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Cellulose Production from *Gluconobacter oxydans* TQ-B2

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Abstract *Gluconobacter oxydans* that produces the cellulose was isolated. In order to confirm the chemical features of cellulose, various spectrophtometeric analysis were carried out using electron microscopy, X-ray diffractogram, and CP/MAS ^{13}C NMR. The purified cellulose was found to be identical to that of *Acetobacter xylinum*. For effective production of cellulose, the various carbon and nitrogen sources, mixture of calcium and magnesium ions, and biotin concentration were investigated in flask cultures. Among the various carbon sources, glucose and sucrose were found to be best for the production of cellulose, with maximum concentration of 2.41 g/L obtained when a mixture of 10 g/L of each glucose and sucrose were used. With regard to the nitrogen sources, when 20 g/L of yeast extract was used, the maximum concentration of bacterial cellulose was reached. The concentration of cellulose was increased with mixture of 2 mM of each Ca^{2+} and Mg^{2+}. The optimum biotin concentration for the production of cellulose was in the range of 15 to 20 mg/L. At higher biotin concentration (25~35 mg/L), the bacterial cellulose production was lower.

Keywords: cellulose, *Gluconobacter oxydans*, red tea fungi

INTRODUCTION

The biosynthesis of bacterial cellulose in some Acetobacteria has been known for more than 100 years. Recently, the bacterial cellulose has received much attention as a new functional material for commercial applications because of its characteristic features such as ultrafine fibrils, high tensile strength, biodegradable polymer, and high water holding capacity. These mechanical properties must be due to unique structure features of the bacterial cellulose [1]. *Acetobacter xylinum* is known as nature's most prolific cellulose-producing bacterium. Ross *et al.* have advanced the model for regulation of cellulose biosynthesis in *A. xylinum* [2,3].

The structural features and properties of bacterial cellulose produced in agitated and static culture with *A. xylinum* have been investigated in detail [4,5]. Chao *et al.* utilized an airlift bioreactor which was energy saving to produce bacterial cellulose by *A. xylinum* [6]. Vandamme *et al.* predicted the application potential of bacterial cellulose [7]. In addition, there have been some studies on breeding bacteria to produced cellulose through genetic engineering [8,9]. Although in many ways *A. xylinum* may be the most thoroughly characterized organism with respect to cellulose synthesis and a wide range of bacteria, including *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Aerobacter*, *Azotobacter*, *Agrobacterium*, *Rhizobium* and *Sarcina species*, can also produce cellulose [2]. A number of studies on improving culture method and structural features of bacterial cellulose [6,4,10] have been reported in the screening of bacterial cellulose producing strains [4]. Bacterial cellulose is also produced in Asia countries from the fermentation of traditional foods such as nata, Red Tea Fungi and Feng Liang Ye [11,12]. Nata is a bacterial cellulose produced by *Acetobacter aceti* ssp. *xylinum* through the fermentation of the fruit juice or plant extract. Red Tea Fungi and Feng Liang Ye are the traditional beverages in China, and their fermentation, the bacterial cellulose is produced as a by-product of the tea.

In this study, a bacterial strain, *Gluconobacter oxydans*, *which* produces cellulose was isolated. In order to confirm the chemical features of its cellulose, various spectrophotometric analyses were carried out using electron microscopy, X-ray diffractogram and MAS ^{13}C NMR. For the effective production of cellulose, the various carbon and nitrogen sources, and mixture of calcium and magnesium ions, and biotin were investigated in flask cultures.

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MATERIALS AND METHODS

Strain Isolation and Culture

Strains were isolated from Feng Liang Ye using a selective medium which were contained 12° Brix wort, 30 g/L of $CaCO_3$, and 30 g/L of ethanol. One kilogram of concentrated malt extract was diluted with 2.6 L of tap water to yield 12° Brix wort. The diluted malt extract was incubated at 45°C for 2 h, and then at 60°C for 3 h. Thereafter, the diluted malt extract was autoclaved at 120°C for 15 min, and passed through filter paper (Whatman, No. 3) to remove the resulting precipitate. For solid medium, agar was used at a concentration of 3%. The ethanol was added after autoclaving. The culture was carried out at 28°C for 72 h. Several cellulose producing bacterial colonies were selected from the plates. Each isolated strain was subsequently transferred to a 250-mL Erlenmeyer flask containing 25 mL of liquid media, and allowed to grow in static culture for 37 h at 28°C. It was observed that the surface of the liquid was covered with a gelatinous membrane. The strain that yielded a high level of bacterial cellulose was selected and identified. This strain was maintained at 4°C on agar slants containing the same medium used for isolation. The composition of medium used in the seed culture was as follows (g/L): glucose, 20; yeast extract, 5; peptone, 5; Na_2HPO_4, 1.5; and citric acid, 0.5. One loop of *Gluconobacter oxydans* was transferred to the slant medium and cultured at 28°C for 4 days. For the production of bacterial cellulose, 10% of the seed was inoculated into a 500-mL Erlenmeyer flask containing 50 mL of the basal medium and cultured at 28°C for 6~7 days.

Analytical Methods

The cell concentration was estimated by measuring the optical density at 660 nm after treating the culture broth with 5% of cellulase at 50°C and pH 5.0 for 60 min. To measure the concentration of cellulose produced, the cellulose in the culture broth was washed twice with distilled water and then treated with 0.1 N NaOH at 80°C for 2 h to dissolve the cell mass and other impurities. The purified bacterial cellulose was washed twice with distilled water and dried at 80°C to get a constant weight. The reducing sugar concentration in the culture broth was measured as glucose using the Somogyi-Nelson method. The bacterial cellulose morphology was observed by the scanning electron microscope (N-2250, Hitachi Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Identification of Organism

About thousand organisms were tested in this study and several strains were isolated. The organism was identified as *Gluconobacter oxydans* based on the morphological and physiological properties as shown in Table 1.

Table 1. Characterization of *Gluconobacter oxydans* TQ-B2

Morphological characteristics	Strain TQ-B2
Gram reaction	Negative
Cell morphs	5~0.8 × 0.9~4.2 μm ellipsoids or rods
Catalase	Positive
Growth in media at pH 3.6	Positive
Growth in media at pH 3.6	Positive
Optimum pH	5.5~6.0
Temperature for growth at 37°C	Negative
Optimum temperature	25~30°C
Glucose fermentation to gluconic acid	Positive
Nitrate reduction	Negative
Liquefaction gelatin	Negative
Production of indole	Negative
Oxidation of acetic acid	Negative
Oxidation of ethanol to acetic acid	Positive
Unessential pantothenic acid	Positive
Essential tibitol	Negative
Essential arabitol	Negative
Oxidation of lactate	Negative

Among these strains, TQ-B2 was observed to be about 0.5~0.8 × 0.9~4.2 μm in size, ellipsoids or rods like in shape, Gram-negative and produced catalase. It also produced gluconic acid from glucose and acetic acid from oxidation of ethanol, respectively. The optimum pH and temperature for the culture ranged from 5.5 to 6.0 and 25 to 30°C, respectively.

Separation of Cellulose

The amounts of cellulose produced by *G. oxydans* TQ-B2 was determined using enzyme digestion and acid hydrolysis and were found to be 72 and 91%, respectively. After the sample of cellulose had been hydrolyzed by cellulase, it was analyzed using paper chromatography. The Rf value of the cellulose was 0.2, which was similar to that of the glucose standard.

Structural Features of Cellulose

In order to confirm the structure features of the cellulose produced, various spectrophotometric analyses were carried out using electronic scanner, X-ray, and ^{13}C NMR. Fig. 1 shows the electron micrograph of the cellulose produced by the *G. oxydans* TQ-B2 strain. The reticular structure consisting of ultrafine cellulose fibrils is shown. Fig. 2 presents the X-ray diffractogram of the cellulose sheet. It was obtained by the reflection method with an automatic diffractomater (D/max-250, Rigaku,

Fig. 1. The electron micrograph of bacterial cellulose.

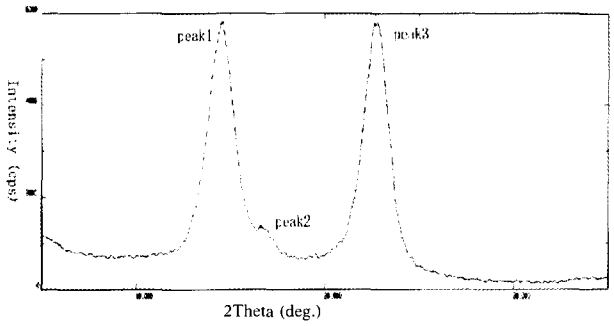

Fig. 2. X-ray diffractograms of bacterial cellulose.

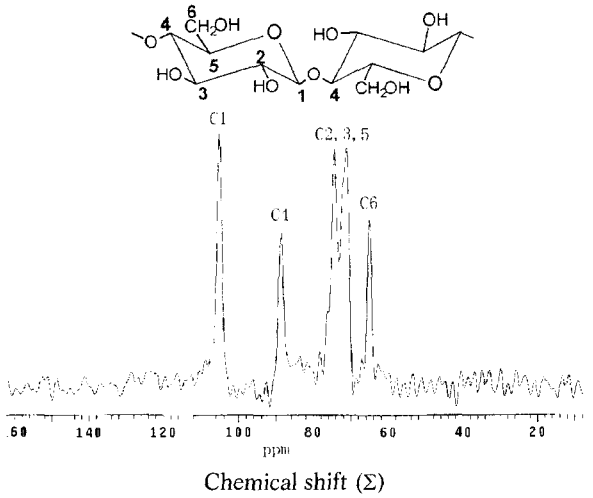

Fig. 3. CP/MAS ^{13}C NMR spectra of bacterial cellulose.

Tokyo) that was operated at between 5° and 40° for 2θ with Cross Polarization. Peak 1:2θ (deg.), 14.56; d-value (A°), 6.08; Intensity (cps), 5808; I/I_0,100. Peak 2:2θ (deg.), 16.60; d-value (A°), 5.34; Intensity (cps), 1358; I/I_0, 23. Peak 3:θ (deg.), 22.64; d-value (A°), 3.92; Intensity (cps), 5760; I/I_0, 99. The result of our experiment fitted closely to those previously reported for bacterial cellulose, which was produced by *A. xylinum* and *G. hansenii* PJK isolated from rotten apples [13,14]. Fig. 3

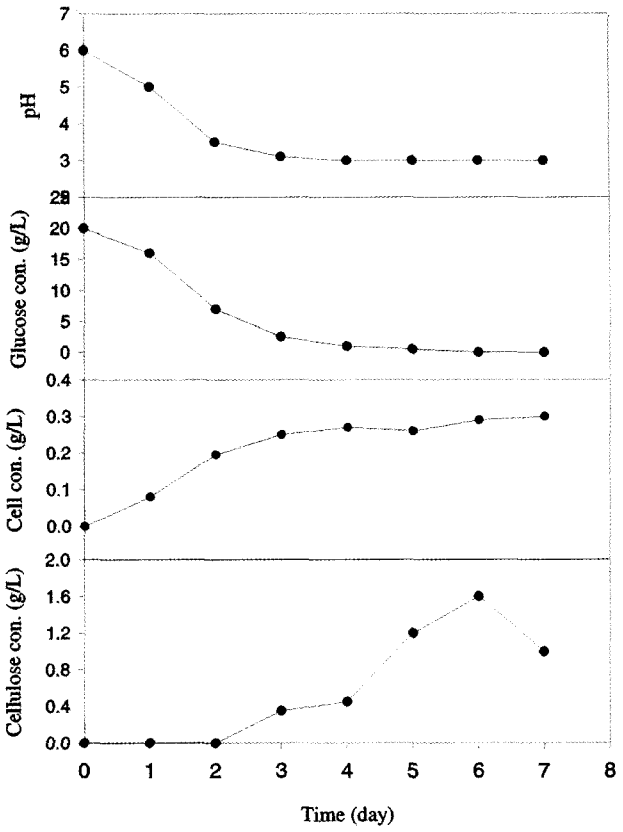

Fig. 4. Production of bacterial cellulose in shake flasks.

shows solid-state ^{13}C NMR spectrum of the cellulose sheet. The measurement of MAS ^{13}C NMR was conducted on the cellulose sheet using a Varian Unity Plus 400 spectrometer. The chemical shifts (ppm) of cellulose appear as follows: C1, 105.1; C4, 88.8; C2, 74.5; C3, 71.2; C5, 69.2; and C6, 65.2. The main peaks of the cellulose biosynthesized by *G. oxydans* TQ-B2 were identical to those of the typical bacterial cellulose [15,16].

Production of Cellulose

The growth pattern of *Gluconobacter oxydans* TQ-B2 and the production of cellulose were observed in a 500-mL Erlenmeyer flask contatining 20 g/L of glucose, 5 g/L of yeast extract, 2.5 g/L of Na_2HPO_4, and 1 g/L of citric acid. The culture was carried out at 28°C for 7 days at 200 rpm. The results are shown in Fig. 4. The cell was grown exponentially from 24 h of culture. The cell concentration was increased slowly between 48 h and 96 h of culture and a maximum concentration of 0.25 g/L was reached after 96 h of culture. The production of cellulose was started from 48 h of culture and was rapidly increased from 96 h of culture. A production of cellulose 1.8 g/L was obtained from an initial glucose concentration of 20 g/L after 6 days of culture. The pH of the culture broth was decreased rapidly from 6.0 at the beginning to below of 3.5 after 48 h of culture. It may have been due to the limitation of cell growth in low pH. The

Table 2. Effect of various carbon sources on the bacterial cellulose production

Carbon sources (g/L)	Cellulose concentration (g/L)
Glucose (20)	1.84
Lactose (20)	1.32
Glucose (10) + Lactose (10)	1.62
Maltose (20)	0.77
Glucose (10) + Maltose (10)	1.27
Fructose (20)	1.56
Glucose (10) + Fructose (10)	1.85
Sucrose (20)	1.81
Glucose (10) + Sucrose (10)	2.41
Dextrin (20)	0.66
Glucose (10) + Dextrin (10)	1.29
Soluble starch	0.59
Glucose (10) + Soluble starch (10)	1.14

pH of the culture broth was maintained at 5.0 with 1 N H_2SO_4.

Effect of Carbon Sources on the Cellulose Production

In order to investigate the effects of different carbon sources on the cellulose production, soluble starch, dextrin, sucrose, glucose, fructose, maltose, and lactose were used. The results are shown in Table 2. When 20 g/L of glucose or sucrose was used, the synthesis of cellulose was enhanced. Moreover, mixtures of glucose and sucrose or glucose and fructose or glucose and maltose or glucose and lactose in various ratios were further studied. When the media containing mixture of glucose and sucrose was used, the cellulose production was higher than that of the glucose or sucrose as the sole carbon source, or other mixtures of the saccharine. The maximum concentration was 2.41 g/L when the mixture of 10 g/L of glucose and 10 g/L of sucrose was used.

Effect of Nitrogen Sources on the Cellulose Production

For investigating the effect of various nitrogen sources on the cellulose production, urea sodium nitrate, soybean meal, bran, corn steep liquor, peptone, and yeast extract were used. Various nitrogen sources were added respectively to the medium at 10 g/L or 20 g/L of concentration. The results are shown in Table 3. Among different nitrogen sources tried, the yeast extract was found to be the best followed by corn steep liquor, peptone, and soybean meal. *G. oxydans* TQ-B2 could not use inorganic nitrogen sources such as urea and sodium nitrate to grow and produce the cellulose. Especially, in the case of 20 g/L of yeast extract, it was optimal for cellulose production (2.78 g/L).

Table 3. Effect of various nitrogen sources on the bacterial cellulose production

Nitrogen sources (g/L)	Cellulose concentration (g/L)
Urea (10)	0
Sodium nitrate (10)	0
Soybean meal (10)	0.32
Soybean meal (20)	0.20
Bran (10)	0.25
Bran (20)	0.26
Corn steep liquor (10)	1.64
Corn steep liquor (20)	1.73
Peptone (10)	1.35
Peptone (20)	1.54
Yeast extract (5)	1.98
Yeast extract (10)	2.39
Yeast extract (15)	2.58
Yeast extract (20)	2.78
Yeast extract (25)	2.75

Table 4. Effect of the mixture of calcium and magnesium ions on the bacteral cellulose production

Mixture of Ca^{2+} and Mg^{2+} (mM)	Cellulose concentration (g/L)
0	2.70
2	2.91
4	3.57
6	3.41
8	3.35
10	3.30

* The mixture ratio of Ca^{2+} and Mg^{2+} is 1:1.

Effect of Calcium and Magnesium Ions on the Cellulose Production

In cellulose biosynthesis, the diguanylate cyclase is a key enzyme, uridine diphosphate (UDP)-glucose and c-di-guanosine 5'-monophosphate (GMP) are precursors of cellulose synthesis [2,3]. While enhancing concentration of c-di-GMP, the diguanylate cyclase was activated by Mg^{2+} and phosphodiesterase was inhibited by Ca^{2+}. Table 4 shows the effect of calcium ion and magnesium ion on the cellulose production. The cellulose concentration reached the maximum at above 4 mM of the mixture

Table 5. Effect of biotin concentrations on the bacterial cellulose production

Biotin concentrations (mg/L)	Cellulose concentration (g/L)
0	3.50
5	3.78
10	4.02
15	4.55
20	4.53
25	4.36
30	4.25
35	4.20

of Ca^{2+} (2 mM) and Mg^{2+} (2 mM). Thus, the addition of calcium ion and magnesium to the culture medium can be increased cellulose production.

Effect of Biotin Concentrations on the Cellulose Production

In *A. xylinum* fermentation, the cellulose can be enhanced by adding biotin [4]. The same phenomenon was observed with *G. oxydans* in our work. The results are shown in Table 5. When 0 to 15 mg/L of biotin was added, the production of cellulose was increased. The maximum production of cellulose occurred at 15~20 mg/L of biotin (4.55 g/L). At higher biotin concentration (25~35 mg/L), the production of cellulose was lower.

CONCULSION

Because the cellulose production by *G. oxydans* TQ-B2 was lower than that of *A. xylinum*, it would be difficult to use *G. oxydans* TQ-B2 for industrialization, directly. At present, *A. xylinum* has been used for the production of cellulose. However, after culturing, the excess culture broth causes environment contamination, if it is directly discharged and the production costs of cellulose must be increased if it is treated. *G. oxydans* TQ-B2 isolated from Feng Liang Ye is a novel strain, which produces the cellulose. In the culture of Feng Liang Ye, the cellulose was produced as a by-product. It is similar to the production of cellulose in the nata de coco culture system. Although the cellulose produced along with the production of Feng Liang Ye was low, it would be a worth researching its cellulose production.

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