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탄소원에 따른 Bacterial Cellulose의 물성

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Properties of Bacterial Cellulose Cultured in Different Carbon Sources

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초록: Bacterial cellulose는 초산균인 *Gluconacetobacter xylinus*에 의해 생산되며, 배양 배지의 표면에 나노섬유상의 막을 형성한다. 본 연구에서는 배지의 조성에서 탄소원을 달리하여 생산한 bacterial cellulose의 결정화도, 점도, 모폴로지와 역학적 물성을 살펴보았다. *Gluconacetobacter* sp. V6 균은 세 종류의 배양 배지에서 정치 상태로 배양되었다. 배양 배지로는 표준 Hestrin-Schramm 배지와 탄소원으로 glycerol 또는 molasses를 첨가한 개질 배지가 각각 사용되었다. 세포 성장과 셀룰로오스 수율은 molasses 배지와 glycerol 배지에서 증가하였다. Glycerol 배지를 사용한 배양은 결정화도와 고유점도, 파단응력과 같은 셀룰로오스의 물성을 향상시켰으나, molasses 배지를 사용한 배양은 셀룰로오스의 결정화도, 미결정의 크기, 고유점도를 감소시켰다. 요약하면, molasses 배지에서 셀룰로오스의 수율은 현저히 향상되었으나, 낮은 구조적 물성을 가졌다.

Abstract: Bacterial cellulose is produced by the bacterium *Gluconacetobacter xylinus*, which forms a nanofibrous pellicle in its culture medium. We studied properties of the bacterial cellulose such as crystallinity, viscosity, morphology, and mechanical properties according to the carbon source. Static cultures of *Gluconacetobacter* sp. V6 were performed in three kinds of media: standard Hestrin-Schramm medium, and modified medium with either glycerol or molasses as carbon sources. Cell growth and cellulose yield were increased in the glycerol and molasses media. The culture in the glycerol medium improved the physical properties of cellulose such as crystallinity, intrinsic viscosity, and breaking stress. However, the culture in the molasses medium decreased crystallinity, crystallite size, and intrinsic viscosity of cellulose. In summary, the cellulose yield was remarkably improved in the molasses medium, but with inferior structural properties.

Keywords: bacterial cellulose, structural property, nanofibrous pellicle, molasses medium, carbon source.

Introduction

Cellulose is the most abundant polysaccharide on earth, being produced by a wide variety of organisms, including vascular plants, marine algae, and prokaryotic organisms.[1-8]

The microorganism *Gluconacetobacter xylinus* produces microbial cellulose, which has a higher purity than plant cellulose, as it does not contain other components such as hemicellulose, lignin, pectin, etc.[9,10] Bacterial cellulose has a unique microstructure, which consists of an aggregated ribbon-like microfibril bundle consisting of nanofibers with high molecular weight.[3-5] It has good Young's modulus, large surface area, and high water-holding capacity and porosity, which makes it useful in fields requiring precise control, such as high-frequency tympanum and artificial skin.[3,4,11]

Studies have been done on various culture media compositions of a static *Gluconacetobacter* culture as a carbon source, with a view to increasing cellulose production. These

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media compositions contained additives such as fructose, galactose, glucose, glycerol, inositol, mannitol, sucrose, etc.[4,12] Cellulose yield is influenced by carbon sources, and the highest cellulose yield has been obtained in the case of glycerol.[13] In addition, different culture media compositions also influenced the structure of the cellulose produced in the media. Tokoh *et al.* reported that acetyl glucomannan led to characteristic variations in cellulose's microstructure and crystalline phase.[14]

Molasses is runoff syrup, a byproduct of the final crystallization stage in the industrial process that makes sugar from sugar cane. About 50% (w/w) of molasses is total sugar consisting of sucrose, glucose, fructose, raffinose, etc.[15,16] Molasses is also an economical carbon source for producing bacterial cellulose. Our previous studies have shown that cell growth and medium composition can be optimized using molasses as a carbon source in bacterial cellulose production.[17] In this study, we investigated in more detail the cellulose yield and structural properties of the bacterial cellulose produced by *Gluconacetobacter* sp. V6 in a glucose medium, glycerol medium, and molasses medium as carbon sources.

Experimental

Bacterial Cultures. *Gluconacetobacter* sp. V6 was used for production of bacterial cellulose. A pre-culture was grown in Hestrin-Schramm (HS) medium for 3 days at 30 ℃ as the basal medium. For the main culture, three kinds of media were used: HS medium (referred to hereafter as "glucose medium") and a modified medium with either glycerol (referred to hereafter as "glycerol medium") or molasses (referred to hereafter as "molasses medium") as carbon sources. A pre-cultured fluid of 5% was inoculated into each of the three media, and was cultured at 30 ℃ for 8 days. The molasses was pretreated by the following procedure.[17] The pH of the molasses solution was adjusted to 7.0 by the addition of 0.1 N NaOH and treated with 1% (w/v) $Ca_3(PO_4)_2$, followed by heating at 100 ℃ for 15 min. Finally, the mixture was cooled and centrifuged at 17479×g for 15 min. Bacterial cellulose was obtained as a pellicle formed on the medium's surface. The optimal media compositions are shown in Table 1.

Purifications. To remove the bacterial cells, cellulose pellicles were soaked in 0.5 N NaOH solution at 90 ℃ for 1 h, washed with distilled water several times, and dried at 105 ℃ for 12 h.

In order to observe the nanofibrous morphologies, the cellulose pellicles were soaked in a 0.1 N NaOH solution at 90 ℃ for 20 min and washed with distilled water. These pellicles were soaked in 1% acetic acid solution to neutralize, washed with distilled water several times, and subsequently soaked in ethanol and then butyl alcohol in order to minimize any changes in dimension. The cellulose pellicles were freeze-dried at −120 ℃ for 24 h.

Analyses. Cell growth was evaluated by measuring the absorbance of the homogenized fermentation broth at 660 nm using a spectrophotometer (Ultraspec 3000, Pharmacia Biotech, Sweden).[17]

Cellulose pellicle specimens were coated with platinum using an ion sputter. Surface morphologies of the cellulose pellicles were examined using a field emission-scanning electron microscope (FE-SEM; HITACHI-S4700, HITACHI corp., Japan).

X-ray diffraction spectra were recorded (XRD; DMAX 2000 V vertical diffractometer, Rigaku Corp., Japan) with reflection method using monochromatic CuKα radiation at 40 kV, 30 mA, and a scan speed of 10°/min.

Fourier transformation infrared spectroscopy (FTIR) was measured in a wavelength range of 400 to 4000 cm^{-1} (FTIR; IRAffinity-1, SHIMADZU Corp., Japan). All cellulose specimens were treated by the KBr pellet method. The mass fraction of cellulose I crystalline phase was obtained by the equations $f_\alpha = 2.55\ f_\alpha^{IR} - 0.32$, $f_\alpha^{IR} = A_\alpha/(A_\alpha + A_\beta)$.[18,19] A_α and A_β showed FTIR absorbances at 750 cm^{-1} and 710 cm^{-1}, respectively.

The density of cellulose was measured by floating position using a density gradient column of the mixed solution of CCl_4 and ethanol at 23 ℃.

Table 1. Compositions of Culture Media as Carbon Sources

Medium name	Inoculation/ Rate	Carbon[a] sources	Nitrogen[a] sources	Inorganic salts[a]	Additional carbon sources[a]	Optimum pH
Glucose	5%	glucose 2%	yeast extract 0.5% polypepton 0.5%	$Na_2HPO_4 \cdot 12H_2O$ 0.675%	citric acid 0.115%	6
Glycerol	5%	glucose 0.5% glycerol 2.5%	yeast extract 1.6%	$Na_2HPO_4 \cdot 12H_2O$ 0.4%	succinic acid 0.3%	6
Molasses	5%	molasses 5%	CSL 4%	$Na_2HPO_4 \cdot 12H_2O$ 0.2% Na_2HPO_4 0.2%	acetic acid 0.2%	6

[a]The composition of each medium was optimized in advance.

The intrinsic viscosity of cellulose was measured by flowing time of a 0.5 g/L cellulose solution of *N,N*-dimethyl-acetamide with 9% LiCl using Ostwald viscometer at 30 ℃.[20]

Mechanical properties were measured by an universal testing machine (UTM; SSTM-1, United corp., USA) at load cell of 5 kg, tensile speed of 5 mm/min, and specimen size of 10 mm×50 mm.

Results

Cell Growth and Cellulose Yield. Figure 1 shows the cell growth of *Gluconacetobacter* sp. V6, and the cellulose yield after 8 days using the glucose medium, glycerol medium, and molasses medium as carbon sources. Cell growth and cellulose yield were increased in the glycerol and molasses media more than in the glucose medium. The cellulose yield in the molasses medium was especially increased, about 3 times more than in the glucose medium.

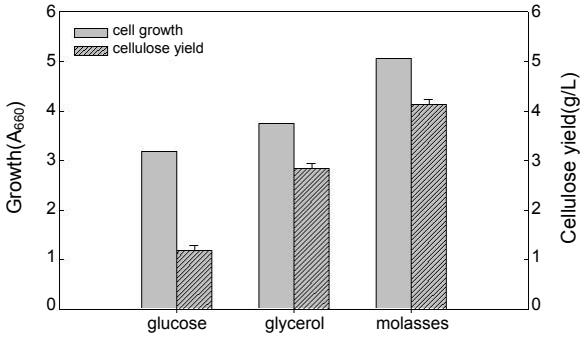

Figure 1. Cell growth and cellulose yields by carbon source in cultures.

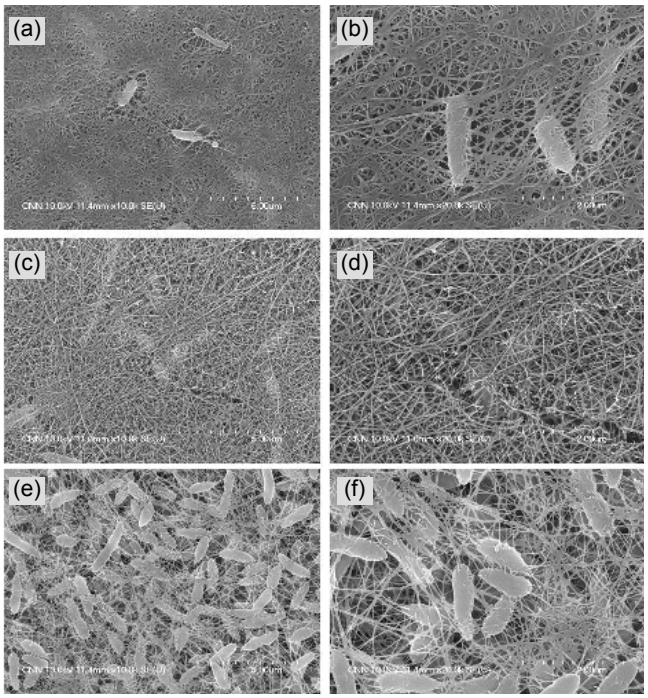

Figure 2. Surface morphologies of bacterial cellulose without purification; carbon sources: (a), (b) glucose, (c), (d) glycerol, (e), (f) molasses; (b), (d), (f) magnified figures of (a), (c), (e).

Morphology. The morphologies of the cellulose nanofiber and bacterial cell in the cellulose pellicle were observed by FE-SEM. Figure 2 shows the surface morphologies of the treated cellulose pellicle without purification. The morphologies of each bacterial cell were observed for all specimens. The apparent number of cells in the media appeared in increasing order of glucose < glycerol < molasses. The bacterial cells in the glycerol medium were buried in the abundantly-produced cellulose fibers. The average thickness of nanofibers produced in the glucose medium was around 50 nm. The cellulose nanofibers produced in the molasses medium were particularly thin and irregular. Surface morphologies of the cellulose pellicles are shown in Figure 3 after bacterial cells were removed by purification. The cellulose fibers of the pellicle formed in the glucose medium were damaged and partially dissolved, although the purification

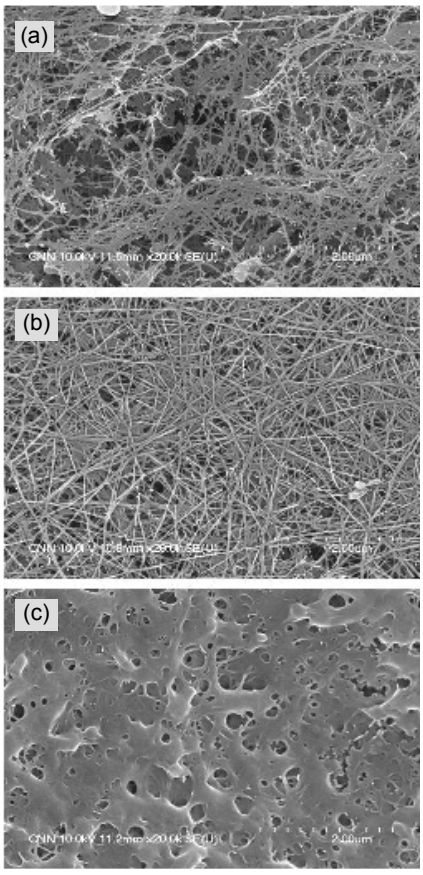

Figure 3. Surface morphologies of bacterial cellulose pellicles after purification, carbon sources: (a) glucose; (b) glycerol; (c) molasses.

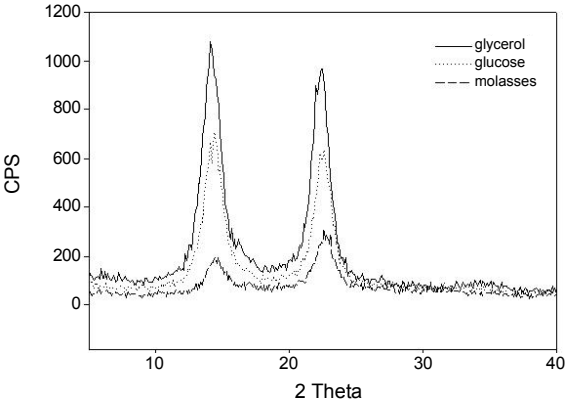

Figure 4. X-ray diffraction profiles of bacterial cellulose by carbon source.

Table 2. Crystallinity, Crystallite Size, and Mass Fraction of Cellulose I in the Crystalline Phase of Bacterial Cellulose

Carbon sources	Crystallinity[a]	Crystallite size (nm)[b]	Mass fraction of cellulose I^c	
			I_α(%)	I_β(%)
Glucose	medium	4.90	89	11
Glycerol	high	4.65	88	12
Molasses	low	3.86	87	13

[a]Estimated by relative value. [b]Determined by X-ray diffraction at crystallographic plane (101). [c]Determined by absorbance ratio of 750 cm^{-1} and 710 cm^{-1} in FTIR spectra.

procedure used for it was the same as for those in the other media. The fibrous morphology of the pellicle formed in the molasses medium disappeared by dissolution, but the cellulose nanofiber formed in the glycerol medium was well-preserved.

Crystallinity. The crystalline structures of the cellulose specimens were analyzed by X-ray diffraction. All specimens exhibited crystal structure of cellulose I appearing at (101) diffraction of 6.02 Å d-spacing and at (002) diffraction of 3.85 Å d-spacing.[21,22] The X-ray diffraction profile of each carbon source are shown in Figure 4. The diffraction intensity of the cellulose produced in the molasses medium was lowest, and that of the cellulose produced in the glycerol medium was highest. The crystallite size calculated at full-width of half-maximum at (101) diffraction using the Scherrer equation[3] is shown in Table 2. The crystallite size of the cellulose pellicle produced in the molasses medium was about 21% lower than that in the glucose medium. In addition, the mass fractions of cellulose I_α produced in the glycerol and molasses media were lower than that produced in the glucose medium.

Viscosity. The intrinsic viscosity of cellulose as measured in dilute cellulose solution is shown in Figure 5. The intrinsic viscosity of cellulose produced in the glycerol medium was slightly higher than that in the glucose medium, and the intrinsic viscosity of cellulose produced in the molasses medium was

Figure 5. Intrinsic viscosities of bacterial cellulose by carbon source.

Table 3. Mechanical Properties of Bacterial Cellulose Pellicles

Carbon sources	Stress at break (MPa)	Elongation (%)	Elastic modulus (MPa)
Glucose	116.21	3.792	3064.58
Glycerol	130.53	5.034	2592.90
Molasses	10.40	3.685	282.09
Filter paper	11.18	1.971	567.20
Foil[a]	65.70	5.738	1145.08

[a]Aluminum foil with 16 μm thickness.

lower than that in the other two carbon sources. The fact that the intrinsic viscosity of a polymer is proportional to its average molecular weight implies that the cellulose produced in the molasses medium has a low average molecular weight.

Mechanical Property. Table 3 shows the mechanical properties of cellulose pellicles measured by UTM. Filter papers and aluminum foils were used for comparison with cellulose pellicle. The cellulose pellicle produced in the glycerol medium had the highest breaking stress over the other carbon sources. The cellulose pellicle produced in the molasses medium had remarkably low breaking stress and an elastic modulus comparable to those from the other carbon sources, and also similar to that of filter paper.

Discussion

The cell growth and cellulose yield of the cultures of *Gluconacetobacter* sp. V6 in glycerol and molasses media were higher than those in the glucose medium, as shown in Figure 1. The bacterial cells were buried by abundantly-produced cellulose, as shown in Figure 2(c) and (d). Improvements in cellulose yield in the glycerol medium have been reported in several studies.[12,13] Cell growth was especially promoted in the molasses medium due to its rich supply of nutrients, which increased the cellulose yield. This is confirmed by the great proliferation of bacterial cells in it, as

shown in Figure 2(e) and (f). Accordingly, since the glycerol and molasses media had increased cell growth and cellulose yield, these media are recommended over HS medium for a productive feed.

As shown in Figure 4, the crystal structures of bacterial cellulose exhibited a cellulose I structure in all carbon sources. The bacterial cellulose produced in glycerol medium had high crystallinity, as shown in Table 2. This is considered to be due to the regularity of the cellulose chain being increased by a high-regularity component supply, and also by a delay of transforming time in cellulose production process due to a slight lag from the pentose cycle into the Krebs cycle.[12] However, the cellulose produced in the molasses medium had relatively low average molecular weight and very low crystallinity, and decreased crystallite size, and decreased I_α mass fraction. It is thought that various saccharide components of molasses may further increase the transforming time into glucose, cause chain irregularities in the cellulose production process, and disturb the cellulose fibril aggregation on the outer wall of the cell in the medium in the cellulose production process of *Gluconacetobacter*. A similar phenomenon has been reported in an acetyl glucomannan medium.[14] It is also confirmed by the difference in dissolution degree in the morphologies among nanofibers given the same purification, as shown in Figure 3. These factors made the cellulose produced in the molasses medium readily dissolve and have low breaking stress.

Conclusions

In this study, we investigated the cellulose yields of bacterial cellulose produced in media with glucose, glycerol, and molasses. Molasses as a carbon source increased the cell growth of *Gluconacetobacter* sp. V6, and considerably increased the cellulose yield. The different carbon sources caused differences in cellulose microstructure, such as crystallinity, crystallite size, cellulose I_α/I_β mass fraction, and in the mechanical properties of the cellulose pellicle. Especially by culture in the molasses medium, cellulose yield was remarkably improved, but its structural properties were deteriorated.

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