Characterizations of Bacterial Cellulose Producing Strain *Gluconoacetobacter hansenii* CGMCC3917

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Abstract

*Gluconoacetobacter hansenii* CGMCC3917 (M438), isolated from inoculums of strain J2, treated by high hydrostatic pressure, has strong ability of producing cellulose as more than three times as that of its initial strain J2. In this paper, in order to further study the effects of high hydrostatic pressure treatment on characterizations of strain J2 on the basis of previous study, properties of these two strains were examined and compared. The results indicated that the mutant strain M438 and its initial strain J2 had different Phenotypic Characterizations in liquid seed medium. The fermentation parameters showed that cell growth rate of strain M438 was relatively higher than that of strain J2, namely, residual sugar, residual nitrogen and acidity of strain M438 were less than that of strain J2. Furthermore, water holding capacity (WHC) and water release rate (WRR) of bacterial cellulose (BC) membranes produced by M438 were both better than those of BC membranes produced by J2. However, SEM imagines suggested that there was no evidence difference in microstructure of BC membranes. Additionally, FT-IR also showed no difference between BC membranes produced by strain M438 and its initial strain J2.

Keywords: *Gluconoacetobacter hansenii*, Bacterial cellulose; Characterizations; High hydrostatic pressure

Introduction

*Gluconoacetobacter hansenii* is gram negative, rod, straight or slightly curved, singly or in pairs, belonging to the family *Acetobacteraceae* within the *Alphaproteobacteria* class of the Proteobacteria phylum [1]. It was reported that a notable feature of this bacterium has been found is to secrete extracellular cellulose in the form of a pellicle at the air–liquid interphase, which is commonly referred to as bacterial cellulose (BC) [1]. BC, being an eco-friendly biomaterial, differs from plant cellulose in chemical and physical features. It exhibits higher purity, higher crystallinity, higher degree of polymerization, higher water absorbing and holding capacity, higher tensile strength, and stronger biological adaptability [2-5]. Due to its unusual physicochemical and mechanical properties, BC presents a potential alternative to plant-derived cellulose for specific applications in bio-medicine, cosmetics, high-end acoustic diaphragms, paper-making, food industry and other applications [6-10].

High hydrostatic pressure (HHP) is a well-known physical stress, causing various effects on a variety of cellular structures and functions. It can interfere with the processes of polymerization and depolymerization of proteins which are essential for the formation and functioning of the mitotic structure and its stability [11]. Additionally, reactivity of some enzymes has been shown to be affected by hydrostatic pressure [12]. Therefore, it has been used to as a useful mutating method to cause mutagenesis to *Escherichia coli*, *Streptococcus lactis* and *Bacillus subtilis* [13]. In our previous work, BC-producing strain J2, screened from homemade vinegar under static condition, was treated by HHP for 15 min at 250 MPa, 25°C and then a mutant strain M438 was obtained. Its BC yield (dry weight) increased from 1.08 g L$^{-1}$ to 3.58 g L$^{-1}$ under the same condition [14,15]. Additionally, the mutant strain M438 and its initial strain J2 were identified as *Gluconoacetobacter hansenii* subsp. nov. and a varietas of Ga. *Hansenii* based on 16S rRNA gene sequences respectively [14].

Figure 1: Colony shape of bacterial strain J2 (a) and M438 (b) in the seed medium.

In the present study, in order to further study the effects of HHP treatment on characterizations of strain J2 on the basis of previous study, colonial morphologies in the liquid medium and fermentation parameters including residual nitrogen, residual sugar, the total acidity and cell density, were determined and compared. In addition, water holding capacity (WHC) and water release rate (WRR) of the bacterial cellulose were measured. Its microstructure was observed using scanning electron microscopy (SEM) and its chemical bonds were determined using Fourier transform infrared spectrometry (FT-IR).
Materials and Methods

Microorganism and BC preparation

The mutant strain $M_{438}$, deposited as CGMCC3917 at China General Microbiological Culture Collection, Beijing, China, was used in the present study and maintained on glucose agar slants containing: 20 g glucose L$^{-1}$, 5 g yeast extract L$^{-1}$, 1 g $K_2HPO_4$ L$^{-1}$, 15 g $MgSO_4\cdot7H_2O$ L$^{-1}$, 15 mL ethanol L$^{-1}$, and 17 g agar L$^{-1}$. The strain was cultured at 30°C for 12–18 h, then stored at 4°C in a refrigerator and sub-cultured every 2 months for inoculum development or stored at −80°C with 20% (v/v) glycerol instead of agar for long-time storage [14].

For producing inoculums, a loop of the strain was transferred from a slant culture into an Erlenmeyer flask (250 mL) containing 100 mL seed medium with the same components as glucose agar slants but without agar. The seed cultures were grown at 30°C and 150 rpm in a rotary shaker (THZ-82A, Jintan Jieruier Electrical apparatus Co. Ltd., Jiangsu, China) for 12–18 h until it reached the logarithmic growth phase. Then inoculum (9%, v/v) was transferred into a glass vessel (300 mL) containing 100 mL of fermentation medium containing: 30 g carbon source L$^{-1}$ (glucose/sucrose = 2:1), 5 g yeast extract L$^{-1}$, 3.6 g $FeSO_4\cdot7H_2O$ L$^{-1}$, 3 g $ZnSO_4\cdot7H_2O$ L$^{-1}$, 0.8 g $K_2HPO_4$ L$^{-1}$, 16.2 g $MgSO_4\cdot7H_2O$ L$^{-1}$, 0.5 g citric acid L$^{-1}$, 7 mL ethanol L$^{-1}$). The starting pH of the medium was adjusted to 5.0. The glass vessel was covered with 8 layers of gauzes, and then statically cultured at 30°C for 14 days [14].

After cultivation, BC membranes were rinsed with running water overnight, soaked in 0.1 M NaOH solution at 80°C for 2 h, and then washed with deionized water several times to completely remove alkali. The purified cellulose was dried at 105°C to constant weight [15].

The medium, culture condition and BC preparation of strain $J_2$ in this study were as same as those of strain $M_{438}$ described above.

Phenotypic characterization

Individual morphologies of strain $M_{438}$ and $J_2$ were examined in their logarithmic growth phase in liquid seed medium at 30°C and 150 rpm in a rotary shaker incubator.

Fermentation parameters

Cell density was measured according to Guo et al. methods [16] and modified. One loop of the slant culture incubated for 24 h was transferred to an Erlenmeyer flask (500 mL) containing 200 mL seed medium, and was cultivated at 30°C for 72 h. 5 mL of cell culture was collected to a test tube under sterile condition at intervals and stored at 4°C. All the collected cell cultures were mixed with phosphate buffer (3 mL, pH = 5.0) containing cellulase (2%, w/v) (60,000 u/g, Wolsen, China) at 55°C for 30 min., and then the mixtures’ optical densities at 600 nm (OD 600) were tested on a UV = 1,700 PharmaSpec spectrophotometer ( Shimadzu, Japan), using culture medium without inoculation as control. Growth curve was drawn with denary logarithm of OD600 x 100 as vertical coordinate and with incubation time as horizontal coordinate [15].

The residual sugar concentration in culture medium was determined by anthrone–sulphuric acid reaction [17].

The residual nitrogen concentration in culture medium was determined by Coomassie brilliant blue (G-250, Sigma) method [18].

The total acidity was measured according to the method of GB/T 12293-90. The result was expressed in the total concentration of acetic acid.

Characteristics of BC membranes

Water holding capacity: For the determination of WHC, wet BC samples were removed from the storage container with tweezers. The samples were shaken twice quickly and then weighed, and dried at 60°C in order to completely remove water in them. WHC was calculated by the following formula [19]:

\[
\text{WHC} = \frac{W_1 - W_2}{W_0} \times 100\%
\]

where $W_1$ is the weight of wet BC sample, $W_2$ is the weight of dry BC sample, and $W_0$ is the initial weight of BC sample.

Figure 2: Cell density of strain $J_2$ and strain $M_{438}$ during 14-day fermentation.

Figure 3: Residual sugar of medium from $J_2$ and $M_{438}$ during 14-day fermentation.
Water Holding Capacity = Mass of water removed during drying (g) / Dry weight of cellulose (g)

Water release rate: To determine WRR, Shezad’s method [20] was used and modified. The wet BC membranes from M438 and J2 were cut into small pieces and dried by a freeze dryer (MCFD5508, SIM International CO) for 48 h. The dried BC samples (1g) were subsequently immersed in distilled water under shaking (100 rpm) condition at room temperature for rewetting. After complete rewetting (stabilized wet weight), samples from M438 and J2 were analysed for WRR by continuously measuring their weights at various time intervals at ambient temperature until complete drying. The loss of water at different time intervals was plotted against time.

SEM observation

For scanning electronic microscope observation, the freeze-dried BC membrane was mounted on a copper stub using double adhesive carbon conductive tape and coated with platinum for 30s using a platinum coating facility (Auto Fine Coater JFC-1300, Jeol, Japan). The SEM photographs were obtained by scanning electron microscope (JSM-6360LV, Jeol, Japan) at room temperature at 15 kV.

FT-IR spectroscopy

FT-IR spectra were recorded on these membranes using a Perkin Elmer FTIR Spectrum 100 System spectrometer equipped with a universal single bounce diamond ATR attachment: the resolution was 4 cm⁻¹ after 32 scans. Spectra were collected from 4000 to 600 cm⁻¹.

Results

Morphology Characterization

In the previous study, strain J2 and M438 were examined by Gram staining and both displayed gram-negative rod, straight, or slightly curved singly or in pairs, and cell shape of both strains was similar [14]. Furthermore, there was still no evident difference in colonies on plating medium. However, colonies of strain M438 were obviously bigger than those of J2, although colonies of both strains M438 and J2 in liquid seed medium displayed transparent spheres after cultivation in a rotary shaker incubator at 150 rpm, 30°C for 24 h, (Figure 1).

Fermentation parameters analysis

Mutant strain M438 and its initial strain J2 were statically cultured at 30°C for 14 days in the same optimal fermentation medium. As the key fermentation parameters, cell density, residual nitrogen, residual sugar and total acidity of strain M438 and J2 were determined in this study.

As shown in Figure 2, the growth process of both strain M438 and J2 in seed medium included lag phase (0-2d), cell growth phase (2-6d), stationary phase (6-10d), and decline phase (10-12d). At the beginning of cell growth phase (2-4d), the growth rate of J2 was faster than that of M438, while the growth rate of mutant strain M438 was significantly faster than that of its initial strain after day 4.

As described in Figure 3, from day 0 to day 4, the residual sugar of both strain M438 and J2 decreased rapidly from 30 g L⁻¹ to 15.47 g L⁻¹ and 14.49 g L⁻¹ respectively. After that, the residual sugar of J2 reduced slightly, then began to flatten and reached 13.78 g L⁻¹. While the residual sugar of M438 reduced significantly until d7 and leveled off at approximate 7.8 g L⁻¹ in the left days. To sum up, during these 14 days, strain M438 consumed more carbon source, compared with J2. Moreover, the residual sugar of J2 was nearly doubled that of mutant strain M438.

As can be seen in Figure 4, the nitrogen consumption trend was similar to sugar consumption trend. During the first 5 days (d0-d5) the residual nitrogen of M438 and J2 declined rapidly from 5g L⁻¹ to 3.36 g L⁻¹ and 3.25g L⁻¹ respectively. After that, residual nitrogen of J2 continued to decrease until day 7, then leveled off and arrived at 2.28 g L⁻¹ on day 14. While during that time strain M438 consumed nitrogen faster than strain J2 and the residual nitrogen was only 0.73g L⁻¹ on day 14. It was observed that residual nitrogen of J2 was nearly three times as that of M438.

![Figure 4: Residual nitrogen of medium from J2 and M438 during 14-day fermentation.](image)

![Figure 5: Acidity of medium from J2 and M438 during 14-day fermentation.](image)
Data from Figure 5 demonstrates that the mutant strain M438 and its initial strain J2 produced much acid during the fermentation period. The acidity increased with time and strain M438 produced more acid than strain J2 during the first 5 days, while after that, strain J2 produced more acid than strain M438 days. Furthermore, the acidity of strain J2 went gradually up to maximal value 17.03 g L\(^{-1}\) on day 14, while the acidity of M438 increased slightly to 12.62 g L\(^{-1}\) on day 14. It could be seen that the maximal acidity of J2 was significantly higher that of M438.

To sum up, mutant strain M438 grew faster than its initial strain J2 (Figure 4), therefore it consumed more carbon and nitrogen sources to supply energy, compared to strain J2. But during fermentation period, strain J2 produced more acid than strain M438, previous studies has demonstrated that high acid will inhibit the BC production [21]. Likely, that is one of the reasons that BC yield from strain M438 was more than three times as that from strain J2 [14]. It can be inferred that strain M438 has better properties compared with strain J2.

**WHC and WRR**

WHC and WRR were determined for estimating the usefulness of BC membranes in some medical areas [22] and food industries [23]. WHC of wet BC sample produced by M438 was found to be 105 times its dry weight, higher than that of BC produced by J2. It was obviously indicated that WHC of BC produced by M438 is better than that of BC produced by J2. The reverse was certainly true for WRR. Release of water from wet BC produced by M438 took more time as compared to that of BC produced by J2 (Figure 6). It needed nearly 60 h to completely release all the water from BC produced by J2, while it took almost 90 h to release all of the water BC produced by M438. The slow release of water from BC is important in biomedical applications and food additives.

**SEM observation and FT-IR**

The microstructure of cellulose membrane was observed by SEM. Although multiple SEM micrographs were taken for the membranes produced from strain J2 and strain M438, only one representative micrograph is presented here (Figure 7). Micrographs obtained for these samples revealed a densely packed network of cellulose fibrils with few subtle differences between each sample. As observed by SEM (Figure 7), it shows that the micro-fibrils of BC membranes from J2 and M438 were randomly arranged with plenty of spaces among them. This arrangement of fibrils results in the formation of pores with different diameters on the surface and through the entire matrix of the BC sheets. So the water molecules can be sandwiched between pores of the thick fibrils, and these fibrils act as a shield for water molecules. Hence, these fibrils display high water holding capacity. Results from SEM images of Figures 7a and 7b suggest that fibrils of BC produced by J2 were the same as those of M438 in fibril size, while there were more pores in the fibrils of BC produced by M438 compared with those of J2 (Figures 7c and 7d). It was reported that BC membranes with more pores have high WHC capacity [24]. Therefore the higher WHC of BC produced by M438 may be due to more pores.

As can be seen in Figure 8, there was no evident differences between these two membranes. And both were consistent with the result reported in the previous literature [25].

**Discussion**

In this study, the different characterizations of M438 and J2 should be induced by HHP. Of course, another possibility was not still removed completely, that is, this mutant strain was from the presence of potential mutant strains in test strains. However, the test strain used every time was from pure culture of a single colony. Therefore, there is very little possibility that the mutant strain was obtained from screening naturally.

As a key physical parameter, the pressure exerts an important influence on the viability and biological activity of organisms. A wide variety of high pressure-induced phenomena in living cells have been reported and reviewed, including changes in cellular morphology, biochemical reactions, genetic mechanisms, and membrane integrity [26,27]. In the previous study, it was found that only 0.5% of strains can survive after strain J2 treated by HHP for 15 min at 25°C, 250 MPa [15], which is the same pressure as that in deep sea of 25,000 m depth where only piezophilic microbes can survive. Previous report showed that G. xylinus ATCC53582 cells were cultivated under culture conditions within 0.1–100 MPa and can grow well without undergoing morphological changes. It also maintains its cellulose producing ability but only produces a little BC [28]. While in this study, Colonies of strain M438 in liquid seed medium were bigger than those of its initial strain J2 and fermentation parameters of strain M438 were different from those of strain J2. Maybe it is probably due to the different magnitude and duration of treatment. Up to date, there are few reports about polysaccharide production by microorganism after treatment by high hydrostatic pressure under the high pressure of 250 MPa, so no one has known the sensitivity of polysaccharide producing protein inside the cells [28,29]. It is widely recognized that cell growth in organisms is inhibited by pressure [13]. However, in the present study the mutant strain M438 treated by HHP grew faster than its initial strain. According to the fermentation parameters, metabolism of strain M438 was faster, compared to those of strain J2.

Additionally, there were no evident differences in the properties of BC membranes to some extent, except for that the BC yield increased significantly after HHP treatment [15]. However, WHC of BC membranes produced by mutant strain M438 was a little higher and wet BC membranes took more time to completely to remove water in BC, compared to those of strain J2. BC is formed on the surface of a bacterium through a linearly ordered array of terminal complexes (TCs) composing of cellulose synthesis protein units that give rise to
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