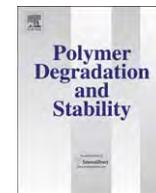




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Structural changes in switchgrass lignin and hemicelluloses during pretreatments by NMR analysis

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ABSTRACT

Switchgrass is currently being developed as a sustainable bio-energy crop due to its broad adaptability, high mass yield and low agricultural input. Its current conversion to biofuels is detrimentally impacted by its native recalcitrance which is typically addressed using chemical and/or biological pretreatments. In this study, extractives free switchgrass was pretreated with steam, dilute H₂SO₄ and lime at 160 °C for 1 h. The degradation and impact of pretreatment was estimated semi-quantitatively by ¹³C–¹H HSQC (heteronuclear single quantum coherence) NMR analysis of ball milled untreated and pretreated switchgrass samples in perdeuterated pyridinium chloride–DMSO-d₆ solvent system. As a result of steam pretreatment the resulting switchgrass was depleted of xylan and a slight degradation of lignin were observed. This was confirmed by the relative decrease of cross peak intensity for β-aryl ether, phenylcoumaran, resinol and dibenzodioxocin units. Significant structural changes observed due to the lime pretreatment of switchgrass was deacetylation/dissolution of hemicellulose and the extent of delignification was less however, a preferential removal *p*-hydroxy of benzoyl ester, ferulate and coumarate type linkages were notified from the HSQC studies. Finally the most significant degradation resulted in acid pretreatment involving ~90% loss of hemicellulose and a substantial degradation of various lignin sub-units. These results are further supported by the composition analysis of the respective switchgrass samples.

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1. Introduction

Lignocellulosic material such as agricultural and forestry residues, herbaceous energy crops and woody biomass, composed of cellulose, hemicellulose and lignin, are attractive feed stocks for renewable biofuels [1–4]. Conversion of lignocellulosics to ethanol is a challenging pathway due to the recalcitrance of biomass. The well documented method for eliminating recalcitrance is pretreatment, which enhances the efficiency of enzymatic hydrolysis by dramatically altering the structure of the plant cell wall, including a decrease in the degree of polymerization of cellulose, increasing the porosity of biomass, removing lignin and hemicellulose [5,6]. A variety of chemical, physical and biological pretreatments have been explored in order to address the technical and economical requirements of effective pretreatment of biomass [7,8]. Biological

pretreatments using polysaccharide and/or lignin degrading enzymes result in partial delignification, dissolution of hemicelluloses and pectin substances in lignocelluloses [9,10]. Whereas, chemical pretreatments have been optimized to solubilize hemicelluloses, disrupt lignin and expose cellulose for enzymatic hydrolysis. Physical pretreatments such as milling and microwave irradiation have been reported to enhance cellulase biomass deconstruction by decreasing cellulose crystallinity and its degree of polymerization [11,12].

Switchgrass is considered a promising lignocellulosic feed stock for bioethanol production because of its excellent productivity, adaptability for various soils, climate conditions and low agricultural inputs [13–17]. Extensive studies are ongoing with switchgrass to alter its chemical composition, genotypic variability, tailored pretreatments, saccharification and fermentation conditions [18,19]. In order to optimize each step in bioethanol production there is a growing need to develop rapid, information rich methods for the structural characterization of native and pretreated biomass.

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Many of the traditional methods used for the characterization of biomass are dependent on time-consuming extraction and purification techniques followed by spectroscopic characterization [20,21]. Alternative methods that utilize whole cell plant analysis such as ^{13}C CP/MAS, NIR and FT-IR are frequently limited by spectral resolution and/or the absence of detailed structural information. Recently, Ralph et al. demonstrated a binary solvent system consisting of 1-methyl imidazole- d_6 /DMSO- d_6 to dissolve lignocelluloses and utilize these systems to acquire high resolution liquids NMR spectral data [22].

They analyzed whole cell samples of Loblolly pine, quaking aspen and kenaf which provided a facile means of characterizing all biomass components without extensive component extraction and isolation. In a subsequent study, they utilized the same ball milled plant material for 2D ^{13}C - ^1H HSQC of cell wall gels in 4:1 DMSO- d_6 and pyridine- d_5 system. The resulting spectral data provided a technique for identifying various lignin sub-units including resinol, phenylcoumaran, β -aryl ether and polysaccharide units including acetylated xylopyranosides and galactopyranosides [23].

Ionic liquids (IL) are green solvents uniquely suited for the dissolution and processing of a host of inorganic and organic materials due to their unique properties including tunable hydrophobicity, polarity, miscibility with other solvents, low vapor pressure and high thermal stability [24,25]. Rogers and co-workers reported that IL are good solvents for cellulose and woody biomass and it was further shown that an IL with a high chloride concentration is responsible for breaking the strong hydrogen bonding network in cellulose which enhances their solubility [26]. Recently, our group investigated anhydrous pyridinium chloride (IL) with DMSO as an excellent solvent for assorted ball milled and Wiley milled biomass samples [27]. The NMR solvent, consisting of 1:3 perdeuterated pyridinium chloride- d_6 and DMSO- d_6 is an effective solvent for Wiley milled biomass [27]. We have applied this solvent system for the in-depth characterization of native switchgrass and poplar by 1D and 2D NMR spectroscopy. As a continuation of this work, in this study we have investigated the chemistry of various pretreatments of switchgrass, estimated the structural changes by 2D ^{13}C - ^1H HSQC NMR spectroscopy and by composition analysis.

2. Experimental section

2.1. Materials

All chemicals used in this study were purchased either from VWR international or Sigma–Aldrich and used as received except 1, 4-dioxane, which was distilled over NaBH_4 prior to use. The lowland cultivar Alamo switchgrass used in this study was harvested from Ardmore, Oklahoma in November 2007, Wiley milled to a particle size of 1–3 mm and received from National Renewable Energy Laboratory (NREL).

2.2. General procedure for pretreatment

The Wiley milled switchgrass was extracted successively with water and dichloromethane for 24 h each, the experimental conditions used for pretreatments are summarized in Table 1. In brief, milled switchgrass was transferred to a 4560 mini-Parr 300 mL pressure reactor with 0.10 M H_2SO_4 solution added at 5% dry solids to solvent (w/w) ratio, and then sealed. The impeller speed was set to about 100 rpm, and the vessel was heated to 160 °C over ~25–30 min (at ~6 °C/min). The reactor was held at the maximum pretreatment pressure (~668 kPa) for 60 min and then quenched in an ice bath (~5 min). The pretreated slurry was

Table 1

Pretreatment conditions on switchgrass.

Pretreatment ^a	Reagent concentration	Time (min)	Temperature (°C)
Steam		60	160
Dilute H_2SO_4	0.05 M	60	160
Lime	0.1 M	60	160

^a Yields ranged between 75 and 85% by dry weight of biomass.

filtered to remove the solid material and washed with an excess of de-ionized (DI) water. The steam and lime pretreatments were performed in an analogous manner. Paramagnetic impurities were removed by washing the solids with a dilute aqueous solution of ethylenediamine tetraacetic acid (EDTA) and DI water. The switchgrass yields after pretreatment ranged between 75 and 85% (w/w) by the dry weight of biomass.

2.3. Carbohydrate and Klason lignin analysis

Klason lignin content was analyzed by a modified literature procedure [28]. In brief, the extracted switchgrass sample was treated with 72 wt% sulfuric acid for 1 h at 30 °C and then diluted to 3 wt% sulfuric acid using de-ionized water and subsequently autoclaved at 121 °C for 1 h. The resulting solution was cooled to room temperature and the precipitate was filtered, dried and weighed to determine the Klason lignin content. The filtrate was used for the detection of sugar composition by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using Dionex ICS-3000 (Dionex Corp., USA). The results obtained are shown in Table 2.

2.4. NMR sample preparation

Ball milled, vacuum dried switchgrass, 0.060 g, was added into an NMR tube flushed with N_2 , containing a small stir bar and 0.60 g anhydrous perdeuterated pyridinium chloride/DMSO- d_6 (1:3, w/w) and sealed. The mixture was stirred to dissolve at 60 °C and once dissolved the magnetic stir bar was removed from the tube using a strong magnet. Finally the tube was flushed with N_2 .

2.5. NMR analysis

Two dimensional (2D) ^{13}C - ^1H heteronuclear single quantum coherence (HSQC) correlation NMR spectra were recorded on a Bruker DRX 500 spectrometer with a 5 mm z-gradient triple resonance probe with inverse geometry at 50 °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 $^1\text{J}_{\text{C-H}}$ coupling constant of 145 Hz, 256 scans, using an F1 spectral width of 31 694 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. Interactive integrations of contours in 2D

Table 2

Composition of switchgrass before and after pretreatment (%).

Switchgrass ^a	Arabinan	Galactan	Glucan	Xylan	Mannan	Klason Lignin
Untreated	2.8	1.4	39.1	20.2	0.4	20.8
Steam	1.3	0.4	45.3	12.8	0.1	29.4
Lime	1.8	0.5	34.9	12.5	0.2	18.0
Acid	0.13	0.1	47.9	1.3	0.2	42.5

^a see Table 1 for experimental conditions.

HSQC plots were carried out using MestReNova v5.2.1 software, and data processing was carried out in Brukers TopSpin 2.5 software.

3. Results and discussion

3.1. Pretreatment

The goal of this pretreatment process is to induce significant structural differences in the plant cell wall for analysis by our recently developed whole cell ionic liquid NMR technique which facilitates the analysis of all major cell wall biopolymers on milligram samples without isolation or derivatization. The pretreatment conditions utilized were taken from the literature [29–33] and modified to some extent to produce comparable time/temperature profiles among the various pretreatments as summarized in Table 1.

Regardless of the particular conditions used, the pretreatment effectively modified the lignocelluloses as can be seen in Table 2 where the chemical composition of switchgrass before and after pretreatment is presented. The structural changes in lignin and hemicellulose were determined by ^{13}C – ^1H 2D HSQC NMR in perdeuterated pyridinium chloride- d_6 /DMSO- d_6 bisolvent system. These investigations were supported by traditional composition analysis techniques.

3.2. Composition of switchgrass

The results of composition analysis are presented in Table 2. Glucan and xylan are the major polysaccharides in switchgrass which agree with values recently reported by Kumar et al. for Alamo switchgrass [34]. After steam pretreatment, the xylan content was decreased by 37% and Klason lignin content was increased by 41%. Lime pretreatment resulted only in a minor, 13% decrease, in Klason lignin content this could be due to the formation of calcium–lignin complexes during $\text{Ca}(\text{OH})_2$ pretreatment which has been reported to inhibit the delignification during pretreatment [36]. As a result of acid pretreatment a ~90% removal of hemicellulose and a considerable increase in Klason lignin content were observed. The significant increase in Klason lignin content during steam and acid pretreatment were in part attributed to the accumulation of condensed degraded polysaccharides during pretreatment [37–39].

3.3. HSQC ^{13}C – ^1H NMR analysis

The whole cell HSQC ^{13}C – ^1H NMR spectra of switchgrass in perdeuterated pyridinium chloride-DMSO- d_6 system has the potential to assign all three of the polymeric structures in plant cell wall namely cellulose, hemicellulose and lignin. Figs. 1–3 represent the whole cell HSQC spectra of untreated and various pretreated

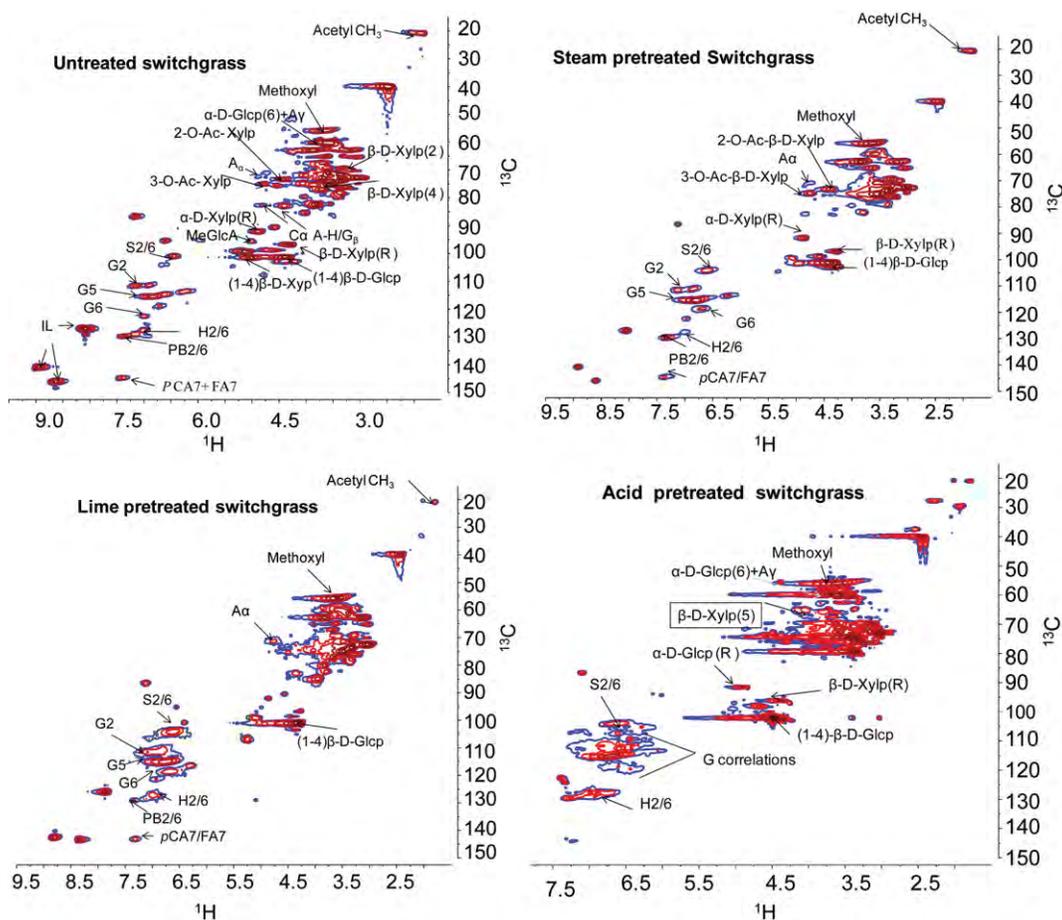


Fig. 1. HSQC spectrum of untreated and pretreated switchgrass in perdeuterated pyridinium chloride–DMSO- d_6 system. A: β -O-4 ether; B: phenylcoumaran; C resinol; D: dibenzodioxocin G: guaiacyl; S: syringyl; CA: coumarate; FA: ferulate; PB: *p*-hydroxybenzoyl; β -D-Xylp: β -D xylopyranoside; β -D-Glcp: β -D glucopyranoside; 2-OAc- β -D-Xylp: 2-Acetyl β -D xylopyranoside; 3-OAc- β -D-Xylp: 3-Acetyl β -D-xylopyranoside; 4-O-MeGlcA: 4-O-methyl- α -D-glucuronic acid; cross peaks at δ 127.5/8.14, 146.4/8.69, 141.4/9.05 are from pyridinium chloride.

switchgrass. Fig. 4 presents the structure of all identified lignin sub-units. Table 3 illustrates the signal assignments for lignin and polysaccharides units based on the literature data in other solvent systems [22,23,40,41].

3.3.1. Structural characterization of untreated switchgrass

The region between δ_C/δ_H 50.0–90.0/3.0–5.1 ppm is overlapped with signals from the side-chain of lignin units and polysaccharides. Nonetheless, the major inter-units of lignin such as β -aryl ether (β -O-4) A, phenyl coumaran B, resinol (β - β) C, dibenzodioxocin D and methoxyl are readily characterized due to the well resolved peaks in the aliphatic side-chain region as illustrated in Figs. 1 and 2. Methoxyl, A_α , B_α , C_α and D_α cross peaks were observed at δ_C/δ_H 56.2/3.6, 72.0/4.8, 87.5/5.6, 85.7/4.5, and 83.0/4.9 ppm respectively (see Fig. 2).

The A_γ and C_γ cross peaks are overlapped in the polysaccharide C_2 – C_6 region. The peaks at δ_C/δ_H 20.4/2.0 ppm represent acetylated groups on hemicellulose and lignin [22,42].

The cross peaks for $S_{2,6}$, G_2 and $H_{2,6}$ were observed at δ_C/δ_H 103.3/6.6, 114.4/7.0 and 128.0/7.2 ppm (see Fig. 3) respectively. The oxidized (α -ketone) structure of syringyl lignin (S') was identified at δ_C/δ_H 106.5/7.0 ppm along with normal $S_{2,6}$ correlations. The S/G ratio for the starting switchgrass was estimated from the integration of the contour $S_{2,6}$ and G_2 , and it was determined to be 0.80, which is comparable to literature value of 0.70 reported by Yan et al. for Alamo switchgrass [42]. The presence of *p*-coumarate and

ferulate esters were confirmed due to the presence of peaks at δ_C/δ_H 145.0/7.5 (p CA7 & FA7) 115/6.4 (p FA8), and 122.5/7.1 (FA6) ppm respectively [42].

The cross peaks for 2-acetylated xylan (2-OAc- β -D-Xylp) and 3-acetylated xylan (3-OAc- β -D-Xylp) H-2/C-2 and H-3/C-3 correlations were readily observed at δ_C/δ_H 74.0/4.5 ppm and 75.0/4.8 ppm, respectively [22] (see Fig. 2). It was observed that the ratio of 2-acetylated xylan versus 3-acetylated xylan is 1:1.2 which was estimated from the respective volume integration of the corresponding cross peaks.

The spectral region between δ_C/δ_H 90.0–115.0/4.0–5.5 ppm are primarily due to polysaccharides-anomeric cross peaks. The most prominent polysaccharides cross peaks can be readily assigned based on the literature data and pure compounds spectra [11,22,40]. The C-1 cross peaks for (1-4) β -D-Glcp from cellulose and (1-4) β -D-Xylp of xylan are observed at δ_C/δ_H 102.5/4.3 ppm and 102.2/4.5 ppm. 4-O-Methyl α -D-glucouronic acid anomeric correlation was observed at δ_C/δ_H 97.5/5.2 ppm (see Fig. 1). The reducing ends of (1-4)- β -D-Xylp yields a δ_C/δ_H signal at 96.5/4.4 ppm. The reducing ends of (1-4)- α -D-Xylp and (1-4) α -D-Galp are present at δ_C/δ_H 92.5/5.0 ppm (see Fig. 1).

3.3.2. Structural changes after pretreatment

Steam pretreatment resulted in partial dissolution and deacetylation of hemicellulose which was clearly observed by the significant decrease in intensities of 2 and 3-acetyl xylopyranoside

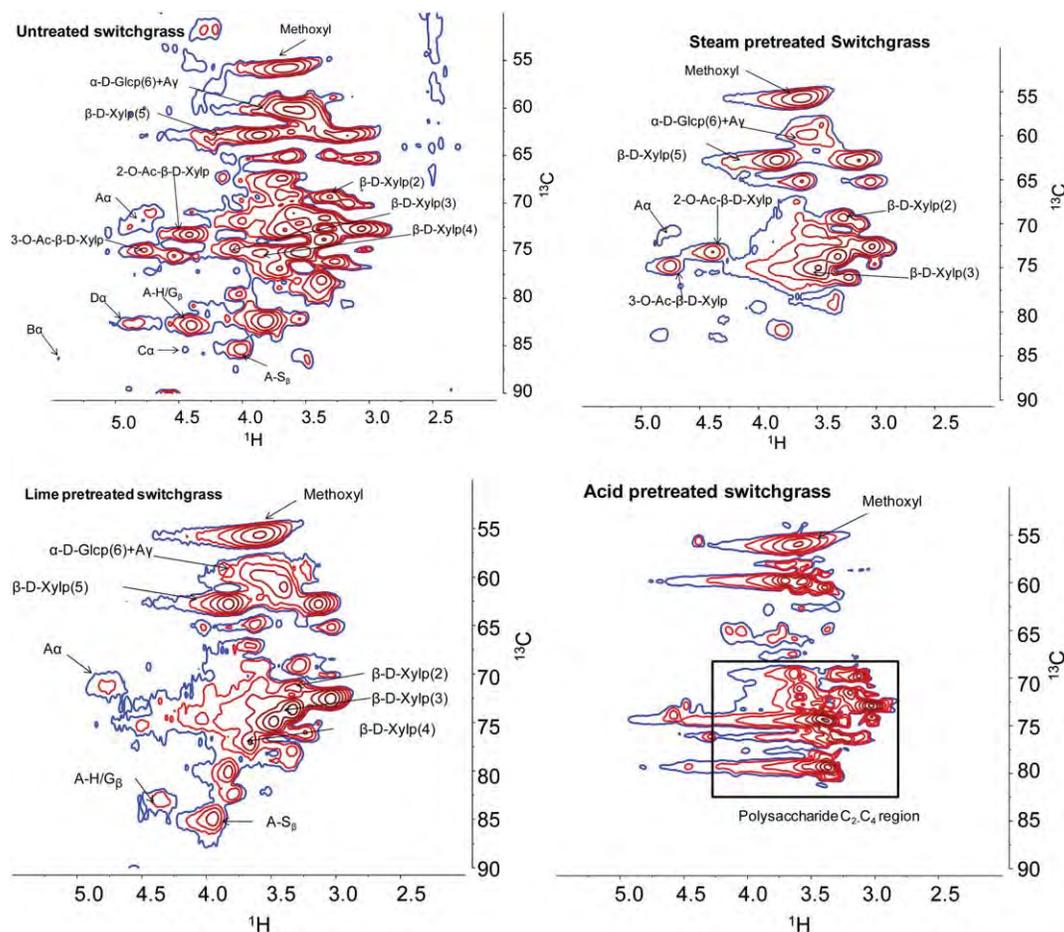


Fig. 2. HSQC spectrum of untreated and pretreated switchgrass (Side chain region). A: β -O-4 ether; B: phenylcoumaran; C resinol; D: dibenzodioxocin; β -D-Xylp: β -D xylopyranoside; β -D-Glcp: β -D glucopyranoside; 2-OAc- β -D-Xylp: 2-Acetyl β -D xylopyranoside; 3-OAc- β -D-Xylp: 3-Acetyl β -D-xylopyranoside; 4-O-MeGlcA: 4-O-methyl- α -D-glucuronic acid.

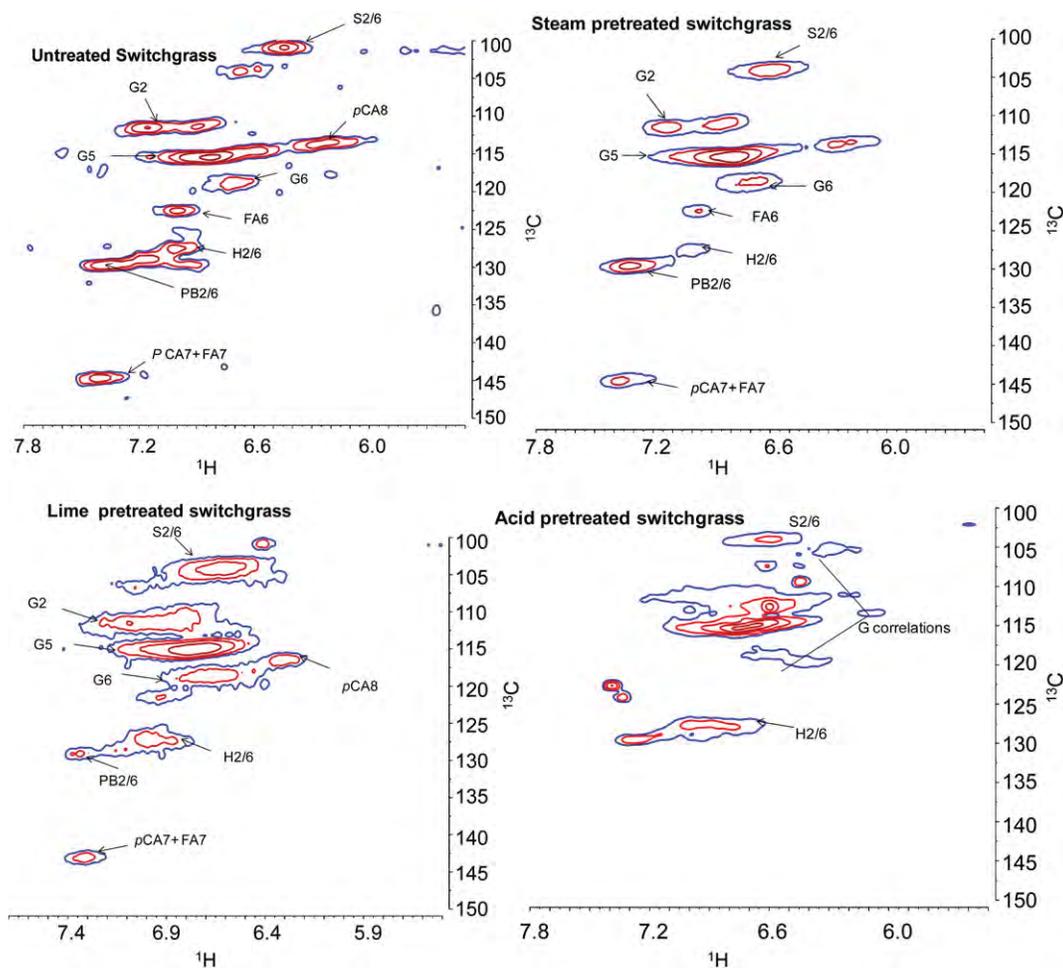


Fig. 3. HSQC spectrum of untreated and pretreated switchgrass (Aromatic region). G: guaiacyl; S: syringyl; CA: coumarate; FA: ferulate; PB: *p*-hydroxybenzoyl.

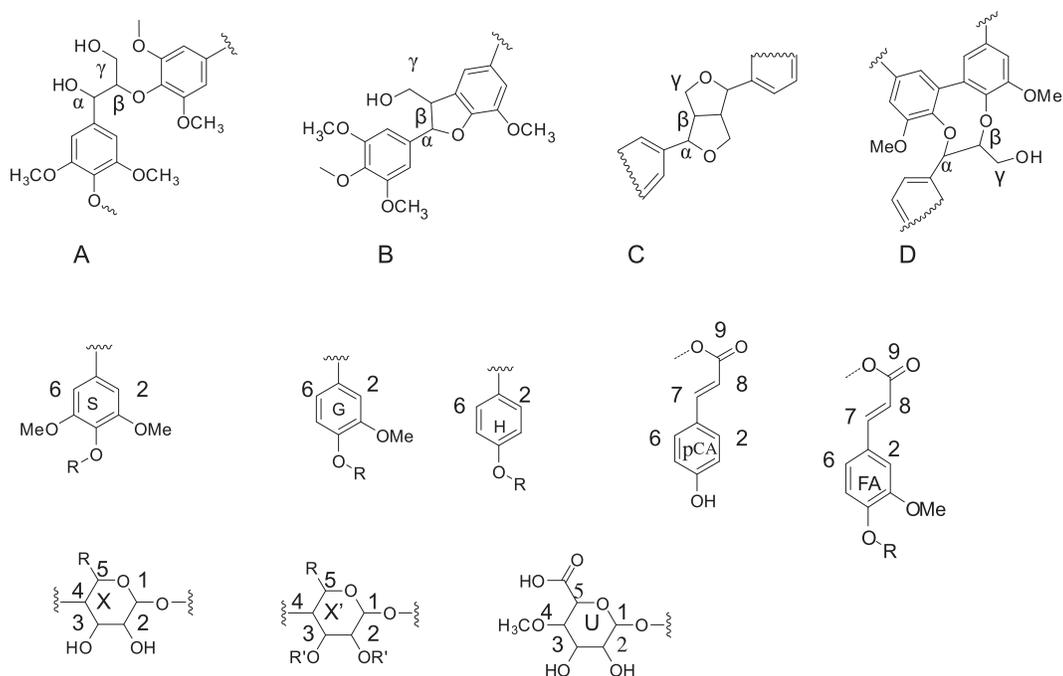


Fig. 4. Structures of identified lignin and hemicelluloses units. (A: β -O-4 ether linkage; B: β -5/ α -O-4 phenylcoumaran; C: resinol; D: dibenzodioxicin; S: syringyl; G: guaiacyl; H: *p*-Hydroxyphenyl; PB: *p*-hydroxy benzoyl, *p*CA: *p*-Coumarate; FA: Ferulate; X: Xylopyranose, R= OH; X': Acetylated xylopyranoses; R' = $\text{CH}_3\text{-C=O}$; U: 4-O-Methyl glucuronic acid.

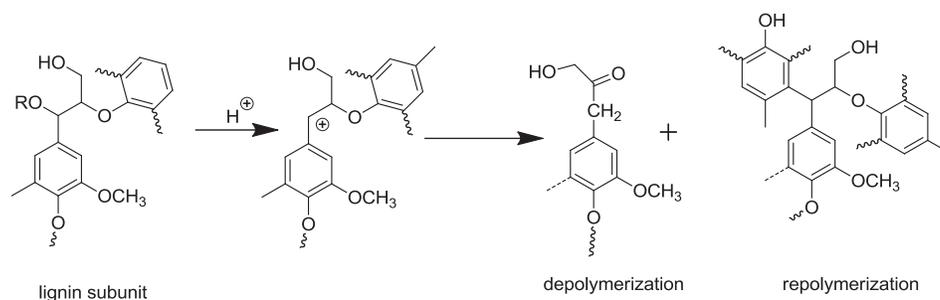
Table 3Assignment of ^{13}C - ^1H correlation signals in the HSQC spectrum for polysaccharides and lignin in switchgrass [21–23,40,41].

Chemical shift $\delta_{\text{C}}/\delta_{\text{H}}$ (ppm)	Assignment
74.0/4.5	C-2/H-2 in 2-OAc- β -D-Xylp
75.0/4.8	C-3/H-3 in 3-OAc- β -D-Xylp
97.5/5.2	4-O-MeGlcA
96.5/4.5	(1-4) β D-Xylp (R)
102.5/4.3	(1-4)- β -D-Glcp (C ₁ H ₁)
102.5/4.5	(1-4)- β -D-Xylp (C ₁ H ₁)
56.2/3.6	OMe
60.1/3.7	β -aryl ether (A γ)
86.0/4.1	(AS β)
72.0/4.8	A(α)
87.5/5.6	Phenylcoumaran (B α)
85.0/4.5	resinol (C α)
83.0/4.9	dibenzodioxocin (D α)
103.3/6.6	C _{2,6} /H _{2,6} syringyl units (S)
106.0/7.0	C _{2,6} /H _{2,6} in oxidized S units (S')
111.4/7.0	C ₂ /H ₂ in guaiacyl units (G)
115/6.7	C ₅ /H ₅ in guaiacyl units (G)
119.5/6.9	C ₆ /H ₆ in guaiacyl units (G)
128.0/7.2	C _{2,6} /H _{2,6} <i>p</i> -hydroxyphenyl units (H)
130.5/7.5	PB2/6
115/6.4	FA8
122.5/7.1	FA6
145.0/7.5	<i>p</i> -CA + FA

and xylan C-1 cross peaks (see Fig. 2). This result was further supported by the observed decrease in xylan content by HPLC composition analysis. The 4-O-methyl α -D-glucuronic acid was completely removed after steam pretreatment. Typically, the mechanism of hemicellulose removal in steam pretreatment involves, at elevated temperature and pressure autocatalysed cleavage of glycosidic bonds in hemicelluloses and ester lignin-hemicelluloses linkages. This facilitates the dissolution of hemicelluloses and increases the accessibility of cellulose to enzymatic hydrolysis. Faga et al. recently reported that as a result of hydrothermolysis of switchgrass, the xylan content was reduced by 89% and the lignin content was increased by 70% which is consistent with our results [35]. The decrease in peak intensities of A α , disappearance of B α , C α and D α cross peak, reduction in contour levels of S_{2/6}, G₂, G₆, PB_{2/6} also accounts for the lignin degradation. However, the composition analysis reflects 41% increase in Klason lignin content after steam pretreatment. Li et al. reported that depolymerization and subsequent repolymerization of lignin happens, with increasing severity of steam pretreatment, which could account for the significant accumulation of acid insoluble material [37]. Nevertheless steam pretreatment facilitates enzymatic hydrolysis most likely the extent of lignin repolymerization inhibit the accessibility of cellulose to the enzymatic hydrolysis.

Lime pretreatment enhanced the deacetylation of xylopyranoside with respect to steam pretreatment which was observed due to the disappearance of the C-2/H-2 correlations for acetylated xylopyranoside and substantial decrease in peak intensity of acetyl peak at $\delta_{\text{C}}/\delta_{\text{H}}$ 20.4/2.0 ppm (see Fig. 1). These changes indicate the base catalyzed hydrolysis of the ester linkages in hemicelluloses which reduces the steric hindrance and increases the accessibility of cellulose to enzymatic hydrolysis. The extent of lignin degradation was not significant however, a selective degradation of ester linkages were confirmed due to the substantial decrease in signal intensities of PB_{2/6}, pCA₈ and pCA₇ correlations (see Fig. 3), which could be due to the saponification of ester linkages in benzoyl esters, ferulates and coumarates which were extensively presented in switchgrass. Xu et al. previously reported that lignin was degraded during alkaline pretreatment of switchgrass although the extent of degradation was shown to be heavily dependent on the residence time and pH [36]. As a result of lime pretreatment partial delignification, deacetylation of hemicelluloses and cleavage of lignin-carbohydrate linkages were occurred however, lignin repolymerization was not observed which could be a favorable factor for enzymatic hydrolysis.

Acid pretreatment of switchgrass resulted in the almost complete deacetylation and dissolution of hemicelluloses which was confirmed by the disappearance of acetylated xylopyranoside cross peaks and very low intensities of C-1 xylan cross peaks in the anomeric region. Lignin degradation was also apparent due to the disappearance of A α , B α , C α , D α and pCA/FA cross peaks and the decreased intensities of S, G, and H correlations (see Fig. 3). The above results indicated that dilute sulfuric acid effectively hydrolyzes hemicelluloses which has resulted in a substantial increase in biomass porosities there by enhance the enzymatic hydrolysis [38,39]. Another major process occurring during acid pretreatment is lignin depolymerization and repolymerization through the formation of a common carbocation intermediate (Fig. 5). It seems the mechanistic pathway for acid pretreatment is similar to that of steam pretreatment. The composition analysis showed a relative decrease in xylan content and a 105% increase in Klason lignin content after acid pretreatment, suggesting the accumulation of condensed degraded polysaccharides as acid insoluble material [43,44]. These lignin like materials are detrimental to enzymatic hydrolysis due to the formation of non-productive binding to enzymes. Accordingly, the wider and less resolved cross peaks for S, G and H aromatic units in the acid pretreated switchgrass may be due to increased levels of condensation between lignin units. These heavily substituted or fused aromatic molecules would have lower degrees of freedom in the ionic liquid solvent system significantly decreasing the transverse relaxation rates and increasing corresponding line-widths generating the observed cross peak appearance.

**Fig. 5.** Mechanistic pathway for lignin depolymerization and repolymerization.

4. Conclusion

Switchgrass was pretreated under acidic, neutral and basic conditions for the detailed characterization and estimation of structural changes after pretreatment by 2D ^{13}C – ^1H HSQC NMR in perdeuterated pyridinium chloride-DMSO- d_6 solvent system. Most of the correlations were identified with literature data and established that switchgrass has acetylated hemicellulose and lignin. The major hemicellulose in switchgrass is xylan and the lignin is composed of H, G and S monolignol units with significant amounts of *p*-coumarate and ferulate. As a result of steam pretreatment, the HSQC spectra indicate partial dissolution of hemicellulose occurs through autohydrolysis and slight degradation in the lignin happens which was confirmed with the decrease in intensity or disappearance of side-chain and aromatic carbon cross peaks. Lime pretreatment resulted in a significant deacetylation, less severe hemicellulose dissolution, but more preferential degradation of *p*-hydroxy benzoyl ester, ferulate and coumarate. Lastly, the spectral data showed acid pretreatment to be the most harsh, causing large amounts of degradation in the hemicelluloses component and significant lignin degradation. Degradation in the lignin component during acid pretreatment was clearly confirmed from the disappearance or considerable diminishing of intensities of the A_α , B_α , C_α and, D_α cross peaks and peaks in the lignin aromatic region. Moreover, *p*-coumarate and ferulates resonances completely deteriorated as a result of acid pretreatment. Whole cell dissolution followed by HSQC NMR analysis is a beneficial structural analysis methodology in the emerging biomass research field for the characterization of numerous biomass samples.

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