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Isolation and Cultivation Characteristics of *Acetobacter xylinum* KJ-1 Producing Bacterial Cellulose in Shaking Cultures

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Abstract Eight strains producing bacterial cellulose (BC) were isolated from rotten fruits and traditionally fermented vinegars. One of the isolated strains from the rotten grape in Gwangju, Korea, maintained a relatively stable BC production in shaking cultures. This isolated strain proved to be *Acetobacter xylinum*, based on several biochemical and morphological tests. It was shown that the slant-baffled flask was more efficient than the conventional flask for the BC production in shaking cultures. To determine the most suitable carbon and nitrogen sources for the production of BC, various compounds were examined. Fructose was found to be the most effective carbon source with an optimal concentration of 2%. Mixed carbon source (glucose:fructose=1:3) was also better than glucose or fructose alone. Optimal nitrogen source, when basal medium was used, was 10% (v/v) corn steep liquor (CSL). When corn steep liquor was used with a mixed carbon source (glucose:fructose=1:3), 4% CSL exhibited the best BC production. Based on these results, a defined medium was developed for the BC production by *Acetobacter xylinum* KJ-1. When this medium was used under optimal culture conditions, the BC production was 7.2 g/l, which was approximately 3 times higher than that with the traditional HS medium.

Key words: Bacterial cellulose, *Acetobacter xylinum*, shaking culture

Cellulose is the most abundant organic compound on earth. It has been estimated that about 10^{11} tons of cellulose are biosynthesized each year, and that the cellulose accounts for about 50% of the bound carbon on earth. Cellulose forms the fibrous component of plant cell walls. The rigidity of cellulose arises from its overall structure. Cellulose molecules are chains or microfibrils, of up to 14,000 units of D-glucose that occur in twisted rope-like bundles held together by hydrogen bonding [14].

In 1886, Brown first reported that a bacterium of *Acetobacter* sp. formed a cellulosic pellicle on the surface of broth in a static culture [1]. This material was found to be bacterial cellulose (BC). Since this discovery, numerous reports have appeared on the cultural, structural, mechanical, and biosynthetic aspects of BC [5, 6, 10, 13, 15, 17, 21].

The structural features and mechanical properties of BC differ from those of plant cellulose and its potentials application in industry is extensive. BC possesses excellent physical properties, including a high degree of polymerization and preferential orientation specificities, plus strong mechanical and absorbent properties. In addition, since BC is composed of pure cellulose without lignin, hemicellulose, and other substances, it can be purified more easily than natural cellulose. At present, practical applications of BC have frequently been found, such as sensitive diaphragms for stereo headphone, additives for food and paper products, thickener for paint, and also as a temporary skin substitute in skin burn treatment [2, 5, 7, 15, 19, 21, 22].

BC production rate by shaking or agitated culture system is lower than that by static culture system. However, a static culture system is inefficient in the industrial aspect, because it requires a very long cultivation period and much manpower. Recently, the mass production of BC based on the shaking and agitated cultures has been studied vigorously [12, 15, 17, 18]. Although *Acetobacter xylinum* has proved to possess a greatest potential for commercialization in industrial applications, its BC production would seem to be poor in a large-scale. For this reason, the subject of how to improve the cellulose productivity of *Acetobacter xylinum* has received increasing attention. Studies have been conducted on isolating a high cellulose-producing strain from nature or developing a stable strain on shear stress through genetic transformation [5, 6, 8, 12, 15].

In this study, we isolated and identified a BC-producing strain, referred to as *Acetobacter xylinum* KJ-1, from rotten

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grapes and investigated the strain's cultivation characteristics under shaking cultures. An attempt was also made to elucidate the effects of various nutritional sources, especially the compounding effects of fructose and corn steep liquor, on the BC production so as to optimize the defined medium composition for BC production.

MATERIALS AND METHODS

Bacterial Strains and Experimental Media

Acetobacter xylinum KJ-1 isolated from rotten grape was selected as the highest BC producer in this study. *A. xylinum* KCCM 40407 [Institute for Fermentation, Osaka (IFO) 3288], KCCM 40274 (IFO 13772), and *Gluconacetobacter xylinus* subsp. *xylinus* KCCM 40129 [IFO 13693; Japan Collection of Microorganisms (JCM) 7664] were used as reference strains in the cultivation and identification tests.

The components of media used in the experiments were as follows; Hestrin & Schramm (HS) medium contained 20 g glucose, 5 g bactopeptone, 5 g yeast extract, 2.7 g Na_2HPO_4, and 1.2 g citric acid monohydrate per liter of distilled water, and it was adjusted to pH 5.25 [3]. The GYC medium comprised 5 g glucose, 5 g yeast extract, and 5 g $CaCO_3$ per liter of distilled water. The YPC medium comprised 5 g yeast extract, 5 g peptone, and 5 g $CaCO_3$ per liter of distilled water. The YEG medium comprised 10 g yeast extract, 30 g glycerol, and 20 g agar per liter of distilled water. The minimum medium comprised 4.4 g KH_2PO_4, 4.8 g Na_2HPO_4, 1 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g ferric ammonium citrate, and 1 g $CaCl_2$ per liter of distilled water. The SM medium comprised 5 g yeast extract and 50 g glucose per liter of distilled water. The Carr medium comprised 30 g yeast extract, 20 g ethanol, 0.02 g bromocresol purple, and 20 g agar per liter, and pH was adjusted to 5.5–6.0.

Culture and Analytical Methods

For preculture, the cell suspension was inoculated in 100 ml HS medium in a 500-ml flask. For shaking cultures in a flask, the cell suspension was first grown under static condition, then it was inoculated in 100 ml HS medium in a 500-ml slant-baffled flask, and cultured in a rotary shaker at 150 rpm and 30°C for 5 days.

Residual glucose concentration and reducing sugars concentration in culture broth were measured by a glucose reagent kit (GAHK-20, Sigma Co. St. Louis, U.S.A.) and the 3,5-dinitrosalicylic acid (DNS) method, respectively [20]. The cell growth was measured by the optical density at 660 nm after the culture broth was treated with 0.1% (w/v) cellulase.

To purify the BC produced, centrifuged pellicle was treated with 0.1 N NaOH at 80°C for 20 min to dissolve microorganisms, filtered on the filter paper, and washed with distilled water. The purified BC was dried at 80°C for 8 h and weighted.

Isolation and Identification of Microorganisms

Samples for bacterial sources were collected from rotten fruits, soils, and traditional vinegars around Gwang-ju in Korea, and screened for BC production. Each sample was diluted with sterilized water and transferred into 10 ml of HS medium, then statically cultured at 30°C in a test tube. After static culture in 10 ml of HS medium in a test tube at 30°C for a maximal 2 weeks, one drop of each culture broth was streaked on the HS agar medium, and maintained at 30°C for 4 to 5 days. Isolated microorganisms which formed typical colonies on the plates were further cultured statically in 10 ml HS medium in the test tubes at 30°C for 5 days, and screened by observing BC pellicle formation. The selected microorganisms were cultured statically in 100 ml of HS medium in 500-ml flask at 30°C for 5 days. The microorganism with the highest BC production was selected.

The morphological and biochemical properties of the isolated strain were determined according to Bergey's Manual of Determinative Bacteriology [18]. The overoxidation of ethanol was examined in the isolate and control strains. The bacterial strains were statically incubated at 30°C for 3–4 days in Carr medium. The overoxidation of ethanol was judged by the change of the medium's color from violet to yellow and then from yellow to violet. The bacterial strains were statically cultured at 30°C for 5–7 days in the GYC agar medium and then their formations of water-soluble brown pigments were checked. In order to estimate the formation of γ-pyrones from D-glucose and D-fructose, the bacterial strains were statically cultured at 30°C for 5 days in the YPC medium, including 3% glucose and 5% fructose. In order to estimate the formation of 5-keto-D-gluconic acid and 2,5-diketogluconic acid from D-glucose, the bacterial strains were incubated with shaking at 30°C for 7 days in the HS medium. The formation of 5-keto-D-gluconic acid and 2, 5-diketogluconic acid were measured by a high-performance liquid chromatography (M 930, Young-Lin, Korea) with a Shodex DE-613 column. The samples were developed with a solvent composed of 2 mM perchloric acid at a flow rate of 1.0 ml per minute. In order to estimate ketogenesis from glycerol, the bacterial strains were incubated at 28°C for 3–5 days in the YEG agar medium. Adding a few drops of Fehling's solution to the medium can prove ketogenesis from glycerol, as the color of the medium changes to orange. In order to find an optimal carbon source, the bacterial strains were statically cultured at 28°C for 5 days in a minimum medium including ethanol, dulcitol, sodium acetate, and methanol. The bacterial growth was evaluated based on the pellicle formation and the increase of turbidity. In order to estimate the growth in L-amino acids such as L-glycine, L-threonine, L-tryptophan, L-glutamine, and L-asparagine in the presence of D-mannitol as the carbon sources, the bacterial strains were statically cultured at 28°C for 5 days

in a minimum medium. The bacterial growth was also evaluated based on the pellicle formation and the increase of turbidity. In order to estimate the growth in the presence of 10% ethanol, the bacterial strains were statically cultured at 30°C for 5 days in the SM medium. The bacterial growth was evaluated based on the pellicle formation and the increase of turbidity. The bacterial strains were statically cultured in the presence of a 30% D-glucose at 30°C for 5 days in the HS medium with 30% glucose. The bacterial growth was evaluated based on the pellicle formation and the increase of turbidity.

RESULTS AND DISCUSSION

Screening of Cellulose Producer from Natural Sources

Eight bacterial strains producing a BC pellicle on the HS medium were obtained from 54 rotten fruits, 11 traditional vinegars, and 10 soil samples (Table 1).

All samples from humus soil and beach formed a thin layer and increased turbidity in the test tube. However, those on the typical BC pellicle were different due to easy adsorption to the test tube wall. This thin layer was most likely a mold living in the soil, which was similar to the colony shape in the HS agar medium. No BC producers were detected in soil samples. When screening persimmon vinegars and rice wine vinegars, only three samples from persimmon vinegar formed thick pellicle of gel shape, while the rest of the vinegar sample formed white powder on the bottom of the test tube. After streaking on the HS agar medium, colony shapes were shown to be similar to those of control strains, which was Gram negative.

All the isolates were examined for the BC productivity in the HS medium. Since strain KJ-1 showed the best productivity, it was used in the subsequent studies.

Table 1. Numbers of screened and isolated strains with respect to sample source.

Sample		Screening sample	Isolated strain
Soil	Humus soil	8	0
	Beach	2	0
Traditional vinegar	Persimmon vinegar	8	4
	Rice wine vinegar	3	0
Fruit	Pear	11	1
	Apple	7	1
	Grape	7	2
	Orange	23	0
	Persimmon	3	0
	Fig	2	0
	Peach	1	0
Total		75	8

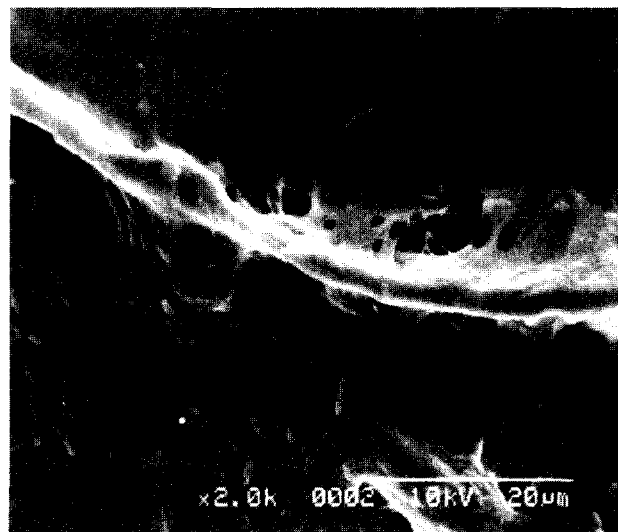

Fig. 1. Scanning electron micrograph of *A. xylinum* KJ-1 and its produced BC.

Morphological and biochemical characteristics of the strain KJ-1 were examined and compared with those of *A. xylinum* 40407 and *A. xylinum* 40274. The scanning electron micrograph of *A. xylinum* KJ-1 showed ellipsoidal to rod shapes as seen in Fig. 1. The strain KJ-1 and control strains were 0.6–0.8×1.0–3.0 µm, g-negative, in pairs, and in chain of cells (Table 2). The overoxidation of ethanol and ketogenesis of glycerol were positive. The strain KJ-1 produced 5-ketogluconic acid from D-glucose, but not 2,5-diketogluconic acids from D-glucose, and grew in the presence of 10% ethanol. When the KJ-1 strain was statically cultivated in culture broth, a thick cellulose pellicle was formed. The unique BC pellicle formation was a good indicator of a cellulose producer [17]. All biochemical tests, except the ethanol tolerance experiment, showed the same result as in Bergey's manual and also control strains. According to the report of Gossele *et al.* (unpublished results), *Acetobacter* sp. showed 100%, 87%, 82%, 58%, and 13% growth in media containing 0%, 1%, 2%, 5%, or 10% ethanol, respectively [9]. Therefore, the strain KJ-1 was estimated to be more tolerant than typical *Acetobacter* sp. Consequently, those characteristics were consistent with the description of the *Acetobacter xylinum* in the Bergey's manual, suggesting that strain KJ-1 should be named as *A. xylinum* KJ-1.

Initial Conditions of Cultivation

To discover the optimal conditions for BC production by *A. xylinum* KJ-1, we investigated effects of initial pH, shaking speed, and carbon and nitrogen sources. *Acetobacter* sp. was grown well in the aerobic condition, which was greatly influenced by oxygen concentration in the culture broth [9, 18]. Toyosaki *et al.* reported that baffled flasks

Table 2. Identification of the isolated strains.

Characteristics	*Acetobacter xylinum**	Isolated strain
Morphological tests		
Size	0.6–0.8×1.0–3.0 μm	0.6–0.8×1.0–3.0 μm
Shape	Ellipsoidal to rod shape	Ellipsoidal to rod shape
Arrangement of cells	Singly, in pairs, in chains	Singly, in pairs, in chains
Gram stain	Negative or variable	Negative
Biochemical tests		
Overoxidation of ethanol	+	+
Formation of water-soluble brown pigments on GYC	-	-
Formation of γ-pyrones from		
D-glucose	-	-
D-fructose	-	-
Formation of 5-Ketogluconic acid from D-glucose	+	+
Formation of 2,5-Diketogluconic acid from D-glucose	-	-
Ketogenesis from glycerol	+	+
Growth in the presence of 10% ethanol	-	-
Growth in the presence of 30% D-glucose	-	-
Growth in carbon sources		
Ethanol	-	-
Dulcitol	-	-
Sodium acetate	-	-
Methanol	-	-
Growth in L-amino acids in the presence of D-mannitol as the carbon source		
L-glycine	-	-
L-threonine	-	-
L-tryptophan	-	-
L-glutamine	-	-
L-aspargine	-	-
Growth in the presence of 10% ethanol	-	+
Growth in the presence of 30% D-glucose	-	-
Production of cellulose	+	+

*Bergey's manual

were better for BC production in shaken cultures than smooth-walled flasks [17]. In the present study, *A. xylinum* KJ-1 was cultured in both the conventional and slant-baffled flasks for 5 days, and BC productivity was found to be 1.4 g/l in the former and 2.1 g/l in the latter, showing higher productivity in the slant-baffled flask. The conventional flask in shaking cultures can not efficiently transfer oxygen to the inside of the culture broth and disperse the solid materials [17]. Therefore, the slant-baffled flask was used in the following shaking cultures.

The effects of the initial pH of the medium on BC production were examined. When *Acetobacter xylinum* KJ-1 was cultured at 30°C for 5 days at various initial pHs of 3, 4, 5, 6, 7, 8, 9, and 10, the BC productivity at initial pH 7.0 was the highest with 2.4 g/l (Table 3). On the other hand, BC production decreased noticeably below initial pH 4 and above pH 8.

The effects of shaking speed were examined at 100, 150, and 200 rpm (Table 3). The maximum BC productivity was observed to be 2.1 g/l at 150 rpm. When shaking at 100 rpm, the shape of the BC pellicle was a very large ellipse. On the other hand, in the case of shaking at 150 and 200 rpm, the shape of the BC pellicle was small compared to that formed at 100 rpm.

Table 3. Effects of initial pH and shaking speed (rpm) on BC production.

Initial conditions		BC productivity (g/l)	Final pH
Shaking speed (rpm)	100	1.8927	
	150	2.0570	
	200	0.0790	
Initial pH	3	0.0690	2.80
	4	0.5930	3.20
	5	2.0570	3.55
	6	2.0620	3.74
	7	2.3880	4.70
	8	1.8600	7.36
	9	1.6370	7.64
	10	0.0520	7.74

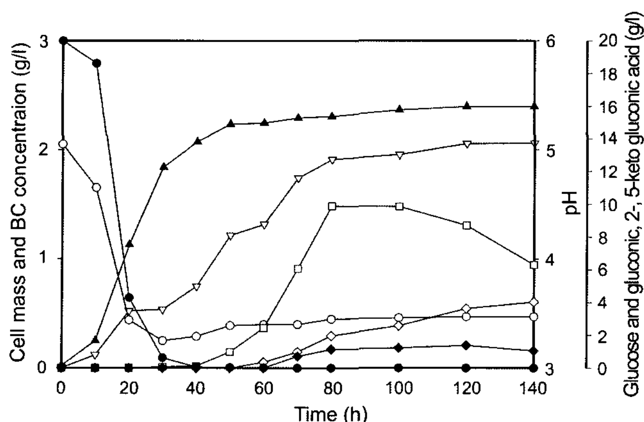

Fig. 2. Time courses of cell mass, BC, pH, gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid, and glucose concentrations in the shaking culture with a baffled flask by *A. xylinum* KJ-1.
Cell mass (▲); BC (▽); pH (○); gluconic acid (□); 2-ketogluconic acid (◇); 5-ketogluconic acid (◆); glucose (■).

Figure 2 shows the time course of BC production by *A. xylinum* KJ-1 for 140 h in a slant-baffled flask. The concentration of residual glucose and pH of the culture broth rapidly decreased in 10 h and then the concentration of residual glucose was negligible in 40 h. On the other hand, the cell mass and BC productivity rapidly increased, and most BC production was obtained in the latter part of the logarithmic growth phase. The glucose was converted to gluconic acid, 2- and 5-ketogluconic acid, by *A. xylinum* KJ-1 in 40 h. D-Glucose as a carbon source is actively converted into (keto) gluconic acids by membrane-bound *Acetobacter* dehydrogenases. This reaction lowers not only the overall cellulose yield, but also the medium pH to sub-optimal levels for cell viability and cellulose synthesis [10, 16].

Effects of Carbon Sources on BC Production

The effects of carbon sources on BC production were examined. When *A. xylinum* KJ-1 was cultured in the medium using fructose for 5 days, the maximum BC productivity was 2.7 g/l (Fig. 3). *A xylinum* KJ-1 grew effectively, while producing BC, in the medium containing

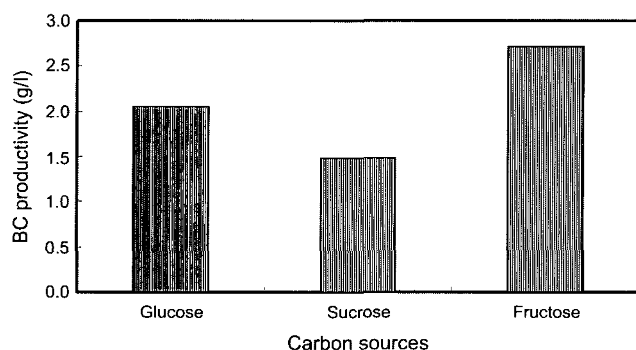

Fig. 3. Effects of carbon sources on BC production.

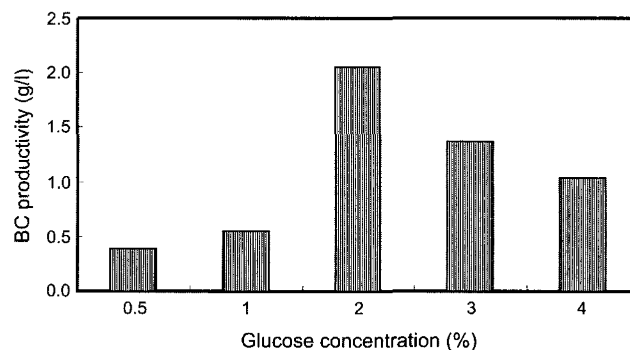

Fig. 4. Effects of glucose concentration on BC production.

2% carbon source such as glucose or fructose (Fig. 3 and Fig. 4), but BC production was low by sucrose or xylose as the carbon source. It has been generally suggested that glucose is more easily converted into gluconic acid and ketogluconic acid than cellulose [10, 22], and cell growth is inhibited in the culture at low pH due to these acids [6, 11], resulting in the lower production of BC with glucose than with sucrose. However, *A. xylinum* KJ-1 in the culture including sucrose as the carbon source gave different results, showing less BC productivity than those including fructose or glucose as shown in Fig. 3. Figure 5 shows the effects of mixed carbon sources with glucose and fructose on the BC production. When the ratio of glucose:fructose (1:3) was added to the medium, BC productivity was maximum with 2.8 g/l. With these results, a mixed carbon source was better than glucose or fructose alone as a sole carbon source.

Effects of Nitrogen Sources on BC Production

Nitrogen sources, such as yeast extract, bactopeptone, and corn steep liquor, have been generally used for the BC production of *A. xylinum* [1, 3, 6, 10, 11, 17]. A cheaper nitrogen source should be preferable in the economical BC production. We investigated a cheap nitrogen source with high productivity for the BC production by *A. xylinum* KJ-1. Figure 6 shows the results with 0.5% yeast extract with 0.5% bactopeptone; 2% (v/v) corn steep liquor; 1%

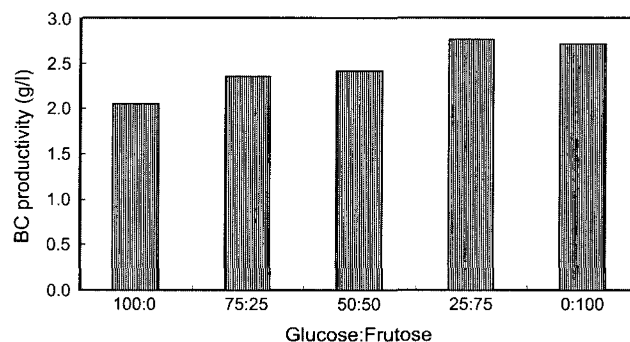

Fig. 5. Effects of mixed carbon sources based on the ratio of glucose to fructose on BC production.

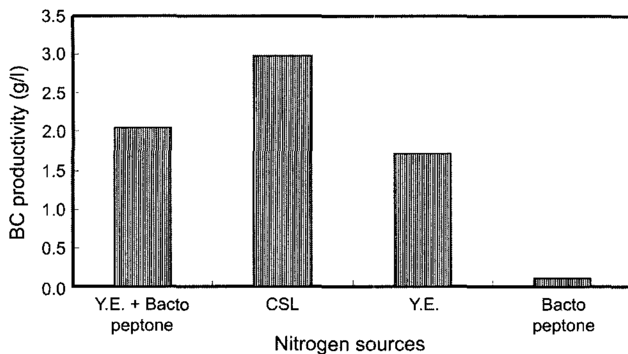

Fig. 6. Effects of nitrogen sources on BC production.

yeast extract; and 1% bactopeptone as nitrogen sources on the BC production. With CSL as nitrogen sources, the BC production was favorable, while bactopeptone alone was not effective for the BC production. When 2% (v/v) CSL was added to the HS medium, BC productivity increased up to 2.98 g/l, while with yeast extract or yeast extract with bactopeptone, the BC production was not much improved. These results indicated that *A. xylinum* KJ-1 seemed to efficiently use the ingredients contained in the CSL, such as lactate, amino acid, organic acid, and vitamin as nitrogen and carbon sources, indicating that CSL could improve the BC production [11].

Therefore, effects of CSL at various concentrations on the BC production were examined. Figure 7 shows that BC productivity increased with the addition of CSL. When 2% to 10% of CSL was added to the HS medium containing glucose as a carbon source, BC productivity increased almost 1.8 times, while by the addition of 12% to 20% CSL, BC productivity decreased, suggesting that CSL could serve more effectively as nitrogen sources of *A. xylinum* KJ-1 to synthesize BC than yeast extract and bactopeptone.

BC Production in Optimal Conditions

As shown in Fig. 7, in the HS medium containing 2% glucose, 0.27% Na_2HPO_4, 0.015% citric acid monohydrate,

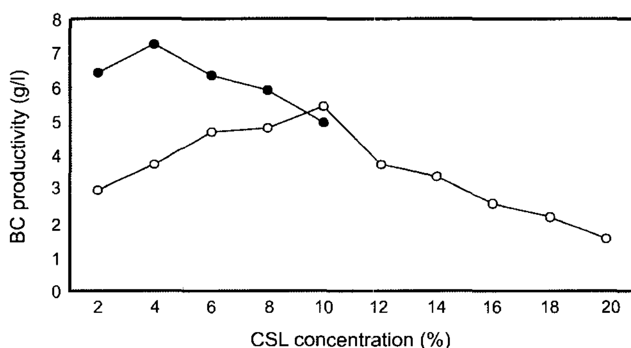

Fig. 7. Effects of CSL concentration on BC production.
Glucose:fructose (1:3) as carbon source (●), and glucose as carbon source (○) are shown.

and various concentration of CSL, BC productivity was maximum at 10% (v/v) CSL. Based on the above results, a medium containing 0.5% glucose, 1.5% fructose, 10% (v/v) CSL, 0.27% Na_2HPO_4, and 0.015% citric acid monohydrate seemed to be the most suitable composition for the BC production. Unexpectedly, however, BC productivity was very low with this composition, because of the presence of fructose. Therefore, the additional effects of CSL at the various concentrations were studied in the HS medium containing 0.5% glucose plus 1.5% fructose. When *A. xylinum* KJ-1 was cultivated at 30°C for 5 days in the medium containing 4% (v/v) CSL, BC productivity showed the maximum of 7.2 g/l (Fig. 7), and the BC productivity was more than 3 times higher than that in the HS medium. This BC productivity was significantly higher than those previously obtained with shaking cultures [10, 17]. Therefore, these optimal conditions could be applied on an industrial scale for *A. xylinum* KJ-1.

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