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Effects of Mixing Conditions on the Production of Microbial Cellulose by *Acetobacter xylinum*

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Microbial cellulose has many potential applications due to its excellent physical properties. The production of cellulose from *Acetobacter xylinum* in submerged culture is, however, beset with numerous problems. The most difficult one has been the appearance of negative mutants under shaking culture conditions, which is deficient of cellulose producing ability. Thus genetic instability of *Acetobacter xylinum* under shaking culture condition made developing a stable mutant major research interest in recent years. To find a proper type of bioreactor for the production of microbial cellulose, several production systems were developed. Using a reactor system with planar type impeller with bottom sparging system, it was possible to produce 5 g/L microbial cellulose without generating cellulose minus mutants, which is comparable to that of static culture system.

Key words: *Acetobacter xylinum*, microbial cellulose, bioreactor design, negative mutants, shaking culture

INTRODUCTION

Acetobacter xyilnum, a gram-negative aerobic bacteria, generally under static culture conditions secretes cellulose fibrils as part of its normal metabolic activity. Under electron microscope microbial cellulose (MC, or bacterial cellulose) 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. Using those characteristics of microbial cellulose, therefore many potential commercial applications are being developed. At present, microbial cellulose has found practical applications such as sensitive diaphragms for stereo headphones, additives for food and composite paper and textile products, thickener for paint, composite membrane, binder and dietary fiber supplement in food processing 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 in industrial applications, reported values of cellulose productivity are too low for large scale production [9-15]. For this reason the subject of how to improve cellulose productivity of *Acetobacter xylinum* has already absorbed interests of many researchers. Investigations have been made on isolating high cellulose-producing strain [16], mechanism of cellulose biosynthesis and genetic structure involving cellulose secretion [3,17-24]. However, relatively few reports have discussed in details the relationship between cellulose production and culture conditions [11,14], a few were about the influence of nutritional sources on cellulose productivity after the first report of Schramm and Hestrin [14]. In the literatures concerning the production of microbial cellulose by *A. xylinum* [3,9-15], peptone, yeast extract and $(NH_4)_2SO_4$ 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 Hestrins medium or made by adding single component such as citrate, ethanol, and so on. Therefore, for these newly developed applications to be economically feasible, a commercial scale fermentation process for large-scale production of microbial cellulose needs to be developed. By static culture of *Acetobacter xylinum*, however, about 2,000 square feet of air-liquid interface area is required for the production of one pound cellulose per day, therefore, static culture system is considered to be inefficient from the industrial point of view [3]. In general, relatively smaller amount of cellulose is produced in shaking and agitated culture than in static culture and this is closely related to the generation of negative mutants which does not produce microbial cellulose. These mutants are known to occur with agitation in the culture. Insertional sequences were found in the genes of negative mutants [25,26]. In addition to the reports on developing culture medium and isolating bacterial strains which is stable under shaking and agitated culture conditions [27-31], some reports have been made on the environmental conditions causing mutant generation [32,33]. However, none was on the bioreactor configuration which can produce microbial cellulose without generating mutants deficient of microbial cellulose producing ability. Developing a submerged fermentation system for the production of microbial cellulose is the subject of this research.

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MATERIALS AND METHODS

Cell Lines and Media

Acetobacter xylinum BRC5, provided by Bioproducts Research Center of Yonsei University, was maintained by serial transfer to fresh medium (pH 6.0) 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 about 5-mm dices, put into 20 mL fresh 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). 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, agar plates having an appropriate number (10 ~ 300) of the grown colony per plate were selected. A complex medium [31] containing 5.0 g of peptone, 20 g of yeast extract, 20 g of glucose, 1.56 g of Na_2HPO_4, 1.8 g of KH_2PO_4, 0.05 g of $MgSO_4$, 0.002 g of $FeCl_3$, 5 g of citric acid and 10 mL of ethanol per liter was used in reactor experiments. Microbial seed was maintained by serial transfer every two weeks. A loop of microbe was inoculated in 10 mL medium and cultivated statically at 30℃ in a plastic cylinder. After 6 days of culture, the pellicle formed at the surface of medium was cut into dices, put into 20 mL medium and homogenized. The homogenate was used as inoculum.

Cellulose Recovery and Treatment

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.

Analytical Methods

Identification of the colonies producing normal amount of cellulose (Cel^+) and cellulose-deficient mutant (Cel^-) was carried out by spreading properly diluted culture broth on agar plate (complex medium plus 2% agar) supplemented with 0.01% Calcoflour White (Sigma Chem. Co.). After growing at 30℃ for 6 days, fluorescence was observed under long wave UV light. Colony of nornal cells producing microbial cellulose fluoresced brightly, while that of negative mutant was markedly darker. Cell number was measured by counting colony number grown on agar plate.

Bioreactor Operation

Bioreactor was operated at 30℃ with water circulation and oxygen requirement was met by supplying air through filter. Main body and controllers of the reactor was purchased from Boksung Engineering Co. (Korea), and Chang Young Science Co. (Korea) made reactor vessels.

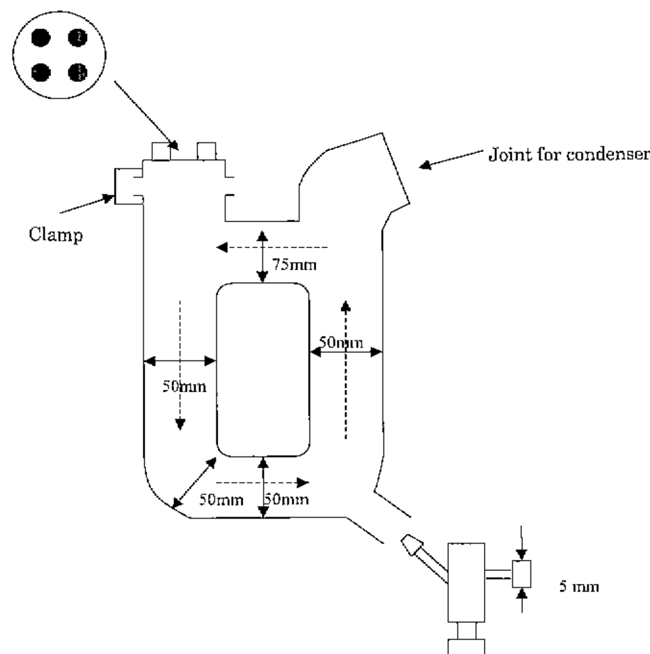

Fig. 1. Schematic diagram of air-lift bioreactor for the production of microbial cellulose.

RESULTS AND DISCUSSION

Production of Microbial Cellulose in a Normal Bioreactor with Rushton Type Flat-blade Disk Turbine as an Impeller

To investigate the effect of shear stress on the production of microbial cellulose, the seed for microbial cellulose production was inoculated in a normal bioreactor with traditional Rushton type flat-blade disk turbine as an impeller. After two days of culture, all the cells in the culture medium were converted to negative mutants, and no microbial cellulose was produced. In a bioreactor with high shear stress caused by Rushton turbine, microbial cellulose production was not obtained.

Production of Microbial Cellulose in an Air-lift Reactor

Airlift bioreactor was designed and prepared to investigate the effect of the reduced shear stress on the production of microbial cellulose (Fig. 1). In the airlift bioreactor, mixing and oxygen transfer were offered by air supply from the bottom of the larger tube. In this airlift bioreactor, with 0.5 L/min of aeration rate for 1.25 L of working volume, no negative mutant was found and 1.5 ~ 2.0 g/L of microbial cellulose production was observed.

Design of a Bioreactor System with Surface Aeration

To reduce the shear stress in a bioreactor, planar

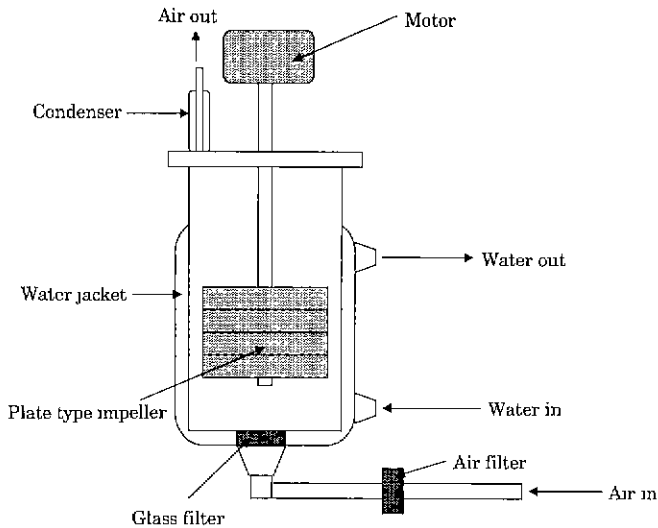

Fig. 2. Schematic diagram of bioreactor with plane-type impeller for the production of microbial cellulose.

impeller (Fig. 2) was designed and due to the impeller structure air was supplied by surface aeration. The agitation rate was 150 rpm and the aeration rate was set initially at 2 L/min for 1 L of working volume. The production of microbial cellulose in this reactor with initial aeration rate was 0.38 g/L and that was even less than that of airlift bioreactor. To increase oxygen transfer rate in the reactor system, agitation rate was increased up to 300 rpm, but no significant increase in the production of microbial cellulose was found. Negative mutant was not found up to 200 rpm but at 300 rpm negative mutant start to appear in the culture. To increase the oxygen transfer rate per culture volume even further, the working volume of the culture was reduced. With decrease in culture volume, the production of microbial cellulose increased to 0.67 and 1.08 g/L at 700 mL and 500 mL of working volume, respectively (Fig. 3). This is still lower than that of airlift bioreactor. This suggests that the oxygen transfer is important and not enough in this reactor system.

Combining Bottom Sparger with Planar Impeller

To increase the oxygen transfer even further, glass screen type air sparger was installed at the bottom of the bioreactor with planar type impeller. The culture volume was 1.5 L in 3-L reactor, agitation rate was 50 rpm, and aeration rate was set at 1 liter per minute. At this operation condition, no cellulose minus mutant was found and the production of microbial cellulose was increased up to 5 g/L (Fig. 4). The results from the above experiments for the production of microbial cellulose with different reactor systems are summarized in Table 1. Under shaking culture conditions, lower yields of microbial cellulose were reported as typical characteristics of *Acetobacter xylinum*. In this study, the culture conditions for cellulose production in bioreactor systems were investigated and cellulose production comparable to that of static culture was obtained, which is much higher than reported values. Batch culture of *Acetobacter xylinum* in an aerobic fermenter with typical Rushton-type turbine at agitating speed of 150 rpm showed that after 48 hr all cells converted to negative mutants and thus resulted in no

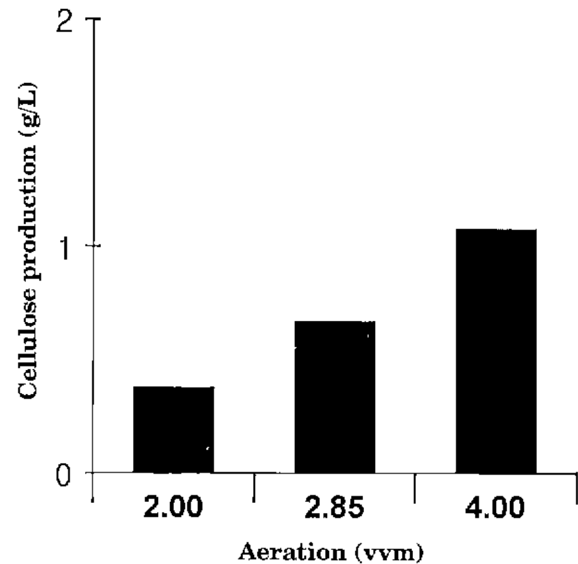

Fig. 3. Production of microbial cellulose in a bioreactor with plane-type impeller and surface aeration.

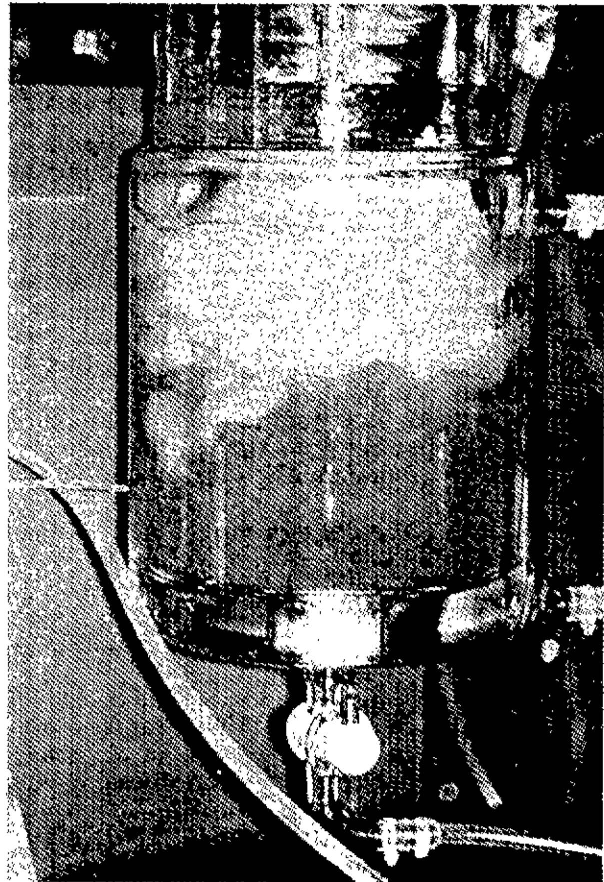

Fig. 4. Production of microbial cellulose in bioreactor with plane-type impeller and bottom-sparging system, where more than half of the reactor space was filled with microbial cellulose.

cellulose production. The conception of the plane-type impeller design was to reduce turbulence in the micro-environment of the cells producing microbial cellulose. The conversion of normal cells producing microbial cellulose to negative mutants might be due to cellular microenvironments in bioreactor. The previous report in shaking cultue flask [34] confirm this observation.

Table 1. Production of microbial cellulose and conversion to cell minus mutants in different bioreactor systems

Mixing aeration	Rushton turbine air sparger	Air-lift bottom sparger	Planar impeller surface aeration	Planar impeller bottom sparger
Production of microbial cellulose	No production	1.5 ~ 2.0 g/L	0.37 ~ 1.08 g/L	~ 5.0 g/L
Cel^- mutants appearance	After 2 days all cells were cenverted to Cel^- mutant	At the aeration rate over 1.5 vvm, Cel^- mutant start to appear	Not found	Not found

We will continue our investigations on effects of culture condition on conversion from normal cells to negative mutants with the aim of solving the problems associated with the scaling up the process of microbial cellulose production into industrial scale.

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