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Production of bacterial cellulose by a static cultivation using the waste from beer culture broth

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Abstract−Bacterial cellulose (BC) was produced by using the waste from beer culture fermentation instead of a chemically defined medium. Static cultivation was superior to the shaking cultivation on the basis of the BC production. The amount of BC produced during 120 hrs of cultivation using the waste from beer fermentation broth (WBFB) by a static cultivation was 4.52 g/L on the dry weight basis and much higher than 0.45 g/L produced from a Buffered Schramm and Hestrin (BSH) chemically-defined medium. The addition of 1% industrial-grade glucose to WBCB increased the production of bacterial cellulose from 8.46 to 13.95 g/L after 336 hrs of cultivation. Water soluble oligosaccharide (WSOS), the by-product obtained during BC cultivation increased to 5.05 g/L at 192 hrs of cultivation and then decreased to 2.18 g/L at 336 hrs.

Key words: Bacterial Cellulose, *Gluconacetobacter hansenii* PJK, Waste from Beer Fermentation Broth, Static Cultivation, Water Soluble Oligosaccharide

INTRODUCTION

Bacterial cellulose (BC) shows unique physical properties and, thus, can be used in new functional materials as well as in diet foods [1-3]. The diameter of BC is only a thousandth that of plant cellulose, which results in a high water retention value, tensile strength, and moldability [4-6]. These unique properties of BC, distinct from those of plant celluloses, have contributed to the development of new materials for high performance speaker diaphragms, tourniquets, diet foods, artificial skin, medical pads, make-up pads, and paint thickeners. BC can also be used as a material in age-resisting cosmetics [2,4-8]. Given all these applications, BC has a high potential for commercialization.

BC has been produced traditionally by a static culture that has a low productivity, because shear stress in the shaking culture converts microbial strains into non-cellulose-producing (*Cel*$^-$) mutants during cultivation, resulting in a decrease of BC production yield [9]. In previous reports [10,11], *Gluconacetobacter hansenii* was isolated from rotten apples. It was possible to preserve the cellular activity of cellulose production without the spontaneous occurrence of *Cel*$^-$ mutants in consecutively shaken-cultures using a medium containing ethanol. In another previous study, an optimum culture condition for maximizing BC production in an agitated culture of a medium containing ethanol without *Cel*$^-$ mutants was found [12]; the productivity of BC was increased slightly by a perfusion system using a spin filter [13].

However, the production of BC from a chemically defined medium is an expensive process, thus hindering its application for commercial purposes. Therefore, it is desirable to investigate the feasibility of using an alternate, cheaper culture media for the production of BC. Waste from beer fermentation broth (WBFB) contains more carbon and nitrogen than a chemically defined medium, a small amount of sulfur and it has more than 4% ethanol [14]. WBFB seems to constitute an attractive feedstock for the production of BC because it has been certified to be an effective nutritional source for the production of water-soluble oligosaccharide (WSOS) [15,16]. The present study was designed to evaluate WBFB with regard to the production of BC by *G. hansenii* PJK.

EXPERIMENTAL

1. Preparation of WBFB

WBFB was supplied by the Hite Co. which is located in the southern part of Korea. WBFB was taken and its pH was adjusted to 5 with 2 M NaOH solution. WBFB was centrifuged at 3,580 g for 20 min in order to remove the yeast cells and other particles [17]. The supernatant obtained was termed as "untreated WBFB." The elemental composition of the untreated WBFB was determined by direct analysis of the broth (liquid state) by using a Fisons EA1108 elemental analyzer. The direct elemental analysis of untreated WBFB in liquid state revealed that it contained 6.49% (w/w) (68.11 g l^{-1}) carbon, 1.64% (w/w) (17.19 g l^{-1}) nitrogen and 11.06% (w/w) (116.14 g l^{-1}) hydrogen. It contained 29.44 g l^{-1} total carbohydrate and 0.67 g l^{-1} total protein content [16]. WBFB was autolyzed or hydrolyzed in order to break the yeast cell walls and increase the level of nitrogen nutrient in the supernatant of WBFB. WBFB was kept in dry oven at 50 °C for 48 hrs after its pH was adjusted to 5 for autolysis [18]. The supernatant obtained by centrifuging at 3,580 g for 20 min was termed as "autolyzed WBFB." WBFB was autoclaved for 15 minutes at 121 °C after adjusting its pH to 2 for hydrolysis [19]. The broth was centrifuged for 20 min after the pH of

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the broth was adjusted to 2. The supernatant was termed as "hydrolyzed WBFB."

Commercial grade glucose was added to untreated WBFB at a concentration of 10 g/L. Untreated WBFB was autoclaved at 121 °C, and the pH was adjusted to 5. After cooling to room temperature, untreated WBFB was inoculated with pre-cultured cells at 5% of the working volume. The sample was then divided into two equal samples. The first untreated WBFB sample was transferred into a shaking incubator and cultured at 30 °C and 200 rpm. The other sample of untreated WBFB was cultured at 30 °C under static conditions. The samples were tested for the following parameters: pH, BC, total cells, and WSOS. Autolyzed WBFB and hydrolyzed WBFB were also supplemented with glucose and autoclaved in the same way.

2. Microorganism and Cell Culture

G. hansenii PJK (KCTC 10505BP), isolated from rotten apples and identified by using the 16S rDNA complete sequencing method [10], was grown on a basal medium containing glucose 10 g/L, yeast extract 10 g/L, peptone 7 g/L, acetic acid 1.5 mL/L, and succinate 0.2 g/L. The agar plates used for keeping strains were prepared by dissolving agar 15 g/L in the basal medium. The pH of the medium was adjusted to 5.0 with NaOH. Colonies of *G. hansenii* were inoculated into a 50 mL medium in a 250 mL flask shaken at 200 rpm and cultured at 30 °C for 24 hours. Five percent of the culture broth was inoculated into a 50 mL of untreated WBFB in 250 mL flask and cultured in either a static mode at 30 °C, or shaken at the rate of 200 rpm at 30 °C.

3. Analysis of Cells, BC and WSOS

BC was produced in a pellet form from the shaking culture and in a plate form in the static culture. In the shaking culture, BC was harvested by centrifuging the culture broth for 20 min at 3,580 g, and washed with distilled water. The process of washing with distilled water and centrifugation was repeated two more times. In the static culture, BC was harvested simply by removing the BC plate from the culture flask and washing it with distilled water more than two times. The BC dry weight, including the microbial cells, was measured after freeze-drying at −50 °C. The BC containing cells were treated with 20 mL of 0.3 M NaOH at 100 °C for 5 min in order to disrupt and dissolve the microbial cells; afterwards, the solution was filtered (pore size: 8 μm) with an aspirator to remove the dissolved materials. The filter cake was rinsed repeatedly with distilled water until the pH of the filtrate became neutral. The BC plate obtained in a static culture was rinsed with distilled water without filtration after dissolving microbial cells with NaOH solution for 10 min. The BC dry weight, without any microbial cells, was measured after freeze-drying at −50 °C. The dry cell weight was taken to be the difference between the weights of the dried BC containing cells and the dried BC after treatment with NaOH.

After removing the BC pellets or the BC plate from the culture broth, 2 ml of supernatant obtained was taken and treated with five volumes (10 ml) of ethanol for 1 hr at 4 °C. The precipitates obtained were separated by centrifugation for 20 minutes. After the supernatant was discarded, these precipitates were dissolved in 2 ml of distilled water and again treated with 10 ml of ethanol at 4 °C for 1 hr. After this the precipitates were again separated by centrifugation. Finally, these precipitates were dried at 60 °C till a constant weight was achieved. The constant weight was considered as the dry weight of WSOS [20].

4. Measurement of CFU and Detection of Cel^- Mutants

The conversion of Cel^+ cells to Cel^- mutants was confirmed by measuring the colony-forming unit (CFU). The culture broth was diluted with saline solution, and then spread onto an agar plate containing the basal medium. The plate was then incubated at 30 °C until colonies formed on the agar plate, after which the cellulose producing (Cel^+) cells and non-cellulose producing (Cel^-) mutants were counted. Cel^- mutants were detected on the plates as smooth-type colonies, while Cel^+ cells formed mucous and rough-type colonies, as reported previously [11,21-23].

RESULTS AND DISCUSSION

1. BC Production in Static Culture

The cellulose film was produced on the surface of untreated WBFB without glucose supplement, and the BC harvested after 336 hrs of cultivation is shown in Fig. 1. As shown in Table 1, the amount of BC produced at 120 hrs of static cultivation is 4.52 g/L and reaches 8.46 g/L at 336 hrs. BC production at 120 hrs in a static cultivation is much higher than 0.6 g/L of BC which was obtained in a shaking cultivation using WBFB [14]. This can be explained by the conversion of Cel^+ cells into Cel^- mutants. An agitated culture converts Cel^+ cells into Cel^- mutants, which become more enriched than the Cel^+ cells because they grow faster, thereby causing a lower productivity of BC in a continuous culture [23,24]. The conversion ratio of Cel^+ cells into Cel^- mutants was less than 3% at 120 hrs of cultivation and 0% at 336 hrs although the cell density at 120 hrs was 0.75×10^6 CFU/mL and increased to 1.06×10^7 CFU/mL, as shown in Table 2. Thus, the static production of BC by using WBFB seems

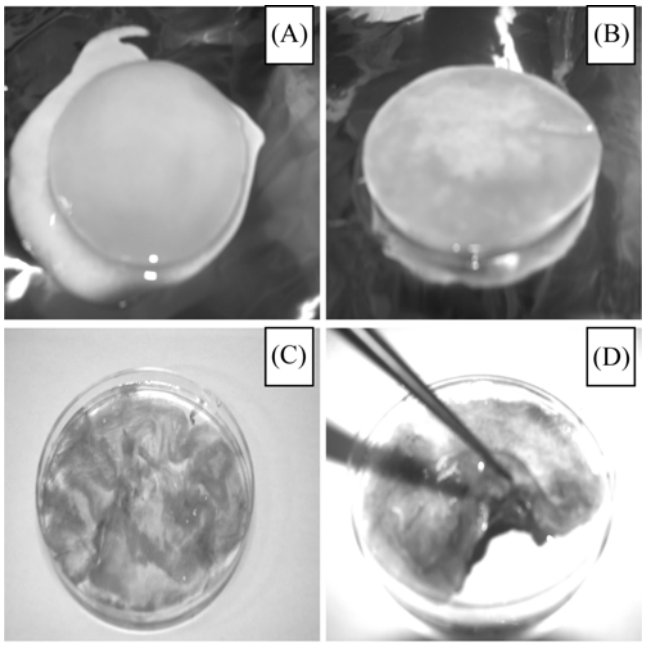

Fig. 1. A photograph of BC produced after 336 hrs of static culture using untreated WBFB (A) and untreated WBFB supplemented with 1% glucose (B). BC obtained from untreated WBFB after treatment with 0.3 N NaOH (C). BC obtained from untreated WBFB supplemented with 1% glucose after treatment with 0.3 N NaOH (D).

Table 1. The production of BC and WSOS using 50 mL of various WBFB in a 250 mL flask at 30 ºC on a static condition

Culture time (h)	Untreated WBFB		Untreated WBFB with 1% glucose		Autolyzed WBFB		Autolyzed WBFB with glucose		Hydrolyzed WBFB		Hydrolyzed WBFB with 1% glucose	
	BC dry wt (g/L)	WSOS dry wt (g/L)	BC dry wt (g/L)	WSOS dry wt (g/L)	BC dry wt (g/L)	WSOS dry wt (g/L)	BC dry wt (g/L)	WSOS dry wt (g/L)	BC dry wt (g/L)	WSOS dry wt (g/L)	BC dry wt (g/L)	WSOS dry wt (g/L)
48	0.67	2.2	1.14	3.3	0.08	5.65	0.06	6.5	0.06	4.63	0.07	4.25
120	4.52	4.8	4.97	4.38	0.15	5.65	0.41	6.75	1.23	4.98	1.25	4.53
192	6.2	5.05	10.51	5.13	0.4	6.8	1.71	7.65	1.31	5.08	1.79	4.5
264	7.05	3.58	12.34	7.15	1.13	5.73	4.38	6.8	1.66	5.1	2.69	4.83
336	8.46	2.18	13.95	5.85	2.00	5.4	7.37	6.4	2.82	5.18	3.64	4.85

Table 2. Conversion of *Cel*$^+$ cells to *Cel*$^-$ mutants in a static cultivation using 50 mL of various WBFB in a 250 mL flask at 30 ºC

Culture time (h)	Untreated WBFB		Untreated WBFB with 1% glucose		Autolyzed WBFB		Autolyzed WBFB with 1% glucose		Hydrolyzed WBFB		Hydrolyzed WBFB with 1% glucose	
	CFU/ mL	*Cel*$^-$ mutants /total cells	CFU/mL	*Cel*$^-$ mutants /total cells	CFU/mL	*Cel*$^-$ mutants /total cells	CFU/mL	*Cel*$^-$ mutants /total cells	CFU/mL	*Cel*$^-$ mutants /total cells	CFU/mL	*Cel*$^-$ mutants /total cells
48	5.5×10^4	0	3.3×10^4	0.028	ND	0	2.25×10^4	0	2.8×10^4	0	8.1×10^4	0
120	7.5×10^5	0.0291	4.42×10^5	0	6.76×10^4	0	1.102×10^5	0	1.225×10^5	0	2×10^5	0
192	1.804×10^7	0.0132	9.072×10^6	0.0108	9.72×10^5	0	2.306×10^6	0	2.242×10^6	0	2.69×10^6	0
264	7.228×10^6	0	2.603×10^7	0	1.466×10^6	0	5.525×10^6	0	8.45×10^5	0	9.432×10^6	0
336	1.057×10^7	0	4.41×10^7	0.05	6.375×10^6	0	1.775×10^7	0	5.375×10^5	0	4.196×10^6	0

to be profitable because of higher productivity rather than a shaking cultivation even though it requires a longer culture period and is more labor intensive.

2. Effect of Glucose Supplement on BC Production

In a previous study, the composition of WBFB was analyzed. More than 5% ethanol was found and glucose was not detectable [16]. Thus, for the improvement of BC production, 1% of glucose was added to WBFB. Yeast cells of WBFB were hydrolyzed and autolyzed in order to increase the amount of nitrogen source in untreated WBFB.

With the addition of 1% glucose to the untreated WBFB, BC production increased about 60% from 8.46 g/L to 13.95 g/L at 336 hrs of static cultivation as shown in Table 1. The cell density during cultivation could explain the increase in BC production. The addition of glucose as a carbon source increased the cell population from 1.06×10^7 CFU/mL to 4.41×10^7 CFU/mL as shown in Table 2, and this seems to result in an increase in BC production.

However, BC production decreased from 8.46 g/L to 2.00 g/L with autolyzed WBFB and decreased to 2.82 g/L with hydrolyzed WBFB. This also can be explained by the decrease in cell density during cultivation. The cell population decreased from 1.06×10^7 CFU/mL to 6.37×10^6 CFU/mL with autolyzed WBFB and decreased to 5.37×10^5 CFU/mL with hydrolyzed WBFB although there was no conversion of *Cel*$^+$ cells into *Cel*$^-$ mutants with autolyzed WBFB and hydrolyzed WBFB. It could be theorized that the nutrient in WBFB was destroyed by the harsh conditions of autolyzing and hydrolyzing treatments even though the source of nitrogen was expected to increase with these treatments.

The addition of 1% glucose to autolyzed WBFB increased the cell population from 6.37×10^6 CFU/mL to 1.78×10^7 CFU/mL without any conversion of *Cel*$^+$ cells into *Cel*$^-$ mutants and resulted in the increase in BC production from 2.00 g/L to 7.37 g/L. The addition of 1% glucose to hydrolyzed WBFB increased the cell population from 5.38×10^5 CFU/mL to 4.20×10^6 CFU/mL and resulted in the increase in BC production from 2.82 g/L to 3.64 g/L.

3. WSOS Production as Byproduct

WSOS is usually co-produced when BC is produced by *Gluconacetobacter hansenii* strains [10,13]. The amount of WSOS produced during culture monotonically increased with the culture time in agitation cultivation [16]. However, in a static cultivation with untreated WBFB, the amount of WSOS increased to 5.05 g/L at 192 hrs of culture and then decreased with culture time and reached 2.18 g/L at 336 hrs as shown in Table 1. The addition of 1% glucose to untreated WBFB increased the amount of WSOS produced during cultivation, and the maximum was found to be 7.15 g/L at 264 hrs. In an agitated cultivation using untreated WBFB, WSOS increased monotonically. The amount of WSOS produced in agitation cultivation with untreated WBFB supplemented with glucose was much higher than that of BC produced as shown in Fig. 2. However, the amounts of BC produced in static cultivation with untreated WBFB supplemented with glucose was much higher than the amount of WSOS produced as shown in Fig. 2. With these reproducible experimental results, it can be concluded that shear stress generated during cultivation converts *Cel*$^+$ cells into *Cel*$^-$ mutants, and thus results in the decrease in BC production and the increase in WSOS production. However, the mechanism for the conversion of WSOS to some other materials during static cultivation should be investigated in future research.

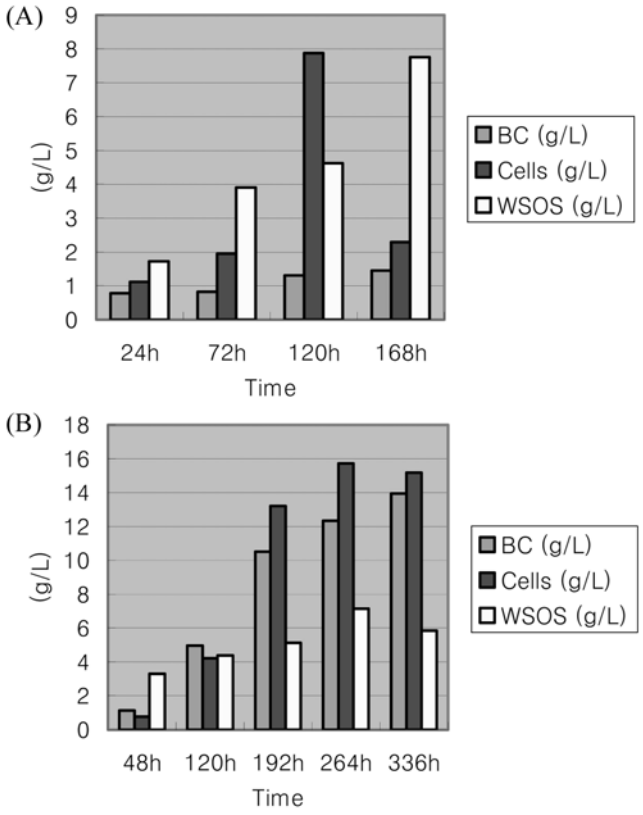

Fig. 2. Time course of BC and WSOS production using untreated WBFB supplemented with 1% glucose in a shaking cultivation (A) and in a static cultivation (B).

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