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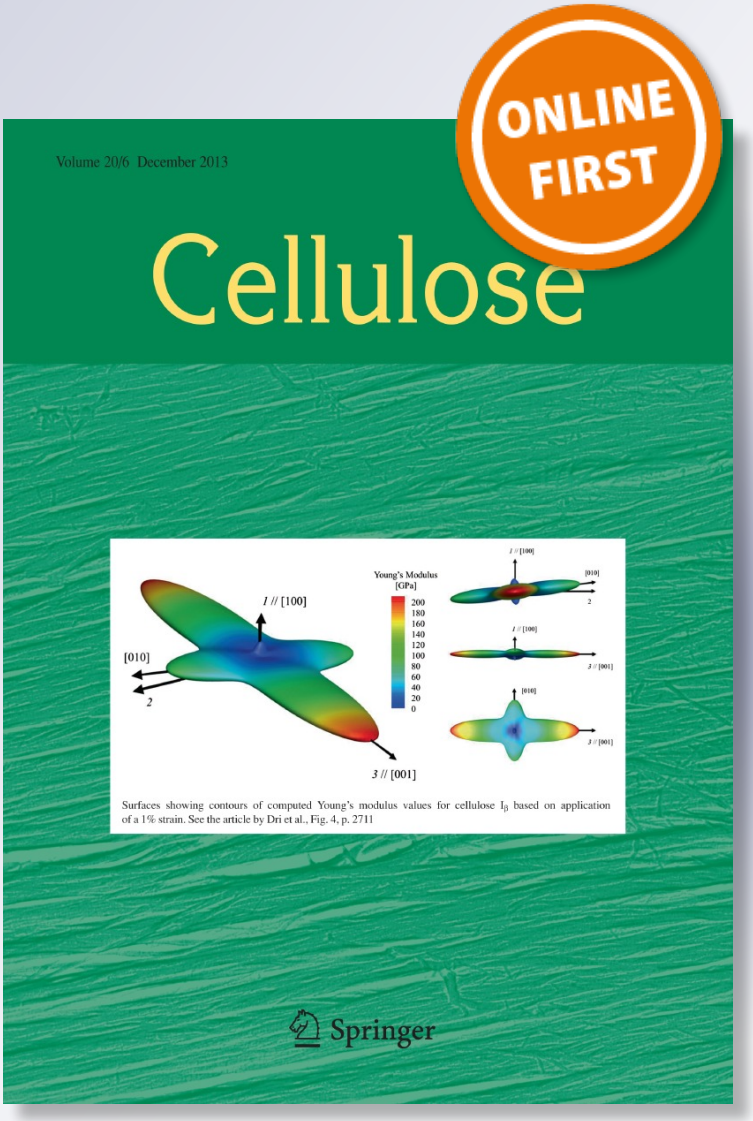

Surfaces showing contours of computed Young's modulus values for cellulose I_β based on application of a 1% strain. See the article by Dri et al., Fig. 4, p. 2711

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ORIGINAL PAPER

Semi-continuous bacterial cellulose production in a rotating disk bioreactor and its materials properties analysis

Shin-Ping Lin · Shu-Chen Hsieh · Kuan-I Chen · Ali Demirci · Kuan-Chen Cheng

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Abstract A rotating disk bioreactor with plastic composite support (PCS) as the solid support was evaluated for bacterial cellulose (BCel) production. Results demonstrated that BCel can be produced in a semi-continuous manner. The BCel productivity reached around 0.24 g/L/day and can be sustained for at least five consecutive runs. Scanning electron microscopy results confirmed that *Gluconacetobacter* can attach on the PCS surface, which eliminates the need of reinoculation. X-ray diffraction patterns and mechanical analysis of BCel produced from this semi-continuous process exhibited lower crystallinity (66.9 %) and mechanical property (Young's modulus of 372.5 MPa) when compared with the BCel obtained from static culture (crystallinity = 88.7 %, Young's modulus of 3,955.6 MPa). Both BCel samples possessed similar water content (98.66 vs. 99.04 %) and thermostability (around 346 °C). In conclusion, the PCS rotating disk bioreactor system can be used to produce BCel in pellicle form with enhanced productivity and, meanwhile, can be scaled up easily to meet commercial need.

Keywords *Gluconacetobacter xylinum* · Bacterial cellulose · Semi-continuous production · Plastic composite support · Bioreactor design · Materials property analysis

Abbreviations

BCel	Bacterial cellulose
PCS	Plastic composite support
PCS-RDB	PCS rotating disk bioreactor
SS-RDB	Stainless steel rotating disk bioreactor
SC	Static culture
SEM	Scanning electron microscopy
XRD	X-ray diffraction

Introduction

Cellulose, a β-1,4 linkage polysaccharide, which is the most abundant material on earth (Brown 2004), can be acquired from plants, microorganisms (Ohad et al. 1962), and animals (Sturcova et al. 2005). Although plant cellulose and bacterial cellulose (BCel) possess the same molecular structure, BCel exhibits the unique physical properties at nanoscale network (Yamanaka et al. 1989; Nishi et al. 1990) (i.e. high water content and high tensile strength (Tanpichai et al. 2012)), and

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does not require extra processing steps to remove impurities such as lignin, pectin, and hemicellulose. Accordingly, BCel has been adopted as biomaterials of wound dressing (Portal et al. 2009), low calorie foods (Okiyama et al. 1992), and composite papers (Brown 2004; Cheng et al. 2011; Trovatti et al. 2012).

Many bacteria can produce BCel including *Achromobacter*, *Alcaligenes*, *Gluconacetobacter*, *Aerobacter*, *Agrobacterium*, *Azotobacter*, *Pseudomonas*, *Rhizobium*, and *Sarcin* (Brown 2004; Deinema and Zevenhui 1971). However, only *Gluconacetobacter xylinum* can produce BCel at commercially viable level due to its high productivity. Traditionally, BCel is produced via static culture (SC) obtaining BCel pellicle (Keshk and Sameshima 2006). Nevertheless, it is not efficient for mass production to meet industrial demand due to its low productivity and long cultivation time (5–12 days) (Lin et al. 2013). Furthermore, BCel production in static culture is proportional to the surface area of the culture medium. Through genetic modification and/or strain selection, several strains can now produce cellulose in an agitated (Kim et al. 2007) or aerated bioreactor (Song et al. 2009). Instead of cellulose pellicle, small BCel pellets are produced under submerged and aerated cultivations (Hu and Catchmark 2010), which, however, are with limited application.

Plastic composite support (PCS), a composite material made of agriculture wastes and nutrients, was noted for providing an ideal surface for biofilm formation and, meanwhile, releases nutrients slowly to the attached microorganisms (Ho et al. 1997b). Many studies have shown that PCS bioreactor can enhance total productivity of ethanol, acetic acid, pullulan, which has been summarized in our previous review article (Cheng et al. 2010). PCS biofilm reactors also demonstrated itself the capability to enhance BCel production (Cheng et al. 2009, 2011). However, most of the produced BCel adhered to agitator and formed large chunks, increasing the difficulty of real-time BCel sampling and eliminating the potential of continuous BCel production, which is the most significant advantage of biofilm reactors.

As for the industrial aspect, scale up of BCel production must meet high productivity, low production cost, and short cultivation time.SC cultivation and submerged fermentation (Toyosaki et al. 1995) were usually employed in traditional bench scale BCel production. However, these methods may be limited for the scale-up purpose due to their low productivity.

In this study, a PCS rotating disk bioreactor (PCS-RDB) was implemented to produce BCel in a semi-continuous manner. The materials properties (morphology, water content, crystallinity, and tensile strength) of BCel produced from PCS-RDB (referred to as PCS-RDB/BCel) were further investigated.

Experimental section

Microorganisms

The bacterial strains used in this study were *G. xylinum* (ATCC 700178) and *G. xylinum* (ATCC 23769), which were obtained from the American Type Culture Collection (Rockville, MD, USA) and Bioresource Collection and Research Center (Hsinchu, Taiwan), respectively. The cell suspensions of two *G. xylinum* strains were stored at −80 °C in a 20 % glycerol solution. Upon cultivation, one mL frozen cell suspension was thawed and added to 50 mL of CSL-Fru medium (descripted below) in a 250 mL flask, and statically cultivated at 28 °C for 3 days. Cellulose pellicle formed on the medium surface was hydrolyzed by cellulase (Sigma-Aldrich, Saint Louis, MO, USA) for 60 min and centrifuged using a centrifuge (Universal 320R Model, Hettich Zentrifugen, Tuttlingen, Germany) at 5,000×*g* for 10 min to collect cell biomass. The cell pellet was resuspended in deionized water and used as an inoculum.

Media

For BCel production, corn steep liquor with fructose (CSL-Fru) medium was slightly modified as previously described (Toyosaki et al. 1995), containing the following constituents per liter: 50 g of fructose, 20 mL of corn steep liquor, 1.0 g of KH_2PO_4, 0.25 g of $MgSO_4{\cdot}7H_2O$, 3.3 g of $(NH)_2SO_4$, 3.6 mg of $FeSO_4{\cdot}7H_2O$, 1.5 mg of $CaC1_2{\cdot}2H_2O$, 2.4 mg of $Na_2MoO_2{\cdot}2H_2O$, 1.7 mg of $ZnSO_2{\cdot}7H_2O$, 1.4 mg of $MnSO_4{\cdot}5H_2O$, 0.05 mg of $CuSO_4{\cdot}5H_2O$, 2.0 mg of inositol, 0.4 mg of nicotinic acid, 0.4 mg of pyridoxine·HCl, 0.4 mg of thiamine·HCl, 0.2 mg of pantothenic acid calcium salt, 0.2 mg of riboflavin, 0.2 mg of *p*-amino-benzoic acid, 0.002 mg of folic acid, and 0.002 mg of biotin.

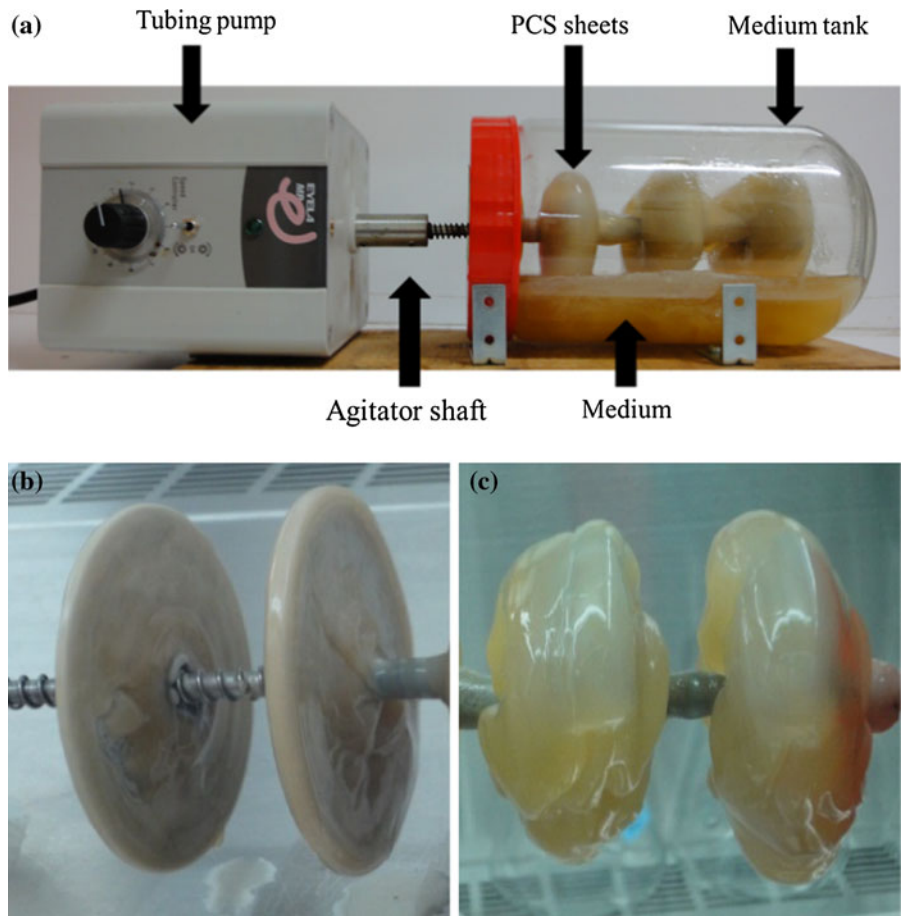

Fig. 1 The illustration of PCS-RDB, which is composed of tubing pump, agitator shaft, PCS sheets and medium tank (**a**), and BCel produced from SS-RDB (**b**) and PCS-RDB (**c**) by *G. xylinus* ATCC 700178 after 5 days of cultivation

Plastic composite support

PCS slice were manufactured using twin-screw extruder (BC 45 model, Clextral Co., Firminy, France) as described by Ho et al. (1997b). Polypropylene [50 % (w/w)] and other ingredients including 35 % (w/w) of soybean hulls, 5 % (w/w) of soybean flour, 5 % (w/w) of yeast extract, 5 % (w/w) of dried porcine red blood cell, 0.272 % (w/w) of sodium acetate, 0.0004 % (w/w) of $MgCl_2 \cdot 6H_2O$, and 0.002 % (w/w) of NaCl were mixed together and extruded at 13 rpm through a medium pipe die with barrel temperatures of 180, 200, and a die temperature of 220 °C. The nutrition composition of PCS (soybean hulls, defatted soy bean flour, yeast extract, dried porcine red blood cell, and mineral salts) was selected as described in our previous study based on the amount of biofilm formation on the PCS (CFU per gram PCS) and BCel production (Cheng et al. 2009). The size of extruded slice is 8 cm in length, 3.5 cm in width and 1.735 mm in height.

Selection of BCel production strains

Gluconacetobacter xylinum ATCC 23769 and *G. xylinum* ATCC 700178 were used to produce BCel in two different cultivation systems, SC and PCS-RDB. In SC, inoculation (10^7 CFU) was added in 250 mL flask with the working volume of 50 mL CSL-Fru medium at 28 °C. In PCS-RDB, three pieces of PCS were fixed in the bioreactor (Fig. 1a) inoculated with (10^7 CFU) in 900 mL CSL- Fru medium at the rotating speed of 5 rpm at 28 °C for 5 days. After being removed from PCS, BCel was treated with 0.1 N NaOH and rinsed with distilled deionized water until the impurities were all removed. The dried weight of

BCel sample was measured for further productivity comparison.

Semi-continuous BCel production

PCS-RDB was implemented for semi-continuous BCel production, which comprises three PCS slices mounted on a stainless steel agitator in a glass vessel. A peristaltic pump (EYELA, Tokyo, Japan) was used to control the rotation speed (Fig. 1a). *G. xylinum* ATCC 700178 was inoculated in 900 mL sterile CSL-Fru medium at a rotation rate of 5 rpm at 28 °C. After each run per 5 days, BCels on the PCS slices and in the suspension were harvested, dried to a constant weight in an oven, and weighed. Stainless steel rotating disk bioreactor (SS-RDB) containing three pieces of stainless steel disk (O.D. = 8.5 cm) with similar conformation was used as control.

Materials properties analysis of BCel

In order to evaluate the effects of PCS-RDB on BCel production by *G. xylinum* ATCC 700178, materials properties of the produced BCel were analyzed including morphology, degree of crystallinity, thermostability, water content, and tensile strength.

Scanning electron microscopy

BCels after removal of cells and other impurities were lyophilized and coated with a thin layer of gold. The morphology was observed by using scanning electron microscopy (SEM) at an accelerating voltage of 15 kV (JSM-5410 model, Jeol, Tokyo, Japan). Imaging magnification was approximately 20,000 to determine surface structure of BCels and PCS.

X-ray diffraction

To determine crystallinity of the produced BCels, X-ray diffraction (XRD) patterns were collected on an x-ray powder diffractometer (X Pert PRO model, Nalytical, Almelo, Netherlands) using a copper x-ray source. Scans were collected at 4 deg per minute from 5°–30° 2θ. BCels were lyophilized overnight by using a freeze dryer (SFD-25 model, Charng Juing Machinery, Kaohsiung, Taiwan) and pressed into a thin and flat layer (~ 1.0 mm thickness) for analysis. The degree of crystallinity was taken as Cr $I = (I_{200} - I_{am})/I_{200}$, where I_{200} represents for the overall intensity of the peak at 2θ about 22.9° and I_{am} stands for the intensity of the baseline at 2θ about 18°, respectively.

Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) spectra of BC samples were acquired on a Spectrum 100 FT-IR spectrometer (Perkin Elmer Inc., Wellesley, MA, USA) equipped with an attenuated total reflectance (ATR) sampling accessory. The investigated spectral range was from 4,000 to 1,000 cm^{-1}. The signal was obtained by averaging 30 scans at resolution of 1 cm^{-1}.

Thermogravimetric analysis and water content analysis

The dynamic weight loss test was conducted on a thermogravimetric analyzer machine (Pyris 1 model, Perkin Elmer, Waltham, MA, USA). For thermal decomposition behavior test, cellulose samples were dried at 80 °C and tests were then conducted in a N_2 purge (40 mL/min) in a temperature range of 80–650 °C at an increase rate of 10 °C/min. Water content was calculated by following equation: $[(W_t - W_0)/W_t] \times 100$ %, where W_0 and W_t represent for the weight of dried BCel and wet BCel, respectively.

Mechanical properties

The strength measurement of dried BCel samples was performed using Texture analyzer (TA-XT2 model, Texture technologies, Westchester, NY, USA). BCels were cut into rectangular strips (20 × 10 × 0.02–0.04 mm). The tests were carried out at the force of 0.1 N/min at the temperature of 28 °C. Stress (σ) was calculated by F/A, where A is the area of sample (measured as width × thickness) and F is force in Newton. Strain (ε) was calculated by $\Delta L/L_0$, where ΔL is exerted extension from the starting point L. Young's modulus was calculated by stress/strain. (All measurements were performed for at least five replications).

Statistical analysis

All experiments were performed in at least triplicates. The significant difference of the results was evaluated

using the Generalized Linear Model (GLM; with $p < 0.05$) of the SigmaPlot (Systat Software, San Jose, CA).

Results and discussion

Selection of BCel production strain

To select the most effective strain with higher BCel yield, the productivity of BCel produced from strains *G. xylinum* ATCC 700178 and *G. xylinum* ATCC 23769, which are both commercial BCel producers, were compared in the conditions of SC and PCS-RDB. In SC, *G. xylinum* ATCC 23769 exhibited higher BCel productivity (0.3 g/L/day) than *G. xylinum* ATCC 700178 (0.24 g/L/day). However, *G. xylinum* ATCC 700178 (0.21 g/L/day) revealed a 1.4-fold BCel productivity when compared to *G. xylinum* ATCC 23769 (0.148 g/L/day) in PCS-RDB (Table 1). Previous study has showed that high shear force from agitating system resulted in an accumulation of *G. xylinus* ATCC 23769 with Cel^- gene mutation (Schramm and Hestrin 1954). Our results also demonstrated that *G. xylinum* ATCC 700178 possesses higher tolerance of shear force than *G. xylinum* ATCC 23769, and is more suitable for this PCS-RDB system. It means *G. xylinum* ATCC 700178 might be avoided to converse to Cel^- gene mutation strain due to its higher shear force tolerance. Therefore, *G. xylinum* ATCC 700178 was selected as the production strain for the following experiments.

Semi-continuous cultivation of BCel in PCS-RDB

PCS-RDB was implemented to establish a semi-continuous culture system with no need of reinoculation. A SS-RDB was used as control. A 6-day cultivation of *G. xylinum* in the PCS-RDB was performed. The results revealed that BCel productivity reached 0.25 g/L/day on the 5th day and started to decrease (Fig. 2). The surface area of PCS slices may restrict the production of BCel after 5 days, resulting in a lower BCel productivity. Therefore, a 5-day cultivation was adopted for the following experiments before further improvement of PCS-RDB is applied. Bungay and Serafica (1999) first introduced the SS-RDB system. The rotating disk bioreactor was designed that half area of its disks was submerged in the medium and the other half exposed to the atmosphere (Serafica et al. 2002; Krystynowicz et al. 2002; Bungay and Serafica 2000). However, the drawback of this system resulted from the need of reinoculation each time after BCel harvest, which reduced the BCel productivity. Our new PCS-RDB system can overcome this shortage. PCS-RDB provides a rough surface that can accumulate more bacteria on it (Fig. 1b). *G. xylinum* attached on the PCS surface and formed biofilm as the starter for next run of BCel production resulting in a higher BCel production. Contrarily, during cultivation, it was observed that the *G. xylinum* barely adhered to the stainless steel disks because of its smooth surface (Fig. 1c). Less BCel was produced under this condition.

This semi-continuous culture system was carried out for five consecutive runs to evaluate its sustainability. Meanwhile, in order to assess the possible reduction of capital input, we kept a total working volume of 900 mL by (a) replacing with fresh medium and (b) replenishing with only distilled deionized water but not fresh medium. The results indicated that the BCel productivity can be kept constant around 0.24 (g/L/day) for 5 runs with medium replacement (Fig. 3). Contrarily, a dramatic decrease of BCel productivity was observed from 0.19 to 0.07 g/L/day after the second batch without medium replacement (Fig. 3). Lacking of sufficient nutrients from the medium may limit the production of BCel. The BCel productivity was kept at the low value with only distilled deionized water replacement (0.05 g/L/day), which implies that the nutrients slowly released from PCS can sustain the growth of *Gluconacetobacter* in the nutrient-deficient environment but not BCel production. Krystynowicz et al. (2002) concluded that RDB system can reduce Cel^- gene mutation, and the BCel productivity reached 0.47 g/L/day. Although the BCel productivity of PCS-RDB (0.24 g/L/day) is lower than RDB (0.47 g/L/day), the culture condition

Table 1 The BCel productivity of *G. xylinum* strains under different cultivation system

Strains	SC/BCel (g/L/day)	PCS-RDB/BCel (g/L/day)
G. xylinum 23769	0.3 ± 0.025^a	0.148 ± 0.047^a
G. xylinum 700178	0.24 ± 0.009^b	0.21 ± 0.044^a

Values (in the same column) not marked by the same letter are significantly different ($p \leq 0.05$; $n = 3$)

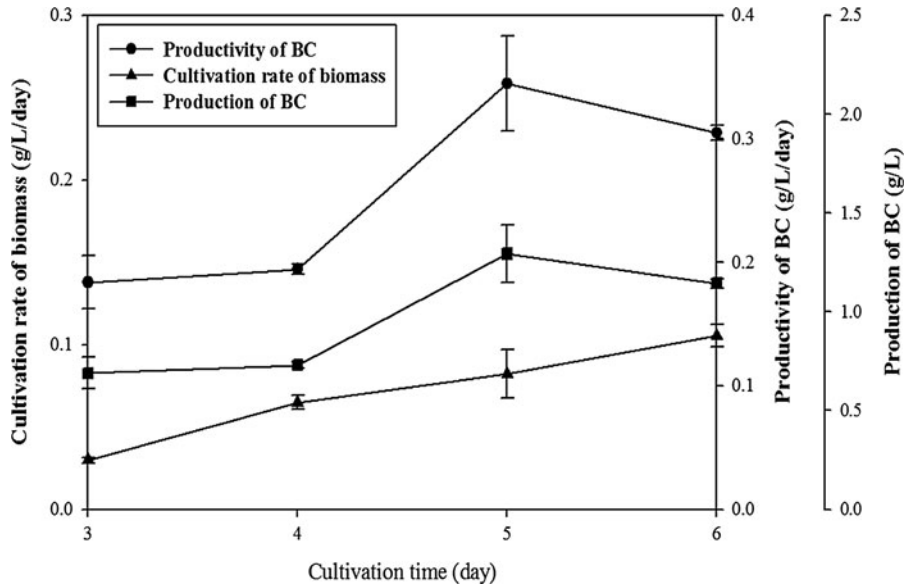

Fig. 2 Cellulose and biomass production of *G. xylinum* ATCC 700178 in PCS-RDB as a function of cultivation time

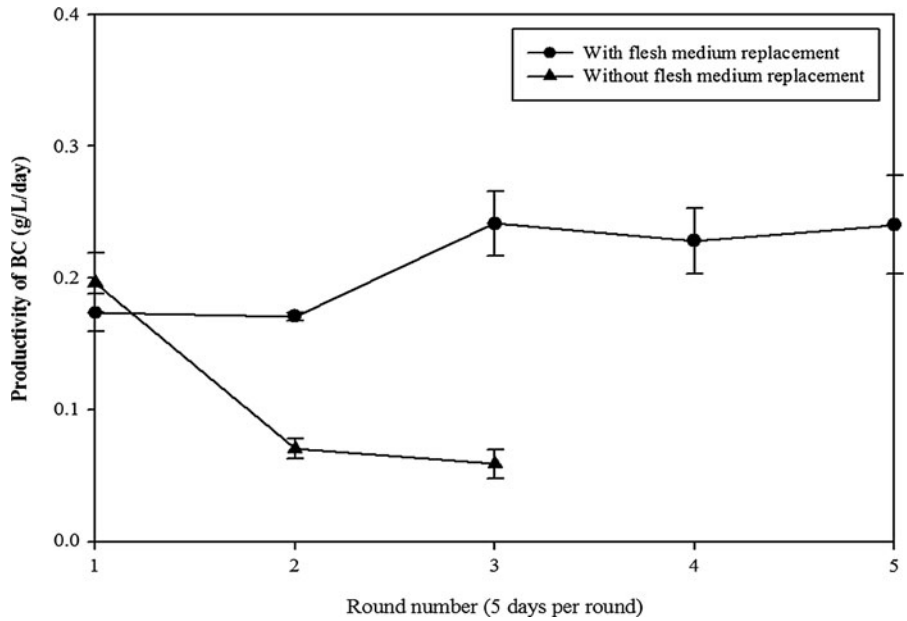

Fig. 3 Semi-continuous BCel productivity in the PCS-RDB system with and without medium replacement

and disc number (3 vs. 24) are different. Further improvement of PCS-RDB system is needed to obtain its highest BCel productivity.

Materials properties analysis of BCel

BCel structure and crystallinity

To determine the effect of the PCS-RDB system on BCel property, SEM and XRD was employed to verify BCel's morphology and crystallinity. SC/BCel was used as control. As shown in Fig. 4, three major peaks standing for crystal plane $\langle -110 \rangle$, $\langle 110 \rangle$ and $\langle 200 \rangle$ were observed in XRD results of both SC/BCel and PCS-RDB/BCel, which suggests that BCel samples were both in cellulose I-β form. The crystallinity of SC/BCel (88.7 %) is higher than that of PCS-RDB/BCel (66.9 %) (Table 2). It has been previously reported that crystallization of cellulose is the rate-limiting step in BCel producing process (Haigler et al.

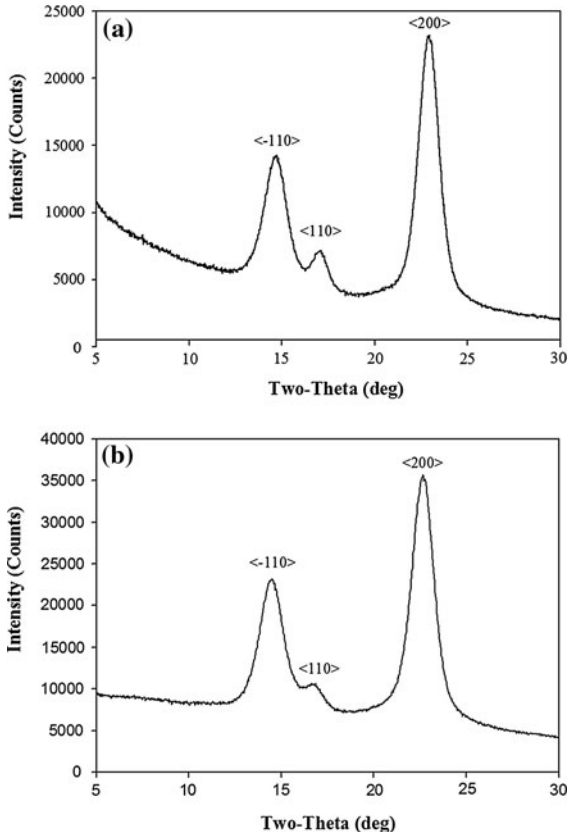

Fig. 4 XRD patterns of BCel samples produced from SC (**a**) and PCS-RDB (**b**)

1982). The enhanced BCel productivity in our study may due to the interference of this step. Moreover, the incorporation of ingredients from PCS may also contribute to the reduction of crystallinity (Cheng et al. 2011).

Previous study revealed that PCS provides its rough surface for cell adhesion (Ho et al. 1997b). To verify the efficacy of cell adhesion on PCS, a PCS slice after semi-continuous culture was examined by SEM, which demonstrated that PCS exhibits rough, porous surface, which is suitable for cell adhesion (Fig. 5a) (Ho et al. 1997a). Figure 5b, c showed that BCel samples produced from PCS-RDB and SC exhibited the same interweaving with similar fiber size (15–30 nm), which suggest the cultivation method did not affect BCel morphology. Moreover, the existence of bacteria attached on PCS surface provided the direct evidence of biofilm formation which realized the semi-continuous BCel production without further reinoculation in a PCS-RDB without the need of reinoculation (Fig. 5d).

Fourier transform infrared spectroscopy (FTIR)

FTIR technique is used to obtain the specific functional groups or chemical bonds in BCel samples. The FTIR spectra of the SC/BCel and PCS-RDB/BCel membranes were measured with the wave number ranging from 4,000 to 1,000 cm^{-1} as shown in Fig. 6. In the FTIR results, the BCel samples showed few important peaks of BCel at 1,646 cm^{-1} (stretching of C=O), 2,894 cm^{-1} (stretching of CH), and 3,348 cm^{-1} (stretching of C=O). The spectra of PCS-RDB/BCel also showed the entire characteristic band of SC/BCel, and without any appearance of new peaks. These results confirmed that SC/BCel and PCS-RDB/BCel exhibit similar chemical binding.

Thermogravimetric analysis and water content analysis

To evaluate the thermostability of BCel samples, thermogravimetric analysis was performed. Roman and Winter (2004) reported that the weight loss of BCel under high temperature condition (between 300 and 350 °C) is considered as BCel degradation process, such as depolymerization, further dehydration, and degradation of the glucopyranosyl units. In Fig. 7, the thermostability analysis depicted a single peak for SC/BCel (348 °C) and PCS-RDB/BCel (346 °C), respectively. This result confirmed that the thermostability of both BCel samples were similar and PCS-RDB/BCel was pure without incorporating of PCS ingredients during production.

Table 2 Materials property analysis of BCel samples

BCel samples origin	Crystallinity (%)	WA (%)	Stress (MPa)	Strains (%)	Young's modulus (MPa)
SC	88.7 ± 0.03^{a}	99.04 ± 0.18^{a}	99.2 ± 22.1^{a}	2.4 ± 0.3^{a}	$3{,}955.6 \pm 310.6^{a}$
PCS-RDB	66.9 ± 0.03^{b}	98.66 ± 0.36^{a}	15.9 ± 3.8^{b}	4.3 ± 1.2^{b}	372.5 ± 42.3^{b}

Values (in the same column) not marked by the same letter are significantly different ($p \leq 0.05$; $n = 3$)

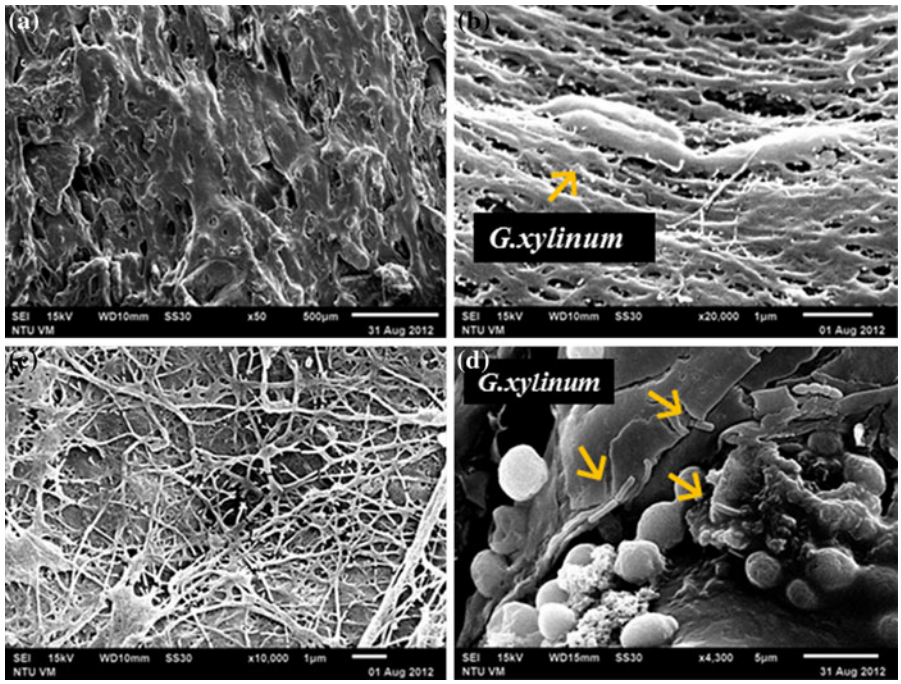

Fig. 5 SEM images of freeze-dried PCS and BCel. **a** PCS surface, **b** BCel/SC, **c** PCS-RDB/BCel, and **d** bacteria adhesion on PCS surface

PCS-RDB/BCel exhibited similar high water retention ability (98.66 %) to SC/BCel (99.04 %), which suggests that PCS-RDB/BCel may be used as a biomaterial in the applications of cosmetics and wound dressing (Table 2).

Mechanical properties of BCel

BCel exhibits several unique properties because of its nano-scaled structure, such as high water content and high tensile strength (Yamanaka et al. 1989; Nishi et al. 1990). However, BCel materials properties may be changed under different production methods (Benziman et al. 1980). As the result, mechanical property test was conducted to reveal the strength of BCel produced from PCS-RDB system. Stress, strain and Young's modulus were determined and summarized in Table 2. The results demonstrated that SC/BCel exhibited a superior stress property (99.2 MPa) than PCS-RDB/BCel (15.9 MPa), whereas PCS-RDB/BCel (4.3 %) showed higher strain property than SC/BCel samples (2.4 %). This might be due to the break of crystal structure of BCel during BCel production, resulting in a reduction of stress in the case of PCS-RDB/BCel. Another possible reason may be the process of microfibril crystallization was disordered by external forces during rotating culture which may cause crystallinity reduction (Watanabe et al. 1998). The Young's modulus of the PCS-RDB/BCel (372.5 MPa) was lower than the SC/BCel (3,955.6 MPa), which indicates that PCS-RDB/BCel exhibits more flexible but lower strength of mechanical property. Several studies also show similar results that BCel produced from rotating disk bioreactor

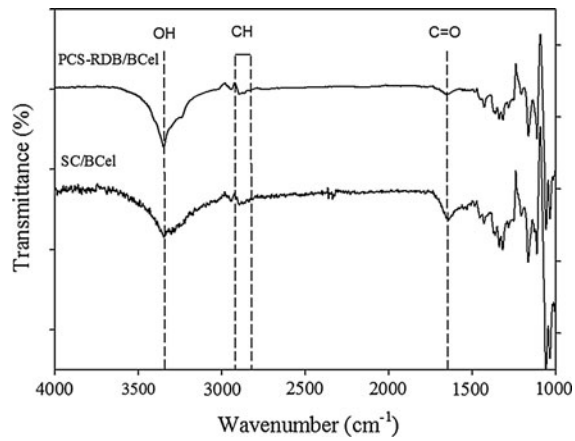

Fig. 6 FTIR spectra of SC/BCel and PCS-RDB/BCel

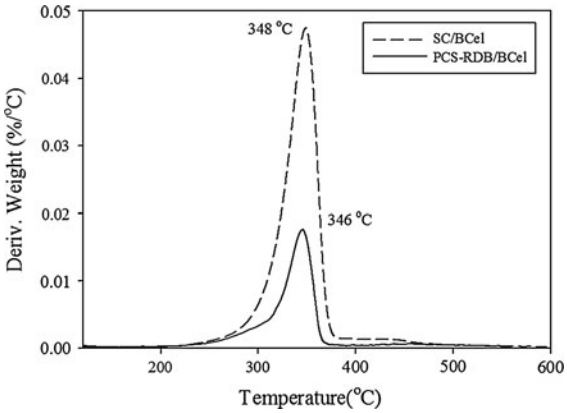

Fig. 7 Thermogravimetric analysis of different cultivation systems

exhibits both lower Young's modulus and much tensile strength compared with SC/BCel (Krystynowicz et al. 2002; Watanabe et al. 1998) which is in accordance with our finding in this work. The result suggests that PCS-RDB/BCel may be a substitute of SC/BCel as wound dressing materials due to its high flexible property and is potentially valuable in the area of biomedical applications.

Conclusion

In this study, a semi-continuous BCel production system was introduced by using PCS-RDB. *G. xylinum* ATCC 700178 can achieve its highest BCel productivity (0.24 g/L/day) in a 5-day cultivation and sustained for at least five consecutive runs. SEM images provided the direct evidence that bacteria can attach onto PCS surface and produce BCel without the need of reinoculation. FTIR, TGA and water content analysis showed similar properties of PCS-RDB/BCel and SC/BCel, which suggests PCS-RDB/BCel retained these important characteristics of BCel. Mechanical property results confirmed that the decrease of crystallinity may lead to less strength, but more flexible mechanical property. In summary, PCS-RDB can be used to produce BCel in a semi-continuous manner and is easy for scaled-up to meet commercial production. Further studies on enhancing BCel productivity in PCS-RDB system and materials properties analysis of PCS-RDB/BCel (i.e. biocompatibility) will be the next challenge for applying aspects.

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