

THE DEVELOPMENT OF *Acetobacter xylinum* GROWTH MEASUREMENT THROUGH TOTAL PROTEIN ANALYSIS

(Pembangunan Kaedah Pengukuran Pertumbuhan *Acetobacter xylinum* Melalui Analisis Jumlah Protein)

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Abstract

Acetobacter xylinum, an aerobic Gram-negative bacterium secretes ribbon-like cellulose polymer through the cell wall during growth. The information available on the growth determination of this organism was largely based on turbidimetric measurements which are inaccurate due to the presence of cellulose fragments in the growth medium. Therefore, this study was aimed at developing a rapid and more accurate method for the determination of *A. xylinum* growth through total protein analysis. The fermentation process was carried out at room temperature in static cultures using Hestrin-Schramm medium at pH 6.4 for 19 days. The methods used for the bacterial growth determination included the total protein content, cell dry weight, viable plate count, and turbidimetry. The total cellular protein content was determined based on the Lowry method after the cells were hydrolyzed in 3% (w/v) of potassium hydroxide (KOH) and boiled for 20 minutes. It was found that the growth profile obtained through the total protein analysis has similar pattern with other conventional methods. However, this method was found to be more accurate and less time-consuming compared to viable plate count and cell dry weight. The growth curve obtained showed four microbial growth phases, namely lag phase (day 0 – day 3), log phase (day 4 – day 12), stationary phase (day 13 – day 16), and finally death phase. The average total cellular protein content of *A. xylinum* was $27.1 \pm 6.8\%$ by weight percent.

Keywords: *acetobacter xylinum*, growth profile, total protein assay, bacterial cellulose

Abstrak

Acetobacter xylinum merupakan bakteria aerobik Gram negatif yang mengeluarkan polimer pita selulosa melalui dinding sel ketika pertumbuhan. Sebahagian besar maklumat tentang kaedah penentuan pertumbuhan organisma ini adalah melalui kaedah turbidimetrik yang kurang tepat akibat kewujudan serpihan selulosa di dalam medium pertumbuhan. Oleh itu, kajian ini bertujuan untuk membangunkan kaedah yang cepat dan lebih tepat dalam menentukan pertumbuhan *A. xylinum* melalui analisis jumlah protein. Proses penapaian dijalankan pada suhu bilik dalam keadaan statik menggunakan medium Hestrin-Schramm pada pH 6.4 selama 19 hari. Kaedah yang digunakan untuk penentuan pertumbuhan bakteria termasuk jumlah kandungan protein, berat kering sel, bilangan sel hidup dan turbidimetri. Jumlah kandungan protein sel ditentukan melalui kaedah Lowry setelah sel-sel dihidrolisis dengan mendidihkannya di dalam 3% (w/v) KOH selama 20 minit. Profil pertumbuhan yang diperolehi melalui analisis jumlah protein mempunyai profil yang sama dengan kaedah konvensional yang lain. Walau bagaimanapun, kaedah ini didapati lebih tepat dan singkat berbanding kaedah bilangan sel hidup dan berat kering sel. Lekur pertumbuhan yang diperolehi menunjukkan empat fasa pertumbuhan mikrob, iaitu fasa lag (hari 0 – hari ke 3), fasa log (hari ke 4 - hari ke 12), fasa pegun (hari ke 13 - hari ke 16) dan akhirnya fasa kematian. Kandungan protein selular keseluruhan *A. xylinum* adalah $27.1 \pm 6.8\%$ mengikut peratus berat sel.

Kata kunci: *acetobacter xylinum*, profil pertumbuhan, penentuan jumlah protien, selulosa bakteria

Introduction

Acetobacter xylinum is an aerobic Gram-negative bacterium that produces extracellular cellulose when grown in the presence of sugars at pH 5 to 7 and at 25 to 30 °C [1]. This was first reported by Brown in 1886 that the resting cells of *Acetobacter* produce cellulose in the presence of oxygen and glucose [2]. *Acetobacter xylinum* has been extensively used as a model for fundamental and applied investigations on cellulose due to its capability to synthesize a relatively high number of polymers when grown on a range of carbon and nitrogen sources [3]. Microbial cellulose or usually known as bacterial cellulose is an exopolysaccharide that is branched, repeating units of sugars or sugar derivatives secreted extracellularly, mainly glucose, galactose, and rhamnose in different ratios [4].

The monitoring of bacterial cell growth is important to provide information on their nutritional and energetic requirements as well as an understanding of the survival and proliferation conditions of different species under different conditions [5]. The number of bacteria cells can be estimated using direct and indirect methods. Traditionally, the estimation of bacteria growth can be performed spectrophotometrically where the turbidity of the bacterial suspension is measured at a wavelength ranging from 540–600 nm. Turbidimetric measurement is an indirect method which is fast and mostly preferred when there are many culture samples to be counted. However, this measurement procedure does not reflect the actual cell numbers, as it is not able to differentiate between living and dead cells. To date, the growth of cellulose producing bacteria has been largely determined by turbidimetric [6] and cell dry weight [7] measurements. The adhesion of the cellulose to the cells will interfere with both turbidimetric and gravimetric measurements.

The viable count method estimates the cell numbers within the sample but the process is laborious and selective. This method counts the target bacteria through the colonies formed on the surface of agar plates where the environment is assumed to be suitable for the targeted bacteria. Even though the method can give accurate results of cell growth, the many dilutions needed prior to plating and the long incubation period makes this method unsuitable when working with a large number of samples.

Bacterial cellulose exists as structures known as microfibrils, which are composed of glucan chains interlocked by hydrogen bonds to produce a crystalline domain. It is preferred over plant cellulose as it can be obtained in high purity and exhibits a higher degree of polymerization and crystallinity index. It also has higher tensile strength and water holding capacity than plant cellulose, making it a more suitable raw material for producing high fidelity acoustic speakers, high quality paper, and dessert foods [4]. Since the produced cellulose is pure, the determination of cell growth can be relied on the protein content of the cells. Typically, 30-70% of the cell's dry weight is protein [8]. Based on this relationship, a more accurate result of *A. xylinum* growth can be determined through total protein assay. Therefore, this study was aimed to investigate the growth profile of *A. xylinum* through total protein analysis. Cell growth was also determined by conventional methods, which include turbidimetry, viable plate count, and cell dry weight as comparison. The findings from this study will provide an alternative and a more reliable method for the measurement of *A. xylinum* growth.

Materials and Methods

The preparation of inoculum

The *A. xylinum* 0416 used in this study was purchased from the Malaysian Agriculture and Research Institute (MARDI), Serdang, Malaysia. Hestrin-Schramm (HS) medium was prepared as follows (% w/v): 4% glucose, 0.5% peptone, 0.5% granulated yeast extract, 0.27% Na₂HPO₄, and 0.115% citric acid in 100 mL of distilled water. The pH of the media was adjusted to 6.4 using either 0.1 M HCl or 0.1 M NaOH before being autoclaved at 121°C for 15 minutes at 15 psi. After the mixture was cooled to 28 ± 1°C, 10% (v/v) of *A. xylinum* 0416 seed culture was aseptically transferred into the sterile culture medium and incubated for 3 days at room temperature (±28 °C).

Fermentation process for growth profile studies

The HS medium was prepared accordingly as described in the inoculum preparation procedures. Then 10% (v/v) of the inoculum was aseptically transferred to the sterile HS medium. The static fermentation process was carried out

for 19 days at room temperature ($\pm 28^\circ\text{C}$). The sampling was done at 24 hours intervals and analyzed for cell dry weight measurement, turbidimetric measurement, viable plate count, and total protein assay.

Cell dry weight measurement

A 1 mL sample of cell broth from 24 h sampling intervals was centrifuged at 4,000 rpm for 20 min at 4°C . The bacteria cell pellets were collected and the supernatants were kept for the determination of glucose consumption. The pellets were washed twice by adding 1 mL of sterile deionized water into the 1.5 mL tubes and were vigorously mixed by using a laboratory vortex mixer. The tubes were then centrifuged at 4,000 rpm for 20 minutes. The supernatants which consisted of the remaining medium and impurities were removed carefully, leaving the washed cell pellets in the tubes. These procedures were conducted twice to ensure the maximum removal of impurities. The cell pellets were then dried in an oven at 80°C for 24 hours. The dried samples were placed in a desiccator for 20 minutes before being weighed using an analytical balance. The cell dry weight was calculated according to the formula given below.

$$\text{Cell dry weight (mg/mL)} = \frac{\text{weight of dry sample in tube (mg)} - \text{weight of empty tube (mg)}}{\text{volume of sample (mL)}} \quad (1)$$

Turbidimetry measurement

Similar to cell dry weight determination procedures, the cell pellets were rinsed before being suspended with 1 mL of sterile deionized water. A 100 μL samples of the cell suspension were pipetted into 96 well of microplate and the turbidity was measured at a wavelength of 600 nm by using microplate reader (ThermoFisher Scientific, model VLB000D0). The remaining cell suspensions were further used for viable plate count.

Viable plate count

HS agar was prepared by adding 1.5% technical agar to the HS medium. The mixture was boiled to solubilize the agar before being autoclaved at 121°C for 15 minutes at 15 psi. The sterile medium was cooled to $45\text{--}50^\circ\text{C}$ before being poured into sterile petri dishes aseptically and left to solidify. The agar plates were stored in a refrigerator until further use. A total of 4 different dilutions were prepared for each bacteria suspension, which were 1:10, 1:100, 1:500, and 1:1000. The diluted samples were aseptically spread on agar plates by using the spread plate technique. The plates were incubated at room temperature for 5 days, and the colonies formed on the agar plates were counted. The colony forming unit (CFU/mL) was calculated by using the following formula.

$$\text{Colony Forming Unit (CFU/mL)} = \frac{\text{colony forming unit (CFU)}}{\text{volume of sample (mL)}} \times \text{dilution factor} \quad (2)$$

Total protein analysis: The preparation of bacterial suspension

The samples of 5 mL of cell broth from 24 hours sampling intervals were centrifuged at 4,000 rpm for 20 minutes at 4°C . The pellets were harvested by removing the supernatants from the microcentrifuge tubes. The pellets were washed twice by adding 5 mL of sterile deionized water into the tubes and were vigorously mixed by using a laboratory vortex mixer. The tubes were centrifuged at 4,000 rpm for 20 minutes. Then the supernatant which consisted of the remaining medium and impurities were removed carefully. Cell suspensions were prepared by adding 5 mL of sterile deionized water to the pellets which were then kept for further analysis.

Protein extraction

The extraction of proteins from *A. xylinum* cells was carried out through enzymatic and alkaline cell lyses prior to the quantification of the total protein content of the cells. The efficiency of protein extraction between the two methods was compared.

Alkaline cell lysis was carried out by boiling 5 mL of cell suspension that was mixed with 4 mL of 3% (w/v) KOH for 20 minutes to release all proteins and nucleic acids. The mixture was cooled down to room temperature, and 1 mL of the boiled sample was pipetted into a clean microcentrifuge tube and was further centrifuged at 10,000 rpm for 20 minutes. The supernatant which contained the cellular proteins was separated and kept for protein quantification.

Enzymatic cell lysis was carried out by using lysozyme from chicken egg white lyophilized powder, protein ≥ 40000 units/mg protein. The cell lysis buffer was prepared by adding 5 mL of 1 M Tris HCl, 0.8 mL 0.5 M MgCl, 0.1 mL of Triton X-100%, and 20 mg of lysozyme into 50 mL of distilled water. The solution was marked up to 100 mL with distilled water and stored in a refrigerator for further use. For cell lysis, 100 μ L of bacterial suspension was mixed with 1 mL of cell lysis buffer and incubated for 40 minutes at room temperature before being centrifuged at 4000 rpm for 20 minutes. The supernatant was collected and kept for protein quantification by using the Lowry method.

Protein quantification

The protein quantification process was carried out using bovine serum albumin (BSA) at a concentration ranging from 1–1000 μ g/mL as the calibration standard as described by Lowry et al. [9]. Spectrophotometric measurements were done by using a microtiter plate reader at 750 nm.

Results and Discussion

The effect of alkaline and enzymatic cell lysis on total protein content

Figure 1 shows the effect of cell lysis method on the total protein extracted from *A. xylinum* cells using alkaline and enzymatic cell lysis. Overall, the total protein concentration profile during the entire fermentation period showed similar pattern in all samples obtained from the alkaline and enzymatic cell lysis, and without the cell lysis. However, the total protein obtained from the alkaline cell lysis was 1.7 fold higher than the enzymatic cell lysis. There was no significant difference of the total protein in the samples obtained from the enzymatic cell lysis and without the cell lysis. The cell lysis by 3% KOH is one of the most commonly used techniques due to its fast, reliable, and relatively clean way to obtain proteins from cells. KOH hydrolyses the cell wall and liberates the DNA, which is a very viscid compound [10]. The OH⁻ ion in alkaline cell lysis reacts with the cell membrane and breaks the fatty acid-glycerol ester bonds and subsequently makes the cell membrane permeable.

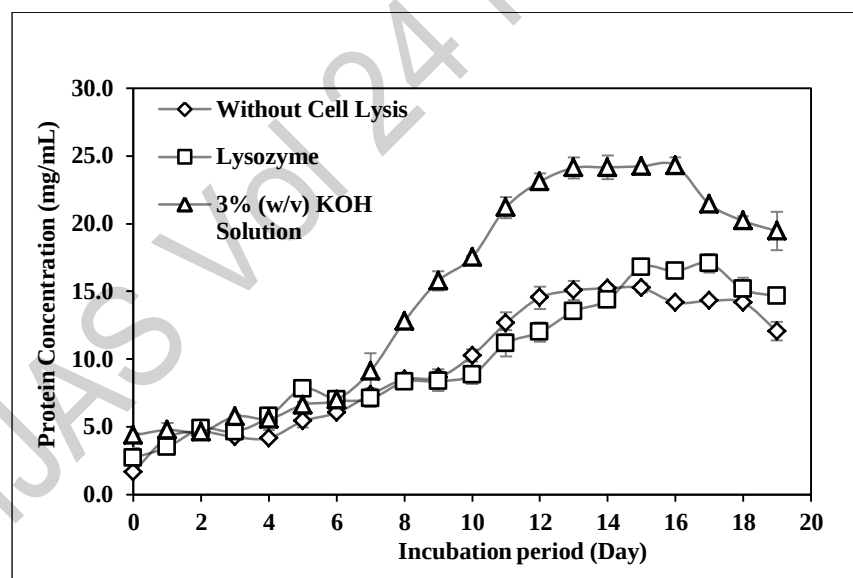


Figure 1. *Acetobacter xylinum* intracellular protein concentration profile during 19 days of fermentation period

On the other hand, lysozyme reacts with the peptidoglycan layer and breaks the glycosidic bonds. For that reason, Gram-positive bacteria can be directly attacked by lysozyme, but in Gram-negative bacteria, the outer membrane needs to be removed before exposing the peptidoglycan layer to the enzyme [11]. Accordingly, lysozyme is usually combined with detergent to break the cell wall and membrane, which later hydrolyses β -1-4-glucosidic bonds in the peptidoglycan of Gram-negative bacteria. However, the low concentration of total protein obtained from the

enzymatic lysis in this study suggested that the modification on the reaction condition, including incubation time, concentration of detergent, and lysozyme might be necessary. A combination of ultrasonication with detergent and lysozyme may also improve the extraction of intracellular protein from *A. xylinum*. The findings obtained from this study suggested that alkaline cell lysis is a good method to extract proteins from the cells.

The comparison of growth profile with conventional methods

Figure 2 shows the results of bacterial growth profile measured by turbidimetric method in comparison to the total intracellular protein concentration profile obtained through alkaline cell lysis. The static fermentation process took 19 days to get the complete phases of bacterial growth. The results obtained showed a typical bacterial growth curve with four distinct phases of growth, namely lag phase, exponential phase, stationary phase, and finally death phase. Both profiles showed similar patterns from the early stages of growth until stationary phase but the death phase was not clearly visible in the optical density profile. The cell debris from the non-viable cells present in the samples might have caused an increase in the optical density values. Moreover, the cellulose excreted might still be attached to the cells that contributed to the optical density values. On the contrary, the total protein content dropped after 16 days of static fermentation, indicating the onset of the death phase as the intracellular protein content also began to drop from day 16 onwards. Other studies have shown similar growth patterns of *A. xylinum* by using total viable count [12-14]. On the other hand, Lestari et al. have reported the growth pattern of *A. xylinum* in shake flask fermentation by the measurement of optical density where the whole fermentation process only took 5 days [6]. However, the bacterial cellulose produced had irregular shapes, which appeared in chunks and aggregate forms [12].

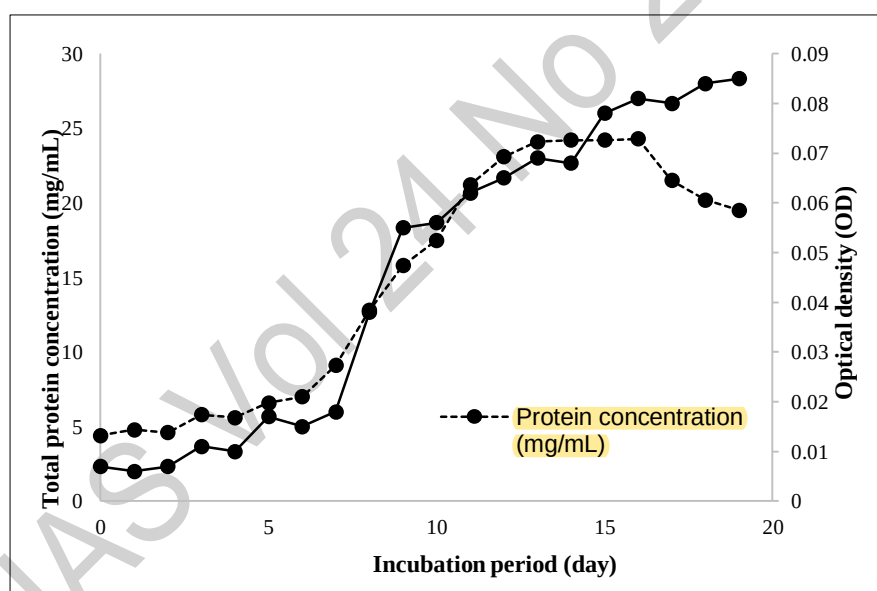


Figure 2. The time course profile of *Acetobacter xylinum* growth by turbidimetry measurement (OD 600) in comparison to the total intracellular protein concentration

In this study, the lag phase began on day 2 of the fermentation before entering the log phase. At this phase, the cellular metabolism started to be expressed and the bacteria cells began to increase in size. However, the cells were not competent to replicate and therefore no increment in cell mass was observed. The lag phase depended directly on the previous growth condition of the bacteria. Upon inoculation, the bacteria started to synthesize their necessary components, such as proteins, vitamins, and co-enzymes needed for growth.

The log phase began at day 3 until day 13 where *A. xylinum* grew rapidly based on the results of the total intracellular protein concentration. At this phase, the growth medium was consumed at a maximal rate and the number of bacteria cells increased logarithmically. All the nutrients in the HS medium were used up by the cells as

the population continued to grow. This resulted in the accumulation of toxic metabolites and waste materials in the medium. Therefore, this shifted log phase to stationary phase which can be seen on day 13 to day 16. Based on the total intracellular protein content, the death phase occurred from day 16 onwards. The depletion of nutrients and accumulation of toxic waste made the environment unsuitable for growth. Nevertheless, some bacteria cells were still viable even though they were starved of nutrients.

Figure 3 shows the results of bacterial growth profile measured by cell dry weight method in comparison to the total protein concentration profile obtained through alkaline cell lysis. Both profiles showed similar growth patterns, except that the death phase was not clearly seen in the cell dry weight method. The presence of dead cells in the biomass pellet may lead to the consistent values of cell dry weight until the end of fermentation period. Moreover, the cellulose excreted from the cells may still be attached to the cells, which therefore might contribute to the cell dry weight values. It can be concluded from this finding that the average protein content of *A. xylinum* is $27.1 \pm 6.8\%$ by weight. This result is closely correlated with the value reported by Kampen [8] who mentioned that typically 30–70% of the cell's dry weight is protein. This correlation can be used as a reference in the future study.

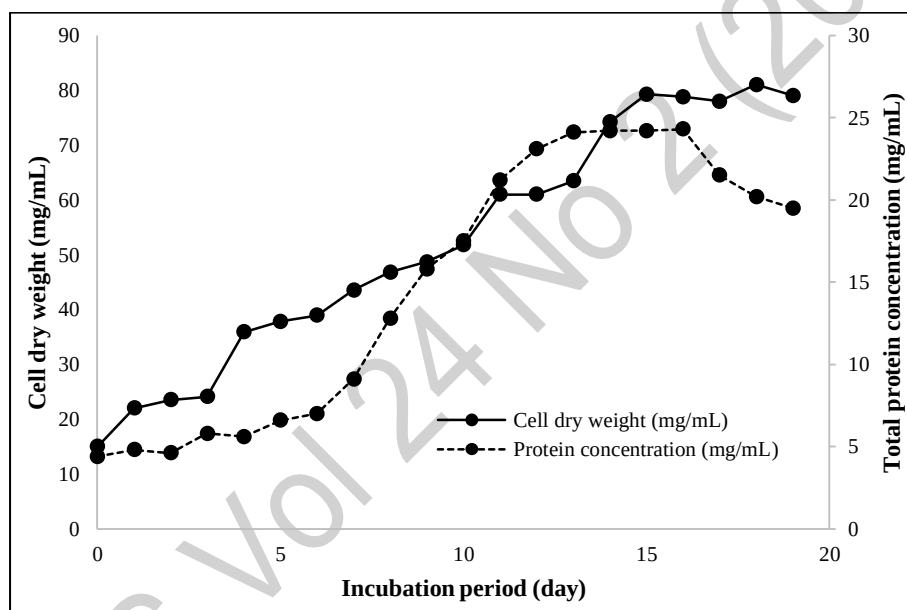


Figure 3. The time course profile of *Acetobacter xylinum* growth by cell dry weight measurement in comparison to the total intracellular protein concentration

Figure 4 shows the results of the bacterial growth profile measured by the viable plate count method in comparison to the total intracellular protein concentration profile obtained through alkaline cell lysis. The results from the viable plate count method showed that *A. xylinum* skipped the lag phase and directly entered the logarithmic phase until day 12 of fermentation. It later entered the stationary phase until the end of fermentation period but the death phase was not clearly seen.

The comparison between the conventional methods and the total protein analysis is summarized in Table 1. Of all the methods performed in this study, it was found that the total protein analysis is the best method to determine the growth of *A. xylinum*. The method can be considered rapid and reliable, as the estimated total protein obtained was solely from the cells. Moreover, it only took 1 hour and 30 minutes to get the final value of the protein.

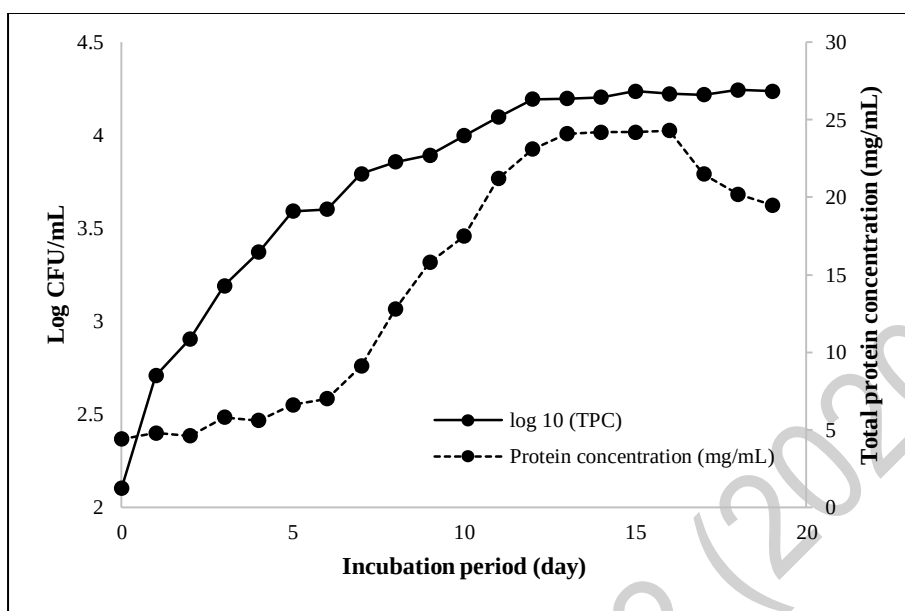


Figure 4. The time course profile of *Acetobacter xylinum* growth by viable plate count measurement in comparison to the total intracellular protein concentration.

Table 1. The comparison of all techniques used for *A. xylinum* growth in this study

	Cell Dry Weight	Viable Plate Count	Turbidimetry Measurement	Total Protein Assay
Procedures	Cell washing, dilution, weighing, drying, cooling, weighing	Cell dilution, agar plate preparation, spread plate, incubation, colony counting	Cell washing, Optical density measurement	Preparation of cell suspension, alkaline cell lysis, protein assay by Lowry method
Estimated time required	1 day 3 hours	3 days 10 hours	45 minutes	1 hour and 30 minutes.
Advantages	Simple steps Low cost	Distinguish between live and dead cells	Simple steps Rapid estimation of bacterial growth	Rapid and reliable method
Disadvantages	Tedious Does not differentiate live, dead cells and BC attached to the cells. Time consuming	Laborious High chance of contamination. Time consuming	Low cost Does not differentiate live, dead cells and BC attached to the cells.	Harsh cell lysis technique which may denature the protein.

Conclusion

The determination of the total protein content was shown to be a reliable and rapid method for the determination of the bacterial cellulose producer, *A. xylinum* growth. Unlike turbidimetric and dry weight measurements, the presence of contaminating cellulose attached to the cells did not cause interference, as only the protein content was measured. Therefore, the use of total protein content as the measurement for the growth of other polysaccharides producers, such as *Sarcina* and *Agrobacterium* can be further explored based on the recent findings. On average, *A. xylinum* contained $27.1 \pm 6.8\%$ protein by weight which may be used as a baseline data for future studies.

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