Production of Bacterial Cellulose by *Acetobacter xylinum* through Utilizing Acetic Acid Hydrolysate of Bagasse as Low-cost Carbon Source

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Bacterial cellulose (BC) is a promising and renewable nanomaterial due to its unique structural features and appealing properties. Intensive study on BC preparation has been mainly focused on biosynthesis by certain bacteria, while the high economic costs of fermentation, especially the carbon sources, remain challenges to its application. In this study, bacterial cellulose was synthesized by *Acetobacter xylinum* with the acetic acid hydrolysate of bagasse used as carbon source. After the bagasse was pretreated by acetic acid, the components in hydrolysate and the removal rate was investigated, and the pretreatment conditions were optimized as follows: temperature of 160 °C, heating time of 60 min, addition of acetic acid of 2.0% (m/m), and solid-to-liquid ratio of 1/5. Prior to *Acetobacter xylinum* cultivation, the hydrolysate was detoxified by activated carbon. The detoxification process was very efficient for BC production, with a yield up to 2.13 g/L when the dosage of activated carbon was 5% (m/V). Furthermore, the obtained BC was characterized by scanning electron microscopy (SEM), which showed that the ribbons width of BC was between 30 and 80 nm. X-ray patterns showed the crystallinity value was 74.6 % and the crystallinity index (CI%) was 66.5 %, which also evidenced the presence of peaks characteristic of Cellulose I polymorph. In conclusion, it is feasible to produce BC from bagasse hydrolysate. This model of waste recycling could aid in the development of sustainable strategies.

Keywords: Bacterial cellulose; Acetobacter xylinum; Acetic acid; Bagasse

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INTRODUCTION

Bacterial cellulose (BC) is a linear homopolymer of D-glucopyranose linked by β-1,4-glycosidic bonds, and it is a promising nano-biomaterial produced by some bacteria, such as acetic acid bacteria (Hong *et al*. 2012; Fu *et al*. 2013; Shah *et al*. 2013; Hu *et al*. 2014). Compared with natural plant cellulose, bacterial cellulose is devoid of other contaminating polysaccharides such as lignin and hemicellulose; it exhibits many superior physicochemical properties such as high purity, high crystallinity, high degree of polymerization, nano-structured network, high wet tensile strength, high water-holding capacity, and good biocompatibility (Hong and Qiu 2008; Li *et al*. 2015; Santos *et al*. 2015; Xu *et al*. 2015). These desirable characteristics are of interest for broad prospective applications bringing tremendous economic and societal benefits in diverse fields including biomedical materials, food matrix, textiles, electronic displays, audio

membranes, functional papers, *etc*. (Iguchi *et al*. 2000; Shah and Brown 2005; Klemm *et al*. 2006; Hu *et al*. 2008). Nevertheless, the high economic cost of culture media as well as low-yield production limit wide scale industrial production and extended applications of BC (Hu *et al*. 2010; Hong *et al*. 2011). Hence, it is challenging and meaningful to develop new approaches to high-yield BC production at the lowest cost possible.

In recent years, an increasing effort has been devoted to ensuring favorable production yields and controlled costs. These efforts include the species and genetic modification of the bacteria used, feedstock type and composition, and the type of reactor used for production (Shi *et al*. 2014). The cost of feedstock normally accounts for as high as 50 to 65% of the total cost of production, and thus it is essential to develop low-cost carbon sources (Thompson and Hamilton 2001; Sani and Dahman 2010). Some relatively cheap agricultural products or waste, such as food process effluents, molasses, konjak glucomannan, and fruit juices, have been developed as cost-effective feedstocks for BC production; the use of these products simultaneously reduces environmental issues related to waste disposal (Sasithorn 2008; Kurosumi *et al*. 2009; Faranak *et al*. 2015).

Bagasse is a crop byproduct whose annual output reaches up to 26 million tons in China. The use of agricultural residue bagasse is attractive due to its sugar-rich hydrolysate. If the abundant bagasse can be converted into a growth medium for microbial fermentation to produce BC, high-value products could be made from the agricultural byproducts and effectively reduce the cost of BC production (Li *et al*. 2012; Jiang *et al*. 2015). Vazquez *et al.* (2013) produced bacterial cellulose by *G. xylinus* using glycerol remaining from grape bagasse as the carbon sources, with the aim of formulating a general, simple and inexpensive medium to produce BC. They obtained 8.0 g/L of BC production and widths values in the range of 35 to 70 nm under optimized conditions. However, some microbial growth inhibitors include phenolic compounds, furan derivatives, and aliphatic acids in the hydrolysate. These compounds inhibit BC production and must be removed through detoxification treatments (Palmqvist and Hahn-Hagerdal 2000; Cheng *et al*. 2015). Different alkaline detoxification treatments include $Ca(OH)_2$, NaOH, and NH_4OH; such treatments are used to remove inhibitors are well established methods to improve the fermentability of hydrolysates for BC production (Mussatto and Roberto 2004; Hong *et al*. 2009; Hong *et al*. 2011).

In this study, bagasse was first pretreated by acetic acid, and the pretreatment conditions were optimized. Hydrolysates were subsequently detoxified by activated carbon to remove microbial growth inhibitors. Thus, bagasse hydrolysates were used as a suitable carbon source to cultivate *Acetobacter xylinum*, which could decrease the cost of BC production, especially on the industrial scale.

EXPERIMENTAL

Materials

Acetobacter xylinum ATCC 23767 was obtained from Guangdong Microbial Culture Center (Guangzhou, China). The bagasse was obtained from farmland in Guangxi, China. The acetic acid glacial (CH_3COOH) was purchased from Guangzhou Jinhuada Chemical Reagent Co., Ltd, (Guangzhou, China), and activated carbon was obtained from Shanghai Aladdin Bio-chem Technology Co., Ltd (Guangzhou, China). All chemicals used in the preparation of culture media were obtained from Tianjin

Benchmark Chemical Reagent Co., Ltd. (Tianjin, China). The water utilized throughout the experimental procedures was deionized (DI) water at room temperature.

Methods

Hydrolysis of bagasse

First, 100 g of absolute dry bagasse (40 to 60 mesh) was impregnated with 500 mL of 0.5% (w/v) acetic acid in a batch reactor overnight. The mixture was treated at 140 °C, 160 °C, and 180 °C respectively, and the pretreatment time was 30 min, 60 min, and 90 min, respectively. Subsequently, the hydrolysate was collected by filtration. To collect enough hydrolysate for later experiments, the hydrolytic process was carried out several times, and all hydrolysates were pooled together as the final hydrolysates. Different dosages of acetic acid including 0%, 2.0%, 4.0%, 6.0%, 8.0%, and 10.0% (m/m) were also added to the hydrolysates to investigate the influence of the composition under the optimum hydrolysis temperature and time.

Detoxification of bagasse hydrolysates

The pH value of the hydrolysate was adjusted to 2.0 by addition of 6.0 M H_2SO_4 and the mixture was incubated at 30 °C for 6 h. The pH was then adjusted to 7.0 by calcium oxide. Next, a certain dosage of activated carbon (AC) was added into the hydrolysate and mixed at 25 °C for 10 min. Finally, the activated carbon was removed from the hydrolysate, and the pH value was adjusted to 7.0 with 0.1 M H_2SO_4 or calcium oxide (Hong *et al*. 2011; Guo *et al*. 2013).

Culture media and growth conditions

The detoxified hydrolysates were combined with culture medium containing 5 g/L peptone, 5 g/L yeast powder, 5 g/L mannitol, 1 g/L magnesium sulfate, and 5 g/L anhydrous ethanol. The pH was adjusted to 6.8 by NaOH, and the media was subjected to high pressure sterilization. The activated strain was inoculated into a 50 mL liquid production medium of 7 v/v% capacity, which was cultivated at 30 °C in a static incubator for 10 days.

Harvest and weighing of BC

After cultivation, bacterial cellulose membranes were collected and impregnated with 0.5 M NaOH at 80 °C for 2 h, followed by repeated soaking and washing in deionized water until the washing liquid reached neutral pH. The BC was filtered and dried to constant weight at 105 °C. The mass of BC was determined, and the BC yield was calculated.

Scanning electron microscopy (SEM) observations of BC

SEM was used to examine the bacterial cellulose produced in different cultures. The samples were fixed to a metal-base specimen holder using double-sided sticky tape. The fixed samples were coated with an approximately 30-Å Au/Pd layer and then observed using a Hitachi S-3700 SEM (Tokyo, Japan).

X-ray diffraction (XRD) analysis

The crystal structures of the two kinds of obtained BC were investigated using an XRD analyzer (D8 ADVANCE X-ray diffractometer) set at 40 kV and 30 mA. Wide-

angle X-ray intensities were collected for 2θ, ranging from 5°to 50°, with a step increment of 0.02.

The crystallinity index (CI) of produced BC was determined using the following equation (Vazquez *et al*. 2013),

$$CI(\%) = \frac{I_{200} - I_{2\theta}}{I_{200}} \times 100$$

where I_{200} corresponds to the maximum intensity of the lattice diffraction, and $I_{2\theta}$ corresponds to the intensity of the peak at $2\theta = 18°$, which accounts for the amorphous part of cellulose. The intensity of the peaks was measured as the maximum value obtained for the peak taking into account a baseline.

RESULTS AND DISCUSSION

Effects of Pretreatment Temperature and Time on the Composition of Hydrolysate

Table 1 shows that the removal rate, total sugar, lignin, and furfuraldehyde content were significantly increased at higher pretreatment temperatures and longer pretreatment times, which resulted in more intense hydrolysis reactions as well as the degradation of cellulose and hemicellulose. When the pretreatment temperature was increased from 140 °C to 180 °C at the pretreatment time of 60 min, the corresponding removal rate, total sugar content, lignin, and the furfuraldehyde content increased by 24.96%, 97.98%, 49.26%, and 298.39%, respectively.

Table 1. Component Analysis and Removal of Hydrolysate from Bagasse

Pretreatment Temperature (°C)	Pretreatment Time (min)	Removal Rate (%)	Total Sugar (g/L)	Lignin (g/L)	Furfuraldehyde (g/L)
180	30	34.93	27.82	5.09	1.85
180	60	37.05	32.47	5.03	2.47
180	90	41.96	21.58	5.78	4.39
160	30	26.10	24.31	3.88	0.36
160	60	33.16	28.72	4.01	1.03
160	90	35.97	29.72	4.89	1.69
140	30	28.98	8.93	2.48	0.18
140	60	29.65	16.40	3.37	0.62
140	90	30.02	24.56	3.63	0.73

When the pretreatment time was increased from 30 min to 90 min at the pretreatment temperature of 160 °C, the corresponding removal rate, total sugar content, lignin, and the furfuraldehyde content increased by 37.81%, 22.25%, 26.01%, and 369.1%, respectively. However, the degradation products of glucose and xylose could be further degraded into furfuraldehyde or other by-products. When the temperature was too high (> 180 °C), there was a high removal rate and total sugar content, but the furfuraldehyde content increased accordingly. In contrast, low temperature (< 140 °C) led to incomplete hydrolysis and incomplete dissolution of hemicellulose. Hence, the optimum pretreatment conditions included the pretreatment temperature of 160 °C and

pretreatment time of 60 min. Under the best conditions, the raw material removal rate was 33.16%, the total sugar content of the hydrolysate was 28.72 g/L, the lignin content was 4.01 g/L, and the furfuraldehyde content was 1.03 g/L.

Effects of Dosage of Acetic Acid on the Composition of Hydrolysate

The effects of acetic acid dosage on the hydrolysate components and raw material removal rate is shown in Fig. 1. The raw material removal rate as well as the content of total sugar, lignin, and furfuraldehyde increased as the dosage of acetic acid was increased. When the dosage of acetic acid increased from 0% to 2.0%, the corresponding total sugar content, furfuraldehyde content, and the removal rate increased by 35.3%, 23.2%, and 27.2%, respectively. However, if the dosage of acetic acid increased continuously from 2.0% to 10%, the increase in the total sugar, furfuraldehyde, and removal rate was 11.1%, 84.7%, and 20.3%, respectively. These results indicated that the increase of acetic acid promotes the hydrolysis of small molecules such as glucose, xylose, or other monosaccharides to furfuraldehyde, *etc*. Hence, 2.0% acetic acid was best for the hydrolysis of bagasse. Generally speaking, it still had enough sugar in the hydrolysate as a carbon source to cultivate bacterial cellulose.

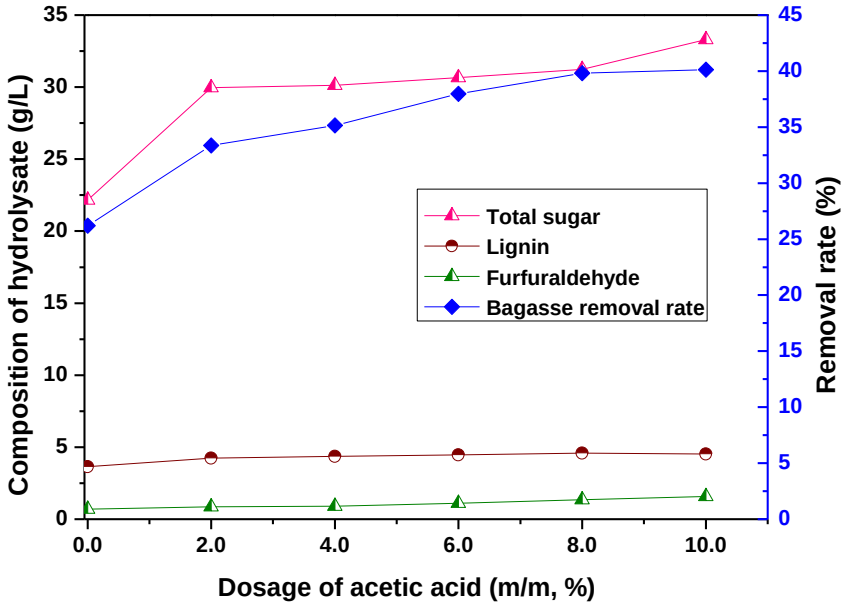

Fig. 1. Component analysis and removal of hydrolysate from bagasse

Effects of Dosage of Activated Carbon on the Detoxification of Bagasse Hydrolysates

The components in the bagasse hydrolysate are shown in Table 2. The optimal pretreatment conditions were selected as follows: the temperature was 160 °C, the heating time was 60 min, the addition of acetic acid was 2.0% (m/m), and solid-to-liquid ratio was 1/5. The main carbon source material was glucose and xylose, corresponding to 29.36 g/L and 4.66 g/L, respectively, while the mannose was only 1.92 g/L. The concentration of furfuraldehyde was a high (2.97 g/L) in the hydrolysate. Thus, it was essential to detoxify the hydrolysate before utilization.

Table 2. Main Components in the Bagasse Hydrolysate

Component	Glucose (g/L)	Xylose (g/L)	Mannose (g/L)	Furfuraldehyde (g/L)	Lignin (g/L)
Concentration	4.66	29.36	1.92	2.97	4.13

Table 3 shows that 59.3% furfuraldehyde and 86.7% lignin were removed at a dosage of 5% (m/V) of activated carbon, while 21.0% glucose and 32.1% xylose were removed at the same time in the optimal pretreatment conditions. The adsorption of components by activated carbon is a physical adsorption process, and the adsorption capacity is affected by concentration and solubility of the adsorption material. The adsorption quantity of xylose by activated carbon was higher than that of the glucose because the concentration of xylose was much higher than glucose, resulting in a higher contact with activated carbon. The adsorption quantity of lignin was the highest, which may be associated with its solubility; materials with lower solubility are more easily removed (Zhang *et al*. 2011).

Table 3. Concentration of Substances after AC Treatment

Dosage of Activated Carbon (m/V, %)	Glucose (g/L)	Xylose (g/L)	Furfuraldehyde (g/L)	Lignin (g/L)
0	1.76	8.31	0.81	1.42
1.0	1.93	8.08	0.67	0.81
2.0	1.82	7.76	0.56	0.45
3.0	1.61	7.29	0.44	0.37
4.0	1.44	5.84	0.38	0.24
5.0	1.39	5.65	0.33	0.19

Bacterial Cellulose Production by *Acetobacter xylinum* from Bagasse Hydrolysates

Figure 2 shows the effect of activated carbon dosage on bacterial cellulose yield.

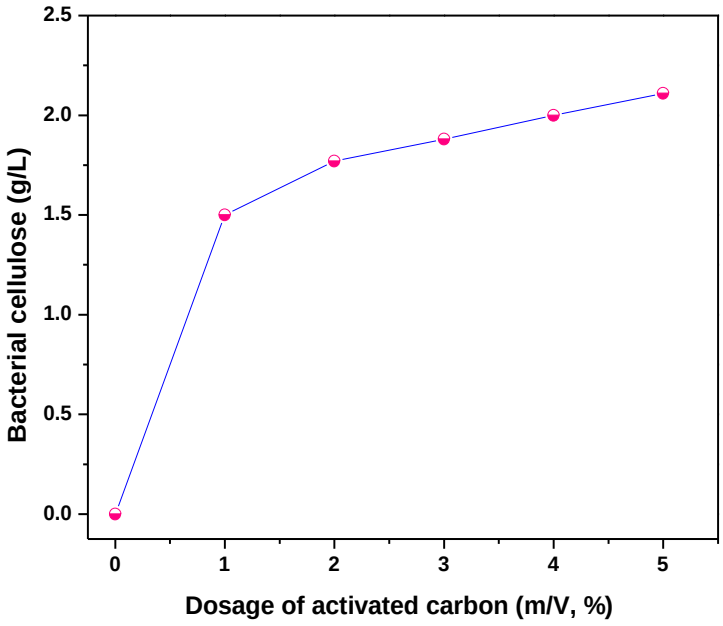

Fig. 2. BC yield with the dosage of activated carbon

The bacterial cellulose yield increased with increasing activated carbon dosage. When the dosage of activated carbon was 0% (m/V), *Acetobacter xylinum* did not produce bacterial cellulose; the furfuraldehyde concentration in the hydrolysate was 0.81g/L, which was beyond the acceptable concentration for BC production. When the dosage of active carbon increased from 1.0% to 5.0% (m/V), the overall utilization of glucose and xylose increased from 10.8% to 22.5%, and the yield of bacterial cellulose reached 2.13 g/L. This result proved that the activated carbon detoxification was effective for the production of bacterial cellulose.

SEM micrographs of the freeze-dried bacterial cellulose in different culture media are shown in Fig. 3. The nano network structure can be clearly seen. The ribbons width of the bacterial cellulose was about 30 to 80 nm, which was consistent with other reports (Esra *et al*. 2015). Comparing the size of BC in the two pictures, the diameter size of BC was smaller under the effect of inhibitors (furfuraldehyde), and it was especially more obvious for the BC produced by hydrolysate (Fig. 2b). Thus, the BC diameter size was affected by the existence of inhibitors.

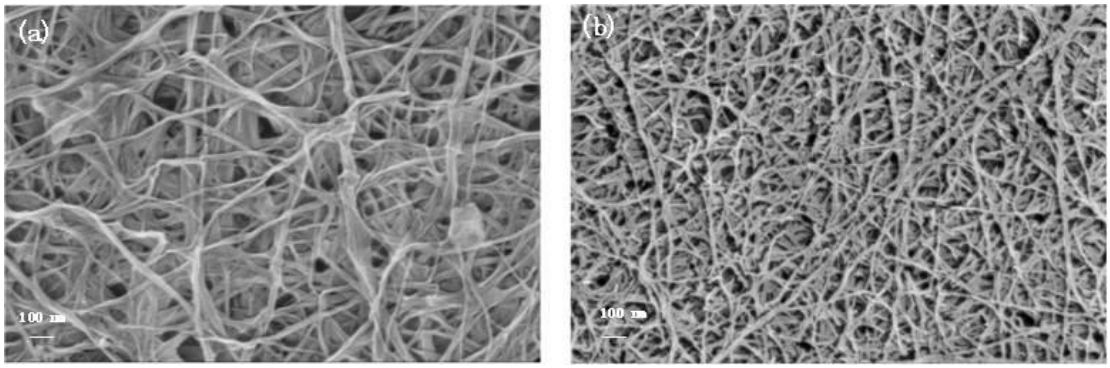

Fig. 3. Bacterial cellulose in the different culture mediums: (a) glucose; (b) bagasse hydrolysate

X-ray diffraction (XRD)

Diffraction patterns obtained for BC obtained from glucose and bagasse hydrolysate are shown in Fig. 4.

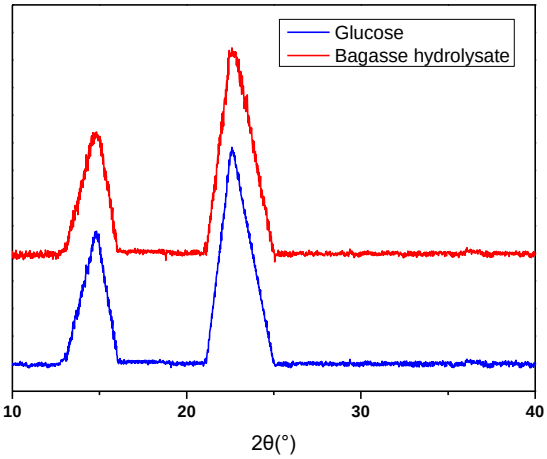

Fig. 4. X-ray diffraction patterns of bacterial cellulose obtained from different culture media: (a) glucose; (b) bagasse hydrolysate

Each pattern exhibited two diffraction dominant peaks, the first one was located between 13° and 16°, and the second one was located between 21° and 25°, which confirmed that only cellulose I including crystalline phase Iα and Iβ was present in the two BC samples. The first peak represents the projection of the planes (100) of fraction Iα and (110 and 010) of fraction Iβ, and the second peak represents the projection of the planes (110) Iα and (200) Iβ (Gea *et al*. 2011; Santos *et al*. 2015).

Calculated crystallinity and crystallinity index (CI %) values are shown in Table 4. It is worth noting that bacterial celluloses obtained from two kinds of carbon sources exhibited almost similar crystallinity values, which was between 74.5 and 76.5. Besides, the CI% obtained with bagasse hydrolysate was slightly lower than that obtained with glucose. The results corresponded to 66.5% of bagasse hydrolysate and 70.3% of the glucose. Such behavior could be ascribed to the small amount of residual inhibitors in the culture medium, which resulted in the hydrogen bonding between the cellulose glucan chains, and there was little reduction in the crystallinity index (Vazquez *et al*. 2013; Li *et al*. 2015).

Table 4. Crystallinity and Crystallinity Index (CI) of BC Obtained by Use of Different Carbon Sources

Carbon source	I_{am}	I_{200}	Crystallinity (%)	Crystallinity Index (CI) (%)
Glucose	470	1583	76.2	70.3
Bagasse hydrolysate	464	1385	74.6	66.5

CONCLUSIONS

1. In this study, activated carbon detoxification was used to treat the acetic acid hydrolysates of bagasse for BC production. The results indicated that a dosage of 5% (m/V) of activated carbon detoxification treatment was effective.
2. Under the optimum pretreatment conditions, the yield of BC was up to 2.13 g/L, and the ribbons width of BC was between 30 to 80 mm. X-ray patterns confirmed the presence of cellulose I crystalline polymorphism. The crystallinity and crystallinity index values determined for bacterial cellulose obtained from bagasse hydrolysate were similar to those obtained from glucose BC. These conditions were a pretreatment temperature of 160 °C, heating time of 60 min, addition of acetic acid of 2.0% (m/m), and solid-to-liquid ratio of 1/5.
3. Bacterial cellulose was produced with the bagasse hydrolysate used as the carbon source. The cost of feedstock was relatively low because bagasse is a cheap, abundant resource throughout the world. Thus, the cost-effective feedstocks are promising new carbon sources for the large scale industrial production of nanostructured BC materials.

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