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High Bacterial Cellulose Production by *Acetobacter xylinum* ATCC 10245 in a New Culture Medium with a Sulfite Pulping Waste Fraction

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Abstract: Higher cellulose production was observed in a new culture medium (named KC medium) consisting of a sulfite pulping waste fraction (named CP powder), glucose, yeast extract, peptone and buffer components as compared with the standard 2% glucose Hestrin-Schramm culture medium (HS medium). *Acetobacter xylinum* ATCC 10245 cultivated in KC medium of 2% glucose gave high cellulose yields (296%), and the highest yield was obtained at the 3% dosage of glucose and 0.5% dosage of the CP powder (326%). The fractionation and the ion exchange experiments suggested that the high molecular lignosulfonate contributed to the yield enhancement.

1. Introduction

Bacterial cellulose is a product of certain microorganisms that can synthesize cellulose. There are a large number of bacteria which can produce cellulose, for example, *Alcaligenes* sp., *Pseudomonas* sp., *Aerobacter* sp., *Rhizobium* sp., *Agrobacterium* sp., and *Acetobacter* sp., [1-3]. However, only the cellulose produced by *Acetobacter xylinum* has been extensively studied as a model for cellulose biogenesis [1, 2, 4]. The cellulose produced by *A. xylinum* resembles the plant cellulose, but shows higher purity and crystallinity [1, 2]. The bacterial cellulose has more excellent qualities than the other fibers; for example (1) outstanding hydrophilicity, (2) affinity for the dye (dyeability) and (3) high tear strength (about 5 times stronger than polyethylene) [5]. Thus, it has been reported to be applicable to many products, such as high-quality paper, fiber-forming materials, food additives, acoustic diaphragms and synthetic skin [1, 6]. However,

the commercialization is limited to a small area because of the extremely high production cost. If the bacterial cellulose could be produced at a moderate price, it should become utilized extensively. We reported that among several *A. xylinum* strains (ATCC 10245, IFO 13772, and IFO 3288), ATCC 10245 showed the highest efficiency for cellulose production in the standard Hestrin-Schramm (HS) medium which contained 2% of glucose [7]. We also showed that *A. xylinum* could produce cellulose membranes from various kinds of inexpensive carbon substrates, such as ethanol, methanol, glycerol and inositol. Among them, glycerol gave the maximum cellulose yield [7, 8].

In our preliminary study, we found an effective new culture medium while we examined the effect of the addition of a sulfite pulping waste fraction (commercial name: SAN PEARL CP, Nippon Paper Industries) to standard HS medium. We named this new culture medium as KC medium after KOCHI. This KC medium was examined

Table 1 Media Composition for Cellulose Production of *Acetobacter xylinum*^{a)}

Medium	Yeast extracts	Peptone	Glucose	CP powder ^{b)}	Buffer system		Initial pH
					Citric acid	Na ₂ HPO ₄	
HS medium	0.5	0.5	2.0	-	0.115	0.27	6.0
KC medium	0.5	0.5	2.0	0.5	0.27	0.115	6.0

^{a)} The values in the table indicate the percentage of contents in the media (w/v).

^{b)} Commercially available wood based sulfite pulping waste liquor fraction isolated by ultra-filtration and spray drying ("SAN PEARL CP", Nippon Paper Industries).

further in this paper.

2. Experimental

2.1 Culture Media

The HS medium[9] is widely used as the standard culture medium for the production of bacterial cellulose. The chemical ingredients of the HS and KC media are shown in Table 1. The KC medium differs from HS medium in the two points. One is the presence of CP powder and the other is the proportion of the buffer components, citric acid and Na_2HPO_4 . The new buffer system of KC medium was found by chance, and its effectiveness was confirmed by several experiments. There still remain some points to be solved for this buffer system, therefore the function of the buffer is being further studied.

In each experiment, 30 mL of a prepared culture medium was added into more than five 100 mL Erlenmeyer flasks and sterilized by autoclaving for 15 min at 121 °C.

2.2 Culture Conditions

A. xylinum ATCC 10245 was grown on 15% agar slant HS medium for 48 h. One loop-full was inoculated to each flask of the liquid culture medium. The culture media in the flasks were incubated under both static and swirling conditions at 29 °C for 7 days. Under swirling conditions, the culture was gently swirled by hand two times a day for 30 sec throughout the incubation period.

2.3 Isolation of Cellulose Membranes

The cellulose membranes were washed with distilled water and pretreated with 1% NaOH at 90 °C for 15 min. After cooling, the membranes were neutralized with 1% acetic acid, thoroughly washed with distilled water and air dried on glass plates. The cellulose yields were measured after drying at 105 °C. The yield data under various conditions were shown in the average from five to fifteen repeated experiments. The variation coefficient was 4.79% in average.

2.4 Gel Filtration of CP Powder Solution on Sephadex Column

The CP powder was fractionated by gel filtration through a Sephadex G-25 fine column (ϕ , 22 × 470 mm), and distilled water as an eluant. Two milliliters of 10% CP powder solution was applied on the top of the column and 5 mL fractions were collected at a flow rate of 1 mL/min. Elution was monitored both by the absorbance

Fig. 1 Comparison of cellulose yields in different culture media under static conditions.

at 280 nm and the sugar determination by the phenol-sulfuric acid method[10].

2.5 Ion Exchange of CP Powder Solution

The ion exchange of the CP powder aqueous solution was performed through AMBERLITE resin IR-120 B column (ϕ , 24 × 300 mm). Distilled water was used as an eluant. Five milliliters of 20% CP powder solution was applied on the top of the column and 500 mL of eluate was collected at a flow rate of 1 mL/min. The eluate was freeze-dried for storage.

3. Results and Discussion

3.1 Comparison of Cellulose Yields in Different Culture Media under Static Conditions

Various culture media were examined for the cellulose production of *Acetobacter xylinum* ATCC 10245. Some typical results obtained for the modified HS media are summarized in Fig. 1. The standard carbon source of the HS medium is 2% glucose (A). Usage of 2% glycerol instead of glucose gave 152% of cellulose yields based on the standard HS medium (B), therefore it was a better carbon source than glucose. When the CP powder which was separated from the sulfite pulping waste liquor was added to the HS medium, the cellulose yields increased to 256% (C). The KC medium which contained 0.5% CP powder and 2% glucose gave the highest yield about 288% (D).

If the KC medium did not contain the CP powder, the yield was only 110% (E), still slightly higher than the standard medium. It is obvious that CP powder has a re-

markable effect on the cellulose production enhancement while the alternation of the buffer system has only slightly positive effect.

3.2 Effect of CP Powder on Cellulose Production under Static and Swirling Conditions

According to the hypothesis of Hestrin and Schramm, *A. xylinum* produces less cellulose under swirling than static conditions [9]. However, we found in the preliminary experiment that the swirling of culture medium showed slightly positive effects on the cellulose production in the HS medium (Fig. 2). This might be due to the increase of oxygen supply to the bacterial cell which promoted the cellulose production. However, in the case of the KC medium without CP powder the swirling conditions showed rather negative effects (Fig. 3). The reason for this is not known, but it might be possible that the buffer change could have affected the oxygen supply system to the bacterial cell. In the KC medium with CP powder under static conditions, the cellulose yields gradually increased as the dosage of CP powder increased, but under swirling conditions the yields were affected adversely. The reason for this is also unknown. The increase stopped at 0.5% dosage of CP powder under static conditions, and further dosage increase did not show any yield improvement (Fig. 3).

3.3 Effects of Glucose Concentration in KC Culture Medium on Cellulose Production

Usually 2% of glucose are used as a carbon source of standard culture medium. The cellulose yields of the HS media with 0.5% CP powder and those of the KC media which also contained 0.5% CP powder with different glucose dosage are shown in Fig. 4. The cellulose yields in both media were gradually increased when the glucose dosage increased and then decreased showing maxima. The highest yield was 326% at 3% glucose dosage in the KC medium. In the HS medium with 0.5% CP powder, it was 256% at 2% glucose. At 1% glucose dosage, the HS medium with 0.5% CP powder gave a slightly higher cellulose yield (132%) than the KC medium. It is obvious that at high glucose concentrations (2-5%) in the KC medium, *A. xylinum* can produce more cellulose than in the HS medium with 0.5% CP powder.

3.4 Fractionation of CP Powder

To investigate the effects of CP powder in detail, the CP powder was fractionated by the gel filtration with Sephadex G-25. CP powder was divided into two frac-

Fig. 2 Comparison of cellulose yields in HS medium with 2% glucose under static and swirling conditions.

Fig. 3 Effect of dosage of CP powder on cellulose production under static and swirling conditions: Yields are on standard HS medium.

Fig. 4 Comparison of cellulose yields in KC and HS media with 0.5% CP powder at different glucose concentrations.

tions, A and B (yields (w/w) were 24.5% and 75.5%, respectively) with different molecular weight distribution as shown in Fig. 5. The detection was based on two methods, one is the UV absorption at 280 nm which is a characteristic absorption maximum of lignosulfonate and the other is the phenol-sulfuric acid method [10] which is based on the color reaction of carbohydrates. The divided fraction A is of higher molecular mass with a low sugar content, which might consist mainly of lignosulfonate as it shows strong UV absorption at 280 nm [11]. On the other hand, the fraction B which shows a strong sugar color reaction and low UV absorption is deduced to consist mainly of low molecular weight carbohydrates and lignosulfonates.

3.5 Effects of Fractions A and B on Cellulose Production

Either fraction A or B was added to the KC medium in place of the whole CP powder based on their yield. The effects of each fraction are shown in Table 2. The effi-

ciencies for the cellulose production were calculated by dividing the yield (mg) by the dosage weight of each fraction (mg).

The maker of CP powder indicated that the chemical components of the CP powder consist of low molecular weight lignosulfonates (about 30% w/w), sugar metamorphic compounds (34%), reducing sugars (34%) and minerals (2%). From the results shown in Table 2, it is clear that the efficiency of fraction A is about three times higher than that of fraction B and the whole CP powder, therefore fraction A of the CP powder is a dominant factor on cellulose production. As fraction A is a purified lignosulfonate fraction, this water soluble lignin might be the cause of the effective bacterial cellulose production enhancement.

The CP powder contains some mineral salts (2%). It was reported that mineral salts are unnecessary to support growth and cellulose production [12]. We tried to eliminate the minerals from the CP powder by AMBER-LITE resin IR-120B. The KC medium with 0.5% of mineral-free CP powder showed the same yield as the untreated CP powder, and the minerals are not the cause of the yield enhancement.

Ross et al. [13] reported that magnesium is an essential element for growth and cellulose synthesis, and it is also reported that for successful functioning of *A. xylinum* cellulose synthase [14] must be activated by guanyl oligonucleotide.

In our system, yeast extracts were added together with the CP powder, and these requirements must have already been achieved.

It is very interesting to know the biochemical effects of the lignosulfonate-carbohydrate complex (LCC) fraction in the CP powder. Inaba et al. have reported that the fraction, almost identical to our fraction B in Fig. 5, could stimulate the mycelial growth and the following

Fig. 5 Gel filtration of CP powder: Eluates were diluted to have absorbance values between 0.5-1.5. Sugars were measured as glucose by phenol-sulfuric acid method.

Table 2 Comparison of Efficiency on Cellulose Production of Fraction A and B

Medium	Fraction weight mg ^{a)} (%)	Cellulose weight mg ^{a)} (%)	Efficiencies ^{b)}
KC medium with fraction A	36.8 (24.5)	48.3 (215)	1.31
KC medium with fraction B	113.2 (75.5)	46.2 (206)	0.41
KC medium with whole CP powder	150.0 (100)	66.2 (296)	0.44
HS medium	-	22.4 (100)	-

^{a)} In mg/30 mL culture medium. ^{b)} Cellulose yields (mg)/fraction dosage (mg).

fruiting body formation in certain edible mushrooms [15]. On the other hand, they observed a negative effect for the high molecular weight lignosulfonate fraction [16].

Some polyelectrolytes including poly(vinyl sulfonate) etc. were found to have similar immunological activities like lignosulfonate [11, 17]. Therefore, the polyelectrolytic nature of lignosulfonate might have some effects on the enhancement of the cellulose production by *A. xylinum*, but experimental support should be shown on this point.

4. Conclusion

The addition of CP powder to either standard or modified HS medium remarkably enhanced the efficiency of the cellulose production of *Acetobacter xylinum* ATCC 10245. The modified HS medium with CP powder was named as KC medium. The KC medium with 3% of glucose and 0.5% of CP powder gave the highest cellulose yield (326% based on the standard HS medium). The high molecular weight sulfonated lignin fraction separated from CP powder by gel filtration showed higher cellulose production efficiency than the other fraction rich in low molecular weight carbohydrates. The lignosulfonate might be the effective substance for the high cellulose production by *A. xylinum*.

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