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PAPER

Pseudo-lignin and pretreatment chemistry

Poulomi Sannigrahi, Dong Ho Kim, Seokwon Jung and Arthur Ragauskas*

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The formation of pseudo-lignin by the combination of carbohydrate and lignin degradation products has been proposed to be responsible for the increased Klason lignin content in biomass pretreated under acidic conditions. Direct evidence for the presence of pseudo-lignin has never been presented. The formation of additional lignin-like material may be detrimental to enzymatic hydrolysis due to the non-productive binding of enzymes with lignin. To investigate the chemistry of pseudo-lignin formation, dilute acid pretreatments were performed on delignified hybrid poplar biomass under conditions of varying severity. The results show a progressive increase in the Klason lignin content of the acid pretreated material with increasing pretreatment severity. NMR and FT-IR spectroscopic characterization shows the development of aliphatic, unsaturated and carbonyl carbon functionalities in the samples pretreated at higher severities. Given the very low Klason lignin content of the starting material, acid catalyzed dehydration of carbohydrates is responsible for the formation of pseudo-lignin.

1 Introduction

Growing worldwide energy demands and increasing concerns of energy security and climate change have led to renewed global efforts in the development of alternative energy sources from renewable sources such as lignocellulosic biomass, to augment and replace fossil transportation fuels.^{1–3} Due to the inherent recalcitrance of biomass towards enzymatic deconstruction,² chemical pretreatments are necessary to make the carbohydrates amenable to enzyme hydrolysis and fermentation.⁴ Dilute acid pretreatment has emerged as one of the leading chemical pretreatment technologies for the conversion of lignocellulosic biomass to bioethanol. These pretreatments are performed over

a temperature range of 120 to 210 °C, with acid concentration typically less than 4 wt%, and residence times from a few seconds to an hour in either flow-through or batch reactors. Sulfuric acid is primarily used, though hydrochloric, nitric and phosphoric acids have also been employed.⁵ The primary effects of acid treatment on biomass are hemicellulose hydrolysis and disruption of the biomass internal structure to allow enzymes to access the cellulose fraction.^{6,7} Acid pretreatment does not result in significant delignification unless accompanied by a subsequent oxygen or lime treatment stage. In fact, several studies have found the acid insoluble (Klason) lignin content of acid treated or steam-exploded biomass to be higher than the starting material.^{8–12} By measuring the Klason lignin content of steam exploded aspen wood before and after extraction with acidic dioxane, a method commonly used for lignin isolation, Li *et al.*¹⁰ suggested that about 50% of the Klason lignin fraction was pseudo-lignin. Jakobsons *et al.*¹³ had inferred that during steam explosion, hemicellulose and lignin degradation products

BioEnergy Science Center, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, 30332, USA. E-mail: arthur.ragauskas@chemistry.gatech.edu; Fax: +1 (404) 894-4778; Tel: +1 (404) 894-9701

Broader context

Pretreatments performed under acidic conditions are frequently employed as an integral step in the process of converting lignocellulosic biomass to bioethanol. These pretreatments serve to remove hemicelluloses and disrupt the lignocellulosic structure, thus making the cellulose accessible to enzymes. In this process, a fraction of the carbohydrates are degraded to furfural and hydroxymethylfurfural. We have investigated the observation of increased acid insoluble lignin in biomass after acidic pretreatments. Partially delignified hybrid poplar biomass has been subjected to acid pretreatment and the resulting solid residue is seen to have a higher content of acid insoluble lignin especially under more severe conditions. Spectroscopic analysis shows that these acid treated residues have lignin-like characteristics and SEM images show the appearance of spherical droplets. These results provide evidence for the presence of alternate carbohydrate degradation pathways, which result in the formation of a lignin-like or pseudo-lignin solid phase. Pseudo-lignin may be detrimental to enzymatic action and pretreatment conditions should be optimized to reduce their formation.

combine to form lignin-like material termed pseudo-lignin. In their study, the pretreated material had a higher proportion of pseudo-lignin compared to real lignin with more severe pretreatment. In the case of pine biomass treated with sulfuric acid, results from solid-state ^{13}C NMR showed an increased proportion of unsaturated carbon species.¹² Together with results from proximate analysis, these carbon functionalities were hypothesized to have come about from either acid catalyzed dehydration of carbohydrates or from substitution reactions on lignin aromatic rings.¹²

While the formation of pseudo-lignin has been reported after steam explosion and dilute-acid pretreatments, it is likely to occur during all pretreatments conducted under low pH conditions. From a bioethanol production perspective, the importance of understanding the fundamental chemistry surrounding this apparent increase in Klason lignin content is important on several accounts. First, the presence of high amounts of lignin-like material is detrimental to the enzymatic deconstruction of biomass due to the non-productive binding of enzymes to this material. Lignin has been shown to inhibit the activity of cellulase and related enzymes such as β -glucosidase and xylanase by non-productive binding.¹⁴ Identification of pretreatment conditions with diminished pseudo-lignin formation will lead to reductions in enzyme requirements and loss of fermentable sugars and hence improve process economics. In addition, ongoing studies to optimize pretreatment chemistry are often guided by lignin or carbohydrate content and the fundamental structural determination of lignin.

To delineate the contributions of polysaccharides to pseudo-lignin, a series of acid pretreatments were performed on poplar holocellulose. Hybrid poplar is a short rotation woody crop with several desirable qualities for biofuels production including high cellulose content, pest and disease resistance and widespread geographic distribution.¹⁵ Using holocellulose, which is essentially free of lignin, ensures that if an increased Klason lignin content is observed after pretreatment, the possibility of the pseudo-lignin being formed from lignin can be eliminated. The solid residue after acid pretreatment of poplar holocellulose was studied using chemical, spectroscopic and imaging techniques. Under the most severe pretreatment conditions, there is a significant mass loss in the absence of the protection afforded by lignin to carbohydrates in biomass. NMR and FT-IR spectroscopic characterization shows that the pseudo-lignin consists of aromatic functionality, methoxyl and carbonyl functional groups. Spherical droplets previously attributed to lignin¹⁶ are seen on the pretreated biomass surface in the SEM images, indicating that pseudo-lignin resembles lignin in physical appearance as well. These results demonstrate that pseudo-lignin can be formed solely from carbohydrate degradation without significant contributions from lignin in the biomass.

2 Experimental

2.1 Biomass

Hybrid poplar (*Populus trichocarpa* \times *deltoides*) milled to pass a 20 mesh screen was obtained from Oak Ridge National Laboratory, Oak Ridge, TN. The sample was air-dried and

extractives were removed by sequential Soxhlet extractions with ethanol and ethanol/benzene (1 : 2, v/v).

2.2 Isolation of holocellulose

Holocellulose was isolated from the extractive-free biomass according to literature procedures by exposure to NaClO_2 (40% by dry weight of biomass) and acetic acid (10% by dry weight of biomass) at 70 °C for 1 h. The process was repeated to ensure maximum lignin removal.

2.3 Acid pretreatment of holocellulose

Holocellulose was treated using the acid pretreatment procedure of Lloyd and Wyman.¹⁷ In brief, 10.00 g holocellulose was soaked in 0.10 M sulfuric acid solution (5% solids), while stirring at room temperature for 4 h. The presoaked slurry was filtered and the solids were washed with excess DI water. The solids were then added to a 0.10 or 0.20 M sulfuric acid solution (5% solids) and transferred to a Parr 4560 mini pressure reactor (300 mL). The reactor was heated to the desired temperature with constant stirring at a heating rate of ~ 6 °C min^{-1} . Pretreatment conditions were chosen to cover a range of combined severity (CS).¹⁸

$\text{CS} = \log [t \exp [(T - T_{\text{ref}})/14.7]] - \text{pH}$, where t is the pretreatment time (min), T is the pretreatment temperature (°C) and T_{ref} is 100 °C.¹⁸

2.4 Isolation of *p*-dioxane lignin from untreated and pretreated holocellulose

The untreated holocellulose and solid residue from acid pretreatment were refluxed with *p*-dioxane/0.10 N HCl (9 : 1, v/v), under nitrogen following literature methods.¹⁹ The samples were filtered and washed with dioxane, and the combined aliquots were neutralized, filtered and concentrated under reduced pressure. The precipitated lignin was washed with water and freeze-dried.

2.5 Characterization of pretreated material

Carbohydrate, Klason lignin and acid soluble Klason lignin contents of the solid material recovered after pretreatment were measured according to NREL standard procedure.²⁰ The solid residue after sulfuric acid hydrolysis was weighed to obtain the Klason lignin content, while the liquid phase was analyzed using a Dionex HP-AEC for carbohydrate profile and with UV spectrophotometry for acid soluble Klason lignin content. Solid-state CP/MAS NMR measurements were carried out on a Bruker Advance-400 spectrometer operating at a ^{13}C frequency of 100.55 MHz. Samples were packed in 4 mm zirconia rotors fitted with kel-F caps and measured at 8 KHz spinning speed, 1 ms contact time. Data were processed with the MestreNova NMR software.

FT-IR analyses were performed after incorporating 2 mg samples of untreated and pretreated holocellulose in KBr pellets. Spectra were acquired on a Nicolet 550 spectrometer over the 4000 to 400 cm^{-1} region absorption range. Unwashed pretreated solids were also characterized using scanning electron microscopy (SEM), after mounting dry samples on aluminium specimen stubs and sputter coating with gold. Images were acquired on

a JEOL-1530 SEM at 10 kV beam accelerating voltage and various resolving powers.

3 Results and discussion

3.1 Lignin and carbohydrates in pretreatment residues

Holocellulose (hemicelluloses and cellulose), with very low Klason lignin content (1.6% by dry wt) isolated from hybrid poplar, was subjected to a two-step sulfuric acid pretreatment covering a range of combined severities (1.7 to 3.7).¹⁷ The reaction conditions used in steam explosion pretreatment of biomass often have combined severity values greater than 3.7.^{17,21} The results of carbohydrate and lignin determination by chemical analysis show that hemicelluloses are increasingly degraded after the more severe pretreatments. For all pretreatment conditions, excluding the least severe (CS = 1.7), the Klason lignin content of the solid residue is higher than the untreated poplar holocellulose (Table 1). The proportion of Klason lignin in the solids increases with increasing pretreatment severity and under the most severe conditions, the pretreated solid comprises 95% Klason lignin. A fraction of the lignin is solubilized during acid hydrolysis to measure Klason lignin and carbohydrate contents and is referred to as the acid soluble Klason lignin content in Table 1. Acid soluble Klason lignin shows a decreasing trend with increasing pretreatment severity.

A significant fraction (25 to 94%) of the total Klason lignin in the acid treated holocellulose was recovered by *p*-dioxane extraction. Extraction with acidic dioxane is used to remove carbohydrates. For the four most severe pretreatments, the *p*-dioxane soluble Klason lignin or pseudo-lignin fraction is larger than that isolated from the starting holocellulose. The relative proportion of pseudo-lignin in the most severely pretreated samples is also higher. This implies that not only are large amounts of pseudo-lignin being formed during the acid hydrolysis, since the starting material has a very low Klason lignin content, but also this 'new' Klason lignin is being formed solely from carbohydrates. The carbohydrates remaining in the solid residue are mostly glucan with small amounts of xylan in some samples (Table 1). Some of the carbohydrates are measured in the liquid phase after acid pretreatment, but with increasing severity a larger fraction is detected in the form of furans, which are formed by carbohydrate degradation. Prior studies have also shown that most of the carbohydrates lost during acid pretreatment are not detected in the form of furans.⁷ Other

competing degradation pathways leading to the formation of non-cellulosic polymeric materials have been suggested.²²

3.2 Scanning electron microscope (SEM) imaging

The physical structure of the untreated and acid treated holocellulose samples was studied by SEM imaging of unwashed samples. The most striking feature in the SEM images of the pretreated solids is the presence of discrete spherical balls or droplets on the surface (Fig. 1). The droplets cover a range of sizes from ~0.3 to 8.0 μm and exhibit a slightly rough exterior. The shapes of some droplets indicate that they have formed from the fusion of two smaller droplets. These structures are not

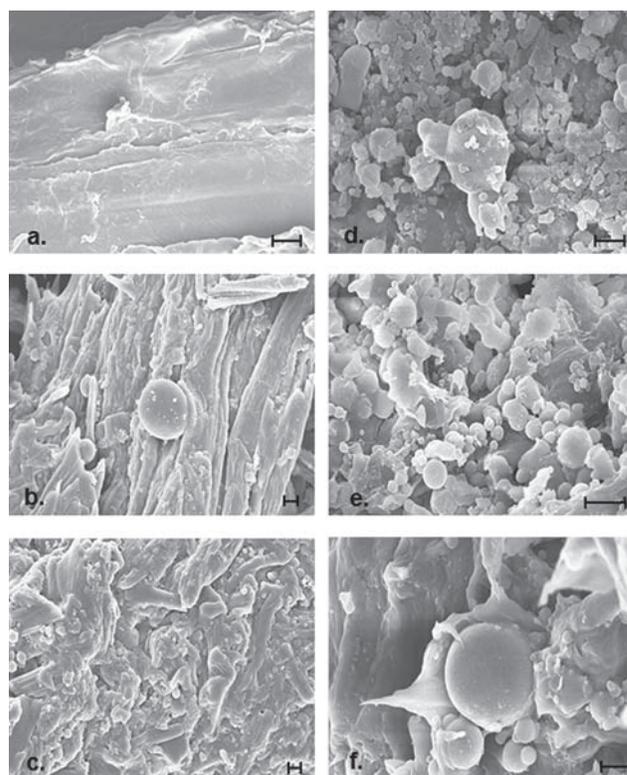


Fig. 1 SEM images of untreated and acid treated holocellulose: (a) untreated, (b) CS = 2.97, (c) CS = 3.27, (d) CS = 3.45, (e) CS = 3.27 at higher magnification, and (f) CS = 2.97 showing details of the surface of the lignin droplet. All scale bars are equivalent to 2 μm.

Table 1 Pretreatment conditions and lignin contents of untreated and acid pretreated poplar holocellulose^a

Sample	Time/min	Temp./°C	SA/M	CS	Mass/g	Klason lignin/g	Acid-soluble Klason lignin/g	Glucan/g	Xylan/g	Dioxane lignin/g
Holocellulose					10.00	0.16	0.66	6.72	2.18	0.21
Pretreatment 1	5.0	160.0	0.1	1.77	5.17	0.04	0.09	n.d.	n.d.	0.06
Pretreatment 2	20.0	170.0	0.1	2.67	5.02	0.08	0.09	n.d.	n.d.	0.16
Pretreatment 3	40.0	170.0	0.1	2.97	2.89	0.88	0.04	1.87	0.004	0.26
Pretreatment 4	40.0	180.0	0.1	3.27	1.91	0.93	0.03	0.003	0.00	0.48
Pretreatment 5	60.0	170.0	0.2	3.45	1.92	1.17	0.02	0.002	0.01	0.45
Pretreatment 6	60.0	180.0	0.2	3.74	2.03	1.93	0.01	n.d.	n.d.	0.47

^a SA: sulfuric acid concentration in second step of pretreatment; CS: combined severity parameter. Mass = mass of solid residue after acid pretreatment of 10.00 g holocellulose; n.d.: not determined; mannose, arabinose and galactose were detected in low concentrations in the untreated holocellulose and their concentrations were below the detection limit in the pretreated holocellulose.

present in the image from the untreated holocellulose surface. Similar spherical structures termed “lignin droplets” have been extensively studied on pretreated maize rinds.¹⁶ These authors isolated these droplets and performed spectroscopic characterization to conclude that they are composed of lignin. During pretreatment, the maize lignin droplets coalesce and migrate to the cell surface where they get deposited.¹⁶ In our study, as the severity of the pretreatments increases, the frequency of occurrence of these pseudo-lignin droplets also increases. The SEM image of the sample from CS = 3.27 is almost entirely covered with pseudo-lignin droplets (Fig. 1c). The newly formed pseudo-lignin yields a Klason lignin value by wet chemical analysis and it also resembles lignin in appearance by forming droplets or balls on the biomass surface. Coupled with results from spectroscopic analysis, pseudo-lignin can thus be broadly defined as aromatic material that yields a positive Klason lignin value and is not derived from native lignin.

3.3 Solid-state CP/MAS ¹³C NMR

The ¹³C NMR spectrum from untreated holocellulose shows peaks from cellulose between 60 and 110 ppm, hemicellulose acetyl groups at 21 and 173 ppm and a small shoulder at 56 ppm from methoxyl group (Fig. 2). NMR signals arising from lignin aromatic or unsaturated C (usually between 109 and 167 ppm) are not seen in the untreated holocellulose, due to its low Klason lignin content. The acetyl groups in hemicelluloses are highly susceptible to acid hydrolysis as is evident from the disappearance of these peaks after the least severe pretreatment (CS = 1.77). Signals from cellulose dominate the NMR spectra from all but the highest severity pretreatment. With increasing pretreatment severity new peaks appear in the NMR spectra of the acid treated holocellulose samples (Fig. 2). In the three most severe pretreatments (CS = 3.27 to 3.74), a broad signal in the aliphatic region (0–50 ppm) appears. In extractive-free biomass there are few signals that absorb in this region.¹² Since the holocellulose used in this study was isolated from extractive free poplar, the

chemistry of pseudo-lignin formation appears to be the source of these groups. A methoxyl signal at 56 ppm progressively increasing with severity is also present in these spectra.²³ In the aromatic/unsaturated C region, a broad peak centered at ~148 ppm is seen in the holocellulose NMR spectra after severe pretreatments. This is most likely associated with substituted aromatic C in pseudo-lignin.²³ In addition to these peaks, the spectrum from the most severe pretreatment shows a carbonyl peak at 200 ppm. These NMR results show that polyphenolic, lignin-like, materials with aliphatic, methoxyl, aromatic and carbonyl resonances develop from poplar holocellulose during acid hydrolysis and could account for the additional lignin detected by wet chemical analysis.

The mechanisms of degradation of glucose and xylose in acidic solutions have been studied using molecular dynamic simulations.^{22,24} These studies have confirmed the presence of sugar degradation pathways that do not lead to the formation of furfural and hydroxymethylfurfural. ¹³C NMR of isotopically labeled glucose added to commercial cellulose subjected to dilute acid pretreatment was very informative in identifying possible reaction pathways.²² These spectra showed that the C-1 in glucose was being converted mostly to sp² carbons and some to carbonyls and sp³ carbons. In the NMR spectra from acid pretreated holocellulose (Fig. 2), sp² carbon peaks are seen in the spectral region from 120 to 160 ppm, the signal from ketone carbonyl groups is seen in the most severely pretreated sample at 206 ppm and the broad aliphatic region can be attributed to the sp³ carbons. The labeled C-2 carbon was observed to be incorporated into the polymer as sp² carbons (141 ppm) and ketone carbonyl carbons (206 ppm).²² These peaks are also seen in the ¹³C NMR spectra from our more severely pretreated holocellulose. It is also possible that HMF and furfural are incorporated into the pseudo-lignin polymer by forming bonds with glucose and xylose or their degradation intermediates respectively.²⁵ Furfural xylose or difurfural xylose are possible condensation products.²⁵ The formation of these compounds can account for the unsaturated C (120 to 160 ppm) and aliphatic C peaks (10 to 60 ppm) seen in the NMR spectra from the pretreated poplar holocellulose.

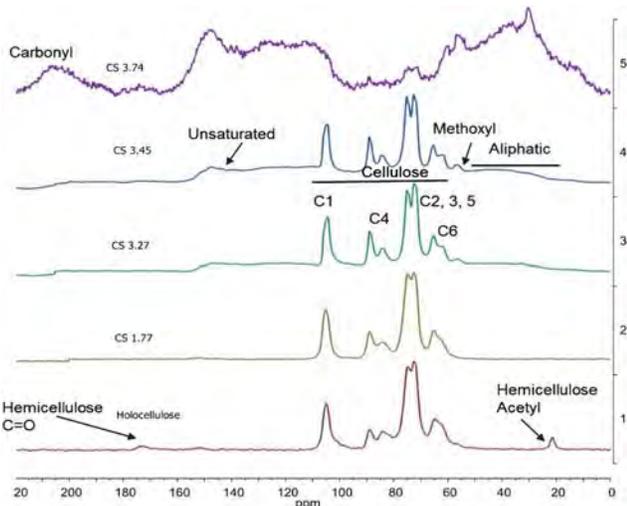


Fig. 2 Solid-state CP/MAS ¹³C NMR spectra from untreated and dilute acid pretreated poplar holocellulose. The combined severity (CS) values of the pretreatments and the peaks of interest are identified in the spectra.

3.4 FT-IR characterization

FT-IR spectra from the untreated and acid hydrolyzed holocellulose provide additional information on the chemical transformations taking place in poplar holocellulose during acid pretreatment (Fig. 3). The signal from C–O stretching in hemicelluloses at 1245 cm⁻¹ is present only in the untreated holocellulose. The broad C–O stretching bands from ring alcohols in carbohydrates around 1050 cm⁻¹ are seen in all but the most severely treated holocellulose. In the samples treated at the four most severe conditions, a peak at around 1720 cm⁻¹ is seen, which can be assigned to carbonyl (C=O) stretching in unconjugated ketones in lignin side chains.²⁶ This confirms the presence of ketone carbonyl groups seen in the NMR spectra (Fig. 2). The samples from the most severe conditions also show a peak at ~1595 cm⁻¹ due to aromatic ring stretching. A small peak at 835 cm⁻¹ arising from C–H out-of-plane deformation in 1,3,5-trisubstituted benzene rings in lignin¹⁶ is seen only in the most severely treated sample.

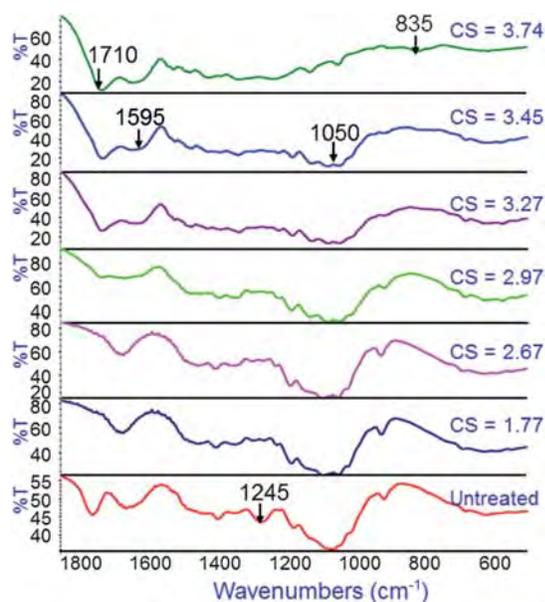


Fig. 3 Fingerprint region of the FT-IR spectra from untreated and dilute acid pretreated poplar holocellulose. The combined severity (CS) values of the pretreatments and peaks of interest are marked in the spectra.

4 Conclusion

Multiple lines of evidence have been presented showing that during acid pretreatment of poplar holocellulose, especially under higher severity conditions, a new Klason lignin material is produced from carbohydrates. SEM images show the formation of spherical structures on the surface of the acid pretreated holocellulose. Results from chemical and spectroscopic analysis presented here show that the carbohydrates in hybrid poplar biomass are being modified by acid catalyzed dehydration or reforming reactions to yield considerable amounts of unsaturated carbon such that the products are polyphenolic in structure and yield a Klason lignin value. Dilute acid pretreatments of biomass should be performed under the mildest reaction conditions possible to avoid the formation of these additional lignin fractions which may be detrimental to enzymatic deconstruction.

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