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Pseudo-lignin formation and its impact on enzymatic hydrolysis

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ABSTRACT

Pseudo-lignin, which can be broadly defined as aromatic material that yields a positive Klason lignin value and is not derived from native lignin, has been recently reported to form during the dilute acid pretreatment of poplar holocellulose. To investigate the chemistry of pseudo-lignin formation, GPC, FT-IR and ^{13}C NMR were utilized to characterize pseudo-lignin extracted from dilute-acid pretreated α-cellulose and holocellulose. The results showed that pseudo-lignin consisting of carbonyl, carboxylic, aromatic and aliphatic structures was produced from dilute acid pretreated cellulose and hemicellulose. Pseudo-lignin extracted from holocellulose pretreated at different conditions had similar molecular weights ($M_n \sim 1000$ g/mol; $M_w \sim 5000$ g/mol) and structural features (carbonyl, carboxylic, aromatic and methoxy structures). These characterizations have provided the pseudo-lignin formation mechanisms during pretreatment. The presence and structure of pseudo-lignin is important since pseudo-lignin decreases the enzymatic conversion.

1. Introduction

Increasing global energy demand, unstable and expensive petroleum resources and concern over global climate changes have led to the development of renewable energy sources such as cellulosic ethanol that can supplement fossil fuels (Himmel et al., 2007; Ragauskas et al., 2006); however, the yield of ethanol production from native lignocellulosic materials is relatively low due to the natural resistance of plant cell walls to decomposition from microbes and enzymes (Himmel et al., 2007). Dilute acid pretreatment has been proven to successfully hydrolyze hemicelluloses and disrupt the lignocellulosic structure thereby reducing its recalcitrance for a wide range of feedstocks including softwoods, hardwoods, herbaceous crops, agricultural residues, wastepaper and municipal solid waste (Zheng et al., 2009). Dilute acid pretreatment does not lead to significant delignification. Instead, several studies have found that the acid-insoluble (Klason) lignin content of dilute acid pretreated material is often higher than that of the starting material (Jung et al., 2010; Mao et al., 2010; Pingali et al., 2010; Sannigrahi et al., 2008). This phenomenon has been hypothesized to be due to repolymerization of polysaccharides degradation products (such as furfural) and/or polymerization with lignin to form a lignin-like material termed pseudo-lignin (Li et al., 2005, 2007).

Sannigrahi et al. (2011) demonstrated that pseudo-lignin can be generated from carbohydrates without significant contribution from lignin during dilute acid pretreatment, especially under high severity pretreatment conditions. Scanning electron microscopy (SEM) studies on dilute acid pretreated holocellulose revealed the presence of spherical droplets on the surface of pretreated holocellulose, which were attributed to pseudo-lignin. ^{13}C CP/MAS NMR analysis of pretreated holocellulose indicated significant peaks originating from carbonyl, aromatic, methoxy and aliphatic structures and attributed to the structure of pseudo-lignin. Furthermore, the intensities of these peaks increased as pretreatment severity increased, suggesting an acid-catalyzed disproportionation mechanism accompanying pseudo-lignin formation.

Although the generation of pseudo-lignin has been proposed by many researchers (Jakobsons et al., 1995; Li et al., 2007; Negro, 2003; Nguyen et al., 2000; Sievers et al., 2009), there has been a lack of understanding of the fundamental chemistry surrounding pseudo-lignin. From a bioethanol production perspective, it is extremely important to understand the mechanisms underlying the generation of pseudo-lignin as these compounds may be detrimental to subsequent enzymatic processing. The objective of the current work is to characterize pseudo-lignin isolated from dilute acid pretreated hybrid poplar holocellulose and α-cellulose, in order to give an insight into the chemical structure and formation mechanism of pseudo-lignin. More importantly, the interaction between pseudo-lignin spheres and cellulases was investigated in order to determine whether the presence of pseudo-lignin may be detrimental to enzymatic hydrolysis of cellulose.

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2. Methods

2.1. Materials

Hybrid poplar (*Populus trichocarpa x deltoides*) was obtained from Oakridge National Laboratory, TN and Wiley milled to pass a 2-mm screen. The sample was air-dried and stored at −20 °C.

2.2. Pseudo-lignin preparation

Extractives from hybrid poplar were removed by Soxhlet extraction with ethanol/toluene (1/2, v/v) for 24 h. Holocellulose was isolated from the extractive-free poplar by sodium chlorite bleaching (Hubbell and Ragauskas, 2010; Wise et al., 1946). α-Cellulose was obtained from the poplar holocelluose according to Tappi method T-203 cm-09. Two-step dilute acid pretreatments with two different conditions for the second step were applied to both poplar holocellulose and α-cellulose (Table 1). The samples were soaked in 0.10 M sulfuric acid (5% solids) while stirring at room temperature for 4 h. The presoaked slurry was filtered through a filter funnel and the solids were washed with excess DI water, added to 0.10 or 0.20 M sulfuric acid (5% solids) and transferred to a Parr 4560 mini pressure reactor (600 mL) for the second pretreatment step. The reactor was heated to the desired temperature with constant stirring at a heating rate of ~6 °C/min. After pretreatment, the slurry was filtered through a filter funnel and the pretreated solids were washed with excess DI water.

The dilute acid pretreated poplar holocellulose and α-cellulose were refluxed with *p*-dioxane/water (9:1, v/v), under nitrogen (Froass and Ragauskas, 1998). The mixtures were filtered through a filter funnel and washed with *p*-dioxane. The combined aliquots were concentrated under vacuum and dissolved in DI water to precipitate pseudo-lignin. Finally, the precipitated pseudo-lignin was freeze-dried and vacuum-dried at 40 °C.

2.3. Preparation of pseudo-lignin on holocellulose samples

Hybrid poplar was subjected to a two-step dilute acid pretreatment with the same protocol as applied to holocellulose A. Pretreated poplar holocellulose was isolated from dilute acid pretreated poplar with sodium chlorite bleaching (Hubbell and Ragauskas, 2010; Wise et al., 1946). Pseudo-lignin extracted from pretreated holocellulose was added to pretreated poplar holocellulose to produce pseudo-lignin on holocellulose samples. In brief, the appropriate amount of pseudo-lignin (10%, 20% and 40% of acid-insoluble lignin value of pretreated poplar) was dissolved in *p*-dioxane/water (10/1, v/v). A sample of pretreated poplar holocellulose (0.50 g dry weight) was added to pseudo-lignin solution, and the mixture was allowed to stir at room temperature for 2 h. The slurry was transferred to an aluminum weigh dish and allowed to air-dry in a fumehood.

Table 1
Conditions for treatment (2nd step) of poplar holocellulose and α-cellulose following initial 1st step treatment of soaking (5% solids) while stirring in 0.10 M H_2SO_4 at room temperature for 4 h.

Sample	2nd step condition
Holocellulose A	180 °C, 0.10 M H_2SO_4, 40 min
Holocellulose B	180 °C, 0.20 M H_2SO_4, 60 min
α-Cellulose A	170 °C, 0.10 M H_2SO_4, 20 min
α-Cellulose B	180 °C, 0.10 M H_2SO_4, 40 min

2.4. Acid-insoluble lignin and carbohydrate analysis

Samples for carbohydrate constituents and acid-insoluble lignin analysis were prepared using a two-stage acid hydrolysis protocol based on Tappi method T-222 om-88 with a slight modification. The first stage utilized a severe pH and a low reaction temperature (72% H_2SO_4 at 30 °C for 1 h). The second stage was performed at lower acid concentration and higher temperature (3% H_2SO_4 at 121 °C for 1 h) in an autoclave. The resulting solution was cooled to room temperature and filtered through a G8 glass fiber filter (Fisher Scientific, USA). The remaining residue, considered as acid-insoluble lignin, was oven-dried and weighed to obtain the acid-insoluble lignin content. The filtered solution was analyzed for carbohydrate constituents by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using Dionex ICS-3000 (Dionex Corp., USA).

2.5. Analysis of pseudo-lignin extracted from dilute acid pretreated holocellulose and α-cellulose

2.5.1. Measurement of molecular weight of isolated pseudo-lignin

The extracted pseudo-lignin sample (20.0 mg) was weighed into a small vial and dried in a vacuum oven at 40 °C overnight. Anhydrous pyridine (0.50 ml) and acetic anhydride (0.50 ml) were added sequentially, the vial was capped, vortexed and allowed to sit at room temperature for 24 h. Ethanol (5.00 ml) was added to quench the reaction. The vial was allowed to sit uncapped overnight in a fumehood to dry. The air-dried sample was placed in a vacuum oven at 40 °C for 4 h to remove residual volatiles. The acetylated pseudo-lignin sample was purified by dissolving in chloroform (1.00 ml) and precipitating into diethyl ether (75.00 ml). The resulting solids were collected by filtration through ultrafiltration and dried in a vacuum oven at 40 °C for 1 h.

Prior to gel permeation chromatography (GPC) analysis, the acetylated pseudo-lignin samples were dissolved in tetrahydrofuran (1.00 mg/ml), filtered through a 0.45-μm filter and placed in a 2-ml auto-sampler vial. The molecular weight distributions of the acetylated pseudo-lignin samples were analyzed by an Agilent GPC SECurity 1200 system equipped with four Waters Styragel columns (HR0.5, HR2, HR4, HR6), Agilent refractive index (RI) detector and Agilent UV detector (270 nm). Tetrahydrofuran was used as the mobile phase (1.0 ml/min) and the injection volume was 30.0 μl. A calibration curve was constructed based on 10 narrow polystyrene standards ranging in molecular weight from 1.2×10^3 to 5.5×10^4 g/mol. Data collection and processing were performed with Polymer Standards Service WinGPC Unity software (Build 6807). The number-average and weight-average molecular weights (M_n and M_w) were calculated by the software relative to the universal polystyrene calibration curve.

2.5.2. FTIR-ATR spectroscopic analysis

The Spectrum One FT-IR system (Perkin Elmer, Wellesley, MA) with a universal attenuated total reflection (ATR) accessory was used to characterize the extracted pseudo-lignin samples. Each sample was pressed uniformly and tightly against the diamond surface using a spring-loaded anvil. FT-IR spectra were obtained by averaging 64 scans from 4000 to 650 cm^{-1} at 4 cm^{-1} resolution. Baseline and ATR corrections for penetration depth and frequency variations were carried out using the Spectrum One software supplied with the equipment.

2.5.3. ^{13}C NMR spectroscopic analysis

NMR experiments were performed using a Bruker AMX-400 spectrometer operating at a frequency of 100.61 MHz for ^{13}C NMR analysis. Quantitative ^{13}C NMR spectrum was acquired using dimethylsulfoxide (DMSO)-d_6 (450 μL) as the solvent for

Table 2
Lignin and carbohydrate contents of holocellulose and α-cellulose (based on oven-dried samples).

Sample	Acid-insoluble lignin (%)	Xylan (%)	Glucan (%)	Recovery from pretreatment (%)	Pseudo-lignin extraction yield (isolated pseudo-lignin/ total acid-insoluble lignin × 100%)
α-cellulose	2.33	1.01	99.42	NA	NA
α-cellulose A	6.61	0	94.01	62.67	0
α-cellulose B	19.95	0	80.41	31.73	44.58
Holocellulose	4.68	22.00	68.09	NA	NA
Holocellulose A	37.47	0	65.31	28.81	51.30
Holocellulose B	86.93	0	6.95	19.19	33.71

Table 3
Molecular weights of pseudo-lignin extracted from pretreated α-cellulose and holocellulose.

Pseudo-lignin extracted from	M_n (g/mol)	M_w (g/mol)	PDI
α-Cellulose (180 °C, 0.10 M H_2SO_4, 40 min)	1.08×10^3	3.44×10^3	3.17
Holocellulose (180 °C, 0.10 M H_2SO_4, 40 min)	1.24×10^3	5.08×10^3	4.09
Holocellulose (180 °C, 0.20 M H_2SO_4, 60 min)	1.19×10^3	5.97×10^3	5.00

pseudo-lignin (120 mg) at 303 K with an inverse-gated decoupling sequence, 90° pulse angle, 12-s pulse delay and 8000 scans. Distortionless enhancement by polarization transfer (DEPT) NMR spectra were recorded using a 135° pulse angle, 3-s pulse delay, and 12000 scans for the same samples from quantitative ^{13}C NMR analysis.

2.5.4. Scanning electron microscopy (SEM)

After mounting dry samples on aluminum specimen stubs and sputter coating with gold, SEM images of pretreated holocellulose and pseudo-lignin on holocellulose samples were acquired on a JEOL-1530 SEM at 5 kV beam accelerating voltage and various resolving powers.

2.6. Enzymatic hydrolysis

Cellulase (4-glucano-hydrolase) from *Trichoderma reesei ATCC* 26921 and Novozyme 188 (β-glucosidase) from *Aspergillus niger* were purchased from Aldrich–Sigma and used as received. The activities of cellulase and β-glucosidase were determined to be 91.03 FPU/ml and 387.70 CBU/ml, respectively according to Ghose (1987). Enzymatic hydrolysis of different samples was performed at a consistency of 1% (w/v) in 50 mM citrate buffer (pH 4.8) with cellulase and β-glucosidase loadings of 20 FPU/g and 40 CBU/g, respectively. The mixture was incubated at 50 °C under continuous agitation at 150 rpm. Prior to the addition of enzymes, the substrate and buffer mixtures were placed on the shaking incubator for 10 min to allow the substrate to disperse uniformly in the buffer. A sample of hydrolysis liquid (1.00 mL) at time intervals of 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 7, 24 and 48 h was withdrawn and the hydrolysis was quenched by submersion for 10 min in a vigorously boiling water bath. The liquid samples were then immediately frozen at −20 °C until analysis on an Agilent 1200 series HPLC system (Agilent Technologies) equipped with an auto sampler and an Aminex HPX-87H column and pre-column (Bio-rad Laboratories). The analysis was carried out at 65 °C using 10 mM nitric acid as eluent at a flow rate of 0.6 mL min^{-1} and with refractive index detection. The calibration of the system was performed with glucose standards (Cateto et al., 2011). The results represented here were from single experiments.

3. Results and discussion

3.1. Pseudo-lignin extraction yields

The lignin and carbohydrate contents of untreated and pretreated α-cellulose and holocellulose are summarized in Table 2. In general, the acid-insoluble lignin content of pretreated samples is higher than that of the starting material, indicating the forma-

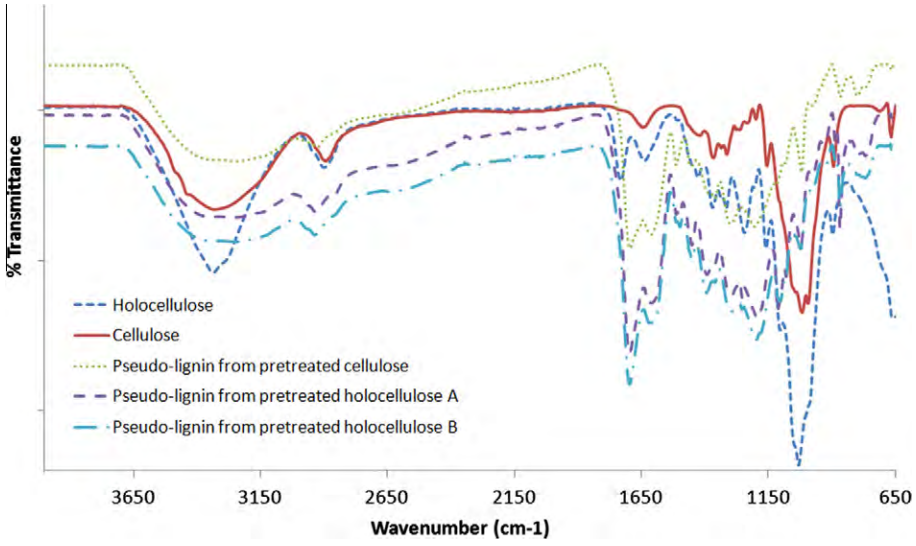

Fig. 1. FT-IR spectra of cellulose, holocellulose, pseudo-lignin extracted from pretreated α-cellulose, holocellulose A (0.1 M) and holocellulose B (0.2 M).

Table 4
Peak assignments for FT-IR spectra of pseudo-lignin.

Wavenumber (cm^{-1})	Assignment
3238	O–H stretching in alcohols, phenols or carboxylic acids
2923	Aliphatic C–H stretching
1697	C=O stretching in carboxylic acids, conjugated aldehydes or ketones
1611, 1512	Aromatic C=C stretching (in ring)
1360	Aliphatic C–H rocking
1299, 1203, 1020	C–O stretching in alcohols, ethers, or carboxylic acids
867, 800	Aromatic C–H out-of-plane bending

tion of pseudo-lignin during pretreatment. The proportion of acid-insoluble lignin and/or pseudo-lignin in the pretreated solids increased as the pretreatment severity increased, while the proportion of carbohydrates retained in the solids showed the inverse trend. Compared to untreated α-cellulose, the increase in acid-insoluble lignin content of α-cellulose pretreated at the least severe conditions (170 °C, 0.10 M H_2SO_4, 20 min) was not significant, leading to no significant amount of isolated pseudo-lignin. Whereas the acid-insoluble lignin content of α-cellulose B increased by more than seven times, and its glucan value declined by 19% compared to untreated α-cellulose. At the most severe conditions (180 °C, 0.20 M H_2SO_4, 60 min), holocellulose B has the highest proportion of pseudo-lignin and the least amount of glucan retained compared to untreated holocellulose. Furthermore, pretreated holocellulose generated higher proportion of pseudo-lignin than pretreated α-cellulose, even under the same pretreatment conditions. Additionally, no significant amount of xylan was retained for all samples after pretreatment. These results suggest that pseudo-lignin can be produced from both acid pretreated cellulose and hemicellulose, and its proportion increases as the pretreatment severity increases.

3.2. Molecular weight analysis of isolated pseudo-lignin

The molecular weights of isolated pseudo-lignin are shown in Table 3. In general, the molecular weights of isolated pseudo-lignin were much lower than those of milled wood lignin (M_n ~4060 g/mol; M_w ~10002 g/mol) from poplar (Kim et al., 2011). Furthermore, pseudo-lignin extracted from holocellulose pretreated at different conditions had similar molecular weights, which were larger than those of pseudo-lignin extracted from pretreated α-cellulose.

3.3. Structural characterization of isolated pseudo-lignin

The FT-IR spectra of α-cellulose, holocellulose, pseudo-lignin extracted from pretreated α-cellulose, holocellulose A and holocellulose B are presented in Fig. 1. The FT-IR spectra of all isolated pseudo-lignin samples show similar profiles but different intensities of the absorption bands, which are significantly distinct from those of the starting materials (α-cellulose and holocellulose). Based on FT-IR characterization, pseudo-lignin consists of hydroxyl, carbonyl and aromatic structures. The hydroxyl stretching peaks at ~3238 cm^{-1} are strong and broad, indicating the presence of hydrogen-bonding in isolated pseudo-lignin (Pavia et al., 2009). The strong bands at ~1697 cm^{-1} and ~1611 cm^{-1}, together with the band at ~1512 cm^{-1} can be attributed to C=O (carbonyl and/or carboxylic) conjugated with aromatic ring, whereas the bands in the 1320–1000 cm^{-1} region correspond to C–O stretching (in alcohols, ethers or carboxylic acids) (Pavia et al., 2009). These observations indicate dehydration and aromatization reactions of carbohydrates take place during the formation of pseudo-lignin. In addition, the peak at ~867 cm^{-1} arising from C–H out-of-plane bending suggests the benzene rings of pseudo-lignin are 1,3,5-trisubstituted (Dyer, 1965). The above peak assignments are summarized in Table 4.

To further characterize the functional groups in pseudo-lignin, ^{13}C NMR spectra of isolated pseudo-lignin were obtained and this data is summarized in Fig. S1 in Supplemental data. A primary qualitative assignment based on literature data is proposed in Table S1 (Pavia et al., 2009). The ^{13}C NMR spectra are predominantly comprised of signals from carbonyl, carboxylic, aromatic and aliphatic structures, which do not contain significant signals from cellulose and/or xylan. The peaks centered at 208–205 ppm and 203–185 ppm can be attributed to C=O in ketones and C=O in aldehydes, respectively. Whereas the peaks centered at 178–172 ppm correspond to C=O in carboxylic acids. This is consistent with FT-IR characterization, indicating the presence of carbonyl and carboxylic groups in pseudo-lignin. In addition, the intensities of carbonyl and carboxylic peaks are stronger for pseudo-lignin extracted from pretreated holocellulose B compared to other samples, which implies more degradation reactions of carbohydrates occur at the more severe conditions. The ^{13}C NMR spectra of isolated pseudo-lignin also present common peaks in the aromatic region (δ 155–96 ppm). The peaks in the 155–142 ppm region are characteristic of aromatic C–O bonds. Whereas the peaks in the 142–125 ppm and 125–96 ppm regions represent aromatic C–C bonds and aromatic C–H bonds, respectively. Among the aromatic signals, aromatic C–O bonds are the most predominant, and the signal at ~66 ppm was due to *p*-dioxane which could not be fully removed after extended time in a vacuum oven. To obtain further precise knowledge of the carbon signals in the 96–0 ppm region, the isolated pseudo-lignin samples were investigated by DEPT (θ = 135 °C) edited ^{13}C NMR spectroscopy, and Fig. S2 represents the DEPT-135 NMR spectrum of pseudo-lignin extracted from pretreated holocellulose B. According to DEPT NMR analysis, the two peaks centered at ~72 ppm and ~63 ppm can be attributed to hydroxylated methylene groups. Signals at 61–59 ppm and ~56 ppm correspond to the methoxy groups connected to aromatic rings. Furthermore, their intensities are stronger for pseudo-lignin extracted from pretreated holocellulose than those from pretreated α-cellulose, which may be due to the presence of 4-*O*-methyl-D-glucuronic acid in the hemicellulose of poplar (Teleman et al., 2000). Pseudo-lignin also possesses aliphatic structures, as can be deduced from the signals in the 50–20 ppm region, where the peaks centered at ~30 ppm and ~26 ppm correspond to CH groups while the signals at ~29 ppm represent CH_2 groups according to the DEPT-135 NMR spectra. In addition, there are two CH_2 groups centered at ~38 ppm and ~28 ppm for pseudo-lignin extracted from holocellulose B. Furthermore, the ^{13}C NMR spectra of pseudo-lignin extracted from pretreated holocellulose are similar, in addition to the similar FT-IR spectra and molecular weights, suggesting they have similar structural features. This is probably due to pseudo-lignin was formed from the same substrate and/or the difference in these two pretreatment severities was not distinct enough to significantly change the pseudo-lignin structure.

Table 5
Lignin and carbohydrate contents of dilute acid pretreated poplar and holocellulose (based on pretreated samples).

Sample	Acid-insoluble lignin (%)	Glucan (%)	Total (%)
Pretreated poplar	56.41	39.75	96.16
Pretreated holocellulose	4.15	91.30	95.45

Table 6
The quantities of pseudo-lignin (based on acid-insoluble lignin value of pretreated poplar) and holocellulose addition for the pseudo-lignin on holocellulose preparation.

Sample	Pseudo-lignin extracted from holocellulose pretreated at	Pseudo-lignin addition level (%)	Pseudo-lignin addition (g)	Holocellulose addition (g)	Total mass (g)
a	180 °C, 0.10 M H_2SO_4, 40 min	10	0.07	0.50	0.57
b	180 °C, 0.20 M H_2SO_4, 60 min	10	0.07	0.50	0.57
c	180 °C, 0.10 M H_2SO_4, 40 min	20	0.14	0.50	0.64
d	180 °C, 0.20 M H_2SO_4, 60 min	20	0.14	0.50	0.64
e	180 °C, 0.10 M H_2SO_4, 40 min	40	0.28	0.50	0.78
f	180 °C, 0.20 M H_2SO_4, 60 min	40	0.28	0.50	0.78
Pretreated holocellulose	NA	NA	NA	0.50	0.50
Pretreated poplar	NA	NA	NA	NA	1.26

3.4. Mechanistic consideration

Although the concept of pseudo-lignin has been applied for several decades (Jakobsons et al., 1995; Li et al., 2007; Negro, 2003; Nguyen et al., 2000; Sievers et al., 2009), there has been a lack of detailed mechanistic studies of pseudo-lignin formation during pretreatment, probably owing to the complexity of the pseudo-lignin structure and the heterogeneity of the reaction media. The data from lignin and carbohydrate analysis indicates that the proportion of pseudo-lignin increases as the amount of carbohydrates retained decreases. Sannigrahi et al. (2011) showed that a large fraction of the carbohydrates was detected in the form of furans after dilute acid pretreatment. In addition to the presence of high proportions of unsaturated carbons in the pseudo-lignin structure, it can conclude that the hydrolysis of polysaccharides to the corresponding monosaccharides, and the subsequent dehydration and fragmentation of sugars take place during dilute acid pretreatment. It is generally accepted that 5-hydromethylfurfural (HMF) and furfural are the first and main dehydration products of hexoses and pentoses, respectively (Li et al., 2005). Further rearrangements of HMF and/or furfural to yield other aromatic compounds have been suggested. For instance, 1,2,4-benzenetriol (BTO) was generated by hydrolytic ring-opening reaction of HMF in yields of up to 46% at 50% HMF conversion (Luijkx et al., 1993). Additionally, 3,8-dihydroxy-2-methylchromone (DMC) was the major aromatic component in the slightly acidic degradation of D-xylose (Popoff and Theander, 1972). These results suggest BTO and DMC as key intermediates for pseudo-lignin formation during dilute acid pretreatment. The subsequent reaction stages involve polycondensation and/or polymerization reactions as BTO can rapidly react with HMF and/or furfural via aromatic electrophilic substitution to produce a three-dimensional polymer under acid catalysis. In the presence of oxygen, oxidative polymerization of BTO to form a poly(phenylene)-like structure is also possible (Yamamoto et al., 1990). The above reaction pathways are summarized in Fig. S3, which suggest high temperature and the presence of acid and oxygen are the crucial conditions for pseudo-lignin formation during dilute acid pretreatment and indicate lower temperature, lower acid concentration and/or anaerobic condition may suppress pseudo-lignin generation during pretreatment.

3.5. Pseudo-lignin/enzyme interaction

Donohoe et al. (2008) observed lignin droplets on the cell wall of biomass after dilute acid pretreatment, which act as a physical barrier to prevent enzyme access to the carbohydrate fraction of biomass during enzymatic hydrolysis. In addition, these lignin droplets tend to irreversibly bind to enzymes through hydrophobic interactions that cause a loss in their activities. Such non-productive binding of enzymes to lignin has been suggested to be responsible for the requirement of high enzyme loadings (Balan et al., 2009; Yang and Wyman, 2006). Since the presence of pseudo-lignin droplets on the surface of pretreated holocellulose has been observed (Sannigrahi et al., 2011), the interactions between pseudo-lignin droplets and enzymes were investigated. Several pseudo-lignin on holocellulose samples were prepared, and the glucose yields of enzymatic hydrolysis of these samples were compared with dilute acid pretreated poplar and holocellulose. The lignin and carbohydrate contents of dilute acid pretreated poplar and holocellulose are tabulated in Table 5. Table 6 shows the quantities of

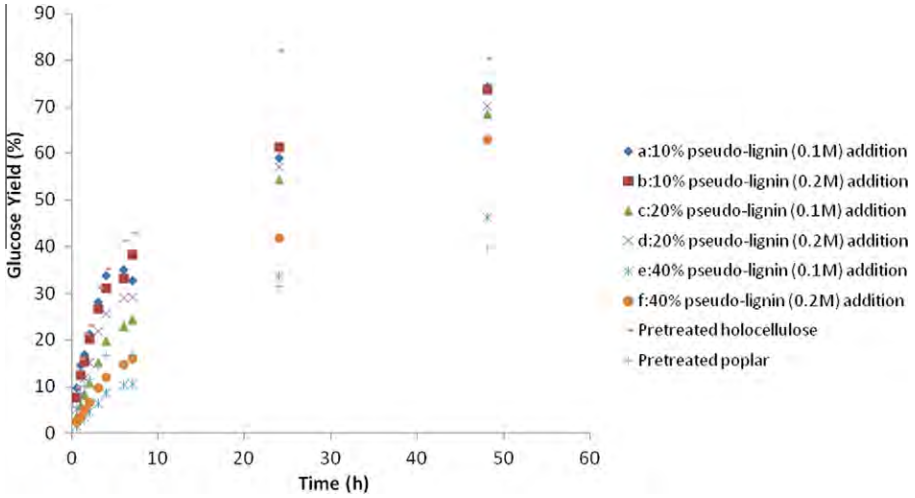

Fig. 2. Time course of glucose yield of various samples after 48 h of enzymatic hydrolysis.

pseudo-lignin and holocellulose addition for the preparation of pseudo-lignin on holocellulose samples. The physical structure of the pseudo-lignin on holocellulose sample and pretreated holocellulose was studied by SEM imaging. Compared to the surface of pretreated holocellulose (Fig. S4 left), discrete spherical droplets representing pseudo-lignin balls were presented on the surface of pretreated holocellulose of the pseudo-lignin on holocellulose sample (Fig. S4 right). These pseudo-lignin droplets could be detrimental to enzymatic hydrolysis of pretreated biomass. Indeed, the enzymatic hydrolysis results (Fig. 2) clearly indicate the presence of pseudo-lignin/enzymes interaction, since the glucose yield declines as the lignin and/or pseudo-lignin content increases. Pseudo-lignin on holocellulose samples at a level of 10% have the highest glucose yields (~74% after 48 h) among all pseudo-lignin on holocellulose samples, whereas samples at 40% addition level have the lowest (~46% after 48 h). In general, pseudo-lignin on holocellulose samples with the same pseudo-lignin content have similar glucose yields, which is probably due to pseudo-lignin samples extracted from holocellulose pretreated at different conditions have similar structural features. Pretreated holocellulose has the highest glucose yield (~80% after 48 h) among all samples while pretreated poplar gave the lowest (~40% after 48 h), which may be due to the lignin content of pretreated poplar under the conditions studied.

4. Conclusions

Results from chemical and spectroscopic analysis indicated that during dilute acid pretreatment, cellulose and hemicellulose in hybrid poplar biomass underwent acid-catalyzed dehydration, fragmentation, rearrangement and polycondensation and/or polymerization reactions to produce an acid-insoluble material termed pseudo-lignin, which consists of carbonyl, carboxylic, aromatic and aliphatic structures. Pseudo-lignin not only yields a positive Klason lignin value, but also significantly inhibits enzymatic hydrolysis of cellulose. This suggests that dilute acid pretreatment should be performed at lower severity and/or anaerobic conditions in order to avoid the formation of pseudo-lignin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.04.037.

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