한국생물공학회지 제 11권 제 5호
Korean J. Biotechnol. Bioeng.
Vol. 11. No. 5. 550-556(1996)

*Acetobacter xylinum*에 의한 미생물 셀룰로오스의 생산을 위한 배지 최적조성

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The Optimal Medium Composition for the Production of Microbial Cellulose by *Acetobacter xylinum*

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ABSTRACT

A complex medium was developed for the production of microbial cellulose by *Acetobacter xylinum* ATCC 23769. The optimum concentration of each nutrient for the production of microbial cellulose was determined to be 10 g peptone, 20 g yeast extract, 5 g glucose, 1.56 g Na_2HPO_4, 1.8 g KH_2PO_4, 0.05 g $MgSO_4$, 0.002 g $FeCl_3$, 5 g citric acid and 10 mL ethanol per liter. With synergistic effects of citric acid and ethanol, cellulose productivity achieved in developed medium was 0.446 gram of cellulose per gram glucose for static culture, which is much higher than reported values. Cell growth and the cellulose production in the developed medium under static culture was also investigated.

INTRODUCTION

Acetobacter xylinum, a gram-negative aerobic bacteria, secretes cellulose fibrils as part of its normal metabolic activity. Under electron microscope, microbial cellulose(MC) characteristically appears as a form of separate ribbon-like fibrils in contrast to the cellulose of high plants consisting of bundles of microfibrils(1). It possesses not only excellent physical properties, such as high degree of polymerization and preferential orientation, but also strong mechanical and absorbent properties, moreover, the fibrils of microbial cellulose are composed of pure cellulose, which is devoid of lignin, hemicellulose, and other substances, thus it can be purified more easily than natural cellulose. At present, microbial cellulose has found practical applications such as sensitive diaphragms for stereo headphones, additives for food and paper products, thickener for paint, and also as a temporary skin substitute in skin burn treatment(2-8).

Although *Acetobacter xylinum* has proved to be the greatest potential for the commercialization

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in industrial applications, reported values of cellulose productivity are too low for large scale production(4, 9-15). For this reason, the subject of how to improve cellulose productivity of *Acetobacter xylinum* has already absorbed many researchers interests. Investigations have been made on isolating high cellulose-producing strain (16), mechanism of cellulose biosynthesis and genetic structure involving cellulose secretion(13, 17-23). However, relatively few reports have discussed in details the relationship between cellulose production and culture conditions(3, 24), none was about the influence of nutritional sources on cellulose productivity after the first report of Schramm and Hestrin in 1954(14).

In the literatures(4, 9-11, 13-15) concerning the production of microbial cellulose by *A. xylinum*, peptone, yeast extract or $(NH_4)_2SO_4$ were used as nitrogen source, while glucose, mannitol, sucrose, fructose, citrate, or ethanol as carbon source, KH_2PO_4 and Na_2HPO_4 as phosphate source, and $MgSO_4$ or $FeCl_3$ as mineral elements. These medium compositions are the simple variations of Schramm and Hestrin's medium(14) or made by adding single component such as citrate, ethanol, and so on. In this article, we attempt to elucidate the influences of various kinds of nutritional sources, especially compounding effects of peptone, yeast extract and ammonium sulfate, synergistic effects of ethanol and citric acid, KH_2PO_4, Na_2HPO_4, $MgSO_4$ and $FeCl_3$, on the cellulose productivity so as to obtain a optimal medium composition.

MATERIALS AND METHODS

Cell Culture

Acetobacter xylinum ATCC 23769, provided by Bioproducts Research Center of Yonsei University, was maintained by serial transfer to fresh Hestrin-Schramm medium(12) every month and stored at 4 ℃ on Hestrin-Schramm agar plate. A loopful of microbe was inoculated in 20 mL Hestrin-Schramm liquid medium and incubated at 30 ℃ for six days. Cellulose pellicle formed at the air-liquid interface was cut into 5 mm dices, put into 20 mL medium and homogenized by a homogenizer(Biospec products, Inc. Germany) for two minutes. Homogenate was used as inoculum for further culture. 0.2 mL of the homogenate was inoculated into 20 mL of medium in a plastic cylinder tube(Volume = 50 mL, Surface area = 0.062 cm^2) and incubated at 30 ℃ for 6 days. The sample was taken from the liquid portion of culture and diluted with fresh medium and each 0.2 mL was applied evenly on an agar plate with a glass spreader. After 4 days incubation at 30 ℃, the number of the colony was counted and the number of living cells was calculated by multiplying the dilution factor. For this observation the agar plates having an appropriate number of the grown colony per plate(10-300) were selected.

Residual glucose

Residual glucose concentration was quantitatively determined by using enzyme assay kit (Sigma Glucose Reagent HK20). 10 μL of diluted culture medium solutions was added into 1 mL of aqueous glucose oxidase solution and mixed by gentle inversion. The mixture was incubated at 30 ℃ for 5 minutes. The optical density of the mixture was measured at 340 nm with glucose oxidase solution as reference. The glucose concentration was calculated by comparing with the absorbance of the standard glucose solution.

Amount of cellulose

Six days after inoculation, cellulose was collected by filtration on a paper filter. Filtered cellulose pellicle was washed with water, suspended in 4 % NaOH solution, and boiled at 100 ℃ for 20 minutes. The product was washed successively with deionized water, 0.5 % acetic acid, and deionized water, then dried overnight at 80 ℃ and weighed after cooling to room temperature. Cellulose productivity was expressed as the amount of cellulose produced per gram of glucose.

RESULTS

Effects of nitrogen source

Different concentrations of peptone, yeast extract and ammonium sulfate were tested for the production of cellulose by *A. xylinum*. Table 1 shows the effects of yeast extract, peptone and ammonium sulfate on production of cellulose as sole nitrogen source and added ammonium sulfate in the medium containing yeast extract or peptone at 5 gram per liter. With yeast extract or peptone as sole nitrogen source, cellulose production was satisfied, while ammonium sulfate alone was not effective for cellulose production. The addition of ammonium sulfate at concentrations of 1 to 5 gram per liter to medium containing 5 gram per liter of either peptone or yeast extract resulted in diminished cellulose production.

Effects of peptone and yeast extract at various concentrations on the cellulose productivity were studied. Fig. 1 shows that cellulose productivity increased with the increase in either peptone or yeast extract concentration. When 2 gram per liter of yeast extract was added to medium containing 5 or 10 gram per liter peptone, cellulose productivity was increased to almost three times, while 5 or 10 gram per liter of yeast extract was supplied, the increase in cellulose productivity was not significant. When yeast extract concentration was increased from 10 to 20 gram per liter of medium without peptone, cellulose productivity was found to be improved almost twice. When peptone concentration increased to 5 or 10 gram per liter in medium without yeast extract, cellulose productivity increased slightly. However, in the presence of 2 to 20 gram per liter of yeast extract, addition of peptone increased the production of cellulose significantly. With these results, yeast extract and peptone were found to together serve as nitrogen source for *A. xylinum* to synthesize glucose into cellulose.

Table 1. Effect of peptone, yeast extract, and ammonium sulfate on the production of microbial cellulose by *Acetobacter xylinum*.

Nitrogen Source(gram per liter)			Cellulose Productivity (gram per gram glucose)
Peptone	Yeast Extract	Ammonium Sulfate	
5	0	0	0.163
5	0	1	0.134
5	0	2	0.130
5	0	3	0.125
5	0	4	0.120
5	0	5	0.100
0	5	0	0.189
0	5	1	0.151
0	5	2	0.103
0	5	3	0.089
0	5	4	0.084
0	5	5	0.058
0	0	3	ND
0	0	5	ND

ND : Not Detected

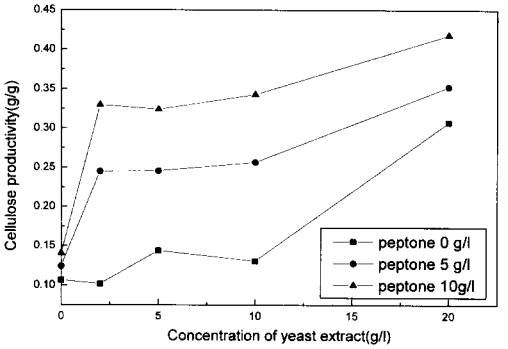

Fig. 1. Effects of peptone and yeast extract concentration on the production of cellulose.

Effects of carbon source

It has been reported that cellulose can be synthesized by *A. xylinum* from various carbon sources, oligosaccharides, starch, alcohol and organic acid(10).

Table 2 shows the cellulose productivity with various carbon sources. *A. xylinum* can utilize fructose, mannose, ethanol, citric acid and succinic acid to produce cellulose, but their cellulose productivity were much lower in comparison with that of glucose in the given medium.

Increasing concentrations of glucose up to 20

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

Carbon sources (10 gram/liter)		Cellulose productivity (relative % to glucose)
Monosaccharides	D-fructose	18.9
	Mannose	9.43
	D-galactose	ND
Disaccharide	Lactose	ND
	Maltose	ND
	Sucrose	ND
Polysaccharide	Starch	ND
Organic Acid	Citric acid	10.8
	Succinic acid	8.96
Others	Ethanol	3.78
	Glycerol	ND

ND: Not Detected.

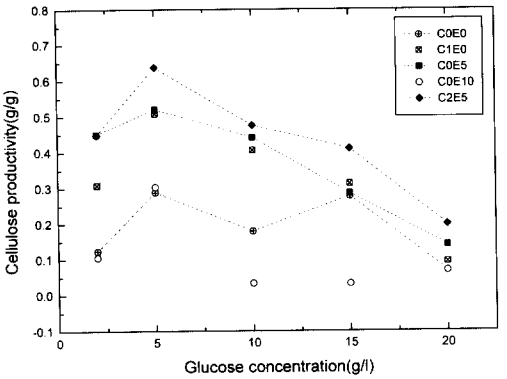

Fig. 2. Effect of glucose on the production of cellulose with various concentrations of citric acid and ethanol.
C: citric acid, E: ethanol
0, 1, 2, 5, 10: concentrations in gram or mL per liter

gram per liter increased the total cellulose production which can be calculated as productivity times glucose concentration, however the productivity of cellulose per glucose showed maximum value at 5 gram per liter(Fig. 2).

Synergistic effect of citric acid and ethanol

The effects of citric acid and ethanol concentration on cellulose production were shown in Fig. 3.

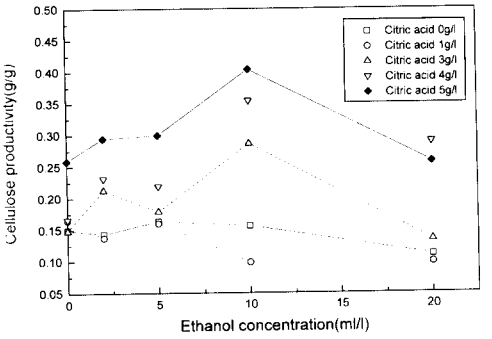

Fig. 3. Synergistic effect of citric acid and ethanol concentrations on cellulose production.

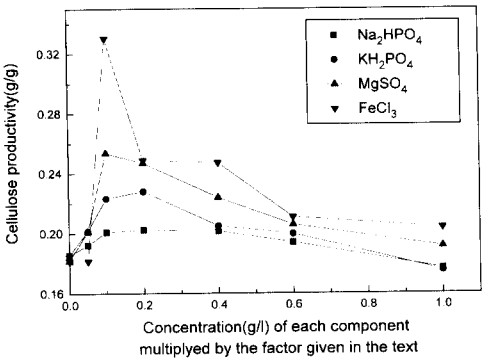

Fig. 4. Cellulose production in static culture on different concentrations of (A) $Na_2HPO_4 \times 1.56$, (B) KH_2PO_4, (C) $MgSO_4 \times 0.5$ and (D) $FeCl_3 \times 0.02$.

Although addition of ethanol alone up to 2 %(v/v) to the medium containing 5 gram of glucose per liter resulted in reduction of cellulose production, addition of citric acid at concentrations between 2 and 5 gram per liter to medium with glucose and ethanol increased cellulose productivity in a synergistic fashion. Fig. 3 shows that 5 gram of citric acid and 10 mL of ethanol per liter give the highest synergistic effects on cellulose productivity.

Effects of phosphate, $MgSO_4$ and $FeCl_3$ on cellulose production

Cellulose productivity at different concentrations of Na_2HPO_4, KH_2PO_4, $MgSO_4$ and $FeCl_3$ in

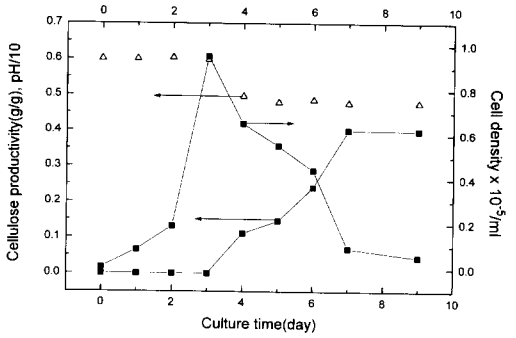

Fig. 5. Change in the cell number, cellulose productivity, and pH of culture broth in static culture of *A. xylinum* ATCC 23769 in optimal culture medium.
On left axis: open triangle-pH/10, closed square-cellulose productivity
On right axis: closed square-cell density
Culture conditions: cells were cultivated statically at 30 ℃ for 6 days.
Volume = 20 mL, Surface area = 0.062 cm^2.

static culture of *A. xylinum* are shown in Fig. 4. With increasing concentrations of these inorganic components, cellulose productivity increased initially, then subsequently decreased steeply. Na_2HPO_4, KH_2PO_4, $MgSO_4$ and $FeCl_3$ at 1.56 g/L, 1.8 g/L, 0.05 g/L and 0.002 g/L, respectively, gave the optimal cellulose productivity.

Cell growth and cellulose production curve under static culture

Fig. 5 shows the time changes of free cell number in liquid medium, cellulose productivity, and pH of culture broth during static cultivation. Cell number in culture liquid increased until detectable amount of cellulose produced very slowly first, then steeply increased on day 3, then decreased gradually until 7th day, followed by a region of no change thereafter. Cellulose productivity did not increase until day 4 and increased relatively rapidly when it reached plateau at day 7 with a production rate of 0.1 g cellulose/liter/day. pH was kept constant around 6.0 until day 3, then decreased to a rather constant value around 4.6.

DISCUSSION

Various nutritional sources for *A. xylinum* ATCC 23769 to produce cellulose were examined in this work. The developed medium contains 10 gram peptone, 20 gram yeast extract, 5 gram glucose, 1.56 gram Na_2HPO_4, 1.8 gram KH_2PO_4, 0.05 gram $MgSO_4$, 0.002 gram $FeCl_3$, 5 gram citric acid and 10 mL ethanol per liter. With synergistic effects of citric acid and ethanol, cellulose productivity achieved in developed medium by *A. xylinum* ATCC 23769 was 0.446 gram of cellulose per gram glucose for static culture, which is much higher than reported values. Table 3 shows cellulose productivity under static culture of *A. xylinum* in medium developed by other researchers. M. Romano(11) reported less cellulose production with medium of high yeast extract concentration than those of others(4, 9-11, 13-15) with lower concentration of yeast extract. The medium components contained in higher production cultures were ethanol, citric acid, phosphates and mineral elements separately in each case in a rather random way. All of which were found in this work to be important for *Acetobacter xylinum* to synthesize glucose into cellulose. Especially, optimal combination of ethanol and citric acid exhibited significant contribution on cellulose production.

We found that complex media containing yeast extract and peptone together were quite satisfactory for both cell growth and production of cellulose, whereas in defined medium *A. xylinum* was able to grow on ammonium sulfate as sole nitrogen source, but no cellulose was detected. Moreover, cellulose productivity was reduced when it was supplemented at concentrations up to 5 gram per liter to complex media. Obviously, NH_4^+ have a inhibitory effect on cellulose biosynthesis.

Our results suggest that glucose concentration up to 20 gram per liter did not cause catabolite repression on cellulose production by *A. xylinum*. However, the cellulose productivity decreased

Table 3. Comparison of cellulose productivity under static culture of *A. xylinum*.

Investigators (Ref. No.)	Strain	Productivity(g/g)
Hestrin & Schramm(12)	*A. xylinum*	0.058
K. Kamide(10)	IFO13693	0.05
S. Masaoka(13)	IFO13693	0.021
M. Romano(11)	ATCC 10821	0.010
T. Oikawa(15)	KU-1	0.031
D. Byrom(9)	ATCC 23789	0.09
This Work	ATCC 23769	0.446

with an increase in initial glucose concentration. Satosh and Tatasuhiko also reported same results (13). In order to determine the reason for the decrease in cellulose yield the concentration of gluconic acid was measured during cultivation. They observed that when the initial glucose concentration was 40 gram per liter gluconic acid accumulated to as high as 20 gram per liter, but addition of gluconic acid, however, did not affect the cellulose production. These results suggest that the decrease in cellulose productivity is ascribed to the partial metabolism of glucose to gluconic acid other than the inhibitory effect of gluconic acid on the cellulose production. A mutant deficient in glucose dehydrogenase activity might be more efficient for cellulose production in a medium with glucose as the sole carbon source. Peter Ross(3) reported a genetically stable strain with a substantially reduced ability to form gluconic acid produced reticulated, highly crystalline cellulose over 70 hours at a rate of 0.26 g/liter/day under shaking culture conditions.

A. xylinum has the ability of utilizing alcohol and organic acid to biogenesis cellulose by gluconeogenesis pathway. Haim and Moshe(25) investigated gluconeogenesis in the form of cellulose synthesis from succinate and pyruvate in resting cells of *A. xylinum*. They found that large amount of pyruvate and succinate was metabolized for respiration and much lower cellulose productivity were achieved. Our results show that when citric acid, ethanol and succinate acid was used as major carbon source cell grew well but produced very little cellulose. The reason for synergistic increase of cellulose productivity when ethanol and citric acid were supplemented together to medium containing glucose is not clear.

Static culture of *A. xylinum* in developed medium showed that cellulose production was not detectable during the logarithmic growth phase. Cellulose production was not effective until cell growth went into the stationary phase. This might mean that cellulose synthesis is effective under poor nutritional environments for bacterial growth. As cellulose being produced, bacterium attached to the cellulose floats to the air-liquid interface, so the cell number in liquid medium decreased gradually during cellulose production phase.

요 약

Acetobacter xylinum ATCC 23769에 의한 미생물 셀룰로오스의 생산을 위한 최적배지가 개발되었다. 미생물셀룰로오스의 생산을 위한 각 영양성분의 최적농도는 리터당 10 g peptone, 20 g yeast extract, 5 g glucose, 1.56 g Na_2HPO_4, 1.8 g KH_2PO_4, 0.05 g $MgSO_4$, 0.002 g $FeCl_3$, 5 g citric acid 그리고 10 mL ethanol로 결정되었다. 개발된 배지에서 수득한 셀룰로오스의 생산성은 구연산과 에탄올의 상승효과로 정치배양시 보고된 결과보다 매우 높은 포도당 단위그람당 0.446 그람이었다. 개발된 배지에서 정치배양시 세포성장 및 셀룰로오스의 생산 또한 조사되었다.

ACKNOWLEDGMENT

This study was supported partly by a research grant from Bioproducts Research Center of Yonsei University and partly by a research grant from Sun Moon University.

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