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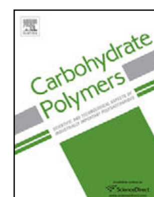

Characterization of cellulose and other exopolysaccharides produced from *Gluconacetobacter* strains

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ABSTRACT

This study characterized the cellulosic and non-cellulosic exopolysaccharides (EPS) produced by four *Gluconacetobacter* strains. The yields of bacterial cellulose and water-soluble polysaccharides were dependent on both carbon source and *Gluconacetobacter* strain. The carbon substrate also affected the composition of the free EPS. When galactose served as an exclusive carbon source, *Gluconacetobacter xylinus* (*G. xylinus*) ATCC 53524 and ATCC 700178 produced a distinct alkaline stable crystalline product, which influenced the crystallization of cellulose. *Gluconacetobacter hansenii* (*G. hansenii*) ATCC 23769 and ATCC 53582, however, did not exhibit any significant change in cellulose crystal properties when galactose was used as the carbon source. Microscopic observation further confirmed significant incorporation of EPS into the cellulose composites. The cellulosic network produced from galactose medium showed distinctive morphological and structural features compared to that from glucose medium.

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1. Introduction

Cellulose is a major component in plant cell walls, and is also produced by a variety of organisms such as algae, fungi and bacteria. Bacterial cellulose (BC) is an exopolysaccharide produced by various species of bacteria, such as the genera *Acetobacter* (reclassified as *Gluconacetobacter*), *Agrobacterium*, *Rhizobium* and *Sarcina* (Ross, Mayer, & Benziman, 1991). The *Gluconacetobacter* species are known to be one of the most effective cellulose producers. The chemical composition of BC is the same as that of plant cellulose, but some of its properties are quite unique such as high purity, water holding capacity, tensile strength and adaptability to the living body (Shoda, 2005). These characteristics give BC promising applications in a wide range of industrial fields including food additives, electronics (such as acoustic transducer diaphragms for headphones) (Iguchi, Yamanaka, & Budhiono, 2000), medical materials for wound healing and tissue regeneration (Fontana et al., 1990; Hu & Catchmark, 2011), and selective permeation membranes (Sokolnicki, Fisher, Harrah, & Kaplan, 2006).

BC has a significant advantage over higher plant cellulose in that it is free of lignins, hemicelluloses and pectins. However, cellulose is not the only product from the *Gluconacetobacter* system. In addition to cellulose, certain *Gluconacetobacter* strains have been reported to produce a variety of water-soluble polymers such as anionic acetan (Couso, Ielpi, & Dankert, 1987; Jansson, Lindberg, Wimalasiri, & Dankert, 1993; Valla, 1981) and neutral mannan (MacCormick, Harris, Cunning, & Morris, 1993). Those EPS produced have different levels of association to BC, and a small portion of them cannot be separated from BC pellicles by the routine purification procedures. Those EPS were defined as hard to extract EPS (HE-EPS) in our previous study (Fang & Catchmark, in press). Different from the free EPS, the presence of HE-EPS in the culture medium could interfere with the alignment of the BC crystals and modulate the bundling of cellulose ribbons.

BC synthesis in *Gluconacetobacter* strains also serves as a model system to study the basic principles that govern the biogenesis of cellulose as well as cellulose interaction with other polymers during synthesis. For example, a commonly used strategy to study plant cell wall assembly is to add purified hemicellulose to *Gluconacetobacter* fermentation medium. Other than hemicellulose, extensive work has already shown that BC fibril assembly and crystallization can be impacted by the adding a variety of biopolymers such as carboxymethyl cellulose, fluorescence brightener, etc. (Haigler, Brown, & Benziman, 1980; Haigler, White, Brown, & Cooper, 1982; Mondal, 2013) The addition of those polymers may modify the BC synthesis in a similar or distinct fashion through competing cellulose–cellulose hydrogen bonding during the early stage of ribbon assembly. The synthesis of EPS, especially those with strong association with cellulose, may impact the interpretation of this prior research. There is a need to explore different *Gluconacetobacter* strains and identify a pure cellulose production system.

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Gluconacetobacter can utilize a range of carbon sources to produce cellulose. The biosynthesis of cellulose in bacteria has traditionally been studied using glucose. Fructose, sucrose and complex carbon sources such as molasses have also been reported to provide constant high yields of cellulose (Bae, 2005; Mikkelsen, Flanagan, Dykes, & Gidley, 2009). Some of those studies demonstrated the composition of the media did not play a significant role in the structure of the cellulose produced. Galactose is also used as a carbon source for cellulose production. The structure of galactose is very similar to that of glucose and the only difference is the configuration of the hydroxyl group at the C4 position. However, previous reports have concluded that galactose is not an effective carbon substrate for BC production (Keshk & Sameshima, 2005; Mikkelsen, Flanagan, Dykes, & Gidley, 2009; Ramana, Tomar, & Singh, 2000). Those studies did not perform the characterization of any product formed using galactose as a substrate.

Further utilization of BC as a model system requires an understanding of *Gluconacetobacter* strains' ability to metabolize different carbon sources and their impact on cellulose and other related polysaccharide production. The main goal of this work is to understand the effect of galactose when used as a carbon source in place of glucose on cellulose structure and other EPS production. The association between BC and EPS produced on the crystal structure of cellulose was also evaluated in this study.

2. Method

2.1. Cell culture conditions

Four *Gluconacetobacter* strains (*G. hansenii* ATCC 23769, *G. xylinus* 53524, *G. hansenii* 53582, *G. xylinus* 700178) obtained from the American Type Culture Collection (Rockville, MD) were used in this study. Cells were cultured in a standard Hestrin–Schramm (HS) medium (Hestrin & Schramm, 1954) containing 2% (w/v) glucose, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.27% (w/v) Na_2HPO4 and 0.12% (w/v) citric acid or in the modified media by replacing glucose with galactose. The final pH was adjusted to 5.0.

Primary inoculates were prepared by transferring a single colony from the HS medium working culture plate into 100 mL of each of the six modified HS media. Cells were grown in 100 mL of HS medium with 0.1% (vol/vol) cellulase (from *Trichoderma reesei*, Sigma, Lot#C2730) at 30 °C with shaking at 125 rpm for three days. Cells were harvested by centrifugation, then resuspended in the culture medium. The main cultures were grown in 250 mL Erlenmeyer glass flasks containing 100 mL medium for 7 days at 30 °C.

2.2. Purification of BC, free EPS and hard to extract (HE) EPS

At the end of incubation, the pellicles were collected and treated with 0.1 M NaOH aqueous solution followed by rinsing until a pH of 7 was achieved. The purified cellulose was lyophilized.

To harvest free EPS, the supernatant of the culture broth was obtained by centrifugation and KCl was added to a final concentration of 1% w/v to solubilize any protein present. The free EPS were precipitated with two volumes of ethanol. The ethanol precipitate was separated by centrifugation and then dissolved in water. This process was repeated at least three times. The HE-EPS were extracted from the BC films. The wet BC films were treated with 4 M NaOH aqueous solutions overnight at room temperature, and the alkali solution was neutralized. Salts were removed by dialysis (3.5 K molecular cut off) against DI water for 24 h. Protein concentrations were less than 1% for all the EPS samples, which were determined by the Bradford method.

2.3. NaOH extraction

BC composites produced from glucose and galactose medium were extracted with 4 M NaOH solutions for 12 h at 25 °C. After extraction, the residues were washed with DI water until neutral and freeze-dried.

2.4. Monosaccharide composition of EPS

The purified EPS were hydrolyzed in an aqueous solution of 2 N Trifluoroacetic acid (TFA) for 2 h at 120 °C in sealed glass vials. The resulting solution was evaporated at 40 °C to dry the EPS, and then the dried product was dissolved in distilled water. The analysis of monosaccharides of the hydrolyzates was performed on a Dionex ICS-5000 Capillary Reagent-Free IC System (Dionex, Sunnyvale, CA) and a CarboPac 20 column (Dionex). Monosaccharides were identified by comparison to the retention times with the monosaccharide standards.

2.5. X-ray diffraction (XRD)

X-ray diffraction diagrams were recorded using PANalyticalX'Pert Pro multi-purpose diffractometer with Cu $K\alpha$ radiation generated at 45 kV and 40 mA. The diffractometer was used in reflection mode with the automatic divergence slit. The data was collected in the 2θ range 5–40° with a step size of 0.026°. Freeze dried BC samples were mounted onto a quartz sample holder.

Crystallinity has been used to describe the relative amount of crystalline material in cellulose. Two different approaches including peak height and peak deconvolution were used to estimate crystallinity of BC. In the peak height method, crystallinity was calculated from the intensity ratio between the (1 1 0) crystalline reflection and the amorphous background near 18.3° (Segal, 1959). In the peak deconvolution method, a pseudo Voigt function was used to profile the peak shape and area, assuming a linear background. A broad peak at around 21.5° was assigned to the amorphous contribution. Crystallinity is calculated from the ratio of the area of all crystalline peaks to the total area (Thygesen, Oddershede, Lilholt, Thomsen, & Ståhl, 2005):

The dimension of the crystal perpendicular to the diffracting planes with $h\,k\,l$ Miller indices, $B_{h\,k\,l}$, was evaluated by using Scherer's expression (Nieduszynski & Preston, 1970):

$$B_{h\ k\ l} = \frac{K\lambda}{\sqrt{(\Delta 2\theta)^2 - (\Delta 2_{\text{inst}})^2}\cos\theta}$$

B_{hkl} is the average crystalline width of a specific phase; K is a constant that varies with the method of taking the breadth ($K = 0.9$); λ is the wavelength of incident X-rays ($\lambda = 0.15418$ nm); θ is the center angle of the peak; $\Delta 2\theta$ is the full width at half maximum (FWHM) of the reflection peak and $\Delta 2\theta_{\text{inst}}$ is the instrumental broadening. The instrumental broadening was determined from the FWHM of four reflections of a silicon standard (NIST Si 640).

2.6. Field emission scanning electron microscopy (FESEM)

FESEM was conducted to observe sample morphology and microstructure. Purified BC samples were lyophilized and sputter coated with gold (approximately 5 nm). A LEO 1530 field emission scanning electron microscope (LEO Elektronenmikroskope, Oberkochen, Germany) was used operating at 5 kV.

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