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Aggregation of ribbons in bacterial cellulose induced by high pressure incubation

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Abstract

The nascent morphology of cellulose produced by *Acetobacter aceti* (AJ12868) incubated at 30 atmospheres pressures was investigated using AFM, TEM, X-ray and optical microscopy. The bacteria survived the elevated pressure and produced cellulose. The ribbons produced at 30 atm had similar crystalline features as the ribbons produced in the control medium but exhibited larger microfibril aggregates.

Keywords: *Acetobacter aceti*; Cellulose I; High pressure; Hydrogen bonding

1. Introduction

The cellulose microfibril is the main constituent of plant cell walls and is an aggregate of cellulose molecules with various degree of order. Cellulose material with large microfibrils and high cellulose crystallinity is found in marine green algae such as *Siphonocladales*. In general terrestrial plants generate thinner microfibrils with lower cellulose crystallinity and furthermore the microfibrils are often intimately associated with encrusting materials such as hemicellulose.

There are two mechanisms to pack molecules in a crystal lattice in native cellulose, cellulose I_α and I_β (Atalla & VanderHart, 1984; Sugiyama, Persson, & Chanzy, 1991; Sugiyama, Vuong, & Chanzy, 1991). All native cellulose microfibrils are now recognized as a mixture of the two crystalline phases, of which one is dominating depending on species (Koyama, Sugiyama, & Itoh, 1997).

The incubation of *Acetobacter* sp. in the presence of hemicellulose causes cellulose molecules to crystallize or assemble into smaller aggregates compared to the control medium. This change is accompanied by crystallographic modifications, where cellulose I_β becomes dominant (Atalla, Hackney, Uhlin, & Thompson, 1993; Tokoh, Takabe, Fujita, & Saiki, 1998). Incubation of *Acetobacter* sp. in a viscous medium (Shibazaki, Saito, Kuga, & Okano, 1998) or at low temperature (Hirai, Tsuji, & Horii, 1997) revealed the formation of cellulose II. In a previous study (Yamanaka, Ishihara, & Sugiyama, 2000), it was demonstrated that the assembly of bacterial cellulose can be stimulated by adding antibiotics in the incubating medium which prevented the bacterial cells to divide and resulted in longer cells. The assembly of cellulose ribbons produced by such cells, resulted in broader microfibrils than those generated under normal condition. The Young's modulus of bacterial cellulose films is affected by the effective cross sectional area of the cellulose microfibrils (Nishi, Uryu, Yamanaka, Watanabe, Kitamura, Iguchi, & Mitsuhashi, 1990) thus a high modulus is achieved for these samples. Based on these results new applications for bacterial cellulose can be expected.

The above mentioned studies suggested that the alternation of the chemical or biochemical condition during the biosynthetic environment would alter the higher order structure of cellulose assemblies. What about the physical environment? With the advent of deep sea exploration, numerous biological lives previously unseen by human eyes have been discovered. One interesting example is *Tevnia jerichonana* that produces tubes made of extremely high crystalline β-chitin (Gaill, Persson, Sugiyama,

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Vuong, & Chanzy, 1992). The origin of its high crystallinity is not known yet, but can be expected to correlate with the high-pressure environment and the self-assembly of polysaccharide molecules.

Given this background, we incubated *Acetobacter aceti* at the high pressure up to 30 atmospheres (atm) to investigate the effect of high-pressure on the higher order structure of cellulose. The structural analyses using by AFM, X-ray and TEM indicated that high-pressure indeed stimulated the association or aggregation of cellulose ribbons.

2. Experimental section

Microorganism. *A. aceti* AJ12868 which was described in the previous paper was used throughout this study (Yamanaka et al., 2000).

Culture medium. The liquid medium contained 50 g/l of sucrose, 5.0 g/l of amino acid compound F made from soybeans and consisting of a mixture of 17 kinds of amino acids (Yamanaka et al., 2000). The pH was adjusted to 5.0 using HCl and the medium was sterilized at 120 °C for 15 min.

Cultivation method. Cultivation of bacterial cellulose at elevated pressures i.e. 10, 20, 30, 50 and 100 atm were carried out in order to investigate the upper pressure limit for cellulose formation. From this study it was concluded that 30 atm was the maximum pressure at which cellulose formation took place. The cellulose mat formed at 30 atm pressures was more transparent compared to the corresponding cellulose mat formed at 1 atm. The experimental setup is shown in Fig. 1.

Sample preparation for TEM. The bacterial celluloses (1 and 30 atms) were treated for 3 h at 100 °C in 1 ml of 0.1 M NaOH in order to remove contaminants like proteins. Thereafter, the bacterial celluloses were disintegrated for 30 s using a Hiscotron double cylinder homogenizer.

Transmission electron microscopy. A 10 ml aliquot of the disintegrated cellulose was mounted on a hydrophilically treated carbon-coated grid. Before complete air-drying, 10 ml of 2% uranyl acetate was deposited and after 1 min excess solution was squeezed out with a filter paper and allowed to dry. The grid was observed with a JEOL electron microscope (JEM 2000-EXII) operated at 100 kV and equipped with a GATAN camera and image intensifier (model 622-0300). All micrographs were taken at a magnification of 5 × K and 20 × K on Fuji FG film.

The selected area electron diffraction experiment was performed using a low dose electron probe of approximately 1 μm diameter area of the specimen at room temperature. All the diagrams were recorded on Mitsubishi MEM film at a camera length of 15 cm.

Atomic Force Microscopy. The gelatinous pellicle was visualized using an atomic force microscope as described in the previous paper (Yamanaka et al., 2000). A wet cellulose mat harvested from a culture broth was washed in about 1 l of distilled water for 60 min under stirring. The washed specimen was directly observed by the AFM (tapping mode) at room temperature.

X-ray diffractometry. X-ray diffraction diagrams were recorded on imaging plates from air dried samples using a vacuum camera equipped with Rigaku RH-200BU, using Cu Kα radiation ($\lambda = 0.1542$ nm). The intensity was radially integrated to obtain 2θ-intensity profiles. Separation of diffraction peaks was done using least-squares profile fitting program, assuming Gaussian function for each peak.

3. Results

Macroscopic observations. To obtain cellulose formation at 30 atm about 10 times of fresh seed culture (2–3 days culture) was found to be necessary compared to obtain cellulose formation at 1 atm since the death rate of the bacteria was higher at elevated pressures. The bacteria were cultivated for 21 days at 30 atm pressures. In Fig. 2, the newly formed pellicle is shown incubated at 1 and 30 atm pressures. As can be seen, the pellicle thickness was smaller after the incubation at 30 atm and after the high pressure incubation the bacteria cellulose were still alive. In this

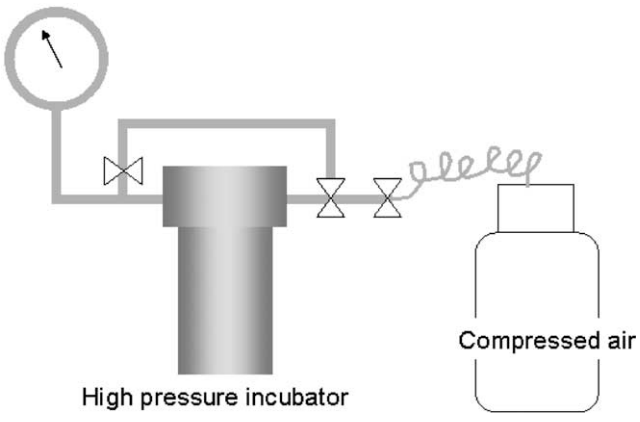

Fig. 1. High pressure incubation apparatus.

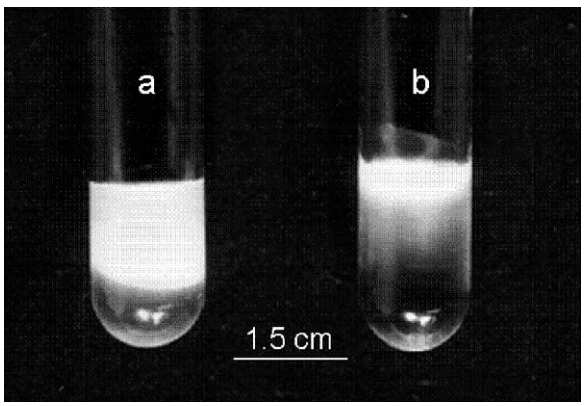

Fig. 2. The pellicle shown (a) after incubation at 1 atm pressure and (b) after incubation at 30 atm pressures.

Table 1
The estimated widths of the ribbons grown at 30 atm pressures and in the control medium

Sample	Bacteria size (nm)	Width, average value (nm)	Crystallinity index (%)	Crystallite size (nm)
Control	3.1(0.7)	36(12)	80	5.1
BC (30 atm)	3.2(0.8)	80(25)	85	5.4

study it was not possible to study the movement of the bacteria. However, the approximate size of the bacteria was not altered (Table 1) before and after the high pressure incubation as shown by the optical micrographs in Fig. 3.

In order to verify that the increase in ribbon size during high pressure incubation was not merely an effect of the elevated pressure, bacterial cellulose was subjected to 30 atm for 21 days. No additional increase in ribbon size was observed.

Morphology and higher order structure. The widths of the ribbons produced at 30 atm pressures and in the control medium of *A. aceti* were measured by AFM which shows the in situ morphology of the ribbons. As can be seen in Fig. 4, the ribbons produced by *A. aceti* at the higher pressure exhibit apparently larger widths compared to the control ribbons. A visual inspection of the AFM-images reveals that *A. aceti* grown at the higher pressure produced

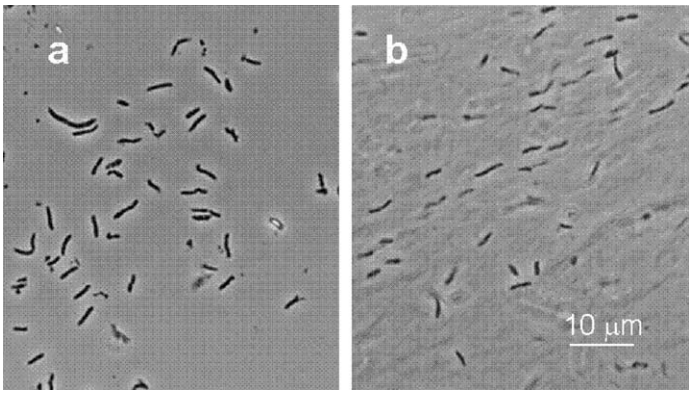

Fig. 3. Optical micrographs showing the bacteria size (a) in the control medium and (b) after incubation at 30 atm pressures.

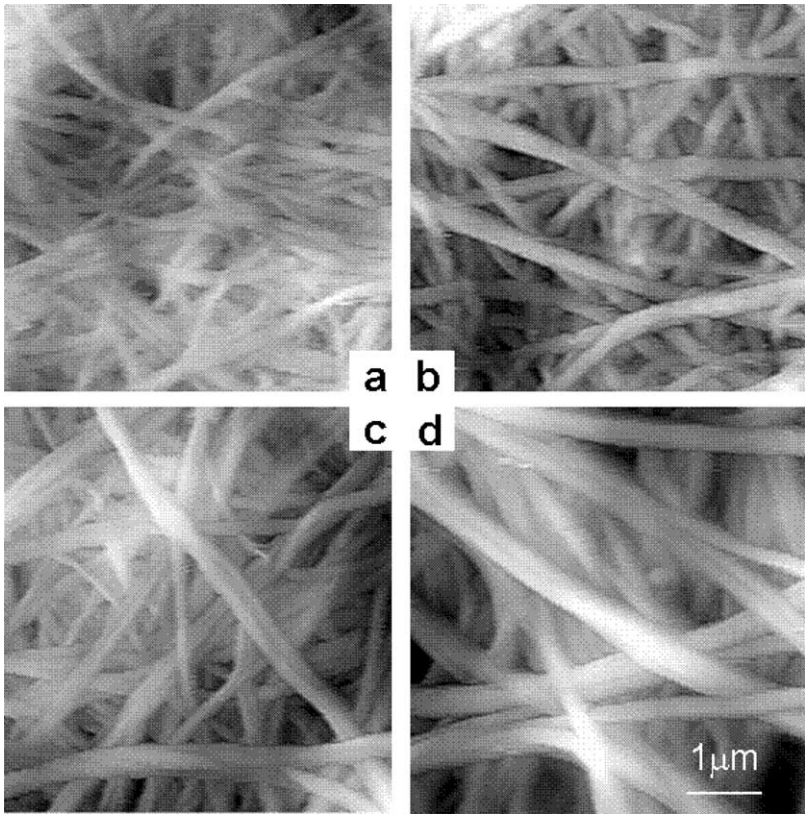

Fig. 4. AFM images showing (a) the ribbons produced in the control medium (b-d) and the ribbons produced during incubation at 30 atm pressures of *Acetobacter aceti*.

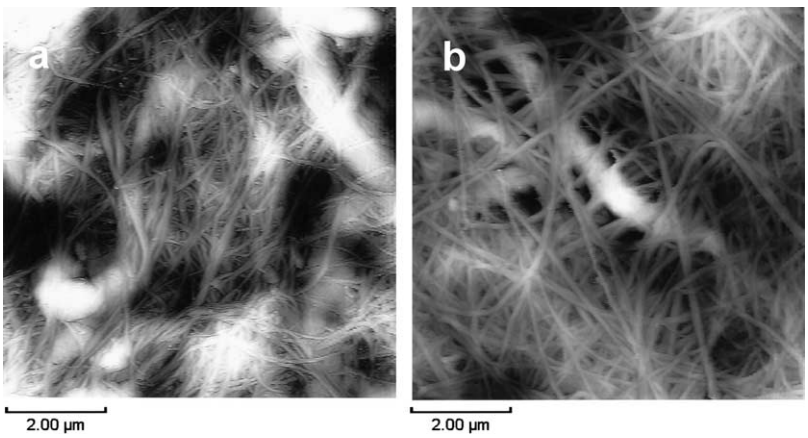

Fig. 5. AFM images showing the bacteria with its produced cellulose ribbons (a) control medium (b) 30 atm pressures..

ribbons that were twice as large compared to the ribbons produced in the control medium. In Fig. 5 are the bacteria shown together with its produced cellulose. As previously shown by optical microscopy (Fig. 3), larger ribbons were generated by the high pressure incubated bacteria compared to the control whereas their sizes remained similar (Fig. 5, Table 1).

The ribbons width were also estimated by electron microscopy. The electron micrographs of the two samples are presented in Fig. 6, and at higher magnification in Fig. 7. As can be seen in Fig. 6 there are large morphological differences between the two samples. It seems as if the ribbons produced at high pressure are stiffer and less entangled compared to the ribbons produced by the control medium. The electron micrographs (Fig. 7) taken at higher magnification confirm results by AFM that the ribbons produced at 30 atm pressures are larger compared to the ribbons produced in the control medium. The wide ribbons produced at high pressure are composed of associated ribbons which further are composed by microfibrils. The width values of the ribbons were estimated using the electron micrographs. The average value for the control was 36 nm (Table 1) results in agreement with previous estimates (Tokoh et al., 1998) whereas the estimated average width value for the ribbons grown at 30 atm pressures was 80 nm.

The electron diffractogram recorded on ribbons produced at high pressure shows a highly ordered diffraction pattern with typical triclinic diffraction spots of $1\,\underline{0}3$ indicating high degree of microfibril aggregation compared to the control medium.

Fig. 8 shows the X-ray diffraction diagrams of the control sample and the 30 atm sample, respectively. The crystallite size from the 110 ($2\theta = 22.6$) reflection and the crystallinity index were calculated and are given in Table 1. The cellulose produced at 30 atm exhibited

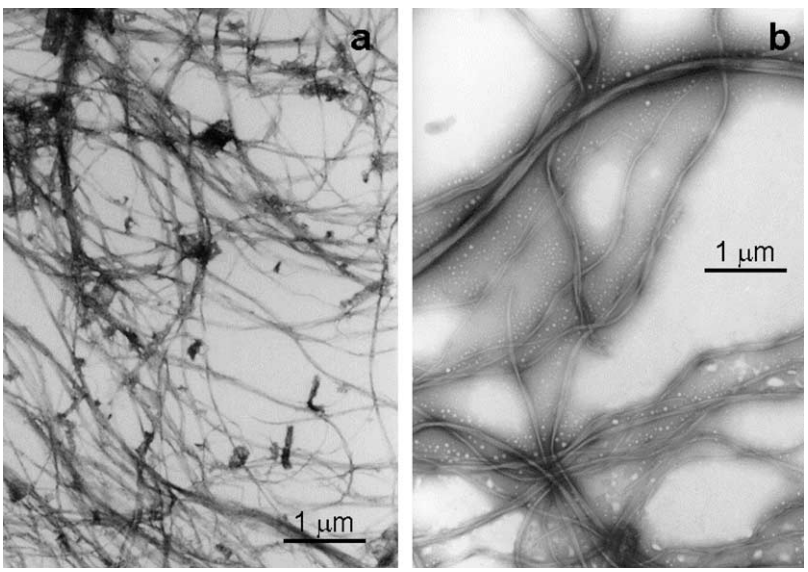

Fig. 6. Low magnification electron micrographs of ribbons produced (a) in control medium (b) at 30 atm pressures.

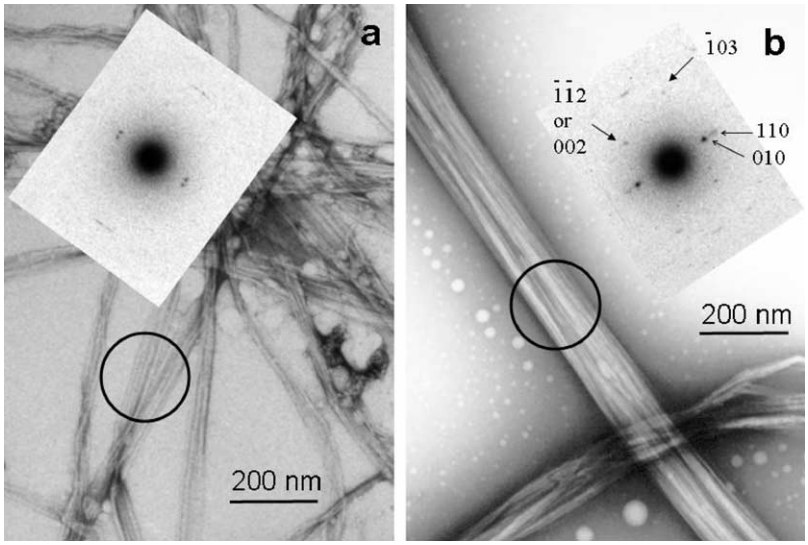

Fig. 7. High magnification electron micrographs of ribbons produced in (a) in control medium (b) at 30 atm pressures. Insert, electron diffraction recorded on oriented cellulose ribbons, as circled. The asymmetrical spots on the second and third layer line originate from the triclinic structure.

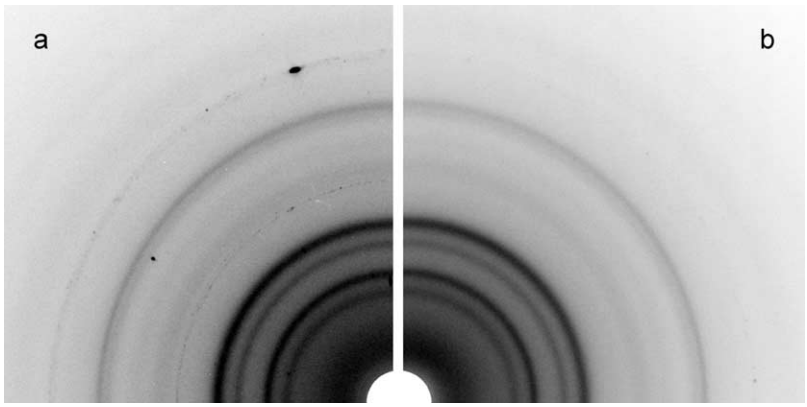

Fig. 8. Typical X-ray diffraction diagrams of (a) ribbons produced in the control medium and (b) ribbons produced at 30 atm.

similar crystallite size and crystallinity index compared to the control (Table 1).

4. Discussion

In this study it was shown that cellulose could be produced at a pressure of 30 atm corresponding to a depth of 3000 m in the ocean. The pressure was too small to induce any changes in crystallinity and crystallite size of the cellulose. However, the ribbons produced at higher pressures appeared larger in size compared to the control as observed by AFM and TEM.

The formation of cellulose ribbons by *A. aceti* incorporates three steps. First, terminal complexes (TCs) synthesize subelementary fibrils which further associates into cellulose microfibrils. Finally, the cellulose microfibrils assemble into ribbons (Cousins & Brown, 1995). In a previous investigation (Watanabe & Yamanaka, 1995), it was shown that an increase in oxygen tension resulted in a denser network of cellulose whereas the average widths of the cellulose remained constant during the entire culture period. In this study, not only the oxygen pressure was increased but also the total pressure to 30 atm which may contribute to shorten the intermolecular distances between the ribbons thus leading to ribbon association.

In a previous study it was demonstrated that the Young's modulus of sheets made from bacterial cellulose having wider cellulose ribbons or aggregates of ribbons increased (Yamanaka et al., 2000) Furthermore, sheets made from kraft pulps with different fibril aggregate sizes exhibited different mechanical properties, i.e. tear index and tensile index (Duchesne et al., 2001) Both studies show the relative importance of aggregate size on the mechanical properties. It can therefore be expected that the cellulose produced at higher pressure exhibit different mechanical properties compared to the control. The cellulose produced at high pressures exhibited larger ribbons with stiffer appearance compared to the control.

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