection in other food systems and not just limited to meat and its co-products.

Conclusion

Four peptides (EVTEFAK, LVVITAGAR, FVIEIR, and TVLGNFAAFVQK) were consistently detected in commercial meat products. Apart from determining the specificity of the peptides in heat-treated meat, the MRM method developed through this study was also able to identify the targeted porcine-specific peptides in processed meat products, which suggests the method's selectivity. Moreover, it has been demonstrated that when collision energy is optimized, unique peptides can be detected by targeting their specific precursor ion and product ion through MRM analysis. Indeed, targeted simultaneous analysis of multiple proteins will enable rapid halal detection of large sample sets of contaminated meat products. Thus, a set of porcine-specific markers was proposed rather than individual markers, which could have increased the specificity and confidence of the detection.

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COMPARISON OF PATTIES PRODUCED USING MEAT FROM VARIOUS ANIMAL SPECIES WITH BLACK-EYED PEAS AS THE PARTIAL MEAT SUBSTITUTE

(Perbandingan Burger yang Dihasilkan menggunakan Daging daripada Pelbagai Spesies Haiwan dengan Kacang Mata Hitam sebagai Pengganti Separa Daging)

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Abstract

Incorporating plant-based ingredients as meat substitutes can be a strategy to reformulate healthier and more environmentally sustainable meat products. However, meat species variations could lead to different physicochemical and sensory characteristics of the final products. This study aimed to evaluate the physicochemical, sensory, and microstructural properties of patties made from different meat species [chicken (CB), beef (BEB), mutton (MB), and buffalo (BFB)] and substituted with 50% black-eyed peas compared to 100% black-eyed peas (BEP) as the control. An array of physicochemical properties was evaluated, encompassing cooking yield, shrinkage, water holding capacity (WHC), pH, proximate composition, texture, gel strength, and colour. Furthermore, scanning electron microscopy and sensory evaluation were employed to elucidate the microstructural modifications and sensory attributes of the samples. The results reveal significant differences in proximate composition, WHC, and textural properties across the meat species. CB exhibited a higher lightness, cooking yield and softer texture than other samples, which displayed better water retention. Whereas BEB and BFP were harder and chewier. The BEB resulted in lower gel strength and less intact structure, as evidenced by microstructure images and texture profile analysis (TPA) results. No significant difference in sensory traits exists between different meat species. Despite the overall acceptability of BEB being the highest, the MB was the lowest. The composition, texture, and sensory features of chicken and beef with the incorporation of BEP make them viable candidates for use in the development of healthy patties.

Keywords: different meat types, meat analogues, meat emulsion, meat substitute, plant-based protein

Abstrak

Menggabungkan bahan berasaskan tumbuhan sebagai pengganti daging boleh menjadi strategi untuk merumuskan semula produk daging yang lebih sihat dan lebih mampan alam sekitar. Namun, variasi spesis daging boleh menghasilkan ciri-ciri fizikokimia dan deria yang berbeza pada produk akhir. Kajian ini bertujuan untuk menilai sifat fizikokimia, deria rasa, dan struktur mikro burger yang diperbuat daripada spesis daging yang berbeza [daging ayam (CB), daging lembu (BEB), daging kambing (MB), dan daging

kerbau (BFB)] yang digantikan dengan 50% kacang mata hitam berbanding dengan 100% kacang mata hitam (BEP) sebagai kawalan. Pelbagai sifat fizikokimia telah dinilai, merangkumi hasil memasak, pengecutan, kapasiti pegangan air (WHC), pH, komposisi proksimat, tekstur, kekuatan gel dan warna. Tambahan pula, penilaian deria dan pengibas mikroskop elektron telah digunakan untuk menjelaskan sifat deria dan pengubahsuaian mikrostruktur sampel. Komposisi proksimat, WHC, pengecutan, kekuatan gel, warna dan profil tekstur adalah berbeza secara signifikan di antara patties yang berbeza. CB menunjukkan nilai kecerahan yang lebih tinggi, hasil masakan yang lebih tinggi, dan tekstur yang lebih lembut daripada sampel lain, yang menunjukkan pengekalan air yang lebih baik. Manakala BEB dan BFP lebih keras dan lebih kenyal. BEB menghasilkan kekuatan gel yang lebih rendah dan struktur yang kurang utuh, seperti yang dibuktikan oleh imej struktur mikro dan hasil analisis profil tekstur (TPA). Tiada perbezaan yang signifikan dalam ciri-ciri deria antara spesis daging yang berbeza. Walaupun penerimaan keseluruhan BEB adalah yang tertinggi, MB adalah yang terendah. Komposisi, tekstur, dan ciri-ciri deria daging ayam dan daging lembu dengan penggabungan BEP menjadikan mereka calon yang berpotensi untuk digunakan dalam pembangunan patties yang sihat.

Kata kunci: analog daging, emulsi daging, jenis-jenis daging yang berbeza, pengganti daging, protein berasaskan tumbuhan

Introduction

According to the United Nations, the world's population has grown more than threefold since the mid-twentieth century, reaching nearly 8 billion by 2022 from 2.5 billion in 1950 [1]. Despite a reduced growth rate, the global population continues to increase. This population growth, along with urbanization and rising incomes in developing countries, has become the main driving force behind the escalating demand for meat products. However, the shift towards a diet dominated by animal products has led to significant external costs. Primarily, the consumption of meat and processed meat products has been directly associated with a higher prevalence of cancer, including colorectal, pancreatic, and prostate cancer [2]. Additionally, these products are major contributors to weight gain due to their cholesterol and saturated fatty acid content [3]. Moreover, meat consumption, particularly from ruminant animals, is a significant source of greenhouse gas emissions, deforestation, and global biodiversity loss [4]. It also contributes to local pollution of water, soil, and air [5]. Furthermore, a substantial portion of the land, energy, and water used to grow feed crops for animals could be more efficiently utilized to produce plant-based foods directly consumed by humans [6]. The rearing conditions of animals for meat production also indirectly contribute to global threats such as antibiotic resistance and zoonotic diseases [7]. In addition to environmental concerns, society is increasingly showing heightened awareness and concern for animal suffering and welfare in the farming industry. This growing concern is reflected in the rising number of vegetarian and vegan consumers in affluent countries [8]. As a result, there is a growing demand for alternative and sustainable sources of protein to meet the nutritional needs of the global population while addressing the challenges posed by conventional meat consumption.

There has been a growing interest in developing plant-based meat replacements or incorporating plant-based substitutes with various meat types to improve human health, contribute to environmental sustainability, and address animal welfare concerns [9,10]. In comparison to traditional meat, plant-based meat production boasts significant advantages, utilizing 72% to 99% less water and 47% to 99% less land, while also causing 51% to 91% less water pollution and greenhouse gas emissions [11]. Recognizing the growing demand for meat alternatives, various companies in Europe and the United States have introduced plant-based meat products, such as Impossible Burger®, Beyond Burger®, and Schnitzel® [12]. By processing ingredients like wheat gluten, soy protein, mushrooms, rice, legumes, and flavour additives, plant-based meat can closely mimic the aesthetic qualities and nutrient content of real meat [13]. Studies indicate that plant-based proteins are particularly favoured among consumers as an alternative to conventional meat [11, 14, 15].

However, despite the potential benefits and consumer interest, the development of plant-based meat products that truly resemble and rival conventional meat in appearance, flavour, and texture remains a significant challenge for food producers [16]. Achieving the desired texture, water-holding capacity, and mouthfeel akin to

real meat is particularly complex, as the microscopic structure of plant-based proteins differs from that of animal muscle fibres [13]. The use of various additives to enhance flavour, texture, and juiciness has raised concerns regarding nutrition, food safety, cost, and consumer acceptance [17]. Therefore, while plant-based meat holds tremendous promise as a sustainable and ethical alternative to conventional meat, overcoming challenges related to texture, flavour, nutrition, and food safety will be crucial to its widespread acceptance and successful integration into global diets.

Black-eyed peas, a highly nutritious legume, are increasingly considered a promising option for a meat substitute in various food products due to their rich composition and health benefits [18]. These legumes belong to the Fabaceae/Papilionaceae family and are characterized by their diversity across four cultivar groups: Unguiculata, Biflora, Sesquipedalis, and Textilis, which vary by seed size, colour, flavour, yield, and maturity [19]. Cultivated widely across warm regions like Southeast Asia, Africa, the Southern United States, and Latin America, black-eyed peas thrive in warm climates with adequate rainfall [20]. Nutritionally, black-eyed peas are laden with both soluble and insoluble dietary fibres, essential minerals, vitamins, particularly those of the B group, and other beneficial compounds such as phenolic acids that are known for their antioxidant properties [21]. These components are crucial not only for general health but also offer preventive benefits against chronic diseases such as cardiovascular diseases, hypercholesterolemia, obesity, diabetes, and various cancers due to their bioactive compounds [22-27].

In addition to health benefits, black-eyed peas contain a significant concentration of bioactive compounds like polyphenols, carotenoids, vitamins, and omega-3 fatty acids that have attracted attention for their potential to prevent several chronic illnesses [28, 29]. The resistant starch and dietary fibre content of seeds contributes to their low glycemic index, beneficial for diabetes management and weight control, by slowing digestion and reducing the intake of glucose by intestinal cells [30, 31]. Moreover, the phenolic content of black-eyed peas, particularly abundant in the seed coat, exhibits strong antioxidant activities, which help mitigate oxidative stress, thus providing further health advantages [32, 33].

The textural properties of black-eyed peas also make them particularly suitable for meat substitution. When cooked, their firm texture and mild flavour allow them to blend seamlessly into dishes traditionally dominated by ground meat [34, 35], making them an ideal ingredient in patties, meatballs, and other textured protein products. Their capacity to absorb flavours and spices used in meat preparations enhances their appeal, providing a satisfying culinary experience for both meat-eaters and vegetarians alike [36]. Given these nutritional, health, and functional advantages, black-eyed peas stand out as a promising option for developing healthier and more environmentally sustainable meat alternatives.

Recent research indicates that partially (10-50%) substituting meat with pulses (including chickpeas, lentils, black beans, or green peas) not only enhances the nutritional profile of meat products but also improves their environmental footprint and potentially reduces production costs. Importantly, these benefits are achieved without compromising the sensory and physical properties of the meat products [36-39]. Building on these findings, this study aims to assess the physicochemical, sensory, and microstructural properties of patties made with different meat species (chicken, beef, mutton, and buffalo) and partially substituted with black-eyed peas, compared to patties made entirely of black-eyed peas as a control. The goal is to maximize the retention of the physicochemical properties and sensory qualities of meat products while developing healthier and more environmentally sustainable alternatives. The findings of this research can contribute to the growing body of knowledge surrounding sustainable protein sources and aid in creating consumer-directed strategies to promote the adoption of plant-based meat substitutes, thereby further reducing the environmental impact of meat consumption.

Materials and Methods

Materials

Frozen deboned beef, beef fat, frozen deboned buffalo, buffalo fat, frozen deboned mutton, mutton fat, and breast chicken with chicken skin were procured from the wholesale market (Seri Kembangan, Selangor, Malaysia). Black-eyed peas, salt, sugar, garlic, black pepper, sodium tripolyphosphate (STPP), shortening, and isolated soy protein (ISP) 90% were purchased from the local market. Four different species of meat (chicken, beef, mutton, and buffalo) were selected as raw materials based on considerations of diversity in consumer preferences, textural variations, nutritional profiles, and environmental impact considerations.

Preparation of patties

The basic formulation of the patties was from the study conducted by Kahar et al. [40], based on the Malaysian Food Regulations 1985, where the amount of meat should be not less than 65% and fat not more than 30%. Nevertheless, this study modified the formulation to incorporate black-eyed peas as the meat substitute. Black-eyed peas were washed, soaked in potable water for 30 minutes, and subsequently boiled for 45 minutes. The boiled black-eyed peas were mechanically crushed into small pieces using a food processor (Panasonic MK-5087M, Malaysia). Meat from beef, chicken, buffalo, and mutton were cut into small pieces and washed separately under running water. Each species of meat was minced individually for approximately 5 minutes by a meat mincer machine (H.L TJ12-A, Henglian, China). The patties were produced following distinct formulations listed in Table 1. The minced meat was placed in a bowl cutter (Mainca CM-21, Spain), and ice water and salt were incrementally added to the mixture, followed by a 2-minute mixing process. Subsequently, all other ingredients were homogeneously mixed for an additional 8 minutes. The resulting mixture was shaped using a patty's moulder, resulting in uniformly sized patties weighing 70g each.

Table 1. Formulation of black-eyed peas patties with partial replacement of meat from different species

Ingredients	Amount (%)				
	BEP	CB	MB	BEB	BFB
Black-eyed peas	75	37.5	37.5	37.5	37.5
Chicken	-	37.5	-	-	-
Mutton	-	-	37.5	-	-
Beef	-	-	-	37.5	-
Buffalo	-	-	-	-	37.5
Chicken fat	-	15	-	-	-
Mutton fat	-	-	15	-	-
Beef fat	-	-	-	15	-
Buffalo fat	-	-	-	-	15
Shortening	15	-	-	-	-
Black pepper	0.5	0.5	0.5	0.5	0.5
Ice water	4	4	4	4	4
Isolated Soy Protein (ISP)	2	2	2	2	2
Salt	1.5	1.5	1.5	1.5	1.5
Sugar	1	1	1	1	1
Crushed garlic	0.75	0.75	0.75	0.75	0.75
STPP	0.25	0.25	0.25	0.25	0.25

BEP: black-eyed peas patties; CB: chicken with black-eyed peas patties; MB: mutton with black-eyed peas patties; BEB: beef with black-eyed peas patties; BFB: buffalo with black-eyed peas patties.

Cooking yield and shrinkage

The patties underwent a cooking process in an electric oven set at 150°C for 10 minutes. Subsequently, the individual patty samples were weighed both before and after cooking. The percentage change in weight from the original weight was computed to determine the cooking yield, following the methodology outlined by Ramle et al. [41] as expressed in Equation 1. Furthermore, the shrinkage of the patties was determined using the approach proposed by Kahar et al. [42], and the corresponding calculation is represented by Equation 2.

$$Cooking\ yield\ (\%) = \frac{Weight\ of\ cooked\ \ patty}{Weight\ of\ raw\ patty} \times 100 \quad (1)$$

$$Shrinkage(\%) = \frac{(Raw\ thickness - Cooked\ thickness) + (Raw\ diameter - Cooked\ diameter)}{Raw\ thickness + Raw\ diameter} \times 100 \quad (2)$$

Water holding capacity (WHC)

Patty samples (1.5g) were placed with absorbent paper in centrifuge tubes, which were then subjected to centrifugation at 4000 ×g at 20 °C for 15 minutes using a Kubota 3740 centrifuge (Japan). The absorbent paper effectively absorbed the water in the centrifuge. WHC was expressed as the amount of retained water per 100 g of water present in the sample before centrifuging (Equation 3) [43].

$$WHC(\%) = \frac{Weight\ before\ centrifugation - Weight\ after\ centrifugation}{Original\ sample\ weight} \times 100 \quad (3)$$

pH measurement

The pH of each 10g sample was measured by homogenizing it with 100 mL of distilled water for five minutes, followed by pH measurement using a Model HI 84530 pH meter (Hanna Instruments Co., USA).

Proximate analysis

Moisture content, ash content, protein content, fat content, and fibre content of the patties were determined using the Association of Official Analytical Chemists (AOAC) techniques [44].

TPA and gel strength

The TPA and gel strength of the cooked patties were determined using specific equipment and procedures. The TPA analysis was conducted using a texture analyser XT Plus (Stable Micro Systems Ltd., Godalming, Surrey, UK) with the P75 probe [43]. The parameters assessed included hardness, gumminess, chewiness, and cohesiveness. For the TPA analysis, a flat square surface probe and a 75 mm probe were utilized, and a 25 kg load cell was employed. The compression test was performed in one cycle, with the pre-test speed set at 1.00 mm/s, the test speed at 0.8 mm/s, and the post-test speed at 5.00 mm/s. The distance for compression was set at 8.00 mm, and the auto-trigger type was set at 5.0 g.

To determine the gel strength of the patties, a texture analyser (TA-XT2, Stable Microsystem System Ltd., UK) was used. The patties were cut into cylindrical shapes with dimensions of 30 mm in height and 30 mm in diameter. A spherical probe of 5 mm (P/5S) was employed for the analysis, with a test speed of 1.1 mm/s, a force of 10 g, and a distance of 5 mm applied to the sample. In the context of TPA, hardness is defined as the peak force observed at the first compression, while the maximum force required to break the polymer matrix in the stressed region is characterized as gel strength. Cooked patty samples were utilized for both TPA and gel strength analyses, and they were cut into square shapes with dimensions of 30 mm in height and 30 mm in diameter.

Colour parameter

The colour of cooked and uncooked patties was evaluated in terms of lightness (L*), redness (a*), and yellowness (b*) using a chromameter (CR-410, Konica Minolta, Japan) [41].

Microstructure evaluation with scanning electron microscopy (SEM)

Small pieces (10mm×10mm) of uncooked patties were horizontally cut, and thin layers were examined and photographed using a scanning electron microscope (LEO 1455 VPSEM, Cambridge, UK) at 250× magnification.

Sensory evaluation

Fifty untrained panellists participated in sensory evaluation, rating different samples of patties based on texture, aroma, colour, flavour, and overall acceptability using a nine-point hedonic scale.

Statistical analysis

All the experiments were performed in triplicate (n=3) except for sensory tests (n=50). They were analysed using one-way ANOVA and Tukey's test with a confidence level of 95% (p <0.05). All data were analysed using Minitab Statistical Software version 19 (MiniTab Inc., USA).

Results and Discussion

Proximate analysis

Table 2 shows the proximate composition of the various patties produced with black-eyed peas and different meat species. The moisture content of the patties showed significant differences (p <0.05). The patties containing chicken breast (CB) exhibited the highest moisture content (62.99%), likely due to the inherent higher moisture content of chicken breast meat (77.19%) [45]. On the other hand, black-eyed peas influenced the moisture content in all patties, with values ranging from 46.69% in black-eyed peas patties (BEP) to 57.04% in mutton patties (MB).

Regarding the ash content, BEP (2.93%), CB (2.96%), and BFB (2.77%) did not show significant differences (p >0.05), but they were lower than BEB (3.11%) and higher than MB (2.68%), which might be attributed to the variations in meat species and the presence of spices and condiments used in the patties [46]. The protein content in the different patties also displayed significant variations (p <0.05). CB (15.41%) exhibited the highest protein content compared to BFB (14.75%), MB (14.12%), BEB (13.53%), and BEP (10.19%). The higher protein content in CB patties can be attributed to chicken meat being a lean source of protein with less fat than red meats, which are relatively higher in fat and calories.

The fat content in BEP patties (19.20%) was the highest among all the patties, likely due to the addition of shortening. On its own, BEP has a low-fat content (1% on a dry basis of average black-eyed peas) [47]. Shortening is a solid fat derived from liquid vegetable oil, processed through hydrogenation, partial-hydrogenation, fractionation, and interesterification [48]. The fibre content in all the patties showed no significant difference (p >0.05), possibly due to the presence of black-eyed peas in all formulations. Legumes like black-eyed peas are known to contain dietary fibre, which can influence the physical, rheological, and sensory properties of food systems based on their physicochemical properties [49]. Mallillin et al. [50] reported 34% dietary fibre (TDF) in cowpea/black-eyed peas, which is comparable to other legumes, supporting the consistent total fibre content observed in all the patties.

Water holding capacity, cooking yield, shrinkage, pH value, and gel strength analysis

Table 3 presents the results of the water holding capacity (WHC), cooking yield, shrinkage, pH value, and gel strength for the various patties prepared with different meat species. Notably, the cooking yield and pH value showed minor differences among the patties, while WHC, shrinkage, and gel strength exhibited more significant variations. CB (29.53%) exhibited the highest WHC ($p < 0.05$), followed by BFB (24.21%), BEB (22.80%), MB (20.4%), and BEP (15.66%), which had the lowest WHC. The contribution of dietary fibre to higher WHC has been reported in the literature [51]. However, in this study, BEP exhibited the lowest WHC, possibly due to its lower moisture content.

Table 2. Proximate analysis of black-eyed peas patties with partial replacement of meat from different species

Proximate Composition (%)	BEP	CB	MB	BEB	BFB
Moisture content	46.69±0.45^{d}	62.99±0.15^{a}	57.04±0.12^{b}	56.45±0.24^{b}	55.77±0.07^{c}
Fat content	19.20±3.3^{a}	12.13±0.54^{c}	7.80±1.45^{bc}	13.05±3.63^{ab}	13.89±0.44^{ab}
Protein content	10.19±0.03^{d}	15.41±0.30^{a}	14.12±0.15^{bc}	13.53±0.31^{c}	14.75±0.27^{b}
Ash content	2.93±0.03^{ab}	2.96±0.07^{ab}	2.68±0.25^{b}	3.11±0.12^{a}	2.77±0.10^{ab}
Fiber content	0.56±0.02^{ab}	0.57±0.02^{a}	0.56±0.03^{ab}	0.53±0.03^{ab}	0.51±0.01^{b}

The values represent means ± SD. Small letters that do not share the same letter are significantly different ($p < 0.05$) in the same row

Cooking yields were consistently high for all patties (p <0.05), ranging from the highest in BEP (92.70%) to CB (92.23%), BEB (91.44%), MB (90.87%), and BFB (89.02%). The low cooking yield reported in some studies can be attributed to moisture and fat loss, as well as the denaturation of meat proteins [52]. In contrast, the higher cooking yield observed for BEP could be attributed to its lower moisture content and the distinctive properties of black-eyed pea protein compared to animal protein. Pea fibre addition to meat products has been found to increase cooking yield without affecting sensorial properties [53,54]. The shrinkage values were lower for BEP (5.02%) compared to CB (11.71%), MB (14.79%), BEB (12.95%), and BFB (13.09%). Heating during the cooking process causes moisture loss and protein shrinkage in the patties [55]. The meat patties tend to shrink due to the loss of water and fat during cooking [56].

The pH values exhibited no significant difference (p <0.05) between BEP (6.53), CB (6.49), and BFB (6.40). MB (6.28) and BEB (6.32) displayed lower pH values (p >0.05). CB (395.14 N/m²) and MB (423.53 N/m²) had higher gel strength compared to BEP (324.80 N/m²). Gel strength is influenced by the molecular weight distribution in the gelatin [57,58], and the lower gel strength of gelatin restricts its application areas in biomaterials [59]. The increase in gel strength could be attributed to the formation of higher molecular weight structures resulting from cross-linking reactions between protein molecules (gelatin components) and polyphenols [60].

Table 3. Water holding capacity, cooking yield, shrinkage, pH value and gel strength of black-eyed peas patties with partial replacement of meat from different species

Properties	BEP	CB	MB	BEB	BFB
Water Holding Capacity (%)	15.66±1.25^{c}	29.53±1.73^{a}	20.42±1.17^{bc}	22.80±2.74^{b}	24.21±2.12^{b}
Cooking yield (%)	92.70±0.28^{a}	92.23±0.20^{ab}	90.87±0.26^{b}	91.44±0.09^{ab}	89.02±1.09^{c}
Shrinkage (%)	5.02±0.38^{c}	11.71±0.54^{b}	14.79±0.67^{a}	12.95±0.46^{b}	13.09±0.59^{b}
pH value	6.53±0.03^{a}	6.49±0.05^{a}	6.28±0.04^{b}	6.32±0.10^{b}	6.40±0.03^{ab}
Gel strength (N/m²)	241.8±32.05^{c}	395.14±58.25^{ab}	423.53±10.78^{a}	324.80±9.74^{bc}	313.44±14.7^{bc}

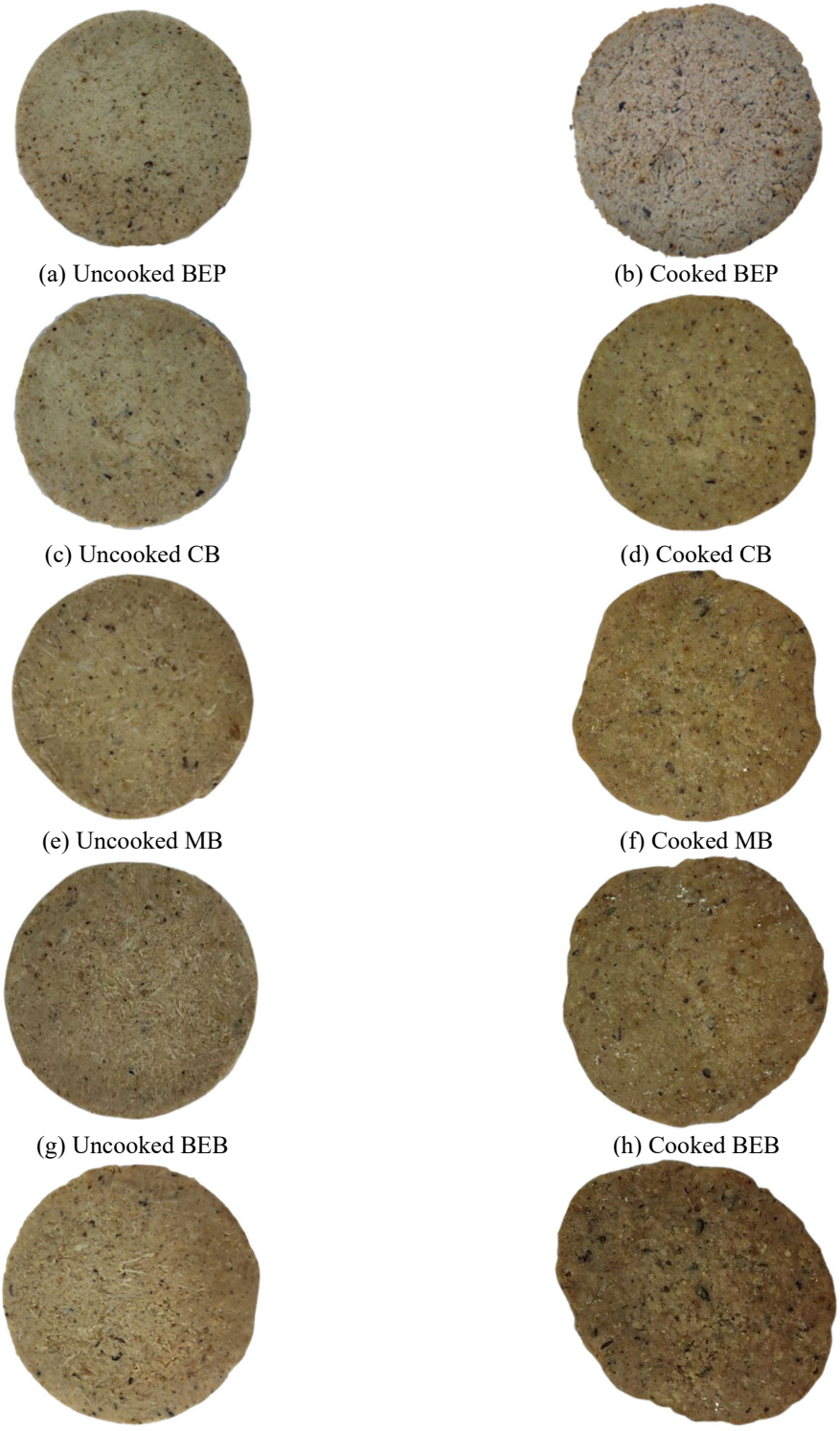
The values represent means ± SD. Small letters that do not share the same letter are significantly different ($p < 0.05$) in the same row

Colour parameter and TPA

Table 4. presents the colour parameters of uncooked and cooked patties, as well as the results of the TPA for patties with partial replacement of meat with black-eyed peas from different species. The colour traits of cooked meat products are influenced by the formulation additives and the inherent pigmentation of the meat [61]. Figure 1 illustrates the comparison of colour parameters for uncooked patties. In terms of lightness (L*), BEP (64.14) exhibited a lighter colour (p <0.05) compared to other patties, followed by CB. This distinction can be attributed to the natural colour of black-eyed peas, while chicken meat is known for its lighter colour compared to other meat species. The L* value reflects the paleness of the meat, and chicken breast, characterized by its light and pale nature [62], exhibited the highest value. The redness (a*) of BEB (6.35), BFB (6.10), and MB (5.23) was significantly higher than CB (3.43) and BEP (3.45). This observation is consistent with the known fact that red meats, such as goat, beef, and buffalo, have higher redness due to the presence of myoglobin [63]. For the yellowness (b*), BEP (18.70) had the highest value, followed by MB (12.70), CB (12.30), BEB (11.21), and BFB (11.03). The vibrant yellow colour of the black-eyed peas used in all patties likely contributed to their yellow appearance.

Regarding the lightness (L*) of cooked patties, BEP (47.58) and CB (44.77) showed similar values (p >0.05) but were lighter (p <0.05) than MB, BEB, and BFB. This finding is consistent with the raw patties, except for CB. In terms of redness (a*), BEB (8.06) displayed the highest redness and was significantly higher than CB. The higher myoglobin content in BEB could account for its greater red colour compared to meat with lower levels of myoglobin. The yellowness of cooked patties was highest in BEP (19.94), followed by CB (15.43), MB (10.47), BEB (10.34), and BFB (9.36). The Maillard reaction, occurring during the cooking process, produces brown pigments by rearranging amino acids and certain sugars, leading to the formation of ring structures that reflect light and give the meat a brown appearance. The protein denaturation during cooking causes the patties to darken in colour. Cooked meats typically have a dull-brown colour due to heat-induced denaturation of myoglobin, the water-soluble sarcoplasmic heme protein responsible for the red colour of fresh meat. Consequently, the biochemistry of cooked colour differs from that of fresh meat colour, where the heme protein is in its original state. However, there is a strong relationship between the chemistry of myoglobin in raw meat and the cooked product, and both are affected by various factors (endogenous and exogenous) [64–66]. Figure 1. provides a more detailed comparison of colour parameters for the cooked patties of different samples.

As for the TPA, no significant differences (p >0.05) were observed between BEP, CB, MB, BEB, and BFB for the hardness, gumminess, chewiness, and cohesiveness of the patties. However, the hardness of BFB was higher compared to other patties. The increased hardness during cooking can primarily be attributed to the denaturation and aggregation of myosin and actin, leading to protein matrix shrinkage and fluid expulsion [67]. This observation also aligns with the gumminess and chewiness of BFB, which were the highest compared to BEB, MB, CB, and BEP [68]. The cohesiveness of the patties was influenced by sarcoplasmic and myofibrillar proteins [69]. BEP exhibited low cohesiveness, while BEB displayed high cohesiveness.



(a) Uncooked BEP (b) Cooked BEP

(c) Uncooked CB (d) Cooked CB

(e) Uncooked MB (f) Cooked MB

(g) Uncooked BEB (h) Cooked BEB

(i) Uncooked BFB (j) Cooked BFB

Figure 1. The visual appearance of raw and cooked BEP, CB, MB, BEB, and BFB

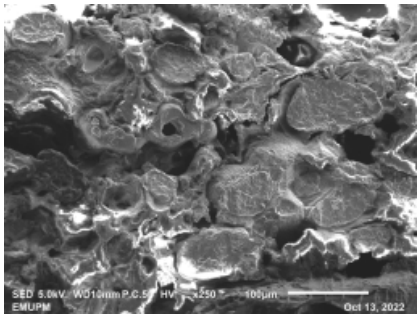
Table 4. Colour parameter of uncooked and cooked patties and textural profile analysis of black-eyed peas patties with partial replacement of meat from different species

Properties		BEP	CB	MB	BEB	BFB
Colour Un-cooked	L*	64.14±0.57^{a}	54.36±1.55^{b}	47.22±0.61^{c}	48.43±2.19^{c}	45.91±1.64^{c}
	a*	3.45±0.07^{c}	3.43±0.10^{c}	5.23±0.42^{b}	6.35±0.57^{a}	6.10±0.59^{ab}
	b*	18.70±0.27^{a}	12.30±0.19^{bc}	12.70±0.24^{b}	11.21±0.38^{c}	11.03±1.03^{c}
Colour Cooked	L*	47.58±0.68^{a}	44.77±2.06^{a}	35.10±0.44^{b}	35.19±0.79^{b}	32.40±0.85^{b}
	a*	6.61±0.17^{ab}	5.36±0.15^{b}	6.83±0.33^{ab}	8.06±1.27^{a}	7.17±0.84^{ab}
	b*	19.94±0.30^{a}	15.43±0.82^{b}	10.47±0.41^{c}	10.34±0.25^{c}	9.36±1.17^{c}
TPA	Hardness (g)	11280±2041^{a}	11876±5721^{a}	13328±11676^{a}	13757±1521^{a}	27202±4507^{a}
	Gumminess (g)	3000±690^{a}	7344±2958^{a}	8468±7400^{a}	8804±627^{a}	11309±1193^{a}
	Chewiness (J)	1226±322^{a}	5638±2362^{a}	6513±5693^{a}	6480±651^{a}	8260±662^{a}
	Cohesiveness	0.27±0.03^{a}	0.63±0.05^{a}	0.42±0.37^{a}	0.64±0.03^{a}	0.42±0.03^{a}

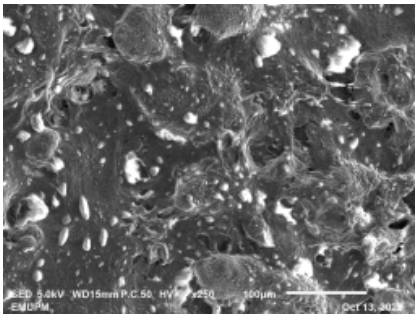
The values represent means ± SD. Small letters that do not share the same letter are significantly different ($p < 0.05$) in the same row

SEM

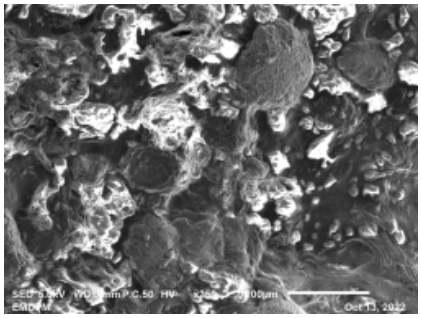
Figure 2. presents the microstructure of the cooked BEP, CB, MB, BEB, and BFB (the magnification scale used was 250× for all samples). Overall, the microstructures of CB, MB, BEB, and BFB exhibited certain similarities. In contrast, the protein matrix of CB is more compact compared to that of BEP. This difference likely results from higher protein content of CB, which enhances the density of the matrix [70]. The protein content in meat formulations significantly affects the texture by influencing the density and cohesion of the protein matrix. Higher protein contents lead to denser and more cohesive structures, which explains the observed difference in the SEM images of the patties [71]. Additionally, BEP exhibited a less intact structure, as evidenced by the presence of several gaps observed in the microscopic image. This observation aligns with the gel strength results, where BEP had the lowest gel strength compared to the other meat species. It indicates that the protein matrix in BEP patties was less cohesive, resulting in a patty with a less structurally compact composition. Studies on the impact of dietary fiber (chickpea fiber) on meat proteins, also reveal that fiber can fundamentally alters the three-dimensional network of myofibrillar protein [72]. This effect of dietary fiber is critical in modifying the structural integrity of patties, influencing their ability to form a cohesive network, which in turn affects their textural properties and gel strength. These findings imply that disparities in protein composition and structural attributes between black-eyed peas and meat-based patties played a pivotal role in determining their capacity to form a cohesive and integral network, thereby influencing both microstructural integrity and gel strength outcomes.



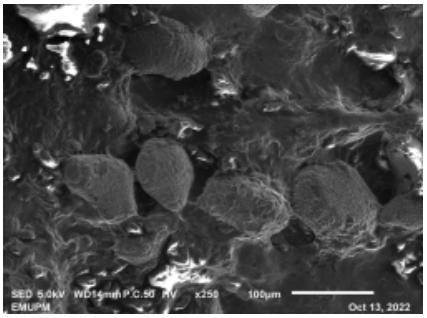
(a) BEP



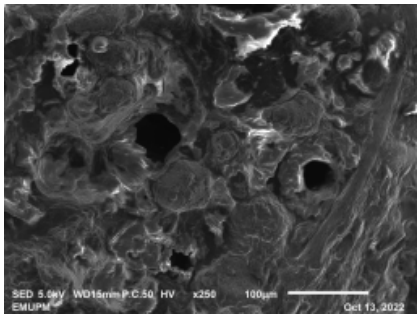
(b) CB



(c) MB



(d) BEB



(e) BFB

Figure 2. The microstructure images of BEP, CB, MB, BEB, and BFB

Sensory evaluation

The results of the sensory evaluations for black-eyed peas (BEP) patties with partial replacement of meat from different species are presented in Table 5. The sensory attributes examined included texture, aroma, colour, flavour, and overall acceptability for BEP, CB, MB, BEB, and BFB. Based on the sensory analysis, no significant differences (p >0.05) were observed in the texture, aroma, colour, flavour, and overall acceptability among the patties prepared with different meat species. However, BEB patties received higher overall acceptability scores compared to the other patties, likely due to favourable evaluations in terms of texture, aroma, and colour. The unique taste characteristics of beef may have been recognized by the panellists during the evaluation, and they may have appreciated the combination of beef with black-eyed peas.

In contrast, the control patty, BEP, received less favourable ratings for texture, aroma, colour, flavour, and overall acceptability (p <0.05) compared to the other patties. These results are in line with the findings from the texture profile analysis, where BEP showed lower values in hardness, gumminess, chewiness, and cohesiveness. The inferior ratings could be attributed to the lower moisture content and reduced gel strength of BEP, as evidenced by the less intact structure observed in Figure 1 and Figure 2.

Table 5. Sensory evaluation of black-eyed peas patties with partial replacement of meat from different species

Properties	BEP	CB	MB	BEB	BFB
Texture	2.90±1.73[b]	6.78±1.80[a]	6.52±1.47[a]	7.08±1.55[a]	6.50±1.61[a]
Aroma	3.98±1.78[b]	6.32±1.97[a]	6.18±1.72[a]	6.44±1.85[a]	6.24±1.65[a]
Colour	4.02±2.20[b]	6.10±2.04[a]	6.10±1.73[a]	6.58±1.84[a]	6.46±1.80[a]
Flavour	3.26±2.13[b]	6.96±1.81[a]	6.96±1.81[a]	6.92±1.69[a]	6.72±1.64[a]
Overall acceptability	3.10±1.71[b]	6.82±1.84a	6.40±1.85[a]	7.04±1.64[a]	6.68±1.65[a]

The values represent means ± SD. Small letters that do not share the same letter are significantly different ($p < 0.05$) in the same row

To sum up, the sensory evaluation revealed that the panellists supported the utilization of black-eyed peas in combination with different meat species as a meat substitute. The patties prepared with partial meat replacement using black-eyed peas received favourable evaluations compared to those with 100% meat replacement by black-eyed peas. These findings indicate the feasibility and acceptability of incorporating black-eyed peas into meat products, offering a promising approach to enhance nutritional value and sustainability in meat-based formulations. However, further studies are warranted to optimize the formulation and processing techniques to ensure the best sensory attributes and consumer acceptance of these innovative patties.

Conclusion

In summary, this study explored the feasibility of using black-eyed peas as a partial meat substitute in various patties containing different meat species. Black-eyed peas significantly influenced the moisture content of the patties, leading to higher moisture levels in chicken breast (CB) patties due to the inherent moisture content of chicken meat. However, all patties incorporated with black-eyed peas demonstrated variations in protein, fat, and fibre content, reflecting the diverse composition resulting from the incorporation of this legume. In terms of sensory evaluations, the patties with partial meat replacement using black-eyed peas received more favourable ratings compared to the control patty made entirely of black-eyed peas (BEP). Specifically, beef patties (BEB) stood out with higher overall acceptability, potentially due to the unique taste of beef combined with black-eyed peas. On the other hand, the control patties (BEP) were perceived less favourably, with lower scores in texture, aroma, colour, flavour, and overall acceptability, aligning with the textural analysis results showing lower hardness, gumminess, chewiness, and cohesiveness. Microstructure analysis revealed differences in the protein matrix, with black-eyed pea patties displaying a less compact structure compared to those with meat species. These differences negatively implicated the overall sensory attributes and acceptance of the patties. Overall, the study suggests that incorporating black-eyed peas as a partial meat substitute offers a promising pathway to enhance the nutritional value and sustainability of meat-based products. By improving the sensory qualities and addressing potential challenges, the food industry can potentially promote the adoption of black-eyed pea-based patties as an environmentally friendly and nutritionally enriched alternative to traditional meat products. In light of these findings, future research should focus on expanding the variety of legumes used as meat replacers and optimizing processing methods to enhance the sensory and nutritional qualities of these products. This approach will not only cater to the growing market for sustainable and healthy food options but also contribute to environmental conservation and public health improvements.

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SCREENING PHYSICAL FACTORS TO ENHANCE BIOETHANOL PRODUCTION IN OIL PALM TRUNK SAP FERMENTATION

(Saringan Faktor Fizikal Untuk Penghasilan Bioetanol Melalui Fermentasi Air Perahan Batang Kelapa Sawit)

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Abstract

Fermentation is a simple process that can produce a high–demand byproduct such as bioethanol. To produce a high yield concentration of bioethanol by *Saccharomyces cerevisiae* (*S. cerevisiae*) in the fermentation of oil palm trunk (OPT) sap as a sole carbon source, an experimental design by using a two–level full factorial design (2^{k-1}) was conducted at a laboratory scale to screen important factor in fermentation. The experiment was conducted to study the effect of pH, temperature, agitation rate, incubation time, and inoculum size as important physical factors in fermentation. The factors were exploited, respectively, at low (–1) and high (+1) level parameter ranges of 3.5 to 7.5 for pH, 20°C to 40°C for fermentation temperature, 0 to 50 rpm for agitation rate, 20 to 48 hours for the time of incubation, and 5% v/v to 15% v/v of inoculum size in the fermentation media. Thirty–two combinations of experimental design with a 2^{5-1} full factorial design reflected in 32 flasks of OPT sap with *S. cerevisiae* were conducted for the fermentation process. The bioethanol yield concentration was investigated in these experiments using gas chromatography with flame ionization detection (GCFID). In this study, the maximum bioethanol yield concentration was 37.8383mg/mL with pH media at 3.5, 5% v/v inoculum size, temperature at 40°C, agitation rate at 50rpm, and incubation length of 48 hours. Temperature, pH, agitation rate, incubation time, and inoculum size in the fermentation media were significant contributing factors in the fermentation of *S. cerevisiae* in OPT sap to produce a high yield concentration of bioethanol. These factors can be further optimized to increase bioethanol yield concentration in the fermentation by *S. cerevisiae* in OPT sap.

Keywords: fermentation, bioethanol, full factorial design, oil palm trunk sap, physical factor

Abstrak

Fermentasi adalah proses yang mudah dan boleh menghasilkan produk sampingan yang sangat diperlukan seperti bioetanol. Untuk menghasilkan hasil bioetanol oleh *Saccharomyces cerevisiae* (*S. cerevisiae*) dalam proses fermentasi cecair perahan batang kelapa

sawit (OPT) sap sebagai sumber karbon utama, satu reka bentuk eksperimen dengan menggunakan reka bentuk faktorial penuh dua peringkat (2^{k-1}) telah dijalankan di skala makmal untuk menyaring faktor penting dalam proses fermentasi. Eksperimen ini dijalankan untuk mengkaji kesan pH, suhu, kadar pengadukan, masa, dan kandungan *S. cerevisiae* sebagai faktor fizikal penting dalam penapaian. Faktor–faktor tersebut dieksploitasi masing–masing pada julat parameter paras rendah (–1), dan tinggi (+1) 3.5 hingga 7.5 untuk pH, 20°C hingga 40°C untuk suhu penapaian, 0 hingga 50 rpm untuk kadar pengadukan, 20 hingga 48 jam untuk masa pengeraman, 5% v/v hingga 15% v/v kandungan *S. cerevisiae* dalam media fermentasi. Tiga puluh dua kombinasi reka bentuk eksperimen dengan 2^{5-1} reka bentuk faktorial penuh yang dijalankan di dalam 32 kelalang yang mengandungi larutan perahan OPT bersama *S. cerevisiae* telah dijalankan untuk proses fermentasi, dan tindak balas hasil bioetanol telah disiasat dalam eksperimen ini. Dalam kajian ini, kepekatan hasil bioetanol maksimum ialah 37.8383 mg/mL dengan media pH pada 3.5, 5% v/v kandungan *S. cerevisiae*, suhu pada 40°C, kadar pengadukan pada 50rpm, dan panjang pengeraman selama 48 jam. Suhu, pH, kadar pengadunan, masa penyejukan, dan saiz inokulum merupakan faktor penyumbang penting dalam fermentasi oleh *S. cerevisiae* dalam sap OPT untuk menghasilkan hasil bioetanol yang tinggi. Kesemua faktor ini boleh dioptimumkan lagi untuk meningkatkan pengeluaran bioetanol dalam proses fermentasi oleh *S. cerevisiae* dalam sap OPT.

Kata kunci: fermentasi, bioetanol, reka bentuk faktorial penuh, air perahan batang kelapa sawit, faktor fizikal

Introduction

The development of biofuels as a sustainable replacement for fossil fuels has become increasingly important in recent years. Various microorganisms can be used in fermentation to make bioethanol used in biofuel [1–2]. Various biomass sources produce bioethanol, such as plant sugars, lignocellulosic materials, and agricultural waste [3]. Employing microorganisms like *Saccharomyces cerevisiae* (*S. cerevisiae*) to ferment carbohydrates is a typical way to make bioethanol in fermentation [4–5]. Carbohydrates (including total sugar content) undergo anaerobic metabolism to produce bioethanol and carbon dioxide [6]. Robust fermentation capabilities make the *S. cerevisiae* strain widely used in bioethanol synthesis and an excellent choice for large–scale bioethanol production [7–8]. *S. cerevisiae* has several beneficial industrial properties, including rapid growth, efficient anaerobic glucose metabolism, and high resistance to various environmental stressors such as high yield concentration of bioethanol, low pH, and low oxygen [9–12]. Thus, it is crucial to understand the influence of physical factors throughout the fermentation process to achieve a high yield concentration of bioethanol.

Physical factors such as temperature, pH, agitation, inoculum size, and inoculum time play crucial roles in *S. cerevisiae* enzyme activity in fermentation. In the optimum temperature range, the bacteria's growth and metabolism speed up as the temperature rises [13]. Therefore, the rate of the fermentation reaction rises. However, the enzymes are inactivated when the temperature exceeds the optimum range and cause mortality of the microbe, the fermentation cycle is shortened, and the yield concentration of bioethanol is lowered [13, 14]. The pH can influence *S. cerevisiae* fermentation by altering enzyme activity and the charge state of cell membranes. This may affect the metabolic and physiological changes produced at high or extreme values, preventing yeast growth [14]. The correlation of these factors may influence the growth and metabolic activity of *S. cerevisiae*. In this work, the Design of experiments (DOE) was employed to screen and determine the significant physical factors that affect *S. cerevisiae*'s ability to produce bioethanol. Understanding their impact on bioethanol production is essential for enhancing process efficiency and yield.

The oil palm trees aged above 25 decrease their oil production; thus, replantation of the oil palm trees is essential to maintaining oil production. Thus, maintaining stable and high levels of palm oil production within the current palm oil extent is a potential strategy to relieve deforestation pressure [15]. The oil palm trunk (OPT) is a byproduct of the oil palm industry and is rich in ready–to–ferment sugars, which can serve as a potential low–cost fermentation medium for bioethanol yield concentration. Selecting a suitable fermentation medium is essential for practical and long–lasting bioethanol production. Shahirah et al. [16] found that glucose (75.51g/L) was the dominant sugar in OPT sap, followed by sucrose (62.68g/L), fructose (29.41g/L), and a small amount of galactose (4.49g/L). OPT sap contains a small amount of micronutrients such

as P (0.001%) and Mg (0.014%) and other micronutrients in sufficient amounts such as Mo, Na, Ca, Zn, and vitamins that promote the growth of yeast cells [16–17]. Liu et al. [18] suggested that the bioethanol yield produced from fermentation improves by adding nutrients such as magnesium sulfate, ammonium sulfate, and disodium hydrogen phosphate. At the same time, these micronutrients were contained in OPT sap liquid as a buffer [19]. Utilizing this resource not only adds value to the oil palm industry but also promotes the use of sustainable feedstocks, reducing the reliance on food crops for bioethanol yield concentration. The felled OPT sap produced by the oil palm industry could become a versatile, affordable, and renewable waste material. However, its effectiveness as a fermentation medium for *S. cerevisiae's* bioethanol yield concentration needs more research.

The optimization of physical factors during fermentation using OTP sap as a medium for *S. cerevisiae* to produce bioethanol was attempted to address in this work. Examining one variable at a time may be acceptable in some circumstances, but it occasionally ignores the combined impact of several factors, mainly when doing experiments involving physiological systems [20–27]. Therefore, DOE was used as a statistical approach for efficient screening and optimization of multiple factors simultaneously. Operational variables interact during fermentation and influence their respective effects on response [28]. The experimental method must account for these interactions so that a set of optimal research conditions can be determined. Full factorial design involves systematically varying all possible combinations of factors, providing comprehensive visions into the interactions between factors and their impact on the response variable. This approach enables the identification of critical process parameters and their optimal levels. Empirical models and statistical analysis are significant, thus providing better control and understanding of the optimization of physical factors to produce bioethanol from OTP sap [21, 26–29]. Hence, this study aimed to optimize the physical factors of bioethanol production by *S. cerevisiae* during fermentation using the DOE full factorial design approach. The fermentation process can be optimized to maximize bioethanol yield concentration by identifying the critical factors and their optimal levels.

Materials and Methods

Preparation of OPT sap

The 25 years old *Elaeis guineensis* oil palm tree from Ladang Sawit Kampung Sungai Ranggam Muar was felled, and OPT was squeezed using a sugar cane press machine within 12 hours to produce sap. Homogenized OPT sap liquid was filtered using a 9.0 filter and autoclaved at 121°C for 20 minutes. Sterilized OPT sap was kept at 4 °C before usage.

Microorganisms and preadaptation

S. cerevisiae, a commercial baker's yeast from AB Mauri Malaysia, was used as a fermenter. *S. cerevisiae* was cultured in 10% w/v yeast extract, 20% w/v peptone, 20% w/v glucose as sole carbon source, and 15% w/v agar and incubated at 30 °C for 24 hours. One colony was recultured onto a new yeast extract peptone glucose agar (YPGA) and incubated at 30 °C for 24 hours. Five colonies from the pure culture plate were introduced into sterile OPT sap and incubated at 30 °C for 18 hours before usage.

Two–level factorial design

Fermentation was performed in sterilized OPT sap media in a 250 mL shake flask with 50 mL fermentation media as working volume. Experimental design and statistical analysis were analyzed using Design–Expert® v12 software. Five physical factors in the fermentation by *S. cerevisiae* were exploited, respectively, at low (–1) and high (+1) level parameter ranges 3.5 to 7.5 for pH (factor A), 20°C to 40°C for fermentation temperature (factor B), 0 to 50rpm for agitation rate (factor C), 20 to 48 hours for time of incubation (factor D) and 5% v/v to 15% v/v of inoculum size (factor E) in the fermentation media. In contrast, the other remaining factors were kept constant. A full factorial design, 25, comprising 32 experimental runs, was performed to evaluate the effect of a physical factor of fermentation by *S. cerevisiae* in producing a high yield concentration of bioethanol by using OPT sap as the sole carbon source medium. Fermentation parameters for each experiment set were performed with five physical factors, as in Table 1.

Samples were collected in all experiments, and bioethanol yield concentration was analyzed.

Determination of bioethanol yield concentration

The bioethanol content was determined using gas chromatography (GC) with flame ionization detection (FID). The Agilent 7890B GC System and ZB–WAX Plus column (60m × 0.25mm × 0.25µm) from Phenomenex, USA, were used. 100 µL of supernatant from the fermentation product was mixed with 900 µL of n–propanol and filtered through a 0.22 µL nylon filter before being injected into GCFID for analysis. The minimum and maximum temperatures were 40 and 200 °C, respectively. The temperature rate in the oven was 15 per minute up to 140 °C and 50 per minute up to 200 °C. Nitrogen was the carrier gas with a flow rate of 1.4 mL/min and a temperature of 250 °C for the injection opening. The result of the data was recorded, and the graphic was created.

Results and Discussion

Five physical factors and parameters expected to influence bioethanol yield concentration were employed in the fermentation of sugar in OPT sap by *S. cerevisiae* to produce a high yield concentration of bioethanol. Table 1 shows the design matrix covering five variables to evaluate their effect on bioethanol yield concentration as the response for bioethanol yield concentration in mg/mL; the runs were randomized for statistical reasons. The physical factor variables included in the screening experiment, their setting, and the results of all 32 experimental two–level factorials are shown in Table 1. Each independent variable was investigated at a high (+1) and a low (–1) level. The variables affecting bioethanol yield concentration were identified based on confidence levels above 94% (p <0.05). The experiments were performed to optimize the physical factor of fermentation by *S. cerevisiae* on producing a high bioethanol yield concentration by using OPT sap as the sole carbon source medium. The variables having the most significant effect on bioethanol yield concentration in the fermentation of OPT sap were identified using a 2–level factorial design.

The experiment showed that bioethanol yield concentration varied from 5.79 mg/mL to 37.84 mg/mL from various combinations of five parameters. The results were analyzed using the analysis of variance (ANOVA) as appropriate to the experimental design used. The regression equation obtained after the variance analysis showed the bioethanol production level as a function of different variables. The interaction of variables pH (A), temperature (B), agitation rate (C), incubation time (D), and inoculum size (E) on bioethanol production in the fermentation of OPT sap were summarized in regression Equation (1) as below:

$$\text{Bioethanol yield concentration (mg/mL)} = 19.9919 - 0.544538A + 3.87677B + 0.512207C + 7.60135D + 0.970862E - 0.756333AB - 0.119174AC - 0.569173AD + 0.686418AE + 0.359868BC + 0.219447BD - 0.254717BE - 0.569908CD - 0.280364CE - 0.610352DE \quad (1)$$

The half–normal plot can be utilized to determine the significant factors affecting bioethanol yield concentration in the fermentation of OPT sap by *S. cerevisiae*. The half–normal plot showed the symbol of factors far away from the linear line as the most significant factors toward bioethanol yield concentration. The half–normal plot of effects revealed two large main effect factors, incubation time (D) and temperature (B), with a p–value of 0.007, as in Figure 1. Incubation time (D) fell far from the line and represented the strongest factor affecting bioethanol yield concentration, followed by temperature (B). Zhang et al. [30] and Silva et al. [31] also reported that incubation time and temperature were some of the most important factors in the fermentation by *S. cerevisiae* to produce bioethanol.

Table 1. Full factorial design of fermentation physical factors

Run Order	Factor					Response Bioethanol (mg/mL)
	A	B	C	D	E	
1	– 1	+ 1	– 1	– 1	– 1	14.42
2	+ 1	+ 1	+ 1	– 1	+ 1	17.92
3	+ 1	+ 1	– 1	– 1	– 1	10.53
4	+ 1	+ 1	+ 1	– 1	– 1	15.68
5	+ 1	+ 1	+ 1	+ 1	+ 1	31.22
6	+ 1	– 1	+ 1	– 1	– 1	6.70
7	– 1	– 1	– 1	– 1	+ 1	7.92
8	– 1	– 1	+ 1	+ 1	+ 1	24.96
9	– 1	+ 1	+ 1	– 1	+ 1	19.54
10	+ 1	+ 1	– 1	– 1	+ 1	17.48
11	+ 1	– 1	+ 1	+ 1	+ 1	22.76
12	+ 1	– 1	– 1	+ 1	– 1	20.64
13	– 1	+ 1	+ 1	+ 1	– 1	37.84
14	+ 1	– 1	– 1	– 1	– 1	8.40
15	– 1	+ 1	– 1	– 1	+ 1	16.43
16	+ 1	+ 1	+ 1	+ 1	– 1	29.19
17	– 1	+ 1	– 1	+ 1	– 1	32.12
18	– 1	– 1	+ 1	+ 1	– 1	22.03
19	+ 1	– 1	+ 1	+ 1	– 1	22.12
20	– 1	– 1	– 1	+ 1	+ 1	23.22
21	+ 1	+ 1	– 1	+ 1	– 1	29.06
22	+ 1	– 1	– 1	+ 1	+ 1	27.38
23	+ 1	+ 1	– 1	+ 1	+ 1	29.46
24	– 1	– 1	+ 1	– 1	+ 1	9.87
25	– 1	– 1	– 1	– 1	– 1	5.79
26	+ 1	– 1	– 1	– 1	+ 1	9.48
27	+ 1	– 1	+ 1	– 1	+ 1	13.13
28	– 1	+ 1	+ 1	+ 1	+ 1	30.16
29	– 1	+ 1	– 1	+ 1	+ 1	34.47
30	– 1	+ 1	+ 1	– 1	– 1	16.38
31	– 1	– 1	– 1	+ 1	– 1	24.86
32	– 1	– 1	+ 1	– 1	– 1	8.57

The conversion process took time, and sufficient incubation was required to ensure that all available sugars were metabolized and converted into bioethanol. The length of the incubation time influenced the growth and activity of *S. cerevisiae*, affecting the bioethanol yield concentration. However, longer incubation times could also lead to the formation of undesirable byproducts and secondary metabolites, such as higher alcohols and esters, which could inhibit *S. cerevisiae* cells and negatively affect bioethanol quality [11]. Incubation temperature played a crucial role in the fermentation process of *S. cerevisiae* for bioethanol yield concentration. At optimal temperatures, *S. cerevisiae* cells could effectively use sugar and convert it into bioethanol. Higher temperatures could speed up yeast metabolism, resulting in faster fermentation rates, but could also increase the production of unwanted byproducts [32]. On the other hand, lower temperatures

could slow yeast metabolism and fermentation, reducing bioethanol yield concentration. Additionally, temperature influences the solubility and diffusion of gases like oxygen and carbon dioxide, which are essential for yeast growth and fermentation. Higher temperatures generally reduced the solubility of gases in the fermentation medium, thus reducing oxygen availability for yeast respiration [33]. Higher temperatures could also increase the rate of carbon dioxide production, impacting pH and fermentation performance. Furthermore, higher temperatures could improve yeast's ability to tolerate higher bioethanol concentrations, allowing for increased bioethanol yield concentration. However, excessive temperatures could cause thermal stress, affecting yeast viability and fermentation efficiency [34].

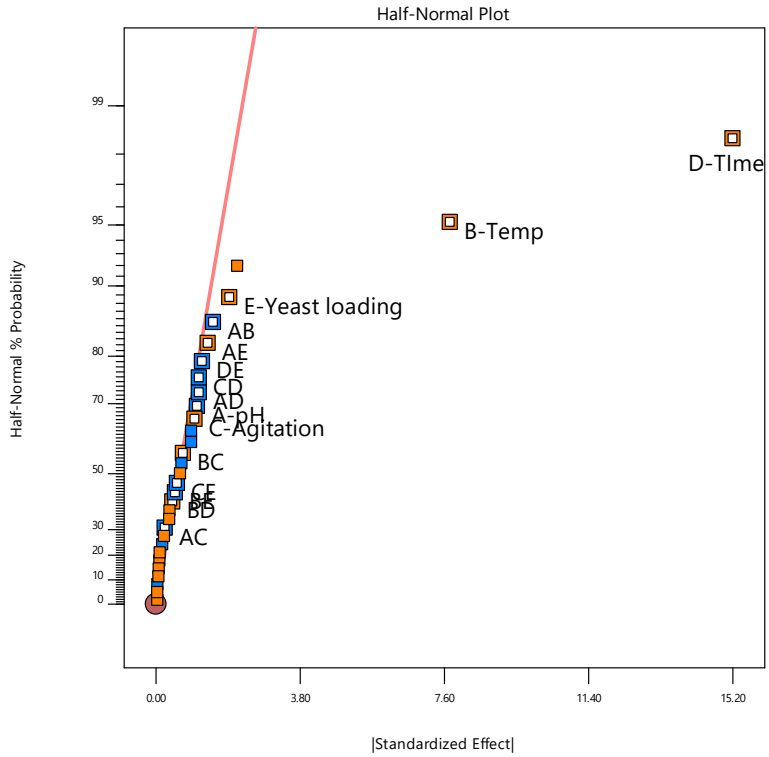


Figure 1. Half–normal plot effect of factor A (pH), B (incubation temperature), C (agitation rate), D (incubation time) and E (inoculum size) in fermentation. Orange dots indicate a positive effect, while blue dots indicate a negative effect

This result could have been further interpreted in the Pareto Chart, as in Figure 2, a graphical tool used to display the magnitudes of the effects from the results obtained from the largest to the smallest effects. Referring to the Pareto charts, orange bars represent a positive effect, and blue bars represent a negative effect. A positive effect meant the response variable increased as the factor level increased. This suggested that higher levels of the factor were beneficial for maximizing the response. On the other hand, an adverse effect meant that the response variable decreased as the factor level increased. In this case, lower factor levels were preferred to maximize the response. The main factors or two–factor interactions were displayed on the top of the bar. The residual's degree of freedom (df) was 16, so the critical t–value was 2.1199. The Bonferroni limit was a more conservative t–value that accounted for the estimated effects by dividing the risk value alpha by the desired probability, producing a value of 3.7880. The Pareto chart showed that incubation time was the most critical factor affecting bioethanol yield concentration, followed by the incubation temperature. Both positively

affected bioethanol yield concentration in the fermentation process by *S. cerevisiae*. These two factors exceeded even the more conservative Bonferroni limit, thus providing a high confidence level greater than 95%. Inoculum size also marked a significant factor that exceeded the t–value line, as shown in Figure 2.

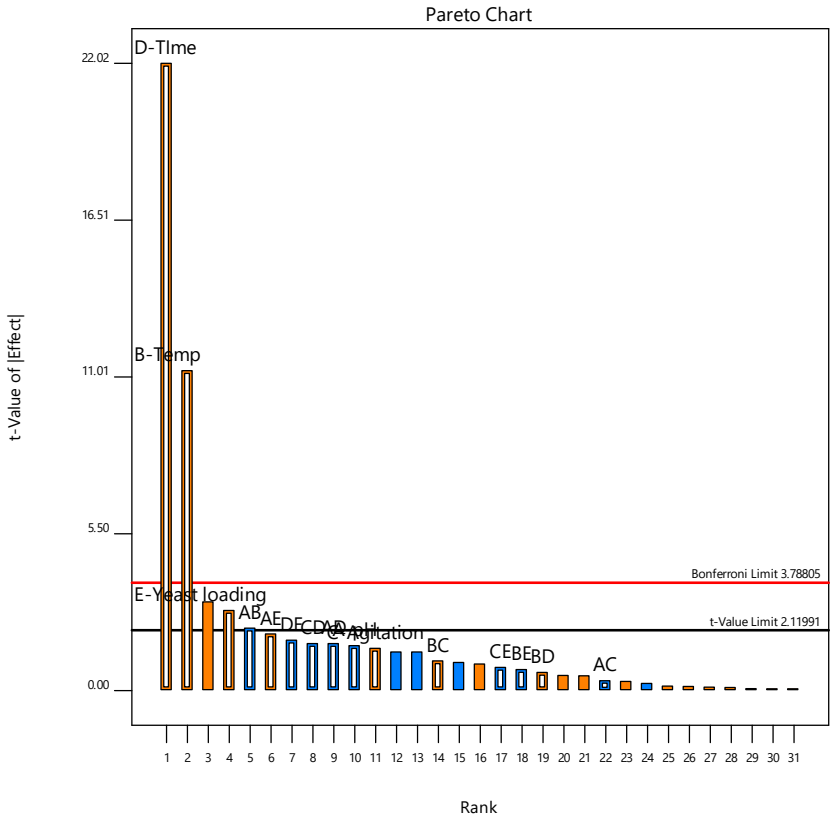


Figure 2. Pareto chart of factors A (pH), B (incubation temperature), C (agitation rate), D (incubation time), and E (inoculum size) that affect fermentation. Orange indicates a positive effect, while blue indicates a negative effect

The ANOVA was used to evaluate the adequacy of the fitted model. It can be used to assess the findings of a full factorial design, which manipulates possible combinations of the factors being studied. Table 2 shows the ANOVA of bioethanol yield concentration for the desired response. The Model F–value of 42.91 implied that the model was significant. There was only a 0.01% chance that an F–value this large could occur due to noise. P–values less than 0.0500 indicated that model terms were significant. Three main factors were found to have a significant effect on the fermentation of OPT sap to produce a high yield concentration of bioethanol by *S. cerevisiae*, which was incubation temperature p–value <0.0001, incubation time p–value <0.0001 and inoculum size with p–value = 0.0125 whereas interaction of two factors, pH and temperature (AB) also shown a significant effect on bioethanol yield concentration in the fermentation of OPT sap by *S. cerevisiae.* The significance of each coefficient was determined using a p–value (p <0.05), and the smallest p–value indicated a high significance of the corresponding coefficient. Variables with the most significant effect were incubation time (D) and incubation temperature (B), followed by inoculum size (E). Chol et al. [35] and Dasgupta et al. [36] both reported that incubation time was one of the significant factors in fermentation to produce a high yield concentration of bioethanol, while El–Gendy et al. [37]

reported that both incubation time and temperature resulted in a significantly high yield concentration of bioethanol product in the fermentation by *S. cerevisiae*. The media's pH did not significantly affect bioethanol yield in the fermentation of OPT sap as media. This is because OPT sap contains a rich composition of amino acids, organic acids, vitamins, and minerals essential for the growth and metabolism of yeast cells. Therefore, this composition may contribute to maintaining pH levels to regulate physiological processes **[38, 39].**

Table 2. ANOVA for bioethanol production as the desired response for unreduced models.

Source	Model	A	B	C	D	E	AB
Sum of Squares	2454.760	9.490	480.940	8.400	1848.970	30.160	18.310
df	15	1	1	1	1	1	1
Mean Square	163.650	9.490	480.940	8.400	1848.970	30.160	18.310
F–value	42.910	2.490	126.090	2.200	484.770	7.910	4.800
p–value	< 0.0001	0.1343	< 0.0001	0.1573	< 0.0001	0.0125	0.0436

Table 3 shows the percentage of factors contributing to the fermentation of OPT sap by *S. cerevisiae*. Higher percentages of contribution indicated that the element has a more significant impact on high bioethanol yield concentration. Small changes in this element have a significant impact on bioethanol yield concentration. The factor contributing the most to bioethanol generation during the fermentation of OPT sap by *S. cerevisiae* was incubation time, at 73.5%, followed by incubation temperature, at 19.1%. Inoculum size (1.2%), pH (0.4%), and agitation rate (0.3%) were the factors that had the most negligible impact on the outcome.

Table 3. Percentage contribution based on the full factorial design for factors in fermentation

Factor	Standardized Effect	Sum of Squares	Percentage Contribution
pH	–1.089	9.489	0.377
Temperature, °C	7.756	480.94	19.117
Agitation rate, rpm	1.024	8.3954	0.334
Time, h	15.203	1848.97	73.495
Inoculum size, % v/v	1.942	30.1623	1.199

Incubation time and incubation temperature were indeed essential factors affecting bioethanol yield concentration by *S. cerevisiae* during fermentation. Research showed that bioethanol yield concentration increased with increasing fermentation time, reaching the maximum bioethanol yield concentration after a certain period, such as 72 hours of incubation [40]. Bioethanol yield concentration increased gradually during fermentation at certain temperatures, with an optimal temperature range and a subsequent decline at higher temperatures [41]. These results were consistent with the broader understanding that bioethanol yield concentration during fermentation depended on several factors, including temperature, incubation time, and other variables [32]. With a longer incubation time, bioethanol yield concentration decreased when the substrate had been depleted, causing the accumulation of waste byproducts. Moreover, the optimal temperature for bioethanol yield concentration by *S. cerevisiae* depended on the strain and fermentation conditions. Most studies reported that the optimal temperature for bioethanol yield concentration by *S. cerevisiae* was between 30 and 40 °C, with some strains reaching the highest bioethanol yield concentration at 30 °C. In contrast, some strains produced more bioethanol yield concentration optimally at higher temperatures, such as 34°C [9, 40, 42, 43]. Temperature significantly influenced yeast cells' enzymatic activity and membrane turgidity. Thus, higher temperatures could cause denaturation of enzymes and ribosomes and problems with membrane fluidity, resulting in reduced bioethanol yield concentration [9].

Figure 3 shows the tendency of the primary effects diagrams when the variable level is different. The two points of the experiment setting were determined at low and high values from the test runs. For overall individual influence, these graphs showed that factors B– and D– produced the least sufficient amounts of bioethanol in fermentation, in contrast to A–, A+, B+, C–, C+, D+, E–, and E+. This resulted in a positive influence on bioethanol yield concentration during fermentation. Variables with steeper slopes calculated the main effects and significantly influenced the data experimentally. Subsequently, the impact of variable D, the incubation time of OPT sap fermentation by *S. cerevisiae*, was determined to be the primary contributing variable compared to other effects.

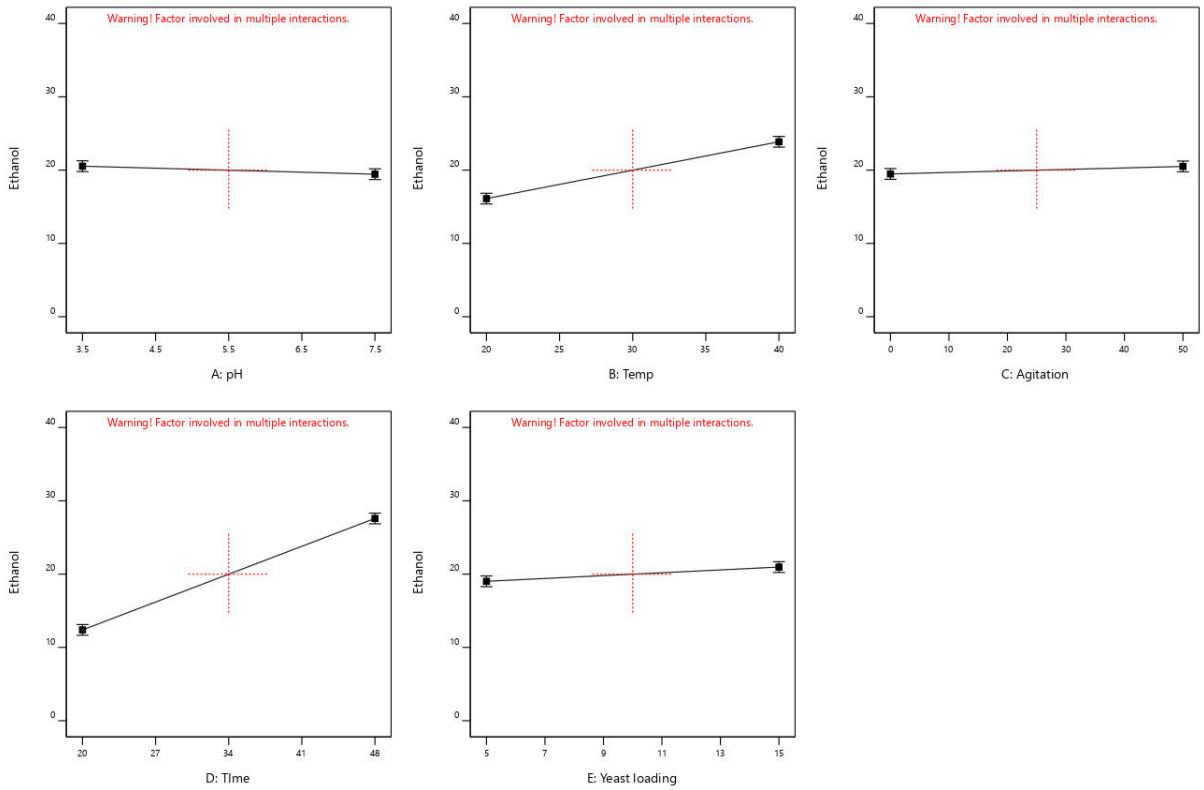


Figure 3. The main impact plots of physical factors of fermentation that produce a high bioethanol yield concentration during the fermentation of OPT sap by *S. cerevisiae*.

Figure 4 shows response prediction for bioethanol yield concentration in mg/mL as a function of fermentation temperature (B) and initial pH (A) of OPT sap and agitated rate (C) for the actual factor of 10% volume over volume (v/v) inoculum size and 34 hours of fermentation. The figure shows that without agitation rate, the temperature from 20 °C to 40 °C increased bioethanol yield concentration during fermentation at initial pH 3.5 from 15.63mg/mL to 24.18mg/mL. In contrast, the bioethanol yield concentration was slightly lower in the same condition, with a difference in initial pH at 7.5. A slight agitation at 50rpm of the fermentation broth provides a better condition for fermentation, which at an initial pH of 3.5, with increased temperature from 20 °C to 40 °C, showed increases of bioethanol yield concentration from 16.18 mg/mL to 26.16 mg/mL. Agitation rate may cause homogenization of fermentation media that can cause the collision of cells and sugar that can drive better conditions for the cell to ferment sugar compared to batch fermentation, where dense cells are sediment at the bottom of the flask and minimize the collation of cells and sugar to happen.

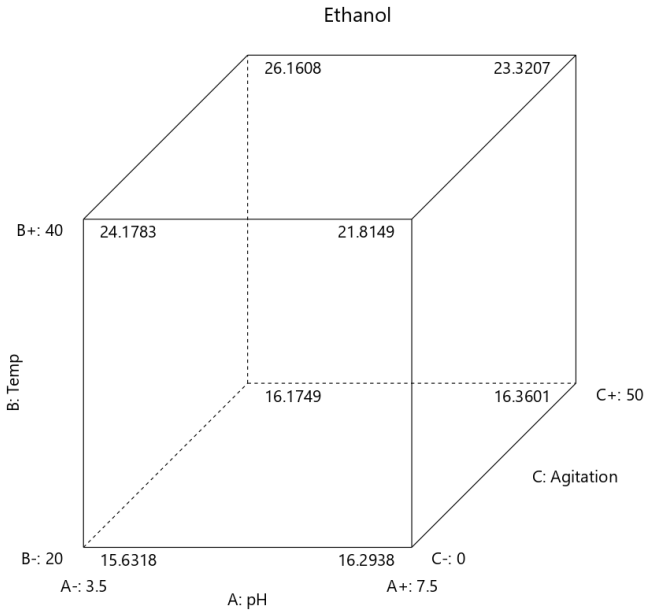


Figure 4. Response prediction for bioethanol yield concentration in mg/L as a function of fermentation temperature (B) and initial pH (A) of OPT sap for fermentation and agitated fermentation (C)

The comparative analysis of bioethanol production across various studies illuminated the critical role of yeast strain selection, incubation parameters, and process conditions in optimizing yield. *S. cerevisiae* emerged as a preferred organism due to its robust fermentative capabilities, yet its bioethanol yield varied significantly across different research efforts (Table 4). This variation underscored the impact of genetic differences within the species and possibly the conditions under which fermentation was conducted. The yields reported by Kumneadklang et al. [44] and Kusmiyati et al. [45] were identical, reflecting a potential for standardization in bioethanol production when conditions were kept consistent. Conversely, Nutongkaew et al. [46] reported a lower yield, emphasizing the influence of yeast strain variability or process conditions on bioethanol production efficiency. The range in yields from 0.350 to 150 g/L, as seen in the study by Edeh [47] further highlighted the potential for optimizing production through strain selection or genetic enhancement.

The incubation duration also played a pivotal role, varying widely across studies. Ezzatzadegan et al. [48] achieved substantial yields within a relatively short time frame, suggesting that bioethanol production efficiency may only sometimes correlate directly with longer incubation times. This insight pointed to the potential for reducing production cycles, thereby enhancing the economic viability of bioethanol as a renewable energy source. Moreover, these studies' agitation speed and temperature conditions suggested a consensus towards higher agitation speeds (around 150rpm) and a temperature range favorable for *S. cerevisiae* activity, generally around 30 ºC to 40 ºC. These conditions reflected the yeast's requirements for optimal oxygen transfer, nutrient uptake, and metabolic activity conducive to bioethanol production. However, the broad temperature range explored in some studies indicated the adaptability of yeast to varying conditions, offering flexibility in industrial bioethanol production settings. The pH levels across the studies remained relatively consistent, with slightly acidic conditions around 4.80, except in the study by Gimbun et al. [49], with a higher pH of 5.79. This consistency underlined the importance of maintaining specific pH levels for optimal yeast performance.

The variation in yields, incubation times, and operation al parameters across these studies illustrated the comple

x interplay of factors influencing bioethanol production . These comparative studies highlighted the importance of strain selection and process optimization and pointed to the potential for further research into fermentation strategies. Optimizing these factors could significantly enhance bioethanol's efficiency, sustainability, and economic feasibility as a renewable fuel source.

Table 4. Recent literature findings on bioethanol production from OPT.

Studies	Yeast Strain	Bioethanol Yield	Incubation Time (hours)	Agitation (rpm)	Temperature (ºC)	pH
[44]	*S. cerevisiae*	2.648%	120	100.00	37.00	4.80
[45]	*S. cerevisiae*	2.648%	120	100.00	37.00	4.80
[46]	*S. cerevisiae* TISTR5055	0.350g bioethanol/g	12–54	150.00	30.00±2	4.80
[47]	*S. cerevisiae*	150.000g/L	–	150.00–200.00	20.00–35.00	4.00–5.00
[48]	*S. cerevisiae*	95.000%	24	150.00	27.34	4.54
[49]	*S. cerevisiae* SC90	44.250g/L	96	150.00	40.00–50.00	4.80
[50]	*S. cerevisiae* NCYC 479 & *Pichia stipitis* NCYC 15411	2.180% (v/v)	120	100.00	30.00	5.00
[51]	*S. cerevisiae* SC90	0.469g EtOH/ g cellulose	72	150.00	40.00	4.80
[52]	*S. cerevisiae* SC901	0.320g/g	24	150.00	40.00	4.80
[53]	*S. cerevisiae*	~77.670%	24	164.38	31.05	5.79

Conclusion

Analysis of the physical factor fermentation of OPT sap by *S. cerevisiae* using a full factorial design reveals that the maximum bioethanol production can be achieved at a longer incubation time of 48 hours. As shown above, incubation time has the most significant effect on bioethanol production during fermentation. The highest bioethanol yield at 37.84 mg/mL was achieved during fermentation of OPT sap by *S. cerevisiae* at 48 hours of incubation with 3.5 for initial pH of OPT sap media, 40°C of incubation temperature, agitation of 50rpm, and 5% vv of inoculum size. Therefore, the significant physical factors in the fermentation of OPT sap by *S. cerevisiae* associated with bioethanol production in this study served as the foundation for further optimization studies to achieve a high bioethanol yield during fermentation.

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PHYSICAL AND MECHANICAL PROPERTIES OF SPENT COFFEE GROUNDS (SCG) IN CONCRETE

(Sifat Fizikal dan Mekanikal Sisa Buangan Kopi dalam Konkrit)

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Abstract

Incorporating waste materials into concrete enhances concrete properties and aligns with sustainable construction concepts. In this study, Spent Coffee Grounds (SCG) were investigated for their potential as an additive material in the cement mortar, to be used as a preliminary investigation for the formation of SCG concrete. SCG were collected from a local traditional Malaysian coffee shop and then undergone oven-drying, grinding and sieving process under controlled laboratory environments to obtain finely grounded SCG powder to be use as additive materials. The study begins with an investigation of the physical and chemical properties of SCG before being applied into cement mortar, through SEM and XRF analysis. Previous literatures have studied the use of SCG as sand substitutes, however, limited studies were conducted in the formation of cement mortar containing SCG additives. Thus, SCG was applied as additives into cement mortar at 6 different percentages of 0%, 1%, 3%, 5%, 7%, and 10%, undergoing 6 different periods of water curing of 7-day, 14-day, 28-day, 35-day, 42-day, and 70-day, tested under compressive strength and flexural strength test. The result of the study shows that under SEM, the SCG tends to form clusters and absorb water, whereas SCG collected for this study contains high carbon content under XRF analysis. The 1 % SCG additive performed the best for both compressive strength and flexural strength outperforming the control mixes. As a conclusion, SCG can be utilized as cement mortar additives when applied at 1%.

Keywords: concrete, spent coffee grounds, cement mortar, additives, sustainability

Abstrak

Menggabungkan bahan buangan ke dalam konkrit meningkatkan sifat konkrit dan sejajar dengan konsep pembinaan mampan. Dalam kajian ini, sisa buangan kopi (SCG) telah dikaji potensinya sebagai bahan tambahan dalam simen mortar, untuk digunakan sebagai penyiasatan awal bagi pembentukan konkrit yang mengandungi SCG. SCG dalam kajian ini dikumpul dari kedai kopi tradisional Malaysia dan proses pengeringan, pengisaran dan penyaringan dijalankan dalam persekitaran makmal untuk mendapatkan serbuk SCG yang dikisar halus untuk digunakan sebagai bahan tambahan konkrit. Kajian dimulakan dengan penyiasatan sifat fizikal dan kimia SCG sebelum digunakan pada mortar simen, melalui analisis SEM dan XRF. Kajian penerbitan SCG dahulu menunjukkan bahawa SCG pernah dikaji sebagai pengganti pasir, namun, kajian SCG sebagai bahan tambahan mortar

simen amat terhad. Oleh itu, kajian ini fokuskan kepada SCG digunakan sebagai bahan tambahan ke dalam mortar simen pada 6 peratusan berbeza iaitu 0%, 1%, 3%, 5%, 7%, dan 10%, menjalani 6 tempoh pengawetan air yang berbeza selama 7, 14, 28, 35, 42, dan 70 hari, akan diuji bawah ujian kekuatan mampatan dan kekuatan lenturan. Hasil kajian menunjukkan bahawa di bawah SEM, SCG cenderung untuk membentuk kelompok dan menyerap air, manakala SCG mengandungi kandungan karbon yang tinggi di bawah pemeriksaan XRF. Bahan tambahan SCG 1% menunjukkan prestasi terbaik untuk kedua-dua kekuatan mampatan dan kekuatan lenturan dimana prestasi lebih tinggi berbanding dengan campuran kawalan. Sebagai kesimpulan, SCG boleh digunakan sebagai bahan tambahan simen mortar apabila digunakan pada 1%.

Kata kunci: konkrit, sisa buangan kopi, simen mortar, bahan tambahan, kelestarian

Introduction

Concretes are made from 4 different important materials; cement, fine aggregates, coarse aggregates, and water [1]. These materials are non-renewable natural resources [2]. Every day, more buildings are being constructed to shelter the increasing global human population, leading to the increasing use of these materials that are hard to replenish [3]. According to Watts [4], the use of concrete has risen so much that concrete is now considered the world's second most consumed substance, after water. According to Nie et al. [5], it was reported that concrete production utilizes cement and to produce cement, huge amounts of carbon dioxide pollutants are released. Throughout every lifecycle of the cement, from production until the end of service life, the carbon footprint left behind by the cement cannot be underestimated [6]. Fine aggregates and coarse aggregates present in the concrete are also made up of natural resources that are hard to replenish, to obtain these aggregates, mining, and quarrying of natural resources are needed to obtain the fragments and rock materials for further processing into coarse aggregates, fine aggregates, and cement [7].

Recently, the United Nations (UN) has outlined 17 Sustainable Development Goals (SDG), and among these 17, SDG 11: Sustainable Cities and Communities, SDG 12: Responsible Consumption and Production, and SDG 13: Climate Action Indicators conveyed the need for action on promoting sustainable concepts into the construction industry [8]. Aligned with that vision, waste materials were also being studied for their application as additives in the concrete to improve the mechanical and durability properties of the concrete [9]. The addition of additives could improve the service life of the concrete and reduce the consumption of concrete materials [10].

Coffee is a common beverage, and the level of coffee consumption is estimated to be around 2.25 billion cups consumed every day [11]. Coffee drinking offers a variety of health benefits, but the environmental effects of the coffee wastes are easily taken for granted [12]. Coffee wastes are by-products formed from coffee brewing. When coffee grounds lose their aroma, taste, and colour after being extracted to brew coffee drinks, spent coffee grounds (SCG) are formed. The common disposal method of SCG is by treating it as general waste and simply landfilling [13]. Decomposition of SCG at high volumes produces high content of nitrogen making it less preferable to be used in large quantities as plant fertilizers [14]. Hence, throwing large quantities of SCG directly into landfills may lead to soil infertility. Together with the increasing human population, the demand for coffee may increase as well, increasing coffee waste generation [15]. Thus, an alternative method is required to better manage the SCG waste and minimize the negative environmental impacts of the SCG waste.

Previous studies have utilized SCG as a material in cement mortar. A study by Saeli et al. [16], identified that SCG could be deployed as fine aggregate replacement in cement mortar. Another study by Roychand et al. [17], identified that SCG could similarly be used as fine aggregate replacement in concretes. While both researchers have investigated the use of SCG as fine aggregate materials, limited research has been conducted on the use of SCG as additive materials in cement mortar and concrete. A study by Chua [18], identified that while SCG is used as additive material in cement mortar, a retarding effect has been observed. Nevertheless, the incorporation could only be effective up to 0.6%. Therefore, from the literature reviewed, no

studies have been conducted yet on the SCG incorporation in concrete. Hence, this study proceeds with identifying the potential use of SCG in concrete as additive materials by conducting a pilot study investigating the properties of the cement mortar containing SCG additives.

The coffee culture of a locality and treatment methods could affect the properties of the SCG produced, mainly due to the wide variations of roasting methods of coffee beans before being extracted to make a drink [19]. Previous literatures studying the utilization of SCG in cement mortar are mainly sourced from western coffee shops. This is deviating from the coffee beans deployed for this study. In this study, coffee beans were collected from traditional Malaysian coffee shops. The Malaysian coffee adopted a method similar to the Torrefacto method of roasting coffee beans, where sugar is added to the coffee beans [20]. Hence, the difference in roasting methods and brewing methods leads to variations in temperature, humidity, and purity of the SCG produced [21]. The differences in genotypes of the coffee bean could also cause variations in the SCG properties [22]. Therefore, while many studies were conducted on SCG obtained from Western coffee beans, there are lack of studies being conducted on Southeast Asia coffee, specifically the Malaysian coffee beans that could be obtained through local Malaysian coffee shops but not Western coffee shops in Malaysia.

With that, the objective of the study is formed, which is to investigate the applicability of Malaysian SCG as an additive material in concrete, enhancing the concrete as well as reducing the volume of SCG waste materials. SCG is selected as an additive material as opposed to sand or cement replacement due to its composition. According to a study conducted by Tapangnoi et al. [23], after conducting Carbon (C), Hydrogen (H), Nitrogen (N), and Sulphur (S) analyzer on the SCG samples, it is reported that SCG contains at least 50 % of carbon. These high carbon properties indicated that SCG is more suited as an additive as SCG lacks the composition of cement.

It is important to study SCG as an alternative for its application into cement mortar due to the high accessibility of SCG wastes. When compared to other industrial wastes that have been previously studied as concrete additive materials, wastes such as palm oil fuel ash (POFA) and rice husk ash (RHA), are wastes generated from plantation derivatives [24, 25]. Countries with cold climate temperatures tend to have increased challenges developing palm oil and rice husk plantations [26, 27]. This eventually resulted in decreased availability and accessibility of these wastes which are not commonly exported. On the other hand, coffee beans are exported and marketed globally. In the year 2019, Brazil recorded a trade export of roughly 8.06 billion kilograms while Vietnam recorded a trade export of 1.38 billion kilograms of unprocessed coffee beans [28]. The European Union recorded roughly 3.05 billion kilograms of unprocessed coffee beans imported in 2019 alone [29]. This indicated that unlike rice or palm oil where only derived products are exported, unprocessed coffee beans, not roasted nor decaffeinated, are a global trade commodity [30], therefore, indicating the significance of SCG as a suitable alternative to be explored as a concrete additive.

In this pilot study, cement mortar mixes will be used as a replication of concrete. This study investigated the physical and chemical properties of the SCG. Noting the basic properties of the SCG, the SCG will then be studied for its effect on the mechanical strength of the cement mortar samples when added through 6 different percentages, 0%, 1%, 3%, 5%, 7%, and 10%. The mechanical strength involved in this study includes compressive strength test and flexural strength test, both complying with the standards set by ASTM [31, 32]. The mechanical strength testing will be conducted at 6 different periods of water curing, that is 7 days, 14 days, 28 days, 35 days, 42 days, and 70 days.

Materials and Methods

Study area

The main focus of the study is to evaluate the efficacy of spent coffee grounds (SCG) as a filler material in cement mortar for concrete applications. Hence, this chapter looks to discuss and present the methodology involved in this study implemented to achieve the objectives of the study. This section covers from the material used, material preparations, test procedures, and test equipment involved in the study.

Materials and preparation methods

The fine aggregates used are river sand. Fine aggregates were first sieved with ASTM E11 Test Sieve No. 8 with a pore opening of 2.36 mm [33]. Only the fine aggregates that pass the 2.36 mm sieve opening will be selected for this study. After the fine aggregates are sieved, the fine aggregates will then be left to dry under the sun before being used for casting the cement mortar. The cement used in this study is ordinary Portland cement (OPC). The OPC used belongs to a Type 1 cement supplied by YTL Corporation Berhad. The OPC used were kept in a plastic container away from heat with silica gel desiccant to avoid the OPC exposure to moisture conditions.

The spent coffee grounds (SCG) used in this study were first collected from traditional Malaysian coffee shops and then brought to the concrete lab at Universiti Malaysia Pahang Al-Sultan Abdullah for processing. Unlike typical Western coffees, the coffee grounds used to make Malaysian coffee are a type of pour-over coffee, requiring a coarse grind to medium coarse ground, resulting in irregularity in shapes, fineness, and sizes. The collected SCG was washed with tap water and any impurities were removed. The washed SCG was then sent to dry in a 100 ˚C oven for a minimum period of 24 hours. SCG wastes are considered dry when consecutive weight readings indicate no changes. The dried SCG were then grounded with a grinding machine and then sent for further crushing with a Los Angeles Abrasion (LAA) Machine. According to BS EN 13139, for a material to be able to be used as a filler, the material will have to pass a sieve size of 63 microns [34]. After the SCG is grounded in the LAA machine, the SCG is sieved and the SCG that has passed the 45-micron sieve size is used for the study. The processed SCG were then stored in a container with silica gel desiccant to keep in a cool and dry condition.

Test equipment and procedures

To grind the SCG into fine powder to be used as an additive in cement mortar, the Los Angeles Abrasion (LAA) machine is used. The LAA machine was used with 21 metal balls crushing at 1500 cycles on the SCG samples [35]. The grounded SCG was then sieved to pass 45 microns. To understand the morphology characteristics of materials before being used into concrete, a Scanning Electron Microscopy (SEM) test was conducted. SEM test is a method of analysis involving the use of a microscopic tool that could allow for high magnifications into a material while producing high resolution images [36]. From the material analysis through SEM, the surface morphology and characteristics of the particles could be determined [37]. In this study, the SCG wastes before application into cement mortar were analysed for the physical characteristics through SEM test. From this test, the shape and size of the finely grounded SCG material are observed and recorded. To obtain the chemical composition of concrete materials, previous literatures have identified the use of SEM coupled with Energy Dispersive X-Ray (EDX) analysis to perform chemical composition determination [38]. However, the EDX analysis coupled with SEM is only possible at specific area of interest in the materials after specific magnifications [39]. When compared to the X-Ray Fluorescence (XRF) test, the elemental and oxide composition of the material could be obtained through an averaged analysis over a bulk specific area [40]. Previous literatures have also reported that the XRF analysis is generally able to provide higher precision and sensitivity when compared to the EDX techniques when analysing bulk samples [41]. Therefore, for this study, to obtain a general chemical composition of the SCG wastes, the SCG is tested through the use of X-Ray Fluorescence (XRF) spectroscopy. The XRF spectroscopy was run for both elemental and oxides analysis.

To obtain a quantifiable analysis of the performance of SCG in concrete, the mechanical performance test is conducted on the cement mortar samples containing SCG. Concretes are renowned for their use as building construction materials. Among all types of forces involved in a concrete structure, compressive forces and flexural forces are crucial to identify the potential of the concrete in supporting load [42]. With limited studies conducted on the use of SCG as additive materials in concrete, as a pilot study, cement mortar samples were adopted as a replication of concrete samples. This is carried out to determine the optimum proportion of SCG to be added into concrete for improved strength

performance. In a study conducted by Al-Jabri et al. [43], it is found that when studying the use of copper slag as fine aggregates, the best performance cement mortar and concrete were observed to have the same proportion of substitute materials. Another study by Son et al. [44] discovered that when studying the cement mortar samples containing substitute materials and the concrete containing similar proportions of substitute materials, the differences in the best compressive strength performance differ by only 6.60%. Hence, previous works of literature have identified that the difference in proportions of additive materials present in the concrete could affect the properties of the concrete and that a pilot study could be conducted on cement mortar samples before their application into concrete. Thus, in this study, the proportions of SCG to be involved in the cement mortar samples are investigated. From here, through the mechanical strength performance investigation, the most optimum content of SCG to be added into the cement mortar can then be determined.

Cement mortar cube samples (50 mm x 50 mm x 50 mm) and cement mortar prism samples (40 mm x 40 mm x 160 mm) were casted and then water-cured in a curing tank for a total of 70 days. Compressive strength tests, per ASTM C109/C109M – 20 were conducted on the cube test samples while flexural strength test, per ASTM C348 – 08 were conducted on the prism test samples. All the cube and prism samples will be tested for its mechanical performance at 6 different periods of water curing, 7 days, 14 days, 28 days, 35 days, 42 days, and 70 days. For each proportion of additive used in the cement mortar, three samples are cast during each of the six different water curing periods, resulting in a total of 108 cube and 108 prism samples. This comprehensive approach ensures accurate results with high repeatability. The compressive strength and flexural strength data are obtained by averaging the strength values of the three cement mortar samples for each percentage and each water curing period.

Spent coffee grounds (SCG) cement mortar mix design

In this study, SCG was added into the cement mortar as filler additives through 6 different percentages, that is 0 %, 1 %, 3 %, 5 %, 7 %, and 10 %. The rationale behind the 6 percentages is mainly due to concrete additives that are usually added into concrete at a range of 0.5 % to 8 % [45, 46]. Chemical agents such as water reducing admixtures and superplasticizer are available in liquid form [47]. Nevertheless, previous studies have identified the use of mineral admixtures, which are available in powder form, studied for its addition into concrete up to 10% [48]. Hence, the percentage of SCG additives to be added into the cement mortar becomes the focus of the study, in order to achieve the objective of finding the most optimum SCG additive proportion for the formulation of an improved performance cement mortar when compared to controlled cement mortar. The controlled cement mortar mix is casted according to the IS 2250 guidelines, utilizing a standard design of 1 part binder to 3 parts of fine aggregates, with a water-cement ratio of 0.5 [49]. Adhering to the standards, the mix design of the cement mortar utilized for this study is listed in Table 1.

All the cement mortar specimens were produced in the laboratory environment under normal room temperature using plastic molds of cubes (50 mm x 50 mm x 50 mm) and prisms (40 mm x 40 mm x 160 mm). The casting process begins with dry mixing the fine aggregates and the cement for about 2 minutes. SCG was then added to the mixing process without the addition of water. Until the materials were dry mixed homogenously, water was slowly added in 3 batches. The wet cement mortar mix will then be poured into the plastic cube and prism molds, which will then undergo a 24-hour setting time under air curing before being removed from the plastic molds to be soaked into water for water curing.

Table 1. Cement mortar mix design applied for this study

Mixes	Cement (kg/m^3)	Fine Aggregates (kg/m^3)	Water (kg/m^3)	SCG (kg/m^3)
SCG-0	50	150	25	0
SCG-1	50	150	25	0.5
SCG-3	50	150	25	1.5
SCG-5	50	150	25	2.5
SCG-7	50	150	25	3.5
SCG-10	50	150	25	5

Results and Discussion

Physical properties of the spent coffee grounds (SCG)

The physical characteristics of the SCG were determined through the use of scanning electron microscopy (SEM) spectroscopy. From the analysis, as shown in Figure 1, the SCG compounds used for this study have been grounded down to sizes finer than 45 microns through the use of the LAA Machine. The SCG material other than having irregularly shaped compounds, tends to form a cluster (as shown in the red rectangle box highlighted in Figure 2), combining with other SCG particles despite being kept away from moist conditions. These results obtained are like the results obtained by Nagaratnam et al. [50] on the microstructure of palm oil fuel ash (POFA) which reported that the material tends to form small clusters and contain irregular shapes. Due to the tendency of the SCG material to form clusters, the SCG materials could be hypothesized to contain a fair amount of carbon composition. This is illustrated by Qing et al. [51], where materials containing high amounts of carbon are reported to form clusters as they have a high-water absorbency with high water retention capacity. Materials with a high tendency to absorb water without utilizing the excess water for other chemical reactions in the concrete results in adverse effects on the inner structure and mechanical strength of the concrete [52]. Noting the hypothesized characteristics of the SCG materials, the content of SCG in the cement mortar will have to be adjusted carefully.

The negative effects of high water retention capacity of concrete materials is reported in a research by Beaudoin and Odler [53], whereby when large amount of water is being absorbed by concrete materials at the initial stage, the cement in the concrete will face water deficit required for hydration process to take place, and when water absorbed by the concrete materials evaporates, this eventually causes void formation in the concrete, causing the cement matrix to loses compaction and ultimately decreasing the mechanical strength of the concrete. Thus, from the physical characteristic investigation using SEM, careful measures of the SCG content should be considered in the cement mortar mix.

Chemical composition of the spent coffee grounds (SCG)

According to ASTM C618-19, a material can be classified as pozzolan if it has a minimum content of 70% by mass of silicon dioxide (SiO_2), aluminum oxide (Al_2O_3), and ferrous oxide (Fe_2O_3) [54]. Pozzolans are materials for the formation of secondary C-S-H gel in the concrete by consuming the calcium hydroxides formed from the cement hydration process, increasing the durability and mechanical strength of the concrete. The chemical composition of the SCG materials were investigated using the X-Ray fluorescence (XRF) test.

From the XRF elemental and oxide analysis, the chemical composition of the SCG is presented as shown in Table 2. Carbon and oxygen are highest in SCG with 51.2% by mass and 46.1% by mass. This indicates that the SCG material does have a high carbon content, confirming the hypothesis formed from the inspection of the physical characteristics of the SCG clumping together. These findings could be supported by the biochar particles, where the high carbon and high porous particles resulted in the biochar having a high tendency to absorb water [55].

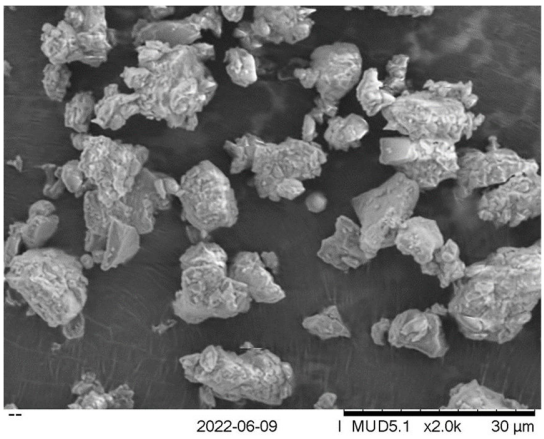


Figure 1. Results obtained from SEM spectroscope at 2000 times magnification.

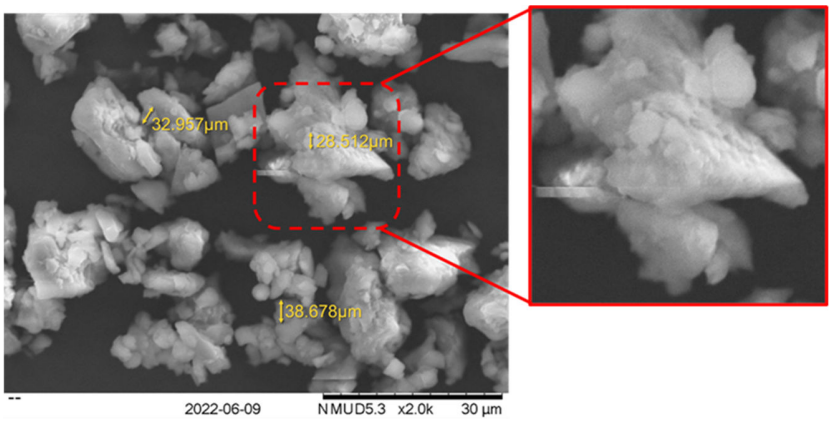


Figure 2. Sizes of the SCG materials from 2000 times magnification (in Red: Clustering of SCG materials).

In the oxide analysis, the highest oxides reported are also carbon and dioxygen with 53.3% by mass and 42.2% by mass, indicating a parallel result with elemental analysis. The SiO_2, Al_2O_3, and Fe_2O_3 compounds in the SCG reported 1.01%, 0.15%, and 0.45%, respectively. The total amount of SiO_2, Al_2O_3, and Fe_2O_3 compounds in the SCG is 1.61%, indicating its pozzolanic properties. However, these properties are expected to make only a marginal difference to the cement mortar mix. When comparing the chemical composition of the SCG to cement, as shown in Table 2, SCG does not possess any similarities with cement's chemical composition [56]. In cement, the highest oxide composition is calcium oxide (CaO) while SCG only contains 0.6% of CaO. This makes SCG unsuitable to be used as a cement substitution.

Hence, from all the findings presented, SCG containing high carbon content can only be used as a filler additive into the cement mortar mix to fill in the pores present in the internal structure of the cement mortar. The SCG in the study reported to not possess any pozzolanic properties and no resemblance to the composition of the cement. The percentages of its use in cement mortar should be low to avoid SCG from absorbing water in the wet mix and releasing it through evaporation, resulting in inefficient use of water in the cement mortar mix.

Table 2. Data excerpt of Elemental and Oxide analysis of SCG compared with cement

Spent Coffee Grounds (SCG)				Cement [56]	
Elemental	**Mass %**	**Oxides**	**Mass %**	**Oxides**	**Mass %**
C	51.20	C	53.30	LOI	5.94
O	46.10	O_2	42.20	O_2	-
K	0.57	SiO_2	1.01	SiO_2	18.68
Si	0.46	K_2O	0.71	K_2O	0.36
Ca	0.42	CaO	0.60	CaO	62.88
Fe	0.31	P_2O_5	0.55	P_2O_5	0.11
P	0.23	Fe_2O_3	0.45	Fe_2O_3	3.31
S	0.17	SO_3	0.44	SO_3	3.78
Cl	0.13	MgO	0.30	MgO	1.04
Mg	0.18	Al_2O_3	0.15	Al_2O_3	5.00
Al	0.07	Na_2O	0.14	Na_2O	0.35
Na	0.11	Cl	0.13	Cl	-

Compressive strength of the spent coffee grounds (SCG) cement mortar

From the compressive strength test result shown in Figure 3, the 1% SCG additive had outperform the 0% controlled cement mortar throughout all 70 days of water curing. At higher percentages 3%, 5%, 7%, and 10%, the SCG additives experienced a huge strength reduction and resulted in a lower strength when compared to the controlled cement mortar. At 7% and 10% additive, the strength was too low for the compressive strength test machine to record a reading, thus the data was not included in Figure 3. Suarez-Riera et al. [57] reported that with the use of biochar, a material high in carbon, the strength of the concrete increased only at small percentages, whereas at higher percentages of additives, the concrete experienced a significant drop in mechanical strength. At 28 days of water curing, where concretes are known to have achieved almost 90% of the concrete strength, the 0% controlled cement mortar has a strength of 24.23 MPa whereas the 1% SCG cement mortar has a strength of 28.23 MPa. Upon reaching 42 days of water curing, the compressive strength of the 0% controlled cement mortar has reached its peak and plateau until the 70-day curing. The 1% SCG cement mortar illustrated an increase in strength throughout the 70 days of curing. The difference in strength between 1% SCG mixes and 0% SCG mixes are illustrated in Figure 4, at 7 days of curing, the 1% SCG has a strength value of 20.95 MPa, which is higher than conventional cement mortar mixes by 9.86%. From the 7th day of curing, the gap of strength difference between 1% SCG mixes and 0% SCG mixes continues to increase. The strength of the 1% SCG cement mortar is 28.23 MPa, higher than the control mixes by 16.51% with a peak at 70 days of curing where the strength difference between 1% SCG and 0% SCG grows to 29.86%. The increase in the gap towards 42-day and 70-day water curing is mainly due to the compressive strength of the 0% SCG mixes plateau after 42 days of curing while the 1% SCG mixes continue to gain strength.

The 3% SCG cement mortar initially has a significantly low compressive strength but witnessed a huge increase from 2.74 MPa at 14 days to 15.28 MPa at 28 days, depicting a strength increase of 45.8%, achieving satisfactory strength at 70 days of curing with a compressive strength of 20.75 MPa. From Figure 3, the compressive strength of the 3% SCG cement mortar continuously gains strength throughout the 70 days of curing. Initially, the strength of the 3% SCG cement mortar is 98.79% lower than that of conventional mixes. Towards the 70-day curing, the difference slowly decreased to 27.91% at the end of the curing period. Though the strength is still lower when compared to the 0 % SCG mixes, the SCG cement mortar continues to gain strength at a quicker rate with the period of curing.

For the 5% SCG cement mortar, the cement mortar increases strength gain with curing time, initially the strength of the 5% SCG cement mortar was 0.17 MPa, and after 70 days of curing, the strength increased to

2.11 MPa. When comparing the differences between the 5% SCG cement mortar and the 0% SCG mixes, the strength differential remains substantial, with a reduction of 92.67% compared to conventional mixes even after 70 days of water curing. Consequently, although the 5% SCG exhibits strength improvement over the curing period, its compressive strength remains unsatisfactory for use in the construction industry. Thus, from this compressive strength test findings, when cement mortar mixes were added with 1 % SCG, the strength generally increased, however, for SCG cement mortar at other percentages of mixes, there is a need for longer curing time.

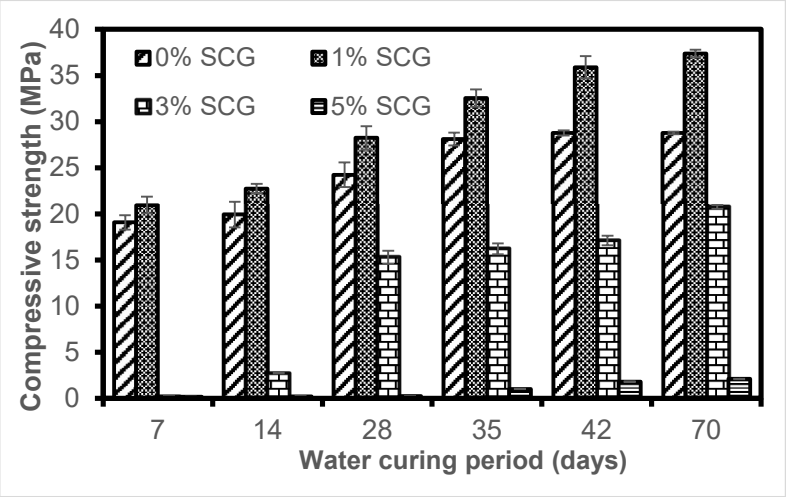


Figure 3. Compressive strength performance of SCG cement mortar

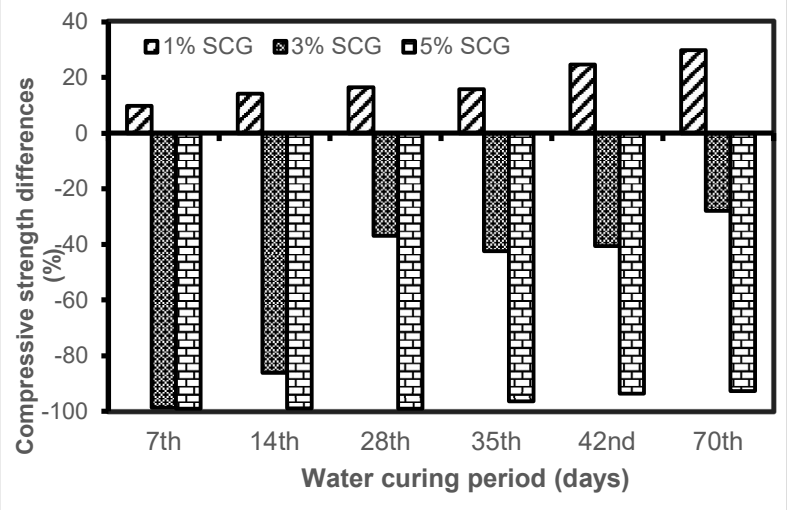


Figure 4. Gap of differences in compressive strength to 0% SCG cement mortar.

Flexural strength of the spent coffee grounds (SCG) cement mortar

From the flexural strength test shown in Figure 5, over the curing period of 70 days, the 1% SCG cement mortar has the highest flexural strength. At 28 days of water curing, the flexural strength of the 1% SCG cement mortar was recorded at 4.75 MPa, which is higher when compared to the 0 % controlled cement mortar specimen at 4.39 MPa. At percentages higher than 1% SCG additives, a decreasing trend was observed for the 3%

and 5% SCG cement mortar samples, like the trend observed in the compressive strength test. At 7% and 10% SCG additives, the cement mortar exhibited low flexural strength, rendering it unable to withstand hand abrasion. As a result, there is no numerical value to report. The difference in strength between 0% SCG cement mortar is shown in Figure 6, 1% SCG cement mortar overall shows higher strength compared to the 0% cement mortar. Unlike the increasing gap from compressive strength test results, the flexural strength indicated a decrease in the gap at 70 days of curing. Hence, cement hardening is still occurring in the beam samples as compared to the cube samples, where pores continue to harden due to the larger volume, filling up the pores internally.

The 3% SCG cement mortar at the early stages of water curing had values lower than the 5% SCG cement mortar, reporting 1.08 MPa and 1.12 MPa, respectively. However, the 3% SCG cement mortar eventually increased in strength gain at a rate quicker than 5% SCG cement mortar, achieving 1.16 MPa for both percentages at 14 days of curing. In Figure 6, the gap of difference between the 0% cement mortar in 3% SCG cement mortar is initially higher compared to the 5% SCG mixes. Starting from the 14th day, the gap of difference is similar. After 28 days of curing, the 3% SCG cement mortar had higher strength values than the 5% SCG cement mortar, and the same trend was observed until the 70-day water curing period, resulting in the gap of 3% SCG cement mortar being slightly higher as compared to 5% SCG mixes. A significant gap was observed at 42 days of water curing, where the 3% SCG cement mortar reported 26.47% lower than 0% cement mortar, while the 5% SCG cement mortar reported a 53.92 % lower than 0 % cement mortar. By 70 days of water curing, the 3 % SCG cement mortar had a flexural strength of 4.35 MPa, while the 5% SCG cement mortar reported only 2.42 MPa, with the biggest difference of roughly 44.37% between the two mixes.

Saeli et al. [16], reported that when deploying SCG as fine aggregate substitute, the flexural strength dropped when compared to that of the controlled cement mortar not containing SCG. Comparing the data obtained from the flexural strength to previous literature by Saeli et al. [16], the flexural strength of the cement mortar containing SCG in this study presented higher values with an improvement of 116%. This indicated that the study is a success in improving the flexural strength of cement mortar while incorporating SCG as part of its mix design. Overall, for all samples, the SCG cement mortar gains strength with the increasing water curing periods, and this trend is similar to the compressive strength test results. Thus, when 1% of SCG was added to the cement mortar mix, the strength generally increased. However, for higher percentages than 1%, there is a need for longer curing time.

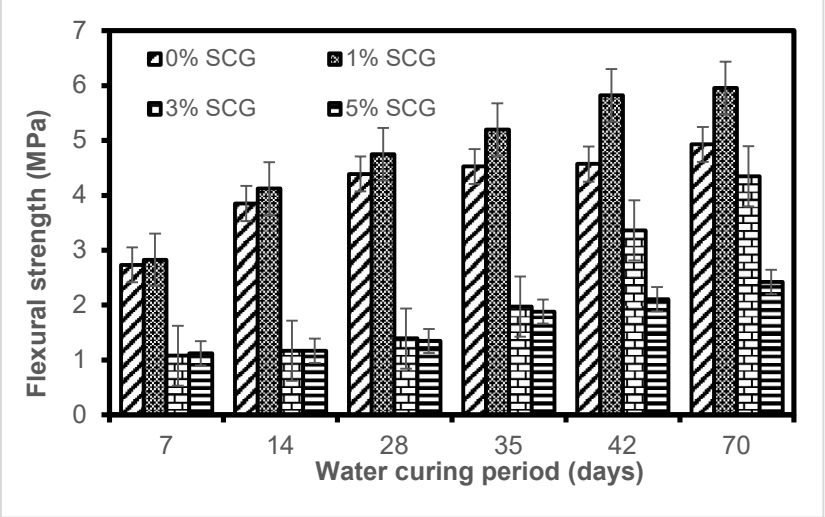


Figure 5. Flexural strength performance of SCG cement mortar

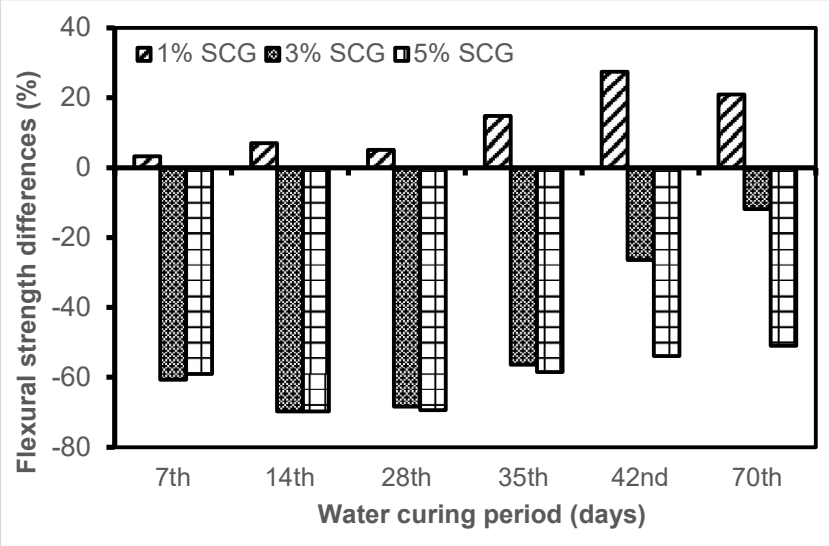


Figure 6. Gap of differences in flexural strength to 0% SCG cement mortar

Mechanical properties of the spent coffee grounds (SCG) cement mortar

From both the compressive strength test and the flexural strength test, a similar trend could be observed for both mixes. Generally, at 1% SCG additive, the strength of the cement mortar would increase. The appearance of the 1% SCG cement mortar is shown in Figure 7. It can also be concluded that at mixes with 3% SCG additive, at longer curing times, the strength of the cement mortar may be significantly increased up to satisfactory mechanical strength. For 5% additives, the strength of the cement mortar up to the 70-day water curing mark has shown non-satisfactory values. At 7% and 10%, the cement mortar does not have sufficient strength to withstand hand abrasion and no data could be recorded from the machine. A study by Gallegos-Villela et al. [58] utilized a curing period of up to 90 days before the concrete containing waste materials indicated an increase in mechanical strength. Hence, a similar methodology may be suggested for future studies on SCG additives into concrete at higher percentages than 1%. Nevertheless, this study concluded that the most optimum percentage of SCG additive would be 1%.

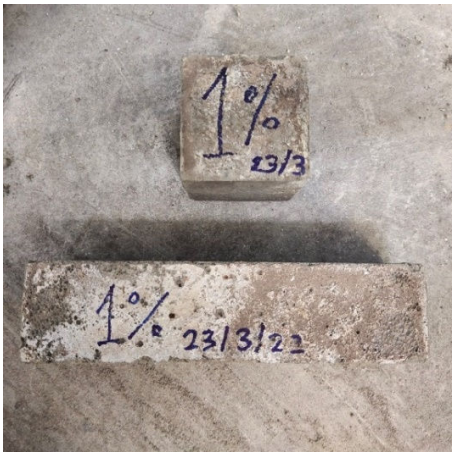


Figure 7. The 1% SCG cement mortar that exhibits the highest strength properties

Implication of the research study

From this research, spent coffee grounds (SCG) could only be utilized in cement mortar up to 1 % as additives. Through this study, the level of addition of SCG into the cement mortar is significantly lower when compared to its other counterparts such as rice husk ash (RHA) and palm oil fuel ash (POFA) [59, 60]. Nevertheless, this study is still significant due to the high availability of SCG wastes around the world. SCG wastes are widely accessible around the world. In regions with extreme climate conditions, under high or low temperatures, paddy and palm oil plantations are suppressed [61-63]. This leads to the lowered availability of these waste products unlike coffee, a common beverage consumed globally unaffected by weather and climate [64]. Though coffee plantation is not practiced worldwide, the coffee beans are exported to all parts of the world resulting in the accessibility to these wastes compared to rice husk ash or palm oil fuel ash that may not be readily available from their exported end products.

Cement mortar mixes use cement and sand in their mixture. Though sand is available in abundance, sand that can be used for construction is still a natural resource that is decreasing and limited in volume [65]. Meanwhile, cement production is a damaging industry with detrimental environmental effects [66]. One study revealed that every ton of cement produces approximately 0.79 tons of carbon emissions [67]. With SCG as additives, the cement mortar could have increased service life, reducing the consumption rate of sand and cement. Previous research on incorporating spent coffee grounds (SCG) into concrete has highlighted their potential application in concrete mixtures. However, no published studies have been identified regarding the utilization of SCG from traditional Malaysian coffee shops, where coffee is prepared using the pour-over method, as additives in concrete. This study initiates an exploration into the use of such SCG as additives in concrete and suggests potential future research directions. These directions include investigating the application of Malaysian SCG in various concrete products such as high-strength concrete, lightweight concrete, architectural concrete, among others.

Sustainable Development Goals are goals outlined to achieve the social and economic development of a nation while protecting environmental values and balancing social equity. Through the involvement of waste materials in the construction industry, reducing demand for non-renewable raw natural resources through repurposing of wastes achieves the UN SDG 12 Responsible Consumption and Production. At the same time, by reducing reliance on environmentally detrimental materials such as cement, the UN SDG 13 Climate Action is achieved, making the incorporation of SCG a sustainable alternative for the construction industry.

Conclusion

As a conclusion of the study based on the experimental results, spent coffee grounds (SCG) sourced from local Malaysian coffee shops could be utilized as an additive into cement mortar up to 1%. The mechanical performance of SCG cement mortar at 1% incorporation indicated improved performance as compared to cement mortar without SCG. The physical properties of SCG after lab processing have a particle size of finer than 45 microns with irregularly shaped particles that have a high tendency to clump together. Chemical properties of SCG, investigated through XRF analysis shows that carbon is the highest chemical composition in the SCG.

The limitations of the study include that the spent coffee grounds (SCG) used may not fully represent all SCG properties globally. Different sources of materials could result in variations in their reactions with concrete and differences in the physical and chemical properties of the SCG. The tests involved in this study utilized casting of cement mortar. To strongly correlate the use of SCG in concrete and its strength properties, concrete mixes have to be tested as there are chances in which the data may differ in this study when SCG is applied into concrete mix. Water curing period conducted in this study is only limited up to 70 days due to the COVID-19 restrictions in lab activities. While there are other studies that reported strength increase only noticeable after 90 days, further studies may be conducted to investigate the strength gain of the SCG additive concrete after 90 days of water curing.

For future studies, it is recommended that SCG additives at 1% can be tested for its application into concrete mixes to confirm if the concrete produced at 1% additives yields the highest in performance. The curing period in this study is limited to 70 days and it is recommended for future studies to carry out further testing and utilize a curing period of up to 90 days and more to observe the performance of strength of the SCG additive in concrete.

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MEASUREMENT UNCERTAINTY IN THE ANALYSIS OF SELECTED POLYCYCLIC AROMATIC HYDROCARBONS IN ENVIRONMENTAL WATER BY MAGNETIC MOLECULARLY IMPRINTED POLYMER-BASED MICRO-SOLID PHASE EXTRACTION- LIQUID CHROMATOGRAPHY

(Ketidakpastian Pengukuran dalam Analisis Hidrokarbon Aromatik Polisiklik Terpilih di dalam Air Persekitaran dengan Pengekstrakan Fasa Pepejal-Mikro berasaskan Polimer Bercetakan Molekul Bermagnet-Kromatografi Cecair)

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Abstract

Sample preparation is important to produce a clean extract to enhance detection sensitivity and minimize instrument maintenance. A micro-solid phase extraction (μ-SPE) technique using magnetic molecularly imprinted polymers (magnetic-MIPs) as the adsorbents combined with micro-high performance liquid chromatography and ultraviolet detection (μ-HPLC-UV) has been developed for the determination of selected polycyclic aromatic hydrocarbons (PAHs), namely benzo[a]pyrene, phenanthrene, fluoranthene and pyrene in environmental water samples. The magnetic-MIPs were characterized using scanning electron microscopy, Brunauer–Emmett–Teller, thermogravimetric analysis and vibrating sample magnetometer to finalize the physical properties of the magnetic-MIPs. The μ-SPE was then optimized using one variable at a time approach to enhance the extraction efficiency. Under the optimal extraction conditions, the feasibility of using magnetic-MIPs as the adsorbents for the extraction of the selected PAHs was proven with a wide linearity range (5-250 μg L^{-1}), good repeatability (relative standard deviation <10%), ultra-trace detection limits (0.01-0.02 μg L^{-1}) and good relative recovery (80.2-119.3%) for the application in the environmental water. The doubt of using the μ-SPE was investigated by calculating the measurement uncertainty. The estimated combined standard uncertainties for the determination of the selected PAHs were in the range of 0.0678-0.0890. It was concluded that the uncertainty of the μ-SPE-μ-HPLC-UV is mainly attributed to its accuracy. Nevertheless, the results showed that the measurement uncertainty of the proposed magnetic-MIPs-based μ-SPE-μ-HPLC-UV was at an acceptable level. The method is beneficial to the routine analysis, especially in providing simple and sensitive determination of the selected PAHs in environmental water. The μ-SPE technique consumes minimal amounts of solvent and traces of adsorbents, which then greatly minimizes the waste and analysis costs in routine analysis.

Keywords: chromatography, measurement uncertainty, molecularly imprinted polymer, PAHs, water

Abstrak

Penyediaan sampel adalah penting untuk menghasilkan ekstrak bersih untuk meningkatkan sensitiviti pengesanan dan meminimumkan penyelenggaraan instrumen. Teknik pengekstrakan fasa pepejal-mikro (μ-SPE) menggunakan polimer bercetakan molekul bermagnet (MIPs bermagnet) sebagai penjerap yang digabungkan dengan kromatografi cecair berprestasi tinggi-mikro dan pengesanan ultraungu (μ-HPLC-UV) telah dibangunkan untuk penentuan hidrokarbon aromatik polisiklik (PAHs) terpilih, iaitu benzo[a]pirena, fenanthrena, fluoranthena dan pirena di dalam sampel air persekitaran. MIPs bermagnet telah dicirikan menggunakan mikroskop elektron pengimbasan, *Brunauer-Emmett-Teller*, analisis termogravimetrik dan magnetometer sampel bergetar untuk memuktamadkan sifat fizikal MIPs bermagnet. μ-SPE kemudiannya dioptimumkan menggunakan pendekatan satu pembolehubah pada satu masa untuk meningkatkan kerberkesanan pengekstrakan. Di bawah keadaan pengekstrakan yang optimum, kebolehlaksanaan menggunakan MIPs bermagnet sebagai penjerap untuk pengekstrakan PAHs terpilih telah dibuktikan dengan julat kelinearan yang luas (5-250 μg L^{-1}), kebolehulangan yang baik (sisihan piawai relatif <10%), had pengesanan ultra-surihan (0.01-0.02 μg L^{-1}) dan pemulihan relatif yang baik (80.2-119.3%), terutamanya untuk aplikasi di dalam air persekitaran. Keraguan menggunakan μ-SPE ini telah disiasat dengan mengira ketidakpastian pengukuran. Anggaran gabungan ketidakpastian piawai bagi penentuan PAHs terpilih adalah dalam julat 0.0678-0.0890. Disimpulkan bahawa ketidakpastian μ-SPE-μ-HPLC-UV disebabkan terutamanya oleh ketepatannya. Namun begitu, keputusan ini menunjukkan bahawa ketidakpastian pengukuran μ-SPE-μ-HPLC-UV berasaskan MIP bermagnet yang dicadangkan berada pada tahap yang boleh diterima. Kaedah ini bermanfaat kepada analisis rutin terutamanya dalam menyediakan penentuan mudah dan sensitif PAHs terpilih di dalam air persekitaran. Teknik μ-SPE hanya menggunakan sedikit pelarut dan penjerap yang dapat meminimumkan sisa dan kos analisis dalam analisis rutin.

Kata kunci: kromatografi, ketidakpastian pengukuran, polimer bercetakan molekul, PAHs, air

Introduction

The emergence of increased industrial, rapid urbanization and agricultural development has led to environmental pollution and poor sanitation water [1]. Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants that originate from both natural and anthropogenic sources. Dry and wet deposition, road runoff, industrial effluent, leaching from creosote-impregnated wood, petroleum spills and fossil fuel combustion are the main route sources producing PAHs [2]. PAHs are semi-volatile and thus they can travel a long distance in air and water [3]. They are absorbed strongly by waterborne organic and inorganic particles. They may eventually be brought down to the bottom of sediment as a sink in the aquatic system. Even under favorable conditions, the sorbed PAHs will be released into the water as an extended source to threaten the aquatic ecosystem through bioaccumulation in food chains [4]. The ubiquitous presence of PAHs in different environmental compartments greatly opens multiple ways for human exposure to PAHs. The United States Environmental Protection Agency (USEPA) has listed sixteen PAHs as the priority pollutants due to their massive occurrence of toxicological profile and suspected carcinogenicity [5].

To date, liquid-liquid extraction (LLE) and solid phase extraction (SPE) are still the most applied worldwide recognized and accredited techniques for the extraction of environmental pollutants in environmental water. These techniques do not fall in the category of green analysis, and they are tedious and consume large amounts of organic solvents. This indirectly causes environmental pollution, is hazardous to human health and demands extra costs for waste treatment. Therefore, microextraction techniques that are friendlier towards the environment are preferred nowadays, where there is more and more research focusing on greener alternatives.

Magnetic micro-solid phase extraction (μ-SPE) is one of the widely employed microextractions because of its ease of application and low consumption of organic solvents. It is based on the use of magnetic nanoparticles that are composed of a magnetic core, covered with an adsorbent phase to bind analytes of interest. Molecularly imprinted polymers (MIPs) are used as adsorbents due to their specific molecular recognition cavities. MIPs are one of the most promising adsorbents when dealing with complex biological and environmental matrices [6-8]. Integrating both features, namely green μ-SPE and

selective MIPs into one technique greatly enhances the extraction performance and simplifies the routine analysis.

Measurement data is produced and used as the basis for product quality determination, environmental pollution and food safety monitoring. All measurements have some degree of uncertainty that may come from all sorts of sources during the determination process. Measurement uncertainty provides an interval within which the true value is likely to fall, thus it is an indication of the reliability of a measurement. Therefore, it is important to report the measurement uncertainty to demonstrate the quality of test results and ensure its validity for any purpose.

In this study, commercial PAHs MIPs were coated on magnetic nanoparticles, particularly iron oxide (Fe_3O_4) to produce magnetic-MIPs. Then, the magnetic-MIPs were characterized physically using a dissecting microscope namely Zeiss Stemi 2000-c Trinocular microscope, scanning electron microscope and Brunauer-Emmett-Teller to analyze the surface morphology and determine the pore volume. The thermal stability of magnetic-MIPs was measured using thermogravimetric analysis and the magnetization properties were analyzed using a vibrating sample magnetometer. The magnetic-MIPs were then employed as the adsorbents in the μ-SPE for the extraction of selected PAHs, namely pyrene, phenanthrene, fluoranthene and benzo[a]pyrene in environmental water. Several extraction parameters such as amounts of magnetic-MIPs, extraction time, types of desorption solvent, volume of desorption solvent and desorption time were thoroughly investigated and optimized to enhance the extraction efficiency of μ-SPE. Next, the μ-SPE technique was validated and applied for the determination of PAHs in environmental water. The sensitivity and selectivity of the recognition site of MIPs were evaluated using different sample matrices. Lastly, the measurement uncertainty of the developed technique was determined to estimate the propagation of errors, mainly from the precision and accuracy involved in the method.

Materials and Methods

Chemical and reagents

The HPLC grade solvents, specifically, acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), isopropanol (IPA) and tetrahydrofuran (THF) were obtained from Merck (Darmstadt, Germany). Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$), glacial acetic acid and ammonia solution (NH_4OH) were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). The selected polycyclic aromatic hydrocarbons (PAHs) reference standards, namely benzo[a]pyrene (BaP), fluoranthene (FLA), phenanthrene (PHE) and pyrene (PYR) were also supplied by Sigma-Aldrich (St. Louis, Missouri, United States). Commercially available molecularly imprinted polymer (MIP) for PAHs, named RENSA PAH MIP 4003 was obtained from Biotage (Uppsala, Sweden). Lastly, deionized water was obtained from Nano Ultra-pure water system purchased from Thermo Fisher Scientific (New Hampshire, United States).

Preparation of standard and sample solutions

The standard stock solutions (500 mg L^{-1}) of each PAH were prepared by weighing individually 0.005 g of each PAH in a 10 mL volumetric flask and diluting to volume with either ACN (PHE and BaP) or MeOH (FLA and PYR). The mixture working standard solution (50 mg L^{-1}) was prepared weekly by diluting the standard stock solutions with methanol. All standard solutions were stored in darkness at 4 °C when not in use.

Four types of environmental water samples were collected from areas surrounding Universiti Malaysia Terengganu, Kuala Nerus, Terengganu. These included river water, tap water, sea water and rainwater. These samples were collected in glass bottles pre-cleaned with decon and kept in a freezer before analysis. The samples were filtered through Whatman filter paper No. 1 obtained from Sigma-Aldrich (St. Louis, MO, United States) to remove the larger particles before extraction. The environmental water samples were applied in the extraction to imitate real matrices in the relative recovery study.

Fabrication of magnetic molecularly imprinted polymers

The fabrication of magnetic-MIPs was adopted from past research with minor modifications on the chemical [9]. $FeCl_3.6H_2O$ (approximately 3.62 g) and $FeSO_4.7H_2O$ (approximately 0.77 g) were dissolved with 50 mL of deionized water in a 250 mL Erlenmeyer flask. The flask was incubated in a beaker containing water at 40 °C and the mixture was vigorously stirred with a hot plate and magnetic stirrer (Corning, United States) for 30 min. Next, the MIPs (0.35 g) were added to 100 mL of 1% (v/v) acetic acid. The resulting solution was then added into the same Erlenmeyer flask and vigorously stirred for 2 h. Next, the ammonia solution (48 mL) was added dropwise over 2 h into the same flask with vigorous stirring. The temperature was maintained at 40 °C until the end of preparation. After the reaction, the product was filtered and washed with deionized water until the filtrate achieved pH 7. The product was then washed with ethanol 3 times. Lastly, the product was dried in an oven at 60 °C for 24 h. The products obtained were iron oxide (Fe_3O_4)-MIPs or magnetic-MIPs.

Characterization of magnetic-MIPs

Physical characterization was performed to observe the morphology of the MIPs deposited on the Fe_3O_4 that functioned as the adsorbent in this study. The morphology of both MIPs and magnetic-MIPs was observed through a disserting microscope namely Zeiss Stemi 2000-c Trinocular Stereo zoom microscope (Germany). Both MIPs and magnetic-MIPs were also scanned with a focused beam of electrons using a scanning electron microscope (SEM) (JEOL 6360 LA, Japan) to obtain information on their surface morphology. The thermal stability of magnetic-MIPs was also investigated by thermogravimetric analysis (TGA) (Mettler-Toledo GmbH TGA/DSC 1, Switzerland). The pore volume and surface area of the magnetic-MIPs were analyzed using Brunauer-Emmett-Teller (BET) (ASAP 2020 Micromeritics, United States). Magnetization properties of magnetic-MIPs were also recorded using a vibrating sample magnetometer (VSM) (Lakeshore 7404, United States).

Magnetic MIPs-based micro-solid phase extraction procedure

The sample solution (10 mL) was pipetted into a 10 mL glass centrifuge tube and magnetic-MIPs (10 mg) were added to the sample solution. The tube was capped and subjected to vortex at 460 rpm for 10 min using a vortex mixer (DLab Scientific, United States). The magnetic-MIPs were then attracted to an external magnet attached to the outer layer of the tube and the sample solution was discarded. Next, the selected PAHs were desorbed from the magnetic-MIPs by sonicating the magnetic-MIPs in THF (120 µL) for 10 min using an ultrasonic bath (Elmasonic, United States). The extract was then filtered through a 0.2 µm nylon syringe filter before quantification using micro-high performance liquid chromatography-ultraviolet detection (µ-HPLC-UV). Figure 1 shows the schematic of the magnetic-MIPs-based micro-solid phase extraction (magnetic-MIPs-µ-SPE) procedure.

Chromatographic conditions

All analyses were performed using µ-HPLC-UV (Agilent Technologies, Milan, Italy). The chromatographic separation of PAHs was carried out on a ZORBAX Eclipse XDB-C18 column (2.1 × 100 mm, 3.5 µm) purchased from Agilent (Italy). The separation was performed using isocratic mobile phase ACN-water (70:30) (v/v) with the column temperature maintained at 30 °C. The flow rate, injection volume and detection wavelength were fixed at 0.2 mL min^{-1}, 2 µL and 254 nm, respectively. Chromatographic data were processed using Agilent ChemStation software.

Optimization of magnetic-MIPs-µ-SPE-µ-HPLC-UV

In this study, several extraction parameters were thoroughly investigated to enhance the analytes' enrichment. The amounts of magnetic-MIPs (5-20 mg), extraction time (1-12 min), types of desorption solvents (MeOH, EtOH, ACN, THF), volume of desorption solvent (50-150 µL of THF) and desorption time (4-12 min) were optimized based on one variable at a time (OVAT) approach and triplicate extraction was performed to finalize the consistency of the results. The OVAT approach involves experiments holding all variables constant and varying only one variable to observe the changes in extraction efficiency.

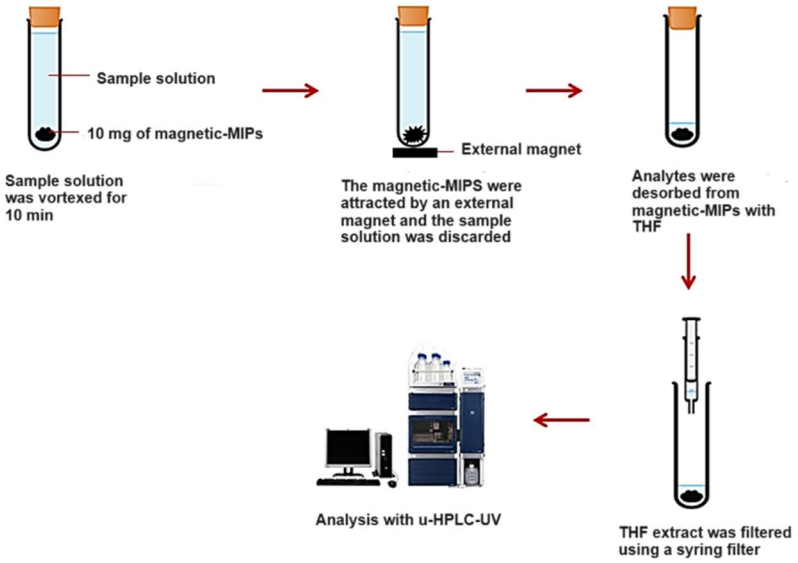


Figure 1. Schematic of magnetic-MIPs-µ-SPE procedure.

Validation of magnetic-MIPs-µ-SPE-µ-HPLC-UV

Validation was carried out to assess the viability of the method, which included linearity, relative recovery, limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were calculated based on signal-to-noise 3:1 and 10:1, respectively.

Measurement uncertainty

Measurement uncertainty (MU) is a measure of the quality and confidence of results for decision-making. MU is important to identify any errors that occur during the experiments and thus the estimation of MU in a method can reduce the errors that exist in data for future analysis. The equation of calculation of the analyte concentration is shown in Equation (1).

The path in calculating the analyte concentration and the error sources were identified and summarized in Figure 2, a fishbone diagram. Several parameters affecting the analyte concentration originated from the preparation of the standard and sample solutions, calibration of glassware and weighing balance, precision and accuracy.

The estimation of the individual uncertainty component was impracticable and tedious. Therefore, all sources were reconciled into bias (systematic errors) and precision (random errors) [10]. The combined standard uncertainty is therefore based on Equation (2).

$$[\mathrm{PAH}] = \frac{(\text{Peak area} \quad)}{\mathrm{m}} \tag{1}$$

where, [PAH] = concentration of the selected PAH, c = y-intercept of linear regression, m = slope of linear regression.

$$\mu^2(\mathrm{y}) = \mathrm{s}^2(\text{precision}) + \mu^2(\text{bias}) \tag{2}$$

where, μ^2 = combined standard uncertainty; s = precision uncertainty; µ = bias uncertainty.

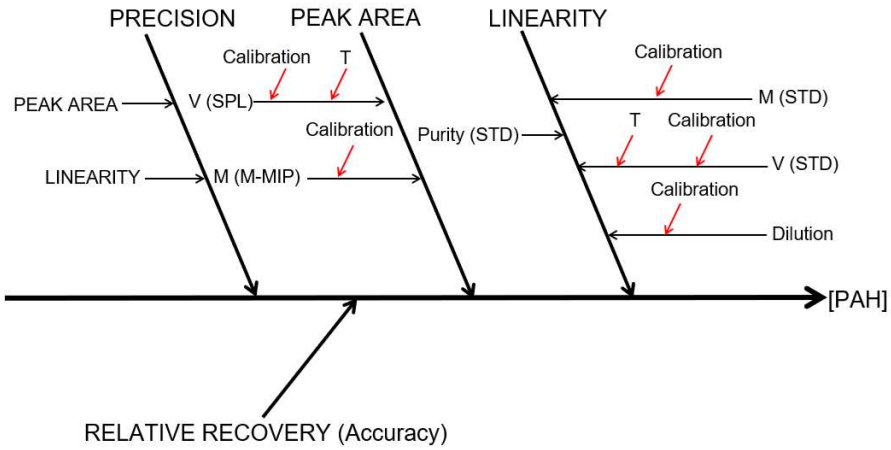


Figure 2. The fishbone diagram indicates error sources in magnetic-MIPs-µ-SPE-µ-HPLC-UV. Abbreviation: standard (STD); sample (SPL); temperature (T); magnetic-MIP (M-MIP); mass (M); volume (V)

The precision uncertainty was obtained from the pooled relative standard deviation (RSD) calculated from the repeatability of the test results (replicate number, n=3) using different analytes spiked at different concentrations using different sample matrices. The pooled RSD equation is shown in Equation (3).

$$\text{Pooled RSD}^2 = \sqrt{\frac{(n_1-1)\ \text{x RSD}_1{}^2+(n_2-1)\ \text{x RSD}_2{}^2+\cdots}{(n_1-1)+(n_2-1)+\cdots}} \quad (3)$$

where, pooled RSD^2 = pooled relative standard deviation and n = the number of data.

$$\mu(\text{bias}) = \sqrt{\text{RMSbias}^2 + \text{RMS}\mu\text{\_ref}^2} \quad (4)$$

$$\text{RMSbias}^2 = \sqrt{(x_1-1)^2 + (x_2-1)^2 + \cdots} \quad (5)$$

where, µ(bias) = bias uncertainty, $RMSbias^2$ = bias in reference standard, $RMS\mu\_ref^2$ = reference material uncertainty, $RMSbias^2$ = bias in reference standard and x = the mean of relative recovery results.

The bias uncertainty was obtained from the relative recovery results, which was also the accuracy study. The uncertainty of bias was calculated using Equation (4) and Equation (5).

Results and Discussion

Fabrication and characterization of magnetic-MIPs

Magnetic-MIPs were fabricated and submitted for morphological characterization using a dissecting microscope namely Zeiss Stemi 2000-c Trinocular Stereo zoom microscope and SEM. The magnetic-MIPs were placed on a petri dish and viewed under a fluorescence microscope at x100 magnification. The dissecting microscope image (Figure 3a) shows the non-homogeneous particle size distribution of MIPs. Nevertheless, it tallied with the manufacturer's analysis, as the particle sizes of MIPs were in the range of 32 to 100 µm.

Figure 3b and Figure 3c show the SEM images of both MIPs and magnetic-MIPs at x1000 magnification, respectively. The results indicated that well-shaped particles with an estimated diameter distribution from 10 µm to 100 µm were obtained for magnetic-MIPs. The MIPs had spherical shapes and smooth surfaces, and the magnetic-MIPs also possessed similar spherical shapes even after being deposited on the magnetite. MIPs

consist of a large surface area leading to more effective imprinting sites [11]. The SEM images of both materials revealed that the particle size distribution was maintained even after deposition of MIPs on the magnetite. This proved that magnetic-MIPs were successfully prepared for the extraction of selected PAHs in this study.

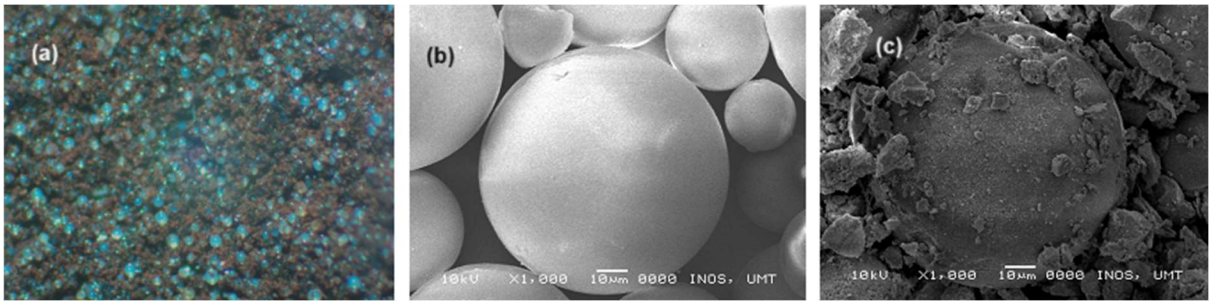


Figure 3. (a) Microscope image of magnetic-MIPs at x100 magnification, (b) SEM images of MIPs and (c) magnetic-MIPs at x1000 magnification

TGA was used to analyze the thermal stability of magnetic-MIPs in the temperature range of 30 °C to 800 °C. There were two stages of degradation behaviour observed on magnetic-MIPs (Figure 4a). The weight loss in the first stage was described as the loss of absorbed and bound trace water between 30 °C and 70 °C. Another weight loss occurred between 330 °C and 400 °C, which was due to the decomposition of organic parts on magnetic-MIPs [12]. The extraction using magnetic-MIPs as the adsorbents in this study was performed at room temperature and thus the magnetic-MIPs were stable throughout the analysis.

The information on pore volume and surface area of adsorbents is important to ensure repeatable and effective adsorption of analyte in trace analysis. The pore volume and surface were determined using BET technique. The pore volumes and surface areas for MIPs and magnetic-MIPs were determined as 1.37 $cm^3 g^{-1}$ and 710 $m^2 g^{-1}$, and 0.38 $cm^3 g^{-1}$ and 224 $m^2 g^{-1}$, respectively. The decline in both pore volume and surface area of magnetic-MIPs indicated that magnetite had occupied parts of the MIPs due to the deposition of magnetite on MIPs. The reduction in surface area for active sites and cavities in MIP might reduce the analytes' adsorption and similar results were also reported in previous studies [13,14]. However, the magnetic-MIPs had greatly simplified the collection of MIPs after the extraction process, and the magnetic-MIPs were still capable of detecting ultra-trace target analytes after a thorough optimization was performed.

VSM analysis showed that the magnetic-MIPs possessed supermagnetism properties. This proved that magnetic-MIPs could be attracted and completely actuated to the external magnet after extraction. Magnetic-MIPs reached a saturation magnetization value at 9.1025 emu g^{-1} with a coercivity of 62.194 G (Figure 4b). The saturation of magnetization value observed for magnetic-MIPs might be less than the pure Fe_3O_4 nanoparticles reported [15]. This could be due to the limited particle surface impact. The magnetic saturation value decreased because of the coating effect into the layer and some of the MIPs might be trapped in the Fe_3O_4 contributing to the degradation of the magnetism moment [16]. This result showed that magnetic-MIPs were successfully prepared and had good magnetism properties that could be applied in µ-SPE for rapid removal of target compounds from sample solution.

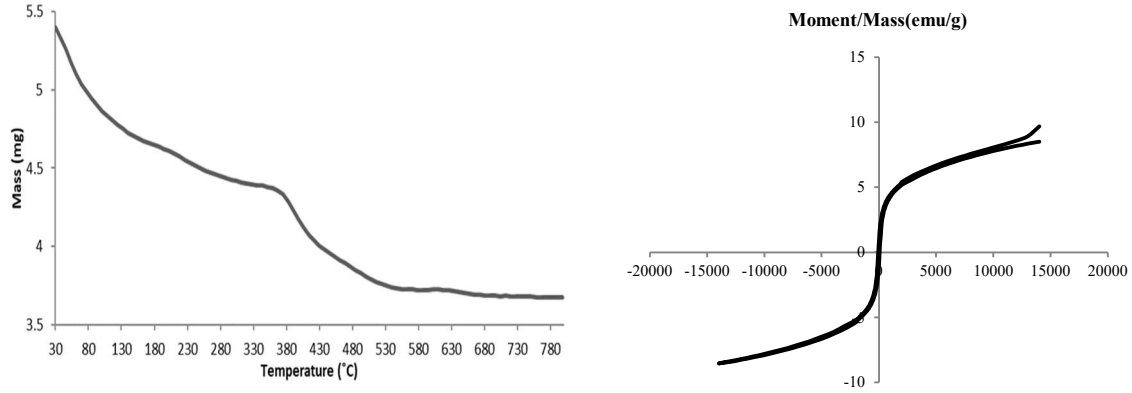


Figure 4. (a) TGA thermogram of magnetic-MIPs, (b) magnetization curves of magnetic-MIPs at room temperature

Optimization of magnetic-MIPs-µ-SPE

Several extraction variables were thoroughly investigated to optimize the magnetic-MIPs-µ-SPE of selected PAHs, namely PHE, FLA, PYR and BaP from water samples. RENSA PAHs MIPs were chosen as the adsorbents in this study due to their selective molecular recognition abilities, which can eliminate the clean-up procedure treated for complicated sample matrices [17]. In this study, the MIPs were deposited on Fe_3O_4 to simplify the collection of MIPs after the extraction. This could eliminate the filtration steps, as well as preventing the loss of microscopic MIPs. The optimization study was performed using deionized water samples that were spiked with each selected PAH at 100 µg L^{-1}. Triplicate extractions were carried out for each variable to ensure results consistency and repeatability.

Amounts of magnetic-MIPs and extraction time

The amounts of magnetic-MIPs were examined to investigate the capacity of magnetic-MIPs in extracting selected PAHs from 10 mL of water sample. In this study, the amounts of magnetic-MIPs in the range of 5 to 20 mg were tested. Based on Figure 5a, the peak areas for all PAHs increased dramatically when magnetic-MIPs were added from 5 to 10 mg in the 10 mL of spiked water sample. However, the decline in peak areas was recorded beyond 10 mg. This meant that the amounts of adsorbents applied achieved the equilibrium mass transfer to lead to the most favorable enrichment [18]. The drop could be due to the agglomeration of magnetic-MIPs occurring in the confined volume, where the relative strength of the van der Waals forces bound the adsorbent particles together [19]. This phenomenon resulted in extraction inefficiency because of the lack of binding sites available for the adsorption. Therefore, 10 mg of magnetic-MIPs were applied in the subsequent optimization experiments.

The effect of extraction time was evaluated by monitoring the peak areas of target analytes from 1 to 12 min of extraction time. The extraction was performed by vortexing the sample solution. The extraction time determines the extraction performance that is dependent on the adsorption equilibrium of the analytes between the sample solution and the adsorbent [20]. The peak areas showed steady increases from 1 to 10 min for all analytes, except for BaP (Figure 5b). There was no significant difference ($p>0.05$) in the peak areas of BaP extracted for 8 min and 10 min. The system achieved complete equilibrium at 10 min. Thereafter, a steep decline in peak areas was observed because of the back-diffusion of analytes from the adsorbent [18]. For this reason, 10 min was chosen as the time to achieve the complete extraction.

Type of desorption solvent, volume of desorption solvent and desorption time

The binding sites in MIPs are mainly dependent on the size, structure and functional groups of the template molecules that have the most similarity with target

analytes [21]. This allows MIPs to possess selective recognition of target analytes and hence the polarity of desorption solvents is important to offer a good polarity match to the target analytes for an efficient desorption [22]. A solvent that can dissolve the target analyte and has a strong affinity to the target analytes is therefore influential. Four polar organic solvents that varied in polarity index (P'), namely ACN (P'=5.8), EtOH (P'=5.2), MeOH (P'=5.1) and THF (P'=4) were investigated in this study. The most effective desorption (Figure 5c) was recorded by the THF. THF possessed the lowest P' and this sustained the release of the hydrophobic PAHs from the imprinted cavities. Therefore, the THF was chosen as the desorption solvent to proceed with the following optimization experiments.

The effect of desorption volume in the range of 50 to 150 µL was investigated. The peak areas increased gradually (Figure 5d) when the selected PAHs were desorbed with 50 to 120 µL of THF, except for BaP, which showed no significant difference ($p>0.05$) in the peak areas extracted using 100 µL and 120 µL of THF. However, when 150 µL of THF was used, a decline in peak areas was observed. This could be due to the dilution effect [23]. Since the 120 µL was sufficient to submerge the magnetic-MIPs and offered better extraction efficiency for selected PAHs, the best-configured volume was therefore chosen to be 120 µL.

Desorption process is an equilibrium-dependent process, thus sufficient time is allocated to the desorption process to optimize the release of analytes from the adsorbents [24]. In this study, desorption time from 4 to 12 min was explored and assisted by ultra-sonication. An enhancement in peak areas of selected PAHs (Figure 5e) was observed when the selected PAHs were desorbed from magnetic-MIPs with THF from 4 to 10 min. When the desorption was performed for longer than 10 min, the peak areas of the selected PAHs diminished. This could be due to the re-adsorption of the analytes on the adsorbents [25]. The 10 min desorption time was therefore chosen for the magnetic-MIPs-µ-SPE.

Validation of magnetic-MIPs-µ-SPE-µ-HPLC-UV

Several parameters were performed for validation, which included establishing linearity range, LOD, LOQ and relative recovery to ensure the viability of the magnetic-MIPs-µ-SPE-µ-HPLC-UV technique for the analysis of selected PAHs in environmental water. Linearity in the range of 5 to 250 µg L^{-1} with correlation coefficients, $r \geq 0.9951$ was established experimentally for the quantification of the selected PAHs. The LODs (0.01-0.02 µg L^{-1}) and LOQs (0.04-0.06 µg L^{-1}) revealed that the µ-SPE-µ-HPLC-UV using the magnetic-MIPs as the adsorbents was capable to offer ultra-trace detection of selected PAHs in water samples.

Relative recovery was performed rather than absolute recovery because the µ-SPE technique is a non-exhaustive extraction technique that depends on the equilibrium time. Environmental samples namely river water, tap water, seawater, and rainwater were chosen to investigate the feasibility of the magnetic-MIPs-µ-SPE for the extraction of selected PAHs in these sample matrices. Each sample was spiked with selected PAH at concentrations of 5, 25 and 100 µg L^{-1}, respectively. Blank samples were analyzed to ensure the blank concentration was deducted from the spiked samples. However, the blank samples chosen were all free from the selected PAHs. The blank magnetite was also used to extract the spiked deionized samples randomly, where the peak areas obtained were negligible. The proposed technique could recover the ultra-trace levels of the selected PAHs spiked in the environmental samples with an acceptable average of relative recoveries in the range of 80.2-119.3% (Table 1) with repeatability <10%. This revealed that magnetic-MIPs with reduced surface area compared to core MIPs were still capable of offering good accuracy in determining the PAH residues in environmental water samples. The matrix effects demonstrated by these environmental water samples were insignificant when the proposed magnetic-MIPs-µ-SPE was applied for the extraction of the selected PAHs in the samples. The technique offers a simple and fast procedure and therefore it can be applied as a greener alternative technique for the analysis of PAHs in environmental water samples.

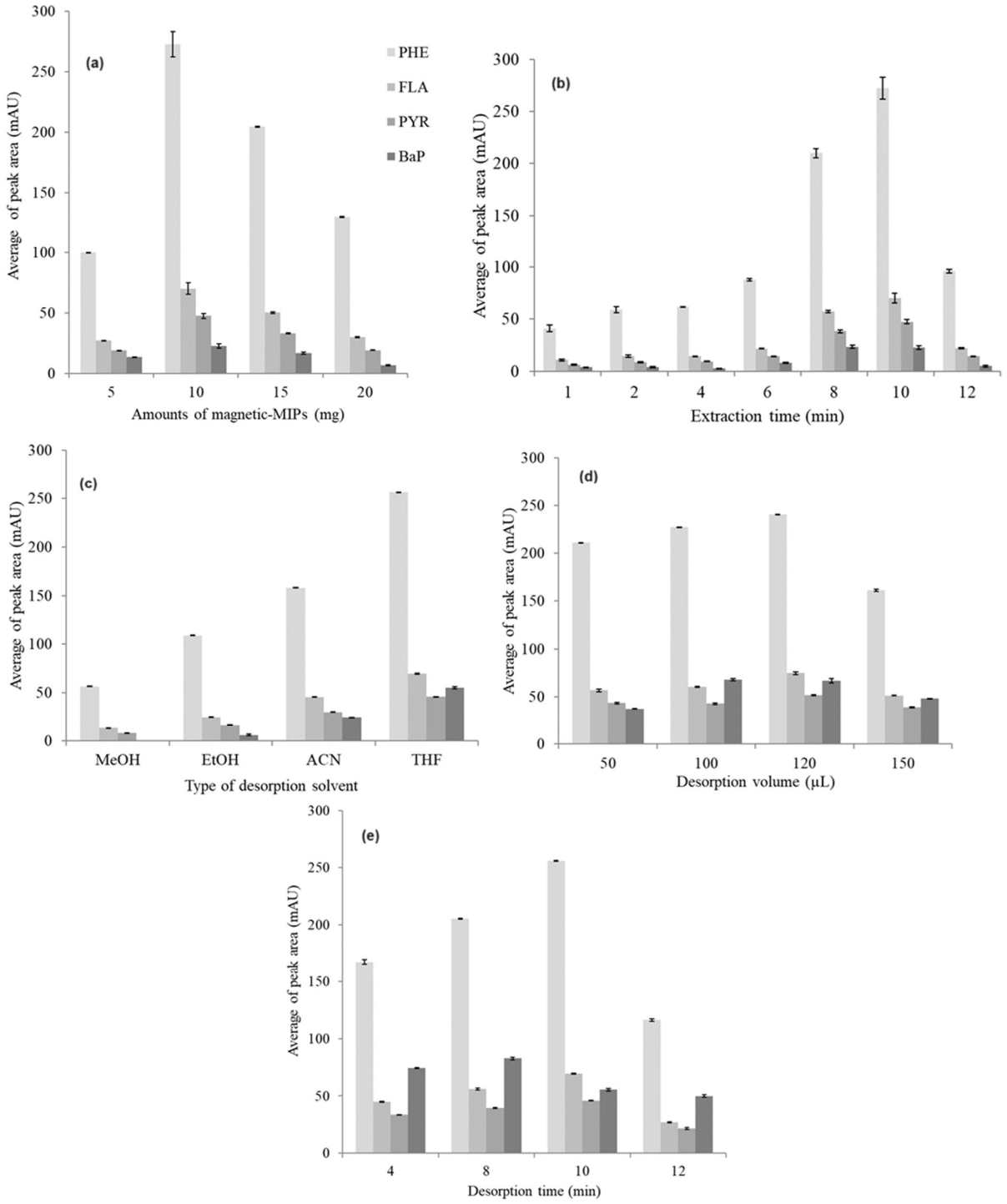


Figure 5. Effect of (a) amounts of magnetic-MIPs, (b) extraction time, (c) type of desorption solvent, (d) desorption volume and (e) desorption time on the extraction efficiency of µ-SPE for the extraction of selected PAHs in spiked deionized water (n = 3 in each case). Initial extraction conditions applied before the optimization were 10 min of extraction time and desorption by 100 µL of acetonitrile for 10 min until an optimal condition was adopted from the experiments. Error bars represent the standard deviations

Table 1. Relative recovery study using magnetic-MIPs as the adsorbent in µ-SPE technique for the extraction of selected PAHs in environmental water samples

Samples	PAH	Average of Relative Recovery, % (RSD%)		
		5.0 µg L^{-1}	25.0 µg L^{-1}	100 µg L^{-1}
River water	PHE	109.6 (0.5)	83.8 (0.5)	106.0 (2.3)
	FLA	113.5 (0.3)	83.1 (0.6)	107.6 (2.1)
	PYR	95.4 (6.7)	81.6 (1.4)	104.6 (0.6)
	BaP	98.4 (0.3)	93.8 (1.5)	108.1 (2.7)
Tap water	PHE	98.0 (1.4)	88.8 (2.2)	99.8 (2.9)
	FLA	92.2 (0.6)	87.4 (2.6)	95.7 (2.7)
	PYR	93.3 (3.4)	98.1 (1.1)	113.9 (2.9)
	BaP	80.2 (4.5)	92.5 (2.9)	96.3 (2.5)
Seawater	PHE	85.5 (10.3)	105.7 (2.3)	92.8 (3.4)
	FLA	84.7 (3.0)	98.3 (4.5)	90.4 (6.5)
	PYR	84.1 (8.0)	94.0 (2.0)	90.8 (3.8)
	BaP	92.0 (5.7)	110.4 (2.8)	109.3 (3.1)
Rainwater	PHE	119.3 (1.7)	108.0 (2.1)	106.9 (1.5)
	FLA	93.3 (2.3)	102.0 (0.9)	105.4 (1.4)
	PYR	116.2 (4.7)	109.7 (3.2)	107.5 (1.7)
	BaP	110.6 (2.3)	104.1 (1.2)	113.0 (1.3)

Measurement uncertainty of magnetic-MIP-µ-SPE-µ-HPLC-UV for the determination of selected PAHs in environmental water

In this study, the combined MU was estimated according to the top-down approach that was best for testing laboratories. The actual dispersion of test results from the validation experiments was observed and included the precision or repeatability, bias uncertainty from reference standard materials, and accuracy or relative recovery results that contributed as the error sources to the uncertainties associated with the outcome. The implementation of the suggested approach was performed using the validation data obtained from the relative recovery study using four types of environmental water samples. In general, the MU in the determination of the concentration of the selected PAHs was associated with systematic and random errors in the method. The combined standard uncertainties for the determination of the PHE, FLA, PYR and BaP were 0.0812, 0.0678, 0.0890, and 0.0778, respectively.

The results showed that the main source contributing to the combined standard uncertainties was the accuracy test performed during method validation, which was termed relative recovery study. The relative recovery study was performed using four different environmental water samples with each spiked at 5, 25 and 100 µg L^{-1}. The relative recovery was calculated relative to the spiked deionized water. This could be the main factor causing the moderate relative standard uncertainty in accuracy because the deionized water was cleaner as compared to other environmental water samples. However, the relative recovery results were still accepted as proposed in the Association of Official Agricultural Chemists validation guidelines, where the recovery studied in micrograms per liter (µg L^{-1}) levels shall be in the range of 80 to 110% and 60 to 115% for 100 µg L^{-1} and 10 µg L^{-1} spiking level, respectively [26] to demonstrate negligible matrix effects. Therefore, the relative recovery average in the range of 80.2-119.3% achieved in this study was satisfactory. In conclusion, the MU of the proposed magnetic-MIP-µ-SPE-µ-HPLC-UV for the determination of selected PAHs in environmental water samples was acceptable.

Conclusion

The integration of magnetism and selective recognition of PAHs into magnetic-MIPs corresponds to a quick and effective way to isolate the selected PAHs from the sample solution by applying an external magnetic field.

The proposed μ-SPE procedure is simple, sensitive and fulfils the green analysis concept in minimizing the use of organic solvents and reducing waste disposal costs. In addition, this technique applies minimal extraction solvent, which is beneficial in the enrichment of analytes. In fact, it is economical and eco-friendly. In conclusion, the combined standard uncertainty for the determination of the selected PAHs is satisfactory and proves that the magnetic-MIP-μ-SPE-μ-HPLC-UV is feasible to be applied as a routine testing procedure for the analysis of selected PAHs in environmental water. The estimation of measurement uncertainty in the analysis revealed that the main source of uncertainty was the accuracy test performed during method validation. By identifying sources of uncertainty, researchers can take steps to reduce uncertainty and improve the accuracy and reliability of the measurements over time.

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ISOTHERM AND KINETIC ANALYSIS FOR THE ADSORPTION OF BISPHENOL S ONTO TEA STALK BASED ACTIVATED CARBON

(Analysis Isoterma dan Kinetik bagi Penjerarapan Bisfenol S Mengunakan Karbon Teraktif Tangkai Teh)

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Abstract

The objective of this investigation is to create activated carbon from tea stalk (TSAC) with the purpose of adsorbing the microplastic Bisphenol S (BPS) from wastewater. Wastewater containing BPS poses a threat to human and aquatic ecosystems, as conventional treatment plants struggle to effectively eliminate this hazardous pollutant. The production of TSAC involves physical activation with CO_2 as the activating agent, resulting in a BET surface area of 712.49 m^2/g and a total pore volume of 0.25 cm^3/g. Scanning electron microscopy (SEM) was employed to analyse both the precursor and TSAC. At an enlargement of 1000×, the SEM image of the precursor revealed a non-porous and dense surface, while the TSAC exhibited a distinctly porous surface. This highly porous adsorbent undergoes the equilibrium study, which demonstrates that as the original concentration of BPS increases from 5 to 50 mg/L, the adsorption uptake rises while the corresponding percentage removal decreases. This study also found that the optimum removal of BPS by TSAC occurs at a solution temperature of 60 °C. Isotherm studies reveal that the BPS-TSAC adsorption system follows the Langmuir model, with a maximum monolayer adsorption capacity (Q_m) of 41.25 mg/g. In kinetic studies, the pseudo-second order (PSO) model provides the best fit for the adsorption of BPS by TSAC. Boyd plot analysis identifies film diffusion as the step that governs the rate of the adsorption process. The CO_2 gasification impact has induced the formation of well-developed pores on TSAC, thereby improving its capacity to adsorb BPS microplastics.

Keywords: activated carbon, adsorption, bisphenol S, isotherm, kinetic

Abstrak

Kajian ini bertujuan untuk mensintesis karbon teraktif dari tangkai teh (TSAC) untuk penjerapan mikroplastik Bisfenol S (BPS) dari sisa kumbahan. Sisa kumbahan yang mengandungi BPS adalah ancaman terhadap manusia dan ekosistem akuatik, kerana rawatan konvensional menghadapi kesulitan dalam menyingkirkan pencemar merbahaya ini secara berkesan. Penghasilan TSAC melibatkan pengaktifan fizikal menggunakan CO_2 sebagai ejen pengaktif telah menghasilkan luas permukaan BET sebanyak 712.49 m^2/g dan jumpah isipadu liang sebanyak 0.25 cm^3/g. Mikrosopi imbasan elektron (SEM) digunakan untuk menganalis

pelopor dan TSAC. Pada pembesaran 1000x, imej SEM pelopor menunjukkan permukaan yang tidak berliang dan padat, manakala TSAC menunjukkan permukaan yang berliang dengan jelas. Penjerap berongga ini menjalani kajian keseimbangan dimana ia menunjukkan bahawa semasa kepekatan asal BPS meningkat dari 5 hingga 50 mg/L, penjerapan turut meningkat manakala peratus penyinkiran pula didapati menurun. Kajian ini juga mendapati penyingkiran optimum BPS oleh TSAC berlaku pada suhu larutan 60 °C. Kajian isoterma mendedahkan bahawa sistem penjerapan BPS-TSAC mengikut model Langmuir, dengan kapasiti penjerapan lapisan tunggal maksima (Q_m) sebanyak 41.25 mg/g. Dalam kajian kinetik, model kinetik pseudo tertib kedua (PSO) memberikan kepadanan terbaik untuk penjerapan BPS oleh TSAC. Analisis plot Byod mendapati bahawa resapan filem adalah langkah pengehad yang mengawal proses penjerapan. Impak gasifikasi CO_2 telah menhasilkan pembentukan liang yang baik pada TSAC, dengan itu meningkatkan kapasiti penjerapan mikroplastik BPS.

Kata kunci: karbon teraktif, penjerapan, bisfenol S, isotherma, kinetik

Introduction

Clean water is essential for sustaining life and maintaining good health, as it plays a crucial role in various bodily functions and hygiene. Access to clean water is not only a basic human right but also a fundamental requirement for thriving communities, agriculture, and overall environmental well-being. Yet, untreated water bodies can harbour various pollutants that harm both humans and the environment, with endocrine disrupting chemicals (EDCs) gaining significance due to their toxic impacts on reproductive and developmental functions in humans and wildlife. These pollutants mainly originate from the extensive use of pesticides, industrial chemicals, plastics, plasticizers, and fuels in environmental matrices [1]. Bisphenol S (BPS), a common substitute for Bisphenol A (BPA), possesses robust O=S=O double bonds, rendering it more resistant to heat and light compared to BPA. This heat resistance makes BPS appealing for use in products like food containers, where less heat-resistant BPA might otherwise leach, especially in tropical regions. Within bisphenols, BPS is more likely to be distributed in the aqueous phase, although there is also a possibility of its adsorption onto sediment. Furthermore, it exhibits a longer half-life in sediments [2]. The primary concern with bisphenols, including BPS, is their potential to mimic hormones in the human body, particularly estrogen. This has raised concerns about their endocrine-disrupting properties and possible health risks. Research suggests that BPS, like BPA, may interfere with the endocrine system, potentially affecting reproductive health and hormone balance.

BPS can enter water sources through various pathways, primarily because of human activities and the widespread use of products containing BPS. Water sources can be contaminated with BPS through the runoff from consumer products. Items such as BPS-containing plastics, thermal paper receipts, and personal care products have the potential to release BPS into the environment. This runoff can find its way into rivers, lakes, and other water bodies. The disposal of products containing BPS, as well as industrial processes that use or produce BPS, can contribute to the presence of this chemical in wastewater. When wastewater is released into rivers or oceans without proper treatment, it can introduce BPS into aquatic ecosystems. Landfills can be a source of BPS contamination in groundwater. When products containing BPS are discarded and end up in landfills, there's the potential for leaching where rainwater or other liquids can carry BPS from the landfill into the surrounding soil and water. Like its counterpart, bisphenol A (BPA), BPS can persist in the environment for extended periods. Additionally, BPS has been found to bioaccumulate in aquatic organisms, which means that it can accumulate in the tissues of fish and other aquatic species. This bioaccumulation can have implications for the broader aquatic food chain. Therefore, it is crucial to treat wastewater containing BPS effectively before its discharge into water bodies.

Many technologies for eliminating EDCs in wastewater are available, each with its own set of pros and cons. For instance, membrane technology is effective but constrained by the high cost of membrane production and fouling issues. The biological approach utilizes bacteria to digest EDC molecules, but it is limited by its inability to effectively degrade certain persistent compounds, potentially resulting in incomplete removal or transformation into harmful by-products. Utilizing

chemical methods to address EDC compounds in wastewater, while effective in some cases, is often considered environmentally unfriendly due to the potential generation of harmful by-products. The adsorption process stands out as one of the most highly efficient yet relatively low-cost means to remediate EDCs in wastewater. This approach offers advantages such as ease of operation, resilience in toxic conditions, and the potential to reuse the adsorbent through the renderability process [3]. Activated carbon (AC) emerges as a prominently acknowledged adsorbent in the adsorption process among all these adsorbents. In the past 10 years, researchers have been vigorously altering biomass waste into AC, with the goal of reducing production costs and effectively addressing waste disposal concerns. In the literature, researchers successfully converted onion waste [4], durian peel [5], date seeds [6], wood residues [7,8], and many others into AC. The effectiveness of AC as an adsorbent stem from its substantial surface area, ranging from 500 to 2000 m^2/g. These expansive surface areas offer ample space for the adsorption of contaminant molecules on the AC's surface. AC's versatility shines through in its capacity to absorb a wide range of pollutants, encompassing heavy metals [9], synthetic dyes [10], microplastics [11], antibiotics [12], and more, making it a universally effective solution.

The manufacturing of AC entails a meticulously designed sequence of steps focused on creating a porous structure with a vast surface area to enhance its adsorption capabilities. The first stage involves the carbonization process, wherein the precursor undergoes pyrolysis at a moderate temperature within an inert atmosphere. Carbonization holds a pivotal role in AC production, marking the commencement of the transformation of carbon-rich precursors into a carbonized state. This pivotal step establishes the foundation for the unique porous structure and notable surface area that define AC. After the carbonization process, the next stage is activation, which falls into three categories: physical, chemical, and physicochemical activation. In physical activation, carbon-rich precursors, usually in the form of char or carbonized material, undergo exposure to high temperatures in the presence of gases like CO_2 or steam. Physical activation yields various advantages, including the ability to control pore structure, achieve a high surface area, ensure thermal stability, simplify the process, exhibit versatility, and potentially enhance energy efficiency. This makes physical activation a valuable method for producing activated carbon for diverse applications. On the other hand, chemical activation involves the treatment of carbonized material with activating agents, following the carbonization step. Physicochemical activation combines elements of both physical and chemical activation, employing heat and activating agents simultaneously. This research selects tea stalk as the precursor for AC generation and employs physical activation with CO_2 as the activating agent. Tea stalks are preferred for AC production because they are readily available as a by-product of tea production, offering a sustainable and plentiful source. Furthermore, tea stalks possess a substantial carbon content, a pivotal factor in the efficient production of activated carbon. The inherent structure of tea stalks, featuring natural pores and voids, plays a key role in fostering an interconnected porous structure within the activated carbon, thereby augmenting its capacity for adsorption.

Materials and Methods

Materials and TSAC preparation

The tea stalk was acquired from a shop in Seberang Jaya, Pulau Pinang. Sigma-Aldrich provided KOH pellet (85%) and HCl (37% assay) and BPS in powder form. MOX Gases Berhad, Malaysia, provided nitrogen gas (N_2) and carbon dioxide gas (CO_2) with 99.99% purity. The precursor tea stalk underwent a meticulous rinse with distilled water and was then dehydrated in the oven for approximately 48 hours. Subsequently, the precursor underwent carbonization at 550 °C for 2 hours under the influence of N_2 gas at 150 cm^3/min to transform it into char. Following this, the resulting char underwent activation at 700°C for 1 hour under the influence of CO_2 gas, also at a rate of 150 cm^3/min. Subsequently, the furnace was enabled to cool down to ambient temperature before collecting and storing the resulting TSAC in an airtight container.

Characterization

The tea stalk precursor and TSAC were characterized to examine both their physical and chemical properties.

The surface area, pore volume, and pore size distribution of the prepared AC were determined from adsorption isotherms of N_2 gas at 77 K using a static volumetric system (Micromeritics ASAP 2020, USA). The calculations for surface area, pore volume, and average pore size were performed by Micropore version 2.46 software, while the Brunauer-Emmet-Teller (BET) equation was employed to determine the specific surface area of the AC. The pore size distribution was determined using the Barren-Joyner-Halenda (BJH) model. Proximate and thermogravimetric analysis (TGA) were performed using a thermogravimetric analyzer (TGA, Model Perkin Elmer STA 6000, USA). To determine the elemental composition of the samples, an elemental analyzer (Perkin Elmer Series II 2400, USA) was used. Meanwhile, the surface morphology of the tea stalk and TSAC was analyzed via scanning electron microscopy (SEM) and carried out using a scanning electron microscope (LEO SUPRA 55VP, Germany). Lastly, Fourier transform infrared (FTIR) spectroscopy was conducted to investigate the functional groups of the tea stalk and TSAC using an FTIR spectroscope (IR Prestige 21 Shimadzu, Japan). Spectrums from 4000 to 400 cm^{-1} were measured, and the analysis was done by the system's software (Spectrum version 5.0.2).

Study of equilibrium, isotherm, and kinetic

The equilibrium study analyzed BPS uptake with consideration for four factors: BPS initial concentration, contact time, pH, and solution temperature. To investigate the impact of the initial BPS concentration, Erlenmeyer flasks were prepared with six dissimilar original concentrations (5, 10, 20, 30, 40,50 mg/L). The BPS solution was prepared by dissolving the purchased BPS powder in distilled water and being stirred using a magnetic stirrer until all the powder was completely dissolved. The flasks were then positioned within a water bath shaker with a rotation speed of 80 rpm. The solution volume, TSAC weight, and solution temperature were maintained at 200 mL, 0.20 g, and 30 °C, respectively, while keeping the solution pH constant. For pH studies, 50 mg/L of BPS solution was prepared at six different pH (2, 4, 6, 8, 10, and 12), while other variable such as solution volume, TSAC weight, and temperature was maintained at 200 mL, 0.20 g and 30 °C. The pH of the BPS solution was adjusted using 0.1 mol/L HCI and NaOH. To investigate the influence of temperature, a BPS solution with an initial concentration of 50 mg/L was employed at three distinct temperatures (30, 45, and 60 °C), while maintaining other parameters constant. The water bath shaker was set according to the temperature investigated, and the adsorption process was continued until equilibrium was reached. Once equilibrium was attained, the TSAC was filtered from the adsorbate solution, and the concentration was measured using a UV-Vis spectrophotometer (Model Shimadzu UV-1800, Japan) at wavelength 245 nm. To ensure the homogeneity of the absorbance values, a calibration curve of absorbance versus BPS concentration was plotted. The BPS uptakes was determined through the utilization of the following equations, respectively:

$$q_e = \frac{(C_o - C_e)V}{W}$$

where q_e is the BPS adsorption uptakes at equilibrium (mg/g), C_o denotes as initial concentration of BPS (mg/L), C_e stands for the concentration of BPS at equilibrium (mg/L), W indicates the weight of TSAC (g) and V is the volume of BPS solution (mL). The isotherm study involved fitting the adsorption data to three models: Langmuir, Freundlich, and Temkin. Their respective formulas are provided as follows:

Langmuir [13]:

$$q_e = \frac{Q_m K_L C_e}{1 + K_L C_e} \quad (2)$$

Freundlich [14]:

$$q_e = K_F C_e^{1/n_F} \quad (3)$$

Temkin [15]:

$$q_e = \frac{RT}{B} \ln (AC_e) \quad (4)$$

where Q_m is the Langmuir adsorption uptakes (mg/g), K_L is the constant of Langmuir which relates to the adsorption capacity (mg/g), K_F stands for Freundlich constant $(mg/g)(L/mg)^{1/n}$). n_F (dimensionless) shows to the ferocity of the surface heterogeneity. A and B refer to the Temkin constants. The kinetics investigation followed a protocol akin to the equilibrium study, with

the measurement of BPS concentration at specified time intervals. Six solutions of BPS, spanning concentrations from 5 to 50 mg/L, a 0.20 g TSAC weight, a solution temperature of 30 °C, the initial pH of the solution, and a rotation speed of 80 rpm were utilized. The kinetic data underwent fitting to three models: pseudo-first order (PFO), pseudo-second order (PSO), and Boyd plot. The corresponding equations are as follows:

Pseudo-first order (PFO) [16]

$$\ln(q_e - q_t) = \ln q_e - k_1 t \quad (5)$$

Pseudo-second order (PSO) [17]:

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (6)$$

Boyd plot [18]:

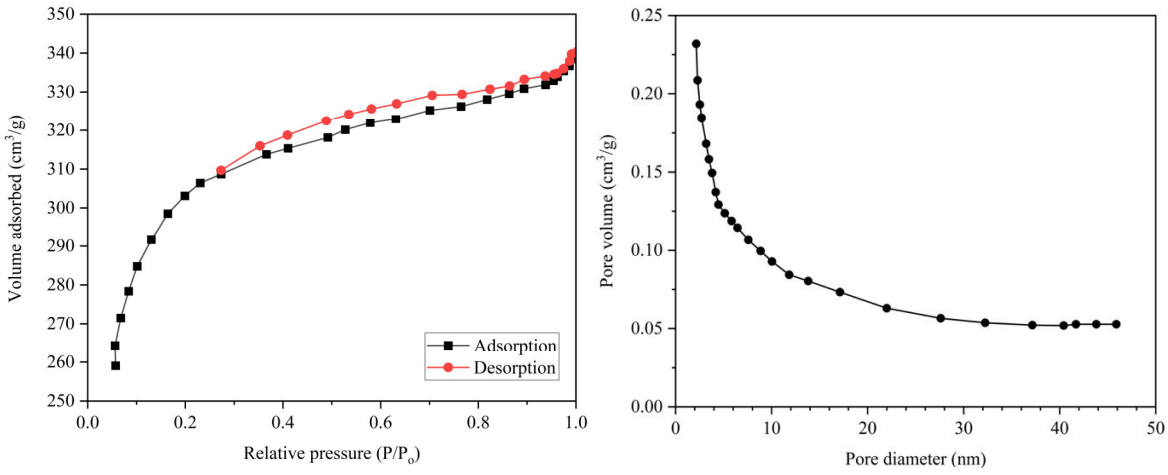
$$B_t = -0.4977 - ln\left(1 - \frac{q_t}{q_e}\right) \quad (7)$$

where k_1 and k_2 are the PFO and PSO rate constant, respectively and B_t denoted as Boyd number.

Results and Discussion

Samples characteristics

The precursor showed a low BET surface area (1.05 m^2/g). After the carbonization and activation process, TSAC exhibited a significant increase in BET surface area (712.49 m^2/g), mesopores surface area (514.71 m^2/g), and total pore volume (0.25 cm^3/g). The average pore diameter for TSAC also expanded from 1.58 nm (micropores) in char to 2.14 nm (mesopores) in TSAC, attributed to CO_2 gasification. A similar effect was observed in the production of teak wood-based activated carbon, where CO_2 aided pore development from 2.64 nm to 2.85 nm [9]. Figure 1(a) shows the adsorption-desorption profile of the TSAC. It was obvious that the isotherm profile belongs to Type IV according to the IUPAC classification. Type IV isotherm pattern suggesting a structure of micropores and mesopores, where capillary condensation is caused by the interaction between gas molecules with the mesopores surface of the adsorbent [19]. Moreover, the desorption path differs from the adsorption in the event of capillary condensation in the mesopores, which accounts for the existence of the type H4 hysteresis loop [20]. Figure 1(b) displays the pore size distribution of the TSAC, which provides significant support for the prepared AC's mesopore structure. The graphic illustrates that the pore diameter ranges from 1.42 to 46 nm. The majority of the TSAC's structures are mesopores, as evidenced by its 2.14 nm average pore diameter [21]. The TSAC's wide pore size distribution suggests a high total pore volume, which will result in good adsorption performance due to its larger surface area.



(a) (b)

Figure 1. (a) BET isotherm profile for TSAC and (b) Pore size distribution of TSAC

Table 1 displays the proximate and elemental analysis outcomes for the samples. The precursor demonstrated a noteworthy elemental C content of 48.71%, a relatively high figure. After the completion of carbonization and activation, the C content in TSAC underwent a substantial increase to 79.54%. In contrast, other elements, namely H, S, and N + O, experienced a reduction, decreasing from 7.41% to 5.86%, 1.03% to 0.08%, and 42.85% to 14.52%, respectively. Proximate analysis revealed a significant decrease in moisture and volatile matter, declining from 4.12% to 1.13% and from 62.87% to 21.66%, respectively. This decrease was credited to the efficiency of the carbonization and activation processes in eliminating water molecules and volatile components like lignin, cellulose, hemicellulose, and tar through evaporation and thermal cracking processes. The percentage of fixed carbon witnessed a significant rise from 27.15% to 74.56%. Considering that fixed carbon plays a role in the matrix structure of TSAC, a higher fixed carbon value was considered advantageous [22]. Finally, the ash percentage in the precursor and TSAC was found to be 5.86% and 2.65%, respectively. The low ash percentage is a favorable trait of AC since ash lacks pores and does not aid the adsorption process [3].

Table 1. Samples' attributes in terms of proximate and elemental analysis

	Proximate Analysis (%)				**Elemental Analysis (%)**			
Samples	**Moisture**	**Volatile Matter**	**Fixed Carbon**	**Ash**	**C**	**H**	**S**	**(N+O)**
Precursor	4.12	62.87	27.15	5.86	48.71	7.41	1.03	42.85
TSAC	1.13	21.66	74.56	2.65	79.54	5.86	0.08	14.52

Scanning Electron Microscopy (SEM) images play a crucial role in unravelling the structural of AC. These high-resolution images provide a detailed visualization of the surface morphology, porosity, and particle size distribution of AC. Understanding these structural aspects is paramount as they directly impact the AC's adsorption efficiency, surface area, and overall performance. SEM images of the precursor and TSAC are illustrated in Figure 2(a) and (b), respectively. As portrayed in Figure 2(a), the exterior of the precursor appeared dense and lacking in pores, consistent with expectations given the presence of components such as lignin, cellulose, hemicellulose, and tar. In contrast, the surface of TSAC in Figure 2(b) displayed enhanced porosity and showcased numerous vacant holes. According to Bakather (2024), these well-developed porous structure provide numerous surface area which facilitated the diffusion of adsorbate into the adsorption sites. These sites were initially filled with volatile matter and moisture components, which evaporated and escaped from the sample during the carbonization and activation process. Research done by Ullah et al. (2024) also mentioned that combined carbonization and physical activation processes does frequently heightened the porosity and surface area of a carbonaceous adsorbent.

FTIR analysis is important to identify the chemical structure of both the precursor and the corresponding activated carbon. Figure 3 presents the FTIR spectrum for raw tea stalk and TSAC. It was observed that the tea stalk displayed diverse functional groups on its surface. The FTIR spectra revealed a broad band at 3315.32 cm^{-1} which corresponds to the intramolecular and intermolecular bending of hydroxyl groups (O-H) from phenolic, alcohol, and carboxylic compounds in the lignin of the precursor [25]. A sharp peak was also observed at 2914.29 cm^{-1} and 2852.59 cm^{-1} represented the C-H asymmetric and symmetric stretch of methylene [26,27]. The peak at 1729.27 cm^{-1} denotes the vibration of C=O stretching in carbonyl, and aliphatic groups [25]. Furthermore, the band at 1595.34 c cm^{-1} is attributed to the olefinic C=C functional group presence in raw tea stalk [28]. The peak at 1426.06 cm^{-1} was assigned to the aromatic vibrations of C-H in the lignin's chain [29]. The small peak at 1241.73 cm^{-1} is a sign of the existence of C-O bonds in the tea stalk structure [30]. Lastly, a sharp peak was observed at 1022.27 cm^{-1} due to the stretching vibration of C-O-C from the ether group in the precursor [31]. On the other hand, the surface chemistry of TSAC has lost most of its peaks due to the thermal decomposition during the carbonization and activation processes. Peaks associated with the hydroxyl

group, C=O stretching, C-H aromatic vibration, and ether group were observed in the TSAC spectra with low intensity and shifted at 3422.72 cm^{-1}, 1632.78 cm^{-1}, 1468.83 cm^{-1}, 1084.88 cm^{-1}, respectively [30, 32].

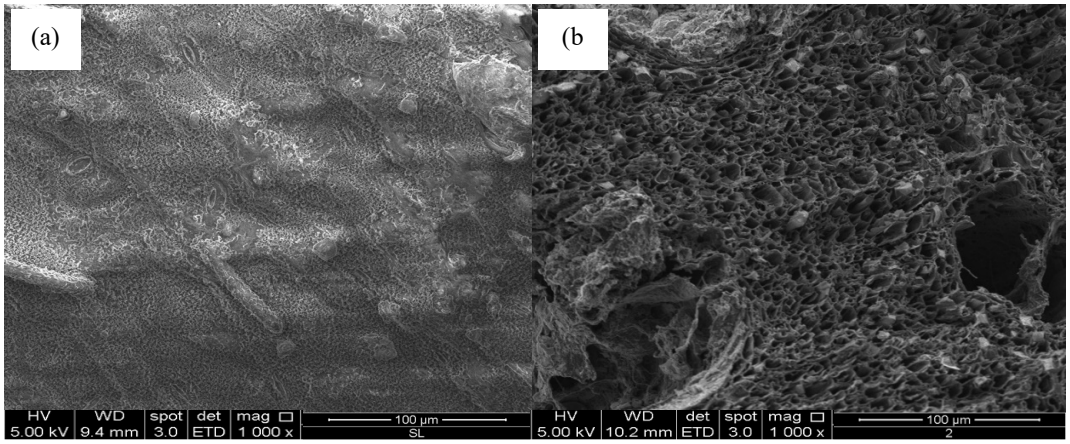


Figure 2. SEM images of (a) precursor (b) TSAC (1000x magnificent level)

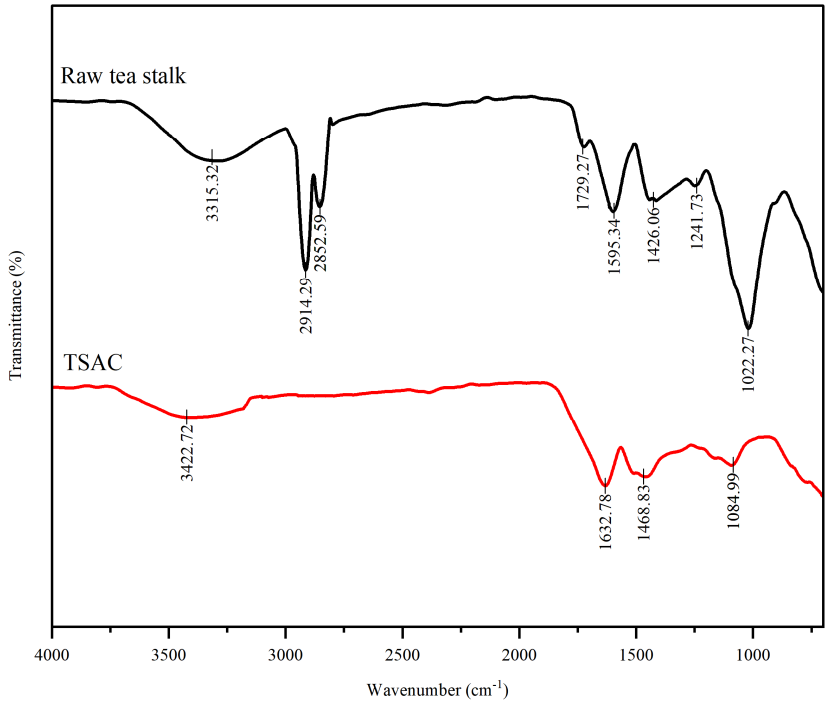


Figure 3. FTIR spectrum of raw tea stalk and TSAC

TGA analysis of the tea stalk and TSAC was reported in Figure 4. The TGA profile displayed a of 9.08 wt.% for the raw tea stalk at temperature below 170°C. The decline was expected due to evaporation process, which removes moisture and water content from the sample [33]. The weight of the tea stalk continues to drop for 59.42 wt.% from 230°C to 550°C, a temperature that may be associated with the thermal decomposition of the biomass's hemicellulose, cellulose, and lignin [34]. Meanwhile, no substantial mass loss was seen above 550°C, signaling a limited degradation of the carbonaceous material in the sample. This is significant

since it shows that the temperature is appropriate for the synthesis of activated carbon. Nonetheless, the TGA curve profile for TSAC exhibits a small weight drop of 18 wt.%, proving that the carbonization and activation procedure was successful in producing thermally stable activated carbon [35].

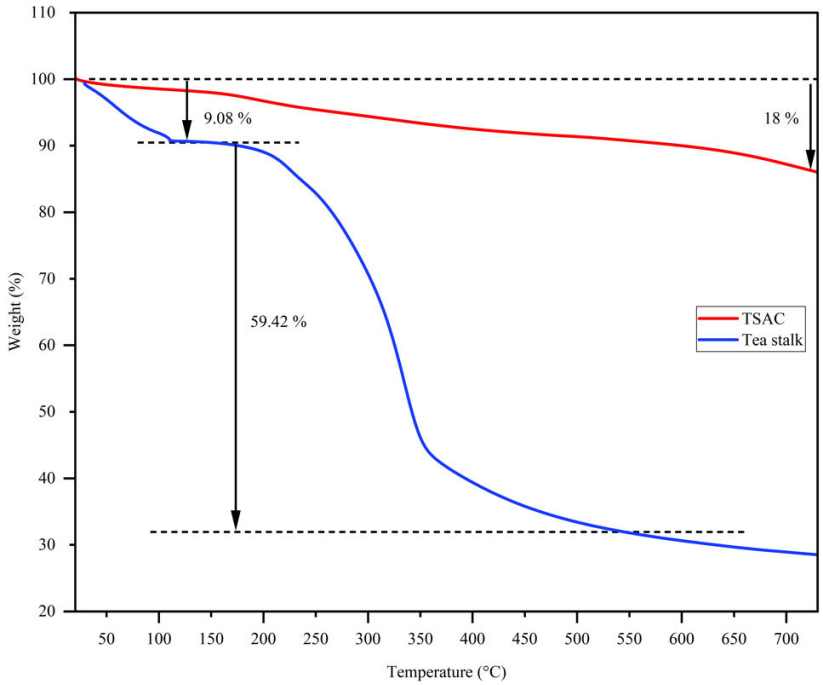


Figure 4. TGA curves for tea stalk and TSAC

Adsorption equilibrium: Effect of BPS initial concentration, contact time, pH, and solution temperature

Figure 5 point out the BPS adsorption uptakes by TSAC at different concentrations and contact time. According to Figure 5, as the original concentration of BPS escalated from 5 to 50 mg/L, the adsorption uptakes of BPS rose from 4.99 to 35.79 mg/g. Meanwhile the percentage removal of BPS was found to be declined from 99.80% to 71.58%, as the initial concentration of BPS increased from 5 to 50 mg/L. This pattern suggests that at greater original concentrations of BPS, there is an increased quantity of molecules within the solution, leading to an increased capacity for BPS adsorption by TSAC. Additionally, at higher initial concentrations, a greater mass transfer driving force is generated, overcoming the mass transfer opposition amongst adsorbate molecules in the bulk (BPS solution) and solid (TSAC's surface) phases [36]. For the study of contact time, it can be seen in Figure 5, where the BPS uptake increased with the increase in contact time up to the point where a constant value was reached (after 3 hour). This indicates that the adsorption capacity of the BPS onto the TSAC had reached its maximum limits and equilibrium. This limit is based on the number of vacant active sites available, as shown in BET and SEM analyses. In the beginning, many active sites were available for adsorption. As time progresses, the number of vacant pores is reduced, and repulsion occurs between the adsorbed molecules and adsorbates in bulk solution, therefore slowing the adsorption process until saturation is reached. Similar trends were observed in the adsorption of BPS on commercial powdered activated carbon by Couto et al. [37].

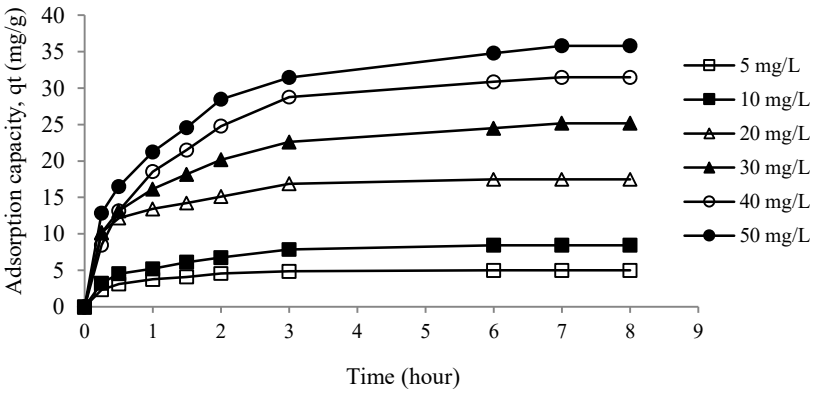


Figure 5. Adsorption uptakes of BPS at different initial concentration and contact time

In the adsorption process, pH plays an important factor that could affect adsorption uptakes. The surface charge of the adsorbent and BPS forms in an aqueous solution are dependent on the pH of the solution. Figure 6 illustrates the adsorption uptake of BPS from an aqueous solution under the influence of pH. According to Figure 6, at pH < 7, the TSAC revealed a high adsorption capacity in the range of 36 mg/g to 36.4 mg/g. However, as the pH of the solution increased, the adsorption capacity decreased significantly to 25.3 mg/g. With the increase in pH solution, deprotonation of functional groups found in previous FTIR analysis such as carboxylic and phenolic occurs [38]. The deprotonation decreases the content of hydrogen donor in the TSAC for the formation of hydrogen bonding with the BPS, therefore decreasing the adsorption capacity at high pH solutions. Moreover, at pH > 10, deprotonation of BPS also occurs and causes repulsion between the anionic species of BPS and the negatively charged adsorbent surface, thereby further decreasing the adsorption capacity [37]. In terms of the study of different temperatures, it was found that as the solution temperature rose from 30 to 60 °C, the adsorption uptake of BPS increased from 35.79 to 41.25 mg/g. This suggests that BPS's adsorption process is more favorable at higher solution temperatures, indicating an endothermic nature.

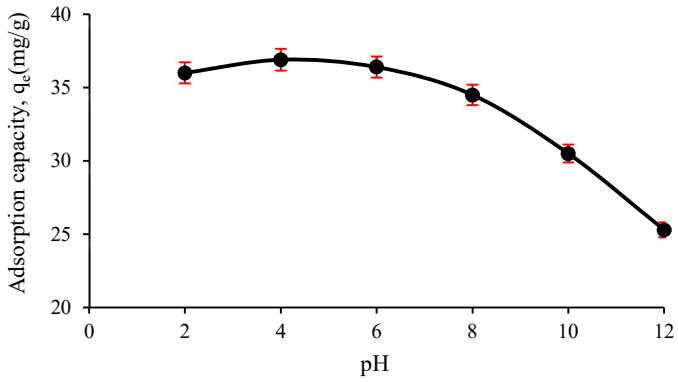


Figure 6. Adsorption uptakes of BPS by TSAC at different pH solution

Adsorption isotherm

Isotherm studies in adsorption are of paramount importance as they provide a comprehensive understanding of the relationship between the amount of adsorbate and its concentration on the adsorbent's exterior surface. By plotting adsorption isotherms, which depict this equilibrium relationship under specific conditions, researchers gain insights into the adsorption mechanism, capacity, and surface characteristics of the adsorbent material**.** Table 2 offers an overview of the isotherm parameters for the BPS-TSAC adsorption system at 30 °C. The data indicates that the Langmuir model closely aligns with the observed behavior, as evidenced by the lowest RMSE of 2.46. This suggests that BPS molecules form a monolayer coverage on the TSAC surface. The heterogeneity factor, denoted as n_F, was determined to be 2.14 for BPS-TSAC. Falling within the range of 1 to 10, this value indicates that the studied adsorption system is considered favorable.

Table 2. Isotherm parameters for BPS-TSAC adsorption system at 30 °C

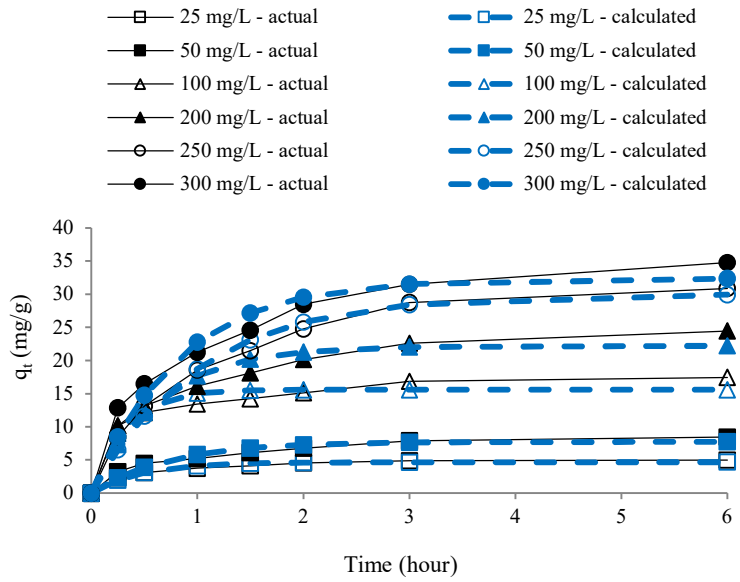
Langmuir		**Freundlich**		**Temkin**	
Q_m (mg/g)	50.27	n_F	2.14	B (L/mg)	3.87
K_L (L/mg)	0.19	K_F (mg/g)(L/mg)$^{1/n}$	10.82	A (L/mg)	119.71
RMSE	2.46	RMSE	2.92	RMSE	6.46

Adsorption kinetic and mechanism study

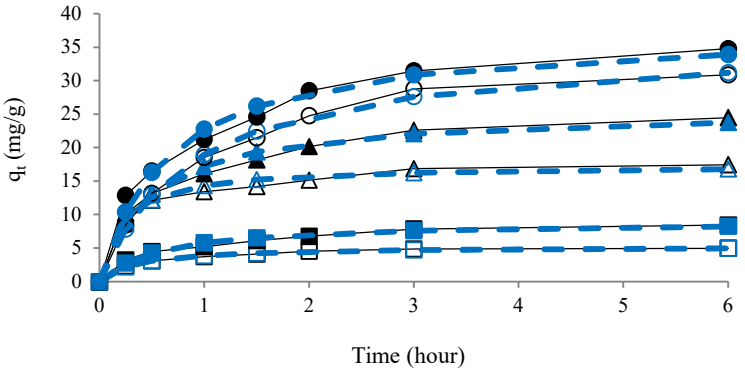
Adsorption kinetics play a crucial role in understanding and optimizing adsorption processes, offering valuable insights into the rate at which adsorption occurs over time. By studying the kinetics of adsorption, researchers can discern the mechanisms and pathways involved in the interplay between the substance being adsorbed and the material serving as the adsorbent. Figures 7(a) and (b) showcase the kinetic plots for PFO and PSO in the BPS-TSAC system, respectively. Table 3 presents a summary of the kinetic parameters. The PSO kinetic model proved to be the best fit for the adsorption system, supported by the lowest RMSE of 0.72. Additionally, the PSO kinetic model was identified as the most suitable for other adsorption systems. With the original concentration of BPS rising from 5 to 50 mg/L, the reaction rate (k_2) decreased from 0.5534 g mg^{-1} min^{-1} to 0.0408 g mg^{-1} min^{-1}. This decline can be ascribed to the higher abundance of BPS molecules in the solution at elevated initial concentrations. The increased concentration heightened the competition among these molecules for adsorption by TSAC, intensifying the competition and ultimately leading to a lower reaction rate [7,8]. The analysis of Boyd plot indicates that film diffusion serves as the controlling factor in the rate-limiting stages for all the examined adsorption systems. This inference is drawn from the observation that these plots do not intersect at the origin.

Conclusions

Effectively produced through physical activation using CO_2 as the gasification agent, TSAC displayed an impressive ability for BPS removal, achieving a supreme adsorption capacity (Q_m) of 50.27 mg/g. The TSAC showcased notable features, including a high BET surface area (712.49 m^2/g), mesopores surface area (514.71 m^2/g), total pore volume (0.25 cm^3/g), and fixed carbon content (74.56%). The porosity of TSAC was also supported by the SEM images where it displayed a highly porous structure compared to dense and compact surface of raw tea stalk precursor. In the equilibrium study, an elevation in the initial concentration of BPS starting 5 to 50 mg/L resulted in higher adsorption uptakes but a decrease in percentage removal. The optimum BPS removal occurred at a solution temperature of 60 °C (41.25 mg/g). The adsorption data for BPS-TSAC exhibited a good fit with the Langmuir isotherm model, and the PSO kinetic model proved to be the most effective. Analysis of the rate-determining step through Boyd plots revealed that film diffusion played a crucial role. Overall, the produced TSAC displayed favourable characteristics, establishing it as an excellent adsorbent for treating BPS-containing wastewater.



(a)



(b)

Figure 7. Kinetic plots for (a) PFO and (b) PSO for BPS-TSAC adsorption system at 30 °C

Table 3. Kinetic parameters for BPS-TSAC adsorption system at 30 °C

Initial Adsorbates Concentration (mg/L)		q_e, exp (mg/g)	Pseudo-First Order			Pseudo-Second Order		
			q_e, cal (mg/g)	k_1 (min^{-1})	RMSE	q_e, cal (mg/g)	k_2 (g mg^{-1} min^{-1})	RMSE
BPS	5	4.99	4.67	2.15	0.31	5.25	0.5534	0.13
	10	8.44	7.74	1.39	0.70	9.00	0.1987	0.39
	20	17.46	15.59	3.44	1.38	17.38	0.2705	0.74
	30	25.15	22.21	1.60	1.92	25.66	0.0799	0.97
	40	31.46	30.02	0.98	1.34	35.80	0.0314	0.70
	50	35.79	32.38	1.22	2.52	37.57	0.0408	1.41
	Average				1.36			0.72

Acknowledgements

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UNVEILING ZN/NI/CO TERNARY MIXED TRANSITION METAL OXIDES COMPOSITE ANCHORED ON GRAPHENE OXIDE AS A POTENTIAL MATERIAL FOR SUPERCAPACITOR ELECTRODE

(Menyingkap Potensi Komposit Logam Peralihan Zn/Ni/Co yang Terikat pada Grafin Oksida sebagai Elektrod Superkapasitor)

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Abstract

Ternary metal oxides continue to draw noteworthy research interest among the energy storage society owing to their remarkable attributes such as upstanding storage capability and cost-effectiveness. Despite that, reduced electrical conductivity and capacity instability tend to hamper the applicability of ternary metal oxides. This work reports a feasible approach to enhance the supercapacitive potential of ternary metal oxide (Zn-Ni-CoO) by incorporating graphene oxide (GO) conductive network. The structural, morphology, and functional groups were deduced via XRD, FESEM, EDS elemental mapping, FTIR, and BET analysis. The metal-carbon hybrid shows astounding specific capacitance value of 608 Fg^{-1} at 5 mVs^{-1}, calculated from three-electrode cyclic voltammetry analysis, with 2 M KOH electrolyte. Significantly, the material is able to preserve 93% of its initial capacitance even after 1000 cycles. This performance is ascribed to the synergistic effect generated by the electric double layer capacitance (EDLC) properties of GO and pseudocapacitance behavior originated from Zn-Ni-CoO. Based on the research findings, Zn-Ni-Co/GO nanocomposite could serve as a favorable active material for supercapacitor electrode.

Keywords: zinc-nickel-cobalt oxide, graphene oxide, electrochemical performance, specific capacitance, supercapacitor

Abstrak

Gabungan tiga elemen logam oksida telah menarik minat para komuniti penyelidik dalam bidang penyimpanan tenaga kerana sifatnya yang luar biasa seperti kemampuan penyimpanan yang tinggi serta keberkesanan kos. Walaupun begitu, kekonduksian elektrik yang berkurang dan ketidakstabilan kapasiti cenderung menghalang penggunaan oksida logam terner. Karya ini melaporkan pendekatan yang wajar untuk meningkatkan potensi superkapasitor logam oksida terner (Zn-Ni-Co O) dengan menggabungkan rangkaian konduktif grafin oksida (GO). Sifat struktur, morfologi, dan fungsional bagi bahan ini disimpulkan melalui analisis XRD, FESEM, EDS-elemen pemetaan, FTIR, dan BET. Hibrid logam-karbon menunjukkan nilai muatan kapasiti spesifik yang setinggi 608 Fg^{-1} pada 5 mVs^{-1}, yang dikira dari analisis voltammetri siklik tiga-elektrod, dengan elektrolit 2 M KOH. Secara keseluruhannya, bahan ini dapat mengekalkan 93% kapasitansi awalnya walaupun selepas 1000 kitaran. Prestasi ini disebabkan oleh kesan sinergi yang dihasilkan oleh sifat kapasitansi lapisan dua elektrik (EDLC) yang datang daripada GO dan

kapasitan yang berasal dari Zn-Ni-Co O. Berdasarkan penemuan penyelidikan, Nanokomposit Zn-Ni-Co / GO boleh berfungsi sebagai bahan aktif yang baik untuk elektrod superkapasitor.

Kata kunci: zink-nikel-kobalt oksida, grafin oksida, prestasi elektrokimia, muatan spesifik, superkapasitor

Introduction

Transition metal oxide (TMO) nanoparticles exhibit distinctive characteristics in their chemical, thermal, optical, magnetic, and electrical properties, which differ from their bulk counterparts. TMO and their composites have been employed in a wide range of disciplines, including photocatalysis [1], sensors [2], and energy storage [3], [4]. The remarkable features of nanoparticles are governed by factors such as their size, structure, morphology, as well as the method of their production. When it comes to morphological aspects, the synthesis method of a nanoparticle plays a significant role in controlling its overall structure. There are great deal of methods that have been previously reported in preparing the hybrids of metal oxides, for instance hydrothermal [5] , solvothermal [6], combustion [7], co-precipitation [8], templated synthesis [9], microwave assisted [10], and sol-gel [11, 12]. Typically, Chen et al. synthesized an urchin-like $ZnCo_2O_4$ via a two-step synthetic route comprising of solvothermal and annealing process [13]. The resulting $ZnCo_2O_4$ are constructed from numerous porous nanorods, with diameters ranging from 80 to 200 nm. These microspheres exhibit a specific surface area of 28 $m^2 g^{-1}$. By altering the volume ratios of water to ethanol, it is possible to effectively manipulate the shapes of the resulting $ZnCo_2O_4$ samples. Meanwhile, Gao et al. implemented a facile hydrothermal method with subsequent calcination procedure to synthesis $NiCo_2O_4$ [14]. Consequently, an enhanced performance of micron-scale $NiCo_2O_4$ with distinct hollow fibrous structure was obtained. This improvement can be attributed to several factors, including a larger specific surface area, increased pore volume, and shorter diffusion path for both electrolyte ions and electrons. In another work conducted by Wei et al. sol-gel route was utilized to prepare $ZnCo_2O_4$ [15]. The obtained particle size was reported to range from 15-20 nm, slightly lower than the value documented in [13]. The synthesis of composite materials comprising mixed TMOs anchored on GO for supercapacitor electrodes has faced numerous obstacles in terms of analytical performance in prior research endeavors. Conventional methods like hydrothermal synthesis and direct chemical precipitation commonly lead to inadequate dispersion and aggregation of metal oxides on the surface of GO [16]. This leads to compromised electrochemical performance and limited charge transfer kinetics. Moreover, these methods often lack precise control over the composition and morphology of the TMO, potentially affecting the specific capacitance and cycling stability of the electrode material.

Specifically in energy storage implementation, TMO often plays an important role as it exhibits a remarkable capacitance. However, despite its promising energy density, TMO encounters obstacles such as low conductivity, uncontrolled volumetric changes, and sluggish ion diffusion within the bulk phase, which then impedes its practical and industrial utility. In general, the desirable characteristics for electrode materials in supercapacitors include excellent conductivity, a high specific surface area, notable thermal stability, significant mechanical strength, and high capacitance [17]. Consequently, there is a necessity to explore functional metal oxides with improved electrochemical properties for supercapacitor (SC) applications. Engineering strategies targeting the composition, fabrication, electroconductivity, and oxygen vacancies of metal oxide nanomaterials have successfully bolstered their inherent properties, encompassing enhancements in conductivity, surface area, stability, and active sites, among other factors. TMO primarily possesses a distinctive characteristic of hosting multiple distinct cations within a single crystalline structure. This coexistence of cations results in a higher number of available electrons compared to metal oxides composed of a single element, thus leading to improved electrical properties. For instance, spinel oxides such as $ZnCo_2O_4$ exhibits a conductivity that is two to three orders of magnitude greater than that of single Co_3O_4 constituent [18]. Simultaneously, carbon-accolades are commonly regarded as superior active materials for supercapacitors. Integration of TMO and carbons are proven to help prevent or minimize severe agglomeration and restacking of the hybrid structure

ensembles. As a result, a larger electrochemically active surface area becomes available, ultimately leading to enhanced electrochemical performance [19].

In this specific work, an effective facile synthesis route of Zn/Ni/Co (ZNC) ternary MTMO anchored on GO has been successfully developed. This technique offers numerous advantages compared to traditional methods. Firstly, it provides superior control over the composition and morphology of the ternary metal oxides by finely adjusting precursor ratios and reaction conditions. Secondly, the sol-gel process ensures the even dispersion and anchorage of metal oxides onto the graphene oxide surface, fostering efficient charge transfer and enhanced electrochemical performance. Furthermore, the co-precipitation stage facilitates the creation of mixed metal oxides with precisely defined crystal structures and heightened synergistic interactions among various metal components, potentially resulting in increased specific capacitance and superior cycling stability. To the best of our knowledge, there are no studies documenting the utilization of ternary MTMO comprising Zn/Ni/Co mixed with GO as active material for supercapacitor electrode. Hence, it is of great importance to study the effect of GO incorporation into the Zn/Ni/Co ternary MTMO.

Materials & Methods

Materials

All the chemicals utilized were used without any further modification. Co $(NO_3)_2 \cdot 6H_2O$, Zn $(NO_3)_2 \cdot 6H_2O$, Ni $(NO_3)_2 \cdot 6H_2O$ (98%) and oxalic acid (99%) were purchased from sigma Aldrich, graphene oxide powder from China Beiihai Building Material Co. LTD, whereas potassium hydroxide (85%) from R&M chemicals.

Preparation of Zn/Ni/Co Oxide-GO

In brief, appropriate amounts of starting materials consisted of Co $(NO_3)_2 \cdot 6H_2O$, Zn $(NO_3)_2 \cdot 6H_2O$, and Ni $(NO_3)_2 \cdot 6H_2O$ were dissolved separately in deionized water. Once the reagents were solvated, an appropriate amount of oxalic acid was added under continuous magnetic stirring at 80 ˚C up until the mixture turned into a gel-like substance. Further, the resulting gel was heated at 110 ˚C to ensure the formation of powder before subsequently being dried and ground. The sample was calcined at 350 ˚C for 3 h afterwards with a heating rate of 5 ˚C/min to acquire the desired ternary metal oxides composites. The procured Zn/Ni/Co Oxide sample (ZNC) was then used further for GO compositing.

Adequate amounts of ZNC powder were dissolved in deionized water and stirred for 30 minutes. In a separate beaker, GO was also dissolved in deionized water and sonicated for 30 minutes to achieve a homogenous solution. Both solutions were then poured together into one beaker and subjected to 24 hours stirring process. Ultimately, the resulting precipitates were gathered through centrifugation, subjected to multiple rinses with deionized water and ethanol, and then dried at 80 °C for a duration of 24 hours to yield the final products.

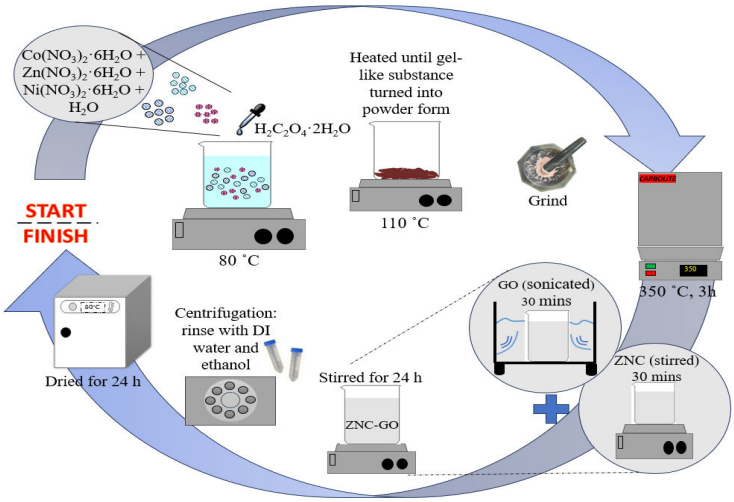


Figure 1. Methodology flowchart

Characterization

The compositions of the as-prepared samples were characterized using X-ray diffraction (XRD), whilst the morphologies and the microstructures of the samples were investigated by imagining with field-emission scanning electron microscope (FESEM) and energy dispersive spectroscopy (EDS) mapping analysis. The presence of chemical bonding was confirmed by Fourier transform infra-red (FTIR) spectroscopy in the range of 450 cm^{-1} – 4000 cm^{-1}. The specific surface area (SSA), pore width, and pore volume was deduced by Brunauer–Emmett–Teller (BET) and Barrett-Joyner-Halenda (BJH) measurements whereas the electrochemical measurements were conducted via a three-electrode system operated by an Autolab PGSTAT204N potentiostat. The setup was using a glassy carbon electrode (GCE) with drop-casted sample on its tip as the working electrode. Ag/AgCl served as the reference electrode while platinum wire was used as the counter electrode, with 2 M KOH as the electrolyte. Cyclic voltammetry was performed over the potential range of 0 – 0.6 V at various scan rates ranging from 20 – 100 mVs^{-1}.

Results and Discussion

X-ray diffraction analysis

XRD analysis was carried out to study the phase structure and the sample's degree of crystallinity. Evident from Figure 2, both ZNC and ZNC-GO samples display a very similar pattern, suggesting that the incorporation of GO does not significantly alter the bulk crystal structure or phase composition of ZNC. The diffraction peaks can be ascribed to the crystallographic data JCPDS no 03-065-3103, with no excrescent peaks detected. The findings indicate a complete conversion of all precursors into pure spinel ternary Zn/Ni/Co O and Zn/Ni/Co-GO nanocomposites. The peak positions are listed in Table 1, with eight apparent peaks indexed to (111), (220), (311), (400), (422), (511), (440), and (533) diffraction planes. Also, a weak diffraction peak at 2θ = 11.09˚ corresponds to (001) diffraction plane due to a very small amount of GO addition and the presence of oxygen functional group [20]. The weak and almost unnoticeable peak is also inferring to the random orientation nature of GO, and high loading mass of ZNC [21]. The resemblance in peak positions seen on both ZNC and ZNC-GO with the stick pattern implies that both samples retain a similar spinel cubic structure. The crystallite sizes and lattice constant can be deduced by implementing Debye Scherrer formulation (Eq.1) and Eq.2, respectively.

$$D = \frac{k\lambda}{\beta cos cos\ \theta} \quad (1)$$

$$a = D\ (h^2 + k^2 + l^2\ )^{\frac{1}{2}} \quad (2)$$

In which D is the crystallite size (nm), k is the Scherrer constant, λ represents the wavelength of the x-ray source, β symbolizes the full width at half maximum (FWHM) of the diffraction peak, θ is the peak positions, and hkl refers to the miller indices. The specific FWHM, crystallite size, and cell parameters of ZNC and ZNC-GO are detailed in Table 2. Based on the displayed data, it is evident that the crystallite size and lattice parameter obtained at the most intense peak (311) for ZNC-GO are slightly below ZNC. The energy storage capacity is significantly influenced by the size and dimension of the crystallites, hence reflecting the material's level of electrical conductivity

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Table 1. The peak positions in XRD patterns of samples

Samples	(111)	(220)	(311)	(400)	(422)	(511)	(440)	(533)
ZNC	19.16	31.33	36.89	44.60	55.39	59.18	65.00	77.18
ZNC-GO	19.23	31.40	37.07	44.83	55.57	59.52	65.18	77.54
JCPDS 03-065-3103	19.07	31.38	36.98	44.97	55.87	59.58	65.49	77.66

Table 2. The FWHM, estimated crystallite size, and cell parameter of ZNC and ZNC-GO using XRD spectra

Samples	(311) Peak Position, 2θ (°)	FWHM (Rad.)	Crystallite Size, d (nm)	Cell Parameter a=b=c, (Å)	Cell Parameter a=b=c, (Å) from Literature
ZNC	36.89	0.79952	10.48	8.063	8.107 [3]
ZNC-GO	37.07	1.33929	6.26	8.043	-

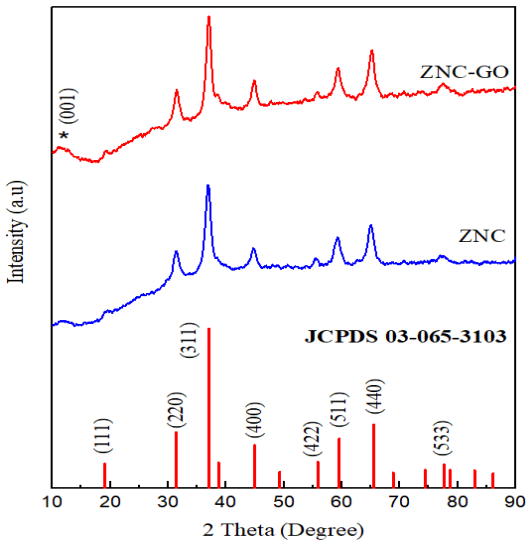


Figure 2. XRD patterns of ZNC and ZNC-GO

Field emission scanning electron microscopy

The morphology of ZNC and ZNC-GO were investigated via FESEM. The image obtained shows heterogenous morphologies considering the hybrid nature of the samples. ZNC micrograph in Figure 3 (a) displays a mixture of petal-like, and sphere-like shapes in various sizing clustered together. These three different elements show different identity shapes, each corresponding to Zn, Ni, and Co elements. Meanwhile, the FESEM image of sample with GO in Figure 3 (b) shows an interesting, wrinkled cabbage-like shape which is believed to be the GO sheet, with fine particles clusters anchored on its surface indicating the attachment of ZNC. The cabbage-like morphology is in good agreement with the FESEM GO-based nanocomposite result documented by Iranshahi & Mosivand [22]. It is also evident that the spherical particles in ZNC are more agglomerated compared to ZNC-GO. These are probably because TMO nanoparticles have high surface energy and attractive forces between particles, which causes them to clump together. When GO is added into the TMO matrices, it acts as a spacer that prevents direct contact between nanoparticles, thus reducing agglomeration. Figure 3 (c) shows the energy dispersive spectroscopy (EDS) mapping analysis, disclosing a congruent distribution of Zn, Ni, Co, O, and C throughout the ZNC-GO structure. This implies a successful synthesis route of ZNC-GO.

Fourier transform infrared

FTIR analysis provides a valuable insight into the functional groups and intermolecular interactions by examining the stretching or bending vibrations of specific bonds. Figure 4 presents the FTIR spectra of ZNC and ZNC-GO with the magnified version presented on the inset. The ZNC-GO retains the same band spectra exhibited by ZNC with a slight shift, owing to their shared MTMO composition with just a dash of GO added. The sharp bands which appear at 648 cm^{-1} and 553 cm^{-1} for ZNC sample whilst at 655 cm^{-1} and 550 cm^{-1} for ZNC-GO are corresponding to metal-oxygen bond [23]. For ZNC, the band arises at 1323 cm^{-1} and 1611 cm^{-1} representing the organic and water molecules, as well as hydroxide ions [6]. The FTIR spectra shown by ZNC-GO displays more distinctive peaks compared to ZNC. The band appears at around 1300 cm^{-1} is

ascribable to the presence of C-O [24]. At ~3800 cm^{-1}, the band is formed due to the stretching vibrations of hydroxyl group [25]. Weak signal at 2100 cm^{-1} is probable to the aliphatic C-H stretching [26]. Furthermore, the IR band appears in 1708 is correlated with the stretching vibration of sp^2 hybridized C=C [27].

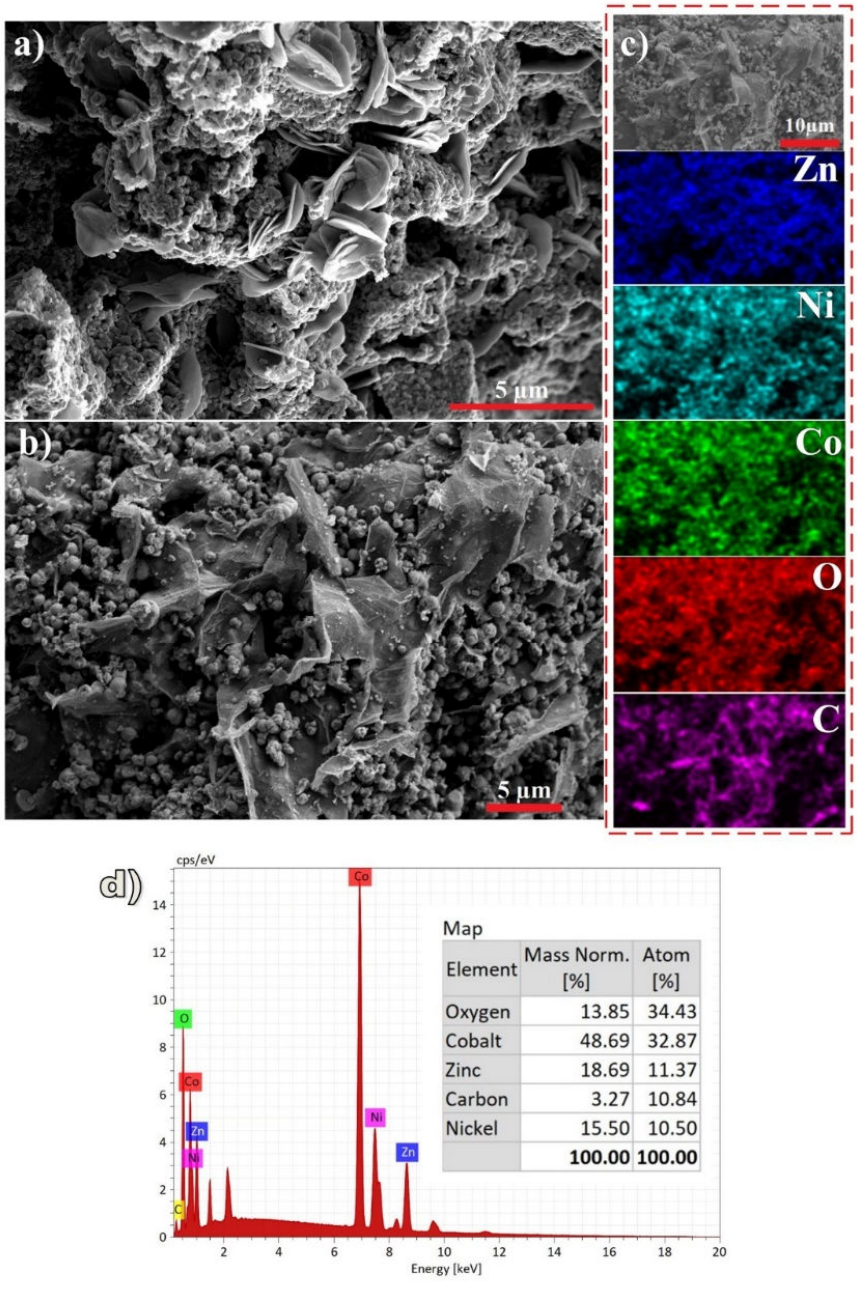


Figure 3. FESEM images of (a) ZNC, (b) ZNC-GO, (c) elemental mapping of ZNC-GO, and (d) EDS elemental peak of ZNC-GO

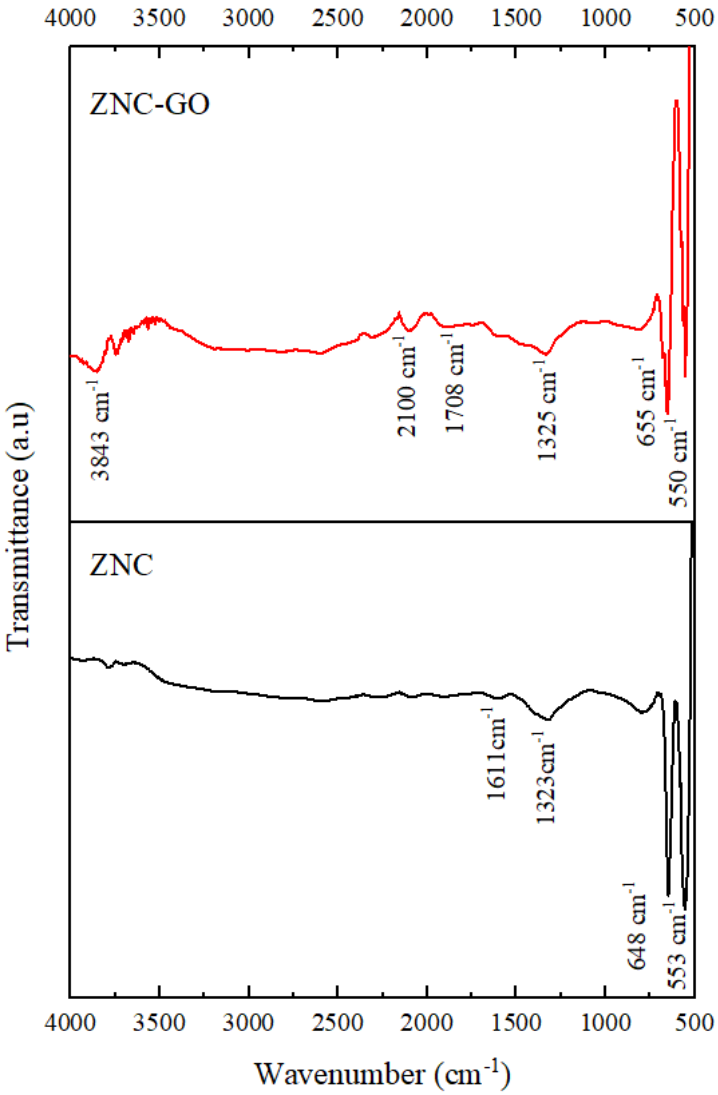


Figure 4. FTIR spectra of ZNC and ZNC-GO

Brunauer–Emmett–Teller

Figure 5 portrays the results of adsorption–desorption measurements, which were conducted to examine the specific surface area (SSA) and pore structure of ZNC-GO sample. The obtained nitrogen adsorption and desorption isotherm verifies the high porosity properties of the prepared metal-carbon nanocomposite. The resulting SSA of Zn/Ni/Co-Go is revealed to be 55.92 m^2 g^{-1}, comparable to the value reported in [28]. The high SSA value facilitates a significant amount of electrochemical active sites and enhances ion–electron interaction, hence leads to an improved electrochemical performance [29]. As displayed in figure 5, a distinct hysteresis loop resembling H3 type is evident, ranging from 0.39 – 1.0 relative pressure (p/p_o). Furthermore, the pore size distribution of ZNC-GO was explored by means of Barrett-Joyner-Halenda (BJH) method, as shown in the inset of Figure 5. The average pore diameter of ZNC anchored on GO sheets was observed from 2 – 6 nm, implying its mesoporous feature. Additionally, according to the measurements, the obtained pore volume for ZNC-GO is 0.1375 cm^3 g^{-1}. The obtained pore volume is slightly higher than the value reported in [30], which also happened to study the material's supercapacitive performance.

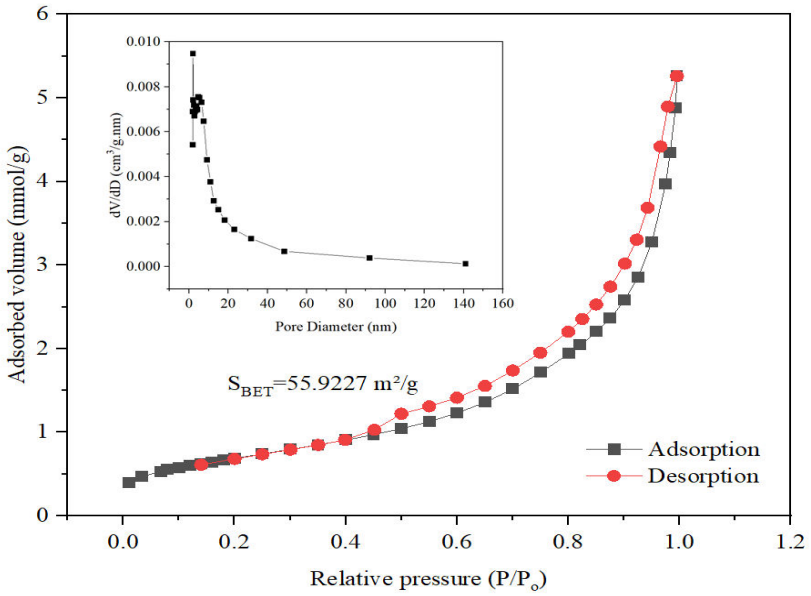


Figure 5. Nitrogen adsorption-desorption isotherm of ZNC-GO and the corresponding pore diameter distribution (inset)

Cyclic voltammetry

CV analysis was employed to analyze the specific capacitance (C_s) value of ZNC and ZNC-GO hybrid. The CV scans were recorded at numerous scan rates ranging from 5 to 100 mVs^{-1}, within the potential window of 0.0 to 0.6 V. Figure 6 (a) displays the CV curves comparison of ZNC and ZNC-GO samples at 50 mVs^{-1}. It is evident that ZNC-GO exhibits higher current response, as well as greater area under the CV curve compared to ZNC. ZNC curve shows a pair of prominent redox peaks, indicating its pseudocapacitive behavior [31]. With the inclusion of GO, vague appearance of redox peaks is witnessed. The broadening of redox peak demonstrates a significant increase of electron transfer and ion diffusion [32]. In Figure 6 (b), the voltammogram shows a progressive size as the scan rates increased. The highest current response can be seen on 100 mVs^{-1} curve while the lowest shown by 5 mVs^{-1}. The graphs show a stagnant shape at all scan rates, implying good electrochemical reversibility [33]. The C_s values were obtained by means of Eq 3.

$$C_s = \frac{\int_{v_a}^{v_c} IV(dV)}{vm\,(V_c - V_a)} \qquad (3)$$

Based on the equation, C_s is the specific capacitance presented in the unit of Fg^{-1}. V_c and V_a indicate the potential window in volts, I(V) demonstrate the response current (A), v is the potential scan rates (Vs^{-1}), and m is the mass of sample drop-casted on the tip of the electrode (g). Upon calculation, the C_s value obtained for ZNC is 113 Fg^{-1} and 318 Fg^{-1} for ZNC-GO at 50 mVs^{-1}, with nearly threefold increment. Figure 6 (c) shows the calculated C_s trend for ZNC-GO sample at various scan rates. Contrast to the progressive pattern of CV curves displays on Figure 6 (b), the value calculated shows a declining trend as the scan rates increase, which is also depicted in Table 3. This pattern was instigated by a complete permeation of electrolyte ions at lower scan rates. Yet, the movement of electrolyte ions is somewhat restricted at higher scan rates due to time limitations [34]. The stability of the ZNC-GO sample was assessed by continuously running CV tests for 1000 cycles, as can be seen on figure 6 d). Based on the obtained data, ZNC-GO retains 93% of its initial C_s value, indicating excellent electrochemical stability.

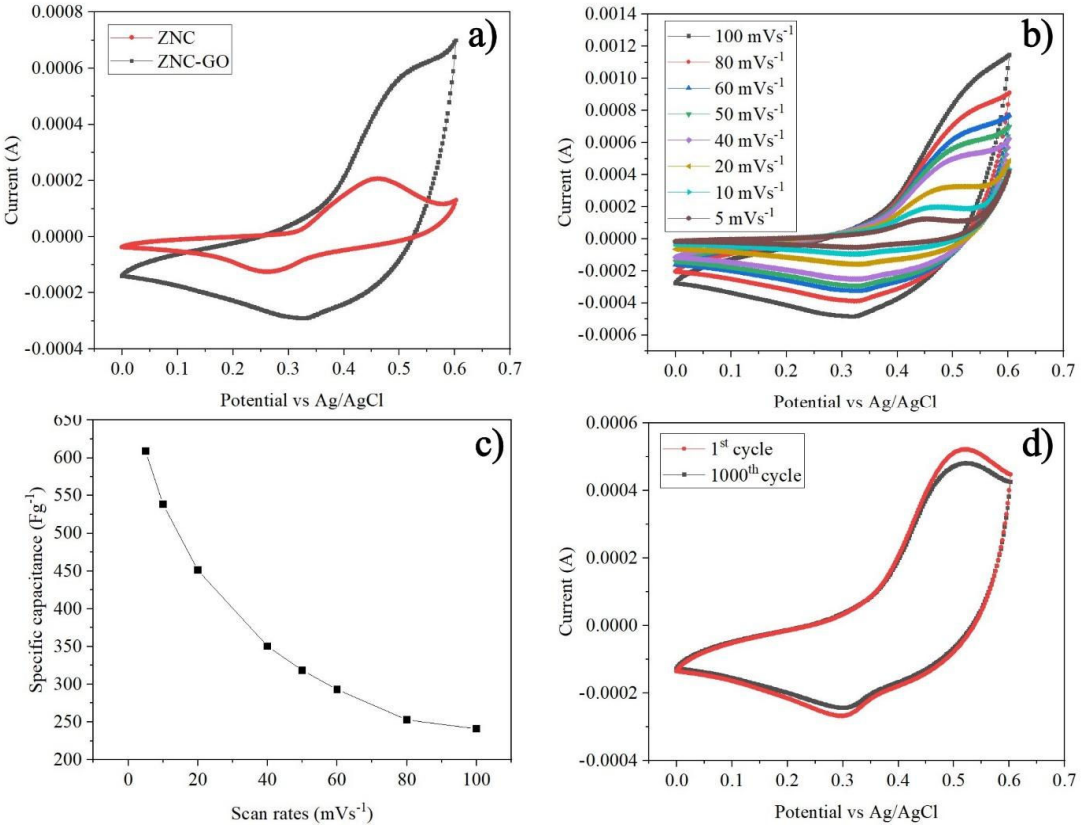


Figure 6. (a) CV curves of ZNC and ZNC-GO at 50 mVs^{-1}, (b) CV curves of ZNC-GO samples at various scan rates, and (c) The C_s trend for ZNC-GO at various scan rates, and (d) Stability test of ZNC-GO at 100 mVs^{-1}

Table 3. Specific capacitance of ZNC, ZNC-GO obtained in this work and other reports in previous works

Samples	Specific Capacitance, Cs (Fg^{-1})	Scan Rate or Current Density	Electrolyte	Reported by Literature
ZNC	113.76	50 mVs^{-1}		
ZNC-GO	240.92	100 mVs^{-1}		
ZNC-GO	252.90	80 mVs^{-1}		
ZNC-GO	292.90	60 mVs^{-1}		
ZNC-GO	318.38	50 mVs^{-1}	1 M KOH	This work
ZNC-GO	350.25	40 mVs^{-1}		
ZNC-GO	450.92	20 mVs^{-1}		
ZNC-GO	538.34	10 mVs^{-1}		
ZNC-GO	**608.86**	5 mVs^{-1}		
Graphene-$NiCo_2O_4$	266.00	2 mVs^{-1}	2 M KOH	[35]
$MgCo_2O_4$/graphene	189.00	5 mVs^{-1}	3 M KOH	[36]
GO/Chitosan/ZnO	137.70	0.5 Ag^{-1}	NA	[37]
Zn-Ni-Co/graphene	502.00	0.2 Ag^{-1}	2 M KOH	[38]

$NiCo_2O_4$ – nickel cobaltite
$MgCo_2O_4$ – magnesium cobaltite
ZnO – zinc oxide

Galvanostatic charge/discharge

Galvanostatic charge-discharge (GCD) is a reliable and efficient method to determine and evaluate the electrochemical capacitance performance of materials. A comparison of GCD curves at 10 Ag^{-1} for ZNC and ZNC-GO can be seen on Figure 7. It should be noted that ZNC-GO displays longer discharge time compared to ZNC, which also indicates higher C_s value [39]. Another vital parameter to determine the practical performance of supercapacitor material are energy density, E (Wh/kg) and power density, P (W/kg). Eq (4) and (5) indicate the equations for energy density and power density, respectively:

$$E = \frac{1}{2} CV^2 \quad (4)$$

$$P = \frac{E}{\Delta t} \quad (5)$$

At the current density of 10 Ag^{-1}, the energy density of ZNC was calculated to be 7.92 Wh/kg at a power density of 208.91 W/kg. The energy density was raised to 11.98 Wh/kg with power density of 382.67 W/kg once GO was incorporated into the ZNC MTMO composites. The GCD curves exhibit a distinct plateau region for every current density, along with a nearly symmetric shape. This behavior suggests that the electrode undergoes a reversible Faradaic redox reaction, demonstrating an ultralow energy loss or an exceptionally high coulombic efficiency. Furthermore, the symmetric nature of the GCD curves indicates ideal pseudocapacitive behavior, which is a desirable characteristic for high-performance energy storage devices [40]. This result is also in good agreement with the CV profile obtained in the previous section. The enhanced electrochemical performance of ZNC-GO can be attributed to the synergistic effect arising from the combination of ZNC ternary MTMO and GO sheet. This synergistic effect increases the number of available oxidation states and shortens the pathways for ion and electron transport. Moreover, the porous and high surface area nature of the composite material, facilitated by GO, provides abundant active sites for redox reactions and ion adsorption/desorption processes. These observations suggest that the ZNC-GO sample can be regarded as a potential electrode material for supercapacitors.

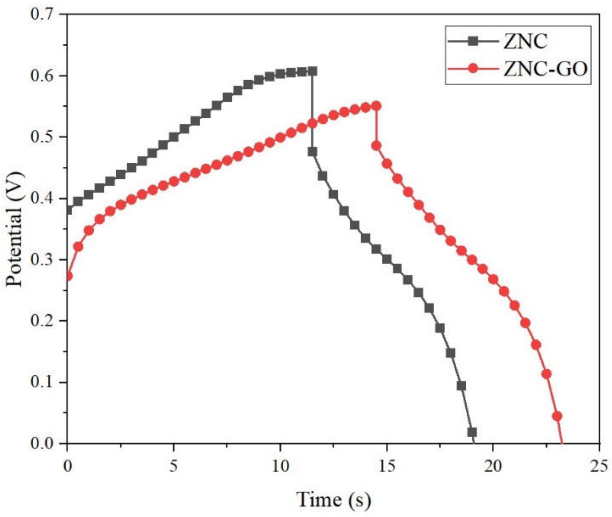


Figure 7. GCD curves of ZNC and ZNC-GO at 10 Ag^{-1}.

Conclusion

In summary, ZNC-GO with heterogenous morphology of flake-lake and sphere like small particles anchored on the cabbage-like sheet was successfully prepared via a facile sol-gel annealing synthesis route. Based on Scherrer's formulation, the estimated crystallite size of ZNC (10.48 nm) became smaller (6.26 nm) after GO addition, with SSA value as high as 55.92 m^2 g^{-1}. The synergistic interaction between high surface area GO and high theoretical capacities ZNC facilitates a significant amount of electrochemical active sites thus enhances ion–electron interaction. Subsequently,

through the integration of GO into the ZNC matrices, the C_s value intensified three-fold from 113 Fg^{-1} to 318 Fg^{-1} at 50 mVs^{-1}, with highest C_s value of 608 Fg^{-1} obtained at a scan rate of 5 mVs^{-1}. The marked improvement of the GO-hybrid performance is also supported by the promising energy and power density enhancement. Ultimately, our work suggests that ZNC-GO is a propitious and favorable material for supercapacitor electrode.

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PHYTOCHEMICAL, DPPH AND BIOASSAY STUDY OF *Mariposa christia vespertilionis* PLANT: A SHORT REVIEW

(Fitokimia, DPPH dan Kajian Bio Asai Terhadap Pokok *Mariposa christia vespertilionis*: Satu Ulasan Ringkas)

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Abstract

Mariposa christia vespertilionis (MCV), colloquially referred to as butterfly wing or Mariposa, has garnered attention in both traditional and modern medicine due to its purported therapeutic properties. This review aimed to consolidate existing knowledge regarding the phytochemical composition, antioxidant potential evaluated through DPPH assay, and bioassay studies of MCV. The plant, widely distributed across China and Southeast Asia, has been traditionally employed for various ailments, including cancer and malaria, with anecdotal evidence supporting its efficacy. In Malaysia, the usage of the green-leafed form of this plant has gained popularity because of customer testimonies about the effectiveness of the plant's water decoction in "curing cancer disease". However, the scientific validation of its medicinal claims against cancer remained unexplored. Current research has identified phytoconstituents such as alkaloids, flavonoids, and quinones in MCV, correlating with its reported pharmacological activities. This review underscores the need for comprehensive research to elucidate the chemical composition and biological activities of MCV, thus providing a foundation for future investigations and potential therapeutic applications.

Keywords: *Mariposa christia vespertilionis*, alkaloids, flavonoids, quinones, cytotoxicity

Abstrak

Mariposa christia vespertilionis (MCV), secara umumnya dirujuk sebagai sayap rama-rama atau Mariposa, telah menarik perhatian dalam perubatan tradisional dan moden disebabkan oleh sifat terapeutik yang diperkatakan. Kajian ini bertujuan untuk mengumpulkan pengetahuan sedia ada mengenai komposisi fitokimia, potensi antioksidan yang dinilai melalui ujian DPPH, dan kajian bioassay MCV. Tumbuhan ini, yang tersebar luas di China dan Asia Tenggara, telah digunakan secara tradisional untuk pelbagai penyakit, termasuk kanser dan malaria, dengan bukti anekdot menyokong keberkesanannya. Di Malaysia, penggunaan bentuk daun hijau tumbuhan ini telah mendapat populariti disebabkan oleh kesaksian pelanggan tentang keberkesanan dekoksi air tumbuhan ini dalam "mengubati penyakit kanser". Walau bagaimanapun, pengesahan saintifik terhadap dakwaan perubatan terhadap kanser masih belum dikaji. Kajian semasa telah mengenal pasti fitokimia seperti alkaloid, flavonoid, dan kuinon dalam MCV, yang berkaitan dengan aktiviti farmakologi yang dilaporkan. Ulasan ini menekankan keperluan penyelidikan menyeluruh untuk menghuraikan komposisi kimia dan aktiviti biologi MCV, seterusnya dapat menyediakan landasan asas untuk penyelidikan masa depan dan aplikasi terapeutik yang berpotensi.

Kata kunci: *Mariposa christia vespertilionis*, alkaloid, falanoid, kuinon, kesitotoksikan

Introduction

There has been a strong bond between plants and humans since ancient times. Plants have been used for decoration, as the primary source of food, and even for therapeutic purposes [1]. Though there is a vast advancement in the field of synthetic medicine, a large number of people as well as researchers throughout the world still rely on herbal medicines [2]. Plant consist of various phytoconstituents that has multiple pharmacological functions, due to which the demand for plant-based or plant-derived drugs has been increasing day by day [3]. Plants have been used as traditional medicine in Indian subcontinent as Ayurvedic medicine, in New Zealand as te rongoā Māori, in China as traditional Chinese medicine, in Australia as Aboriginal medicine, and several other parts of the world [4].

Mariposa christia vespertilionis (MCV) is one of the herbs that have been broadly used in traditional medicine practice [5]. The plant species originated in tropical Southeast Asia and is distributed particularly in Thailand, Vietnam, Cambodia, Indonesia, Malaysia, and Myanmar [6]. The MCV plant is found in two varieties: red-leafed (RMCV) and green-leafed (GMCV) (Figure 1). In Malaysia, GMCV is currently well-known for traditional medicine while the RMCV is another type of plant that is abundantly available. The MCV is an ornamental herb, commonly known as butterfly wing plant due to its vibrant features and unique shape. It is locally referred to as “Rerama” in Malay [7]. The plant can grow up to 60 - 120 cm tall and has 3 leaflets with purplish red leaves, red stripes, and green leaves [8, 9]. Apart from decorative usage, it has been documented in traditional medicine as a treatment for a variety of illnesses and injuries, including tuberculosis, bronchitis, cold, scabies, poor blood circulation, snake bites, bone fractures, and muscular weakness [10, 11]. Several studies have shown that the plant is, anti-inflammatory, antiproliferative, cytotoxic, antimalarial, antidiabetic, and antioxidant [5, 12-16]. The dried leaves can be used to make tea drinks. Recently, in Malaysia, the usage of the green-leafed form of this plant has gained popularity because of customer testimonies about the effectiveness of the plant's water decoction in "curing cancer disease”. There are several in vitro studies exploring the plant extract’s anticancer effects [7, 17-19]. The Bunong people in the Mondulkiri province of Northeast Cambodia used MCV in several methods, the most common way is by crushing the plant with water and smearing it as topical medicine [13].

Over time, numerous researchers have delved into the pharmacological characteristics of this plant, conducting both in vitro and in vivo experiments to unveil its potential applications in contemporary medicine [1]. Despite its widespread usage; little is known about the phytochemical components of this plant's leaf metabolome and the bioassay studies that have been carried out. This review will provide knowledge and information on the chemicals and biological activities of this plant that would be beneficial for future research. Thus, given the medical significance of the plant leaves and the importance of quality control, whether for product manufacturing or regulatory purposes, a better understanding of the leaf metabolome is a logical and critical extension of the research and development activities that should be conducted on this plant [20]. Therefore, in this study, the detailed literature that revealed the use of MCV leaf extract in phytochemical study will be known such as, phenolic content, DPPH, and bioassay.



Figure 1. Morphology of leaf of (a) RMCV (b) GMCV [6, 7]

Sample extraction

Prior to the phytochemical analysis sample extraction was carried out using diverse solvents like (Water (Aq), Ethanol, Methanol (M), Ethyl acetate: Methanol (EM), Hexane (H), Hexane: Ethyl acetate (HE), and Ethyl acetate (E)) [5-7, 15, 21, 22]. For example, Zambari et al. [21] extracted a total of 1g of dried powdered sample of each type of MCV leaves with 200 mL of solvent (ethanol) using the Soxhlet apparatus for 8 h which was then filtered and evaporated using a rotary evaporator. The dry weight was determined from 1 mL of the plant extract and the extraction yield of each extract was calculated by following the formula for extraction yield.

$$\text{Extraction yield (\%)} = (m^2/m^1) \times 100 \qquad (1)$$

Where m^1 is the mass of dry weight of MCV leaf (g); and m^2 is the mass of crude extract of MCV leaf (g).

In a recent study by Yasin and his co-workers [16] exhibited that usage of different solvent polarity lead to variation in the yield of extraction, with a tendency toward EM > M > HE > H > E. Nawaz et al. [23] stated that the extraction yield and the antioxidant activity of phenolic compounds in plant material is remarkably affected by the solvent polarity. The polarity-dependent increase in extraction yield, antioxidant activity, reducing properties and free radical scavenging activity perhaps due to the high affinity of antioxidant compounds towards more polar solvents as compared to nonpolar ones. It is also reported that the phytochemical compounds extracted in polar solvents are pharmaceutically more important due to comparatively higher values of antioxidant activity, reducing properties and free radical scavenging activity [23]. However, the higher values of TPC and TFC in non-polar solvents could be assigned to the non-polar nature of phenolic compounds. Govardhan et al. [24] highlighted the importance of the solvent used to extract phytochemical compounds in producing a high yield of phytochemical compounds. Apart from that, the extraction method also influences the analytical quality of phytochemical compounds. Hence selecting a proper solvent for the extraction process plays a key role in providing better results.

Phytochemical Study

The metabolites present in the plant are generally classified as primary and secondary metabolites [25]. Primary metabolites include carbohydrates, fatty acids, amino acids, and similar compounds which support the plant's growth [26]. In contrast, secondary metabolites such as alkaloids, flavonoids, and terpenoids impart specific characteristics to plants, like coloration or defense against predators [27, 28]. The MCV plant has been gaining tremendous attention because of its therapeutic usage (e.g. anticancer, antimalarial, anti-inflammatory, antidiabetic etc.) [5, 7]. The medicinal plants are known to be fascinating source of novel drugs which are the key ingredients in traditional medicine, pharmaceutical intermediates, modern medicines, and prime compounds in synthetic drugs. The actual reason behind their use as medicine is that they hold chemical components with therapeutic properties that contain defense mechanism and guard against diverse illnesses. Usually, the medicinal value of plants lies in the secondary metabolites which leads to definite physiological action in the human body [29]. Hence, there is a need to determine the phytochemical constituents especially, secondary metabolites present in

the plant. Therefore, in the present study, we focused on reviewing the phytochemical studies that has been done against the MCV plant. Phytochemical studies can be carried out as qualitative and quantitative determination.

Qualitative phytochemical analysis

The preliminary phytochemical analysis of the medicinal plant extract by using different types of solvents can be carried out by using the standard protocols available to identify the presence and absence of metabolites such as flavonoid (ammonia test), Alkaloid (Mayer's test), saponin (foam test), tannin ($FeCl_3$ test), cardenolide (sodium picrate test), cardiac glycoside (Keller-Kiliani test), phenolic ($FeCl_3$ test), terpenoid (Salkowski test), steroid (Libermann-Burchard test) etc. [21, 30, 31]. From Table 1 it can be deduced that MCV leaf contains secondary metabolites like alkaloids, flavonoids, proteins, glycosides, tannins, phenols, saponins, diterpenes, coumarins, steroids, and quinones. Based on studies conducted by Lee et al. [22] the H extract of MCV did not include any phenol or tannins. Only traces of flavonoids were found in HE and E extracts, as well as M extracts. However, moderate levels of flavonoids were present in the H and EM extracts. The presence of coumarin was moderate in all the extracts, except for the M extract, which had a low abundance of the compound. The MCV plant extracts from the E, EM, and M had measurable amounts of tannins. On the other hand, there were none observed in H and HE extracts. Meanwhile, the glycoside concentration of the M extract was the highest, followed by E and EM extracts and the lowest are H and HE extracts [22].

From Table 1, it can be highlighted that the M extract comprises maximum number of phytochemical compounds followed by E extract and ethyl acetate: methanol EM. It is noteworthy that flavonoids are found in the MCV leaf extract derived from all types of solvents and saponins are found in aqueous extract. On other hand, it should be noticed that highest number of phytochemicals are found in methanolic extract although, chemical constituents like carbohydrates, amino-acids, anthraquinone, Phytosterols, resins, saponins, and phlobatannins have not been identified through this extract. The phytochemical screening results for aqueous MCV leaf extracts from the previous study show similar results for the similar extract used in the phytochemical test [5-7, 22]. Simple phytochemical screening can only expose the chemical class of the compound in a broad sense. However, further advancement in the qualitative phytochemical analysis include identification and separation of the compounds from different MCV extracts using high end techniques like TLC, HPLC, LCMS, and GCMS (Table 2) [29]. Additionally, the compound identification with exact chemical structure can be elucidated using techniques like UV, IR, ^{1}H-, ^{13}C-NMR, and MS [15]. The previous findings related to the phytochemical constituents of MCV plant extracts along with their role in therapeutic application are shown in Table 2. [32].

Table 1. Preliminary phytochemical screening of MCV extract using different solvents

	Water (Aq)	**Ethanol**	**Methanol (M)**	**Ethyl acetate: Methanol (EM)**	**Hexane (H)**	**Hexane: Ethyl acetate (HE)**	**Ethyl acetate (E)**
Alkaloids	+	+	+	NA	NA	NA	NA
Flavonoids	+	+	+	+	+	+	+
Carbohydrates	-	NA	-	NA	NA	NA	NA
Proteins	NA	NA	+	NA	NA	NA	NA
Amino acids	NA	NA	-	NA	NA	NA	NA
Glycosides	NA	+	+	+	+	+	+
Tannins	-	+	+	+	-	-	+
Phenols	+	+	+	+	-	+	+
Saponins	+	+	-	NA	NA	NA	NA
Anthraquinone	NA	NA	-	NA	NA	NA	NA
Phytosterol	NA	NA	-	NA	NA	NA	NA
Resin	NA	NA	-	NA	NA	NA	NA
Diterpene	NA	NA	+	NA	NA	NA	NA

	Water (Aq)	Ethanol	Methanol (M)	Ethyl acetate: Methanol (EM)	Hexane (H)	Hexane: Ethyl acetate (HE)	Ethyl acetate (E)
Coumarins	NA	NA	+	+	+	+	+
Quinone	NA	NA	+	NA	NA	NA	+
Steroids	-	+	+	NA	NA	NA	NA
Phlobatannins	-	NA	NA	NA	NA	NA	NA
Terpenoid	NA	-	NA	NA	NA	NA	NA
Cardenolide	NA	+	NA	NA	NA	NA	NA

(+) = presence of phyto-constituents in MCV; (−) = no phyto-constituents in MCV; (NA)= data not available

Table 2. Phytochemical compounds found in MCV plant and their activity

Chemical Class	Name of the Compound	Chemical Formula	Analytical Technique	Source/ Solvent	Activity	Ref.
Alkene hydrocarbon	1-octadecene	$C_{18}H_{36}$	GCMS	RMCV (leaf)/ ethanol	Antibacterial, antioxidant, anticancer	[32]
aliphatic ether alcohol	4-O-methylmannose	$C_7H_{14}O_6$		RMCV (leaf)/ ethanol and methanol	Antibacterial	[32]
Phenolic ester	Phenol, 3,5-bis(1,1-dimethyl ethyl)	$C_{14}H_{22}O$	GCMS	RMCV (leaf)/methanol	Antiseptic, disinfectant, flavoring, antibacterial	[32]
Carboxylic acid ester	Acetic acid, butyl ester	$C_{16}H_{12}O_2$	GCMS	RMCV (leaf)/ methanol	Antifungal, Antitumor	[32, 33]
	1-butanol, 3-methyl-, acetate	$C_7H_{14}O_2$			Antimicrobial	
Fatty acid	n-hexadecanoic acid	$C_{16}H_{32}O_2$	GCMS	RMCV (leaf)/ ethanol and methanol GMCV (leaf)/ ethanol	Anti-inflammatory, antibacterial, antioxidant, and antiandrogenic	[21, 32]
Polyunsaturated omega-6 fatty acid	Linoleate	$C_{18}H_{31}O_2$	LC-MS	GMCV and RMCV (leaf)/ 5% ethanol	Anti-inflammatory, acne reductive, and skin moisturizer	[9]
	Olivetol	$C_{11}H_{16}O$		RMCV (leaf)/ 5% ethanol	Anti-septic and repellent	

Chemical Class	Name of the Compound	Chemical Formula	Analytical Technique	Source/ Solvent	Activity	Ref.
	17β-estradiol	$C_{18}H_{24}O_2$			Help in the female reproductive tissues' development and maintenance	
	Mycophenolate	$C_{17}H_{19}O_6$			Immunosuppressant used to prevent rejection in organ transplantation	
Flavonoid	Quercetin	$C_{15}H_{10}O_7$	LC-MS/MS	MCV (root)/ ethyl acetate	Anticancer, Antidiabetic, antioxidant	[22, 34]
	Kaempferol	$C_{20}H_{18}O_6$				
	Sternbin	$C_{16}H_{14}O_6$			-	
	Kaempferol	$C_{15}H_{10}O_6$			-	
	Kuchecarpins C 8-C-Prenyl	$C_{17}H_{16}O_7$			-	
	Kuwanon L	$C_{35}H_{30}O_{11}$			-	
	3,4-Dihydro-4-(40-hydroxyphenyl) -5,7-dihydroxy coumarin	$C_{15}H_{12}O_5$			-	
Flavonoid glycosides	quercetin-3-O-glucoside	-	NMR, ESI-MS, Hyphenated LC-PDA-MS, HRMS	RMCV (root, stem and leaf)/ ethyl acetate	Antioxidant, antidiabetic	[15]
	catechin-3-O-ß-D-glucopyranoside	-		RMCV (root, stem and leaf)/n-butanol		
Isoflavonoids	2'-hydroxygenestin	-	NMR, ESI-MS, Hyphenated LC-PDA-MS, HRMS	RMCV (Root, stem, and leaf)/ ethyl acetate	Immunosuppressive, antiinflammatory, anticancer	[15, 35]
	Orobol	-				
	2,3-dihydro-2'-hydroxygenestin	-				
Quinones	Fallacinol	$C_{16}H_{12}O_6$	LC-MS/MS	MCV (root)/ ethyl acetate	-	[22]
	Alizarin	$C_{14}H_8O_4$			-	
	Purpurin	$C_{14}H_8O_5$			-	
	Rhein	$C_{15}H_8O_6$			Anticancer	

Chemical Class	Name of the Compound	Chemical Formula	Analytical Technique	Source/ Solvent	Activity	Ref.
	Denbinobin	$C_{16}H_{12}O_5$			Anticancer	
Coumarins	5,7-Dihydroxy chromone	$C_9H_6O_4$	LC-MS/MS	MCV (root)/ ethyl acetate	Anticancer	[22]
	Wedelolacetone	$C_{15}H_{12}O_5$	LC-MS/MS	MCV (root)/ ethyl acetate		
Sterols	Stigmasterol	$C_{29}H_{48}O$	UV, IR, ^{1}H-, ^{13}C-NMR, MS, Co-TLC	MCV (root, stem and leaf)/ n-hexane	Antidiabetic, antiinflammatory, and anticancer	[15, 33]
	ß-sitosterol acetate	-				
	ß-sitosterol	-				
Phenolic acids	Sanleng acid	$C_{18}H_{34}O_5$	LC-MS/MS	MCV (root)/ ethyl acetate	Anticancer	[22]
	2'-Hydroxydecanylpentadec-5,8,10,12-tetraenoate (christanoate)	$C_{25}H_{42}O_3$	NMR, ESI-MS, Hyphenated LC-PDA-MS, HRMS	RMCV (root, stem, leaf)/ *n*-hexane	-	[15]
Pentacyclic triterpenes	D:C-friedoolean-8-en-29α-ol	-	UV, IR, ^{1}H-, ^{13}C-NMR, MS	RMCV (root, stem, leaf)/ *n*-hexane	Antiinflammatory, anticancer, antidiabetic, antioxidant, antibacterial effects	[15, 36]
	Ursolic acid methyl ester	-				
	Oleanolic acid methyl ester	-				
	Erythrodiol	-				
Monoterpene	Geraniol	-	UV, IR, ^{1}H-, ^{13}C-NMR, MS, co-TLC	RMCV (root, stem, leaf)/ *n*-hexane	Antimicrobial, antioxidant, anti-inflammatory	[15, 37]
Sesquiterpene	Zerumbone	$C_{15}H_{22}O_1$	LC-MS	GMCV (leaf)/ 5% ethanol RMCV (leaf)/ 5% ethanol	Anticancer particularly leukemia	[9]
	7-isopropylidene-1-methyl-1, 2, 6, 7, 8,	$C_{14}H_{20}$	NMR, ESI-MS, Hyphenated	RMCV (root, stem,	Anti-plasmodial	[15]

Chemical Class	Name of the Compound	Chemical Formula	Analytical Technique	Source/ Solvent	Activity	Ref.
	9-hexahydronaphthalene (christene)		LC-PDA-MS, HRMS	leaf)/ *n*-hexane		
Nonsteroidal anti-inflammatory drug (NSAID)	Ibuprofen	$C_{13}H_{17}O_2$	LC-MS	GMCV (leaf)/ 5% ethanol	Anti-inflammatory	[9]
Others	Nicotinamide	$C_6H_6N_2O_1$	LC-MS	GMCV (leaf)/ 5% ethanol	Vitamin (food supplement)	[9]
	Psoralen	$C_{11}H_6O_3$			Ultraviolet light therapy for eczema, graft-versus-host disease, psoriasis, vitiligo, large-plaque parapsoriasis, mycosis fungoides, and cutaneous T-cell lymphoma	
	phytol	$C_{20}H_{40}O$	GCMS	RMCV and GMCV (leaf)/ ethanol	Anti-cancer, antinociceptive, antioxidant, and antiarthritic	[22]
	9, 12, 15-octadecatrienoic acid, (Z, Z, Z)-	$C_{18}H_{30}O_2$			Anti-inflammatory, cancer preventive, and antiarthritic	
	squalene	$C_{30}H_{50}$			Antioxidant, antitumor, chemopreventive effect, and Anti-inflammatory	

Quantitative phytochemical analysis

Quantification of phytochemical constituents of the MCV plants has been carried out to check the differences in the concentrations of Alkaloids, Flavonoids, Phenol, Saponin, Steroids and Tannins. The total phytochemical content can be quantified in the MCV plant extract prepared using suitable solvent. According to Mokhtar et al. [38], standard anti-inflammatory agents like Gallic acid and quercetin were used as standards with some modifications to the Folin-Ciocalteu method. whereas, tannic acid was used as a standard anti-inflammatory agent according to the method described by Folin-Ciocalteu, slightly altered by Mesfin and Won [39]. Shimadzu UV-1700 spectrophotometer (Tokyo, Japan) was used to measure the absorbance of extracts.

The total phenolic content was identified using the standard gallic acid solution in the dilutions from 0.0-1.0 mg/mL. Nearly 600 µL of Folin's reagent with 1.0 mL of 7.5% (w/v) sodium carbonate (Na_2CO_3) was added into 200 µL of the MCV extract. After the incubating the mixture for 2 h in the dark, the absorbance was measured at 765 nm against the different dilutions of the standard solution which gives the total phenolic content that is expressed in terms of gallic acid in mg GAE/mL of

extract. Figure 1 shows the total phenolic content of MCV in free phenolic, soluble bound, and insoluble bound extracts [40]. According to the results, the insoluble bound extract (4.94 0.042 mg GAE/g sample) contained the most phenolic extracts, followed by soluble bound extracts (3.16 0.28 mg GAE/g sample) and free phenolic extracts (0.98 0.07 mg GAE/g sample). There was a significant difference ($p < 0.05$) between free phenolic and soluble bound extracts, free phenolic and insoluble bound extracts, and between soluble bound extracts and insoluble bound extracts within group [40].

The total flavonoid content was determined by mixing 600 µL of methanol (CH_3OH), 40 µL of 10% (w/v) aluminum chloride ($AlCl_3$), 40 µL of 1 M potassium acetate (CH_3COOK), and 1.12 mL of Milli-Q water with 200 µL of the extract. The mixture was then incubated for 30 minutes at room temperature. Absorbance was measured at 420 nm and compared to a standard solution of quercetin dilutions ranging from 0.0 to 1.0 mg/mL. To estimate the total tannin content, 250 µL of Folin's reagent, 500 µL of 35% (w/v) Na_2CO_3, and 3.75 mL of distilled water were combined with 500 µL of the extract, followed by a 30-minute incubation at room temperature. The absorbance was then measured at 725 nm using tannic acid dilutions (0.0-1.0 mg/mL) as a standard solution. The total tannin content was quantified in terms of tannic acid equivalent (TAE) concentration in mg/mL of extract [21]. Table 3 indicates the total phenolic content, total flavonoid content, and total tannin content by Zambari et al. [21]. From Table 3 it can be noticed that the total phenolic content in ethanolic extracts of GMCV and RMCV were found to be 29.25 ± 0.50^a and 24.16 ± 0.50^a respectively. The total flavonoid content (mg QE/mL) values of ethanolic extract of GMCV was 1.57 ± 0.03^a while for RMCV was 1.31 ± 0.04^a. for respectively which clearly indicate that the flavonoid content in GMCV is higher than RMCV. The total tannin content in mg TAE/mL was found to be 22.70 ± 3.15ns in GMCV and 17.19 ± 0.32ns in RMCV extract. Overall, it can be deduced that in the phytoconstituent values for ethanolic extracts of GMCV were higher than the RMCV extract.

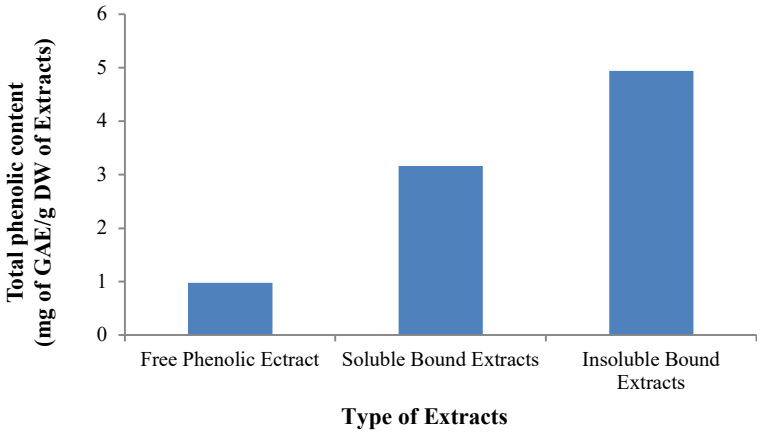


Figure 1. The total phenolic content (TPC) of C. vespertilionis in free phenolic, soluble bound and insoluble bound extracts [40]

Table 3. Total phenolic, flavonoid and tannin content in GMCV and RMCV ethanolic leaf extracts

MCV Leaf Extract	Total Phenolic Content (mg GAE/mL)	Total Flavonoid Content (mg QE/mL)	Total Tannin Content (mg TAE/mL)
Green	29.25 ± 0.50^a	1.57 ± 0.03^a	22.70 ± 3.15^{ns}
Red	24.16 ± 0.50^a	1.31 ± 0.04^a	17.19 ± 0.32^{ns}

All values are triplicate with mean ± SD. a, refer to $p < 0.05$, a significant difference between green and red leaf extract, using a two-way ANOVA test, while ns is a non-significant

Bioassay Studies

2, 2-diphenyl-1-picrylhydrazyl (DPPH) study

Antioxidant activity is often quantified and evaluated using stable DPPH scavenging activity. This method is beneficial over others because it requires less time to reduce the DPPH form to the DPPH non-radical state through hydrogen atom donation [41]. Reduced DPPH can be evaluated by the antioxidants' ability to reduce the absorbance at 517nm. The physical change that occurs during DPPH assessment is the shift in color from purple to yellow [24]. Polyphenols with hydroxyl groups have antioxidant capabilities that scavenge the DPPH radical. The hydroxyl group, specifically, functioned as the main antioxidant. Another screening investigation was conducted using several solvent extracts (hexane, hexane: ethyl acetate, ethyl acetate, and ethyl acetate: methanol), with the ethyl acetate: methanol extract showed the greatest DPPH scavenging activity and the lowest IC_{50} value of 0.549±0.02 mg/mL [5]. Table 4 summaries the antioxidant activity of the MCV extracts and fractions as determined by the DPPH radical scavenging activity based on the IC_{50} values for the DPPH radical scavenging activity [22]. The ethyl acetate root extract and the F6 fraction were found to have the greatest levels of free radical scavenging activity [22]. By comparing the results of the test to previous studies, ascorbic acid (vitamin C) and tocopherol (vitamin E) were used as natural standards to verify the analysis [42].

Table 4. Scavenging activity of C. Vespertilionis crude Extracts, fraction, and standards DPPH free radicals [23]

DPPH IC_{50} (µg/mL)			
	Root Extract		**Leaf Extract**
Hexane	>2000		>2000
Chloroform	338.07 ± 3.32 [b]		679.43 ± 4.72 [a,A]
Ethyl acetate	70.16 ± 1.49 [a]		644.90 ± 20.09 [a,A]
Methanol	421.73 ± 5.40 [c]		716.37 ± 16.46 [a,B]
Ethyl Acetate Root Fraction			
F1	>2000	F5	85.28 ± 1.13 [a]
F2	1786 ± 14.73 [e]	F6	76.71 ± 0.29 [a]
F3	169.63 ± 4.89 [b]	F7	235.63 ± 11.75 [c]
F4	101.53 ± 1.47 [a]	F8	1157.33 ± 22.50 [d]
Standards			
Ascorbic	16.99 ± 1.22	α-tocopherol	10.49 ± 0.98

Antiplasmodial and Antimalerial activity

The *in vitro* anti-plasmodial assays are normally assessed to evaluate the antimalarial activity (Table 5). Upadhyay et al. [15] performed anti-plasmodial assays of MCV root, leaf, and stem extracts (prepared using methanol and aqueous-methanol in 1:4, v/v) against *P. falciparum* (NF-54 strain) that was cultured in human type O-positive red blood cells while the dose response curves prepared from percentage inhibition values versus concentration (Graph Pad Prism (Version 4.0)) revealed the IC_{50} values. It is found out that the aqueous-methanolic (1:4, v/v) extract of the stem was the most powerful [15]. The *in vitro* anti-plasmodial activity exhibited relatively similar TLC profiles even when different extracts were used. Thus, *in vivo* assessment of antimalarial activity of the combined extracts of MCV plant was carried out using *Plasmodium berghei* K173 (sensitive to chloroquine at 10 mg/Kg) [15] that showed suppressed parasitaemia by 87.8%, compared to chloroquine, which suppressed parasitaemia completely on day 8 [15]. Another study by Nguyen-Pouplin et al. [13], MCV plant exhibited anti-plasmodial activity with IC_{50} values ranging from 10 to 20 µg/ml [13].

Malarial SYBR Green I fluorescence-based (MSF) assay is another method was used to determine the antimalarial activity of MCV. MCV methanol extract (CVME) was used in the subsequent antimalarial and anticancer activity assays [16]. NF-54 strains of *P. falciparum* used against CVME for inhibition activity. A graph plotted as the logarithm of CVME concentration versus the percentage of parasite inhibition was generated and the half-maximal inhibitory concentration (IC_{50}) of CVME was determined by using probit

regression analysis with GraphPad Prism software (Version 6) represented in Figure 2. The antimalarial activity of a plant extract can be classified as promising (IC_{50} < 15 µg/mL), moderate (15 < IC_{50} < 50 µg/mL) and inactive activity (IC_{50} > 50 µg/mL). CVME has a moderate antimalarial activity (IC_{50} = 43.87 ± 2.04 µg/mL). Artemisinin as a standard antimalarial drug was potent in inhibited the parasite growth with an IC_{50} value of 4.0 ± 0.22 µg/mL. The methanolic leaf extract has been proven to yield antimalarial activity [16].

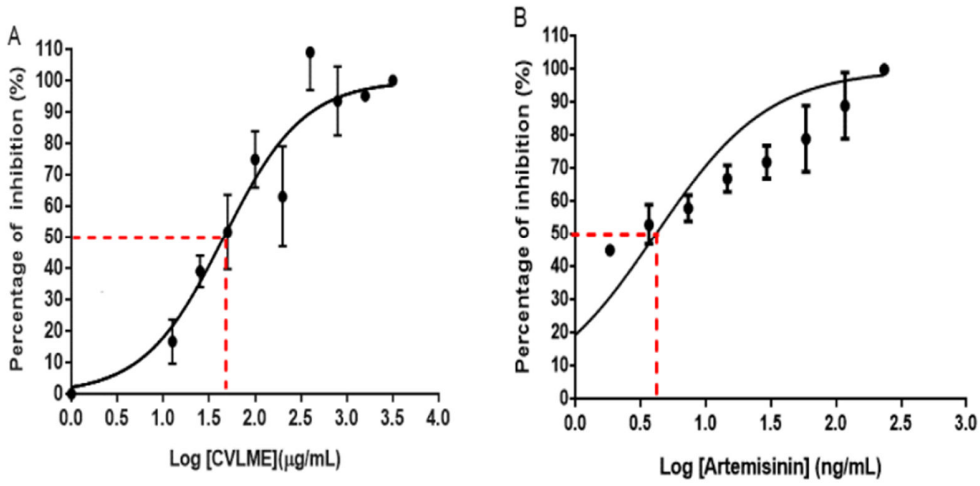


Figure 2. In vitro susceptibility of P. falciparum to (A) CVME and (B) artemisinin [16]

Table 5. Anti-plasmodial activity against NF-54 Plasmodium falciparum strain [15]

SI No.	Extract/compound	IC_{50} *(µg/mL)
1	Methanolic extract of roots (MR)	60.5
2	Methanolic extract of leaf (ML)	32.0
3	Methanolic extract of stem (MS)	62.0
4	Aqueous-methanolic extract of roots (AR)	78.5
5	Aqueous-methanolic extract of leaf (AL)	63.0
6	Aqueous-methanolic extract of stem (AS)	7.5
7	Compound 1	9.0
8	Compound 4	72.0
9	Compound 13	84.0
10	Chloroquine (Standard)	0.024
11	Artemisinin (Standard)	0.0088

Cytotoxicity

As helpful as medicinal plants are, some of them may also show harmful effects depending on the type of toxicant present and its abundance. As a result, it is critical to assess the toxicity of the therapeutic plant extracts being investigated for its pharmacological activities. In a 28-day subacute oral toxicity trial, the researchers used an ethanolic extract of MCV leaf at doses of 75, 125, and 250 mg/kg. Both treated and control rats had no significant changes in serum biochemical and hematological markers. Moreover, histological examination of the kidney and liver revealed no substantial lesions in the kidney tissues of any of the treatment groups. However, mild to severe hepatic necrosis was detected in all three doses, respectively. Apart from that, all three groups showed moderate hepatic deterioration and eventual hepatitis. The plant may include toxicant(s) that contributed to these

observations, or it may contain chemicals that interact antagonistically [16]. A study by Lee et al. [22] suggested that the F3 fraction of the ethyl acetate root extract of MCV consist of flavonoids, quinones, and coumarins that were responsible toward the cytotoxicity specifically against MDA-MB-231 cells, indicating anti-breast cancer activity (Figure 3) [22].

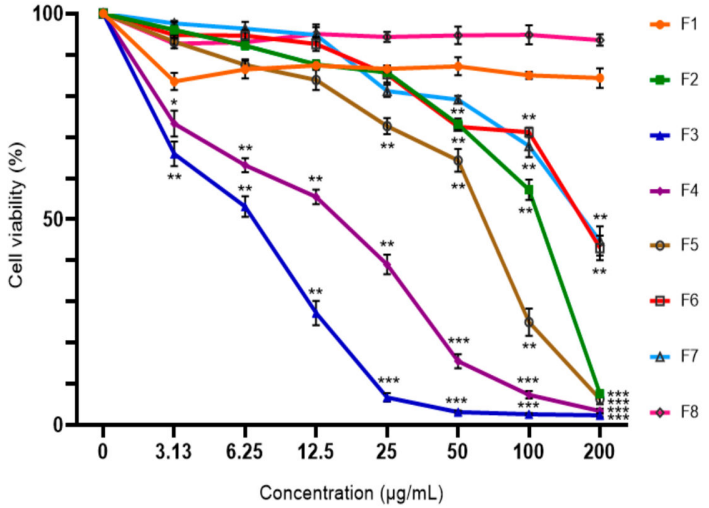


Figure 3. Cytotoxicity of MCV ethyl acetate root fractions (F1–F8) determined using MTT assay on MDA-MB-231 (All values are expressed as mean ± SD and data with asterisks are significantly different at $p < 0.05$ when compared with the untreated group)

Antidiabetic activity

The inhibition of the intestinal enzyme, a-glucosidase will reduce postprandial hyperglycemia which is a common problem for diabetic people. The a-glucosidase inhibitory assay test was adapted from Murugesu et al. and carried out as an improved method [5] (Table 6). Quercetin, a natural a-glucosidase enzyme inhibitor was used as the positive control while PNPG (4-Nitrophenyl-β-D-glucopyranoside) used as the substrate. The linear regression analysis method was used to determine the half maximal inhibition concentration (IC_{50}), in which the concentration of the extract required to inhibit 50% of a-glucosidase activity under the assay conditions was calculated [43]. The HE extracts of MCV shows the highest a-glucosidase inhibitory with IC_{50}: 0.195 mg/mL while M extract expressed the least potential with IC_{50}: 0.416 mg/mL). These results are influenced by the compound present in the extract most probably terpenoids. Outwardly, polarity of solvent at different level may attract various class of compounds that tend to exhibit their distinctive pharmacological properties. Less interference of antagonistic occur between the phytoconstituents in the matrix [5]. Hence, the MCV leaf extracts can be recommended as potential medicine against antidiabetic.

Table 6. AGI IC_{50} value of α-glucosidase inhibitory activity of MCV leaves extracts [5]

SI No.	Extract	AGI IC_{50} (mg/mL) ± SD
1	Hexane (H)	0.201 ± 0.024^{c}
2	Hexane- Ethyl Acetate (HE)	0.195 ± 0.005^{c}
3	Ethyl Acetate (E)	0.211 ± 0.011^{c}
4	Ethyl Acetate- Methanol (EM)	0.370 ± 0.015^{b}
5	Methanol (M)	0.416 ± 0.015^{a}
6	Ascorbic Acid (AA)	nd
7	Quercetin	0.005 ± 0.001^{d}

nd – not detected

Ferric reducing antioxidant activity

The antioxidant activity of the MCV leaf extracts was determined using FRAP (Ferric Reducing Antioxidant Power) assay method adapted Alam et al. [43] with some improvements. The FRAP reagent consist of 0.001M of TPTZ dissolved in 0.004 M HCl, 0.002 M $FeCl_3$ (2.5 mL) and 0.1 M acetate buffer (25 mL) with pH of 3.6, incubated for 10 min at 37 °C [5]. Ascorbic acid was used as the positive control. The highest ferric reducing antioxidant power exhibited by the hexane: ethyl acetate extract. The FRAP value measured to be ranged from 190.60 to 271.67 AAE/g with the HE extracts (271.67 AAE/g) showing the potential ferric reducing antioxidant power. Thus, it can be proved that MCV leaves contain potent antioxidant's ability.

Table 7. FRAP value of MCV leaf extracts [5]

SI No.	Extract	FRAP (AAE/g) ± SEM
1	Hexane (H)	190.60 ± 1.94^{e}
2	Hexane- Ethyl Acetate (HE)	271.67 ± 0.93^{a}
3	Ethyl Acetate (E)	234.05 ± 0.89^{c}
4	Ethyl Acetate- Methanol (EM)	245.55 ± 1.67^{b}
5	Methanol (M)	205.16 ± 1.67^{d}
6	Ascorbic Acid (AA)	194.32 ± 1.39^{e}

Anticancer activity

Research on MCV examines its anticancer potential against various cell lines such as HaCaT (keratinocyte), HepG2 (liver carcinoma), MCF-7 (breast cancer), WRL68 (normal liver), and CRL 2522 (fibroblast). Ethanolic leaf extract showed viability against multiple cell lines, with promising results in combination with cyclophosphamide. Isoorientin, a prominent compound, contributes to its activity, inducing apoptosis and altering cell pathways. Similarly, ethyl acetate and chloroform root extracts demonstrated dose-dependent cytotoxicity against breast cancer cell lines, possibly through different apoptosis mechanisms involving flavonoids and triterpenes. The anticancer potential of MCV root extracts, specifically ethyl acetate and chloroform extracts, was assessed on hormone receptor-positive (MCF-7) and triple-negative (MDA-MB-231) breast adenocarcinoma cell lines. Both extracts exhibited dose-dependent cytotoxic effects, with IC_{50} values of 11.34 ± 1.20 and 29.58 ± 3.80 µg/mL against MDA-MB-231, and 44.65 ± 5.78 and 54.55 ± 9.51 µg/mL against MCF-7, respectively. The differential efficacy observed may stem from varying apoptosis mechanisms attributed to bioactive compounds in the extracts. Flavonoids likely undergo metabolism primarily via the CYP1A1 pathway in MCF-7, while MDA-MB-231 metabolizes them through the CYP1B1 pathway. Additionally, previous research suggests triterpenes induce apoptosis in breast cancer cells through P53-dependent and P53-independent signaling in MCF-7 and MDA-MB-231, respectively. Overall, these findings suggest MCV extracts as potential anticancer agents [34].

Anti-Inflammatory activity

MCV leaf extract has been reported to have phytoconstituents responsible for anti-inflammatory activity. Oyedapo [44] also stated that phenolic compounds, such as flavonoids and tannins, are primarily responsible for anti-inflammatory activity. As protease plays key a role in inflammation and is one of the biological mediators exhibited during inflammation thereby regulating the inflammation complex system via cleaving chemokines, cytokines, and immune components. Therefore, the anti-inflammatory activity can be deduced by correlating the protease inhibition assay with the total phenolic content. According to Zambari et al [21] out of the 2 plant variations of MCV (GMCV and RMCV), GMCV leaf extract has known to contains a greater number of phytoconstituent that lead to suppress inflammation. To test the anti-inflammatory activity the 2 varieties of MCV extract was subjected to *in vitro* protease inhibition activity assay. Table 8 shows the comparative values for GMCV and RMCV against protease inhibition. High protease inhibition value indicates the capability of the substance to treat inflammation. From Table 8, it is clear that the GMCV showed a higher inhibition of 66.55 ± 2.59% and IC50 value of 284.59 ± 10.63 µg/mL, compared to RMCV with 62.63 ± 2.49% inhibition and IC50 value of 226.59

± 10.35 µg/mL. The correlation analysis of the protease inhibition assay with total phenolic content has also revealed positive relationships [21]. Thus, it can be inferred that the phenolic compounds in MCV leaf extract particularly the GMCV extract, contribute to the anti-inflammatory activities.

Table 8. Protease inhibition activity assay in GMCV and RMCV extract with aspirin as a control [21]

Sample	Inhibition (%)	IC_{50} values (µg/mL)
GMCV	66.55 ± 2.59	284.59 ± 10.63[a]
RMCV	62.63 ± 2.49	226.59 ± 10.35[a,b]
Aspirin	75.66 ± 0.99	275.72 ± 6.93[b]

All values are triplicate with mean ± SD. a and b; each refers to p <0.05, a significant difference between different samples, using a two-way ANOVA test

Conclusion

MCV, a commonly available herb in Malaysia is found in 2 varieties namely GMCV and RMCV. Both varieties show comparable beneficial phytoconstituents like alkaloids, flavonoids, proteins, glycosides, tannins, phenols, saponins, diterpenes, coumarins, steroids, and quinones that can be effectively contributed to pharmacological actions. The maximum phytoconstituents were found using methanol followed by ethyl acetate. However, their activity varies based on the solvent utilized for extraction. The MCV plant extract has proven its anticancer, antioxidative, antimalarial, anti-inflammatory, and antidiabetic activities through various bioassays. Although, current review states several therapeutic properties of chemical compounds present in MCV plants, there is a still lot of scope to study several other chemical compounds particularly the flavonoids groups whose pharmacological activities has not been reported. Hence, further studies on this plant can be carried out for the bio-guided fractionation, structural elucidation of the active compounds and need to be extended in every aspect for future pharmaceutical applications.

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CHARACTERIZATION OF CHEMICAL PROFILING, CYTOTOXICITY, AND ANTIMICROBIAL ANALYSES OF CAROB HONEY FROM NORTHERN CYPRUS

(Ciri-Ciri Profil Kimia, Sitotoksik, dan Analisis, Antimikrob Madu Karob Dari Cyprus Utara)

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Abstract

Currently, the preferred options (chemotherapy, radiotherapy, etc.) for the treatment of breast cancer indiscriminately attack healthy cells and cause hair loss. There is also a global concern about the rise of antibiotic resistance by infectious microorganisms. Therefore, searching for an alternative with fewer side effects and improved activity becomes paramount. This study explored the chemical components, cytotoxicity, and antimicrobial potentials of carob honey from Northern Cyprus. Gas chromatography-mass spectrometry was used for the identification of the chemical constituents. Well diffusion assay was used for antimicrobial assessment, and MTT assay was employed to determine the MDA-MB-231 cell viability. A total of 69 compounds, which include hydroxymethyl furfural, quercetin, and furan carboxaldehyde were detected in the honey. At 8000 µg/mL, the zones of inhibition are 13±1.7, 12±1.0, 11±1.7, 10±2.0, 8±1.0, 9±2.6, and 7±1 mm for *S. typhi, E. coli, K. pneumoniae, S. aureus, S. pneumoniae, A. niger* and *A. flavus* respectively. Only *E. coli* and *K. pneumoniae* responded at 2000 µg/mL. Carob honey also suppressed the proliferation of MDA-MB-231 in a concentration and time-dependent manner. At 300 µg/mL, 20 %, 50 % and 55 % of the MDA-MB-231 cells were inhibited after 24, 48 and 72 hours respectively. However, the overall cytotoxic effect of carob honey on the MDA-MB-231 cell line is substantially low.

Keywords: bioactivities, Carob honey, MTT assay, volatile constituents

Abstrak

Pada masa ini, pilihan rawatan yang disukai (kemoterapi, radioterapi, dan lain-lain) untuk rawatan kanser payudara menyerang sel-sel yang sihat tanpa diskriminasi dan menyebabkan keguguran rambut. Terdapat juga kebimbangan global mengenai peningkatan rintangan antibiotik oleh mikroorganisma berjangkit. Oleh itu, mencari alternatif dengan kesan sampingan yang lebih sedikit dan peningkatan aktiviti menjadi penting. Kajian ini meneroka komponen kimia, ketoksikan sitotoksik, dan potensi antimikrobial madu carob dari Cyprus Utara. Kromatografi gas-spektrometri jisim digunakan untuk mengenal pasti konstituen kimia. Ujian difusi telaga digunakan untuk penilaian antimikrobial, dan ujian MTT digunakan untuk menentukan daya hidup sel MDA-MB-231. Sebanyak 69 sebatian, termasuk hidroksimetil furfural, kuersetin, dan furan karboksaldehid telah dikesan dalam madu tersebut. Pada 8000

µg/mL, zon perencatan adalah 13±1.7, 12±1.0, 11±1.7, 10±2.0, 8±1.0, 9±2.6, dan 7±1 mm masing-masing untuk *S. typhi, E. coli, K. pneumoniae, S. aureus, S. pneumoniae, A. niger* dan *A. flavus*. Hanya *E. coli* dan *K. pneumoniae* yang memberi tindak balas pada 2000 µg/mL. Madu carob juga menekan percambahan MDA-MB-231 dengan cara bergantung kepada kepekatan dan masa. Pada 300 µg/mL, 20%, 50% dan 55% sel MDA-MB-231 dihalang selepas 24, 48 dan 72 jam masing-masing. Walau bagaimanapun kesan sitotoksik keseluruhan madu carob terhadap garis sel MDA-MB-231 adalah rendah.

Kata kunci: aktiviti bio, madu carob, ujian MTT, konstituen meruap

Introduction

Honey is a rich liquid substance used for centuries in human nutrition and medicine. It is enriched with water, sugar, minerals, vitamins, enzymes, organic acids, flavonoids, and phenols. These compounds are responsible for the remarkable biological properties of honey [1]. Some of these properties include; antioxidant [2], antibacterial [3], anti-diabetic [4], and anti-inflammatory [5] activities. The antibacterial potential of honey has been largely attributed to its high osmolarity, low pH, production of hydrogen peroxide, and bee defensing-1 formation. Many bacterial and fungal strains are vulnerable to honey [6-9], depending on its type and botanical origin [10]. Additionally, some honey such as Manuka honey and its flavonoids exhibit anti-proliferative activity against MDA-MB-231[11], similar to acacia honey from Malaysia against MCF-7 [12]. In addition, a dose-dependent cytotoxic activity of acacia honey against the MCF-7 cell line was reported by [13].

Over 2.2 million women have been diagnosed with breast cancer in 2020 alone, and it is expected that 2.6 million fresh cases may set in by 2030, which is quite alarming [14]. Triple-negative breast cancer (TNBC) accounts for 10-20% of all the breast cancers diagnosed in women, it lacks the expression of estrogen receptor (ER-), progesterone receptor (PR-), and human epidermal growth factor receptor (HER2) [14-16]. The absence of these receptors makes TNBC non-responsive to hormonal treatment and difficult to diagnose [17, 18]. MDA-MB-231 is a TNBC cell line used by researchers as a good model for studying potential breast cancer therapeutic agents [19]. The current treatment options for breast cancer such as chemotherapy and radiotherapy, have serious side effects on the patients. For instance, chemotherapeutic drugs may indiscriminately target healthy cells [20]. Therefore, the search for an alternative treatment for breast cancer is getting popular among the scientific community.

Carob honey (CH) is made by honeybees that take nectar from the flowers of *Ceratonia siliqua L*, also called carob tree. It possesses numerous bioactive substances [21], however, the data regarding the effects of CH, originating from Northern Cyprus, on breast cancer and pathogenic microorganisms is limited. We therefore aim to evaluate the effect of Cypriot CH on the MDA-MB-231 cell line, and its antimicrobial activity on *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi*, *Aspergillus niger,* and *Aspergillus flavus*.

Materials and Methods

Collection of CH

CH sample was obtained aseptically in December 2021, in the Kyrenia district of Northern Cyprus, an area rich in carob trees. The honey was brought to the lab in a dry and clean glass container and was authenticated by Assoc. Prof. Dr. Erkay Özgör of Cyprus bee and bee products research center, Cyprus International University.

Analysis of chemical composition of Cypriot CH using GC-MS

About 1g of CH extract was mixed with 10 mL ethanol. The mixture was filtered with filter paper (0.45 µm), and then with a syringe filter. The appropriate volume of the sample was transferred to the GC-MS vial for analysis. The method of [22] was adopted with minor modifications. The analysis was carried out using a gas chromatography coupled mass spectrometer. HP-5MS fused-silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) was employed for the analysis. The carrier gas was helium at a constant pressure of 14.5 psi. One microliter (1 µL) of the sample was injected using a splitless mode at an injector temperature of 250 ºC. The oven temperature was ramped from 35 to 280 ºC (1-minute hold) at a rate of 25 ºC/min. The GC-MS interface temperature was set to 280 ºC. Mass spectrometry mode was used during analytical scanning from 20-650 atomic mass units. The ion source

temperature was set to 250 ºC. The blank was first injected, followed by the sample injection. The chromatograms obtained from the total ion count (TIC) were integrated without any correction for co-eluting peaks and the results were expressed as total abundance. All the peaks were identified based on mass spectral matching from the Wiley library.

Antimicrobial assay of CH: Sample preparation

Stock solution (10 mg/mL) of CH ethanol extract was prepared in dimethyl sulfoxide (DMSO (20% v/v)), from which the working concentrations were prepared (1000-8000 µg/mL). The prepared samples were stored at 4°C until use.

Microbial strains

The bacterial strains used are *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella typhi,* and the fungi are *Aspergillus niger,* and *Aspergillus flavus.* The bacteria were maintained in Mueller-Hinton agar, while Sabouraud Dextrose agar was used to grow the fungi. The test isolates were inoculated in a sterile physiological saline and were compared to 0.5 Mcfarland standard to get $1.5x10^8$ CFU/mL [23].

Well diffusion assay

Well labelled petri dishes containing appropriate agar were carefully and evenly covered with $1.5x10^8$ CFU/mL microbial strains, using a sterile swab. Wells (6 mm) were made using cork-borer. The sample (50 µL) was then added to the appropriate wells. The plates were then incubated at 37 ºC for 24 hours, and the zone of inhibition was measured in mm [23]. Ciprofloxacin (30 µg) was used as a standard antibiotic for bacteria, and ketoconazole (200 µg) was used for fungi.

Cell culture

MDA-MB-231 was taken from the cell culture laboratory, Biotechnology Research Center, Cyprus International University. It was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco by Life Technologies™, USA) supplemented with 4mmol/L L-glutamine and 5% fetal bovine serum (FBS) and incubated at 37 °C, 5% CO_2 and 100% relative humidity. At 80% confluence, MTT assay was performed to assess the cell's viability [24] at various concentrations of CH.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cell viability assay

MTT assay as described by [25] was performed, with minor changes. The cells ($1x10^4$) were seeded in a 96-well plate and incubated overnight. Various concentrations (10 – 300 µg/mL) of CH were then added to the cells for 24–72 hr period. After the due period, MTT (5 mg/mL) was added to all the wells and incubated at 37 ºC for 3 hr. Formazan crystal produced by the live cells was dissolved by DMSO. The absorbance of the sample and control (only DMEM) were read using a multiwell plate reader (ELx800, Biotek Instruments) at 490 nm. The experiment was done in triplicate, and the cell viability percentage was determined from the formula;

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \quad (1)$$

Statistical analysis

Kruskal-Wallis and Mann-Whitney U tests were carried out to check for statistical significance between the test concentrations and the control drug. The significance level was set at $p<0.05$. The results were presented as mean±SD. The experiments were done in triplicate.

Results and Discussion

Chemical composition of Cypriot CH identified by GC-MS analysis

GC-MS is a technique that separates organic compounds. It uses gas chromatography to separate the components of a mixed substance, and a mass spectrometry, to decode the molecular structure of the relevant compound [26]. CH was prepared in ethanol and subjected to GC-MS analysis to determine its chemical components. After careful selection, a total of 69 compounds with at least 80% similarity match, upon data correlation on the WILEY 7 library, were detected. The GC-MS chromatogram showing the retention time and intensity is shown in Figure 1, and the molecular weights with the relative abundance (%) of each compound are shown in Table 1. Some of the detected compounds include; 2-Furancarboxaldehyde, 2-Furanmethanol, Dihydroxyacetone, 2-Furancarboxaldehyde, Benzene acetaldehyde, decanal, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 5-(Hydroxymethyl)furfural, benzene, (3-methylbutyl)-, acetaldehyde, hydrazine carboxamide, 2-(2-

methylcyclohexylidene)-, 1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester, 9-Octadecenamide, (Z)-, Quercetin 7,3',4'-trimethyl ether and Squalene.

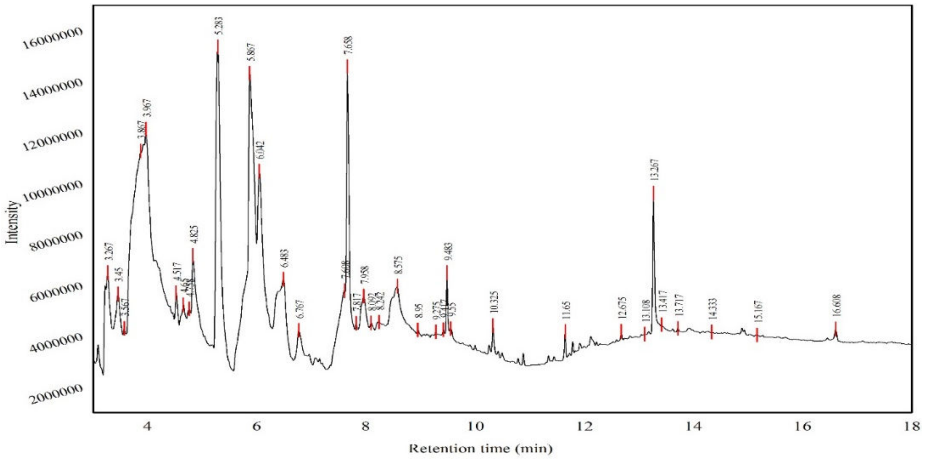


Figure 1. GC–MS chromatogram of the phytoconstituents present in the ethanol extract of carob honey

Butanoic acid, ethyl ester and furfural, detected in the CH are also present in carob fruit of Cyprus cultivars [27]. Moreover, acetaldehyde was also detected in the original *Apis mellifera* and *dorsata* honeys [28]. Linalool oxide detected in our CH was similarly found in carob tree honey samples from Portugal [29] and Morocco [30], and in the flower of the carob tree [31]. Furthermore, benzene acetaldehyde and decanal were also present in bell heather and lavender honey samples respectively [29].

Interestingly, some of the identified chemical components are pharmacologically active. For instance, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 2-Furancarboxaldehyde, and 5-hydroxmethyl, also reported in marjoram honey, Trifolium honey, and citrus honey [32], have antioxidant, anti-inflammatory, and antifungal activities [33]. In addition, quercetin detected in the honey has remarkable antioxidant properties [34] besides its anticancer activity [35].

Antimicrobial Activity of CH

The zones of inhibition, measured in millimeters (mm), of the tested bacterial and fungal strains when exposed to antibiotics (ciprofloxacin, 30µg/disc and Ketoconazole, 200 µg/disc), and varying concentrations of CH (1000-8000 µg/mL) are shown in Tables 2 and 3, and in Figure 2. CH at 8000 µg/mL shows zones of inhibition in all the treated bacteria, with the highest activity against *S. typhi* (13±1.7 mm), and the lowest against *S. pneumoniae* (8±1.0 mm). CH at 4000 µg/mL show reduced inhibition against all the bacteria but *S. aureus* resisted the treatment. At 2000 µg/mL, only *K. pneumoniae* and *E. coli* responded with zones of 10±1.0 mm and 8±1.7 mm respectively. No effect observed at 1000 µg/mL. Generally, gram-negative bacteria show more sensitivity to CH than gram-positive bacteria. Gram-negative *E. coli and K. pneumoniae* respond to the treatment at 2000-8000 µg/mL, *S typhi* responds at 4000-8000 µg/mL and has the widest zone of inhibition. Although gram-positive *S. aureus* has a wider zone of inhibition than *S. pneumoniae*, it responds only at 8000 µg/mL, while the latter responds at both 8000 and 4000 µg/mL concentrations of CH.

Stronger antifungal activity was noted in *A. niger* than *A. flavus*. However, CH could not inhibit both the fungi at concentrations less than 8000 µg/mL. The antibacterial effect increases with an increase in CH concentration. This trend agrees with that of [36], who reported a dose-dependent antibacterial activity of Saudi honey from Asir region and [37], who also found that buckwheat and Manuka honeys exerted dose-dependent antibacterial activity.

Table 2. Antibacterial activity of CH in comparison with ciprofloxacin antibiotic

Carob Honey (CH) (µg/mL)/ Antibiotic (30 µg/disc)	Zone of Inhibition (mm)				
	Bacterial Strains				
	S. aureus	***E. coli***	***K. pneumoniae***	***S. pneumoniae***	***S. typhi***
Ciprofloxacin	16±2.0	21±4.0	25±2.6	15±2.0	16±3.0
CH (8000)	10±2.0*	12±1.0*	11±1.7*	8±1.0	13±1.7
CH (4000)	-	10±2.6*	11±2.0*	8±2.6	11±3.5
CH (2000)	-	8±1.7*	10±1.0*	-	
CH (1000)	-	-	-	-	-

Results are presented as mean±SD of zones of inhibition (mm). *$p<0.05$ vs control (antibiotic). (-) indicate the absence of antibacterial activity. $p>0.05$ for *S. pneumoniae* and *S. typhi* at 8000 and 4000 µg/mL vs positive control

Salmonella species cause salmonellosis, a lethal infection in both humans and animals [38]. *E. coli* is not only responsible for causing gastrointestinal infections but also infection of the urogenital tract [39]. *S. aureus* is implicated in the promotion of wound infection thus causing delayed wound healing [40]. It is the most virulent among the *Staphylococci* family, which causes lethal infections like meningitis, pneumonia and endocarditis [41].

Gram-negative bacteria develop resistance to many antibiotics due to their unique outer membrane structure. As a result, it is capable of causing serious infections in humans, especially immune-deficient individuals [42-44]. Although some studies have shown gram-positive bacteria as more susceptible to inhibition than gram-negative bacteria [45-49], our CH show better antibacterial activity against gram-negative strains, *S. typhi* at 8000 µg/mL (13±1.7 mm) and 4000 µg/mL (11±3.5 mm), *E. coli* (8000 µg/mL (12±1.0 mm), 4000 µg/mL (10±2.6 mm), and 2000 µg/mL (8±1.7 mm), and *K. pneumoniae* (8000 µg/mL (11±1.7 mm), 4000 µg/mL (11±2.0 mm), and 2000 µg/mL (10±1.0 mm) than the gram-positive bacterial strains.

A similar result was reported in honey bee honey, in which a higher zone of inhibition was obtained in gram-negative *E.coli* (26±0mm), *S. typ*hi (14±0mm) and *K. pneumoniae* (17±0mm) than gram-positive *S. aureus* [50]. Similar studies confirmed a better inhibitory effect of honey against *E. coli* than *S. aureus* [51, 52]. Moreover, gram-negative *E. coli* and *S. typhi* were found to be more susceptible to ethanol extract from raw and processed honey than gram-positive *S. aureus* [52]. Furthermore, *E. coli* showed the highest sensitivity to Dharm-1 honey (80%) with a zone of inhibition of 15.33 ± 1.1 mm [36]. A study conducted on Malaysian *Trigona* honey revealed a higher zone of inhibition in *E. coli* and *S. typhi* than *S. aureus* [53]. Algerian honeys were, however, found to have better activity against gram-positive bacteria with a zone of inhibition ranging between 14 to 38 mm than gram-negative bacteria with a zone of inhibition ranging between 8 to 28 mm [54]. In the same vein, honey sample from Adamawa state of Nigeria showed that gram-positive *S. aureus* has the highest susceptibility (13.6 mm) followed by gram-negative *K. pneumoniae* (12.8 mm) and *E. coli* (12.4 mm) [55].

The proposed mechanism of destruction caused by honey on gram-negative bacteria is the destruction of the microbial cell wall and lipopolysaccharide which leads to increased cellular permeability and ultimately cell lysis [56]. In addition, many bioactive components contribute to the antibacterial properties of honey, which include flavonoids and polyphenols [57-59]. Some important components detected in the Cypriot CH may be responsible for the recorded antimicrobial activities. These include; benzene acetaldehyde [60], 2, 3 dihydro-3,5-di-hydroxy-4H-pyran-4-one [61], 5-hydroxymethylfurfural [62], and quercetin [63]. Increased honey acidity, due to the presence of phenolic acids, adds to the honey's flavor, and boosts its antibacterial activity [57]. Generally, the type and amount of bioactive compounds in honey depends on the geographical location, botanical origin, processing and storage conditions [64].

Table 3. Antifungal activity of carob honey against *Aspergillus niger* and *Aspergillus flavus* and Ketoconazole

Carob Honey (CH) (µg/mL)/ Antibiotic (200 µg/disc)	**Zone of Inhibition (mm) Fungal Strains**	
	Aspergillus flavus	***Aspergillus niger***
Ketoconazole	16±4.3	15±1.7
CH (8000)	7±1*	9±2.6*
CH(4000)	-	-
CH(2000)	-	-
CH(1000)	-	-

Results are presented as mean±SD of zones of inhibition (mm). *p<0.05 vs control (antibiotic). (-) indicate the absence of antifungal activity

According to our results, *A. niger* is more sensitive to the tested CH than *A. flavus*. However, the antifungal inhibition occurred only at 8000 µg/mL and no inhibition was recorded at lower concentrations (4000-1000 µg/mL). Similar to our findings, the effects of ethanol, methanol, and ethyl acetate extracts of processed honey against *A. niger*, have shown 11, 15 and 10.5 mm inhibition diameters for the respective extracts [65]. Related research revealed the antifungal activity of Romanian honey against *Aspergillus spp*, with an inhibition diameter from 7 to 12 mm [66]. These findings conformed to the studies on honey samples from Nigeria, India and Pakistan [67-69].

Honeys have generally been shown to exhibit antifungal properties against diverse species of fungi [70]. Our study shows that Cypriot CH has potential antimicrobial activity, which may be attributed to its phytoconstituents. The antimicrobial activities of honey are ascribed to the wide range of bioactive components, such as phenolic acids, flavonoids, and other biomolecules. Phenolic compounds are the notable components responsible for most biological activities of honey. Their antimicrobial role is mainly due to their ability to cause protein denaturation [71].

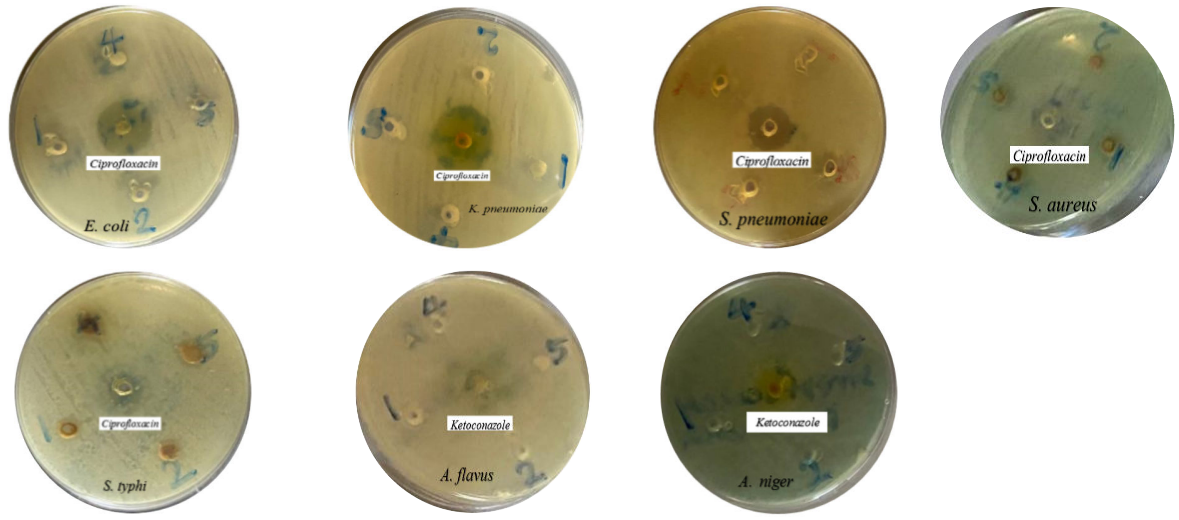


Figure 2. Zones of inhibition of carob honey at 1=8000 µg/mL, 2=4000 µg/mL, 4=2000 µg/mL, 5=1000 µg/mL

Effect of CH on MDA-MB-231 cell viability

Figure 3 below shows a concentration and time-dependent anti-proliferative activity of CH. MDA-MB-231 was treated with CH at five different concentrations (10, 25, 75, 150 and 300 µg/mL). The cell viability (%) was found to decrease according to the concentration applied and the time taken for sample activity. After 24 hours of treatment, the cells showed 80% viability with 20% inhibited. After 48 hours, the viable cells were 50% with 50% attenuated and after 72 hours the viable cells were found to be 45%, indicating 55% inhibition. However, the IC_{50} values are substantially high, 300 µg/mL and 70 µg/mL after 48 and 72hr respectively.

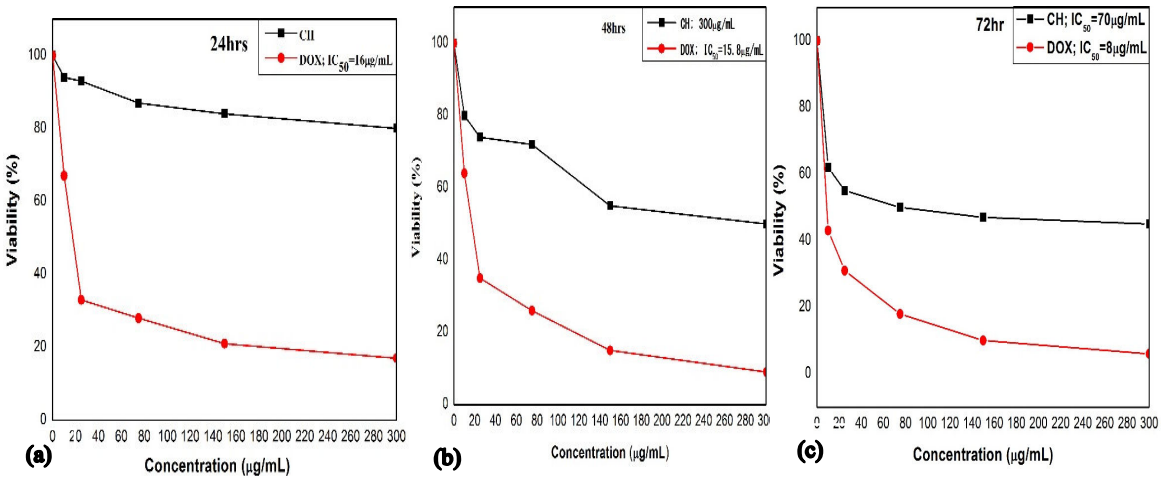


Figure 3. MDA-MB-231 inhibition at different CH concentrations, after A) 24 B) 48 and C) 72 hr. CH=Carob honey. DOX=Doxorubicin

Breast cancer poses a great threat to women due to difficulties in prognosis and uncertainties of the available treatments. Among the breast cancer types, TNBC is the most aggressive and it does not respond to hormonal or HER2 receptor therapy. Although chemotherapy, radiotherapy and surgical procedures have been used to treat it, huge risks are associated with the processes [72]. Honey is a natural dietary ingredient that has been used for centuries in treating many medical conditions. Honey's bioactive components have been linked to antitumor effects at various stages of cancer [73]. Biological activities such as anti-inflammation, antioxidant, immune-modulation and anti-proliferation are all reported in honey [74], hence honey is a great candidate to exploit in search of alternative cancer therapies.

The cytostatic effects of honey can be attributed to its ample phenolic compounds, which possess antitumor and antimicrobial activities [73, 75]. In addition, organic acids, vitamins and trace minerals contribute to the anticancer and antimicrobial effects of honey [69, 76-77]. For example, Manuka honey inhibits the proliferation of MCF-7 breast cancer cell line in a concentration-dependent manner, and this effect correlates with its phenolic compounds [78]. Tualang honey was used as a promoting agent with tamoxifen, and the combination was found to have a considerable cytotoxic activity on MDA-MB-231 and MCF-7 cell lines [79]. Cedar and pine honey from Turkey also have inhibitory effects on MCF-7, MDA-MB-231 and SKBR3 cell lines at high dose [80]. Chestnut honey and cedar honeys were reported to exert 50% inhibition on the MCF-7 cell line. Cedar honey could only exert this inhibition at a higher concentration [81]. Treatment of Sidr and wild honeys changed the appearance of the MDA-MB-231 breast cancer cell line after 48 hr indicating their cytotoxic effects against the breast cancer cell [82]. The differences in the anticancer activities of various honeys may be due to geographical locations, storage conditions, differences in the amount and type of minerals, amino acids, hydrogen peroxide and phenolic contents [83].

Conclusion

The current research assessed the chemical compositions, and antimicrobial and breast cancer inhibitory potentials of CH from Northern Cyprus. Some of the bioactive components detected are quercetin, furan methanol, squalene and 4H-Pyran-4-one. CH inhibited the growth of both gram-negative and gram-positive bacteria as well as the fungi used in this research. CH also exhibited a dose and time-dependent anti-proliferative effect against the MDA-MB-231 breast cancer cell line. These biological activities may be due to the presence of various bioactive compounds detected in the honey in the GC-MS analysis. Further research on carob honey safety, toxicity, and other pharmacological potentials is highly recommended.

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DUAL SACCHARIDES: HOW DO THEY INFLUENCE BACTERIAL CELLULOSE PRODUCTION AND MECHANICAL PROPERTIES?

(Dwi-Sakarida: Bagaimana Ia Mempengaruhi Pengeluaran dan Sifat Mekanik Selulosa Bakteria?)

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Abstract

Bacterial cellulose (BC) production has become more popular over the past few years and has been widely used as an alternative in diverse applications. The most prominently discussed is biomedical applications. Due to its popularity and high demand for biomaterials in that particular application, BC has been focusing on its production and mechanical properties that align with the industries' biomedical demands. This study aimed to investigate the effects of dual-saccharides on BC production and its mechanical properties. Monosaccharides and disaccharides such as glucose, fructose, sucrose, lactose and maltose were used as carbon source combinations and how these dual-saccharides affect the production and properties of BC. Bacterial *K. xylinus* can utilise dual-saccharides glucose+lactose to produce BC, based on the BC coefficient (g/g). The saccharide combinations of glucose+lactose gave the highest BC production with 0.15 (g) of final weight. Bacterial *K. xylinus* can fully utilise dual-saccharides glucose+lactose based on the BC coefficient, 0.007 (g/g). Upon drying, the purified BC was characterised using scanning electron microscope (SEM), Fourier transform infrared (FTIR) and X-ray diffraction (XRD) methods. Hence, combining dual-saccharides into the culture medium can enhance BC's production and mechanical properties for wound healing applications.

Keywords: bacterial cellulose, dual-saccharides, properties, biomedical applications, *Komagataeibacter xylinus*

Abstrak

Pengeluaran selulosa bakteria (SB) menjadi lebih terkenal sejak beberapa tahun kebelakangan ini dan telah digunakan secara meluas sebagai alternatif dalam pelbagai aplikasi. Aplikasi bioperubatan merupakan aplikasi yang kerap dibincangkan. Disebabkan popularitи dan permintaan yang tinggi untuk biobahan dalam aplikasi tertentu, SB telah berfokus pada pengeluaran dan sifat mekanikalnya yang sejajar dengan permintaan industry bioperubatan. Kajian ini bertujuan untuk mengkaji kesan dwi-sakarida ke atas pengeluaran SB dan sifat mekanikalnya. Monosakarida dan disakarida seperti glukosa, fruktosa, sukrosa, laktosa dan maltosa digunakan sebagai gabungan sumber karbon dan bagaimana dwi-sakarida ini mempengaruhi pengeluaran dan sifat SB itu sendiri. Bakteria *K. xylinus* boleh menggunakan dwi-sakarida glukosa+laktosa untuk menghasilkan SB, berdasarkan pekali SB (g/g). Gabungan sakarida glukosa+laktosa menghasilkan SB tertinggi dengan 0.15 (g) berat akhir. Bakteria *K. xylinus* boleh menggunakan sepenuhnya dwi-sakarida glukosa+laktosa berdasarkan pekali SB, 0.007 (g/g). Selepas pengeringan, SB yang telah ditulenkan menjalani pencirian menggunakan kaedah mikroskop imbasan elektron (SEM), transformasi fourier inframerah (FTIR) dan kaedah pembelauan sinar-X (XRD). Oleh itu, penggabungan dwi-sakarida dalam medium kultur boleh meningkatkan pengeluaran SB dan sifat mekanikal untuk aplikasi penyembuhan luka.

Kata kunci: selulosa bakteria, dwi-sakarida, ciri-ciri, aplikasi biomedikal, *Komagataeibacter xylinus*

Introduction

Cellulose is an abundant polysaccharide on Earth. This polysaccharide is commonly derived from the plant cell walls and composed of repeated β-1,4 glycosidic bonds (shown in Figure 1) that link the glucose monomer together [1, 2]. Belongs to the group of carbohydrates, cellulose consists of numerous hydroxyl groups that exist as a linear polymer chain [3]. The capability of hydroxyl groups to hold the hydrogen bonds together is crucial for some properties such as microfibrillar structure, high crystallinity and high cohesive nature [4]. This biomaterial has recently drawn enormous interest among researchers due to its versatility and purely biotechnological processing approach aligned with the green chemistry principle [5]. Rather than plant cellulose, bacterial cellulose (BC) is most favourable as this polysaccharide offers excellent mechanical properties that meet the market demands. Hence, BC was chosen over plant cellulose due to its purity, which frees off contaminants such as pectin, lignin, and hemicellulose. Previous studies have addressed that the *Komagataeibacter* genus can utilise various carbon sources such as sucrose, fructose, glucose, galactose, xylose and mannose to synthesise cellulose [6]. In this study, *Komagataeibacter xylinus* was chosen as the BC producer due to this strain's ability to synthesise BC at a high yield [7]. Carbon source becomes the main contributor that affects BC production. At present, several attempts have been made to improve BC productivity, including combining carbon sources into the fermentation media. Based on studies conducted by Anguluri et al. [8], combining two types of carbon sources can enhance BC productivity using strain *Gluconacetobacter persimmonis*. However, utilising dual-sugar has not yet been discovered to synthesise BC by strain *Komagataeibacter xylinus* and its mechanical properties on wound healing applications. According to Digel et al. [9], strain *Komagataeibacter* can utilise different types of carbon sources and can produce BC in high yield.

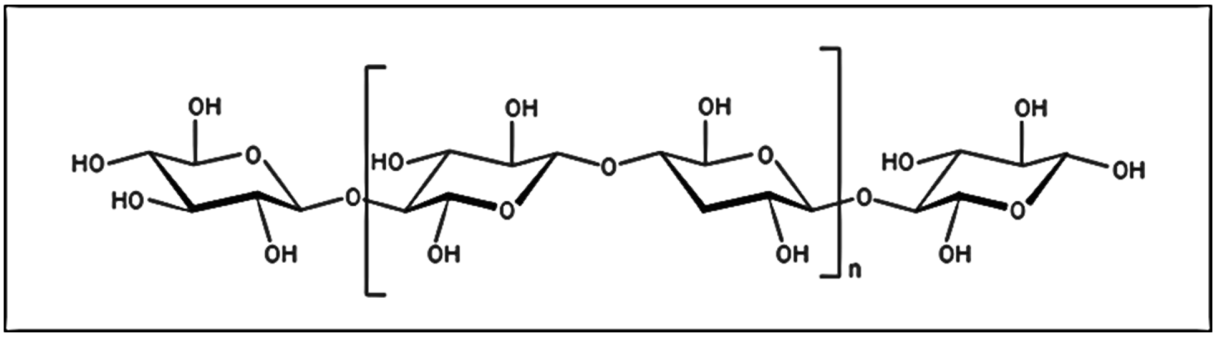


Figure 1. Chemical structure of cellulose made up of β-1,4 glycosidic bond. Adapted and modified from [10]

Materials and Methods

Microorganisms

Bacterium *Komagataeibacter xylinus* ATCC 53524 (American Type Culture Condition) strain was purchased and used as BC producer in this experiment owing to its capability to synthesise cellulose in a large amount [11, 12].

Culture media preparation

Culture media was prepared using the [13] method with some modifications. A standard Hestrin–Schramm (HS) medium, composed of 0.5% (w/v) yeast extract, 0.5% (w/v) peptone, 0.115% (w/v) citric acid, 0.2% (w/v) disodium hydrogen phosphate and filter-sterilised of 50% (v/v) glucose was used as fermentation medium to synthesise BC. The pH value of the culture medium was adjusted until it dropped to 4-5. The overall procedure of BC biosynthesis is illustrated in Figure 2 below.

Preparation of inoculum

These methods followed [4] with slight modifications. The bacterial strain was initially grown on HS agar slant for five days and incubated at 30°C. A loop of newly developed bacterial strains was grown into a sanitised 250-mL conical flask of 75-mL of liquid growth media and incubated overnight in agitated conditions (150 RPM ~ 30°C). This process was run until the culture media became cloudy.

Bacterial cellulose biosynthesis

Approximately 10% of the inoculum was transferred into the fresh 75-mL HS media for fermentation. The fermentation process was run in triplicates, in a static condition, for seven days until a layer of white pellicle was present on the upper layer of the media.

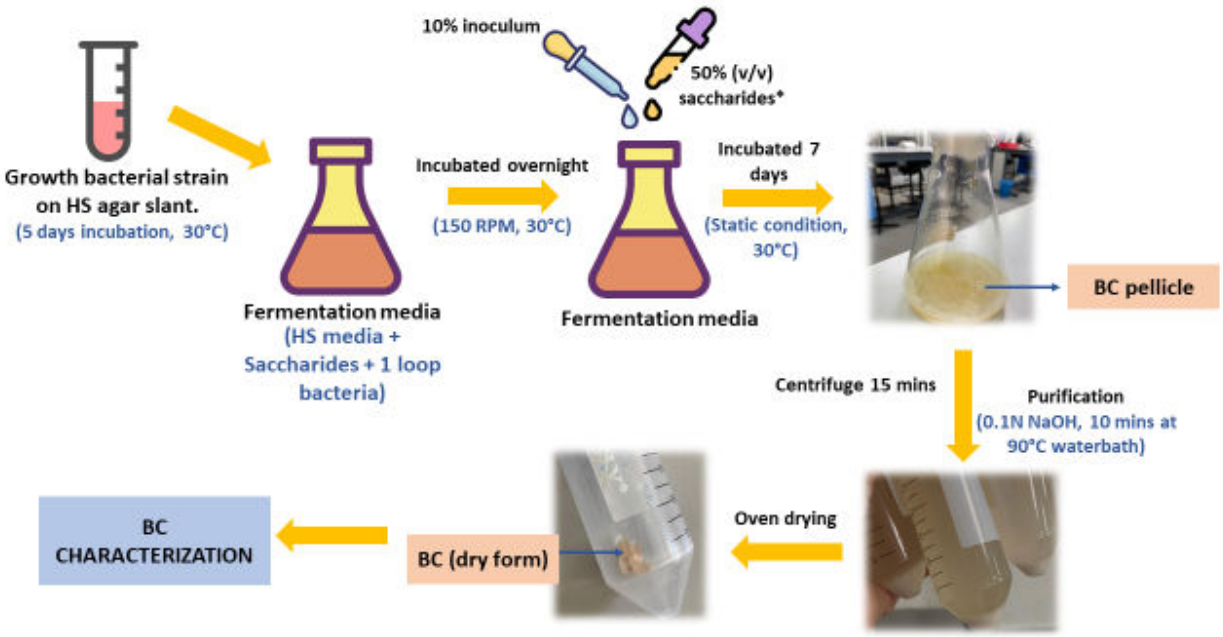


Figure 2. Overall illustration of BC biosynthesis produced by *Komagataeibacter xylinus.* (*) represent studied variables

Effect of carbon source combinations on BC yield

This experiment was conducted to study the effect of dual-sugar on BC production. The inoculum was transferred into the 250 mL flask containing HS media as described previously, and 50% (v/v) of dual-sugar was filter-sterilised. Different combinations of sugar were used, such as 25% glucose + 25% fructose, 25% glucose + 25% maltose, 25% glucose + 25% lactose, 25% fructose + 25% lactose, 25% fructose + 25% maltose and 25% fructose + 25% sucrose were added into the fermentation media. The pH value of the broth was changed as previously described. The fermentation flasks were incubated at 30°C for seven days in static conditions.

Purifications of BC

After fermentation, the native BC pellicles formed were harvested and put into a sterile 50-mL Falcon tube and centrifuged at 4000 RPM for 15 mins to obtain the residue. The residue was immersed into 0.1 M NaOH and was placed in the 90°C water bath for 10 mins. This process was conducted to remove the unwanted cells that were obtained during fermentation. Only purified BC was purified and dried in the oven at 60-70°C until constant weight was achieved. The weight of pure BC was measured.

Characterisations of BC: Scanning Electron Microscope (SEM) analysis

The dried BC obtained was cut into small pieces based on the desired cross-section. The dried pure BC surface morphology was accessed using the JEOL JSM6360LA (Japan) scanning electron microscope. The morphology of the BC structure was observed at an acceleration voltage of 10 kV. All BC samples were covered with gold sputter to enhance conductivity [14].

Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR analysis was conducted according to Sing et al. [15] method using Bruker Invenio-S (Germany) to determine the functional groups and chemical bonds in

the dried BC membranes. The dried BC membrane was put onto the sample stage after being cleaned with acetone to avoid contaminations. The FTIR spectral analysis was then performed over the wavenumber range of 500-4000 cm^{-1}.

X-ray Diffraction (XRD) analysis

Upon drying process, the crystallinity of BC was analysed using X-ray diffraction techniques. The characterisations of the dried BC were analysed using Rigaku Smartlab SE (Japan). The XRD patterns were generated at a voltage of 40 kV at the filament emission of 30 mA and Cu Kβ radiation. The BC pellicles were characterised at the 2θ range, between 5° and 60°.

Results and Discussion

Effect of dual-saccharides treatments on BC production

The effect of dual-saccharides on BC production (dry weight) is depicted in Figure 2 above. There were six types of dual-saccharides treatments utilised by *K. xylinus*, namely glucose+maltose, glucose+lactose, glucose+lactose, fructose+maltose, fructose+sucrose, and fructose+lactose. [9] mentioned that the strain *K. xylinus* can utilise different types of carbon sources other than being able to synthesise BC in high yield. In this experiment, glucose and fructose were used as controls. According to the findings, the dual-saccharides treatments of glucose+lactose gave the highest yield (0.418 g), followed by fructose+lactose (0.132 g), glucose+fructose (0.083 g), fructose+sucrose (0.064 g), fructose+maltose (0.06 g) and glucose+maltose gave the lowest yield (0.022 g). This is because maltose is considered an inadequate carbon source since it does not lower the fermentation media's pH value. A higher pH indicates low BC production since *K. xylinus* works effectively in acidic conditions due to its nature (acid-tolerance bacteria) [16]. Glucose, which acts as a sugar control, gave the maximum yield of 0.127 g, while another sugar control, fructose, gave a yield of 0.092 g. Nevertheless, the findings were contradicted with [4] where BC can be produced at maximum yield when fructose is used as a carbon source.

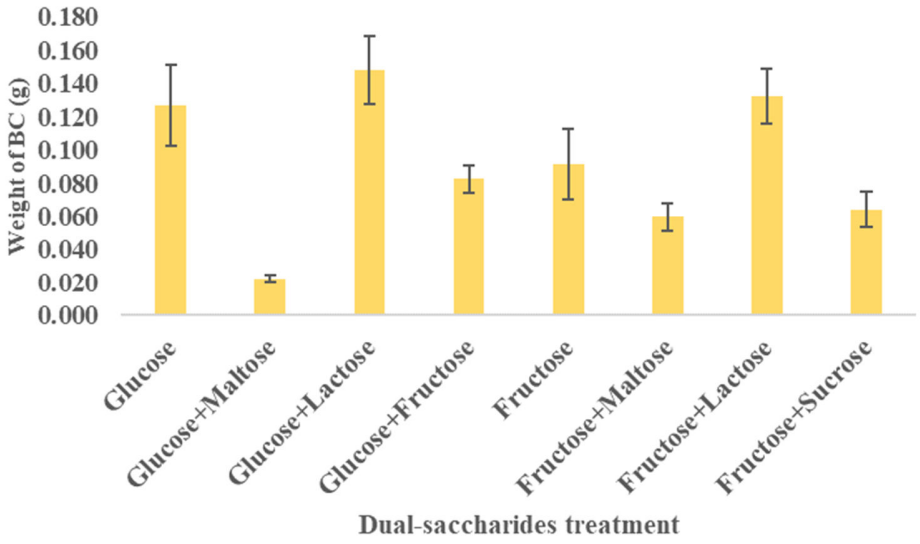


Figure 2. Graph effect of dual-saccharides treatments on BC weight (g)

Effects of dual-saccharides treatments on BC coefficient

The following equation was used to determine the bacterial yield coefficient of *K. xylinus* in BC production.

$$\text{Growth yield coefficient} = \frac{Dry\ weight\ of\ BC\ (g)}{Amount\ of\ C\ source\ consumed\ (g)} \quad (1)$$

Bacteria *K. xylinus* has the capability to break down carbon sources to produce cellulose. This strain can also

produce cellulose by absorbing glucose and fructose as carbon sources [14]. In addition, the prospect of synthesising BC from carbon sources other than glucose has been discovered by researchers for a long time [17]. The findings in Figure 3 above depict the effects of various dual-saccharides treatment on the BC coefficient. Based on the results, the bacterium has utilised 20 g of carbon sources throughout the fermentation process to produce BC. The combined sugar of glucose and maltose proved that the bacteria *K. xylinus* has the lowest efficiency in breaking down those sugar molecules. This is because when bacteria are grown in a combination of two distinct carbon sources, their growth behaviour is altered [18]. Maltose does not cause the pH of the media to be decreased at the end of the fermentation. Since this *K. xylinus* need acidic conditions to break down carbon sources to synthesise BC, the results indicated the strain was unable to utilise maltose to produce BC as it is known as a weak carbon source for the synthesis [16].

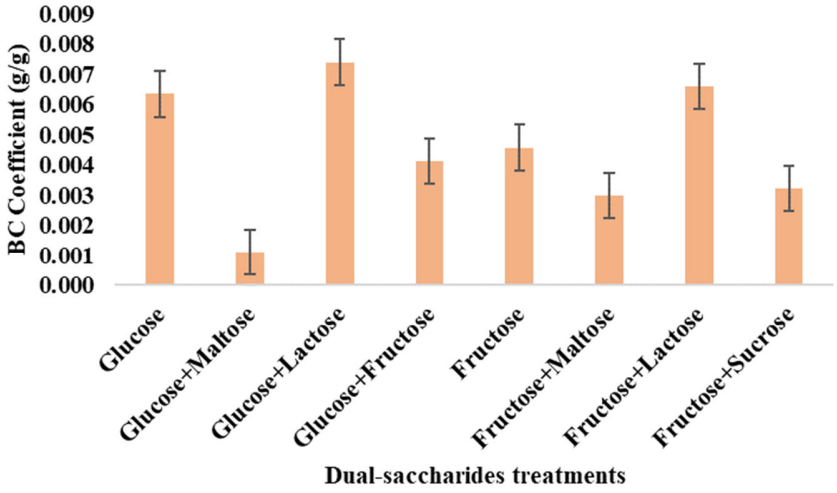
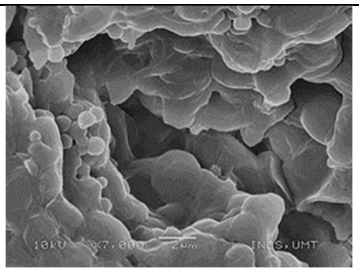
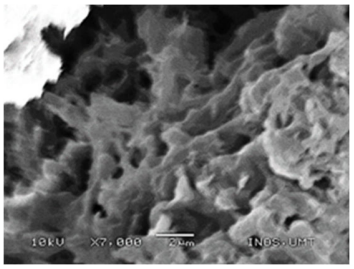
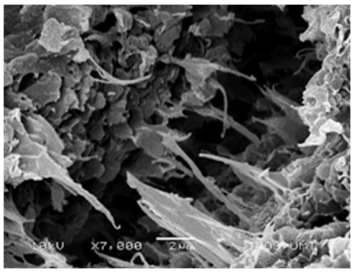
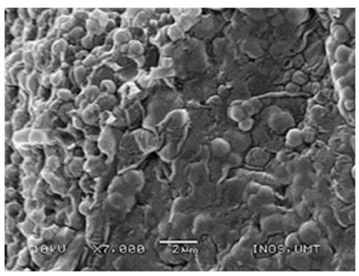
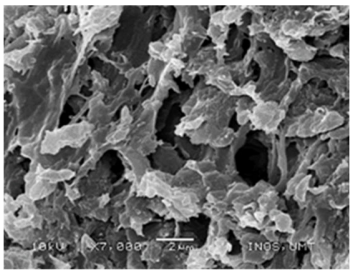


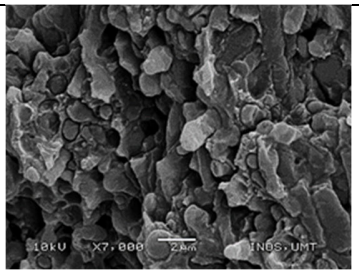
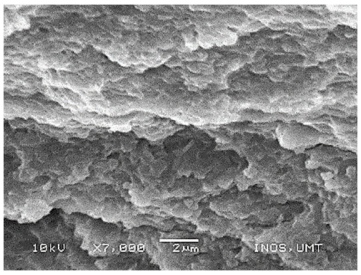
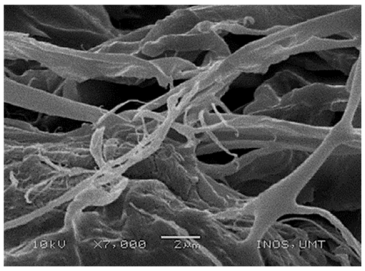
Figure 3. Graph effects of dual-saccharides treatments on BC coefficient (g/g)

Characterisation of BC: SEM analysis

The intricate structure of BC morphology produced by *K. xylinus* using six different treatments of carbon sources are displayed in Figure 2 below. The SEM helps to provide the surface morphology of the BC [19]. The SEM images revealed that all the BC samples have microporous structures with smooth surfaces and highly open pores. The use of different dual-sugar treatments has an influence on BC morphology based on SEM images observed. Based on the surface morphology, it can be seen that BC with the combination of fructose + lactose [Table 1(A)] appeared relatively rigid and had blunt edges. The SEM images in Table 1 (B), (C), (D), (E) and (F) showed some differences in surface structures when sugar combinations were applied. The images in [Figure 5(B)] showed that it has a smudge-like structure and some fibrous-like structure [Table 1 (C)], while Table 1 (D), (E) and (F) have porous appearance. Since dual-sugar was implied into the fermentation media and incorporated into BC fibrils, the reticulated structure may be assigned to the existence of different types of sugars in the broth. However, the findings above were compared with the morphology of the BC membranes when glucose and fructose were used as the only sources of carbon (Table 1 (G) and (F)). The SEM images from Figure 3 and Figure 4 revealed that glucose and fructose lead to a different interwoven pattern in BC. The results were determined from both the top and bottom surfaces of BC membranes. There was a difference in the morphology of BC in G and H, whereas G is more compact and denser, while H shows a fibrous-like structure. H exhibited BC morphology with the thinnest strand, higher porosity and more fibrous compared to G. Based on the results obtained, BC is composed of a nano-fibrillar network of pores, which helps in the absorption of wound exudates. This feature in BC accelerates the wound healing process since it provides a favourable temperature for the wound surface, which is vital in wound healing applications [13].

Table 1. SEM morphology of cellulose produced by *K. xylinus* under six different treatments of dual-sugar

Carbon Sources		SEM Images
Fructose+Lactose	**A**	
Fructose+Maltose	**B**	
Fructose+Sucrose	**C**	
Glucose+Lactose	**D**	
Glucose+Fructose	**E**	

Carbon Sources		SEM Images
Glucose+Maltose	**F**	
Glucose	**G**	
Fructose	**H**	

The SEM helps to provide the surface morphology of the BC [19]. The SEM images revealed that all the BC samples have microporous structures with smooth surfaces and highly open pores. The use of different dual-sugar treatments has an influence on BC morphology based on SEM images observed. Based on the surface morphology, it can be seen that BC with the combination of fructose + lactose [Table 1(A)] appeared relatively rigid and had blunt edges. The SEM images in Table 1 (B), (C), (D), (E) and (F) showed some differences in surface structures when sugar combinations were applied. The images in [Figure 5(B)] showed that it has a smudge-like structure and some fibrous-like structure [Table 1 (C)], while Table 1 (D), (E) and (F) have porous appearance. Since dual-sugar was implied into the fermentation media and incorporated into BC fibrils, the reticulated structure may be assigned to the existence of different types of sugars in the broth. However, the findings above were compared with the morphology of the BC membranes when glucose and fructose were used as the only sources of carbon (Table 1 (G) and (F)). The SEM images from Figure 3 and Figure 4 revealed that glucose and fructose lead to a different interwoven pattern in BC. The results were determined from both the top and bottom surfaces of BC membranes. There was a difference in the morphology of BC in G and H, whereas G is more compact and denser, while H shows a fibrous-like structure. H exhibited BC morphology with the thinnest strand, higher porosity and more fibrous compared to G. Based on the results obtained, BC is composed of a nano-fibrillar network of pores, which helps in the absorption of wound exudates. This feature in BC accelerates the wound healing process since it provides a favourable temperature for the wound surface, which is vital in wound healing applications [13].

FTIR analysis

FTIR spectra represent compound and functional groups present in bacterial cellulose (BC). The purified BC formed when dual-sugar used as carbon sources were determined. The FTIR spectra were scanned, ranging from 500 to 4000 cm^{-1} (Figure 5). The FTIR analysis of BC pellicles formed from various dual-saccharides treatments were compared to the standard sugar, glucose, and fructose. According to the findings in Figure 5 below, the peaks obtained by BC are almost similar in each dual-saccharides treatment and sugar control. Fructose and glucose act as carbon source controls in this experiment. All the peaks were determined in the same position at a similar intensity. Generally, the native BC has plenty of hydroxyl groups, causing the molecule to be hydrophilic [9]. Owing to the plenty of –OH groups present in BC and nanofibril network makes this biopolymer suitable for the current wound-dressing material since it forms strong intramolecular and intermolecular hydrogen bonds that have a significant impact on tensile strength and elastic modulus of BC [20, 13]. According to Choi et al. [19], -OH groups might help in water absorption, and the nanofibril itself can provide a good skin environment for wound healing. Hence, the presence of hydroxyl groups was proven by the FTIR analysis. The FTIR spectra formed also proved that the material produced by strain *K. xylinus* was cellulose [14]. Functional groups obtained from the BC are listed in Table 2 below. Generally, BC itself possessed plenty of hydroxyl groups and oxygen atoms, summarised in Table 2. These create the inter and intra of hydrogen bonding in BC, which helps in improving the mechanical strength of the biopolymer [21], which might be favourable in the wound healing process.

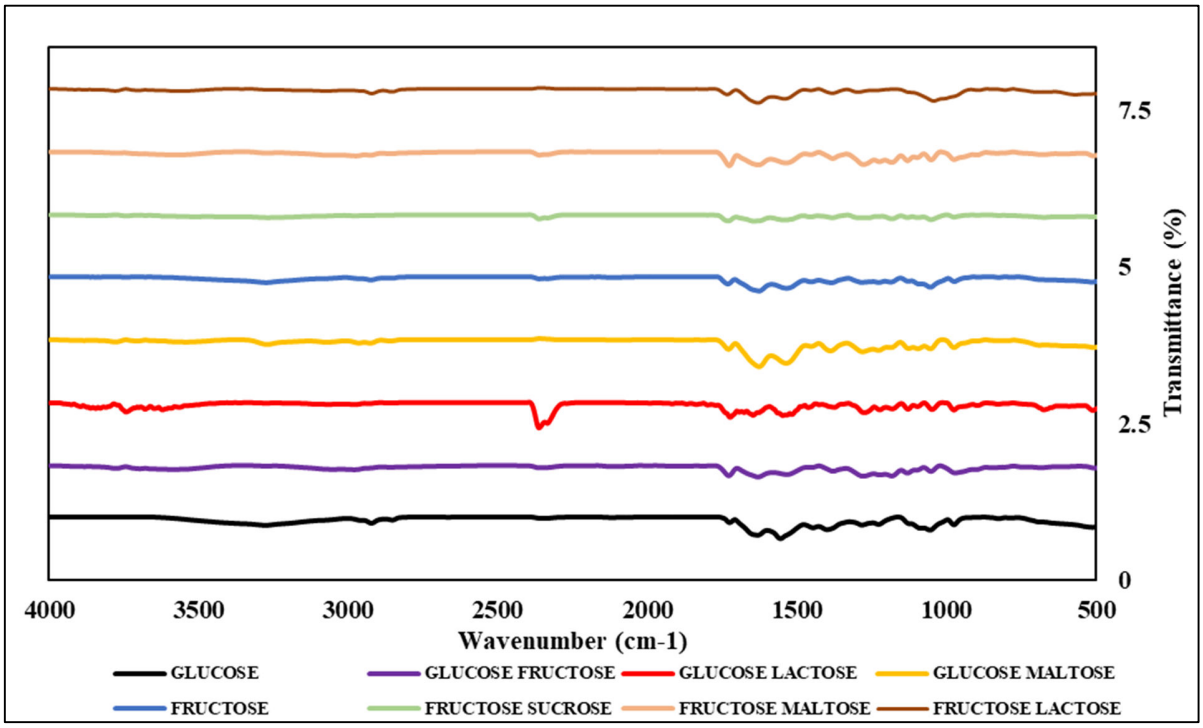


Figure 5. FTIR spectra of BC when dual-saccharides treatments were used

Table 2. FTIR spectra for dried pure BC after different treatments of sugar were applied

Sugar Treatment	Peaks (cm^{-1})	Peak Details	Functional Group Obtained	Class
Glucose	~1052; ~1557; ~1630	Strong; medium; medium	C-O-H bonding; C=C, C-H stretching	Polysaccharide; alkene;
Glucose+Fructose	~1728; ~1635; 970	Strong; medium	C=O stretching; C=C stretching; OH bonding	Carbonyl; alkene; alcohol
Glucose+Lactose	~2360; ~1724; ~1535	Strong; medium	C=C; C=O; C-O-H stretching	Alkene; carbonyl; carboxyl
Glucose+Maltose	~1627; ~1534; ~1383	All strong	O-H bending; C-O-H and O-H stretching	H_2O [14]; carboxyl; and alcohol
Fructose	~1627; ~1527; ~1399	Strong, medium, strong	O-H bond; C-O-H bending; O-H stretching	Water; carboxyl; and alcohol
Fructose+Sucrose	~1731; ~1628; ~1053	All medium	C=O stretching; C-H stretching [13]; C-O-C stretching [13]	Carbonyl; aldehyde and ester
Fructose+Maltose	~1723; ~1533; ~1277	Strong, medium	C=O stretching [13]; C-O-H stretching; and O-H bending	Acetyl, carboxyl and alcohol
Glucose+Maltose	~1728; ~1626; ~1387	Strong and medium	C=O, C=C bending and C-H stretching	Aldehyde, alkene and aldehyde

XRD analysis

It is reported by [22] that BC is a homogeneous polycrystalline macromolecular compound that consists of ordered crystalline and minimum ordered amorphous regions. In order to determine the crystalline structure of cellulose produced from *K. xylinus*, all BC samples were analysed using XRD. Figure 6 shows the XRD pattern of BC under the treatment of various dual-saccharides, and peaks of BC were determined at 2θ angles ranging from 0° to 60°. The findings show that the native BC has a crystalline structure [19]. Analysis of XRD is used to identify the characteristics of cellulose Iα since BC is composed of cellulose Iα [11]. According to Figure 6, BC pellicles from dual-saccharides fructose+lactose, fructose+sucrose, fructose+maltose, glucose+fructose and glucose+lactose had distinct peaks at 2θ angles in the range of 28° to 30° while glucose+maltose formed prominent peaks at range 10° to 30°. The control sugar glucose formed three distinct strong intensity peaks [22], which shows that the typical cellulose produced

from *K. xylinus* contains Iα cellulose. All the results obtained indicate that BC implemented with dual-saccharides has high crystallinity. BC pellicles formed from fructose proved that BC has high crystallinity, although the peaks were produced with low intensity. The crystallinity of the dried BC membranes determined under the various dual-saccharides treatments was reported in Table 2. In general, BC has higher crystallinity than plant cellulose [23], and the crystallinity value can be up to 90% [20]. Based on findings in Table 2, the results indicate that BC with dual-saccharides treatments gave higher crystallinity, parallel to the native BC except for fructose+sucrose and fructose+maltose. The higher crystallinity of BC indicates that the biopolymer has a high degree of polymerisation, making the properties suitable for wound healing material [13]. According to the observations, the unique feature of BC favoured in wound healing material has been proven.

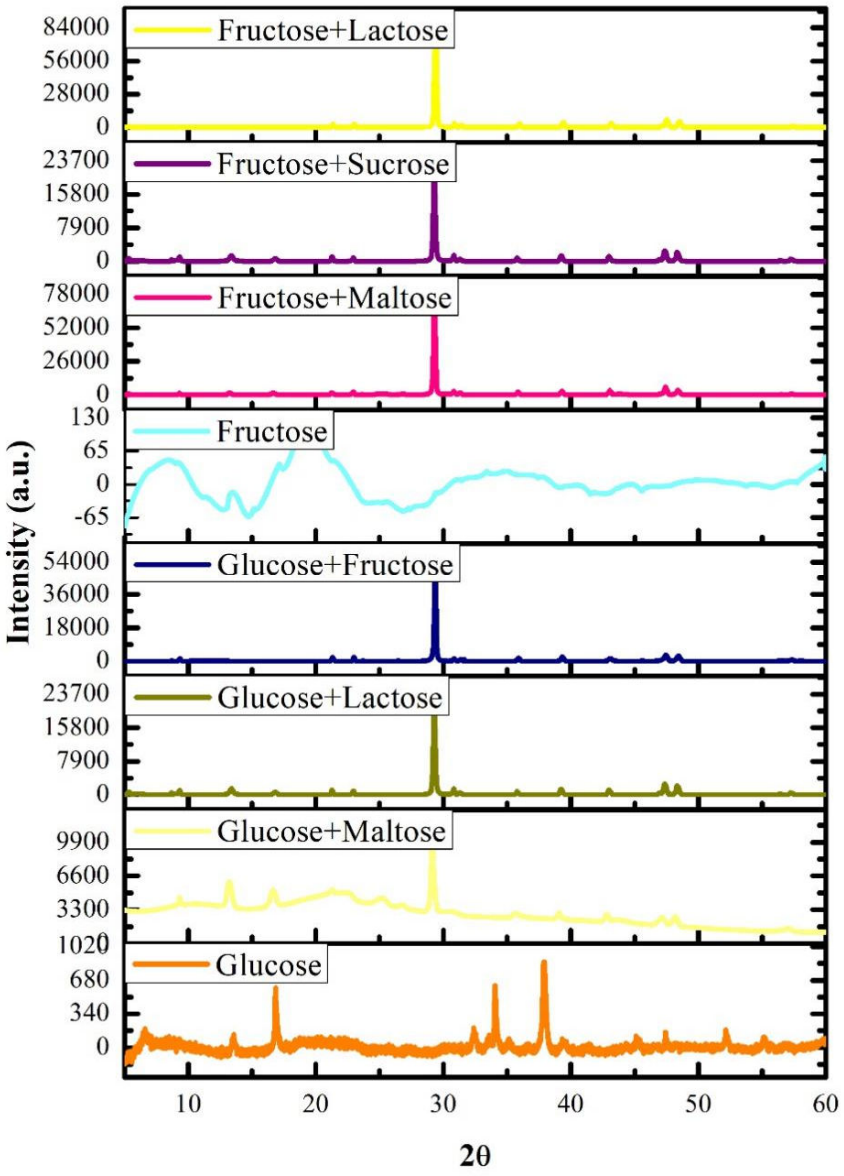


Figure 6. XRD patterns of BC when dual-saccharides treatments were applied

Table 2. Crystallinity % for dried BC after different dual-saccharides treatments were applied

Dual-Saccharides Treatments	%Crystallinity
Glucose	100
Glucose+Fructose	58.8
Glucose+Maltose	1.58
Glucose+Lactose	12.1
Fructose	100
Fructose+Sucrose	0
Fructose+Maltose	0
Fructose+Lactose	2.2

Conclusion

This study demonstrates that the combination of dual-saccharides, specifically glucose and lactose, significantly enhances the production and mechanical properties of bacterial cellulose (BC) produced by *K. xylinus*. The optimal combination of glucose and lactose yielded the highest BC production, confirmed by a BC coefficient of 0.007 g/g. Characterization of the purified BC using SEM, FTIR, and XRD further validated its suitability for biomedical applications, particularly in wound healing. This finding highlights the potential of dual-saccharides to improve BC production, aligning with the increasing demand for high-performance biomaterials in the biomedical field.

Acknowledgements

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IN SILICO AND MS/MS-BASED APPROACHES TO INVESTIGATE PROTEIN-PROTEIN INTERACTION NETWORKS IN *Staphylococcus aureus* BIOFILM

(Pendekatan Berasaskan *In Silico* dan MS/MS Untuk Mengkaji Jaringan Interaksi Protein-Protein dalam Biofilem *Staphylococcus aureus)*

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Abstract

Staphylococcus aureus is a Gram-positive pathogen inhabiting soft tissues like the epidermis and nasal cavity. Currently, there is limited knowledge of the protein-protein interaction (PPI) networks in *S. aureus* biofilm. The present study aimed to characterize *S. aureus* proteins and their interaction networks using an *in silico* approach and to identify the proteins expressed in *S. aureus* biofilm using tandem mass spectrometry. Initially, a preliminary characterization of the PPI networks in *S. aureus* was conducted using the STRING 12.0 database. Subsequently, *S. aureus* biofilm was developed in a 6-well microplate and harvested at 6 h, 12 h, 18 h, and 24 h. The expression of proteins in *S. aureus* biofilm was determined using a combination of one-dimensional SDS-PAGE and HPLC-ESI-MS/MS. The *in silico* results demonstrated that 147 biological processes, 46 molecular functions, 17 cellular components, and 15 biological pathways were significantly enriched (p <0.05) in the PPI networks of *S. aureus*. *S. aureus* biofilm proteins identified from the SDS-PAGE gel bands, such as L-lactate dehydrogenase (quinone), chaperone protein DnaK, and serine hydroxymethyltransferase, corroborated the findings obtained from the preliminary *in silico* work. In conclusion, the formation of biofilm by *S. aureus* may involve complex PPI networks.

Keywords: biofilm, *in silico*, protein-protein interaction network, *Staphylococcus aureus*, tandem mass spectrometry

Abstrak

Staphylococcus aureus adalah patogen Gram-positif yang mendiami tisu lembut seperti epidermis dan rongga hidung. Pada masa ini terdapat pengetahuan terhad tentang rangkaian interaksi protein-protein dalam biofilm *S. aureus*. Kerja-kerja ini dilakukan untuk mencirikan protein *S. aureus* dan rangkaian interaksinya menggunakan pendekatan dalam siliko dan untuk mengenal pasti protein yang dinyatakan dalam biofilm *S. aureus* menggunakan spektrometri jisim tandem. Pencirian awal rangkaian interaksi protein-protein dalam *S. aureus* telah dilakukan menggunakan pangkalan data STRING 12.0. Kemudian, biofilm *S. aureus* telah dibangunkan dalam plat mikro 6-telaga dan dituai pada 6 jam, 12 jam, 18 jam dan 24 jam. Ekspresi protein dalam biofilm *S. aureus*

ditentukan menggunakan gabungan SDS-PAGE satu dimensi dan HPLC-ESI-MS/MS. Keputusan kajian *in silico* menunjukkan bahawa terdapat 147 proses biologi, 46 fungsi molekul, 17 komponen selular, dan 15 laluan biologi didapati diperkaya dengan ketara ($P<0.05$) dalam rangkaian interaksi protein-protein *S. aureus*. Protein biofilm *S. aureus* yang dikenalpasti daripada jalur gel SDS-PAGE seperti L-lactate dehydrogenase (quinone), protein pendamping DnaK, dan serine hydroxymethyltransferase, mengesahkan penemuan yang diperoleh daripada kajian awal *in silico*. Kesimpulannya, pembentukan biofilm oleh *S. aureus* mungkin melibatkan rangkaian interaksi protein-protein yang kompleks.

Kata kunci: biofilem, *in silico*, jaringan interaksi protein-protein, *Staphylococcus aureus*, spektrometri jisim ganda

Introduction

Biofilm formation is a vital adaptation and survival mechanism widely utilized by some microorganisms. It initiates with cellular attachment to a surface, followed by multiplication, maturation, and synthesis of a polymeric extracellular matrix, microbial dispersion, and ultimately, colonization of new surfaces. Biofilms are organized microbial sessile cell populations bound to surfaces, comprising polysaccharides, DNA, and other components within a self-produced extracellular matrix [1]. They exhibit heightened resistance to antimicrobial agents, significantly impacting the treatment of biofilm-related pathogens. Typically, biofilms are highly heterogeneous due to the mixture of single-layer and multilayer cells [2, 3]. According to Chajęcka-Wierzchowska et al. [4], this heterogeneous property often contributes to the survival of biofilms during antimicrobial therapy.

One of the important biofilm-forming pathogens is *Staphylococcus aureus*, an aerobic-anaerobic, non-motile, Gram-positive bacterium that is catalase-positive and does not form spores. In humans, this pathogen often causes life-threatening diseases such as endocarditis and toxic shock syndrome. Due to its role as a common etiological agent of food intoxications, understanding and investigating *S. aureus* is crucial for safeguarding food safety and preventing outbreaks of gastroenteritis, particularly given its prevalence in many countries worldwide as a cause of foodborne illnesses. Diagnostic procedures for *S. aureus* commonly include coagulase, hemolysins, thermostable deoxyribonuclease tests, PCR, ELISA, and ELFA [5]. Common surface proteins of *S. aureus* include Sdr proteins, which are cell wall-associated proteins characterized by large serine-aspartate repeat domains that play a role in the adhesion process [6]. The subfamilies of Sdr protein in *S. aureus*, namely SdrC, SdrD, and SdrE, share a conserved structural organization; however, only SdrC is essential for biofilm growth via homophilic interaction [7, 8]. Previous *in silico* work has demonstrated that several antigenic proteins of *S. aureus* possess multiple functional linkages in a complex protein-protein interaction (PPI) network [9].

Proteins rarely perform their functions in isolation. Instead, the coordination of diverse cellular processes arises from functional interactions among several proteins and other biomolecules. Therefore, understanding PPI networks is crucial to identify which groups of proteins form functional units and underlie a wide range of metabolic and developmental processes [10]. The PPI networks in *S. aureus* biofilm have partially been elucidated using an *in silico* approach [11]. Investigating these networks provides insights into the mechanisms underlying biofilm formation and offers potential targets for developing novel antimicrobial strategies to combat biofilm-associated infections. However, there is still a lack of information about networks associated with major metabolic pathways in *S. aureus* biofilm. Therefore, the present study aimed to characterize *S. aureus* proteins and their interaction networks using an *in silico* approach, and to identify the proteins expressed in *S. aureus* biofilm using SDS-PAGE combined with tandem mass spectrometry.

Materials and Methods

Preparation of proteome dataset

A list of all proteins (799 proteins) expressed in *S. aureus* was obtained from the World-2DPAGE Repository, while the FASTA sequences of all *S. aureus* proteins were downloaded from UniProtKB [9]. Antigenicity, subcellular localization, and functional categories of all *S. aureus* proteins were analysed using VaxiJen, CELLO2GO, and UniProtKB, respectively.

***In silico* analysis of protein-protein interaction networks**

A protein-protein interaction network was constructed using the STRING database, where functional linkages were predicted based on neighbourhood, co-expression, fusion-fission events, text mining, occurrence, and physical interactions [12]. Topography, functional linkages, and hub proteins were analysed based on the constructed network. The predicted functional linkages based on protein co-expression were validated by analysing the whole-cell proteome expression using a combination of one-dimensional SDS-PAGE and HPLC–ESI-MS/MS.

Preparation of test microorganism

S. aureus ATCC 33592 obtained from Microbiology Laboratory, Faculty of Applied Sciences, UiTM Shah Alam, Selangor was grown at 37°C in tryptic soy broth. The bacterial density was adjusted to 0.7 at 600 nm for biofilm formation assay.

Microscopic analysis

S. aureus and glass coverslip were incubated in a 6-well microplate for 6 h, 12 h, 18 h, and 24 h at 37 °C. After incubation, glass coverslips were rinsed with distilled water and submerged in 1 % crystal violet solution for 10 min. Then, glass coverslips were blot dry at room temperature and observed under light microscopy at 100x magnification [13]. The microscopic analysis was performed in triplicates.

Whole-cell protein extraction

Staphylococcus aureus was grown in 6-well microplates for 6 h, 12 h, 18 h and 24 h at 37 °C. Then, whole-cell protein extraction was performed as previously described [12]. The nutrient medium containing stationary-phase planktonic cells was discarded whilst biofilm fraction was rinsed with distilled water twice to remove non-adherent cells. The biofilm fraction was then suspended in 1mL of 0.9 % NaCl and was scratched out from the wells. The solution was transferred into a 1.5mL microcentrifuge tube and centrifuged at 10000 rpm for 10 min at 4 °C. The supernatant was discarded, and the remaining pellet was vigorously dissolved in 1 mL of lysis buffer (25 mM Tris, 150 mM NaCl, 0.5 % SDS) containing 0.01 mM PMSF, and then vortexed, before being incubated at 95 °C for 15 min in a heater block, followed by another centrifugation at 10000 rpm for 10 min at 4 °C. The resulting supernatant was kept at -20 °C until further use.

Acetone precipitation

Protein samples were kept on ice to minimize degradation. Two hundred fifty microliters of protein samples and 500 µL of cold acetone were transferred into 1.5 mL microcentrifuge tubes and incubated overnight at -20 °C. Samples were then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatants were discarded, and protein pellets at the bottom of the microcentrifuge tube were stored at -20 °C. Protein concentration was determined using the standard Bradford assay protocol.

SDS-PAGE

Protein samples were heated for 5 min in boiling water, cooled to room temperature, and loaded (10 µg) onto precast 10% NuPAGE® Bis-Tris gels (8x8 cm, 1.0 mm). Electrophoresis was conducted at 200 V for 60 min using the XCell SureLock Mini-Cell system (Invitrogen, Life Technologies). BenchMark™ Protein Ladder (Novex®, Life Technologies) estimated molecular weights. Gels were stained with SimplyBlue™ SafeStain (Life Technologies) and visualized on the ChemiDoc™ MP System (Bio-Rad). This experiment was conducted in triplicates. Selected protein bands were digested using trypsin, and proteins were identified using HPLC–ESI-MS/MS.

Trypsin digestion

Protein bands were excised, destained in 50:50 acetonitrile: dH_2O with 25 mM ammonium bicarbonate (three times), vacuum-dried, and stored at -20 °C. Then, 10 µL of trypsin digest solution (12.5 µg/mL trypsin, 25 mM ammonium bicarbonate) was applied and incubated overnight at 37 °C with each gel slice. Digested peptides were isolated, dried with Speed-Vac concentrate, desalinated with C18 ZipTip (Millipore), and their concentration was measured using the NanoDrop spectrophotometer for subsequent MS/MS analysis.

HPLC–ESI-MS/MS

Peptides were separated on a Dionex EASY-Spray 75 µm × 10 cm column packed with PepMap C18 3 µm, 100 Å (Thermo Fisher Scientific) using solvent A (0.1% formic acid) and solvent B (0.1% formic acid in 100% ACN) at a flow rate of 300 nL/min with a 60 min gradient in Dionex Ultimate 3000 RSLC nano system. Peptides were analysed on a Q Exactive instrument with an EASY nanospray source at an electrospray potential of 1.5 kV. Raw data files were processed and searched using Proteome Discoverer 2.1 or 2.2, followed by data searching using the Mascot algorithm to identify proteins. The entire procedure was conducted in triplicates.

Results and Discussion

***In silico analysis* and experimental validation**

In the present study, *S. aureus* proteome was characterized in terms of antigenicity, subcellular localization, biological process, molecular function, and functional protein interactions using the *in silico* approach. The expression of characterized proteins and the PPI networks in *S. aureus* biofilm was further verified using a combination of 1D SDS-PAGE and HPLC-ESI-MS/MS. The approach taken in the present study agrees with Rao et al. [14] stating that *in silico* data obtained from the prediction of PPI networks can be verified by various experimental methods including a combination of SDS-PAGE and mass spectrometry.

Antigenicity of protein

A total of 497 *S. aureus* proteins (62%) were predicted to be antigenic proteins, including enolase, phosphomevalonate kinase, elongation factor Tu, chaperone protein DnaK, catabolite control protein A and serine hydroxy methyltransferase. *S. aureus* presents a unique challenge for vaccine development. Moreover, the emergence of antibiotic-resistant strains of *S. aureus* has led to significant economic loss and ineffective treatment in hospitalised patients. As a result, there is a need for the creation of a vaccine that protects against *S. aureus* [15]. For a vaccine strategy to be effective, the vaccine must contain antigens that are expressed in the host microenvironments and elicit immune responses in humans. Identifying all those antigens that are recognized by the human immune system is a prerequisite for the selection of candidates for the development of a potent subunit vaccine. Potential vaccine candidate antigens must be present in the genome of circulating isolates, highly conserved, and expressed by *S. aureus* during natural infection. In a previous study, adding *S. aureus* protein antigens to a capsular conjugate vaccine enhanced its efficacy in the prevention and treatment of experimental *S. aureus* osteomyelitis in rats [16].

Subcellular localization

Figure 1 shows the prediction of subcellular localization of protein expressed in *S. aureus*. Cytoplasmic (93.74%) was predicted to be the major subcellular localization, followed by extracellular (4.38%), membrane (1.75%) and cell wall (0.13 %).

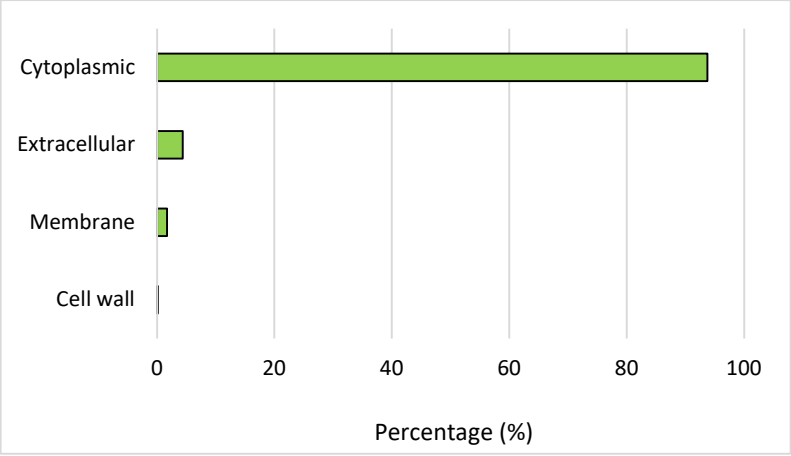


Figure 1. Subcellular localization of *S. aureus* proteins

A protein's subcellular localisation can give important information about its function. Knowledge of subcellular localisation of proteins in a cell is required to disclose the complicated pathways at the cellular level. In the present study, a total of 749 *S. aureus* proteins were predicted to be cytoplasmic including UDP-galactose 4-epimerase, phosphomevalonate kinase, ferrichrome transport ATP-binding protein and probable N-acetylglucosamine-6-phosphate deacetylase. Most of the cytoplasmic proteins are involved in cellular metabolism. Thus, proteins localized in the cytoplasm represent potential therapeutic targets [17].

Biological process

Based on Figure 2, 335 proteins (42%) of *S. aureus* were found to be involved in the cellular processes, 320 proteins (40%) in metabolic, 56 proteins (7%) in biological regulation, and 40 proteins (5%) in response stimulus. Other proteins were found to be involved in localization (24%), signalling (16%), and developmental process (8%).

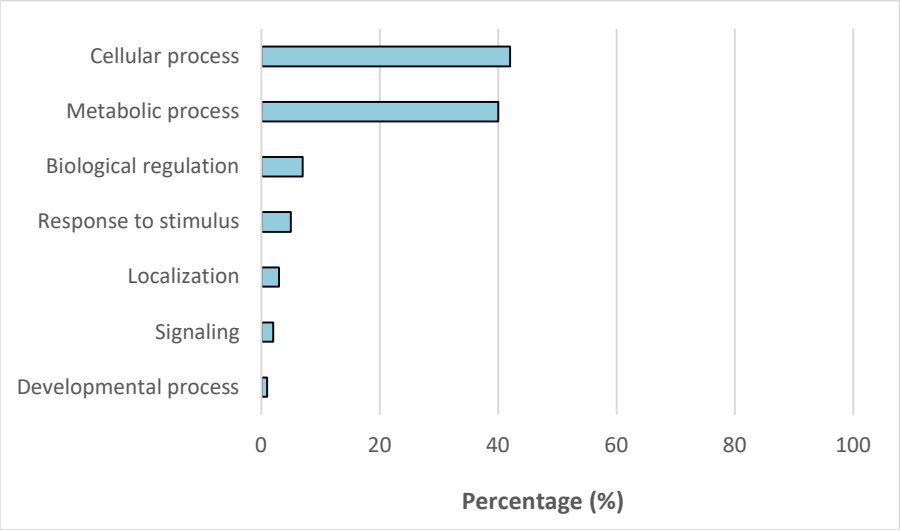


Figure 2. Function classification of *S. aureus* proteins based on biological process

S. aureus proteins involved in the cellular processes include glucokinase, cell division protein FtsA, citrate synthase and GTP-sensing transcriptional pleiotropic repressor CodY. The enzymatic reaction mediated by glucokinase is an energy-consuming mechanism. *S. aureus* is known to preserve energy by decreasing the expression level of this enzyme to suppress glycolysis [18].

Protein-protein interaction networks

A total of 799 *S. aureus* proteins were used as input data in the STRING database. Out of this number, only 437 proteins were successfully identified in the STRING database for the construction of PPI networks as the information on *S. aureus* proteome is incomplete in the STRING database. A total of 430 nodes and 958 functional interactions were produced in the network (Figure 3). Meanwhile, the clustering coefficient value was predicted to be 0.411.

STRING is an online analysis tool used to analyse PPI networks which are often depicted as graphs, with nodes representing the various proteins and edges representing the physical interactions between proteins. In a scale-free network, most proteins participate in just a few interactions (referred to as 'nodes'), but a few proteins (referred to as 'hubs') participate in more than 10 functional interactions, showing that a few hubs bind many tiny nodes. The node diameter represents the network's degree parameter, which represents the number of connections formed by the nodes [19]. Nodes with a higher degree are more vital in the network since removing these nodes will cause the network to collapse [20]. In this study, complex functional interactions in *S.*

aureus were demonstrated. The PPI networks of different metabolism pathways in methicillin-resistant *S. aureus* (MRSA) have previously been reported [21]. On the other hand, average clustering coefficient measures the degree of interconnectivity in the neighbourhood of a node. It shows the overall tendency of nodes to form clusters. According to Hao et al. [22], topological measures of network density such as the clustering coefficient is influenced by hub proteins. The high clustering coefficient values shown in the present study (>0.4) may promote cell self-organization in the natural networks, which are important to make cells more robust to perturbation [23].

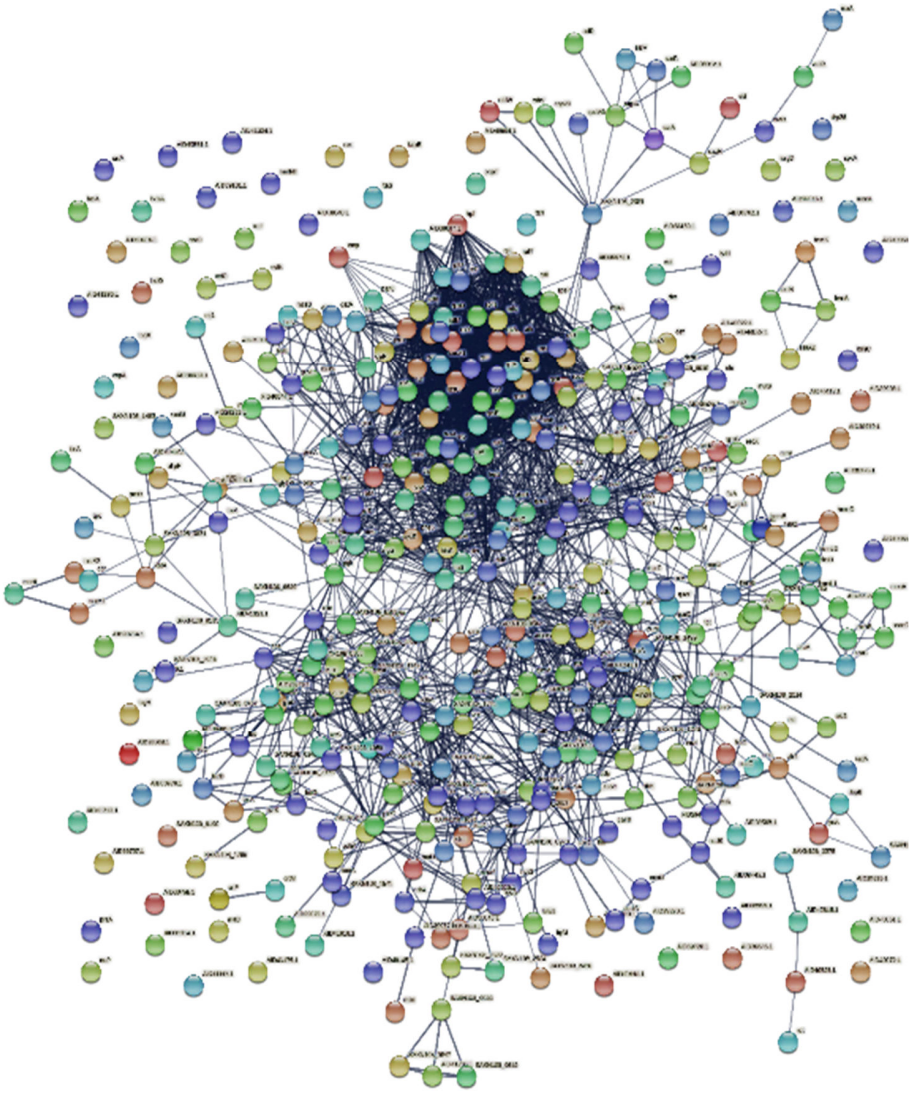


Figure 3. Functional interactions among *S. aureus* proteins

Hub proteins in *S. aureus*

There were 421 (96 %) proteins predicted as hub proteins as they showed more than 10 functional interactions with other proteins in the network, including 50S ribosomal protein L21, elongation factor Tu and uridine kinase. Hubs, by definition, bind to many proteins. Previously, proteins with high connectivity were shown to interact to proteins with low connectivity,

and they typically bind to proteins from the same evolutionary period. These proteins play critical roles in closely linked subnetworks related to specific biological processes [24]. Hub proteins in the PPI network of MRSA represent new attractive drug development opportunities, as the discovery of specific inhibitors has the potential to expand our arsenal of antibiotics that may help address the problem of increasing antibiotic resistance [25]. It has been understood that the PPI network provides multiple drug targets, which are often sufficient to control diseases. According to Csermely et al. [26], drugs with multiple targets have a better chance of affecting the complex equilibrium of whole cellular networks than drugs that act on a single target.

Functional enrichment

A total of 147 biological processes, 46 molecular functions, 17 cellular components and 15 biological pathways were significantly ($P<0.05$) enriched in the PPI networks of *S. aureus*. Table 1 shows the enriched biological processes, including the cellular nitrogen compound metabolic process, small molecule metabolic process, gene expression, and aromatic compound biosynthetic process. Table 2 shows the enriched molecular functions in *S. aureus*, including catalytic activity, binding, and transferase activity. Table 3 shows enriched cellular components including cellular anatomical entity, intracellular, cytoplasm and cytosol. Cellular anatomical entity and intracellular were the most predominant cellular component. Table 4 shows the enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways including metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments and carbon metabolism.

Table 1. Representative enriched biological processes in *S. aureus*

Functional Enrichment Biological process	**P-value**
Nitrogen compound metabolic process	$7.97E^{-37}$
Organic substance biosynthetic process	$1.00E^{-36}$
Gene expression	$3.76E^{-18}$
Translation	$3.65E^{-16}$
Ribose phosphate metabolic process	$2.72E^{-14}$
Carbohydrate derivative metabolic process	$7.89E^{-14}$
Nucleic acid metabolic process	$2.10E^{-05}$
Carbohydrate metabolic process	$9.54E^{-05}$
ATP metabolic process	$1.90E^{-03}$
Glycolytic process	$4.20E^{-03}$
RNA modification	$1.25E^{-02}$
Glucose metabolic process	$1.44E^{-02}$
Lipid biosynthetic process	$1.44E^{-02}$
RNA biosynthetic process	$1.65E^{-02}$
Cellular lipid metabolic process	$3.04E^{-02}$
Cellular response to stress	$3.65E^{-02}$

Table 2. Representative enriched molecular functions in *S. aureus*

Functional Enrichment **Molecular function**	**P-value**
Heterocyclic compound binding	$1.41E^{-22}$
Small molecule binding	$5.00E^{-17}$
Nucleotide binding	$5.25E^{-16}$
Ion binding	$1.85E^{-15}$
Carbohydrate derivative binding	$7.24E^{-14}$
Adenyl nucleotide binding	$2.88E^{-12}$
ATP binding	$3.18E^{-12}$
RNA binding	$1.49E^{-10}$
Ligase activity	$1.78E^{-10}$
Nucleic acid binding	$1.26E^{-07}$
Transferase activity	$9.92E^{-07}$
Metal ion binding	$1.27E^{-06}$
GTP binding	$2.07E^{-02}$
Zinc ion binding	$2.97E^{-02}$
Ribonucleoprotein complex binding	$4.21E^{-02}$
Oxidoreductase activity, acting on a sulfur group of donors	$4.61E^{-02}$
Kinase activity	$4.62E^{-02}$

Table 3. Representative enriched cellular components in *S. aureus*

Functional Enrichment **Cellular component**	**P-value**
Intracellular	$4.35E^{-70}$
Cytoplasm	$5.36E^{-59}$
Cellular anatomical entity	$2.47E^{-32}$
Cytosol	$1.55E^{-31}$
Protein-containing complex	$5.68E^{-11}$
Ribosomal subunit	$7.50E^{-07}$
Ribosome	$1.23E^{-06}$
Cytosolic ribosome	$1.23E^{-06}$
Organelle	$1.23E^{-06}$
Intracellular organelle	$1.68E^{-06}$
Intracellular non-membrane-bounded organelle	$2.34E^{-06}$
Large ribosomal subunit	$4.55E^{-05}$
Cytosolic large ribosomal subunit	$6.64E^{-05}$
Catalytic complex	$2.00E^{-03}$
Small ribosomal subunit	$1.60E^{-02}$
Cytosolic small ribosomal subunit	$2.10E^{-02}$
Proton-transporting ATP synthase complex	$3.36E^{-02}$

Table 4. Representative enriched biological pathways in *S. aureus*

Functional Enrichment KEGG Pathway	P-value
Metabolic pathways	$1.80E^{-15}$
Biosynthesis of secondary metabolites	$1.39E^{-08}$
Aminoacyl-tRNA biosynthesis	$9.99E^{-07}$
Ribosome	$8.20E^{-06}$
Microbial metabolism in diverse environments	$2.29E^{-05}$
Carbon metabolism	$2.29E^{-05}$
One carbon pool by folate	$2.20E^{-03}$
Glycolysis / Gluconeogenesis	$2.60E^{-03}$
Purine metabolism	$2.60E^{-03}$
Pyruvate metabolism	$1.11E^{-02}$
Pentose phosphate pathway	$1.56E^{-02}$
Arginine and proline metabolism	$1.74E^{-02}$
D-Alanine metabolism	$2.51E^{-02}$
Methane metabolism	$2.62E^{-02}$
Citrate cycle (TCA cycle)	$4.05E^{-02}$

In this study, the rank-based Gene Ontology (GO) term enrichment of PPI networks identified biological processes and molecular function annotations associated with *S. aureus* proteins. The selection criterion was p-value; the closer the values were to zero, the more significant the enrichment. Enriched biological processes, such as cellular metabolic process, and translation in the PPI networks of *S. aureus* have previously been reported [27].

Surface coverage of biofilm

Figure 4 shows the images of biofilm formation by *S. aureus* on glass coverslips under light microscope at 100x magnification. The surface coverage increased with increasing incubation time.

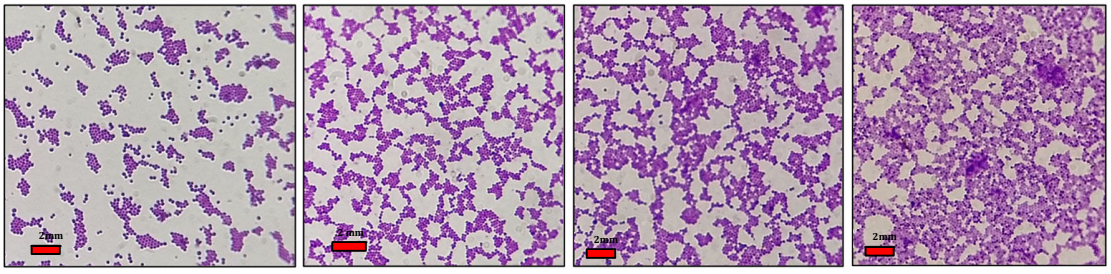


Figure 4. Formation of *S. aureus* biofilm on glass coverslip under light microscope (100x magnification). A) 6 h; B) 12 h; C) 18 h; D) 24 h

Light microscope images depicted the structure of biofilm architecture. The formation of biofilm began with the attachment of free-floating microorganisms to surfaces leading to formation of a sessile population.

Biofilms were then observed to adhere to the surface of glass coverslips and form thick aggregates. The observation shown in the present study agrees with Bai et al. [13] who also observed the clumping of complex biofilm under a light microscope.

Proteome profile of *S. aureus* biofilm

Figure 5 shows the protein profile of *S. aureus* biofilm at different incubation periods. The longer incubation period showed more protein bands in *S. aureus* biofilm. Major protein bands in each biofilm stage were as follows: 6 h – 36 kDa, 26 kDa; 12 h: 47 kDa, 37 kDa; 18 h: 47 kDa, 36 kDa, 33 kDa; 24 h: 80 kDa, 60 kDa, 58 kDa, 47 kDa, 35 kDa, 32 kDa, 30 kDa, 19 kDa. Protein bands of 27 kDa and 36 kDa were found to be expressed in 6h biofilm but no other biofilm stages. Protein bands of 80 kDa, 60 kDa, 58 kDa, 47 kDa, 35 kDa, and 32 kDa expressed in 24 h biofilm were selected for protein identification using MS/MS as they were found to be consistent across all experimental replicates.

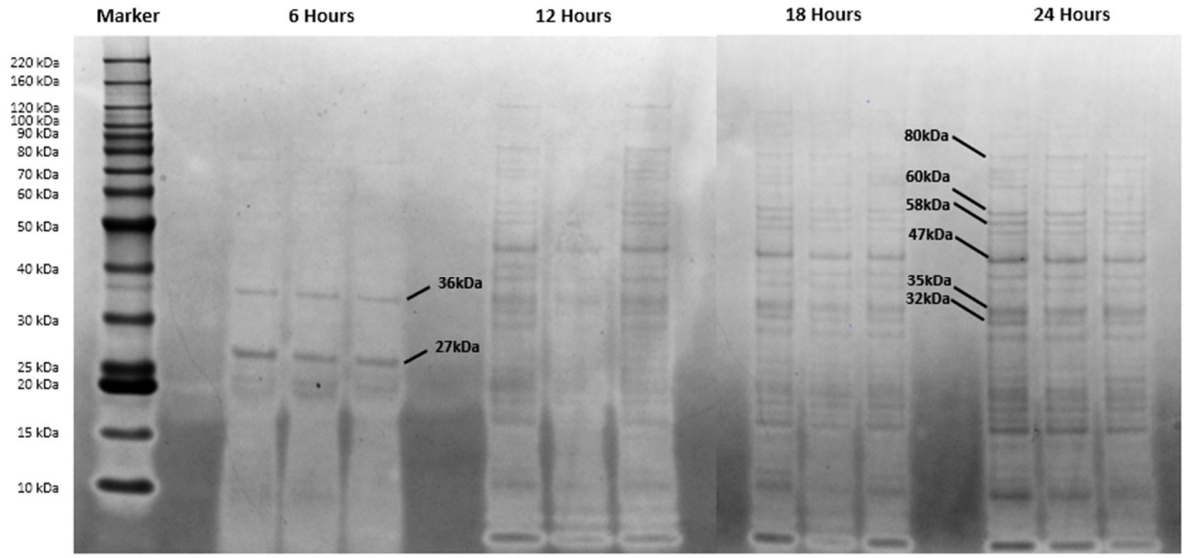


Figure 5. Whole cell protein expression in *S. aureus* biofilm at 6 h, 12 h, 18 h, and 24 h

One-dimensional SDS-PAGE is widely acknowledged as a useful technique for separating all types of proteins, including hydrophobic and low-molecular-mass proteins. The use of this approach in biofilm research is vital since biofilms comprise a diverse spectrum of hydrophobic proteins [12]. SDS-PAGE analysis was successfully used to obtain the whole-cell protein profile of *S. aureus* biofilm in the present study. Protein bands of *S. aureus* biofilm detected in the range between 25 – 80 kDa herein have also been reported elsewhere [28]. The banding pattern of *S. aureus* biofilm proteins was slightly changed across different developmental stages (6 – 24 h). This finding is in line with Lee et al. [29] who found that the protein banding pattern of *Vibrio vulnificus* biofilm slightly changes due to the different heat shock conditions.

MS/MS-based identification of *S. aureus* biofilm proteins

Based on Table 5, a total of 55 proteins were identified in the 24 h biofilm. Of these, 18 identified proteins were previously characterized in the preliminary *in silico* analysis including enolase, protein translation elongation factor G, citrate synthase, and isocitrate dehydrogenase.

Table 5. A list of *S. aureus* proteins expressed in 24 h biofilm. The biofilm proteins characterized in the preliminary *in silico* analysis are indicated by (*)

QUERY ACCESSION	MASCOT SCORE	PEPTIDE MATCH	SEQUENCE COVERAGE	PROTEIN NAME	E-VALUE	% IDENTITY
80kDa-57kDa						
P65424 *	109	6	13.05	L-lactate dehydrogenase (quinone)	0	99.80
A7WYS8	31	1	2.42	lysyl-tRNA synthetase	0	100.00
Q2FK44	18	1	2	formate C-acetyltransferase	0	100.00
T1YCX2 *	116	3	10.18	Malate:quinone oxidoreductase	0	100.00
A0A1Q8DF98	32	1	3.46	2-oxo acid dehydrogenase subunit E2	0	100.00
A0A033UQ54	27	1	1.98	ATP synthase subunit alpha	0	100.00
A0A033V5S8 *	14	1	0.95	carbamoyl-phosphate synthase large chain	0	100.00
A1A766 *	147	11	29.31	chaperone protein DnaK	0	57.69
A4WDC0 *	59	3	10.31	serine hydroxymethyltransferase	$1.00E^{-158}$	57.66
A0A133Q6X4 *	62	3	5.76	enolase	$7.00E^{-06}$	100.00
P64028 *	122	4	12.18	protein translation elongation factor G	$2.00E^{-07}$	100.00
O68883 *	217	5.00	14.75	citrate synthase	$3.00E^{-11}$	100.00
P08200 *	41	3	12.02	isocitrate dehydrogenase [NADP]	$6.00E^{-179}$	63.57
47kDa-32kDa						
A7X0T9	79	6	6.45	Bifunctional autolysin	0	100.00
Q6GDG7	61	2	5.35	Arginine deiminase	0	100.00
Q2YU83	60	2	6.84	Bi-component leukocidin LukGH subunit H	0	100.00
Q2YSE8	57	5	11.29	Phosphopyruvate hydratase	0	100.00
P78386	56	2	4.93	Hypothetical protein	$3.00E^{-24}$	63.75
Q2YVT4	54	2	4.18	Autolysin/adhesin Aaa	$2.00E^{-148}$	99.67
P99063	51	3	13.23	Alpha-ketoacid dehydrogenase subunit beta	0	99.69
Q6G723	47	1	5.95	CHAP domain-containing protein	$7.00E^{-107}$	99.26
Q2YXZ4	43	1	3.10	Glycine glycyltransferase FemB	0	100.00
P63871	40	4	13.55	TPA: cysteine synthase A	0	99.35
Q6GE15	40	2	6.41	Immunoglobulin-binding protein Sbi	0	100.00
Q2FZ75	36	1	4.44	Aspartate carbamoyltransferase catalytic subunit	0	100.00
Q4L6B1	31	1	2.06	Toxic anion resistance protein	0	92.54
B7JX26 *	26	1	3.79	S-adenosylmethionine synthase	$5.00E^{-172}$	59.85
B0UWL1	22	1	4.79	Recombinase RecA, partial	$4.00E^{-153}$	73.78
P68820 *	21	2	6.31	Phosphoglycerate kinase	0	100.00
O86793	21	1	2.94	tRNA	$1.00E^{-75}$	49.38

Q2FXM9 *	20	2	3.76	Pyruvate kinase	0	100.00
Q2YXZ5	19	1	4.52	Factor essential for expression of methicillin resistance	0	100.00
A6U4Y2 *	19	3	10.66	L-lactate dehydrogenase	0	100.00
Q6GAU2	16	1	4.11	Beta-ketoacyl-ACP synthase II	0	100.00
Q56194 *	18	2	3.65	Catabolite control protein A	0	83.89
Q2FY06 *	20	1	6.02	GTPase Era	0	100.00
A0A0U1MF82 *	183	6	16.24	Elongation factor Tu	0	100.00
A0A0E1X6S7	176	6	6.96	Mmannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	0	100.00
A0A0E1X9I3	149	2	3.77	N-acetylmuramoyl-L-alanine amidase Sle1	$2.00E^{-166}$	100.00
A0A0E0VM62	122	3	8.31	Beta-cyano-L-alanine synthase	$3.00E^{-177}$	99.68
A0A380DJ06	66	1	3.69	Acid phosphatase	0	100.00
Q9AFB0	59	1	6.29	Leukocidin S subunit	0	99.65
A0A7U7EZ63	49	3	6.25	Methionine adenosyltransferase	0	100.00
I0B738	47	1	5.82	ScaC, partial	$2.00E^{-85}$	100.00
A0A380DRF9	45	1	7.98	Lipoprotein, putative	$2.00E^{-96}$	100.00
A0A380DZ84	43	1	3.08	FemB, factor involved in methicillin resistance / Glycine interpeptide bridge formation	0	100.00
A0A0H3JYS2	42	1	4.58	Alpha/beta hydrolase	0	100.00
A0A0E0VSM0	34	1	3.40	F0F1 ATP synthase subunit beta	0	100.00
A0A033V625	31	1	2.00	Hypothetical protein V070_00476	0	100.00
A0A0U1MI53 *	29	1	4.32	Pyruvate dehydrogenase E1 component subunit alpha	0	100.00
A0A0E0VMF5	26	1	4.97	Hypothetical protein ST398NM01_0409	0	100.00
A0A1Q8DG34 *	24	1	3.32	Histidine--tRNA ligase	0	100.00
A0A2S6DGJ5	23	1	3.87	Type I glyceraldehyde-3-phosphate dehydrogenase	0	100.00
A0A0D1IBE9	23	1	4.64	ABC transporter substrate-binding protein	0	100.00
A0A1Q8DCE4	21	1	3.22	tRNA (adenosine(37)-N6)-Threonylcarbamoyltransferase complex transferase subunit TsaD	0	100.00

HPLC–ESI-MS/MS data acquisition is nearly fully automated, which enables analysis in a high throughput mode leading to low variations, thus, improving the confidence of the results. Romeu et al. [30] have used this protein identification approach to determine the amount and the distribution of proteins expressed in cyanobacterial strains in planktonic and biofilm stages. In our study, one of MS/MS-identified proteins is enolase, which was consistently expressed in the 24 h biofilm. This finding suggests that enolase may be helpful for *S. aureus* adhesion. This suggestion is supported by the fact that enolase functions as a surface sensing receptor and clumping factor, initiating surface colonization [31]. The significantly higher transcript level of enolase in the first hours of growth under biofilm conditions compared to growth under planktonic conditions suggests the importance of this gene for the first phase of biofilm growth, in which bacterial cells interact with the extracellular ligands of a host [32].

Protein-protein interaction networks of MS/MS-identified proteins

A total of 18 identified proteins of *S. aureus* biofilm were used as input data in the STRING database. Only 15 proteins were successfully identified in the STRING database for PPI network construction. Our results showed that 15 nodes and 13 functional interactions of nodes and functional interactions, respectively were produced in the network. Table 6 shows the GO enrichment analysis. A total of 9 biological processes, 9 molecular functions, 2 cellular components and 5 KEGG pathways were significantly ($P<0.05$) enriched in the PPI networks of *S. aureus* biofilm. Several enriched biological processes, molecular function and cellular component revealed by the network (Table 6) appear to verify the findings obtained from the preliminary *in silico* analysis (Figure 3, Table 1).

Table 6. Functional enrichment of PPI networks constructed using MS/MS-identified proteins of *S. aureus* biofilm. The biological process, molecular function, cellular component, and pathway characterized in the preliminary *in silico* analysis are indicated by (*)

Functional Enrichment	**P-value**
Biological process	
Generation of precursor metabolites and energy *	$2.40E^{-04}$
Glycolytic process *	$2.40E^{-04}$
Ribonucleotide metabolic process *	$5.20E^{-04}$
Carboxylic acid metabolic process *	$2.20E^{-03}$
Organic substance catabolic process *	$7.20E^{-03}$
Cellular response to organic substance	$1.18E^{-02}$
Small molecule metabolic process *	$1.28E^{-02}$
Organonitrogen compound metabolic process *	$1.29E^{-02}$
Cellular nitrogen compound metabolic process *	$1.74E^{-02}$
Molecular Function	
GTPase activity	$2.07E^{-02}$
Purine ribonucleotide binding *	$2.07E^{-02}$
Purine ribonucleoside triphosphate binding *	$2.07E^{-02}$
Small molecule binding *	$2.07E^{-02}$
Anion binding *	$2.07E^{-02}$
Catalytic activity *	$2.34E^{-02}$
Ion binding *	$2.34E^{-02}$
Protein-containing complex binding	$3.28E^{-02}$
Translation elongation factor activity	$3.53E^{-02}$
Cellular Component	
Intracellular *	$1.88E^{-02}$
Cytoplasm *	$3.35E^{-02}$
KEGG Pathway	
Glycolysis / Gluconeogenesis *	$4.40E^{-04}$
Carbon metabolism *	$4.40E^{-04}$
Microbial metabolism in diverse environments *	$6.50E^{-04}$
Biosynthesis of amino acids	$7.20E^{-04}$
Biosynthesis of secondary metabolites *	$3.10E^{-03}$
Pyruvate metabolism *	$4.32E^{-02}$

Cherkasov et al. [25] systematically identified the PPI networks for the hospital-acquired MRSA-252 strain using a high-throughput pull-down strategy combined with quantitative proteomics. They identified 13,219 interactions involving 608 MRSA proteins and revealed highly connected hub proteins. Interestingly, many existing clinical and experimental antimicrobial targets in the PPI networks of *S. aureus* were found to occupy peripheral positions with relatively few interacting partners. The hub proteins identified in MRSA's PPI networks, essential for network integrity and stability, have largely been overlooked as drug targets. However, Cherkasov et al. [25] did not correlate the observed protein interaction networks with the MRSA biofilm stage. Kang et al. [11] reported PPI networks among differentially expressed genes in various biofilm stages of *S. aureus*, with GO results indicating enrichment in biological processes such as cell adhesion and pathogenicity. However, their study lacked comprehensiveness, utilizing only 32 differentially expressed genes to construct the network. Our study demonstrated enriched metabolic pathways in the PPI networks of *S. aureus* biofilm, including glycolysis, amino acid biosynthesis, secondary metabolite biosynthesis, and pyruvate metabolism. These findings provide the first evidence of a more comprehensive PPI network of *S. aureus* biofilm, potentially aiding in the identification of novel antimicrobial drug targets to control *S. aureus* infections.

General discussion

The PPI networks play a crucial role in understanding the pathogenic mechanisms of infectious agents. These networks elucidate the complex web of interactions between pathogen proteins and host proteins, shedding light on the molecular mechanisms underlying infection [35]. By mapping out these interactions, researchers can identify key virulence factors and host targets, offering insights into potential drug targets for therapeutic intervention [36]. The PPI networks also facilitate the prediction of protein functions and pathways, aiding in the characterization of novel virulence factors and their roles in pathogenesis [37]. Identification of drug targets based on the PPI networks for other biofilm-forming pathogens such as *Salmonella Typhi* [38], *Pseudomonas aeruginosa* [39], and *Vibrio parahaemolyticus* [40] has also been reported.

Conclusion

Our *in silico* analysis has shown that the PPI networks in *S. aureus* involve various biological pathways and numerous hub proteins. Further MS/MS-based identification of *S. aureus* biofilm proteins, including L-lactate dehydrogenase (quinone), malate: quinone oxidoreductase, carbamoyl-phosphate synthase large chain, and chaperone protein DnaK, corroborates the findings from the preliminary *in silico* analysis. This study is the first comprehensive effort to characterize the PPI networks of *S. aureus* biofilm, potentially aiding in drug and vaccine development. Future investigations into the specific protein complexes or pathways identified in these networks may enhance the development of targeted therapeutic strategies for virulence and antibiotic resistance. Furthermore, integrating proteomic data with other omics approaches, such as transcriptomics and metabolomics, could provide a comprehensive understanding of the molecular mechanisms underlying biofilm formation.

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MOLECULAR DYNAMICS SIMULATION OF DTPA WITH $CaCO_3$ and FeS

(Simulasi Dinamik Molekul DTPA Dengan $CaCO_3$ dan FeS)

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Abstract

In the production of oil and gas, calcium carbonate ($CaCO_3$) and iron sulphide (FeS) are among mineral scale deposits mainly found in tubing and valves located at surface facilities, which have been a nuisance in the oil flow during processing. Diethylenetriaminepentaacetic acid (DTPA) has a greater affinity to form stable divalent metal ion complexes during chelation to facilitate the dissolution of oilfield solid scale. Octadentate DTPA chelating ligand occupies five carboxylic acids, and three amine groups provide potential binding sites. The interaction between the molecules in the system can be replicated through molecular dynamics simulation explicitly using a COMPASS force field and the Ewald summation method available in the Material Studio software. The radial distribution function (RDF) in simulation trajectory files was utilised to study intermolecular interactions. The RDF results showed strong hydrogen bonding between O—H_2O and H_5—DTPA at a distance of 1.75 Å. The intermolecular interaction of DTPA with H_2O in the existing $CaCO_3$ and FeS denotes the interaction shift from water to the metal ion. The carbonyl group of DTPA exhibited a more significant interaction at a radial distance of 2.25 Å and intensity of 8.81 for Fe^{2+} but lower in $Ca^{2+,}$ which is at 1.47. The amine in DTPA analysis confirmed the low intensity of $CaCO_3$ at a distance of 5.75 Å and intensity of 1.07, and a distance of 2.25 Å and intensity of 1.01 for FeS. Meanwhile, amines in DTPA—CO_3^{2-} and DTPA—S^{2-} systems demonstrated the low interaction at the same distance of 4.75 Å. The interaction of Ca^{2+} with CO_3^{2-} in the DTPA system exhibited a sharp peak and high-intensity interaction at a distance of 2.25 Å and 13.71 intensity. Nevertheless, a sharp and low-intensity peak appeared on the Fe^{2+}—S^{2-} in the DTPA system at a distance of 4.75 Å and 2.18 intensity. In conclusion, these findings suggest that the carbonyl group of DTPA has a stronger interaction with Fe^{2+} than Ca^{2+}. Meanwhile, the hydroxyl group of DTPA shows the highest intensity of interaction with CO_3^2. Additionally, Ca^{2+} ions form more significant interactions with CO_3^{2-} ions in the DTPA systems.

Keywords: diethylenetriaminepentaacetic acid, radial distribution function, molecular dynamics simulation, compass, calcium carbonate

Abstrak

Dalam pengeluaran minyak dan gas, kalsium karbonat ($CaCO_3$) dan besi sulfida (FeS) ialah mendapan kerak mineral yang boleh didapati terutamanya dalam tiub dan injap yang berada di kemudahan permukaan dan menyebabkan gangguan dalam aliran minyak semasa pemprosesan. Asid diethylenetriaminepentaasetik (DTPA) mempunyai afiniti yang lebih tertarik untuk membentuk kestabilan untuk kompleks ion logam divalen semasa pengkelatan untuk memudahkan pelarutan kerak pepejal medan minyak. Oktadentat ligan pengkelat DTPA menduduki lima asid karboksilik, dan tiga kumpulan amina menyediakan tapak pengikatan yang

berpotensi. Interaksi antara molekul dalam sistem boleh direplikasi melalui simulasi dinamik molekul secara jelas menggunakan medan daya COMPASS dan kaedah penjumlahan Ewald yang tersedia dalam perisian Material Studio. Fungsi pengedaran jejari (RDF) dalam fail trajektori simulasi telah digunakan untuk mengkaji interaksi antara molekul. Keputusan fungsi taburan jejari menunjukkan ikatan hidrogen yang kuat antara O—H_2O dengan H_5—DTPA pada jarak jejari 1.75 Å. Interaksi antara molekul DTPA dengan H_2O dalam $CaCO_3$ dan FeS sedia ada menandakan peralihan interaksi daripada air ke ion logam. Kumpulan karbonil DTPA menemui interaksi yang lebih ketara pada jarak 2.25 Å dan keamatan 8.81 untuk Fe^{2+} tetapi lebih rendah untuk Ca^{2+}, iaitu pada 1.47. Amina dalam analisis DTPA mengesahkan keamatan rendah $CaCO_3$ pada jarak 5.75 Å dan keamatan 1.07, manakala FeS pada jarak 2.25 Å dan keamatan 1.01. Sementara itu, amina dalam sistem DTPA—CO_3^{2-} dan DTPA—S^{2-} menggambarkan interaksi rendah pada jarak yang sama iaitu 4.75 Å. Ca^{2+}—CO_3^{2-} dalam sistem DTPA menunjukkan interaksi puncak yang tajam dan berintensiti tinggi pada jarak 2.25 Å dan keamatan 13.71. Namun begitu, puncak tajam dan keamatan rendah muncul pada Fe^{2+}—S^{2-} dalam sistem DTPA pada jarak 4.75 Å dan keamatan 2.18. Secara keseluruhan, penemuan ini menunjukkan bahawa kumpulan karbonil DTPA mempunyai interaksi yang lebih kuat dengan ion Fe^{2+}. Kumpulan hidroksil DTPA menunjukkan interaksi intensiti tertinggi dengan CO_3^{2-}. Selain itu, ion Ca^{2+} membentuk interaksi yang lebih signifikan dengan ion CO_3^{2-} dalam sistem DTPA.

Kata kunci: asid diethylenetriaminepentaasetik, fungsi taburan jejari, simulasi dinamik molekul, COMPASS, kalsium karbonat

Introduction

Oilfield solid scale accumulation in petroleum pipelines, which generates flow control issues while transferring crude oil and natural gas, has agained a research focus [1]. Scale formation in oil and gas pipeline networks creates the conditions for various possible disasters. Among them include valves faltering in their duty, flow succumbing to restrictive barriers, equipment suffering the scars of damage, and the corrosion of pipes or the concealed erosion of tube surfaces beneath the scale [2]. Calcium carbonate ($CaCO_3$) scale is one of the primary prevalent solid scales, particularly in tube wells [3]. The precipitation of $CaCO_3$ occurs when a conversion in temperature and pressure causes the discharge of dissolved carbon dioxide (CO_2) from aqueous to gaseous form from the circulating fluid [4, 5]. A real-scale sample revealed the presence of calcium ions (Ca^{2+}) in the precipitated silica sand solid scale of an oil well. Other elements like oxygen (O), aluminium (Al), and iron (Fe) are present in the complex combination of metal elements [6]. Meanwhile, hydrogen sulphide and iron reaction produce iron sulphide (FeS) scales [7]. Bacterial activity, thermal degradation of sulphate, and gas lift activities are the sources of hydrogen sulphide [8, 9]. Scaling or dissolving scales is accomplished using mechanical or chemical handling [10]. Nevertheless, the mechanical approach is costly and unreliable when the solid scale is difficult to retrieve and might harm the tubing [11, 12]. An inappropriate chemical selection in the cleaning procedure will accelerate the scale return and deteriorate pipe corrosion. Recently, chelating agents have been an attractive and viable alternative to organic and inorganic acids for scale removal. Chelating compounds are less corrosive to well tubes, tubular, and other downhole tools, readily biodegradable, and more ecologically friendly [13]. Chelating agents have an extremely minimal corrosion rate compared to hydrochloric acid, which is their primary attraction [14]. Fewer corrosion inhibitors are needed as there is less corrosion [15, 16]. Chelating agents with carboxylic and amine functional groups are the most prevalent dissolvers and inhibitors in solid scale dissolution [17].

This study modelled the molecular interactions associated with the dissolution of ($CaCO_3$) and iron sulphide (FeS) utilising diethylenetriaminepentaacetic acid (DTPA). For a dissolution solvent, it is essential to comprehend how the functional groups of the dissolver and the solid scale interact at the molecular level. This can give molecular insights that are used to predict how the solid scale and the solvent of interest will interact. Currently, simulation work for chelating agents and solid scale interactions is still lacking; thus, molecular dynamics simulation is required as an approach to better understand the capability of chelating agents to interact with solid scale. An intense intermolecular interaction between the functional groups of the chelating agent reflects good solid scale removal. DTPA is an octodentate ligand where three amines and five carboxylic groups can form a more stable metal-ion complex [18]. It has impressive formation constants

among the several chelating agents utilised in the petroleum industry. Synthesis and evaluation of glutamic acid hydrochloride as a potential dissolver for $CaCO_3$ exceeded 3,000 ppm dissolution at low concentrations [19]. The simulation showed a strong intermolecular interaction between the carbonyl group and Ca^{2+} ions in GLDA and Fe^{2+} ions in GLDA and GLDA-Na_4 [20]. Furthermore, according to the radial distribution function (RDF) analysis, there is evidence of hydrogen bonding between the solvent molecules and patchoulol solutes. The hydroxyl functional groups of patchoulol, specifically the oxygen atom (O1P) and hydrogen atom (H1P), played a significant role in this interaction [21].

Understanding the dissolution of solid scale removal is a crucial concern in oil and gas research. The reliance on experimental methods is insufficient for characterising the molecular-level interactions that impede the dissolution of a specific solid scale by a particular dissolver. Exploring different configurations at an atomic scale and dynamic processes, which are rigorously studied using simulation methods, are great tools for molecular dynamics modelling. The computational method known as molecular dynamics involves the treatment of atoms as individual particles that follow classical mechanics principles. This work applied molecular dynamics simulations to examine the interaction of DTPA with $CaCO_3$ and DTPA with FeS systems. The results feature improved dissolver formulas for creating new solid scale removal techniques with improved dissolution.

Materials and Methods

Molecular dynamics simulation

The exploration of atomic configuration and motion of molecules in solids and liquids is substantially aided by molecular dynamics techniques in computer simulations [22]. This method has shown to be a handy tool. The simulation of intermolecular interactions was performed using molecular dynamics simulations with the Accelrys Material Studio version 7.0 software provided by Accelrys, Inc., based in San Diego, USA. The simulation processes incorporated the Condensed-Phase Optimized Molecular Potentials for Atomistic Simulation Studies (COMPASS) force field in all instances. The processes were upgraded by incorporating the state-of-the-art-third-generation force fields, specifically the COMPASS [23]. The Verlet-velocity algorithm integrates the equations governing atomic motion numerically [24]. Separated pairs of atoms interact via the non-bonded interactions, which comprise a van der Waals (vdW) element expressed in an LJ-12-6 function and an electrostatic interaction described by a Coulombic function as per Equations (1) and (2), respectively [25, 26]. The three-dimensional structures of DTPA, $CaCO_3$, FeS, and H_2O molecules exploited in the simulation were downloaded from the ChemSpider database [27, 28, 29, 30, 31]. Table 1 summarises the total number of DTPA, $CaCO_3$, FeS, and H_2O molecules used in this research. The expression for energy in the COMPASS force field is readily summed up in Equation (3):

$$E_{vdw} = 4\varepsilon\left[\left[\frac{\sigma}{r}\right]^{12} - \left[\frac{\sigma}{r}\right]^{6}\right] \quad (1)$$

$$\sum_{i=1}^{N_A}\sum_{i=1}^{N_B}\frac{q_i q_j}{4\pi q_o r_{ij}} \quad (2)$$

$$E_{tot} = E_{bond} + E_{angle} + E_{torsion} + E_{\iota} + E_{cross} + E_{elec} + E_{LJ} \quad (3)$$

Using the COMPASS force field and the Smart algorithm, the molecular geometry was optimised for electrostatic and vdW interactions using an atom-based summation technique. This optimisation stage aimed to create a stable molecular geometry for the following stages of modelling and simulation. The simulation box was assembled utilising the Amorphous Cell module within the Material Studio software, encompassing the chelating agent (DTPA), solid scale $CaCO_3$/FeS, and water components, as illustrated in Figure 1. The system underwent minimisation using a 10,000-step (fine) Smart Minimizer. Molecular dynamics simulations were

organised by engaging the COMPASS force field and the Ewald-based summation method to account for electrostatic and vdW interactions [32, 33]. Ewald summation was employed to handle long-range electrostatic interactions [34]. The initial molecular dynamics simulation employed a microcanonical ensemble (NVE) for 1,000 ps, starting with a minor time step of 0.5 fs, and the time step was progressively increased in the early stages of the simulation to 1 fs. After that, the system was simulated for a further 1000 ps at a timestep of 1 fs using an isothermal-isobaric ensemble (NPT), which replicated the conditions in a well reservoir at 136 atm and 90°C. The temperature and pressure were maintained using Nose thermostats [35, 36, 37] and Berendsen barostats [38, 39]. Throughout the equilibration and dynamic steps, the trajectory file for each simulation system was recorded and saved every 100 ps.

Table 1. Details on the simulation parameters for the DTPA with $CaCO_3$ and DTPA with FeS intermolecular interaction systems

	Number of Molecules				Temperature,	Pressure (NPT),
System	**DTPA**	**$CaCO_3$**	**FeS**	**Water**	**°C**	**atm**
System 1	30	-	-	270	90	136
System 2	30	60	-	270	90	136
System 3	30	-	60	270	90	136

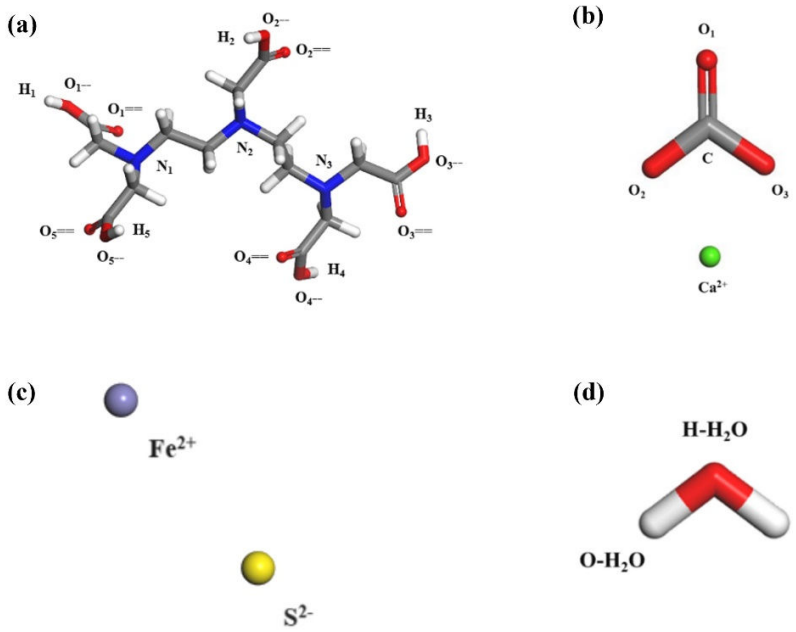


Figure 1. Diethylenetriaminepentaacetic acid (DTPA), Calcium carbonate ($CaCO_3$), Iron sulphide (FeS), Water (H_2O)

Radial distribution function analysis

The RDF was applied to the trajectory output file to examine the relationships between the assigned functional groups, which is presented in Equation (4):

$$g_{xy}(r) = \frac{\langle N_y\,(r,r+dr)\rangle}{\rho_r 4\pi r^2 dr} \quad (4)$$

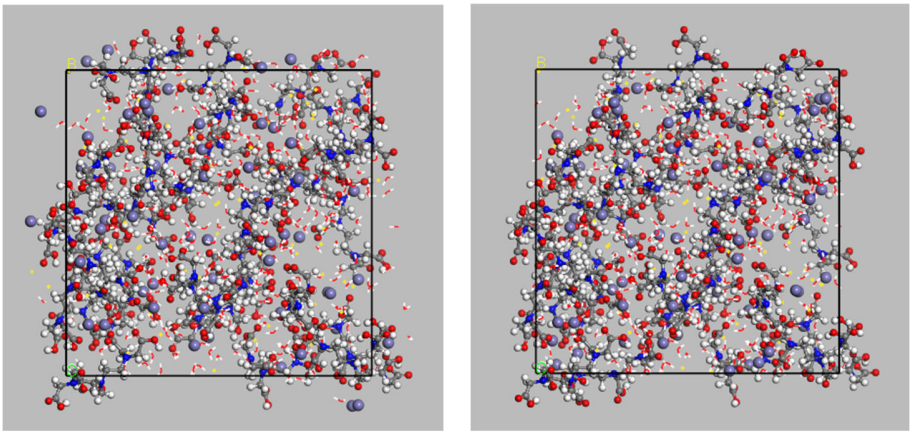
where r is the radius, ρ_y is the density of the y atom, and N_y (r, r + dr) is the number of y atoms.

Figure 1 depicts the labelling of the compounds employed in this simulation for chemical recognition. The identification numbers for the carboxyl groups of the DTPA molecule were 1 to 5. The carboxylic groups of DTPA are more practically classified into two subgroups: hydroxyl and carbonyl. The oxygens in the hydroxyl groups of DTPA were designated as O_1—, O_2—, O_3—, O_4— and O_5—. The dash character (—) describes the single bond in the hydroxyl group. Meanwhile, H_1, H_2, H_3, H_4, and H_5 were assigned to hydrogens in the hydroxyl group. The carbonyl group oxygen atoms were regarded as O_1==, O_2==, O_3==, O_4==, and O_5==. The equals sign indicates the double bond in the carbonyl group (=). The amines in DTPA were identified as N_1—DTPA, N_2—DTPA, and N_3—DTPA. The calcium ion in $CaCO_3$ and the iron ion in iron (II) sulphide were represented by Ca^{2+} and Fe^{2+}, respectively. Three oxygen carbonate ions were labelled O_1, O_2, and O_3, whereas the sulphide ion was labelled S.

Results and Discussion

Investigating the interactions between a solvent and a solid surface at a molecular level is crucial. The strength of these interactions directly correlates with the solvent ability to dissolve the solid effectively. Therefore, in this research, the RDF was employed to model the interactions between neighbouring atoms to predict the extent of intermolecular interactions. Temperature plays an essential role in improving the efficiency of metal ion complexes as heat can render the attraction of metal ions with chelating agents, thus increasing the dissolution.

From Figure 2, the DTPA molecules are initially uniformly dispersed and surrounded by water molecules. The DTPA molecules are in an extended configuration, with their carboxylate and amine groups towards the aqueous environment. Following 1,000 ps simulation at constant pressure and temperature (NPT), the DTPA molecules exhibit aggregation, resulting in a uniformly distributed DTPA and H_2O. This behaviour reflects the dissolution of DTPA in H_2O.



(a) Initial configuration (before simulation) (b) After simulation (NPT 1,000 ps)

Figure 2. Initial and final configurations of the DTPA—H_2O system

DTPA with H_2O

This section details the RDF plots illustrating water intermolecular interactions with the hydrogen and oxygen atoms within the carboxylic groups of DTPA. In DTPA with the water system, there is a strong intermolecular interaction between O—H_2O and H_5—DTPA at a distance of 1.75 Å and intensity of 2.57, with g(r) of 1.16 and 2.10 between H_1 and H_3 atoms, as shown in Figure 3. The first peak corresponds to the closest neighbour interaction between the specific types of atoms in DTPA solution, where the oxygen atoms of water show higher intensity interaction than hydrogen at a distance of 3.25 Å and 1.23 intensity. Therefore, the RDF pattern explains that the closest neighbouring interaction occurs between O—H_2O and H_5—DTPA atoms, representing strong hydrogen bonding in DTPA molecules [40, 41]. In addition, the effect of intramolecular hydrogen bonding in DTPA occurs

between a terminal acetate and a central nitrogen [42, 43]. The strength of these interactions can also be explained by water oxygen being more electronegative than hydrogen [44]. Apart from that, the hydrogen atoms of DTPA are bound to carbon and nitrogen atoms, which are less electronegative than oxygen [45, 46]. Meanwhile, the interaction still occurs for the remaining atoms of H_1 and H_3 but is lower than H_5—DTPA. At a range exceeding 9.25 Å, the intensity approaches 1.00, signifying the non-existence of long-range ordered interaction. The second peak displays a relatively weak contact between H—H_2O and O—DTPA, with a distance of 3.25 Å. An extended top curve with reduced intensity indicates that H—H_2O and O_5—DTPA interact with a g(r) value of 1.23. Both interaction results agree with the previously reported findings on the interaction of DTPA with water at 60 °C [47]. An expanded study that includes other polyaminocarboxylic acids, such as ethylenediaminetetraacetic acid and triethyl-enetetraminehexaacetic acid, is more comprehensive in demonstrating the hydrogen bonding effect. Specific evidence on the analysis for this chelating agent molecule has been proven in Raman, infrared, and nuclear magnetic resonance spectra [48, 49, 50]. Furthermore, this result implies that the sequence of the electronegativity between O-H of water and DTPA extensively agrees with the previous study [51].

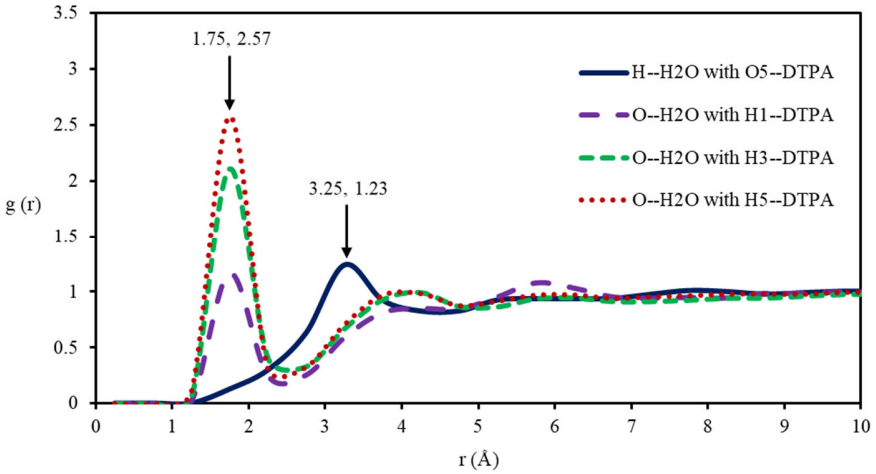


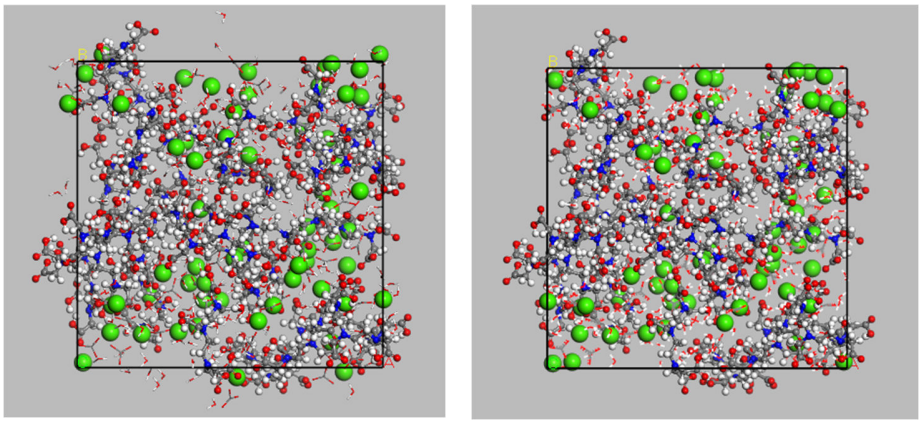
Figure 3. The g(r) plots of DTPA in water, intermolecular interactions in O--H_2O with H—DTPA and H—H_2O with O—DTPA

From Figure 4, DTPA molecules are initially disseminated randomly in an aqueous solution containing $CaCO_3$ surfaces. This dispersion phenomenon results from an extended molecule conformation, which introduces functional groups. Notable changes appear after 1,000 ps of constant pressure and temperature (NPT). The DTPA molecules on the $CaCO_3$ solid scale likely reflect the intermolecular interactions between DTPA-$CaCO_3$ molecules and water molecules.

DTPA with H_2O in the existing $CaCO_3$

This passage describes the outcome of the analysis of DTPA intermolecular interactions with water in the presence of $CaCO_3$ scale. The H_2O—DTPA interaction, namely O—H_2O with H_5—DTPA in the presence of $CaCO_3$, is shown in Figure 5. A slightly decreasing difference was detected between the O—H_2O and H_5—DTPA interaction patterns compared to Figure 6. The intensity dropped from 2.57 to 0.98 at a distance of 1.75 Å. The RDF pattern indicates that $CaCO_3$ has modified the shifting of O—DTPA with Ca^{2+} ions. This is because the O—DTPA molecule is self-stabilised on solute-solvent structures in the water. These changes also manifest the start of the chelation stage on metal ion complexes between the solvent molecules, DTPA, and solute molecules, $CaCO_3$, during dissolution. At a distance of 3.25 Å, the RDFs started to increase, and at

4, it started to fall. The intensity equals 1.00 for distances larger than 4, suggesting no long-range order exists, which indicates less of locating an interaction between hydroxyl water molecule and DTPA. An electrostatic interaction represents a long-range interaction, while a short-range interaction is signified by the vdW interaction between DTPA and H_2O. The adsorption energy of carboxymethyl inulin on calcite (104), (110), and (012) surfaces is primarily influenced by electrostatic interactions among charged atoms, hydrogen bonding, and vdW forces [52]. The discussion also addressed how the high adsorption energies of CO_2 on graphite are influenced by pore size and surface functional groups, employing both density functional theory and grand canonical Monte Carlo simulations [53]. The second peak, however, indicates a weak interaction between H—H_2O and O_5—DTPA, with a distance of 3.25 Å. By looking at a stretched top bend with moderate probability g(r) at 1.20, it is possible to demonstrate a slight difference in Figure 5.



(a) Initial configuration (before simulation) (b) After simulation (NPT 1,000 ps)

Figure 4. Initial and final configuration of the DTPA—$CaCO_3$—H_2O system

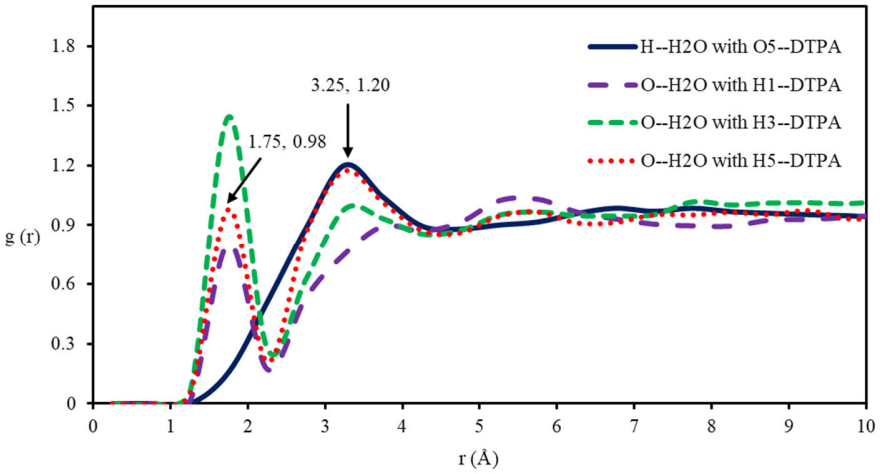


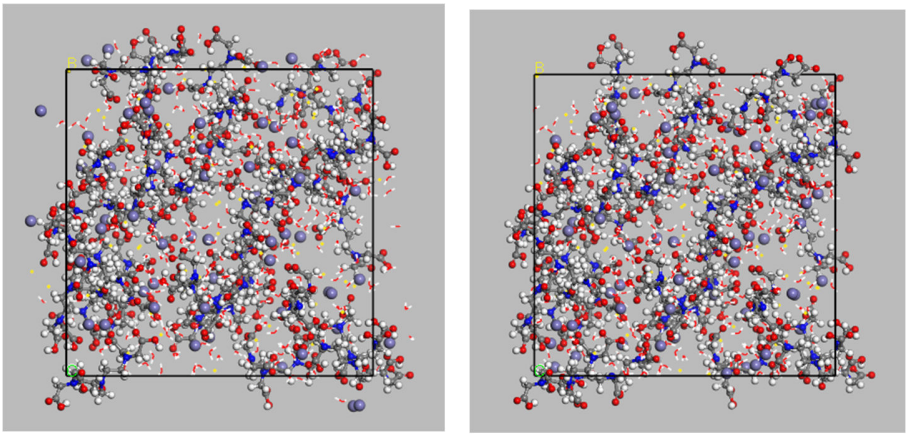
Figure 5. The g(r) plots of DTPA in water in $CaCO_3$ presence, intermolecular interactions in O—H_2O with H—DTPA and H—H_2O with O—DTPA

From Figure 6, in the first configuration of the DTPA—FeS—H_2O system, DTPA molecules expand conformation with exposed carboxylate and amine groups as they scatter randomly in the water with FeS surfaces. Following 1,000 ps simulation in a constant-pressure and temperature (NPT) ensemble, the DTPA

aggregates are formed and driven by interactions between DTPA and water molecules.

DTPA with H_2O in the existing FeS

Similar to the $CaCO_3$ interaction in Figure 8, the RDF for the interaction of DTPA with water in the existing FeS is demonstrated in Figure 7. Based on figure, the interactions between the hydrogen atoms of DTPA, which are H_1, H_3, and H_5, with the oxygen atoms in H_2O, are low, similar to all graph patterns. The distance is 3.25 Å with g(r) at 0.95 for H—H_2O with O_5—DTPA interaction. About 11.7% of the intensity value dropped significantly from 2.57 in water (Figure 6) to 0.30 for H_5—DTPA. Shifting in the water of the hydroxyl group to a lower intensity indicates bonding of the polarised O-H bond, possibly with the iron ion, Fe^{2+}. At an elevated temperature of 90 °C, this result is likely due to the weakening of hydrogen bonds between water molecules [54]. The reduction of RDF intensity affecting O-H and C=O functional groups signals the formation of a metal-ligand soluble complex [55]. Therefore, Fe^{2+} was chosen as the complex metal ion chelating with DTPA [56]. The RDF reveals the long-range disorder correlation between particles of all cases, as its values converge to 1.00 at distances greater than 4.75 Å. The non-bond interactions are used for interactions between DTPA and H_2O in the presence of FeS. It includes a Coulombic function for the electrostatic interaction and an LJ-9-6 function for the vdW term. Chemicals and rocks interact via a process known as adsorption, in which a chemical binds to the surface of a rock via vdW force interaction [57]. In addition, the Derjaguin-Landau-Verweye-Overbeek (DLVO) prediction of colloid stability has been cited in the literature to describe the adhesion phase in a fouling process. Other forms of contact, such as hydrophobic, steric, or ion bridging, can engage in addition to DLVO forces (vdW and electrostatic), and the net result is a mix of all the possible interactions [58].



(a) Initial configuration (before simulation) (b) After simulation (NPT 1,000 ps)

Figure 6. Initial and final configuration of the DTPA—FeS—H_2O system

DTPA with (calcium or iron) ion

According to the literature, the metal ion complexes event will be characterised by strong intermolecular interactions associated with the sharp and closest radial distance peaks in g(r) [20]. Figure 8 (a) shows the carbonyl and hydroxyl of DTPA. The triple carbonyl, which is O_1==, O_3==, and O_5==, has more intensity interaction g(r) than hydroxyl. The O_5== shows the highest interaction with Ca^{2+} ion at a distance of 2.25 Å, 1.47. Meanwhile, the hydroxyl group shows the RDF point with minimal intensity at a distance of 3.75 Å, 1.51 for O_5—DTPA with Ca^{2+} interaction. Although the interactions at a distance of 2.25 Å in this simulation exhibit less strength associated with O—H_2O and H_5—DTPA interaction in Figure 6, specifically at the initial radial distance of 1.75 Å, the presence of an abundant hydroxyl group in DTPA is noteworthy. This abundance could facilitate hydrogen bond formation, serving as a hydrogen donor or acceptor. As part of the metal-ion complexes process, the hydroxyl group will lose its

proton, H^+, to oxygen, which can bind and hold on to the metal ion [20]. The hydroxyl group is deprotonated with a delocalised electron form on carboxylate ions [-COO] [60]. The correlation of the long-range fluctuations of carbonyl and hydroxyl waves within the interaction with Ca^{2+} is slow with distances, as demonstrated by the peak convergence of metal ion complexes from g(r) 5.25 to 1.00 Å.

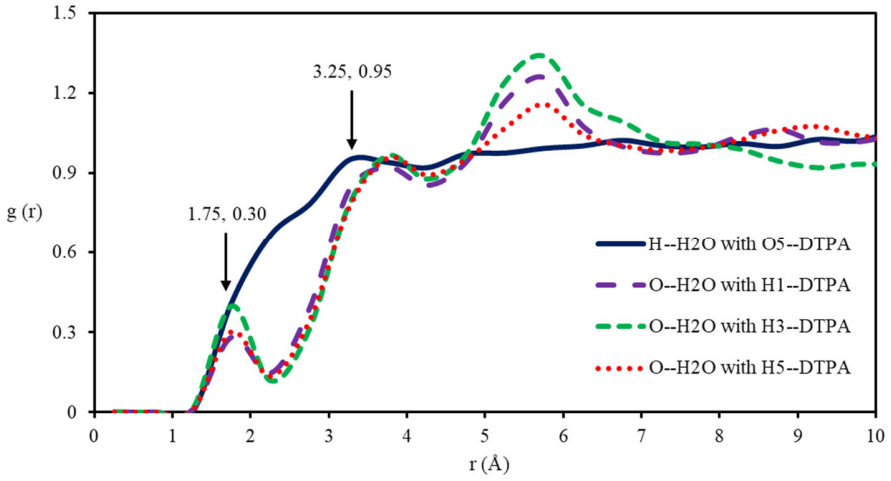


Figure 7. The g(r) plots of DTPA in water in FeS presence, intermolecular interactions in O—H_2O with H—DTPA and H—H_2O with O—DTPA

As shown in Figure 8 (b), the carbonyl group intermolecular interactions with iron are primarily significant. The first peak is identified at a distance of 2.25 Å with an intensity value of g(r) of 8.81 for the O_1==Fe^{2+} interaction. The interaction on O_3== and O_5== also shows the highest values at 7.87 and 7.90, respectively, close to O_1== intensity. The DTPA-iron interaction discloses that the carbonyl groups have more promising interactions than hydroxyl. The hydroxyl in the second peak demonstrates the broad peak, and low intensity occurs at a distance of 4.25 Å in the 2.26 – 2.65 range values. All interactions exhibit a straight line at the same point r, 4.75 Å. This simulation revealed that all carbonyls (C=O) in DTPA are more attracted to interacting with metal ions, $Fe^{2+,}$ than with H—(H_2O) in Figure 8 (b). The carboxylate groups (COO^-) in DTPA have a pair of electrons that form covalent bonds with the metal ion Fe^{2+}. The adsorbed H ions are expected to form associations with the sulphide ions of the lattice, which initiate the dissolution process [61]. The carbonyls interact on the surface at the iron lattice site and generate a charge disparity imbalance, leading to the extraction of Fe^{2+} from the FeS.

Amine in DTPA with (calcium, iron, carbonate, or sulphide) ion

Figure 9 (a) illustrates the RDF pattern depicting the intermolecular interaction between the amine functional group of DTPA and calcium ions. Three amines, N_1, N_2, and N_3, associated with DTPA exist at a distance of 5.75 Å. The findings of simulations reflect the low degree of intensity by intermolecular hydrogen bonding between amine and carboxylic groups. The results help justify the interaction of metal ions that tend to interact with the carbonyl in Figure 8 rather than the amines of DTPA itself in this simulation The DTPA carboxylic acid groups are linked to the chelating agent 'arms' and bind to calcium ions by 'grabbing' them from the solution [62]. In contrast, the nitrogen group is typically found at the molecule's centre. The study found that the metal ions in M(Glu), M(Asp), and some metal ions can form chelates over amine groups, leading to the selectivity of metal ions and their transfer through building complexes [63]. As an example, when there are positively charged amines, intramolecular hydrogen bonds are created (as seen in $C_3N\cdots H\cdots O_2C$), which can result in the acid groups of DTPA becoming unavailable for binding to barium sites on the surfaces of crystals [64].

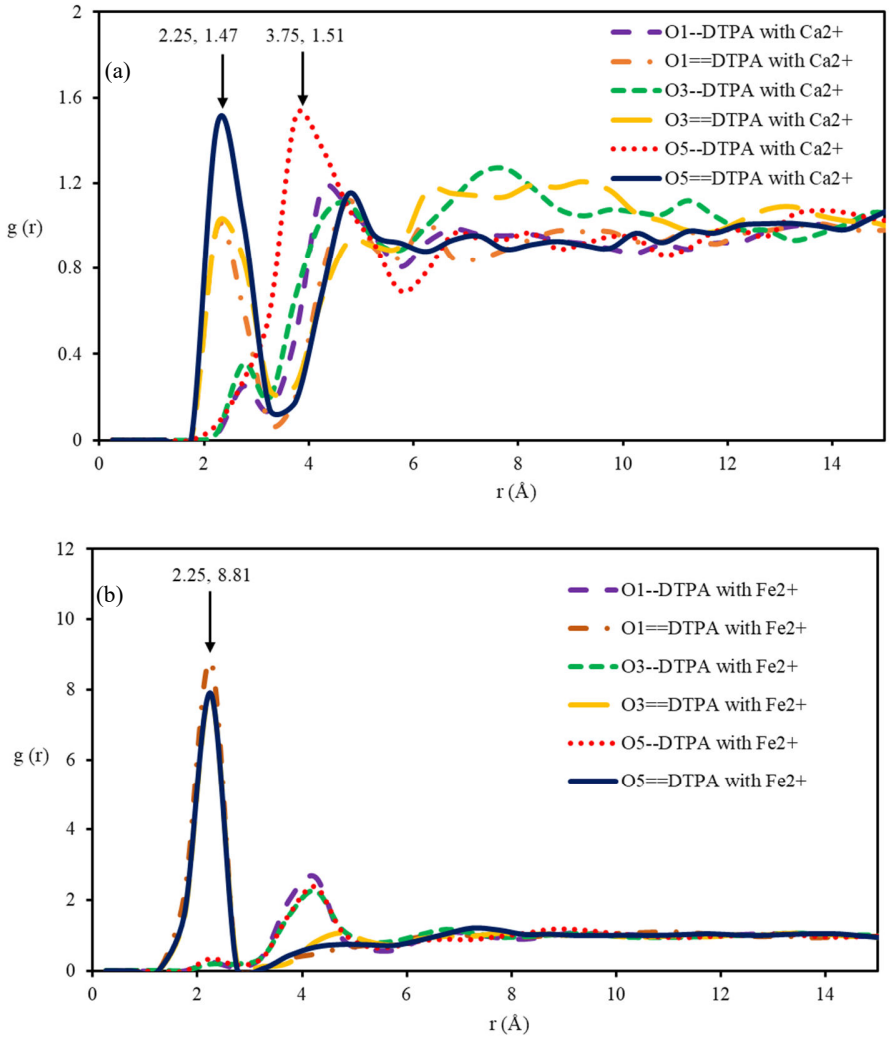


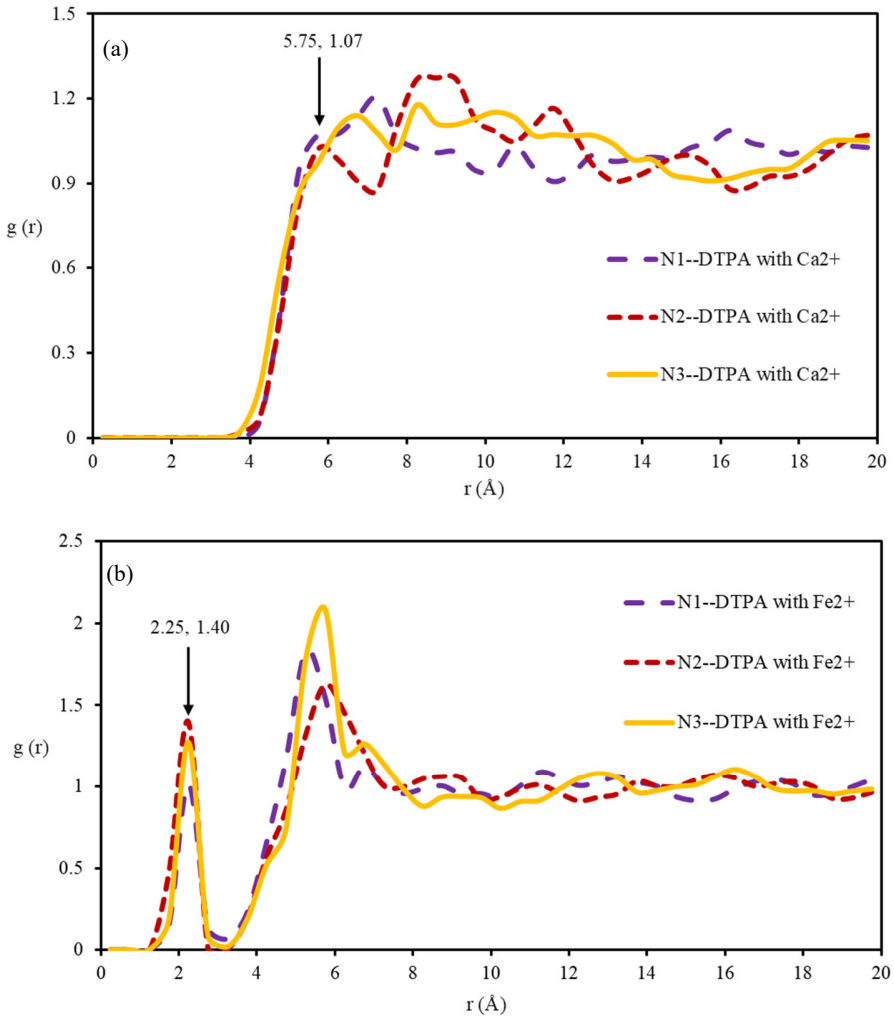
Figure 8. The g(r) plots of the DTPA carboxylic group with (a) Ca^{2+} and (b) Fe^{2+} ion.

Figure 9 (b) elucidates the RDF pattern describing the intermolecular interaction involving the amine functional group of DTPA with FeS. The association of amine, specifically N_1 in DTPA, was observed with a g(r) value of 1.01 for iron at a distance of 2.25 Å. The N_2 and N_3 also show higher g(r) values at 1.40 and 1.27, respectively, with the same distance. The findings of simulations reflect the low degree of intensity stimulated by the intermolecular hydrogen bonding between the carboxylic and amine groups in DTPA. Additionally, lone pairs of electrons are present in the nitrogen atoms of the amine groups (NH_2). Although these nitrogen atoms can engage in coordination chemistry, in the case of DTPA, the carboxylic acid groups are frequently linked to significant chelation sites in Figure 8.

In Figure 9 (c), the amine functional group has a weaker intermolecular interaction between carbonates. Compared to Figure 9 (a), amine favours calcium ions with more vital intermolecular interaction than carbonate ions The chelation of calcium ions by DTPA results in changes to the solubility of $CaCO_3$. Hydrogen bonding is a mechanism by which amine groups and carbonate ions interact. In the case of the amine group

of DTPA and the carbonate ion of $CaCO_3$, the amine group contains a nitrogen atom with a lone pair of electrons. The carbonate ion (CO_3^{2-}) has three oxygen atoms, and one carries a partial negative charge. These two charges, partially positive on the hydrogen atom in the amine group and partially negative on the oxygen atom in the carbonate ion, attract each other, creating the hydrogen bond.

In Figure 9 (d), the amine functional group has a weaker intermolecular interaction between sulphides. In comparison to Figure 9 (b), amine prefers iron with stronger intermolecular interaction. Amine groups are present in DTPA. Nitrogen (N) atoms bound to hydrogen (H) atoms comprise an amine group. As nitrogen only possesses two electrons that are not employed in bonds and are focused on the nitrogen atom, it is known as having a lone pair of electrons. The lone pair of electrons on the nitrogen is attracted to the partial positive charge on the hydrogen atom in the S—H bond of the sulphide ion.



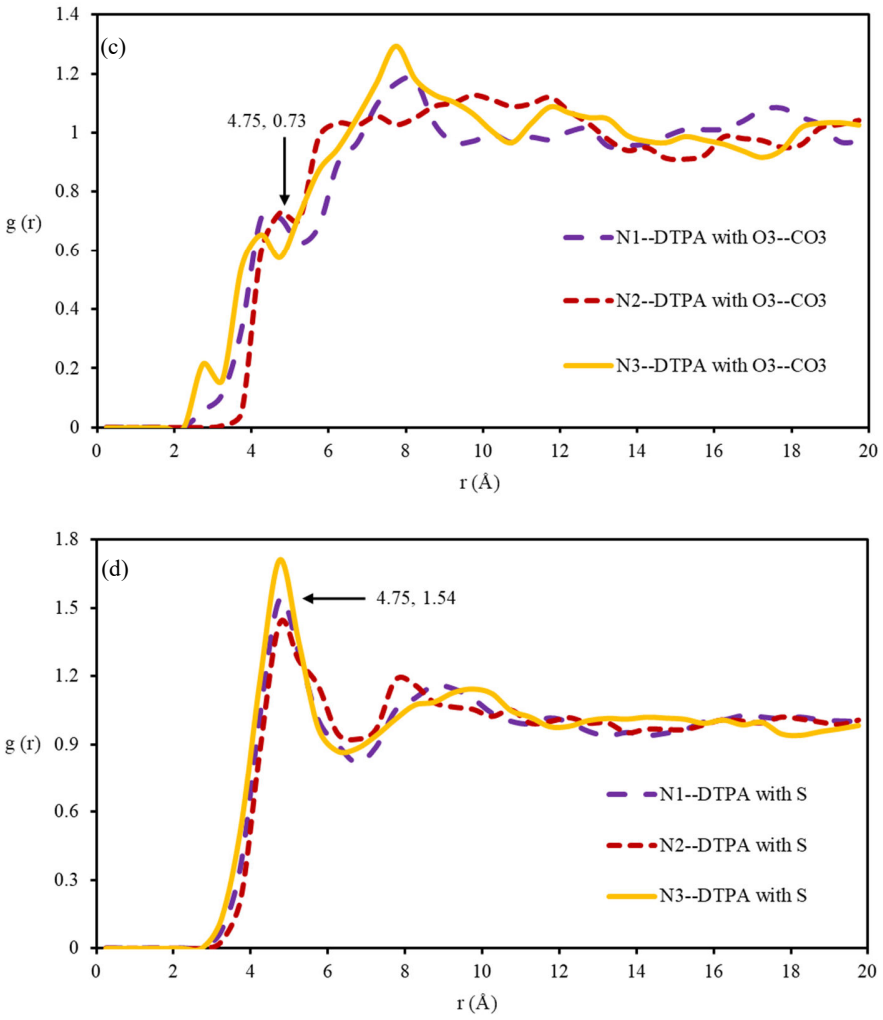


Figure 9. Interaction between the amine in DTPA and (a) Ca^{2+}, Fe^{2+}, (c) carbonate, O_3^{2-} ion of $CaCO_3^{2-}$ and (d) sulphide, S^{2-} ion of FeS

Calcium with carbonate ions in DTPA

Figure 10 shows the intermolecular interaction of Ca^{2+} with CO_3^{2-} of $CaCO_3$ molecule in the DTPA system. A sharp and high peak intensity occurred at 13.71 for O_1—CO_3 interaction at a distance of 2.25 Å. The O_2—CO_3 and O_3—CO_3 illustrate similar intensity values at 9.07 and 9.05, respectively, at 2.25 Å. The highest intensity of the interaction on Ca^{2+} with CO_3^{2-} is explained by the low metal ion complexes of Ca^{2+} ion in Figure 8 (a), whereby the DTPA molecules exist in the acid form. The low pH values do not effectively chelate metal ions because the hydrogen ions occupy the coordinating functional groups [65]. Aminopolycarboxylic acid chelating agents can most efficiently form complexes with metal ions in alkaline solutions. The surface chelation mechanism, as it pertains to the dissolution of $CaCO_3$, describes the process in which DTPA adsorbs onto the surface of $CaCO_3$ and establishes complexes with calcium ions. This adsorption results in an accelerated dissolution rate. The mechanism follows Langmuir-Hinshelwood kinetics, where the chelating agent attacks the calcium component of the calcite lattice rather than the carbonate component [66].

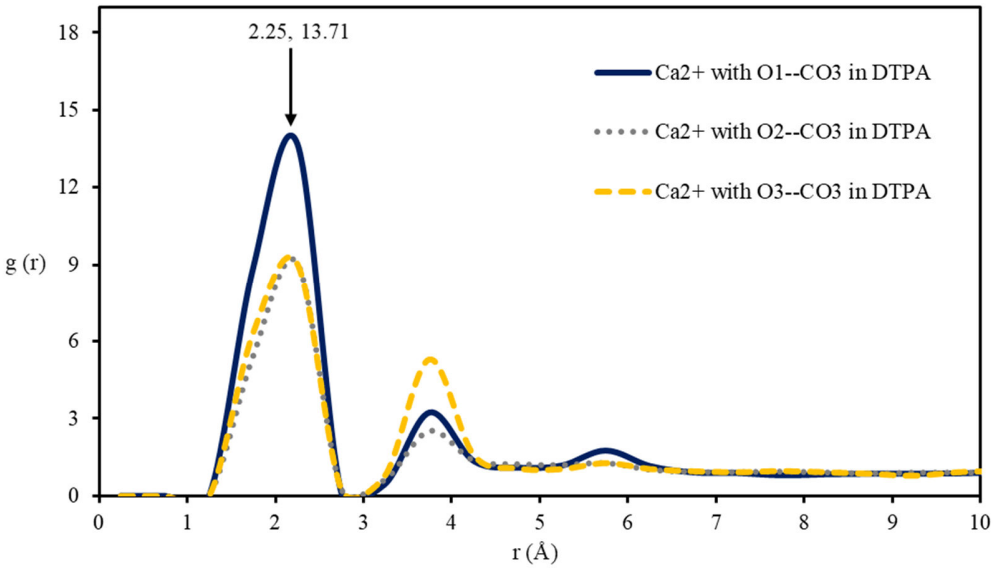


Figure 10. RDF plot of Ca^{2+} and CO_3^{2-} ions interaction that exists in DTPA

Iron with sulphide ions in DTPA

In Figure 11, in separate FeS in DTPA simulation systems, the Fe^{2+}—S^{2-} scale intermolecular interaction in the systems is at 4.75 Å with 2.18 intensity, showing a modest interaction. The interaction between iron ions (Fe^{2+}) and sulphide ions (S^{2-}) in the context of FeS in DTPA primarily involves the complexation of the metal ions by DTPA. The complexation involves binding DTPA carbonyl groups to the iron ions, effectively locking the iron ions within the chelate ring structure of DTPA in Figure 8 (b) [67]. The complexation of iron ions by DTPA prevents the formation of FeS precipitates [68]. The resultant complex between DTPA and iron ions is generally soluble in water, which retains the iron ions in a soluble form, preventing the formation of solid particles and retaining the metal ions in a distributed condition in the solution [69].

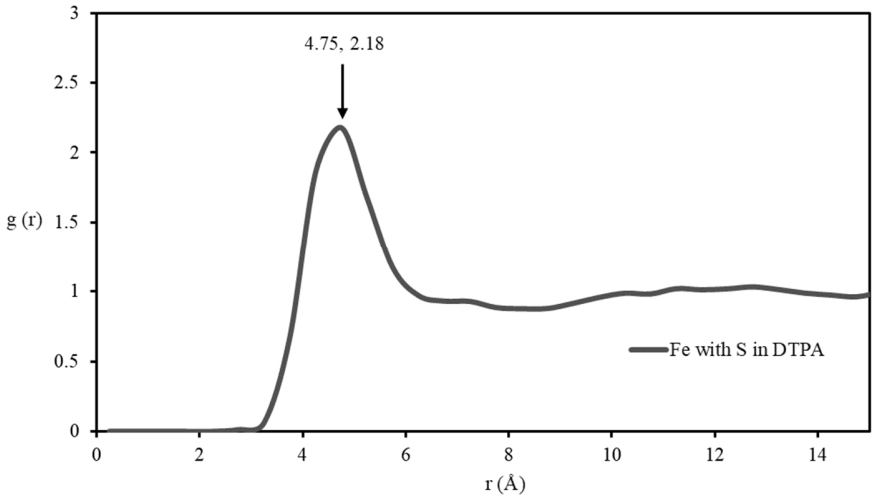


Figure 11. RDF plot of Fe^{2+} and S^{2-} ions interaction that exists in DTPA

Hydrogen in DTPA with (carbonate or sulphide)

Figure 12 (a) depicts the RDFs of hydrogen-atom-containing DTPA molecules in the carboxylic group of DTPA interacting with the carbonate of $CaCO_3$. The H_2 peaks in DTPA have extraordinary intensity values of 11.79 when the distance is 1.25 Å. The positively charged protons (H^+) on DTPA can combine with the negatively charged carbonate ion (CO_3^{2-}) to generate hydrogen carbonate ions (HCO_3^{2-}). This reaction produces hydrogen carbonate ions when the carbonate ion takes up a proton from the DTPA molecule. In summary, the transfer of protons that create hydrogen carbonate ions is the interaction between DTPA and carbonate ions. However, chelating metal ions are the primary function of DTPA. and its interaction with carbonate ions is only a minor aspect of its chemistry.

Figure 12 (b) shows the RDFs of DTPA molecules with hydrogen atoms in the carboxylic group of DTPA with non-metal ions sulphur of FeS interactions. All the peaks show lower probability values of approximately 1.00 with a distance of 3.25 Å. Hydrogen bonding is the mechanism in which the sulphur of FeS and the hydrogen of DTPA interact. There is a chance that the sulphur atom in FeS and the acidic hydrogen atoms in the carboxylic acid groups of DTPA will form hydrogen bonds. However, compared to the coordination bonds that can form between the Fe^{2+} in Figure 8 (b) while the S anion stays constant, the intensity of this interaction in Figure 9 (d) is predicted to be comparatively moderate.

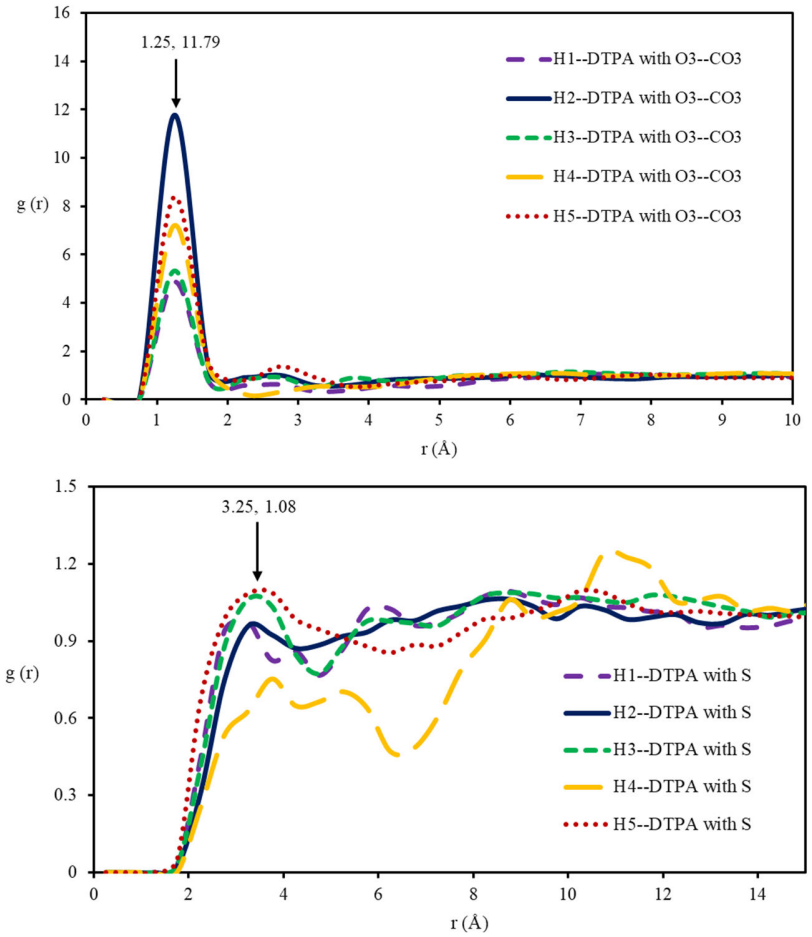


Figure 12. RDF plot for the intermolecular interaction between H in DTPA and (a) O^{2-} in $CaCO_3$ and (b) S^{2-} in FeS

Conclusion

This study elucidates the molecular mechanism of DTPA—H_2O, DTPA—$CaCO_3$—H_2O, and DTPA—FeS—H_2O systems. The analysis of RDF plots for H_2O interactions with hydrogen and oxygen atoms in the DTPA carboxylic groups system reveals strong hydrogen bonding between O—H_2O and H_5—DTPA. The interaction of DTPA—H_2O in the presence of the $CaCO_3$ system modifies the interaction patterns, indicating the interaction shift from water to metal ions. For the DTPA—H_2O in the presence of the FeS system, hydrogen bonding between water and DTPA deteriorates, suggesting the formation of a metal-ligand soluble complex with Fe^{2+} ion. Meanwhile, in the metal ion complexes, DTPA—$CaCO_3$ and DTPA—FeS system interactions show that the carbonyl groups of DTPA exhibit more significant interactions than hydroxyl groups. Amines in DTPA—$CaCO_3$ and DTPA—FeS systems reveal low-intensity interactions, demonstrating a preference for interactions of Ca^{2+} and Fe^{2+} ions with the carbonyl groups of DTPA. The analysis of the amine group in DTPA—CO_3^{2-} and DTP—S^{2-} systems indicates weaker intermolecular interactions, signifying amine preference for Ca^{2+} and Fe^{2+} ions. The ionic interactions within Ca^{2+} and CO_3^{2-} exhibit the highest intensity and shorter radial distance than Fe^{2+} and S^{2-} in the DTPA system. For H—DTPA interactions, H_2—DTPA with O_3—CO_3^{2-} appears expressive, whereas for interactions of H—DTPA with S^{2-}, H_5—DTPA with S^{2-} appears at low intensity. These findings contribute to a better understanding of the chemical processes involved in metal ion complexation (Ca^{2+}/Fe^{2+}) during solid scale dissolution with the chelating agent (i.e., DTPA).

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