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Impact of hemicelluloses and pectin on sphere-like bacterial cellulose assembly

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ABSTRACT

It has been shown previously that certain strains of the bacterium *Gluconacetobacter xylinus* produce a spherical form of cellulose where the cellulose was formed in a layered fashion. The spherical cellulose was used as a model system to study cellulose–hemicellulose and cellulose–pectin composite formation. Cultures were produced in the presence of 0.5% (w/v) xyloglucan, xylan, arabinogalactan and pectin under agitating conditions. Cellulose samples with xyloglucan and pectin had different macro structures compared to other culture conditions. The micro structures showed that these two samples formed dense cellulose layers and had fewer cellulose fiber connections between layers. Cellulose samples with xylan and xyloglucan were found to contain more Iβ cellulose as found in higher plants, and exhibited decreases in crystallinity and crystalline sizes according to X-ray diffraction patterns. IR spectroscopy confirmed the changes in crystal allomorph. Cellulose was also grown in cultures containing different blends of both xyloglucan and pectin. Results show that xyloglucan had the dominant impact on the assembly of cellulose, suggesting that xyloglucan and pectin may interact with cellulose at different points in the assembly process, or in different regions. Bacterial cellulose and biomass yields indicated that xyloglucan and pectin could also stimulate the growth of cellulose.

1. Introduction

The plant cell wall is a dynamic and highly ordered macromolecular complex (Vian, 1982). This complex consists of various polysaccharides and structural proteins (Albersheim, Darvill, Roberts, Sederoff, & Staehelin, 2010; Obembe, Jacobsen, Visser, & Vincken, 2006). Cellulose is the major structural and load-carrying component of the primary cell wall. The cellulose microfibrils are embedded in a matrix consisting of other components of the wall, including hemicelluloses and pectin (Albersheim et al., 2010). A three-dimensional network is formed by linking the cellulose and hemicelluloses. Another independent network consists mainly of pectin (Albersheim et al., 2010).

Cellulose from *Gluconacetobacter xylinus* (formerly *Acetobacter xylinum*) has been used as a model system to study cellulose synthesis for decades. The cellulose produced by the bacterium is highly pure without any hemicellulose or pectin, in contrast to that from higher plants. It is rich in triclinic (Iα) cellulose rather than monoclinic (Iβ) as in higher plants. Detailed structural characteristics of these two crystal forms were described by CP/MAS (cross polarization/magic angle sample spinning) ^{13}C NMR spectroscopy (Vanderhart & Atalla, 1984), Fourier transform infrared (FT-IR) spectroscopy, electron diffraction (Sugiyama, Persson, & Chanzy, 1991a; Sugiyama, Vuong, & Chanzy, 1991b) and X-ray diffraction (Wada, Sugiyama, & Okano, 1993). Two methods are used to synthesize bacterial cellulose: (1) static culture, which produces a cellulose pellicle on the surface of the medium; and (2) agitated culture or shaking culture, which results in the cellulose synthesized in various forms including fibers, pellets, spheres or irregular masses in the media (Chao, Ishida, Sugano, & Shoda, 2000; Czaja, Romanovicz, & Brown, 2004; Hu & Catchmark, 2010; Watanabe, Tabuchi, Morinaga, & Yoshinaga, 1998). The agitated condition could cause a slight reduction in the crystallinity of the cellulose (Iwata, Indrarti, & Azuma, 1998). The Iα mass fraction of cellulose from agitated culture is lower than that from static culture (Czaja et al., 2004; Whitney et al., 2006).

Avoiding the complexity associated with many polymers being synthesized together in the cell wall, bacterial cellulose has been used in model systems in order to understand the synthesis of cellulose and its interactions with other wall components. In both bacterial and plant cells, cellulose is synthesized by membrane bound cellulose synthases. Most other plant cell wall polymers are synthesized in the Golgi apparatus and secreted in vesicles to the extracellular environment (Taiz & Zeiger, 2002). To mimic this process, test polymers have been added into the fermentation medium of *G. xylinus* culture. For instance, it has been shown that the assembly of cellulose microfibrils or bundles is disrupted by adding Calcofluor (a fluorescent brightener) or carboxymethylcellulose (Cheng, Catchmark, & Demirci, 2009; Haigler

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& Benziman, 1982; Haigler, Brown, & Benziman, 1980; Haigler, White, Brown, & Cooper, 1982). A cross-bridging microstructure similar to that observed in plant cell walls was observed under electron microscopy in the presence of xyloglucan (Whitney, Brigham, Darke, Reid, & Gidley, 1995). The mechanical performance of the cell wall could be predicted by addition or removal of the cell wall polysaccharides using bacterial cellulose system (Chanliaud, Burrows, Jeronimidis, & Gidley, 2002). This system allows the use of defined components with known structure, molecular weight and concentration, which is not achievable in *in vivo* conditions.

Xyloglucan is the most abundant hemicellulose in the primary wall of dicotyledons and non-graminaceous monocotyledons (Taiz & Zeiger, 2002). This polysaccharide has a β-1,4-linked D-glucose backbone with frequent substitution of α-D-xylosyl residues at C-6 position. Some substitutions like galactose, fucose and arabinose are also present (Sims, Munro, Currie, Craik, & Bacic, 1996; Vincken, York, Beldman, & Voragen, 1997; Whitney et al., 2006). Some xyloglucans are acetylated (Ebringerová et al., 2005). It is one of the best-studied examples of hemicellulose and *G. xylinus* cellulose interaction. Some researchers believe that the xyloglucan cellulose interaction is based on the structural similarity between the xyloglucan backbone and cellulose chain (Hanus & Mazeau, 2006). The effect of side chains and molecular weight is also extensively studied and contradictory conclusions were made concerning the role of fucose in cellulose binding (Whitney et al., 2006).

Xylans are found in many primary cell walls. In some monocots, xylans are the linking polymers (Carpita & Gibeaut, 1993). They are also the major hemicellulosic component in the secondary cell wall (Ebringerova & Heinze, 2000). The backbone of xylan consists of β-1,4-linked D-xylose with varying extent substitutions of the glucuronyl, acetyl and arabinosyl groups at the O-2 or O-3 position (Ebringerová et al., 2005). Studies of glucuronxylan, *O*-acetyl-glucuronoxylan, arabinoglucuronoxylan and arabinoxylan showed that xylans were blended into the cellulose preventing the assembly of cellulose microfibrils and changed the crystal structure of cellulose (Iwata et al., 1998; Tokoh, Takabe, Sugiyama, & Fujita, 2002; Uhlin, Atalla, & Thompson, 1995; Winter, Barakat, Cathala, & Saake, 2006). The adsorption of xylans to cellulose is also dependent on their side chains, molecular weights and level of impurities.

Mannan-based hemicelluloses are found in large quantities in some plant species. Studies of the interaction of mannan-based hemicelluloses and bacterial cellulose led to the conclusion that mannan lowered the crystallinity of cellulose by decreasing the crystal size (Uhlin et al., 1995). The cellulose–mannan interaction is also affected by the Man:Gal/Glc ratio and degree of polymerization (Whitney, Brigham, Darke, Reid, & Gidley, 1998). Other hemicelluloses such as arabinogalactan, which is classified as gum as well as hemicellulose (Ebringerová et al., 2005), was also studied using bacterial cellulose systems and had low affinity to cellulose (Iwata et al., 1998).

Pectin is the gel-forming component in primary cell walls and intercellular regions of higher plants. It is a structural heteropolysaccharide containing partially methyl-esterified homogalacturonan, rhamnogalacturonan I and II (RGI and RGII) (Albersheim et al., 2010). Studies of pectin-cellulose composites indicated that pectin was phase separated with cellulose although it stayed close to cellulose ribbons (Tokoh et al., 2002). Pectin forms independent networks and networks involving cellulose but it is dependent on the methyl esterification levels of the polysaccharide and calcium concentration in the culture medium (Chanliaud & Gidley, 1999). The role of lignin in secondary cell walls has also been studied using bacterial cellulose fermentation systems (Iwata et al., 1998). Moreover, dehydrogenation polymers were synthesized and characterized using bacterial cellulose pellicles (Cathala et al., 2005; Touzel, Chabbert, Monties, Debeire, & Cathala, 2003).

The goal of this study was to evaluate the impact of hemicelluloses and pectin on the novel sphere-like bacterial cellulose. This system may be an improved model system to understand cellulose assembly over static culture. The potential advantages of this system include: (1) The effect of cell wall components could be investigated on both macro and micro scales, and (2) The interaction of additive to cellulose could be rapidly indentified. The crystallinity and crystal structure of bacterial cellulose were compared among different culture conditions using X-ray diffraction (XRD), the chemical structure of the cellulose was investigated using infrared (IR) spectroscopy, and the yield of biomass and cellulose was measured under different culture conditions.

2. Materials and methods

2.1. Hemicelluloses and pectin

Xyloglucan (from tamarind seeds, Megazyme, Lot #90102a) mainly consists of a β-1,4-linked D-glucosyl backbone which is highly substituted with α-D-xylosyl or β-D-galactosyl-1,2-linked-α-D-xylosyl residues at position C-6 (Whitney et al., 2006). To determine the carbohydrate composition, the polysaccharide was hydrolyzed to mono sugars following Sluiter et al. (2008). A high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex, ICS-5000) and a CarboPac 20 column (Dionex) was used to separate the mono sugars. The composition is xylose 28.2%, glucose 51.4%, galactose 18.6%, and arabinose 1.8%. The molecular weight of the xyloglucan is 202 kDa determined by Megazyme using multi angle laser light scattering.

Xylan (from birchwood, Sigma, Lot # X4252) consists of β-1,4-linked D-xylopyranosyl residues in the backbone. A few xylose residues are substituted by α-1,2-linked-4-*O*-methylglucuronic acid or contain an *O*-acetyl group at C-2 and/or C-3 (Teleman, Tenkanen, Jacobs, & Dahlman, 2002). The molecular weight of xylan was distributed widely from several thousands Da to tens of thousands Da according to high performance size exclusion chromatography (HPSEC) analysis (Freixo & de Pinho, 2002). The main peak was approximately 10,000 Da. The carbohydrate composition is rhamnose 0.8%, arabinose 0.5%, galactose 1.3%, glucose 1.6% and xylose 95.9% determined by HPAEC-PAD.

Arabinogalactan (from larch wood, Sigma, Lot # 10830) is usually used as a dietary fiber. It is a highly branched macromolecule. The backbone chain consists of β-1,3/6-linked D-galactopyranosyl residues. The L-arabinofuranosyl and L-arabinopyranosyl residues are attached to the backbone as side chains (Currier, Lejtenyi, & Miller, 2003). The carbohydrate composition is arabinose 10.4%, galactose 87.8% and glucose 1.8%. The molecular weight is $7.2–9.2 \times 10^4$ Da determined by Sigma.

Pectin (from apple, Sigma, Lot# P8471) was characterized by Chanliaud and Gidley (1999). The molecular weight is 190 kDa determined by HPSEC. Apple pectin composes mainly linear homogalacturonan of α-1,4-linked D-galactosyluronic acid and a rhamnogalacturonan. The methyl residue content is 5% of esterified galacturonic acid residues. The carbohydrate composition is fucose 0.4%, rhamnose 2.3%, arabinose 4.0%, galactose 7.2%, glucose 3.0%, xylose 2.6%, and galacturonic acid 80.4%.

2.2. Cell culture conditions

G. xylinus JCM 9730 (ATCC 700178, provided by the bioresource center of American Type Culture Collection) was cultured in an agitated medium (pH = 5, 30 °C, 125 rpm) in 250 mL Erlenmeyer glass flasks containing 100 mL medium following Hu and Catchmark (2010).

Hemicellulosic polysaccharides and pectin were water soluble and added to the medium at the beginning or 5 days after cultivation. The concentration of hemicelluoses and pectin was 0.5% (w/v) in the medium. For the xyloglucan and pectin blend cultures, the total additive was still 0.5% (w/v), but the ratio of xyloglucan to pectin was made to be 1:0, 3:1, 1:1, 1:3 and 0:1, respectively. After 7 days, cellulose particles were harvested, rinsed with DI water several times and stored at 4 °C until use.

2.3. Field emission scanning electron microscopy (FESEM)

Cellulose samples were frozen (−80 °C) and sectioned to preserve the microstructure, freeze dried and coated with a thin platinum film (around 5 nm). A field emission scanning electron microscope (LEO 1530, Leo Co., Oberkochen, Germany) operating at 5 kV was used for examination of the samples.

2.4. X-ray diffraction spectroscopy

Bacterial cellulose was washed with 0.1 M sodium hydroxide at 80 °C for 1 h and repeated once. Then the samples were washed with DI water until the pH reached neutral. Thin sheets were obtained by lyophilizing and pressing the samples under 2000 lbs force. X-ray diffraction spectroscopy (PANalyticalX'Pert Pro multipurpose diffractometer) with Cu Kα radiation generated at 45 kV and 40 mA was used to obtain the crystal structure of the cellulose samples. X-ray scanning was performed at a rate of 2° per minute from 5° to 70°. The instrumental broadening was determined from the full width at half maximum (FWHM) of four reflections of a silicon standard (NIST Si 640). The degree of crystallinity index was calculated following Park, Baker, Himmel, Parilla, and Johnson (2010) using a program named PeakFit (www.systat.com). Gaussian function was used to fit the peaks and the broad peak at around 21.5° was assigned to be the amorphous contribution.

MDI Jade 9 software (Materials Data, Inc., Livermore, CA) was used to get the crystal size of sample following Cheng et al. (2009). The crystal size was calculated by the Scherrer equation:

$$B_{h\,k\,l} = \frac{K\lambda}{\sqrt{(\Delta 2\theta)^2 - (\Delta 2\theta_{\text{inst}})^2 \cos\,\theta}} \quad (1)$$

where $B_{h\,k\,l}$ is the average crystalline width of a specific phase ($h\,k\,l$, 110 in this case); K is a constant that varies with the method of taking the breadth ($0.89 < K < 1$, usually 0.9 is used); 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 FWHM of the reflection peak and $\Delta 2\theta_{\text{inst}}$ is the instrumental broadening.

To distinguish which crystal allomorph was dominant in the cellulose sample, a method was taken from Wada, Okano, and Sugiyama (2001). They classified typical native celluloses into the algal–bacterial (Iα-rich) type and the cotton-ramie (Iβ-rich) type using a function: $Z = 1693d_1 - 902d_2 - 549$, where d_1 is the d-spacing of 100 plane and d_2 is the spacing of 0 1 0 plane. If $Z > 0$, the cellulose is classified as Iα-rich type. If $Z < 0$, the cellulose is classified as Iβ-rich type.

2.5. Fourier transform infrared spectroscopy (FTIR)

The samples were prepared in the same way as for X-ray analysis. A high pressure clamp was used to press the samples on the ATR sensor of the FTIR spectrometer (Nicolet 8700, Thermo Fisher Scientific Inc.). The FTIR instrument operated from 4000 to 500 cm^{-1} using co-addition of 32 scans with a resolution of 4 cm^{-1}. The baseline correction and normalization of FTIR spectra was done with OMNIC software (Thermo Electron Corporation). The Iα content of the cellulose was calculated following Yamamoto, Horii, and Hirai (1996).

2.6. Total product and biomass yields

The total product and biomass yields were obtained following Hu and Catchmark (2010). Briefly, the biomass was determined by treating the sample with 2% cellulase (v/v) (Sigma C2730) in pH 5.0 buffer of citric acid–sodium phosphate dibasic at 50 °C and measuring the dry weight of remaining cells. The total yield was obtained by lysing the cells with 0.1 M sodium hydroxide at 80 °C and weighing the remaining dry material. The yield (g/g) ratio of total product to biomass was calculated as follows:

$$Y_{\text{TP/B}}\ \text{(g/g)} = \frac{\text{dry weight of total product (g/L)}}{\text{dry weight of biomass (g/L)}} \quad (2)$$

2.7. Statistical analysis

Each data point was averaged from three replicas. The significant difference of the data was evaluated using the Generalized Linear Model (with $p < 0.05$ confident interval) (Minitab Statistical Software; Release 15.1; Penn State University, University Park, PA). All the variations are standard deviations.

3. Results

3.1. Changes in bacterial cellulose structure

It has been shown that the *G. xylinus* JCM 9730 (ATCC 700178) is able to produce sphere-like cellulose under agitated conditions (Hu & Catchmark, 2010). The speed of the agitation can affect the structure and size of the sphere potentially due to differing shear forces present at the surface of the forming spheres. Based on previous results, 125 rpm rotation speed using 250 mL glass flasks filled with 100 mL medium was chosen as our culture conditions to obtain spheres. The diameter of most cellulose spheres formed during culturing was 6–8 mm at approximately 5 days and remained constant until the end of the culture (Fig. 1a). Spheres with larger or smaller diameters were also found but in a very small amount. The culture medium became turbid after about 3 days and continued to be more turbid even after 5 days indicating the cell and biomass was still increasing although the size of the cellulose spheres remained constant. The edge of the sphere was found to be denser than the center. Under electron microscope imaging, the cross section of the sphere was found to have two different structures (Hu & Catchmark, 2010) (Fig. 1S, see supplementary data). The edge of the sphere formed a multi-layered structure (Fig. 2a) while the center was composed of loose cellulose bundles. The distance between layers was about 20 μm with variations. The cellulose bundles formed loose connections between layers and densely overlapped in random directions within the layers (Fig. 2b). Bacterial cells were found in the layered structure. The plant cell wall also forms a multi-layered structure under electron microscope imaging (Vian, 1982). The microfibrils have the same directions within the layer and the layers are densely packed. Although the thickness of the cell wall is in the range of tens of nanometers (Cosgrove, 1997), it is just equal to one layer in the bacterial cellulose sphere which is a few cellulose fibrils thick. Cellulose assembly at a large scale can be examined in this novel system.

Preliminary results have shown that when hemicelluloses and pectin were added to the culture medium at the beginning, the cellulose produced was very different from the control in some hemicelluloses/pectin medium compositions (Gu & Catchmark, 2011). Cellulose produced in the presence of xyloglucan formed aster-like structures (Fig. 1b). The size of the particle was about

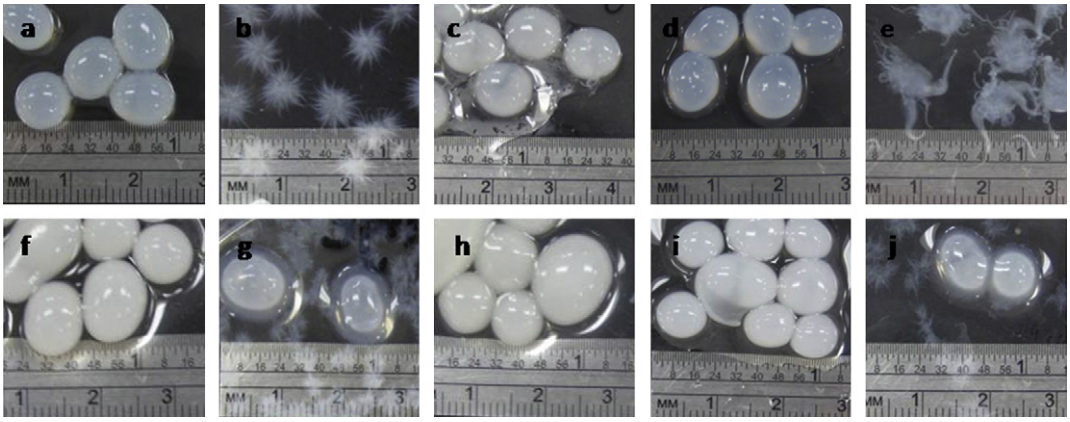

Fig. 1. Optical images of cellulose assembly formed in the presence of hemicelluloses and pectin: (a–e) cellulose culture with: (a) control; (b) xyloglucan; (c) xylan; (d) arabinogalactan; and (e) pectin added at the beginning of the cultivation. (f–j) Cellulose culture with: (f) control; (g) xyloglucan; (h) xylan; (i) arabinogalactan and (j) pectin added 5 days after the cultivation.

4–5 mm without an obvious central core. Electron microscopy analysis showed that there were much fewer connections between layers as compared to the control. The layers were not parallel to each other or may have been deformed during dissection (Fig. 2c). Effort was taken to avoid any deformation by dissecting the samples while frozen then freeze drying as described above. The cellulose bundles were packed more densely within the layers than the control (Fig. 2d).

Bacterial cellulose could still form spheres in the xylan medium (Fig. 1c). The diameter of the spheres was also about 7–8 mm like the control, but on the surface of the spheres, a few tails formed. The inside structure of the sphere was similar to the control under low magnification. Loose bundles were observed in the center region and a layered structure was found at the edge. However, the detailed layered structure was slightly different from the control (Fig. 2e). The space between the layers was about 30–100 μm. Less connections formed between the layers and the layers were not as dense but clearly contained bundles (Fig. 2f).

The arabinogalactan medium had only a minor effect on the bacterial cellulose. Cellulose spheres formed in the exact same way as in the control medium, but the growth rate was a little lower than the control and the diameter of the sphere was a little smaller (Fig. 1d). The cross section of the sphere was very similar to the control (Fig. 2g and h). The cellulose cross linking network between layers was also found in the sample.

Bacterial cellulose formed in the presence of pectin showed significant changes in structure. Particles also appeared somewhat aster-like as in the case where the xyloglucan was added to the medium, but the long macro-scale bundled cellulose features extending from the center of the particle were irregular, much thicker and longer, and varied in both length and thickness (Fig. 1e). The central region of the particle measured 5–6 mm in width and the extensions varied from 2 mm to nearly 1 cm. The inside structure of the central region and extensions were very similar. Dense layers were produced with few cellulose bundles linking the layers (Fig. 2i). On the surface of the layers, some cellulose bundles were extruded (Fig. 2j). The layers were not parallel with each other but overlapped.

Hemicelluloses and pectin were also added 5 days after cultivation. Fig. 1(f–j) shows the structure of bacterial cellulose when harvested on the 7th day. On the 5th day, the spheres fully formed in the medium. The addition of arabinogalactan and xylan did not have any significant effect on the morphology of the cellulose microspheres. However, the addition of xyloglucan and pectin resulted in new cellulose formation on the surface of the spheres, where small bundles of cellulose formed which extended from the surface of the spheres into the media. This formation resembled the respective aster-like fiber extensions which formed in the case where the additive was introduced at the start of the culture. In addition, new cellulose particles also formed in the media apart from the spheres after xyloglucan and pectin were added. These particles also resembled the structures which formed in the case where the additive was introduced at the start of the culture. Electron microscopy imaging showed a dense cellulose network in the new particles similar to that shown in Fig. 2(c, d, i and j) respectively (data not shown).

3.2. X-ray diffraction patterns

Fig. 3 shows the X-ray diffraction pattern of bacterial cellulose in the presence of hemicellulose or pectin as well as the control without any additive. Cellulose I is the dominant form of natural cellulose. Bacterial cellulose is a mixture of Iα and Iβ and the content of Iα is typically more than Iβ. Three characteristic peaks of cellulose Iα: 100, 010 and 110 peaks were found in the control sample. Preliminary data has shown that the spectra of cellulose with xyloglucan and xylan appeared different from the control, while the spectra of bacterial cellulose in the presence of arabinogalactan and pectin were similar to the control sample (Gu & Catchmark, 2011). Table 1 summarizes the X-ray data obtained from the X-ray spectroscopy. D-spacing results were consistent with those obtained previously (Wada et al., 2001). The peak positions and ratio of peak intensities all indicated changes in the crystal structure. It is hard to distinguish 1 0 0 and 0 1 0 in the xyloglucan sample but they appeared to be closer than the control which indicated that the dominate Iα form of cellulose shifted to the Iβ form (Wada et al., 1993). The width of the 1 1 0 peak increases. In the presence of xylan, the peaks 1 0 0 and 0 1 0 were closer than the control. In the control sample, the height of 0 1 0 peak was about half the height of 1 0 0 peak while the additives caused the height of 1 0 0 peak to be more comparable to 0 1 0 peak. This was an indication of a less oriented crystalline structure (Uhlin et al., 1995).

Analysis of calculated d-spacing is an effective way to distinguish Iα to Iβ. Fig. 4a shows the change of Iα/Iβ ratio (based on the calculation presented in Section 2.4). The presence of xyloglucan and xylan in the culture medium could decrease the Iα content dramatically. Arabinogalactan did not have an obvious effect while pectin had a small effect. The crystallinity and crystalline size of bacterial cellulose were also decreased in the presence of all the hemicelluloses and pectin (Fig. 4b). The effect of the additive decreases in the following order: xyloglucan, xylan, pectin then arabinogalactan. The xyloglucan and xylan changed the crystallinity significantly ($p < 0.05$) while all of the additives changed the crystal

Table 1
Indices, position, d-spacing and intensities of x-ray diffraction peaks.

	Control	0.5% xyloglucan[a]	0.5% xylan	0.5% arabinogalactan	0.5% pectin
Peak position (2θ)					
1 0 0	14.63 ± 0.19	15.32 ± 0.03	14.80 ± 0.03	14.68 ± 0.10	14.85 ± 0.15
0 1 0	16.91 ± 0.07	17.22 ± 0.12	16.95 ± 0.01	16.93 ± 0.05	17.11 ± 0.20
1 1 0	22.79 ± 0.07	22.76 ± 0.10	22.80 ± 0.08	22.87 ± 0.07	22.99 ± 0.13
d-spacing (nm)					
1 0 0	0.607 ± 0.004	0.571 ± 0.002	0.590 ± 0.002	0.604 ± 0.004	0.595 ± 0.005
0 1 0	0.523 ± 0.002	0.521 ± 0.011	0.525 ± 0.003	0.521 ± 0.002	0.517 ± 0.002
1 1 0	0.390 ± 0.001	0.392 ± 0.003	0.389 ± 0.002	0.389 ± 0.001	0.386 ± 0.003
Peak relative intensities					
0 1 0/1 0 0	0.41 ± 0.05	1.04 ± 0.13	0.67 ± 0.07	0.57 ± 0.07	0.63 ± 0.06
1 1 0/1 0 0	1.82 ± 0.42	3.43 ± 1.09	2.55 ± 0.81	2.53 ± 0.37	3.04 ± 0.38

[a] The peaks of 1 0 0 and 0 1 0 are broad and hard to distinguish as shown in Fig. 3.

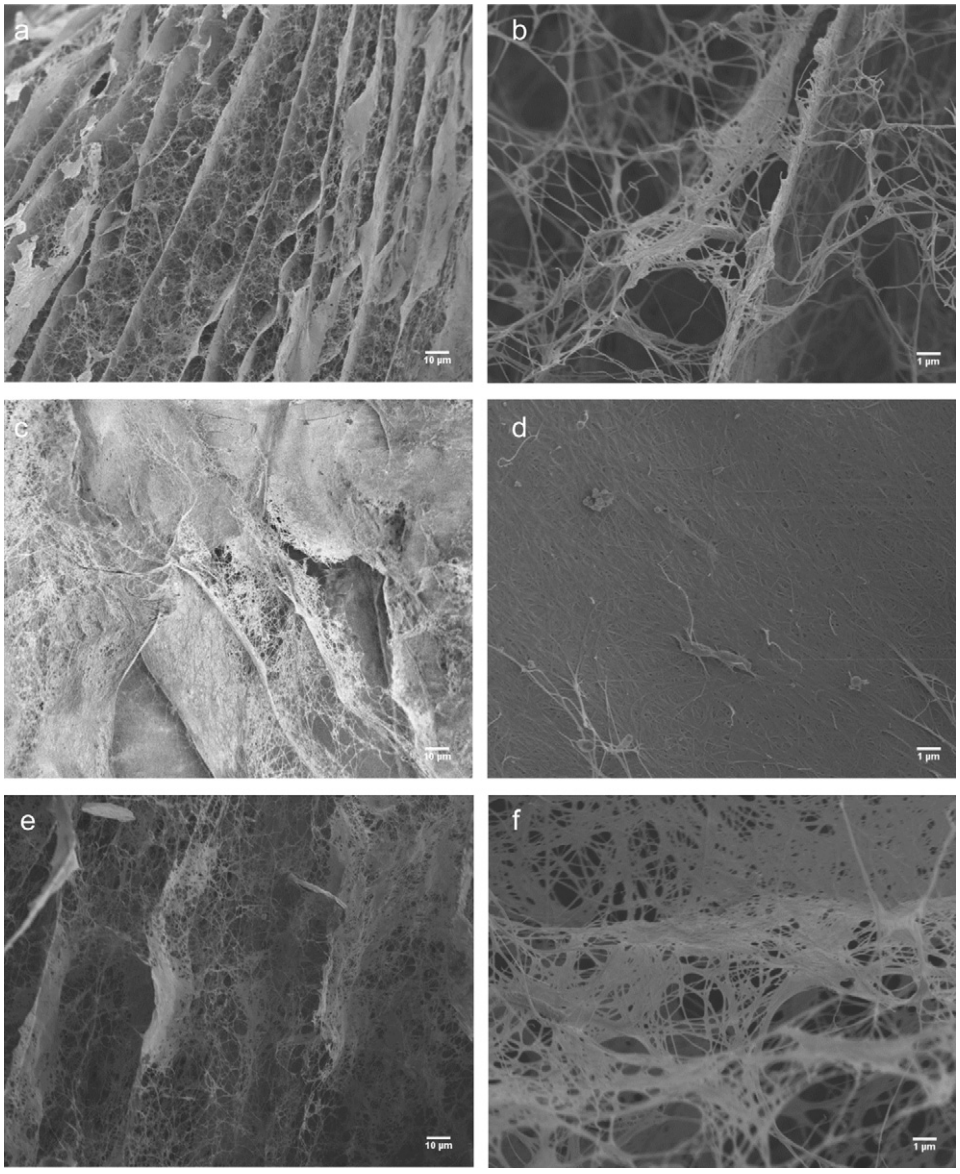

Fig. 2. SEM images of cellulose cultured with: (a) control: layered structure at the edge of cross section under medium magnification; (b) control: cellulose linkage between layers under high magnification; (c) xyloglucan: layered structure under medium magnification; (d) xyloglucan: densely packed cellulose bundles under high magnification; (e) xylan: layered structure at the edge of the sphere under medium magnification; (f) xylan: pore structure of cellulose bundles under high magnification; (g) arabinogalactan: layered structure at the edge of the sphere under medium magnification; (h) arabinogalactan: cellulose linkage between layers under high magnification; (i) pectin: layered structure under medium magnification; (j) pectin: densely packed cellulose bundles under high magnification.

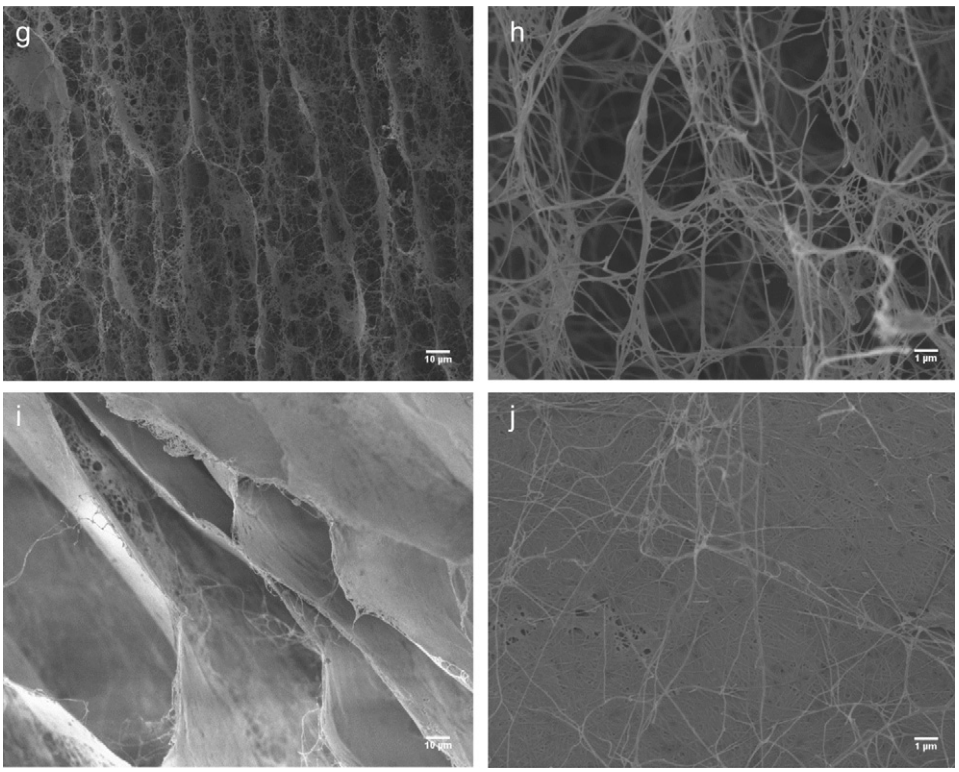

Fig. 2. (*Continued*).

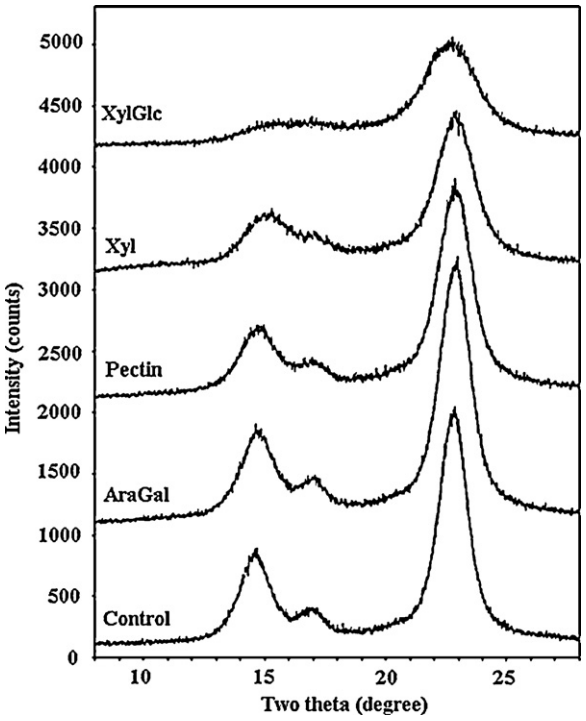

Fig. 3. X-ray diffraction patterns of bacterial cellulose in the presence of 0.5% hemicelluloses/pectin. XylGlc: cellulose cultured with xyloglucan; Xyl: cellulose cultured with xylan; AraGal: cellulose cultured with arabinogalactan. From left to right are peaks 1 0 0, 0 1 0 and 1 1 0.

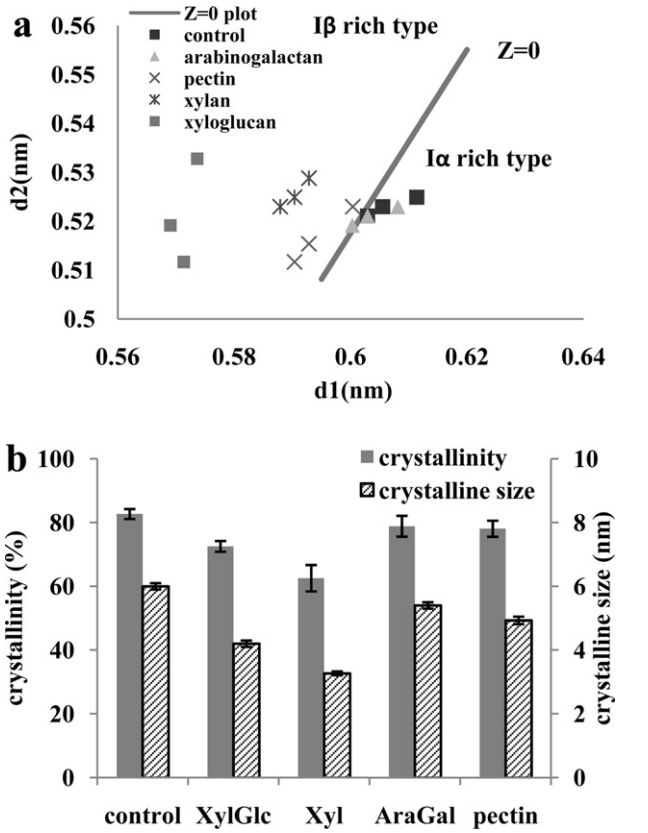

Fig. 4. Analysis of X-ray diffraction patterns. (a) Z-plot of cellulose cultured with hemicellulose or pectin. The cellulose samples are classified to algal–bacterial (Iα rich) type and cotton-ramie (Iβ rich) type. (b) Crystallinity and crystalline size (based on width at half height of 1 1 0 peak) of cellulose samples.

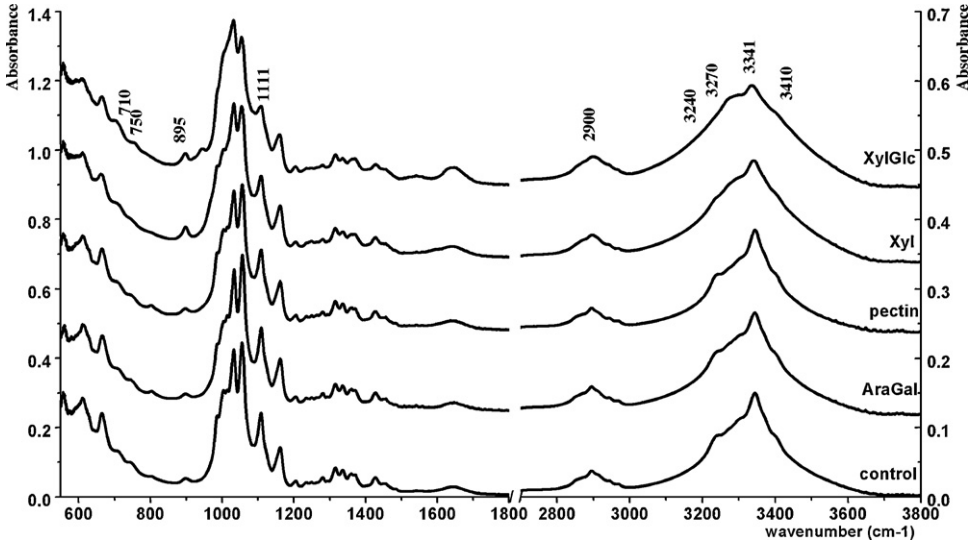

Fig. 5. FTIR spectra of cellulose samples in the presence of hemicellulose or pectin. See Fig. 3 for abbreviations. The peaks of interest are marked with the wavenumber. In order to optimize the representation, the region of 1800–2700 cm^{-1} is omitted. The left hand absorbance scale corresponds to the spectra between 550 cm^{-1} and 1800 cm^{-1} and the right hand absorbance corresponds to the spectra between 2700 cm^{-1} and 3800 cm^{-1}.

size significantly ($p < 0.05$). The lateral dimension of cellulose crystallites decreased in the presence of the additives, particularly with xylan and xyloglucan.

3.3. Fourier transform infrared (FT-IR) spectroscopy

Fig. 5 depicts the FTIR spectra of bacterial cellulose samples. The peaks near 3270 cm^{-1} and 710 cm^{-1} indicated the presence of cellulose Iβ while the peaks near 3240 cm^{-1} and 750 cm^{-1} are specific to Iα (Yamamoto et al., 1996). The Iβ peaks are very clear in each spectrum. The cellulose sample obtained in xylan medium gave only a shoulder at 750 cm^{-1} which indicated less Iα content. The peak near 3240 cm^{-1} is hard to identify. The relative intensity of peak 750 cm^{-1} to 710 cm^{-1} is lower in cellulose sample from xyloglucan medium than the control. The peak near 3240 cm^{-1}, which represents Iα, disappears while the peak near 3270 cm^{-1} is higher than the control. The intensities of Iα and Iβ peaks are similar in cellulose samples obtained from control medium, pectin medium and arabinogalactan medium. These results are consistent with our previous studies and studies from other groups (Gu & Catchmark, 2011; Tokoh et al., 2002). The relative intensities of peaks near 750 cm^{-1} and 710 cm^{-1} have a linear relation with the relative mass ratio of cellulose Iα to Iβ (Yamamoto et al., 1996). The mass fraction of cellulose Iα is calculated as: $f_\alpha = 2.55 f_\alpha^{IR} - 0.32$, where $f_\alpha^{IR} = A_\alpha/(A_\alpha + A_\beta)$. A_α and A_β are the integrated intensities of the Iα and Iß peak, respectively. According to this formula, the Iα content was calculated for all the samples (Table 2). The relative amount of Iα was decreased significantly in cellulose samples obtained from xylan and xyloglucan media compared with the control. This result is consistent with X-ray diffraction studies.

Except the allomorph change from Iα to Iß, other IR bands also show interesting differences when the cellulose samples were cultured with xyloglucan and xylan as compared to the control. The intensity of the peak near 1111 cm^{-1} (C–O stretch) is decreased in cellulose samples obtained from xylan and xyloglucan media while the peak near 895 cm^{-1} (group C1 frequency) is increased. These changes are consistent with a shift from order to disorder due to the change in hydrogen bonding patterns (Nelson & O'Connor, 1964). Also, the peaks representing O–H stretching (especially bands near 3341 and 3410 cm^{-1}) are decreased and become broader in cellulose samples from xyloglucan and xylan media. This indicates weakened or ruptured hydrogen bonding in the samples (Marechal & Chanzy, 2000). The crystalline region of cellulose may be disrupted through the disruption of hydrogen bonding. This is consistent with X-ray diffraction studies.

Table 2
Cellulose Iα content in different culture conditions.

Media type	Iα content
Control	0.60 ± 0.01
0.5% xylan	0.51 ± 0.03
0.5% xyloglucan	0.54 ± 0.01
0.5% arabinogalactan	0.59 ± 0.00
0.5% pectin	0.59 ± 0.02

3.4. Bacterial cellulose and biomass yields

Fig. 6a summarizes the total product (after NaOH treatment) and cell (biomass) concentration at the end of the cultivation when the additives were introduced at the start of the culture. As shown in the preliminary results (Gu & Catchmark, 2011), the cell concentration did not change much in the presence of xyloglucan and arabinogalactan, but the pectin and xylan media appeared to have a higher cell production but not significantly different from the control ($p > 0.05$). The total product concentration was significantly higher in xyloglucan and pectin media ($p < 0.05$). Xylan medium also caused an increase in production although not significant. The yield ratio is significantly higher for xyloglucan ($p > 0.05$) as compared to the control. The pectin medium also appeared to have a higher yield ratio than control although not significantly. It indicates that in the presence of xyloglucan and pectin, more product was produced although the cell concentration was comparable.

To accurately quantify the amount of cellulose produced, the concentration of hemicellulose or pectin remaining after the sodium hydroxide treatment was analyzed using mono sugar analysis as described in Section 2.1. The results are summarized in Table 3. A small amount of xylose and galactose were detected in the cellulose sample obtained in xyloglucan medium. Based on the sugar analysis result of pure xyloglucan in Section 2.1, roughly 25–35% weight of the cellulose composite was xyloglucan. However, after subtracting 35% of the dry weight, the cellulose production in xyloglucan medium was still 80% higher than the control. In cellulose samples obtained from arabinogalactan and xylan medium, no other neutral sugars were detected except glucose. For

Table 3
Carbohydrate compositions[a] of cellulose composites in different culture media after water and sodium hydroxide treatment.

	Control	0.5% xyloglucan	0.5% xylan	0.5% arabinogalactan	0.5% pectin
Arabinose	–	–	–	–	0.4
Galactose	–	6.5	–	–	1.4
Glucose	100	85.0	100	100	82.5
Xylose	–	8.5	–	–	6.9
Galacturonic acid	–	–	–	–	15.0

–, Amount not detectable.
[a] All values are given on relative percent-weight basis.

cellulose sample obtained from pectin medium, galacturonic acid was detected which indicates about 20% weight of the cellulose composite was made of pectin. After subtracting 20% of the dry weight, the cellulose production in pectin medium was 46% higher than the control. The calculated values for the total cellulose yield is shown in Fig. 6b. These results show that xyloglucan and pectin could stimulate the rate of cellulose synthesis.

3.5. Viscosity of culture media

To investigate whether viscosity of culture medium affects sphere-like cellulose formation, the viscosity of different media was measured (Table 1S, see supplemental data). The viscosity of 0.5% xyloglucan medium was 7 times larger than the control. Also, the viscosity of 0.5% pectin and 0.5% xylan was significantly higher than the control. Arabinogalactan did not have detectable impact on the viscosity of the medium. Culture medium with 1.5% alginate had a relative viscosity compatible to 0.5% xyloglucan and was used as a control. Sphere-like bacterial cellulose still formed in the culture media with 0.5–1.5% alginate (data not shown). It is clear that viscosity did not have a significant impact on morphology change of sphere-like bacterial cellulose assembly. The crystal structure of cellulose samples under alginate culture condition did not change as compared to the control using XRD analysis (data not shown).

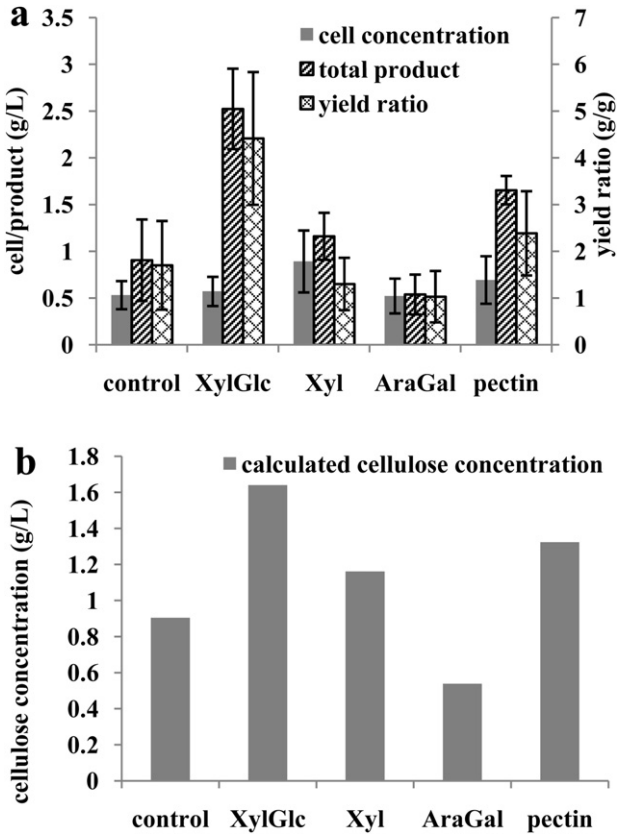

Fig. 6. Bacterial products in different culture conditions: (a) cell concentration, total product (including cellulose and other insoluble polymers) and yield ratio; (b) calculated cellulose concentration.

Table 4
Cellulose Iα content in xyloglucan pectin blend media.

xyloglucan/pectin ratio	Iα content
1:0	0.56 ± 0.00
3:1	0.55 ± 0.01
1:1	0.56 ± 0.01
1:3	0.55 ± 0.01
0:1	0.61 ± 0.01
0:0	0.60 ± 0.01

3.6. Assembly of bacterial cellulose in the xyloglucan/pectin/cellulose ternary systems

Cellulose samples with pure xyloglucan or pectin formed structures different than cellulose assembly without any additive as studied above (Fig. 1a, b and e). The morphology of the bacterial cellulose was changed from spheres to aster-like structures. Cellulose samples formed in the presence of both xyloglucan and pectin were more like cellulose formed in the binary xyloglucan/cellulose system (Fig. 7). Depending on the ratio of xyloglucan to pectin, the diameter of the assembly increased with increased pectin concentration. From XRD studies, the diffraction pattern of samples from any ternary system was the same to the patterns in the binary xyloglucan/cellulose system (data not shown). The Iα/Iβ ratio of cellulose samples was estimated based on the d-spacing of the crystals as above. Iα content was decreased in all samples compared to the control especially for the samples with xyloglucan (Fig. 8a). The crystallinity and crystalline size of the cellulose samples were decreased to the same degree as long as xyloglucan was present in the medium (Fig. 8b). The IR spectra of all these samples were also collected as described above. The peaks near 3270 cm^{-1} and 710 cm^{-1} which indicated the presences of cellulose Iβ were obvious for all samples while the peaks near 3240 cm^{-1} and 750 cm^{-1} specific to Iα changed for the samples with xyloglucan present. The samples with any amount of xyloglucan had similar spectra as samples with pure xyloglucan (data not shown). The Iα content of all the samples are summarized in Table 4. The results of IR analysis confirm the XRD analysis that as long as xyloglucan was present in the culture medium, the crystal allomorph of cellulose was changed to the same degree.

4. Discussion

This study has shown the impact of different hemicelluloses and pectin on the structure of sphere-like cellulose assembly. The formation of sphere-like cellulose has not been studied extensively and is not well understood. It has been hypothesized that bacterial cells first exist at the air–water interface under agitated conditions (Czaja et al., 2004). As the culture continues to grow, the shear force

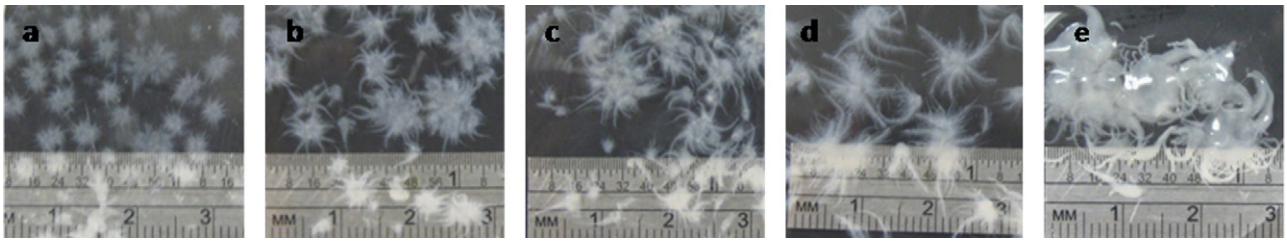

Fig. 7. Optimal images of cellulose assembly harvested from 0.5% (total) xyloglucan and pectin blend medium after 7 days. (From left to right (a–e), xyloglucan: pectin = 1:0, 3:1, 1:1, 1:3, 0:1).

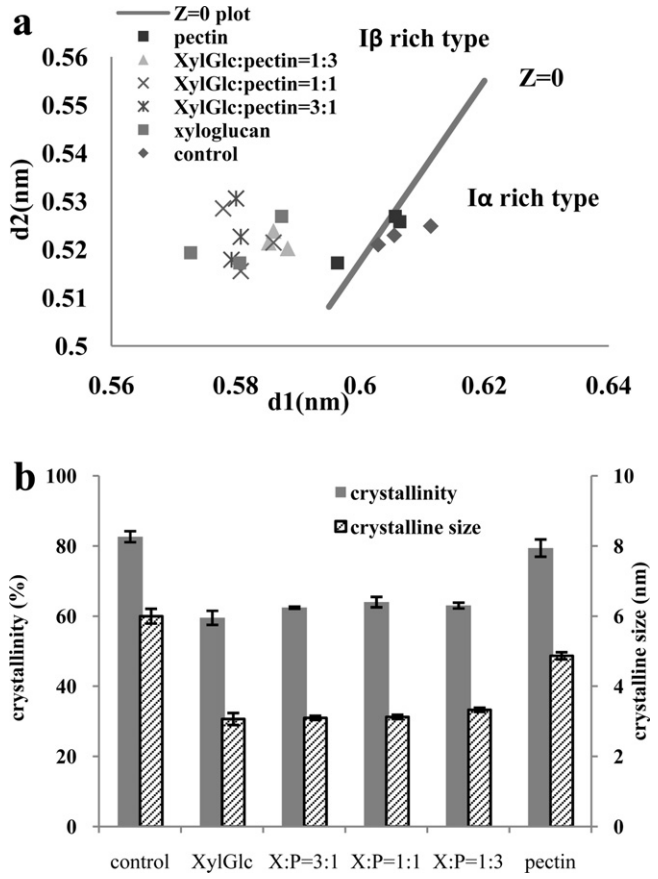

Fig. 8. Analysis of X-ray diffraction patterns of cellulose samples from blend media. (a) Z-plot of cellulose samples from xyloglucan pectin blend media. The cellulose samples are classified to algal–bacterial (Iα rich) type and cotton-ramie (Iβ rich) type. (b) Crystallinity and crystalline size (plane 1 1 0) of cellulose composites culture in xyloglucan-pectin blend media. X:P indicates the ratio of xyloglucan(X) to pectin (P).

causes the cells to separate from the surface of the cellulose layers, resulting in the formation of cellulose spheres (Czaja et al., 2004).

How hemicelluloses and pectin changed sphere-like cellulose is summarized qualitatively in Table 5. Cellulose has an interconnected layered structure under control conditions. In the presence of xyloglucan and pectin, these regular layers were interrupted and much fewer interconnecting fibers between layers were observed in comparison to the control (Fig. 2c, d, i and j). It is possible that the xyloglucan or pectin links the forming cellulose bundles to the existing layer resulting in fewer linkages between layers. Intact xyloglucan with high molecular weight was reported to link the cellulose microfibrils (Whitney et al., 2006). In our study, the molecular weight of xyloglucan was lower (202 kDa as compared to 880 kDa, both using light scattering to measure the molecular weight) and the linkage is hard to see under scanning electron microscope imaging. Xylan and arabinogalactan had minor effects on the microscopic structure of the cellulose spheres. When xyloglucan and pectin were added five days after cultivation, the aster-like structure was observed at the surface of the existing spheres. It indicated that cellulose was continuously synthesized around the layered structure and that xyloglucan and pectin interacted with the cellulose during the process of assembly but had less impact on the existing structures.

Xyloglucan and xylan were able to interact with cellulose microfibrils during aggregation according to X-ray diffraction and IR analysis. They caused an increase in disorder of the cellulose structure. The cellulose Iα content is about 0.7 in static culture (Tokoh, Takabe, Fujita, & Saiki, 1998; Whitney et al., 2006). Cellulose produced under agitated condition always has less Iα component than in static culture (Czaja et al., 2004). Different strains used and the culture conditions may also result in different Iα/Iβ ratios. Addition of hemicelluloses and pectin led to a further decrease in Iα content. These results are consistent with previous studies using cellulose with xylan and xyloglucan under static and agitated culture conditions (Uhlin et al., 1995; Whitney et al., 2006). However, Bootten, Harris, Melton, & Newman, 2008 showed that it was possible to include xyloglucan into *G. xylinus* culture without modifying the cellulose allomorph. Since a heating step is usually used in sample preparation, Bootten et al. proposed that including the xyloglucan into cellulose samples potentially lowered the critical temperature for conversion of cellulose Iα to Iβ. Although pectin changed the morphology of cellulose assembly dramatically, the crystallinity of cellulose with pectin was not significantly lower than the control. The crystalline size was also not deceased as much as cellulose grown with xylan and xyloglucan present.

Morphology, XRD and IR analysis indicated that xyloglucan, xylan and pectin interacted with cellulose in different ways. The backbone of the xyloglucan is the same as cellulose and thus can hydrogen bond with cellulose resulting in decrease of crystallinity as shown in X-ray and IR studies. The backbone of xylan consists of xylose instead of glucose, but it is also β-1,4-linked. The addition of xylan also changed the hydrogen bonding pattern of cellulose. Pectin has an α-linkage which is different from cellulose, potentially causing it to exist in close proximity to the cellulose bundle, but has been shown to have a minor effect on the aggregation of cellulose microfibrils (Chanliaud & Gidley, 1999). Arabinogalactan had the smallest effect on the cellulose assembly. The backbone chain of arabinogalacatan consists of β-1,3/6-linked D-galactopyranosyl residues which is very different from cellulose. The side chain of arabinogalactan may also hinder its binding to cellulose.

The molecular weight and viscosity of xyloglucan and pectin are significantly higher than the other two polysaccharides, initially suggesting it may be the cause of changes in micro structure. In Section 3.5, it is shown that culture media with the same viscosity as 0.5% xyloglucan did not affect the formation of cellulose spheres. The change of cellulose morphology and/or crystal structure was not caused by the increasing viscosity of the media. It is possible that in this model system, the relatively high-molecular-weight

Table 5
Summary of SEM images, XRD studies, cell growth and viscosity data.

	Control	0.5% xyloglucan	0.5% xylan	0.5% arabinogalactan	0.5% pectin
Layer density	0	+	−	−	++
Layer interconnection	0	−	−	0	−
Iβ/Iα	0	+	+	0	0/+
Crystallinity	0	−	−	0	0
Crystalline size	0	−	−	−	−
Cell conc.	0	0	0/+	0	0/+
Cellulose conc.	0	++	0/+	0/−	+
Cellulose yield ratio	0	+	0	0	0/+
Molecular weight	−	202 k	10 k	70–90 k	190 k
Media viscosity	0	++++	+	0	++
Affinity to cellulose[a]	−	++++	+++	+	++++

+, increase of certain properties comparing to control or significantly increased for numbers; −, decrease of properties comparing to control or significantly decreased for numbers; 0/+, increased, but not significantly; 0/−, decreased, but not significantly.

[a] The affinity assessment is a qualitative comparison based on data from literature (Chanliaud & Gidley, 1999; Iwata et al., 1998; Zykwinska et al., 2008) and sugar analysis in Section 3.4.

xyloglucan and pectin linked microfibrils on different scales. Pectin may be able to aggregate larger numbers of microfibrils or microfibrils which are positioned within a larger volume. This would explain the more dense aggregation of the cellulose within the layer and smaller change of sphere structure. The xylan studied here, which also interacts with cellulose, exhibits a shorter chain length (lower molecular weight) which may limit the binding to multiple cellulose chains, thus not causing a significant change in sphere structure.

From the cellulose and biomass yield study, the cellulose production was higher than the control in the xyloglucan, xylan and pectin medium, respectively (Fig. 6b). Xyloglucan and xylan may stimulate the cellulose production by reducing the crystallinity and crystalline size the same way as Calcofluor or carboxymethlycellulose (Cheng et al., 2009; Haigler & Benziman, 1982). However, viscosity of the medium also impacts cellulose yield. Higher viscosity has been shown to improve cellulose yield in other studies since high viscosity may result in better dispersal of the cells and enhance the nutrients and oxygen consumption rate (Ishida, Sugano, Nakai, & Shoda, 2002). This was observed in our study. The cellulose production was increased in the order of xylan, pectin and xyloglucan and it is consistent with the order of increased viscosity (Table 1S). Xyloglucan increases the viscosity of the medium dramatically and thus has the highest impact on cellulose production, followed by pectin and xylan. Since xyloglucan increases viscosity of the medium and decreases crystallinity of cellulose, its impact is much higher than others.

To further understand the different roles of xyloglucan and pectin on cellulose assembly, xyloglucan/pectin/cellulose ternary systems with various ratios of xyloglucan and pectin were studied. Previous studies have shown that in ternary systems, the binding behavior of one additive could affect the other (Chanliaud et al., 2002; Zykwinska, Thibault, & Ralet, 2008). Zykwinska et al. assumed that pectin and xyloglucan were in competition to bind to cellulose. The xyloglucan and pectin used in this study have a similar molecular weight, providing a more direct comparison. Macro-scale shapes of the cellulose assemblies from media with different xyloglucan/pectin ratios were slightly different from each other which indicated that both xyloglucan and pectin interacted with the cellulose. The change in crystal structure properties relative to the control was comparable when any concentration of xyloglucan was present in the media, suggesting that the effect of xyloglucan is dominant. Since the presence of pectin did not exclude the binding of xyloglucan to cellulose, pectin may prefer to bind the more crystalline regions of cellulose or to the xyloglucan. Another possibility is that cellulose interacts with the xyloglucan first and then the pectin. When the cellulose is extruded from the bacterial cell, it is not completely crystallized (Benziman, Haigler, Brown, White, & Cooper, 1980). During the crystallization process, xyloglucan, but not pectin, may prefer to interact with pre-crystalline cellulose.

5. Conclusions

The impact of different hemicelluloses and pectin on sphere-like cellulose assembly has been studied in this work. This system is able to evaluate the effects of additives on different scales. Interaction of additives to the cellulose culture could be observed directly on the macro scale during fermentation. The interconnection between layers of cellulose is easily disrupted if the additive is able to bind to cellulose. Xyloglucan and pectin caused the largest morphology change in cellulose assembly, perhaps due to their higher molecular weight, which permits improved binding between different cellulose fibrils. Xyloglucan and xylan, but not pectin or arabinogalactan, increased the Iβ content of the bacterial cellulose by approximately 10%. Xyloglucan and xylan also decreased the cellulose crystallinity as shown by XRD analysis. It is first demonstrated that not only xyloglucan, but pectin is able to stimulate the growth of cellulose, perhaps due to the higher viscosity of the culture medium. Results from studies on cellulose grown in xyloglucan and pectin blends suggest xyloglucan and pectin may interact with cellulose at different points in the assembly process or different regions of cellulose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.12.040.

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