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ORIGINAL PAPER

Viability and cellulose synthesizing ability of *Gluconacetobacter xylinus* cells under high-hydrostatic pressure

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Abstract The effect of pressure on viability and the synthesis of bacterial cellulose (BC) by *Gluconacetobacter xylinus* ATCC53582 were investigated. *G. xylinus* was statically cultivated in a pressurized vessel under 0.1, 30, 60, and 100 MPa at 25°C for 6 days. *G. xylinus* cells remained viable and retained cellulose producing ability under all the conditions tested, though the production of cellulose decreased with increasing the pressure. The BCs produced at each pressure condition were analyzed by field emission scanning electron microscopy (FE-SEM) and Fourier Transform Infrared (FT-IR). FE-SEM revealed that the widths of BC fibers produced under high pressure decreased as compared with those produced under the atmospheric pressure. By FT-IR, all the BCs were found to be of Cellulose type I, as the same as typical native cellulose. Our findings evidently showed that *G. xylinus* possessed a piezotolerant (barotolerant) feature adapting to 100 MPa without losing its BC producing ability. This was the first attempt in synthesizing BC with *G. xylinus* under elevated pressure of 100 MPa, which corresponded to the deep sea at 10,000 m.

Keywords *Gluconacetobacter xylinus* · Bacterial cellulose · High pressure · Size of microfibrils · Viability of cell

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Introduction

Cellulose is a major polymer on earth and is indispensable globally due to its wide application in a number of industries. The ability of *Gluconacetobacter xylinus*, also known as *Acetobacter xylinum*, in synthesizing cellulose extracellularly dates back from the report of Brown (1886) about a bacterium producing thick films, later identified as cellulose (Hibbert and Barsha 1931), in carbohydrate medium. Thereafter, *G. xylinus* has been a model organism for cellulose biosynthesis (Ross et al. 1991). Bacterial cellulose (BC) has remarkable features that are of advantage over plant-derived cellulose. BC is highly pure containing no hemicelluloses and lignin, and possesses high strength of ultra fine crystalline structure as compared to those of the plant cellulose. These excellent physical properties led to the development of BC production for the material industry (Yamanaka et al. 1989; Nishi et al. 1990).

Bacterial cellulose is formed on the surface of a bacterium through a linearly ordered array of terminal complexes (TCs) consisting of cellulose synthesis protein units that give rise to sub-elementary fibrils (Brown Jr and Montezinos 1976; Zaar 1979). These fibrils are extruded out of TC sub-units via the pores aligned on the lipopolysaccharide membrane of the bacteria. Subsequently,

the sub-elementary fibrils are aggregated into microfibrils by hydrogen bonding and are finally spun to form a ribbon-like assembly of cellulose. The structure of the microfibrils varies among cellulose producing organisms depending on the array of TCs on their cell surface (Okuda et al. 1994).

The fibril size and molecular conformation of BC from *G. xylinus* depends on the medium composition and culture condition. High-viscosity medium or low-temperature drove the bacteria to produce cellulose type II, which is composed of anti-parallel microfibril (Hirai et al. 1997; Shibazaki et al. 1998). Normal static atmospheric condition led to the production of type I cellulose consisting of parallel microfibrils. Addition of an antibiotic and a chemical reagent in the culture medium caused changes to the morphology of BC (Yamanaka et al. 2000). A higher-order structure of cellulose ribbon was obtained when the pressure of the gas phase was raised to 3 mega Pascal (MPa) during *Acetobacter aceti* cultivation (Hult et al. 2003).

The physical properties of BC, which are influenced by the ultrastructure of the cellulose ribbons, are very important factor for its applications in various industries. In this paper, as a first attempt to elucidate the effect of pressure in the formation of higher-ordered structure of BC, *G. xylinus* was cultivated under pressure magnitudes of 0.1, 30, 60, and 100 MPa. The magnitude of 100 MPa corresponds to 10,000 m in depth from the sea surface. There has been no report of existing terrestrial organisms, like *G. xylinus* (Gage and Tyler 1991). Here we showed that the ability of cellulose synthesis by *G. xylinus* was maintained under high-hydrostatic pressure up to 100 MPa and that the three-dimensional structure of cellulose produced under these severe conditions was changed.

Materials and methods

Microorganism

Gluconacetobacter xylinus (*A. xylinum*) ATCC53582 was used throughout this study.

Media and growth conditions

Gluconacetobacter xylinus was grown in liquid medium containing 50 g l^{-1} of sucrose, 5 g l^{-1} of yeast extract, 3.0 g l^{-1} of KH_2PO_4, 2.4 g l^{-1} of $MgSO_4$-$7H_2O$, and 1.0 g l^{-1} of $(NH_4)_2SO_4$. The effect of pressure on BC production was evaluated at 30, 60, and 100 MPa with the culture under ambient pressure (0.1 MPa) serving as the control. Precultures were grown at 25°C for 3 days in 300-ml Erlenmeyer flasks containing 100 ml of medium adding 0.2% cellulase (Cellusoft, Novo, Franklin, NC, USA) using rotary shaker at 110 rpm, then cells were centrifuged at 3,000 rpm, and were resuspended in 1.0 l fresh medium. About 50 ml aliquot of medium was divided to each plastic bag. Each plastic bag was statically incubated at 25°C for 6 days in a titanium pressure vessel, which was filled with water. The pressure in the vessels was controlled at constant pressure, 30, 60, and 100 MPa, using the oil pump. These instruments were borrowed from Japan Agency for Marine-Earth Science and Technology (JAMSTEC) (Kato et al. 1994).

To assess the viability of the bacterial cells cultured at 0.1–100 MPa for 6 days, the diluted cultures in phosphate-buffered saline (PBS) were spread on agar plates, and incubated for 5 days at 25°C at atmosphere. Then the numbers of colonies grown on the plates were counted to estimate the cell growth. This experiment was repeated three times and it was evaluated as average value.

Preparation of BC

Bacterial cellulose was prepared as previously reported (Amano et al. 2001). The harvested BCs cultured under the high pressure were treated with 0.5 mol l^{-1} NaOH at 100°C for 1 h to remove the bacterial cells and medium components. The cellulose fibers were then washed thoroughly with distilled water to remove residual NaOH and used for the structure analysis. The amount of cellulose produced should be measured, but the amount of cellulose was too little to weigh directly. So it was semiquantitatively estimated by the amount of insoluble matter only by naked eyes.

Field emission scanning electron microscopy (FE-SEM)

The morphology of BC produced by *G. xylinus* was observed by field emission scanning electron microscopy (FE-SEM) S-4100 (Hitachi Ltd., Tokyo, Japan) at 3,000 and 30,000 magnifications. The washed BCs produced under the high pressure were freeze-dried before coated with gold particles using Twin Coater JEC-550 (JEOL, Tokyo, Japan). The spattering of gold was carried out at 1.5 kVA for 2.5 min. The morphology of cell was also observed by FE-SEM after cell was fixed with successive, 2.0% glutaraldehyde and 1.0% osmium tetra oxide.

Fourier transform infrared (FT-IR) spectroscopic analysis

For IR analysis, the BCs produced at 0.1, 30, 60, and 100 MPa were first washed as described above and freeze-dried and crushed into powdered form. The powdered BC was then mixed with potassium bromide and the mixture was pressed into tablet. Finally, the tablet was analyzed by

using the FT/IR-4200 spectrometer (JASCO, Tokyo, Japan). The IR spectra of the BC produced at 30, 60, and 100 MPa were compared to that produced at ambient pressure (0.1 MPa), which served as the control.

Results

Effect of pressure on the BC synthesis of *G. xylinus*

Gluconacetobacter xylinus ATCC53582 was cultured at 25°C for 6 days under elevated hydrostatic pressures of 30, 60, and 100 MPa. Cultures grown at ambient pressure (0.1 MPa) were used as control. Interestingly, BC was synthesized by the aerobic *G. xylinus* even under hydrostatic pressure as high as 100 MPa (Fig. 1b), although a concomitant decrease in the amount of BC was observed. The higher was the pressure imposed to the bacterial culture, the lesser was the amount of BC produced by *G. xylinus*. Hence, BC produced at 100 MPa was significantly decreased to a very minimal amount as compared to that produced at 30 and 60 MPa. The amounts of cellulose produced even at atmosphere were too little to measure the weights of them directly. In this case, the growth was significantly limited because of no supply of oxygen as the plastic bags were steeped in the water.

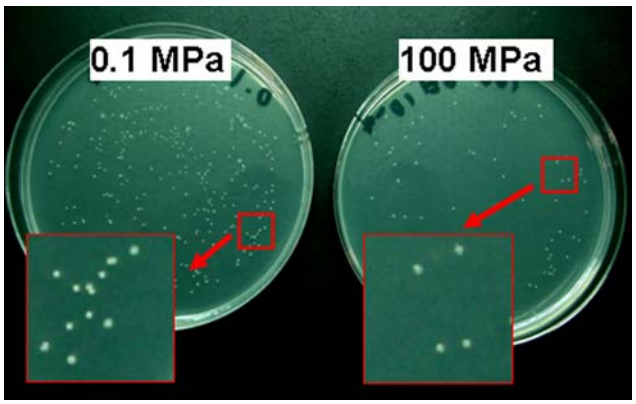

Fig. 1 The viability of *G. xylinus* ATCC53582 cells following incubation at 0.1 and 100 MPa (**a**) and FE-SEM image of BC produced at 100 MPa (**b**)

The marked decrease in the quantity of BC from *G. xylinus* at 100 MPa was further investigated taking into consideration the viability of the bacteria and the cellulose synthesis capability. To evaluate whether cell death attributed to the observed discrepancy in BC synthesis, we streaked cell obtained from culture at 0.1 and 100 MPa hydrostatic pressure on an agar plate. As shown in Fig. 1a, the *G. xylinus* survived at 100 MPa were viable as those of the control (0.1 MPa), though the number of colonies decreased apparently. The average numbers of viable cells after cultured at 0.1 and 100 MPa were estimated at 2.7×10^7 and 8.2×10^6 CFU ml^{-}, respectively, when 1.8×10^7 CFU ml^{-} cells were inoculated in the medium initially. From these results, it is suggested that cell growth was suppressed even under the normal pressure, compared with the culture using an Erlenmeyer flask. On the other hand, the number of viable cells decreased after cultured at 100 MPa. In addition, morphology of the bacterial cells cultivated under 0.1 and 100 MPa revealed rod shape cells and no significant difference as observed under FE-SEM (Fig. 2). These results indicate that the main reason why the amount of cellulose decreased when cultured at 100 MPa is the decrease in the viable cells. However, it is noteworthy that viable cells existed and the ability for cellulose synthesis was maintained at high pressure such as 100 MPa.

Morphology of BC

To elucidate the effect of pressure to the morphology of BC, the spun cellulose ribbons making up the microfibril aggregates of the sub-elementary fibril commencing from the TC sub-units of the bacterial cells were analyzed by FE-SEM and FT-IR.

Elemental analysis of each of the harvested cellulose ribbons by scanning electron microscope revealed profound morphological differences among the cellulose ribbons. As shown in Fig. 3a, all the BC had the three-dimensional knitting structure. The microfibrils of the

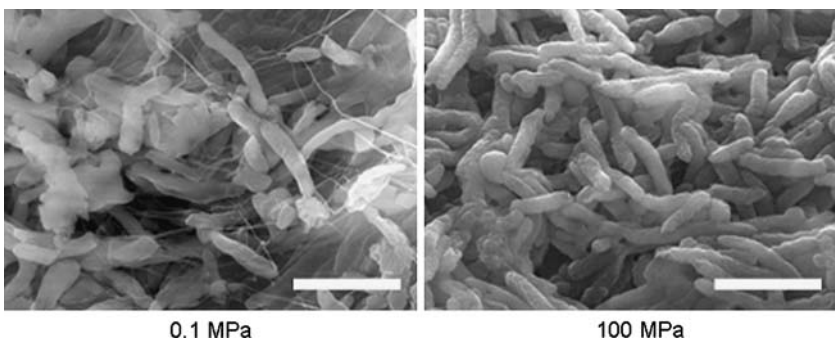

Fig. 2 FE-SEM images of cell morphology after cultivation at 0.1 and 100 MPa. *White bars* corresponds to 3.0 μm

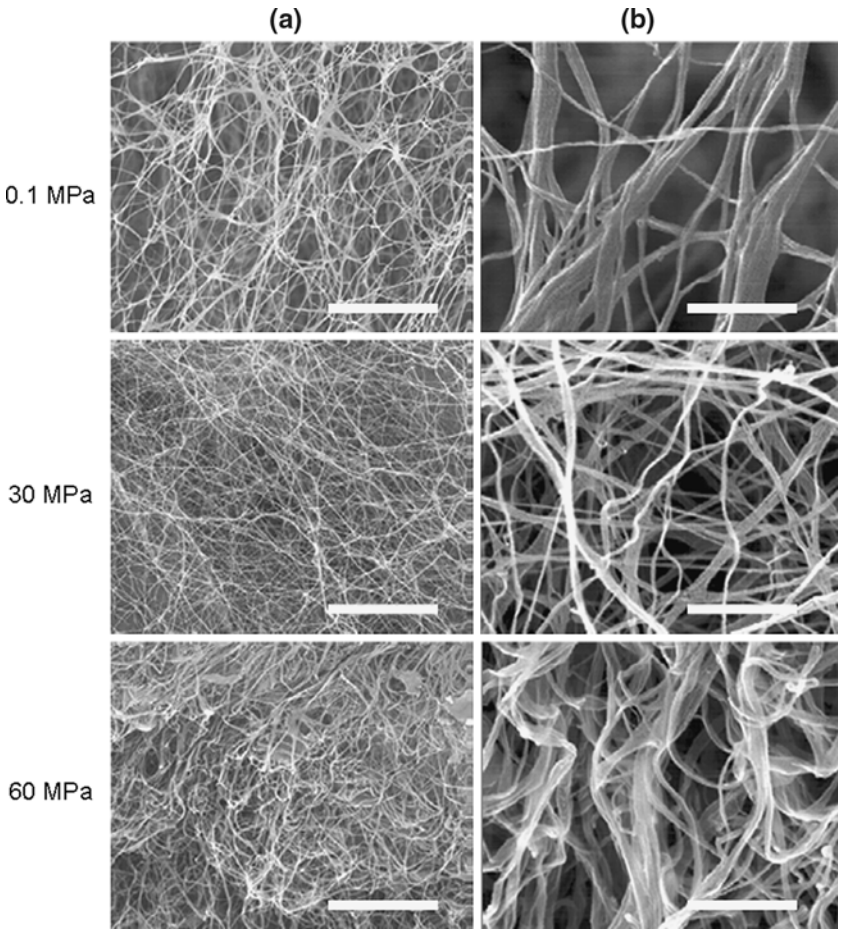

Fig. 3 FE-SEM images of BC ribbons produced at 0.1, 30, and 60 MPa at magnifications of 3,000-fold (**a**) and 30,000-fold (**b**). *White bar* corresponds to 6.0 μm (**a**) and 600 nm (**b**), respectively

ribbon from the 0.1 MPa culture were closely entangled rendering a marked networking structure. With increasing pressure, the microfibrils became denser yielding a crowded structure. When observed at higher magnification, the images revealed that at elevated pressure, most of the microfibrils overlaps increasing the number of glucan sheets, which run preferable on the longitudinal axis with diminished interweaving among the fibers (Fig. 3b). Unexpectedly, although the width of the ribbons varied significantly at different pressure conditions, the effect is not proportional with the increase in the magnitude of the pressure. The width of the cellulose ribbon obtained from 0.1 MPa culture was about 70 ± 45 nm (30 fibers), while those from 30 to 60 MPa cultures were about 47 ± 21 nm (30 fibers) and 57 ± 21 nm (30 fibers), respectively.

To determine the types of BC synthesized by *G. xylinus* at high pressure, the BCs were analyzed by FT-IR (Fig. 4). The adsorption spectra of the BC prepared from cultures at 30, 60, and 100 MPa generated peaks similar to the crystalline cellulose obtained from the control (culture at 0.1 MPa). All the samples elicited signals at the area around 3,700–3,000 cm^{-1} and 3,000–2,850 cm^{-1} corresponding to the absorption spectra of the O–H and C–H bonds, respectively, of typical cellulose type I.

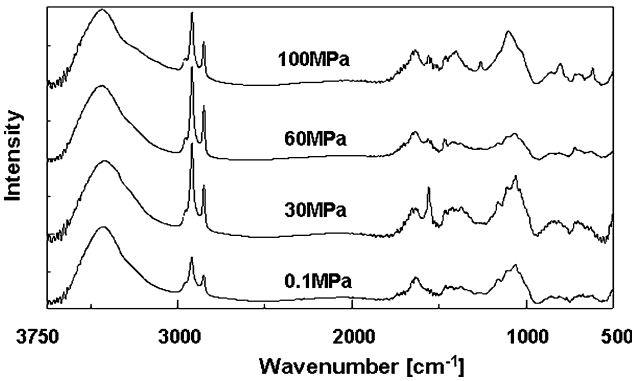

Fig. 4 FT-IR spectra of BCs harvested from the cultures under 0.1, 30, 60, and 100 MPa. The preparation of KBr tablets was described in Materials and methods

Discussion

Gluconacetobacter xylinus ATCC53582 is well-known as a prolific producer of BC, yet culture conditions greatly influence cell growth, cell morphology, and cellulose structure. Herein, we clearly show that *G. xylinus* can produce BC at various magnitudes of hydrostatic pressures

within the range of 0.1–100 MPa (Fig. 3). The ability of *G. xylinus* to synthesize BC even at 100 MPa, which is the same pressure as that in deep seas such as 10,000 m in depth where only piezophilic microbes can survive (Yayanos 1995), is noteworthy. Previous report showed that *Escherichia coli* K-12 strain W3110 cells cultivated at 40 MPa failed to grow on agar plate and its morphology was subsequently altered into extended fiber type (Ishii et al. 2004). On the other hand, piezophilic bacteria from the deep sea like *Shewanella benthica* DB21MT-2 and *Moritella yayanosii* DB21MT-5 grew well under pressures of 70–80 MPa but not at <50 MPa (Kato et al. 1998; Nogi and Kato 1999). In contrast, *G. xylinus* ATCC53582 cells not only can grow well under culture conditions within 0.1–100 MPa (Fig. 1) without undergoing morphological changes, it also maintains its cellulose producing ability (Fig. 3). To date, a few reports have dealt with growth and survival of bacteria at high pressures. *E. coli* MG1655 survived by residing within fluid inclusions in ice IV crystal at pressure several hundreds above 100 MPa (Sharma et al. 2002). *G. xylinus* ATCC53582 cells can survive and produced cellulose even in 100 MPa (Fig. 1) without undergoing morphological changes (Fig. 2). Likewise, mutants of *E. coli* MG1655 (Hauben et al. 1997) and *E. coli* NCTC 8164 (Casadei et al. 2002) were tolerant to pressure above 100 MPa. Whether *G. xylinus* could extends its adaptability and sustainability at pressures higher than 100 MPa is a future prospect, especially in corollary with BC synthesis. There is no report about polysaccharide production by microorganism under the high pressure such as 100 MPa, so no one has known the sensitivity of polysaccharide producing protein inside the cells. We showed the stability of cellulose producing protein complex called terminal complex at 100 MPa, and make it sure important study for cellulose production.

The effects of high pressure in cell growth and viability are still unclear because the equipments for cultivation of microorganisms under the high pressure were limited. It is not easy to supply the oxygen in the vessel under the high pressure over 100 MPa if we use the closed system. In the deep sea water can circulate and supply soluble oxygen to the organisms, and therefore aerobic microorganisms can grow. In our cases, it is suggested that *G. xylinus* cell can grow only early stages in the closed vessel and produce a small amount of cellulose without the supply of oxygen. However, it is very interesting that this bacterium may survive and produce cellulose under the condition of deep sea. How could *G. xylinus* survived at a high pressure of 100 MPa? *Gluconacetobacter* might be equipped with a defense mechanism through its own product, the BC. BC is very hydrophilic holding over 100 times its weight in water (Ross et al. 1991; Brown Jr 1993). *Gluconacetobacter* sp. excretes nascent cellulose through its TCs, which are

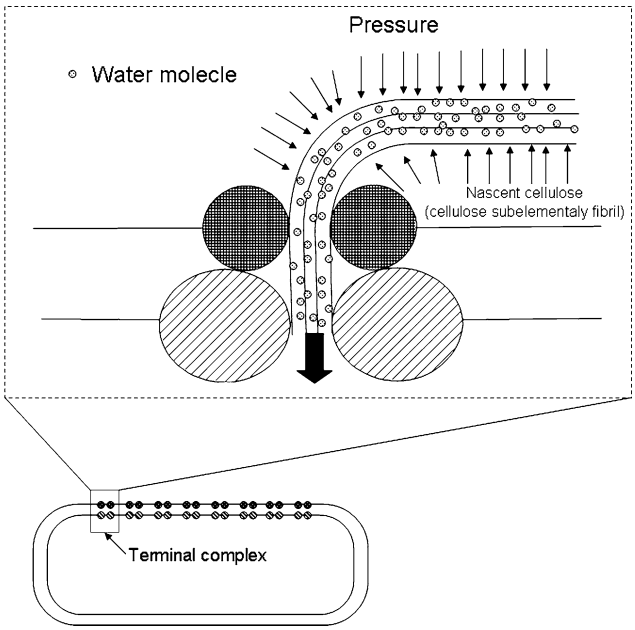

Fig. 5 The possible mechanism for the tolerance against high pressure in *Gluconacetobacter* cell. The structure of terminal complex was described (Brown Jr and Montezinos 1976; Zaar 1979) and it was suggested water molecules could pass through the pores formed with nascent cellulose

composed of some proteins concerning to cellulose synthesis, located at the cell membrane (Brown Jr and Montezinos 1976; Zaar 1979). The nascent cellulose fibrils are excreted in extracellular through TCs as shown in Fig. 5. The nascent cellulose before crystallization holds a relatively large amount of water (Kai and Koseki 1985). Water molecules are speculated to run through the non-crystallized cellulose, thereby balancing the extracellular high pressure with the intracellular pressure accordingly. Such mechanism of a pressure balance might enable cells of *G. xylinus* to survive even at a pressure of 100 MPa. Alternatively, the membrane fluidity may contribute to the resistance of the bacteria at elevated pressure, as previously suggested (Casadei et al. 2002).

In this study, *G. xylinus* at high-pressures synthesizes cellulose type I consisting of parallel microfibrils with the same crystal allomorph as those synthesized at normal static atmospheric condition (Fig. 4). The structures of BC may depend on various factors including the rheological behavior in culture (Shibazaki et al. 1998). Lipids are particularly sensitive to pressure effects, being compressible on pressure (Kamimura et al. 1993). As mentioned above, many deep-sea organisms and pressure-resistant bacteria modulate their membrane fluidity and composition in response to pressure (Barlett 2002; Casadei et al. 2002). *G. xylinus* has a well known envelope structure composed of an outer lipopolysaccharide membrane on which situated a long row of large pores, each corresponding to about

100 Å particle (Zaar 1979). The pores were proposed to act as an ''assemblyase'' or represent the organization of terminal complex (Brown Jr and Montezinos 1976) operating as a catalyst promoting crystallization of the microfibril. On the other hand, they may instead function as the spinning machines spinning glucan chains into a crystalline microfibril (Zaar 1979). Based on the results, it is possible that at high pressure, the lipopolysaccharide membrane might have compressed, affecting the pore size or number of pores on the membrane, subsequently resulting in rearrangements or reorientation of the terminal complex. The reorientation of TCs may in turn influence the array of the sub-elementary fibrils and accordingly the structure of the synthesized BC. In fact, we often observed thinner ribbons in the BC produced under the high pressure than normal ones (Fig. 3).

Overall, this is a first report showing the synthesis of BC at 100 MPa by an acetic acid bacterium, *G. xylinus*. Whether *G. xylinus* can survive above 100 MPa remains should be addressed, considering the limitation of the system employed in this study. Nevertheless, the adaptability and sustainability of *G. xylinus* at 100 MPa promised a new method for BC production. The BC produced at 100 MPa might warrant a novel BC with improved quality for therapeutic and industrial applications.

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