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Newly developed medium and strategy for bacterial cellulose production

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ABSTRACT

Bacterial cellulose (BC) has unique properties, such as high crystallinity, a high degree of polymerisation, high tensile strength and high purity, compared with native cellulose. In this study, a previously determined BC production medium was improved in static culture, and the production cost was evaluated and compared with molasses and with other defined media, such as Hestrin–Schramm, Zhou, Yamanaka and Park, using *Gluconacetobacter xylinus*. In addition to this analysis, because the surface area/volume ratio is an important parameter in static culture, different surface area/volume ratios were analysed in the range of 0.2–1.46. The defined medium (M1A05P5) and culture type contained glucose (10 g/L), yeast extract (10 g/L), peptone (7 g/L), acetic acid (1.5 ml/L), and ethanol (5 ml/L), and the pH was adjusted to 5.0 in static culture. The highest productivity was observed in the M1A05P5 medium that was 5-fold higher than either molasses or Park's medium. Although the molasses medium was proposed as a cost-effective medium, the production price of BC was the lowest in the M1A05P5 medium. Therefore, the newly developed medium and strategy were highly promising candidates for the industrial-scale production of BC.

1. Introduction

Cellulose, the most abundant biopolymer in the biosphere, is composed of glucose monomers with β-1,4-glucosidic bonds. Cellulose is commonly used in food, biomedical, textile and biotechnology areas. However, to obtain cellulose from plant, separation and purification processes require harsh chemicals, such as alkali and acidic compounds to remove hemicellulose and lignin structures. The wastes of these processes cause environmental pollution and damage the structure of native cellulose. Bacterial cellulose (BC), which is generated by microorganisms from monosaccharides, has unique properties, such as high crystallinity, a high degree of polymerisation, high tensile strength and high purity, compared with native cellulose [1,2]. Because of these properties, BC is preferable for specific pharmaceutical, biomedical and biotechnology applications.

In the literature, various BC production media have been proposed by researchers. These variations are based on combinations of bacteria, mixing type and production methods. Hestrin–Schramm medium, HS, is the most preferable medium for the growth of bacteria and for the production of BC [3]. HS medium contains a small amount of carbon source, an enriched nitrogen source and a small amount of citric acid, whereas Yamanaka's medium has a large amount of carbon source and enriched mineral contents [4]. Zhou's medium is prepared using a combination of HS and Yamanaka's media [5]. In addition to these media, corn steep liquor (CSL)-fructose medium, which is a fully enriched medium with minerals, inositol, nicotinic acid, thiamine and pantothenic acid, has been proposed and widely used by researchers for large-scale production [6]. The use of date syrup and molasses has also been reported for the production of BC, and the results were highly competitive with those of traditional media, such as HS and Yamanaka media [7,8]. The aim of using these types of cheap sources is to decrease the production cost of BC. Industrial productions of BC are commonly done using HS and CSL-fructose media. However, depend on bacterial strain, the other media, such as Yamanaka's, Zhou's, molasses and date syrup, BC production may be performed.

Acetobacter and *Gluconacetobacter* strains are preferable for BC production; however, some of the subspecies tend to generate biomass instead of BC. Therefore, Park et al. and Zhou et al. made minor changes to the HS medium to decrease cell growth and to increase the yield of BC production [5,9]. Their attempt was successful for increasing the productivity of BC compared with HS medium.

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The mixing type and bioreactor design are also important parameters for the generation of BC. To increase the production amount of BC, a large-scale fermenter design and mixings were proposed. The most cited design is a rotating disc bioreactor that has many discs that are submerged in production medium [10]. During the fermentation, discs rotate at a low speed, and cellulose fibrils are extruded and attached to the discs. Different types of composite support material are used in large scale BC production to enhance cell growth and to become a surface for BC generation in a stirred tank fermenter [11]. Moreover, the usage of different shapes of containers assists in forming the desired shape of BC during production [12]. The most well-known example of this system is the artificial vein formation of BC that can be used in biomedical applications [2]. The productivity of BC is widely distributed by mixing type, which can be agitated or static cultures. Depending on the microorganism strain and on the medium composition, the effect of the mixing type varies. For example, Zhou medium in which mannitol and sucrose was distinctively used was investigated for this purpose. Medium containing sucrose showed a higher activity for BC production in an agitated culture than in a static culture; however, there was no significant difference observed in mannitol containing Zhou medium [13].

In this study, BC production was investigated using *Gluconacetobacter xylinus* FC01 under different mixing conditions, such as static or agitated, and under different medium compositions, such as HS medium, Park medium, their modifications and molasses. The yield, structural features and strength of BC were determined.

2. Materials and methods

2.1. Bacterial strain and culture

Gluconacetobacter xylinus FC01 strain was previously isolated and identified using 16S rRNA sequence analysis. The composition of the HS medium included the following materials (g/L): glucose, 20; peptone, 5; yeast extract, 5; disodium phosphate (anhydrous), 2.7; and citric acid (monohydrate), 1.15, with the pH adjusted to 5 using HCI or NaOH [3]. The Zhou medium was composed of the following materials (g/L): glucose, 18; sucrose, 21; corn steep liquor, 20; $(NH_4)_2SO_4$, 4; KH_2PO_4, 2; and $MgSO_4{\cdot}7H_2O$, 0.4, with an initial pH value 6.0 [5]. Yamanaka medium was composed of (g/L) sucrose, 50; yeast extract, 5; $(NH_4)_2SO_4$, 5; KH_2PO_4, 3; $MgSO_4{\cdot}7H_2O$, 0.05 [4]. The composition of Park medium was (g/L): glucose, 10; peptone, 10; yeast extract, 7; acetic acid, 1.5; succinic acid, 2 [9]. Improved medium, M1A05P5, was fundamentally composed of (g/L): glucose, 10; yeast extract, 10; peptone, 7; acetic acid, 1.5 and different amount of ethanol in inoculation and production segments with different pH values. Microbial cultures were incubated for 6 days at 30 °C under static or agitated cultures at 150 rpm. After the incubation period, cellulose was collected by centrifugation at $3600 \times g$ for 20 min. After samples were freeze-dried, samples were treated with NaOH (0.3 M) at 80 °C for 1 h. Then, samples were freeze-dried again to calculate the cell dry weight (CDW; g/L) via the difference of weights during the freeze-drying processes [3]. The production of BC was performed in different volume of Erlenmeyer flasks containing various volumes which dimensions were measured using ruler.

2.2. Treatment of molasses

The molasses used in this study was supplied by a local company (Torku Şeker, Turkey). To prepare physically treated molasses, centrifugation was performed at $6000 \times g$ for 20 min after crude molasses was diluted 2-fold (w/v) with distilled water. Chemically treated molasses was prepared using heat and sulphuric acid treatment. The crude molasses was diluted 2-fold (w/v) with distilled water and adjusted to pH 3.0 with 6 M H_2SO_4. Then, the molasses was heated at 60 °C for 1 h at pH 3.0 and for 2 h at pH 1.0. Solid materials were removed by centrifugation at $6000 \times g$ for 20 min. Before the sterilisation of the molasses, the solutions were adjusted to pH 7.0 with 10 M NaOH [14].

2.3. Fourier-transform infrared spectroscopy

FT-IR spectroscopy was completed using a Perkin-Elmer Spectrum 100 Spectrometer. Scans were completed between 4000 and 450 cm^{-1}. Baselines for each sample spectrum were normalised using the Spectrum software.

2.4. Scanning electron microscopy

The samples were mounted and gold-coated in preparation for SEM imaging. SEM was performed using a Carl Zeiss EVO-40 instrument under high vacuum at high potential, 10 kV.

2.5. Mechanical testing of BC

Mechanical properties (tensile strength, Young's modulus) of BC were analysed using an Instron 5900. The samples (0.2 g) were weighted and prepared as previously described by Demirci et al. [15].

2.6. Statistical analysis

All determinations and experiments reported here were performed in triplicate, and Student's *t*-test was performed for the correction of experiments.

3. Results

3.1. Characterisation of BC

At the end of BC production, BC was collected and treated by NaOH at a mild temperature to remove microorganisms and substrates from the BC sheets. The SEM images showed that BC was obtained as fibril structures in all productions (Fig. 1a–c). A dense fibril web was clearly seen during the molasses medium production compared with HS and M1A05P5 media.

FT-IR spectra of the BC from M1A05P5, HS, Zhou and molasses media are shown in Fig. 1d. The pattern of the FT-IR spectra of production from different media was similar, such as at 1664 and 1431 cm^{-1} for carboxylate groups; at 2999 cm^{-1} for the stretching of CH_2; at 1058 cm^{-1} in the functionalities of C—O—C ether bonds; at 3415 cm^{-1} for hydroxyl groups and at between 3230 and 3455 cm^{-1} for inter- and intra-molecular hydrogen bounds. These spectra showed that these compounds obtained in different media were cellulose [16]. There was a slight peak at 2360 cm^{-1} in BC in molasses medium.

3.2. The effect of the combination of ethanol, pH and the mixing type

The effect of ethanol addition on the inoculant and/or on the production was investigated. BC production was observed between 0.4 and 2.2 g/L in the inoculant without ethanol addition (Fig. 2). BC production in the static culture was almost 2-fold higher than in the agitated culture and it increased by increasing the pH without ethanol during production. However, there were no similar changes in agitated cultures. In addition, the BC yield was higher when the pH level was lower than 6.0 in the static culture; the reverse situation was observed in the agitated culture when the pH level was