Carbohydrate Polymers 106 (2014) 7–13

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Improvement production of bacterial cellulose by semi-continuous process in molasses medium

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ARTICLE INFO

Article history:
Received 27 October 2013
Received in revised form 21 January 2014
Accepted 28 January 2014
Available online 14 February 2014

Keywords:
Bacterial cellulose
Semi-continuous process
Gluconacetobacter xylinus
Molasses

ABSTRACT

Bacterial cellulose (BC) has unique properties such as structural, functional, physical and chemical. The mass production of BC for industrial application has recently become attractive to produce more economical and high productive cellulose. In this study, to improve the productivity of bacterial cellulose (BC), BC production by *Gluconacetobacter xylinus* FC01 was investigated in molasses medium with static semi-continuous operation mode. Cell dry weight, polysaccharide, sugar and cellulose concentrations were monitored and cellulose was characterized by Fourier transform infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM). The highest cellulose yield (1.637 g/L) was obtained in SCP50-7d, which molasses of 1/2 ratio for 7 days by static semi-continuous operation mode. The results show that BC can be highly produced by *G. xylinus* in molasses with static semi-continuous process than batch process. We claimed that low-cost medium with semi-continuous operation mode in static culture is a good candidate for industrial scale BC productions.

1. Introduction

Cellulose, the most abundant biopolymer in nature, is composed of glucose monomers with β-1,4 glucosidic bonds. Cellulose is traditionally extracted from plants and/or their wastes. To obtain pure cellulose, compounds of hemicellulose and lignin should be effectively removed. Even if different chemical and biological extraction processes are being continuously developed, chemical processes which cause environmental pollution that consists of harsh acid and alkali treatments are commonly preferred. Plant cellulose consists of both hemicellulose and lignin, however, bacterial, or microbial cellulose comprises pure cellulose possessing unique properties such as high crystallinity, high degree of polymerization, high tensile strength and high purity that widely produced by some of *Acetobacter* strains (Delmer, 1999; Yamanaka et al., 1989). Among the *Acetobacter* strains, *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) is the most studied and well-known bacterium since it has a high level of bacterial cellulose production in liquid culture (Ross, Mayer, & Benziman, 1991).

Because of its unique properties, BC is used in various industrial applications including foods, biomedical, textile, and biotechnology (Klemm, Schumann, Udhardt, & Marsch, 2001). In traditional methods, static cultivation has been used for the BC production, by forming pellicles on the surface production medium. Although the amount of cellulose production is relatively high on static culture, it is not applicable for large-scale production due to the needs of large area and a long culture time (Okiyama, Shirae, Kano, & Yamanaka, 1992).

There have been several reports on both static (Hutchens, León, O'Neill, & Evans, 2007; Keshk & Sameshima, 2006; Yamanaka et al., 1989) and agitated (Kim, Kim, Wee, Park, & Ryu, 2006; Son et al., 2003; Zhou, Sun, Hu, Li, & Yang, 2007) cultures for cellulose production. Hestrin–Schramm (HS) (Hestrin & Schramm, 1954) medium is the most widely used for producing cellulose by using pure sugar. Ruka, Simon, and Dean (2012) have investigated the modified Zhou and HS medium in static culture. They have been reported that the amount of cellulose production was higher in Zhou rather than HS medium; however, the production of cellulose by static culture was more inefficient than agitated culture. Moreover, to increase the productivity of BC, fed-batch and continuous operation modes were performed and it was found that efficiency of fed-batch mode was generally higher than batch and continuous modes (Bae & Shoda, 2004).

Cost of the fermentation medium, 30% of total cost, plays a critical role over total cost in microbial fermentations (Rivas, Moldes, Domínguez, & Parajó, 2004). Thus, one of the important aspects in the fermentation process is finding a new cost-effective culture

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http://dx.doi.org/10.1016/j.carbpol.2014.01.103
0144-8617/

medium to get the highest yield of bacterial cellulose. In most of the studies, pure sugars including glucose, sucrose, mannitol, fructose and arabitol are used as fermentation media (Chao, Sugano, & Shoda, 2001; Oikawa, Ohtori, & Ameyama, 1995; Son et al., 2003). However, these carbon sources are not economical to use in industrial scale production of BC. New carbon sources for low cost and high cellulose yield needs to be found to produce BC.

Molasses is a by-product of the final stage of crystallization of sugar production process that can be a promising candidate for being a low cost carbon source in microbial industry. Molasses has been used as a fermentation medium in production of various industrial products such as the lactic acid (Kotzamanidis, Roukas, & Skaracis, 2002), polyhydroxybutyrate (Beaulieu, Beaulieu, Melinard, Pandian, & Goulet, 1995), ethanol (Sheoran, Yadav, Nigam, & Singh, 1998), pullulan (Lazaridou, Roukas, Biliaderis, & Vaikousi, 2002), xanthan gum (Kalogiannis, Iakovidou, Liakopoulou-Kyriakides, Kyriakidis, & Skaracis, 2003), and cellulose (Bae & Shoda, 2004). It contains suspended particles and complex structures which cause heterogeneity in medium and affect the cell growth rate. Therefore, many types of molasses treatment have been proposed to prepare the unique molasses medium for identical microorganism strains (Bae & Shoda, 2004).

In this study, we investigated the bacterial cellulose production by *G. xylinus* FC01 with semi-continuous operation mode in static culture using molasses medium in order to improve the production of BC. The structural features of BC fibrils were examined. In addition, total and reduced sugars, as well as by-products like polymers were analyzed.

2. Materials and methods

2.1. Bacterial strain and culture

G. xylinus (FC01) strain used in this study was previously isolated and identified by 16S rRNA sequence analysis in our laboratory. HS medium was used for basal medium. *G. xylinus* was grown on the following medium (%, w/v): glucose, 2.0; peptone, 0.5; yeast extract, 0.5; disodium phosphate (anhydrous), 0.27; citric acid (monohydrate), 0.115; pH adjusted to 5 with HCl or NaOH. Zhou medium was contained (%, w/v): glucose, 1.8; sucrose, 2.1; corn steep liquor, 2.0; $(NH_4)_2SO_4$, 0.4; KH_2PO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.04; initial pH value 6.0 (Zhou et al., 2007). Microbial cultures were incubated for 5–7–10–15 days at 30 °C under static conditions in treated molasses that was prepared with 120 ml medium in 500 ml flask. Volume changing ratios (VCR) were set to 1/3 (40 ml), 1/2 (60 ml) and 2/3 (80 ml) for semi-continuous processes that have been named the 1/3, 1/2 and 2/3 of VCRs as SCP33, SCP50 and SCP66, respectively, according to semi-continuous process and volume changing ratio in percent. After each incubation period, cellulose was collected from the surface and the volume was changed depending on the process continuously until liquid medium was depleted. These experiments were generally focused on 30 days of production.

2.2. Analysis of bacterial cellulose (BC), total sugar, reduced sugar and polysaccharide

After the incubation period, BC was collected from the culture broth by centrifugation for 20 min at 3600 × *g* and washed twice with distilled water. Then, BC was measured by using repetitive lyophilization after degradation of bacteria with treatment of 0.3 M NaOH for 1 h at 80 °C. To analyze reduced sugar and total carbohydrate, DNS assay (Miller, 1959) and phenol–sulphuric acid assay (Masuko et al., 2005) were used. To analyze the polysaccharide, 2 ml culture supernatant incubated with 6 ml ethanol for 1 h at 4 °C. The solution was centrifuged at 3600 × *g* for 10 min and the pellet was incubated with distilled water for 1 h at 50 °C. Finally, 0.2 ml prepared solution and 0.2 ml 5% (w/v) phenol solution was incubated with 1 ml H_2SO_4 on ice for 2 min. Last mix was read at 492 nm wavelength with spectrophotometer.

2.3. Treatment of molasses

The molasses used in this study was supplied by a local company (Torku Şeker, Turkey). 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, molasses was heated at 60 °C for 1 h. The pH was then adjusted pH 1.0 and continuously heated at 60 °C for 2 h. The molasses solution was centrifuged at 6000 × *g* for 20 min to separate solid materials. Before sterilization of molasses, the solution was adjusted to pH 7.0 with 10 M NaOH. This treatment was designated the H_2SO_4–heat treatment and the supernatant was termed as H_2SO_4–heat-treated molasses.

2.4. Fourier-transform infrared spectroscopy

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

2.5. 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.

2.6. Statistical analysis

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

3. Results

3.1. Batch operation mode for cellulose production

Molasses was initially prepared by 2-fold dilution and centrifugation steps. However, BC production was not observed in clarified molasses (data not shown). To increase the clarification and the sucrose degradation, concentrated H_2SO_4 and heat treatment were applied and precipitated solid content was then removed by centrifugation. Before using the chemically treated molasses, pH of molasses should be adjusted to desired pH that is taken in the range of 5.0–6.5 for bacterial growth. Initially, different BC production media, HS, Zhou, and treated molasses, were tested for efficiency of BC production and cell growth in batch operation mode and different incubation periods (Fig. 1). The production yield in HS medium was higher than other media up to 10 days incubation. After 7 days incubation, BC production in molasses medium drastically increased the concentration of cellulose to the highest as 0.5 g/L in batch mode. Besides, there was not any significant increase at Zhou and HS media but molasses medium had a strong effect for cellulose production. The BC production in Zhou and HS showed that the kind of media was not directly related to the cultivation period. However, BC production was increased during the elevated incubation period in molasses medium. As to cell dry weight, molasses medium has most cells among cultures and they grow from 5 to 15 day cultures.