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Disparate roles of solvent extractable lignin and residual bulk lignin in enzymatic hydrolysis of pretreated sweetgum

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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The roles of solvent extractable lignin and residual bulk lignin in enzymatic hydrolysis of Avicel and lignocellulosic biomass were distinguished in this study. Solvent extractable lignin removal reduced the 72 h hydrolysis yields of dilute acid pretreated sweetgum (DASG) and organosolv pretreated sweetgum (OPSG) from 38.1% to31.8% and from 69.9 to 49.3%, respectively. On the contrary, residual bulk lignin removal enhanced the 72 h hydrolysis yields of DASG and OPSG to 91.7% and 90.5%, respectively. The isolated lignins were added into enzymatic hydrolysis of Avicel, which revealed the positive effect of extractable lignin and the negative effect of residual bulk lignin on enzymatic hydrolysis. The cellulase distribution during the hydrolysis and cellulase adsorption indicated that the extractable lignin could counter the negative effect of residual bulk lignin by reducing the non-productive binding between cellulase and bulk lignin.

Introduction

Lignocellulosic biomass (such as agriculture residues, forestry biomass and energy crops) is the most abundant carbohydrate resource. It has great potential to be used for producing liquid biofuels and valuable chemicals.[1] In the typical biorefinery process, fermentable sugars are released from biomass by enzymatic hydrolysis, and then fermented to fuels and chemicals by microorganisms. Cost-effective enzymatic hydrolysis is one of the main bottlenecks in commercializing lignocellulosic biorefinery. Significant progress has been made in reducing enzyme cost and finding new oxidative enzyme (such as lytic polysaccharide monooxygenases (LPMOs)), boosting enzymatic conversion.[2] However, the enzymatic hydrolysis is still limited by several critical enzyme and biomass substrate factors.[3] The factors related to enzyme include enzyme non-synergistic action, enzyme deactivation, and product inhibition.[4, 5] The factors related to biomass substrate include residual lignin content and type, fiber particle size, surface area, cellulose crystallinity and cellulose accessibility.[5-7]

The residual lignin contributes significantly to the biomass recalcitrance, by physically blocking and non-productive binding. A strong inverse association has been observed between residual lignin content and poplar digestibility, when the syringyl to guaiacyl ratio is low.[8] Selectively removing a fraction of residual lignin from steam exploded Douglas-fir resulted in 30% increase of hydrolysis yield.[9] The residual lignin from steam-pretreated spruce showed significant inhibition on endoglucanase and exoglucanase activities by inactivating bound enzymes,[10] the acid hydrolysis residual lignin was more inhibitory than enzymatic hydrolysis residual lignin. Organosolv dissolved lignin has also been reported to be more repressing than enzymatic hydrolysis residual lignin.[11] In addition to residual lignin, soluble lignin degradation compounds have been recently reported to inhibit enzymatic hydrolysis.[12-15] Non-volatile compounds from steam pretreated willow have been found to severely affected the enzymatic hydrolysis.[16] Phenolic compounds from hot water pretreatment of maple could reduce the hydrolysis yield by 20%.[14] Tannic, gallic, hydroxycinnamic and hydroxybenzoic acids were able to deactivate cellulases or β-glucosidases by 20-80%.[15]

Much of previous research has been mainly focused on the negative effect of residual lignin and soluble lignin-derived compounds on enzymatic hydrolysis.[17] However, the positive effect of lignin on enzymatic hydrolysis has been observed recently by several research groups.[18, 19] Lignosulfonate has been found to enhance enzymatic hydrolysis of lignocellulose by acting as a surfactant to reduce the non-productive binding. The hardwood organosolv lignins have been discovered to increase the 72 h hydrolysis yield of woody biomass by 39-47%.[20] In contrast, the softwood organosolv lignins decreased the hydrolysis yield by 8-15%. Moreover, the effect of residual lignin content on enzymatic hydrolysis has been a controversial issue. Previously, it was noted that steam pretreated softwood with higher lignin content resulted in better digestibility.[21] It was possible that certain lignin fraction in residual lignin was not inhibitory, but stimulatory to enzymatic hydrolysis. However, the critical knowledge on lignin inhibition is lacking in identifying the inhibitory or stimulatory effect of specific lignin fraction, because it is not clear which lignin fraction is inhibitory and which lignin fraction could be stimulatory. Can the

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†Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

residual lignin in pretreated substrates be separated into different fractions to distinguish their positive or negative effects?

Consequently, the objective of this study is to fractionate the residual lignin from pretreated biomass into two fractions, and identify their distinct effects on enzymatic hydrolysis. It is hypothesized that solvent extractable lignin, one decomposed lignin with lower molecular weight, plays a positive role in enzymatic hydrolysis, while the residual bulk lignin, one condensed lignin with higher molecular weight, plays a negative role. We first examined their effects on enzymatic hydrolysis by comparing the hydrolysis yield of pretreated substrates with and without removing extractable lignin and residual bulk lignin in substrates. The extractable lignin was removed by solvent extraction, which has been used to collect various pure lignin fractions.[22] The residual bulk lignin was removed by sodium chlorite delignification.[23] To further identify the disparate roles of lignin fractions in enzymatic hydrolysis, three types of lignin fractions (extractable lignin, cellulolytic enzyme lignin and ethanol-washed cellulolytic enzyme lignin) were isolated from organosolv pretreated sweetgum, and their effects on enzymatic hydrolysis of Avicel were also assessed. Langmuir adsorption isotherms were used to determine the affinity between cellulase enzymes and isolated lignins. Finally, the interaction between extractable lignin and residual bulk lignin in cellulase adsorption was also determined and a potential mechanism for the positive effect of extractable lignin was proposed in this study.

Experimental

Enzymes

Commercial cellulase (Novozym 22C) was kindly provided by Novozymes (Franklinton, NC) and used for the enzymatic hydrolysis. Its filter paper activity, and β-glucosidase activity was 100 FPU/mL and 343 IU/mL, respectively. Cellulase (C2730) from *Trichoderma reesei* ATCC 26921 was purchased from Sigma-Aldrich (St. Louis, MO) and used for cellulase adsorption isotherm determination and enzyme adsorption experiments. Its filter paper activity and β-glucosidase activity was 75 FPU/mL and 24 IU/mL, respectively. Protease from *Streptomyces griseus* was also purchased from Sigma-Aldrich and used for cellulytic enzyme lignin preparation.

Organosolv pretreatment and dilute acid pretreatment

Sweetgum (*Liquidambar styraciflua*) has been used as one lignocellulosic feedstock to produce biofuels in our study, because it is abundant and widespread in the Southern U.S.[24] Sweetgum wood chips with the size of 1.0 × 1.0 cm (L × W) were collected by Forest Products Laboratory at Auburn University. For organosolv pretreatment, 80 g (dry weight) wood chips were soaked in 25% ethanol solution with 1.0% sulfuric acid (based on biomass) in a solid to liquid ratio of 1: 7 overnight. For dilute acid pretreatment, wood chips were soaked in sulfuric acid (1.0%) only, in the same solid to liquid ratio as organosolv pretreatment. Then the mixtures of wood chips and cooking liquor were loaded into 1 L Parr reactor, and cooked at 160 °C for 1 h, respectively. After cooking, the reactor was cooled down in a water bath. The pretreated substrates were washed at least three times by tap water, and collected by filtration. The resultant organosolv pretreated sweetgum and dilute acid pretreated sweetgum were designated as OPSG and DASG, respectively.

Solvent extraction and chlorite delignification

To remove extractable lignin, solvent extraction was conducted on organosolv pretreated sweetgum (OPSG) and dilute acid pretreated sweetgum (DASG). Briefly, the pretreated substrates were first mixed with 95% ethanol in the solid to liquid ratio of 1: 10 at room temperature for 5 min, and then the liquid was removed by filtration. This ethanol washing process was repeated for three times. After that, the collected substrates were further washed with water to remove the residual solvent and collected by filtration. The resulting solid from organosolv pretreated sweetgum and dilute acid pretreated sweetgum were designated as ethanol-washed organosolv pretreated sweetgum (OPSG-EW) and ethanol-washed dilute pretreated sweetgum (DASG-EW), respectively. The filtrate was collected for isolating the solvent extractable lignin later.

To further remove the residual bulk lignin, sodium chlorite delignification was carried out on ethanol-washed substrates (ethanol-washed OPSG and ethanol-washed DASG) at room temperature.[23] For ethanol-washed OPSG, sodium chlorite (0.6 g/g substrate) and acetic acid (0.1 mL/g substrate) were added into the solution with a solid to liquid ratio of 1: 20. Due to the higher acid insoluble lignin content in ethanol-washed DASG, higher sodium chlorite dosage (0.8 g/g substrate) was used in the delignification of ethanol-washed DASG. The delignification reactions were performed for 2 h, with gentle manual mixing each half hour in the fume hood. After that, the delignified substrates were washed with warm water until the solution was colorless. Then the substrates were collected by filtration, and designated as delignified OPSG (OPSG-DL) and delignified DASG (DASG-DL), respectively.

Isolation of solvent extractable lignin and cellulolytic enzyme lignin from OSPG

Solvent extractable lignin (SEL) was precipitated from the previous ethanol-washing solution (filtrate) of organosolv pretreated sweetgum by adding 3-fold volumes of water and adjusting the pH to 4. The precipitated solvent extractable lignin was collected on Whatman NO. 1 filter paper, washed by warm water at least three times, and air dried in the fume hood.

Cellulolytic enzyme lignin (CEL) was isolated from the enzymatic hydrolysis of organosolv pretreated sweetgum. To achieve nearly complete glucan hydrolysis, two-stage enzymatic hydrolysis was carried out on organosolv pretreated sweetgum. In brief, the substrate at 2% glucan (w/v) was mixed with 20 FPU/g glucan of cellulase, and incubated at pH 4.8, 50 °C and 150 rpm for 72 h. The enzymatic hydrolysis residue was recovered by centrifugation, and the supernatant was discarded. To restart the enzymatic hydrolysis, the residue was suspended with fresh buffer, supplemented with another 20 FPU/g glucan of cellulase, and incubated for another 72 h. After this two-stage enzymatic hydrolysis, the enzymatic hydrolysis residue was recovered by centrifugation. Due to the high cellulase usage, cellulase adsorption on enzymatic hydrolysis residue was unavoidable, thus the enzyme protein was removed as previously described.[11] The residue was re-suspended in water,

sonicated for 60 min, and washed three times by distilled water. The washed residue was then subjected to the protease treatment overnight at 37°C in 50 mM phosphate buffer, pH 7, with 1 U/mL protease (Sigma-Aldrich). After that, the residue was incubated at 90 °C for 1 h, to deactivate the protease enzyme. The residue was washed with phosphate buffer and distilled water, and then air dried. Protease treatment has been suggested as an effective way to reduce the adsorbed enzyme on lignin.[11, 23] The resultant residue was collected as cellulolytic enzyme lignin, which contained both solvent extractable lignin and residual bulk lignin. To obtain the real residual bulk lignin, the solvent extractable lignin was removed by 95% ethanol washing as previously described. The final solid residue was collected as ethanol-washed cellulolytic enzyme lignin (CEL-EW). The nitrogen content was 0.52% and 0.69% in CEL and CEL-EW, respectively.

Enzymatic hydrolysis of pretreated sweetgum and Avicel

Enzymatic hydrolysis was performed in 50 mL of 50 mM sodium citrate buffer (pH 4.8) at 2% glucan (w/v) with commercial enzyme (Novozym 22C), and incubated at 50 °C and 150 rpm for 72 h. To examine the effects of solvent extractable lignin removal and bulk lignin removal, enzymatic hydrolysis of organosolv pretreated sweetgum (OPSG), ethanol-washed OPSG, and delignified OPSG was conducted with 5.0 FPU/g glucan of enzyme loading. Similarly, enzymatic hydrolysis of dilute acid pretreated sweetgum (DASG), ethanol-washed DASG, and delignified DASG was compared, but with 10.0 FPU/g glucan of enzyme loading. The samples were taken from the hydrolysis reaction at various time intervals (3, 6, 12, 24, 48, and 72 h) to monitor the sugar release, and analyzed by HPLC with Aminex HPX-87P column. The hydrolysis yields of the substrates were calculated from the released glucose or xylose content, as a percentage of the theoretical sugars available in the substrates. Initial hydrolysis rates were calculated based on the released sugars in the first 3 h of enzymatic hydrolysis.

To investigate the effect of three lignin preparations (solvent extractable lignin, cellulolytic enzyme lignin, and ethanol-washed cellulolytic enzyme lignin) on enzymatic hydrolysis, 4 g/L of lignin was added into enzymatic hydrolysis of pure cellulose (Avicel), respectively. The samples were taken from the hydrolysis reaction at various time intervals to monitor the sugar concentration and also the free enzyme concentration. The hydrolysis yields and initial hydrolysis rates were calculated as described previously. The free enzyme concentration in supernatant was determined by Bradford assay, and presented in the percentage of the total protein concentration. All enzymatic hydrolysis experiments were carried out in duplicate.

Enzyme adsorption isotherms on pretreated substrates and isolated lignins

To determine enzyme adsorption onto pretreated sweetgum and Avicel, cellulase C2730 was incubated with substrates at 2% (w/v) glucan at 4 °C and 150 rpm for 2 h. A range of enzyme concentration from 0.01 to 2.0 mg/mL was added in 50 mM citrate buffer (pH 4.8) with the substrates. While for enzyme adsorption onto lignin samples (solvent extractable lignin, cellulolytic enzyme lignin, and ethanol-washed cellulolytic enzyme lignin), cellulase C2730 was incubated with 2% (w/w) lignin at 4 °C and 150 rpm for 3 h. A range of enzyme concentration was from 0.01 to 0.4 mg/mL, due to the less protein adsorption capacity of lignin. After reaching equilibrium, the sample was taken and centrifuged to collect the supernatant. The enzyme concentration in the supernatant was determined by Bradford assay as free enzyme in the solution. The classical Langmuir adsorption isotherm was used to fit the cellulase enzyme adsorption on substrates and lignins. In this case, the surface concentration of adsorbed enzyme (Γ) was given by

$$\Gamma = \Gamma_{max} KC/(1+KC).$$

Where Γ_{max} is the surface concentration of protein at full coverage (mg/g substrate); K is the Langmuir constant (mL/mg); and C is the free enzyme in solution (mg/mL). Moreover, the distribution coefficient (R) can be expressed as

$$R = \Gamma_{max} \cdot K.$$

Extractable lignin counters the negative effects of bulk lignin

Cellulase (C2730) adsorption on lignin samples (solvent extractable lignin and ethanol-washed cellulolytic enzyme lignin) was performed at pH 4.8, 50 °C, 150 rpm for 3 h and 24 h respectively. Cellulase enzyme (0.05 mg/mL of protein concentration) was mixed with 1% (w/v) solvent extractable lignin or ethanol-washed cellulolytic enzyme lignin respectively. To investigate the countering effect of solvent extractable lignin on enzyme adsorption onto ethanol-washed cellulolytic enzyme lignin, 1% (w/v) extractable lignin and 1% (w/v) ethanol-washed enzyme lignin was mixed together with cellulase enzymes. The free enzyme concentration in the supernatant was determined by Bradford assay. The adsorbed enzyme concentration (as percentage of total enzyme) was calculated as the difference between the free enzyme concentration and the initial enzyme concentration.

Results and discussions

Effects of solvent extraction and chlorite delignification on chemical composition of pretreated biomass (organosolv and dilute acid pretreated sweetgum) and their enzyme adsorption isotherms

To examine the effects of extractable lignin and residual bulk lignin on enzymatic hydrolysis of biomass, solvent extraction and acid chlorite delignification were used to remove extractable lignin and bulk lignin from organosolv pretreated sweetgum (OPSG) and dilute acid pretreated sweetgum (DASG).The chemical composition of six pretreated substrates (OPSG, ethanol-washed OPSG, delignified OPSG, DASG, ethanol-washed DASG and delignified DASG) was compared with unpretreated sweetgum in Table 1. Organosolv pretreatment significantly removed lignin and xylan, resulting in high glucan content (61.9%) in OPSG. The Klason lignin was reduced significantly from 23.6% (raw biomass) to 10.8% in OPSG; while the xylan was reduced from 16.2% (raw biomass) to 6.0% in OPSG. It was observed that the solvent extractives content was very high (12.3%) in OPSG. As we reported previously, the high extractives content was probably due to the precipitation of dissolved lignin on the pretreated substrates, and this dissolved lignin was extractable in solvent.[18] Subsequently, after solvent extraction, the extractives (extractable lignin) content was reduced dramatically to 2.9%, while the glucan in ethanol-washed OPSG increased to 70.6% and the

Table 1 Chemical composition of organosolv and dilute acid pretreated biomass and lignin samples (%)

Biomass and lignin	Ethanol extractives	Klason lignin	Acid soluble lignin	Glucan	Xylan	Mannan
UPSG[a]	0.99±0.09	23.56±0.28	2.24±0.02	41.19±0.73	16.18±0.50	3.33±0.49
OPSG[b]	12.30±0.20	10.83±0.26	1.13±0.01	61.93±0.14	6.00±0.17	1.11±0.12
OPSG-EW[c]	2.87±0.07	11.98±0.34	1.11±0.01	70.57±0.26	7.09±0.21	1.23±0.08
OPSG-DL[d]	0.68±0.08	4.41±0.07	2.54±0.08	78.40±0.89	6.70±0.17	1.99±0.20
DASG[e]	9.27±0.04	22.89±0.20	1.48±0.02	56.43±0.31	7.81±0.23	1.97±0.08
DASG-EW	1.53±0.13	24.70±0.09	1.49±0.00	60.08±0.15	8.10±0.06	1.90±0.01
DASG-DL	0.64±0.07	9.03±0.06	3.92±0.04	72.30±0.40	6.49±0.18	2.06±0.10
SEL[f]	95.15± 0.62	-	1.26± 0.02	0.10± 0.02	0.34± 0.13	0.89± 0.12
CEL[g]	40.71± 0.92	43.84± 0.36	1.50± 0.06	6.88± 0.25	1.46± 0.20	2.78± 0.86
CEL-EW[h]	-	75.85± 0.60	1.20± 0.08	11.92± 0.34	2.06± 0.31	3.92± 0.77

[a]UPSG refers to unpretreated sweetgum. [b]OPSG refers to organosolv pretreated sweetgum. [c]OPSG-EW refers to ethanol washed OPSG. [d]OPSG-DL refers to sodium chlorite delignified OPSG-EW. [e]DASG refers to dilute acid pretreated sweetgum. [f]SEL refers to solvent extractable lignin from OPSG. [g]CEL refers to cellulolytic enzyme lignin from OPSG. [h]CEL-EW refers to ethanol-washed CEL lignin.

Klason lignin was increased slightly to 11.9%. This indicated that the solvent extraction only removed the extractable lignin, not the bulk lignin (Klason lignin). On the other hand, the acid chlorite delignification was used to remove the bulk lignin. Consequently, the Klason lignin was reduced significantly to 4.4%in delignified OPSG. Meanwhile, the glucan content increased to 78.4%, due to the lignin removal in delignified OPSG.

As for the dilute acid pretreated sweetgum (DASG), the glucan (56.4%) was lower than that in organosolv pretreated sweetgum. The Klason lignin content was very high (22.9%). This agreed well with previous report that lignin removal was less effective in dilute acid batch pretreatment.[25] The extractives content was also high (9.3%) in DASG. After further solvent extraction, the extractives (extractable lignin) content decreased significantly to 1.5% in ethanol-washed DASG, while the Klason lignin (bulk lignin) slightly increased to 24.7%. In delignified DASG, the Klason lignin content was reduced to 9.0% by acid chlorite delignification.

The cellulase adsorption on pretreated substrates with lignin removal (ethanol-washed OPSG, delignified OPSG, ethanol-washed DASG and delignified DASG) was compared with that on pretreated substrates without lignin removal (OPSG and DASG)(Fig. 1 and Table 2).The results showed that extractable lignin removal increased the distribution coefficient (*R*) slightly in both ethanol-washed OPSG and ethanol-washed DASG, while bulk lignin removal increased the distribution coefficient significantly in both delignified OPSG and delignified DASG (Table 2). Specifically for OPSG substrates, extractable lignin removal increased the adsorption capacity (Γ_{max}) from 52.9 (OPSG) to 60.4 mg/g (ethanol-washed OPSG), but did not change the Langmuir constant (*K*=2.5 mL/mg). Subsequently, bulk lignin removal significantly increased the adsorption capacity to 82.9 mg/g and Langmuir constant to 3.8 mL/mg on delignified OPSG, respectively. Similarly for DASG substrates, extractable lignin removal increased the adsorption capacity by 18.8% from 30.5 (DASG) to 36.2 mg/g (ethanol-washed DASG), and increased the

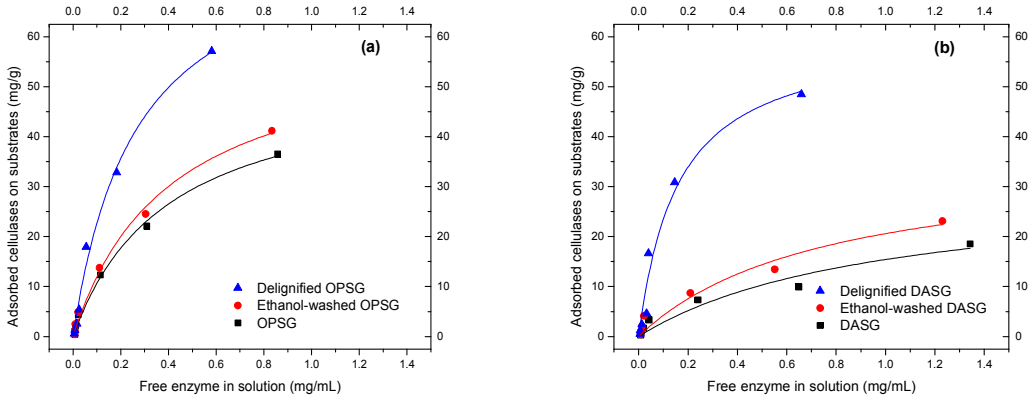

Fig.1 Cellulase adsorption isotherms on organosolv pretreated sweetgum (a) and dilute acid pretreated sweetgum (b).

Table 2 Langmuir adsorption parameters of cellulase adsorption on pretreated biomass and lignins

Samples	Γ_{max} (mg/g)	K (mL/mg)	R (L/g)
OPSG[a]	52.92	2.51	0.133
OPSG-EW[b]	60.43	2.47	0.149
OPSG-DL[c]	82.98	3.76	0.312
DASG[d]	30.50	1.02	0.031
DASG-EW	36.22	1.32	0.048
DASG-DL	60.99	6.26	0.381
Avicel	51.87	5.63	0.292
SEL[e]	1.13	6.50	0.007
CEL[f]	4.90	14.12	0.069
CEL-EW[g]	4.37	35.01	0.153

[a]OPSG refers to organosolv pretreated sweetgum. [b]OPSG-EW refers to ethanol-washed OPSG. [c]OPSG-DL refers to sodium chlorite delignified OPSG-EW. [d]DASG refers to dilute acid pretreated sweetgum. [e]SEL refers to solvent extractable lignin from OPSG. [f]CEL refers to cellulolytic enzyme lignin from OPSG. [g]CEL-EW refers to ethanol-washed CEL lignin.

Langmuir constant by 29.4% from 1.0 (DASG) to 1.3 mL/mg (ethanol-washed DASG). Extractable lignin removal appeared not to enhance cellulase adsorption considerably. Bulk lignin removal doubled the adsorption capacity on delignified DASG, and increased the Langmuir constant by six times. This actually indicated that extractable lignin did not control substrate accessibility, but the bulk lignin did.

Comparing cellulase adsorption on OPSG/OPSG-EW and DASG and DASG-EW revealed an inverse correlation between adsorption capacity (Γ_{max}) and residual bulk lignin content. The Γ_{max} of OPSG/OPSG-EW was two-fold higher than those from DASG/DASG-EW, while the Klason lignin in DASG/DASG-EW was two-fold higher than those in OPSG/OPSG-EW. This further suggested bulk lignin governs the substrate accessibility. Removing the bulk lignin would increase the cellulose surface accessibility for enzymes significantly, which has been demonstrated by the following hydrolysis performance of delignified substrates (Fig. 2).

Effects of extractable lignin and bulk lignin removal on enzymatic digestibility of pretreated biomass

Enzymatic digestibility of OPSG, ethanol-washed OPSG and delignified OPSG was compared under 5 FPU (Fig.2a). The result showed that extractable lignin removal surprisingly decreased enzymatic digestibility of ethanol-washed OPSG. On the contrary, the residual bulk lignin removal significantly enhanced enzymatic digestibility of delignified OPSG. Specifically, after extractable lignin removal, the 72 h hydrolysis yield of ethanol-washed OPSG decreased by 29.5%, as compared to the hydrolysis yield of OPSG. However the bulk lignin removal increased the 72 h hydrolysis yield from 49.3% (ethanol-washed OPSG) to 90.5% (delignified OPSG). Similar higher digestibility of delignified cellulose has been reported before.[14] For the initial hydrolysis rate, extractable lignin removal decreased the initial rate from 0.81 g/L/h (OPSG) to 0.75 g/L/h (ethanol-washed OPSG), however, bulk lignin removal increased the initial rate to 1.36 g/L/h (delignified OPSG). These results indicated the extractable lignin played a positive role in enzymatic hydrolysis but residual bulk lignin played a negative role. The extractable lignin was analysed by ^{13}C NMR, the spectra showed similar signals in aliphatic and aromatic regions as the milled wood lignin.

Similarly, enzymatic digestibility of DASG, ethanol-washed DASG and delignified DASG was compared under 10 FPU (Fig. 2b). The results showed that extractable lignin removal in ethanol-washed DASG decreased the 72 h hydrolysis yield of ethanol-washed DASG by 16.5%. On the contrary, bulk lignin removal in delignified DASG increased the 72 h hydrolysis yield from 31.8% (ethanol-washed DASG) to 91.7% (delignified DASG). As for the initial hydrolysis rate, extractable lignin removal decreased the initial rate from 0.52 g/L/h (DASG) to 0.43 g/L/h (ethanol-washed DASG). However, bulk lignin removal increased it from 0.43 g/L/h (ethanol-washed DASG) to 1.65 g/L/h (delignified DASG). Similar to delignified OPSG, the bulk lignin removal significantly enhanced the substrate digestibility in delignified DASG. However, the change on 72 h hydrolysis yield was relatively small in ethanol-washed DASG (-

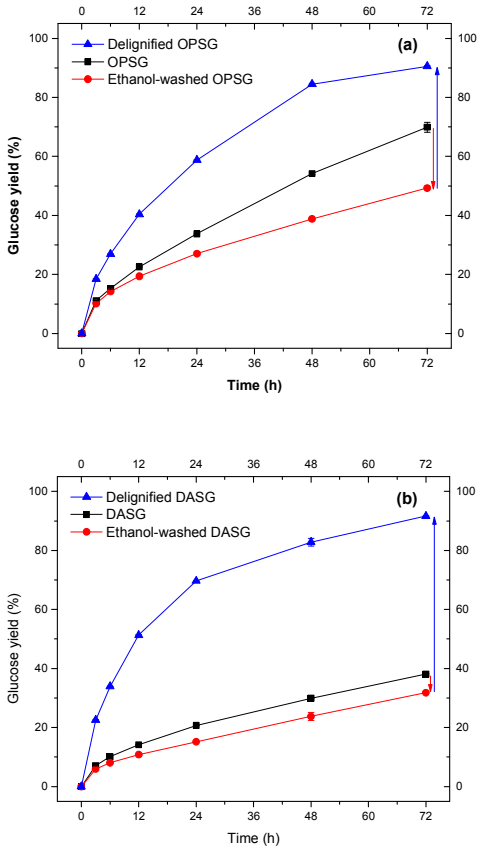

Fig.2 Effects of extractable lignin removal and bulk lignin removal on enzymatic hydrolysis of organosolv pretreated sweetgum (a) and dilute acid pretreated sweetgum (b).

16.5%) after extractable lignin removal, compared to that in ethanol-washed OPSG (-29.5%). This indicated that extractable lignin from organosolv process could be more stimulatory than that from dilute acid process. This probably is due to the lignin etherification reaction in organosolv process, which changed lignin functional groups and made them more hydrophilic, thus more stimulatory.[26]

In summary, the extractable lignin removal reduced the 72 h hydrolysis yields by 29.5% in OPSG, and 16.5% in DASG, respectively. This result was consistent with our previous study that the 72 h hydrolysis yield decreased in OPSG as extractable lignin content reduced. And a strong correlation between extractable lignin contents and 72 h hydrolysis yields was observed, which suggested the extractable lignin in OPSG played a positive role in enzymatic hydrolysis.[18] Whereas, the residual bulk lignin removal significantly increased the 72 h hydrolysis yields and the initial hydrolysis rates in both ethanol-washed OPSG and ethanol-washed DASG. These results partially supported the traditional thinking that lignin retarded cellulose hydrolysis through either physical blocking or non-productive binding of the cellulases.[23] Compared to residual bulk lignin, the extractable lignin from OPSG and DASG showed no negative effect on enzymatic hydrolysis, but the positive effect. This agreed well with previous observation that the precipitated lignin interfered less with enzyme action than bulk lignin.[25] Moreover, it should be noted that both OPSG and DASG substrates was washed with water at the end of pretreatment process. Therefore, the potential inhibition or deactivation from soluble lignin-derived phenolic compounds was minimized.[14] In the real biorefinery process with dilute acid pretreatment, the soluble inhibitors in pretreatment slurry have to be removed although the insoluble extractable lignin showed positive effect and could be kept.

Effects of isolated solvent extractable lignin and residual bulk lignin on enzymatic hydrolysis of Avicel

To confirm the disparate roles of extractable lignin and bulk lignin on enzymatic hydrolysis, the extractable lignin and bulk lignin were isolated from OPSG respectively and examined further on enzymatic hydrolysis of Avicel. Specifically, the extractable lignin was isolated from OPSG by solvent washing and referred as solvent extractable lignin (SEL). The cellulolytic enzyme lignin (CEL) was collected from the nearly complete enzymatic hydrolysis of OPSG. To remove the extractable lignin out of cellulolytic enzyme lignin, the ethanol washing was conducted on cellulolytic enzyme lignin, which resulted in the isolated residual bulk lignin (ethanol-washed cellulolytic enzyme lignin, CEL-EW).

The chemical composition of three lignin samples (solvent extractable lignin, cellulolytic enzyme lignin and ethanol-washed cellulolytic enzyme lignin) was compared in Table 1. The carbohydrates (glucan, xylan and mannan) content was very low (1.3%) in solvent extractable lignin, the extractable lignin content was 95.2%.For cellulolytic enzyme lignin, small amount of glucan (6.9%) was left in this lignin sample. It was observed that extractable lignin content (40.7%) was very high in cellulolytic enzyme lignin, which is similar to the Klason lignin content (43.8%). Since high extractable lignin in cellulolytic enzyme lignin, it was not classified as bulk lignin in this study. For ethanol-washed cellulolytic enzyme lignin, the Klason lignin was 75.9% and glucan was 11.9% in this lignin sample. The ethanol-washed cellulolytic enzyme lignin was referred as bulk lignin in this study, because extractable lignin was negligible in ethanol-washed cellulolytic enzyme lignin.

The cellulase adsorption isotherm on solvent extractable lignin, cellulolytic enzyme lignin and ethanol-washed cellulolytic enzyme lignin was evaluated respectively (Table 2). The adsorption capacity (Γ_{max}) of cellulase on solvent extractable lignin, cellulolytic enzyme lignin and ethanol-washed cellulolytic enzyme lignin was 1.1, 4.9 and 4.4 mg/g, which was much lower than those in Avicel or pretreated biomass (30-60mg/g). However, the Langmuir constant of cellulase on solvent extractable lignin (K=6.5 mL/mg), cellulolytic enzyme lignin (K=14.1 mL/mg) and ethanol-washed cellulolytic enzyme lignin (K=35.0 mL/mg) was much higher than those in pretreated biomass (1.0-6.3 mL/mg. Similar observations have been reported previously on binding strength between ethanol organosolv lignin and cellulase.[20, 27] Solvent extractable lignin showed the lowest affinity to cellulase enzymes (R=0.007 L/g), which was similar with ethanol organosolv lignin from sweetgum (R=0.004 L/g) in our previous study.[20] However, ethanol-washed cellulolytic enzyme lignin showed the highest affinity to cellulase enzymes (R=0.153 L/g), which was very close to the R value in cellulase adsorption onto enzymatic residual lignin (R=0.151 L/g) from steam pretreated Douglas-fir.[28] It has been suggested that enzymatic residual lignin had the higher degree of condensation than ethanol organosolv lignin.[28] As for cellulolytic enzyme lignin, the affinity of cellulase enzymes was relatively low (R=0.069 L/g), this probably was due to the presence of 40.7% extractable lignin in cellulolytic enzyme lignin. The extractable lignin fraction in cellulolytic enzyme lignin probably reduced the binding between cellulase enzymes and the bulk lignin (ethanol-washed cellulolytic enzyme lignin).

The effects of solvent extractable lignin, cellulolytic enzyme lignin and ethanol-washed cellulolytic enzyme lignin on enzymatic hydrolysis of pure cellulose (Avicel) were compared (Fig. 3a). It was observed that the addition of solvent extractable lignin enhanced the 72 h hydrolysis yield by 5.3% (P value <0.05), from 60.4% (Avicel control) to 63.6%. Whereas, the addition of residual bulk lignin (ethanol-washed cellulolytic enzyme lignin) decreased the 72 h hydrolysis yield of Avicel by 13.4%.This confirmed that the extractable lignin played a positive role in enzymatic hydrolysis and bulk lignin played a negative role in enzymatic hydrolysis. The xylan content in SEL, CEL and CEL-EW were very low (<2%), thus the xylan inhibition can be negligible in the hydrolysis of Avicel. It should be noted that the positive effect of extractable lignin on enzymatic hydrolysis of Avicel appeared to be much lower than that on enzymatic hydrolysis of pretreated sweetgum. The level of enhancement was indeed related to the lignin content in the substrates.[19] The lignosulfonate improved pretreated hardwood and softwood significantly, however the enhancement was much lower on enzymatic hydrolysis of filter paper. Lignosulfonate acted as a surfactant to block bound lignin from binding cellulases non-productively. This also suggested that the solvent extractable lignin might improve enzymatic hydrolysis also by reducing the non-productive binding of enzyme on the residual bulk lignin.

Interestingly, it was found that cellulolytic enzyme lignin (containing both extractable lignin and bulk lignin) increased the 72 h hydrolysis yield by 4.1%. There was 1.5% xylan and 2.8% mannan

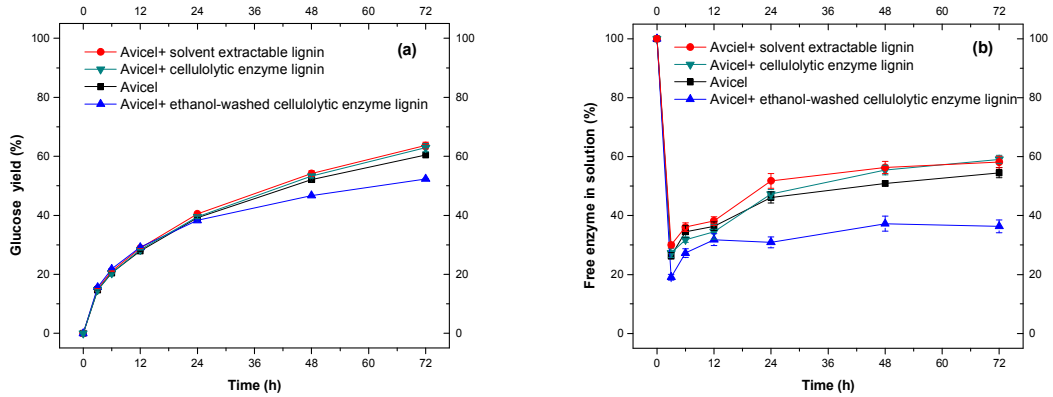

Fig. 3 Effect of solvent extractable lignin (SEL) and residual bulk lignin (CEL-EW) on enzymatic hydrolysis of Avicel (a) and cellulase distribution (b).

in cellulolytic enzyme lignin, which did not appear to inhibit enzymatic hydrolysis. This indicated that extractable lignin could counter the negative effect of bulk lignin on enzymatic hydrolysis. This agreed well with our previous findings that ethanol organosolv lignin from hardwood could offset the negative effect of ethanol organosolv lignin from softwood.[20]

To examine whether the disparate roles of solvent extractable lignin (SEL) and residual bulk lignin (CEL-EW) were related to cellulase distribution during hydrolysis, the free enzyme concentration was determined in the hydrolysis of Avicel with the addition of solvent extractable lignin, cellulolytic enzyme lignin and ethanol-washed cellulolytic enzyme lignin (Fig. 3b). The results showed that the addition of solvent extractable lignin increased the free enzyme and ethanol-washed cellulolytic enzyme lignin decreased the free enzyme in solution. Specifically, addition of solvent extractable lignin increased the free enzyme percentage slightly from 26.6% (control) to 30.0% of its initial at 3 h, but increased the free enzyme considerably from 54.5% (control) to 58.2% of its initial at 72 h. On the contrary, the addition of ethanol-washed cellulolytic enzyme lignin decreased the free enzyme percentage to 19.1% of its initial at 3 h, and decreased the free enzyme percentage to 36.4% of its initial at 72 h. The drop of free enzyme in solution with ethanol-washed cellulolytic enzyme lignin probably was due to the non-productive binding between bulk lignin and cellulase. As for the addition of cellulolytic enzyme lignin, the free enzyme percentage (27.2%) did not changed at 3 h, but increased to 59.1% at 72 h. This indicated the extractable lignin in cellulolytic enzyme lignin countered the non-productive binding between residual bulk lignin and cellulase. In this study, a strong correlation between 72 h free enzyme content in solution and 72 h hydrolysis yield was observed (r^2=0.99), which indicated the disparate roles of extractable lignin and bulk lignin were correlated with their effects on the change of free enzyme in the hydrolysis. Moreover, the different cellulase enzyme components have different binding affinities to lignins. Endoglucanase was considered to have the higher affinity to lignin, due to the more open binding domain structure compared to the tunnel-shaped binding site of exoglucanase.[29] β-Glucosidase was least adsorbed on lignin, among these three enzyme components.[11] Therefore, the extractable lignin and bulk lignin will affect the enzyme profiles differently in the enzymatic hydrolysis. It should be noted that the activity profiles of the free enzymes in solution were not determined in this study. The effects of extractable lignin on free enzymes activity could be different from cellulase distribution.

Extractable lignin counters the negative effects of bulk lignin by reducing the non-productive binding

To further confirm the countering effect of extractable lignin on bulk lignin, effect of solvent extractable lignin addition on the adsorption of cellulase (C2730) onto ethanol-washed cellulolytic enzyme lignin was determined in a batch process. The result showed that solvent extractable lignin addition (1%) reduced enzyme adsorption on ethanol-washed cellulolytic enzyme lignin significantly (Fig. 4). Specifically, after 3 h of incubation, 20.9% of cellulase enzymes adsorbed onto solvent extractable lignin and

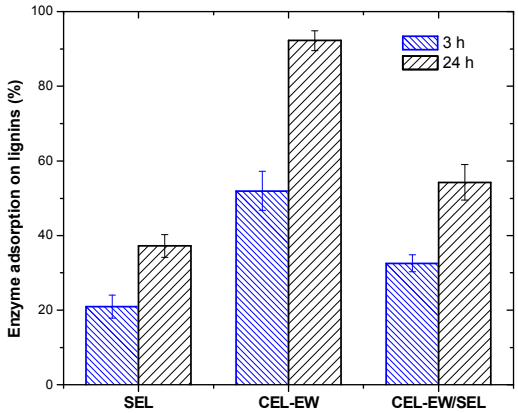

Fig. 4 Effect of extractable lignin (SEL) on cellulase adsorption onto bulk lignin (CEL-EW)

51.9% of cellulase enzymes adsorbed onto ethanol-washed cellulolytic enzyme lignin. Ethanol washing changed chemical composition in CEL-EW, which could potentially increase the bulk lignin surface area. However, the addition of solvent extractable lignin into ethanol-washed cellulolytic enzyme lignin resulted in only 32.6% of cellulase enzyme adsorbed onto lignin samples (containing both solvent extractable lignin and ethanol-washed cellulolytic enzyme lignin). This number was much lower than the sum of cellulase adsorption onto two lignins (72.8%), and even lower than that onto ethanol-washed cellulolytic enzyme lignin. This indicated that the addition of solvent extractable lignin significantly reduced the cellulase adsorption by 37% as comparing to the adsorption onto ethanol-washed cellulolytic enzyme lignin. This confirmed that extractable lignin countered the binding between bulk lignin and cellulase, which most likely offset the negative non-productive binding. As incubation time extended to 24 h, similar results were observed and solvent extractable lignin reduced the cellulase adsorption onto ethanol-washed cellulolytic enzyme lignin by 41%. This suggested that the countering effect could be larger when the incubation or hydrolysis time was longer. This also could be used to explain the higher enhancement at 72 h, not in the initial hydrolysis phase. It should be mentioned different cellulase component will adsorb on extractable lignin and bulk lignin in a different way and they may affect each other on adsorption as well.

Conclusions

Disparate roles of solvent extractable lignin and residual bulk lignin on enzymatic hydrolysis were observed. The solvent extractable lignin played a positive role and residual bulk lignin played a negative role in enzymatic hydrolysis of pretreated biomass (OPSG and DASG). The solvent extractable lignin could counter the negative effects on residual bulk lignin by relieving the non-productive binding between residual bulk lignin and cellulase. This research suggested that solvent extractable lignin should be kept in the pretreated biomass to enhance enzymatic hydrolysis. Future work will focus on the chemical structure difference between solvent extractable lignin and bulk lignin, and identify the functional groups on solvent extractable lignin which are responsible for the positive effect on enzymatic hydrolysis.

Acknowledgements

The study was supported in part by grants from Alabama Agricultural Experimental Station, Sun Grant and USDA NIFA (Grant NO. 2011-68005-30410). Lai's study was also partially supported by Jiangsu Planned Projects for Postdoctoral Research Funds (1402062B).

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