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Understanding the Changes to Biomass Surface Characteristics after Ammonia and Organosolv Pretreatments by Using Time-of-Flight Secondary-Ion Mass Spectrometry (TOF-SIMS)

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Surface characteristic changes to poplar after ammonia and organosolv pretreatments were investigated by means of time-of-flight secondary-ion mass spectrometry (TOF-SIMS) analysis. Whereas normalized total polysaccharides and lignin contents on the surface differed from bulk chemical compositions, the surface cellulose ions detected by TOF-SIMS showed the same value trend as the cellulose content in the biomass. In addition, the lignin syringyl/guaiacyl ratio according to TOF-SIMS results showed the same trend as the ratio measured by means of NMR spectroscopic analysis, even though the ratio scales for each method were different. A similar correlation was determined between the surface cellulose and glucose release after enzymatic hydrolysis. These results demonstrate that surface characterization using TOF-SIMS can provide important information about the effects of pretreatment on biomass properties and its hydrolysis.

Lignocellulosic biomass is the most abundant and renewable natural resource on earth. Utilization of cellulose, hemicelluloses, and lignin in the biomass can facilitate in the production of biofuels and/or bio-based products. Biomass recalcitrance, like the structural complexity and heterogeneity of its chemical components within the cell wall, contributes to the plant's natural defense against biological degradation, but it also plays a negative role in biomass conversion and must be overcome to achieve efficient biomass utilization.

One way to reduce the recalcitrance is to change the physicochemical characteristics of the biomass through pretreatments. Physical, chemical, and/or biological pretreatments can break down or solubilize one or more biomass components, thereby making the utilization of the treated biomass easier. Traditionally, cellulose-rich pretreated solids show higher yields and faster conversion rates during enzymatic hydrolysis and fermentation when compared to the untreated biomass.

Characterization of biomass is an essential step for designing effective biomass conversion processes, such as pretreatment and fermentation. In particular, biomass characterization techniques are capable of providing insight into how the pretreatment impacts the physical structure and chemistry of the biomass. Chemical composition, syringyl (S)/guaiacyl (G) lignin ratio, molecular weight of each biomass fraction (cellulose, hemicellulose, and lignin), and cellulose crystallinity are typical biomass characteristics measured using high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectroscopy, gel permeation chromatography (GPC), and other instrumentation.

Another biomass characterization method is surface characterization, which examines the morphology and chemistry of the plant cell walls on the surface of a sample. Surface characteristics of biomass are crucial factors for enzymatic hydrolysis and fermentation of biomass, because enzymes and microorganisms directly interact with the substrate's surface. Surface analysis to reveal the erosion and relocation of cell-wall matrix components has assisted in explaining how enzyme accessibility increased and enzymatic hydrolysis was improved in pretreated biomass.[1] Scanning electron microscopy (SEM),[2] transmission electron microscopy (TEM),[3] Raman spectroscopy,[4] Fourier-transform infrared spectroscopy (FTIR),[3–5] and atomic force microscopy (AFM)[6] are commonly used for biomass surface analysis.

Time-of-flight secondary-ion mass spectrometry (TOF-SIMS) is another analysis technique for surface characterization that produces high mass resolution spectra and spatial mapping of the surface chemical ions. This analytical technique has been applied in the analysis of the surface of neurobiological systems,[7] drug delivery,[8] and polymer films.[9] Recently, biomass

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characterization studies using TOF-SIMS have been carried out.[10] In the early stage, these studies focused on identifying key fragmentation peaks associated with cellulose and lignin.[10a,11] Application of TOF-SIMS characterization was expanded to understand the surface changes of woody biomass after pretreatments.[5,12] Groacher et al. developed a list of approximately 40 secondary-ion peaks that correlate with polysaccharides and lignin found within lignocellulosic biomass.[10a,13] These values contain the common fragmentations for cellulose (*m/z* 127, 145), the lignin G unit (*m/z* 137, 151), and the lignin S unit (*m/z* 167, 181). The ion intensities (or counts) for each mass peak were normalized to the total ions to determine compositional ratio of specific peaks detected on the surface, including polysaccharide peak fraction, lignin peak fraction, and the lignin S/G ratio.

To date, TOF-SIMS analysis has been applied to only a few pretreated biomass, such as hydrotropic-pretreated birch and pine and dilute-acid-pretreated poplar.[5,12] Herein, we expand the application of TOF-SIMS analysis to ammonia-treated and organosolv-treated poplar to determine the advantages and limitation of this analytical method relative to traditional methods of analysis, including wet chemical compositional analysis by HPLC through two-step acid hydrolysis, lignin S/G ratio by 2D heteronuclear single quantum coherence (HSQC) ^{13}C–^{1}H NMR spectroscopic analysis, and sugar release by enzymatic hydrolysis. Ammonia and organosolv pretreatments are considered to be lignin-targeting pretreatments,[14] but each has its own advantages and limitations. For instance, ammonia pretreatment partially removed lignin with minimal carbohydrate loss,[14a,b] whereas organosolv pretreatment significantly removed both lignin and hemicellulose.[14c] Ammonia pretreatment showed effective delignification, particularly on herbaceous plants or agricultural residues,[15] whereas organosolv pretreatment was applicable towards diverse biomass, including woody biomass.[16]

The impact the two pretreatments had on the surface and bulk chemical composition were determined using TOF-SIMS and wet chemical composition analysis methods. A TOF-SIMS bismuth ion beam was rastered across six different locations on each of the 60 μm-thick untreated (extractive-free) or pretreated cross-section poplar samples, and forty secondary-ion peaks associated with polysaccharides and lignin from the TOF-SIMS total ion spectra of the poplar samples were quantified,[10a,13] normalized, and averaged. The polysaccharide and lignin peak fractions (Figure 1) reveal a 9% decrease in polysaccharides to the total polysaccharides and lignin ratio for the ammonia-treated poplar relative to the untreated sample, whereas the organosolv pretreatment resulted in a slightly higher fraction (2%). The ammonia-treated poplar cross-section possessed a higher lignin peak fraction than the others.

Untreated and pretreated samples also underwent a two-step sulfuric acid hydrolysis process to quantify the bulk composition of polysaccharides and lignin using an ion chromatography system. Compositional information based on the oven-dry weight of untreated samples (Figure S1 in the Supporting Information) showed a removal of 40% hemicellulose and 19% lignin with minimal cellulose loss (≈2%) for the ammonia-treated sample. Organosolv pretreatment removed 73% of hemicellulose and 42% of lignin, but it also caused 15% cellulose loss. The results indicated that organosolv pretreatment possessed a greater efficiency in deconstructing and removing hemicellulose and lignin in poplar than ammonia pretreatment, but it solubilized more of the cellulose. For a better understanding of the chemical compositions in the pretreated poplar solids, the results were calculated on the basis of the oven-dry weight of each solid residue (Figure S2). The values represent the actual amount of each composition in the pretreated solid residues; these values were then used for calculating the yields of fermentable sugars from each pretreated biomass. Total hemicellulose contents (xylan, arabinan, and galactan) in ammonia-treated and organosolv-treated poplar were 5.3% and 11.2% lower, respectively, than the untreated poplar. Lignin contents of both pretreated poplar solids were also slightly lower than the content of untreated poplar. As a result of the changes in hemicellulose and lignin contents, the relative cellulose content increased from 48.6% to 56.9% and 63.2% after ammonia pretreatment and organosolv pretreatment, respectively (Figure S2).

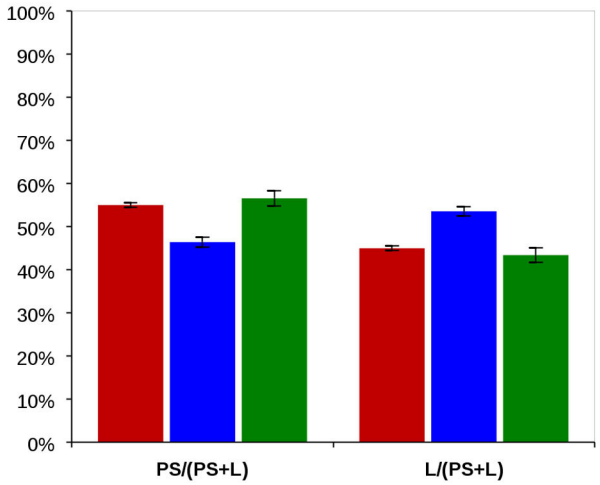

Figure 1. Polysaccharide and lignin peak fraction observed from untreated (red), ammonia-treated (blue), and organosolv-treated (green) poplar cross-sections. Derived from the sum of polysaccharide (PS) and lignin (L) TOF-SIMS peaks.[10a,13].

For direct comparison of the surface composition and the bulk composition of poplar, the aforementioned chemical compositional analysis results (Figure S2) were normalized using the sum of cellulose, hemicellulose, and lignin, excluding other compositions. Table 1 presents the normalized polysaccharide and lignin fractions for the untreated, ammonia-treated, and organosolv-treated poplar samples as determined by TOF-SIMS and chemical compositional analysis. The normalized polysaccharides and lignin contents by chemical compositional analysis for each sample did not vary significantly (< 3%), whereas the normalized compositions from the surface chemistry using TOF-SIMS presented up to an 11% difference. The contents of total polysaccharides and lignin by TOF-SIMS analysis did not correlate with the results from chemical compositional analysis. However, the cellulose peak intensities (*m/z* 127 and 145) on

Table 1. Comparison of normalized polysaccharides and lignin contents as determined by TOF-SIMS analysis and chemical composition analysis for untreated, ammonia-, and organosolv-pretreated poplar samples.

Composition	Analysis method	Untreated [%]	Ammonia-treated [%]	Organosolv-treated [%]
Polysaccharide	TOF-SIMS	55	46	57
	Chemical	73	74	76
Lignin	TOF-SIMS	45	54	43
	Chemical	27	26	24

the poplar surface as determined by TOF-SIMS (Figure S3) showed the same tendency as the bulk cellulose contents (Figure S2). The cellulose content of organosolv-treated poplar was greater than that of ammonia-treated poplar, and both were greater than that of the untreated poplar sample.

Besides the changes to the compositions of the bulk and biomass surface, other modifications to the chemistry could have occurred during biomass pretreatments. Lignin content based on the residual solids, for example, might not appear to be significantly altered from the previous analysis, but the structural and compositional information of lignin, like the lignin S/G ratio, could have changed. Studies have shown that the lignin S/G ratio can impact cellulose accessibility and/or sugar release during enzymatic hydrolysis.[17] Specifically, the blocked C-5 position of S lignin allows for S-rich lignin to form more linear chains and minimizes cross-linkages that are more common with G-rich lignin, which allows microbes greater access to desired chemicals on the surface.[17]

Therefore, the surface lignin S/G ratio for the untreated and pretreated samples were analyzed by means of TOF-SIMS and compared to the lignin S/G ratios as determined by NMR spectroscopic analysis. The TOF-SIMS lignin S/G ratios were calculated from the normalized ion intensities of the sum of S lignin peaks (*m/z* 167, 181) and the sum of G lignin peaks (*m/z* 137, 151). The pretreatments increased the detected lignin ion intensities on the surface of the samples; the ammonia-treated poplar showed G and S lignin ion intensities that were two and four times higher, respectively, than the untreated sample (Figure S4). The changes in lignin composition (S and G contents) on the surface of ammonia-treated poplar resulted in the highest lignin S/G ratio (1.72). The S and G lignin ion intensities from the organosolv-treated surface also increased notably (Figure S4), and the resulting lignin S/G ratio of the organosolv-treated poplar (1.15) was higher than that of the untreated sample (0.93).

NMR spectroscopic analysis is a typical analytical method for understanding the structural characteristics of biomass, including the lignin S/G ratio. Semiquantitative analysis using ^{13}C–^{1}H HSQC NMR spectroscopic analysis was applied to monitor the changes of the lignin S/G ratio of the poplar samples. Aromatic regions of the NMR spectra from untreated, ammonia-treated, and organosolv-treated poplar (Figure S5) revealed syringyl (S), guaiacyl (G), and *p*-hydroxybenzoate (PB) contents. The correlation for $S_{2/6}$ and α-oxidized $S_{2/6}$ were assigned at $\delta_C/\delta_H = 104.0/6.74$ and 106.3/7.25 ppm. The correlations of the G unit (G_2, G_5, and G_6) were found at $\delta_C/\delta_H = 111.0/7.03$, 114.7/6.84, and 118.8/6.84 ppm, respectively. Changes in the relative contents of the S and G units in the pretreated biomass were observed by means of NMR spectroscopic analysis by quantifying the S and G units using $S_{2/6}$ and G_2. The S and G unit contents were 58 and 42% in the untreated poplar, respectively. Organosolv pretreatment increased the S content to 70% with 30% G-unit content, therefore the lignin S/G ratio increased to 2.35. Ammonia pretreatment changed this ratio further to result in a 3.20 lignin S/G ratio.

According to both the TOF-SIMS analysis results from the cross-section of poplar samples and the NMR spectroscopic analysis results from the whole plant cell wall, ammonia and organosolv pretreatments increased the lignin S/G ratio. Although the scale in each analytical method was different, the lignin S/G ratio from each analysis method shows a strong positive correlation ($R^2 = 0.9223$; Figure 2). The order of the lignin S/G ratio for these poplar samples was ammonia-treated poplar > organosolv-treated poplar > untreated poplar for both analytical methods.

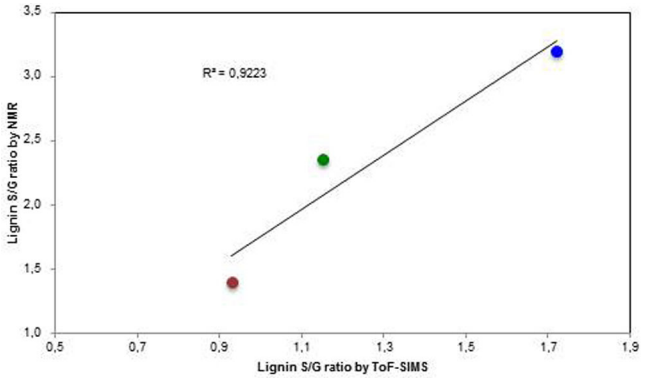

Figure 2. TOF-SIMS lignin S/G ratio versus NMR spectroscopic lignin S/G ratio for untreated (brown point), organosolv-pretreated (green point), and ammonia-pretreated (blue point) poplar samples.

The changes in the compositions and the lignin S/G ratio as a result of ammonia and organosolv pretreatments were evaluated by a sugar-release test. The glucose-release performance of untreated, ammonia-treated, and organosolv-treated poplar was tested by means of enzymatic hydrolysis. Glucose-release performances of pretreated poplar samples had a faster rate of increase at the beginning of hydrolysis, and the difference between the samples' rates became larger over time (Figure 3). For instance, glucose releases at 6 h were 23, 26, and 27 mg from untreated, ammonia-treated, and organosolv-treated poplar, respectively, and the releases reached 40, 92, and 133 mg, respectively, after 72 h enzymatic hydrolysis. The significant improvement in the sugar release of the pretreated samples indicates that the pretreatments assisted in overcoming some inhibiting barriers, possibly the presence of lignin and/or other characteristics of the biopolymer. The glucose release followed the same trend as the enzyme-accessible cellulose on the biomass surface as detected by TOF-SIMS (Fig-

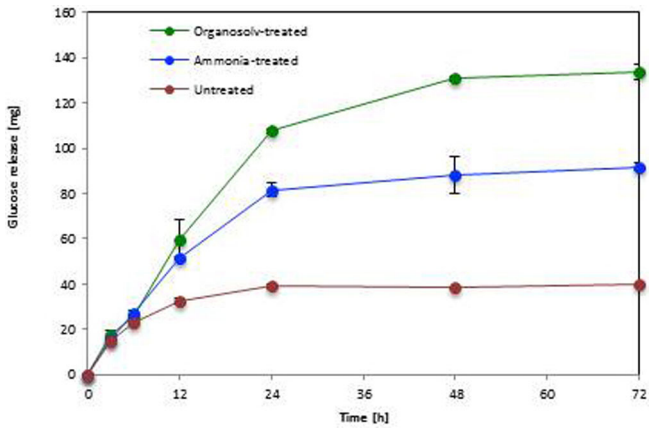

Figure 3. Glucose release of untreated, ammonia-treated, and organosolv-treated poplar.

ure S3) and cellulose content as detected by bulk compositional analysis (Figure S2). Further surface characterization studies that analyzed enzymatically hydrolyzed biomass at various times might provide deeper insight into how enzymes consume cellulose on the biomass surface.

The information about the surface chemistry of a biomass sample can differ significantly from that of the whole plant cell wall. This might explain the results that revealed that the TOF-SIMS and bulk chemical composition for polysaccharides and lignin did not correlate well with each other. However, the surface and bulk cellulose content did follow a similar trend, which also corresponded to the order of higher glucose release for the three samples. Although these two methods cannot necessarily be used in place of each other, both surface and bulk chemistry provide important, but different information. In addition, a positive correlation was detected between the lignin S/G ratios as determined by TOF-SIMS and NMR spectroscopic analyses. Overall, TOF-SIMS provides a different perspective in understanding the pretreatment effects on the properties of biomass and its sugar release, and it could be a good predictor of cellulose content, glucose release, and the lignin S/G ratio.

Experimental Section

Sample Preparation

Six-month-old *Populus deltoides* stems were harvested from the Oak Ridge National Laboratory greenhouse. The poplar stem was cut into 38–50 mm-long sections. The stem sections were freeze-dried at −80 °C for three days. A laboratory-scale Thomas–Wiley Mill machine was used to mill the poplar stems to 0.42 mm or less. A Leica CM 1850 cryostat was used to cut the stem into 60 μm cross-sections using an acetone-cleaned disposable steel blade and embedding material (OCT Compound, Tissue-TEK) to hold the base of the stem to the metal stage. Both milled and cross-sectioned poplar samples were Soxhlet-extracted with dichloromethane for 16.5 h. A previous study by Jung et al. reported that analysis of milled and cross-sectioned biomass produced comparable compositional analysis and FTIR results.[5]

Pretreatments

Poplar samples were prepared by two different pretreatment methods (ammonia and organosolv). Milled (0.75 g) and cross-sectioned (0.012 g) poplar samples were loaded with pretreatment solution (25 mL) in a batch Parr reactor (internal volume: 300 mL). Ammonia pretreatment was conducted with 5.0% ammonia hydroxide, and organosolv pretreatment used 65.0% ethanol with 1.0% sulfuric acid as an acid catalyst. The same reaction temperature (180 °C) and reaction time (20 min) were applied in both pretreatments. After the pretreatment, solid residues were filtered using Whatman #1 filter paper and rinsed by deionized (DI) water until the pH reached approximately 7. The pretreated cross-sections were separated from the milled biomass, rinsed, and air-dried between glass slides for TOF-SIMS analysis. The milled pretreated samples were collected and air-dried for chemical compositional and NMR spectroscopic analyses.

TOF-SIMS Analytical Method

TOF-SIMS spectra of the untreated and pretreated poplar samples were obtained using a TOF-SIMS V instrument with a bismuth (Bi_3^{2+}) liquid-metal ion gun as the primary ion source. The instrument was operated under positive bunch mode, whereby pulsed primary ions were randomly rastered across the surface. To reduce site specificity, six spectra were acquired from different locations on the sample. A raster size of 500 μm^2 was used for all data acquisitions. The spectra were calibrated to CH^+, CH_2^+, CH_3^+, and $C_2H_3^+$. The ion intensities of selected peaks were normalized to the total ions detected and the average value for each ion fragmentation was used for further calculations.

Chemical Composition Analytical Method

Untreated and pretreated poplar samples were treated by two-step acid hydrolysis process adapted from NREL/TP-510-42618 to analyze carbohydrate and lignin composition.[18] In the first step, biomass (ca. 150 mg) was hydrolyzed by 72% sulfuric acid (1.5 mL) at 30 °C for 1 h, and then the mixture was diluted to 4% sulfuric acid for the second step. The second step was conducted in the autoclave at 121 °C for 1 h. After the two-step hydrolysis, solid residues and hydrolysate were separated by using a crucible with a glass filter. The hydrolysate was analyzed using a Dionex ICS 3000 ion chromatography system for quantifying carbohydrates. Acid-soluble lignin was analyzed by UV/Vis spectroscopy. The solid residues were further washed with DI water until neutralized and oven-dried to measure the acid-insoluble lignin.

NMR Spectroscopic Analytical Method

The extractive-free biomass, as prepared by the aforementioned Wiley milling and solvent extraction, was ground using a planetary ball mill (Retsch PM 100) at 580 rpm with zirconium dioxide (ZrO_2) vessels (internal volume: 50 mL) and ZrO_2 ball bearings (10 mm × 10) for 2 h and 30 min (5 min grinding and 5 min break) for NMR spectroscopic analysis of the whole cell wall. Ball-milled biomass (ca. 50 mg, either untreated or pretreated) and [D_6]DMSO/[D_{18}]HMPA (4:1, v/v; ≈0.5 mL) were loaded in a 5 mm NMR spectroscopic tube as described in a previous study.[19] The biomass samples were well dispersed and dissolved by vortexing and sonication for 1–2 h. NMR spectra of each sample were obtained by using a Bruker Avance III 400-MHz spectroscopy equipped with a 5-mm Broadband Observe probe (5-mm BBO 400 MHz W1 with

Z-gradient probe, Bruker). Two-dimensional (2D) ^{13}C–^{1}H HSQC spectra were collected at 298 K using a Bruker standard pulse sequence ('hsqcetgpsi2') with the following parameters: spectral width of 11 ppm in F2 (^{1}H) with 2048 data points and 190 ppm in F1 (^{13}C) with 256 data points; 128 scans (NS) and 1 s interscan delay (D1). The chemical shift was calibrated with the central DMSO solvent peak at $\delta_H/\delta_C = 2.49/39.5$ ppm. Volume integration of contours in HSQC spectra was conducted using Bruker's TopSpin 3.5 software.

Sugar-Release Test

Untreated, ammonia-treated, and organosolv-treated poplar samples were tested for sugar-release measurement. Poplar samples (250 mg oven-dry weight) were loaded in citrate buffer solution (25 mL, 50 mM, pH 4.8) with Novozymes CTec2 (10 FPU per gram biomass; Franklinton, NC). The enzymatic hydrolysis was conducted at 50 °C with 200 rpm in an incubator shaker. The hydrolysate was periodically collected at 0, 3, 6, 12, 24, 48, and 72 h. Released sugars in each hydrolysate were analyzed using Dionex ICS-3000 ion chromatography system. Each analysis was conducted in duplicate.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biomass · carbohydrates · ToF-SIMS · pretreatment · surface analysis

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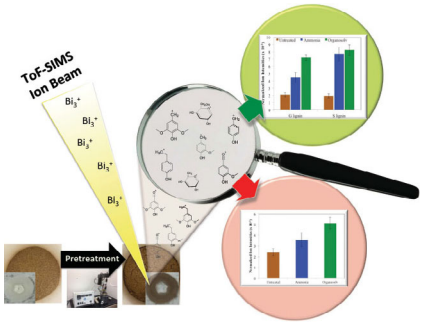

Elucidating biomass surface: Time-of-flight secondary-ion mass spectrometry (TOF-SIMS) analysis is an effective tool to understand the surface characteristics of biomass (see figure). Investigated here are the surface characteristics of untreated, ammonia-pretreated, and organosolv-pretreated *Populus deltoides* wood samples by means of TOF-SIMS. Other analytical methods are also discussed.