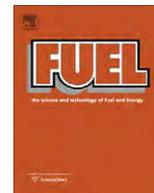


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HSQC (heteronuclear single quantum coherence) ^{13}C – ^1H correlation spectra of whole biomass in perdeuterated pyridinium chloride–DMSO system: An effective tool for evaluating pretreatment

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ABSTRACT

Perdeuterated pyridinium chloride–DMSO- d_6 is an effective solvent system for whole cell biomass dissolution and NMR characterization. Employing this solvent system, semi-quantitative ^{13}C – ^1H heteronuclear single quantum correlation (HSQC) spectroscopy of untreated, steam, dilute acid and lime pretreated poplar biomass samples was readily accomplished. In an effort to demonstrate the efficacy and usefulness of this fairly new characterization technique, relative spectral intensities of the untreated and pretreated biomass samples were evaluated and compared. From the relative signal intensities of hemicelluloses in each system it was observed that hemicelluloses are being removed in various pretreatment conditions, but complete dissolution of hemicellulose was observed only with acid pretreatment. The relative changes in lignin subunits after pretreatment were estimated from the volume integration of resolved cross peaks of various lignin subunits. The degradation of lignin was observed in all pretreatments, though more significant changes were noticed after dilute acid and lime pretreatment. HSQC analysis results were in agreement with the composition analysis of pretreated biomass samples. Thus, this methodology broadens the application of whole cell NMR analysis in biofuel research.

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1. Introduction

Renewable alternative fuel research has increased significantly over the past years in light of societal concerns for climate change, energy security and general sustainability issues [1–6]. Lignocellulosic materials such as agricultural residues and dedicated energy crops are attractive feedstocks due to high availability, low cost and avoidance of food or fuel concerns [7–9]. However the presence of lignin and hemicelluloses in lignocellulosics complicates the conversion of cellulose to ethanol [10]. The goal of pretreatment is to reduce the native recalcitrance of plants by altering the amount, distribution and structure of lignin and hemicellulose in the plant cell wall and thereby facilitate the enzymatic hydrolysis of cellulose to fermentable sugars [11,12]. A variety of pretreatment technologies involving physical, biological and chemical processes are available today [13,14]. The detailed chemical analysis of the structure of biomass polymers due to pretreatment typ-

ically requires tedious biomass isolation techniques that require the separation and isolation of cellulose, lignin and hemicellulose followed-by characterization of the individual components [15–17]. Not only are these techniques extensively time and manpower consuming but more than likely alter the native structure of the cell wall polymers. Nondestructive spectroscopic methods that can be employed for biomass characterization include solid-state NMR, infra-red (IR) and near IR spectroscopy. However, they are frequently hampered by poor resolution and lack of detailed structural determinations [18].

Ralph et al. reported the solution state NMR spectroscopy of whole plant cell samples by employing an NMR solvent system consisting of a 1:4 mixture of 1-methyl imidazole and dimethyl sulfoxide. This solvent system was shown to effectively dissolve ball-milled wood and facilitating high-resolution HSQC NMR spectroscopy of the plant cell wall biopolymers [19]. Recently the same group studied various biomass characterization by 2D NMR studies of ball-milled biomass gel samples in DMSO- d_6 and pyridine- d_5 solvent system [20]. Utilizing these approaches, the researchers were able to identify several plant cell wall components.

Ionic liquids (ILs) are low melting, thermally stable organic salts capable of dissolving organic, inorganic and polymeric materials

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that provide an alternative approach to the dissolution and characterization of biomass [21–23]. Following an initial report by Fort et al. that 1-N-butyl-3-methylimidazolium chloride could dissolve cellulose, several alternative ionic liquid systems have been developed to solubilize and derivatize cellulose [24–28]. Pu et al. reported that ionic liquids such as [hmim][CF₃SO₃], [mmim][MeSO₄] and [bmim][MeSO₄] are effective solvents for lignin and can be used for NMR analysis of its structure [29]. Xie et al. used ionic liquids for the homogenous chemical modification of wood and the resulting material was shown to be highly substituted with unique and distinctly different thermal and morphological properties than the starting wood [30].

In our previous study, we have reported that a novel bi-solvent system consisting of perdeuterated pyridinium molten salt and DMSO-d₆ is an effective solvent for biomass dissolution and NMR characterization [31]. This solvent can dissolve ball-milled and Wiley-milled biomass and readily facilitate NMR analysis of plant cell walls. Direct ¹H, ¹³C and 2D HSQC NMR analysis of poplar and switchgrass dissolved in this bi-solvent system was reported. The unique property of this system is its direct dissolution and characterization of Wiley-milled sample (i.e., non-ball-milled biomass). In the present paper, we describe the application of this procedure to characterize the changes in the fundamental chemical structure of poplar before and after pretreatment and estimate the quantitative changes in lignin and hemicelluloses. The primary goal being to show that this type of analysis can be particularly useful in bio-fuel research and improving bioprocessing methodology by providing detailed structural information on milligram samples of intact pretreated cell wall material.

2. Experimental section

2.1. Substrates

Populus trichocarpa x *deltoides* was harvested between late 2007 at area 0800 at Oak Ridge National Laboratory, TN. The biomass samples extractives were removed by Soxhlet extraction in methylene chloride (3 × 4 h). The extractive free samples were grinded in a Retsch mixer mill MM 200 to reduce the particle size (~80 μm) and dried under vacuum to constant weight prior to NMR analysis. Methanol-d₄, DMSO-d₆, diethyl ether, and acetyl chloride was supplied by Aldrich Chemical Company.

2.2. General procedure for pretreatment

The experimental conditions for pretreatments are summarized in Table 1. In brief, dilute sulfuric acid pretreatment was conducted in a 1 L stirred tank reactor made of Hastelloy (4520 Series, Parr Instruments, Moline, IL), equipped with a double stacked pitch blade impeller. The acid pretreatment were carried out at 170 °C with a 5% w/w dry biomass to solvent ratio. The stirring speed was set to 100 rpm, and the agitator rotated in a direction that directed the biomass downward. The reactor was heated in a fluidized sand bath, in which the temperature was set to 400 °C. The target temperature was maintained by floating the reactor a small distance above the undulating surface (Lloyd and Wyman 2005).

Table 1
Various Pretreatments conditions on poplar.

Pretreatment ^a	Reagent concentration	Time (min)	Temp (°C)
Steam		60.0	160
Dilute Acid	0.05 M H ₂ SO ₄	5.4	170
Lime	0.09 M Ca(OH) ₂	60.0	120

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

The reactor was cooled to 80 °C by submerging the reactor in a cold water bath. The pretreated slurry was vacuum filtered (filter paper: Whatman No. 4, Fisher Scientific) to recover the solid material which was then washed with DI-water using three times the volume of the pretreatment slurry. The pretreated lignocellulosic sample was subsequently dried overnight.

The experimental procedure for lime pretreatment utilized size-reduced poplar transferred to a 4560 mini-Parr 300 mL pressure reactor with ~0.09 M Ca(OH)₂ solution, 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 120 °C over ~25–30 min (at ~6 °C/min). The reactor was held at the maximum pretreatment temperature (~668 kPa) for 60 min. The reactor was then quenched in an ice bath (~5 min). The pretreated slurry was filtered to remove the solid material and washed with an excess of deionized (DI) water. The steam pretreatment was performed in an analogous manner except no external base/acid was added and the maximum temperature was 160 °C.

Paramagnetic impurities were removed by washing the solids with a dilute aqueous solution of ethylenediamine tetraacetic acid (EDTA) and DI-water. The poplar yields after pretreatment ranged between 75% and 85% w/w by the dry weight of biomass.

2.3. Carbohydrates and Klason lignin analysis

The preparation and analysis of samples for carbohydrate and Klason lignin analysis were based on methods described in Tappi T-249 [32]. Carbohydrate and Klason lignin analysis were repeated three times on the untreated and pretreated lignocellulosics. The typical error values associated with the Klason lignin and carbohydrate analysis was ±0.5 and ±1 respectively.

2.4. Synthesis of pyridinium chloride -d₆ [31]

A mixture of methanol-d₄ (20 mmol) and pyridine-d₅ (20 mmol) in anhydrous diethyl ether (10.00 mL) was added to a round bottom flask and this was immersed in ice water for 2 min. Then, a solution of acetyl chloride (1.57 g, 20.0 mmol) in anhydrous diethyl ether (5.00 mL) was added into the reaction mixture over a 5 min period. The reaction mixture was stirred for 30 min and filtered to recover a white solid which was dried under vacuum to afford pyridinium chloride-d₆. The molar yield of reaction was calculated as 90%.

2.5. NMR analysis

HSQC experiments were carried out at 60 °C in a Bruker Advance-500 spectrometer equipped with xyz-gradient triple resonance indirect detection probe using a gradient enhanced sequence. The spectral widths were 11.0 and 180.0 ppm for the ¹H and ¹³C dimensions, respectively. The number of scans was 204, and 256 increments in the ¹³C dimension. The recycle delay was 1.5 s. NMR samples were prepared as follows: 60 mg milled dry poplar sample was added to 0.60 g perdeuterated pyridium chloride–DMSO-d₆ (1:3, w/w) solution and stirred at 60 °C for 3 h. The resulting solution was transferred directly into a 5 mm NMR tube.

3. Results and discussion

The biological conversion of biomass to biofuels is dependent upon reducing the recalcitrance of biomass via a pretreatment stage. Over the past few years several differing pretreatments have been developed tailored to specific feed stocks whereas the fundamental pretreatment chemistry has been understudied. The use of deuterio ionic liquids and 2D HSQC NMR facilitates the detailed

Table 2
Composition of poplar before and after pretreatment.

Poplar ^a	Arabinan	Galactan	Glucan	Xylan	Mannan	Klason lignin
Untreated	1.0	1.1	32.5	32.7	4.5	20.3
Steam	0.0	0.1	44.1	6.4	1.6	47.7
Lime	0.3	0.3	55.7	14.8	2.1	26.6
Acid	0.0	0.0	72.5	1.2	0.5	25.8

^a see Table 1 for experimental conditions

analysis of biomass without the need for time-consuming isolation of plant biopolymers [31]. These benefits suggest it may be well suited for analyzing the chemistry associated with pretreatment. To explore this application, poplar was subject to an acidic, alkaline and neutral pretreatment as summarized in Table 1.

3.1. Carbohydrate and Klason lignin analysis

In order to determine whether the 2D HSQC NMR effectively describes the chemical changes occurring during pretreatment, HPLC-based monosaccharide anionic exchange chromatography was used to establish a baseline for comparison.

The results of the sugar analysis and Klason lignin content measurements before and after pretreatment are presented in Table 2. The composition of the untreated poplar indicates that glucose and xylose are the prominent monosaccharides. The major hemicellulose in poplar is xylan however; small amounts of arabinan, galactan and mannan were present. The sum of this entire component is considered as the total hemicelluloses content present in the poplar. The index of hemicelluloses we described are in terms of the total hemicelluloses present in the poplar. The hemicelluloses content of the poplar somewhat decreases as a result of steam and lime pretreatment whereas after acid pretreatment hemicelluloses were completely removed.

It is also clear that the relative Klason lignin content increased after pretreatment. This was attributed, in part, because of the hydrolysis of hemicelluloses and also due to the accumulation of non-lignin based material formed by acid catalyzed condensation of polysaccharides during pretreatment [15,33].

3.2. Characterization of untreated poplar by HSQC analysis

Extractive-free, dried, native and pretreated poplar samples were dissolved in perdeuterated pyridinium chloride/DMSO- d_6

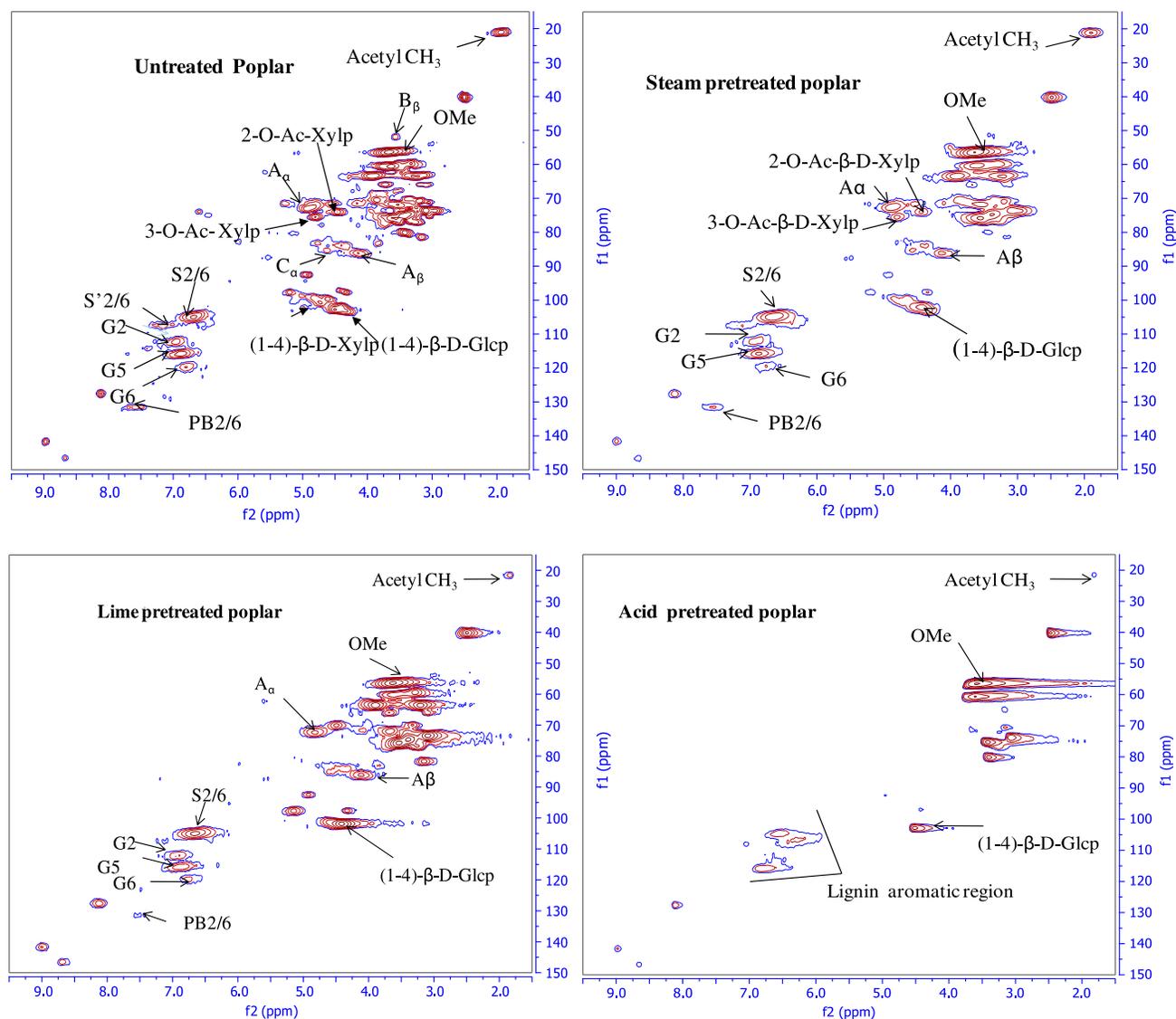


Fig. 1. HSQC spectrum of untreated and pretreated poplar in perdeuterated pyridinium chloride–DMSO- d_6 system.

(1:3) solvent system at 60 °C and analyzed by 2D ^{13}C - ^1H HSQC. The HSQC NMR spectra of poplar samples and their expanded aromatic ranges are presented in Figs. 1–3 and signal assignments are based on the literature and summarized in Table 3 [19,20,34].

HSQC NMR spectra of untreated poplar in perdeuterated pyridinium chloride/DMSO- d_6 yielded characteristic cross peaks for all components namely, cellulose, hemicellulose and lignin. Cross-peaks attributed to polysaccharide C_2 – C_6 and some of the lignin side chain contours are overlapped in the region δ_c/δ_H 59–82/3.0–4.1 ppm. Prominent correlations for 2-acetylated xylan (2-O-Ac- β -D-Xylp) and 3-acetylated xylan (3-O-Ac- β -D-Xylp) were also observed at δ_c/δ_H 73.5/4.5 (C-2/H-2) and 75.0/4.8 ppm (C-3/H-3), respectively (See Fig 2). The relative ratio of 2-O-Ac- β -D-Xylp: 3-O-Ac- β -D-Xylp was estimated from the contour integration as 1.1:1.0. In the polysaccharide anomeric region (δ_c/δ_H 90–105/4.4–5.5 ppm) the signals were fairly well resolved. The partial characterization of glucan (cellulose) and xylan (hemicellulose) was accomplished by the cross peaks for internal anomeric of the (1–4) linked β -D-glucopyranoside (β -D-Glcp) at δ_c/δ_H 102.8/4.4 ppm and (1–4) linked β -D xylopyranoside (β -D-Xylp) at 102.4/4.5 ppm respectively. The clusters of cross peaks at δ_c/δ_H

60.6/3.6, 72–76/2.9–3.7, 81.8/3.5, 102.5/4.5 are attributed to be of C_6 , C_{2-3} C_5 , C_4 and C_1 carbons of polysaccharides (cellulose and hemicelluloses) respectively [35,36].

The typical lignin inter-unit linkages in biomass are shown in Fig 4. The HSQC spectra of poplar samples demonstrated that ^{13}C - ^1H correlation due to the methoxyl of lignin was prominent and observed at δ_c/δ_H 55.7/3.8 ppm. Whereas A_γ (i.e., β -O-aryl ether in lignin, see Fig 2) and C_γ (i.e., resinol in lignin) correlations were overlapped in the aliphatic spectral region. From the HSQC spectra, β -O-aryl ether linkage in lignin was confirmed by the cross peaks at δ_c/δ_H 72.0/4.8, 86.0/4.2 ppm for α and β C-H side-chain correlations, respectively. The cross peaks at δ_c/δ_H 87.4/5.5 and 53.2/3.5 ppm are attributed to α and β correlations respectively for phenyl coumaran (B). Finally resinol (C) units were also detected due to the presence of C_α -H correlations at δ_c/δ_H 85.7/4.6 ppm. The relative ratio of various lignin side chain units A, B and C are semi-quantitatively estimated from the volume integration of A_α , B_α and C_α cross peaks and determined to be \sim 82:7:11.

Presence of syringyl and guaiacyl units in lignin were confirmed by the separate contour for syringyl and guaiacyl at δ_c/δ_H 103.3/6.6 ($\text{S}_{2/6}$), 111.4/7.0 (G_2), 115.0/6.8 (G_5), 119.5/6.9 (G_6) ppm,

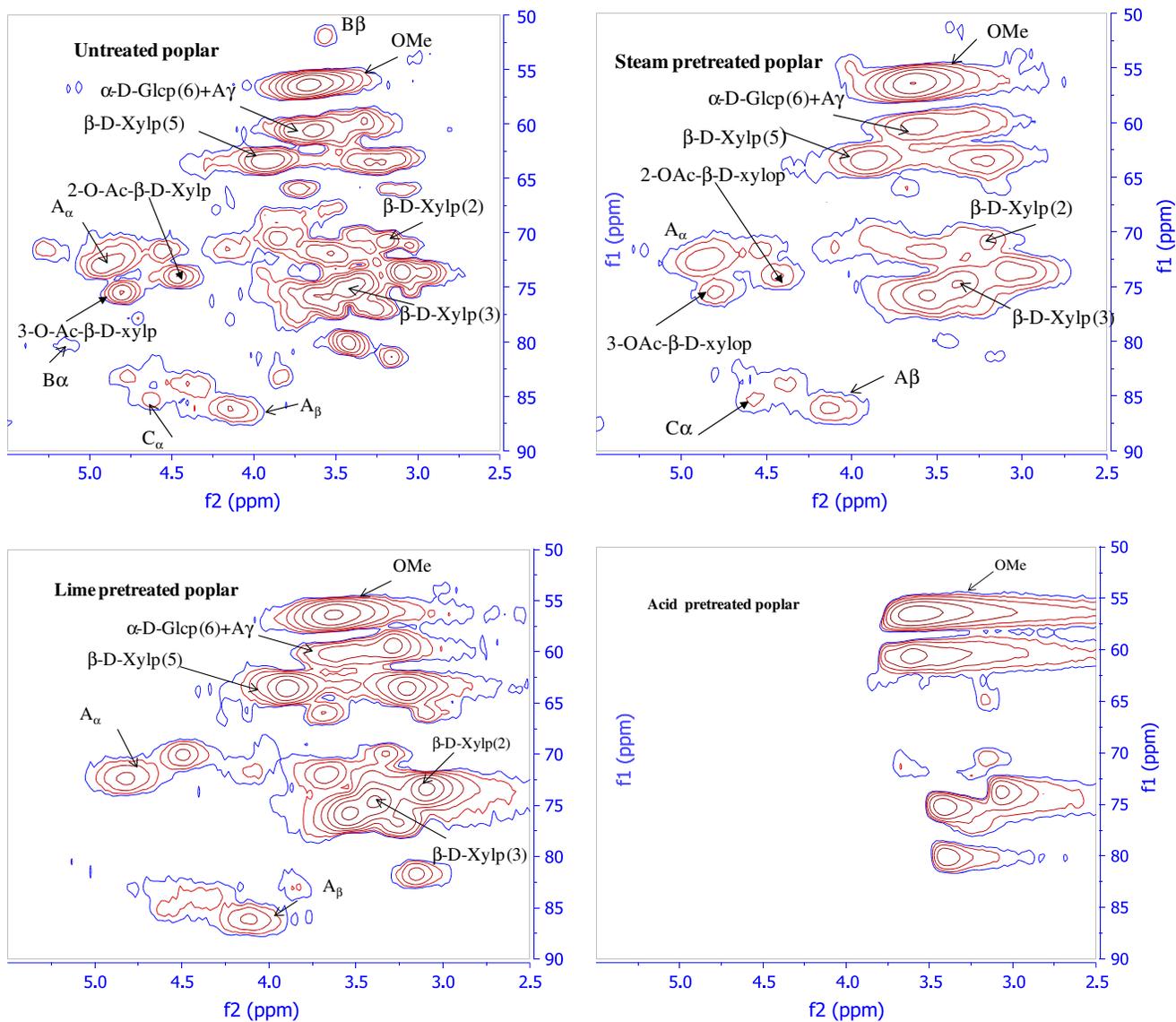


Fig. 2. HSQC spectrum of untreated and pretreated poplar (side chain region).

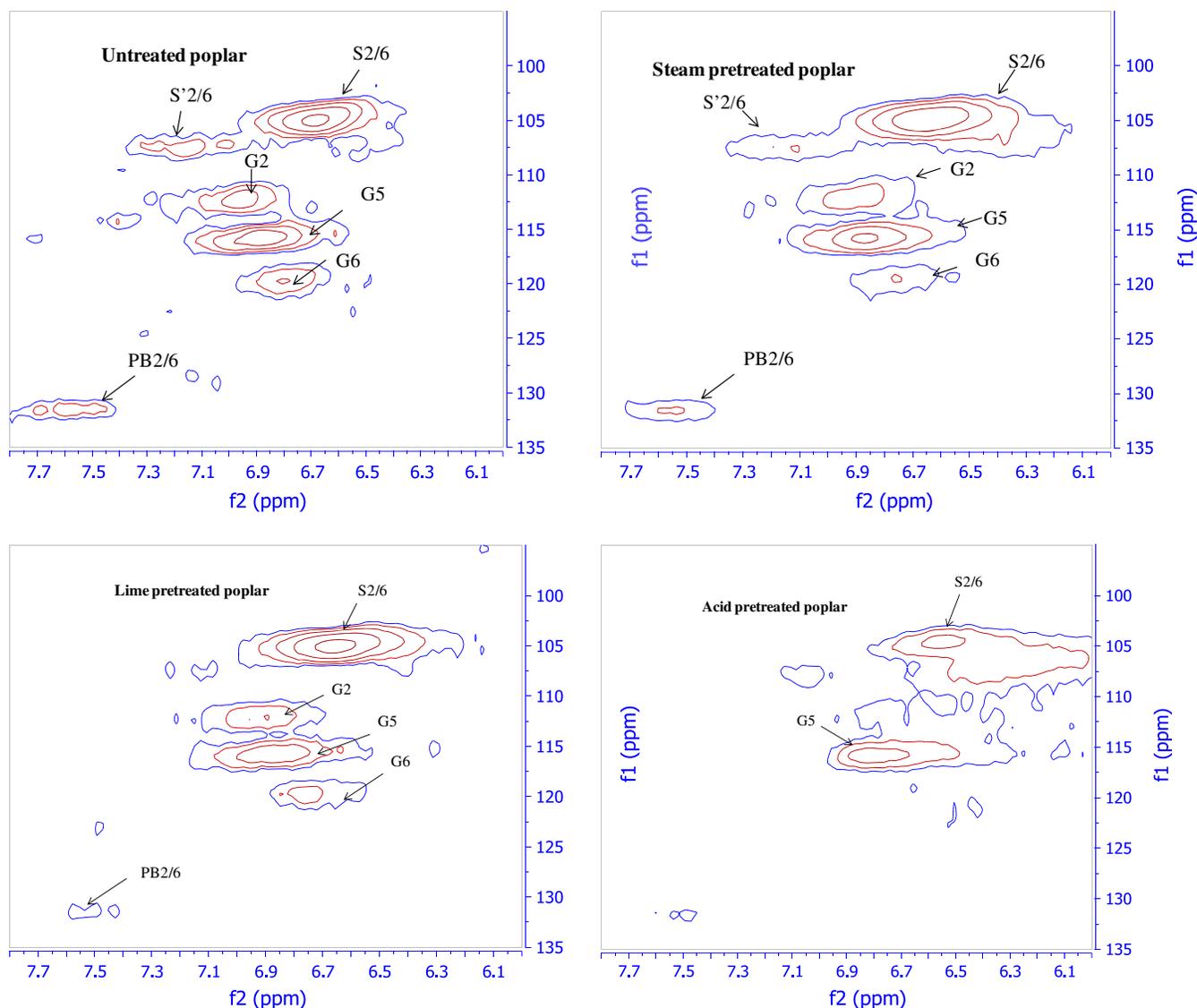


Fig. 3. HSQC spectrum of untreated and pretreated poplar (Aromatic region).

respectively. The presence of the *p*-hydroxy benzoates (PB) structure was confirmed by the cross peaks at δ_c/δ_H 131.5/7.6 ppm. From the relative volume integration of $S_{2/6}$, G2 and $PB_{2/6}$ cross peaks it was observed that lignin in poplar dominated by syringyl units with S/G ratio 2.1.

3.3. Characterization of pretreatment changes in poplar

Fig. 2 illustrates the changes in side chain region as a result of various pretreatments. The relative decrease in the spectral complexity and number of overlapping resonances in the aliphatic/lignin chain region after all pretreatments (see Fig 2) indicates the dissolution and degradation of polysaccharides and lignin during pretreatments. As a result of steam pretreatment the HSQC spectral data indicates that the relative amount of residual hemicelluloses was tremendously reduced and which was confirmed by the decrease in volume integration of 2 and 3-acetyl xylopyranoside resonances.

Based on the HSQC results, the chemistry governing steam pretreatment of biomass in fact seems similar to that of acid pretreatment. Under high temperature and pressure water causes an auto-catalyzed cleavage of glycosidic bonds in hemicelluloses

and lignin–hemicelluloses linkages. This along with the production of acetic acid from acetyl groups facilitates the removal of hemicelluloses from the plant cell wall, increasing the enzyme accessibility to cellulose which has been shown to directly reduce biomass recalcitrance [37–39].

The reduced contour intensity of A_α cross peaks, the disappearance of B_β cross peaks and decrease in intensity of C_α peak indicate the degradation of β -aryl ether, phenyl coumaran and resinol units during steam pretreatment. The lignin aromatic region displays a significant reduction in the oxidized syringyl units ($S'_{2/6}$) while a noticeable decrease in the relative intensities of the $S_{2/6}$, G2 and $PB_{2/6}$ resonances were also observed (see Fig 3). The decrease in lignin side chain units indicate that depolymerization of lignin occurring during pretreatment. However, previous work has shown that there is some amount of repolymerization which can happen and the extent of that processes greatly depend on hydrothermal pretreatment time and temperature [33].

After lime pretreatment the HSQC spectrum indicates a ~95% removal of acetylated xylopyranosides attributed to the disappearance of peaks at δ_c/δ_H 73.5/4.5 (C-2/H-2) and 75.0/4.8 ppm (C-3/H-3), and was further supported by the low contour level of acetyl peaks at δ_c/δ_H 19.5/2.0 ppm (see Fig 1). Lime pretreatment can

Table 3Assignment of ^{13}C - ^1H correlation signals in the HSQC spectrum of poplar biomass. (19–20, 34).

δ_c/δ_H (ppm)	Assignment
53.2/3.5	C_{β}/H_{β} in phenyl coumaran substructure (B)
55.7/3.8	CH in methoxyl group
60.6/3.6	C-6 polysaccharide + A γ
72.0/4.8	C_{α}/H_{α} in β -O-4 CC α /H α in β -O-4 linkage (A)
73.5/4.5	C-2/H-2 in 2-OAc- β -D-Xylp
75.0/4.8	C-3/H-3 in 3-OAc- β -D-Xylp
86.0/4.2	C_{β}/H_{β} in β -O-4 linkage (A)
87.4/5.5	C_{α}/H_{α} in phenyl coumaran (B)
85.7/4.6	C_{α}/H_{α} in resinol substructure(C)
102.8/4.4	(1-4)- β -D-Glcp
102.4/4.5	(1-4) (β -D-Xylp)
103.3/6.6	$C_{2,6}/H_{2,6}$ in syringyl units(S)
106.1/7.2	$C_{2,6}/H_{2,6}$ in oxidized syringyl units (S')
111.4/7.0	C_2/H_2 in guaiacyl units (G)
115/6.8	C_5/H_5 in guaiacyl units (G)
119.5/6.9	C_6/H_6 in guaiacyl units (G)
131.5/7.6	$C_{2,6}/H_{2,6}$ in p-hydroxybenzoate (PB) units

Note: A: β -O-4 ether linkage, B: β -5/ α -O-4 phenylcoumaran, C: resinol, G: guaiacyl unit; S: syringyl unit, S': oxidized syringyl with $C_{\alpha} = \text{O}$, β -D-xylp: β -D xylopyranoside units; β -D-Glcp: β -D glucopyranoside units, 2-OAc- β -D-Xylp: 2-Acetyl β -D xylopyranoside units, 3-OAc- β -D-Xylp: 3-Acetyl β -D-xylopyranoside units.

readily remove acetyl groups from hemicelluloses, thereby reducing the steric hindrance and increasing the cellulose enzymatic accessibility and enhancing carbohydrate digestibility [40–42]. There was also a noticeable amount of lignin degradation which was confirmed from the decrease in volume integration of A_{α} and $PB_{2/6}$ cross peaks and (see Fig 3). These spectral observations suggest a preferential removal of PB units which was attributed to the saponification of the ester linkages in the p-hydroxy benzoyl ester linkages during alkaline pretreatment. Kumar et al. recently reported lime pretreatment facilitates delignification and enzymatic hydrolysis; however extensive delignification highly depends on temperature and presence of oxygen [43] and loss of the groups reported above contributes to this effect.

Lastly, dilute acid pretreatment resulted in an almost complete removal of hemicelluloses, it was confirmed by the disappearance of acetylated xylopyranoside and C_2 – C_5 hemicelluloses cross peaks in the region δ_c/δ_H 60.0–80.0/3.0–4.5 ppm. Comparing the volume integration of A_{α} cross peaks in untreated and acid pretreated poplar (see Fig 1) indicated almost complete degradation of β -aryl ether linkages occurred. The disappearance of C_{α} and B_{α} cross peaks

also suggest the significant degradation of phenyl coumaran and resinol subunits. In Fig 3, by comparing the volume integration of $S_{2/6}$, G_2 , G_5 and G_6 cross peaks; it was evident that there was a substantial degradation of lignin aromatic units as a result of acid pretreatment.

The above results illustrate that during acid pretreatment hemicelluloses are effectively hydrolyzed, which can contribute to the reported increase in pore expansion sizes recently reported by Foston et al. [44]. This increased pore size effect has been suggested to enhance the digestibility of pretreated cellulosic biomass [45,46]. The other leading process occurring due to acid pretreatment is fragmentation, recondensation and redistribution of lignin units. This along with the subsequent condensation of degraded polysaccharides, accumulate as acid insoluble material or 'pseudo lignin' [33,47], presumably this could be the reason for the removal of the majority of side chain resonances in the HSQC spectrum of acid pretreated switchgrass.

Hence, whole cell HSQC analysis of plant cell wall material provides an in-depth characterization of biomass which is consistent with published composition analysis of raw and pretreated poplar. This study has shown this technique to be a particularly useful new tool for the characterization of changes in plant cell wall chemical structure due to pretreatment.

4. Conclusions

Poplar biomass was pretreated with steam, lime and dilute sulfuric acid. Extractives-free samples were readily dissolved in perdeuterated pyridinium chloride–DMSO- d_6 solvent system and ^{13}C - ^1H HSQC correlation spectra of untreated and pretreated poplar was accomplished. All major cell wall components in untreated and pretreated poplar, were readily characterized in detail on milligram quantity samples without component isolation. The HSQC spectral data provided a means to identify and semi-quantitatively estimated acetylated xylan structures, ratio of acetate contents, the amount of various lignin monolingnol units and side chain subunits. HSQC analysis confirmed that as a result of steam pretreatment, significant lignin and hemicellulose degradation occurred where as lime pretreatment resulted mainly in hemicellulose dissolution and selective PB lignin degradation while dilute sulfuric acid pretreatment completely removed the hemicelluloses and displaying noticeable lignin degradation in both aromatic and side chain moieties. Composition analysis of the untreated and pretreated samples clearly indicated an enrichment of Klason lignin content after all pretreatments and this could be due to the

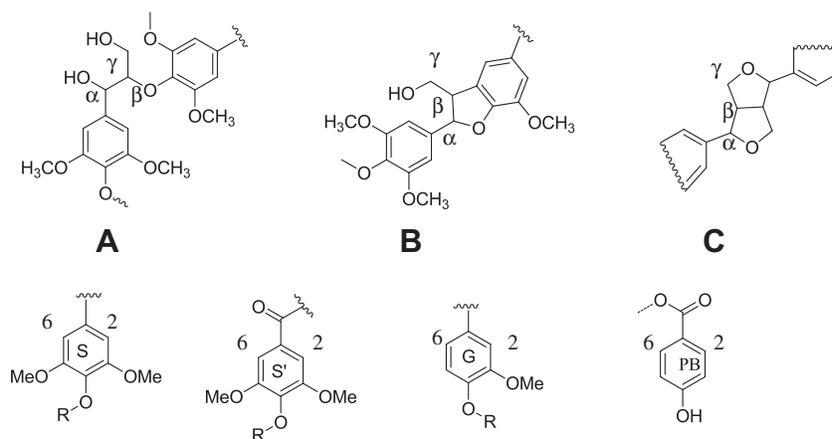


Fig. 4. Structures of identified lignin units. A: β -O-4 ether linkage; B: β -5/ α -O-4 phenylcoumaran; C: resinol; S: syringyl unit; S': syringyl (oxidized α -ketone), G: guaiacyl unit; PB: p-hydroxy benzoyl.

accumulation of non-lignin degraded polysaccharide material during pretreatment. Accompanying these changes, was approximately 99–75% decrease in xylan content for the acidic and auto-hydrolysis pretreatment whereas the lime pretreatment decreased the xylan content approximately 55%. These results seemingly support the structural changes indicated by the spectral data, validating the use of this methodology as a means of characterizing both native and pretreated biomass for the purposes of improving biomass processing and biofuel production technologies.

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