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Recalcitrance and structural analysis by water-only flowthrough pretreatment of ^{13}C enriched corn stover stem

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HIGHLIGHTS

- Understanding plant cell wall recalcitrance.
- Continuous flowthrough pretreatment of ^{13}C enriched corn stover.
- Application of time-resolved NMR aided by isotope enrichment.

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ABSTRACT

This study presents high temperature water-only continuous flowthrough pretreatment coupled with nuclear magnetic resonance (NMR) as a promising analytical tool to examine the plant cell wall, to understand its recalcitrance (i.e., cell wall resistance to deconstruction), and to probe the chemistry occurring during batch pretreatment of biomass. ^{13}C-enriched corn stover stems were pretreated at 170 °C for 60 min with 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 various NMR techniques to determine the degree and sequence of fragments released, as well as, the chemical composition, molecular structure, and relative molecular weight of those released fragments.

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 and 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

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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 1980s 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 and 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 and Wyman, 2003, 2004a, b, 2005; Yang and 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 and Wyman, 2003, 2004a,b, 2005; Yang and 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 and Wyman, 2003, 2004a,b, 2005; Trajano et al., 2015; Yang and Wyman, 2004). For example, Liu et al. found removal of xylan and lignin from corn stover was nearly linearly related (Liu and 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 and 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 and 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 *Poplulus* (DeMartini et al., 2011a). They extracted the untreated and pretreated *Poplulus* 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 *Poplulus*. 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.

Similar to sequential extraction, this study found that flowthrough pretreatment is an ideal method to systematically deconstruct (i.e., fractionate) the plant cell wall in a relatively facile, controlled, and repeatable manner for analytical purposes. In this study, flowthrough pretreatment was applied to ^{13}C enriched corn stover stems at 170 °C for 60 min with a hot-water flow rate of 20 mL/min to investigate the degree and sequence of plant cell wall fractionation. 1D ^{13}C and ^{1}H NMR, ^{1}H diffusion ordered spectroscopy (DOSY) NMR, and 2D ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) NMR methods were applied to not only establish the time-resolved release of carbohydrate and lignin fragments from biomass but also probe which chemical and molecular moieties on those fragments were cleaved, retained, and/or modified upon release from the cell wall as well as during its residence time in the reactor. Techniques used in other similar flowthrough pretreatment studies, including high-performance liquid chromatography (HPLC) and ultraviolet (UV) spectroscopy, were also applied to support the NMR analysis. Herein, this study demonstrates how flowthrough pretreatment on ^{13}C enriched biomass coupled with time-resolved NMR can provide insights into the level of structural rigidity and recalcitrance of the eluted chemical and molecular moieties. However, the unique capability afforded (the chemical and molecular analysis of cell wall carbohydrate and lignin fragments under controlled fractionation) by this methodology can be applied, for example in future studies, to understand 1) why one species or genotype of plant might be more recalcitrant than another or 2) what chemistry occurs in batch pretreatments. In both cases by detecting specific changes in the degree, sequence, and chemical/molecular structure of eluted cell wall fragments as a function of biomass feedstock, pretreatment conditions (i.e., temperature, pH, etc.) and/or pretreatment flow rate (which controls the residence time of fragment in the reactor).

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 Iso*Life* (Iso*Life*, 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 × 152 mm length) to process ~0.7 g of corn stover per run. Pretreatment was conducted at 170 °C for 60 min with 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 were 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 post-hydrolysis conditions of 4 wt.% sulfuric acid at 121 °C for 1 h (Yang and Wyman, 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-d_6 (99.9 atom% D) in a 5 mm NMR tube. All NMR spectra were recorded on a Bruker Avance-500 spectrometer. One-dimensional ^{1}H 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 ^{13}C 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 NMR analysis liquid hydrolyzate samples were prepared in DMSO-d_6 as described above and performed on a Bruker DRX 500 spectrometer with a 5 mm z-gradient triple resonance probe with inverse geometry at 35 °C. The HSQC analysis was performed with a Bruker phase-sensitive gradient-edited HSQC pulse sequence 'hsqcetgpsi.2' using 1024 data points for a 0.11 s acquisition time, a 1.5 s recycle delay, a ^{1}JC-H coupling constant of 145 Hz, 16 scans, using an F1 spectral width of Hz (210.00 ppm), and acquisition of 256 data points in the F1 dimension. Processing used zero-filling to 2048 points and a typical squared sine-bell apodization in both F2 and F1 dimensions. ^{1}H 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 degree and sequence 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. Fig. 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. Fig. 1a demonstrates differences in cell wall composition between ^{13}C enriched and natural abundant corn stover; however, this difference is consistent with differences observed within genotypes. Moreover, there is a large body of literature on stable isotope labeling of plants that indicate ^{13}C enriched biomass is chemically representative of natural abundant biomass (Foston et al., 2012; Kikuchi et al., 2004). Fig. 1b indicates that ~60% of the starting solids were solubilized as a result of flowthrough pretreatment at 170 °C for 60 min 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, Fig. 1b indicates that the glucan, or at least a fraction of glucan, is especially recalcitrant.

3.2. Liquid hydrolyzate analysis

Fig. 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.

The diamond data points in Fig. 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 Fig. 3a. Figs. 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 and 16 min). After ~40 min

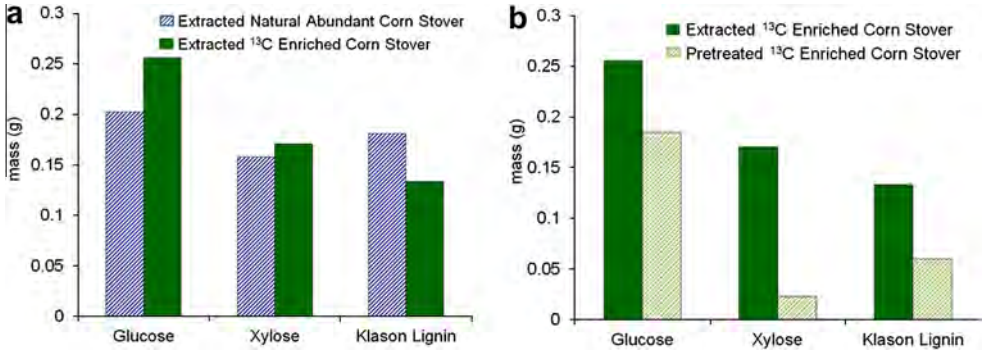

Fig. 1. (a) Glucose, xylose, and Klason lignin percent distribution of extracted natural abundant and ^{13}C-enriched corn stover stem samples. (b) Glucose, xylose, and Klason lignin percent distribution of extracted ^{13}C-enriched corn stover stem samples before and after pretreatment.

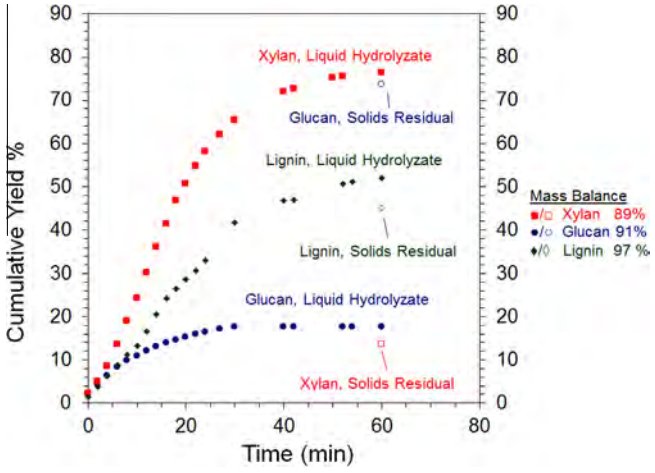

Fig. 2. Cumulative yield of aqueous soluble lignin (◆/◇), xylan (■/□), and glucan (●/○) fragments as a function of pretreatment time for extracted ^{13}C enriched corn stover stem samples. Closed symbols represent the cumulative yields as determined via the reactor effluent fractions. Open symbols represent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

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., 2010, 2011).

The square data points in Fig. 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 xylo-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, Fig. 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 can be characterized as a volcano plot and 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 and 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.

The total yield of glucose as both monomers and oligomers in the liquid hydrolyzate is plotted as a function of pretreatment time in Fig. 2. The glucan mass balance closure is 91%, lower than that of lignin but slightly higher than that of xylan. Fig. 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 Fig. 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 and 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 inaccessible 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

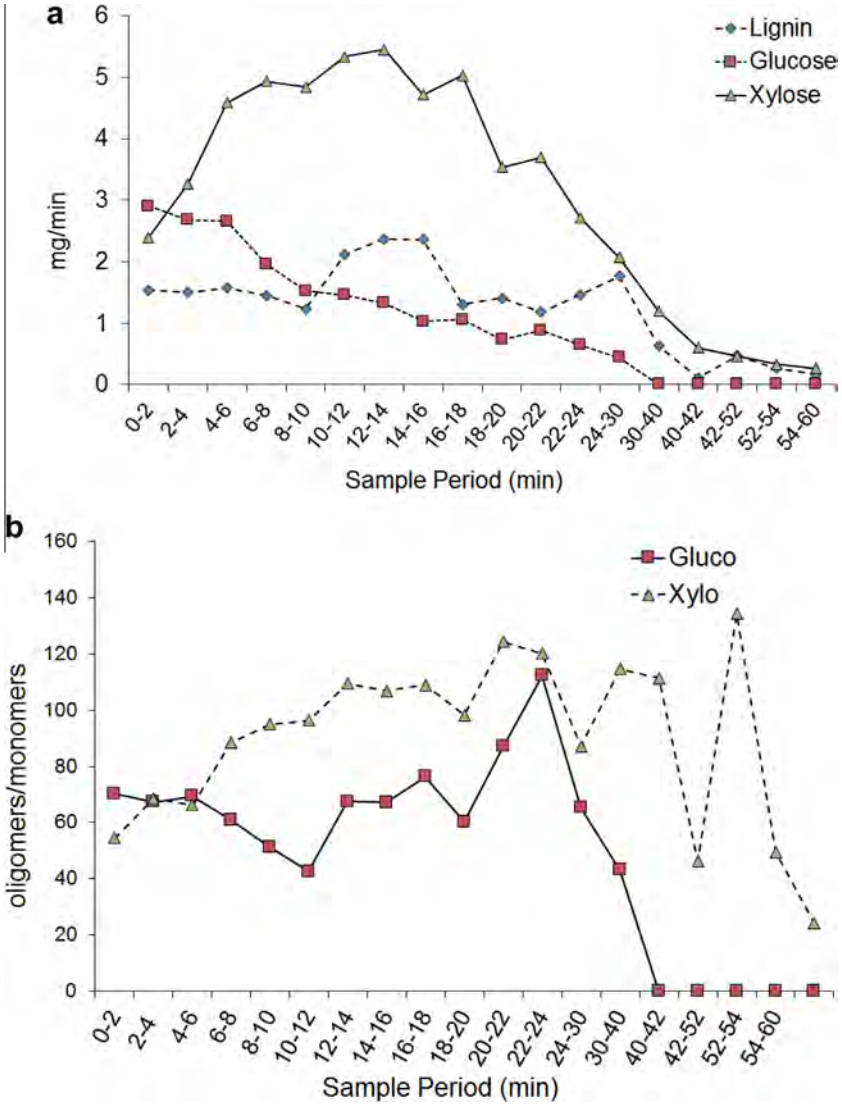

Fig. 3. (a) Yield rate (mg/min) by sampling interval for aqueous soluble lignin and carbohydrate fragments expressed as monomeric glucose and xylose. (b) Ratio of oligomeric monomers to monomers for released carbohydrates by sampling interval.

pretreatment time. This minimum in the ratio of gluco-oligomers to free glucose coincided with a significant deceleration in the rate of glucan release.

3.3. Nuclear magnetic resonance (NMR)

Other researchers have conducted similar flowthrough pretreatment studies (Liu and Wyman, 2003, 2004a,b, 2005; Yang and Wyman, 2004) and observed similar results with respect to yields and oligomer/monomer ratios. However, those studies and their results only profile the yields of carbohydrates and their monosaccharide compositions; and do not account for the chemical complexity of lignin and carbohydrate functionality, substructures, and linkages in the released fragments. 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 from 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

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

Chemical shifts δ_C (ppm)	Assignments
173–168	Aliphatic-*C*OOR
168–166	Conjugated-*C*OOR
162–160	pCA_4
155–142	$S_{3,5}$ and $G_{3,4}$
132–122	$S_{4,1}$ and G_1
120–117	G_6
117–113	G_5
112–110	G_2
108–104	$S_{2,6}$
104–95	C_1 carbohydrates
89–80	C_4 carbohydrates and lignin side chains: C_β in β–O–4, C_α
83–70	$C_{2,3,4}$ xylan, $C_{2,3,5}$ 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*CA: *para*-coumaric acid; G: guaiacyl; S: syringyl.

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 ^{1}H NMR, with and without solvent suppression techniques to remove residual water signals, was utilized to acquire NMR spectra of collected fractions (Supplementary Fig. 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, 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 Fig. 4 is a representative quantitative ^{13}C NMR spectrum of the freeze-dried hydrolyzate following 6–8 min of pretreatment.

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 Fig. 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

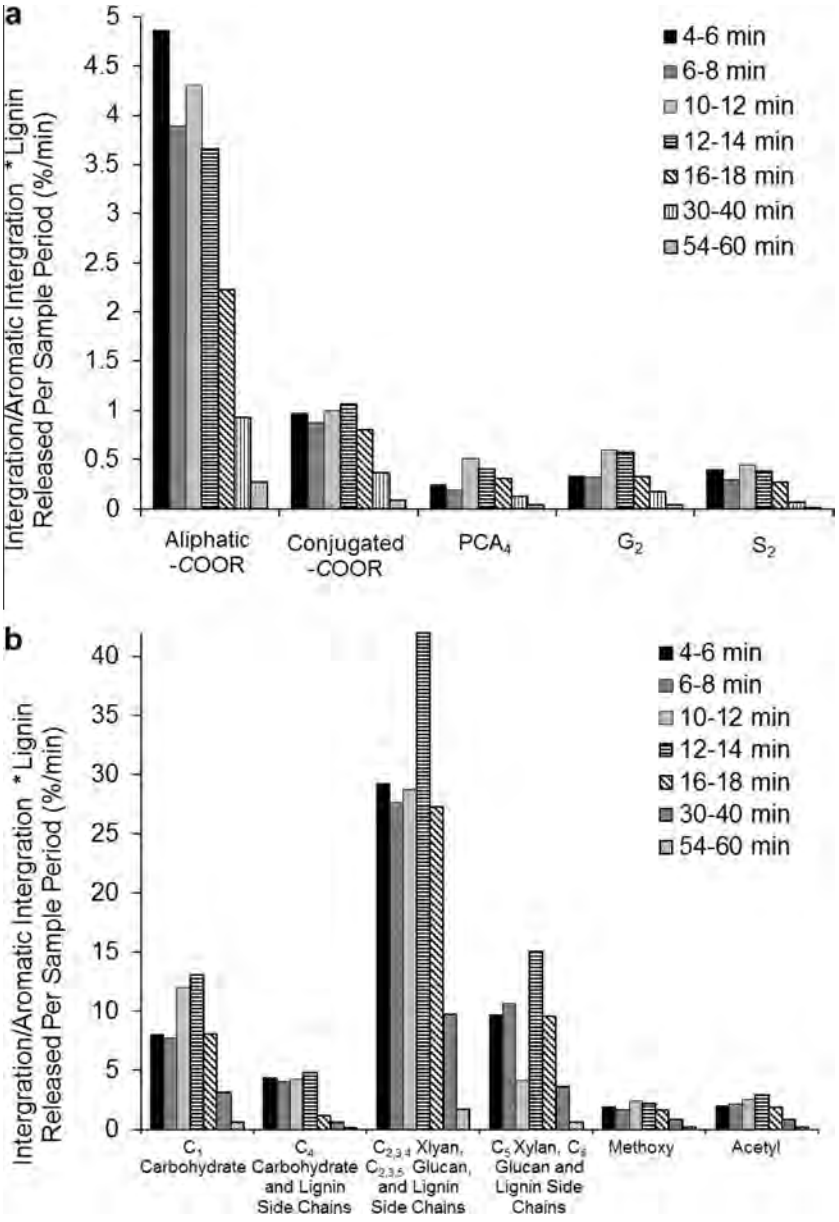

Fig. 4. Integrations of select chemical shift ranges in 1D ^{13}C NMR spectra of liquid hydrolyzate normalized by integration of the total aromatic region (δ_C 162–104 ppm) and the percent lignin released per sampling period. (a) Carbonyl and aromatic resonances solely associated with lignin and hemicellulose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

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-postion (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 ($S_{2,6}$) 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 Fig. 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. Fig. 4a illustrates the release profiles of carbonyl and aromatic resonances solely associated with lignin and hemicellulose fragments. Fig. 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 Fig. 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 Fig. 5, which is a bar plot of the monolignol ratio in each sample as determined by the 1D ^{13}C NMR spectra.

Fig. 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 (Fig. 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.

3.4. Two-dimensional NMR

2D ^{1}H–^{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 ^{1}H–^{13}C NMR in the literature (Kim and Ralph, 2010; Kim et al., 2008; Samuel et al., 2011) as summarized in Supplementary Table 1. Fig. 5 is a representative 2D ^{1}H–^{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 δ_C/δ_H 130.0/7.3, 115.0/6.7, and 103.3/6.8 ppm

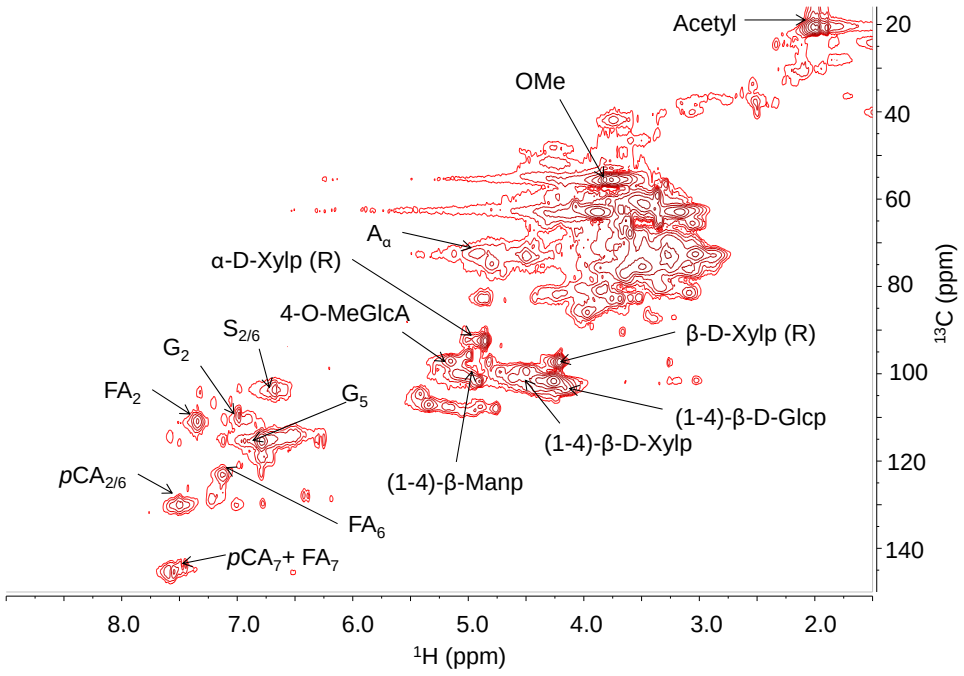

Fig. 5. 2D ^{1}H–^{13}C HSQC NMR spectrum of liquid hydrolyzate collected for flowthrough pretreatment (6–8 min) of extracted ^{13}C enriched corn stover stem.

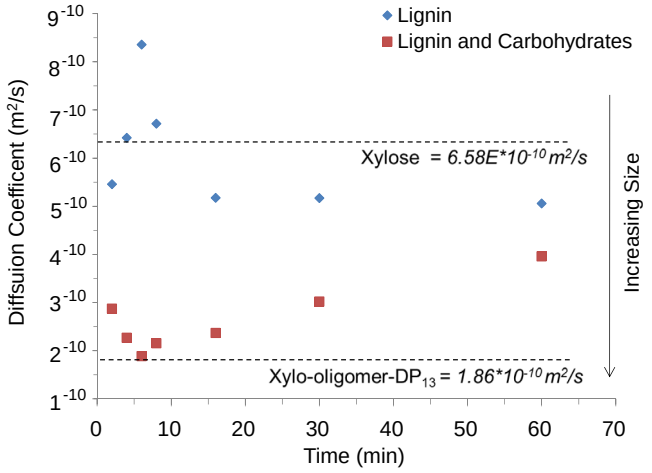

Fig. 6. Average self-diffusion coefficients of protons associated with lignin-only fragments and with lignin and carbohydrate fragments collectively as determined by ^{1}H diffusion-ordered spectroscopy (DOSY) NMR (water has a self-diffusion coefficient of $2.27 * 10^{-9}$ m^2/s).

respectively, as well as ferulate acid (FA) units at δ_C/δ_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 → 4-β-D-Glc*p*) at $\delta c/\delta_H$ 102.5/4.3 ppm and (1–4)-linked-β-D-xylopyranoside (1 → 4-β-D-Xyl*p*) at $\delta c/\delta_H$ 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 $\delta c/\delta_H$ 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 ^{1}H–^{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.

3.5. 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 ^{1}H 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).

^{1}H 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). Accordingly, all DOSY curves were fit to a single exponential echo decay using a discrete average diffusion coefficient, as shown in Fig. 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.

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 inter-unit linkages such as glucopyranoside, xylopyranoside, β-aryl ether, and phenyl coumaran linkages in 1D ^{13}C and 2D ^{1}H–^{13}C NMR indicates the presence of native lignin and carbohydrate bonds in fragments that elute with a flow rate of 20 mL/min. ^{13}C NMR data suggests that lignin fragments composed of S units were less recalcitrant than G and hydroxyphenyl-like units.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.08.065.

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