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PRODUCTION OF BIOCELLULOSE BY *ACETOBACTER XYLINUM* 0416 USING PINEAPPLE PEEL

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Abstract

Biocellulose produced by *Acetobacter xylinum* displays unique properties far and better than the plant cellulose, including a unique nanostructure, high water holding capacity, degree of polymerisation, mechanical strength, and crystallisation. The production of biocellulose, however, is costly due to the carbon sources and fermentation feedstocks. Pineapple peel waste has potential to be used as the carbon source since it is cheap and easy to obtain. This study was conducted to determine the composition of reducing sugar in pineapple peel using the high performance liquid chromatograph (HPLC) to produce and compare biocellulose derived from pineapple peel with biocellulose derived from commercial Hestrin-Schramm (HS) medium and other agricultural wastes. Moreover, this study also aimed to produce the process flow diagram and process and instrumentation diagram for large production of biocellulose using pineapple peel. Pineapple peel has approximately 0.58 g/mL of glucose and 0.78 g/mL of fructose, which is suitable to be used as fermentation medium for biocellulose production by *Acetobacter xylinum*. The dry weight of biocellulose that was produced using pineapple peel was 15.67% heavier than biocellulose produced using HS medium. Based on findings of the current study, it can be concluded that pineapple peel is suitable to be used as fermentation medium for high production of biocellulose.

Keywords: *Acetobacter xylinum*, Biocellulose, Fermentation, Pineapple peel, Process and instrumentation, Process flow diagram.

1. Introduction

Biocellulose is an organic compound with formula $(C_6H_{10}O_5)_n$ which is produced by bacteria. Biocellulose and plant cellulose have an identical chemical structure, but different physical and chemical properties [1]. Biocellulose consists of glucose monomers, and it shows better characteristics than plant cellulose, such as a unique nano-structure [2], high mechanical strength [3], high level of polymerisation [4], and high degree of crystallinity [5]. The basic structure of biocellulose is built from β-1-1-glucan chains with molecular formula $(C_6H_{10}O_5)_n$. These chains are connected with hydrogen bonds. Structure of biocellulose is 100 times smaller compared to the plant cellulose [6].

The unique characteristics of biocellulose make it suitable for various applications. Biocellulose can be eaten raw as a near-zero calorie delicacy, known as nata de coco and can be used as a food additive, cosmetic face mask, wound dressing, artificial skin, textile, and composite material [7]. In contrast with production of cellulose using methods, such as acid hydrolysis, oxidation, and enzymatic reaction [8, 9], biocellulose can be synthesised by various genera of bacteria, such as: *Gluconacetobacter*, *Aerobacter*, *Sarcina*, *Rhizobium*, *Azotobacter*, *Agrobacterium*, *Pseudomonas,* and *Alcaligenes* through fermentation process [7].

Acetobacter xylinum or previously known as *Gluconacetobacter xylinus* is the earliest discovered biocellulose-producing bacteria [7]. It is a gram-negative rod-shaped bacterium with 2-10 microns length and 0.5-1 microns width [10]. *Acetobacter* xylinum is classified as acetic acid bacteria with aerobic properties which requires oxygen to grow. The ability to polymerise glucose into cellulose with one process synthesis is the most favourable feature of this bacteria [10]. *Acetobacter xylinum* can survive in unstable conditions, such as sudden change of medium level, difference in pH, presence of toxic substances, and pathogenic organisms [11].

In industrial scale production, Hestrin-Schramm (HS) medium is usually used to grow biocellulose-producing bacteria like *Acetobacter xylinum*. However, HS medium as the carbon source proves to be costly and has low productivity. Several studies using agriculture wastes, such as pineapple waste [5], oil palm waste, matured coconut water [12], sago waste [13], and tobacco waste [14] have been conducted to replace the HS medium.

Malaysia is one of the largest exporters of pineapple worldwide and high global demand caused the pineapple plantation to increase from the width of 1,342 hectares in 2017 to 3,500 hectares in 2020 [15]. Out of the total yield, 84% is used for domestic, 10% for fruit processing, and 6% is exported as fresh fruit [16]. However, during the fruit processing, almost 70-75% of the original weight is thrown as agricultural waste, which causes environmental pollution that needs lengthy time of disposal [17]. Pineapples infected with plant disease caused by *Phytophthora sp*. [18], for example, cause additional accumulation of pineapple waste. Fortunately, it was found that pineapple peels contain 10% of reducing sugar and 13% non-reducing sugar, which is suitable for bacterial growth [19].

Therefore, in this study, pineapple peel extract was evaluated and compared with HS media to determine its suitability as the carbon source for production of

biocellulose by *Acetobacter xylinum*. The sugar content in pineapple peel was measured using HPLC and compared with previous studies. A process flow diagram (PFD) and a process and instrumentation diagram (P&ID) were drafted for large scale production of biocellulose. Apart from that, comparisons of biocellulose productivity between pineapple peel and different types of agricultural wastes, such as tobacco waste and coconut water were also included in this study. The results were compared based on differences in the physical properties, such as thickness, colour, and the dried weight of biocellulose.

2. Methods

2.1. Materials

Bacteria used in this study which is *Acetobacter xylinum* 0416 was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Serdang. Pineapple peels were collected from a fruit stall in Kolej Pendeta Zaaba, Universiti Kebangsaan Malaysia (UKM), Bangi. Other chemical substances, such as glucose, peptone, extract yeast, citric acid, disodium phosphate, water, and magnesium sulphate were available in the Chemical Engineering and Process Laboratory.

2.2. Apparatus

The apparatuses used in this project were shake incubator, oven, laminar flow, HPLC, autoclave, centrifuge, blender, pH metre, electronic weighing scale, beaker, measuring cylinder, test tube, and conical flask.

2.3. Preparation of inoculum

HS medium was prepared first by using 500 mL of distilled water, 10 g of glucose, 1.35 g of disodium phosphate, 2.5 g of peptone, 2.5 g of yeast, and 0.57 g of citric acid. The medium was then passed through an autoclave to ensure sterility, and 10 mL of *Acetobacter xylinum* was transferred into 90 mL of HS medium. Then, the inoculum was put in the incubator shaker for three days at a temperature of 30°C and 150 rpm [20].

2.4. Preparation extract of pineapple peel

Pineapple peel of 250 g was weighed using electronic weighing scale. Pineapple peel was blended with water at a ratio of 1:1 to obtain the pineapple peel extract. The extract was filtered with muslin cloth and centrifuged to separate solid waste from the extract [21].

2.5. Analysis of sugar content

High performance liquid chromatography (HPLC) was used to identify the sugar content in the pineapple peel. Two types of sugar, glucose and fructose were analysed using HPLC. Standard solution for both glucose and fructose were prepared at five different concentrations, which were 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 1.0 M. The extract of pineapple peel and standard solution of glucose and fructose was then transferred into vials. Thereafter, a total of 11 vials were sent for HPLC analysis.

2.6. Fermentation process to produce biocellulose

Six empty conical flasks were prepared since the fermentation process involved two types of medium that included the pineapple peel extract medium and HS medium. Pineapple peel medium was prepared using 500 mL of pineapple peel extract, 1.35 g of disodium phosphate, 2.5 g of peptone, 2.5 g of yeast, and 0.57 g of citric acid. HS medium was prepared using 10 g of glucose, 1.35 g of disodium phosphate, 2.5 g of peptone, 2.5 g of yeast, 0.57 g of citric acid, and 500 mL of distilled water. Both the mediums were autoclaved. Then, 10 mL of inoculum was transferred into six empty conical flasks. Pineapple peel extract of 90 mL was added into three conical flasks, and another three conical flasks were filled with HS medium. All the conical flasks were incubated for five days at 30°C. Once the pellicle of biocellulose was formed, it was rinsed with water and heated in 1% of sodium hydroxide for 30 minutes at 90°C. The pellicle was then fully dried in the oven and the dry weight of biocellulose was recorded [20].

2.7. Comparison with previous studies

The results from HPLC analysis and fermentation process were then compared with previous studies. Figure 1. shows the process flow of this study.

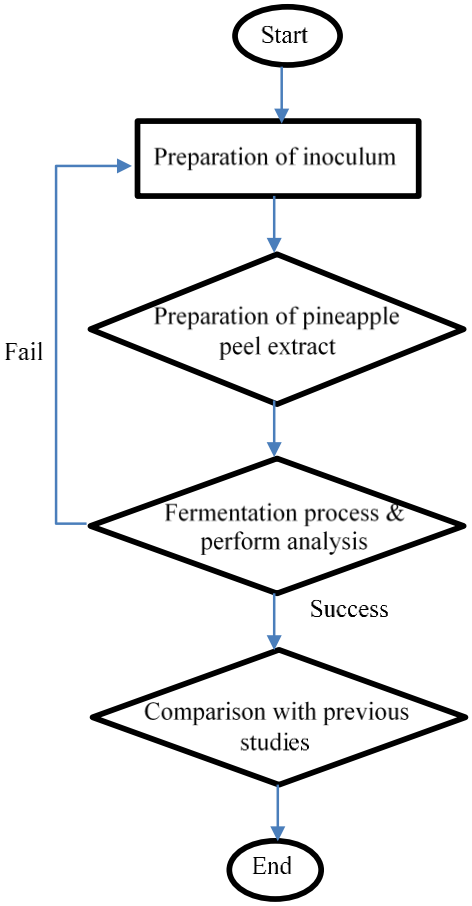

Fig. 1. Process flow of this study.

3. Results and discussions

3.1. Identifying reduced sugar content in pineapple peel

HPLC was used in this study to identify, measure, and purify glucose and fructose in pineapple peel extract [22]. According to previous studies, it was found that glucose is the dominant component in pineapple peel [23]. The concentration of glucose and fructose can be calculated using the standard graph. In this study, it was found that fructose content in pineapple peel was higher than glucose content. Based on the comparison with previous studies, it was observed that the sugar content in pineapple peel was different and depended on several factors. Factors that influenced the sugar content are maturity and species of the fruits, and types of soil used [23]. Maturity of the fruit depended on the hydrolysis process and this reaction produced more sugar content in the pineapple peel [23]. Sugar content as well as the value of reducing sugar, such as glucose and fructose can be measured using the dinitrosalicylic acid (DNS) method [24]. Table 1. shows the sugar content in pineapple peel.

Table 1. Comparison of sugar content in pineapple peel.

Component	Volume (mL)	Result	Previous study	
			[23]	[24]
Glucose (g/mL)	500	0.58	0.12	0.10
Fructose (g/mL)	500	0.78	0.10	0.08

The comparison of sugar content was also performed by using different carbon sources. In a study conducted by Ye et al. [14], tobacco waste was used as the culture medium in biocellulose production. The study used a different ratio of tobacco and water, such as 1:4, 1:6, 1:8, 1:10, and 1:12 to determine the most optimal sugar content for producing biocellulose. It was found that the ratio of 1:6 (22.43 mg/mL) produced high sugar content. However, during the fermentation process, productivity of biocellulose was higher in the medium with a ratio of 1:10 (18.67 mg/mL). This was due to the presence of nicotine in the tobacco waste, which caused sugar content to decrease. Nicotine is a harmful component for bacteria, and thus it was removed through the process of extraction. Nicotine acted as an inhibitor and sugar acted as a substrate during fermentation [14].

3.2. Production of biocellulose from pineapple peel

3.2.1. Stoichiometry equation and process flow diagram

Based on the current study, the stoichiometry equation for fermentation process of biocellulose can be formed. Carbon source (pineapple peel), nitrogen source (disodium phosphate), and oxygen reacted with each other to produce biocellulose, biomass, water, and carbon dioxide. The stoichiometry equation is shown below:

$$C_6H_{10}O_6 + 0.1\ NH_3 + 2.3\ O_2 \rightarrow 0.1\ C_6H_{10}O_5 + 5\ CH_{1.66}N_{0.02} + 1.5H_2O + 0.4\ CO_2$$

Mass balance for the fermentation process can be calculated using the following equation:

For total mass balance,

$$\frac{dV}{dt} = \rho_1 F_1 + \rho_2 F_2 - \rho_3 F_3 \quad (1)$$

For material balance of glucose,

$$V\frac{dc_A}{dt} = F_1(c_{o\ glucose} - c_{i\ glucose}) - \alpha r \quad (2)$$

For material balance of disodium phosphate,

$$V\frac{dc_B}{dt} = F_2(c_{o\ disodium\ phosphate} - c_{i\ disodium\ phosphate}) - \alpha r \quad (3)$$

For material balance of biomass,

$$V\frac{dc_c}{dt} = F_3(c_{o\ biomass} - c_{i\ biomass}) - \alpha r \quad (4)$$

For material balance of biocellulose (product),

$$V\frac{dc_P}{dt} = F_3(c_{o\ product} - 0) - \alpha r \quad (5)$$

Process flow diagram of the biocellulose production in industrial scale was prepared based on the laboratory work. Production of biocellulose involved batch process and was divided into three parts: pre-treatment, fermentation, and separation. In the pre-treatment process, pineapple peel entered a juice extractor to separate the solid waste from pineapple peel extract. The solid waste then entered the hammer mill to obtain the remaining extract of pineapple peel. Centrifugation process further separated the solid particles in the pineapple peel extract. The extract was then transferred into a storage tank before continuing with the next process.

Inoculum was prepared before the fermentation process could begin. Water, disodium phosphate, citric acid, yeast, glucose, and peptone were transferred into the mixer to form HS medium. *Acetobacter xylinum* and HS medium entered the seed fermenter at a temperature of 28°C and an acidic pH. Impeller in seed fermenter was used to ensure that the medium, bacteria, and oxygen were mixed uniformly. During the fermentation process, only 10% of inoculum from seed fermenter, and 90% of pineapple peel from storage tank were fed into the main fermenter with the presence of oxygen at 28°C. Biocellulose that was formed through the fermentation process underwent filtration to separate the product from another by-product. Biocellulose was transferred into sodium hydroxide and soaked in a water bath at 90°C for 30 minutes. Sodium hydroxide was used to neutralise and wash the biocellulose from the residual of fermentation process. Finally, it passed through a dryer to dry the biocellulose at 50°C.

Previously, a study on the design and control of biocellulose production using *Acetobacter xylinum* had been performed. Compared to this study, commercial glucose as raw material was used instead of carbon source from agricultural waste. The usage of agricultural waste can help to reduce cost of raw materials since it can be obtained easily. Besides, in the previous study, sedimentation process was used to separate the components. Filter could be used to replace the sedimentation tank during separation process. Figures 2 and 3 show the process flow diagram (PFD), and process and instrumentation diagram (P&ID) of the fermentation process.

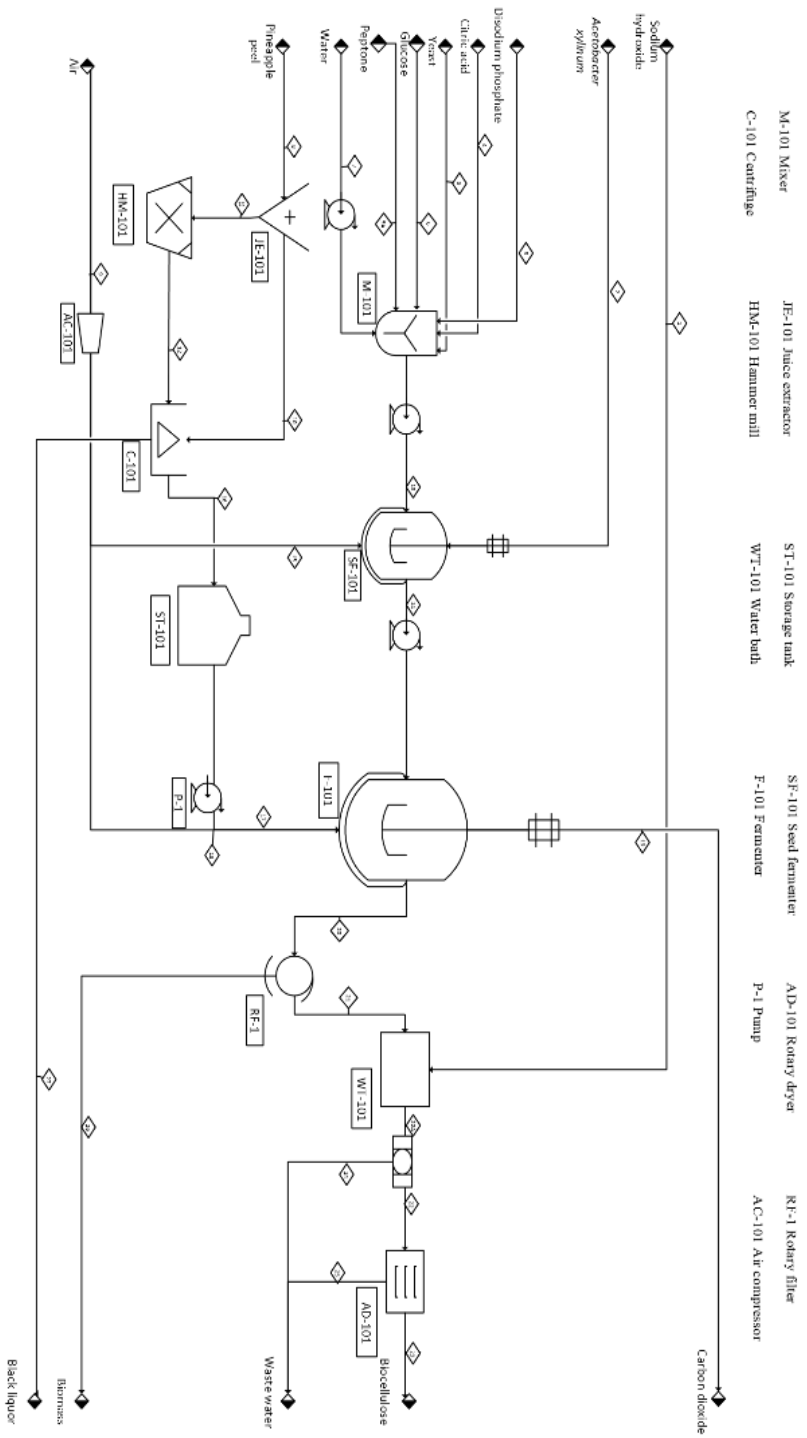

Fig. 2. Process flow diagram of biocellulose production from pineapple peel waste using bacteria *Acetobacter xylinum*.

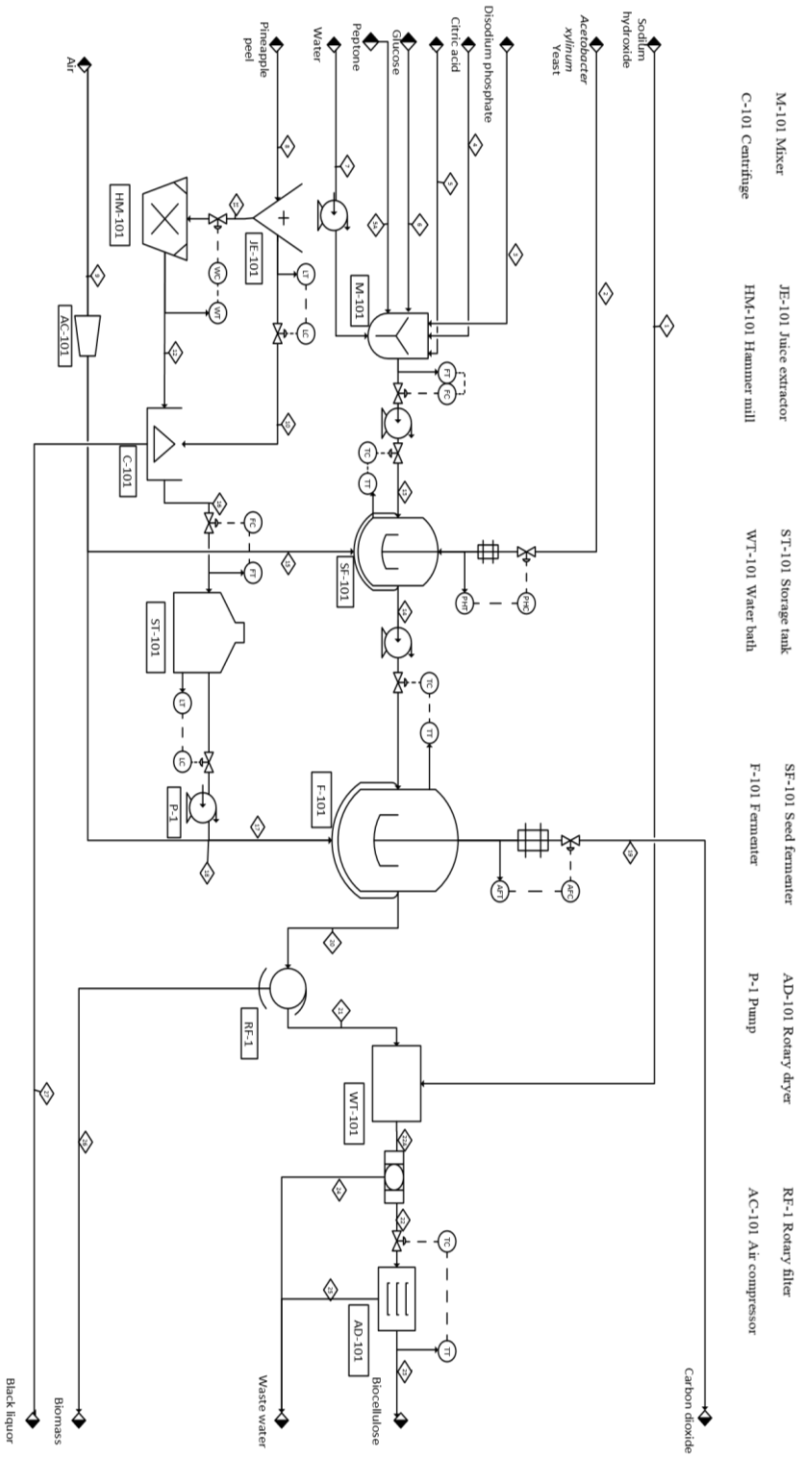

Fig. 3. Process and instrumentation diagram of biocellulose production from pineapple peel waste using bacteria *Acetobacter xylinum*.

From the perspective of a cost analysis, by replacing glucose with pineapple peel extract, the cost of raw material was reduced by USD 1.09/kg with respect to the price of glucose per kg [25] and 15.67% higher productivity (Table 2.) was achieved. The increased productivity enhanced the profit gain by 0.16 fold per batch.

3.2.2. Production of biocellulose from fermentation process

In this study, two different types of fermentation medium were used, which were HS medium and pineapple peel extract medium. In a previous study, 40% of pineapple peel extract was used as the medium [5]. Compared to the previous study, this project used 100% of pineapple peel extract and 100% of medium HS. The result from the fermentation process is shown in Fig. 4.

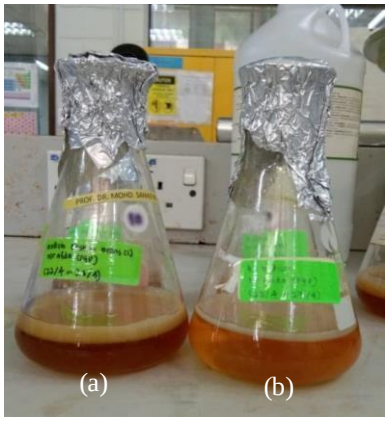

Fig. 4. Biocellulose pellicle from the fermentation in (a) pineapple peel extract and (b) HS medium.

After five days of fermentation, it was found that the productivity of biocellulose pellicle in pineapple peel extract was thicker than in HS medium, which signified that more biocellulose was formed in the pineapple peel extract. Morphologically, the colour of biocellulose that was formed in the pineapple peel extract was yellowish, while in HS medium it was more towards transparent. The difference in colour was due to the increase of pH that was caused by production of ammonia [26]. The biocellulose pellicles were later dried and weighed to determine its dry weight. Table 2. shows the dry weight of biocellulose pellicles formed in pineapple peel extract and HS medium, respectively.

Table 2. Dry weight of biocellulose in different medium.

Fermentation medium	Dry weight (g)
100% pineapple peel extract	2.51
100% HS medium	2.17

Unfortunately, during the drying process, there was fungi contamination found on the surface of the biocellulose pellicles, as depicted in Fig. 5. The fungi contamination occurred due to the moist surface of the biocellulose pellicle, which

stimulated the fungi growth on the area. This observation showed that biocellulose produced through fermentation was an organic material, which was safe and non-toxic for living organisms use and can be degraded naturally. These characteristics proved that biocellulose is suitable to be utilised by various industries, such as food, cosmetic, and pharmaceutical, as food additive, face mask, and wound dressing, respectively [7]. However, producing a sterile biocellulose is important to ensure safety of consumers. Therefore, the procedure of this study should be altered and improved with additional sterilisation steps, such as autoclaving the biocellulose in distilled water before drying it in a sterile laminar flow instead of using oven as performed by Nagmetova et al. [27].

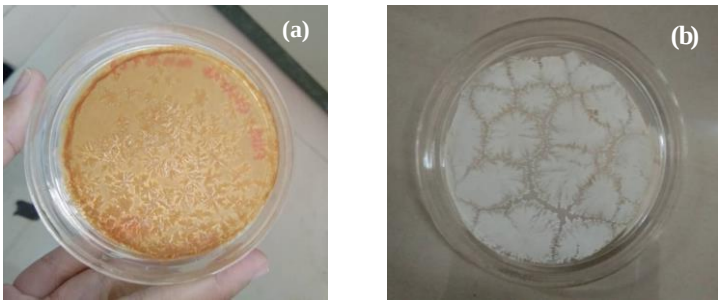

Fig. 5. Fungi contaminated biocellulose pellicles from the fermentation in (a) pineapple peel extract and (b) HS medium.

In a previous study conducted by Ch'ng et al. [21], the fermentation process to produce biocellulose was conducted in two different conditions, which were in static and agitated condition. Based on the study, the dry weight of biocellulose produced in the static and agitated condition were,0.16 g and 0.19 g, respectively. As a conclusion, it was found that the fermentation process in agitated condition could produce more biocellulose than in static condition due to the better presence of dissolved oxygen for bacterial growth in fermentation medium [21].

Optimisation study of biocellulose production from pineapple waste using *Acetobacter xylinum* by Zakaria and Nazeri [5] showed the influences of temperature, pH, and medium concentration on biocellulose production. The parameter of the study ranged from 40-100% of medium concentration, 28°C-32°C of temperature, and 4.5-8.5 of pH. At the end of the study, the dry weight of biocellulose produced before optimisation was 3.39 g and increased to 3.44 g after optimisation. It was proven that the most optimal condition for biocellulose production was using 83.32% pineapple waste medium at 30.51°C temperature and pH 5.15 [5].

3.3. Comparison of biocellulose production using other agricultural waste

Besides using pineapple waste, coconut water, palm oil extract, and sago waste are attractive options to be used as fermentation medium for biocellulose production. Phruksaphithak et al. [12] used coconut water and palm oil extract at a ratio of 50:50, 0:100, and 100:0 to maximise the production of biocellulose. As a result, biocellulose produced at a ratio of 50:50 showed the highest production due to high sugar content in the mixture, which is suitable for bacterial growth [12]. Factors, such as pH, temperature, and duration of fermentation process also affected the

biocellulose production. Table 3. shows the dry weight of biocellulose produced in different ratios of fermentation medium.

Table 3. Dry weight of biocellulose produced in different ratio of fermentation medium.

Fermentation medium	Dry weight of biocellulose (g/L)
Coconut water and palm oil extract (50:50)	4.97
Coconut water and palm oil extract (0:100)	3.65
Coconut water and palm oil extract (100:0)	3.79

Source: Phruksaphithak et al. [12].

In another study carried out by Voon et al. [13], three different types of fermentation medium (i.e., HS medium, sago waste, and coconut water) had been used to produce biocellulose using bacteria *G.xylinus* and *B.fluminensis*. The results of the study, as summarised in Table 4., show competitive production of biocellulose using sago waste and coconut water medium in comparison to well-established HS medium. The sago waste medium seems to have higher potential to replace HS medium as a carbon source for production of biocellulose compared to coconut water medium [13]. Apart from that, the bacteria *G.xylinus* also showed better performance than *B. fluminensis* in producing biocellulose.

Table 4. Total production of biocellulose using different bacteria and fermentation medium.

Bacteria	Total production of biocellulose (g/L)		
	HS medium	Coconut water medium	Sago waste medium
B.fluminensis	0.52	0.45	0.47
G.xylinus	1.57	1.00	1.55

Source: Voon et al. [13].

In conclusion, various types of agriculture wastes can be exploited as carbon source for production of biocellulose. In conjunction to reduce waste accumulation and pollution caused by agriculture industry, high value biocellulose could be produced for the benefits of human life, either as food, cosmetic or pharmaceutical products. Further study to optimise the production of biocellulose should be conducted in future to improve its production capabilities.

4. Conclusion

Biocellulose could be produced by *Acetobacter xylinum* using pineapple peel as the fermentation medium. Sugar content evaluation using HPLC showed that the pineapple peel contained 0.58 g/mL glucose and 0.78g/mL fructose, which vary from the studies conducted by other researchers. The sugar content in pineapple peel is affected by maturity of the fruit, species of the fruit, and types of soil used.

A process flow diagram and process and instrumentation diagram were designed for production of biocellulose in the industrial scale based on the conducted laboratory work. The fermentation that took place using two types of

media (i.e., HS medium and pineapple peel medium) produced different morphology of biocellulose pellicle. Unfortunately, during the drying process, it was found that there was fungi contamination on the surface of biocellulose pellicle. However, the dry weight of biocellulose produced in the pineapple peel medium (2.51 g) was found to be higher than in HS medium (2.17 g).

Comparison study conducted also found that other agricultural wastes, such as coconut water, palm oil extract, and sago waste were suitable to be used as fermentation media for biocellulose production.

As a conclusion, pineapple peel and other agriculture wastes could be used in biocellulose production to replace HS medium, which is considered as a high cost raw material with low productivity. Exploitation of these agriculture wastes could lessen production cost of biocellulose and help in minimisation of polluting agriculture wastes.

Nomenclatures

F_1	Inlet flowrate of glucose (L/hr)
F_2	Inlet flowrate of disodium phosphate (L/hr)
F_3	Outlet flowrate of product (L/hr)
V	Volume (L)
ρ_1	Density of glucose (g/L)
ρ_2	Density of disodium phosphate (g/L)
ρ_3	Density of product flowrate (g/L)
α	Stoichiometry coefficient
r	Rate of reaction (g/hr)
dC_A/dt	Change of glucose concentration with time (g/L.hr)
dC_B/dt	Change of phosphate concentration with time (g/L.hr)
dC_C/dt	Change of biomass concentration with time (g/L.hr)
dC_P/dt	Change of biocelullose concentration with time (g/L.hr)
dV/dt	Change of volume with time (L/hr)
c_i	Total inlet concentration (g/L)
c_o	Total output concentration (g/L)

Greek Symbols

α	Stoichiometry coefficient
ρ_1	Density of glucose (g/L)
ρ_2	Density of disodium phosphate (g/L)
ρ_3	Density of product flowrate (g/L)

Abbreviations

DNS	Dinitrosalicylic acid
HPLC	High performance liquid chromatograph
HS	Hestrin-Schramm
MARDI	Malaysian Agricultural Research and Development Institute
P&ID	Process and instrumentation diagram
PFD	Process flow diagram
UKM	Universiti Kebangsaan Malaysia
USD	United States dollar

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