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ORIGINAL PAPER

Production of bacterial cellulose with various additives in a PCS rotating disk bioreactor and its material property analysis

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Abstract Previous studies have demonstrated that bacterial cellulose (BC) can be semi-continuously produced by utilizing the plastic composite support-rotating disk bioreactor (PCS-RDB). In this study, different additives, such as microcrystalline cellulose (Avicel was used in this study), carboxymethylcellulose (CMC), agar and sodium alginate, were added to the PCS-RDB culture medium to improve the BC productivity and material properties. The produced BC was then analyzed by Fourier transform infrared spectroscopy (FTIR), scan electron microscopy (SEM), thermogravimetric analysis, X-ray diffraction (XRD) and strength analysis. Adding CMC and Avicel can increase the production of BC in PCS-RDB. The highest BC production reached (0.64 g/slice) when 0.8 % Avicel was added. Data from FTIR, XRD and SEM indicated that CMC and Avicel were incorporated into the BC during production, creating a disordered BC structure and thus reducing crystallinity. Both BCs and additive-altered BCs exhibited similar high water retention abilities (98.6–99 %). Additive-altered BCs exhibit similar strain but lower stress. BC production in PCS-RDB was improved by incorporating different additives, while the material properties of the produced BCs were also modified.

Keywords *Gluconacetobacter xylinus* · Bacterial cellulose · Plastic composite support · Rotating disk bioreactor · Materials property analysis

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Introduction

Cellulose is the most abundant macromolecule on earth, being found in large quantities in nature (Brown 2004). It is predominantly generated by vascular plants and algae (Ohad et al. 1962) but can also be synthesized by bacteria (Brown 1886). Although plant cellulose is widely used, it also has limitations. The impurities of plant cellulose may decrease both the water content and mechanical strength of this material. In contrast, bacterial cellulose (BC) possesses excellent physical (water content, thermostability and tensile strength) and biological (biodegradation and biocompatibility) properties; it also can be grown into

any desired shape based on the culture methods (Nishi et al. 1990). Due to its special material property, BC is now being applied in the textile industry (Wan et al. 2006) as well as food processing (Okiyama et al. 1993) and pharmaceutical applications (Schumann et al. 2009; Meftahi et al. 2010; Lin et al. 2013b).

In the traditional producing protocol, static culture is the main manufacturing method. Static cultivation provides a relatively simple approach and low shear force environment during production. However, the productivity of this method does not meet industry needs nowadays. Hence, a novel cultivation approach with high BC productivity should be developed.

Several factors, such as the nutrients, trace elements, pH, viscosity of the medium, shear force and oxygen penetration, may influence the BC production during fermentation (Aydın and Aksoy 2014; Keshk and Sameshima 2005; Mohammadkazemi et al. 2015; Ruka et al. 2012). The incorporation of various additives was found to regulate these factors and result in an increase of BC production by disarranging the crystallization procedure (Haigler et al. 1980; Tomita and Kondo 2009), regulating the cellulose synthesis pathway of microorganisms (Hu and Catchmark 2010), increasing the medium viscosity for shear force reduction (Kouda et al. 1996; Bae et al. 2004), changing the type of BC (Hu et al. 2013) and switching the cell type of the BC producer (Park et al. 2003). The additives may also alter the structure of BC during production and modify its material properties, including water retention, thermostability, biocompatibility, crystallization and mechanical strength (Cheng et al. 2009a; Ruka et al. 2013; Yang et al. 2014). Previous studies indicated that the production of BC was significantly increased along with rising concentrations of carboxymethylcellulose (CMC) in the medium. Furthermore, the CMC-altered BC also possessed a different structure, resulting in decreased stress and strain (Cheng et al. 2011; Chen et al. 2011).

In our previous study (Lin et al. 2013a), we developed a semi-continuous cultivation system to produce BC by utilizing a plastic composite support-rotating disk bioreactor (PCS-RDB). PCS-RDB can produce BC without re-inoculation, consequently retaining its productivity for at least five cycles. The goal of this study is to evaluate the effects of different additives on BC production by PCS-RDB. Material property analysis of the produced BC was also carried out to reveal the possible mechanism of enhanced BC production.

Experimental section

Microorganisms

The bacterial strain used in this study was *Gluconacetobacter xylinus* ATCC 700178, purchased from the American Type Culture Collection (Rockville, MD). The cell suspension of *G. xylinus* strain was stored at −80 °C in a 20 % glycerol solution. Upon cultivation, 1 ml frozen cell suspension was thawed and added to 50 ml corn steep liquor with fructose medium (CSL-Fru medium) in a 250-ml flask and statically cultivated at 28 °C for 1 day. A cellulose pellicle formed on the medium surface was hydrolyzed by cellulase (Sigma-Aldrich, Saint Louis, MO, USA) for 3 h and centrifuged using a centrifuge (Universal 320R Model, Hettich Zentrifugen, Tuttlingen, Germany) at 5000*g* for 10 min to collect the cell biomass. The cell pellet was resuspended in deionized water and used as an inoculum.

Media

For static culture of BC production, CSL-Fru medium was slightly modified as previously described (Toyosaki et al. 1995), containing the following constituents per liter of deionized water: 50 g fructose, 20 ml corn steep liquor, 1.0 g KH_2PO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 3.3 g $(NH)_2SO_4$, 3.6 mg $FeSO_4{\cdot}7H_2O$, 1.5 mg $CaC1_2{\cdot}2H_2O$, 2.4 mg $Na_2MoO_2{\cdot}2H_2O$, 1.7 mg $ZnSO_2{\cdot}7H_2O$, 1.4 mg $MnSO_4{\cdot}5H_2O$, 0.05 mg $CuSO_4{\cdot}5H_2O$, 2.0 mg inositol, 0.4 mg nicotinic acid, 0.4 mg pyridoxine·HCl, 0.4 mg thiamine·HCl, 0.2 mg pantothenic acid calcium salt, 0.2 mg riboflavin, 0.2 mg *p*-aminobenzoic acid, 0.002 mg folic acid and 0.002 mg biotin. For BC production in the plastic composite support-rotating disk bioreactor (PCS-RDB), the modified CSL-Fru medium (the concentration of fructose was reduced from 50 to 10 g/l) was used in the following experiment. The additives were purchased from Sigma-Aldrich (Saint Louis, MO, USA), including sodium alginate, agar, carboxymethylcellulose (CMC) and microcrystalline cellulose (MCC; Avicel was used in this study).

Plastic composite support

The PCS slices were manufactured using a twin-screw extruder (BC 45 model, Clextral Co., Firminy, France) as described by Ho et al. (1997). Polypropylene (50 % (w/w)) and other ingredients including 35 % (w/w) soybean hulls, 5 % (w/w) soybean flour, 5 % (w/w) yeast extract, 5 % (w/w dried porcine red blood cells, 0.272 % (w/w) sodium acetate, 0.0004 % (w/w) $MgCl_2{\cdot}6H_2O$ and 0.002 % (w/w) NaCl were mixed together and extruded at 13 rpm through a medium pipe die with barrel temperatures of 180 and 200 °C and a die temperature of 220 °C. The nutrient 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 BC production (Cheng et al. 2009b). The extruded slice size was 8 cm long, 3.5 cm wide and 1.7 mm high.

BC production with various additives

The effects of various additives on BC production in PCS-RDB were evaluated. PCS-RDB is a semi-continuous BC producing system developed in our previous study (Lin et al. 2013a). In PCS-RDB, six pieces of PCS were fixed in the bioreactor inoculated with *G. xylinus* (5 % v/v) in 900 ml CSL-Fru medium with or without different additives at different concentrations (0.2–1.0 %). The experimental conditions were 5 rpm for the rotating speed at 28 °C for 5 days (Lin et al. 2013a). BC detached from PCS was treated with 0.1 N NaOH and rinsed with deionized water until the impurities were completely removed. BC samples were then lyophilized using a freeze dryer (Manifold Freeze Dryer HCS-T11, HCS, Taipei, Taiwan), and its productivity and material properties were evaluated.

Material property analysis of BC

In order to estimate the effects of different additives on BC production in PCS-RDB, material property analyses were performed on the morphology, crystallinity, thermostability, water retention and tensile strength of the produced BC.

Scanning electron microscopy

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

X-ray diffraction

To determine the crystallinity of the produced BCs, X-ray diffraction (XRD) patterns were collected on an X-ray powder diffractometer (X Pert PRO model, Nalytical, Almelo, The Netherlands) using a copper X-ray source. Scans were collected at 4° per minute from 5°–30° 2θ. BCs were later lyophilized overnight by a freeze dryer (SFD-25 model, Chang Juing Machinery, Kaohsiung, Taiwan) and ground into fine powders by a grinder (RT-02B; Yuan-Shen Co., Taipei, Taiwan) for analysis. The degree of crystallinity was taken as $\mathrm{Cr}I = (I_{200} - I_{\mathrm{am}})/I_{200}$, where I_{200} represents the overall intensity of the peak at 2θ at about 22.9° and I_{am} the intensity of the baseline at 2θ at about 18°.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectra of BC samples were acquired on a Spectrum 100 FTIR spectrometer (PerkinElmer Inc., Wellesley, MA, USA) equipped with an attenuated total reflectance (ATR) sampling accessory. The spectral range was investigated from 4000 to 1000 cm^{-1}. The signal was obtained by averaging 30 scans at 1 cm^{-1} resolution.

Thermogravimetric and water content analysis

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

Tensile strength

The strength measurement of dried BC samples was performed using the Texture analyzer (TA-XT2 model, Texture Technologies, Westchester, NY). BCs were cut into rectangular strips (60 × 10 × 0.02–0.04 mm). The tests were carried out at 0.1 N/min force at 28 °C temperature. Stress (σ) was calculated by F/A, where A is the area of the sample (measured as width × thickness) and F is the force in Newtons. Strain (ε) was calculated by $\Delta L/L_0$, where ΔL is the exerted extension from the starting point $L\sigma$. Young's modulus was calculated by stress/strain. All measurements were performed in at least five replications.

Statistical analysis

Statistical evaluation of all experimental data (variation from basal values) was performed using ANOVA. Post hoc tests with negative control were performed with the Tukey test. Statistical analysis was conducted with IBM SPSS Statistics 20 ($p < 0.05$).

Results and discussion

Effects of various additives on BC production

In this study, BC was produced from CSL-Fru medium with various additives in PCS-RDB. In the culture medium, 0.2 and 0.5 % (w/v) additives, including CMC, sodium alginate, agar and Avicel, respectively, were added to improve the production of BC. Figure 1 shows that the BC production of both the agar and control groups was kept at a similar level (0.3 g/slice). In this set of samples, the produced BC showed a great water content capacity. The results of the sodium alginate, CMC and Avicel addition groups showed that the BC production was significantly higher compared to the control group. In the 0.2 and 0.5 % sodium alginate addition groups, the production of BC reached 0.49 g/slice, which is consistent with Zhou et al.'s (2007) findings. BC production reached 6.0 g/l when 0.04 % (w/v) sodium alginate was added to the medium. In the CMC and Avicel addition groups, higher productions of BC were also obtained. CMC and Avicel are both cellulose derivatives that are not only used to improve the production of BC, but also to

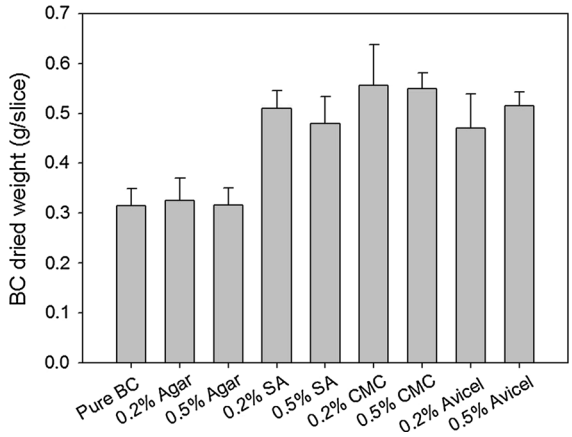

Fig. 1 BC production in CSL-Fru medium including different additives ($n = 3$)

modify BC's material properties (Cheng et al. 2011). Therefore, various concentrations (0.2, 0.5, 0.8 and 1.0 %) of CMC and Avicel were studied to check whether a dose-dependent relation exists between BC production and the concentration of additives. The results (Fig. 2) demonstrated that both 0.8 % CMC and Avicel additions achieved the highest BC production, 0.55 and 0.64 g/slice, respectively. These results indicate that using CMC and Avicel as additives in the medium can effectively enhance the BC production. The reason that BC production decreased when 1.0 % CMC or 1.0 % Avicel was applied may be the incorporation of additives. The incorporation of additives hinders the adhesion of the produced BC onto PCS because of its softness and fragility. The direct evidence for this hypothesis is that the stress of the produced BC decreased with the concentrations of CMC and Avicel (Table 1). Lee and Zhao (1999) tried to improve BC production using a static culture system by adding different addtives. Unfortunately, in this system, insoluble additives fell to the bottom and caused nonuniform BCs with limited applications. However, in our system, the insoluble additive (Avicel) was continuously agitated. Furthermore, the high viscosity of the medium kept the additives suspended therein. Kouda et al. (1996) also found that the improvement of BC production in an agitating bioreactor with 2 % CMC addition is due to non-Newtonian behavior, which increases the viscosity at low shear rates. Therefore, these suspended additives will permit incorporation during BC production, resulting in an additive-altered BC.

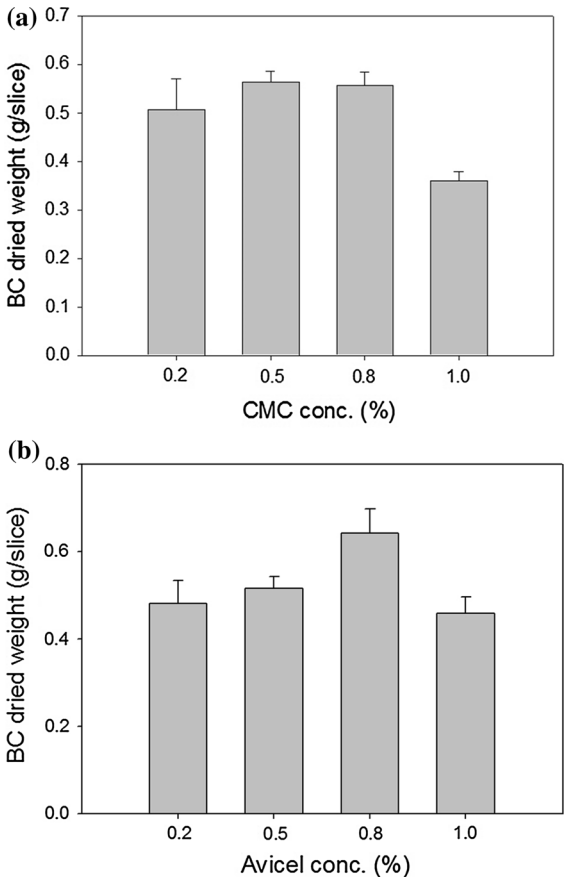

Fig. 2 BC production in CSL-Fru medium with different concentrations of **a** CMC and **b** Avicel ($n = 3$)

Fourier transform infrared spectroscopy (FTIR)

Figure 3 depicts ATR-FTIR spectra of BC with different concentrations of incorporated additives. In the CMC addition group (Fig. 3a), absorption of the carboxyl group (R-COOH) was observed at 1572 cm^{-1}, which was absent in pure cellulose samples, thus indicating that CMC was incorporated into BC during cultivation. Avicel, a microcrystalline cellulose, is derived from purified wood α cellulose (Battista and Smith 1962; Doelker 1993). In Avicel treatment, the modified cellulose exposed a many hydroxyl groups because of the disrupted intermolecular hydrogen bonds. Therefore, the amount of hydroxyl groups can be adopted as a signal for detecting Avicel incorporation. Figure 3b agrees with our assumption that the absorption intensity of 3348 cm^{-1} (stretching of O–H) increased with the ratio of Avicel to BC concentration. FTIR results provided direct evidence that CMC and Avicel were incorporated or adsorbed by the produced BC. This might explain why the BC production increased after additive addition, as discussed previously (Cheng et al. 2009a, 2011).

X-ray diffraction

X-ray diffraction (XRD) was used to analyze the crystal structure and crystallinity of BC samples. The degree of crystallinity influences the biomaterial's tensile strength (El-Hadi et al. 2002; Retegi et al. 2010) and water retention (Huang et al. 2010; Wan et al. 2009). El-Hadi et al. (2002) reported that poly-3-hydroxyalkanoate (PHA), a bacterial thermoplastic polyester, was modified to improve its tensile strength. Huang et al. (2010) mentioned that decreased BC crystallinity may also influence its water retention ability. Figure 4 shows the XRD patterns of BC and additive-altered BCs with different additive concentrations. Three major peaks from the <100>, <010> and <110> planes of BC were observed, suggesting that both BC and additive-altered BC were in the

Table 1 Material property analysis of additive-altered BCs

	Crystallinity	Stress	Strain	Water content
Pure BC	78.4	43 ± 7.9[a]	2.4 ± 0.5[a]	98.8 ± 0.05[a]
0.2 % CMC	72.8	46.1 ± 6.2[a]	2.3 ± 0.6[a]	98.6 ± 0.03[b]
0.5 % CMC	71.1	35.8 ± 5.4[a]	1.4 ± 0.6[a]	98.6 ± 0.01[b]
0.8 % CMC	65.6	22.1 ± 6.5[b]	1.7 ± 0.7[a]	98.8 ± 0.01[a]
1.0 % CMC	63.5	16.2 ± 3[b]	1.6 ± 0.5[a]	98.7 ± 0.04[c]
0.2 % Avicel	72.8	18.2 ± 3.9[b]	2.6 ± 0.5[a]	98.9 ± 0.04[d]
0.5 % Avicel	71.8	17.6 ± 4.5[b]	2.3 ± 0.3[a]	99 ± 0.02[d]
0.8 % Avicel	66.9	14.3 ± 2.2[b]	2.8 ± 0.5[a]	99 ± 0.04[d]
1.0 % Avicel	64.3	13.8 ± 2.7[b]	2.7 ± 0.6[a]	99 ± 0.06[d]

* Values for each sample with different superscripts are significantly different ($p < 0.05$; $n = 3$)

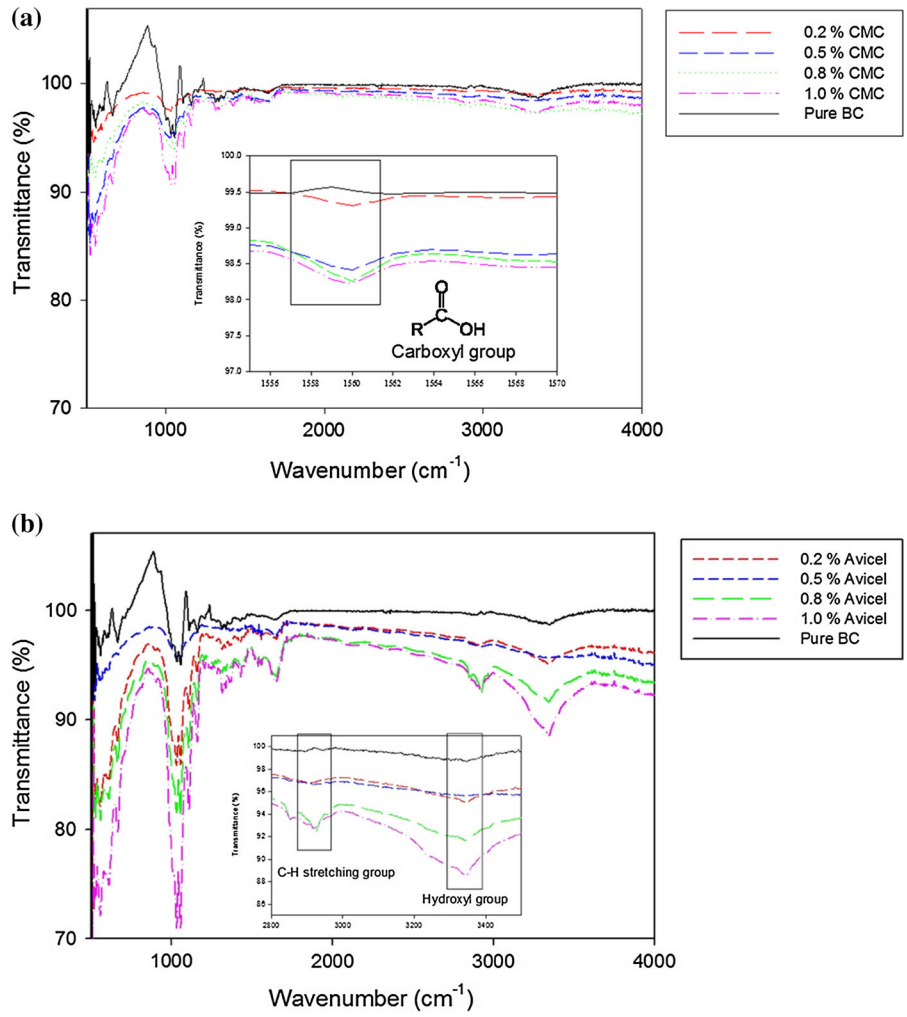

Fig. 3 FTIR spectra of BC prepared in the presence of different concentrations of **a** CMC and **b** Avicel

cellulose *Iα* form for randomly oriented crystallites (French 2014). The crystallinity data (Table 1) revealed that the BC crystallinity decreased with increased concentrations of CMC and Avicel. The slight decrease of crystallinity indicates that the additives may attach onto the microfibrils during crystallization, or even after crystallization (Zhou et al. 2007; Huang et al. 2010; Yamamoto et al. 1996). Previous studies have also suggested similar results. Haigler et al. (1980) demonstrated that Calcofluor White ST addition decreased BC crystallinity and increased glucose polymerization, resulting in the enhanced BC productivity. This may explain why BC production increased after the crystallization had been disturbed.

Morphology of BCs

The surface morphology of the pure and additive-altered BCs was observed by SEM (Fig. 5). A straight nano-scaled fibrillar structure was observed in the pure BC (Fig. 5a). Figure 5b–e indicates that the additives may disturb the network structure of BC, allowing the adsorption of additives on the fiber. In the 0.2 % CMC addition group (Fig. 5b), the small amount of CMC adsorbed onto the BC fibers led to a relatively large pore size. Conversely, in the highest CMC concentration group (Fig. 5c), the pore size decreased as large amounts of CMC were adsorbed onto the BC fibers. A possible explanation is that the incorporation of CMC into BC may result in the repulsion of fibers. CMC-

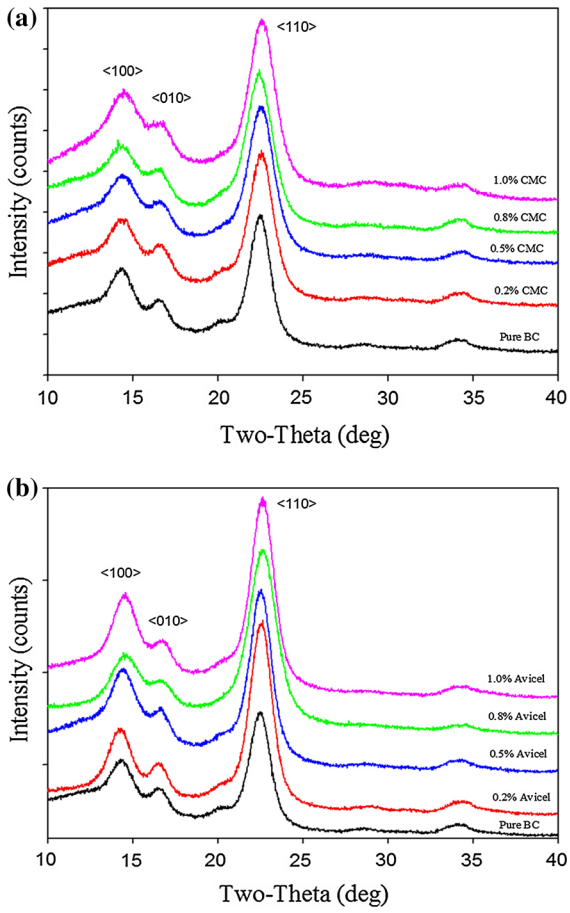

Fig. 4 X-ray diffraction patterns of **a** CMC-altered BC and **b** Avicel-altered BC at different concentrations

altered BC fibers were both positively charged and repelled each other, resulting in a disordered structure. In the 1.0 % CMC addition group, however, a high concentration of CMC was blended with the BC fibers and decreased the pore size. In the Avicel-altered BC group (Fig. 5d–e), Avicel for both the 0.2 and 1.0 % groups presented higher adsorption, resulting in decreased pore size. This might be because of Avicel's electric neutrality, as Avicel was incorporated into BC fibers and they did not repel each other. This could explain why the production of BC with Avicel addition is higher than that with CMC. Furthermore, the fiber size of additive-altered BC also increased (Fig. 6). The Avicel-altered BC fiber exhibited a larger fiber size than the CMC-altered BC (150 vs. 105 nm). Chen et al. (2011) also mentioned that CMC discontinuously adhered on the BC surface when BC was produced in situ. These results provide direct evidence that CMC and Avicel were absorbed into the BC fiber in the PCS-RDB system.

Thermogravimetric analysis and water content analysis

Thermogravimetric analysis (TGA) was performed to study the thermal degradation behavior of the composite samples. The results (Fig. 7) showed that both additive-altered BCs exhibited a single weight loss peak at the 355–370 °C range. Figure 7a shows that the 0.2 % CMC addition group exhibits a weight loss peak at 362 °C, and the weight loss temperature decreases with the CMC concentration (0.5, 0.8 and 1.0 % CMC addition groups present a weight loss temperature at 360, 358 and 355 °C). The correlation between weight loss temperature and CMC concentrations revealed that CMC addition during BC production may interfere with the network structure of BC and decrease its thermostability. Cheng et al. (2009a) found that CMC-modified cellulose hydrogel exhibited a lower weight loss temperature than the pure cellulose hydrogel, suggesting that the incorporated CMC decreases the thermostability of BC. Conversely, the Avicel groups (Fig. 7b) showed a similar weight loss temperature at 367–370 °C, slightly higher than that of the pure BC group (362 °C) ($p > 0.05$). CMC, which incorporates fibers with positive charges, may repel each other and result in an unstable structure of CMC-altered BC. However, the addition of Avicel with electric neutrality did not influence the structure of Avicel-altered BC. This may explain why CMC and Avicel addition had opposite influences on the TGA results.

The water content results indicated that the additive-altered BC exhibited a similar high water retention ability (98.6–99 %) to pure BC (98.8 %), suggesting that additive-altered BC can also serve as a good biomaterial for medical applications (e.g., wound dressing) (Kirdponpattara et al. 2015; Kwak et al. 2015).

Strength measurement

BC can be applied in many fields because of its special material properties (Lin et al. 2013b). Tensile strength plays an important role among these material features. BC with a specific tensile strength is crucial because of its versatile applications, such as filter paper (Chen

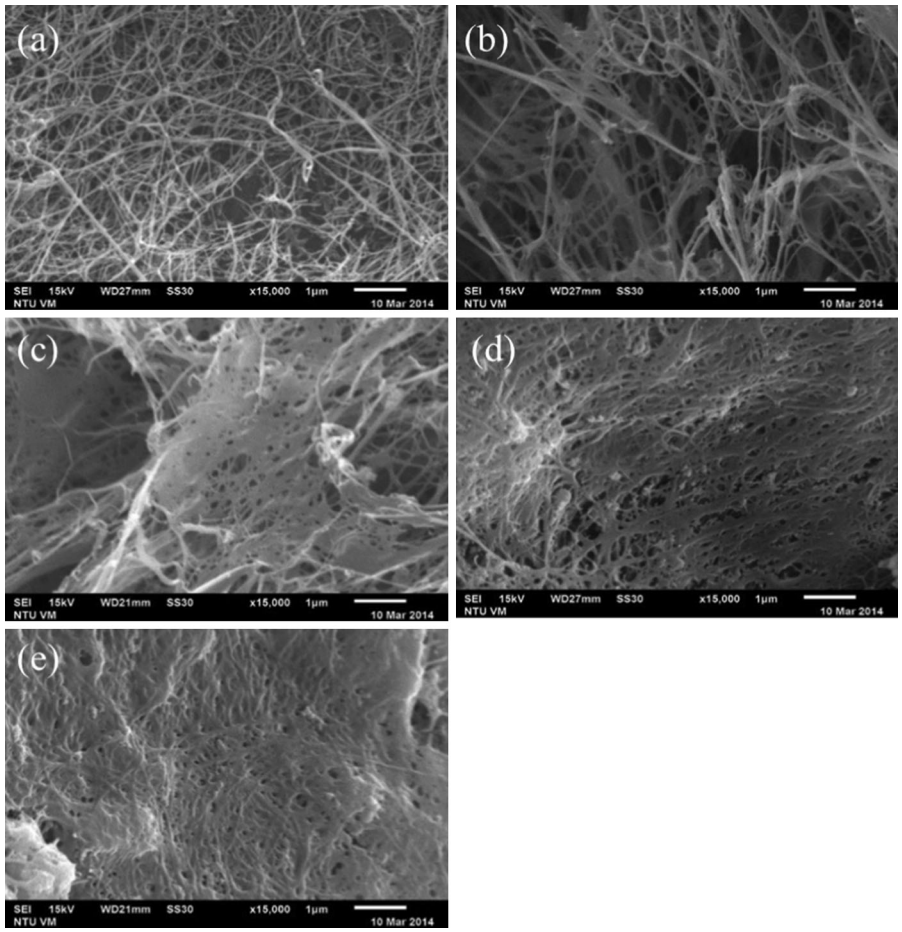

Fig. 5 Visualization of additive-altered BC. **a** Without additives, **b** 0.2 % CMC addition, **c** 1.0 % CMC addition, **d** 0.2 % Avicel addition and **e** 1.0 % Avicel addition

et al. 2009), packaging (George and Siddaramaiah 2012) and magnetic paper. The results for the tensile strength of pure and additive-altered BC are summarized in Table 1. In the CMC and Avicel addition groups, the stress decreased with increased CMC concentrations. The crystallinity also showed the same tendency, suggesting that CMC and Avicel incorporation may disrupt the crystalline structure and decrease the stress of additive-altered BCs. The results for the fiber size (Fig. 6) showed that Avicel-altered BCs exhibited a larger fiber size than those with CMC. This may explain why the stress of Avicel-altered BC is lower than that of CMC-altered BC. The results in strain analysis demonstrated that the strain of CMC-altered BCs decreased with increased CMC concentrations, but that of the Avicel addition groups presented a constant value at about 2.3–2.8 % (no significant difference). In conclusion, CMC- and Avicel-altered BCs still retain their strain, but with decreased stress. Yang et al. (2014) used potato starch as a scaffold to produce modified BC in situ. They found that the potato starch added as an interfering substance into the culture media could significantly decrease the crystallinity and stress of modified BC, but did not change its strain, and this result also supports our findings.

Conclusions

In this study, a novel PCS-RDB system was applied for BC production. CMC and Avicel can enhance BC production when they are introduced into culture medium. The addition of 0.8 % CMC and Avicel

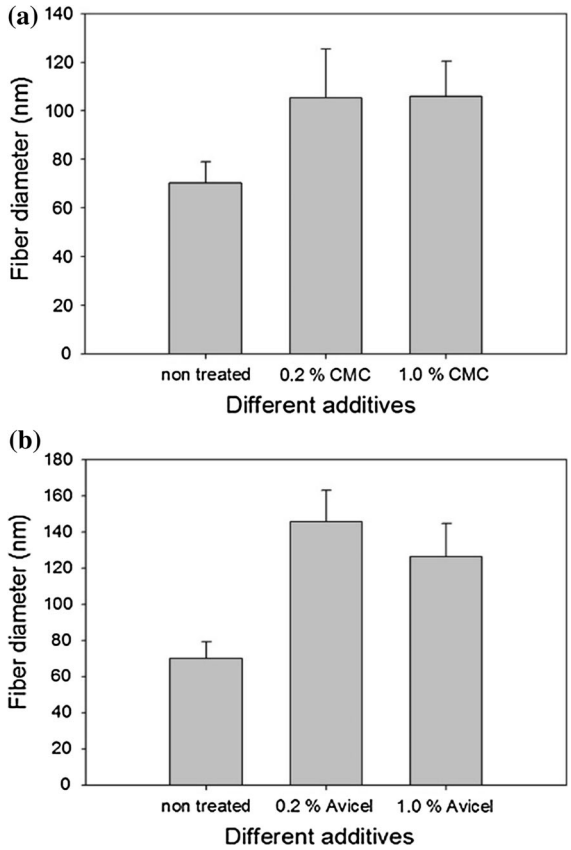

Fig. 6 Fiber size of **a** CMC-altered BC and **b** Avicel-altered BC ($n = 5$)

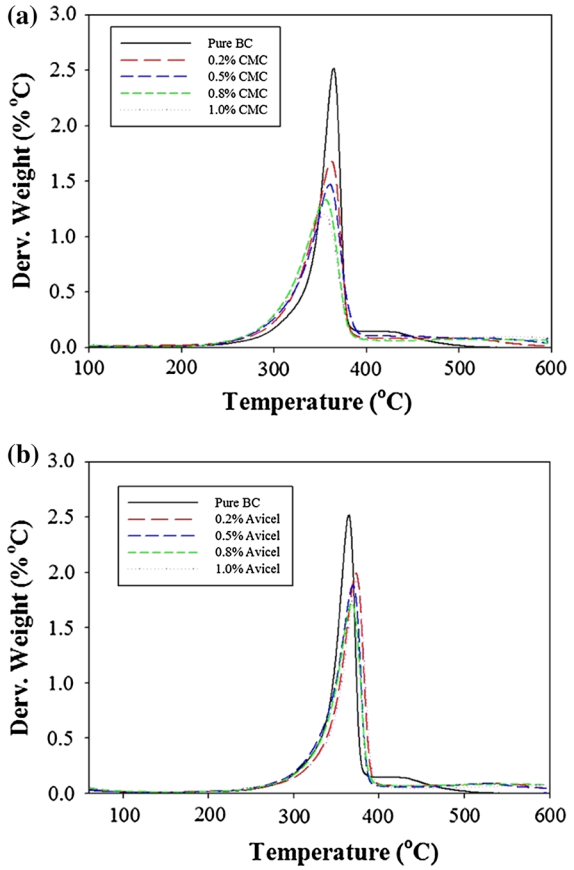

Fig. 7 Thermogravimetric analysis of BC with different concentrations of **a** CMC and **b** Avicel

reached the highest BC production, about 80 and 113 % more when compared with the control, respectively. In material analysis, the XRD and FTIR results provided direct evidence that CMC and Avicel were incorporated onto BC fibers and decreased the BC crystallinity. The SEM results show that the fiber size and morphology of additive-altered BC are also changed when CMC and Avicel are incorporated. The strength and water content results demonstrate that additive-altered BCs present a similar strain and water retention ability but lower stress depending on the additive concentration. In summary, we have introduced a BC production model featuring a low shear force, high oxygen penetration and adequate agitation rate for producing additive-altered BC. In addition to BC production, the system can modify the material property of the produced BC. Further studies on optimizing the cultivation conditions (e.g., rotating speed, PCS sheet number and inoculum) in PCS-RDB with Avicel and CMC addition will be the next challenge for industrial applications.

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