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Surface Characterization of *Populus* during *Caldicellulosiruptor bescii* Growth by TOF-SIMS Analysis

Allison K. Tolbert,[†,§,#] Jenna M. Young,[‡,§,#] Seokwon Jung,[†,§] Daehwan Chung,[‡,§] Ali Passian,[§,∥] Janet Westpheling,[‡,§] and Arthur J. Ragauskus[*,§,⊥,▽]

[†]School of Chemistry & Biochemistry and Renewable Bioproducts Institute, Georgia Institute of Technology, 500 10th Street NW, Atlanta, Georgia 30332, United States

[‡]Department of Genetics, University of Georgia, 120 Green Street, Athens, Georgia 30602, United States

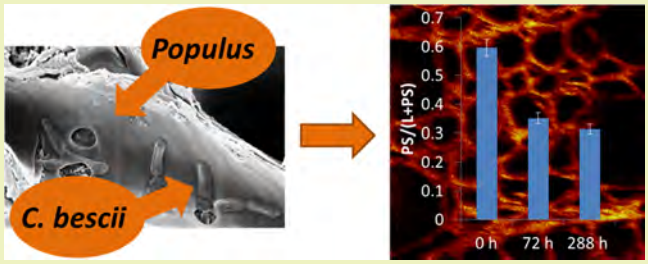
[§]BioEnergy Science Center, Biosciences Division, Oak Ridge National Laboratory, 1 Bethel Valley Road, Oak Ridge, Tennessee 37831, United States

[∥]Computational Science and Engineering Division, and [⊥]Joint Institute of Biological Science, Biosciences Division, Oak Ridge National Laboratory, 1 Bethel Valley Road, Oak Ridge, Tennessee 37831, United States

[▽]Department of Chemical and Biomolecular Engineering and Department of Forestry, Wildlife, and Fisheries, Center for Renewable Carbon, University of Tennessee, Knoxville, Tennessee 37996, United States

Supporting Information

ABSTRACT: *Caldicellulosiruptor bescii* is a thermophilic, anaerobic bacterium that is capable of utilizing unpretreated biomass in addition to breaking down cellulose and hemicellulose into simple sugars. Despite the fact that *C. bescii* must first bind to the surface of the biomass, there has been no analysis of the morphological or chemical changes to the biomass surface as a result of incubation with the microorganism. To understand more about *C. bescii* growth, juvenile poplar stems were sectioned (80 μm thick) and incubated with *C. bescii* beyond the typical 24 h experiment length. Monitoring the cell counts during incubation revealed a biphasic growth pattern. The impact the micro-organism had on the surface was determined by scanning electron microscopy (SEM) and time-of-flight secondary ion mass spectrometry (ToF-SIMS), which showed physical crevices in the cell wall caused by the *C. bescii* along with a decrease of polysaccharide ions and an increase in lignin ions on the poplar surface. Employing infrared microspectroscopy, the decreasing trend was corroborated.



KEYWORDS: *Caldicellulosiruptor bescii, Poplar, ToF-SIMS, SEM, Lignocellulosic biomass*

INTRODUCTION

Lignocellulosic biomass is an abundant and renewable carbon resource that can be used to create biofuels. One challenge in producing cost-efficient biofuels is the natural recalcitrance of the biomass. Traditional methods to overcome recalcitrance incorporate various pretreatments to increase cellulose and hemicellulose accessibility by enzymes.[1] In place of standard purified enzymes cocktails, researchers have recently focused on engineering microorganisms for consolidated bioprocessing (CBP), a process that combines hydrolysis and fermentation of biomass to ethanol in one step with cellulase producing microorganisms, thus increasing efficiency and reducing costs.[2]

Members of the genus *Caldicellulosiruptor* are thermophilic, anaerobic bacteria known for their cellulolytic capabilities, making them potential CBP microorganisms.[3,4] Members of this genus have the specific ability to utilize complex polysaccharides such as crystalline cellulose and unpretreated plant biomass.[3,5,6] *Caldicellulosiruptor bescii* is one of the most cellulolytic strains within this genus,[4] and recent genetic advances have resulted in engineering a strain capable of converting unpretreated biomass directly to ethanol.[7] The *C. bescii* genome contains 52 glycoside hydrolases, five pectate lyases, and seven carbohydrate esterases in combination with 22 carbohydrate binding modules contributing to their ability to break down plant biomass.[4,5,8] Many of these enzymes are multifunctional enzymes that contain two catalytic modules connected by linker regions and CBM domains. In particular, *C. bescii* has a gene cluster (Cbes1853−1867) that together contains six multifunctional enzymes linked with CBM3s capable of assisting in the binding of 12 separate catalytic domains to insoluble substrates.[8] The cellulases are multifunctional, somewhat similar to the cellulosome of organisms such as *Clostridium thermocellum*,[9,10] but they act as noncomplexed free enzymes.[8] The enzymes appear capable of degrading plant biomass by combining the ease of substrate access of the free cellulases with collective catalytic activities in one protein product.[11] Recently, a pectinase gene cluster (Cbes1853-1856)

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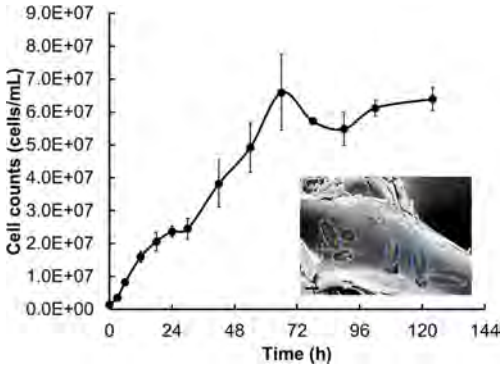

Figure 1. Growth properties of *Caldicellulosiruptor bescii* on poplar slices. Growth measured by cell counts of cultures stained with Acridine Orange after growth on poplar slices as a sole carbon source in LOD medium. Inset: SEM image of *C. bescii* (denoted by blue circles) attached to cellulose walls at 48 h (scale bar 1 μm).

and a combination exo-/endoglucanase (CelA; Cbes1867) were both shown to play an important role in overcoming plant biomass recalcitrance.[12,13] CelA is the most abundant extracellular protein produced by *C. bescii* and was found to be more active than commercial enzyme cocktails.[14]

While *C. bescii* is known to bind to the surface of the biomass,[8] the mechanism of how cells and their enzymes deconstruct the biomass is not well understood. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) is a surface analysis technique that requires little sample preparation in order to detect secondary ions[15] from cellulose and lignin. High-resolution spectra and high spatial imaging provide valuable insight into biomass chemistry through normalized ion intensities, or counts, and image mapping of the fragmentation ions. Goacher et al.[16] developed an approach using ToF-SIMS to assess both enzyme activity on biomass and any subsequent biomass degradation. Here, we go beyond the use of enzyme cocktails and apply a similar process using a potential CBP organism, *C bescii*. We primarily utilize scanning electron microscopy (SEM), FTIR microscopy, and ToF-SIMS to gain insight into the physical and chemical impacts of *C. bescii* and the extensive array of enzymes it produces on the surface of juvenile poplar stems.

EXPERIMENTAL SECTION

Strain and media. *Caldicellulosiruptor bescii* wild type was used for this study. *C. bescii* was grown anaerobically at 75 °C in low osmolarity defined (LOD) medium,[17] as previously described, with maltose

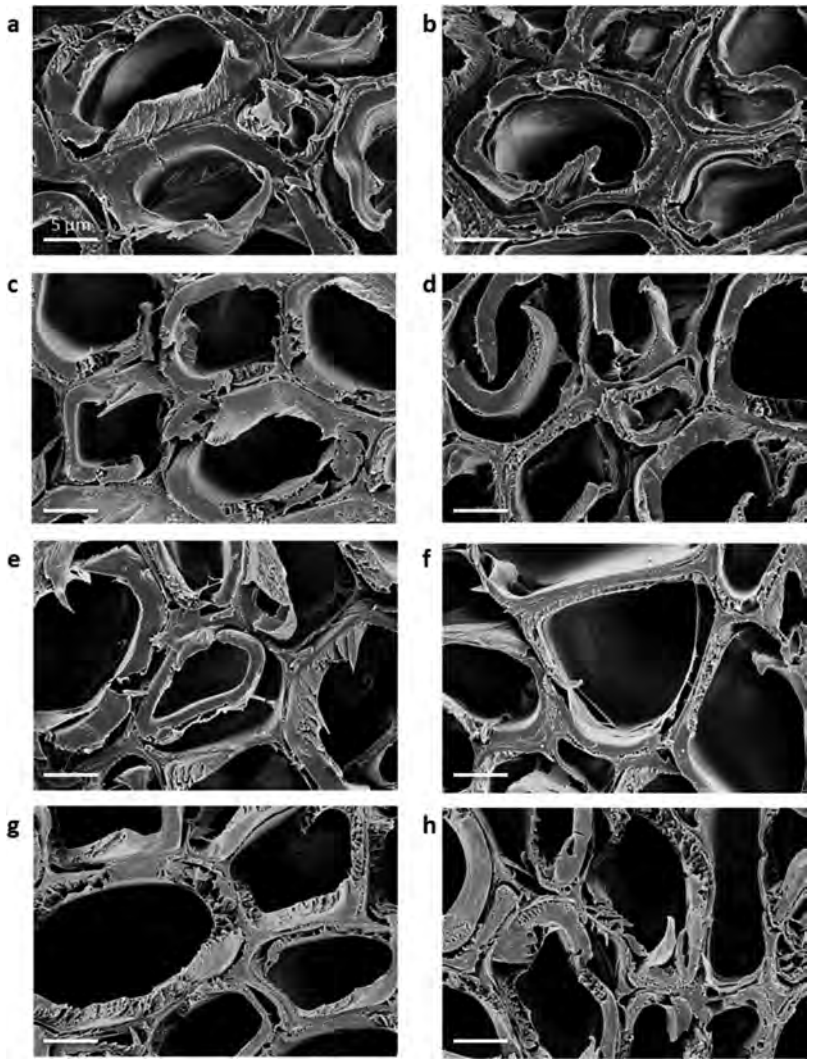

Figure 2. Electron micrograph of poplar slices after incubation in LOD medium at 75 °C after 72 h without (a–b) and with *C. bescii* (c–d) and after 288 h without (e–f) and with *C. bescii* (g–h).

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(0.5 wt %/v; catalog no. M5895, Sigma) as the sole carbon source for routine growth or juvenile poplar stem cross sections (see below for biomass preparation). The defined medium contained 40 μM uracil. This concentration of uracil does not support growth of *C. bescii* as the sole carbon source.

Plant material and growth conditions. Juvenile poplar stems were harvested from a six month-old *Populus. deltoides x nigra* (DN34) clone provided by NREL for this study. Poplar stems were sectioned into 80 μm thick discs from the debarked stem using a LEICA CM 3050S cryostat (Leica Microsystems, Wetzlar, Germany) with a disposable steel blade and embedding material (OCT compound, Tissue-TEK). The embedding material surrounded approximately 0.5 cm of the stem, adhering it to a metal plate for the duration of the sectioning process and avoiding chemical contamination to the samples. The cryostat functioned under a chamber temperature of approximately −8 °C with a cutting speed under manual control. Extractives were subsequently removed from the sectioned samples through Soxhlet extraction with dichloromethane (~150 mL) at a reflux rate of 6 solvent cycles/h for 4 h. The sectioned samples were then air-dried before *C. bescii* treatment. Poplar stems were then used as a sole carbon source for *C. bescii*, and uninoculated controls were used to account for the effect of the growth conditions over time (75 °C in LOD media). To monitor growth on the poplar slices (0.5% (w/v)), cultures were periodically sampled and fixed in 3.7% formaldehyde, vortexed, and stored at −20 °C for cell counts. Samples were appropriately diluted and stained with 0.1% Acridine Orange before visualizing using an epifluorescent microscope at 100× (oil immersion). Cell counts from 15 to 20 fields were averaged.

Characterization. After growth with *C. bescii* or control treatment at 75 °C, the poplar sections were thoroughly washed with DI water (3 × 50 mL) and ethanol (2 × 50 mL). Each section was air-dried between glass slides before further surface characterization. TOF-SIMS analysis was performed using an ION-TOF TOF·SIMS V instrument (ION-TOF, Münster, Germany) equipped with a bismuth liquid metal ion gun (LMIG) as a primary ion source. With a pulsing Bi_3^{2+} primary ion gun (25 keV), positive charged secondary ions were collected and used to form either high mass resolution spectra or high spatial resolution images. High mass resolution spectra resulted from primary ions randomly rastering a 500 × 500 μm area on the sample for a total of 200 scans at 128 × 128 pixels. Three data points per sample were acquired from three replicates to reduce any site specificity. The normalized ion intensities from the fragmentation ions of cellulose (m/z 127, 145), G lignin (m/z 137, 151), and S lignin (m/z 167, 181) at the nine different locations were averaged together, and standard deviation error analysis was conducted to determine the normalized ion intensity data. The samples underwent similar Bi_3^{2+} primary ion beam bombardment to form 100 × 100 μm spatial images at 256 × 256 pixels. The images for the different fragmentations were combined to form a cellulose ion image and a lignin ion image. The lignin ion image was given a green color and overlaid on the total ion image. For scanning electron microscopy images, all cross-sectioned samples were coated with gold for 20 s by an SPI Module Sputter/Carbon Coater System. SEM images were acquired via a Leo 1525 Field Emission (FE) SEM at 3 kV and various resolving powers. FTIR studies were performed using an infrared microscope (BRUKER OPTIK GmbH) allowing for spatially resolved absorption spectra of the biomass across a grid spanning a region typically 100 μm × 100 μm. A spectral resolution of 4 cm^{-1}, a spatial resolution of ~5−10 μm, and a spectral averaging up to 64 times were utilized to generate spectra covering the wavenumber range 4000−500 cm^{-1}. Several spectral features, previously reported[18,19] as signatures of the various transitions in hemicellulose, cellulose, and lignin, may be utilized for the study of the treated samples. The poplar spectral features selected for our studies include the following: 1155 cm^{-1} band of cellulose/hemicellulose, 1270 cm^{-1} of guaiacyl lignin, and 1327 cm^{-1} of syringyl lignin.

RESULTS AND DISCUSSION

***C. bescii* exhibits biphasic growth on poplar biomass.** Growth of *C. bescii* on poplar stems was monitored by cell counts using fluorescence microscopy after staining with Acridine Orange.[12,13] In the first 24 h, *C. bescii* grew quickly before temporarily plateauing around 24 h (Figure 1). We attribute this primary growth to utilization of easily accessible sugars, most likely soluble components of the biomass (such as xylan) released at high temperatures or by initial enzymatic degradation. Previous work has shown that incubation of poplar in liquid at 75 °C can release about 7% soluble materials (by weight).[6] *C. bescii* is capable of growing on xylan as a sole carbon source and has the ability to utilize a wide range of sugar substrates found in plant biomass.[6,20]

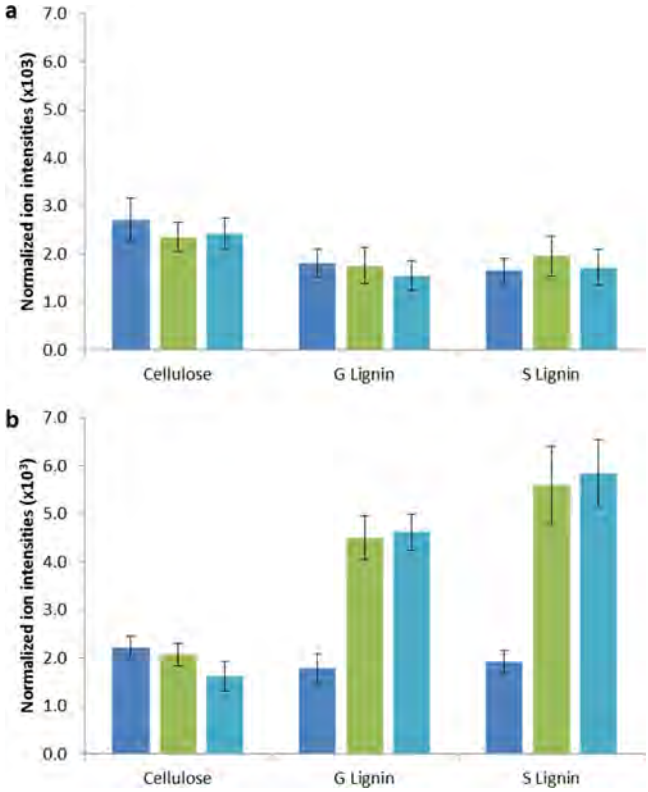

Figure 3. Normalized ion intensities for the surface components (cellulose, G- and S-lignin) by ToF-SIMS for (a) uninoculated controls for 0 h (blue), 72 h (green), and 288 h (teal) and (b) poplar cross sections after growth with *C. bescii* for 0 h (blue), 72 h (green), and 288 h (teal).

We observe a secondary growth phase starting around 30 h and peaking at 72 h. This is attributed to degradation of the more insoluble portion of the biomass, as the enzymes produced during the primary growth phase provide a new release of usable carbon source. Washed materials and spent materials (residual substrate after previous growth with *C. bescii*) are both able to support growth of *C. bescii*.[6] In fact, we see cells attaching to the substrate at 48 h during this secondary growth phase (Figure 1 inset), potentially to provide better access to sugars on the surface. The proximity of the microbe and the enzymes it produces to the substrate has been shown to create a favorable microenvironment for cellulose hydrolysis,[21] presumably by increasing the local concentration of the enzymes trapped between the cell and the substrate. A structurally unique, noncatalytic protein has been described in *Caldicellulosiruptor* spp. involved in attachment to cellulose[22] and is specific to the strongly cellulolytic strains within this genus, suggesting a link between attachment and cellulose degradation.

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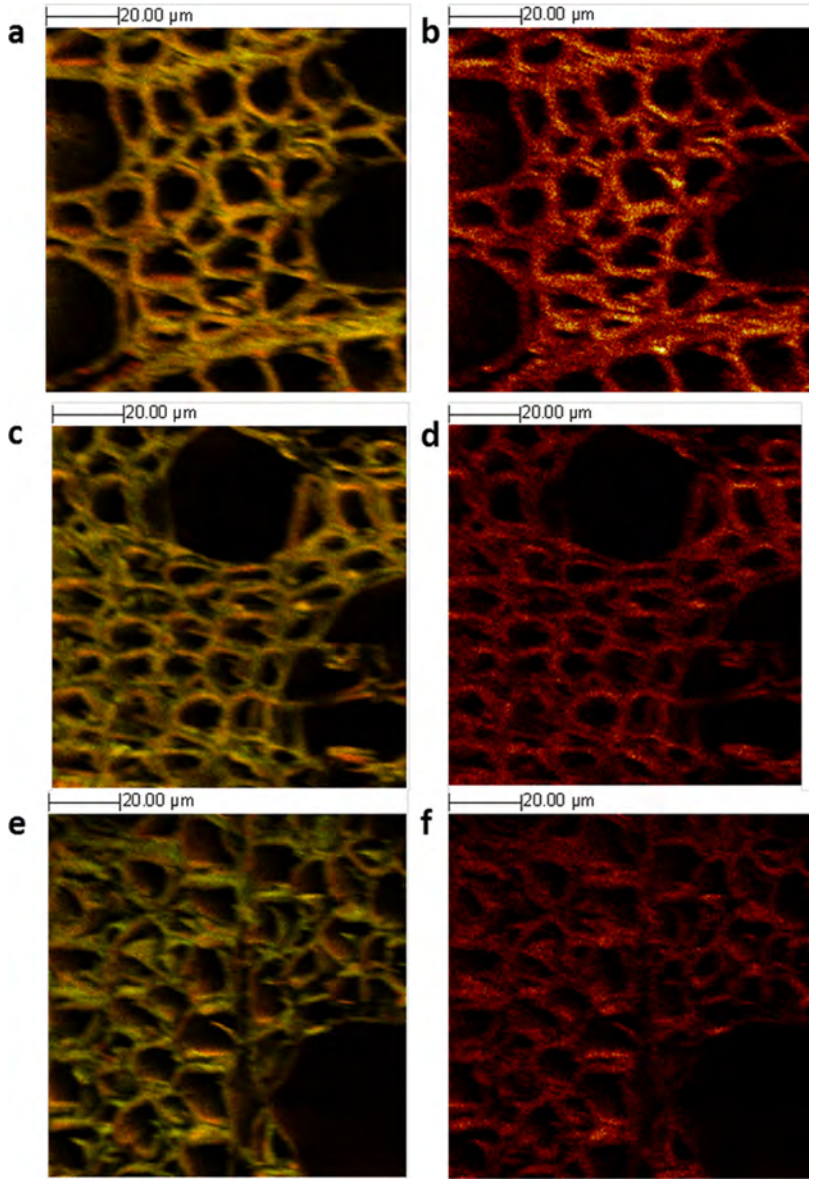

Figure 4. ToF-SIMS images of poplar cross sections incubated with *C. bescii* in LOD medium at 75 °C for (a–b) 0 h, (c–d) 72 h, and (e–f) 288 h. The left column (a,c,e) shows the lignin ions image (green) overlaid on the total ion image (red). The right column (b,d,f) is the cellulose ion image. Scale bar = 20 μm.

Cell wall morphological changes. To determine the impact of *C. bescii* on cell wall structure, SEM was used to observe the surface morphology of the sectioned samples. To ascertain if the LOD medium or incubation temperature (75 °C) contributed to cell wall damage, uninoculated control samples were also analyzed, and underwent the same sample incubation conditions and incubation only without *C. bescii*. These controls do appear to have some surface roughness along the cell wall, likely the result of the sectioning process (Figure 2a–b, e–f); however, they lack the degradation observed in the samples grown with *C. bescii* (Figure 2c–d, g–h). After 72 h of incubation with *C. bescii* (Figure 2c–d), the poplar cell walls develop crevices that are not seen in the uninoculated controls (Figure 2a–b). This corresponds well with the knowledge that CelA, the most abundant excreted enzyme of *C. bescii*,[23] is able to create cavities during digests of Avicel, a crystalline cellulose.[24] These crevices become even more prevalent at 288 h (Figure 2g–h) and are primarily localized within the primary and secondary cell walls. This indicates that *C. bescii* enzymes have continued to digest the biomass after 72 h. *In vitro* work with CelA demonstrates that the thermophilic enzymes produced by *C. bescii* remain active up to at least 160 h at 75 °C.[24] The proteomics of *Caldicellulosiruptor* spp. have also shown that the highest levels of extracellular proteins are observed in the stationary phase.[23] Enzymes produced in the stationary phase likely remain active for some time and would account for the continued degradation of the poplar slices after the end of the growth cycle.

Chemical changes to the cell wall. We also measured changes in the surface chemical composition during growth of *C. bescii* using ToF-SIMS. Average normalized ion intensities for cellulose (m/z 127 and 145), guaiacyl (G) lignin (m/z 137 and 151), and syringyl (S) lignin (m/z 167 and 181)[15] were obtained from high resolution spectra for the uninoculated controls and samples incubated with *C. bescii* for 0, 72, and 288 h. The normalized ion intensities for the controls (Figure 3a)

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showed very little variation, indicating that the high incubation temperature and LOD medium alone do not impact the amount of cellulose and lignin measured on the surface. The changes in the cellulose and lignin ion count after incubation with *C. bescii* can be seen in Figure 3b. Most notable was the increase in lignin ions between samples treated for 0 and 72 h for G lignin and S lignin, 150% and 190%, respectively. This trend was supported by the lignin ion images (Figure 4a,c,e), where the pixels representing the lignin ions were scaled green and overlaid on the red total ion image. It is clear that, for the 72 and 288 h samples (Figure 4 c,e), there is an increase in lignin ions within the middle lamellas and the cell corners of the plant cell wall. The increase in lignin can be attributed to the removal of other chemical components (hemicellulose and cellulose), allowing more lignin ions to be measured at the surface.

While the cellulose ion counts appear to remain relatively level over time (Figure 3b), there was actually a decrease of 27% from 0 to 288 h. This was supported with the decrease in cellulose ion intensity of the ToF-SIMS images (Figure 4b,d,f). In order to take into account the potential hemicellulose removal in addition to the cellulose, a more extensive analysis occurred to determine the sum of the polysaccharide (PS) and lignin (L) peaks.[16] The normalized ion intensities of approximately 30 ToF-SIMS peaks from each location were used to calculate the average polysaccharide peak fraction (PS/(PS+L)),[16] seen in Figure 5. From 0 to 72 and 288 h,

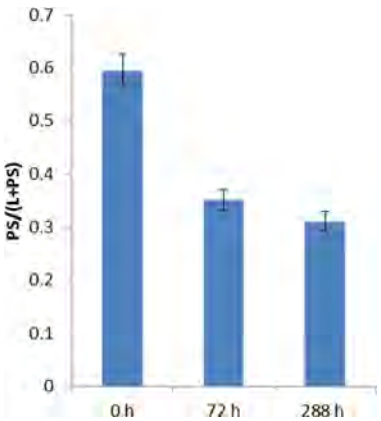

Figure 5. Average ToF-SIMS polysaccharide peak fraction (PS/(PS+L))[16] for poplar cross sections incubated with *C. bescii* for 0, 72, and 288 h.

there was a drop of 25% and 29% in PS/(PS+L), respectively. The decrease in surface cellulose and polysaccharide peak fraction together with the sharp increase in surface lignin indicates that the microorganism is successfully removing both hemicellulose and cellulose. Using FTIR microscopy, it was possible to compare polysaccharide-to-lignin ratios based on the peak heights from the absorption spectra for the 72 h and 288 h samples (Figure S1) with the ToF-SIMS data. Specifically, using the cellulose and hemicellulose peak height at 1155 cm^{-1} (C−O−C asymmetrical stretching)[18] divided by the sum of the lignin peak heights at 1270 cm^{-1} (guaiacyl lignin) and 1327 cm^{-1} (syringyl lignin),[19] the polysaccharide-to-lignin ratios at the cell wall corners for 72 and 288 h were reduced from 0.92 to 0.48, respectively. The depletion of polysaccharides in addition to the increase in lignin detected on the surface both contribute to the decrease in ratio.

CONCLUSIONS

This study has shown that the potential CBP organism, *C. bescii*, significantly alters the surface of unpretreated poplar biomass over time, as visualized by SEM and measured by ToF-SIMS. The physical degradation of the cell walls can be observed in the formation of crevices, which is not a result of temperature or LOD medium. The ToF-SIMS analysis revealed a decrease in cellulose ions and polysaccharide peak fractions as length of incubation time increased, which indicates that *C. bescii* and its enzymes are targeting cellulose and hemicelluloses. As polysaccharides are removed from the surface, an increase in the amount of lignin becomes visible; this is seen in the significant increase in G lignin and S lignin by 150% and 190%, respectively. While the detection of more lignin on the surface signifies that the microorganism is removing hemicelluloses and cellulose, it could pose a problem by hindering further access to the sugars by *C. bescii*. Overall, surface characterization is an underutilized technique in traditional analytical studies. ToF-SIMS analysis provided additional understanding of the consumption of untreated biomass by *C. bescii* by studying the surface chemistry.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.6b01877.

FT-IR microscopy spectra and image, FT-IR peak heights, and numerical data for the cell growth and ToF-SIMS graphs (PDF)

AUTHOR INFORMATION

Corresponding Author

*Office phone: 865 576 0635. Fax: 865 241 1555. E-mail: aragausk@utk.edu.

Author Contributions

#A.K.T. and J.M.Y. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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