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Production of Bacterial Cellulose by *Gluconacetobacter* sp. RKY5 Isolated From Persimmon Vinegar

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Abstract

The optimum fermentation medium for the production of bacterial cellulose (BC) by a newly isolated *Gluconacetobacter* sp. RKY5 was investigated. The optimized medium composition for cellulose production was determined to be 15 g/L glycerol, 8 g/L yeast extract, 3 g/L K_2HPO_4, and 3 g/L acetic acid. Under these optimized culture medium, *Gluconacetobacter* sp. RKY5 produced 5.63 g/L of BC after 144 h of shaken culture, although 4.59 g/L of BC was produced after 144 h of static culture. The amount of BC produced by *Gluconacetobacter* sp. RKY5 was more than 2 times in the optimized medium found in this study than in a standard Hestrin and Shramm medium, which was generally used for the cultivation of BC-producing organisms.

Index Entries: Bacterial cellulose; fermentation; *Gluconacetobacter*; optimization; persimmon vinegar.

Introduction

Cellulose (poly-β-1,4-glucose) is the most abundant biological macromolecules in the world, with an estimated production of 10^{11} t/yr. It forms a structural matrix of cell walls of several fungi, algae, and nearly all plants, in which the cellulose forms semi-crystalline microfibrils of several nm in diameter *(1,2)*. In general, most of the plants generate thinner microfibrils with lower cellulose crystallinity, and the microfibrils are often intimately associated with the encrusted materials including hemicellulose *(3)*. The bacterium, *Acetobacter xylinum*, is known to be able to produce an extracellular cellulose, which is called bacterial cellulose (BC) that was first described by Brown in 1886 *(4,5)*. The cellulose synthesized by *A. xylinum* is structurally identical to that made by plants or algae. The microbial

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cellulose synthesis may offer a more cost-effective means of supply, because the microorganism secretes cellulose which is pure and free of lignin, pectin, hemicellulose, and phytate found in plant cellulose as a crystalline pellicle, making its recovery simple and relatively inexpensive *(6)*. Because of high-tensile strength and water-holding capacity of BC, it has been used as a raw material for manufacturing of high-fidelity acoustic speaker, high-quality paper, and dietary foods.

It is well known that the cellulose-producing bacteria are subject to spontaneous mutation yielding cellulose-negative mutant, Cel^-, when exposed to strong shear stress fields or serial cultivation. Many strategies for the production strains capable of producing cellulose with a high yield have been attempted including chemical mutation, gene modification, development of improved fermentation technique, and improved components in culture broth. However, the productivity of BC is still quite low, which makes the production costs extremely high. Therefore, it is necessary to isolate a novel microorganism for stable production of BC and to establish a production medium for mass production of BC.

In this study, we presented an optimized medium composition for the efficient production of BC by batch fermentation of *Gluconacetobacter* sp. RKY5. Furthermore, under the optimized culture medium, we compare the fermentation efficiencies between static and agitated cultures of *Gluconacetobacter* sp. RKY5.

Materials and Methods

Microorganism

The microorganism used in this study was *Gluconacetobacter* sp. RKY5 KCTC 10683BP, which was previously isolated from persimmon vinegar in Korea. Based on the Bergey's Manual of Determinative Bacteriology *(7)*, several biochemical tests were carried out such as gram staining, ability to oxidize ethanol, acetate and lactate, and ketogenic activity for glycerol. In addition, morphological test was carried out using scanning electron microscope and the produced BC was identified by X-ray diffractometer *(4,8–10)*. According to the results from the above tests, this isolate was suggested to be *Acetobacter* sp. and eventually identified as *Gluconacetobacter* sp. through 16*s* rDNA sequence analysis *(11)*. It was maintained by monthly transfer on Hestrin and Shramm medium (HS medium) and kept at –20°C with 50% (v/v) glycerol.

Cultivation Conditions

The standard medium used in this study was HS medium, consisting of 20 g/L glucose, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L Na_2HPO_4, and 1.15 g/L citric acid monohydrate *(12)*. For seed culture, 1% (v/v) of the stock culture was inoculated into 50 mL of HS medium in a 250-mL Erlenmeyer flask, which was then cultivated at 30°C and 150 rpm for 48 h in a shaking

incubator (KMC-8480SF, Vision Scientific, Daejeon, Korea). After that, the culture broth was vigorously shaken in order to release the cells from the BC pellicle. The resulting cell suspension was then filtered through 12 layers of sterilized gauze, and 2% (v/v) of the filtrate was inoculated into 50 mL of fermentation medium in a 250-mL Erlenmeyer flask for static and agitated culture.

Optimization of Culture Medium

Based on HS medium as a standard medium, several carbon, nitrogen, phosphate sources, and secondary substrates were tested in sequence to determine the optimal composition of culture medium for the production of BC. After one component was chosen, the test for its optimum concentration followed. After all components were selected and its concentrations were determined, these were used for further experiment. The amounts of BC produced were measured in static and agitated culture conditions under the optimized medium.

Analytical Methods

Cell growth was evaluated by measuring the optical density at 660 nm using a UV-1700 spectrophotometer (Shimadzu, Kyoto, Japan), after the culture broth was treated with 0.1% (v/v) cellulase (Celluclast 1.5 L, Novozymes A/S, Bagsvaerd, Denmark) at 50°C with shaking at 150 rpm. The dry cell weight (g/L) was then calculated by using a predetermined calibration curve.

In order to measure the amount of BC produced, the gelatinous membrane of BC that was formed on the surface of the culture was picked up with tweezers, and the pellets of the agitated culture broth were centrifuged at 9940*g* for 15 min *(8)*. After the separation of BC from the culture broth, the pellicles or pellets were washed with distilled water to eliminate the medium components, which was treated with 0.1 *N* NaOH at 80°C for 20 min in order to dissolve the cells *(13)*. After the neutralization with 0.1 *M* acetate buffer, the BC was rinsed again with distilled water, and then filtered. The purified BC was dried at 80°C under a vacuum until a constant weight was obtained. For each investigation, two independent experiments were carried out and each sample was analyzed in three replicates, from which we obtained mean values.

Results and Discussion

Effect of Carbon Sources on BC Production

To investigate the effect of carbon sources on the production of BC, 2% (w/v) of several carbon sources were added to the glucose-free standard medium. As shown in Fig. 1A, a high level of BC production was observed when glycerol, fructose, or sucrose was used as a carbon source.

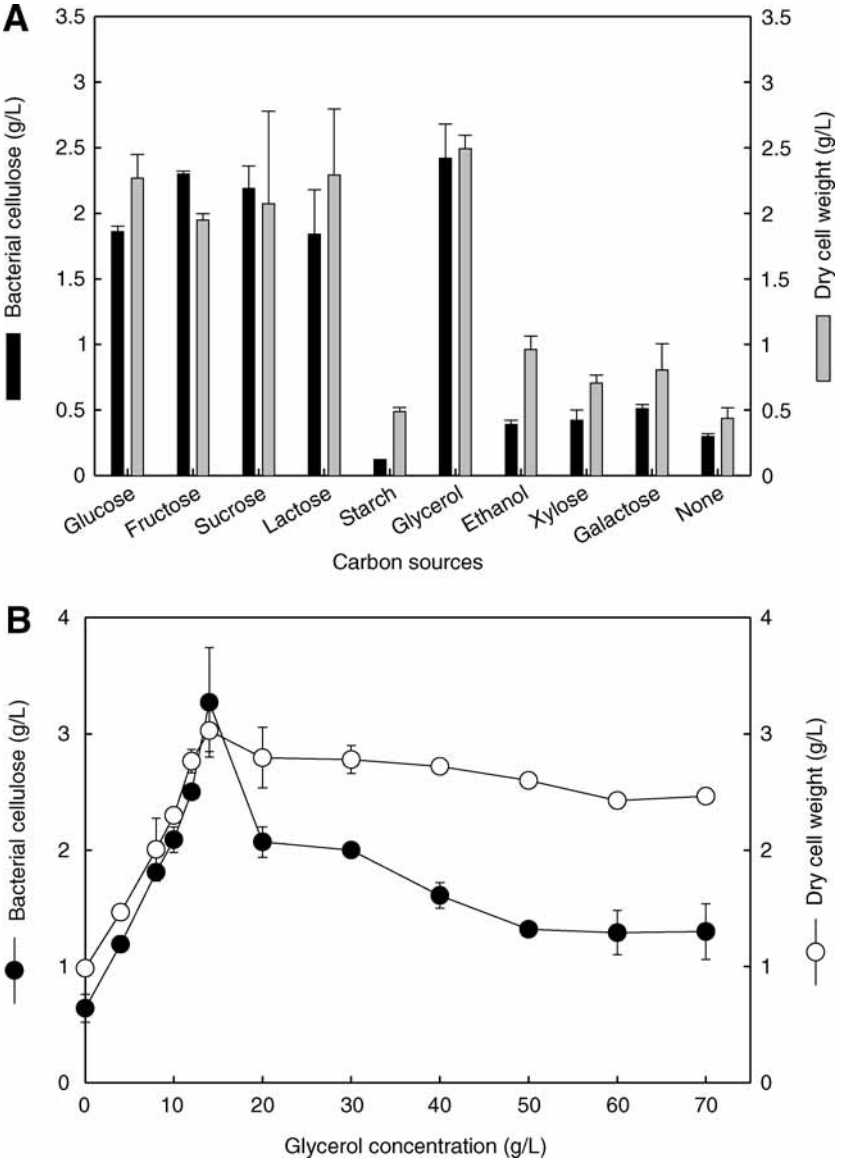

Fig. 1. Effect of various carbon sources **(A)** and glycerol concentrations **(B)** on the production of bacterial cellulose. The cultivation medium was composed of 20 g/L carbon source, 5 g/L yeast extract, 5 g/L peptone, 2.7 g/L Na_2HPO_4, and 1.15 g/L citric acid monohydrate. Data are presented as the means of two replicates and error bars indicate the standard deviation.

However, when starch, ethanol, xylose, or galactose was used as a carbon source, poor cellulose production was observed. It might be expected that, when the glycerol was used as a carbon source, cell growth was much higher than that of other carbon sources because the culture pH during the

fermentation using glycerol as a carbon source did not significantly drop. Therefore, the amount of BC produced from glycerol as a carbon source was highest. Although BC-producing bacteria generally metabolize glucose or fructose for the production of BC, glycerol was found to be the best carbon source for cultivation of *Gluconacetobacter* sp. RKY5. Therefore, it is supposed that *Gluconacetobacter* sp. RKY5 has another metabolic pathway to synthesize cellulose from C_3 component, whereas *Acetobacter* species synthesize cellulose via such pathway as conversion of glucose to uridine diphosphate glucose (UDP-glucose) *(14)*. Therefore, the glycerol was selected as an optimum carbon source for BC production, and the effects of glycerol concentration on the production of BC was investigated. As shown in Fig. 1B, the amount of BC produced increased with increases in glycerol concentrations up to 14 g/L, and decreased beyond this value. The maximum BC production (3.27 g/L) was observed at 14 g/L of glycerol. Yields might be improved in batch fermentation if the cultures are initiated with a low glycerol concentration, because high initial glycerol concentrations resulted in low amount of BC production.

Effect of Nitrogen Sources on BC Production

Several nitrogen sources were added to the standard medium at a level of 1% (w/v) to investigate the effect of nitrogen sources on the production of BC. As shown in Fig. 2A, when yeast extract was added to the medium, the highest amount of BC (4.49 g/L) was obtained. Tryptone and corn steep liquor (CSL) were also tested for BC production and yielded 3 g/L and 2.16 g/L of BC, respectively. The other nitrogen sources resulted in poor BC production. The addition of yeast extract considerably stimulated BC production, probably owing to its abundant nutrients as well as several growth factors. To find the optimum concentration of yeast extract, various concentrations ranging from 0 to 20 g/L were tested. As shown in Fig. 2B, the BC production increased gradually with increases in yeast extract concentrations up to 8 g/L, and remained constant beyond this value. Therefore, 8 g/L of yeast extract was selected as an optimum nitrogen source concentration for the production of BC by batch fermentation of *Gluconacetobacter* sp. RKY5.

Effect of Phosphate Sources and Secondary Substrates on BC Production

To determine the effect of phosphate sources on the production of BC, various phosphate sources were tested by addition to the standard medium in the same range of concentration. All phosphate sources except $(NH_4)_2HPO_4$ exhibited similar BC production and cell growth, but K_2HPO_4 was found to be the best (Fig. 3A). To assess the effect of K_2HPO_4 concentrations on BC production, various concentrations of K_2HPO_4

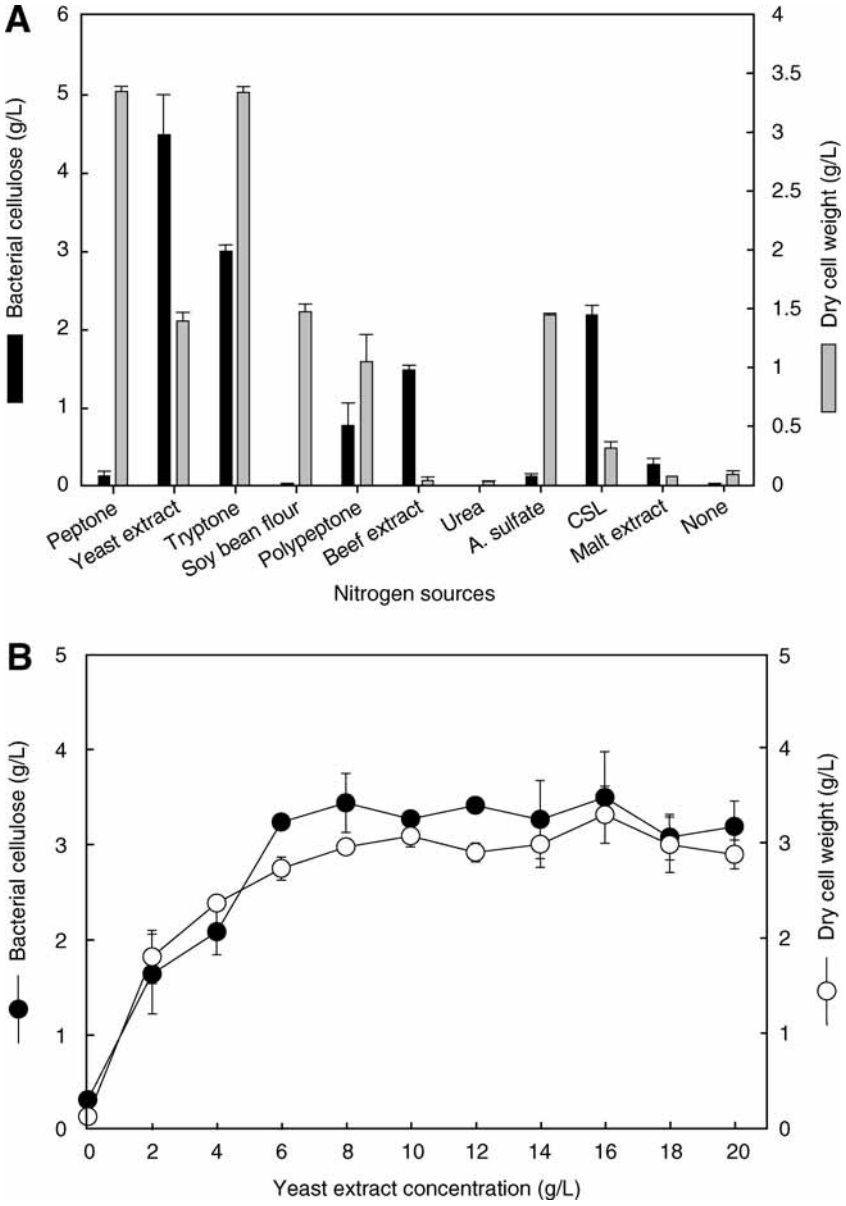

Fig. 2. Effect of various nitrogen sources **(A)** and yeast extract concentrations **(B)** on the production of bacterial cellulose. The cultivation medium was composed of 15 g/L glycerol, 10 g/L nitrogen source, 2.7 g/L Na_2HPO_4, and 1.15 g/L citric acid monohydrate. Data are presented as the means of two replicates and error bars indicate the standard deviation.

ranging from 0 to 5 g/L were added to the medium. As shown in Fig. 3B, the maximum yield of BC (3.21 g/L) was observed in the medium containing 3 g/L of K_2HPO_4, but the production of BC decreased slightly beyond this value.

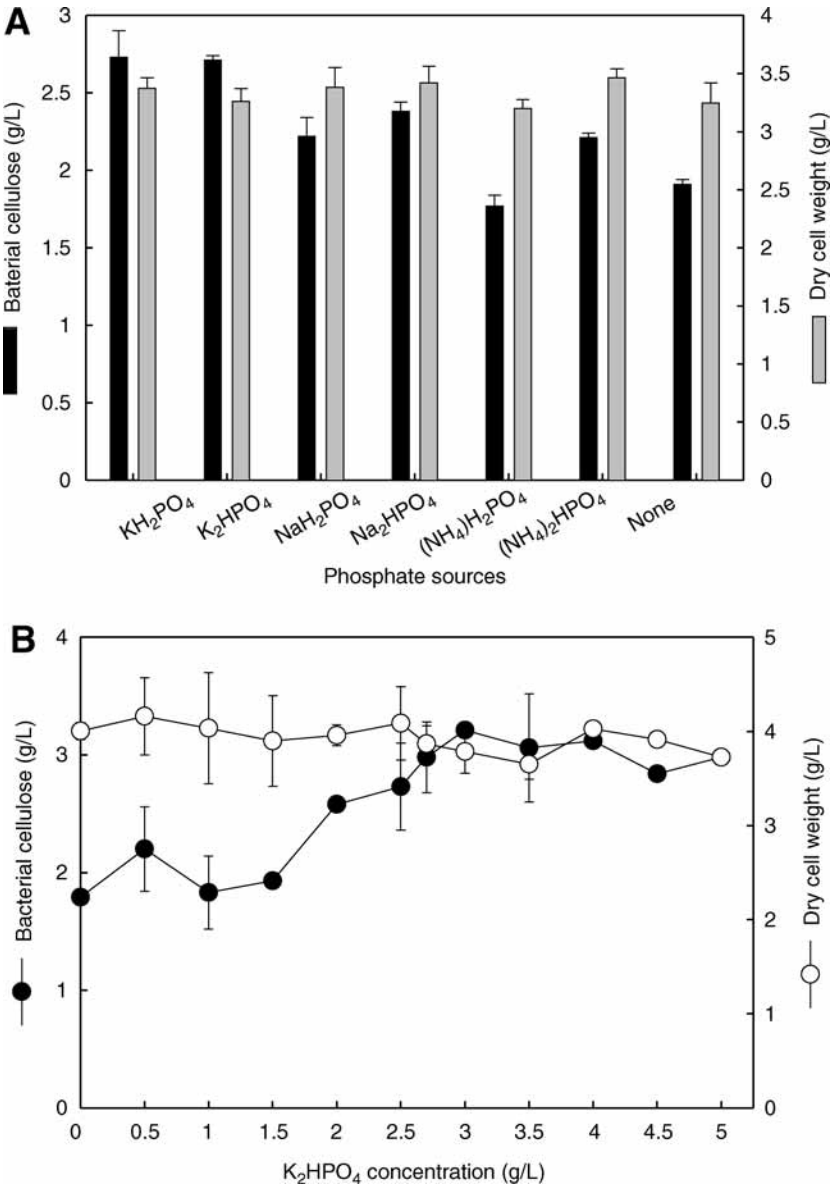

Fig. 3. Effect of various phosphate sources **(A)** and K_2HPO_4 concentrations **(B)** on the production of bacterial cellulose. The cultivation medium was composed of 15 g/L glycerol, 8 g/L yeast extract, 2.7 g/L phosphate source, and 1.15 g/L citric acid monohydrate. Data are presented as the means of two replicates and error bars indicate the standard deviation.

It is well known that the addition of secondary substrate, including some organic acids or ethanol, to the fermentation medium stimulates the production of BC. Therefore, several organic acids and ethanol were tested for the investigation of their effects on the production of BC by

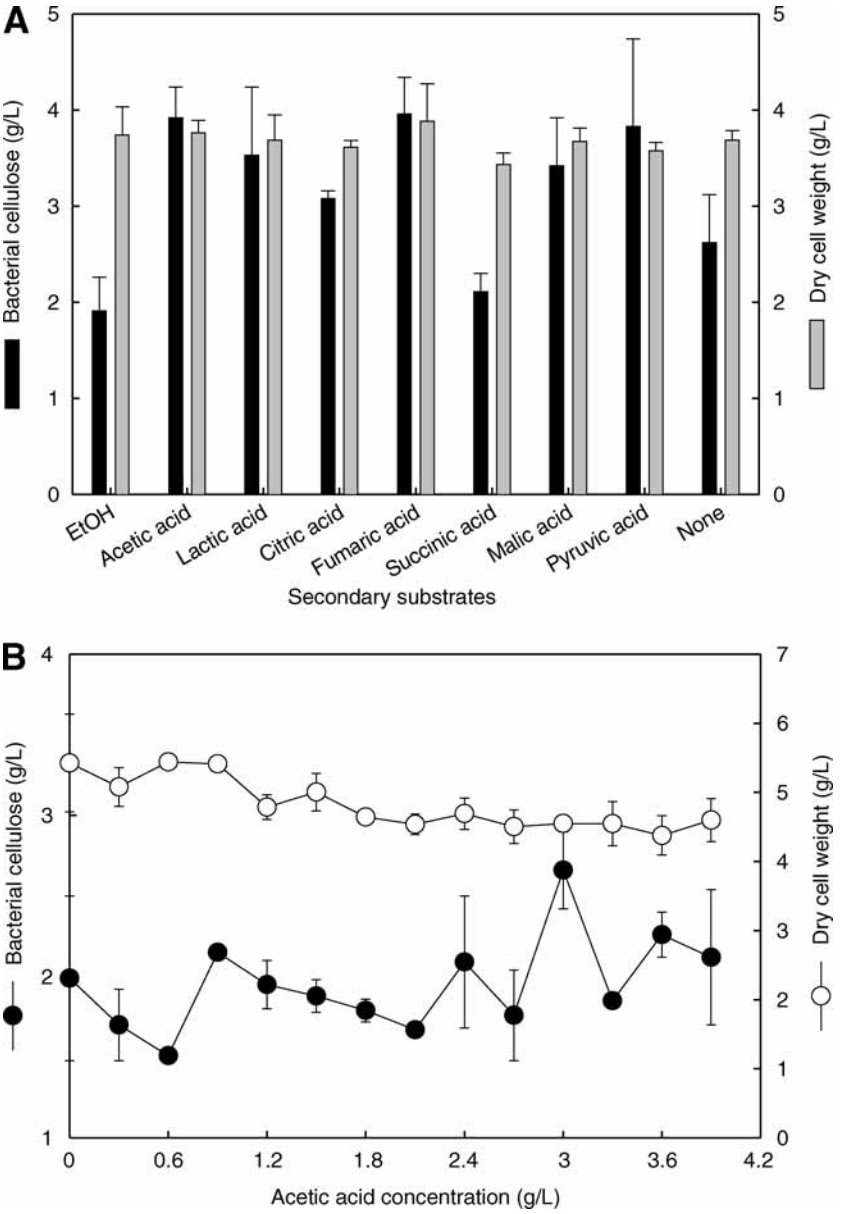

Fig. 4. Effect of various secondary substrates **(A)** and acetic acid concentrations **(B)** on the production of bacterial cellulose. The cultivation medium was composed of 15 g/L glycerol, 8 g/L yeast extract, 3 g/L K_2HPO_4, and 1.15 g/L secondary substrate. Data are presented as the means of two replicates and error bars indicate the standard deviation.

Gluconacetobacter sp. RKY5. As can be seen in Fig. 4A, all the compounds added except ethanol and succinic acid enhanced the production of BC. Among the compounds tested, acetic and fumaric acids were the best for BC production. According to the previous reports, the addition of lactic

acid to the medium stimulated the production of BC by *A. xylinum* subsp. *sucrofermentans* BPR 2001 *(5)*, and Son et al. *(15)* improved the production of BC by adding the ethanol to the medium in batch culture of *Acetobacter* sp. A9. However, for the production of BC, *Gluconacetobacter* sp. RKY5 used in this study preferred acetic acid and fumaric acid to other compounds tested, which is a distinguishing characteristic of *Gluconacetobacter* sp. RKY5 from the other strains. To investigate the effect of acetic acid concentrations on BC production, various concentrations of acetic acid ranging from 0 to 3.9 g/L were added to the medium. As shown in Fig. 4B, the maximum yield of BC (3.92 g/L) was observed in the medium containing 3 g/L of acetic acid, but acetic acid concentrations below or above this value resulted in low amount of BC. However, the yields of cellulose were quite variable.

BC Production in Optimal Medium

As investigated earlier, the optimized culture medium for BC production by *Gluconacetobacter* sp. RKY5 was found to be 15 g/L glycerol, 8 g/L yeast extract, 3 g/L K_2HPO_4, and 3 g/L acetic acid. This media composition was designated as modified HS (MHS) medium. Figure 5 shows the time-course of BC production and cell growth during static (Fig. 5A) and agitated (150 rpm, Fig. 5B) cultivations of *Gluconacetobacter* sp. RKY5 in the MHS medium. In a static culture condition, the amount of BC produced gradually increased, and the maximum amount of BC (4.59 g/L) was obtained after 144 h of cultivation. In case of an agitated culture condition, the BC produced was poor before 48 h of cultivation, but it sharply increased after 48 h of cultivation. When mean values were compared, the maximum amount of BC produced (5.63 g/L) was observed at 144 h of cultivation. Because the production of BC started during exponential cell growth, BC production by *Gluconacetobacter* sp. RKY5 might be initially associated with cell growth. However, maximum production of BC occurred in stationary growth phase. Because the agitated culture condition has more shear stress than the static culture condition, this might cause the delay in production. On the other hand, in the static culture condition, the mobility of the microorganism decreases compared to those in the agitated culture condition. Therefore, the uptake of carbon source may be dependent on diffusion permitting more glycerol to go to cellulose-synthesizing enzyme. However, for the production of BC, the agitated culture condition was better than the static culture condition in this study. It seemed that the cellulose-synthesizing mechanism of *Gluconacetobacter* sp. RKY5 yielded more BC in MHS medium for agitated culture, probably as a result of better aeration. In conclusion, the amount of BC produced was approximately more than 2 times in the optimized medium established in this study than that in a standard HS medium.

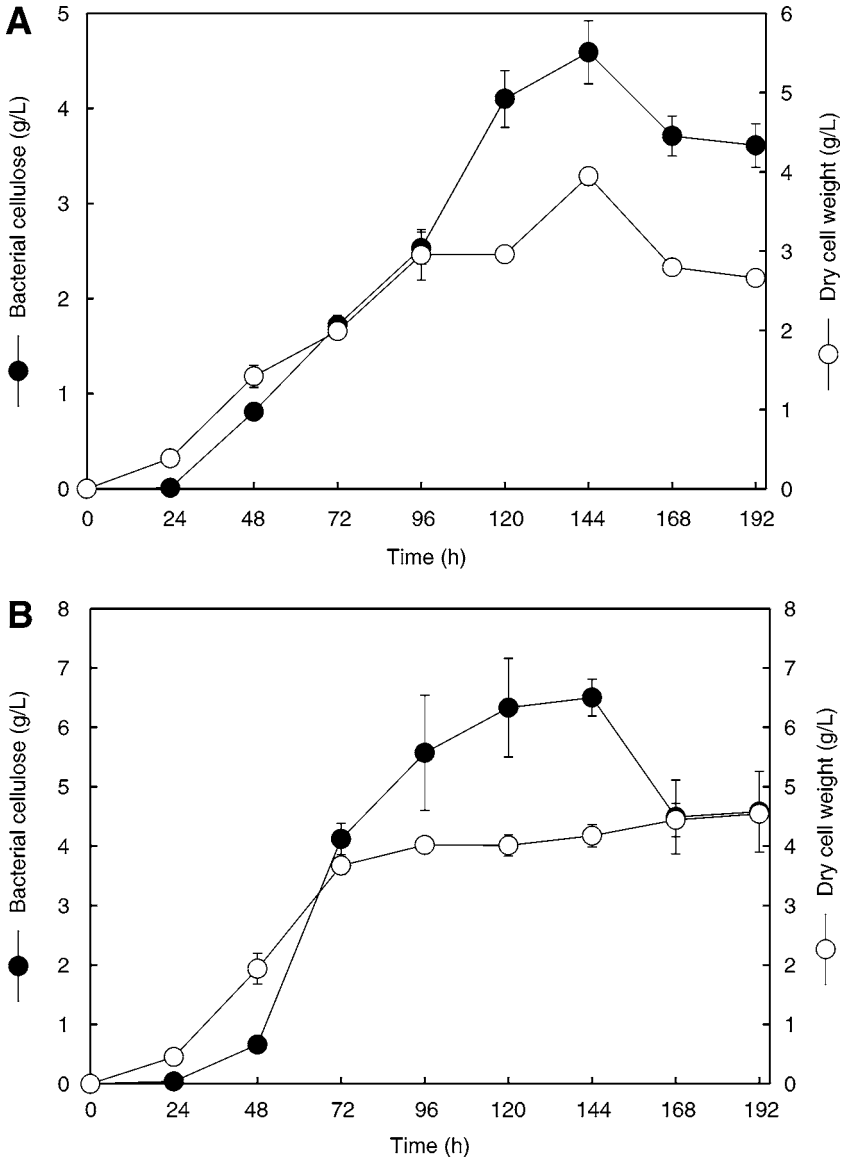

Fig. 5. Time-course of bacterial cellulose production and cell growth using MHS medium. The cultivation medium was composed of 15 g/L glycerol, 8 g/L yeast extract, 3 g/L K_2HPO_4, and 3 g/L acetic acid. **(A)** static culture condition; **(B)** agitated culture condition. Data are presented as the means of two replicates and error bars indicate the standard deviation.

References

1 Astley, O. M., Chanliaud, E., Danald, A. M., and Gidley, M. J. (2001), *Int. J. Biol. Macromol*. **29,** 193–202.
2 Jonas, R. and Farah, L. F. (1998), *Polym. Degrad. Stabil.* **59,** 101–106.
3 Hult, E. L., Yamanaka, S., Ishihara, M., and Sugiyama, J. (2003), *Carbohydr. Polym.* **53,** 9–14.

4 Brown, A. J. (1886), *J. Chem. Soc.* **49,** 432–439.
5 Matsuoka, M., Tsuchida, T., Matsushita, K., Adachi, O. A., and Yoshinaga, F. (1996), *Biosci. Biotechnol. Biochem.* **60(4),** 575–579.
6 Wulf, P. D., Joris, K., and Vandamme, E. J. (1996), *J. Chem. Techchol. Biotechnol.* **67,** 376–380.
7 Buchanan, R. E. and Gibbons, N. E. (1974), *Bergey's Manual of Determinative Bacteriology*, 8th edition, Williams and Wilkins Co., Baltimore.
8 Park, J. K., Park, Y. H., and Jung, J. Y. (2003), *Biotechnol. Bioprocess Eng.* **8,** 83–88.
9 Toyosaki, H., Naritomi, T., Seto, A., Matsuoka, M., Tsuchida, T., and Yoshinaga, F. (1995), *Biosci. Biotech. Biochem.* **59,** 1498–1502.
10 Yamada, Y., Hoshino, K., and Ishikawa, T. (1997), *Biosci. Biotech. Biochem.* **61,** 1244–1251.
11 Ryu, H. W., Wee, Y. J., Kim, S. Y., Kim, J. N., and Yun, J. S. (2004), *Korea Patent Application No.* 10-2004-0104790.
12 Schramm, M. and Hestrin, S. (1954), *Biochem. J.* **56,** 163–166.
13 Son, C., Chung, S., Lee, J., and Kim, S. (2002), *J. Microbiol. Biotechnol.* **12(5),** 722–728.
14 Tonouchi, N., Tsuchida, T., Yoshinaga, F., Beppu, T., and Horinouchi, S. (1996), *Biosci. Biotechnol. Biochem.* **60(8),** 1377–1379.
15 Son, H. J., Heo, M. S., Kim, Y. G., and Lee, S. J. (2001), *Biotechnol. Appl. Biochem.* **33,** 1–5.