Evaluation of inoculum size and fermentation period for bacterial cellulose production from sago liquid waste

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Abstract. The production of bacterial cellulose (BC) from sago liquid waste was examined. In this study, the influence of different inoculum size (5, 10, 15, 20, 25 and 30%) and fermentation period (5, 10, 15 and 20 days) for BC production were evaluated. The yield of BC was examined based on dry weight. The growth of bacteria in the fermentation medium was enumerated using pour plate technique. The profile of viable bacterial cell *Acetobacter xylinum* LKN6 on the BC pellicles was analyzed by scanning electron microscopy. The results showed that the inoculum size and fermentation period had an effect on bacterial cellulose production from sago liquid waste. The inoculum size of 25% (v/v) produced highest yield of bacterial cellulose (13.85 g/L). The yield of BC increased with fermentation time and the optimum fermentation period was 15 days. The pattern of bacterial growth was related to BC production. The cell of *A. xylinum* LKN6 embedded in the bacterial cellulose pellicle and it was formed fibril cellulose secreted through bacteria cell wall. Therefore, the factors of inoculum size and incubation period affected production of bacterial cellulose from sago liquid waste.

1. Introduction

Bacterial cellulose (BC) is an organic compound derived from fermentation process by certain types of bacteria including the genera *Acetobacter, Gluconacetobacter*, *Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Rhizobium, Sarcina* and *Salmonella* [1]. Unlike the cellulose from wood pulp, bacterial cellulose is devoid of other contaminating polysaccharides and its isolation and purification are relatively simple, not requiring energy- or chemical intensive processes [2, 3], hence the BC can be used for many purposes.

Production of Bacterial cellulose is relatively expensive, so that its production is low on an industrial scale [4]. Utilization of cheap and readily available substrates such as sago liquid waste, can reduce the BC production cost. The sago liquid waste is a waste from processing sago starch and it is produced along the year in Southeast Sulawesi. In the current research, the sago liquid waste was very potential to be used as fermentation medium for BC production [5].

Bacterial cellulose production appears to depend on a complex relationship involving a variety of factors like inoculum size, fermentation period, carbon source, nitrogen source, pH and temperature. The inoculum is bacterial culture that is the main component in BC production, therefore the size and age of inoculum may affect on the formation of BC. The effect of fermentation period for batch

culture system, needs to be studied to enhance the BC production. In batch culture system, depleted sources of nutrients reduce the number of cells in the medium throughout fermentation process in turn reduce the production rate utilizing pineapple waste as medium [4]. Therefore, the main objective of this research was to evaluate the effects of inoculum size and fermentation period (days) to enhance the production of bacterial cellulose by *A. xylinum* LKN6 using sago liquid waste as medium.

2. Material and Methods

2.1. Preparation of Inoculum Acetobacter xylinum LKN6

Acetobacter xylinum LKN6 was prepared and maintained in coconut water. The culture broth was incubated at room temperature (28-30°C) for 3 days for further study.

2.2. Preparation of Fermentation Medium

Preparation of fermentation medium using a 1 L sago liquid waste has been described in detail by Yanti *et al*. in Paten No. P00201709533 [6]

2.3. Determination of Inoculum Size

Acetobacter xylinum LKN6 inoculum was inoculated aseptically into 1 L fermentation medium. The inoculum concentration was added in fermentation medium i.e. 5, 10, 15, 20, 25 and 30% (v/v). Culture was incubated statically in ambient temperature for 14 days. At the end of incubation time, the bacterial cellulose was harvested. The experiment was repeated twice to evaluated of BC yield.

2.4. Determination of Fermentation Period

A 25% (v/v) of inoculum was poured aseptically into the 1 L fermentation medium. Culture was incubated statically in ambient temperature. The experiment was repeated twice for different fermentation periods, which were 5, 10, 15 and 20 days. At the end of incubation time, the bacterial cellulose was harvested to evaluated of BC yield and the number of bacteria inoculum in the fermentation medium.

2.5. Measurement of Dried Weight of Bacterial Cellulose

The yield of bacterial cellulose (BC) was evaluated based on the dried weight. The measurement of the dried weight of bacterial cellulose produced followed a method described by [7]. The bacterial cellulose was harvested from fermentation medium and rinsed with distilled water. The BC then was soaked in 200 mL of 2 M NaOH solutions for 2 h to eliminate bacteria. Bacterial cellulose was rewashed with of distilled water until NaOH residue removed or until pH became 7.0, and was dried with tissue paper. The bacterial cellulose was dried in an oven at 45◦C for 48 h to obtain the constant dried weight. Bacterial cellulose was weighed using an electronic balance (BOECO, Germany)

2.6. Enumeration of Bacteria Inoculum

Enumeration of bacteria cell was done to determine the growth pattern of *A.xylinum* LKN6 in the fermentation medium, sago liquid waste. The number of viable cells in the medium was determined at timed intervals of 0, 5, 10, 15, and 20 days by the pour plate technique on CARR Agar medium (3 g glucose; 10 g $CaCO_3$; 0.04 g bromothymol blue; 10 g yeast extract; 20 g agar and ethanol 17.5 ml/L in 1000 ml of pH 6.8. [8]. The medium was autoclaved at 121◦C for 15 min. Samples was serial diluted and poured on each of the two media poured. Culture was grown for 3 days at 30°C. Bacterial colonies were counted. Cell number was expressed as colony-forming units (CFU) per mililiter (cfu/ml).

2.7. Determination of pH

The samples of 5 ml was taken carefully. The pH of the samples was checked in interval time of 0, 5, 10, 15, 20 days using an electronic pH meter 691 (Metrohm).

2.8. Scanning Electron Microscopy Analysis of Bacterial Cellulose

SEM analysis was performed to know the presence of bacterial cell in the bacterial cellulose dried sheets. The dried sheet was cut (1 × 1 cm^2) and placed on a sample grid. Thin layers of the samples were coated with gold using an ion sputter. The coated samples were viewed and photographed using the SEM (model 5526, Cambridge, UK) at 10 kv.

3. Result and Discussion

3.1. Effect of Inoculum Size on Bacterial Cellulose production

The inoculum concentration plays an important role in cellulose production. The bacterial cellulose production was observed in all inoculum concentration however optimum inoculum concentration was 25% yielded 13.85 g/L of bacterial cellulose (Figure 1).

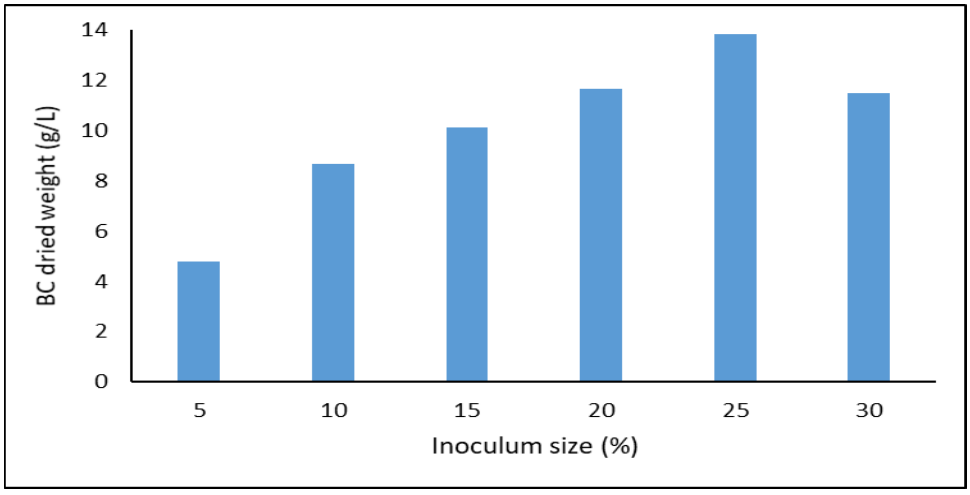

Figure 1. Effect of inoculum size on bacterial cellulose production from sago liquid waste by *Acetobacter xylinum* LKN6 after 14 days fermentation

The result indicated that optimization of inoculum size is as important for producing BC using a new media and organism. [9] reported that the culture conditions have a crucial influence, in particular inoculum size to achieve high yield cellulose production by the new organism. The inoculum concentration lower or higher than 25% (v/v) decreased BC production. The addition with an excess inoculum in the fermentation medium, lead competition between bacteria in using nutrients so that bacterial growth and production of BC were disrupted. These results are similar to those reported by [10].

3.2. Incubation Period for Bacterial Production in sago liquid waste medium

The fermentation time of BC production by *Acetobacter xylinum* LKN6 was observed at the optimal inoculum size (25% v/v) of 5 to 20 days at ambient temperature (30°C) in constant culture condition. Figure 2 displays the BC yield (BC dried weight) and cell inoculum growth during fermentation. The BC produced in the culture medium at 5 day of incubation time was 7.5 g/L, and production gradually increased with time until its reach maximum production (13.85 g/L) at 15 day of incubation. Cellulose production becomes constant thereafter **(**Figure 2). The same result was shown in BC production from Kombucha by *Acetobacter xylinum* [11].

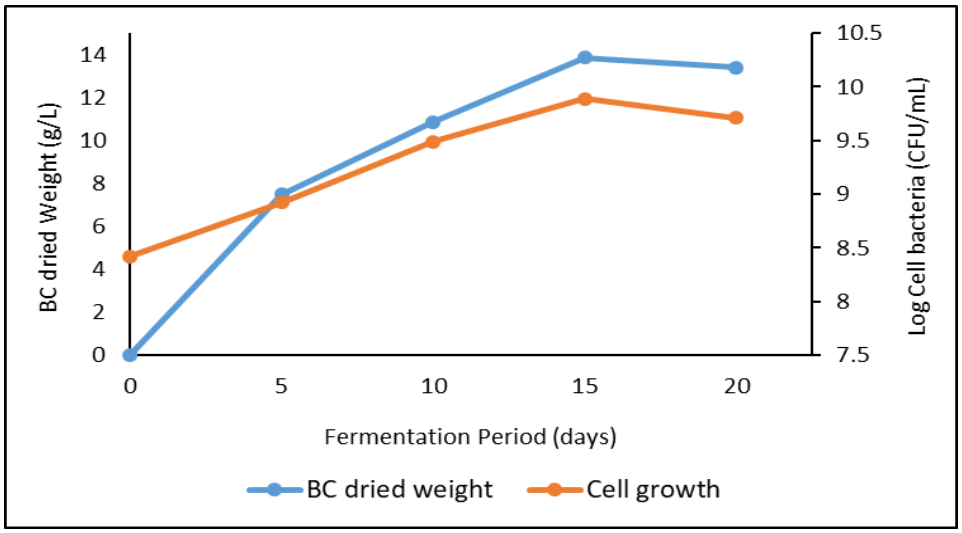

Figure 2. Bacterial cellulose yield and bacteria cell growth on sago liquid waste medium during fermentation

The bacterial cellulose production increased during fermentation, with a maximum production at 15 days. According to [11], this pattern occurred because by day 17–18 of fermentation, glucose in medium was almost exhausted and the metabolites had reached maximum production. The maximum BC yield reached 13.85 g/L at 15 days and was decreased to 13.41 g/L at 20 days. Similar profiles have been observed in static fermentation experiments conducted by other researchers [11, 12, 13]. They reported that the yield of bacterial cellulose increased sharply after a few days of induction until the rate reached a maximum after 2 weeks. Different researchers have obtained maximum BC production in different time periods depending upon the strain used and culture conditions [4, 14, 15]. The mechanism of bacterial cellulose formation has been described in detail by [1, 2].

Figure 2 also showed that bacterial growth exponentially increased, reaching maximum at 15 days of cultivation and after that was decreased. These pattern similar with the pattern of BC yield. The time fermentation of more than 2 weeks, the number of bacterial cells showed a decreasing trend, As a result, the BC yield also decrease. These results are similar to those reported by [1] and [4] states that the longer of incubation time, causes the nutrients in the medium decreases so that bacterial growth and BC production decreases.

3.3. Changes in pH during bacterial cellulose synthesis

The pH plays an important role in microbial growth and bacterial cellulose synthesis [1]. Figure 3 shows the changes in pH for the fermented BC with different inoculum size during incubation. Generally, the changes in pH with inoculum at concentrations of 20, 25, and 30% (v/v) showed a similar trend, pH dropped gradually as the fermentation proceeded. These results are consistent with some of the earlier findings [12, 16, 17]. [12] reported that *A. xylinum* is unique in its family for being able to convert carbohydrates to acetic acid as a by-product of cellulose, which influences the decreased pH in the culture medium. [1] and [16] also reported that the pH decreased during fermentative BC production because of the accumulation of gluconic, acetic or lactic acids in the culture broth.

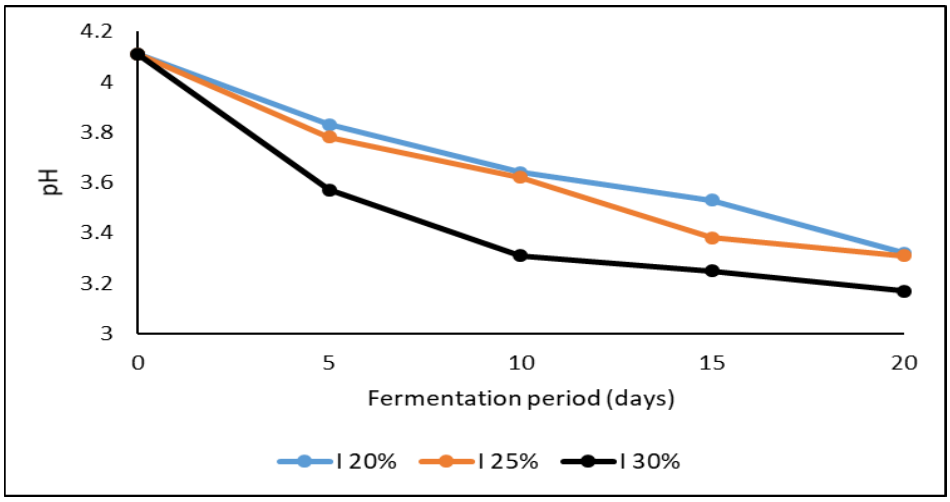

Figure 3. Change of pH value for BC synthesis on sago liquid waste medium with different inoculum size

Figure 3 shows that the pH of a culture medium at high inoculum concentration (I 30%) tends to drastically reduce. These results might be due to the large number of *A. xylinum* bacteria on the medium produced more organic acid thereby decreasing the pH of the medium. [18] stated that *A. xylinum* is also known as acetic acid bacteria which are able to form acid from glucose, in the presence of oxygen and forming acid in the medium fermentation will decrease pH medium. [17] reported that the rapid decrease of pH during fermentation was mainly due to the increase in the organic acid content as metabolites.

According to [1], the optimum pH of the culture medium for bacterial cellulose production is in the range of 4.0 to 6.0, the yield of cellulose decreasing below pH=4. However, these results showed that pH culture medium decreased from 4.1 became 3.17-3.3 (Fig. 3). These results indicated that *Acetobacter xylinum* LKN6 were able to grow and produce cellulose at pH < 4. This discrepancy in results might be due to the variability of the *Acetobacter xylinum* strain [18].

3.4. Profile of Viable cell of Acetobacter xylinum LKN6 in Bacterial Cellulose Pellicles.

The distribution of the bacterial cell in the bacterial cellulose network is highly complex. Therefore, this study was conducted to investigate the viable bacterial cell on the BC pellicle and formation of fibril cellulose by *Acetobacter xylinum*. Figure 4 shows the viable cell of *A. xylinum* embedded in the bacterial cellulose pellicles harvested on the 15th day of fermentation.

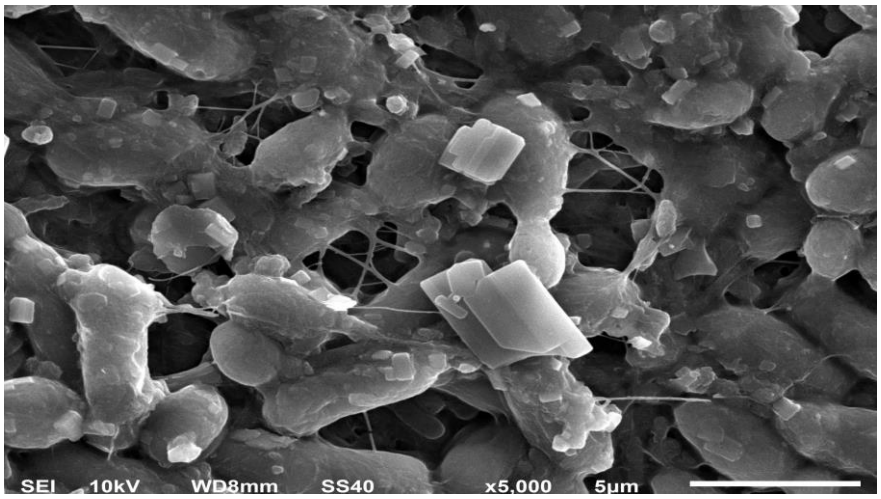

Figure 4. Scanning electron microscope image of bacteria generated bacterial cellulose network

Figure 4 illustrates an overview of BC network produced by bacteria from sago liquid waste. The bacteria cell synthesized fibril cellulose which secreted through cell wall and aggregate together forming cellulose ribbons (Fig. 4). [2] and [3] reported that during the synthesize process, protofibrils of glucose chain are secreted through bacteria cell wall and their assembly outside the cells forming cellulose ribbons. The pre cellulosic polymer molecules synthesized in the interior of bacterial cell are spun out of the cellulose export components to form a protofibril [1, 2, 3, 19].

4. Conclusion

Inoculum size and fermentation period showed an effect on bacterial cellulose production from sago liquid waste. The optimum inoculum size in BC fermentation was 25% (v/v) and the optimum fermentation period was 15 days.

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