Impact of Pseudolignin versus Dilute Acid-Pretreated Lignin on Enzymatic Hydrolysis of Cellulose

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S *Supporting Information*

ABSTRACT: To evaluate the inhibition effects of pseudolignin to enzymatic hydrolysis of cellulose in comparison to lignin, enzymatic mild acidolysis lignin (EMAL) was isolated from poplar after an 8 min pretreatment at 170 °C using 0.5% H_2SO_4. Fourier transform infrared (FT-IR) and ^{13}C NMR characterization revealed that the poplar lignin was partially degraded during the pretreatment and did not contain detectable amounts of pseudolignin. Holocellulose was treated with varying amounts of pseudolignin and/or EMAL dissolved in *p*-dioxane and then dried. The treated and control holocellulose was then treated to a standard cellulase treatment, and the results from enzymatic hydrolysis of these samples showed that the dilute acid-pretreated lignin inhibited hydrolysis in the initial stage but had a negligible impact on the overall cellulose-to-glucose conversion yield. In contrast, pseudolignin significantly reduced the overall enzymatic conversion yield of cellulose to glucose. This study suggests that pseudolignin formation needs to be avoided because it is more detrimental to enzymatic hydrolysis of cellulose than dilute acid-pretreated lignin.

KEYWORDS: *Poplar, Pseudolignin, Dilute acid pretreatment, Lignin, Holocellulose*
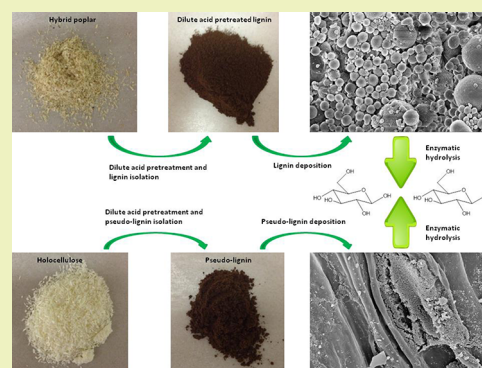
INTRODUCTION

Increasing global energy demand, unstable and expensive petroleum resources, and concern over global climate changes lead to the development of biofuels from lignocellulosics, which are relatively inexpensive, abundant, and based on sustainable feedstocks.[1,2] To convert lignocellulosics to ethanol, plant polysaccharides need to be deconstructed into their corresponding monosaccharides, which subsequently are biologically fermented to ethanol. Utilization of enzymes to produce fermentable sugars is regarded as the most viable strategy, because enzymatic hydrolysis of lignocellulosics offers several advantages including higher yield, lower byproduct formation and energy requirement, mild operation conditions, and environmentally benign processing compared to conventional chemical hydrolysis.[3] However, native lignocellulosics are recalcitrant to decomposition from enzymes because of their physical features and chemical compositions/structures.[2] Furthermore, lignin has been viewed as one of the major factors contributing to this recalcitrance. During enzymatic hydrolysis, lignin acts as a physical barrier to prevent enzyme access to the carbohydrate fraction of biomass and tends to irreversibly bind to enzymes through hydrophobic interactions that cause a loss in their activities.[4,5]

Pretreatment of lignocellulosic biomass is thus an essential step to overcome recalcitrance and increase overall fermentable sugar yield. Dilute acid pretreatment (DAP) has been proven to successfully hydrolyze hemicelluloses and disrupt the lignocellulosic structure for a wide range of feedstocks. Generally,

DAP does not lead to significant delignification. Several studies instead reported that the acid-insoluble (Klason) lignin content of dilute acid-pretreated material was often higher than the starting material.[6−11] This phenomenon has been hypothesized to be due, in part, to the formation of a lignin-like material termed pseudolignin.[12,13]

The formation of pseudolignin was only recently confirmed by Sannigrahi et al.[14] Their work demonstrated that pseudolignin can be generated from carbohydrates without significant contribution from lignin during DAP, particularly under high-severity pretreatment conditions. Additionally, Hu et al.[15] isolated and characterized pseudolignin produced from dilute acid-pretreated poplar holocellulose and α-cellulose. They showed that pseudolignin was polymeric with a $M_w \approx$ 5000 g/mol and contained carbonyl, carboxylic, aromatic, and aliphatic structures, which were produced from both dilute acid-pretreated cellulose and hemicellulose. Equally important, these studies indicated that the presence of pseudolignin on the surface of pretreated biomass can significantly inhibit enzymatic hydrolysis of cellulose. In the present study, pseudolignin and dilute acid-pretreated lignin were isolated, and their inhibition properties on enzymatic deconstruction of poplar holocellulose were evaluated and compared.

EXPERIMENTAL SECTION

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.

Pseudolignin 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.[16] A two-step dilute acid pretreatment with the first step: soaking in 1.0% sulfuric acid (5% solids) while stirring at room temperature for 4 h was performed on poplar holocellulose. The presoaked slurry was filtered, and the solids were washed with excess deionized (DI) water. The solids were then added to 1.0% sulfuric acid (5% solids) and transferred to a Parr 4560 mini pressure reactor (600 mL) for the second step of the pretreatment (180 °C, 40 min). 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 and the pretreated solids were washed with excess DI water.

The dilute acid-pretreated poplar holocellulose was refluxed with *p*-dioxane/water (9:1, v/v), under nitrogen.[17] The mixtures were filtered and washed with *p*-dioxane. The combined aliquots were concentrated under vacuum and then dissolved in DI water to precipitate pseudolignin. Finally, the precipitated pseudolignin was freeze-dried and vacuum-dried at 40 °C.

EMAL Preparation. Ball-milled dilute acid-pretreated poplar powder (dry weight: 21.42 g; pretreatment conditions: 170 °C, 0.5% H_2SO_4, 8 min; combined severity factor (CS): 1.68) was subjected to enzymatic hydrolysis by Cellulysin cellulase at an enzyme loading of 40 mg/g of biomass. The enzymatic hydrolysis was carried out at 35 °C over a period of 48 h at pH 5.0 (20 mM acetate buffer solution) and a consistency of 3%. After enzymatic hydrolysis, the impure enzymatic hydrolysis lignin was centrifuged and washed twice with acidic deionized water at pH 2.0 (HCl) and freeze-dried.

Impure enzymatic hydrolysis lignin (dry weight: 16.26 g) was suspended in 326.00 mL *p*-dioxane/acidified deionized water solution 85:15 v/v, containing 0.01 M HCl and stirred at 87 °C under N_2 for 2 h. The obtained solution was filtered, and the lignin solution was collected. The solid residue was sequentially washed with fresh *p*-dioxane/deionized water solution (85:15 v/v) 2−3 times. The total filtrates solution was neutralized with sodium bicarbonate and stirred for 3 h. The neutralized solution was then rotary-evaporated until a thick solution was obtained. This thick solution was carefully dropped into a large quantity of acidified deionized water (pH 2.0, HCl), and the precipitated lignin was isolated by centrifugation, washed, and freeze-dried.

Lignocellulosic Samples Preparation. Pseudolignin (Pseudo-L) or dilute acid-pretreated lignin (EMAL DAP) was added to poplar holocellulose to produce various lignocellulosic samples. In brief, 12%, 22%, and 36% of Pseudo-L or EMAL DAP (Supporting Information, Table S1) was dissolved in *p*-dioxane/water (10/1, v/v). A sample of poplar holocellulose (dry weight: 0.50 g) was added to the solution, and the mixture was stirred at room temperature for 2 h. The slurry was then transferred to an aluminum weigh dish and allowed to air-dry in a fumehood.

FT-IR-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 pseudolignin and dilute acid-pretreated 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.

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. A quantitative ^{13}C NMR spectrum was acquired using dimethylsulfoxide (DMSO)-d_6 (450 μL) as the solvent for the samples (120 mg) at 298 K with an inverse-gated decoupling sequence, 90° pulse angle, a 12 s pulse delay, and 8000 scans. 2D heteronuclear single quantum coherence (HSQC) correlation NMR analysis was performed using a standard Bruker pulse sequence with a 90° pulse, 0.11 s acquisition time, a 1.5 s pulse delay, a $^1J_{C-H}$ of 145 Hz, and acquisition of 256 data points.

Scanning Electron Microscopy. After mounting dry samples on aluminum specimen stubs and sputter-coating with gold, scanning electron microscopy (SEM) images of holocellulose and various lignocellulosic samples were acquired on a JEOL-1530 SEM at 5 kV beam accelerating voltage and various resolving powers.

Enzymatic Hydrolysis. Cellulase 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 the literature methods.[18] 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. A sample of the hydrolysis liquid (1.00 mL) at time intervals of 1, 2, 4, 7, 10, 24, 48, and 72 h were 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 high-performance liquid chromatography (HPLC) system (Agilent Technologies) equipped with an autosampler and an Aminex HPX-87H column and precolumn (Bio-Rad Laboratories). The analysis was carried out at 65 °C using 10 mM nitric acid solution 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.[19] All experiments were performed in duplicate, and the results represented the mean values of two independent experiments. The standard deviation associated with the glucose yield at each time interval was in the range of ±0−10%.

RESULTS AND DISCUSSION

Structural Comparison between Pseudo-L and EMAL DAP. The FT-IR spectra of pseudolignin and dilute acid-pretreated lignin are presented in Supporting Information, Figure S1. The peak assignments are summarized in Supporting Information, Table S2. Both samples had a hydroxyl stretching peak centered at ∼3300 cm^{-1}, but the hydroxyl stretching peak of pseudolignin is broader than that of dilute acid-pretreated lignin. Furthermore, pseudolignin and dilute acid-pretreated lignin exhibited aromatic absorptions. Different intensities of the absorption bands at ∼1600 and 1500 cm^{-1} suggest different aromatic structures and differing aromatic substitution patterns between pseudolignin and dilute acid-pretreated lignin. Pseudolignin also possesses carbonyl and carboxylic groups, which can be observed from the strong band at ∼1697 cm^{-1} in its FT-IR spectrum.

To further compare the structures of pseudolignin and dilute acid-pretreated lignin, ^{13}C NMR spectra of these two samples were obtained and this data is summarized in Supporting Information, Figure S2. A primary qualitative assignment based on literature is proposed in Supporting Information, Table S3. The peaks centered at 208−205, 203−185, and 178−172 ppm from pseudolignin can be attributed to C=O in ketones, aldehydes, and carboxylic acids, respectively,[15] whereas the small peak centered at ∼166 ppm corresponding to conjugated carboxylic ester group is shown in the ^{13}C NMR spectrum of dilute acid-pretreated lignin.[20] This indicates that pseudolignin contains more carbonyl and carboxylic acid groups than dilute acid-pretreated lignin, consistent with the FT-IR characterization. Both the pseudolignin and dilute acid-pretreated lignin possess aromatic structures. The peaks in the 162−142 ppm region are characteristic of aromatic C−O bonds. Whereas the peaks in the 142−125 ppm and 125−102 ppm regions represent aromatic C−C bonds and aromatic C−H bonds,
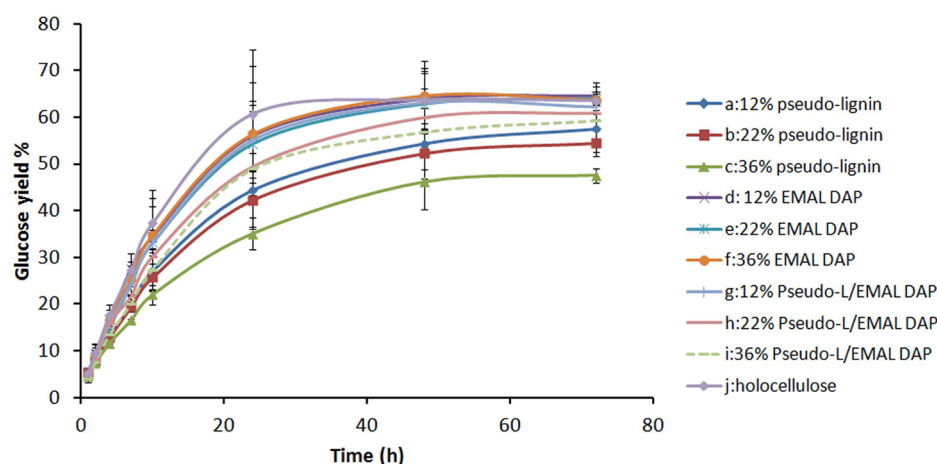
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dx.doi.org/10.1021/sc300032j | *ACS Sustainable Chem. Eng.* 2013, 1, 62−65

Figure 1. Time course of glucose yield of various samples after 72 h of enzymatic hydrolysis.

respectively.[20] Additionally, the characterization of guaiacyl (G) and syringyl (S) units in dilute acid-pretreated poplar lignin was identified by the peaks in the regions 125−110 and 109−103 ppm, respectively.[21] 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 an extended time in a vacuum oven. With respect to aliphatic structures, pseudolignin consists of more oxygenated aliphatic structures that can be observed by the additional peaks at ∼72 ppm, ∼63 ppm, and aliphatics in the region 50−20 ppm. The ^{13}C NMR spectra of these two samples also present common peaks at ∼60 and ∼56 ppm.

To obtain further knowledge of pseudolignin and dilute acid-pretreated lignin structures, their HSQC spectra are presented in Supporting Information, Figure S3. The S units in dilute acid-pretreated lignin show a major cross-peak for the $C_{2,6}/H_{2,6}$ correlation at δ_C/δ_H 103.5/6.7 ppm, and the G units show correlations at δ_C/δ_H 110.6/7.0 and 115.1/6.8 ppm.[22] A considerable amount of p-hydroxybenzoate can be observed from $C_{2,6}/H_{2,6}$ correlation at δ_C/δ_H 131.3/7.7 ppm.[23,24] Compared to dilute acid-pretreated lignin, pseudolignin shows much weaker C/H correlation signals at different chemical shifts in the aromatic region. The common peak at ∼56 ppm can be attributed to the methoxy groups according to the C/H correlation signals at δ_C/δ_H 56.0/3.8 ppm.[22] The C/H correlation in β-O-4 substructure of dilute acid-pretreated lignin was observed for the γ-C position at δ_C/δ_H 60.0/3.7 ppm,[22] whereas this signal from pseudolignin was assigned to methoxy group according to the distortionless enhancement by polarization transfer (DEPT) NMR analysis.[15] The structural characterization shows that the poplar lignin was partially degraded during DAP at 170 °C, 0.5% H_2SO_4, and 8 min (CS: 1.68), and it did not contain a significant proportion of pseudolignin after the pretreatment. This is consistent with literature indicating insignificant pseudolignin formation during DAP at combined severity lower than 1.77.[14] On the other hand, pseudolignin is a lignin-like aromatic material but is not derived from native lignin, which appears to be generated at higher acidic severity conditions. During DAP, the hydrolysis of polysaccharides, which leads to some release of monosaccharides, and their subsequent dehydration reactions to form furfural and 5-hydromethylfurfural (HMF) takes place. Further rearrangements of furfural and/or HMF may produce aromatic compounds, which undergo further polymerization and/or polycondensation reactions to form pseudolignin.[15]

Enzymatic Hydrolysis Results. When preparing the lignocellulosic samples, pseudolignin or dilute acid-pretreated lignin was solubilized in p-dioxane before the holocellulose sample was added to form a slurry. The purpose of this effort is to incorporate pseudolignin or dilute acid-pretreated lignin into the cellulose and hemicellulose structures, rather than creating a dry physical mixture. Compared to the poplar holocellulose, it was visually evident that pseudolignin or dilute acid-pretreated lignin had coated the cellulose fibers, as the resulting samples became darker with increasing amounts of pseudolignin or EMAL. Upon examination of the lignocellulosic samples by SEM (Supporting Information, Figure S4), pseudolignin or dilute acid-pretreated lignin droplets were successfully deposited on the holocellulose surface. These droplets have different diameters and were postulated to inhibit enzymatic hydrolysis of cellulose. The enzymatic conversion yields of cellulose for various lignocellulosic samples are summarized in Figure 1. The data in Supporting Information, Table S4 indicated that both pseudolignin and dilute acid-pretreated lignin inhibited enzymatic hydrolysis of holocellulose in the initial stage (before 24 h of hydrolysis), and hydrolysis inhibition generally increased with pseudolignin or dilute acid-pretreated lignin content. However, the inhibition effect of dilute acid-pretreated lignin was much less significant than that of pseudolignin. In addition, dilute acid-pretreated lignin alone had a negligible inhibition after 48 h of hydrolysis, which is consistent with literature reports of a limited effect of dilute acid-pretreated lignin on enzymatic hydrolysis of cellulose.[25−27] In contrast, pseudolignin alone had a strong inhibition of 9.5−25.1% on the overall enzymatic conversion yield of cellulose, whereas this inhibition was modest (1.9−6.7%) for the samples with a 50/50 mixture of pseudolignin and dilute acid-pretreated lignin. It is well-known that lignin has nonproductive association with cellulases due to its hydrophobic structural features including hydrogen bonding, methoxy groups, and polyaromatic structures.[28] The structural functionality of methoxy and polyaromaticity of pseudolignin revealed by the FT-IR and ^{13}C NMR analyses suggest its hydrophobicity. Indeed, pseudolignin is insoluble in water. We speculate that the hydrophobic structural functionality of pseudolignin accounts for its nonproductive association with cellulases, resulting in the inhibition effects to enzymatic hydrolysis of cellulose. These results suggest that, although pseudolignin that

is not derived from native lignin is even more detrimental to enzymatic hydrolysis of cellulose than dilute acid-pretreated lignin, its formation should be avoided.

CONCLUSIONS

Results from the spectroscopic analysis indicated that, although pseudolignin and dilute acid-pretreated lignin have some common structural features, the poplar lignin pretreated at 170 °C, 0.5% H_2SO_4, for 8 min did not contain detectable amounts pseudolignin; on the other hand, pseudolignin is not derived from native lignin but is a lignin-like aromatic material. The enzymatic hydrolysis studies revealed that the impact of pseudolignin on enzymatic deconstruction of cellulose was much more detrimental than that of dilute acid-pretreated lignin. This study suggests that dilute acid pretreatment should be performed at less severe conditions, thereby avoiding the formation of pseudolignin.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information on FT-IR, ^{13}C NMR and HSQC NMR spectra, and SEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

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