Valorization of pineapple peel waste: Investigating cultivation conditions for bacterial cellulose production

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Abstract. A fresh pineapple typically produces about 40 to 50% of pineapple peels and cores. If these fruit residues are not properly treated, they can cause severe environmental issues. In fact, pineapple peels are rich in sugar content, which is suitable for use as carbon source in microbial fermentation to produce valuable products like bacterial cellulose (BC). BC is a versatile biomaterial with diverse applications because of its excellent physical and mechanical characteristics. This research aimed to use pineapple peel extract as a substitute for the commercial glucose usually used in the medium. Fermentation uses waste as a substrate for microbial growth and product formation under static conditions, certain growth conditions are necessary to ensure the desired product yield and properties. This study showed that peel juice concentration, the medium pH, the fermentation temperature and the duration significantly influenced both cells and BC concentrations. The best condition for producing BC was recorded when the experiment was set up at a waste juice concentration of 80% (w/v), medium pH 4.0, fermentation temperature of 30°C, and 12 days of fermentation time. This study will enable the efficient utilization of pineapple peel wastes from industry and could help reduce the costs of BC production.

1 Introduction

Pineapple is one of the most widely produced fruit, being commercialized and consumed all over the world. The Malaysian Pineapple Industry Board focuses on expanding pineapple production and generating quality canned pineapple. However, the canning process in the pineapple industry results in substantial waste, comprising approximately 40-50% of the total fresh pineapple weight, primarily consisting of peels and cores [1]. As the production of pineapple products increases, so does the generation of significant amounts of waste. This waste is rich in organic matter and suspended solids, making it prone to microbial spoilage. If not managed well, it can lead to significant pollution problems due to its high biochemical and chemical oxygen demand [2].

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This has led researchers to explore ways to turn pineapple waste into valuable products. Siti Roha et al. [3] highlight that pineapple waste contains high levels of simple sugars like sucrose, glucose, and fructose, with pineapple peels alone having a sugar content of 8.09% (w/v). This nutrient-rich material has potential for conversion into a fermentation medium for producing valuable products like bacterial cellulose (BC). BC is a type of insoluble exopolysaccharide. Unlike plant-derived cellulose, BC lacks hemicellulose and lignin, which contributes to its remarkably pure form of cellulose [4]. Despite sharing an identical chemical structure with plant cellulose, BC exhibits higher crystallinity, better water holding capacity, increased hydrophilicity, higher polymerization degree, greater porosity, and stronger mechanical strength [5]. These characteristics make BC suitable for various applications in the paper, textile, pharmaceutical, and food industries.

Various bacterial species, including those from genera like *Acetobacter*, *Achromobacter*, *Aerobacter*, *Agrobacterium*, and *Azotobacter*, are known to produce BC [6]. However, the cost of the fermentation medium accounts for about one-third of the total production cost [7], which limits large-scale production and commercial use of BC products. To reduce these costs, researchers are looking into using food and agro-industrial wastes as alternative sources for BC production [8]. However, the composition of the fermentation medium, especially when derived from different waste sources, greatly affects the yield and quality of BC [9]. A specific concentration of nitrogen and carbon sources is crucial for high BC production and enhanced properties, while exceeding these concentrations can reduce cell yield due to environmental adaptation challenges [10-11].

Adjusting the composition of the waste medium, as well as controlling pH, temperature, and fermentation time, is key to improving bacterial growth and BC production. Previous studies have indicated that high BC production typically occurs within a pH range of 4.0 to 7.0, though this can vary with different bacterial strains [11-13]. Additionally, maintaining the appropriate fermentation temperature is important, as it influences the energy available for both cell growth and the cellulose biosynthetic pathway [14]. Typically, the incubation temperature is maintained between 25 and 30°C, varying based on the strain and the characteristics of the BC being targeted [15]. Moreover, the duration of cultivation directly affects the final BC yield, with a 7-day growth period reported as best result by Sheykhnazari et al. [16], while Feng et al. [17] observed that an 11-day incubation led to the highest BC yield. These varying results highlight the need to understand how different factors influence BC production, particularly when fermentation utilizes waste as a substrate. This study, therefore, aims to investigate the effects of pineapple peel waste juice concentrations, pH levels, temperature, and fermentation duration on cell growth and BC yields. It is anticipated that using pineapple waste for BC production offers a sustainable approach for both waste reduction and the generation of valuable product.

2 Materials and methods

2.1 Materials

Figure 1 illustrates the experimental setup used to produce BC. Pineapple peel waste was the raw material for this study. *Gluconacetobacter xylinus* ATCC® 700178™ (*G. xylinus*) was obtained from the American Type Culture Collection (located in Manassas, VA, USA). Chemical such as D-glucose, calcium carbonate, and sodium phosphate dibasic were purchase from Biobasic, Canada. Yeast extract was obtained from Pronadisa, Spain. Bacteriological agar, cellulase from *Trichoderma Reesei*, and citric acid were purchased from Sigma-Aldrich, US. Chemical bought from Merck, US are including acetic acid, and sodium hydroxide.

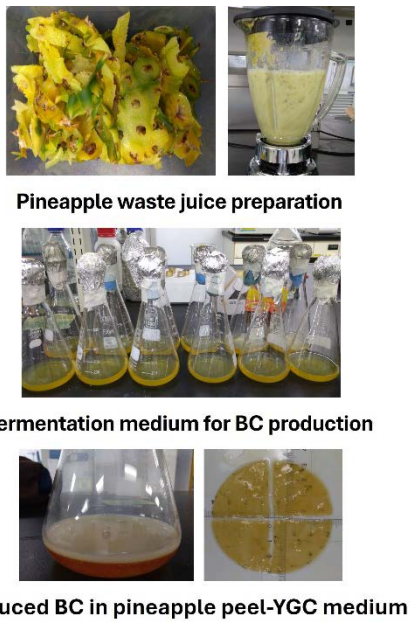

Fig. 1. Production of BC by *Gluconacetobacter xylinus* (ATCC® 700178) in pineapple peel-YGC medium.

2.2 Pineapple peel waste collection and preparation

Pineapple peel waste was collected from fruit store. The peels were chopped into small pieces and combined with water in a household blender at a 1:1 ratio, blending for 3 min. The blended mixture was filtered through a cotton cloth strainer to separate the solids from the liquid, which was further separated using centrifugation at 10,000 ×g for 10 min at 4°C. After centrifugation, supernatant (waste juice) was collected and stored at -20°C for subsequent use [18].

2.3 Bacterial glycerol stock preparation

G. xylinus bacterial strain was cultivated in ATCC® Medium 459: yeast extract-glucose-calcium carbonate (YGC) medium. This medium contains glucose at 50 g/L, yeast extract at 5 g/L, and calcium carbonate at 12.5 g/L. YGC agar reagents (YGC medium with addition 15 g/L of agar) were dissolved in distilled water, and the mixture pH was adjusted to 5.0 by adding glacial acetic acid, continuing with sterilization at 121°C for 15 min. *G. xylinus* was streaked on the sterized agar medium and incubated in an incubator at 28°C for 3 days. The colony of *G. xylinus* was then transferred from agar medium into sterized YGC medium (pH 5.0) and incubated for 7 days at 28°C [10]. After incubation, the culture was mixed with sterized glycerol at final concentration of 20% (v/v), aliquoted into tubes and stored at -80°C.

2.4 Inoculum preparation

YGC medium (pH 5.0) was prepared and autoclaved at 121°C for 15 min. The sterilised medium was then inoculated with 1% (v/v) glycerol stock of *G. xylinus* and incubated

statically at 30°C for 7 days [19]. After the incubation, the inoculum was further used for BC production by using pineapple peel waste juice.

2.5 Varying fermentation conditions for bacterial cellulose production

Fermentation medium for BC production were prepared by mixing the substituting the commercial glucose in YGC medium by pineapple peel waste juice. The pH of the mixture was adjusted accordingly by using glacial acetic acid and sterilized at 121°C for 15 min. After sterilization, the culture medium was inoculated with 10% (v/v) inoculum and incubated statically at in an incubator [19]. BC production was conducted at different waste juice concentrations (ranging from 0, 20, 40, 60, 80, and 100% w/v), medium pH levels of 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5, the temperature of 26, 28, 30, 32, and 34°C, and the duration of fermentation of 3, 5, 7, 10, 12, and 14 days. Duplicate runs were implemented for each factor intervals.

2.6 Determination of cell production

After fermentation, the culture underwent centrifugation at 12,000 ×g for 5 min. The supernatant was discarded, and the resulting pellet was subjected to incubation in a buffer solution (pH 5.0, composed of citric acid – sodium phosphate dibasic, with 2% (v/v) cellulase from *Trichoderma Reesei*) at 50 °C with a rotational speed of 200 rpm for 2 h to degrade the cellulose. The mixture was then centrifuged at 12,000 ×g for 5 min and the collected cell pellet was dried at 80 °C for 24 h. After the drying process, the mass of dried cell pellet was weighted, and the cell production was calculated using equation (1) [20]. All sample runs were measured in duplicate.

$$Cell\ concentration\ \left(\frac{mg}{mL}\right) = \frac{mass\ of\ dried\ cell}{initial\ volume\ of\ culture\ medium} \quad (1)$$

2.7 Determination of bacterial cellulose production

Following fermentation, the culture underwent centrifugation at 12,000 ×g for 5 min, and the supernatant was removed. The collected pellet was then incubated in 0.1 M sodium hydroxide at 80 °C with agitation at 100 rpm for 1 h to lyse the bacterial cells. This lysis procedure was conducted twice to ensure all cells have decomposed. The mixture was centrifuged again under the same conditions, and the resulting BC pellet was dried at 80 °C for 24 h. After drying, the mass of dried BC pellet was measured, and the BC production was determined using equation (2).

$$BC\ concentration\ \left(\frac{mg}{mL}\right) = \frac{mass\ of\ dried\ BC}{initial\ volume\ of\ culture\ medium} \quad (2)$$

2.8 Determination of bacterial cellulose thickness

The BC harvesting and processing steps described by Jagannath et al. [10] was used in the present study. Following fermentation, the BC was gathered and subjected to multiple washes with water to eliminate any remaining culture medium. To remove the sour odor, the BC was soaked in water for 24 h, with the water being changed multiple times. After wiping-off

excess liquid, the BC thickness was measured at various points using a digital vernier caliper, with measurements taken in duplicate.

3 Results and discussion

3.1 Effect of waste juice concentration

In this study, the effect of various concentration of pineapple peel waste juice on microbial growth and BC production were examined through a series of fermentation with *G. xylinus*. BC samples were produced using different concentration of pineapple waste juice [0, 20, 40, 60, 80 and 100% (w/v)], and the resulting cell and BC concentrations, as well as BC thickness were compared (Table 1). At zero and 20% (w/v) of waste juice concentration, only very thin and soft BC was produced on the medium surface. BC was unable to maintain the shape for measurement and hence the BC thickness was not able to be measured. In this study, the commercial glucose in YGC medium was replaced by pineapple peel waste juice. Fermentation with no or at a low waste juice concentration in the medium produced low concentrations of cells and BC because there was absence of carbon source for bacterial growth and BC production.

Table 1. Effect of pineapple peel waste juice concentration on cell concentration, BC concentration and BC thickness produced by *G. xylinus* (ATCC® 700178™). Standard deviation (SD): duplicate experimental runs with duplicate measurements.

Waste Juice Concentration (% w/v)	Cell Concentration[a] (mg/mL)	BC Concentration[a] (mg/mL)	BC Thickness[a] (mm)
0	0.61 ± 0.06	0.31 ± 0.09	n.d.[b]
20	0.25 ± 0.08	0.48 ± 0.05	n.d.[b]
40	1.04 ± 0.12	1.69 ± 0.02	0.83 ± 0.20
60	1.77 ± 0.21	2.60 ± 0.01	0.74 ± 0.05
80	1.96 ± 0.31	2.83 ± 0.27	1.01 ± 0.15
100	2.07 ± 0.31	2.64 ± 0.48	1.35 ± 0.21

[a] mean ± SD

[b] n.d. = not determined

For the waste juice concentration ranging from 0 to 80% (w/v), the cell and BC concentrations were increased. The BC thickness follows a similar pattern, with noticeable increases as the waste juice concentration rises. In the process of BC synthesis, tiny subfibrils combine to form microfibrils, which then bundle together into larger cellulose ribbons that eventually merge by hydrogen bonds and Van der Waals forces to create a dense, continuous layer of cellulose [21]. The juice derived from peel waste serves as a source of sugar (sucrose, glucose, and fructose) in the culture medium [3]. These microfibrils are the cellulose chain that polymerized from sugar units in fermentation [22]. The cellulose chain length increased with increasing sugar content due to the conversion of more sugar into 1, 4-β-glucan chains, which polymerize together to form cellulose [23].

Using pineapple waste juice for BC fermentation is significantly more cost-effective than using pure pineapple juice. Besides, it also reduces environmental issues such as methane gas formation and the degradation of soil organism protein caused by pineapple waste. Table 1

indicates that increasing the waste juice concentration beyond 80% (w/v) results in relatively stable cell and BC concentrations, suggesting a possible saturation point where the cells growth and BC production stabilize despite higher juice concentrations. Therefore, 80% (w/v) pineapple waste juice have been chosen for the pH study due to its cost-effectiveness.

3.2 Effect of medium pH

The experiment demonstrated that varying the initial pH levels in the fermentation medium (from 4.0 to 6.5) significantly impacted cell production, BC production and BC thickness (Table 2). At the acidic initial pH of 4.0, the cells seemed to thrive the most, producing the highest amount of cells and BC compared to other pH levels. However, the production of cells and BC decreased when the initial medium pH was increased. The thickness of BC also followed this trend, being thicker at the initial pH of 4.0 and much thinner when the initial pH was higher. After 14 h of fermentaion, BC thickness was not measurable when the starting medium pH was set above 5.5.

Table 2. Effect of medium pH on cell concentration, BC concentration and BC thickness produced by *G. xylinus* (ATCC® 700178™). Standard deviation: duplicate experimental runs with duplicate measurements.

Medium pH	Cell Concentration[a] (mg/mL)	BC Concentration[a] (mg/mL)	BC Thickness[a] (mm)	Final Culture pH[a]
4.0	13.29 ± 1.31	9.23 ± 0.31	7.12 ± 0.32	6.87 ± 0.28
4.5	7.57 ± 0.08	4.02 ± 0.49	2.69 ± 0.24	7.27 ± 0.18
5.0	5.33 ± 1.03	1.61 ± 0.46	1.31 ± 0.13	7.69 ± 0.06
5.5	5.31 ± 0.07	1.48 ± 0.19	1.33 ± 0.25	7.78 ± 0.08
6.0	5.19 ± 0.11	0.18 ± 0.03	n.d.[b]	8.18 ± 0.06
6.5	2.98 ± 0.30	0.07 ± 0.02	n.d.[b]	8.23 ± 0.10

[a] mean ± SD

[b] n.d. = not determined

The fermentation medium in this study was composed of waste juice, yeast extract, and calcium carbonate, with the initial pH reduced by adding acetic acid. Acetic acid, being a metabolic product in the metabolic network of *G. xylinus* [24], was present in significant amounts in the medium along the fermentation. Combination of acetic acid and calcium carbonate produced calcium acetate, water and carbon dioxide. Vandamme and colleagues [25] suggested that acetic acid functions to regulate pH directly within the system. It is converted into acetyl-CoA, which then enters the tricarboxylic acid cycle. During this process, it decomposes into carbon dioxide and water, leading to the production of ATP. The produced ATP, along with the role of acetic acid in gluconeogenesis [26], enhances glucose availability, ultimately improving sugar utilization for cellulose production. The present study indicates that a low pH medium is best for both cell the BC production. This supports the findings of Chen et al. [27], which suggest that cellulose-producing bacteria grows in acidic environments rather than alkaline conditions. In industrial fermentation processes for BC production, this situation is particularly advantageous as it helps minimize microbial contamination.

Dirisu et al. [28] found that pH levels of 4.0 and 5.0 were most effective for cell growth and cellulose production by *G. xylinus* in a Moringa oleifera tea-sugar medium. Previous studies, such as those by Jagannath et al. [10] and Verschuren et al. [13], also noted that a pH of 4.0 is favorable for static fermentation with *Acetobacter xylinum* (*A. xylinum*), achieving

the maximum thickness of BC. In fact, the fermentation process involving *A. xylinum* generates both cellulase and bacterial cellulose. Tahara et al. [29] reported that when the cultivation is carried out at a lower pH of 4.0, the production of cellulase is lower, resulting in a higher degree of cellulose polymerization. Conversely, at pH 5, cellulase activity is elevated, leading to a reduction in the polymerization level of cellulose. The polymerization degree of cellulose synthesized by *A. xylinum* is reduced when cellulase is produced by the same strain. Therefore, adjusting the pH of the culture medium can regulate cellulase activity and help maintain the polymerization degree of BC.

In addition, the final pH of the residual culture medium was determined at the end of fermentation. The initial medium pH, which ranged from 4 to 6.5, had risen to above 6.8 (see Table 2). Previous studies showed that pH usually drops after fermentation due to glucose being converted into gluconic acid [21]. However, this study observed a rise in pH after fermentation, likely because the acidic acid in the medium was consumed. Literature suggests that organic acids positively influence BC biosynthesis [30], with acetic acid in the medium enhancing this process by reducing the production of gluconic acid [24].

3.3 Effect of fermentation temperature

Table 3 illustrates the effect of fermentation temperature on the cells production, BC production, and thickness, with temperatures ranging from 26 to 34°C. At a temperatures of 26°C, low productions of cell and BC were observed, likely due to the metabolic activity of the bacteria involved in the fermentation. Cooler conditions tend to slow down bacterial metabolism, leading to decreased enzymatic activity, which makes the bacteria less efficient at converting nutrients into BC, resulting in the observed lower output.

Table 3. Effect of fermentation temperature on cell concentration, BC concentration and BC thickness produced by *G. xylinus* (ATCC® 700178™). Standard deviation: duplicate experimental runs with duplicate measurements.

Fermentation Temperature (°C)	Cell Concentration[a] (mg/mL)	BC Concentration[a] (mg/mL)	BC Thickness[a] (mm)
26	8.02 ± 0.57	13.09 ± 0.52	3.72 ± 0.12
28	11.78 ± 2.32	20.40 ± 1.42	7.52 ± 0.08
30	12.98 ± 0.63	19.96 ± 0.80	7.88 ± 0.12
32	13.57 ± 2.83	20.40 ± 1.42	6.30 ± 0.09
34	9.45 ± 0.14	12.61 ± 3.17	4.03± 0.15

[a] mean ± SD

[b] n.d. = not determined

As the temperature increases to 28°C, there is a noticeable improvement in both the concentration of cells and BC produced and the thickness of the BC film. The increased trend of cells continues as the temperature reaches 30°C, where the higherst BC thickness of 7.88 mm was obtained. This suggests that the conditions at 30°C are best for the fermentation process. This finding aligns with previous research by Son et al. [31]. The data reveal that fermentation temperature between 28°C to 32°C result in comparable BC concentrations, suggesting that these mid-range temperatures are favorable for bacterial activity in BC synthesis. Controlling the temperature during fermentation plays a crucial role in the energy supply needed for cell growth and the synthesis of cellulose, which requires energy to convert

glucose into BC [14]. At these temperatures, the enzymatic activities and metabolic processes are likely functioning efficiently, which enables the bacteria to consistently produce similar amounts of BC.

However, when the temperature exceeds 32°C, a decline in both BC production and thickness was observed again. This reduction can be attributed to the fact that temperatures above the optimal range may begin to denature essential proteins and enzymes within the bacteria, thereby inhibiting their ability to produce cellulose effectively. This suggests that while a moderate increase in temperature enhances bacterial productivity, crossing a certain threshold (around 32°C) results in diminishing returns due to thermal stress on the cells. Beyond 32°C, bacterial activity diminishes or may cease altogether, inhibiting the conversion of glucose into polysaccharide. High temperature prevents the growth of bacteria and pellicle formation, consistent with findings by Chen et al. [27]. Additionally, the previous study of the production of biocellulose using A. *xylinus* have identified the best between 28 to 31°C, with temperatures above 31°C causing damage and death of cells [32]. Hence, fermentation temperature is an important factor with significant influence on microbial growth and product synthesis.

3.4 Effect of fermentation duration

The experiment investigated how varying fermentation durations, from 3 to 14 days, influenced the activiy of cells and the production of BC. Table 4 illustrates that both the quantity and thickness of BC increased in direct proportion to the growth of *G. xylinus*. The bacteria convert sugar into acetic acid as primary metabolites, and subsequently produces BC as a secondary metabolites. In the initial 3 to 5 days of fermentation, the data indicates a moderate increase in both cell growth and BC production, suggesting that the cells experienced lag phase. This period reflects the time required for the cells to acclimate to the conditions of the growth medium. Cellular metabolism accelerates to increase cell size and synthesize the enzymes needed for cell division. However, actual cell reproduction is minimal at this stage, leading to a limited increase in total cell mass. Abdullah et al. noted comparable findings, where cell production was also lower within the first 5 days of fermentation.

Table 4. Effect of fermentation duration on cell concentration, BC concentration and BC thickness produced by *G. xylinus* (ATCC® 700178™). Standard deviation: duplicate experimental runs with duplicate measurements.

Fermentation Duration (day)	Cell Concentration[a] (mg/mL)	BC Concentration[a] (mg/mL)	BC Thickness[a] (mm)
3	0.61 ± 0.03	1.73 ± 0.13	0.52 ± 0.04
5	2.28 ± 0.19	5.48 ± 0.06	0.85 ± 0.08
7	7.27 ± 0.08	12.99 ± 0.68	4.10 ± 0.11
10	8.06 ± 1.51	15.16 ± 0.22	5.42 ± 0.29
12	11.54 ± 1.77	22.41 ± 3.99	7.53± 0.15
14	12.98 ± 0.63	19.96 ± 0.80	7.88± 0.12

[a] mean ± SD

[b] n.d. = not determined

Extending the fermentation period from 5 to 7 days significantly increased cell concentration (Table 4). This abrupt increase in cell mass is attributed to *G. xylinus* entering its exponential growth phase during cultivation. Initially, the culture medium contains high nutrient levels, which allows a higher metabolic rate in *G. xylinus*, allowing for substantial cell proliferation. As fermentation progresses, cellulose is released into the medium, gradually forming visible BC sheets. Nugroho et al. also found that *G. xylinus* entered the log phase on the sixth day, a process monitored using image processing to track BC formation. Longer fermentation durations allow *G. xylinus* to secrete additional BC fibers, leading to a more robust and thicker BC sheet. Beyond 7 days, although cell concentration continued to rise slightly, the growth rate slowed significantly. This reduction is likely due to the depletion of nutrients in the medium, which slows the conversion rate of glucose to polysaccharide BC. These results are similar to the previous report by Chen et al. [27].

After 12 days of fermentation, both cell growth and BC production approach saturation likely due to limited oxygen availability. In static culture, oxygen required for aerobic cells is transported by diffusion. The accumulation of BC on the surface of medium during fermentation restricted the oxygen supply, which is vital for cell growth. Additionally, the presence of BC in the fermentation medium increased its viscosity, thereby hindering oxygen transfer. An effective oxygen supply is crucial for maximizing BC yield, and this can be improved in agitated culture. Moreover, a slight decrease in BC concentration observed on the 14th day of fermentation (Table 4) could be due to the BC serving as a nutrient source. Bacteria are surrounded in a network of cellulose they produce. When other nutrients are depleted, microorganisms start consuming the BC itself. Sheykhnazari et al. [16] also confirmed that there is no significant increase in BC formation beyond 14 days of fermentation. Hence, a 12 days fermentation period is the best for BC production, achieving highest BC concentration while avoiding the drawbacks associated with extended fermentation times.

4 Conclusion

Our present research successfully demonstrated the production of BC from *G. xylinus* fermentation using pineapple peel wastes. It is clearly seen in the results presented above that varying the fermentation condition of waste juice concentration, medium pH, fermentation temperature and duration can lead to significant impact on the yield of cells and BC. In this study, the best conditions for producing BC are maintaining at a waste juice concentration of 80% (w/v), medium pH 4.0, fermentation temperature of 30°C, and 12 days of fermentation time. This yield cell concentration of 11.54 mg/mL and BC concentration of 22.41 mg/mL.

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