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Production of Bacterial Cellulose by *Gluconacetobacter hansenii* PJK Isolated from Rotten Apple

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Abstract A cellulose-producing strain isolated from rotten apples was identified as *Gluconacetobacter hansenii* based on its physiological properties and the 16S rDNA complete sequencing method, and specifically named *Gluconacetobacter hansenii* PJK. The amount of bacterial cellulose (BC) produced by *G. hansenii* PJK in a shaking incubator was 1.5 times higher than that produced in a static culture. The addition of ethanol to the medium during cultivation enhanced the productivity of bacterial cellulose, plus the supplementation of 1% ethanol into the culture medium made the produced BC aggregate into a big lump and thus protected the bacterial-cellulose-producing *G. hansenii* PJK cells in the shear stress field from being converted into non-cellulose-producing (*Cel*) mutants. Cells subcultured three times in a medium containing ethanol retained their ability to produce BC without any loss in the production yield.

Keywords: bacterial cellulose, 16S rDNA complete sequencing method, *Gluconacetobacter hansenii*, ethanol

INTRODUCTION

Cellulose is the most abundant polysaccharide in nature and the major component of higher plants. Industrial uses of cellulose include many products, such as paper-related items, diet foods, artificial skin, and so on. However, the increasing demand for cellulose and thus increased consumption of wood as the raw material of cellulose are causing deforestation and creating an environmental problem. Therefore, research on the development of substitutes for plant cellulose is very urgent. The traditional production of polysaccharides from plants and seaweed is inadequate to satisfy the required mass production of polysaccharides. As such, there has been a recent increased interest in the cellulose produced by microbial cells, BC. In particular, the production of BC by *Acetobacter* strains has become a very important subject in the area of new materials with high functions and for food-related industries, as BC includes unique properties that are distinct from those in plant cellulose [1-3].

The BC produced by *Acetobacter* strains is free of hemicellulose, pectin, lignin, and other biogenetic products associated with plant cellulose, as reported by Brown [4]. BC also requires less energy and fewer chemicals than a pulp processing, and when contrasted with plant cellulose, BC has a large specific surface area, higher water retention value, moldability, and high tensile strength [5], since the diameter of a BC fibril is generally 0.1 μm, which is 300 times smaller than that of wood fibrils. According to previous literature [6], the water retention value for BC is about 17 times higher than that for cotton linter. Thus, based on these remarkable physical properties, current industrial applications include using BC as the raw material for speaker diaphragms, tourniquets, and diet foods. BC is also used in medical pads, make-up pads, and artificial skin, as the smooth membrane of BC can be easily fused with human skin, plus it can retain a significant amount of water over a long period of time [3, 6]. With a large effective area and high durability, BC can immobilize many times more enzymes than any plant cellulose. Plus, the addition of 80% BC fibrils to a plant pulp makes the strength and elasticity coefficient of the intermixed paper 2.5 times higher than that of general paper [7,8].

As mentioned above, the eco-friendly BC produced by *Acetobacter* strains has a high potential for commercialization. However, BC is traditionally produced using a static culture, which requires a long culture period and is labor intensive, resulting in a low productivity. Meanwhile an agitated culture converts *Acetobacter* strains into *Cel*⁻ mutants, which become more enriched than the wild type because they grow faster, thereby causing a lower productivity of BC in a continuous culture [9]. Although many good cellulose-producing strains suitable for agitated cultures have been developed, the productivity of BC is not high enough to be industrialized. Accordingly, further research is required to isolate genetically stable cellulose-producing strains and identify the optimal conditions for fermentation that protect the microbes from being converted into *Cel*⁻ mutants.

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Table 1. Physiological properties of microbial strain isolated from rotten apples

	Isolated strain	*A. xylinum* IFO 15237*	*A. hansenii* ATCC 35959*	*A. liquefaciens* ATCC 14835*	*A. pasteurianus* ATCC 33445*	*A. aceti* ATCC 15973*
Formation of acetic acid from ethanol	+	+	+	+	+	+
Oxidation of lactate	+	+	+	+	+	+
Oxidation of acetate	+	+	+	+	+	+

* Data from reference [9]

MATERIALS AND METHODS

Isolation and Identification of Cellulose-producing Bacteria

Cellulose-producing bacteria were isolated using the modified method described by Toyosaki *et al.* [10]. Samples from rotten apples were inoculated into a BSH medium (20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L Na_2HPO_4, 1.15 g/L citric acid·H_2O, pH 5.0) [11] containing 2 mL/L acetic acid, 5 mL/L ethanol, and 0.1 g/L cycloheximide and cultured statically at 30°C. The culture broth in which pellicles were formed was diluted with saline and then spread onto an agar plate containing the BSH medium. The plates were then incubated at 30°C until colonies formed. Thereafter, cellulose-producing single cells were isolated after repeating the static culture more than 5 times, as described above. Identifying the single cells was carried out using the 16S rDNA complete sequencing method. The base sequence alignment was performed according to the method suggested by Juke and Cantor [12] and the percent similarity and phylogenetic tree were analyzed following the procedure suggested by Saito and Nei [13].

Cell Culture

The BSH medium [11] was used the basic medium for all the cell cultures. The cells were kept on 15% agar plates containing the BSH medium, then the colonies were inoculated into 50 mL of the medium in a 250 - mL flask and cultured at 30°C for 24 h in a shaking incubator rotated at 200 rpm. The culture broth was then filtered with a sterilized mesh (38 μm) and 5% of the filtrate was inoculated into 50 mL of the medium in a 250 - mL flask for the next batch culture. To increase the stability of the cells in a shear stress field, ethanol was added to Son's medium [14], composed of 10 g/L glucose, 10 g/L yeast extract, 7 g/L peptone, 1.5 mL/L acetic acid, and 0.2 g/L succinate. To identify the isolated cells, their ability to oxidize ethanol, acetate, and lactate, like *Acetobacter* strains, was tested. The oxidation of ethanol was confirmed by observing the change in color of the Carr medium [15], composed of ethanol 20 mL/L, yeast extract 30 g/L, bromocresol green 0.022 g/L, and agar 20 g/L. A medium [16] composed of sodium acetate or sodium lactate 2 g/L, yeast extract 2 g/L, peptone 3 g/L, bromothymol blue 0.02 g/L, and agar 20 g/L was used to check the oxidation of lactate or acetate, as the color of the medium changes from yellow to green when lactate or acetate is oxidized.

Analysis of BC

The BC was harvested by centrifuging the culture broth for 20 min at 3,580 *g* and washing twice with distilled water. The dry weight of the BC, including the microbial cells, was measured after freeze-drying at -50°C. Next, the BC containing cells was treated with 20 mL of 0.3 N NaOH at 100°C for 5 min to disrupt and melt the microbial cells and thereafter, the solution was filtered using an aspirator to remove the dissolved materials. The filter cake was repeatedly rinsed with distilled water until the pH of the filterate became neutral. The dry weight of the BC without any microbial cells was measured after freeze-drying at –50°C. As such, the dry cell weight was considered to be the difference between the weights of the dried BC containing cells and the dried BC after treatment with NaOH. The glucose concentration in the medium was measured using a glucose reagent kit (Sigma no. 510-A). An X-ray diffractogram (XRD) of the BC was obtained using an X-Ray Diffractometer (X' Pert-APD, Philips) operated between 5 and 40° (2θ) to facilitate an indirect comparison of the structure of the BC with that of commercial plant cellulose and other known BC. A scanning electron micrograph (SEM) of the BC was also obtained using a Field Emission Scanning Electron Microscope (S-4200, Hitachi).

RESULTS AND DISCUSSION

Characteristics and Identification of Cells Isolated from Rotten Apples

In previous studies, *Acetobacter* strains, including *Acetobacter pasteurianus, Acetobacter xylinum, Acetobacter aceti, Acetobacter liquefaciens,* and *Acetobacter hansenii,* have been identified as capable of producing BC [10,11, 17] and able to oxidize ethanol, lactate and acetate [10, 15,16]. Therefore, to determine whether the strains isolated from the rotten apples belonged to the *Acetobacter* genus, their ability to oxidize ethanol, lactate, and acetate was tested. As such, the isolated strains were inoculated onto an agar medium, including bromocresol green and bromothymol blue, and the color changed from green to yellow and then returned to the initial green color as the culture time proceeded. As shown in Table 1, the strains

Table 2. Similarity analysis of strain isolated from rotten apples

Strain	% Similarity
Gluconacetobacter hansenii NCIMB 8746 (T)	99.27
Gluconacetobacter europaeus DSM 6160 (T)	98.40
Gluconacetobacter xylinus subsp. *sucrofermentans* BPR2001	98.33
Gluconacetobacter xylinus subsp. *xylinus* NCIMB 11664 (T)	98.25
Acetobacter intermedius DSM 11804 (T)	98.03
Acetobacter oboediens DSM 11826 (T)	98.03
Gluconacetobacter liquefaciens IFO 12388 (T)	96.72
Gluconacetobacter diazotrophicus ATCC 49037 (T)	96.58
Acetobacter aceti JCM 7641 (T)	94.61
Acetobacter pomorum DSM 11825 (T)	93.95
Acetobacter pasteurianus LMG 1262 (T)	93.80

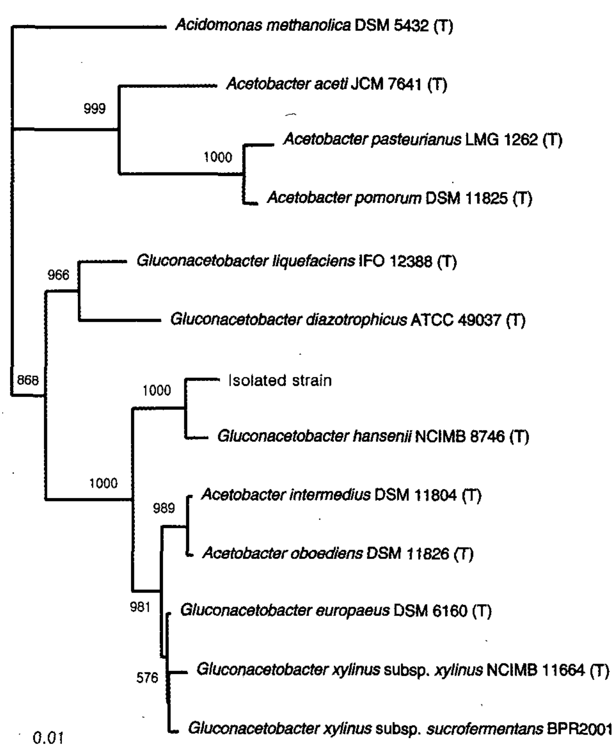

Fig. 1. Phylogenetic tree representing position of strain isolated from rotten apples.

Table 3. Effect of adding ethanol to medium at beginning of cell cultivation on cellulose production

	Production of BC (g/L)		
Number of subculture	1	2	3
With ethanol	2.27	1.66	1.74
Without ethanol	1.46	0	0

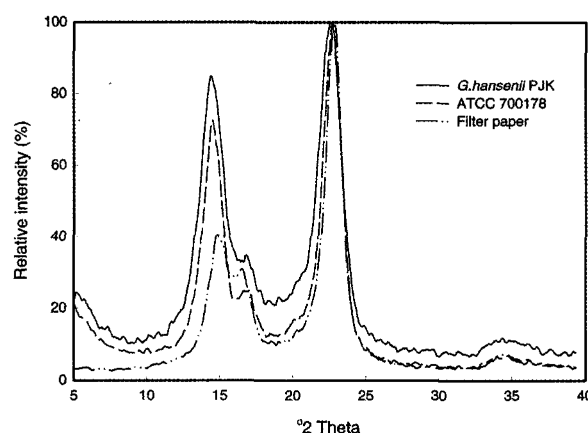

Fig. 2. X-ray diffractogram of BC produced by *G. hansenii* PJK.

were found to produce acetate from ethanol and then oxidize acetate and lactate like strains of the genus *Acetobacter*.

For a more accurate identification of the isolated strains, base sequences of 1377 bp were analyzed using the 16S rDNA complete sequencing method, their similarity examined, and a phylogenetic tree created. As shown in Table 2 and Fig. 1, the isolated strains exhibited a high similarity value to *Gluconacetobacter hansenii* and were identified as belonging to *G. hansenii* based on a 1000 bootstrap value [18]. Therefore, the strain isolated from the rotten apples was identified as *G. hansenii* and named *G. hansenii* PJK.

BC Production in Static Culture

The cellulosic film produced by *Acetobacter xylinum* in a static culture is located on the air/liquid surface, and its thickness increases with the culture time [19]. Similarly, the film formed by *G. hansenii* PJK on the surface of the culture broth also became thicker with an increased culture time. An X-ray diffractogram of the BC film produced by *G. hansenii* PJK was compared with that of the cellulose produced by *A. xylinus* subsp. *sucrofermentans* ATCC 700178. As shown in Fig. 2, the X-ray diffraction profile was typical of BC [10]. The X-ray diffraction profile of the cellulose produced by *G. hansenii* PJK was very similar to that of filter paper (Adventec Toyo) made of

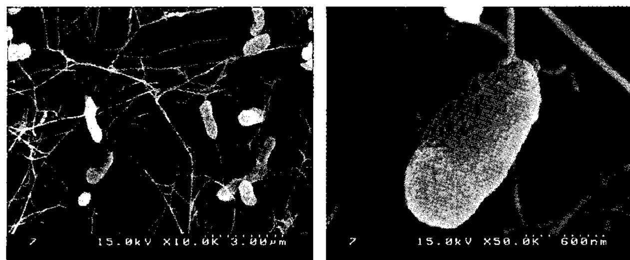

Fig. 3. Scanning electron micrograph of BC produced by *G. hansenii* PJK. The white elliptical mass is a microbe embedded in BC.

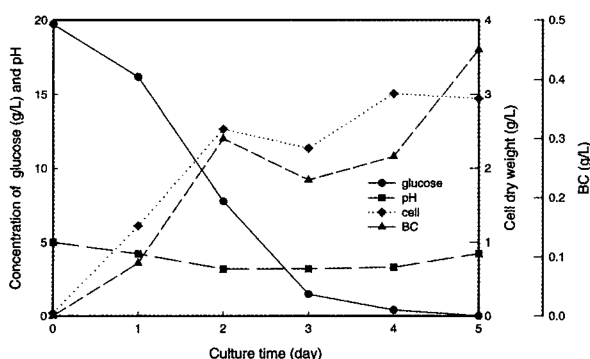

Fig. 4. Time course of BC production by *G. hansenii* PJK in flask culture. The cells were cultivated at 30℃ under static conditions.

highly-purified cellulose.

The morphology was also essentially the same as that of the BC produced by cellulose-producing *Acetobacter* strains in a static culture [2,20,21], as shown in the scanning electron micrograph of the BC produced by *G. hansenii* PJK (Fig. 3.). Therefore, the above-mentioned X-ray diffraction profile and SEM confirmed that the BC produced by *G. hansenii* PJK was pure cellulose and free of any other impurities.

A BSH medium [11] is generally used for the production of BC. The cell growth rate and BC production rate of *G. hansenii* PJK in a static culture were measured, as presented in Fig. 4. The stationary phase appeared after 2 days of cultivation and the cell dry weight reached a maximum value of 3 g/L. The amount of BC produced increased in accordance with the cell growth, as for a growth-associated production type, and reached 0.35 g/L after 4 days of cultivation. The glucose concentration in the medium steeply decreased for 3 days of cultivation, then the microbial cell dry weight became constant after 4 days when almost all the glucose had been consumed, although BC still produced after the fourth day of cultivation. This result was very different from the time course of BC production by *A. xylinus* subsp. *sucrofermentans* ATCC 700178, where the dry weight of the produced BC reaches the maximum value (0.4 g/L) on the second day of cultivation and then becomes constant with an increased cultivation time. Another remarkable difference in the pattern of BC production by *G. hansenii* PJK was that no BC was produced when using fructose as the carbon source, whereas *A. xylinus* subsp. *sucrofermentans* ATCC 700178 can produce BC based on consuming fructose [22]. Thus, more research is required to investigate the metabolic pathway of the BC production by the isolated *G. hansenii* PJK. The pH of the growth medium decreased from 5 relative to the culture time and then increased after the third day. A similar trend was reported in the literature and explained based on the path of glucose consumption where some glucose, as the carbon source, was converted into gluconate by membrane-bound glucose dehydrogenase from the microbial cells, thereby lowering the medium pH [7]. Thereafter, the sequential consumption of gluconate by the microbial cells increased the medium pH [14].

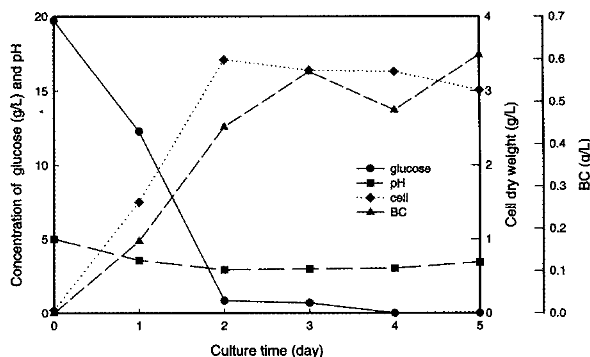

Fig. 5. Time course of BC production by *G. hansenii* PJK in flask culture. The cells were cultivated at 30°C in a shaking incubator rotated at 200 rpm.

BC Production in Shaking and Agitated Culture

A static cultivation method is inappropriate for the commercial production of BC, as static cultivation is labor intensive and requires a long culture period. Hence, an attempt was made to cultivate *G. hansenii* PJK in a shaken or agitated vessel. As shown in Fig. 5, in contrast to the static culture, the amount of BC produced in the shaking culture increased for 3 days of cultivation and reached 0.6 g/L. The dry weight of the cells produced during the 5 days of cultivation was nearly equal to that produced in the static culture, yet the dry weight of the resulting BC was 1.5 times higher than that produced in the static culture. This was due to the higher mass transfer rate of the nutrients in the shaking vessel, although the BC produced in the shaking vessel formed pellets, which then aggregated as the culture time increased. The glucose concentration in the medium decreased sharply during the first 2 days of cultivation and the pattern of pH change was similar to that in the static culture. It is generally known that the shear stress generated in a shaking cultivation causes *Acetobacter* strains to convert into *Cel*$^-$ mutants [23]. In the current study, *Cel*$^-$ mutants were also formed after 2 days of cultivation. The forma-

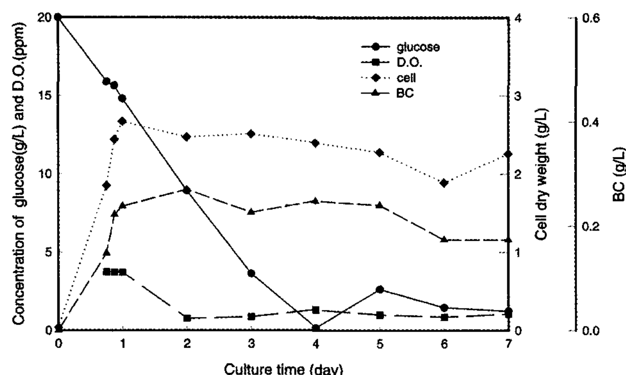

Fig. 6. Time course of BC production by *G. hansenii* PJK in 5L jar-fermentor with working volume of 3L. The cultivation was carried out at 30℃ with an initial pH of 5.0, aeration rate of 0.5 vvm, and impeller speed of 100 rpm.

tion of *Cel*⁻ mutants was indirectly confirmed based on the formation of smooth-type colonies on an agar medium inoculated with the culture medium. The culture medium became turbid when *Cel*⁻ mutants were formed. However, the dry weight of the produced BC continued to increase for 5 days of culture. As such, it would seem that not all the microbial cells were converted into *Cel*⁻ mutants in the shear stress field during the cultivation

The rate of glucose consumption by *G. hansenii* PJK in the jar fermentor was lower than that in the static culture, and the glucose was still not completely consumed on the seventh day of culture (Fig 6). The dry weight of the produced BC increased in proportion to the cell growth rate and reached a maximum value of 0.25 g/L. The amount of BC produced in the jar fermentor was only 44% of that produced in the shaking culture. A previous literature [7] reported that a highly increased dissolved oxygen level in an agitated culture diminished the cellulose yield, implying that the dissolved oxygen level in the fermentation medium should be controlled at 30% of the saturated level. We measured the volumetric oxygen transfer coefficients (K_La) in the jar fermentor using on the gassing out method [24]. K_La for 3 days old BC culture broth was 3.24 h^{-1}, which was 1.8 times higher than that for *E. coli* culture broth in the shaking incubator [25]. As shown in Fig. 6, the dissolved oxygen concentration in the medium was maintained at 10% of the saturated level although that remained at 50% of the saturated level on the first day of cultivation when almost all the BC was produced. The reduction in BC production in a jar-fermentor might be caused by the high dissolved oxygen concentration on the first day of fermentation. However, there was another remarkable observation in this study. The turbidity of the broth culture also rapidly increased on the first day. More than 95% of the cells cultivated for 1 day in a jar-fermentor formed smooth-type colonies, whereas the cells harvested on the second day of a shaking culture formed rough-type colonies. This may be due to the conversion of the cells in a jar-fermentor to *Cel*⁻ mutants on the first day of culture. It was also observed that the cells produced 0.3 g/L soluble polysaccharide for 5 days in a jar-fermentor although they produced 0.1 g/L in a shaking incubator. Thus, it would appear that the higher shear rate generated by the impeller tip in the fermentor accelerated the conversion of the microbes into *Cel*⁻ mutants, thereby partially causing the decrease in the production of BC in the fermentor.

Effects of Ethanol on Maintenance of *Cel*⁺ Genetics in Shaking Culture

It is well known that the addition of a small amount of ethanol into a culture medium usually increases the amount of produced BC [22]. Thus, 1% ethanol was added to the culture medium, resulting in an increased harvest of BC. It was also found that the BC formed pellets, which then aggregated into a big lump on the second day of cultivation. Eventually, the culture medium became clear after 5 days of cultivation. Five percent of the supernatant of the culture broth was introduced into a new medium containing 1% ethanol and cultured under the same conditions described previously. The microbial cells, *G. hansenii* PJK, produced BC and the culture broth was clear at the end of the cultivation. The dry weight of the BC produced in the second batch was nearly equal to that produced in the first batch. The operation of a subsequent third batch was also successful, as shown in Table 3, although the *G. hansenii* PJK cells did not produce any BC in the third batch without the addition of ethanol, and the culture medium became turbid. This result was very interesting and distinct from observations in previous literature [9], where *Cel*⁻ mutants of *A. xylinum* formed spontaneously when the wild type was repeatedly transferred in a shaking flask culture, plus the *Cel*⁻ mutants grew faster than the wild type. Valla and Kjosbakken [9] also reported that *Cel*⁻ mutants form smooth-type colonies and cellulose-producing cells form mucoid rough-type colonies. In the current study, the cells cultivated for 5 days in an ethanol-supplemented medium formed rough-type colonies, whereas the cells cultivated in a non-ethanol-supplemented medium formed mostly smooth-type colonies. It is possible that the formation of the big lump in the medium containing 1% ethanol may have effectively protected the microbes from the shear stress in the shaking culture. However, further research is needed to investigate the mechanism that preserves the cellular activity of cellulose production in a medium with ethanol.

CONCLUSION

A cellulose-producing strain, *G. hansenii* PJK, that can produce highly-purified BC free from impurities was isolated from rotten apples and identified by the 16S rDNA complete sequencing method. When cultivated in a shaking culture, *G. hansenii* PJK was found to produce more BC than in a static culture. However, the larger shear rate produced at the end of the impellers in the fermentor seemed to promote the conversion of the cellulose-

producing cells into *Cel⁻* mutants, resulting in a decrease in the amount of BC produced in the fermentor. The supplementation of ethanol into the medium appeared to prevent *G. hansenii* PJK from converting into *Cel⁻* mutants in the subculture, however, the mechanism involved in preserving the cellulose-producing cells in the medium containing ethanol still needs to be elucidated.

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