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Study on the Production of Bacterial Cellulose from *Acetobacter xylinum* using Agro-Waste

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Abstract

Bacterial cellulose (BC), produced by *Acetobacter xylinum*, has been given a great attention due to its high potency for many industrial applications. An optimized substrate is important for higher BC production; thus, an alternative natural product, as a carbon source, should be determined. This study aims at investigating the BC of *A. xylinum* cultured in coconut water and pineapple juice-based media and to predict its fermentation kinetics. The BC was produced on two stages of fermentation system, the shaking culture for propagation and the static culture fermentation for BC production. *A. xylinum* exhibited exponential phase at 48 h which showed BC production associated with its cell growth on both substrates. Fermentation kinetics of *A. xylinum* using coconut water and pineapple juice revealed Rp 0.117 and 0.051 g/l/h, Rx 0.309 and 0.133 g/l/h, Rs 0.079 and 0.215 g/l/h, Yx/s 1.408 and 0.240 g biomass/g glucose, Yp/s 3.612 and 0.599 g cellulose/g glucose, Yp/x 2.235 and 2.452 g cellulose/g biomass, μmax 0.0132 and 0.0082/h, σ 0.028 and 0.0173/h, respectively. Overall parameters of fermentation kinetics revealed a high rate of BC formation and efficient conversions of glucose to biomass and BC by *A. xylinum* on coconut water substrate. Thus, coconut water proved to be a more suitable substrate to produce BC in comparison with pineapple juice.

Keywords: *Acetobacter xylinum*, Bacterial cellulose, Coconut water, Fermentation kinetic, Agro-waste.

1. Introduction

Cellulose is a linear glucose polymer of ß-1,4-glycosidic bond with various polymerizations. Cellulose is the main component of plant cell walls (Hardjo *et al.*, 1989), which is generally used as a raw material for paper, board, or rayon fibers in industrial manufactures. At present, wood becomes the major source of cellulose because of its quite high content. However, cellulose from wood pulp is still contaminated with polysaccharides, such as hemicellulose and lignin. Thus, it is necessary to find other alternative sources of high purity cellulose.

Acetobacter xylinum is a type of bacteria that produces cellulose with favorable physical properties (Suwannapinunt *et al*., 2007). *A. xylinum* is identified as a gram negative bacterium with short rod, which is capable of oxidizing glucose to gluconic acid and organic acids simultaneously. This bacterial cellulose (BC) has been known as secondary metabolite from glucose with the release of acetic acid into the environments (Tomita and Kondo, 2009). Unlike the cellulose from wood pulp, the BC has a high purity, unique strength, an ultra-fine structure and is biodegradable. Isolation and purification of this BC are also simple. These properties allow BC to be used as a substitute for wood raw material in the high-quality paper industry, low-calorie foods membrane ingredients (ultra filtration) and other materials (Iguchi *et al*., 2000).

Several studies have dealt with BC biosynthesis using various strains and cultivations systems (Ishikawa *et al*., 1995; Chao *et al*., 2000; Kongruang, 2008). The BC has been produced using different carbon sources (Ramana *et al.*, 2000; Chawla *et al.*, 2009), various nitrogen sources (Budiono *et al*., 1999; Melliawati *et al*., 2006) and other environmental factors (Chao *et al.*, 2001; Ishida *et al*., 2002). The cultivation method and the growth media influence the ability of *A. xylinum* to produce BC (Coban and Biyik, 2011). The high nutritious media induce *A. xylinum* cells growth with higher BC being formed. However, a standard media solely for producing BC is expensive even though a kind of sorbitol, glucose, lactose and mannitol are good

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as a carbon source. Thus, more investigations using natural resources could be useful for the efficiency of BC production.

A number of fruit extracts, including orange, apple, pear, pineapple and coconut, is available every season. Because of the vitamin content and the proteolytic enzymes present in the pineapple, this fruit is good for food uses (Hebbar *et al*., 2008). The increasing production of pineapple processed items, results in a massive waste which is unsuitable for human consumption. Coconut juice is discarded from many applications of agro-industries in Southeast Asian countries. Because the residues still contain carbon and nitrogen sources, they could be utilized as a substrate for producing a good quality BC (Kongruang, 2008; Kurosumi *et al*., 2009). In Indonesia, abundant coconuts and pineapples are produced and need to be used efficiently. Before we determine them as fermentation media for producing BC, their function as the best substrate should be defined. Therefore, the present study aims at determining an efficient substrate from agro-residues for the production of BC. The fermentation kinetics of *A. xylinum* and related parameters were investigated.

2. Materials and Methods

2.1. Bacteria and Culture Media

A. xylinum strain, used in this study, was obtained from Bioprocess Lab., at the Faculty of Industrial Technology, Bogor Agricultural University. We prepared media of starter and fermentation for *A. xylinum.* A starter medium of Hassid-Barker was made per 1 liter of distilled water containing 10% sucrose, 0.25% yeast extract, 0.5% K_2HPO_4, 0.6% $(NH_4)_2SO_4$, and 0.2% $MgSO_4$. Fermentation medium for the BC production is the Hassid-Barker enriched with 0.25% ammonium sulfate and 0.25% calcium sulfate using two types of solvents as treatment, coconut water and pineapple juice. Pineapple solvent was made by filtering blended pineapple and mixing it with coconut water at ratio of 1:3. The media pH was adjusted using glacial acetic acid to 4.0 and sterilized. The composition of coconut water and pineapple juice-based media are presented in Table 1.

Table 1. Composition of fermentation media used in this study.

Coconut water-based medium		Pineapple-based medium	
Sucrose	100 g/l	Sucrose	100 g/l
Yeast extract	2.5 g/l	Yeast extract	2.5 g/l
K_2HPO_4	5 g/l	K_2HPO_4	5 g/l
$(NH_4)_2SO_4$	6 g/l	$(NH_4)_2SO_4$	6 g/l
$MgSO_4$	2 g/l	$MgSO_4$	2 g/l
Ammonium sulfate	2.5 g/l	Ammonium sulfate	2.5 g/l
Calcium sulfate	2.5 g/l	Calcium sulfate	2.5 g/l
Coconut water	1 l	Pineapple juice	333,33 ml
		Coconut water	666,66 ml

2.2. Bacterial Growth Pattern

A single colony of *A. xylinum* from stock agar was inoculated on nutrient agar and incubated for 4 days at 28ºC for regeneration. The four-day old bacterial cells (Pae *et al*., 2011) were inoculated into 50 ml of starter medium of Hassid- Barker, then incubated with shaking at 125 rpm for 48 hours at 28ºC. At the end of the incubation, the 50 ml of inoculums were added to the 500 ml of fermentation medium of Hassid-Barker in flask, homogenized and shaked for the next 5 days. To investigate the growth pattern of *A. xylinum*, culture was sampled periodically in a certain period and determined its Optical Density (OD_{620}) using spectrophotometer.

2.3. Fermentation Process

BC production was carried out with two cultivation steps, starting with shaking culture for microbial propagation and fermentation without shaking (static condition). Propagation step was prepared in 50 ml of starter liquid medium of Hassid-Barker and incubated according to pre-determined time of *A. xylinum* growth. The 50 ml propagated culture was transferred into 500 ml fermentation medium depending on the treatment of substrates and incubated in a static condition for 12 days. The lids of the flasks containing *A. xylinum* culture were kept loose to ensure the transfer of oxygen. Several parameters, such as pH, biomass which was represented by cell dry weight, and reducing sugars, were performed. Reducing sugar in the form of glucose was determined using a 3.5-Dinitrosalicylic Acid (DNS) and BC yield with gravimetric method (AOAC, 1984). Based on the value of reducing sugar, the parameters below were further determined:

- Used sugar (%) = (IS-FS) / IS x 100
- Sugar converted into BC (%) = Y / (IS-FS) x100, where: IS = initial sugar concentration (g / l), FS = final sugar concentration (g / l), Y = BC yield (g / l).

2.4. Fermentation Kinetics

To identify the efficiency of using substrate, the fermentation kinetics during BC production was measured. Parameters, such as X as biomass (g/l) is obtained from the cell dry weight, S is the substrate by

reducing sugar content in the form of glucose (g/l) and P is the weight of wet BC product per volume of fluid (g/l). Fermentation kinetics parameters were then measured as follows:

Rx = the growth rate or biomass formation (g / l / h) = dx / dt

Rs = the utilization rate of glucose substrate (g / l / h) = ds / dt

Rp = the formation rate of BC (g / l / h) = dp / dt

Yx / s = the biomass yield toward glucose substrate (g / g) = (X-Xo) / (So-S)

Yp / s = the BC yield toward glucose substrate (g / g) = (P-Po) / (So-S)

Yp / x = the BC yield toward biomass (g / g) = (P-Po) / (Xo-X)

μmax = the maximum specific growth rate (per h) = slope of ln X = f (t)

σ = the specific rate of BC formation (per h) = slope of ln P = f (t)

3. Results and Discussion

3.1. Determination of the Optimum Growth Phase of A. xylinum for Inoculum

Bacterial growth can be represented by biomass or cell number which is estimated with optical density (OD_{620nm}). In this study, the growth of *A. xylinum* was observed to determine its maximum cell number at log phase. *A. xylinum* showed a rapid exponential phase from beginning (OD: 0.201) to 36 h incubation as expected. The growth increased slower before reaching the highest OD (1.182) after 48 h, suggesting that its logarithmic phase could support for optimum *A. xylinum* growth from propagated culture to static cultivation system. The growth pattern *A. xylinum*, which was preceded by a short adaptation (lag) phase, indicates enough growth-induced substrate (Figure 1). Since *A. xylinum* cells consume nutritional products in parallel with metabolites secretion at specific period before stationary phase, thus, Hassid-Barker medium which was combined with carbon source is assumed to be enough for *A. xilynum* growth.

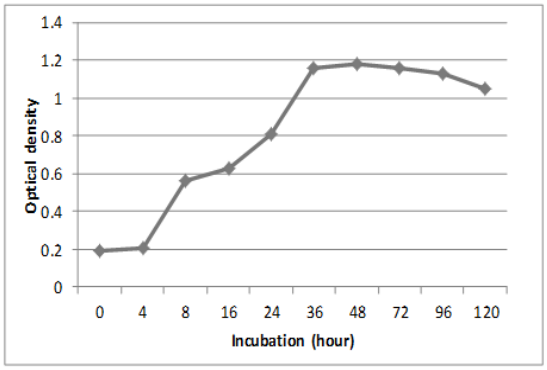

Figure 1. The growth curve of *A. xylinum* in the stirring cultivation to determine the exponential phase.

Based on this result, the carbon source concentration in fermentation medium seems to affect the *A. xylinum* growth cells. Sucrose 10% and coconut water contribute to cells regeneration, which is consistent with a previous study showing that coconut water in HS medium promotes *A. xylinum* growth rate (Kamarudin *et al*., 2013). Additionally, the adaptation phase of bacteria, including *A. xylinum*, may be influenced by the number of inoculated cells, age and physiological state (Rolfe *et al*., 2012). This initial investigation of *A. xylinum* growth is important in order to obtain the optimum BC production, thus, 48 h was determined as the best timing to transfer *A. xylinum* culture into fermentation process in this study.

3.2. pH Changes

The pH value of the fermentation media decreased on both coconut water and pineapple juice substrates, reaching approximately pH 3.5 as demonstrated in this study (Figure. 2). The pH value decreased faster in the early period of incubation up to 48 h which was proportional with the lag phase. Decrease in the pH value in this study is relatively faster than that in other formulated media with coconut water which was the only one carbon source and the other inorganic carbon sources-based media (Kamarudin *et al*., 2013). It is believed that it is more likely due to the released gluconic acid, acetic acid and acidic-by product (Ndoye *et al*., 2007; Kongruang, 2008) which lead to the inhibition of BC production because acidic medium can be toxic to *A. xylinum* cells. Furthermore, organic acids from coconut and pineapple juices may contribute to decline pH due to spontaneous fermentation.

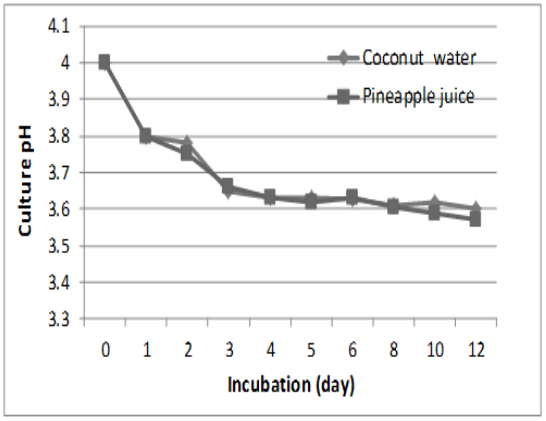

Figure 2. Pattern of pH change of *A. xylinum* culture on static culture fermentation.

These results agree with a previous study using two different strains of *A. xylinum* which revealed higher fold of acetic acid levels in Hestrin-Schramn medium with coconut juice as substrate than that with pineapple juice (Kongruang, 2008). The acidity of liquid fermentation of *A. xylinum*, however, can be adjusted with a buffer solution for optimum BC production (Pae *et al*., 2011), though it is still difficult to be controlled (Ishikawa *et al*., 1995). Overall, a decrease in the pH of culture medium is very influential on cell growth and productivity of BC from *A. xylinum* (Mathew *et al*., 2005; Kongruang, 2008).

3.3. Pattern of BC Formation

A. xylinum grown in coconut water and pineapple substrates showed a similar pattern of cell biomass, BC yield and reducing sugar (Figure 3). In both substrates, BC yield increased in parallel with cell biomass, but in contrast to declining of reducing sugars during the fermentation process. Cell biomass representing bacterial growth increased to be the greatest after 6 days, and showed higher on coconut water (19.34 g/l) than on pineapple (10.04 g/l). Even though *A. xylinum* grew relatively slow in pineapple substrate, but this fruit waste is also promising as carbon source for producing BC (Kurosumi *et al*., 2009; Upadhyay *et al*., 2010). Especially in this study, both substrates had a similar decrease in pH which is in the range of optimum production of BC, 3.5 to 6 (Pae *et al*., 2011). Moreover, the time course of maximum growth of *A. xylinum*, in our study, is relatively comparable with other *A. xylinum* strain in static culture fermentations (Coban and Biyik, 2011).

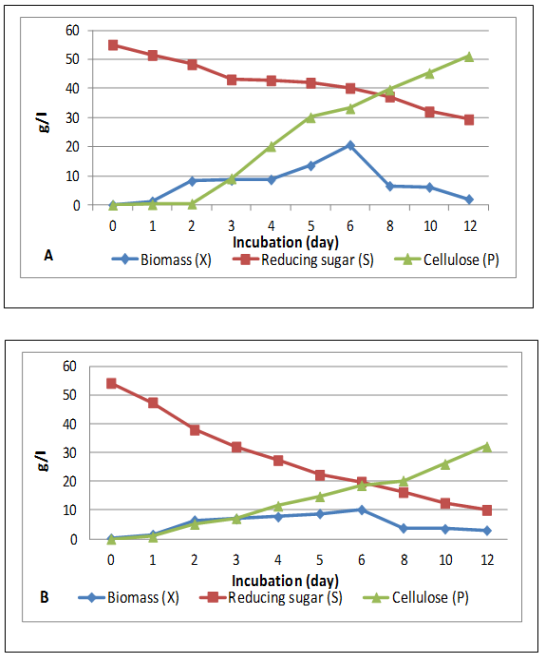

Figure 3. The relationship between the cell dry weights, BC products of *A. xylinum* and reducing sugar in cultivation system two stages. A) coconut water, B) pineapple juice

This study illustrates the *A. xylinum* growth with decreasing substrate concentration as represented by reducing sugar. Reducing sugars were found to have decreased in media containing pineapple juice more than that in media containing coconut water. *A. xylinum* used sugar in coconut water was lower in comparison with that in pineapple substrate, showing 50.8% and 81.1%, respectively. It seems that sugar converted by *A. xylinum* on coconut water was much higher (184.9%) than in pineapple juice (72%). Thus, it reveals that *A. xylinum* had higher efficiency of 2.6-fold of metabolization for monosaccharide and disaccharide in coconut water than in pineapples subtrate, which supports previous findings in the same subtrate (Kongruang, 2008; Kamarudin *et al*., 2013). *A. xylinum* presumably uses glucose from pineapple substrate more for cell metabolism than for the formation of BC.

Formation of BC can be predicted based on the pattern of substrate utilization. This study demonstrated that the BC yield achieved in maximum on day 12 when utilization of sugar on substrate was to be stationary as expected. In coconut water, *A. xylinum* produced 1.6-fold of BC yield and 0.6-fold of cell biomass with more efficient consumption of substrate of 2.9-fold than those in pineapple substrate during fermentation (Figure 3). Thus, we found out that coconut water in Hassid-Baker medium is clearly more efficient that pineapple-based medium to produce BC, convincing the flexibility of coconut water as substrate of *A. xylinum* (Kamarudin *et al*., 2013).

Our results could also explain that in the BC formation, glucose and fructose from decomposition of sucrose and other carbon sources were possibly polymerized to form BC which usually occurs via oxidative major pathway of *A. xylinum* (Ross *et al*., 1991). There is a phenomenon in which *A. xylinum* uses a specific substrate like coconut water which leads the cells to get adapted to consume the substrate. Formation of BC, which was also positively correlated with bacterial cells, indicating that the BC begins to form early cell metabolism is in line with prior study (Kongruang, 2008). Ishikawa and coworkers (1995) also reported that *A. xylinum* subp. *sucrofermentans* in the static culture fermentation formed BC pellicle and then bacterial cells were absorbed in the pellicle. Instead of various chemical nitrogen (such as casein hydrolisate, ammonium sulfate, yeast extract) and carbon sources (monosaccharides, oligosaccharides, alcohols, sugar alcohols and organic alcohols) (Jung *et al*., 2010; Coban and Biyik, 2011), coconut water, therefore, could replace carbon source to maximize the BC production with low cost.

3.4. Fermentation Kinetics

Microbial growth can be viewed as a series of biochemical reactions that convert a substrate for the synthesis of cell material and extracellular products. Analysis of the kinetics of *A. xylinum* growth is needed to determine the growth rate, the rate of substrate utilization and the product formation rate. *A. xylinum* fermentation kinetic parameters, indicating the difference between the substrates of coconut and pineapple juices, are shown in Table 2.

Table 2. Parameters of fermentation kinetics of *A. xylinum.*

Parameters	Substrate	
	Coconut water	Pineapple juice
R_p	0.117 g/l/h	0.051 g/l/h
R_x	0.309 g/l/h	0.133 g/l/h
R_s	0.079 g/l/h	0.215 g/l/h
$Y_{x/s}$	1.408 g biomass/g glucose	0.240 g biomass/g glucose
$Y_{p/s}$	3.612 g BC/g glucose	0.599 g BC/g glucose
$Y_{p/x}$	2.235 g BC/g biomass	2.452 g BC/g biomass
μ_{max}	0.0132/h	0.0082/h
σ	0.0258/h	0.0173/h

The rates of *A. xylinum* growth and of BC formation were 2.32- and 2.29-fold higher on coconut water than those in pineapple juice, respectively. Surprisingly, the greatest growth and BC productivity of *A. xylinum* on coconut water substrate were followed by much lower rate of glucose utilization (Rs:0.079 g/l/h) than that in pineapple juice (Rs:0.215 g/l/h). Based on yield values, the conversion of substrate to biomass and BC were 5.87- and 6.03-fold greater, respectively, in coconut water (Yx/s:1.408 g biomass/g glucose and Yp/s:3.612 g BC/g glucose) than in pineapple juice (Yx/s:0.24 g biomass/g glucose and Yp/s:0.599 g BC/g glucose). The more convincing results were the maximum specific growth rate (μ_{max}) in the exponential phase which was 1.61-fold higher in coconut water compared to pineapple juice. The specific rate of BC formation of *A. xylinum* was also higher in coconut water (σ:0.0258/h) than that in pineapple (σ:0.0173/h). Compared with the study of Kongruang (2008) which used the bigger scale up of cultivation, the growth kinetics parameters in our study were somewhat lower; however, our result with small laboratory scale was able to determine the efficiency of agro-residues as substrate for *A. xylinum*. Thus, it could be noted that coconut water may be preferred for a better fermentation of *A. xylinum* than other agro-wastes. However, taken together, abundant pineapple fruit waste in addition to coconut water should be further investigated for their best formulation in conjunction with cultivation methods for production of BC.

4. Conclusion

Maximum growth of *A. xylinum* reached in exponential phase at 48 h and was determined to be the best time to transfer the propagation culture to static fermentation system. BC produced by *A. xylinum* was positively correlated with the cell growth and negatively related to the reducing sugar. Overall parameters of fermentation kinetics suggest that coconut water is more efficient to be used as a substrate for producing BC by *A. xylinum* compared with pineapple juice.

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