RSC Advances

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Investigation of the fate of poplar lignin during autohydrolysis pretreatment to understand the biomass recalcitrance†

Cite this: *RSC Advances*, 2013, **3**, 5305

Received 19th November 2012,
Accepted 4th February 2013

DOI: 10.1039/c3ra40578h

www.rsc.org/advances

Reichel Samuel,[a] Shilin Cao,[a] Birinchi K Das,[a] Fan Hu,[a] Yunqiao Pu[b] and Arthur J. Ragauskas*[ab]

Lignocellulosic biomass is the most abundant renewable resource for the potential replacement of fossil fuels, though to fully realize this vision, an improved understanding of the chemical structures of its components within the biomass and after bioprocessing is critical. In this study, we investigated the fate of isolated poplar lignin during autohydrolysis pretreatment at different temperatures and subsequently analyzed the structural changes by gel permeation chromatography, ^{13}C–^{1}H HSQC and phosphorylation/^{31}P NMR. Our results suggested that an increase in temperature and time of autohydrolysis of lignin resulted in an increase in phenolic hydroxyl groups coupled with a decrease in aliphatic hydroxyl groups. This may be attributed to the cleavage of β-*O*-4 linkages *via* acidolysis. Molecular weight determination revealed that lignin depolymerization predominates over condensation. Our results also highlight that the cleavage of lignin side-chain units is relatively fast in lignin autohydrolysis compared to the autohydrolysis of biomass. This study provides an enhanced understanding of the fundamental autohydrolysis pretreatment lignin chemistry and will facilitate improved methodology to reduce biomass recalcitrance.

Lignocellulosic biomass such as forest residues, agricultural waste and energy crops are promising renewable resources for the production of second generation bioethanol, produced from the fermentation of sugars derived from cellulose and hemicelluloses.[1–3] The major constituents of biomass, cellulose, hemicelluloses and lignin, exist as a closely associated, rigid network in plants. Lignin is a polyphenolic, highly cross linked macromolecular polymer which is the second most abundant biopolymer in plants and it is covalently linked to polysaccharides to form lignin-carbohydrate complexes (LCCs). This native structural construct inhibits biomass decomposition by enzymes and microbes, which is referred to as recalcitrance.[4,5] Generally, cellulosic ethanol production involves three steps: pretreatment, enzymatic hydrolysis and fermentation. The pretreatment stage is considered to be the most expensive component for 2nd generation biofuels but it leads to significant benefits by reducing the recalcitrance of biomass by decreasing the degree of polymerization (DP) of cellulose/hemicelluloses, increasing the porosity of the biomass and disrupting lignin and hemicellulose networks which benefit the 'unlocking' of plant polysaccharides for enzymatic hydrolysis.[3] Though these treatments facilitate enzymatic hydrolysis they have several disadvantages such as severe reaction conditions, generation of fermentation inhibiters such as furfural, and high capital investments. A complete understanding of the complex chemical reactions occurring for lignin and polysaccharides is essential for optimizing sugar yields and improving the overall pretreatment effects.[6]

Some of the commonly used pretreatment methods are dilute acid, lime, steam explosion, ammonia percolation and organosolv.[7–12] One of the promising pretreatment technologies for second generation ethanol is autohydrolysis, since it is a hot water only pretreatment which increases the digestibility of the biomass. During autohydrolysis, hydronium ions generated from water and *in situ* generated organic acids lead to the hydrolysis of hemicelluloses and the disruption of lignin, resulting in an increase in cellulose reactivity with cellulase.[13–16]

Several researchers have studied the autohydrolysis pretreatment of biomass and the accompanying structural changes in lignin and polysaccharide structure under these conditions and established its positive impact on subsequent enzymatic hydrolysis.[17,18] To examine in greater detail the reactions of lignin under autohydrolysis conditions without competing reactions from plant polysaccharides, we carried out the autohydrolysis of poplar cellulolytic enzyme lignin[19,20] under varying autohydrolysis conditions (*i.e.* 150–200 °C, 0–30 min residence time) and examined the structural changes in the lignin by ^{13}C–^{1}H HSQC, ^{31}P NMR[21] and GPC[22] analysis. The use of cellulolytic enzyme lignin was guided by the consideration of utilizing a lignin that was minimally altered by the isolation process and still retained some lignin–carbohydrate complexes which this method certainly achieved.[23] To the best of our knowledge this is the first report on the investigation of the structural changes in lignin under autohy-

[a]*BioEnergy Science Center, School of Chemistry and Biochemistry, 500 10th St., Atlanta, GA 30332, USA. E-mail: arthur.ragauskas@chemistry.gatech.edu*
[b]*Institute of Paper Science and Technology, Georgia Institute of Technology, 500 10th St., Atlanta, GA 30332, USA*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ra40578h

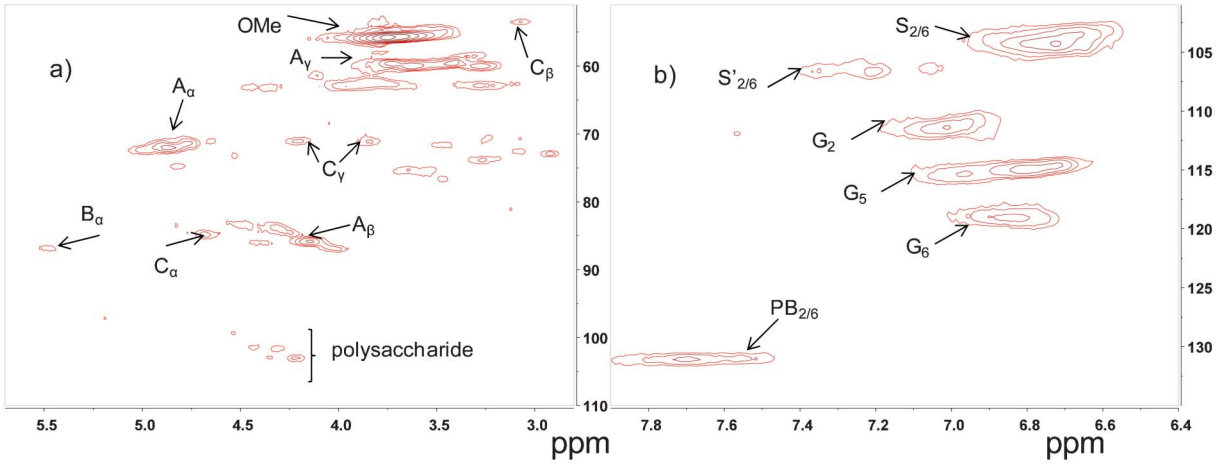

Fig. 1 HSQC spectra of poplar enzyme lignin in DMSO-d_6 (a) side chain region (b) aromatic region A: β-*O*-4 ether linkage; B: phenylcoumaran; C: resinol; S: syringyl units S′ (oxidized α-ketone), G: guaiacyl unit; PB: *p*-hydroxybenzoate.

drolysis pretreatment conditions apart from the biomass matrix which was anticipated to provide fundamental information as to the chemistry of lignin under autohydrolysis conditions in the absence of plant polysaccharide cell wall polymers.

HSQC NMR analysis of untreated and autohydrolyzed lignin samples provided evidence of structural changes in lignin sub-units after autohydrolysis. The HSQC spectra of poplar enzyme lignin are given in Fig. 1 and the spectra are annotated with peak assignments based on the literature (Table 1 in the ESI†).[24–26] Fig. 2 depicts the structures of identified lignin sub-units.

The inter-unit linkages in poplar lignin, β-*O*-4 aryl ether (A), phenylcoumaran (B) and resinol (C) were identified due to the presence of cross peaks at δ_C/δ_H 71.8/4.8 (A_α), 85.8/4.2 (A_β), 87.1/5.5 (B_α), 84.7/4.7 (C_α) and 53.6/3.1 (C_β) ppm, respectively (see Fig. 1). The aromatic lignin units syringyl (S), guaiacyl (G) and *p*-hydroxybenzoate (PB) units showed prominent correlations at δ_C/δ_H 104.3/6.7 ($S_{2/6}$), 111.4/7.0 (G_2), 119.3/6.8 (G_6), 130.0/7.7 ($PB_{2/6}$), respectively (Fig. 1).[24] Minor amounts of oxidized syringyl units were detected due to the presence of a correlation at δ_C/δ_H 105.5/7.2 ($S'_{2/6}$).

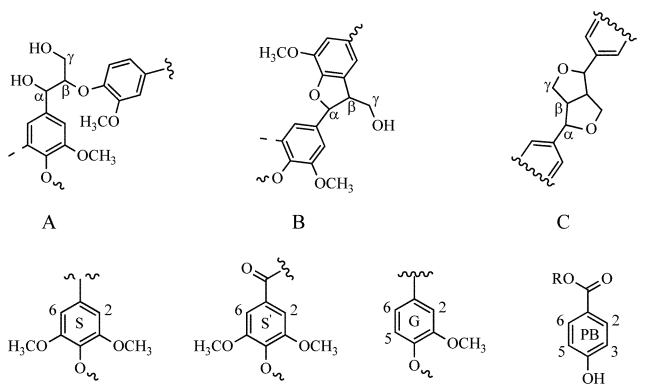

Fig. 2 Identified poplar lignin sub-units.

The HSQC spectra of autohydrolysed lignin samples illustrate the relative degradation of lignin side chains and aromatic units with increasing autohydrolysis temperature. Based on the relative signal intensities of corresponding peaks at various temperatures it was observed that the cleavage of β-*O*-4 aryl ether was fairly significant even at 150 °C. Fig. 3 represents the HSQC spectrum of autohydrolysed lignin at 200 °C with 30 min residence time. The reduced contour intensity of A_α, A_β, and A_γ cross peaks indicates ~90% of β-*O*-4 aryl ether was removed under these experimental conditions. The lignin aromatic region displays a considerable reduction in syringyl, guaiacyl and *p*-hydroxy benzoyl units, which was confirmed by the reduced signal intensities of the $S_{2/6}$, G_2 and $PB_{2/6}$ peaks, which indicates the decrease in protonated aromatic carbon and presumably leads to the condensation of lignin aromatic units. Interestingly, the ^{13}C–^1H HSQC NMR spectra clearly indicate the presence of the polysaccharides associated with lignin-carbohydrate linkages due to the presence of correlations in the region δ_C/δ_H 95.0–105.0/4.2–4.5 in the untreated enzymatic lignin which appeared to degrade only at 200 °C.

The acetylated lignin/hemicellulose was substantially reduced with increasing severity of autohydrolysis conditions, which was confirmed from the decrease in volume integration of the acetyl cross peaks at δ_C/δ_H 20.0/1.9 ppm (not shown). In our previous studies, after the hot water pretreatment of poplar sawdust at 160 °C with a one hour residence time, ^{13}C–^1H HSQC NMR analysis demonstrated that the degradation of lignin was not significant.[24] However in the present lignin autohydrolysis study, ~60–80% of lignin side chain units were removed at 150–200 °C with zero residence time, which demonstrates that lignin degradation is the major process occurring under the experimental conditions.

^{31}P NMR spectroscopy is a facile and direct analysis tool for quantifying the major hydroxyl groups in lignin.[27] The hydroxyl groups of lignin are *in situ* phosphitylated using 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) and the phosphitylated groups are quantitatively estimated by ^{31}P NMR spectroscopy based on the integration area of the respective peaks and an internal standard (*N*-hydroxyl-norbornene-2,3-dicarboximide).[28]

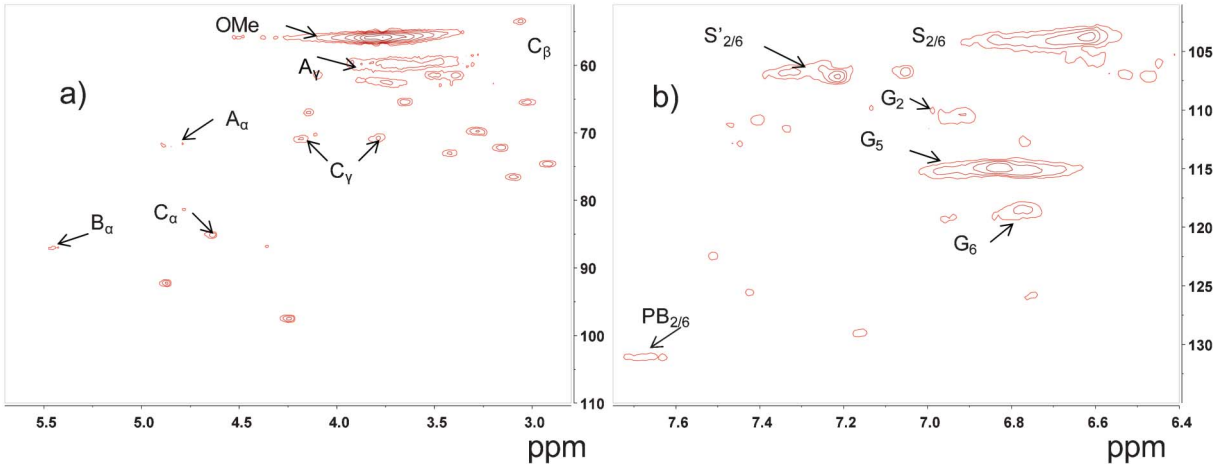

Fig. 3 HSQC spectra of autohydrolyzed poplar enzymatic lignin at 200 °C with a 30 min residence time.

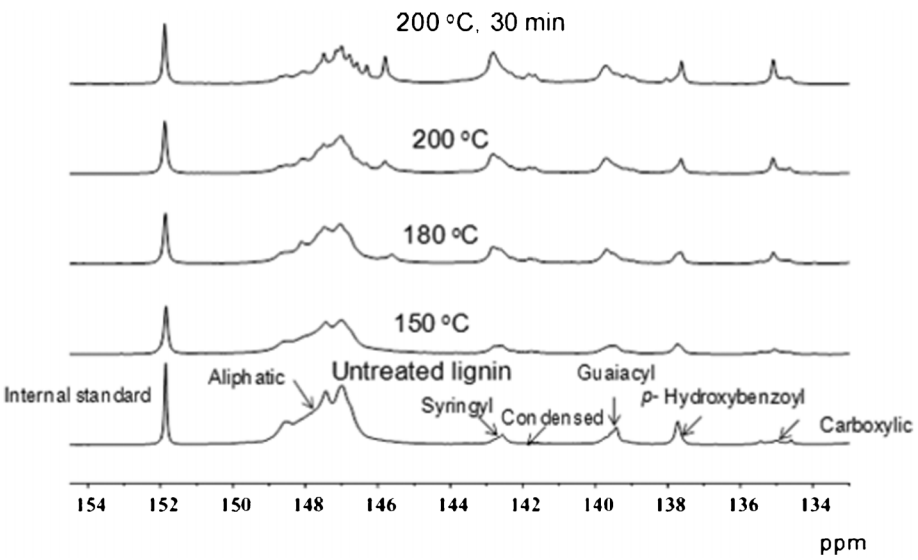

Fig. 4 NMR spectra of untreated and pretreated poplar lignin.

Fig. 4 provides typical ^{31}P NMR spectra of phosphitylated poplar lignin and Table 1 provides the different hydroxyl group contents.

Table 1 shows that the majority of the hydroxyl groups in poplar lignin come from the aliphatic side-chain of lignin and carbohydrate sugars. The observed amounts of syringyl, guaiacyl and *p*-benzoyl hydroxyls in poplar lignin are consistent with literature values.[29] The aliphatic hydroxyl content is significantly higher than the reported values, this is attributed to the polysaccharide hydroxyl groups associated with the enzyme isolated lignin.

Table 1 Estimation of the concentration of hydroxyl groups in lignin by ^{31}P NMR analysis

Chemical shift	Assignment	Untreated lignin (mmol g^{-1})	150 °C[a] (mmol g^{-1})	180 °C[b] (mmol g^{-1})	200 °C[c] (mmol g^{-1})	200 °C[d] (mmol g^{-1})
145.5–150.0	Aliphatic	6.90	4.80	4.40	3.70	3.60
141.4–142.2	Condensed	0.09	0.10	0.18	0.20	0.25
142.5–143.1	Syringyl	0.37	0.41	0.85	0.97	1.62
138.7–140.2	Guaiacyl	0.59	0.45	0.68	0.71	1.10
137.2–138.1	*p*-Benzoyl	0.41	0.27	0.29	0.29	0.42
134.5–135.7	Carboxylic	0.27	0.30	0.34	0.37	0.53

[a] Autohydrolysis at 150 °C, zero residence time. [b] Autohydrolysis at 180 °C, zero residence time. [c] Autohydrolysis at 200 °C, zero residence time. [d] Autohydrolysis at 200 °C, 30 min residence time.

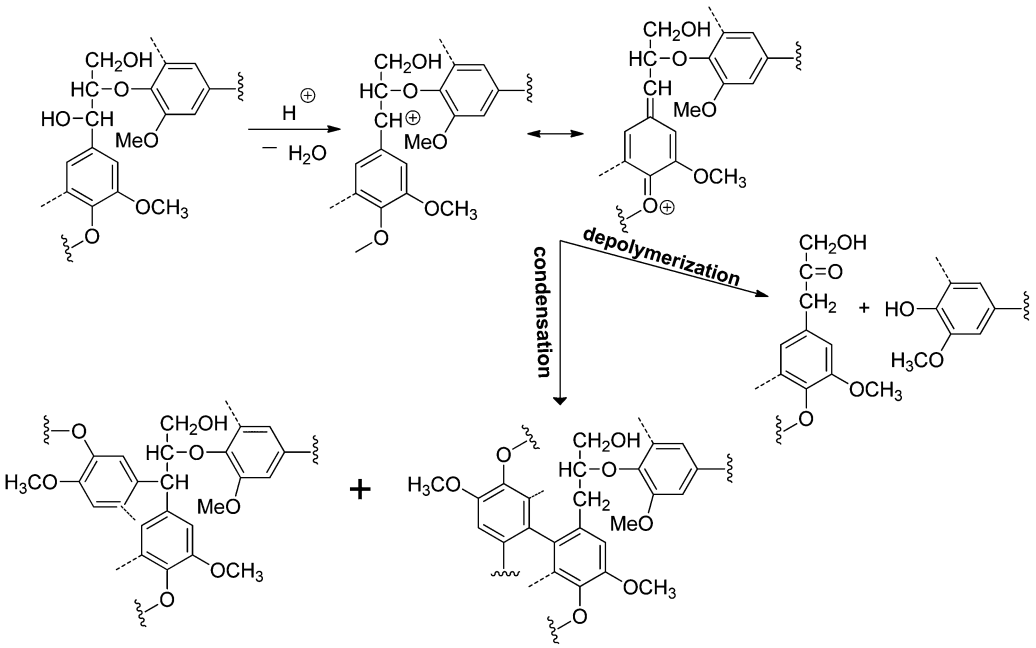

Fig. 5 Possible lignin degradation mechanism during autohydrolysis pretreatment.

As a result of autohydrolysis (200 °C, 30 min residence time), we observed a 48% decrease in aliphatic hydroxyl groups, whereas the total phenolic and carboxylic OH groups increased to 130% and 96% respectively. A possible mechanistic pathway associated with these structural changes during autohydrolysis is presented in Fig. 5. The acidic conditions lead to the formation of a carbocation by elimination of water (alcohol) from the benzylic position and subsequent cleavage of β-*O*-4 aryl ether linkage leads to the formation of Hibbert ketones.[30–33] The observed increase in condensed units was further supported by the decrease in aromatic CH correlations in the ^{13}C–^{1}H HSQC NMR analysis as well. The increase in carboxylic OH groups is believed to be a result of the formation of acids due to the autocatalyzed cleavage of ester linkages in lignin.

To determine the effect of autohydrolysis on the degree of polymerization (DP) of lignin, the starting isolated lignin and autohydrolysis samples were acetylated and subsequently analyzed by gel permeation chromatography. Table 2 illustrates the drastic changes in molecular weight of the autohydrolysed lignin with respect to temperature and extent of autohydrolysis.

Table 2 Molecular weights of poplar lignin

Lignin	Untreated lignin	150 °C[a]	180 °C[b]	200 °C[c]	200 °C[d]
Mn	2959	1594	530	458	448
Mw	9938	3507	1421	987	868
PDI[e]	3.3	2.1	2.7	2.1	1.9

[a] Autohydrolysis at 150 °C zero residence time. [b] Autohydrolysis at 180 °C zero residence time. [c] Autohydrolysis at 200 °C zero residence time. [d] Autohydrolysis at 200 °C 30 min residence time.
[e] Polydispersity index (Mw/Mn).

It can be seen that with an increase in autohydrolysis temperature, the lignin macromolecule undergoes depolymerization and the recovered lignin at higher temperature has a tremendous decrease in molecular weight (Table 2). Several researchers have reported the evaluation of lignin structural changes during autohydrolysis pretreatment of biomass and the results suggest that there is no significant decrease in the molecular weight of lignin. This was attributed to the partial protection of lignin by polysaccharides in the compact biomass matrix, which may facilitate the simultaneous re-condensation process occurring along with depolymerization.[30,34,35] The results of this study strongly support this hypothesis, since in the absence of the plant polysaccharide matrix the molecular weight of lignin is significantly decreased.

Conclusions

The present study demonstrates the impact of autohydrolysis conditions on lignin. Our results suggest that with increasing temperature and time of autohydrolysis there is a decrease of lignin hydroxyl groups associated with aliphatic side chains, which is accompanied by a considerable increase in phenolic hydroxyl groups. This may be attributed to the autocatalyzed elimination of water from the benzylic position and the resultant carbonium ion facilitates the cleavage of β-*O*-4 aryl ether linkages and the subsequent formation of phenol. The molecular weight determination confirmed that apart from in whole biomass pretreatment, lignin autohydrolysis results in depolymerization as the pivotal reaction rather than recondensation.

Another interesting aspect of this data is that it could be used to guide the autohydrolysis chemistry of lignin isolated from cellulase treated biomass. For example, prior studies by

Sannigrahi and Ragauskas[36] have characterized the lignin residues from cellulosic ethanol processing streams and this study suggests that a hot water treatment of lignin could be a means of generating low molecular weight components for subsequent conversion to chemicals and fuels.

Acknowledgements

We are grateful to the Bioenergy Science Centre (BESC) for financial support. BESC is a U.S. Department of Energy Bioenergy Research Center supported by the office of Biological and Environmental Research in the DOE office of Science. BKD thanks USIEF, New Delhi for a Fulbright-Nehru Research Fellowship award enabling him to be involved in research work at Georgia Institute of Technology during his sabbatical leave from Gauahti University, India.

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