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

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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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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.

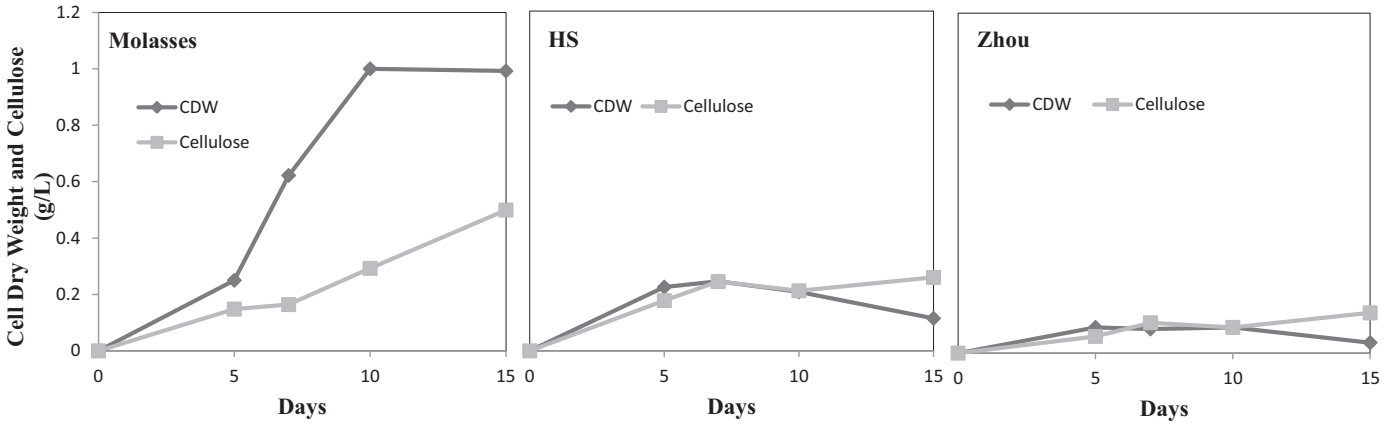

Fig. 1. BC and biomass concentration for 5, 7, 10 and 15 days in batch operation mode in molasses, HS and Zhou medium.

3.2. Semi-continuous operation mode at different volume changing ratios (VCRs)

After the observation of the efficiency of molasses medium; three different VCRs were performed in semi-continuous operation mode that were 1/3 (SCP33), 1/2 (SCP50) and 2/3 (SCP66), respectively (Fig. 2). To observe the optimum VCRs value, production of BC was initially performed as 5 days (equals to 1 stage) semi-continuous culture. The BC production was almost same in each semi-continuous process at the end of the 1st stage. After the 1st stage, BC production was increased according to VCRs values. However, SCP33 and SCP66 started to decrease dramatically after the 3rd stage (15 days) while an SCP50 steadily changed for BC production. Although, the highest cellulose concentration (0.36 g/L) was obtained at SCP66 on the 3rd stage; total cellulose concentration was the highest (1.243 g/L) in SCP50 process. Pattern of cell growth in each process was not different. After the 2nd stage of process, cell growth stopped and remained steady in SCP33, it then decreased in 4th stage; in SCP50, cell growth was stopped and immediately decreased in the 3rd stage, after that it continuously increased. Cell growth in SCP66 sharply decreased after the 2nd stage which was the highest value (0.62 g/L) among all processes.

3.3. Semi-continuous operation mode at different incubation periods

After 4 days incubation, BC formation was clearly observed on the liquid surface. To investigate the incubation period; SCP50, the highest BC production was observed as shown in Fig. 2, incubated in longer days as 7, 10 and 15 days of SCP50-7d, SCP50-10d and SCP50-15d, respectively (Fig. 3). BC generation of SCP50-5d and SCP-7d was followed in the same pattern. After the 2nd stage (20 days) of SCP50-10d, the amount of cellulose increased. In SCP50-15d, BC generation directly decreased after the 1st stage (15 days). BC concentrations were almost the same in the 2nd stage (14 days) of SCP50-7d and the 1st stage (15 days) of SCP50-15d. By reason of the existence of the 1st stage and higher BC values of the 3rd and 4th stages of SCP50-7d and decreasing of the 2nd stage of SCP50-15d, SCP50-7d was found to be a better choice than SCP50-15d for production.

3.4. Characterization of BC

Morphological structure and FT-IR graphs are shown in Fig. 4. Most common media of BC production, HS, in 15 days incubation showed a light fibril structure (Fig. 4a) in comparison to molasses medium (Fig. 4b). According to incubation period, BC formation changed from thin fibril (Fig. 4c) to web-like structure (Fig. 4d). Production of BC in molasses medium in batch mode showed dense fibril structure.

FT-IR spectrum of the BC from HS medium, Molasses and SCP50-7d are shown in Fig. 4e, in this order. The similarities were observed in carboxylic acid and carboxylate groups (at 1664 and 1431 cm^{-1}), in stretching of CH_2 (at 2999 cm^{-1}), in functionalities of C—O—C ether bounds (at 1058 cm^{-1}), in the presence of hydroxyl groups (at 3415 cm^{-1}) and inter- and intra-molecular hydrogen bounds (between 3230 and 3455 cm^{-1} regions) of FT-IR graphs (Oh et al., 2005). These spectra of standard cellulose and produced ones showed that these compounds obtained from different media and operation modes were cellulose.

3.5. Polysaccharide and reduced sugar analysis

Reduced sugar and production of polysaccharide were compared with BC production as shown in Fig. 5. Polysaccharide production in the SCP systems was common at low values, except 5 days incubation systems. As shown in Fig. 5, cellulose concentration was 100-fold higher than polysaccharide concentration in SCP50-7d; at the same time in SCP50-5d, cellulose concentration was still higher than polysaccharide concentration, but it was between 5- and 10-fold. Polysaccharide concentrations of SCP50-10d and SCP50-15d were lower than SCP50-7d, but in comparison to other processes; polysaccharide concentration of SCP50-7d was still at low values (Table 1).

The total production results of cell dry weight, polysaccharide and cellulose are summarized in Table 1. According to these results, SCP50-7d showed the highest BC productivity, which is an important parameter for industrial scale productions. Moreover, total BC concentration was higher in all semi-continuous processes than batch processes in defined media. Although the productivity of SCP50-10d and molasses in batch mode was almost the same, total cellulose concentration of SCP50-10d was 2-fold higher than molasses in batch model; therefore, it can be concluded that 5 and 7 day cultures of semi-continuous system may be preferable for cellulose production among others.

4. Discussion

So far, several studies have been reported on the productions of BC in both agitated and static cultures including different kinds of bioreactors. Many of these studies used only purified sugar (HS,

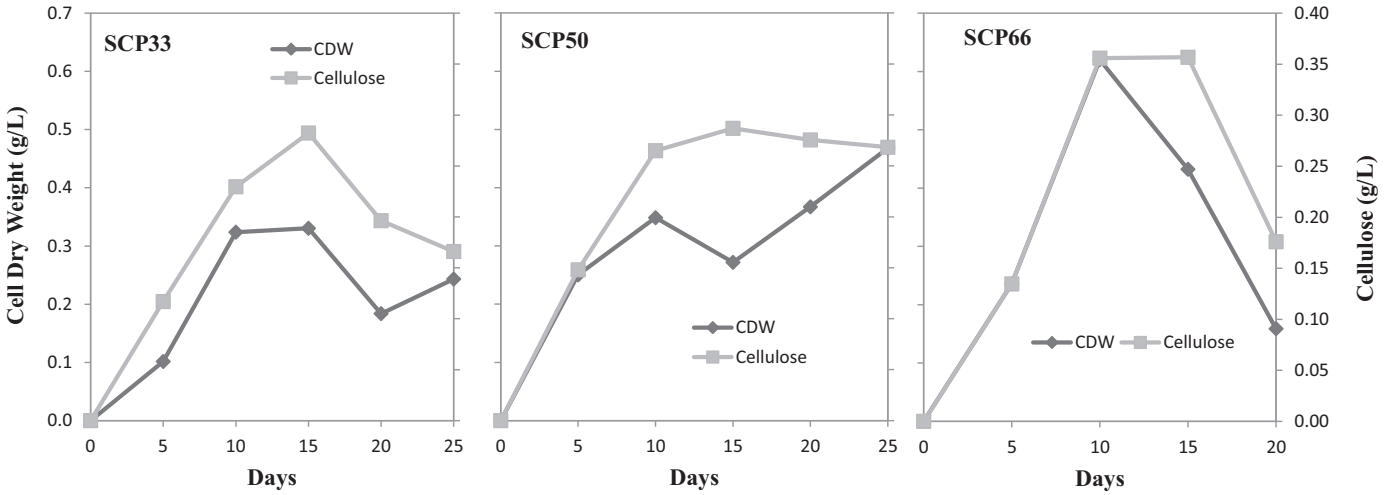

Fig. 2. BC and biomass concentration for 5 days of SCP33, SCP50 and SCP66 in molasses medium with semi-continuous operation mode.

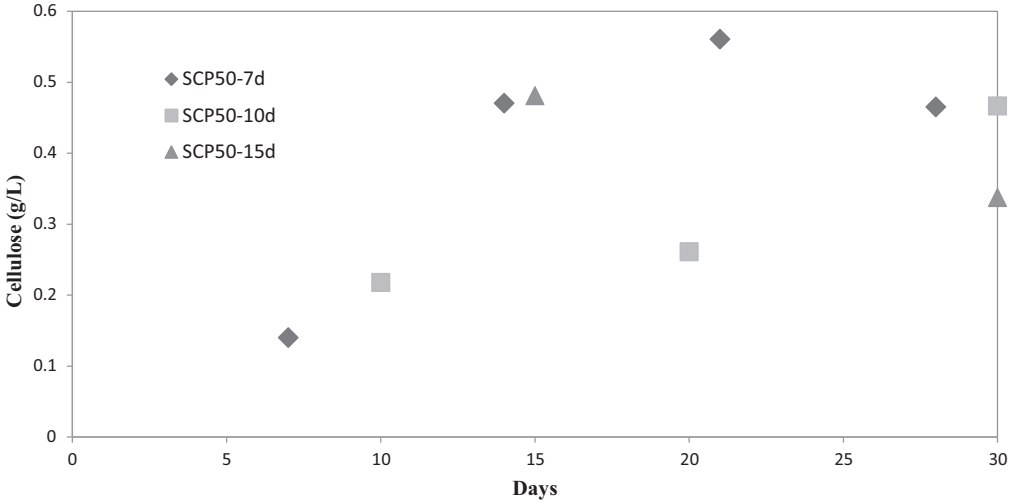

Fig. 3. BC concentration of SCP50 for 7, 10 and 15 days in semi-continuous operation mode in molasses medium.

Zhou, Yamanaka, etc.), which could dramatically increase the cost of production of cellulose in industry. Therefore, the use of less expensive carbon sources is necessary for practical BC production. In this study, molasses, which is widely used as a carbon source, was used in commercial production for BC production by *G. xylinus*. Although, Zhou has been advised for static cultures by some researchers, minimum cellulose production was observed in this study. In addition, a semi-continuous system was conducted by molasses to increase the amount of cellulose. However, molasses medium also affect the cell growth rate that may cause complexity in NaOH treatment for disrupting cells which were entrapped in cellulose fibers. Therefore, semi-continuous processes may be a better choice for BC production which depending on the total BC preparation process.

Because, there was not enough amount of liquid to continue the process caused by higher volume changing ratio and small amount of liquid medium penetration in cellulose fibrils removed when cellulose was discarded from process, the semi-continuous

Table 1
Comparison of cell dry weight and the concentration of polysaccharide, total cellulose and productivity of cellulose in various mediums and different operation mode.

Medium and process	CDW (g/L)	Polysaccharide concentration (g/L)	Total cellulose concentration (g/L)	Productivity of cellulose (g/L day)
Zhou (batch)	0.037	0.003	0.142	0.009
HS (batch)	0.115	0.049	0.259	0.017
Molasses (batch)	0.992	0.006	0.499	0.033
SCP33-5d	1.183	0.221	0.991	0.040
SCP66-5d	1.447	0.192	1.023	0.041
SCP50-5d	1.705	0.249	1.243	0.050
SCP50-7d	3.459	0.024	1.637	0.058
SCP50-10d	3.137	0.015	0.946	0.032
SCP50-15d	1.733	0.009	0.819	0.027

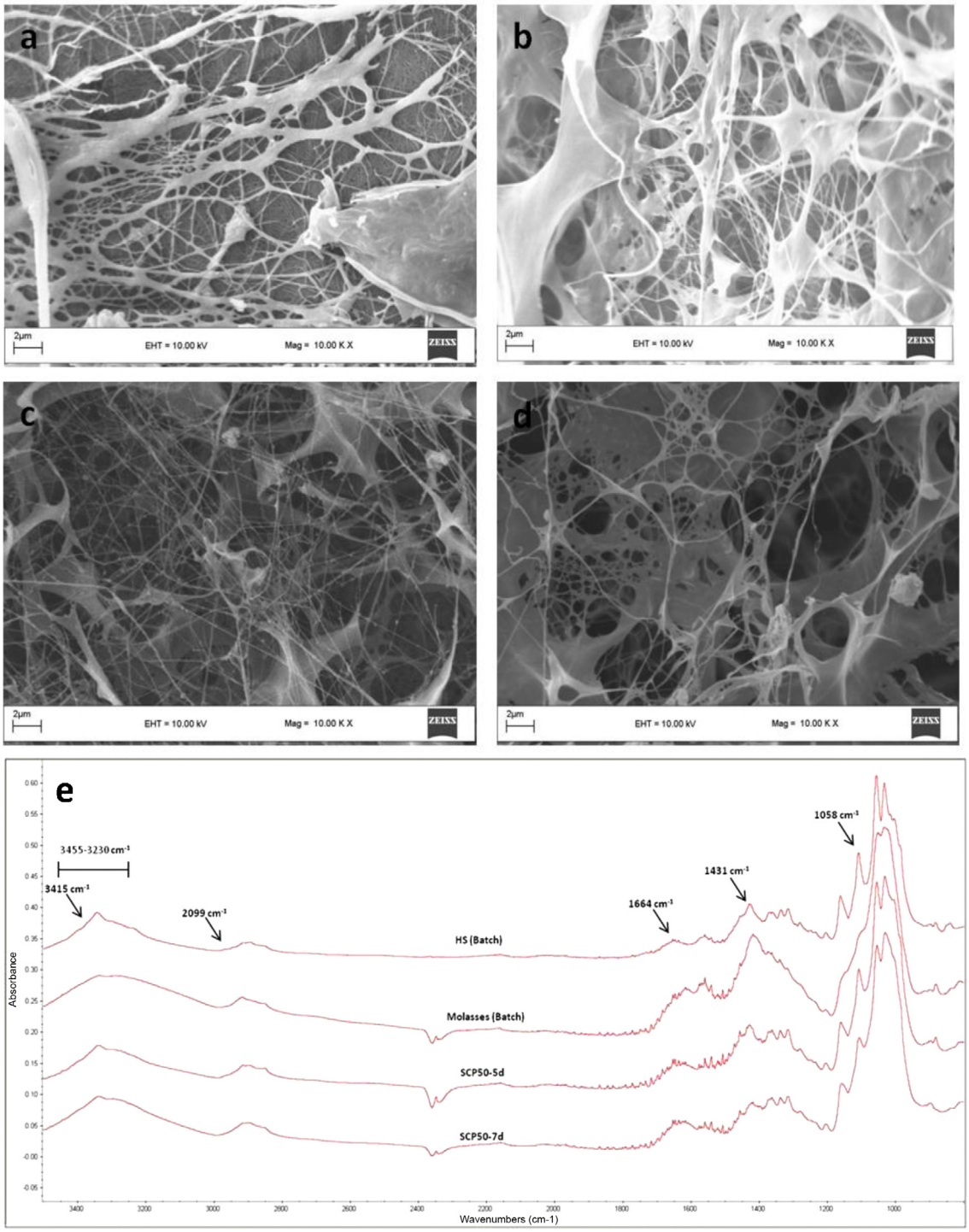

Fig. 4. SEM images of cellulose pellicles: (a) HS (batch), (b) molasses (batch), (c) SCP50-5d, (d) SCP50-7d and FT-IR spectra of cellulose: (e) HS (batch), molasses (batch), SCP50-5d, SCP50-7d produced by *G. xylinus* in various media.

process halted after the 4th stage of SCP66. In addition to this, after generated much amount of cellulose, microorganism was also trapped in cellulose fibrils and hence biomass was drastically decreased after 10 days incubation that was the point of the highest production of BC. It directly affected to the BC production and generation of BC decreased at 15 days of process. BC production of SCP50 was kept steady after the 2nd stage and resulted in higher total cellulose production. Hence, it is suitable for large-scale applications.

The longest semi-continuous process, SCP50-15d, had a lower BC productivity than molasses in batch mode. Therefore, we can conclude that between 5 and 10 days incubation is better for semi-continuous processes. In Fig. 3, there was an increasing trend in SCP50-10d after 2nd stage, this study focused on 1-month production capacity of BC; therefore, SCP50-10d was halted after 3rd stage of process.

Amount of reduced sugar pointed to production capacity for cellulose and/or polysaccharide in the system. As shown in Fig. 5,

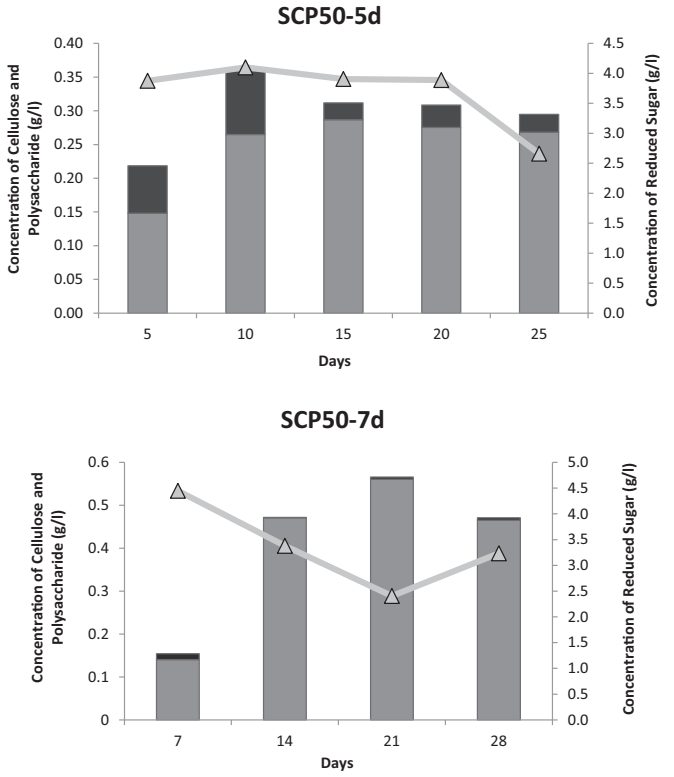

Fig. 5. Production of BC (■) and polysaccharide (■) and consumption of reduced sugar (▲) by *G. xylinus* using treated molasses of SCP50-5d and SCP50-7d.

reduced sugar concentration was changing steadily in SCP50-5d but polysaccharide concentration was high. SCP50-7d showed lower reduced sugar concentration, except at the 1st stage, and it pointed that bacteria just produced BC. Because of cell degeneration, BC production decreased after 3rd stage of SCP50-7d.

In industrial BC production, there are three important parameters which are efficiency of sugar conversion into cellulose, easy to handle the BC in desired form and the productivity of BC. Although, SCP50-7d was the best in productivity (0.058 g/L d), the highest cell growth (3.5 g/L) was observed in this process that may cause the difficulty in cell degradation via alkali treatment. The second choice might be SCP50-5d having the second highest productivity (0.050 g/L d) and lower cell amount (1.7 g/L); however, because of the high polysaccharide amount (0.2 g/l), the efficiency of sugar conversion was lower than others.

5. Conclusion

When production of BC at industrial scale is considered, semi-continuous processes are better choices than batch processes. Because of its complexity, an agitated culture was not performed in this study. The advantages of basic structure of reactor and not included moving parts as impeller or rotating discs, static type bioreactor is preferable. We have tried to increase the productivity of BC in static culture with this study. Although removing the cells from cellulose structure was difficult, the highest productivity of BC was obtained from a 1/2 of VCR and 7 days incubation in semi-continuous process that is the most preferable one. We concluded that semi-continuous process in static culture has potential for BC production at industrial scale.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.01.103.

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