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Cite this: *RSC Adv.*, 2014, **4**, 4317

Suppression of pseudo-lignin formation under dilute acid pretreatment conditions†

Fan Hu and Arthur Ragauskas*

Received 11th June 2013
Accepted 6th December 2013

DOI: 10.1039/c3ra42841a

www.rsc.org/advances

Pseudo-lignin is formed during dilute acid pretreatment (DAP), particularly under high-severity conditions, and has been shown to significantly inhibit enzymatic hydrolysis of cellulose. To suppress its formation, DAP was modified by performing it under O_2 or N_2; adding surfactant (Tween-80) to the reaction mixture; or using a water–dimethyl sulfoxide (DMSO) mixture as reaction medium. Pseudo-lignin analysis showed that only the addition of DMSO to DAP reaction medium can effectively suppress pseudo-lignin formation. This was attributed to DMSO preferentially solvating and stabilizing 5-hydroxymethyl furfural (HMF) which is the key intermediate to form pseudo-lignin, thereby reducing the overall yield of pseudo-lignin. Furthermore, the addition of DMSO was shown not to reduce pseudo-lignin molecular weight or change any of its structural features significantly. Therefore, pseudo-lignin generated from aqueous DMSO DAP had similar inhibition properties as compared to that acquired from routine DAP at equal mass dosages. This study is the first demonstration that the amount of pseudo-lignin formed during DAP can be reduced, which contributes to further optimization of DAP technology.

Introduction

As global energy consumption is continuing to rise, the world is pursuing the development of renewable energy sources such as lignocellulosic ethanol to address vital strategic, economic and environmental issues related to the depleting fossil fuels.[1,2] Lignocellulosic biomass such as wood, grass, and agricultural and forest residues are an inexpensive and abundant feedstock for sustainable production of fuels and chemicals.[1] To convert biomass to ethanol, plant polysaccharides must be deconstructed into their corresponding monosaccharides, which subsequently are biologically fermented to ethanol.[3] Utilization of enzymes to produce fermentable sugars is viewed as the most viable strategy, because enzymatic hydrolysis of biomass offers several advantages such as higher yield, lower byproduct formation and energy requirement, mild operation conditions, and environmentally benign processing compared to conventional acid hydrolysis.[4] Unfortunately, the yield of ethanol production from native lignocellulosic biomass is relatively low due to the natural resistance of plant cell walls to decomposition from microbes and enzymes.[2]

Pretreatment of lignocellulosic biomass is an essential step to overcome recalcitrance and increase overall fermentable sugar yield. It utilizes various technologies such as chemical treatment to alter plant cell wall structural features so that the polysaccharide fractions become more accessible and amenable to enzymatic hydrolysis. Dilute acid pretreatment (DAP) has been considered to be among the leading and most promising pretreatment technologies that can enhance fermentable sugar release performance.[3,5] DAP involves the treatment of biomass with a combination of an acidic solution, heat and pressure with residence times ranging from less than a minute to 1 h, which is generally carried out using 0.4–2.0 wt% H_2SO_4 at a temperature of 140–200 °C.[7] Generally, DAP does not lead to significant delignification and several studies have reported that the acid-insoluble (Klason) lignin content of dilute acid-treated 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 pseudo-lignin[12,13] and its formation was confirmed by Sannigrahi *et al.*[14] Their work demonstrated that pseudo-lignin can be generated from carbohydrates without significant contribution from lignin during DAP, particularly at high-severity pretreatment conditions. In addition, Hu *et al.*[15] isolated and characterized pseudo-lignin produced from dilute acid-treated poplar holocellulose and α-cellulose. They showed that pseudo-lignin was polymeric and contained carbonyl, carboxylic, aromatic, and aliphatic structures, which were produced from both dilute acid-treated cellulose and hemicellulose. In a continued study, Hu *et al.*[16] reported that pseudo-lignin is not derived from native lignin but is a lignin-like aromatic material, which is even more detrimental to enzymatic deconstruction of cellulose when compared to dilute acid-treated lignin. This latter study clearly

BioEnergy Science Center, School of Chemistry and Biochemistry, Institute of Paper Science and Technology, Georgia Institute of Technology, 500 10th Street, Atlanta, Georgia 30332, USA. E-mail: arthur.ragauskas@chemistry.gatech.edu; Fax: +1 404 894 4778; Tel: +1 404 894 9701

† Electronic supplementary information (ESI) available: 2D heteronuclear single quantum coherence (HSQC) NMR spectra of pseudo-lignin samples. See DOI: 10.1039/c3ra42841a

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indicated that the formation of pseudo-lignin during DAP should be avoided. During DAP, the hydrolysis of polysaccharides leads to the release of some monosaccharides, and subsequent dehydration reactions lead to the formation of furfural and 5-hydromethylfurfural (HMF). Further rearrangements of furfural and/or HMF may produce aromatic compounds, which undergo further oxidative polymerization and/or polycondensation reactions to form pseudo-lignin.[16] Oxygen thus may play a role in pseudo-lignin formation. In addition, using a surfactant such as Tween-80 during DAP has been shown to significantly increase lignin removal[17] and thus could potentially assist in the solubilization of pseudo-lignin. It has also been reported that addition of dimethyl sulfoxide (DMSO) to the reaction medium containing dilute HCl solution can suppress undesired HMF side reactions during the process of HMF production.[18] With these issues in mind, we modified the DAP process with different methods including performing DAP under O_2 or N_2; adding surfactant (Tween-80) to the reaction mixture; and using water–DMSO mixture as reaction medium, with the intention of exploring new methods of suppressing pseudo-lignin formation. These studies demonstrated that using a water–DMSO mixture in the DAP process can significantly reduce pseudo-lignin formation at high-severity pretreatment conditions. To the best of our knowledge, this is the first report demonstrating that the amount of pseudo-lignin generated during DAP can be reduced, and this study contributes to further understanding of fundamental principles behind DAP technology, in order to develop a more effective, economic and environmentally benign DAP technology.

Results and discussion

Screen of modified dilute acid pretreatment conditions

The detailed mechanism of how pseudo-lignin is formed during DAP is still unclear. Previous studies strongly suggested that during DAP, the hydrolysis of polysaccharides (*e.g.* xylan and cellulose) leads to some release of monosaccharides (*e.g.* xylose and glucose), which are then converted to furfural and HMF. Further rearrangements of furfural and/or HMF are responsible for the formation of pseudo-lignin.[15,16,19] One can thus conclude that furfural and especially HMF are the key intermediates for pseudo-lignin formation, and their stabilization from further reactions may effectively suppress pseudo-lignin formation. DMSO has recently been shown to prevent undesired side reactions of HMF in HMF production,[18] thus we hypothesized that DMSO could suppress pseudo-lignin formation during DAP. Furthermore, in the presence of oxygen, oxidative reaction of compounds produced from further rearrangements of HMF is one of the potential reactions to form pseudo-lignin,[15] thus DAP performed under O_2 or N_2 may facilitate or curb pseudo-lignin formation. In addition, surfactants are well-known pretreatment additives, and Qing *et al.*[17] reported that dilute acid pretreating corn stover with Tween-80 could remove more lignin from the solids, probably by forming emulsions that reduce lignin redeposition back on the biomass surface through interaction of Tween-80 with hydrophobic lignin during pretreatment. Pseudo-lignin is also hydrophobic,[16] and thus it is possible to reduce pseudo-lignin deposition by pretreating with surfactant.

Table 1 Acid-insoluble lignin (K-lignin) and carbohydrate contents of various samples (based on dried samples)

Sample	Solids recovery %	K-Lignin %	Xylan %	Glucan %	Total %
Holocellulose	NA	4.3	2.9	93.4	100.6
Control DAP	18.9	42.0	0	54.4	96.4
A (O_2)	18.1	89.2	0	4.3	93.5
B (N_2)	19.1	48.7	0	47.1	95.8
C (DMSO)	37.8	14.7	0	86.2	100.9
D (Tween)	13.3	52.1	0	45.0	97.1

To test our hypotheses, a series of DAP conditions using O_2/N_2; Tween-80 or DMSO were conducted, and pseudo-lignin and carbohydrate analysis of solids recovered from various DAP conditions was performed. This data was summarized in Table 1. The starting material (*i.e.* untreated poplar holocellulose) had very low acid-insoluble lignin content, thus the dramatic increase in acid-insoluble lignin content of pretreated samples strongly suggests the generation of pseudo-lignin during DAP. As expected, solids recovered from DAP performed under O_2 consisted mainly of pseudo-lignin (~89%). However, DAP conducted under N_2 did not diminish pseudo-lignin formation, in contrast, it resulted in moderately higher (~6%) pseudo-lignin content when compared to the control DAP. These results suggest that oxidative reaction is one but not the only one reaction that leads to produce pseudo-lignin during DAP. As a result, DAP with O_2 could facilitate pseudo-lignin formation very likely through promoting oxidative reactions, while N_2 could suppress oxidative reactions but cannot curb other reactions to produce pseudo-lignin from furfural and/or HMF, or prevent carbohydrates from forming furfural and HMF. In the DAP study with Tween-80, this resulted in the lowest solids recovery yield (~13%), and (~10%) higher pseudo-lignin content than the control DAP. This indicates that pseudo-lignin is hydrophobic just like lignin, but its formation cannot be curbed by dilute acid pretreating with surfactants. In contrast to N_2 and Tween-80, addition of DMSO to DAP reaction medium effectively reduced pseudo-lignin content by approximately 30% and increased solids recovery yield by around 20%. The effectiveness of DMSO was further supported by the FT-IR analysis of recovered solids (Fig. 1). For instance, the FT-IR spectrum of sample C (DMSO) resembles that of holocellulose, implying a minor proportion of pseudo-lignin in recovered solids. On the other hand, all the remaining spectra of control DAP, sample A (O_2), B (N_2) and D (Tween) showed C=O stretching and aromatic C=C stretching peaks at ~1705 cm^{-1} and ~1615 cm^{-1}, respectively, which are the characteristic structural features of pseudo-lignin.[15,16]

In the presence of DMSO, it has been suggested that the coordination of HMF with water can be reduced due to the competition between water oxygen and DMSO oxygen to be in the first solvation shell of HMF, with DMSO having a stronger interaction; and the hydrophobic nature of the sulfur atom and methyl groups of DMSO.[20,21] The hydrophobic parts of DMSO in

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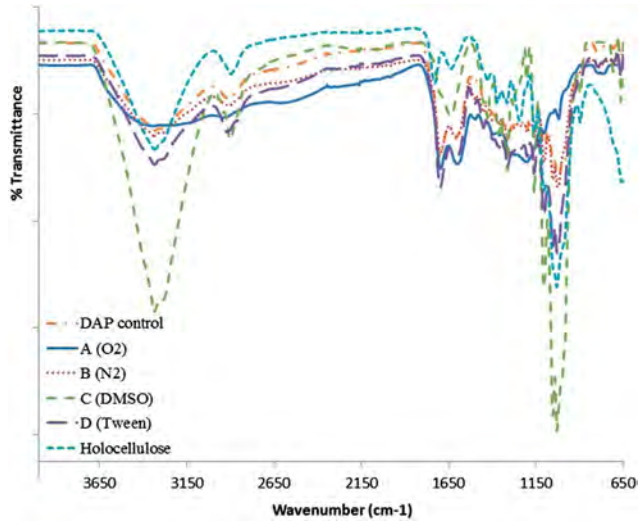

Fig. 1 FT-IR spectra of solids recovered from various DAP conditions.

the first solvation shell could also push the second water solvation shell farther away from the HMF molecule.[20] In addition, the carbonyl carbon atom (C1) of HMF was shown to be the most strongly and well-coordinated carbon atom with DMSO, and C1–DMSO interaction even prevails over the C1–H_2O interaction.[20] As the cleavage of C1 atom of HMF eventually leads to form pseudo-lignin, the reduction of HMF–water coordination and the preferential solvation of the C1 carbon by DMSO can protect the HMF molecule from further reactions to form pseudo-lignin in the aqueous medium.

Table 2 Acid-insoluble lignin (K-lignin) and carbohydrate contents of solids recovered from DAP in water–DMSO mixture (based on dried samples)

Sample	Solids recovery %	K-Lignin %	Glucan %	Total %
Control DAP (no DMSO)	18.9	42.0	54.4	96.4
H_2O–DMSO (4/1; v/v)	37.8	14.7	86.2	100.9
H_2O–DMSO (10/1; v/v)	24.2	34.8	62.0	96.8
H_2O–DMSO (12/1; v/v)	24.6	30.6	65.1	95.7
H_2O–DMSO (15/1; v/v)	27.2	31.2	66.7	97.9
H_2O–DMSO (20/1; v/v)	19.1	37.4	59.1	96.5

Further optimization of the amount of DMSO in dilute acid pretreatment

The pseudo-lignin content of solids recovered from DAP in water–DMSO mixture (4/1; v/v) was the lowest among all DAP examined conditions. However, it is difficult to extract any pseudo-lignin from pretreated solids at such low proportion, and the volume of DMSO used was relatively high. Therefore, in order to isolate sufficient pseudo-lignin for structural characterization, the amount of DMSO used in DAP was further optimized with the intention of lowering DMSO volume while still maintaining moderate pseudo-lignin content. We screened water–DMSO volume ratio ranging from 4 to 1 to 20 to 1, while maintaining all other experimental parameters (*i.e.* 180 °C, 1.0 wt% H_2SO_4 and 40 min), and analyzed pseudo-lignin contents of recovered solids. These results were summarized in Table 2. In general, increasing the volume ratio of DMSO led to

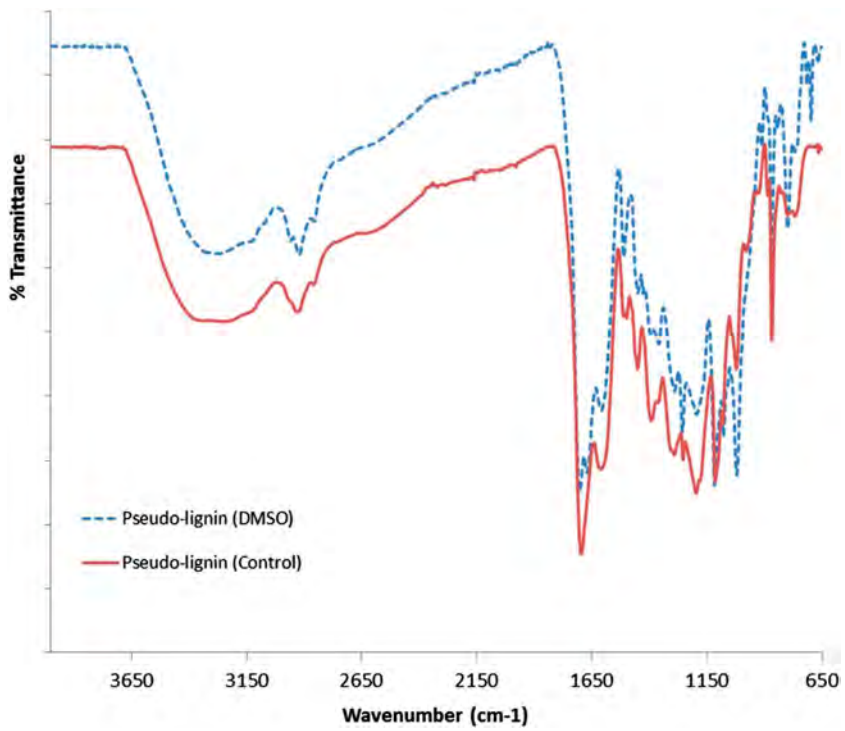

Fig. 2 FT-IR spectra of pseudo-lignin (DMSO) and pseudo-lignin (control).

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Table 3 Peak assignments for FT-IR spectra of pseudo-lignin samples

Wavenumber (cm^{-1})	Assignment
3333	Hydrogen-bonded O–H stretching in alcohols or phenols
2940	Aliphatic C–H stretching
1705	C=O stretching in carboxylic esters, conjugated aldehydes or ketones
1665	α,β-Unsaturated aldehydes or ketones
1615, 1516	Aromatic C=C stretching (in ring)
1457	Aliphatic C–H bending
1320–1000	C–O stretching in alcohols, ethers or carboxylic esters
870	Aromatic C–H out-of-plane bending

a reduction in pseudo-lignin proportion and an increase in solids recovery yield. At water–DMSO: 20/1 ratio, both the pseudo-lignin content and the solids recovery yield were comparable to the control DAP, suggesting the amount of DMSO was too low to suppress pseudo-lignin formation. At low DMSO concentration condition, the solvent coordination around HMF is dominated by the interaction between the oxygen atoms of water and the carbon atoms of HMF. In addition, water can form hydrogen bonds with the oxygen atom attached to C1 atom of HMF.[20] The acidic aqueous condition thus leads to further rearrangements of HMF and/or furfural to yield other aromatic compounds which in turn can be converted to pseudo-lignin. Reducing the DMSO ratio from water–DMSO: 12/1 to 15/1 resulted in only a slightly increase in pseudo-lignin content and even higher solids recovery yield. As a result, water–DMSO: 15/1 ratio was chosen as the pretreatment condition to isolate pseudo-lignin and investigate if DMSO could change any structural features of pseudo-lignin.

Structural characterization of isolated pseudo-lignin

Pseudo-lignin samples generated from DAP in water–DMSO (15/1; v/v) mixture (pseudo-lignin (DMSO)) and from control DAP conditions (pseudo-lignin (control)) were isolated following a literature protocol.[15] Their FT-IR spectra were presented in Fig. 2 and the peak assignments were summarized in Table 3.[22] The spectra suggest that pseudo-lignin produced from the two conditions had similar functionalities, particularly in the case of hydroxyl, aliphatic C–H, C=O (carbonyl and/or carboxylic) and aromatic functional groups. The hydroxyl stretching peaks at ~3333 cm^{-1} are strong and broad, indicating the presence of hydrogen-bonding in pseudo-lignin samples. The strong bands at ~1705 cm^{-1} conjugated with ~1615 and ~1516 cm^{-1} can be attributed to C=O conjugated with aromatic ring. Perhaps the biggest difference between these two samples was the presence of strong stretching peak at ~1665 cm^{-1} corresponding to α,β-unsaturated aldehyde or ketone in pseudo-lignin (DMSO) sample, whereas this peak was absent in pseudo-lignin (control) sample. These observations indicate dehydration and aromatization reactions of carbohydrates took place during the formation of pseudo-lignin.

To further characterize and compare the functional groups in isolated pseudo-lignin samples, their ^{13}C NMR spectra were obtained and the spectra were presented in Fig. 3. The ^{13}C NMR spectra are predominantly comprised of signals from carbonyl, carboxylic, aromatic and aliphatic structures. The peaks in the 153–142 ppm region are characteristic of aromatic C–O bonds, whereas the peaks in the 142–102 ppm regions represent aromatic C–C bonds and aromatic C–H bonds.[23] The signal at δ 66 ppm was due to *p*-dioxane which could not be fully removed after extended time in a vacuum oven. To obtain better knowledge of NMR signals besides aromatic carbons, distortionless enhancement by polarization transfer-135 (DEPT-135) NMR spectra of pseudo-lignin samples were obtained (Fig. 4). DEPT-135 is a useful tool to determine the presence of primary, secondary and tertiary carbon atoms, which gives positive CH and CH_3 signals while negative CH_2 signals. According to the DEPT-135 NMR analysis, the CH peaks centered at ~177 pm correspond to conjugated aldehyde groups, which is further confirmed by the C/H correlation at δ_C/δ_H 177.2/9.6 ppm (please see the ESI†), while the signal at ~162 ppm is attributed to the

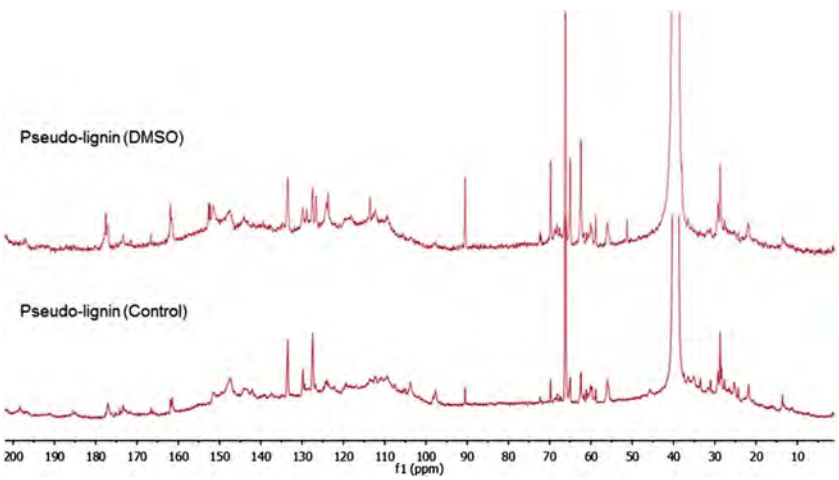

Fig. 3 ^{13}C NMR spectra of pseudo-lignin (DMSO) and pseudo-lignin (control).

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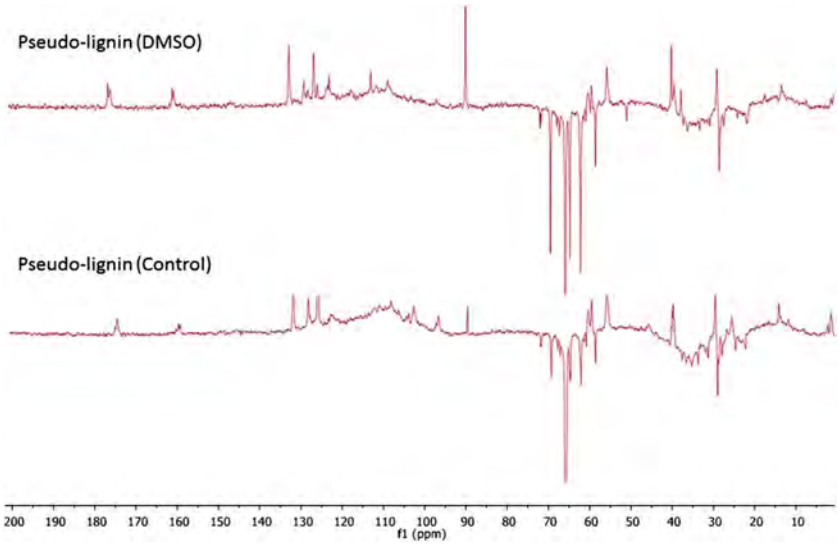

Fig. 4 DEPT-135 NMR spectra of pseudo-lignin (DMSO) and pseudo-lignin (control).

tertiary carbon belonging to the carboxylic group of formate, which is confirmed by the C/H correlation at δ_C/δ_H 161.7/8.2 ppm (see ESI†). Furthermore, the DEPT NMR analysis revealed that the peaks in the 133–102 ppm region are characteristic of aromatic C–H bonds, thus aromatic C–C bonds belong to the 142–133 ppm region. In the case of aliphatic structures, most secondary carbon atoms are located in the 72–62 ppm region, suggesting these CH_2 groups belong to ethers, alcohols or carboxylic esters. In addition, the methoxy groups of pseudo-lignin are identified by the peaks centered at ~60 ppm and ~56 ppm, which are also observed by the C/H correlations at δ_C/δ_H 60.2/3.8 ppm and δ_C/δ_H 56.0/3.8 ppm, respectively (please see the ESI†). The origin of the methoxy group of pseudo-lignin was postulated to come from 4-*O*-methyl-D-glucuronic acid in the poplar hemicellulose,[15] and the low intensity of the methoxy peak could support this postulate because of the low xylan content in untreated poplar holocellulose. The NMR characterization of pseudo-lignin samples is consistent with the FT-IR analysis, showing pseudo-lignin samples produced from the two conditions had similar structural features. This suggests that although DMSO can suppress the pseudo-lignin formation, it does not involve a change in the mechanism of pseudo-lignin formation during the course of DAP.

Molecular weight analysis of isolated pseudo-lignin

The molecular weights of pseudo-lignin (DMSO) and pseudo-lignin (control) were represented in Table 4. In general, the molecular weights of isolated pseudo-lignin samples were much lower than those of milled wood lignin (M_n ~ 4060 g mol^{-1}; M_w ~ 10 002 g mol^{-1}) from poplar.[24] Furthermore, pseudo-lignin samples produced from two conditions had similar molecular weights, which is very likely attributed to their similar structural features. In addition, polydispersity (PDI) values greater than 1 indicate that pseudo-lignin is not homogeneous.

Table 4 Molecular weights of isolated pseudo-lignin samples

Sample	M_n (g mol^{-1})	M_w (g mol^{-1})	PDI (M_w/M_n)
Pseudo-lignin (DMSO)	1.78×10^3	3.36×10^3	1.89
Pseudo-lignin (control)	1.84×10^3	3.74×10^3	2.03

Enzymatic hydrolysis results

When preparing the lignocellulosic samples, pseudo-lignin was incorporated into the cellulose and hemicellulose structures by dissolution and evaporation rather than physically mixing with holocellulose. This protocol has been frequently used in literature, leading to a successful deposition of pseudo-lignin droplets on the holocellulose surface.[15,16,25] These pseudo-lignin droplets have been shown to inhibit enzymatic hydrolysis of cellulose, probably by blocking cellulases surface binding sites; and/or through non-productive association with cellulases due to the hydrophobicity of pseudo-lignin.[16,26] The inhibition property of pseudo-lignin generated from aqueous DMSO DAP on enzymatic deconstruction of poplar holocellulose was evaluated and compared with that of pseudo-lignin acquired from routine DAP. The enzymatic conversion yields of cellulose for various lignocellulosic samples were represented in Fig. 5. The data indicates that neither the presence of pseudo-lignin (DMSO) nor pseudo-lignin (control) had a major impact on the initial cellulose conversion (before 4 h of enzymatic hydrolysis). However, the inhibition effect became more pronounced as the hydrolysis time was extended, which reached the maximum reduction in glucose yield around 20% at 24 h and 48 h of hydrolysis time for pseudo-lignin (DMSO) and pseudo-lignin (control), respectively. Furthermore, the results suggest that these two pseudo-lignin samples had similar inhibition effects, which is very likely due to their similar molecular weights and structural features. The inhibition effects of pseudo-lignin to enzymatic hydrolysis of cellulose is enhanced as its content increases,[15,16] thus although DMSO did not reduce the

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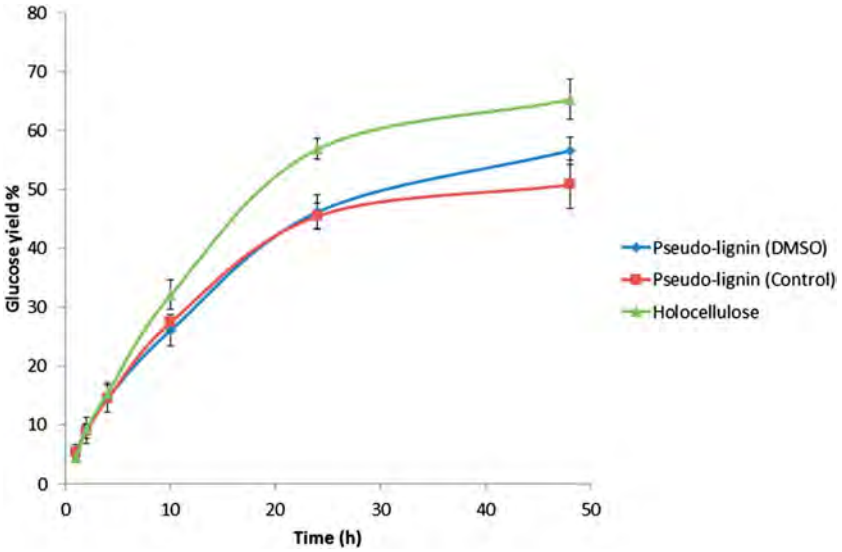

Fig. 5 Time course of glucose yield of various samples after 48 h of enzymatic hydrolysis.

inhibition effects of pseudo-lignin, it can significantly suppress pseudo-lignin formation during DAP, which in turn can increase the enzymatic digestibility of cellulose after the pretreatment.

Conclusions

In summary, we have demonstrated that addition of DMSO to the DAP reaction medium can effectively suppress pseudo-lignin formation, even under high-severity pretreatment conditions. Although DMSO has exceptional pseudo-lignin suppression property, it did not change the pseudo-lignin molecular weight or any of its structural features significantly. In addition, although DMSO cannot reduce the inhibition effect of pseudo-lignin to enzymatic deconstruction of cellulose, its pseudo-lignin suppression effect can in turn increase the enzymatic digestibility of cellulose after DAP. This study showed that the amount of pseudo-lignin formed during DAP is controllable, which is a good contribution to development of more economic and effective DAP technology.

Experimental

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.

Dilute acid pretreatment

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.[22] A two-step dilute acid pretreatment with the first step soaking in 1.0 wt% 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 DI water. The solids were then added to 1.0 wt% sulfuric acid (5% solids) and transferred to a Parr 4560 mini pressure reactor (600 mL) for the second step of the pretreatment (Table 5). Experiments conducted with O_2 or N_2 were (1) 4 min gas bubbling through the reactor (inlet tube releases gas at the very bottom of the reactor, and mixing distributes gas for better saturation); (2) 5 min gas bubbling; and (3) 10 min gas bubbling plus 100 psi gas pressure over atmospheric pressure. The other parameters were the same as in control DAP. In the DAP study with surfactant, 5% (w/w) of Tween-80 was added based on dry biomass weight. The reactor was heated to the desired temperature with constant stirring at a heating rate of ~6 °C min^{-1}. After pretreatment, the slurry was filtered and the pretreated solids were washed with excess DI water.

Table 5 The conditions for the second step in two-step DAP of poplar holocellulose

Sample	Second step pretreatment conditions
Control DAP	180 °C, 1.0 wt% H_2SO_4, 40 min
A (O_2)	180 °C, 1.0 wt% H_2SO_4 at 100 psi O_2, 40 min
B (N_2)	180 °C, 1.0 wt% H_2SO_4 at 100 psi N_2, 40 min
C (DMSO)	180 °C, 1.0 wt% H_2SO_4 (H_2O–DMSO: 4/1; v/v), 40 min
D (Tween)	180 °C, 1.0 wt% H_2SO_4 with 5% (w/w) Tween-80, 40 min

Pseudo-lignin preparation

Poplar holocellulose samples recovered from various DAP conditions were refluxed with *p*-dioxane–water (9 : 1, v/v), under nitrogen following a literature method.[27] The samples were filtered and washed with *p*-dioxane, and the combined filtrates were concentrated under vacuum and then dissolved in DI water to precipitate pseudo-lignin. Finally, the precipitated pseudo-lignin was freeze-dried and vacuum dried at 40 °C.

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Lignocellulosic samples preparation

Pseudo-lignin generated from DAP in water–DMSO mixture (pseudo-lignin (DMSO)) or produced from control DAP conditions (pseudo-lignin (control)) was added to poplar holocellulose to produce various lignocellulosic samples. In brief, a sample of pseudo-lignin (dry weight: 0.10 g) 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.

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^{-1} and 387.70 CBU mL^{-1}, respectively, according to the literature methods.[28] 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^{-1} and 40 CBU g^{-1}, 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, 10, 24, and 48 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. 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 ±0–4%.

Acknowledgements

The authors are grateful for financial support from the Bio-Energy Science Center (BESC), the Paper Science & Engineering (PSE) Fellowship program at Institute of Paper Science & Technology (IPST) at Georgia Institute of Technology.

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