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Production and Characterization of Cellulose by *Acetobacter* sp. V6 Using a Cost-Effective Molasses–Corn Steep Liquor Medium

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Abstract In order to reduce of the manufacturing cost of bacterial cellulose (BC), BC production by *Acetobacter* sp. V6 was investigated in shaking culture using molasses and corn steep liquor (CSL) as the sole carbon and nitrogen sources, respectively. The highest BC production was obtained with $Ca_3(PO_4)_2$-treated molasses. Maximum BC yield (2.21±0.04 g/l) was obtained at 5% (*w*/*v*) total sugar in molasses. In improved medium containing molasses and CSL, BC production was observed in the medium after 1 day of incubation and increased rapidly thereafter with maximum yield (3.12±0.03 g/l) at 8 days. This value was approximately twofold higher than the yield in the complex medium. Physical properties of BC from the complex and molasses media were studied using Fourier-transform infrared (FT-IR) spectroscopy and X-ray diffractometer. By FT-IR, all the BC were found to be of cellulose type I, the same as typical native cellulose. The relative crystallinity of BC produced in the complex and molasses media were 83.02 and 67.27%, respectively. These results suggest that molasses and CSL can be useful low-cost substrates for BC production by *Acetobacter* sp. V6 without supplementation with expensive nitrogen complexes such as yeast extract and polypeptone, leading to the reduction in the production costs.

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Introduction

It is known that some *Acetobacter* strains produce cellulose. This cellulose is called bacterial cellulose (BC) and is expected to be a new industrial material, because of its unique properties, such as mechanical strength, high purity, and biodegradability [1]. For example, there are plans to use it in the production of artificial skin, ultrafiltration membrane, a cover-membrane for glucose biosensors, as culture substrate for mammalian cells, and in binders for powders and thickeners for paint, ink, and adhesive [2].

The traditional processes for the production of BC have used static cultivation methods, with pellicles of BC being formed on the surface of the static culture. However, this requires a large area and a long culture time [3]. Therefore, for economical BC production, it is necessary to establish a mass production system using shaking culture technique. However, relatively smaller amount of BC is produced in shaking and agitated culture than in static culture and this is closely related to the generation of mutants which do not produce BC. Insertional sequences were found in the genes of these mutants [4]. Thus, developing a submerged fermentation system for large-scale production has been a major research interest in recent years.

In microbial fermentations, the cost of the fermentation medium can account for almost 30% of the total cost [5]. Therefore, one of the key aspects in the fermentation process is the development of a cost-effective culture medium to obtain maximum product yield. Most of the studies on the BC production by *Acetobacter* strains have been carried out in media containing pure sugar as carbon source, such as glucose, sucrose, fructose, mannitol, and arabitol [6–8]. The majority of studies on BC production have also used media containing complex nitrogen and vitamin sources such as yeast extract and polypeptone [6–8]. However, these nutrient sources are economically unfavorable, because these substrates are very expensive. The manufacturing cost of BC must be significantly reduced if it could be possible to use a waste product such as molasses and corn steep liquor (CSL) as a raw material for the production of BC.

Molasses is a byproduct of the sugar industry readily available at relatively low cost. It contains approximately 50% (*w*/*w*) of total sugars (sucrose, glucose, fructose, raffinose) [5], and therefore, is a very attractive carbon source for BC production from an economic point of view. Molasses has already been used for the production of a number of industrially important chemicals, such as ethanol [9], lactic acid [10], citric acid [11], and polysaccharide [12]. CSL is a byproduct of corn wet-milling, and contains a rich complement of important nutrients to support robust microbial growth and fermentation [13]. As a fermentation medium supplement, CSL can be viewed either as a complete source of nitrogen or as a source of vitamins and other trace elements [13]. At present, very little published information is available on the simultaneous utilization of molasses and CSL as a raw material to produce BC.

Recently, we have reported on the isolation of new *Acetobacter* strains, which are able to produce BC under shaking culture conditions [14]. Following this, we attempted to improve the ability of the strains to produce BC in complex and synthetic media [8, 15]. In this work, we tested the potential use of molasses as the carbon source for BC production using *Acetobacter* sp. V6. The effect of supplementing CSL on BC production from molasses was also examined. Besides, we studied structural features of BC produced from an improved medium.

Materials and Methods

Microorganism

The *Acetobacter* sp. V6 strain used in this study was isolated from the traditionally fermented vinegar in Korea. It has been generally known that the ability to produce BC in *Acetobacter* strains is highly sensitive to shearing force [16]. *Acetobacter* sp. V6, however, did not lose this ability even when it was cultivated at an agitation of 200 rpm in a flask containing complex medium, indicating that the cellulose-producing ability of this bacterium is resistant to shearing force [14].

Pretreatment of Molasses and CSL

Molasses and CSL were obtained from the local companies in Korea. The molasses was diluted with distilled water in order to obtain 10% (*w*/*v*) total sugar concentration. The molasses was pretreated before use. The following two methods were tried with a view to test its ability to support BC production [17]. Firstly, the pH of the molasses solution was adjusted to 3.0 by adding 1 N H_2SO_4. This was allowed to stand for 24 h and then centrifuged at 17,478×*g* for 30 min. Secondly, the pH of the molasses solution was adjusted to 7.0 by the addition of 1 N NaOH and treated with 1% (*w*/*v*) $Ca_3(PO_4)_2$ followed by heating at 100°C for 15 min. The mixture was cooled and centrifuged at 17,478×*g* for 15 min. These supernatants collected were added as the carbon source instead of pure sugar in complex medium.

The CSL was diluted to the desired concentration, centrifuged at 17,478×*g* for 30 min followed by autoclaving. Precipitates were removed aseptically by centrifugation and the supernatant was used as the nitrogen source for the basal medium.

Culture Conditions

For shaken culture in flasks, stock culture was inoculated into 50 ml Hestrin and Shramm (HS) medium in a 250-ml conical flask and incubated for 72 h under static conditions. The resulting seed culture was shaken vigorously to release cells from the pellicle. The suspension was passed through 16 layers of sterile gauze [8, 15]. This cell suspension was used as inoculum and seeded into the culture medium at a level of 5% (*v*/*v*). Shake-flask fermentations were carried out in 250-ml conical flasks containing 75 ml of culture medium. Cultivations were performed at 30°C and 200 rpm for 7 days in a rotary shaker unless stated otherwise.

A complex medium developed previously, containing 4% (*w*/*v*) glucose, 0.1% (*w*/*v*) yeast extract, 0.7% (*w*/*v*) polypeptone, 0.8% (*w*/*v*) $Na_2HPO_4{\cdot}12H_2O$, 1.4% (*v*/*v*) ethanol with pH 6.5 [15], was used as a basal medium. HS medium used comprised the following: 2% (*w*/*v*) glucose, 0.5% (*w*/*v*) yeast extract, 0.5% (*w*/*v*) polypeptone, 0.675% (*w*/*v*) $Na_2HPO_4{\cdot}12H_2O$ and 0.115% (*w*/*v*) citric acid monohydrate in distilled water (pH 6.0) [18].

FT-IR Spectroscopy

Fourier-transformed infrared (FT-IR) spectrometry was used to identify the chemical structure of the BC. The FT-IR spectrum of the BC was measured at wave numbers ranging from 4,000 to 400 cm^{-1} with an IRAffinity-1 FT-IR spectrometer (Shimadzu Corp., Japan).

X-ray Diffractometry

X-ray measurement was carried out to analyze the change in crystallinity of the BC by a Rigaku III X-ray diffractometer (Rigaku Corp., Japan) with reflection geometry and Cu Kα radiation (λ=0.154 nm) operated at 40 kV and 30 mA. The scanning was made through 2θ=5–40° with a scanning speed of 10°/min. The relative crystallinity index was estimated by Segal's method [19], using the following equation:

$$\mathrm{CrI}^{\mathrm{XRD}} = [(I_{002} - I_{\mathrm{am}})/I_{002}] \times 100$$

where, I_{002} is the peak intensity corresponding to the (002) plane at 2θ=22.8° for cellulose *I*, and I_{am} is the peak intensity of amorphous fraction at 2θ=18° for cellulose *I*.

Measurement of Cell Growth and BC Concentration

Cell growth was evaluated by measuring the absorbance at 660 nm using a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Sweden). Prior to measurement, the fermentation broth was homogenized aseptically, the homogenate filtered through the filter paper and the filtrate was then analyzed [15]. To purify BC, the culture broth was washed with distilled water to remove medium components and treated with 0.5 N NaOH at 90°C for 1 h to eliminate bacterial cells. The BC was rinsed extensively with distilled water until the pH of water became neutral [15]. The purified BC was dried to constant weight at 105°C and then weighed. Total sugar concentration was quantified as glucose equivalent by the phenol–sulfuric acid method [20]. All experiments were repeated three times. The data presented in the Tables correspond to mean values ± standard deviation.

Results and Discussion

Effect of Pure Sugar on BC Production

Acetobacter sp. V6 had been isolated from the traditionally fermented vinegar in Korea using HS medium containing glucose as the sole carbon source in our laboratory [14]. This strain could produce 1.12–1.51 g/l BC when cultivated in media with the initial glucose concentrations of 1–5% (*w*/*v*) [8].

Molasses is cheaper than other carbon sources, and is mainly composed of sucrose, fructose, and glucose. Thus, we investigated the effect of these sugars on the BC production by *Acetobacter* sp. V6 in the complex medium containing yeast extract and polypeptone as complex nitrogen source. As shown in Table 1, the culture produced 1.12±0.02–1.51±0.02, 1.22±0.05–1.29±0.02, and 0.38±0.04–0.57±0.02 g/l BC from glucose, fructose, and sucrose, respectively, at the concentration range from 1% to 4% (*w*/*v*). In cases of glucose and sucrose, concentrations above 5% (*w*/*v*) resulted in a decrease in the BC production (data not shown).

Effect of Molasses and CSL on BC Production

Since glucose, fructose, or sucrose was a potential substrate for BC production, molasses could be the substrate of choice. Molasses contains heavy metals and minerals that have deleterious effects on microbial growth and product synthesis [17]. These components have

Table 1 Effect of pure sugar as the carbon source on cellulose production by *Acetobacter* sp. V6.

Carbon source	BC yield (g/l)	Final pH
None	0.20±0.03	5.8±0.1
Glucose 1%	1.12±0.02	3.1±0.0
2%	1.29±0.05	2.8±0.1
3%	1.36±0.03	2.7±0.0
4%	1.51±0.02	2.6±0.0
Fructose 1%	1.22±0.05	5.4±0.1
2%	1.23±0.03	5.2±0.0
3%	1.29±0.02	5.1±0.0
4%	1.25±0.01	4.9±0.0
Sucrose 1%	0.38±0.04	6.0±0.0
2%	0.45±0.01	5.9±0.1
3%	0.52±0.01	5.8±0.0
4%	0.57±0.02	5.7±0.1

to be removed from the molasses, which could help in realizing increased yield of desired products. To examine the effect of pretreatment of molasses on BC production, molasses was prepared by two different methods as indicated in the "Materials and Methods" section. Pretreated molasses was provided at the total sugar concentration of 4.0% (*w*/*v*). As shown in Table 2, H_2SO_4- and $Ca_3(PO_4)_2$-treated molasses gave higher BC production compared with untreated molasses. The highest BC production (2.09±0.03 g/l) was obtained with $Ca_3(PO_4)_2$-treated molasses, while the above value was lower by 32% with untreated molasses. This suggests that molasses contained undesirable substances which may affect the production of BC. Otherwise, heavy metals and suspended impurities of molasses could be removed by H_2SO_4 treatment, which has been used to increase the fermentative production of polysaccharide [17]. BC production of *Acetobacter* sp. V6 achieved in the pretreated molasses medium was also comparable with that in the complex medium containing pure glucose as the carbon source. It is well known that *Acetobacter* strains, BC-producing bacteria, oxidize glucose to gluconic acid [21]. The conversion of glucose to gluconic acid leads to a significant drop in pH of the culture broth and inhibits BC production [21]. In this study, therefore, a decrease of BC production in the complex medium containing glucose as the sole carbon source may be a result of the low cultural pH value (Table 2) due to gluconic acid formation. Moreover, the presence of a lower amount of glucose [17] in the molasses components would lead to less gluconic acid in the molasses medium; consequently, the pH of molasses culture would be maintained at 5.5–5.6 (Table 2), resulting in improved BC production. In order to study this, the effect of

Table 2 Cellulose production from pretreated molasses by *Acetobacter* sp. V6.

Treatment	BC yield (g/l)	Final pH
Complex medium	1.53±0.03	2.6±0.1
Untreated molasses	1.51±0.02	5.6±0.0
H_2SO_4-treated molasses	1.93±0.05	5.5±0.0
$Ca_3(PO_4)_2$-treated molasses	2.09±0.03	5.6±0.1

initial pH on the BC production was tested in the range of pH 3.0 to 8.0. A high level of BC production was observed over a pH range between 5.5 and 6.5, and was maximum at pH 6.5 (data not shown). This result confirms the conclusion presented above. It is evident from these results that molasses is a good carbon source to support the BC production. Further experiments on BC production were carried out using $Ca_3(PO_4)_2$-treated molasses as a substrate. On the other hand, Bae and Shoda [22] reported that the maximum BC concentration by H_2SO_4-heat treated molasses increased to 76% more than that achieved using untreated molasses, indicating that substances in molasses inhibitory to growth and BC production were removed to some degree. These authors also reported that sucrose in molasses was hydrolyzed by H_2SO_4-heat treatment into glucose and fructose, which also stimulated BC production [23].

A few reports have showed that the sugar concentration in molasses could influence metabolites production [9, 12]. Therefore, the effects of initial concentrations of total sugars in the molasses on the BC production were tested. As shown in Fig. 1, the production of BC depended on the initial concentration of total sugar in molasses. BC production increased with the increase of initial total sugar concentration up to 5% (*w*/*v*), but then decreased beyond this value. Maximum BC yield obtained was 2.21±0.04 g/l at an initial total sugar concentration of 5% (*w*/*v*). On the other hand, the decline in polysaccharide production encountered with high sugar concentrations in the medium is probably due to osmotic effects; a lower level of water activity as well as plasmolysis events could decrease the fermentation rate and polysaccharide synthesis [24].

Yeast extract and polypeptone are the most commonly used nitrogen source in BC production as it provides convenient nitrogens and growth factors for *Acetobacter* strains [6–8]. However, the high cost of yeast extract and polypeptone has a negative impact on the economics of its use in industrial-scale processes. In order to improve the economic parameters of BC production by using cheaper and comparable nitrogen source, CSL was tested. Five different concentrations of CSL (2, 4, 6, 8, and 10% (*v*/*v*) were tested in comparison with a molasses medium containing 0.1% (*w*/*v*) yeast extract and 0.7% (*w*/*v*) polypeptone. As shown in Fig. 2, BC production at all CSL concentrations tested was comparable with that (2.21±0.04 g/l; Fig. 1) obtained with yeast extract and polypeptone. Maximum yield of BC (2.36±0.02 g/l) was obtained with 4% (*v*/*v*) CSL in the medium after 7 days of incubation. Further increase of CSL slightly

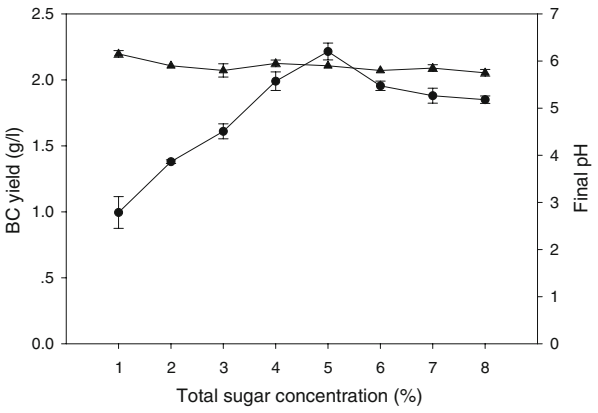

Fig. 1 Effect of molasses concentration (as total sugar) on cellulose production by *Acetobacter* sp. V6. *filled circle* BC yield, *filled upright triangle* final pH

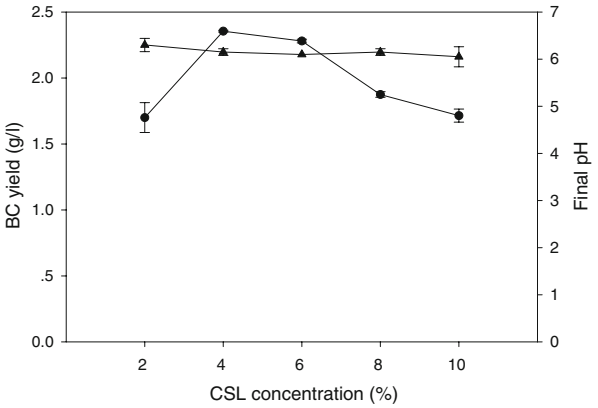

Fig. 2 Effect of CSL concentration on cellulose production by *Acetobacter* sp. V6. *filled circle* BC yield, *filled upright triangle* final pH

decreased BC production. The addition of CSL also maintained the pH range at 5.9–6.2. Therefore, it is thought that CSL is buffered against pH change. The buffering action of CSL may be an important additional factor in producing high BC production by maintaining the cultural pH value within the optimum range for BC synthesis. These results indicate that the addition of CSL at 4–6% (*v*/*v*) is beneficial to BC production, both for the buffer effect and from the nutritional point of view, and CSL could be a good substrate for rather expensive yeast extract and polypeptone. The results were in agreement with the previous studies of Noro et al. [25], who reported a role of CSL in the production of BC by *Acetobacter xylinum* BPR2001. However, taking into account the fact that CSL is an industrial waste and its composition is therefore dependent on both the raw material used (corn) and the process conditions, the optimal concentration of CSL to be used in *Acetobacter* sp. V6 medium must be defined for each particular sample of this material.

To determine the effect of phosphate concentration on the production of BC, various concentrations of $Na_2HPO_4{\cdot}12H_2O$ ranging from 0 to 1.0% (*w*/*v*) were added to the medium. Maximal BC production was found in the medium containing 0.2% (*w*/*v*) $Na_2HPO_4{\cdot}12H_2O$. $Na_2HPO_4{\cdot}12H_2O$ concentrations above or below this value resulted in a decrease in BC production (data not shown). On the other hand, we also found that more BC was obtained when NaH_2PO_4 (0.2%, *w*/*v*) was added to the $Na_2HPO_4{\cdot}12H_2O$ (0.2%, *w*/*v*)-containing medium (data not shown).

Previously, we observed stimulation of the BC production by addition of some secondary substrates to the medium [8, 15]. Therefore, various compounds were tested for their effects on BC production by *Acetobacter* sp. V6. Several organic acids instead of ethanol in the medium were provided in the medium containing molasses and CSL. As a result, acetic acid, citric acid, lactic acid, pyruvic acid, and malic acid showed better BC production as compared to ethanol. Among them, acetic acid had the greatest effect (data not shown). Figure 3 shows that the high BC production was observed in the medium containing 0.1–0.2% (*v*/*v*) acetic acid. Further increases with acetic acid up to 1% (*v*/*v*) decreased BC production. Cell growth was also decreased with increasing concentrations of acetic acid (data not shown). BC yields at 0.1% (*v*/*v*) and 0.2% (*v*/*v*) acetic acid were 3.08± 0.03 g/l and 2.76±0.02 g/l, respectively. Thus, the 0.2% (*v*/*v*) was the concentration selected for following experiments.

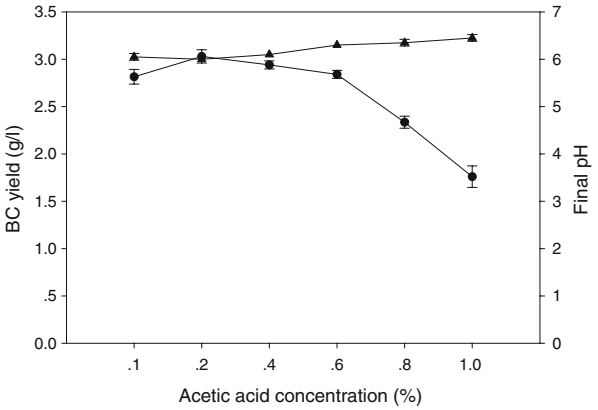

Fig. 3 Effect of acetic acid concentration on cellulose production by *Acetobacter* sp. V6. *filled circle* BC yield, *filled upright triangle* final pH

As a result of these studies, a cost-effective improved medium was developed. The medium contained 5% (*w*/*v*) total sugar in molasses, 4% (*v*/*v*) CSL, 0.2% (*w*/*v*) $Na_2HPO_4{\cdot}12H_2O$, 0.2% (*w*/*v*) NaH_2PO_4, and 0.2% (*v*/*v*) acetic acid with an initial pH 6.5. Figure 4 shows the comparison of time courses of growth and BC production of *Acetobacter* sp. V6 in the improved molasses and complex media. When *Acetobacter* sp. V6 was grown in the complex medium, the pH value of the culture medium rapidly decreased from 6.5 to 2.8 after 2 days of incubation, and slightly decreased to pH 2.6 thereafter. Cell growth increased with increase of culture time, reaching a maximum at 4 days, and then remained constant during prolonged cultivation. BC production increased from 1.32±0.05 g/l at 5 days to 1.57±0.08 g/l at 7 days, and remained relatively constant thereafter. In contrast, the pH of the improved molasses medium decreased from 6.5 to about 5.3 in 4 days and then slightly increased to 5.8 by 8 days. Cell growth increased gradually as the culture time was increased up to 7 days. BC production also increased with increase of culture time, reaching a maximum at 8 days. BC production in the improved molasses medium was around 3.12±0.03 g/l, which was approximately twofold higher than the yield in the complex medium.

FT-IR Spectroscopy

The conformational characterization of the BC from the complex and molasses media was carried out by FT-IR spectroscopy. Figure 5 shows FT-IR spectra of the BC produced from the molasses and complex media that are very similar to each other. The band at around 3400 cm^{-1} corresponded to the O–H stretching frequencies of cellulose. Since BC contains a large number of highly polar hydroxyl groups, the molecular chains interact by intramolecular and intermolecular hydrogen bonds [26]. Thus, the band at 3400 cm^{-1} is attributed to the intramolecular hydrogen bond for 3O…H-O5 [27]. It is particularly useful for elucidating hydrogen-bonding patterns because, in favorable cases, each distinct hydroxyl group gives a single stretching band at a frequency that decreases with increasing strength of hydrogen bonding [28]. In this study, it became the broader in BC from the molasses medium, indicating there are more hydrogen-bonding patterns in BC from the molasses medium. On the other hand, it is reported that in BC (S-BC) from static culture, there is significant peak at 3,240 cm^{-1}, which is attributed to the intermolecular hydrogen

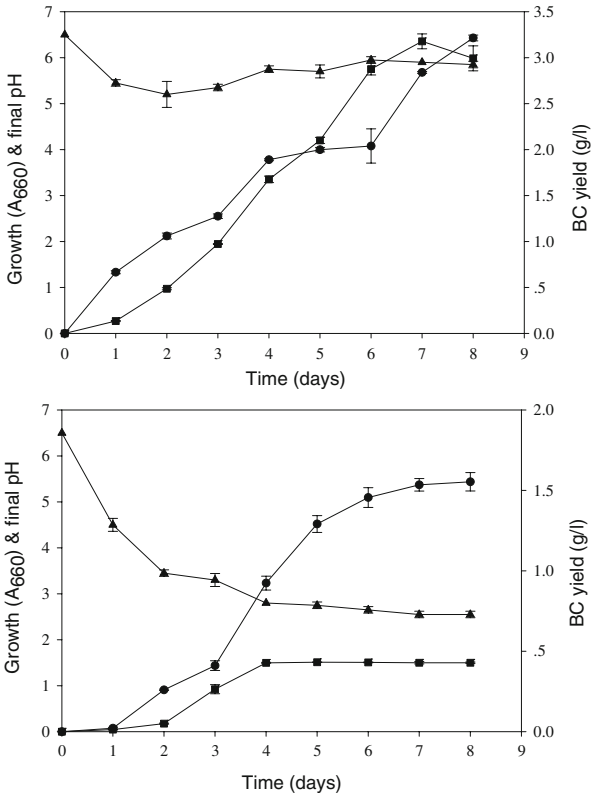

Fig. 4 Time courses of cellulose production by *Acetobacter* sp. V6 in complex (*lower part*) and improved molasses (*upper part*) media. *filled circle* BC yield, *filled upright triangle* final pH, *filled square* cell growth

bond, corresponding to the contributions from cellulose Iα [29]. In this study, however, there was no absorbance peak near 3,240 cm^{-1} in BC (SH-BC) from shaking cultures. Therefore, it is guessed that BC from shaking culture in this study has less cellulose Iα content. It implies that the shaking stress interferes with the formation of intramolecular and intermolecular hydrogen bonds in sub-elementary cellulose fibrils and disturbs their aggregation and crystallization. All the samples elicited signals at the area around 2,900 cm^{-1} corresponding to the absorption spectrum of the C–H bond of typical cellulose type I. The band at 1,370 cm^{-1} is assigned to CH_2 bending vibration [30]. The band at 1,060 cm^{-1} is attributed to the C–O–C stretching vibration [31]. The intensity at 1,060 cm^{-1} in BC from the complex medium was higher relative to that in the BC from the molasses medium. It suggests that there are stronger intramolecular hydrogen bonds for 3O…H-O5 in BC from the complex medium, which is consistent with the FT-IR spectrum in the O–H stretching vibration region.

X-ray Diffractometry

In order to compare the microstructural changes in BC from the different media and especially to estimate if the molasses causes any disturbance in the crystallization process,

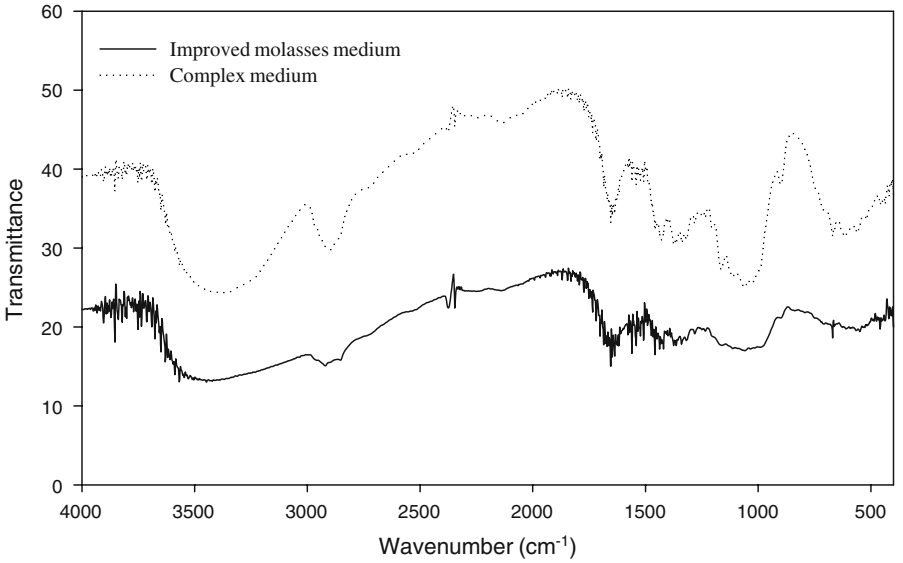

Fig. 5 FT-IR spectra of celluloses produced in complex and improved molasses media

X-ray diffraction was used. Figure 6 shows the X-ray diffraction patterns of BC produced in the molasses and complex media. In the diffraction diagram of BC from the complex medium, the peaks of the (101), ($10\bar{1}$), and (002) planes particular to cellulose I occurred at 2θ: 14.6°, 16.4°, and 22.6°. The diffraction angles 2θ of these peaks for the BC synthesized in the molasses medium were the same as those in the complex medium. The peak ($10\bar{1}$), although coincident in terms of position, was less distinguishable. It indicates that these BC are the typical crystalline forms of cellulose I [32]. The relative degrees of crystallinity of

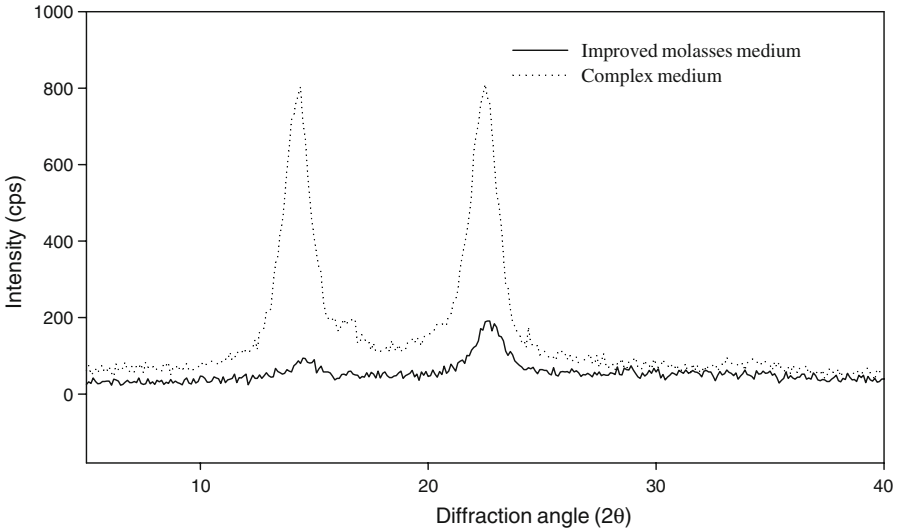

Fig. 6 X-ray diffractograms of celluloses in complex and improved molasses media

BC produced in the complex and molasses media were 83.02 and 67.27%, respectively (data not shown). That is, the usage of molasses instead of glucose has decreased the crystallinity index values of BC, but the same peaks with no change in positions indicated the preservation of the same planar spacings in the crystalline regions. Thus, it was confirmed that the medium compositions gave some influence on the crystalline phase of the BC. These results agreed with those of Keshk and Sameshima who found that use of molasses resulted in a small decrease in crystallinity index of BC produced by *A. xylinum* strains [33]. On the other hand, Watanabe et al. [34] and Iwata et al. [35] reported that BC produced in agitated culture had lower crystallinity index than that in static culture. It is thought that as it greatly affected by shearing force during shaking culture, hydrogen bonding between fibrils is reduced so that the degree of polymerization and the degree of crystallinity are both reduced [36].

Conclusion

BC is one of the most promising biological-based materials with broad prospective applications. However, the high economic costs of carbon and nitrogen sources become a drawback for BC's industrial production and extended applications. The present study investigated approach to prepare a low-cost medium using molasses and CSL for BC production. Molasses as the sole cheap carbon source was found to support substantial production of the BC by *Acetobacter* sp. V6 in shaking culture. CSL was also a suitable substitute for yeast extract and polypeptone. These findings are valuable toward the development of a cost-effective fermentation method for the production of BC. In order to establish the usefulness of these substrates on growth and BC production, further studies are being made in a 5-l fermentor.

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