Carbohydrate Polymers 117 (2015) 518–523

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Production of bacterial cellulose using different carbon sources and culture media

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ARTICLE INFO

Article history:
Received 28 July 2014
Received in revised form
23 September 2014
Accepted 3 October 2014
Available online 25 October 2014

Keywords:
Bacterial cellulose
Gluconacetobacter xylinus
Carbon source
Culture media

ABSTRACT

In this work, the effects of carbon sources and culture media on the production and structural properties of bacterial cellulose (BC) have been studied. BC nanofibers were synthesized using *Gluconacetobacter xylinus* strain PTCC 1734. Media used were Hestrin–Schramm (H), Yamanaka (Y), and Zhou (Z). Five different carbon sources, namely date syrup, glucose, mannitol, sucrose, and food-grade sucrose were used in these media. All the produced BC pellicles were characterized in terms of dry weight production, biomass yield, thermal stability, crystallinity and morphology by thermogravimetric analysis (TGA), x-ray diffraction (XRD), and field emission scanning electron microscopy (FE-SEM). The obtained results showed that mannitol lead to the highest yield, followed by sucrose. The highest production efficiency of mannitol might be due to the nitrogen source, which plays an important role. The maximum improvement on the thermal stability of the composites was achieved when mannitol was used in H medium. In addition, the crystallinity was higher in BC formed in H medium compared to other media. FE-SEM micrographs illustrated that the BC pellicles, synthesized in the culture media H and Z, were stable, unlike those in medium Y that were unstable. The micrographs of BC produced in media containing mannitol and sucrose provided evidence of the strong interfacial adhesion between the BC fibers without noticeable aggregates.

1. Introduction

Cellulose is one of the most common carbohydrate polymers found in the world and has been widely studied during the past decades (Lin, Lopez-Sanchez, Li, & Li, 2014). Although it is a major structural biopolymer of all plants, various bacteria are also able to produce it (Tanskul, Amornthatree, & Jaturonlak, 2013). Cellulose from plants is normally mixed with lignin and hemicelluloses, but cellulose from bacteria or bacterial cellulose (BC) contains sets of parallel chains composed of β-D-glucopyranose units interlinked by intermolecular hydrogen bonds, which is identical in chemical composition to plant cellulose (Dahman, Jayasuriya, & Kalis, 2010). BC displays many unusual physicochemical and mechanical properties, including higher purity, higher crystallinity, higher degree of polymerization (Ashori, Sheykhnazari, Tabarsa, Shakeri, & Golalipour, 2012), higher water absorbing and holding capacity (Saibuatong & Phisalaphong, 2010), higher tensile strength (Castro et al., 2011) and stronger biological adaptability (Ul-Islam, Khan, & Park, 2012). Therefore, BC represents a promising alternative to plant-derived cellulose for specific applications in bio-medicine, cosmetics, high-end acoustic diaphragms, papermaking, food industry and other applications (Shah, Ul-Islam, Khattak, & Park, 2013; Lin et al., 2014).

In recent years, much interest has developed in producing BC on a large commercial scale (Czaja, Young, Kawechi, & Brown, 2007; Castro et al., 2011). However, compared with other popular commercial organic products, BC is still expensive, therefore, its use is limited (Sheykhnazari, Tabarsa, Ashori, Shakeri, & Golalipour, 2011). There have been many reports of cellulose being produced by both Gram negative bacteria such as *Gluconacetobacter xylinus*, *Agrobacterium*, *Achromobacter*, *Aerobacter*, *Azotobacter*, *Pseudomonas*, and *Rhizobium*, and Gram positive bacteria such as *Sarcina* (Tanskul et al., 2013). Among the mentioned genera, *G. xylinus* is one of the most commonly studied sources of BC (Nguyen, Flanagan, Gidley, & Dykes, 2008). It has been used for producing commercial quantities of cellulose and has become the most popular strain so far for application not only as a food component but also for drug-delivery systems (Tanskul et al., 2013), wound

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http://dx.doi.org/10.1016/j.carbpol.2014.10.008
0144-8617/

healing and tissue regeneration (Czaja et al., 2007), flexible display screens (Nakagaito, Nogi, & Yano, 2010), composite reinforced, electronic paper (Jonas & Farah, 1998), and so on. However, it is difficult to obtain a high productivity using this bacterium in a large-scale fermentation system due to its low yield under agitated conditions. It is important to develop methods to produce BC at the lowest possible cost. The significant factors that many researchers have investigated are the optimal medium, the culture conditions and their interaction effects. Additionally, determining growth conditions that produce high amounts of cellulose is necessary to complete further research using BC as reinforcement for biodegradable polymers as well as understanding any effects such conditions have on the basic materials' morphology and properties (Ruka, Simon, & Dean, 2012). There have been several reports of different media used in the literature, as well as various carbon sources (El-Saied, El-Diwany, Basta, Atwa, & El-Ghwas, 2008; Jung et al., 2010; Ruka et al., 2012). Some authors have reported that the structure of cellulose is not affected by changing the carbon or nitrogen source (Mikkelsen, Flanagan, Dykes, & Gidley, 2009), whereas others have reported differences. El-Saied et al. (2008) reported that a medium containing corn steep liquor and molasses medium resulted in a higher degree of crystallization over carbon and nitrogen sources such as glucose, mannitol, yeast extract and peptone, whereas Jung et al. (2010) reported a decrease in crystallinity in molasses medium compared to a complex medium control.

This work aimed at studying the effects of various culture media and carbon sources in order to formulate a general, simple and inexpensive medium to produce BC. More common carbon sources such as glucose, mannitol, sucrose, date syrup and food-grade sucrose were used in this study. Carbon sources were compared in terms of biomass and production yield, and the morphology and structure of the BC obtained were studied. In this context, BC nanofibers produced in the selected media were characterized by means of x-ray diffraction (XRD), field emission scanning electron microscopy (FE-SEM) and thermogravimetric analysis (TGA).

2. Experimental

2.1. Bacterial strain

The organism used was a native strain of *G. xylinus* obtained from the Persian Type Culture Collection (PTCC 1734), Iran. The strain was cultured on glucose yeast extract (GYE) agar containing 100 g D-glucose, 10 g yeast extract, 5 g peptone, 20 g $CaCO_3$, 25 g agar per liter at 28 °C for 3 days. Working cultures were routinely prepared on GYE and stored at 4 °C until use.

2.2. Media and carbon sources

Three different types of media that have been previously reported to have optimized concentrations and are used to cultivate *G. xylinus* were selected from the literature and modified for the present study. Media used were Hestrin–Schramm (H), Yamanaka (Y) and Zhou (Z). The chemical compositions of the media are described in Table 1. Date syrup, glucose, mannitol, sucrose, and food-grade sucrose were used as different carbon sources in these media. All the media were inoculated at a concentration of 10% (v/v) after being autoclaved at 121 °C for 15 min. Ethanol 1% (v/v) was added to all media and the pH was adjusted to 5.5 with H_2SO_4.

2.3. Pellicles production and purification

Cultures were incubated at 28 °C, 150 rpm, for 7 days. After incubation, the pellicles produced on the surface of each medium were harvested and washed with water and 1% NaOH solution at 80 °C for 1 h and then washed with distilled water to remove the former.

2.4. Dry weight and yield of BC

BC production was recorded as the dry weight of cellulose within the volume of medium in liter ($g\,L^{-1}$). To determine the dry weight of cellulose sheets, the sheets were oven dried at 45 °C for 3 days until reaching constant weight. The pH of the remaining medium was measured after the cellulose sheets were harvested. The yield of biosynthesis process was calculated as follows:

$$\text{Yield}(\%) = \left(\frac{m_0}{C}\right) \times 100 \quad (1)$$

where m_0 is dry weight of cellulose (g) and C is the weight of carbon source (g).

2.5. Thermogravimetric analysis (TGA)

Thermogravimetric analysis of samples was done using a TGA instrument 931 thermal analyzer (TA Instruments). The average weight of samples was approximately 0.6 mg. Scan rates of 20 °C min^{-1} over a temperature range of 20–620 °C were applied. All tests were carried out under inert (N_2) atmosphere.

2.6. X-ray diffraction (XRD)

Dried films of cellulose nanofibers were X-rayed using an X'Pert pro MPD (multi-purpose diffractometer, Model PW3040/60). X-ray diffraction patterns were recorded at the CuKα radiation wavelength (λ = 1.54 Å), generated at a voltage of 40 kV and a filament emission of 30 mA. Samples were scanned from 0–40° 2θ-range at scan speed of 0.5° min^{-1}. The crystallinity (Cr) and crystallite size (CrS) were calculated based on X-ray diffraction measurements. Crystallinity was calculated from the following Eq.:

$$\text{Cr}(\%) = \frac{S_c}{S_t} \times 100 \quad (2)$$

where S_c is sum of net area and S_t is sum of total area. The Cr*S* was determined using Scherrer equation as following:

$$\text{Cr}S = \frac{K\lambda}{\beta \cos\theta} \quad (3)$$

where K is the shape factor (0.9), λ is the x-ray wavelength (1.54 Å), β is the full width at half maximum height (FWHM), and θ is the Bragg's angle.

2.7. Field emission scanning electron microscopy (FE-SEM)

The samples were mounted and gold-coated in preparation for field emission scanning electron microscopy (FE-SEM) imaging. FE-SEM was performed using the field-emission SEM Hitachi SU 8090. FE-SEM experiment was conducted at an accelerated voltage of 5 kV and magnification of 20k.

3. Results and discussion

3.1. BC production and yield

Fig. 1a shows the amounts of BC production from various carbon sources. After 7 days incubation, the production of the BC was confirmed from three media. However, more BC cultured in media Zhou (Z) and Hestrin-Schramm (H) was produced, respectively. Moreover, it was confirmed that *G. xylinus* could not produce BC in the medium consisting of Yamanaka (Y). The nitrogen sources such as yeast extract and pepton were indispensable for the cell

Table 1
Components and concentrations of media.

Components	Media (%, w/v)		
	Hestrin–Schramm (H)	Yamanaka (Y)	Zhou (Z)
Glucose	2	5	4
Corn steep liquor	–	–	2
Yeast extract	0.5	0.5	–
Peptone	0.5	–	–
Na_2HPO_4	0.27	–	–
Citric acid. H_2O	0.115	–	–
$(NH_4)_2SO_4$	–	0.5	0.4
KH_2PO_4	–	0.3	0.2
$MgSO_4.7H_2O$	–	0.005	0.04

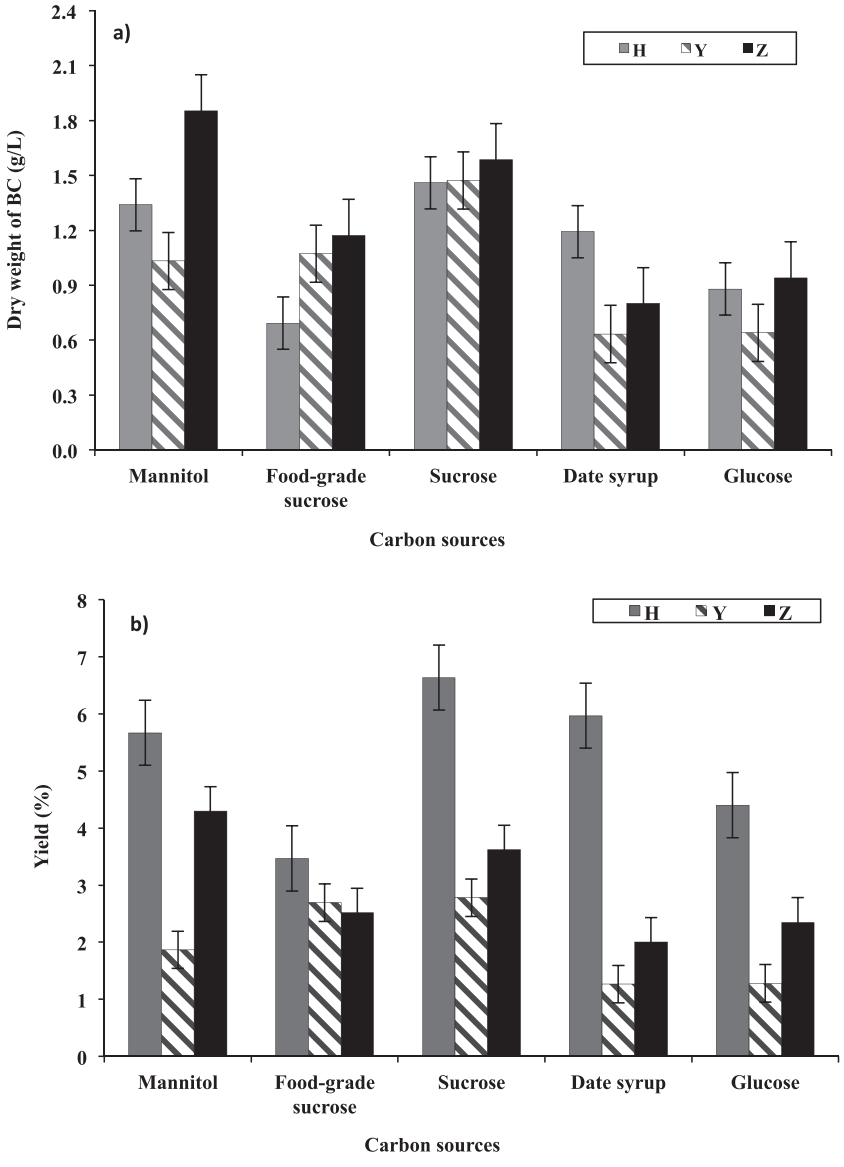

Fig. 1. Bacterial cellulose production (a) and yield (b) from various carbon sources on media H, Y and Z.

growth and BC production. It is therefore suggested that such nitrogen sources should be included in the media. The dry BC weight produced from the medium that consisted of mannitol and sucrose was the maximum. Although the production of sucrose in all media is the same (Fig. 1), the BC was slightly produced in Zhou medium (1.59 g L^{-1}). Therefore, it is thought that the mannitol and sucrose could consist of promoting substance for production of the BC. From the above-mentioned observations, it is thought that the mannitol and H was the most suitable condition for the BC production.

Fig. 1b shows comparison of yield of BC yield in various media. When the yields of the BC produced with by H and Y media was compared, the yields of the BC with Y medium were very low and the yields with H medium were higher in all the samples. Yield of the BC production in H was 3 times more than that with medium Y for mannitol. This yield was in same range of those reported in

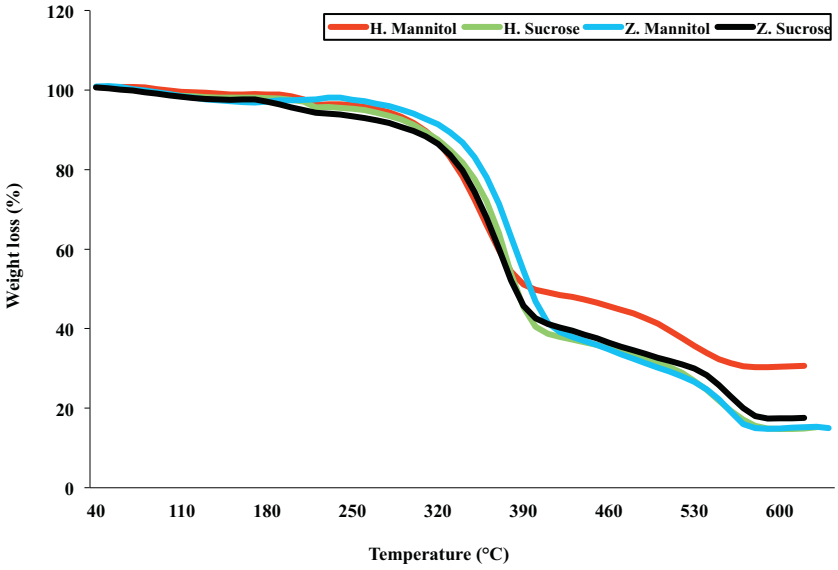

Fig. 2. TG curves of bacterial cellulose from different culture media and carbon sources.

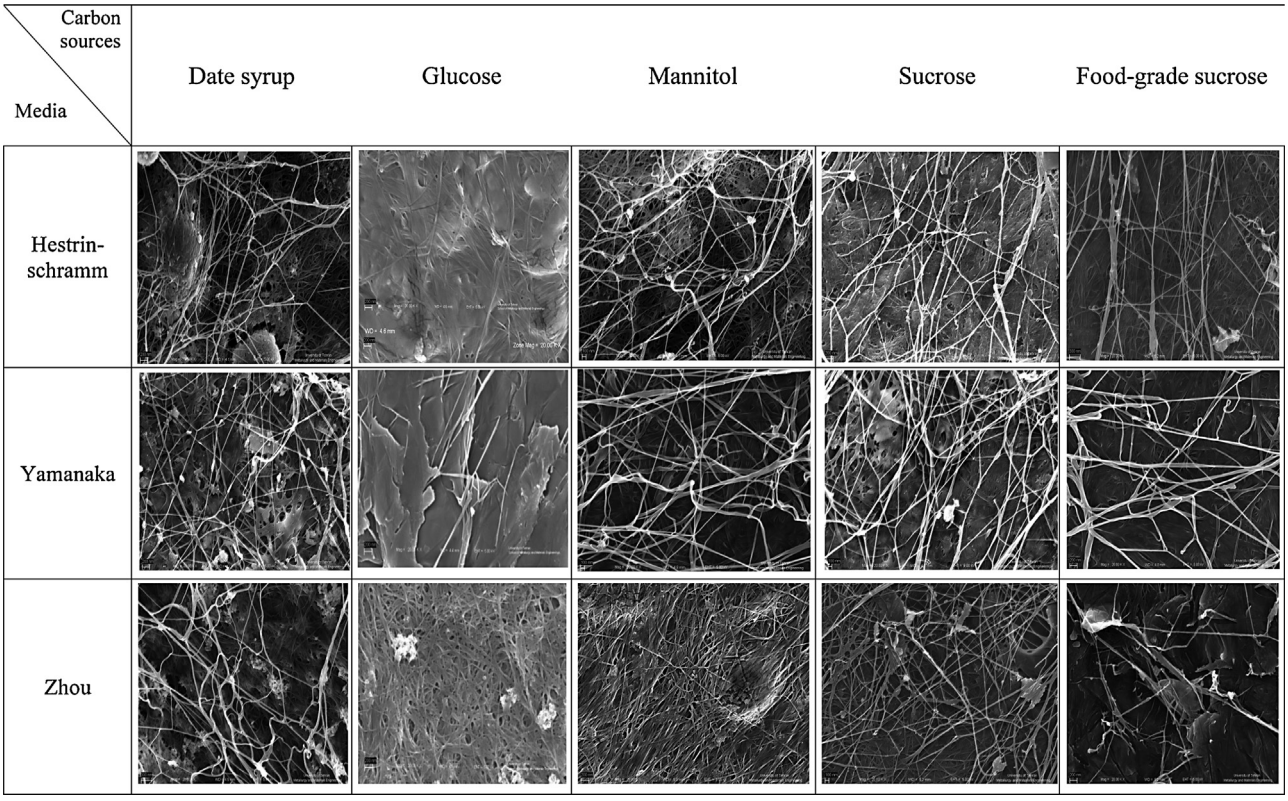

Fig. 3. FE-SEM images of bacterial cellulose produced under different conditions (all views with 20,000× magnification).

the literature using the same medium (El-Saied et al., 2008; Carreira et al., 2011). Yields of the BC produced with the medium H that used date syrup, glucose, sucrose, and food-grade sucrose were 4.7, 3.4, 2.4 and 1.3 times, respectively, more than those with Y medium. Therefore, the addition of the nitrogen source to the carbon source was very effective in the BC production. Compared with the yield of the BC with H medium, the yields of the BC with Z medium decreased.

3.2. TGA

Thermal stability of the samples might be important for some applications, and might provide some clues on BC fiber interactions (Stumpf, Pértile, Rambo, & Porto, 2013). Fig. 2 shows the TG degradation curves of the dried pellicles obtained using the different carbon sources assayed, in terms of percent weight of the original sample versus temperature. As usual, during the initial

thermal treatment, samples showed a small weight loss starting at room temperature up to 100 °C due to loss of water. No significant differences between BC samples were observed in this region. This indicates that there is no difference in moisture content between these samples. BC shows a second mass loss event that is attributed to a decomposition process at temperatures between 200 and 250 °C. At around 360–390 °C the decomposition of the samples resulted in a significant weight loss (70–80%). This event also may be associated with a degradation of cellulose, including depolymerization, dehydration and decomposition of glucose units, followed by the formation of a carbon residue (Vazquez, Foresti, Cerrutti, & Galvagno, 2013). Results obtained for the mannitol supplemented H medium showed temperature values higher than the other BC pellicles. The maximum decomposition temperature is a criterion of thermal stability. Thermal degradation behavior is known to be affected by some structure parameters such as molecular weight, crystallinity and orientation of the fibers (Barud et al., 2007; Vazquez et al., 2013).

In the case of mannitol-based produced BC, XRD results (Table 2) show that it had the highest crystallinity, which could be the reason for thermal behavior observed. In the case of the mass loss profile of BC obtained from sucrose, the sharp decrease in weight evidenced for this sample could be ascribed to its low crystallinity.

3.3. BC morphology characterization

Detailed morphological characterization of different BC samples was carried out using FE-SEM. A set of selected micrographs of the surface of BC materials are shown in Fig. 3. For each carbon source and medium, the BC fibers dispersion and the interfacial adhesion are shown. It is notable that the BC pellicles, which were synthesized in the culture media H and Z, were stable, unlike those in medium Y that were unstable. A close observation revealed that most uniaxially oriented cellulose ribbons were formed in culture media H and Z. The micrographs in Fig. 3 show the rod-shape of nanofibers bundles. This compact cellulose network structure is made up of a random assembly of fibrils. A rich medium containing yeast extract and peptone (H medium) has been found to be good for BC production (Tanskul et al., 2013).

The micrographs of BC produced in mannitol and sucrose-containing media provided evidence of the strong interfacial adhesion between the BC fibers, as shown by the good dispersion within the matrix, without noticeable aggregates. Obviously, in the case of data syrup and food-grade sucrose, only the small mat fragments, and not the isolated fibrils, were observed. The open structure and large amount of smaller cellulose nanocrystal aggregates are seen. Image analysis was made manually in order to distinguish between widths, apparent thickness of edged on ribbons, and "intermediate" views. The analysis performed did not show the existence of significant differences among the dimensions of nanofibers obtained from the different carbon sources assayed, with nanofibers widths in the range of 29–77 nm, and thickness values in the 15–31 nm intervals. The FE-SEM image illustrates that void space dominates with the average fiber diameter of 18–35 nm.

Table 2
Crystallinity (Cr) and crystallite size (CrS) of the used bacterial cellulose samples.

BC type	Cr (%)	CrS (nm)
H. Mannitol	65.5	6.3
H. Sucrose	63.2	5.8
Z. Mannitol	53.3	6.4
Z. Sucrose	46.7	6.2

4. Conclusions

In this study, the production of BC using *G. xylinus* from five categories of carbon sources (date syrup, glucose, mannitol, sucrose, and food-grade sucrose) was examined. Some conclusions are as follows:

- A reference native strain used in this study that was known to produce good yields of cellulose was *G. xylinus* 1734, obtained from the Persian Type Culture Collection (PTCC), Iran.
- The food-grade sucrose and date syrup were not suitable carbon sources for the growth of *G. xylinus*, so BC was not produced very well. In contrast, mannitol and sucrose could produce more BC in H medium.
- The addition of the nitrogen source to the mannitol was very effective for the production of BC.
- From X-ray diffractometer, the crystallinity of BC in presence of H medium had higher values than those from other media.
- The BC pellicles, which were synthesized in the culture media H and Z, were mostly uniaxially oriented ribbons. In addition, the micrographs of mannitol and sucrose provided evidence of the strong interfacial adhesion between the BC fibers.

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