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

Reichel Samuel [a,c], Marcus Foston [a,c], Nan Jaing [a,c], Shilin Cao [a], Lenong Allison [a,c], Michael Studer [c], Charles Wyman [c], Arthur J. Ragauskas [a,b,c,*]

[a] School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, United States
[b] Institute of Paper Science and Technology, Georgia Institute of Technology, Atlanta, GA, United States
[c] BioEnergy Science Center, CE-CERT and Chemical and Environmental Engineering Department, Bourns College of Engineering, University of California, Riverside, CA, United States

ARTICLE INFO

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–^{1}H 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.

© 2011 Elsevier Ltd. All rights reserved.

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 typically 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

* Corresponding author at: BioEnergy Science Center, CE-CERT and Chemical and Environmental Engineering Department, Bourns College of Engineering, University of California, Riverside, CA, United States. Tel.: +1 404 894 9701; fax: +1 404 894 4778.
E-mail address: Art.Ragauskas@chemistry.gatech.edu (A.J. Ragauskas).

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][CF$_3$SO$_3$], [mmim][MeSO$_4$] and [bmim][MeSO$_4$] 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-d$_6$ 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 ^{1}H, ^{13}C 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 biofuel 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-d$_4$, DMSO-d$_6$, 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).

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 −d$_6$ [31]

A mixture of methanol-d$_4$ (20 mmol) and pyridine-d$_5$ (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-d$_6$. 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 ^{1}H and ^{13}C dimensions, respectively. The number of scans was 204, and 256 increments in the ^{13}C 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-d$_6$ (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 deutero ionic liquids and 2D HSQC NMR facilitates the detailed

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.

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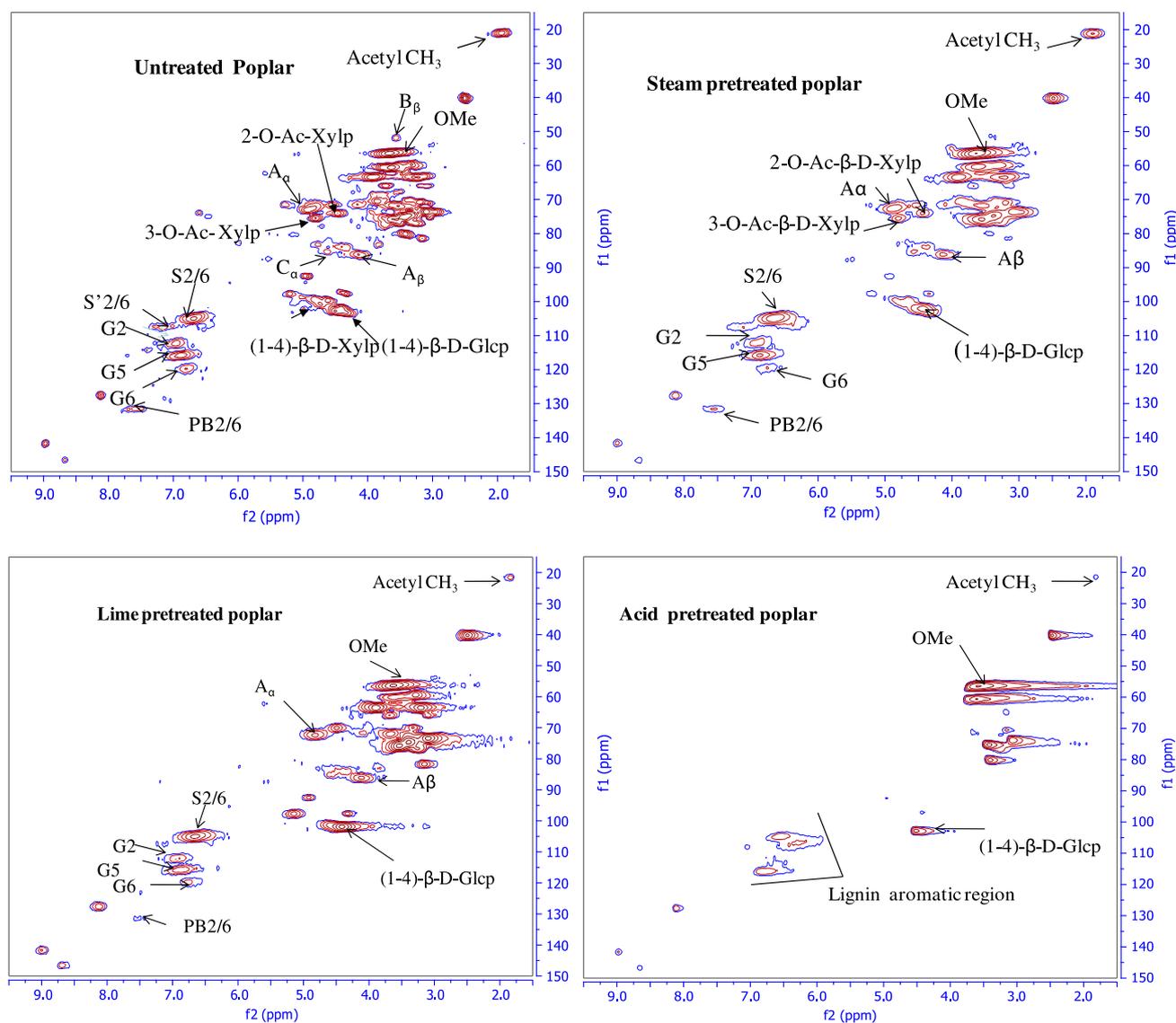
Fig. 1. HSQC spectrum of untreated and pretreated poplar in perdeuterated pyridinium chloride–DMSO-d_6 system.

(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. Crosspeaks 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 anomerics 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 ~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 ($S_{2/6}$), 111.4/7.0 (G_2), 115.0/6.8 (G_5), 119.5/6.9 (G_6) ppm,
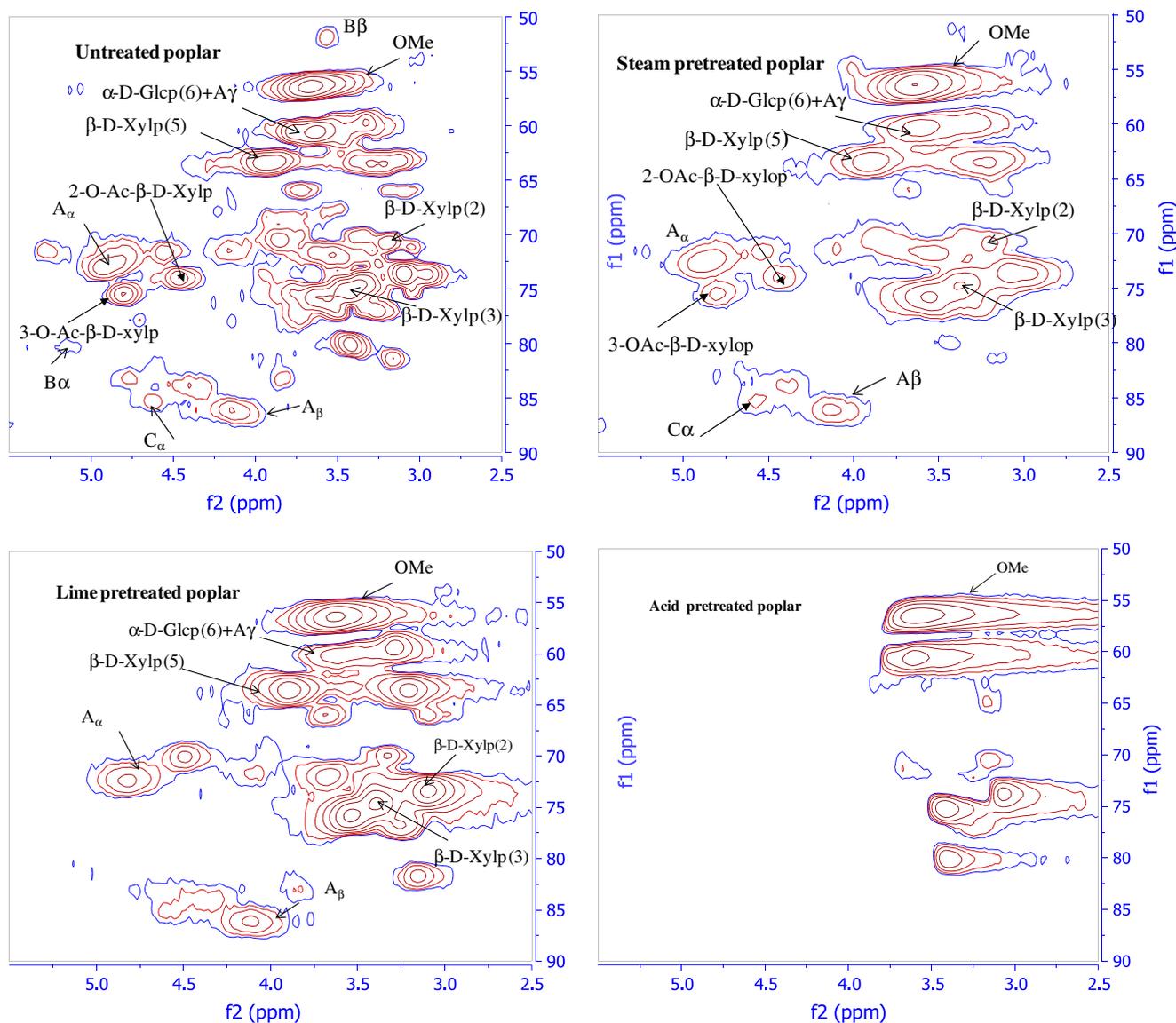
Fig. 2. HSQC spectrum of untreated and pretreated poplar (side chain region).
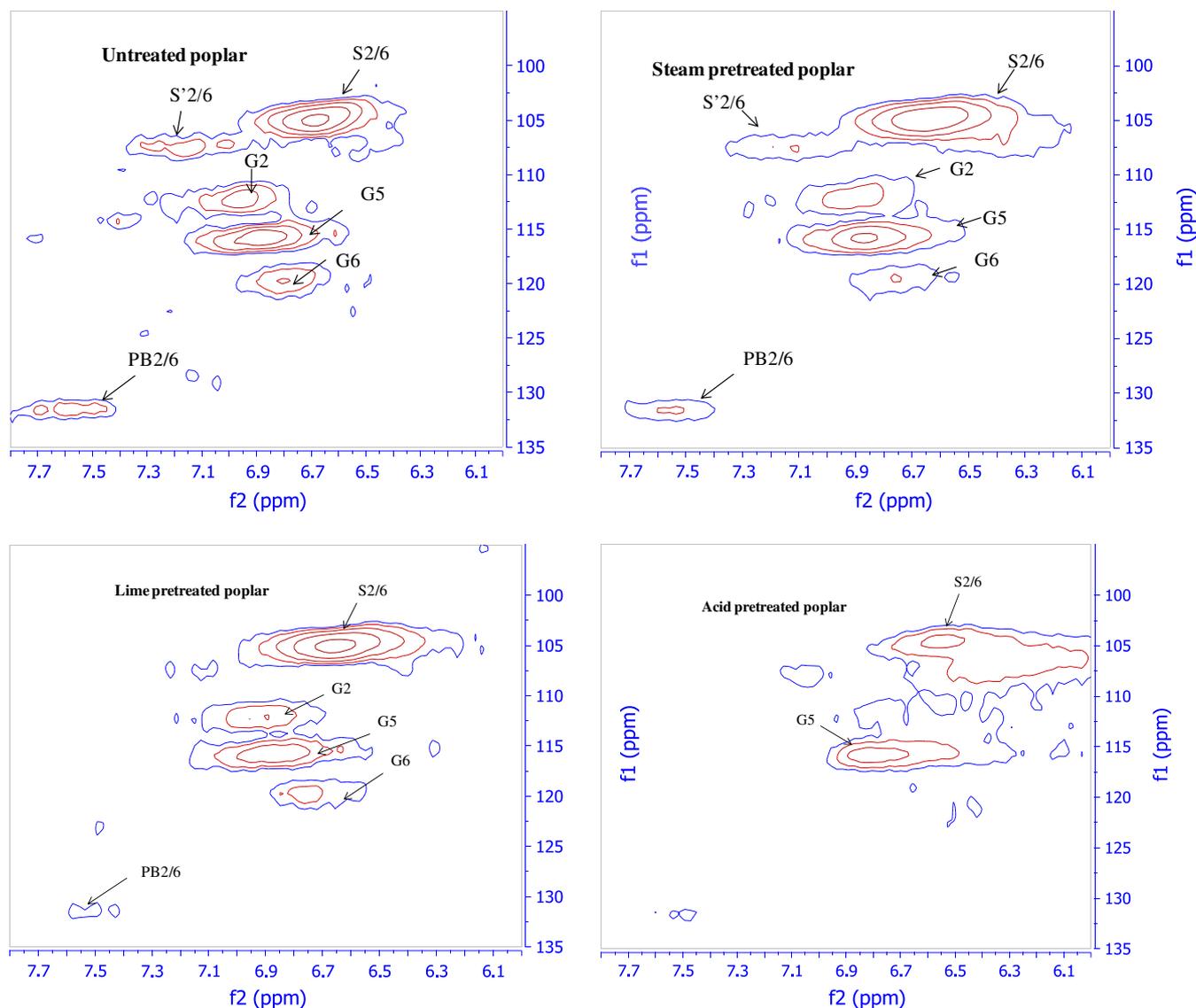
Fig. 3. HSQC spectrum of untreated and pretreated poplar (Aromatic region).

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 $S_{2/6}$, G_2 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. Charectarization 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 $S_{2/6}$, G_2 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 happened 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 3
Assignment 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 + Aγ
72.0/4.8	$C\alpha/H\alpha$ 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 phenylcoumararan, C: resinol, G: guaiacyl unit; S: syringyl unit, Ś: oxidized syringyl with C_α = O, β-D-xylop: 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 occured. 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 perdeuterted 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
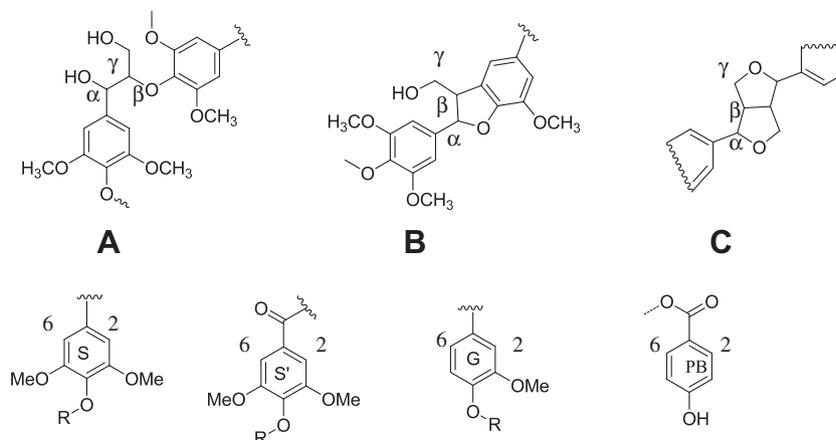
Fig. 4. Structures of identified lignin units. A: β-O-4 ether linkage; B: β-5/α-O-4 phenylcoumaran; C: resinol; S: syringyl unit; S′: syringyl (oxidized α-ketone), G: guaiacyl unit; PB: p-hydroxy benzoyl.

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.

Acknowledgement

References

[1] Ragauskas AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, et al. The path forward for biofuels and biomaterials. Science 2006;311:484–9.

[2] Clark JH. Green chemistry for the second generation biorefinery – sustainable chemical manufacturing based on biomass. J Chem Technol Biotechnol 2007;82:603–9.

[3] Shiwu Li, Yunpeng W, Shengwu D, Yang C, Fenghua C, Fang C, et al. Biodiesel production from Eruca Sativa Gars vegetable oil and motor, emissions properties. Renewable Energy 2009;34(1):1871–6.

[4] Shi-Zhong Li, Catherine CH. Ethanol production in the People's Republic of China: potential and technologies. Appl Energy 2009;86(Suppl.1):S162–9.

[5] Huber GW, Corma A. Synergies between bio- and oil refineries for the production of fuels from biomass. Angew Chem Int Ed 2007;46:7184–201.

[6] Corma A, Iborra S, Velty A. Chemical routes for the transformation of biomass into chemicals. Chem Rev 2007;107:2411–502.

[7] Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 2002;83:1–11.

[8] Jacques K, Lyons TP, Kelsall DR. The alcohol text book. 3rd ed. Trowbridge, Wiltshire: Redwood Books; 1999.

[9] Brown RC. Products from biorenewable resources in biorenewable resources: engineering new products from agriculture; 2003. p. 109–38.

[10] Himmel M, Ding S, Johnson D, Adney W, Nimlos M, Brady J, et al. Biomass recalcitrance: engineering plants and enzymes for biofuels production. Science 2007;315:804–7.

[11] Ding SY, Himmel ME. The maize primary cell wall microfibril: a new model derived from direct visualization. J Agric Food Chem 2006;54(3):597–606.

[12] Chundawat SPS, Venkatesh B, Dale BE. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. Biotechnol Bioeng 2007;96:219–31.

[13] Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. Coordinated development of leading biomass pretreatment technologies. Bioresour Technol 2005;96:1959–66.

[14] Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresour Technol 2005;96:2026–32.

[15] Samuel R, Pu Y, Raman B, Ragauskas A. Structural characterizations and comparison of switchgrass lignin before and after dilute acid pretreatment. Appl Biochem Biotechnol 2010;162(1):62–74.

[16] Sannigrahi P, Ragauskas AJ, Miller SJ. Effect of two stage dilute acid pretreatment on the structure and composition of lignin and cellulose in Loblolly pine. Bioenergy Res 2008(3–4):205–14.

[17] Hult EL, Larsson PT, Iversen T. A comparative CP/MAS ^{13}C–NMR study of the supramolecular structure of polysaccharides in sulphite and kraft pulps. Holzforschung 2002;56:179–84.

[18] Bardet M, Emsley L, Vincendon M. Two-dimensional spin-exchange solid-state NMR studies of ^{13}C-enriched wood. Solid State Nucl Magn Reson 1997;8:25.

[19] Yelle DJ, Ralph J, Frihart CR. Characterization of non derivatized plant cell walls using high-resolution solution-state NMR spectroscopy. Magn Reson Chem 2008;46:508–17.

[20] Kim H, Ralph J. Solution –state 2D NMR of ball-milled plant cell wall gels in DMSO-d$_6$/pyridine-d$_5$. Organic Bimolecular Chem 2010;8:576–91.

[21] Swatloski RP, Spear SK, Holbrey JD, Rogers RD. Dissolution of cellulose with ionic liquids. J Am Chem Soc 2002;124:4974–5.

[22] Fort DA, Remsing RC, Swatloski RP, Moyna P, Moyna G, Rogers RD. Can ionic liquids dissolve wood? Processing and analysis of lignocellulosic materials with 1-N-butyl-3-methylimidazolium chloride. Green Chem 2007;9:63–9.

[23] Liu Q, Janssen MHA, Rantwijk V, Sheldon RA. Room-temperature ionic liquids that dissolve carbohydrates in high concentrations. Green Chem 2005;7:39–42.

[24] Wu J, Zhang J, Zhang H, He J, Re Q, Guo M. Homogeneous acetylation of cellulose in a new ionic liquid. Biomacromolecule 2004;5:266–8.

[25] Phillips DM, Drummy LF, Conrady DG, Fox DM, Naik RR, Stone MO, et al. Dissolution and regeneration of Bombyx mori silk fibroin using ionic liquids. J Am Chem Soc 2004;126:14350–1.

[26] Xie H, Li S, Zhang S. Ionic liquids as novel solvents for the dissolution and blending of wool keratin fibers. Green Chem. 2005;7:606–8.

[27] Xie H, Zhang S, Li S. Chitin and chitosan dissolved in ionic liquids as reversible sorbents of CO_2. Green Chem 2006;8:630–3.

[28] Fukaya Y, Hayashi K, Wada M, Ohno H. Cellulose dissolution with polar ionic liquids under mild conditions: required factors for anions. Green Chem 2008;10(1):44–6.

[29] Pu Y, Jiang N, Ragauskas AJ. Ionic liquid as a green solvent for lignin. J Wood Chem Techno 2007;27:23–33.

[30] Xie H, King A, Kilpelainen I, Granstrom M, Argyropoulos DS. Thorough chemical modification of wood-based lignocellulosic materials in ionic liquids. Biomacromolecule 2007;8(12):3740–8.

[31] Jiang N, Pu Y, Samuel R, Ragauskas AJ. Perdeuterated pyridinium molten salt (ionic liquid) for direct dissolution and NMR analysis of the plant cell walls. Green Chem 2009;11(11):1762–6.

[32] Pronto M. Carbohydrate analysis of pulp using high-performance anion-exchange chromatography with pulsed amperometric detection. Pulping Conference; 1998.

[33] Li J, Henriksson G, Gellerstedt G. Lignin depolymerization/repolymerization and its critical role for delignification of aspenwood by steam explosion. Bioresour Technol 2007;98:3061–8.

[34] Rio JCD, Rencoret J, Marques G, Gutierrez A, Ibarra D, Santos JI, et al. Highly (acetylated and/or p-coumaroylated) native lignins from diverse herbaceous plants. J Agric Food Chem 2008;56:9525–34.

[35] Samuel R, Pu Y, Foston M, Ragauskas AJ. Structural modification on switchgrass polysaccharides after dilute acid pretreatment. Biofuels 2010;1(1):85–90.

[36] Sun JX, Sun XF, Zhao H, Sun RC. Isolation and characterization of cellulose from sugarcane bagasse. Polym Degrad Stabil 2004(84):331–9.

[37] Grous WR, Converse AO, Grethlein HE. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. Enzyme Microb Technol 1986;8:274–80.

[38] Morjanoff PJ, Gray PP. Optimization of steam explosion as method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification. Biotechnol Bioeng 1987(29):733–41.

[39] Youngmi K, Nathan MS, Michael LR. Enzymatic digestion of liquid hot water pretreated hybrid poplar. Biotechnol Prog 2009;25(2):340–8.

[40] Karr WE, Holtzapple T. Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover. Biomass Bioenergy 2000;18:189–99.

[41] Scalbert A, Monties B. Holzforschung 1986;40:249.

[42] Kumar R, Wyman CE. Access of cellulase to cellulose and lignin for poplar solids produced by leading pretreatment technologies. Biotechnol Prog 2009;25(3):807–19.

[43] Kumar P, Barrett DM, Delwiche MJ, Stroeve P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 2009;48:3713–29.

[44] Foston M, Ragauskas AJ. Changes in the structure of the cellulose fiber wall during dilute acid pretreatment in populus studied by ^{1}H and ^{2}H NMR. Energy Fuels 2010;24:5677–85.

[45] Kumar R, Mago G, Balan V, Wyman CE. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresour Technol 2009;100(17):3948–62.

[46] Yun-Chin C, Alan B, Michael PH. Enzymatic saccharification and fermentation of xylose-optimized dilute acid-treated lignocellulosics. Appl Biochem Biotechnol 2005;124(1):947–61.

[47] Sannigrahi P, Kim DH, Jung S, Ragauskas J. Pseudo-lignin and pretreatment chemistry. Energy Environ Sci; 2011.