Cellulose produced by *Gluconacetobacter xylinus* strains ATCC 53524 and ATCC 23768: pellicle formation, post-synthesis aggregation and fiber density

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Abstract

The pellicle formation, crystallinity, and bundling of cellulose microfibrils produced by bacterium *Gluconacetobacter xylinus* were studied. Cellulose pellicles were produced by two strains (ATCC 53524 and ATCC 23769) for 1 and 7 days; pellicles were analyzed with scanning electron microscopy (SEM), X-ray diffraction (XRD), vibrational sum-frequency-generation (SFG) spectroscopy, and attenuated total reflectance infrared (ATR-IR) spectroscopy. The bacterial cell population was higher at the surface exposed to air, indicating that the newly synthesized cellulose is deposited at the top of the pellicle. XRD, ATR-IR, and SFG analyses found no significant changes in the cellulose crystallinity, crystal size or polymorphic

32 distribution with the culture time. However, SEM and SFG analyses revealed cellulose
33 macrofibrils produced for 7 days had a higher packing density at the top of the pellicle,
34 compared to the bottom. These findings suggest that the physical properties of cellulose
35 microfibrils are different locally within the bacterial pellicles.

36 Keywords: Bacterial cellulose; cellulose microfibril aggregation, fiber density.

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1. Introduction

Some species of Gram-negative bacteria can produce cellulose when grown in a culture medium containing glucose, forming pellicles at the top of the medium. *Gluconacetobacter xylinus* (*G. xylinus*) is the most extensively studied microorganism which produces cellulose under aerobic conditions (Romling, 2002). The bacterial cellulose has hierarchical structures over multiple length scales from nanometers to hundreds of microns. Cellulose chains are linear polymers consisting of β -1, 4-linked glucosyl monomer units. Individual cellulose chains are packed by hydrogen bonding into elementary microfibrils which are ~10–20 nm in diameter and are excreted into the culture media (Haigler, White, Brown & Cooper, 1982). The higher order organization of bacterial cellulose is the aggregation of multiple elementary microfibrils forming thick fibrils that are called macrofibrils (Ding, Liu, Zeng, Himmel, Baker & Bayer, 2012). According to previous studies, macrofibril formation occurs approximately 24–48h after synthesis (Brown, Willison & Richardson, 1976). The width of the macrofibril can vary with cultivation time (Haigler, White, Brown & Cooper, 1982). The three dimensional macrofibril network can vary depending on the strains used, culture time and chemical additives present in the culture media (Czaja, Romanovicz & Brown, 2004; Hu & Catchmark, 2010).

Because of the unique physical properties of *G. xylinus* cellulose, it is being used for many applications including wound care, tissue engineering, filtration, papermaking, engineered foods, and reinforcement fibers for polymer composites (Huang, Zhu, Yang, Nie, Chen & Sun, 2014). Additionally, *G. xylinus* cellulose has been studied as a model system to understand cellulose-hemicellulose interactions during the plant cell wall formation (Benziman, Haigler, Brown, White & Cooper, 1980; Whitney, Brigham, Darke, Reid & Gidley, 1995). In plants, cellulose is produced and embedded in a matrix of amorphous wall polymers with varying

molecular structures and amounts, depending on the plant growth stage and environment (Sarkar, Bosneaga & Auer, 2009). These factors make structural analysis of cellulose in plant cell walls extremely difficult. In contrast, *G. xylinus* produces nearly pure cellulose and extrudes it into the liquid medium. Thus, by adding matrix polymers in the culture medium, it is possible to study how cellulose-matrix polymer interactions alter the post-synthesis crystallization of bacterial cellulose (Gu & Catchmark, 2014; Gu & Catchmark, 2012; Park et al., 2014; Whitney, Brigham, Darke, Reid & Gidley, 1995). It is also possible that non-cellulosic exopolysaccharides secreted by *G. xylinus* could be associated with bacterial cellulose in the pellicle (Fang & Catchmark, 2014).

This study addresses whether the *G. xylinus* strain and culture time affect the pellicle formation, crystallinity, crystal size, polymorphism and bundling of bacterial cellulose produced under static culture conditions. Two of the most widely used strains, ATCC 23769 and 53524, were chosen for this work. The former has been used to study the mechanism of cellulose synthesis and assembly (Benziman, Haigler, Brown, White & Cooper, 1980; Haigler & Benziman, 1982) and its genome sequence was determined (Iyer, Geib, Catchmark, Kao & Tien, 2010). The latter is reported to be genetically stable during the agitated culture conditions and thus could be used for large-scale cellulose production (Atalla, Hackney, Uhlin & Thompson, 1993; Czaja, Romanovicz & Brown, 2004). Static culture conditions were used in this study to distinguish the bacterial cellulose produced in the first and last days of culture. Detailed information of cellulose microfibril and bundle formation is important to understanding the mechanical properties of bacterial cellulose pellicles as well as interactions between bacterial cellulose and matrix polymers.

2. Experimental

2.1 Bacterial cell culture conditions

Two *G. xylinus* strains (ATCC 53524 and ATCC 23769) were obtained from the bioresource center of American Type Culture Collection. *G. xylinus* cells were cultured in 100 mL test tubes with 30 mL media at 30°C under static conditions. One liter culture medium contained 20.0 g glucose, 5.0 g yeast extract, 5.0 g bacterial peptone, 2.7 g sodium phosphate dibasic, 1.2 g citric acid, 1 g magnesium sulfate, 2 g ammonia sulfate and 1 mL of corn steep liquor supernatant. The initial pH of the medium was adjusted to 5.0 with 3 M HCl. The bacterial cellulose pellicles were formed at the liquid-air interface. The samples were harvested at two points during cultivation: (1) after 1 day when the pellicles were barely formed and (2) when the pellicles were fully formed, at about 7 days.

2.2 Field-Emission Scanning Electron Microscopy (FESEM)

FESEM was used to observe the distribution of bacterial cells in the pellicle and the morphology of cellulose network. The pellicle samples were either boiled in DI water for 90 s to sterilize the bacterial cells or washed 3 times with 0.1M NaOH for 30 min to remove the bacterial cells. The samples were then thoroughly rinsed with DI water, frozen at -80 °C and freeze-dried. Before the SEM imaging, a thin layer of gold (~5 nm) was deposited on the samples. The SEM (LEO 1530, Leo Co., Okerkochen, Germany) was operated at 5 kV and 2 mm working distance. Statistical analysis was performed using ImageJ 1.43u (NIH) and only single bacterial cellulose macrofibrils were selected to obtain the mean and standard deviation of their diameter ($25 \leq n \leq 60$).

2.3 X-Ray Diffraction (XRD)

XRD was used to obtain the crystallinity index (CrI) and crystal size (CrS) of cellulose produced by *G. xylinus*. The pellicles were prepared in the same way as for SEM analysis with NaOH treatment. They were then pressed into thin films at 2000 lb and placed on a zero-background quartz sample holder for XRD analysis. A diffractometer (PANalytical Empyrean, Westborough, MA) with 0.15406 nm wavelength (Cu- $K\alpha$ radiation generated at 45 kV and 40 mA) was used to analyze the crystal structure of the cellulose samples. X-ray scanning was performed at a rate of 2 degrees per minute from 10° to $40^\circ 2\theta$. The instrumental broadening was determined from the full width at half maximum (FWHM) of reflection peaks of a silicon standard (NIST Si 640) and corrected. The CrI was calculated using the peak deconvolution method (Park, Baker, Himmel, Parilla & Johnson, 2010). The detailed fitting process could be found in a previous work (Gu & Catchmark, 2012). A Gaussian-shape function was used to fit the peaks and the broad peak centered around 21.5 degrees was assigned to be the amorphous contribution. The crystal size along the (110) direction was calculated by the Scherrer equation $D_{110} = K \cdot \lambda / \beta \cdot \cos\theta$ where K is the Scherrer constant (0.9), λ is the X-ray wavelength, θ is the Bragg angle and β is the FWHM of the (110) peak from the fitting procedure (Gu & Catchmark, 2012; Klug & Alexander, 1954). The significant difference of the data was evaluated using the student t-test (with $p < 0.05$ confident interval).

2.4 Vibrational Sum frequency Generation (SFG) Spectroscopy

The details of our SFG spectrometer and experimental setup used for the SFG measurements were the same as previously published (Barnette et al., 2011). Sodium hydroxide treated bacterial cellulose samples were freeze dried overnight and lightly pressed on a glass slide. Spectra were taken at $4 \text{ cm}^{-1}/\text{step}$ in the CH stretching region ($2700\text{--}3050 \text{ cm}^{-1}$) and $8 \text{ cm}^{-1}/\text{step}$ in the OH stretching region ($3096\text{--}3800 \text{ cm}^{-1}$). The probe volume was estimated to be

~150 × 200 μm² wide and ~20 μm deep from the external surface. The effective probe depth of ~20 μm was governed by the IR beam attenuation in the sample (Kafle et al., 2014). SFG spectra of multiple locations (n = 5) on each sample were analyzed. Spectral data were fitted with Lorentzian-shape functions to find the peak center positions shown in Figure S1 in the supplementary information (Lee, Kafle, Park & Kim, 2014; Park et al., 2014).

A separate set of experiments were performed to verify that the SFG intensity in the OH stretching region (3000–3800 cm⁻¹) did not vary depending on the exposure of celluloses to humid air (Figure S2 in the supplementary information). Avicel PH-101 and *Arabidopsis thaliana* inflorescence stems were placed in a sample cell under a continuous flow of humid air at 0, 25, 50 and 100% humidity during acquisition of SFG spectra. There was no difference in the CH/OH intensity ratio or SFG intensity. Additionally, the OH SFG intensity in bacterial cellulose is due to the OH groups in the crystal core because the bacterial cellulose were fully freeze dried before SFG analysis under ambient conditions.

2.5 Attenuated Total Reflection Fourier-Transform Infrared (ATR-FTIR) Spectroscopy

Infrared spectra were collected using a Nicolet 8700 FTIR spectrometer (Thermo Scientific) equipped with a KBr beam splitter, and a deuterated triglycine sulfate (DTGS) detector. NaOH-treated and freeze-dried pellicles were pressed on a diamond ATR crystal and spectra were collected in the region 800–4000 cm⁻¹ with a 2 cm⁻¹ resolution and averaged over 100 scans. The signal intensities were normalized over the 1032 cm⁻¹ peak.

3. Results

3.1. Bacterial cell population and bacterial cellulose pellicle morphology from SEM analysis

Both *G. xylinus* strains (ATCC 53524 and ATCC 23769) formed visibly noticeable pellicles at the surface of the culture medium after 20–30 h under static conditions. The bacterial cellulose pellicle produced by ATCC 53524 continued to grow thicker after 7 days, but the pellicle thickness of ATCC 23769 did not increase after 7 days of cultivation. In order to see if the morphology of the bacterial cellulose network changes over culture time, pellicles were harvested after 1 or 7 days of cultivation and analyzed with FESEM.

In order to visualize the distribution of bacterial cells, the pellicles were boiled in DI water to kill the bacteria without disturbing the shape of the pellicle and then washed to remove the culture medium. The SEM images of the water-washed samples from ATCC 53524 and 23769 are shown in Figure 1 a-d and k-n, respectively. Most bacterial cells (rod-shaped, about 2 μm long) are found at the top surface in contact with the air (Fig. 1 a, c, k, and m) rather than the bottom surface facing the culture medium (Fig. 1 b, d, l, and n). At the beginning of the culture, the *G. xylinus* cells consume oxygen dissolved in the culture medium. It is well known that the aerobic bacteria such as *G. xylinus* proliferate mostly at the air/solution interface where oxygen is readily supplied, although culture nutrients are more available in the liquid media (Iguchi, Yamanaka & Budhiono, 2000; Klemm, Schumann, Udhardt & Marsch, 2001). Within the pellicle, there might be concentration gradients of oxygen and nutrients (including glucose). The marking experiment by Klemm et al. clearly showed that the newly synthesized cellulose is likely located at the top surface of the pellicle (Klemm, Schumann, Udhardt & Marsch, 2001). This means that *G. xylinus* proliferates better in the oxygen-rich and nutrient-lean top region of the pellicle, rather than the oxygen-lean and nutrient-rich bottom region. Cellulose production is primarily dependent on oxygen supply (Hestrin, Aschner & Mager, 1947).

Fig. 1. SEM images of bacterial cellulose produced from (a-j) ATCC 53524 and (k-r) ATCC 23769. Images of (a-d) and (k-n) are the water-washed samples and images of (e-j) and (o-r) are the NaOH-treated samples. (a,e,k,o) top surface of the pellicle harvested after 1 day; (b,f,l,p) bottom surface of the pellicle harvested after 1 day; (c,g,i,j,m,q) top surface of the pellicle harvested after 7 days; (d,h,n,r) bottom surface of the pellicle harvested after 7 day.

There were some impurities such as cell debris and non-cellulosic extracellular polysaccharides secreted by the bacteria (Embuscado, BeMiller & Marks, 1996). Treatment with sodium hydroxide is commonly used to remove all impurities as well as bacterial cells and purify the bacterial cellulose samples (Iguchi, Yamanaka & Budhiono, 2000). The SEM images of the NaOH-treated bacterial cellulose samples show that the impurities were almost completely removed (Fig. 1 e-j and o-r). Moderate treatment with sodium hydroxide did not appear to alter the network of bacterial cellulose.

Individual microfibrils were not observed in Figure 1, their average diameters are known to be approximately 10–20nm. However, macrofibrils were observed and the average diameter at the bottom of the 7-day pellicle is slightly thicker than those found at the top of the 7-day pellicle as well as the 1-day pellicle surfaces (Table 1). The increase in individual macrofibril diameter could be associated with either the binding between individual cellulose microfibrils (Brown, Willison & Richardson, 1976) or possibly as a result of exopolysaccharides secreted by the bacteria (Fang & Catchmark, 2014).

Table 1. Average diameter of *individual* macrofibrils.

Samples	ATCC 53524	ATCC 23769
1day, Top	32 ± 13 nm	27 ± 7 nm
1day, Bottom	37 ± 9 nm	28 ± 7 nm
7day, Top	28 ± 6 nm	26 ± 7 nm
7day, Bottom	45 ± 12 nm	39 ± 13 nm

The areal packing density of cellulose macrofibrils varied significantly with the culture history. Both top and bottom surfaces of the 1-day sample consisted of a random porous network of macrofibrils (Fig. 1 e,f and o,p). For the 7-day samples, the bottom surface was homogeneous

consisting of only loosely bundled macrofibrils, similar to the 1-day sample (Figure 1 h and r). The similarity of the 1-day old sample surface and the bottom of the 7-day old sample also supported the idea that the bacteria are mostly populated at the top (air-side) of the pellicle during the culture.

In contrast, the top surfaces of the 7-day samples were quite heterogeneous, consisting of porous regions as well as regions with densely-packed macrofibrils (Fig. 1 c and m). Even after the NaOH treatment, some regions remained heterogeneous; an example of the ATCC 53524 pellicle is shown in Fig. 1 g and i. Under low magnification (100X), the dark locations on the top surface of the 7-day cultured pellicle consisted of densely-packed macrofibrils (Fig. 1g). At high magnification (5000-10,000X), most of fibers appear as *bundles of multiple macrofibrils* (Fig. 1 j, q). The bright spots on the top surface of the pellicle consisted of a loosely-packed cellulose network spanning several micrometers (Figure 1i). The total area of the bright spots varied, but was usually less than 15-20% of the entire pellicle. These results clearly showed that the density of macrofibrils are significantly higher for the portion synthesized near the end of culture (top region of the 7-day sample) compared to the region produced at the beginning of the culture (day 1 as well as bottom region of the 7-day sample).

3.2. Crystallinity and crystal size from XRD analysis

Figure 2 shows the XRD analysis results of bacterial pellicles harvested at 1 and 7 days of culture. Three diffraction peaks at $2\theta = 14.5^\circ$, 16.6° and 22.7° can be attributed to the (100), (010) and (110) planes of cellulose I α or the ($\bar{1}\bar{1}0$), (110), and (200) planes of cellulose I β (Wada, Sugiyama & Okano, 1993). Since these peak positions of two allomorphs are so close to each other, it is not easy to distinguish two allomorphs based on the XRD peak positions only. However, it is noted that the intensity of the 14.5° peak is larger than that of the 16.6° peak,

which is characteristic of typical cellulose samples produced by *G. xylinus* which contain mostly $I\alpha$ (Tokoh, Takabe, Fujita & Saiki, 1998). Cellulose crystallites with a square cross sectional shape should have a nearly equal intensities of the (100) and (010) reflections (French, 2013). The slightly stronger (100) reflection in Figure 2 indicated that the cellulose crystallites are in a rectangular shape. It has been hypothesized that the shape of the cellulose crystallites must reflect the arrangement of the cellulose synthesis enzymes at the cell wall membrane (Tsekos, 1999).

Fig. 2. X-ray diffraction patterns of bacterial cellulose pellicles produced by ATCC 53524 and 23769 for 1 day and 7 days of culture. The crystallinity index (CrI, %) and crystal size (CrS, nm) are shown in the figure. The diffraction peaks for cellulose $I\alpha$ are labeled.

There was no clear difference or trend in the peak width or relative intensities among the samples from two different strains and two different cultivation times. Thus, the estimates for the

crystal size and crystallinity, shown in Figure 2 at the top of each XRD pattern, did not vary significantly ($p>0.05$).

3.3. Cellulose polymorphism, density and bundling from SFG and ATR-IR analysis

The SFG spectra of bacterial cellulose produced by two strains (ATCC 53524 and 23769) of *G. xylinus* are shown in Figure 3. The SFG spectral features in Figure 3 are consistent with the previous reports on SFG analysis of cellulose pellicles produced by *G. xylinus* (Lee, Kafle, Park & Kim, 2014; Park et al., 2014). It is noted that there were small changes in the alkyl/hydroxyl intensity ratio with the cultivation time. The lowest alkyl/hydroxyl ratio was observed for the pellicles harvested after 1 day (0.31–0.37). The alkyl/hydroxyl ratio of the day 1 samples was closer to those of the bottom of the 7-day cultured pellicle (0.32–0.44) as compared to the top of the day 7 pellicle (0.52–0.53).

Fig. 3. SFG spectra of bacterial cellulose produced after 1 day or 7 days from (a) ATCC 53524 and (b) ATCC 23769. Spectra were normalized at 3320 cm^{-1} .

In the alkyl (CH and CH₂) stretching regions, a weak peak near 2850 cm⁻¹ and triplet peaks at 2920, 2944 and 2968 cm⁻¹ were observed. The 2850 and 2944 cm⁻¹ peaks were tentatively assigned to the CH₂ symmetric and asymmetric stretching vibrations, respectively (Lee, Mohamed, Watts, Kubicki & Kim, 2013). The origins of the 2920 and 2968 cm⁻¹ peaks are not yet known; but the presence of three peaks in the 2920 – 2968 cm⁻¹ region signified a random ordering of cellulose crystallites at the mesoscale (Lee, Kafle, Park & Kim, 2014), which is consistent with the random orientation observed with SEM (Figure 1).

In the SFG spectra, the 3100–3800 cm⁻¹ region consists of the stretching vibration peaks of the hydroxyl groups involved in intra- and inter-chain hydrogen bonds within the cellulose crystal (Lee, Mohamed, Watts, Kubicki & Kim, 2013). The strongest peak was centered at 3330 cm⁻¹ and there were two small peaks at 3240 and 3370 cm⁻¹ as well as a broad shoulder near 3450 cm⁻¹ (Lee, Kafle, Park & Kim, 2014). The deconvolution of the OH peaks, shown in the supporting information (Figure S1), clearly showed the presence of a peak at 3270 cm⁻¹. The 3240 cm⁻¹ and 3270 cm⁻¹ peaks are characteristic of cellulose I α and I β , respectively (Sugiyama, Persson & Chanzy, 1991). The presence of a shoulder peak at 3270 cm⁻¹ can also be seen in the ATR-IR analysis (Figure 4). Thus, both SFG and ATR-IR analyses confirmed that the bacterial cellulose pellicles of both strains contain both cellulose I α and I β polymorphs.

Fig. 4. ATR-IR spectra of bacterial cellulose produced after 1 day or 7 days from (a) ATCC 53524 and (b) ATCC 23769. Spectra were normalized at 1032 cm⁻¹.

4. Discussion

The aerobic *G. xylinus* requires oxygen supply for proliferation (Klemm, Schumann, Udhardt & Marsch, 2001). The SEM images showed that the bacterial cells were mainly populated at the top face of the cellulose pellicle in contact with the air (Figure 1 a,c,k,m). Bacterial cells at the top of the pellicle could maintain their metabolic activity (Iguchi, Yamanaka & Budhiono, 2000). As the cell population increases near the liquid culture/air interface, the oxygen transport in the deeper regions of the pellicle will be more limited (Shoda & Sugano, 2005) and the bacteria near the top surface would be more active. Additionally, the cellulose synthesis in pellicle formation is mostly limited by oxygen supply (Hestrin, Aschner & Mager, 1947). Hence the 7 day grown pellicle contains newly synthesized cellulose predominantly at the upper regions of the pellicle compared to the deeper or bottom regions, which is consistent with Klemm et al. (Klemm, Schumann, Udhardt & Marsch, 2001). Thus, the

post-synthesis aggregation of bacterial cellulose macrofibrils became denser with culture time at the top surface of the pellicle. This could explain why a greater density of cellulose macrofibrils as well as a higher cell population was observed at the top of the 7 day culture pellicle compared to the bottom (Figure 1).

SFG is intrinsically sensitive to the mesoscale packing of cellulose crystallites over the SFG coherence length which is estimated to be hundreds of nanometers up to a micron (Lee, Kafle, Park & Kim, 2014). In this study, the major difference in the SFG spectra of 1- and 7-day cultured pellicles was the alkyl/hydroxyl intensity ratio (Figure 3). Since there is no significant changes in cellulose content, crystallinity and crystal size (Figure 2), as well as cellulose macrofibril orientation (Figure 1), the relative intensities of alkyl and hydroxyl stretching peaks can be interpreted in terms of the lateral packing of cellulose macrofibrils (Lee, Kafle, Park & Kim, 2014). When cellulose macrofibrils are packed without specific orientations, the alignment of multiple crystallites over the SFG coherence length (hundreds of nanometers to microns) is likely to be antiparallel, on average. In this case, the OH dipoles in the neighboring crystallites or macrofibrils would be cancelled, resulting in lower OH SFG intensities. Thus, the larger alkyl/hydroxyl intensity ratio in SFG could be related to the higher density of random macrofibrils.

When the pellicles are collected after 1 day cultivation, the porous network of macrofibrils is observed for both the top and bottom surfaces of the pellicle (Figure 1); the SFG peak shape and alkyl/hydroxyl intensity ratio were quite similar for the top and bottom surface of the 1-day pellicle (Figure 3). After 7 days in culture, the bacterial cellulose pellicles contained a porous network at the bottom surface (Figure 1 h and r) and densely packed macrofibrils at the top surface (Figure 1 j and q). In the 7-day pellicles, the value of the alkyl/hydroxyl ratio at the

bottom surface was closer to the 1-day pellicles, whereas the top surface showed an increased alkyl/hydroxyl ratio suggesting more antiparallel aggregation of bacterial cellulose crystals in the pellicle (Figure 3). The alkyl/hydroxyl ratio also varied more at the top of the 7-day pellicle (standard deviation = 0.13–0.14) which was consistent with the SEM images which showed that the top surface contained regions of high packing density and more porous regions (Figure 1i). Although the size of *individual* macrofibrils was slightly larger at the bottom of 7-day pellicles (Table 1), the cancellation of the OH SFG signal due to antiparallel packing is expected with higher macrofibril density over the length scale of hundreds of nanometers. This is because the SFG signal is sensitive to the interactions of the propagating lights (IR, visible, and SFG photons) over the coherence length. These results supports the notion that the alkyl/hydroxyl SFG intensity ratio correlates with the packing and density of bacterial cellulose macrofibrils(Lee, Kafle, Park & Kim, 2014). Thus the relative intensities of the alkyl and hydroxyl SFG peaks can be used to discern the arrangements of cellulose macrofibrils which is over hundreds of nanometers.

Understanding cellulose fibril aggregation and network formation under the influence of growth conditions is fundamentally important for optimizing bacterial cellulose properties for many applications. Structural composites composed of bacterial cellulose typically require high mechanical modulus and strength, while bacterial cellulose used for biomedical, cosmetic or even food applications may require lower modulus and higher porosity. SFG analysis may provide new insights to the ordering and assembly of bacterial cellulose, aiding in the controlled production of bacterial cellulose with desired properties.

5. Conclusions

The structure of bacterial cellulose after 1 day and 7 day cultivation has been examined by SEM, XRD, ATR-IR and SFG. After 7 days, the network of microfibrils formed near the air-media interface exhibited increased density. Although no difference in cellulose crystal structure was observed by IR and XRD, SFG was sensitive to changes in microfibril packing. Microfibril assembly can have a significant impact on the physical properties of bacterial cellulose. As an established product in the food, cosmetics and healthcare industries, and new emerging applications, bacterial cellulose produced in large quantities with controlled physical characteristics is becoming more essential. This paper demonstrates the use of complementary characterization techniques to assess microfibril formation, an important component in the structure of cellulose biofilms.

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References

- Atalla, R. H., Hackney, J. M., Uhlin, I., & Thompson, N. S. (1993). Hemicelluloses as structure regulators in the aggregation of native cellulose. *Int. J. Biol. Macromol.*, 15, 109-112.
- Barnette, A. L., Bradley, L. C., Veres, B. D., Schreiner, E. P., Park, Y. B., Park, J., Park, S., & Kim, S. H. (2011). Selective detection of crystalline cellulose in plant cell walls with sum-frequency-generation (SFG) vibration spectroscopy. *Biomacromolecules*, 12, 2434-2439.

354 Benziman, M., Haigler, C. H., Brown, R. M., White, A. R., & Cooper, K. M. (1980). Cellulose
 355 biogenesis: Polymerization and crystallization are coupled processes in *Acetobacter*
 356 *xylinum*. *Proc Natl Acad Sci U S A*, 77, 6678-6682.

357 Brown, R. M., Willison, J. H. M., & Richardson, C. L. (1976). Cellulose biosynthesis in
 358 *acetobacter-xylinum* - visualization of site of synthesis and direct measurement of invivo
 359 process. *Proc Natl Acad Sci U S A*, 73, 4565-4569.

360 Czaja, W., Romanovicz, D., & Brown, R. M. (2004). Structural investigations of microbial
 361 cellulose produced in stationary and agitated culture. *Cellulose*, 11, 403-411.

362 Ding, S.-Y., Liu, Y.-S., Zeng, Y., Himmel, M. E., Baker, J. O., & Bayer, E. A. (2012). How does
 363 plant cell wall nanoscale architecture correlate with enzymatic digestibility? *Science*, 338,
 364 1055-1060.

365 Embuscado, M. E., BeMiller, J. N., & Marks, J. S. (1996). Isolation and partial characterization
 366 of cellulose produced by *Acetobacter xylinum*. *Food Hydrocolloids*, 10, 75-82.

367 Fang, L., & Catchmark, J. M. (2014). Characterization of water-soluble exopolysaccharides from
 368 *Gluconacetobacter xylinus* and their impacts on bacterial cellulose crystallization and
 369 ribbon assembly. *Cellulose*, 21, 3965-3978.

370 Gu, J., & Catchmark, J. (2014). Roles of xyloglucan and pectin on the mechanical properties of
 371 bacterial cellulose composite films. *Cellulose*, 21, 275-289.

372 Gu, J., & Catchmark, J. M. (2012). Impact of hemicelluloses and pectin on sphere-like bacterial
 373 cellulose assembly. *Carbohydr. Polym.*, 88, 547-557.

374 Haigler, C., & Benziman, M. (1982). Biogenesis of cellulose I microfibrils occurs by cell-
 375 directed self-assembly in *Acetobacter xylinum*. In R. M. Brown (Ed.). *Cellulose and other*
 376 *natural polymer systems* (pp. 273-297). New York and London: Plenum.

377 Haigler, C. H., White, A. R., Brown, R. M., Jr., & Cooper, K. M. (1982). Alteration of in vivo
 378 cellulose ribbon assembly by carboxymethylcellulose and other cellulose derivatives. *J*
 379 *Cell Biol*, 94, 64-69.

380 Hestrin, S., Aschner, M., & Mager, J. (1947). Synthesis of cellulose by resting cells of
 381 *Acetobacter xylinum*. *Nature*, 159, 64-65.

382 Hu, Y., & Catchmark, J. M. (2010). Formation and characterization of spherelike bacterial
 383 cellulose particles produced by acetobacter xylinum jcm 9730 strain. *Biomacromolecules*,
 384 11, 1727-1734.

385 Huang, Y., Zhu, C., Yang, J., Nie, Y., Chen, C., & Sun, D. (2014). Recent advances in bacterial
 386 cellulose. *Cellulose*, 21, 1-30.

387 Iguchi, M., Yamanaka, S., & Budhiono, A. (2000). Bacterial cellulose - a masterpiece of nature's
 388 arts. *J. Mater. Sci.*, 35, 261-270.

389 Iyer, P. R., Geib, S. M., Catchmark, J., Kao, T.-h., & Tien, M. (2010). Genome sequence of a
 390 cellulose-producing bacterium, *Gluconacetobacter hansenii* ATCC 23769. *J. Bacteriol.*,
 391 192, 4256.

392 Kafle, K., Xi, X., Lee, C. M., Tittmann, B. R., Cosgrove, D. J., Park, Y. B., & Kim, S. H. (2014).
 393 Cellulose microfibril orientation in onion (*Allium cepa* L.) epidermis studied by atomic
 394 force microscopy (AFM) and vibrational sum frequency generation (SFG) spectroscopy.
 395 *Cellulose*, 21, 1075-1086.

396 Klemm, D., Schumann, D., Udhardt, U., & Marsch, S. (2001). Bacterial synthesized cellulose -
 397 artificial blood vessels for microsurgery. *Prog. Polym. Sci.*, 26, 1561-1603.

398 Klug, H. P., & Alexander, L. E. (1954). *X-Ray diffraction procedures for polycrystalline and*
 399 *amorphous materials*. New York: Wiley Interscience.

400 Lee, C. M., Kafle, K., Park, Y. B., & Kim, S. H. (2014). Probing Crystal Structure and
 401 Mesoscale Assembly of Cellulose Microfibrils in Plant Cell Walls, Tunicate Tests, and
 402 Bacterial Films Using Vibrational Sum Frequency Generation (SFG) Spectroscopy. *Phys.*
 403 *Chem. Chem. Phys.*, *16*, 10844-10853.

404 Lee, C. M., Mohamed, N. M., Watts, H. D., Kubicki, J. D., & Kim, S. H. (2013). Sum-
 405 frequency-generation vibration spectroscopy and density functional theory calculations
 406 with dispersion corrections (dft-d2) for cellulose Ia and Ib. *J. Phys. Chem. B*, *117*, 6681-
 407 6692.

408 Park, S., Baker, J. O., Himmel, M. E., Parilla, P. A., & Johnson, D. K. (2010). Cellulose
 409 crystallinity index: measurement techniques and their impact on interpreting cellulase
 410 performance. *Biotechnol. Biofuels*, *3*, 10.

411 Park, Y. B., Lee, C. M., Kafle, K., Koo, B. W., Park, S., Cosgrove, D. J., & Kim, S. H. (2014).
 412 Effects of plant cell wall matrix polysaccharides on bacterial cellulose structure studied
 413 with vibrational sum frequency generation (SFG) spectroscopy. *Biomacromolecules*, *7*,
 414 2715-2724.

415 Romling, U. (2002). Molecular biology of cellulose production in bacteria. *Res. Microbiol.*, *153*,
 416 205-212.

417 Sarkar, P., Bosneaga, E., & Auer, M. (2009). Plant cell walls throughout evolution: towards a
 418 molecular understanding of their design principles. *J. Exp. Bot.*, *60*, 3615-3635.

419 Shoda, M., & Sugano, Y. (2005). Recent advances in bacterial cellulose production. *Biotechnol.*
 420 *Bioprocess Eng.*, *10*, 1-8.

421 Sugiyama, J., Persson, J., & Chanzy, H. (1991). Combined Infrared and Electron-Diffraction
 422 Study of the Polymorphism of Native Celluloses. *Macromolecules*, *24*, 2461-2466.

423 Tokoh, C., Takabe, K., Fujita, M., & Saiki, H. (1998). Cellulose Synthesized by Acetobacter
 424 Xylinum in the Presence of Acetyl Glucomannan. *Cellulose*, 5, 249-261.
 425 Tsekos, I. (1999). The sites of cellulose synthesis in algae: diversity and evolution of cellulose-
 426 synthesizing enzyme complexes. *J. Phycol.*, 35, 635-655.
 427 Wada, M., Sugiyama, J., & Okano, T. (1993). Native celluloses on the basis of two crystalline
 428 phase (I α /I β) system. *J. Appl. Polym. Sci.*, 49, 1491-1496.
 429 Whitney, S. E. C., Brigham, J. E., Darke, A. H., Reid, J. S. G., & Gidley, M. J. (1995). In-vitro
 430 assembly of cellulose/xyloglucan networks - ultrastructural and molecular aspects. *Plant J.*,
 431 8, 491-504.
 432