Recalcitrance and Structural Analysis by Water-Only Flowthrough Pretreatment of ¹³C Enriched Corn Stover Stem

Marcus Foston^{a,f,*}, Heather L. Trajano^{b,f}, Reichel Samuel^{e,f}, Charles E. Wyman^{c,f} Jian He^a, and Arthur J. Ragauskas^{d,e,f}

^a*Department of Energy, Environmental & Chemical Engineering, Washington University, 1 Brookings Drive, Saint Louis, MO, 63130, USA*

^b*Chemical and Biological Engineering, The University of British Columbia, 3620 East Mall, Vancouver, V6T 1Z3, Canada*

^c*Department of Chemical and Environmental Engineering, Bourns College of Engineering, University of California, Riverside, 1084 Columbia Avenue, Riverside, CA 92507, USA*

^d*Department of Chemical and Biomolecular Engineering, University of Tennessee, 5 323-B Dougherty Engineering Bldg., Knoxville, TN 3796, USA*

^e*Georgia Institute of Technology, School of Chemistry and Biochemistry, Institute of Paper Science and Technology, Atlanta, GA, USA*

^f*BioEnergy Science Center, Division Biological Sciences, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA*

Received (in XXX, XXX) Xth XXXXXXXXXX 20XX, Accepted Xth XXXXXXXXXX 20XX

DOI: 10.1039/b0000000x

*To whom correspondence should be addressed.

Email: mfonton@wustl.edu;

Office: (314) 935-7866

Fax: (314) 935-7211

Abstract

This study presents high temperature water-only continuous flowthrough pretreatment as a promising analytical tool to examine the plant cell wall and understand its recalcitrance, that is, cell wall resistance to deconstruction. ¹³C-enriched corn stover stems were pretreated at 170 °C for 60 min at a hot-water flow rate of 20 mL/min to control fractionation of the cell wall. This approach helped elucidate the nature of plant cell wall chemical recalcitrance and biomass pretreatment chemistry by tracking cell wall fragmentation as a function of time. Fractions of the reactor effluent were collected in a time-resolved fashion and characterized by high-performance liquid chromatography (HPLC), ¹³C nuclear magnetic resonance (NMR), ¹H diffusion ordered

spectroscopy (DOSY) NMR, and ultraviolet (UV) spectroscopy to determine the chemical composition, molecular structure, and molecular weight of the fragments released.

Keywords: Recalcitrance; Corn Stover; Hot-Water Flowthrough Pretreatment; NMR; DOSY; HSQC

1. Introduction

Low-cost production of fermentable sugars from the deconstruction of plant cell wall carbohydrates will play a vital role in addressing long-standing and growing concerns over the global energy demand, dependency on conventional natural resources, and their environmental impacts.(Ragauskas et al., 2006) Fermentable sugars can be converted to a variety of downstream products; however, current emphasis is primarily focused on generation of renewable liquid transportation fuels, mainly second-generation bioethanol.(Hsu et al., 1980) The plant cell wall contains three major biopolymers: cellulose, hemicellulose, and lignin.(Fengel & Wegener, 1983) In plant cell walls, a combination of complex and interrelated chemical and molecular features serve to protect the plant and its cell wall carbohydrates, mainly cellulose and hemicellulose, from environmental and biological mediated degradation.(Mansfield et al., 1999) This resistance to deconstruction constitutes the major obstacle to low-cost conversion of lignocellulosic biomass to ethanol or other fermentation products.

The inherent resistance of the plant cell wall to polysaccharide degradation or deconstruction is referred to as plant cell wall recalcitrance. A variety of strategies have been researched to overcome plant cell wall recalcitrance; however, the exact origins and mechanisms of this recalcitrance have yet to be fully resolved. Thus, these strategies have not sufficiently increased sugar yields and lowered processing cost in such a way to make large-scale biofuel production from lignocellulosic resources more attractive than fuel derived from petroleum sources.(Wyman, 2007) Therefore, a critical component of future biofuel research is continued improvement of analytical methodologies targeted at a fundamental understanding of biomass recalcitrance.

Initially investigated in the late 1980's as a way of reducing recalcitrance prior to enzymatic saccharification, hot-water flowthrough pretreatment uses aqueous media with a specific flow rate and temperature profile.(Bonn et al., 1983; Hörmeyer et al., 1988) The liquid is continuously passed through a reactor containing a bed of biomass at temperatures above 100 °C. In contrast to batch pretreatment systems, these continuous flowthrough configurations have shown the advantages of producing 1) more enzymatically digestible biomass substrates, 2) near theoretical hemicellulose recovery, 3) greater lignin removal, and 4) fewer hydrolysis/fermentation inhibitors.(Yang & Wyman, 2004) A variety of system configurations (e.g., co-current,

countercurrent, and flowthrough fixed solids) have been tested in an attempt to further enhance these advantages.(Bonn et al., 1983; Hörmeyer et al., 1988; Mosier et al., 2005)

More recently, Wyman et al. revisited flowthrough pretreatment, conducting a series of studies, mainly on corn stover, with flowing hot-water and dilute acid.(Liu & Wyman, 2003; Liu & Wyman, 2004a; Liu & Wyman, 2004b; Liu & Wyman, 2005; Yang & Wyman, 2004) In these investigations, operating conditions such as flow rates, temperature, and pH were varied to assess the benefits of flowthrough pretreatment while attempting to mitigate its limitations. Although the results clearly demonstrated the ability of flowthrough pretreatment to significantly increase sugar yields during pretreatment and via subsequent biological deconstruction, large-scale commercial implementation was deemed unattractive because of the significant consumption of water and power along with challenges related to scaling up the flowthrough configuration.(Liu & Wyman, 2003; Liu & Wyman, 2004a; Liu & Wyman, 2004b; Liu & Wyman, 2005; Yang & Wyman, 2004) Despite these limitations, Wyman et al. were able to combine flowthrough pretreatment with modern analytical techniques to provide unique insights into the mechanisms of hot-water pretreatment.(Liu & Wyman, 2003; Liu & Wyman, 2004a; Liu & Wyman, 2004b; Liu & Wyman, 2005; Trajano et al., 2015; Yang & Wyman, 2004) For example, Liu et al. found removal of xylan and lignin from corn stover was nearly linearly related.(Liu & Wyman, 2003) This result suggests that a major fraction of released xylan and lignin fragments were linked in some fashion, possibly covalently through lignin-carbohydrate linkages (LCCs).(Liu & Wyman, 2003) It was also suggested that in batch pretreatment (unlike what was observed for flowthrough pretreatment), released fragments reacted further to form insoluble species that precipitated out of solution upon reactor cooling, explaining the formation of re-precipitated lignin “droplets”(Donohoe et al., 2008) and the much lower solubilization of lignin and other cell wall components(Liu & Wyman, 2003).

Chemical characterization of biomass has long been considered difficult due to its heterogeneous and insoluble nature.(Beecher et al., 2009) A strategy to overcome this problem is to systematically fractionate the plant cell wall and then analyze the chemistry of released soluble components. One such fractionation methodology is termed sequential extraction, where a sequence of different reactive solvents target removal of specific cell wall components for analysis.(DeMartini et al., 2011a; Ziebell et al., 2010) DeMartini et al. utilized sequential extraction and glycome profiling to analyze untreated and batch hydrothermal pretreated *Populus*.(DeMartini et al., 2011a) They extracted the untreated and pretreated *Populus* with a

series of increasingly “harsh” reactive solvents (i.e., oxalate, carbonate, 1.0 M KOH, 4.0 M KOH, chlorite, and then a post-chlorite 4.0 M KOH solution).(DeMartini et al., 2011a) The resulting release of different fractions from the cell wall revealed chemical differences between untreated and pretreated *Populus*. In addition, the increasing “harshness” of reactive solvents applied over the extraction sequence and the corresponding fragments released was used to further elucidate possible origins of plant cell wall recalcitrance.

For example, epitopes for xylans, fucosylated xyloglucans, and non-fucosylated xyloglucans were detected in all extractions for untreated *Populus*, but especially in the 1.0 M KOH, 4.0 M KOH, chlorite, and post-chlorite 4.0 M KOH extractions.(DeMartini et al., 2011a) As the severity of batch hydrothermal pretreatment of *Populus* increased, detection of those epitopes decreased in number and intensity.(DeMartini et al., 2011a) The most significant increase in enzymatic hydrolysis sugar release (or decrease in plant cell wall recalcitrance) occurred following the initial 11 min of pretreatment. The sequential extraction and glycome profiling for the 11 min pretreated solids (unlike the untreated sample) showed no xylans and fucosylated xyloglucans epitopes in the chlorite extraction, an extraction designed for the removal of lignin.(DeMartini et al., 2011a) Thus, the most significant increases in enzymatic hydrolysis of poplar (and decrease in its recalcitrance) was attributed to the removal of lignin-bound xylans and fucosylated xyloglucans, rather than just the more commonly cited removal of bulk hemicelluloses.

Similar to sequential extraction, this study found that flowthrough pretreatment is an ideal method to systematically deconstruct the plant cell wall in a relatively facile, controlled, and repeatable manner for analytical purposes. In this study, flowthrough pretreatment was applied to ¹³C enriched corn stover stems at 170 °C for 60 min at a hot-water flow rate of 20 mL/min to investigate the rate and sequence of plant cell wall fractionation. Furthermore, the chemical composition, molecular structure, and molecular weight of the released components were analyzed as a function of pretreatment time using HPLC, NMR, and UV/Vis techniques to provide insights into the level of structural rigidity and recalcitrance of the eluted chemical moieties. The time-resolved determination of the chemical composition, molecular structure, and molecular weight of the released components via flowthrough pretreatment will be used in this study to understand biomass component structural rigidity and their contributions to biomass chemical recalcitrance, thus affording further insights into pretreatment chemistry for process optimization.

2. Methods

2.1. Feedstock

Ground (0.147 – 0.837 mm) > 97 atom% ^{13}C enriched corn stover and natural abundant control (*Zea mays*) stems were purchased from IsoLife (IsoLife, Wageningen, Netherlands). Extractives were subsequently removed from ground samples by placing the biomass into an extraction thimble in a Soxhlet extraction apparatus. The extraction flask was filled with 1:2 ethanol:toluene mixture (150 mL) and then refluxed at a boiling rate which cycled the biomass for at least 24 extractions over a 4 h period. This extraction process was repeated with distilled de-ionized water (150 mL).

2.2. Pretreatment

Extracted and ground ^{13}C enriched and natural abundant corn stover stems were subjected to hot-water-only flowthrough pretreatment using the flowthrough system described previously by Trajano et al. In this study, the flowthrough reactor was used (12.7 mm OD x 152 mm length) to process ~0.7 g of corn stover per run. Pretreatment was conducted at 170 °C for 60 min at a hot-water flow rate of 20 mL/min. Liquid hydrolyzate was sampled every 2 min for 24 min. Liquid hydrolyzate was then sampled in alternating 6, 10, and 2 min intervals until the reaction was completed. Following 60 min of pretreatment, the reactor was cooled. The residual solids were collected by filtration and washing with an excess of distilled water.

2.3. Residual Solids Analysis

Glucan, xylan, and acid-insoluble residue (which closely approximates Klason lignin) content was determined for both the untreated and pretreated solids using a downscaled compositional analysis described elsewhere. (DeMartini et al., 2011b) This analysis is based on conventional wet chemistry techniques to determine biomass composition but is scaled down by a factor of 100.

2.4. Liquid Hydrolyzate Analysis

The soluble lignin content of the liquid hydrolyzate was determined using ultraviolet (UV) spectroscopy. (Maekawa et al., 1989) The absorbance of the hydrolyzate at 205 nm, using an

extinction coefficient of 110 L/g*cm is used to calculate the amount of soluble lignin present. Aliquots were sampled for high-performance liquid chromatography (HPLC) analysis of sugars.(Kesler, 1967) Sugar monomers in the liquid portion were analyzed quantitatively by a Waters HPLC Alliance 2695 system equipped with a 2414 refractive detector and a Waters 2695 auto-sampler (Waters Co., Milford, MA). A Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) was employed for analyzing sugar monomers using an elution media of 0.005 M sulfuric acid and column heating at 65 °C. Total xylose and glucose concentrations in the liquid fraction were measured before and after post-hydrolysis of each liquid fraction sample with 4 wt. % sulfuric acid at 121 °C for 1 h.(Yang B, 2008) Oligomeric sugar content was determined from the difference between monomer sugar content before and after post-hydrolysis.

The remaining portion of the liquid hydrolyzate was freeze dried to remove water. The liquid hydrolyzate samples were prepared for NMR by adding ~10 mg freeze dried solids to 0.5 mL of DMSO-d₆ (99.9 atom% D) in a 5 mm NMR tube. All NMR spectra were recorded on a Bruker Avance-500 spectrometer. One-dimensional ¹H spectra were acquired using both a standard Bruker one pulse and gradient 3-9-19 pulse water suppression sequence with a 10.0 s recycle delay, 32 scans and acquisition of 32 K data points at 500.13 MHz. One-dimensional ¹³C spectra were acquired using Bruker inverse-gated sequence with waltz-16 decoupling, 20.0 s recycle delay, 32 scans and acquisition of 65 K data points at 125.75 MHz. Two-dimensional HSQC analysis on liquid hydrolyzate NMR samples prepared in DMSO-d₆ was performed as described above on the whole cell wall ionic liquid NMR samples of residual solids. ¹H 2D diffusion order spectroscopy (DOSY) experiments at room temperature employed bipolar gradient pulses for diffusion using a one spoil gradient where 16 scans were recorded with a recycle delay of 10.0 s.

3. Results and discussion

This study examined the rate and sequence at which the cell wall of corn stover stems fractionate during flowthrough hot-water pretreatment at 170 °C for 60 min with a flow rate of 20 mL/min. Prior to pretreatment, naturally abundant and isotopically enriched corn stover stems were Soxhlet-extracted with an ethanol-toluene mixture followed by water to remove starch and other small molecule extractives that could obscure analytical results. The liquid hydrolyzate (i.e., pretreatment effluent) fractions from the flowthrough hot-water pretreatment were collected in a

time-resolved manner, and were characterized along with the biomass solids before and after pretreatment to develop a detailed description of the sequence and rate of fractionation and to better understand biomass recalcitrance and pretreatment chemistry.

3.1.Solid Residue Analysis

Analyzing the solid corn stover samples before and after pretreatment provides a snapshot of the cell wall prior to and following fractionation. HPLC-based anion-exchange chromatography(Kesler, 1967) was performed on acid-hydrolyzed corn stover stem samples. Figure 1 summarizes the variations, both before and after pretreatment, in glucose (attributed mainly to cellulose), xylose (attributed to hemicellulose), and Klason lignin, first for extracted natural abundant and ^{13}C -enriched corn stover stem samples (only before pretreatment) and then comparing extracted ^{13}C -enriched corn stover stem samples before and after pretreatment. Figure 1a clearly demonstrates no significant cell wall compositional effects due to ^{13}C enrichment, while Figure 1b indicates that ~60% of the starting solids were solubilized as a result of flowthrough pretreatment at 170 °C for 60 minutes with a hot-water flow rate of 20 mL/min. This ~60% mass loss was correlated to ~50% removal of the lignin and removal of the majority of the xylan. Lastly, Figure 1b indicates that the glucan, or at least a fraction of glucan, is especially recalcitrant.

[Insert Figure 1]

3.2.Liquid Hydrolyzate Analysis

Figure 2 displays the cumulative yield of aqueous soluble lignin, glucan, and xylan related fragments as a function of flowthrough pretreatment time for the extracted ^{13}C -enriched corn stover stem samples. The data points are the average of two pretreatment trials on extracted ^{13}C -enriched corn stover stem samples. A pretreatment under the same conditions was performed on the extracted natural abundant corn stover stem to further establish that ^{13}C enrichment had little effect on cell wall biosynthesis and recalcitrance. The similarity in cumulative yield profiles of aqueous soluble lignin, glucan, and xylan fragments (see Supplementary Fig. 1) for both ^{13}C -enriched and natural samples suggest that ^{13}C enrichment had little to no effect on cell wall biosynthesis and recalcitrance.

[Insert Figure 2]

The diamond data points in Figure 2 summarize the lignin fragmentation occurring during pretreatment, as determined by ultraviolet (UV) spectroscopy (Maekawa et al., 1989) monitored at 205 nm. Because the sum of 1) Klason lignin content in the residual solids after pretreatment and 2) cumulative yield of eluted soluble lignin fragments was 97% of the Klason lignin content in the untreated solids (before pretreatment), it is reasonable to assume that not only does UV spectroscopy provide an accurate accounting of lignin fragmentation, but also that flowthrough pretreatment does little to degrade lignin fragments or more specifically their UV-absorbing moieties (i.e., aromaticity). The rate of lignin release in mg/min during each sampling period is plotted in Figure 3a. Figures 2 and 3a shows that the lignin release profile was characterized by an oscillating rate regime, that on average indicates a fairly constant generation of lignin fragments (though a maximum rate of release was observed between 10-16 min). After ~40 min of pretreatment, the rate of lignin fragment release drops significantly. This result suggests that the remaining lignin fraction was more difficult to fragment, or possibly, that hydrothermal pretreatment conditions altered the chemistry of lignin that has not been solubilized in such a fashion that it becomes more recalcitrant. Other studies show that hydrothermal pretreatment conditions can cause the formation of condensed (cross-linked) lignin linkages and support the latter hypothesis. (Samuel et al., 2011; Samuel et al., 2010)

The square data points in Figure 2 present, as a function of pretreatment time, the cumulative yield of xylan-related fragments (as both monomers and xlyo-oligomers) in the liquid hydrolyzate fractions, which were determined by an HPLC sugar analysis before and after post-hydrolysis. The xylan mass balance is 89%. The lack of complete material balance closure for xylan suggests that despite the reduced residence time of soluble fragments in the flowthrough reactor, some degradation may have still occurred. It also suggests that released xylose and xlyo-oligomers were more reactive than lignin fragments. Another factor that may contribute to the lower xylan mass balance is the linking of xylan fragments with lignin fragments through LCCs. These xylan-lignin linked fragments would have reduced solubility after elution from the reactor, and as a result may have been precipitated prior to sampling for sugar analysis.

As expected, Figure 2 indicates that ~75% of the xylan was released after 60 min of pretreatment, consistent with it being one of the least recalcitrant macromolecular structures in the cell wall. The xylan release rate profile increased initially for ~10 min, at which point it reached its maximum rate of release: ~5.4 mg/min., twice that of lignin (see Fig. 3a). The rate of xylan

release then dropped until the end of the pretreatment. A greater deceleration in xylan release was observed after ~40 min of pretreatment. The cumulative release of lignin and xylan fragments as a function of pretreatment time was linearly dependent (see Supplementary Fig. 2), thus indicating that removal of xylan and lignin was related and that either xylan removal made lignin less recalcitrant and/or that lignin removal made xylan less recalcitrant. This phenomena is consistent with observations by Liu et al.(Liu & Wyman, 2003), who postulated that the majority of released xylan and lignin fragments are linked via LCCs. As measured by the difference in the monomer content of the hydrolyzate before and after post-hydrolysis, xylo-oligomers were found to be the primary form of released xylan (see Fig. 3b). The ratio of xylan released as xylo-oligomers to free xylose increased to a maximum at 22-24 min before beginning to drop with increasing pretreatment time. This relatively high oligomer recovery is consistent with reduced fragment degradation during flowthrough pretreatment. This result also suggests that a longer hydrolysate residence time is needed to produce monomeric xylose from oligomers, indicating that xylan fragmentation occurs via a series of depolymerizing reactions at internal glycosidic bonds in the liquid phase.

[Insert Figure 3]

The total yield of glucose as both monomers and oligomers in the liquid hydrolyzate is plotted as a function of pretreatment time in Figure 2. The glucan mass balance closure is 91%, lower than that of lignin but slightly higher than that of xylan. Figure 2 suggests that, like xylose and xylo-oligomers, released glucose and gluco-oligomers were more susceptible to degradation than lignin fragments. However, the glucan release did not correlate with the lignin release (see Supplementary Fig. 2). As seen from Figure 2, glucan was much more recalcitrant than xylan and lignin; the total yield after 60 min was ~16%. The glucan release rate was greatest between 0-2 min at ~2.9 mg/min (see Fig. 3a). The rate of release then continuously dropped until ~30 min, after which no further glucan release was detected. This result suggests that: 1) glucan recalcitrance can be thought of in terms of two fractions, a readily released glucan (e.g., likely amorphous cellulose) and a more recalcitrant glucan (e.g., crystalline or solvent inaccessible cellulose), 2) the release of the less recalcitrant glucan fraction had little to do with the presence, or release of xylan or lignin, which are commonly cited as physically blocking glucan fragmentation or release, and 3) based on the glucan level off plateau, the solvent accessible cellulose in the corn stover sample was ~84%. Glucan was released mainly in the form of gluco-

oligomers; the ratio of gluco-oligomers to glucose dropped to a minimum at 10-12 min of pretreatment and then increased with increasing pretreatment time. This minimum in the ratio of gluco-oligomers to free glucose coincided with a significant deceleration in the rate of glucan release.

2.4. Nuclear Magnetic Resonance (NMR)

Other researchers have conducted similar flowthrough pretreatment studies (Liu & Wyman, 2003; Liu & Wyman, 2004a; Liu & Wyman, 2004b; Liu & Wyman, 2005; Yang & Wyman, 2004) and observed similar results with respect to yields and oligomer/monomer ratios. However, those studies and the results just presented did not account for the chemical complexity of the cell wall. Thus, to better account for this complexity, NMR was employed to examine cell wall fragmentation and increase the level of chemical and molecular detail obtained for the liquid hydrolyzate. NMR is well-suited for the analysis of complex chemical systems such as the plant cell walls because it is not only responsive to chemical functionality but also to connectivity and environment. The liquid hydrolyzate samples were freeze-dried and dissolved in deuterated dimethyl sulfoxide (DMSO- d_6). All fractions demonstrated reasonably good solubility in DMSO, indicating that the resulting NMR spectra are representative of the fractions' complete composition, although any highly volatile compounds were likely removed during drying.

$1D^1H$ NMR, with and without solvent suppression techniques to remove residual water signals, was utilized to acquire NMR spectra of collected fractions (Supplementary Figure 3). However, because the majority of these NMR resonances overlapped highly, it was difficult to attribute spectral intensity to any particular functionality or molecular structure. Consequently, $1D^{13}C$ NMR was the major spectroscopy tool in this study. ^{13}C NMR has a much wider chemical shift dispersion, spreading chemical functionality out over a wider shift range, further resolving NMR resonances. Many of the key chemical shift assignments for the ^{13}C NMR associated with intact plant cell wall functionality have been identified in the literature (Holtman et al., 2006; Robert, 1992; Samuel et al., 2010; Teleman et al., 2000; Teleman et al., 2002) as compiled in Table 1. Still, carrying out quantitative ^{13}C NMR can be technically difficult as a result of its inherently long relaxation times and low sensitivity. Because of this challenge, time-resolved ^{13}C NMR studies, in particular on systems of limited sample size for analysis (as in the current study), are not often conducted. To circumvent the technical limitations of ^{13}C NMR, ^{13}C -enriched corn

stover was utilized. Shown in Supplementary Figure 4 is a representative quantitative ^{13}C NMR spectrum of the freeze-dried hydrolyzate following 6-8 min of pretreatment. Komatsu et al. recently applied a multidimensional solution NMR method to ^{13}C -enriched corn stover, assigning up to 119 resonances and characterizing structures in the cell wall of lignocellulose. (Komatsu & Kikuchi, 2013) Many of these resonances showed good agreement with established chemical shifts of NMR resonances for naturally abundant samples, in this case, suggesting that ^{13}C enrichment had little to no effect on cell wall-related ^{13}C chemical shifts.

[Insert Table 1]

An adjusted integration of the chemical shift ranges in Table 1 from the spectra of fractions collected at intervals of 4-6, 6-8, 10-12, 12-14, 16-18, 30-40, and 54-60 min of pretreatment are plotted in Figure 4. Specifically, ^{13}C NMR can resolve the carbon attributed to aliphatic and conjugated carboxylic acids or esters in the region δ_{C} 173–168 and 168-166 ppm, respectively. The conjugated carboxylic esters resonances at δ_{C} 168-166 ppm are related possibly to LCC coumaric and ferulic linkages. Individual resolved resonances belonging to the 4-carbon on *para*-coumaric acid (*pCA*₄) monolignol units show up at ~161 ppm, while δ_{C} 120-117, 177-113, and 112-110 represent the guaiacyl 6-position (G_6), 5-position (G_5), and 2-position (G_2) carbons respectively. In addition, peaks at δ_{C} 108-104 ppm represent the syringyl 2- and 6-position ($\text{S}_{2,6}$) carbons. Carbon at the 1-position of sugar monomers was observed at δ_{C} 104-95 ppm, clearly indicating the presence of pyranoside and 4-O-methyl-D-glucuronosyl units. Overlapping resonances from carbohydrate-associated carbon and carbon in the aliphatic portions of lignin can be observed at δ_{C} 89-59 ppm. Lastly, strong resonances at ~ δ_{C} 55 and 21 ppm indicate the presence of methoxy and acetyl functionality, respectively.

The y-axis of Figure 4 is the spectral integration, in the chemical shift range of interest, normalized by the integration of the total aromatic region (δ_{C} 162-104 ppm) adjusted by the percent of lignin released per sampling period. Normalizing ^{13}C NMR data by the integration of the total aromatic region is commonly utilized for a comparative analysis of the chemical structures in lignin, eliminating concentration and NMR acquisition (e.g., excitation angle, off-resonance, relaxation, etc.) effects on spectral intensity. (Samuel et al., 2010) This ^{13}C NMR lignin data is typically represented as the amount of carbon in the analyte moiety (corresponding to the chemical shift range of interest) per aromatic carbon or ring. This type of spectral data representation is fine when analyzing macromolecular systems with respect to structural differences; however, it does

little to reflect the changes occurring for eluted fragments over time. Basically, due to the fact hydrolyzate was sampled over varying sampling intervals, an additional normalization was required. The product of the above ratio (i.e., the integration of the chemical shift range of interest divided by the integration of the total aromatic region) with the percent lignin released per sampling period allowed for an instantaneous estimation of relative quantity, even during sampling periods of different length, while also eliminating the aforementioned NMR acquisition effects on spectral intensity. This normalization procedure allowed for comparisons of the freeze-dried hydrolyzate ^{13}C NMR data for sampling periods of different length even for the polysaccharide related fragments.

Figure 4a illustrates the release profiles of carbonyl and aromatic resonances solely associated with lignin and hemicellulose fragments. Figure 4a indicates that the release of fragments containing carbonyls within aliphatic carboxylic acids or esters decelerated much more rapidly than the release of other lignin and hemicellulose related fragments containing functional groups, such as conjugated carboxylic acids or esters, aromatic ring, and acetyl-related groups. This result almost certainly suggests that: 1) aliphatic carboxylic acid or ester functionality was associated not only with hemicellulose but also with lignin fragments, and 2) not all aliphatic carbonyls were part of the acetyl functionality. The release of fragments containing conjugated carboxylic acids or esters displayed a more constant profile, with a sharp drop after 18 min of pretreatment. More interestingly, the proportion of conjugated carbonyls was roughly equal to that of aromatic ring units, an additional indication that aromatic-containing lignin fragments include ester-containing LCCs. Though there is no direct method to confirm whether these carbonyls were generated by the pretreatment itself, the relatively high mass balances reported in Figure 2 suggest that these observed functional groups were most likely not associated with degradation products. The fragments containing *p*CA, S, and G were released in such a fashion to suggest that the S units seem less recalcitrant than the G units, which are both, in turn, less recalcitrant than the *p*CA units. At 4-6 min of pretreatment, the percentage of released monolignol fragments was 41, 34, and 25% S, G, and *p*CA units respectively, which in a continuous fashion shifted to 28, 42, and 30% at 12-14 min of pretreatment. In the last sampling period of 54-60 min, the percentage of released monolignol fragments for S, G, and *p*CA units was 8, 46, and 46%. This trend is also evident in Supplementary Figure 5, which is a bar plot of the monolignol ratio in each sample as determined by the 1D ^{13}C NMR spectra.

[Insert Figure 5]

Figure 5b focuses on the release profiles of *O*-alkyl, methoxy, and acetyl-related methyl resonances associated with lignin, hemicellulose, and cellulose fragments. Displaying a similar release profile as that determined by HPLC (Figure 3a), fragments containing position-1 carbons in monomeric sugars show a maximum yield at ~12 min of pretreatment. The strong correlation with HPLC validates the NMR spectral analysis procedure and interpretation. Lastly, the release profiles of fragments containing methoxy and acetyl-related methyl resonances are very similar to those of an aromatic unit containing fragments. This is reasonable, as the methoxy functionality should be primarily associated with the aromatic rings of monolignols.

2.5. Two-Dimensional NMR

2D ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) NMR experiments are not quantitative but can help resolve a variety of overlapping spectral features over two dimensions (i.e., carbon and proton spectral dimensions) and provide more information about the presence of a wide array of chemical moieties. Again, many of the key chemical shift assignments associated within intact plant cell wall functionality have been identified via 2D ^1H - ^{13}C NMR in the literature (Kim & Ralph, 2010; Kim et al., 2008; Samuel et al., 2011) as summarized in Supplementary Table 1. Figure 5 is a representative 2D ^1H - ^{13}C HSQC NMR spectrum of the freeze-dried hydrolyzate (6-8 min of pretreatment). The 2D spectra can distinguish individual resonances for *p*CA, S, and G monolignol units at $\delta_{\text{C}}/\delta_{\text{H}}$ 130.0/7.3, 115.0/6.7, and 103.3/6.8 ppm respectively, as well as ferulate acid (FA) units at $\delta_{\text{C}}/\delta_{\text{H}}$ 123.5/7.2 ppm. Identifying cellulose- and hemicellulose-related fragments can also be accomplished by means of the cross peaks for the anomeric signals of position-1 carbon of (1-4)-linked- β -D-glucopyranoside (1 $\rightarrow$ 4- β -D-Glcp) at $\delta_{\text{C}}/\delta_{\text{H}}$ 102.5/4.3 ppm and (1-4)-linked- β -D-xylopyranoside (1 $\rightarrow$ 4- β -D-Xylp) at $\delta_{\text{C}}/\delta_{\text{H}}$ 102.5/4.5 ppm. Additionally detected fragments containing polysaccharide functionality include 2- or 3-acetylated β -D-xylopyranoside, β -D-mannosyl, 4-O-methyl-D-glucuronosyl, reducing-end β -D-glucopyranoside, and reducing-end β -D-xylopyranoside. Lastly, functionality related to major aliphatic lignin inter-unit linkages such as β -aryl ether (A) and phenylcoumaran (B) linkages at $\delta_{\text{C}}/\delta_{\text{H}}$ 72.0/4.8 (A_{α}) and 87.5/5.3 (B_{α}) respectively, can be observed. An indication of the presence or absence of these structures in the liquid hydrolyzate of NMR-analyzed fractions is provided in Supplementary Table 1. The most important observation resulting from 2D ^1H - ^{13}C HSQC NMR

spectra was that lignin and polysaccharide fragments appear linked via native (observed in untreated biomass) inter-unit linkages in significant amounts.

[Insert Figure 5]

2.6. Diffusion Ordered Spectroscopy (DOSY) NMR

To probe the molecular weight of the carbohydrate and lignin fragments released in each liquid hydrolyzate and to potentially quantify the proportion of fragments containing LCC linkages, a series of ^1H DOSY NMR experiments in DMSO- d_6 was conducted. DOSY NMR uses a series of spin-echo spectra measured with different pulsed field gradient strengths to separate the NMR resonances of different species according to their diffusion coefficient. When analyzing mixtures of compounds, DOSY NMR is in many ways preferable to techniques such as size-exclusion chromatography because DOSY NMR can resolve differences in both analyte molecular weight and chemical structure.(Viel et al., 2003)

^1H DOSY curves were developed by integration of chemical shift ranges δ_{H} 7.8-6.0 ppm (associated with lignin-only fragments)(Lundquist, 1992) and δ_{H} 5.8-3.5 ppm (associated with both hemicellulose, cellulose, and lignin fragments)(Lundquist, 1992). Initially, DOSY curves 1) for the chemical shift range of lignin-only fragments were analyzed using a bi-component (i.e., free lignin and LCC containing fragments) exponential echo decay and 2) for the chemical shift range that included hemicellulose, cellulose, and lignin fragments were analyzed using a tri-component (i.e., free carbohydrate, free lignin, and LCC containing fragments) exponential echo decay. The weighting parameters for free carbohydrate and free lignin components in the DOSY curve was set using results from HPLC and UV-Vis analysis; however, the results unfortunately could not be confirmed due to a high level of uncertainty associated with the non-linear multiple regression. However, the results did suggest that the free carbohydrate and free lignin fragments have similar diffusion coefficients and that there was a slower diffusing component, most likely the LCC-containing fragments, present in at least the carbohydrate/lignin fragment chemical shift range. Effort were made to apply an inverse Laplace transformation that assumes a continuous distribution of molecular weights (i.e., diffusion coefficients). However, the similar nature and magnitude of the different components that comprise the DOSY decay curve resulted in the production of a single wide distribution, rather than two or three narrow distributions, as expected.

Accordingly, all DOSY curves were fit to a single exponential echo decay using a discrete average diffusion coefficient, as shown in Figure 6. The average diffusion coefficients determined

for the lignin-only fragment chemical shift range were on the order of the diffusion coefficient for xylose in DMSO (determined experimentally as a standard) and indicate that aromatic-containing lignin fragments exist primarily as monomers. The average diffusion coefficients determined for the carbohydrate/lignin fragment chemical shift range, on the other hand, were on the order of the diffusion coefficient for 13-mer xylo-oligomer in DMSO (determined experimentally as a standard) and indicate that larger oligomers of xylan, glucan, and/or LCC fragments are present.

[Insert Figure 6]

4. Conclusions

First, xylan or hemicellulose fragments are much more readily extracted than lignin and in turn, lignin fragments are more readily extracted than glucan. In addition, glucan can be viewed as composed of two fractions: a recalcitrant fraction and a less recalcitrant fraction. The observation of common aliphatic, monolignol inter-unit linkages such as β -aryl ether and phenyl coumaran linkages in 1D ^{13}C and 2D ^1H - ^{13}C NMR indicates the presence of lignin dimers linkage through native lignin bonds. ^{13}C NMR data suggests that lignin fragments composed of S units were less recalcitrant than G and hydroxyphenyl-like units.

Acknowledgments

This work was supported and performed as part of the BioEnergy Science Center (BESC). The BioEnergy Science Center is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. ORNL is managed by UT-Battelle, LLC, under contract DE-AC05-00OR22725 for the U.S. Department of Energy. The publisher, by accepting the article for publication, acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, world-wide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes. The Department of Energy will provide public access to these results of federally sponsored research in accordance with the DOE Public Access Plan (<http://energy.gov/downloads/doe-public-access-plan>). We also wish to thank Dr. Lynnea Brumbaugh of Washington University's Engineering Communication Center for her editing suggestions.

References

1. Beecher, J.F., Hunt, C.G., Zhu, J. 2009. Tools for the characterization of biomass at the nanometer scale. *The nanoscience and technology of renewable biomaterials*, 1st edn. Singapore: Blackwell Publishing. p, 61-80.
2. Bonn, G., Concin, R., Bobleter, O. 1983. Hydrothermolysis—a new process for the utilization of biomass. *Wood Science and Technology*, **17**(3), 195-202.
3. DeMartini, J.D., Pattathil, S., Avci, U., Szekalski, K., Mazumder, K., Hahn, M.G., Wyman, C.E. 2011a. Application of monoclonal antibodies to investigate plant cell wall deconstruction for biofuels production. *Energy & Environmental Science*, **4**(10), 4332-4339.
4. DeMartini, J.D., Studer, M.H., Wyman, C.E. 2011b. Small-scale and automatable high-throughput compositional analysis of biomass. *Biotechnology and bioengineering*, **108**(2), 306-312.
5. Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B. 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. *Biotechnology and Bioengineering*, **101**(5), 913-925.
6. Fengel, D., Wegener, G. 1983. *Wood: chemistry, ultrastructure, reactions*. Walter de Gruyter.
7. Holtman, K.M., Chang, H.m., Jameel, H., Kadla, J.F. 2006. Quantitative ¹³C NMR characterization of milled wood lignins isolated by different milling techniques. *Journal of wood chemistry and technology*, **26**(1), 21-34.
8. Hörmeyer, H., Schwald, W., Bonn, G., Bobleter, O. 1988. Hydrothermolysis of birch wood as pretreatment for enzymatic saccharification. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood*, **42**(2), 95-98.
9. Hsu, T., Ladisch, R., Tsao, G. 1980. Alcohol from cellulose. *chemical intermediates*, **1203**(3), 3.
10. Kesler, R.B. 1967. Rapid quantitative anion-exchange chromatography of carbohydrates. *Analytical Chemistry*, **39**(12), 1416-1422.
11. Kim, H., Ralph, J. 2010. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d₆/pyridine-d₅. *Organic & biomolecular chemistry*, **8**(3), 576-591.
12. Kim, H., Ralph, J., Akiyama, T. 2008. Solution-state 2D NMR of ball-milled plant cell wall gels in DMSO-d₆. *BioEnergy Research*, **1**(1), 56-66.
13. Komatsu, T., Kikuchi, J. 2013. Comprehensive signal assignment of ¹³C-labeled lignocellulose using multidimensional solution NMR and ¹³C chemical shift comparison with solid-state NMR. *Analytical chemistry*, **85**(18), 8857-8865.
14. Liu, C., Wyman, C.E. 2003. The effect of flow rate of compressed hot water on xylan, lignin, and total mass removal from corn stover. *Industrial & Engineering Chemistry Research*, **42**(21), 5409-5416.
15. Liu, C., Wyman, C.E. 2004a. The effect of flow rate of very dilute sulfuric acid on xylan, lignin, and total mass removal from corn stover. *Industrial & engineering chemistry research*, **43**(11), 2781-2788.
16. Liu, C., Wyman, C.E. 2004b. Impact of fluid velocity on hot water only pretreatment of corn stover in a flowthrough reactor. *Proceedings of the Twenty-Fifth Symposium on Biotechnology for Fuels and Chemicals Held May 4–7, 2003, in Breckenridge, CO*. Springer. pp. 977-987.
17. Liu, C., Wyman, C.E. 2005. Partial flow of compressed-hot water through corn stover to enhance hemicellulose sugar recovery and enzymatic digestibility of cellulose. *Bioresource Technology*, **96**(18), 1978-1985.

18. Lundquist, K. 1992. Proton (1H) NMR spectroscopy. in: *Methods in Lignin Chemistry*, Springer, pp. 242-249.
19. Maekawa, E., Ichizawa, T., Koshijima, T. 1989. An evaluation of the acid-soluble lignin determination in analyses of lignin by the sulfuric acid method. *Journal of wood chemistry and technology*, **9**(4), 549-567.
20. Mansfield, S.D., Mooney, C., Saddler, J.N. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnology progress*, **15**(5), 804-816.
21. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource technology*, **96**(6), 673-686.
22. Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., Liotta, C.L. 2006. The path forward for biofuels and biomaterials. *science*, **311**(5760), 484-489.
23. Robert, D. 1992. Carbon-13 nuclear magnetic resonance spectrometry. in: *Methods in lignin chemistry*, Springer, pp. 250-273.
24. Samuel, R., Foston, M., Jaing, N., Cao, S., Allison, L., Studer, M., Wyman, C., Ragauskas, A.J. 2011. HSQC (heteronuclear single quantum coherence) 13 C-1 H correlation spectra of whole biomass in perdeuterated pyridinium chloride-DMSO system: an effective tool for evaluating pretreatment. *Fuel*, **90**(9), 2836-2842.
25. Samuel, R., Pu, Y., Raman, B., Ragauskas, A.J. 2010. Structural characterization and comparison of switchgrass ball-milled lignin before and after dilute acid pretreatment. *Applied biochemistry and biotechnology*, **162**(1), 62-74.
26. Teleman, A., Lundqvist, J., Tjerneld, F., Stålbrand, H., Dahlman, O. 2000. Characterization of acetylated 4-O-methylglucuronoxylan isolated from aspen employing 1 H and 13 C NMR spectroscopy. *Carbohydrate research*, **329**(4), 807-815.
27. Teleman, A., Tenkanen, M., Jacobs, A., Dahlman, O. 2002. Characterization of O-acetyl-(4-O-methylglucurono) xylan isolated from birch and beech. *Carbohydrate research*, **337**(4), 373-377.
28. Trajano, H.L., Pattathil, S., Tomkins, B.A., Tschaplinski, T.J., Hahn, M.G., Van Berkel, G.J., Wyman, C.E. 2015. Xylan hydrolysis in *Populus trichocarpa* × *P. deltoides* and model substrates during hydrothermal pretreatment. *Bioresource technology*, **179**, 202-210.
29. Viel, S., Capitani, D., Mannina, L., Segre, A. 2003. Diffusion-ordered NMR spectroscopy: a versatile tool for the molecular weight determination of uncharged polysaccharides. *Biomacromolecules*, **4**(6), 1843-1847.
30. Wyman, C.E. 2007. What is (and is not) vital to advancing cellulosic ethanol. *TRENDS in Biotechnology*, **25**(4), 153-157.
31. Yang B, Wyman CE. 2008. Characterization of the Degree of Polymerization of Xylooligomers Produced by Flowthrough Hydrolysis of Pure Xylan and Corn Stover with Water. *Biores Technol*, **99**, 5756-62.
32. Yang, B., Wyman, C.E. 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. *Biotechnology and bioengineering*, **86**(1), 88-98.
33. Ziebell, A., Gracom, K., Katahira, R., Chen, F., Pu, Y., Ragauskas, A., Dixon, R.A., Davis, M. 2010. Increase in 4-coumaryl alcohol units during lignification in alfalfa (*Medicago sativa*) alters the extractability and molecular weight of lignin. *Journal of Biological Chemistry*, **285**(50), 38961-38968.

1
2
3
4 571
5 572
6 573
7 574
8 575
9 576
10 577
11 578
12 579
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Fig. 1. (a) Glucose, xylose, and Klason lignin percent distribution of extracted natural abundant and ^{13}C -enriched corn stover stem samples. **(b)** Glucose, xylose, and Klason lignin percent distribution of extracted ^{13}C -enriched corn stover stem samples before and after pretreatment.

Fig. 2. Cumulative yield of aqueous soluble lignin (◆/◇), xylan (■/□), and glucan (●/○) fragments as a function of pretreatment time for extracted ^{13}C enriched corn stover stem samples. Closed symbols represent the cumulative yields as determined via the reactor effluent fractions. Open symbols represent residual yields remaining in the solids.

Fig. 3. (a) Yield rate (mg/min) by sampling interval for aqueous soluble lignin and carbohydrate fragments expressed as monomeric glucose and xylose. **(b)** Ratio of oligomeric monomers to monomers for released carbohydrates by sampling interval.

Fig. 4. Integrations of select chemical shift ranges in 1D ^{13}C NMR spectra of liquid hydrolyzate normalized by integration of the total aromatic region (δ_{C} 162-104 ppm) and the percent lignin released per sampling period. **(a)** Carbonyl and aromatic resonances solely associated with lignin and hemicellulose fragments. **(b)** *O*-Alkyl, methoxy and acetyl resonances associated with lignin, hemicellulose, and cellulose fragments.

Fig. 5. 2D ^1H - ^{13}C HSQC NMR spectrum of liquid hydrolyzate collected for flowthrough pretreatment (6-8 min) of extracted ^{13}C enriched corn stover stem.

Fig. 6. Average self-diffusion coefficients of protons associated with lignin-only fragments and with lignin and carbohydrate fragments collectively as determined by ¹H diffusion-ordered spectroscopy (DOSY) NMR (water has a self-diffusion coefficient of 2.27·10⁻⁹ m²/s).

Table 1. 1D ^{13}C NMR chemical shift assignments for liquid hydrolyzate for extracted ^{13}C enriched corn stover stem samples.

Chemical Shifts δc (ppm)	Assignments
173-168	Aliphatic -COOR
168-166	Conjugated -COOR
162-160	$p\text{CA}_4$
155-142	$\text{S}_{3,5}$ and $\text{G}_{3,4}$
132-122	$\text{S}_{4,1}$ and G_1
120-117	G_6
117-113	G_5
112-110	G_2
108-104	$\text{S}_{2,6}$
104-95	C_1 Carbohydrates
89-80	C_4 Carbohydrates and Lignin side chains: C_β in $\beta\text{-O-4}$, C_α
83-70	$\text{C}_{2,3,4}$ Xylan, $\text{C}_{2,3,5}$ Glucan and Lignin side chains: C_α , C_γ
68-59	C_5 Xylan, C_6 Glucan and Lignin side chains: C_β
58-55	Methoxy (OMe)
23-20	Acetyl (OAc)

$p\text{CA}$: *para*-coumaric acid; G: Guaiacyl; S: Syringyl