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Tubular bacterial cellulose gel with oriented fibrils on the curved surface

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Abstract

On static cultivation, *Acetobacter xylinum* synthesizes bacterial cellulose (BC) that has a gel-like fibril network with inappropriate orientation at the air/liquid interface. This can be easily molded into desired shapes and sizes during synthesis. Here, we report a simple technique to synthesize tubular BC (BC–TS) gel with proper fibril orientation. We found that culturing BC in oxygen-permeable silicone tubes with inner diameter <8 mm yields a BC–TS gel of the desired length, inner diameter, and thickness with uniaxially oriented fibrils. The fibrils are oriented along the longitudinal axis of the silicone tube, independent of gravity, oxygen availability, and the morphology of the inner surface of the silicone tube but dependent on the curvature of the silicone tube. The degree of orientation (Δn) of the BC–TS fibrils, as revealed by their birefringence, increases with decrease in the inner diameter of the silicone tube. BC–TS with a uniaxially oriented fibril structure has excellent mechanical properties and holds promise for use as a microvessel or soft tissue material in medical and pharmaceutical applications.

Keywords: Bacterial cellulose; Curvature; Gel

1. Introduction

Bacterial cellulose (BC) is a form of cellulose produced by bacteria such as *Acetobacter xylinum* (*A. xylinum*). The structure and mechanical properties of BC differ from those of plant cellulose, despite their identical chemical compositions. BC has high mechanical properties including tensile strength and modulus, high water-holding capacity, high moldability, high crystallinity, and high biocompatibility [1,2]. BC has been widely used in foods [3,4], in acoustic diaphragms for audio speakers and headphones [5], and for making unusually strong paper [6]. Currently, BC also has received much attention in medical, pharmaceutical, and prosthetic applications in the form of wound dressings [7], artificial skin [8,9], artificial blood vessels [10], special membranes [11], mammalian cell culture substrate [12], and scaffold for cartilage tissue engineering [13].

BC gel synthesized by the aerobic bacterium *A. xylinum* and cultivated under static immersion conditions has an inappropriately oriented fibril network structure in the form of a gel at the air–liquid interface of the culture medium. With regard to the structure of BC gel, the production of BC gel having a well-oriented fibril arrangement could prove beneficial for its use as a substitute material in medical and pharmaceutical applications. It is well known that formation of a highly oriented structure can improve the performance of materials; for example, crystalline polymers having well-oriented fibril structures have better stiffness and strength [14,15] as compared to other materials that lack proper structural orientation. Thus far, there have been limited attempts to produce BC gel that have an oriented structure by (1) addition of chemicals such as lipids and polysaccharides during cultivation of the bacterium *A. xylinum* [16] or (2) use of a surface with oriented polysaccharide chains [17]. We reported a novel one-pot method for the production of BC gel having a highly oriented fibril structure by cultivation of BC on oxygen-permeable polydimethylsiloxane (PDMS) substrates with ridges of various sizes [18].

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As demonstrated in the previous paper, the BC gel can be easily molded into desired shapes and sizes during its synthesis, depending on the cultivation technique and conditions. One such interesting shape is tubular BC that can be synthesized directly in the culture medium without the need for subsequent treatment. This type of BC is a material that holds promise for use as a microvessel or a soft tissue material in different fields of medicine, such as internal medicine, urology, gynecology, otolaryngology, maxillofacial surgery, or plastic surgery. Klemm and his group successfully produced tubular BC by a matrix reservoir cultivation vessel technique [1]. In this technique, a cylindrical glass matrix is immersed in a larger volume of the culture medium. Tubular BC is produced in the culture medium that enters between the outer and inner matrices; BC is then supplied with oxygen through the second opening that opens to the air surface. This technique requires a longer time, or probably, it is difficult to produce long tubular BC by this method. In addition, this method also requires inner and outer glass matrices of several sizes in order to produce the desired tubular BC having controlled inner diameter (ID) and wall thickness. In this paper, we report a simple technique to synthesize tubular BC gel having a desired length, inner diameter, and thickness along with an oriented fibril structure in a shorter cultivation time. A silicone tube was used as the mold for the BC gel because of its high oxygen permeability, which is crucial for BC cultivation. The tubular BC gel thus produced was analyzed by scanning electron microscopy (SEM) and crosspolarizing microscopy (CPM), and its mechanical properties were determined by tensile strength measurement.

2. Experimental section

2.1. Materials

The microorganism used was *A. xylinum* (American Type Culture Collection (ATCC) 53582). We used a culture medium that was based on Hestrin–Schramm's medium [19], the constituents of which were as follows (wt%): 5 g bacto-peptone (Difco Bacto-Peptone; Becton Dickinson and Co., USA), 5 g yeast extract (Difco, Becton Dickinson and Co.), 2.7 g disodium hydrogen phosphate (Na_2HPO_4; Junsei Chemical Co. Ltd.), 1.15 g citric acid (Wako Pure Chemical Ltd.), 20 g D(+) glucose (Wako Pure Chemical Industries Ltd.), and HCl. An aqueous solution of NaOH was used for purification. Silicone tubes (Shin-etsu) of various diameters and sizes were used as molds.

2.2. Preparation of tubular BC

We prepared 10-cm-long silicone tubes with varying IDs (range, 1–20 mm) and wall tube thicknesses (WTs; range, 1–3 mm) that were used as molds for producing BC; these tubes were not subjected to any prior treatment. After washing and drying, these tubes were sterilized in an autoclave at 121 °C for 20 min.

Hestrin–Schramm's medium (pH 6) and a cell suspension prepared from an *A. xylinum* culture (ATCC 53582) at a volume ratio of 10:1 were poured into sterile tubes. Both the edges of the tubes were covered with silicone rubber and were sealed. These samples were incubated at 28 °C for 7 days under static conditions and in 2 different incubation positions—vertical and horizontal. Fig. 1 shows the schematic representation of the production of BC in the silicone tube.

The gel-like, tubular BC thus obtained (hereafter referred to as BC–TS) was purified by soaking in a large amount of distilled water for 1 day (the water was changed 3 times) followed by autoclaving in a 1% (w/v) aqueous solution of NaOH at 121 °C for 20 min to remove the bacterial cell debris and alkali-soluble components. It was then cooled to room temperature and was washed several times with distilled water followed by (1) soaking in distilled water for a long period of time so that it attained a pH of 7 and (2) storage in distilled water at room temperature prior to use.

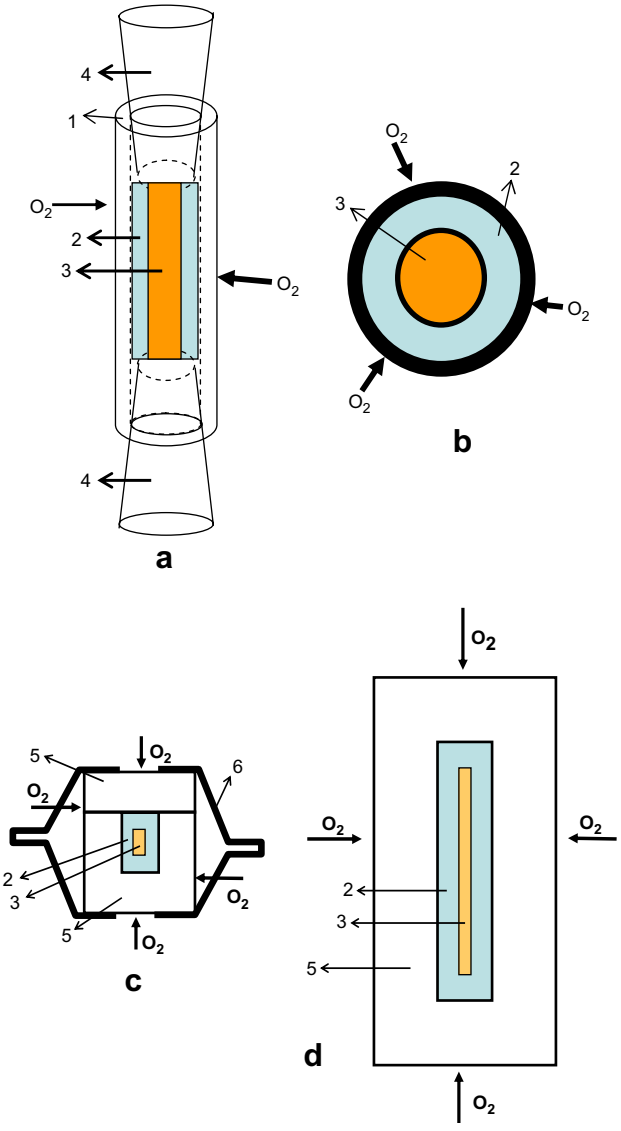

Fig. 1. Schematic representation of BC–ST produced in the silicone tube: (a) lateral view and (b) top view, and BC–ST produced in a rectangular silicone mold: (c) lateral view, (d) top view; (1) wall of the silicone tube; (2) bacterial cellulose; (3) culture medium; (4) plug; (5) rectangular silicone mold and (6) clip.

BC gel was also cultured in a Petri dish (diameter, 8 mm) such that it had 2 different interfaces: the air/liquid interface in which the culture medium was directly in contact with air (hereafter referred to as BC–air) and the silicone sheet (size, 5 × 5 × 1 mm)/liquid interface (hereafter referred to as BC–SS) as the control, i.e., the gel that was used for comparison with BC–TS. In addition, we also prepared a BC gel in smooth rectangular silicone molds of various sizes (length, 5 cm, ranging from 1 to 8 mm and thickness, 1 mm) for comparison with BC–TS. All processes involved in the production and purification of these BC gels were similar to those of BC–TS.

2.3. Measurement

The water content of the BC gels was determined from the degree of swelling (q)—defined as the ratio of the weight of the as-prepared swollen sample to that of the dry sample. The dry BC gels were obtained by drying the samples in vacuum at 60 °C until a constant weight was obtained.

Polarized light microscopic observation was performed using an Olympus BH2 microscope. A 1-cm-long sample of BC–TS was cut from 1 side and was placed on a precleaned micro-slide glass plate (length, 76 mm; breadth, 26 mm; thickness, 0.8–1.0 mm; Matsunami) with its inner side in contact with the plate, and the outer free surface was observed. The birefringence, i.e., $\Delta n = n_{\text{parallel}} - n_{\text{perpendicular}}$, of BC–TS was measured using a crosspolarizing microscope equipped with Berek compensator [20]. The direction of orientation of the sample was determined by rotating the sample in various directions and observing it under the crosspolarizing microscope. Under these conditions, Δn was measured from the retardation. The images were obtained at 0°, 45°, 90°, 135°, and 180°. The average Δn was determined by measuring Δn several times for each sample. All observations were conducted on wet samples.

The morphology and microstructure of BC–TS were examined using a Scanning Electron Microscope (HITACHI-S-2250N). Prior to observation by SEM, small pieces of the BC gels were freeze-dried (VirTis Advantage AD2. 0XL-SC) for 1 day and were mounted on the stub of the scanning electron microscope using a double tip needle, and were then sputter-coated with palladium (60–90 s) by ion sputtering (HITACHI-E 1010) to avoid electrical arcing. The images were obtained at 15 kV acceleration voltage.

A tensile strength tester (TENSILON; Orientec Co) was used to measure the mechanical properties of the BC–TS gel at room temperature. We stretched BC–TS in 2 different directions, i.e., lengthwise and breadthwise. For lengthwise stretching, a 25-mm-long piece of BC–TS was prepared, leaving 5 mm from both the edges covered with Whatman filter paper; it was then attached to a holder and was stretched at a strain rate of 10% min^{-1}. For breadthwise stretching, a 6-mm-long piece of BC–TS was used. Further, 2 wires of a U-shape were inserted through a hole in the sample, attached to the holder, and stretched at a strain rate of 10% min^{-1}. In both instances, stretching measurements were repeated at least 3 times. The strain rate was determined according to the initial length of the specimens. The failure point of the tensile strength was determined from the peak of the stress–strain curve. The elastic modulus, E, was determined by averaging the slope of the stress–strain curve over a strain range of 0–10%. The cross-sectional surface area of BC–TS was calculated from the BC–TS wall thickness.

3. Results and discussion

A silicone tube is an oxygen-permeable material that allows the aerobic bacterium *A. xylinum* to proliferate and produce BC gel. Under static incubation conditions, *A. xylinum* started to produce BC gel all around the interface between the inner surface of the silicone tube and the culture medium and thus produced a tubular BC gel, i.e., BC–TS. This indicated that *A. xylinum* were located on the inner surface of the silicone tube in order to obtain oxygen. An approximately 1.2-mm-thick layer of BC–TS was obtained after incubation for 3 days in a silicone tube with a wall thickness of 1 mm, and the thickness of the layer increased with an increase in the duration of incubation (Fig. 2). The growth of BC–TS in the silicone tube was similar to that of the BC gels cultured on silicone sheets (BC–SS). The thickness of BC–TS and BC–SS was approximately one-third of the BC gel cultured on the air/liquid interface (BC–air) under normal atmospheric conditions over the same duration of incubation. It was reported that the rate of BC production was proportional to the oxygen concentration in the culture medium [21]. The rate of BC production decreased linearly with an increase in the wall thickness of the silicone tube. Furthermore, the cellulose content per unit volume also tended to decrease from 0.005 g/mL to 0.002 g/mL with an increase in the wall thickness of the silicone tube from 1 to 3 mm.

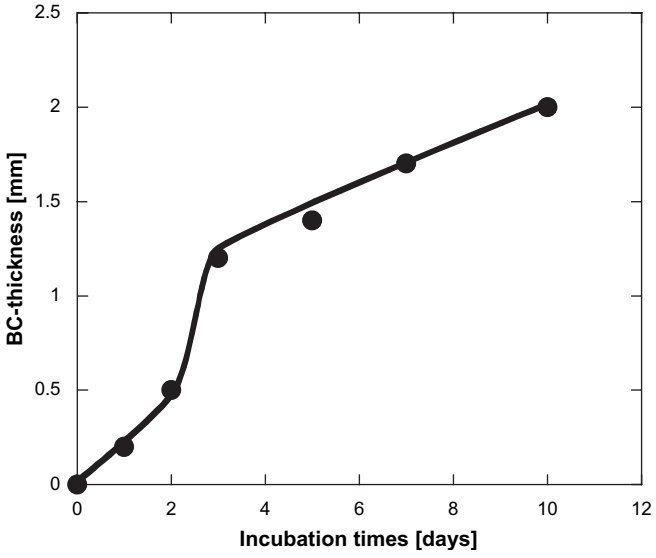

Fig. 2. The effect of the duration of incubation on the thickness of the BC–TS gel layer produced in the silicone tubes with an inner diameter of 5 mm and a wall thickness of 1 mm.

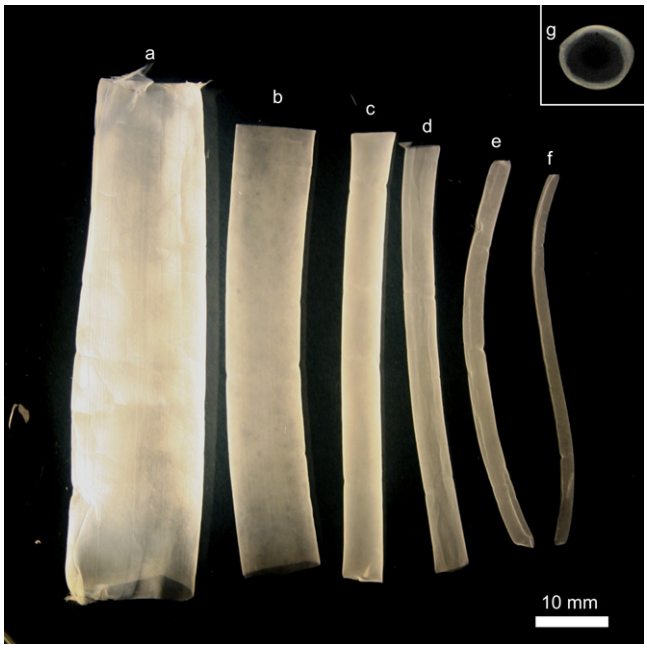

Fig. 3. The BC–TS gels cultured in the silicone tubes of various inner diameters: (a–f) lateral view of BC–TS with an outer diameter of 20, 10, 5, 4, 3, and 1 mm, respectively, and (g) top view of BC–TS with an outer diameter of 10 mm.

Fig. 3 shows the BC–TS gels cultured for 3 days in silicone tubes of various sizes but with a wall thickness of 1 mm. The semitransparent appearance of BC was inherited by BC–TS. By extending the duration of incubation, BC–TS with a greater wall thickness and a smaller ID can be obtained. However, on removal from the water, the BC–TS gels were unable to retain their tubular shape (the walls collapsed against each other). Furthermore, by incubation for 3 days using a smaller silicone tube with an ID as small as 1 mm or 0.5 mm, BC–TS having a rope-shaped morphology without openings could be produced.

Fig. 4 shows the crosspolarizing microscopy images of BC–air (Fig. 4a), BC–SS (BC cultivated on a 1-mm-thick silicone sheet) (Fig. 4b), and BC–TS (cultivated in silicone tubes with a wall thickness of 1 mm and diameters of 1 mm and 8 mm, (Fig. 4c and d, respectively)). A weak birefringence was observed for BC–air, while BC–SS and BC–TS showed strong birefringence with colorful images. The birefringence of BC–SS was isotropic, indicating that locally oriented BC fibrils were randomly positioned in all possible directions, similar to liquid crystal-like domain orientation [22]. In contrast, BC–TS was strongly anisotropic, exhibiting distinct dark and bright alternating bands when the sample was being rotated under the crosspolarizing microscope: complete extinction was observed when the direction of orientation was parallel or perpendicular to the polarizing direction, and maximum brightness was observed when the angle between the direction of orientation and the polarizing direction was 45°. This indicated the existence of BC–TS fibrils that were well-oriented uniaxially along the axis of the tube.

Further detailed observation by SEM revealed the width and orientation of the BC–TS fibrils. Although it was difficult

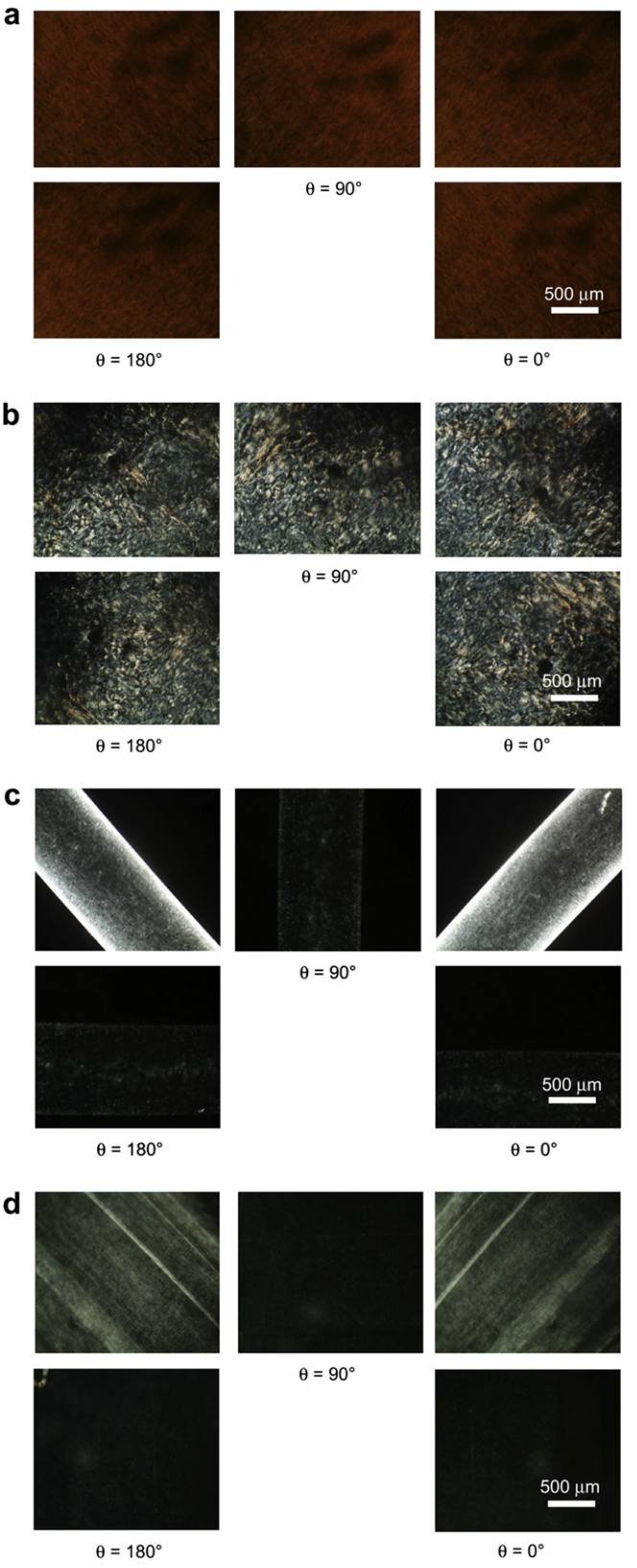

Fig. 4. Polarized light microscopy images of the BC gels cultured on the liquid–air interface (BC–air) (a), 1-mm-thick flat silicone sheet (BC–SS) (b), and in silicone tubes (BC–TS) with a wall thickness of 1 mm and an inner diameter of 1 mm (c) and 8 mm (d).

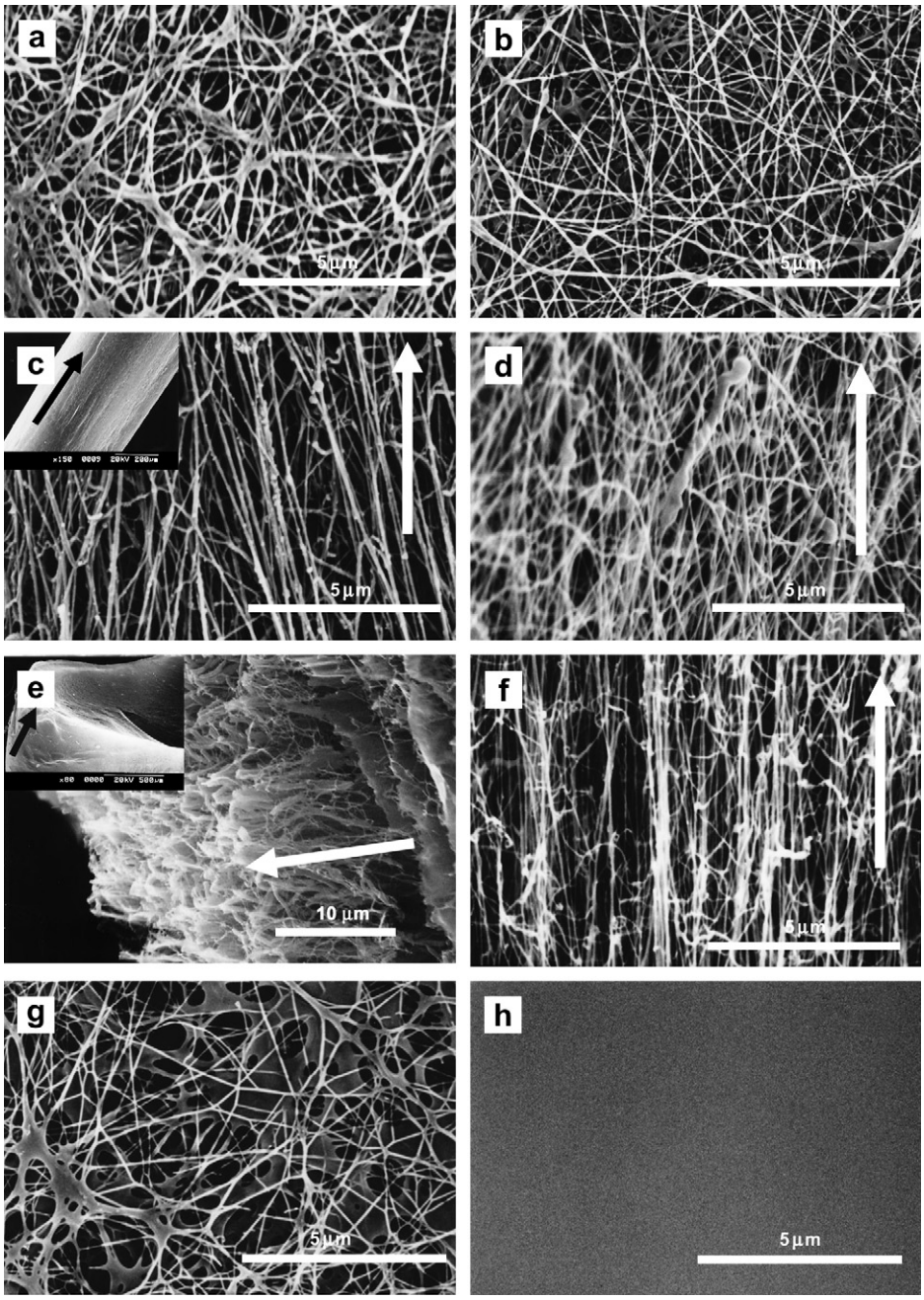

Fig. 5. SEM images of (a) BC–air, (b) BC–SS, (c) BC–TS (diameter, 0.5 mm; vertical incubation), (d) BC–TS (diameter, 8 mm; vertical incubation), (e) cross-section of BC–TS (diameter, 0.5 mm; vertical incubation), (f) BC–TS (diameter, 0.5 mm; horizontal incubation), (g) BC-rectangular (width, 1 mm), and (h) inner surface of the silicone tube. Arrow indicates the direction of the longitudinal axis of the tube.

to accurately estimate the size of each fibril in the limited viewing area after coating with palladium, we calculated the width of the BC–TS gel that appeared to be in the range of 45–150 nm. This width was similar to that of the BC–SS and BC–air gels. In the BC–SS gel, the fibrils were less ramified than that in the BC–air gel and were partially aligned along a common axis (Fig. 5a and b). However, the BC–TS gel fibrils were well aligned uniaxially parallel to the length of the silicone tube, instead of being aligned perpendicular to it (Fig. 5c–f).

The structural orientation of the BC–TS gel fibrils resulted in anisotropy in its mechanical properties. BC–TS was stretched in 2 directions—lengthwise and breadthwise. The typical tensile stress–strain curves are shown in Fig. 6. It is evident that the fracture stress of BC–TS during lengthwise stretching (0.59 MPa) was approximately 1.6 times higher than that during breadthwise stretching (0.37 MPa). Furthermore, the elastic modulus also showed a similar tendency (0.06 and 0.02 MPa during lengthwise and breadthwise stretching, respectively). The higher values of the fracture stress and elastic modulus during lengthwise stretching as compared to breadthwise stretching were in agreement with the structural observation on SEM that the fibrils mainly align along the length of the tube with few ramifications.

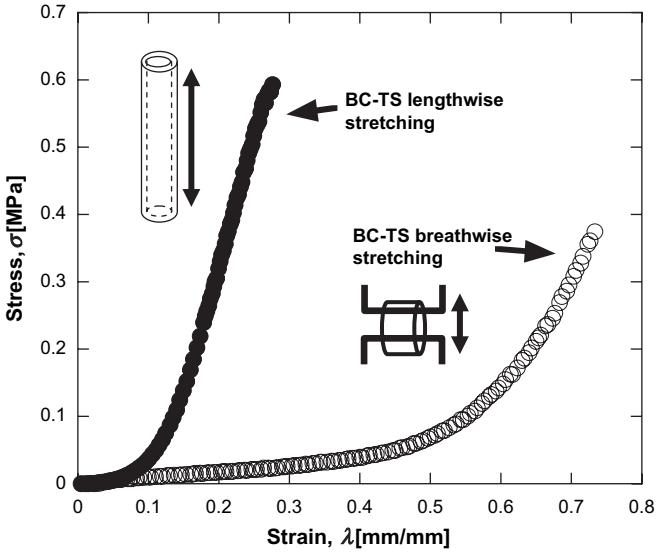

Fig. 6. Tensile stress–strain curves of the lengthwise (●) and breadthwise (○) stretching of BC–TS produced in the silicone tube with an inner diameter and wall thickness of 6 mm and 1 mm, respectively. The duration of incubation was 3 days.

The effect of ID on the degree of orientation (Δn) of BC–TS was studied by culturing the BC–TS gel in silicone tubes of various IDs (d) ranging from 0.5 to 20 mm. ID of the silicone tube was the outer diameter of the BC–TS gel. The relationship between $1/d$ and the degree of orientation (Δn) is shown in Fig. 7—Δn of BC–TS increased almost 5 times when $1/d$ was changed from 0.125 mm^{-1} to 0.1667 mm^{-1}. When $1/d$ was 1 mm^{-1}, Δn was 9.8×10^{-5}, which was 20 times higher than that of BC–SS (5×10^{-6}) or 43 times higher than that of BC–air (2.3×10^{-6}). The direction of orientation of the BC–TS gel fibrils was found to be parallel to the longitudinal axis of silicone tubes having ID of up to 8 mm (Fig. 5d). When the ID of the silicone tube was >8 mm, no further orientation could be observed, and the fibril alignment became similar to BC–SS. This result was surprising, since our previous study showed that on cultivation, geometric confinement of the BC fibrils was effective only when the characteristic spatial size (that is, the ridge size of PDMS) was comparable to the size of the bacterial cell, ca. 4.5 μm. In the previous study, we reported that uniaxially oriented fibrils of the BC gel could be obtained on the ridges of PDMS, where the degree of orientation of fibrils increased with a decrease in the ridge size of PDMS and peaked when the ridge size was 4.5 μm, that is almost equal to the contour length of the bacterial cell. This BC gel showed the highest birefringence (Δn), the highest fracture stress (σ), the highest degree of swelling (q), the lowest elastic modulus (E), and the thickest fibril, as compared to the BC gels cultivated on PDMS with other ridge sizes, either larger or smaller than 4.5 μm [18].

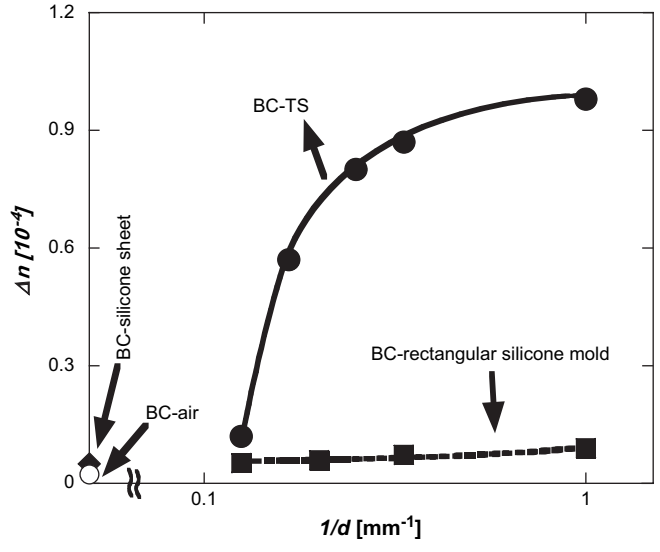

Fig. 7. The dependence of the birefringence (Δn) of BC–TS on the curvature of the silicone tube ($1/d$) in which BC was cultivated (●). The wall thickness of the silicone tube was 1 mm. BC–SS (◆), BC produced on the 1-mm-thick silicone sheet; BC–air (○), BC produced at the liquid–air interface; and (■), BC produced in the 1-mm-thick rectangular silicone mold are also shown in the figure for comparison (d, diameter of the silicone tube or the width of the rectangular mold).

In the present study, the diameter of the silicone tube that effectively brought about the orientation of BC fibrils was in the order of 1 mm, i.e., 1000 times larger than the diameter of the *A. xylinum*. To understand this enigma, several effects that might cause such a uniaxial orientation have been studied, including the morphology of the inner surface of the silicone tube, gravity, oxygen availability, and diffusivity through the wall of the silicone tube, and the effect of radial confinement of the tube.

First, the morphology of the inner surface of the silicone tube was investigated. Fig. 5h shows an SEM image of the inner surface of the silicone tube with a diameter of 1 mm. A smooth surface without any structural features was observed. A similar smooth morphology was also observed in case of tubes of other sizes and silicone sheets. This result indicated that the orientation of the BC–TS fibrils was not caused by the morphology of the inner surface of the silicone tube.

The effect of gravity was studied by culturing the BC–TS gel in silicone tube with 2 different positions—vertical and horizontal. For vertical incubation, the silicone tube was made to stand in an upright position in the incubator, and for horizontal incubation, it was laid down horizontally. Fig. 5c shows the SEM images of BC–TS incubated vertically. The BC–TS fibrils were oriented parallel to the longitudinal axis of the tube. A similar fibrillar alignment was also found in the horizontally incubated sample (Fig. 5f). The similarity between fibril orientation in both the vertically and the horizontally incubated samples indicated that *A. xylinum* preferred to move on the inner surface and along the longitudinal axis of the tube and secreted fibrils parallel to the longitudinal axis of the tube, rather than move around the inner surface of the tube in a circular fashion and secrete fibrils perpendicular to the longitudinal axis of the tube. This implies that gravity did not affect the direction of orientation of the BC–ST fibrils during cultivation.

Next, the effect of oxygen diffusivity through the wall of the silicone tube was studied by culturing BC in silicone tubes

with different wall thicknesses, ranging from 1 to 3 mm. It was also found that oxygen availability and diffusivity did not significantly affect the direction of orientation of the BC fibrils. The BC–TS fibrils remained oriented along the longitudinal axis of the silicone tube. However, as mentioned above, oxygen availability only affected the rate of production of BC–TS, i.e., the thickness of the BC–TS layer decreased with an increase in the wall thickness of the silicone tube.

Since the inner surface morphology, gravity, oxygen availability, and diffusivity did not affect the direction of orientation of the BC–TS fibrils, it might be possible to explain the phenomenon of the orientation of the BC–TS fibrils in terms of the effect of the curvature of the silicone tube. To study the effect of the curvature of the tube on fibril orientation, we compared the structure of BC cultured in silicone tubes of various IDs with BC cultured in smooth rectangular silicone molds of various sizes (length, 5 cm; breadth ranging from 1 to 8 mm; and thickness, 1 mm). It was mentioned above that the fibrils of BC–TS aligned along the longitudinal axis of the silicone tube and the degree of orientation (Δn) decreased with an increase in the ID of the silicone tube. On the other hand, BC cultured in the rectangular silicone molds did not show fibril alignment even at a width of 1 mm (Fig. 5g), which was similar to BC–SS.

The above results indicated that it was not the lateral spatial extent but the curvature of the silicone tube that affected the direction of orientation of the BC–TS fibrils. Although it is presently difficult to explain the exact mechanism of how the curvature affects the orientation, one possible explanation is that the actual length of the bacterial cells themselves persisted during the synthesis of the fibrils. During synthesis, the BC fibrils would preferentially get arranged in a proper manner and in the easiest and most comfortable way on the surface. Besides secreting cellulose fibrils, *A. xylinum* also duplicated themselves. We assumed that the *A. xylinum* (0.6–0.8 μm × 1.0–4.0 μm) with the cellulose ribbon (in mm) that was secreted from the terminal complex of the outer membrane of the bacterial cell comprised a stiff rod-like structure with a certain persistent length in millimeters. If the curvature of the inner surface of the tube was larger than the inverse of this persistent length of cellulose, it would restrict the movement of the stiff rod-like structure in a circular fashion, leading to a preferential orientation of the fibril synthesis in the longitudinal direction.

4. Conclusions

Using a silicone tube, we successfully produced tubular BC–TS gel having a uniaxially oriented fibril structure on a curved surface. The degree of orientation of the fibrils depended on the diameter of curvature itself. An increase in the diameter of curvature led to a decrease in the degree of BC fibril orientation. In case of the silicone tube, the diameter of curvature refers to the ID of the tube. Uniaxially oriented BC–TS fibrils could be observed in silicone tubes with ID <8 mm. The direction of orientation of the fibrils in the BC–TS gel followed the dimensions of the silicone tube. It did not depend on gravity, oxygen availability, and the morphology of the inner surface of the silicone tube; in fact, it depended on the curvature of the silicone tube. Further investigations to improve the orientation of the BC fibrils, the mechanical properties of BC, and the fibrillar orientation mechanisms are in progress.

Acknowledgments

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