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Silicone Rubber Membrane Bioreactors for Bacterial Cellulose Production

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Abstract Cellulose production by *Acetobacter pasteurianus* was investigated in static culture using four bioreactors with silicone rubber membrane submerged in the medium. The shape of the membrane was flat sheet, flat sack, tube and cylindrical balloon. Production rate of cellulose as well as its yield on consumed glucose by the bacteria grown on the flat type membranes was approximately ten-fold greater than those on the non-flat ones in spite of the same membrane thickness. The membrane reactor using flat sacks of silicone rubber membrane as support of bacterial pellicle can supply greater ratio of surface to volume than a conventional liquid surface culture and is promising for industrial production of bacterial cellulose in large scale.

Keywords: bacterial cellulose; surface culture; silicone rubber membrane; *Acetobacter pasteurianus*

INTRODUCTION

Bacterial cellulose (BC) attracts researchers' attention because the fibril is superior to that found in plant cellulose in purity and its firm supra molecular structure [1]. The typical size of fibers of BC is 0.1 micrometer thick, which is about one-hundredth that of wood fibers. Also the reticulated network of the fine fibrils provides a wet BC pellicle with a large surface area, high water retaining capacity, moldability, and a dried BC sheet, strong tear resistance [2]. These properties of BC have been applied in the manufacture of high fidelity acoustic speakers [3], and high quality paper [4]. Attempts have been made to use it as artificial skin [5], ultrafiltration membrane [6], culture substrate for mammalian cells [7], cover-membrane for glucose biosensors [8], as well as binders for powders and thickeners for paint, ink and adhesives [9].

Bacterial cellulose production had been studied extensively from 1992 to 1998 in Japan [10]. Among them some biochemical engineering studies on cellulose production were done using aeration and agitation culture [11], and airlift fermentor [12] Although most of fermentative production by aerobic microorganisms were performed in the agitated and/or aerated reactor, Nata (a cellulosic food product) is produced in industrial scale by liquid surface culture. This is because most of the cellulose-producing bacteria are unstable in their ability in agitated or bubbled culture. For large-scale fermentation in static culture, large area of air-liquid interface is necessary. If we can prepare static air-liquid surfaces in the medium of a fermentor, static cellulose production will improve greatly. There are many kinds of oxygen-permeable membrane of synthetic as well as natural substances. Some of the membranes may be used as a substitute of air-liquid interface.

In our previous paper we reported that BC was produced on some oxygen permeable membranes, each of which was put into the bottom of a static culture vessel [13]. Among the membranes tested, silicone rubber sheet was best for developing the cellulose pellicle. We found in another study that a bioreactor with a bundle of silicone tubes submerged in the culture liquid could provide high oxygen absorption rate, and was suitable for oxidation of ethanol to acetic acid by acetic acid bacteria [14]. In the present study we used silicone tubes as a support of BC pellicle in a bioreactor expecting they might provide larger membrane-liquid interfacial area than a bottom silicone rubber sheet. However BC production on outer surface of silicone tubes was much less than that of flat membranes. Using some other silicone rubber membranes of different geometrical form and size, we investigated the effect of membrane morphology on BC production to look for optimum shape of silicone rubber membrane in practical bacterial cellulose production.

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MATERIALS AND METHODS

Bacterial Strains and Culture Medium

Acetobacter pasteurianus AP and APSK, and *Acetobacter xylinum* DA were used. *A. pasteurianus* AP was isolated as a single colony from the supernatant of a static liquid culture of *Acetobacter pasteurianus* ATCC 10245. Strain

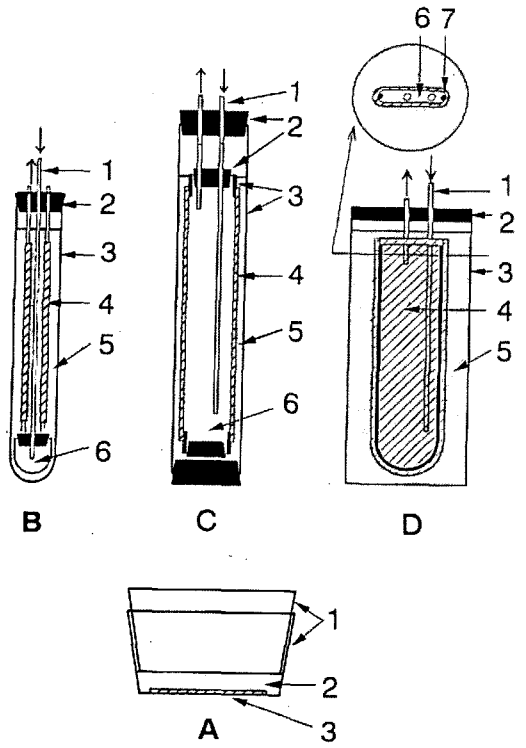

Fig. 1. Culture vessels for growing cellulose-producing bacteria on surface of silicone rubber membrane. Shape of silicone rubber membranes is Flat sheet (A), Tube (B), Cylindrical balloon (C), and Flat sack (D). For the details see the text.

APSK was a mutant resistant to kanamycin (30 mg/dm^3) and streptomycin (10 mg/dm^3) of *A. pasteurianus* AP [13]. *Acetobacter xylinum* DA was an acetic acid-resistant strain isolated from a continuous surface culture for acetic acid production [15]. The composition of the medium [16], consisted (per liter) of 5 g yeast extract (Difco, Detroit, USA), 5 g Polypepton (Nihon Seiyaku, Tokyo, Japan), 2.7 g Na_2HPO_4, 1.15 g citric acid monohydrate and 20 g glucose (pH 6.0). *Aspergillus oryzae* IAM2958 was used in a reference experiment.

Culture

Fig. 1 shows the schematic illustration of the culture vessels used to grow the cellulose-producing bacteria on the surface of silicone rubber membrane. Four types of silicone rubber membranes were used: flat sheet (A), tube (B), cylindrical-balloon(C), and flat-sack (D).

The vessel A in Fig. 1 has a flat sheet of silicone rubber membrane (40 mm diameter, 0.5 mm thickness) at the bottom of a polypropylene cup (60 mm top diameter, 50 mm bottom diameter, 27 mm height). The down side of the membrane was exposed to the atmosphere. The medium of 0.026 dm^3 was put in the vessel and the liquid surface was covered with another cup of the same size. The vessel B is a test tube (26 mm od, 200 mm height) in which four pieces of silicone tube (3.2 mm od, 2.2 mm id, 105 mm long, 0.5 mm thickness) were inserted through a rubber plug as shown in Fig. 1(B). The medium volume was 0.073 dm^3. Air was passed through the tubes at a flow rate of 0.2 dm^3/min. The vessel C is a glass cylinder (50 mm id, 250 mm height) with rubber plugs at the ends. A tubular silicone rubber membrane (32 mm od, 170 mm long, 0.15 mm thickness) with rubber plugs at the ends was submerged into the medium (0.15 dm^3) in the glass cylinder. By flowing air through the tubular membrane at a flow rate of 0.2 dm^3/min, it inflated like a long balloon as shown in Fig. 1(C). At an airflow rate less than 0.2 dm^3/min, it deflated and flattened. The medium volume was increased to 0.3 dm3 for maintaining the appropriate contact between the membrane and the medium. The vessel D is a glass beaker (2.4 dm^3 medium volume). A tubular silicone rubber membrane (0.14 mm thickness) was spun on a U-wire (130 mm long width, 42 mm short width, 8 mm wire diameter) to make a flat sack. The sack was submerged in the medium. The air was flowed through the sack as shown in Fig. 1(D).

The membranes were supplied from Nagayanagi Co. (Tokyo, Japan). The thickness of the membranes was 0.5 mm (A and B) and 0.14-0.15 mm(C and D). A supernatant of the liquid surface culture incubated for 7 d at 30°C using 0.03 dm^3 medium in a 0.1 dm^3 conical flask was inoculated into each culture vessel at a ratio of 5 v/v%. All the culture experiments were carried out at 30°C for 7days (except mentioned otherwise) under static conditions.

Measurement of Cellulose Production

The cellulose pellicle of *Acetobacter pasteurianus* grown on liquid or membrane surfaces was harvested and treated with 1 N NaOH for 1 day at room temperature to disrupt the cells in the pellicle. It was then washed with running tap water for 1 day. Cellulose in the pellicle was measured as dry-weight after drying at 105°C to a constant weight.

Assay of Glucose and Gluconic Acid

The glucose concentration of the culture supernatant was measured colorimetrically by the Glucostat method using glucose oxidase, peroxidase, and o-dianisidine. Gluconic acid was assayed using a gluconic acid analysis kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Electron Microscopy

Microscopic structure of bacterial pellicle was observed with a scanning electron microscope (JSM-5200 LV, Tokyo, Japan). Dehydrated bacterial pellicle was prepared by rinsing a small piece (1 × 1 × 0.3 cm) of intact bacterial pellicle with flowing tap water and soaking into 0.1 dm^3 of 99.5 v/v% ethanol for a few weeks and then drying at room temperature for two days on a filter paper No. 5A (Toyoroshi, Tokyo, Japan).

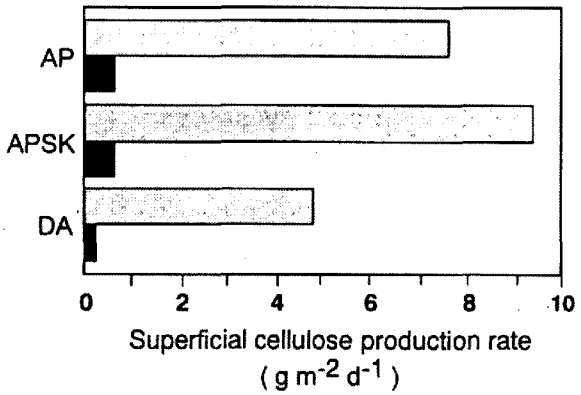

Fig. 2. Cellulose production on surfaces of silicone tube (black bar) and silicone rubber sheet (shaded bar). AP, APSK and DA denote *Acetobacter pasteurianus* ATCC 10245, a mutant of AP resistant to streptomycin and kanamycin, and *Acetobacter xylinum* DA, respectively.

RESULTS AND DISCUSSION

Bacterial Cellulose Production on Tubes Versus Flat Sheet of Silicone Rubber

The two strains of *Acetobacter pasteurianus* (AP and APSK) and A strain of *Acetobacter xylinum* (DA) was cultured statically for one week using the vessels A and B shown in Fig. 1. The amount of cellulose produced on a flat silicone rubber sheet (Fig. 1(A)) and silicone tubes (Fig. 1(B)) of the same thickness (0.5 mm) was determined. The result shown in Fig. 2, estimates superficial cellulose production rate as the amount (g) of cellulose produced per unit surface area (m^2) per day. The rate on silicone tubes was as low as one-tenth that on a flat silicone rubber sheet, although culture conditions were the same. This tendency was observed for each of the three bacterial strains, AP, APSK and DA. Only strain APSK was used in the following experiments, as its cellulose production was relatively stable and better.

Fig. 3 shows the time course of static cultures of *Acetobacter pasteurianus* APSK using silicone tubes and a flat silicone rubber membrane as the support for the cellulose pellicle. The time-dependent curves for remaining glucose, concentration of gluconic acid produced from glucose, and culture pH were almost the same in both cultures. Nevertheless the superficial cel-lulose production rate differed remarkably between the two cultures. As stated previously, the rate for the static culture using silicone tubes was about one-tenth lower than that using a flat silicone rubber membrane. Con-sequently, the cellulose yield was very small (0.023 g on g-glucose consumed) in the culture using silicone tubes in comparison with a flat silicone rubber sheet (0.193 g g^{-1}), although the membrane surface area in unit volume of the culture liquid was almost the same in both cultures.

In an attempt to elucidate the difference in cellulose production on flat and tubular silicone rubber membrane,

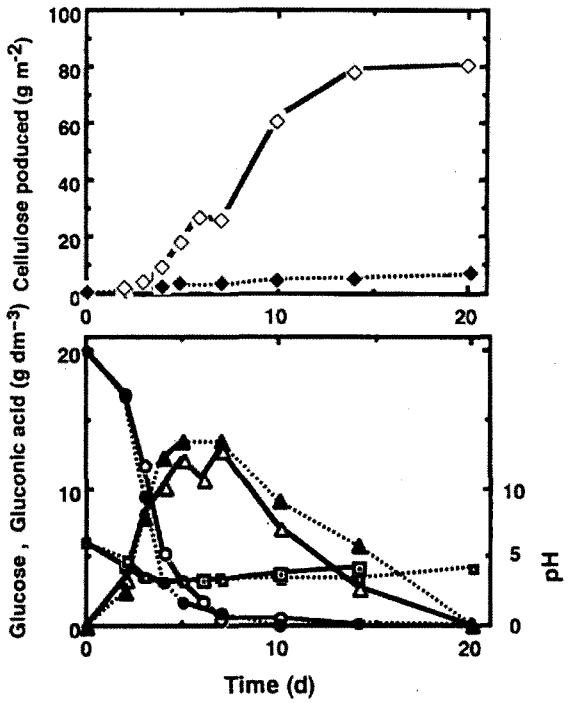

Fig. 3. Time course of static liquid cultures of *A. pasteurianus* APSK on silicone rubber sheet (open symbols) and silicone tubes (closed symbols). Measurements were done with cellulose (diamonds), glucose (circles), gluconic acid (triangles), and pH (squares).

we calculated effectiveness factor of oxygen diffusion in the growing cellulose pellicle on each surface by a model simulation. The model includes the partial differential equations of concentration distribution for oxygen and substrate in cylindrical coordinates. The reaction term for oxygen consumption was assumed as Monod kinetics concerning both oxygen and substrate.

Effectiveness factors for the pellicles of 2 mm thickness on the tubes of 2 and 4 mm outer diameters were estimated as 0.49 and 0.65 respectively while it was unity for that on a flat membrane. This estimation is consistent with the result of an experiment (data not shown) in which mycelial growth rate of *Aspergillus oryzae* IAM 2958 was half on a silicone tube (3.2 mm outer diameter) than on a liquid surface. However we cannot relate the difference (1/2 to 2/3) in the calculated effectiveness factors with the larger difference (about one-tenth) in the superficial cellulose production rates between on a silicone tube and on a flat silicone rubber membrane (Fig. 2). Why flat surface is superior to non-flat one concerning bacterial cellulose production is left for further investigation.

Effect of Tube Size and Surface Processing of Silicone Tube on Cellulose Production Rate

We observed that tube type membrane was inferior to sheet type as a support for cellulose production. How-

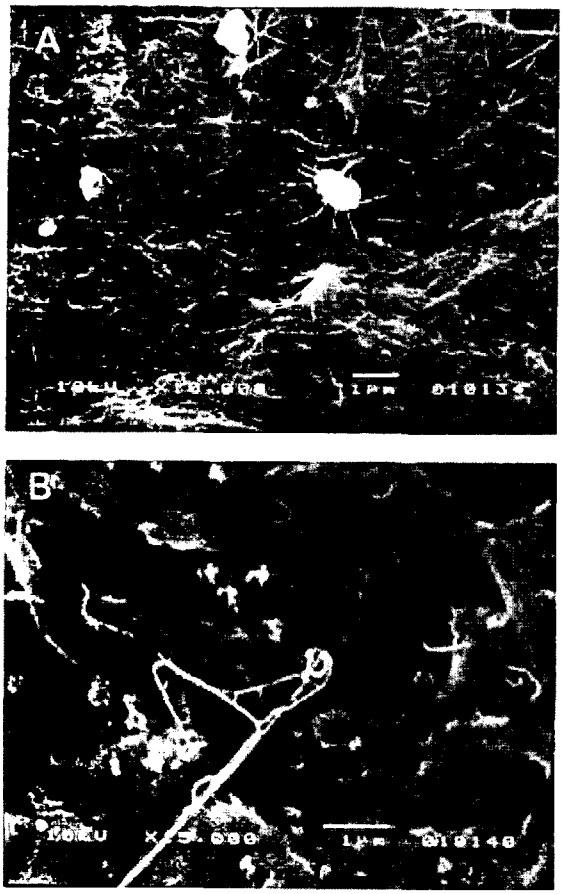

Fig. 4. Electromicrograph of bacterial pellicle grown on flat (B) and tubular (A) silicone rubber membranes.

ever silicone tube is considerably suitable for supplying oxygen by flowing air through its inner space and easy to handle. We examined the possibility of using silicone tube for static cellulose production further.

Silicone tube of 3.2 mm outer diameter was used in most of the experiment. Larger and smaller tubes were used to investigate the effect of tube size on cellulose production: outer diameter of 0.8 to 4.0 mm with constant thickness of 0.25 mm. The tube size did not affect superficial cellulose production rate in the range tested (data not shown).

Bacterial cellulose pellicle grew radially on a tube surface that differed from unidirectional growth on a flat surface. This might be one of the factors deteriorating cellulose production on a tube surface. More energy is required for a bacterial pellicle to develop in radial direction because the cellulose fibrils in the pellicle must expand against the firm tensile strength of the circular network of cellulose fibrils. We attached a neoprene rectangular bar (1 mm × 2 mm, 105 mm long) or a silicone tube (2 mm od, 1 mm id, 105 mm long) along the outer surface of the silicone tube (4.0 mm od, 3.5 mm id) to reduce the geometrical restriction against bacterial growth on a tube surface. The cellulose pellicle

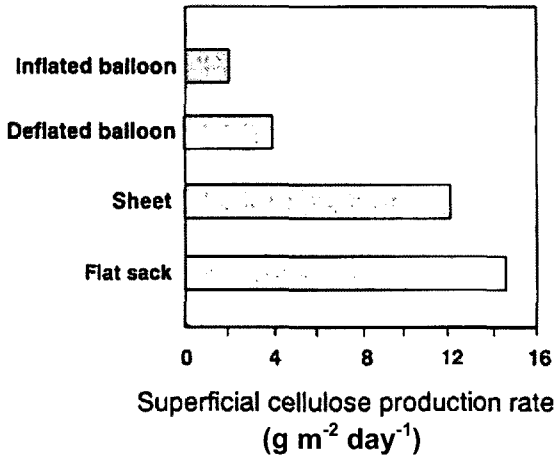

Fig. 5. Cellulose production of *A. pasteurianus* APSK on silicone rubber membrane of flat sheet, flat sack and cylindrical balloon.

grown on the surface was easily peeled off while that on the intact tube surface was not. However superficial cellulose production rate was not affected by the processing of the tube surface.

Electromicrograph of Cellulose Pellicles

We peeled cellulose pellicles from both the surfaces of tubular and flat silicone rubber membranes, and dehydrated them. Microphotographs of the dry pellicles are shown in Fig. 4. With the cellulose pellicle developed on a flat membrane cellulose, microfibrils were hardly observed probably due to close assembly of cellulose microfibrils (Fig. 4(B)). In contrast, the loose networks of cellulose fibrils were observed in the pellicle developed on a tubular surface (Fig. 4(A)).

Cellulose Production on Cylindrical Balloon and Flat Sack of Silicone Rubber Membrane

In the previous experiment, we used silicone tubes of 0.8 to 4.0 mm in outer diameter to investigate how tube curvature affects bacterial cellulose production. There was no significant difference in cellulose production rate among the tubes. The rate was much lower than that on a flat membrane. To examine whether the tube diameter affects cellulose production rate, a silicone rubber cylinder of 30 mm in diameter and 0.15 mm in thickness was used to support the cellulose pellicle in the following experiment. The cylinder was immersed into a liquid medium after closing the ends with neoprene plugs (Fig. 1(C)). By flowing air through the cylinder at a flow rate of 0.95 dm^3/min, it inflated like a long balloon in the liquid. At a lower air flow rate (0.5 dm^3/min), the balloon deflated and partly wrinkled. We used another shape of silicone rubber membrane: a flat silicone rubber sack (Fig. 1(D)) that was made by covering a U-wire with silicone rubber cylinder and closing

Table 1. Effect of membrane expansion on cellulose production by bacteria grown on flat sack (Fig. 1(D))

Membrane expansion (%)	<100	100	112	114	131	164	165
Specific cellulose production rate ($g\ m^{-2}\ day^{-1}$)	2.1-4.0	10.1	9.9	10.1	10.3	6.7	6.6

the two ends with an adhesive. Superficial rates of cellulose production on the surfaces of the above three shapes of silicone rubber membrane were determined and compared with that on a flat silicone membrane (Fig. 1(A)). As shown in Fig. 5, cellulose production on the surface of an inflated balloon was about half of that on a deflated one and one-sixth that on a flat surface. In the case of the deflated balloon, only a portion of the whole surface was available for developing the cellulose pellicle. This was probably due to the channeling of air flow in the cylinder. Cellulose production on the cylindrical silicone rubber membrane extended to the shape of a flat sack using a U-wire was as greater as that on a flat sheet. This result also indicates that flatness of the surface is prerequisite for a good production of cellulose. The flat silicone rubber sack is more suitable for large-scale production of bacterial cellulose than the flat rubber membrane spread on the bottom of a vessel. Total surface area for growing the bacterial pellicles can increase by simply using more sacks in each vessel. The efficient production of bacterial cellulose will yield benefits in many areas thus finding the best culture condition is of great importance.

Effect of Surface Tightness of Flat Membrane on Cellulose Production

Cellulose production was best on the surface of the flat sack (see Fig. 1(D)). We examined the effect of surface tightness of the flat sack membrane on cellulose production. The degree of membrane expansion was varied in the range of less than 100 to 165% which was calculated from the lengths of the tube circle (112-264 mm) and the width (50-89 mm) of U-shape wire. Cellulose production was greater on a tight membrane surface than on a loose one as shown in Table 1. However it reduced on excessively expanded membrane due to some wrinkles caused on the surface. Not only curvature of a silicone tube (Fig. 2) but also unevenness of a flattened silicone rubber membrane resulted in deterioration of bacterial cellulose production.

In recapitulation, bacterial cellulose production using silicone rubber membrane as static surface depends greatly on surface morphology of the membrane. We reported previously [13] microscopic structures of the membrane surface influenced greatly on the rate and yield of cellulose production: higher on gross surface than on emboss surface. The present investigation shows macroscopic structures (flat and tubular) also affect cellulose productivity significantly. Why strictly smooth and planar surface is necessary for good cellulose production is not yet elucidated. Most plausible explanation is that the difference of oxygen permeation rate in a flat and annular pellicle may vary the bacterial growth within the spaces. However, as shown in Fig. 3, the metabolic activities such as glucose consumption and gluconic acid production were almost the same, only cellulose production activity was repressed when we used tubular membrane. This suggests the sites of cellulose synthesis suffer some mechanical stress more in the radial development of a bacterial pellicle. According to the reports cellulose is produced in a cell by the apparatuses for cellulose synthesis [17] and extruded from pores on cell surface into the environment [18] to form microfibrils as bundles of cellulose molecules. Most of the microfibrils are still attached to the cell surface (see Fig. 4(A)). Presumably manner of the microfibril deformation affects the activity of cellulose synthesis.

CONCLUSION

Silicone rubber membrane can be used as an oxygen permeable support for bacterial cellulose production in place of liquid surface in static culture. However, It is essential to use a flat wrinkleless membrane rather than tubular membrane for obtaining high productivity. Among the silicone rubber membrane bioreactors tested, one submerged with a fat sack type membrane (Fig. 1(D)) gives best result in cellulose production rate. This type of membrane bioreactor must be useful for large-scale static culture as it can increase surface to volume ratio in a limited area for reactor installation.

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