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Development of Fed-Batch Strategy to Produce Bacteria Cellulose

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Bacteria Cellulose (BC) is a type of biopolymer widely used in areas such as biomedicine and paper manufacturing industries. The batch fermentation mode of BC is less cost-effective as the yield is low. This study aims to determine the best fed-batch cultivation strategy to produce BC of high yield and quality. First, batch fermentation mode was carried out to determine the glucose consumption profile of *Gluconacetobacter xylinus* using Yeast Extract Glucose Chloramphenicol Medium (YGC). By using the information, the fed-batch strategies with feeding frequencies of 1 time and 3 times per 14-day total fermentation period and varied feed composition (YGC medium and pure glucose solution) were set up. The yield, water holding capacity, morphology, and strength of BC were monitored. It is found that BC produced from fed-batch modes has a higher yield (3.75 g/L for low feed frequency and 4.79 g/L for high feed frequency) compared to batch mode (2.24 g/L). The BC produced with pure glucose solution has the highest water holding capacity (87.34 g water/g sample) among the different modes. The FE-SEM micrographs showed that BC produced in the high feeding frequency with YGC medium fed-batch mode has the highest microfibril density and, thus, the best mechanical strength from this mode. To conclude, the fed-batch mode with higher feeding frequency (3 days intervals for a total of 14 days) using YGC medium has the best BC yield and quality.

Keywords: Bacteria cellulose; fed-batch cultivation strategy; feeding frequency; feed composition

I. INTRODUCTION

Cellulose is a type of long-chain biopolymer and is typically found as the building units of plant cell walls and bio-secretions of certain bacteria strains such as *Gluconacetobacter xylinus*. It is an important material used in various fields for numerous products, e.g., the paper and pulp industry and the textile industry. Nonetheless, bacterial cellulose (BC) is gaining popularity due to its higher purity, unique mechanical strength, higher crystallinity, high biodegradability, exceptional water-holding capacity, and so on. These characteristics increase its demand in the biomedicine, food, and cosmetic industries.

BC may be produced in batch mode, fed-batch mode, or continuous fermentation mode. In batch fermentation, selected strains of bacteria are inoculated into a fixed volume of medium with a pre-determined amount of carbon source and nutrients (Bae & Shoda, 2004). As bacteria reproduce and secrete BC, nutrients in the medium are depleted and thus, the environment is constantly changing chemically (Ying & Ma, 2019). The changing environment slowly deviates from the optimum conditions, leading to the retarded growth of bacteria and a reduction of BC production. Furthermore, the batch cultivation mode is not suitable for producing BC at large-scale industrial production due to the long cultivation time and expensive carbon source required (Portela *et al.*, 2019).

In fed-batch fermentation, microorganisms are inoculated using a batch regime for a certain amount of time and fresh, nutrient-containing medium will be added periodically (Tsouko *et al.*, 2015). Generally, fed-batch fermentation will be more productive as BC may be produced in a more continuous style. In this case, a carbon source will be added continuously or intermittently to the reactor (Sharma *et al.*,

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2021) to meet the minimum demand of the cells, making it more effective in reducing cell growth inhibition caused by high substrate concentration (Hsieh *et al.*, 2016). Feeding strategy is, therefore, critical in determining the success of a fed-batch fermentation.

An unsuitably low feeding rate risks causing the bacteria strain population to decrease as the microorganisms, like all living things, require nutrients to survive. However, an excessively high feeding rate risks flooding the reactor and increasing unnecessary production costs. In addition, excessive glucose concentration was found to inhibit BC production as a high concentration of gluconic acid will be produced as a by-product, which reduces the medium pH below optimal levels (Hsieh *et al.*, 2016). Bacterial growth and BC production were hampered by low medium pH. Meanwhile, low glucose concentration limits the amount of carbon source supplied to the bacteria strain, thus reducing the BC production rate. Therefore, the right amount of nutrient feed at a suitable time is necessary to be identified. As such, an economical fed-batch fermentation procedure is required for the mass production of quality and usable BC to cater to the demand in various biotechnology fields. As a result, this study aims to determine the feeding rate and composition of the medium so that the bacteria strain used, *G. xylinus*, can engage in continuous production of BC.

II. MATERIALS AND METHOD

Glucose, yeast extract, calcium carbonate, 99% purity glacial acetic acid, and 95.5% reagent grade concentrated sulphuric acid were purchased from Sigma Aldrich, USA. The sodium hydroxide solution was obtained from Merck, Germany.

A. Preparation of Fermentation Medium for Batch Fermentation Mode

The batch mode was done before commencing the fed-batch mode to determine the glucose consumption profile of *G. xylinus*. For batch mode, YGC was used as the base medium, containing 25 g/L of glucose, 5 g/L of yeast extract, and 12.5 g/L of calcium carbonate. The medium pH was adjusted to 5.0 using glacial acetic acid and measured with a pH meter (SevenEasy pH Meter, Mettler Toledo AG, Switzerland) before being sterilised (Autoclave HVE-50, Hirayama Manufacturing Corp, Japan) at 121°C for 15 minutes.

B. Preparation of Inoculum for Batch Fermentation Mode

To prepare the inoculum, 1% (v/v) glycerol stock with *G. xylinus* was transferred into 99 mL of the sterile medium and incubated at 30°C for 3 days (Raiszadeh-Jahromi *et al.*, 2020). The inoculum-containing medium was then well shaken to wash off bacteria cells attached to the BC formed. The BC was discarded, and the inoculum medium was subjected to a centrifugation process (5810 R, Eppendorf, Germany) at 10,000 rpm for 10 minutes to separate bacteria cells from the medium. The cell pellet was then weighed with an analytical balance (HR-250AZ, A&D Company Limited, Japan), and the mass of cells required for fermentation at a fixed cell density of 0.05 g/L for 200 mL of fermentation broth was determined using Eq. (1).

$$Mass\ of\ Cells\ (g) = \frac{Volume\ of\ medium(L)}{0.05\ g/L} \quad (1)$$

C. Glucose Consumption Profile of G. xylinus in Batch Fermentation Mode

Two sets of batch mode fermentation, with each set in triplicate, were run. A 10% (v/v) inoculum from Section IIB was inoculated into YGC medium and incubated for 7 days at 30°C. The sample was withdrawn on days 1, 2, 3, and 7 for glucose concentration determination and the average values were reported. Access to the laboratory was restricted during the COVID-19 pandemic, preventing sampling on days 4, 5, and 6.

To determine the concentration of glucose, the phenol-sulfuric method was used (Dubois *et al.*, 1951). One millilitre of the sample mixture was pipetted into a test tube, followed by 0.5 mL of 5% (w/w) phenol solution. Then, 2.5 mL of concentrated sulphuric acid was added to the test tube rapidly. The mixture was vortexed and incubated in a water bath (Waterbath WNB 14, Memmert GmbH, Germany) at 30°C for 30 minutes. The absorbance of the mixture was then measured by a UV-Vis spectrophotometer (GENESYS 50 UV-Vis Spectrophotometer, Thermo Fisher Scientific, US) at 490 nm for hexoses.

D. Development of Fed-Batch Fermentation Strategy

The intermittent fed-batch fermentation (IBF) method was implemented using the information obtained from the glucose consumption profile of *G. xylinus*. Four sets of experiments, each implementing a different fermentation strategy, were prepared and all modes were run in triplicate. The runs were labelled as follows:

- FBA – Fed-batch mode with **low** feeding frequency, **high** feed volume, using YGC medium as the initial medium and feed medium.
- FBB – Fed-batch mode with **high** feeding frequency, **low** feed volume, using YGC medium as the initial medium and feed medium.
- FBNG – Fed-batch mode with **low** feeding frequency, **high** feed volume, using pure glucose solution as the initial medium and feed medium.
- Batch – Batch mode as a control set to the other modes, with YGC medium as the initial medium.

The YGC medium used in FBA, FBB and the Batch mode was prepared using the steps in Section IIA. The pure glucose solution used in the FBNG mode was prepared by using 50 g/L of glucose. The glucose concentration in all modes in this section was doubled to 50 g/L. The prepared media were then sterilised at 121°C for 15 minutes.

For all modes, the initial medium volume was set at 300 mL, and the cultures were started as batches and incubated at 30°C. Figure 1 shows the summary of the runs and the feeding strategy employed. To compare the effects of feeding frequency on the quality of BC produced, the product in FBA mode was compared with BC in FBB mode. On the other hand, to compare the effects of initial medium and feed composition on the BC quality, the BC from FBA mode was compared with the BC in FBNG mode. The culture period was 14 days for the fed-batch fermentation mode.

E. Purification of BC Samples

To isolate the BC products from the fermentation medium, Whatman no.1 filter paper was used to filter the BC samples. Then, distilled water was used to rinse the BC several times. The rinsed BCs were then soaked in 1 N sodium hydroxide and heated in an oven at 80°C for 15 minutes. The BC samples were then washed with distilled water and soaked in 4% glacial acetic acid for 5 minutes at room temperature. When the pH of the solution turns neutral, the BC samples were rinsed thoroughly with distilled water and kept in petri dishes. The dishes were then labelled accordingly.

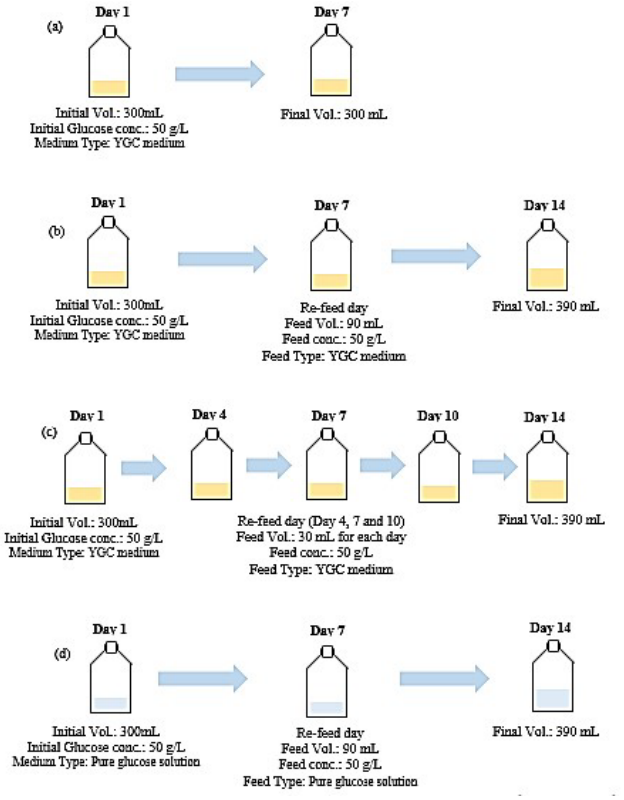


Figure 1: Feeding strategy for (a) Batch mode (b) FBA mode (c) FBB mode and (d) FBNG mode.

F. Yield of BC and Water Holding Capacity of BC Produced

The yield of BC produced in all fermentation was determined using Eq. (2).

$$BC\ Yield\ \left(\frac{g\ sample}{L\ initial\ medium}\right) = \frac{Mass\ of\ Dry\ BC\ Produced\ (g)}{Volume\ of\ Initial\ medium\ Used\ (L)} \quad (2)$$

Fresh, wet BC samples were first weighed using an analytical balance to obtain the wet sample mass. The BC samples were then dried for 12 hours at 60°C (Shezad *et al.*, 2009). Then, the dry mass of the BC samples was obtained, and the mass of water removed during drying was calculated. The WHC of all samples was then calculated using Eq. (3).

$$Water\ Holding\ Capacity\ \left(\frac{g\ water}{g\ dry\ BC}\right) = \frac{Mass\ of\ Water\ Removed\ During\ Drying(g)}{Dry\ Weight\ of\ Cellulose\ (g)} \quad (3)$$

G. Field Emission Scanning Electron Microscope (FE-SEM) Micrographs

FE-SEM (JSM-7800F, Jeol, Ltd., Japan) was conducted to obtain micrographs of the BC surface. The BC samples were treated using vacuum pre-treatment and coated with a platinum layer to ensure the quality of the micrographs. The micrographs were taken at ×2,000, ×10,000, and ×20,000 magnifications with a 5.0 kV LED tube.

H. Fourier-Transform Infrared Spectroscopy (FT-IR) Analysis

A small piece of dried BC sample (1 cm × 1 cm) was cut from each sample in all runs and analysed using the FT-IR analysis. The analysis was done using the Fourier Transform Infrared Spectrometer (Nicolet iS5 FTIR Spectrometer, Thermo Fisher Scientific, US; OMNIC Software). The spectral range used for this analysis was 4000 to 400/cm. The beam splitter used is the KBr/Ge mid-infrared optimised type, and the source type is the Mid-infrared Ever-Glo.

I. Crystallinity and Particle Size – X-Ray Diffraction (XRD) Analysis

To analyse the crystallinity and particle size, 1 cm × 1 cm samples from the BC samples in each run were used. The cut samples were scanned at 30 kV, 15 mA using a fixed monochromator as diffracted beam (monochrome) and the detector used was the MiniFlex2 counter. Duration time was 1 second and the scan range was 3.0 to 40.0 deg. The incident slit was at 0.625 deg and receiving slit was at 1.25 deg. The obtained data were analysed using the Origin-Graphing and Analysis Software (Origin 2020b, 64-bit, OriginLab, Corporation, US). To obtain the crystallinity, the sum of the area under each peak was obtained. Then, it was divided by the total area under the curve and multiplied by 100% (Molina-Ramírez *et al.*, 2018).

J. Mechanical Strength – Tensile Strength Test

To determine the tensile strength and Young's Modulus of the BC produced, 60 mm × 40 mm of BC stripes was first cut from the BC samples from each run. The samples were then set on the texture analyser (CT3 Texture Analyser, AMETEK Brookfield Engineering Labs. Inc.; TexturePro CT V1.8 Build 31 Software). The target distance was set at 80 mm, the data rate was set at 13 points/sec, and the load cell used was 50,000 g. The sample was tested for its tension and elongation at break, and the final graph generated was analysed.

III. RESULTS AND DISCUSSION

Batch fermentation was carried out to determine the glucose consumption rate of the bacteria strain *G. xylinus*. The period of batch mode was 7 days. After plotting the Glucose consumption profile of *G. xylinus* in the YGC medium shown in Figure 2, the equation of best fit was used to predict the concentration of glucose in the medium on day 4, which is one of the planned re-feed days for Fed-batch mode. Two sets (Set A and B) of batch mode fermentation were done, with each set sampling in triplicate samples (A1 to A3 and B1 to B3), and average values were used to plot the curve. The error bars were also shown in the graph.

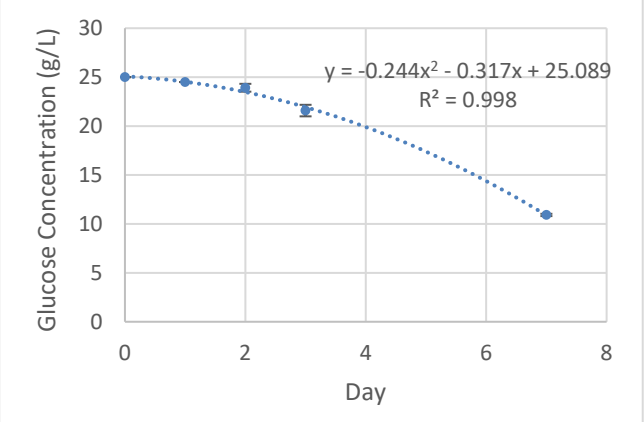


Figure 2: Glucose consumption profile of *G. xylinus* in YGC medium.

Based on the curve, the glucose concentration decreased slowly, which is slightly different from the typical glucose consumption profile of bacteria. The average concentration of glucose on the last day (the seventh day) is 10.92 g/L, whereas the concentration on the fourth day was obtained using the equation of best fit, which returns a concentration value of 19.77 g/L. These values are quite close to the glucose concentration curves provided by Liu *et al.* (2018) and Zhang *et al.* (2014). The values are provided in Table 1. The culture medium used by the former was composed of 23 g/L glucose, 10 g/L peptone, 7.5 g/L yeast extract, and 10 g/L disodium phosphate at a pH of 6.0, while the medium composition used by the latter contained 3.0 g/L yeast extract, 5.0 g/L tryptone,

and 25 g/L glucose. With the best-fit equation obtained in this batch experiment, the glucose consumption rate could be obtained, and the feeding strategy could be developed.

A. Fed-Batch Fermentation Mode Strategy Development

During a feeding process, a large volume of feed medium will be required to return the concentration of the medium in the incubation vessel to the initial concentration. For example, if the initial concentration is 25 g/L and on the fourth day, when the concentration is 19.81 g/L, the volume of feed medium required to increase the glucose concentration up to 25 g/L will be large according to the equation $C_1V_1 + C_2V_2 = C_3V_3$, where C = concentration in g/L and V = volume in L. However, to ensure a higher yield of product, sufficient aeration in the culture medium is necessary as *G. xylinus* is an aerobic bacterium. If the feed volume is too large, air supply to the lowest part of the incubation flask may be reduced, thus inhibiting bacterial growth.

Figure 1 shows the feeding strategy for different modes. As compared to the 25 g/L glucose concentration used in the Batch mode in Section IIIA, 50 g/L was used in the following sections to ensure consistent glucose concentration in media for all modes (Batch, FBA, FBB, and FBNG). In the pure glucose solution fed-batch mode (FBNG), it was thought that 25 g/L of glucose solution without any other nutrients might be less suitable for *G. xylinus* to produce BC. In other words, the concern was that 25 g/L of pure glucose solution might not be able to produce enough BC yield for further analyses. Thus, the glucose was doubled to 50 g/L. Figure 3, Section IIIB1, was used to discuss the similarity of the glucose concentration trend of *G. xylinus* in 25 g/L and 50 g/L glucose concentrations.

Table 1: Glucose consumption profile of *G. xylinus* in different works.

Initial Glucose Concentration (g/L) / Volume (mL)	**Glucose concentration (g/L)**							**References**
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	
25.00 / -[a]	24.50	19.60	16.50	10.00	8.50	9.50	6.20	Zhang *et al.,* 2014
23.00 / 200	17.50	16.25	15.50	13.45	9.66	9.90	8.70	Liu *et al.,* 2018
25.00 / 300	24.53	23.48	21.94	19.92	17.40	14.40	10.91	Line of best fit in Figure 2

[a] Initial medium volume is not given in the source. All methods and results were given in terms of concentration only.

For this experiment, the effects of feeding frequency and feed composition on the yield and quality of BC were identified. The feeding strategy type used was intermittent feeding. One-litre Schott bottles were used to contain the broth and cotton stoppers were used to plug the openings of the bottles. To set up for the comparison of feeding frequency effects, FBA was compared with FBB, where FBA was fed at a lower frequency (1 time) and FBB was fed at a higher frequency (3 times). On the other hand, the effects of feed composition were determined by comparing FBA mode (YGC as initial medium and feed medium) with FBNG mode (pure glucose solution as initial medium and feed medium). Referring to Figure 1, the BC harvest day will be on Day 7 for Batch and Day 14 for FBA, FBB, and FBNG mode. To ensure the feed medium does not over-concentrate the medium, in FBA mode the feed was set to be 90 mL of 50 g/L. In FBB mode, a 90 mL feed was divided into three feeding times, which are on Days 4, 7, and 10. For FBNG mode, the feed frequency was the same as that in FBA mode. All modes were done in triplicates.

B. Fed-Batch Fermentation Mode

In fed-batch fermentation mode, the glucose consumption rate of the bacteria strain was determined for all sets of experiments. The product BC produced from each set was also analysed in terms of yield, surface morphology (FE-SEM imaging), and physical and chemical characteristics (water holding capacity, crystallinity, FTIR test, and tensile strength).

1. Glucose consumption profile, yield, and water-holding capacity (WHC)

Figure 3 shows the glucose consumption profile of *G. xylinus* in different modes. To obtain the concentration of each set, the glucose sample was taken from above the BC layer after feeding. Samples were taken from the same spot in the Schott bottle for each sampling session. In fed-batch set A (FBA), a low feeding frequency was implemented, whereas in fed-batch set B (FBB), a high feeding frequency was used. As compared to FBA and FBB, where YGC medium was used as the feed medium, pure glucose solution was used as the initial medium and fed in fed-batch set NG (FBNG). FBA was compared with FBB mode to determine the effects of feed frequency on the yield and quality of the BC produced, while FBA and FBNG were paired to compare the effects of initial medium and feed composition. Batch mode (Batch) was done as a control set for the other three sets. The average of triplicate sample sets was reported.

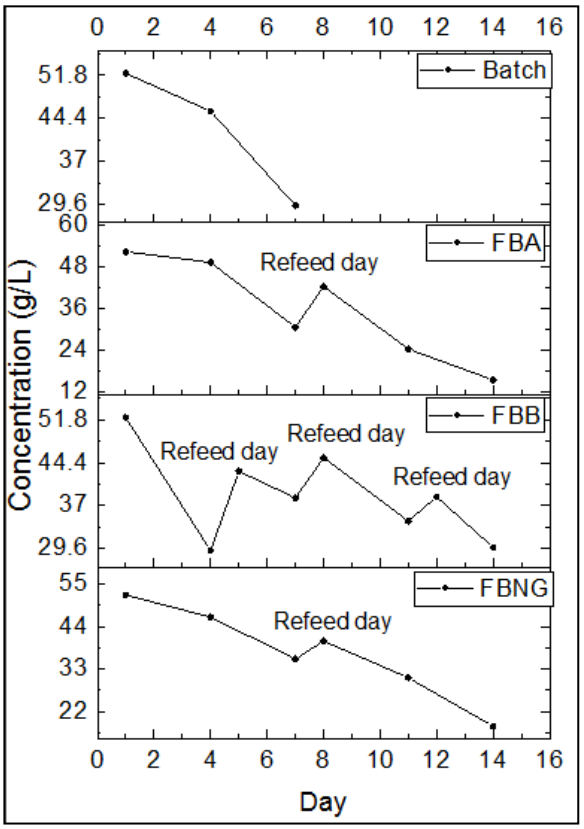


Figure 3: Glucose consumption profile of *G. xylinus* in different modes.

From Figure 3, the glucose concentration in the medium of FBA and FBNG shows similar trends, where the concentrations were fed to approximately 80% of the original concentration after the feeding day. On the other hand, the trend in FBB showed that the feed composition was able to re-concentrate the medium to around 80% of the original, thus exhibiting the fluctuating curve in Figure 3. However, the sharp decrease before the first feeding day in FBB may be due to the sample being taken at different locations in the Schott bottle. This causes a deviation among the three FBB glucose samples, and thus, in future studies, the Schott bottle may be marked so that all samples will be taken from the same location. The Batch concentration trend was roughly the same as that shown in Figure 2. This shows that under different glucose concentrations, the glucose consumption rate of strain *G. xylinus* was similar, provided additional nutrients, such as nitrogen sources, were provided.

Figure 4 shows the yield of BC in each fermentation mode. According to Figure 4, the differences in yield between Batch, FBA, and FBB are small. However, FBNG recorded the lowest yield with 0.06 g BC/L initial medium volume. Fed-batch modes (FBA and FBB) generally produce a higher yield of BC compared to Batch mode as the fermentation period is longer for FBA and FBB modes (7 days for Batch mode, 14 days for FBA and FBB modes). The yield of the fed-batch modes is roughly double that of the Batch modes due to the longer fermentation period. FBB also produces a higher yield compared to FBA, even though the total feeding volume is the same. This may be explained by the tendency of *G. xylinus* to migrate toward the surface of the medium. *G. xylinus* produced BC to propel the bacteria strain upward to the air-liquid interface, where oxygen concentrations are high. Thus, BC-producing *G. xylinus* is generally found on the upper part of the BC layer formed (Cakar *et al.*, 2014).

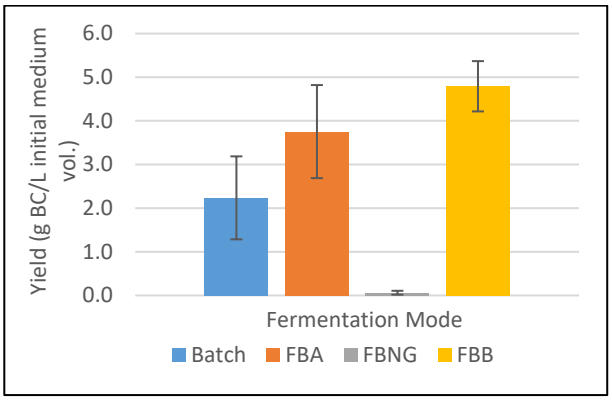


Figure 4: Yield of BC in different fermentation modes.

Over time, as the nutrients in the upper part of the BC layer deplete, the rate of BC production decreases. The bacteria on the upper part of BC floating on the medium may keep on producing BC if a sufficient amount of nutrients is provided to the upper part of the BC layer. According to Hsieh *et al.* (2016), the time for new pellicle formation when culturing *G. xylinus* for 30 days in Schramm & Hestrin's medium is roughly proportional to the distance between the existing pellicle and the new air-liquid interface to some extent. If the feed volume is large, the distance between the newly formed air-liquid interface and the BC upper layer will be large. This causes the bacteria to take a longer time to diffuse from the BC layer to the new air-liquid interface, allowing them to continue producing new BC layers at the new air-liquid interface (Hsieh *et al.*, 2016). This condition is illustrated in Figure 5. In FBA mode, the feeding volume is high, and the feed frequency is low. This condition is illustrated in Figure 5(b), whereas the FBB mode may be depicted in Figure 5(a). Even though in FBB mode, the feed frequency is high, each feed has a lower feed volume, and thus the bacteria strain can diffuse to the new air-liquid interface to produce BC within a shorter time. This explains the higher yield of the FBB mode as compared to the FBA mode, as the BC production rate in the FBB mode is higher due to the lower feed volume.

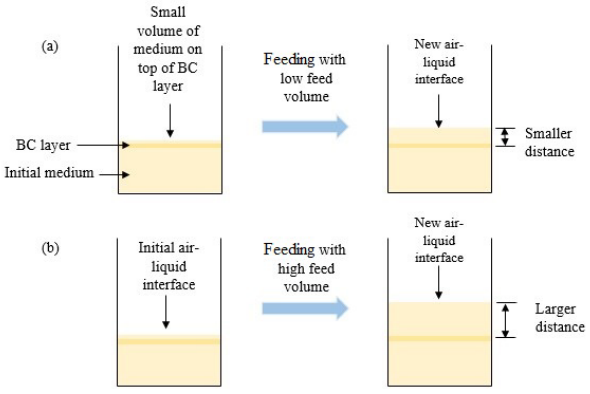
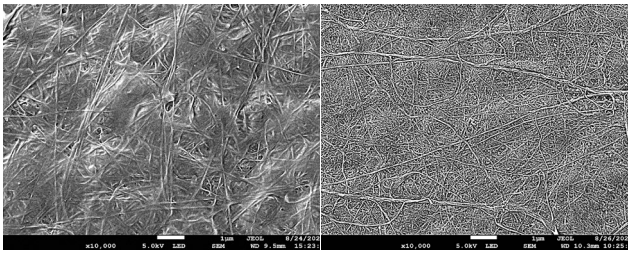


Figure 5: Relationship of feed volume and layer-interface distance with (a) low feeding volume and (b) high feeding volume.

On the other hand, yeast extract contained in the YGC medium acts as the nitrogen source for cells in Batch, FBA, and FBB modes. According to Yodsuwan *et al.* (2012), yeast extract is a very suitable nitrogen source for BC production by the strain *G. xylinus*. The absence of yeast extract, even though other nitrogen sources such as (NH4)2SO4 are used, directly affects the yield of BC production (Yodsuwan *et al.*, 2012; Aswini *et al.*, 2020). In terms of morphology, FBNG also produces BC with a lower BC strand density compared to the FBA mode, even though the same volume of feed and feeding frequency were used for these modes. This is illustrated in Figure 6, where BC from FBA mode are more compact and plump, whereas BC formed in FBNG mode have a flatter appearance. The BC microfibrils in FBNG mode are also generally thinner compared to those in FBA mode. This may be due to the lack of a nitrogen source in FBNG mode (Yodsuwan *et al.*, 2012; Aswini *et al.*, 2020).



(a) (b)

Figure 6: FE-SEM images of BC from (a) FBA mode and (b) FBNG mode at ×10,000 magnification.

Figure 7 depicts the water-holding capacity exhibited by BC produced in all four modes. Similar to the yield of BC, the WHC of BC produced in Batch, FBA, and FBB is found to be of not much difference (29.30, 23.42, and 24.87 g water/g sample, respectively). However, the WHC of BC produced in FBNG was calculated to be 87.34 g water/g sample, which deviates greatly from other modes. This may be due to the lower BC strand density formed in FBNG. The WHC obtained in this work, however, was relatively lower compared to the WHC reported by Portela *et al.* (2019) (60-700 g water/g sample). According to Bechki *et al.* (2019), the WHC property of cellulose is due to the ability of the OH functional groups to form hydrogen bonds with water molecules. As the strand density is lower, there are more spaces between the strands, and a larger total surface area is exposed to the water phase. Thus, more OH functional groups are exposed to the water phase to form more hydrogen bonds with the water molecules and entrap more water molecules within the BC structure (Hubbe & Rojas, 2008).

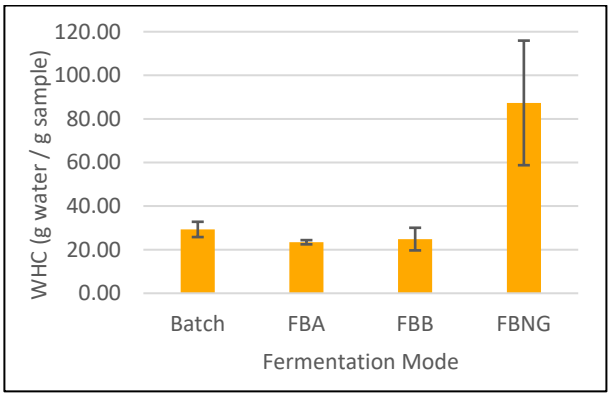
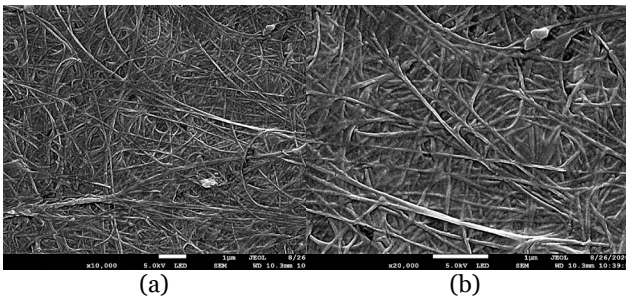


Figure 7: Water holding capacity (WHC) of BC in different modes.

2. Surface morphology - Field emission scanning electron microscope (FE-SEM) micrographs

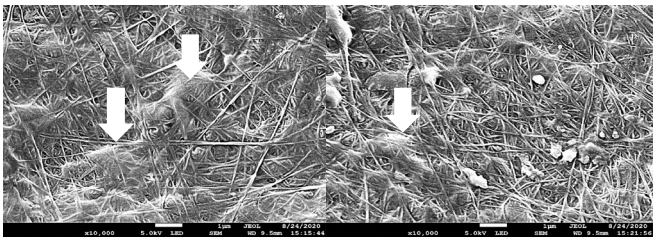
Figure 8 shows the FE-SEM micrographs of the BC produced by Batch mode. The BC samples were treated to separate the cellulose microfibers into clear, individual cellulose strands. From the images, spaces are visible between the microfibers of the BC, which contributes to the high BC total surface area in the sample. This enables the BC to retain a high volume of water per BC mass as more hydroxyl groups (OH) are exposed to the water phase. The hydrogen bonds formed between the hydroxyl groups and water molecules, however, are mostly formed only at the outer layer of the BC, where the BC is exposed to the water phase (Hubbe & Rojas, 2008).



(a) (b)

Figure 8: FE-SEM micrographs of BC produced by Batch mode at (a) ×10,000 magnification (b) ×20,000 magnification.

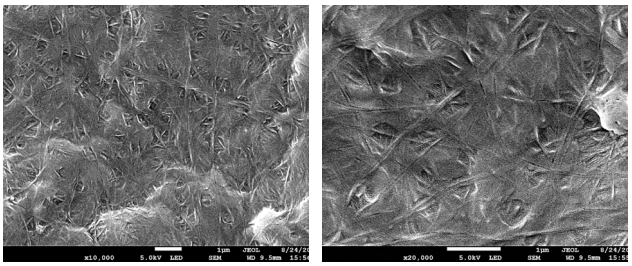
The micrographs shown in Figure 9 show that FBA mode produces denser BC compared to Batch mode (Figure 8(a)), and there are more microfibril 'lumps' as shown. This is due to the longer fermentation period in FBA mode (14 days) compared to Batch mode (7 days). The BC produced by this mode, however, is more uneven in terms of microfibril layering, as shown in Figure 9(a). This may be due to the disturbances caused in the medium during feeding sessions, as accidental medium agitation may cause uneven cell population distribution (Parte *et al.*, 2020). As *G. xylinus* diffuses to the air-liquid interface by using the buoyancy of BC formed, agitation during feeding may cause some newly formed BC clusters to overlap, and as the BC layer thickens, the overlapped BC part may become denser compared to other parts of the BC layer. This may be the cause of the formation of 'lumps' on the BC.



(a) (b)

Figure 9: FE-SEM micrographs of BC produced by FBA mode at ×10,000 magnification. These images were taken from the same sample but on different locations. Arrows show the BC 'lumps' where microfibril density are higher than other parts on the BC.

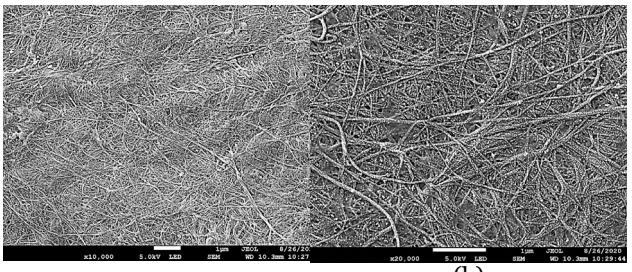
Figure 10 shows that the BC formed using the FBB (high feed frequency) method has denser, less even microfibril layering than the Batch mode (Figure 8(a)), which is similar to the BC formed using the FBA (low feed frequency) mode (Figure 9(a)). Compared to FBA mode, BC in FBB mode demonstrates uneven microfibril layout to a higher degree (more 'lumps' were visible as compared to FBA), with a higher microfibril density. This means in FBB mode, the bacteria strain was able to produce microfibrils at a higher rate to produce BC with a higher microfibril density than in FBA mode within the same period. This supports the theory that lower feed volume ensures a higher BC microfibril production rate (denser BC is produced) as bacteria strains take less time to diffuse to the new aeration zone, which is the new air-liquid interface formed after the feeding session, as discussed in Section IIIB1.



(a) (b)

Figure 10: FE-SEM micrographs of BC produced by FBB mode at (a) ×10,000 magnification (b) ×20,000 magnification.

Figure 11 depicts the FE-SEM micrographs of BC formed in FBNG mode. BC from FBNG (pure glucose solution feed) is less dense than BC formed in FBA and FBB. The microfibrils are also visibly thinner than those formed in the other three modes. This may be due to the lack of a nitrogen source in FBNG mode. Nitrogen is the main component in protein compounds in cells and comprises 30 - 80% of the dry cell mass of bacteria (Nyyssölä *et al.*, 2022). It is a major element vital in ensuring proper cell metabolism and BC production. Also, as the thickness is thinner when the BC is immersed in the water phase, the total surface area exposed to water molecules is significantly higher than BC from the other three modes. In other words, more hydrogen bonds may be formed between the cellulose strands in BC. This contributes to the high water holding capacity, as shown in Figure 7, Section IIIB1 (Hubbe & Rojas, 2008).

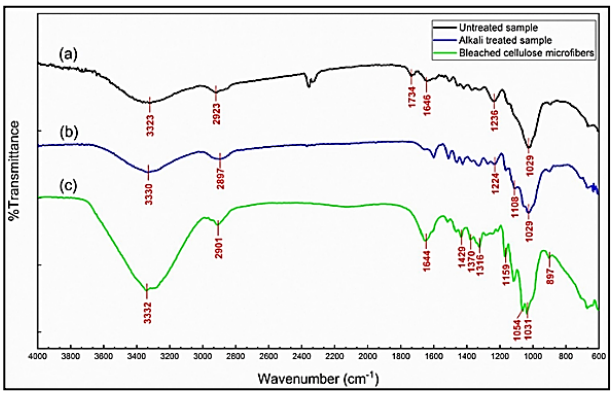


(a) (b)

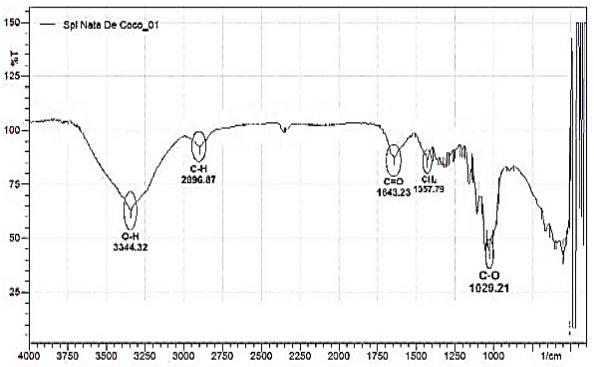
Figure 11: FE-SEM micrographs of BC produced by FBNG mode at (a) ×10,000 magnification (b) ×20,000 magnification.

3. Fourier-transform infrared spectroscopy (FT-IR) analysis

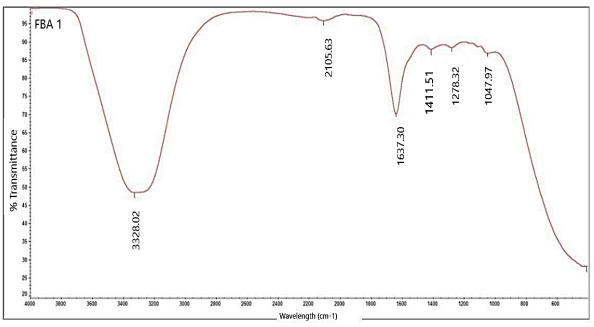
FT-IR is currently one of the most important techniques used to analyse chemical composition changes that occur during chemical treatments (Maryana *et al.*, 2014). It can detect the presence of different functional groups in samples. Figures 12(a) and 12(b) show comparisons of the IR spectra of plant cellulose and nata de coco samples obtained by Bechki *et al.* (2019) and Elfiana *et al.* (2018) to the IR spectra of BC produced in FBA mode (Figure 12(c)). It can be seen that BC produced in FBA mode have the characteristic functional groups of cellulose, which are similar to the nata de coco and plant cellulose. Thus, the fed-batch mode didn't affect the BC composition.



(a)



(b)



(c)

Figure 12: IR spectra of (a) plant cellulose from *Retama raetam* (Bechki *et al.,* 2019); (b) nata de coco (Elfiana *et al.,* 2018); (c) BC produced from FBA mode.

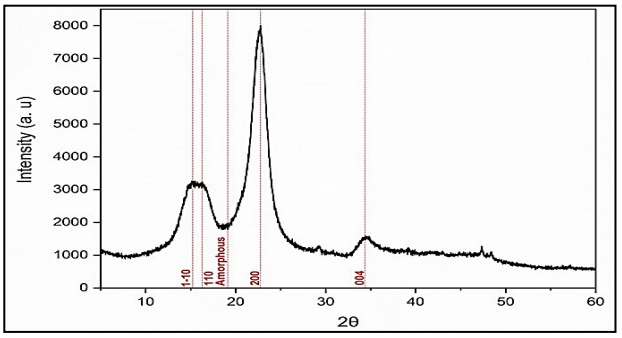
Figure 12(c) shows the BC peaks at 3328.02, 2105.63, 1637.30, 1411.51, 1278.32, and 1047.97/cm. The presence of the characteristic OH functional group stretching in the BC is indicated by the peak at 3328.02/cm. This is in the range of 3200 to 3500/cm as reported by Luo *et al.* (2014). Compared with plant cellulose, the peak at 3328.02/cm is much deeper than that in the plant cellulose (untreated sample). This is due to the higher number of OH groups present in the BC sample compared to the plant cellulose sample (Bechki *et al.*, 2019). Another characteristic band of BC and other types of cellulose is found at around 2923.00/cm for plant cellulose (Figure 12(a)) and 2896.87/cm for nata de coco (Figure 12(b)). This band represents the presence of CH_2 group stretching in the cellulose structure (Bechki *et al.*, 2019). The absence of this band in the BC spectra may be due to overlapping with the intermolecular hydrogen-bonded OH stretching frequency (Biyik & Coban, 2017).

C-O-C bonds, on the other hand, are represented by the intensity band at 1047.97/cm, the peak on the right in Figure 12(c). This characteristic band may be attributed to the antisymmetric bridge stretching for the C-O-C groups in cellulose (Atykyan *et al.*, 2020). This is comparable to the IR spectra of plant cellulose and nata de coco, with peaks at 1029.00/cm and 1029.11/cm, respectively. For BC, the peak at 1637.30/cm corresponds to the presence of carboxylic acid groups stretching (COOH) and the peak at 1411.51/cm represents the carbonyl functional group (CH_2) present in the structure (Gayathry & Gopalaswamy, 2012), whereas the peak at wavelength 1278.32/cm is attributed to the C-C and C-O skeletal vibrations (Gao *et al.*, 2014).

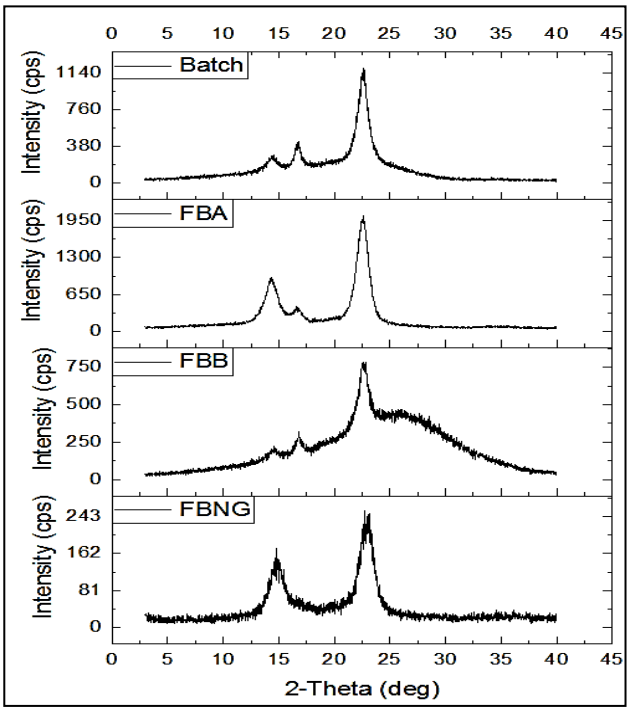
4. Crystallinity and particle size

BC samples from each mode were analysed using the X-ray Diffraction (XRD) method to obtain the crystallinity and calculate the particle size. As a comparison, Figure 13(a) shows the XRD diffractogram for plant cellulose obtained by Bechki *et al.* (2019). The area under peaks represents the crystallinity of the sample, whereas the total area under the curve represents the area of crystallinity and amorphosity. These steps were done by using the Origin software, which aids in plotting curves and calculating areas under curves.

Figure 13(b) shows the XRD diffractograms of different fermentation modes.



(a)



(b)

Figure 13: XRD diffractograms for (a) cellulose of the plant *R. raetam*. (Bechki *et al.,* 2019); (b) BC from different fermentation mode.

Figure 13(b) depicts a similar trend in the XRD curves of the BC samples from Batch, FBA, and FBB, with three peaks at approximately theta = 14.0, 17.0, and 22.5. This shows that BC has similar crystallinity as plant cellulose. However, the intensity of the first two peaks for BC varied. This may be caused by the different sample texture orientations, as each sample may have a different preferred orientation. On the other hand, FBNG produces BC with a peak intensity significantly lower than the other three modes. Also, there are only two peaks instead of three. This indicates that the BC has fewer planes that produce constructive interference

of X-ray monochromatic beams, which are scattered at specific angles (Park *et al.*, 2010). Table 2 shows the crystallinity of BC from each mode. According to Bechki *et al.* (2019), the crystallinity of *R. raetam* plant is 77.80%, and the particle size is 3.62 nm.

Table 2: Crystallinity results of each mode.

Mode	Total Area under Peaks	Total Area under Curve	Crystallinity	Crystallite Size
Batch	3539.7	4244.9	83.4%	7.2 nm
FBA	4830.1	5731.5	84.3%	6.3 nm
FBB	5035.3	6390.7	78.8%	7.5 nm
FBNG	742.4	957.9	77.5%	5.7 nm

Based on Table 2, the crystallinity of BC is generally higher than that of plant cellulose. This is due to the absence of lignin and hemicellulose, which are often present in plant cellulose (Kamal & AlZubaidy, 2019). The high crystallinity also contributes to the high tensile strength (Gao *et al.*, 2019; Yu *et al.*, 2020). Furthermore, BC produced in FBB has the highest crystallite size compared to the other modes. These results are comparable to reported sizes of cellulose particle sizes, which generally range between 4 and 7 nm (Park *et al.*, 2010). The values are slightly higher than the BC crystallite size reported by Singhsa *et al.* (2018), obtained through culturing in glucose-yeast extract medium with 100 g/L glucose (4.8 to 6.6 nm).

5. Mechanical strength - Tensile strength test

Figure 14 shows the stress-strain curve for BC of all four modes. The curves showed that BC produced by all modes displayed overall viscoelastic behaviour when stretching forces are applied. At the low strain region of 0 to approximately 0.025, the stress calculated was found to be less dependent on the applied tensile strain. A near-linear plastic region (approximately 0.025 to 0.15) was then observed following the low-strain region. The Young's Modulus for each fermentation mode was obtained as the gradient of this region (Chen *et al.*, 2018). Figure 15 and Table 3 show the mechanical properties of BC produced in each mode.

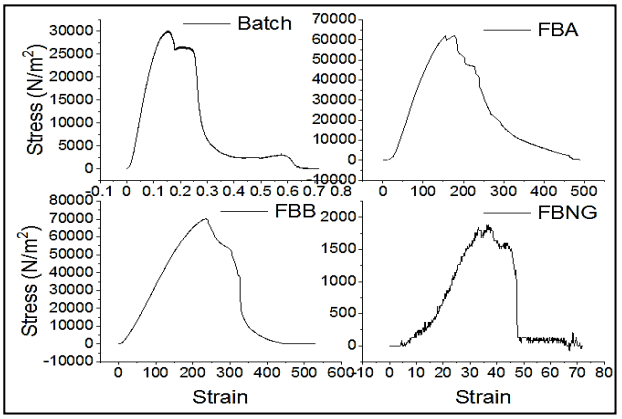


Figure 14: Stress-strain curve for Batch, FBA, FBB and FBNG fermentation mode.

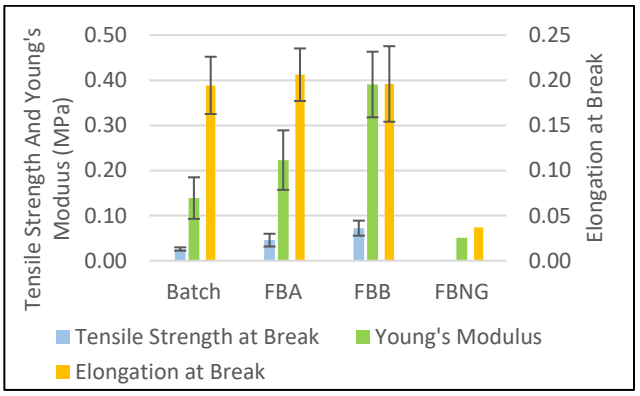


Figure 15: Tensile strength at break, Elongation at break and Young's Modulus of BC produced in all modes.

The tensile strength test was run in triplicate samples for all modes. The BC produced in the FBB mode has significantly higher tensile strength and elongation compared to the other modes. On the other hand, BC from FBA mode has the highest Young's modulus among all modes. This shows that the longer fermentation period in fed-batch mode (14 days) produces BC with higher mechanical strength due to the high density of cellulose microfibrils produced (Chen *et al.*, 2018). For FBNG, since the second and third samples produced are in powdered form due to drying, only one sample was used for the tensile strength test. The analysis illustrated that nitrogen sources such as yeast extract are essential in producing BC with high quality in terms of mechanical strength. Therefore, peptone or corn steep liquor may be added at specific concentrations to improve BC yield and tensile strength (Costa *et al.*, 2017). The results of this work were relatively lower compared to

those reported by Tsouko *et al.* (2015), which were 72.3 to 132.5 MPa for tensile strength and 0.97 to 1.64 GPa for Young's modulus. This may be due to the differences in sample preparation, as Tsouko *et al.* (2015) used hydrated, lyophilised BC samples produced by using an HS medium, and the glucose source was substituted with crude glycerol. On the other hand, the BC used in this text was dried completely in an oven before the tensile strength test. According to Pa'e *et al.* (2014), different drying methods also yield BC with different tensile strengths.

Table 3: Mechanical characteristics of BC produced in all modes.

Fermentation Mode	Tensile Strength at Break (MPa)	Elongation at Break	Young's Modulus at Break (MPa)
Batch	0.026 ± 0.0037	0.1943 ± 0.0317	0.1388 ± 0.0461
FBA	0.0457 ± 0.0142	0.2062 ± 0.0291	0.2230 ± 0.0660
FBB	0.0723 ± 0.0166	0.1959 ± 0.0419	0.3906 ± 0.1727
FBNG	0.0019[a]	0.0370[a]	0.0509[a]

[a]Standard deviation was not included as out of three FBNG samples, only one sample is thick enough to remain in film form after drying.

IV. CONCLUSION

Based on the study results, it is clear that fed-batch cultivation is a highly effective approach for producing high-quality and high-yield bacterial cellulose (BC). Specifically, a higher frequency of feeding is desired to achieve better yield and quality of BC produced. In terms of feeding composition, the study found that using a YGC medium is essential for producing BC at a higher yield, due to its nitrogen source. However, the study also found that BC produced using pure glucose solution has the highest water-holding capacity, making it an excellent option for certain applications. In addition, BC produced through fed-batch high feeding frequency mode was found to have denser microfibrils in FE-SEM micrographs, as well as the highest mechanical strength and elongation at break. The study also found that the fed-batch mode with low feeding frequency has the highest Young's Modulus. The FT-IR analysis showed that the chemical composition of BC, in terms of the presence of functional groups, is similar for all modes. XRD analysis results also showed that the crystallinity of BC produced in all modes is similar, ranging from 77.50% to 84.27%.

Overall, the study suggests that utilising fed-batch mode is a highly feasible and effective approach for producing high-quality BC at a greater yield, while still maintaining economic value. This method could replace batch mode, which is less cost-effective due to lower production rates. The best feeding mode is high-frequency intermittent feeding with YGC medium at 3-day intervals for up to 14 days. Additionally, it is suggested that utilising alternative nitrogen sources, such as food wastes could be further explored in future studies to maximise the efficiency of the fed-batch fermentation mode. In conclusion, the findings from this study provide strong evidence that utilising fed-batch mode for producing BC is highly effective, and can produce superior-quality BC with a higher yield. The study's results provide important insights for researchers and industry professionals seeking to maximise the economic value of BC production while maintaining high-quality standards.

V. ACKNOWLEDGEMENT

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VI. CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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