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Conversion of *G. hansenii* PJK into Non-cellulose-producing Mutants According to the Culture Condition

Joong Kon Park*, Seung Hun Hyun, and Jae Yong Jung

Department of Chemical Engineering, Kyungpook National University, Daegu 702-701, Korea

Abstract The conversion of a cellulose-producing cell (*Cel*$^+$) from *Gluconacetobacter hansenii* PJK (KCTC 10505 BP) to a non-cellulose-producing cell (*Cel*$^-$) was investigated by measuring the colony forming unit (CFU). This was achieved in a shaking flask with three slanted baffles, which exerted a strong shear stress. The addition of organic acid, such as glutamic acid and acetic acid, induced the conversion of microbial cells from a wild type to *Cel*$^-$ mutants in a flask culture. The supplementation of 1% ethanol to the medium containing an organic acid depressed the conversion of the microbial cells to *Cel*$^-$ mutants in a conventional flask without slanted baffles. The addition of ethanol to the medium containing an organic acid; however, accelerated the conversion of microbial cells in the flask with slanted baffles. The *Cel*$^+$ cells from the agitated culture were not easily converted into *Cel*$^-$ mutants on the additions of organic acid and ethanol to a flask without slanted baffles, but some portion of the *Cel*$^+$ cells were converted to *Cel*$^-$ mutants in a flask with slanted baffles. The conversion ratio of *Cel*$^+$ cells to *Cel*$^-$ mutants was strongly related to the production of bacterial cellulose independently from the cell growth.

Keywords: bacterial cellulose, cellulose-negative mutant, *Gluconacetobacter hansenii*, ethanol, organic acid, shear stress

INTRODUCTION

Cellulose is the most abundant polysaccharide in nature and is a major component of higher plants. Cellulose can also be produced by some bacterial strains [1-2], and bacterial cellulose (BC) consists of pure cellulose, with a diameter only a thousandth that of plant cellulose [3]. BC has unique properties, such as a high degree of crystallinity, water retention value, tensile strength and moldability, which are distinct from those of plant cellulose [4-10]. The production of BC by *Acetobacter* or *Gluconacetobacter* strains has become a very important subject in the area of new materials with high functional needs and in food-related industries.

Eco-friendly BC, produced by microbial strains, has a high potential for commercialization. However, BC has been produced traditionally by static cultures, which require long culture periods and are labor intensive [11], thus resulting in low productivity. Meanwhile, an agitated culture converts microbial strains into *Cel*$^-$ mutants, which become more enriched than the wild type, as they grow faster, thereby causing lower productivity of BC in continuous cultures [12]. Although many good cellulose-producing strains suitable for agitated cultures have been developed, the productivity of BC is not far enough advanced for industrialized production. Park *et al.* [13] reported the possibility of preserving the cellular activity of cellulose production, without the spontaneous occurrence of non-cellulose-producing (*Cel*$^-$) mutants, in consecutive shake-cultures using a medium containing ethanol, although it is generally known that the shear stress generated in shaking cultivation causes the *Acetobacter* strains to convert into *Cel*$^-$ mutants [14]. It is important to note that the mechanism involved in preserving the cellulose-producing cells in the medium containing ethanol still requires elucidation [1]. Park *et al.* also insisted that 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 [1]. Coucheron [15] noted that the insertion sequence element was associated with the inactivation of cellulose production in an *Acetobacter xylinum* strain, and the cellulose deficiency in mutants might be due to insertions, (i) in the region of the operon outside the 9.9-kb *Hin*dIII fragment, (ii) in genes representing known functions in the production of cellulose, but which are not part of the cellulose synthase operon, or (iii) in genes whose role in this formation remains to be identified.

Accordingly, further research is required to investigate the optimum fermentation conditions, including nutrient components and medium mixing modes, in an incubator producing a large amount of eddies, resulting in the minimum conversion rate of microbial strains and the maximum production of BC in an agitated cultivation. In

***Corresponding author**
Tel: +82-53-950-5615 Fax: +82-53-950-6615
e-mail address: parkjk@knu.ac.kr

Table 1. The media recipes used in this study

	Composition of each medium (weight %)					
	M	MA	MG	ME	MAE	MGE
Glucose	1	1	1	1	1	1
Yeast extract	1	1	1	1	1	1
Peptone	0.7	0.7	0.7	0.7	0.7	0.7
Succinate	0.02	0.02	0.02	0.02	0.02	0.02
Acetic acid		0.15			0.15	
Glutamic acid			0.15			0.15
Ethanol				1	1	1

this study, we tried to investigate the effect of operating conditions including the shear stress generated at the slanted baffles on the flask wall on the conversion of *Gluconacetobacter hansenii* PJK cells and BC production as the basic research in order to obtain optimum fermentation condition in the future study for BC production in an agitated vessel.

MATERIALS AND METHODS

Microorganism and Cell Culture

Gluconeacetobacter hansenii PJK (KCTC 10505BP), isolated from rotten apples [1], was grown on a basal medium containing 10 g glucose/L, 10 g yeast extract/L, 7 g peptone/L, 1.5 mL acetic acid/L and 0.2 g succinate/L, which had previously been used for the culture of an *Acetobacter* strain [16]. A medium containing ethanol was prepared by the addition of 1% (v/v) ethanol to the autoclaved basal medium. The compositions of the four kinds of media used in this study are shown in Table 1. The pH of the medium was adjusted to 5.0 with NaOH. Colonies of *G. hansenii* were inoculated into 50 mL of the medium in a 250-mL flask, shaken at 200 rpm and cultured at 30°C for 24 h. 5% of the supernatant of the culture broth was inoculated into 50 mL of the medium in a 250-mL flask for the next batch culture. Then, 5% of the culture broth supernatant was inoculated into 50 mL of the medium in a conventional 250-mL flask for batch culturing, with another 5% of the supernatant inoculated in a flask with three slanted baffles [17], which maintained an adequate shear stress for the dispersion of the solid materials.

Detection of Cellulose-negative Mutants

The culture broth was spread onto an agar medium and incubated at 30°C until colonies formed on the agar plate. Cel^- mutants were detected on the plates as smooth-type colonies, while Cel^+ cells formed mucous and rough-type colonies, as reported previously [1,12].

Preparation of Cellulose-producing Strains from an Agitated Culture

The culture broth, cultivated at 30°C for 3 days in a 5-L jar fermentor with a working volume of 3 L of basal medium operated at an impeller speed of 100 rpm and an aeration rate of 0.5 vvm, was filtered with a sterilized mesh (38 μm). The filtrate was spread onto an agar medium and incubated at 30°C for 5 days. Rough-type colonies were inoculated into 50 mL of the medium in a 250-mL flask, shaken at 200 rpm and cultured at 30°C for 24 h for the next culture.

Analysis of Cell, BC, and Glucose

BC was harvested by centrifuging the culture broth for 20 min at 3,580 × g, and washed twice with distilled water. The dry weight of BC and 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 melt the microbial cells; thereafter, the solution was filtered (pore size: 8 μm) using an aspirator to remove the dissolved materials[1,2,4-9,11-13,15,16,18]. The filter cake was rinsed repeatedly with distilled water until the pH of the filtrate became neutral. The BC dry weight, 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 of the medium was measured using a glucose reagent kit (Sigma no. 510-A).

RESULTS AND DISCUSSION

Ethanol Supplementation in an Agitated Culture

In previous studies, *Gluconacetobacter hansenii* PJK strains produced BC in a shaking flask with the medium containing 1% ethanol without conversion of Cel^+ cells to Cel^- mutants [1,13]. Thus, the amount of BC produced in a medium with ethanol was about 50% greater than that produced without ethanol.

In order to certify the effect of the ethanol supplementation in an agitated culture, attempts were made to produce BC in a 5-L jar fermentor with a working volume of 3 L of MA medium, agitated with an impeller speed of 100 rpm. The microbial cells grew for 3 days until all the glucose had been consumed, as shown in Fig. 1, but BC was still being produced on the 4th day of cultivation, as

Table 2. Production of BC and occurrence of *Cel*$^-$ mutant in flask and agitated cultures. Cells were cultivated in 50 mL of medium in a 250 mL flask shaken at 200 rpm for 4(±1) days at 30°C (in particular, 10 days culturing in a conventional flask using MAE and MGE). Cells were also cultivated at 30°C for 4 days in a 5-L jar fermentor, with a working volume of 3 L of medium, operated with an impeller speed of 100 rpm and an aeration rate of 1.7 vvm. All data are duplicated values

Medium	Shaking culture in a conventional flask			Agitated culture in a fermentor			Shaking culture in a flask with three slanted baffles		
	Dry wt. of BC (g/L)	Dry wt. of cells (g/L)	*Cel*$^-$ mutant/ total cells	Dry wt. of BC (g/L)	Dry wt. of cells (g/L)	*Cel*$^-$ mutant/ total cells	Dry wt. of BC (g/L)	Dry wt. of cells (g/L)	*Cel*$^-$ mutant/ total cells
MA	1.42	2.26	0.27	1.12	3.01	0.95	0.54	1.64	0.99
MAE	2.28	2.93	0	0.11	0.44	1	0.65	1.70	0.99
MG	0.86	2.04	0.91				1.14	2.48	0.11
MGE	1.90	3.79	0.07				0.82	2.00	0.99

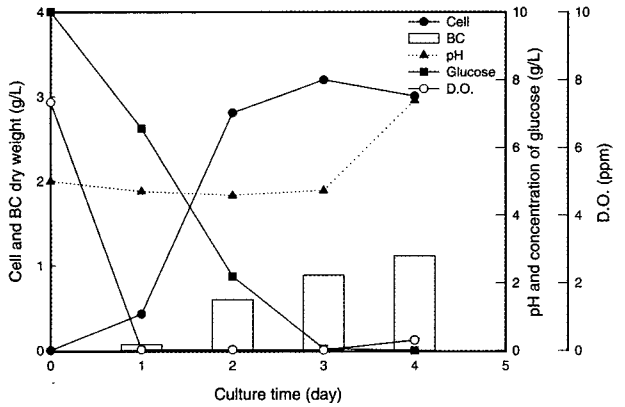

Fig. 1. Time course of BC production by *G. hansenii* PJK in a 5-L jar fermentor with a working volume of 3 L. The cultivation was carried out at 30°C with an initial pH of 5.0, aeration rate of 1.7 vvm and agitation rate of 100 rpm.

reported in our previous study [1]. This lead to speculation that BC might be produced not only from glucose, but also from other carbon sources in the medium. The amount of cells harvested on the 4th day of cultivation increased from 1.14 to 3.01 g/L with the increase in the rate of aeration from 0.5 to 1.7 vvm. An increase in the aeration rate also resulted in an increase of BC production from 0.33 to 1.12 g/L. The dissolved oxygen concentration decreased to 0 ppm after 1 day of cultivation at the aeration rate of 1.7 vvm, as shown in Fig. 1. This means that the oxygen transferred from the air bubbles to the liquid medium was completely consumed by microorganisms, and the cell growth was accelerated by the large amount of oxygen supplied. This resulted in a high cell density in the fermentor, which was about 33% larger than the shaking culture, as shown in Table 2. However, the amount of BC produced in the jar fermentor with an aeration rate of 1.7 vvm was 1.12 g/L, which was about 80% of that produced in a shaking flask culture. The reduction in BC production in the jar fermentor could be easily explained from the literature [14], which reported that the shear stress generated during cultivation caused *Acetobacter* strains to convert into *Cel*$^-$ mutants.

The amount of BC produced in a jar fermentor containing MAE medium, *i.e.* MA medium containing 1% ethanol, was only 0.11 g/L. This means that the addition of ethanol to MA medium in the jar fermentor resulted in a reduction in BC production, unlike cultivation in a shaking flask culture. As shown in Table 2, the ratio of *Cel*$^-$ mutants to the total number of cells after 4 days of cultivation in a jar fermentor was much greater than that in a shaking flask. To my knowledge, why the addition of ethanol should prevent the conversion of microorganisms into *Cel*$^-$ mutants in a shaking flask is unknown. Also, why the addition of ethanol should promote the conversion of microorganisms in a jar fermentor remains to be discovered. It is known from the literature [15] that the conversion of microorganisms to *Cel*$^-$ mutants is caused by the transposon of the insertion sequence element, but to date little has been published on this mechanism. Thus at this stage, there is a need to investigate which components of the culture medium and what culture conditions accelerate the conversion of microorganisms to *Cel*$^-$ mutants in order to produce a greater amount of BC in a jar fermentor.

Effects of Mixing Modes on BC Production

Two of the most important differences in the culture conditions between agitated and flask cultures are the aeration speed, in other words, the rate of oxygen transfer from air bubbles to a liquid medium, and the shear force exerted by the fluid motion. The volumetric oxygen transfer coefficient (k_La) in the jar fermentor was 3.24 h^{-1} with an aeration rate of 0.5 vvm, and that in the shaking flask incubator was 1.8 h^{-1} [1]. The flow pattern in a shaking flask is a circulating flow, without violent lateral mixing, but that in a conventional fermentor there is a circulating flow, with violent lateral and vertical mixing, which is caused mainly by the turbulence exerted by the tip of the impeller. Thus, a flask with three slanted baffles was used in our experiments, as previously used by Toyosaki *et al.* [17], in order to maintain a similar k_La nearly

to that of the shaking flask incubator to obtain a comparable shear force as exerted by the slanted baffle on the flask wall. The k_La in the flask with three slanted baffles was less than 2.0 h^{-1} under all experimental conditions.

The dry weight of the cells grown for 3 days in the flask with three slanted baffles containing MA medium was 1.64 g/L, which was much smaller than the 2.26 g/L in a conventional flask culture, as shown in Table 2, but much greater than the 1.14 g/L in a jar fermentor with an aeration rate of 0.5 vvm. The BC produced in the flask with three slanted baffles was 0.54 g/L, which was also smaller than the 1.42 g/L in a conventional flask culture, but greater than the 0.33 g/L in a jar fermentor. From this, the shear force exerted by the slanted baffles seems to effectively repress the production of BC by the microorganisms, although the shear force is weaker than that exerted by a six-bladed flat turbine impeller. However, a fast oxygen transfer rate due to a high aeration speed of 1.7 vvm could overcome the deficiency caused by the shear force exerted by the tip of impeller. The amount of BC produced in the jar fermentor with an aeration rate of 1.7 vvm was greater than that produced in the flask with three slanted baffles, as shown in Table 2.

The addition of ethanol to the MA medium (and thus, MAE) in the three slanted baffle flask introduced a slight increase in the production of BC, although 99% of the microorganisms were converted to *Cel*$^-$ mutants after 4 days of cultivation with a rotating speed of 200 rpm, as shown in Table 2. However, the amount of BC produced in the MAE medium in the three slanted baffle flask was 0.65 g/L, which was also smaller than the 2.28 g/L obtained in the MAE medium in the conventional flask, but much greater than the 0.08 g/L with an aeration rate of 0.5 vvm in a fermentor. The amount of BC produced in the flask with slanted baffles decreased with increasing rotation speed (data not shown). Thus, it is speculated that the increase in BC production caused by ethanol supplementation would be reduced with the strength of the shear force, regardless of the rate of oxygen supply with in the range used in our experiment.

Glutamic Acid Instead of Acetic Acid

One of the main roles of acetic acid in the medium for BC production is to inhibit contaminant growth [17]. Glutamic acid is also known to be an effective inhibitor [18], and is sometimes used as a component of the medium for BC production by microorganism [19]. How substituting acetic acid for glutamic acid affects the BC production in a flask culture, with and without slanted baffles, was investigated. In a conventional flask culture, both the cell growth and BC production in the medium containing glutamic acid (MG medium) were inferior to those in the medium containing acetic acid (MA medium), as shown in Table 2.

However, the supplementation of ethanol into the MG medium (and thus, MGE medium) was much more effective in promoting the cell growth and BC production. The amount of BC produced in the MGE medium increased 2.2 times, reaching 1.90 g/L with 1% ethanol supplementation, although this was smaller than the 2.28 g/L in the MAE, as shown in Table 2. The cell growth in the MGE increased 1.86 times, reaching 3.79 g/L, which was much higher than that in the MAE. It also became apparent that the addition of glutamic acid to the basal M medium (and thus, MG medium) was much more effective at inhibiting the conversion of microbial cells to *Cel*$^-$ mutants with a vigorous shear stress in a slanted baffle flask compared to the culture in the MA medium. The conversion ratio of microorganisms to *Cel*$^-$ mutants in the MG medium was only 0.11, although that in the MA medium was 0.99, as shown in Table 2. Thus, this lead to both high cell growth and BC production in the slanted baffle flask using the MG medium. However, the supplementation of 1% ethanol to the MG medium (and thus, MGE medium) could not effectively prevent the conversion of microorganisms to *Cel*$^-$ mutants at the shear stress exerted by the slanted baffles, and the conversion ratio became 0.99. Thus, the BC production was decreased by 28%, from 1.14 to 0.82 g/L, by the addition of 1% ethanol to the MG medium. The addition of 1% ethanol to the MG medium in a slanted baffle flask seemed to be preferred, as the amount of BC produced in the MGE medium was much greater than the 0.65 g/L produced in the MAE medium.

Conversion of Microorganisms During Cultivation

It is commonly known that the shear stress exerted in a culture vessel during cultivation converts microbial cells to *Cel*$^-$ mutants, resulting in a decrease in the BC production. As shown in previous sections, the conversion ratio of microorganisms to *Cel*$^-$ mutants varied with the culture conditions, such as the medium components and culture types. How the microorganisms were converted to *Cel*$^-$ mutants was investigated in relation to the culture time and medium components, such as acetic acid, glutamic acid and ethanol, and also the culture type, such as flasks with and without slanted baffles, and the origin of the *Cel*$^+$ cells, such as wild type or fermentor by counting the CFU. The data for the above are shown in Table 3.

There was no significant conversion of microorganisms in the basal medium (M) without organic acid in a conventional flask culture without slanted baffles. The addition of ethanol to the basal medium (and thus, ME) does not promote the conversion of microorganisms from the wild type to *Cel*$^-$ mutants without the organic or amino acids, acetic acid or glutamic acid, respectively, in a conventional flask. The addition of acetic acid to the basal medium (and thus, MA) induced the conversion of microorganisms to *Cel*$^-$ mutants, and the addition of glutamic acid (and thus, MG) accelerated the conversion of microorganisms. This lead to greater BC production in the MA than the MG medium, as shown in Table 2. The supplementation of 1% ethanol to the MA medium (and thus, MAE) efficiently prevented (nearly completely) the conversion of microorganisms to *Cel*$^-$ mutants in the conventional flask culture. The addition of 1% ethanol to the MG medium (and thus, MGE) partially prevented the conversion of microbial cells and reduced the conversion

Table 3. Effects of ethanol supplementation and culture broth flow pattern on the occurrence of *Cel*$^-$ mutants. Cells were cultivated in 50 mL of medium in a 250 mL-flask shaken at 200 rpm for 5 days at 30°C. The culture broth was spread onto an agar medium, and then incubated at 30°C until colonies formed on the agar plate. (- : means not forming a colony)

Culture broth age (day)	*Cel*$^-$ mutants / total cells																			
	Cel$^+$ cells from the wild type										*Cel*$^+$ cells from the agitated culture broth									
	Shaking culture in a conventional flask						Shaking culture in a flask with three slanted baffles				Shaking culture in a conventional flask						Shaking culture in a flask with three slanted baffles			
	M	MA	MG	ME	MAE	MGE	MA	MG	MAE	MGE	M	MA	MG	ME	MAE	MGE	MA	MG	MAE	MGE
1	0	0.078	0.435	0	0.068	0.251	0.353	0.001	0.958	0.200	0	0.006	0.004	0.006	0.006	0.014	0.455	0.010	0.321	0.614
2	0.001	0.636	0.944	0	0	0.455	0.876	0.043	0.619	0.528	0.001	0.019	0.072	0.019	0	0	0.916	0.469	0.500	0.674
3	0.002	0.273	0.864	0	0.002	0.362	1	0.105	0.691	0.494	0	-	0.052	0.048	0.019	0.032	0.988	0.318	0.872	0.640
4	0	-	0.912	0	0	0.320	-	0.067	0.988	0.962	0	-	0.036	0.438	0	0.053	-	0.227	0.985	0.984
5	0	-	0.948	0	0	0.155	-	0.043	-	0.995	0	-	0.035	0.558	0	0	-	0.385	1	1

ratio from 0.864 to 0.362 on the second day of cultivation, as shown in Table 3. This seemed to result in a greater increase in the ratio of BC production in the MGE medium, as shown in Table 2.

However, the turbulence field exerted by the slanted baffles on the flask wall increased the conversion of microbial cells to *Cel*$^-$ mutants in the MA medium, but decreased the conversion ratio in the MG medium. The exact reason why or how the shear stress exerted by the slanted baffles prevented the conversion of microorganisms to *Cel*$^-$ mutants in the MG medium remains unknown, but seemed to result in an increase in the BC production, despite the turbulence field in the MG medium, as shown in Table 2. The addition of 1% ethanol to the MG medium (and thus, MGE) promoted the conversion of microorganisms to *Cel*$^-$ mutants, resulting in a decrease in the BC production in the slanted baffle flask. The supplementation of 1% ethanol to the MA medium (and thus, MAE) affected the conversion of microorganisms in the turbulence field in a complex manner. With unknown reason, the ratio of *Cel*$^-$ mutants to total cells decreased to less than 70% at second and third days of cultivation and increased again to 99% at fourth day. This might have lead to the slight increase in the BC production, as shown in Table 2.

The conversion ratio of *Cel*$^+$ cells from the agitated culture broth in the conventional flask culture was very low compared to that obtained from *Cel*$^+$ cells from the wild type, regardless of the nutrient component, as shown in Table 3. All conversion ratios were less than 0.1, with the exception of those on the 4 and 5th days of cultivation in the ME medium. This would seem to be the reason why many researchers have reported the production of excellent *Cel*$^+$ cells with agitated broth cultivation. However, a portion of the *Cel*$^+$ cells from the agitated culture broth were converted to *Cel*$^-$ mutants due to the turbulence field exerted by the slanted baffles on the flask wall. The supplementation of 1% ethanol to the MA medium (and thus, MAE) seemed to slightly depress the conversion of *Cel*$^+$ cells to *Cel*$^-$ mutants in the agitated culture.

CONCLUSION

It is well known that *Cel*$^+$ cells are converted to *Cel*$^-$ mutants in a shear stress field. There has only been one report that the conversion was related to the transposon of the insertion sequence element. However, the mechanism how the *Cel*$^+$ cells convert to *Cel*$^-$ mutants, until now, remained to be completely understood on the basis of gene. Thus, at this stage, how the conversion of *Cel*$^+$ cells from wild type to *Cel*$^-$ mutants occurs was investigated in relation to the culture conditions. The experi mental results seemed to be slightly cumbersome but a clear trend could be found. In a one-dimensional circulating medium flow, organic (acetic acid) and amino (glutamic acid) acids induced the conversion of microbial cells, and the supplementation of ethanol seemed to prevent the conversion of microbial cells. However, in a turbulent field, the addition of ethanol to the medium containing organic or amino acids accelerated the conversion of *Cel*$^+$ cells from the wild type to *Cel*$^-$ mutants. The *Cel*$^+$ cells from the agitated culture were not easily converted to *Cel*$^-$ mutants in the medium containing acetic acid, glutamic acid and ethanol, but a large portion of the cell population was converted to *Cel*$^-$ mutants in a turbulent field. The conversion ratio of microbial cells to *Cel*$^-$ mutants was strongly related to the production of bacterial cellulose. The focus of this study was not directed towards the industrial mass production of BC, but this result will be a good guideline for developing technology to effectively produce BC on an industrial scale.

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