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# Structural changes of corn stover lignin during acid pretreatment

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**Abstract** In this study, raw corn stover was subjected to dilute acid pretreatments over a range of severities under conditions similar to those identified by the National Renewable Energy Laboratory (NREL) in their techno-economic analysis of biochemical conversion of corn stover to ethanol. The pretreated corn stover then underwent enzymatic hydrolysis with yields above 70 % at moderate enzyme loading conditions. The enzyme exhausted lignin residues were characterized by $^{31}$P NMR spectroscopy and functional moieties quantified and correlated to enzymatic hydrolysis yields. Results from this study indicated that both xylan solubilization and lignin degradation are important for improving the enzyme accessibility and digestibility of dilute acid pretreated corn stover. At lower pretreatment temperatures, there is a good correlation between xylan solubilization and cellulose accessibility. At higher pretreatment temperatures, lignin degradation correlated better with cellulose accessibility, represented by the increase in phenolic groups. During acid pretreatment, the ratio of syringyl/guaiacyl functional groups also gradually changed from less than 1 to greater than 1 with the increase in pretreatment temperature. This implies that more syringyl units are released from lignin depolymerization of aryl ether linkages than guaiacyl units. The condensed phenolic units are also correlated with the increase in pretreatment temperature up to 180 °C, beyond which point condensation reactions may overtake the hydrolysis of aryl ether linkages as the dominant reactions of lignin, thus leading to decreased cellulose accessibility.

**Keywords** Corn stover · Pretreatment · Hydrolysis · NMR spectroscopy · Lignin

## Introduction

Pretreatment of the raw biomass feedstock is the most important processing step to overcome biomass recalcitrance and improve enzymatic digestibility. Many different pretreatment technologies have been extensively studied and reported in the literature. Although it is generally agreed upon that the content of both lignin and xylan in the plant cell wall affect the degree of enzymatic hydrolysis, there is still debate on the relative importance of the removal of one of these components over the other. In acid-catalyzed and autohydrolysis pretreatment, removal of structural xylan appears to have a linear correlation with enzymatic digestibility up to approximately 80 % conversion at which point other unknown factors play a stronger role in cellulose accessibility [15]. Within the domain of alkaline pretreatment chemistries, there is some debate as to whether delignification has a more significant impact on enzymatic hydrolysis and enzymatic digestibility [16, 23] or if decreasing cellulose crystallinity and increasing surface area are a more important contributor [25]. It is clear that the relationship between lignin and hemicellulose in the cell wall superstructure and how it pertains to cellulose accessibility are not entirely understood, as recent work at the National Renewable Energy Laboratory (NREL) suggests that near complete removal of both lignin and xylan dramatically reduces enzyme accessibility [14].

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NMR spectroscopy is a popular technique for experimental studies on lignin as it allows for detailed analysis of lignin structural bonds, functional moieties, and can even be quantitative [3, 8, 13]. As such there have been many publications on the application of NMR spectroscopy to understanding lignin structure and its association with cellulose and hemicellulose in plant cell walls [5]. A majority of the studies in existing literature have primarily applied these methods to lignin isolated from softwood or hardwood biomass [1, 10, 11, 13, 28]. Furthermore, there is evidence to support that differences in distribution of lignin in the plant cell wall and composition of lignin between woody biomass and herbaceous biomass can have a significant effect on enzyme–lignin interactions during hydrolysis [24]. More recently, the trend has been to apply the same techniques to lignocellulosic biomass feedstocks in the context of a biochemical conversion process to better understand the role of lignin in biomass recalcitrance. Such studies have considered dilute acid pretreated switchgrass lignin [27], wheat straw lignin [9], and even a model *Arabidopsis* lignin after hot water pretreatment [20], but there has been very little work carried out with moderate enzyme loadings on a well-characterized model feedstock such as corn stover. While bioenergy crops such as switchgrass or giant miscanthus may eventually offer increased ethanol yields, drought resistance, or broader growing conditions, corn stover has been the most widely studied substrate for bioethanol processes owing to its ready availability and proximity to existing ethanol production facilities. In this study, the relationship between xylan solubilization and lignin degradation during dilute acid pretreatment of corn stover was compared against the effect on enzymatic digestibility of the cellulose solids and changes of the lignin chemical structure as determined by NMR.

## Materials and methods

### Feedstock collection and preparation

Corn stover residue consisting of leaves, stalks, and husks that remained after harvest of grain was cut 18 inches above the ground and collected in Franklin County, NC. The raw corn stover was dried at 45 °C for 72 h and milled in a Thomas Model 10 Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA) with a nominal screen size of 2 mm. The 2-mm milled stover was thoroughly washed with tap water to remove any silica or non-stover debris from the substrate. The washed stover was then allowed to dry in a 45 °C convection oven until the moisture content of the solids was above 90 % total solids (% TS). Finally, the dried solids were sieved through a #60 mesh screen to ensure a uniform size distribution of the particles, and the fines were discarded.

### Pretreatment using Dionex ASE 350

To screen dilute acid pretreatment conditions, a Dionex Accelerated Solvent Extractor (ASE) 350 (Dionex, Sunnyvale, CA, USA) instrument was used. Approximately 20.0 g of the washed, ground, dry, sieved corn stover was packed into a 100-mL stainless steel extraction cell (Dionex, Sunnyvale, CA, USA) for each of the pretreatment conditions tested to ensure consistent solids loading during the pretreatment. The ASE 350 was programmed to run in pressure solvent saver mode for all the pretreatments; in this mode the instrument will load the extraction cell into the heating chamber and fill the cell with the selected solvent until the back-pressure of the cell reaches 1,500 psi, at which point the pump will shut off and no additional liquid will be pumped into the cell. After the cell has been filled the heating chamber will begin to heat the cell to the desired temperature. Although the operation of the instrument did not allow for precise control over the volume of acid in the reaction cell throughout the pretreatment, owing to safety valves that vented pressure from the cell at 1,700 psi, it was found to be highly reproducible between runs. The initial total solids content in each of the reaction cells was calculated to be between 21.3 and 22.0 % after the cells were filled with the appropriate acid solution at the start of the pretreatment. Ramp-up heating curves have been precalculated by Dionex, on the basis of the geometry of the selected extraction cells, and are automatically accounted for in the operation of the instrument. Static pretreatment reaction time began the moment the internal temperature of the extraction cell reached thermal equilibrium with the heating chamber. At the end of the static residence phase, the pretreatment was stopped by immediately releasing the pressure on the extraction cell and rinsing the cell with 10 % cell volume (i.e., 10 mL) of the extraction solvent. After the rinse stage, the cell was purged with nitrogen gas for 60 s, and the pretreatment liquor was collected in a designated glass vial. The extraction cell was then immediately removed from the ASE 350 and quenched in an ice bath with a false bottom to allow water to drain from the bath. After the extraction cell cooled to room temperature, the pretreated solids were removed from the cell and collected. A small aliquot of the pretreatment liquor (ca. 5 mL) was reserved for HPLC analyses to profile soluble sugars and degradation products as described below, and the remainder of the pretreatment liquor was reslurried with the corresponding pretreated solids.

The pretreatment conditions tested were selected as being similar to the dilute acid pretreatment conditions

used in NREL's 2011 report on process design and economics for corn stover to ethanol biochemical processes [13]. Pretreated corn stover was generated at temperatures between 140 and 190 °C with residence times between 1 and 5 min and in the presence of 1.0 % (v/v) $H_2SO_4$.

### Enzymatic hydrolysis at hybridization incubator scale

Enzymatic hydrolysis of the pretreated corn stover (PCS) was performed in 40-mL Nalgene Oak Ridge centrifuge tubes with sealing caps (Thermo Scientific, Pittsburg, PA, USA). The PCS slurry was first adjusted to pH 5.0 by addition of 28 % wt. ammonium hydroxide solution and then buffered to a final concentration of 75 mM with sodium acetate/acetic acid buffer. The final total solids content during hydrolysis was adjusted to 10 % (w/w) with the addition of tap water. Cellic CTec or an equal weight of additional tap water as a control was dosed at 2.5 % (wt-product/g-cellulose) in each of the samples in duplicate. The hydrolysis reactions were performed in a hybridization incubator (Combo-D24, FINEPCR®, Yang-Chung, Seoul, Korea) with inversion mixing at 50 °C for 144 h with 0.5-g samples taken at 0, 72, 120, and 144 h to monitor hydrolysis performance. Each of the samples taken throughout hydrolysis was transferred into a Costar Spin-X centrifuge filter tube with 0.2-μm nylon membrane (Corning Life Sciences, Lowell, MA, USA) and centrifuged at 14,000×*g* for 5 min. The filtrate was acidified with 5 μL of 40 % (w/v) sulfuric acid to deactivate any residual enzyme activity and then analyzed by HPLC as described below. After 120 h of total hydrolysis time each of the remaining hydrolysate samples was dosed with an additional 35 % (wt-product/g-cellulose) Cellic CTec or tap water and returned to the hybridization incubator under the same conditions for 48 h to determine the maximum enzymatic cellulose digestibility of each sample. Total hydrolysis yields were calculated by the method described by Zhu et al. [35].

### Feedstock composition, sugars and degradation products quantification

The total solids content, fraction of insoluble solids, structural carbohydrates composition, and lignin content of both the raw and dilute acid pretreated corn stover solids were determined following the NREL standard laboratory analytical procedures [12, 30–32].

Monomeric sugars were measured by an Agilent 1,200 series modular HPLC with quaternary pump, thermostatted autosampler, temperature-controlled column compartment, and refractive index detector (Santa Clara, CA, USA). Separation was performed at 80 °C with a Bio-Rad Aminex HPX-87P column, 300 × 7.8 mm (Bio-Rad, Hercules, CA, USA) with anion/cation Micro-Guard De-Ashing cartridges and Micro-Guard Carbo-P cartridges. The separation was run isocratically with a mobile phase of deionized water (≥18.2 MΩ cm) at a flow rate of 0.6 mL/min. The concentrations of glucose, mannose, xylose, galactose, and arabinose were measured in each sample on a peak area basis by refractive index with an external calibration set made up of the same compounds.

The profile of degradation products in the pretreatment liquor was quantified in a similar manner to the monomeric sugars above but with the following differences. Separation was performed at 65 °C with a Bio-Rad Aminex HPX-87H column, 300 × 7.8 mm with Micro-Guard Cation H cartridge. The separation was run isocratically at 0.6 mL/min with a mobile phase of 5 mM sulfuric acid in deionized water that was then filtered through a 0.2-μm PTFE membrane filter. A standard curve consisting of glucose, xylose, arabinose, formic acid, acetic acid, levulinic acid, ethanol, 5-hydroxymethylfurfural, and 2-furaldehyde (Sigma-Aldrich, St. Louis, MO, USA) was used to measure sample concentrations on a peak area basis by refractive index.

### Collection of residues from PCS prepared under varying severity

The hydrolysate residues from the different PCS preparations were collected after completion of enzymatic hydrolysis and washed with acid and salt solutions to remove residual carbohydrates and enzymes from the lignaceous residues. Each sample was washed by mixing 20 parts hydrolysate residues to 20 parts 2.5 mol/L KCl solution and 1 part 4.0 % $H_2SO_4$ and vortexing to ensure adequate mixing before being centrifuged at 3,000×*g* for 10 min after which the samples were decanted. Subsequently the hydrolysate residues were washed with an additional 50 parts of a prepared solution of 1.2 mol/L KCl and 1.9 % $H_2SO_4$ to 20 parts residue which was then vortexed and centrifuged as above with the supernatant decanted and discarded. The same procedure was repeated an additional three times replacing the acidified KCl solution with 0.01 % $H_2SO_4$. Finally the washed residues were placed in an isotemp vacuum oven (Thermo Fisher Scientific, Waltham, MA, USA) at 50 °C under 22 in Hg vacuum for 72 h before being pulverized and mixed.

### Lignin phosphitylation for $^{31}$P NMR characterization

The phosphitylation procedure was adapted from Argyropoulos et al. [2, 4]. Approximately 45 mg of the dried, washed lignaceous residue from each pretreatment severity was weighed into a tared glass vial with the weight recorded to the nearest 0.1 mg to which was added a small

magnetic stir bar. The samples were phosphitylated by reacting each of the residues stepwise with the addition of 300 μL *N*,*N*-dimethylformamide (DMF) and 200 μL deuterated pyridine ($PyD_5$) which was left to mix for 2 h at room temperature before adding 200 μL of a prepared internal standard solution, 125 μL of the derivitizing agent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP), 50 μL chromium acetylacetonate ($Cr(acac)_3$) solution, and 125 μL deuterated chloroform ($CDCl_3$). The internal standard solution used was prepared by mixing 9.05 mg *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide (e-HNDI) in 1 mL of $PyD_5/CDCl_3$ (1.6:1, v/v). The $Cr(acac)_3$ relaxation agent solution consisted of 11.4 mg chromium(III) acetylacetonate in 1 mL of $PyD_5/CDCl_3$ (1.6:1, v/v). After phosphitylation, each sample was centrifuged and the supernatant collected and transferred to a 5-mm NMR tube (Kimble Chase, Vineland, NJ, USA).

### Quantitative $^{31}P$ NMR spectroscopy characterization

Quantitative $^{31}P$ NMR spectra of lignins were obtained using a Varian 400-MHz spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a Quad probe dedicated to $^{31}P$, $^{13}C$, $^{19}F$, and $^{1}H$ acquisition. The instrument operates in the Fourier transform mode at 161.9 MHz and uses an inverse-gated decoupling sequence. A pulse width causing a 90° flip angle was used. The delay time was 3 s between pulses, owing to the use of $Cr(acac)_3$ as a relaxation agent, and the number of acquisition scans was 768. All the chemical shifts reported are relative to the reaction product of water with TMDP, which gives a sharp signal at 132.2 ppm relative to the external standard of 85 % $H_3PO_4$.

## Results and discussion

### Dilute acid pretreatment of corn stover

Pretreatment severity is often defined as the combined effect of acid charge, reaction temperature, and residence time on a lignocellulosic feedstock during a thermochemical reaction [29]. In this experiment the acid charge was kept constant at 1.0 % (v/v) $H_2SO_4$ and therefore severity only varied with residence time and reaction temperature within the confines of this setup. In Fig. 1, there appears to be a correlation between monomeric xylose and glucose solubilized from the corn stover cell wall and the severity of the pretreatment. This relationship between glucan and xylan removal and pretreatment severity agrees with previous studies conducted under similar conditions [17]. Monomeric arabinose concentrations remained largely constant throughout the range of severities indicating that the arabinoxylan linkages were the most acid-labile cell wall polysaccharide bond in corn stover and were completely solubilized under the lowest severity conditions included in the scope of this work. No degradation of soluble arabinose in the pretreatment liquor was detected. Xylose solubilization generally increased with severity to a maximum concentration of 24.6 g/L at pretreatment solids

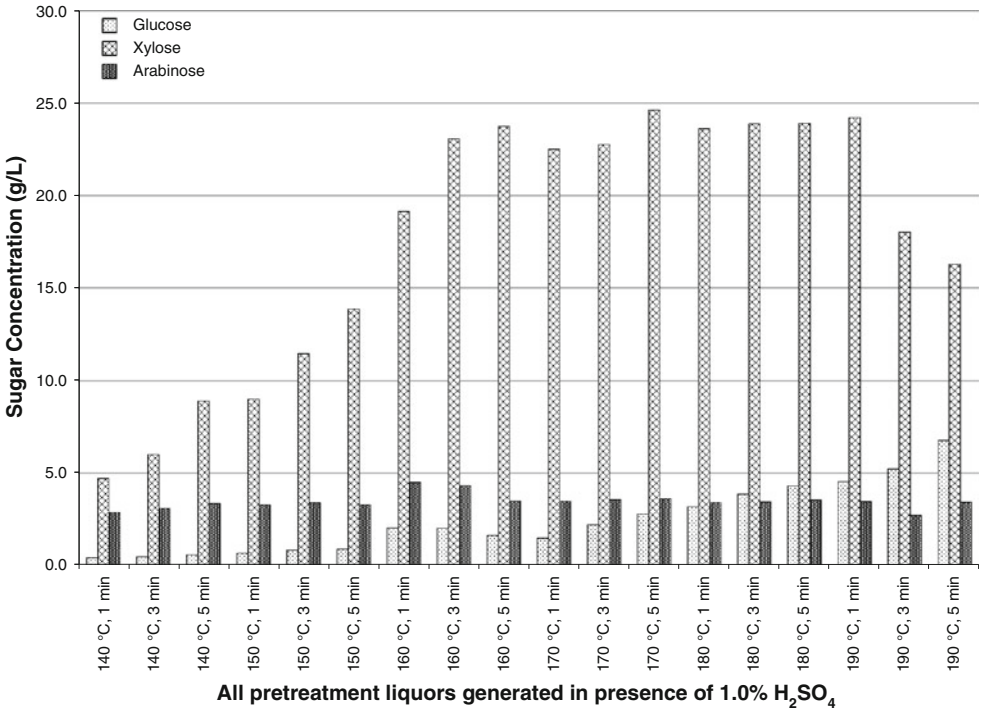


**Fig. 1** Monomeric sugar concentration in dilute acid pretreatment liquor of corn stover. All pretreatment liquors generated in presence of 1.0 % $H_2SO_4$

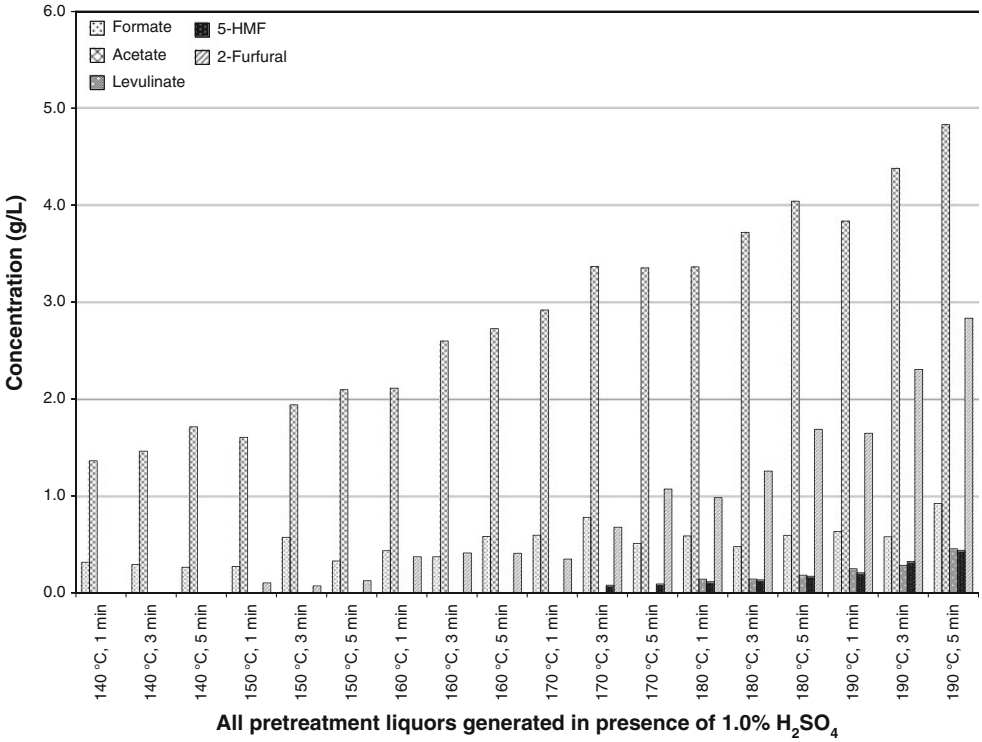


**Fig. 2** Degradation product profile concentration in dilute acid pretreatment liquor of corn stover. All pretreatment liquors generated in presence of 1.0 % $H_2SO_4$

loading of 22.0 %, likely representing complete hydrolysis of the hemicellulose at all pretreatment conditions above 160 °C, 3 min residence time, and 1.0 % (v/v) $H_2SO_4$ charge. The pretreatment severity window that allowed efficient hemicellulose solubilization without significant monomeric xylose degradation extended in this experiment to 190 °C, 1 min residence time, and 1.0 % (v/v) $H_2SO_4$ charge, after which the rate of xylose degradation significantly increased and was marked with a proportionally large increase in 2-furfural concentration (Fig. 2). A degradation product profile of formic acid, acetic acid, levulinic acid, 5-(hydroxymethly)furfural (HMF), and 2-furaldehyde (furfural) was used as a measure of overall sugar degradation, representing the major primary and secondary degradation products of pentoses and hexoses in corn stover. Furfural is the primary degradation product of xylose upon heating in the presence of sulfuric acid [22] and was observed in all the pretreatment liquor samples produced from conditions exceeding 150 °C with concentrations most rapidly increasing at temperatures above 180 °C.

## Enzymatic hydrolysis

Optimal cellulose conversion of the PCS solids (76.4 % after 144 h) was achieved with pretreatment conditions of 180 °C, 1 min residence time, and 1.0 % (v/v) $H_2SO_4$ charge (Fig. 3). All pretreatment conditions screened between 170 and 180 °C within the pretreatment residence times examined allowed for cellulose conversions greater than 70 %, although a pretreatment temperature of at least 180 °C was necessary to achieve complete cellulose digestibility (Fig. 4). The highest severities produced solids which were more recalcitrant than those from lower severities indicating that some chemical or morphological change had occurred to the cellulose or lignin that had either decreased the enzyme affinity to the cellulose substrate or decreased the cellulose surface area available for enzymatic hydrolysis. Monomeric xylose concentration in the pretreatment liquors is a good indication of the degree of xylan hydrolysis that occurred from pretreatment and appeared to be related to cellulose digestibility, indicating that the structural xylan does play a role in the physical exclusion of cellulases from the cellulose surface (Fig. 4). These results are consistent with those presented by Zhu et al. [34] who showed that the residual xylan content of dilute acid pretreated aspen wood chips strongly correlated to the enzymatic digestibility of the resultant solid substrates over a wide range of pretreatment severities. The maximum concentration of monomeric xylose corresponded to the pretreatment conditions 160 °C, 3 min, and 1 % $H_2SO_4$; however, maximum cellulose digestibility was only achieved at higher severity pretreatments suggesting that other factors such as lignin condensation reactions, crystallinity of the cellulose, or enzyme–lignin interactions also play a role in enzymatic cellulose digestibility. Zhu et al. [33] illustrated that optimizing both glucan and xylan

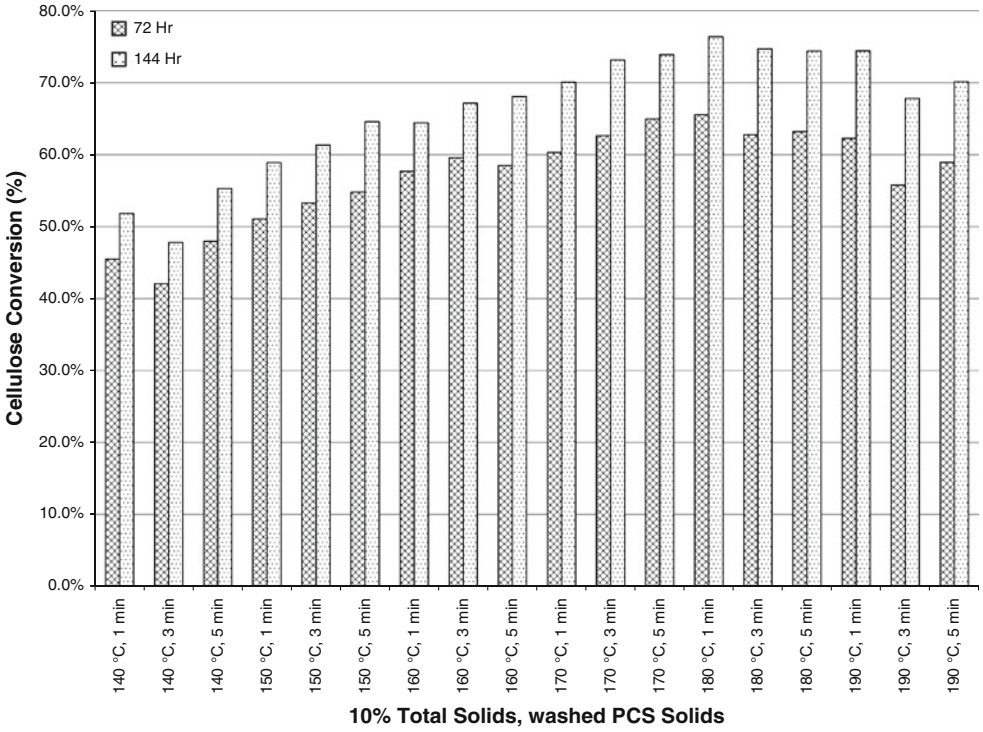


**Fig. 3** Cellulose conversion of washed PCS in enzymatic hydrolysis assay with low enzyme dose. 10 % total solids, washed PCS solids

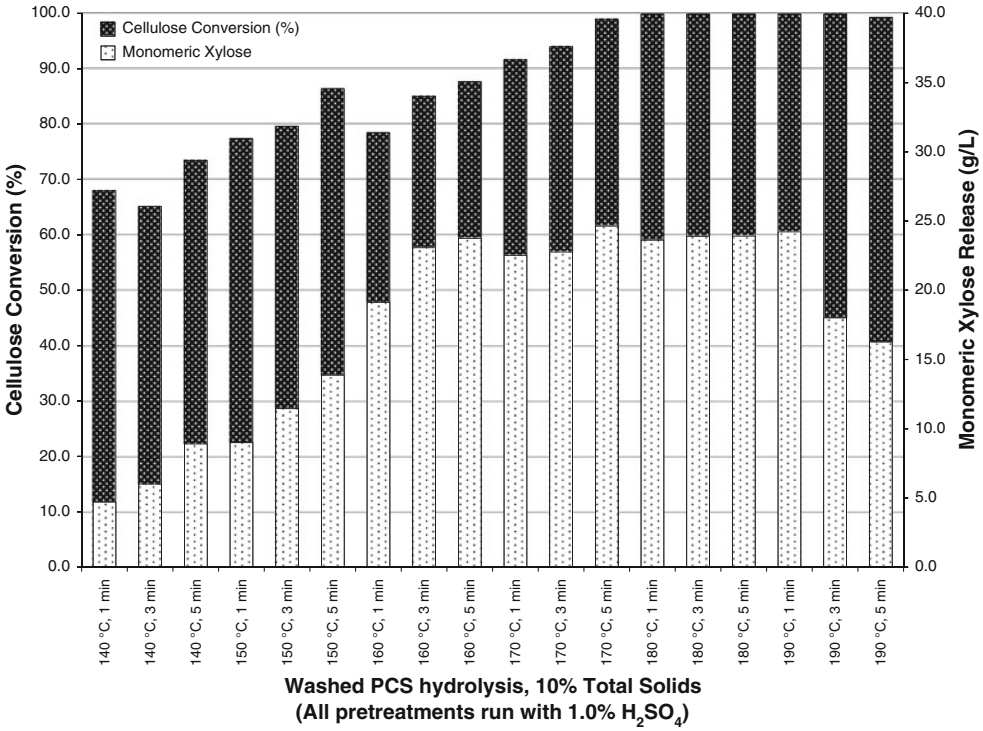


**Fig. 4** Relationship between xylan solubilization during dilute acid pretreatment and total cellulose digestibility after continuous hydrolysis with high enzyme dose. Washed PCS hydrolysis, 10 % total solids. All pretreatments run with 1.0 % $H_2SO_4$

yields without sacrificing monomeric sugars to degradation in a single-stage dilute acid pretreatment system is not possible. Efficient dilute acid pretreatment of corn stover is therefore not only a balance between hemicellulose solubilization, reduction of cellulose microfibril crystallinity, and minimization of degradation product formation but also a function of lignin and cellulose structural changes that occur during pretreatment.

## Lignin characterization by $^{31}$P NMR of pretreated samples of varying severity

It is well understood that the lignin in lignocellulosic material can decrease cellulolytic activity of enzymes on pretreated biomass via both physical mechanisms such as blocking the enzyme from the substrate or non-productive binding of the enzyme to the lignin [7, 16]. Berlin et al. [6] demonstrated that the chemical composition of lignin also affected enzymatic hydrolysis of cellulose and xylan. Preparations of softwood organosolv lignin were reported to be significantly more inhibitory to endoglucanase, xylanase, and $\beta$-glucosidase complexes compared to residual lignin from the same feedstock. It was believed that this effect was due to ionic-type lignin–enzyme interactions in the presence of charged or partially charged carboxyl or hydroxyl functional groups. From this perspective it would be interesting to investigate the changes in the lignin chemical functionalities as they relate to the severity of the pretreatment and to correlate enzyme performance on pretreated corn stover. Lignin residues from pretreated corn stover produced at various severities were collected after extensive enzymatic hydrolysis and characterized by $^{31}$P NMR. Only the pretreated corn stover hydrolysate produced from 1.0 % (v/v) $H_2SO_4$, 3 min reaction time, and temperatures between 140 and 190 °C was considered for NMR analysis. The isolated lignin was phosphitylated with TMDP to label the hydroxyl groups prior to the quantitative $^{31}$P NMR, following procedures adapted from Argyropoulos et al. [2, 4]. This lignin NMR characterization allows for the identification and quantification of aliphatic, phenolic hydroxylic, and carboxylic functional groups. Within the phenolic groups it is possible to distinguish the syringyl (S), guaiacyl (G), *p*-hydroxyphenyl (H), and condensed structural units by their relative chemical shift as compared to a standard of 85 % $H_3PO_4$. In Fig. 5, a lignin structure proposed by Sakakibara in 1980 [26] is presented and indicates the different type of hydroxyl groups that can be phosphitylated for $^{31}$P NMR quantitative characterization.

The quantity of aliphatic hydroxyl moieties in the isolated lignins showed no significant variation with the pretreatment severity, whereas the total phenolic hydroxyl groups increased with severity (Fig. 6). This may be a result of cleavage of the aryl ether lignin bonds, leading to lower molecular weight lignin fragments. These trends were also observed in the steam exploded pretreatments of aspen, investigated by Li et al. [19]. The carboxylic moieties in the isolated lignins increased with the pretreatment severity (Fig. 6) which is believed to be at least partially due to the cleavage of the ester bonds from lignin–carbohydrate complexes, thus releasing the carboxylic functionalities. Another

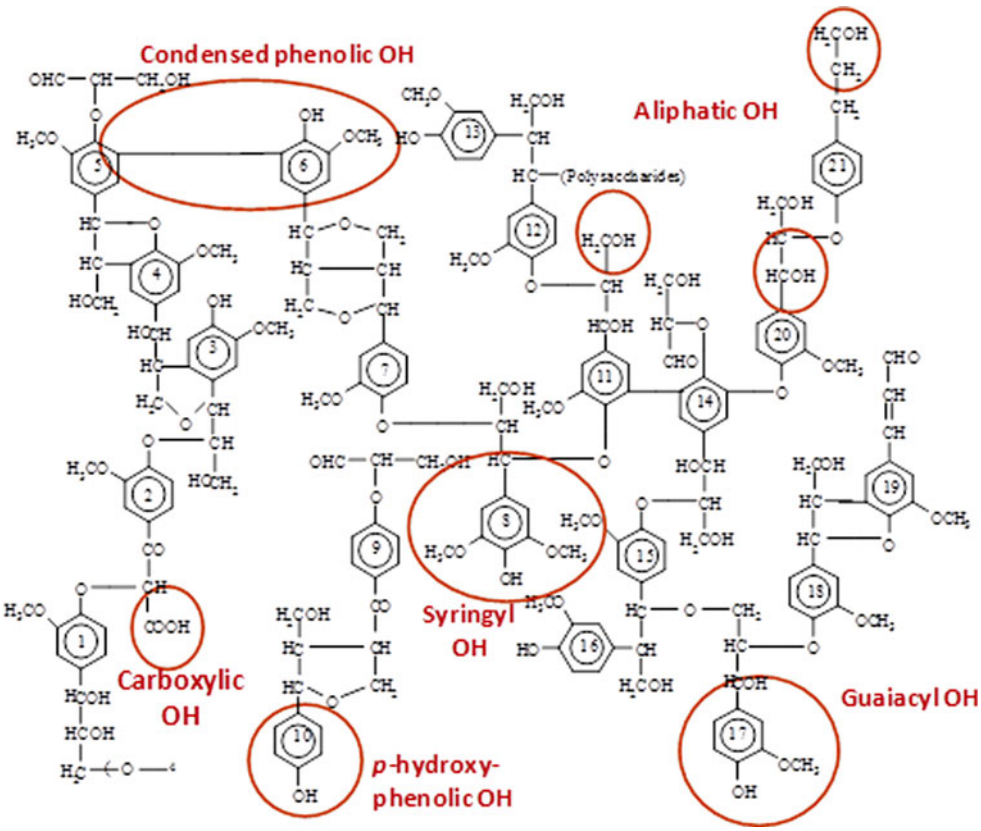


**Fig. 5** Lignin structure proposed by Sakakibara [26] and the different types of hydroxyl groups that can be phosphitylated for $^{31}$P NMR quantitative characterization

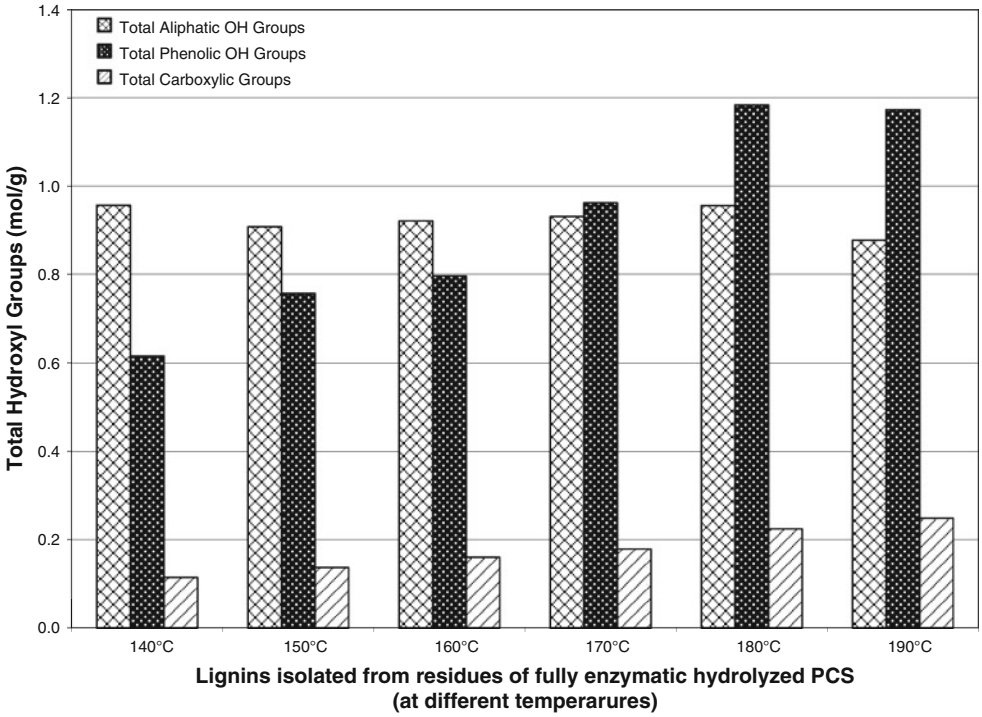


**Fig. 6** $^{31}$P NMR quantified hydroxyl groups (total aliphatic, total phenolic, and total carboxylic hydroxyl moieties) in lignins isolated from the residues of fully enzymatic hydrolyzed PCS (at different temperatures)

possible reason for the increase of carboxylic groups could be oxidation, but in this case the pretreatments were performed mainly in an inert atmosphere (nitrogen blanketing). There was a linear correlation between the measured amount of carboxylic moieties in the isolated lignin and enzymatic hydrolysis yields of the washed pretreated solids (Fig. 6) which agrees with the findings presented by Nakagame et al. in 2010 [21] where the presence of carboxylic acid isolated from softwood lignin improved cellulose digestibility in an Avicel hydrolysis assay. It has been suggested that the presence of carboxylic acids might reduce the negative effects of lignin on enzymatic hydrolysis yields, but an alternative explanation may be that carboxylic moieties resulting from cleavage of the lignin structure are an indicator of reduction of the molecular weight of the polymer owing to lignin degradation and thereby allow increased enzymatic accessibility to the cellulose (Fig. 7).

Quantification of the different non-condensed phenolic groups (i.e., syringyl, guaiacyl, *p*-hydroxyphenolic, and catechol) and condensed phenolic moieties in the enzymatic hydrolysis PCS lignin residues is presented in Fig. 8. Among the different phenolic functional groups there was a general trend of linearly increasing with the pretreatment severity. However the rate of increase between the function groups was not consistent, as the rate of increase of syringyl groups was greater than that for guaiacyl moieties. Another way to describe this tendency is to examine the ratio between syringyl and guaiacyl (S/G) with the increase in severity. At the lower severity range, the S/G ratio was less than 1, whereas with the increase in severity, the S/G ratio gradually increased to greater than 1 ($S/G < 1 \rightarrow S/G > 1$). It is likely that this was due to the degradation of $\beta$-O-4 linkages and consequently the increased severity caused a release of more syringyl units than guiacyl units. It has been reported that the $\beta$-aryl ether model compound of syringyl type reacted much faster than the guaiacyl model by a factor of 2.4 [18]. Another possibility is that perhaps more guaicyl units and *p*-hydroxylphenol units were involved in condensation reactions on the $C_6$ position owing to less steric hindrance. Condensation reactions in lignin during neutral pretreatments were also suggested by Li et al. [19]. All of the phenolic units (S, G, and H) reached a maximum at pretreatment conditions corresponding to 180 °C, 3 min and then started to decline with further increases in severity. However, even at severities above this condition the condensed phenolic lignin units still increased somewhat. This suggests that the severity/condition of 180 °C, 3 min was the transition point at which degradation of the lignin dominates condensation reactions. At lower severities, degradation reactions were favored, whereas at higher severities there were more condensation reactions. It is conceivable that the optimal pretreatment should limit excessive condensation reactions, as the repolymerization could prevent enzyme access to the cellulose surface. As shown in Fig. 5, the enzyme digestibility of cellulose decreased when the pretreatment temperature increased from 180 to 190 °C which could be partially explained by lignin condensation reactions.

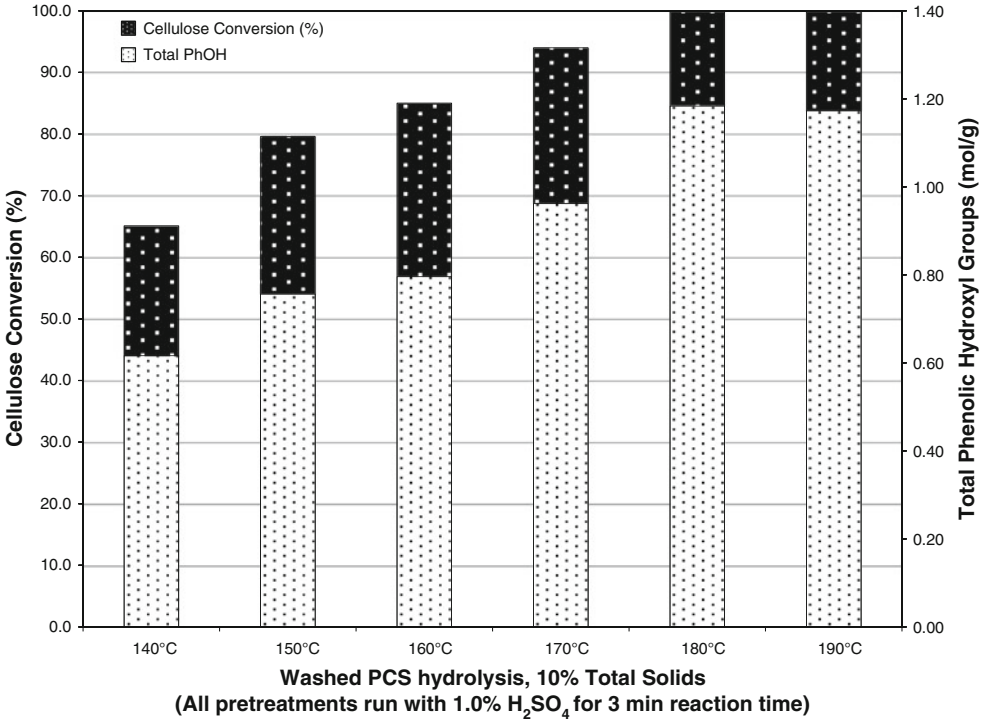


**Fig. 7** Relationship between total cellulose accessibility and total phenolic hydroxyl groups of lignin after dilute acid pretreatment. Washed PCS hydrolysis, 10 % total solids. All pretreatments run with 1.0 % $H_2SO_4$ for 3 min reaction time

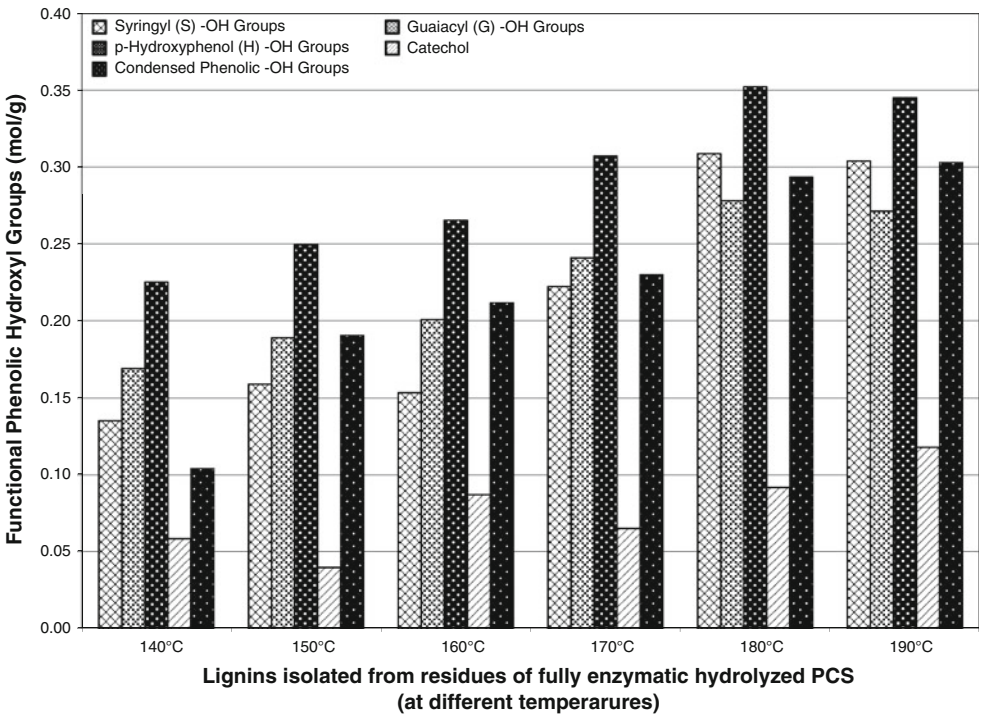


**Fig. 8** $^{31}P$ NMR quantified syringyl (S), guaiacyl (G), *p*-hydroxyphenolic (H), catechol, and condensed phenolic hydroxyl moieties in lignin isolated from residues of fully enzymatic hydrolyzed PCS (at different temperatures)

The role of xylan versus lignin degradation

Figure 4 shows the significance of xylan degradation on enzyme digestibility of the substrate. The digestibility of the cellulose to enzymes increased with xylan degradation, which is consistent with more monomeric xylose present in the liquor. However, the concentration of xylose leveled off at pretreatment conditions of 160 °C for 3 min and the enzymatic digestibility continued to increase with an increase in severity. Enzymatic digestibility was correlated to total phenolic groups shown in Fig. 7. These findings suggest that degradation of $\beta$-O-4 units and formation of phenolic units are very important to open up the recalcitrant lignin structure and improve the enzymatic

digestibility of lignocellulosic substrates. Perhaps within the lower severity range, xylan degradation is more important. However, at the higher severity range, especially after maximum xylan removal is obtained, lignin degradation becomes more critical for increased enzyme digestibility.

## Concluding remarks

Cellulose digestibility increased steadily with pretreatment severity and reached a maximum at a critical temperature independent of residence time. These findings suggest that the effect of xylan modification and/or removal should be correlated to enzymatic hydrolysis performance. Characterization of the lignin from hydrolyzed PCS residues with $^{31}$P NMR has revealed several insights into the nature of enzyme–lignin interactions in pretreated corn stover. While no significant variation of the number of aliphatic hydroxyl groups with the pretreatment severity existed, the number of phenolic hydroxyl groups increased with the pretreatment severity, likely resulting from the cleavage of the aryl ether lignin bonds and leading to lower molecular weight lignin fragments. This appeared to be significant for the degree of enzymatic digestibility of the cellulose and in turn overall hydrolysis yields. Additionally, the number of carboxylic moieties increased with the pretreatment severity, owing to the plausible cleavage of the ester bonds from lignin–carbohydrate complexes.

**Conflict of interest** The authors declare that the experiments comply with the current laws of the USA. The authors also declare that they have no conflicting interests.

## References


1. Ämmälahti E, Brunow G, Bardet M, Robert D, Kilpeläinen I (1998) Identification of side-chain structures in a poplar lignin using three-dimensional HMQC-HOHAHA NMR spectroscopy. J Agric Food Chem 46:5113–5117
2. Argyropoulos DS (1994) Quantitative phosphorus-31 NMR analysis of six soluble lignins. J Wood Chem Technol 14:65–82
3. Argyropoulos DS (2010) Heteronuclear NMR spectroscopy of lignins. In: Heitner C, Dimmel DR, Schmidt JA (eds) Lignin and lignans: advances in chemistry. CRC, Boca Raton, pp 245–265
4. Argyropoulos DS, Bolker HI, Heitner C, Archipov Y (1993) 31P NMR spectroscopy in wood chemistry. Part IV. Lignin models: spin lattice relaxation times and solvent effects in 31P NMR. Holzforschung 47:50–56
5. Balakshin M, Capanema E, Gracz H, Chang HM, Jameel H (2011) Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy. Planta 233:1097–1110
6. Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J (2006) Inhibition of cellulase, xylanase and $\beta$-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198–209
7. Berlin A, Gilkes N, Kurabi A, Bura R, Tu M, Kilburn D, Saddler J (2005) Weak lignin-binding enzymes. Appl Biochem Biotechnol 121:163–170
8. Capanema EA, Balakshin MY, Kadla JF (2004) A comprehensive approach for quantitative lignin characterization by NMR spectroscopy. J Agric Food Chem 52:1850–1860
9. Crestini C, Argyropoulos DS (1997) Structural analysis of wheat straw lignin by quantitative 31P and 2D NMR spectroscopy. The occurrence of ester bonds and $\alpha$-O-4 substructures. J Agric Food Chem 45:1212–1219
10. Ede RM, Brunow G (1992) Application of two-dimensional homo- and heteronuclear correlation NMR spectroscopy to wood lignin structure determination. J Org Chem 57:1477–1480
11. Guerra A, Filpponen I, Lucia LA, Saquing C, Baumberger S, Argyropoulos DS (2006) Toward a better understanding of the lignin isolation process from wood. J Agric Food Chem 54:5939–5947
12. Hames B, Ruiz R, Scarlata C, Sluiter A, Sluiter J, Templeton D (2008) Preparation of samples for compositional analysis. NREL/TP-510-42620
13. Humbird D, Davis R, Tao L, Kinchin C, Hsu D, Aden A, Schoen P, Lukas J, Olthof B, Worley M, Sexton D, Dudgeon D (2011) Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol: dilute-acid pretreatment and enzymatic hydrolysis of corn stover
14. Ishizawa C, Jeoh T, Adney W, Himmel M, Johnson D, Davis M (2009) Can delignification decrease cellulose digestibility in acid pretreated corn stover? Cellulose 16:677–686
15. Jeoh T, Ishizawa CI, Davis MF, Himmel ME, Adney WS, Johnson DK (2007) Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnol Bioeng 98:112–122
16. Kim S, Holtzapple MT (2006) Effect of structural features on enzyme digestibility of corn stover. Bioresour Technol 97:583–591
17. Kobayashi T, Kohn B, Holmes L, Faulkner R, Davis M, Maciel GE (2011) Molecular-level consequences of biomass pretreatment by dilute sulfuric acid at various temperatures. Energy Fuels 25:1790–1797
18. Lai Y-Z (1990) Chemical degradation. In: Hon DNS, Shiraishi N (eds) Wood and cellulosic chemistry. Marcel Dekker, New York, pp 455–523
19. Li J, Henriksson G, Gellerstedt G (2007) Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. Bioresour Technol 98:3061–3068
20. Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C (2010) Lignin monomer composition affects *Arabidopsis* cell-wall degradability after liquid hot water pretreatment. Biotechnol Biofuels 3:1–7
21. Nakagame S, Chandra RP, Kadla JF, Saddler JN (2011) Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. Biotechnol Bioeng 108:538–548
22. Oefner PJ, Lanziner AH, Bonn G, Bobleter O (1992) Quantitative studies on furfural and organic acid formation during hydrothermal, acidic and alkaline degradation of D-xylose. Monatshefte für Chemie/Chem Mon 123:547–556
23. Pan X, Xie D, Gilkes N, Gregg D, Saddler J (2005) Strategies to enhance the enzymatic hydrolysis of pretreated softwood with high residual lignin content. Appl Biochem Biotechnol 124:1069–1079
24. Piccolo C, Wiman M, Bezzo F, Lidén G (2010) Enzyme adsorption on SO2 catalyzed steam-pretreated wheat and spruce material. Enzym Microb Technol 46:159–169
25. Rollin JA, Zhu Z, Sathitsuksanoh N, Zhang Y-HP (2011) Increasing cellulose accessibility is more important than removing lignin: a comparison of cellulose solvent-based lignocellulose

fractionation and soaking in aqueous ammonia. Biotechnol Bioeng 108:22–30
26. Sakakibara A (1980) A structural model of softwood lignin. Wood Sci Technol 14:89–100
27. Samuel R, Pu Y, Raman B, Ragauskas A (2010) Structural characterization and comparison of switchgrass ball-milled lignin before and after dilute acid pretreatment. Appl Biochem Biotechnol 162:62–74
28. Sannigrahi P, Ragauskas A, Miller S (2008) Effects of two-stage dilute acid pretreatment on the structure and composition of lignin and cellulose in loblolly pine. BioEnergy Res 1:205–214
29. Schell D, Farmer J, Newman M, McMillan J (2003) Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor. Appl Biochem Biotechnol 105:69–85
30. Sluiter A, Hames B, Hyman D, Payne C, Ruiz R, Scarlata C, Sluiter J, Templeton D, Wolfe J (2008) Determination of total solids in biomass and total dissolved solids in liquid process samples. NREL/TP-510-42621
31. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D (2008) Determination of structural carbohydrates and lignin in biomass. NREL/TP-510-42618
32. Sluiter A, Payne C, Hyman D, Wolfe J (2008) Determination of insoluble solids in pretreated biomass material. NREL/TP-510-42627
33. Zhu J, Verrill S, Liu H, Herian V, Pan X, Rockwood D (2011) On polydispersity of plant biomass recalcitrance and its effects on pretreatment optimization for sugar production. BioEnergy Res 4:201–210
34. Zhu W, Houtman CJ, Zhu JY, Gleisner R, Chen KF (2012) Quantitative predictions of bioconversion of aspen by dilute acid and SPORL pretreatments using a unified combined hydrolysis factor (CHF). Process Biochem 47:785–791
35. Zhu Y, Malten M, Torry-Smith M, McMillan JD, Stickel JJ (2011) Calculating sugar yields in high solids hydrolysis of biomass. Bioresour Technol 102:2897–2903