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Optimization of Culture Conditions for Bacterial Cellulose Production by *Acetobacter* sp. 4B-2

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Abstract: One of the bacterial cellulose (BC) application problems in industry is its low productivity. So, the researchers have tried to increase the productivity of bacterial cellulose using various biochemical. In this study, production of BC using *Acetobacter* sp. 4B-2 by two categories of carbon sources (monosaccharide and disaccharides) were examined in the modified HS (Hestrin-Shramm) medium by simply replacing D-glucose with other carbon sources. Sucrose gave the highest yield, followed by glucose, xylose and lactose. The highest production of sucrose might be because of the rate of its consumption (80%), which is lower than that of glucose (93.5%). The best yield was achieved by using of 1.5% sucrose. The optimum pH of BC production was 7 and the optimum of temperature for producing BC was 30°C. After optimization of culture conditions, production was reached up to 11.98 g L^{-1} BC.

Key words: Carbon sources, (Hestrin-Shramm) HS medium, bacterial cellulose, *Acetobacter* sp. 4B-2, optimization

INTRODUCTION

Bacterial cellulose (BC) which is produced by some strains of the genera *Acetobacter* differs from plant cellulose with respect to its size, crystallinity and purity. Because of these physicochemical properties, recently there has been interest in new fields of application and development of new methods for its mass production (Bae *et al*., 2004). Static cultivation methods have used for the production of BC, with pellicles of BC being formed on the surface of the static culture (Son *et al*., 2003). In most reports, the cellulose productivity decreased in agitated culture (Hwang *et al*., 1999). One of the BC application problems in industry is its low productivity. Therefore, researchers have tried to increase the productivity of bacterial cellulose using various biochemical (Keshk and Sameshima, 2005). Further investigations of culture conditions are essential to achieve industrial levels of cellulose production. We describe here the production of the BC from two categories of carbon sources and examined their efficiencies to produce the BC. The relationships between the BC yield and the final pH of the culture media during and after the incubation period were examined. Also, the optimization of sugar concentrations used in production of cellulose membrane and optimization of nitrogen source concentrations, initial pH of the medium and temperature were studied.

MATERIALS AND METHODS

Culture media and conditions: The *Acetobacter* sp. 2-4B strain used in this study was recently isolated from the traditionally fermented vinegar in Iran that according to the 16 S rRNA sequencing this wild type isolate is belong to the *Acetobacter* sp. Most of the chemicals which used in this study were provided from Merck company except I, I, 4 -β- D- endoglucanase from *Trichoderma reesei* (EC 3. 2. 1. 4.) (Sigma-aldrich) and I, I, 4- β- D- exoglucanase from *Aspergillus niger* (EC 3. 2. 1. 91) (Serva). Medium of Hestrin and Schramm (%, w/v): glucose, 2.0; peptone, 0.5; yeast extract, 0.5; disodium phosphate, 0.27; citric acid, 0.115; pH adjusted to 6.0 with dil. HCI or NaOH or in the modified media by simply replacing D-glucose with other carbon sources. The prepared 30 mL culture medium in 100 mL Erlenmeyer flasks were sterilized by autoclaving and were inoculated from the solid agar culture of the bacterium and incubated at 30°C for 8 days. All the conditions for optimization include sugar concentration; nitrogen sources, pH and temperature were performed at least in triplicate and were represented by the average. SPSS software 14.0 version was used for statistical analysis. Deviation from the average never exceeded 10%. Since, the concentration of BC in culture broth was determined by the weight of purified BC dried in a vacuum oven, for decreasing error optimization of pH was done at the ranges of 4.0, 5.0, 6.0, 7.0 and 8.0 and optimization of temperature was done at the ranges of 27, 30, 37, 40 and 45°C.

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Purification of pellicles: The samples of culture broth were centrifuged at 4000 rpm for 20 min. The precipitated leathery pellets were treated with 0.5 N NaOH solution at 80°C for 30 min to remove the bacterial cells and medium components. The pellets were then rinsed with deionized water.

This procedure was repeated three times (Bae *et al*., 2004). The concentration of BC in culture broth was determined by the weight of purified BC dried in a vacuum oven at 80°C (Naritomi *et al*., 2002). Supernatants were combined and kept for analysis.

Analytical methods: Hydrolysis of bacterial cellulose was carried out by enzyme. After alkali treatment, the pellet were washed seven times by resuspension in excess of distilled water followed twice by resuspension in 0.5 mL NaAc buffer, pH 5 and stored at 5°C. I, I, 4-β-D-endoglucanase from *Trichoderma reesei* (EC 3.2.1.4.) (Sigma-aldrich) and I, I, 4- β- D-exoglucanase from *Aspergillus niger* (EC 3. 2. 1. 91) (Serva) were used. Enzymatic hydrolysis experiments were carried out in tubes by incubating of producing pellicle treated suspension in 500 μL 0.05 M NaAc buffer, pH 5.0 with 200 μL of each of enzyme diluted with 0.05 M NaAc buffer. Each of enzyme mixture should contain 0.5 U. The reaction was initiated by addition of enzyme followed by vortex mixing for 10 sec in 5 h but the incubation was continued for 24 h to make sure of the complete hydrolysis. Compositional sugar analysis was made by addition of 1 mL of DNS reagent. The cellulose residue was pelleted by centrifugation (13000 g, 5 min) and the concentration of the total sugar in the suspernatant was determined by spectrophotometer (Perkin-elmer junior model 35). Each time point was analyzed for total sugar at least in triplicate and was represented by the average. Deviation from the average never exceeded 10%. The reducing sugars produced were determined by the method of using DNS reagent (Yongchao *et al*., 2007). The amount of released sugars and the amount of cellulose production was calculated using a standard curve recorded for pure cellulose (sigma-aldrich) hydrolysis. Consumption of glucose in the medium after incubation was calculated by using DNS reagent and consumption of sucrose in the medium after incubation was calculated by using anthrone reagent.

RESULTS

BC production, sugar consumption and pH changing during cultivation of *Acetobacter* sp. 4B-2 in glucose and sucrose media is shown in Fig. 1 and 2. The final pH of sucrose medium was 5.46 and the final pH of glucose medium was 4.48.

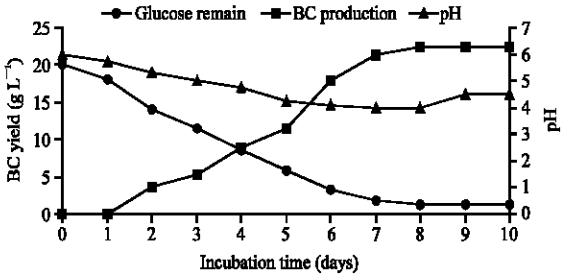

Fig. 1: Change in BC yield, glucose remaining and pH during cultivation period of *Acetobacter* sp. 4B-2 after three replicates

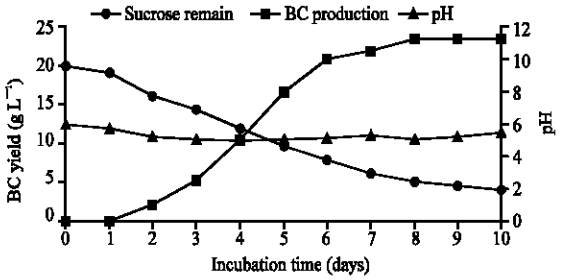

Fig. 2: Change in BC yield, sucrose remaining and pH during cultivation period of *Acetobacter* sp. 4B-2

Table 1: The effect of various carbon sources on BC production

	Yield (%)* (Reference)		
			Yield
Carbon source	Keshk and Sameshima (2005)	Jonas and Farah (1998)	(%) 2-B4
Monosaccharides			
Glucose	100	100	100.00
Galactose	24	15	21.26
Arabinose		14	15.87
Xylose	38	11	53.17
Disaccharides			
Lactose	22	16	53.17
Maltose	25	7	24.45
Sucrose	69	33	187.41

BC yield of glucose was set as 100%

From the consumption percentage and the yield of the cellulose membrane after eight days incubations, the BC production efficiencies have been calculated for the two main substrate. The consumption of sucrose was 80% while the consumption of glucose was 93.5%. The sucrose is the best substrate for cellulose production with the efficiency of 70% and the production efficiency of glucose 34%. Sucrose, glucose, D-xylose and lactose were found to be suitable for optimum levels of cellulose production. Table 1 shows the effect of various carbon sources on BC production and comparison between the strain 4B-2 and other BC producing ATCC strains.

Because sucrose had higher yield than glucose, the optimization was continued by this sugar as the better carbon source. The change in yield is shown in Fig. 3 as

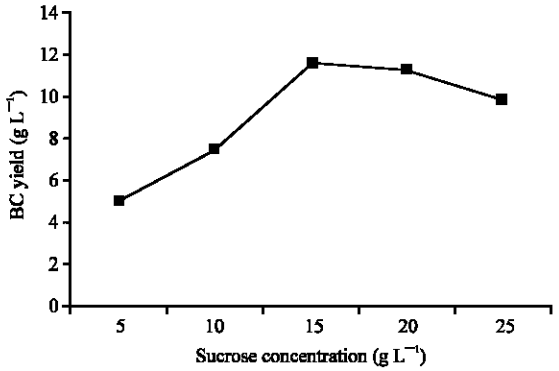

Fig. 3: BC yield from culture at various concentrations of sucrose

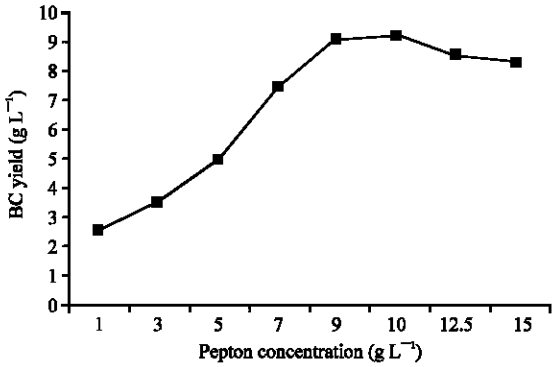

Fig. 5: BC yield from 1.5% sucrose culture at various concentrations of peptone

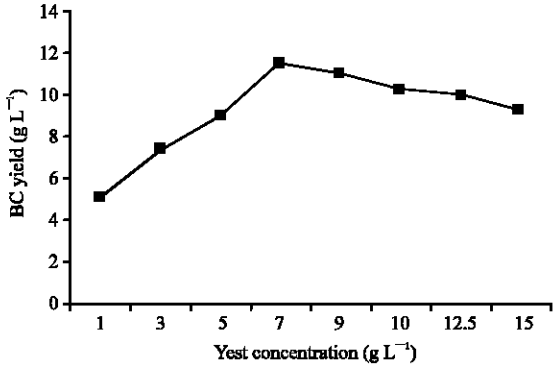

Fig. 4: BC yield from 1.5% sucrose culture at various concentrations of yeast extract

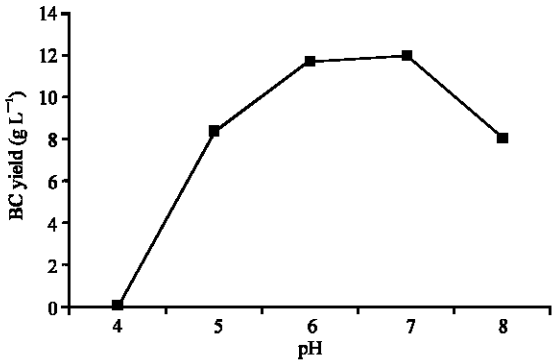

Fig. 6: BC yield from 1.5% sucrose 0.7% peptone and 0.7% yeast extract culture at various pH

Table 2: Various concentrations of yeast extract and peptone used simultaneously

	Yeast extract				
Peptone	2	4	6	8	10
2	4.49	4.65	7.75	10.45	4.50
4	5.30	5.15	8.80	10.20	4.00
6	5.10	6.60	8.50	11.43	11.00
8	5.50	5.89	7.74	11.65	10.58
10	8.85	7.70	8.00	11.15	10.25

The unit of all data in this is g L^{-1}

the function of the percentage of the sucrose concentration. The BC production was enhanced with increasing amounts of sucrose of up to 1.5%, but decreased when it was above 2% of sucrose.

In general, a nutritionally rich medium containing yeast extract and polypeptone supports good BC production of *Acetobacter* strains. Addition of amino acids did not affect BC production (Son *et al*., 2003). The different concentration of yeast extract and peptone were used separately as shown in Fig. 4 and 5. Yeast extract was very effective on BC production and the highest yield was obtained at 7 g L^{-1} of yeast extract and 9 g L^{-1} peptone. But at another experiment using different

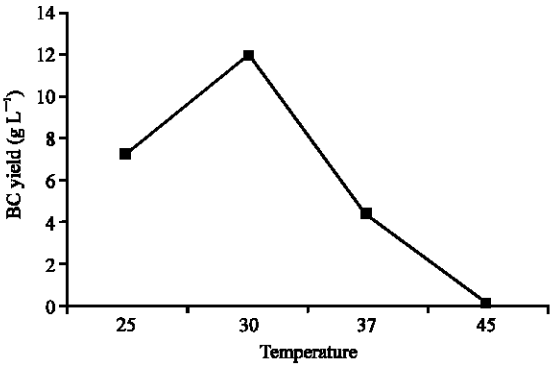

Fig. 7: BC yield from 1.5% sucrose and 0.7% peptone and 0.7% yeast extract culture at various temperature

concentrations of yeast extract and peptone the highest yield was obtained at 7g L^{-1} of each of these nitrogen sources as was shown on Table 2.

The highest yield was obtained at pH 7 as shown in Fig. 6. the lowest yield was obtained at pH 4.

The optimum temperature was 30°C as shown in Fig. 7. The lowest yield was obtained at 45°C.

Table 3: The comparison between the results of different cultural conditions used by different researchers

Volume (L)	Yield (g L^{-1})	System	Temp. (°C)	pH	Time (h)	References
-	9.7	Shaking culture	-	-	7	Tsuchida and Yoshinaga (1997)
30	20	Shaking culture	30	5	42	Kouda *et al.* (1998)
2	15	Shaking culture	30	5/5	50	Hwang *et al.* (1999)
Tubes	3	Static culture	30	5/6-7/5	4 weeks	Ishihara *et al.* (2002)
0.075	16.4	Shaking culture	30	5/6	192	Son *et al.* (2002)
0.61	21	Shaking culture	30	5	50	Naritomi *et al.* (2002)
0.1	12.8	Shaking culture	30	5	72	Bae *et al.* (2004)
0.03	-	Static culture	28	6	168	Keshk and Sameshima (2005)

DISCUSSION

During the cultivation, the appearance of the two acids gluconic acid and 5-ket-gluconic acid are responsible for the decrease of the pH-value of the culture medium during the first cultivation days. Monosaccharides are also converted by membrane-bound *Acetobacter* dehydrogenase into (keto) gluconic acids. The conversion of glucose to (keto) gluconic acids is not beneficial for overall cellulose productivity. The sharp decrease in the medium-pH probably not only cellulose formation, but also lowers the medium pH to suboptimal levels for cell viability and cellulose synthesis (Klemm *et al*., 2001). The pH of the glucose medium was lower than the sucrose medium and its yield was lower than also. Since, initial high glucose concentrations resulted in low yields of cellulose, it is desirable that batch cultures be started with a low glucose concentration (Hwang *et al*., 1999). The pH changes of the culture might be the indicator of the side reactions taking place in the cellulose production (Keshk and Sameshima, 2005). Keshk and Sameshima (2005) stated that the sucrose culture showed only slight decrease in final pH. Lactose showed negligible deviation from the initial pH of 6.0 (Keshk and Sameshima, 2005). That was corresponding to the result of this study. Glucose was consumed rapidly in the early stage of incubation and almost completely after 7 days of incubation while sucrose was not. D-xylose is the second most abundant sugar in nature next to D-glucose (Ishihara *et al*., 2002) and the 4B-2 strain used this carbon source very better than other strains and it produced 53.17% of BC during 8 days of cultivation. Masaoka *et al*. (1993) reported that the yield of cellulose, relative to the amount of glucose consumed, decreased with increase in the initial glucose concentration (Masaoka *et al*., 1993). According to Son *et al*. (2003) BC production was enhanced with increasing amount of glucose of up to 1/5%, but decreased when it was about 2% of glucose (Son *et al*., 2003). However, Keshk and Sameshima (2005) reported that the maximum yield was obtained at 1% concentration whereas, the minimum yield was observed at both 2 and 3% concentration (Keshk and Sameshima, 2005). But in this study, the maximum yield was observed at 1.5% sucrose. As suggested by Fontana *et al*. (1997) and Hestrin and Schramm (1954), a nutritionally rich medium containing yeast extract and polypeptone supports good BC production of *Acetobacter* strains (Hestrin and Schramm, 1954; Son *et al*., 2003). According to the result of this research both yeast extract and peptone were necessary for high production of BC. It is generally accepted that optimal pH range for cellulose production is 4-7. Most researchers used pH 5 or 6 in their researchers studies. At first, we used pH 6. But after optimization the best yield was obtained at pH 7. Jonas and Farah (1998) stated that the optimal growth temperature for cellulose production is 25-30°C, although most researchers used 28-30°C . With 30°C we observed better cellulose yield. The comparison between the results of different cultural conditions used by different researchers was shown at Table 3. While in this study we used static culture by inoculating *Acetobacter* sp. 4B-2 at HS medium that after optimization, it produced 11.98 g L^{-1} BC. The results obtained from this report should help design a better strategy for the production of BC.

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