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Distinct Roles of Residual Xylan and Lignin in Limiting Enzymatic Hydrolysis of Organosolv Pretreated Loblolly Pine and Sweetgum

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Supporting Information

ABSTRACT: The interactions between xylan/lignin and cellulase enzymes play a key role in the effective hydrolysis of lignocellulosic biomass. Organosolv pretreated loblolly pine (OPLP) and sweetgum (OPSG) were used to quantitatively elucidate the distinct roles of residual xylan and lignin on enzymatic hydrolysis, based on the initial hydrolysis rates and the final hydrolysis yields. The initial hydrolysis rates of OPLP and OPSG were 1.45 (glucose) and 1.19 g/L/h (glucose), respectively, under the enzyme loading of 20 FPU/g glucan. The final glucan hydrolysis yields of OPLP and OPSG at 72 h were 76.4 and 98.9%, respectively. By correlating the amount of residual lignin and xylan to the initial hydrolysis rate and the final hydrolysis yield in OPLP and OPSG, a more accurate fundamental understanding of the roles of xylan and lignin in limiting the enzymatic hydrolysis has been developed. The higher amount of residual xylan (9.7%) in OPSG resulted in lower initial hydrolysis rate (1.19 g/L/h). The higher amount of residual lignin in OPLP (18.6%) resulted in lower final hydrolysis yield of glucan (76.4%). In addition, we observed in the simultaneous saccharification and fermentation (SSF) that ethyl xyloside was produced by the enzymatic catalysis of xylose/xylan and ethanol.

KEYWORDS: *xylan, lignin, enzymatic hydrolysis, Langmuir adsorption isotherm, organosolv pretreatment*

INTRODUCTION

The biochemical conversion process consists of multiple stages converting biomass to fermentable sugars for the production of advanced biofuels (such as butanol and terpenes). Biomass feedstock is first pretreated and then subjected to enzymatic hydrolysis with subsequent microbial fermentation [or simultaneous saccharification and fermentation (SSF)] of sugars to alcohols or terpenes by yeast or bacteria.[1−4] The recalcitrant structure of cellulose and its close association with the lignin and hemicellulose matrix make it highly resistant to enzymatic hydrolysis.[5] Therefore, lignocellulosic substrates must be either physically or chemically pretreated to improve the accessibility to enzymes for hydrolysis.[6] Various pretreatment methods including steam explosion, dilute acid, hot water, ammonia fiber expansion (AFEX), and organosolv processes are currently being assessed for their ability to improve enzymatic hydrolysis.[7,8] Among these pretreatment processes, organosolv pretreatment has shown good potential for producing lignocellulosic substrates from hybrid poplar and lodgepole pine with high digestibility.[9,10] However, the residual xylan in organosolv pretreated biomass was still an important factor affecting the glucan digestibility significantly.[11] Furthermore, residual lignin also plays an important role in limiting enzymatic hydrolysis of cellulose, although lignin content is often low in the organosolv pretreated substrates.[12] Pan et al.[12] found a strong correlation (R^2 = 0.91) between the residual lignin content and the final hydrolysis yield for four organosolv pretreated substrates from softwood. As a result, the amounts of residual xylan and lignin are two important factors limiting enzymatic hydrolysis, although other factors (such as crystallinity, acetyl group, pore size, and surface area accessibility) have been suggested to affect the initial hydrolysis rate and extent of enzymatic hydrolysis.[13−15]

It is typically accepted that lignin limits the enzyme accessibility to elementary cellulose fibril by wrapping the cellulose and hemicellulose within microfibril.[16−18] Pretreatment generally disrupts the close association of lignin to microfibril. However, the high lignin content in pretreated substrates still affects the enzymatic hydrolysis due to physical blocking and nonproductive binding between enzyme and lignin.[19−21] Previously, we have suggested using surfactants to reduce nonproductive binding and improve enzymatic hydrolysis of steam exploded lodgepole pine.[19] Lignin removal generally is thought to be able to increase the ultimate hydrolysis yield, not the hydrolysis rate.[17] Hemicellulose removal (especially xylan removal) can increase the initial hydrolysis rate significantly.[22,23] Xylanase supplementation has been suggested to reduce effects of xylan on enzymatic hydrolysis of pretreated biomass and improve the final hydrolysis yield.[23,24] Because most of xylan in biomass is highly acetylated, deacetylation has been found to greatly improve the cellulose accessibility and the hydrolysis rate.[25−27] However, few studies have focused on quantitatively distin-

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Table 1. Chemical Composition of Untreated Biomass and the Pretreated Biomass

compositions	untreated loblolly pine (%)	untreated sweetgum (%)	OPLP substrate (%)	OPSG substrate (%)
acetone extractives	1.64 ± 0.08	0.99 ± 0.09	8.53 ± 0.19	8.47 ± 0.21
acid-insoluble lignin	28.48 ± 0.04	23.56 ± 0.28	18.38 ± 0.17	8.16 ± 0.03
acid-soluble lignin	0.26 ± 0.03	2.24 ± 0.02	0.23 ± 0.06	0.87 ± 0.03
glucan	41.33 ± 0.49	41.19 ± 0.73	63.32 ± 1.24	69.80 ± 1.78
xylan	6.34 ± 0.13	16.18 ± 0.50	2.93 ± 0.79	9.74 ± 0.53
galactan	2.16 ± 0.09	1.87 ± 0.32	NA	NA
arabinan	1.30 ± 0.11	0.83 ± 0.11	0.90 ± 0.21	NA
mannan	12.17 ± 0.39	3.33 ± 0.49	4.50 ± 0.59	3.01 ± 0.28
total	93.69	90.20	98.79	100.05

guishing the distinct roles of residual xylan and lignin in limiting enzymatic hydrolysis.

In this study, we used ethanol organosolv pretreatment to fractionate loblolly pine (*Pinus taeda*) and sweetgum (*Liquidambar styraciflua*) under the same conditions. The resultant substrates with different xylan and lignin contents were used to distinguish the effects of residual xylan and lignin on enzymatic hydrolysis of pretreated substrates. Scanning electron microscopy (SEM) was used to characterize the cell wall disruption and surface properties of pretreated biomass. The Langmuir adsorption isotherm was used to characterize enzyme affinity to the pretreated substrates and the initial hydrolysis rate. Quantitative information on enzymatic hydrolysis was evaluated based on the initial hydrolysis rate and the final hydrolysis yield. The correlation between the amount of residual xylan and the initial hydrolysis rates and the correlation between the amount of residual lignin and the final hydrolysis yields were established. Effects of residual xylan and lignin on ethanol yields were also compared on pretreated softwood and hardwood in the SSF process.

MATERIALS AND METHODS

Woody Biomass and Pretreatment Methods. Loblolly pine (*P. taeda*) and sweetgum (*L. styraciflua*) wood chips were collected from a forest products laboratory at Auburn University. The initial moisture content of these wood chips was approximately 9.0 wt %. Wood chips were ground by a Wiley mill (Thomas Scientific, Philadelphia, PA), and the wood powder between 20 and 40 mesh was collected for chemical composition analysis. The wood chips were reduced to an average size of 1.0 × 2.0 × 0.3 cm^3 (L × W × H) by a Waring commercial blender (Dynamics Corporation of America, New Hartford, CT) prior to organosolv pretreatment. Organosolv pretreated loblolly pine (OPLP) and sweetgum (OPSG) were prepared in a 4 L Parr batch reactor (Parr Instrument Co., Moline, IL) as previously described.[19] Briefly, wood chips (400 g) were loaded into a reactor (7:1 liquor/solid ratio) and treated at 170 °C for 60 min with 65% ethanol and 1.1% (w/w) sulfuric acid. After pretreatment, the slurry was fractionated into a solid fraction and liquid fraction (prehydrolysate) by filtration. The prehydrolysate was collected and stored at 4 °C for later use, while the solid materials (200 g) were homogenized with a blender (Qster, Milwaukee, WI) for 30 s and washed with aqueous water (600 mL) at room temperature. The chemical composition of original raw biomass and pretreated biomass is listed in Table 1.

SEM Analysis of Pretreated Substrates. SEM analysis of raw biomass (40 mesh), OPLP, and OPSG substrates was performed with a field emission SEM, JEOL 7000F operated at 20.0 KV. The samples were coated with a very thin gold layer (50 nm) using a PELCO SC-6 Sputter Coater.

Chemical Analysis of Raw Biomass and Pretreated Substrates. The extractives content of raw biomass and pretreated substrates (wood powders, 20−40 mesh) was determined using acetone extraction according to the standard method as described previously.[28] The lignin and carbohydrate composition of loblolly pine were determined using the extractive-free samples according to National Renewable Energy Laboratory protocol.[29] A Shimadzu (LC-20A) HPLC system consisting of a degasser, autosampler, LC-20AD pump, and RID-10A detector equipped with a 300 mm × 7.8 mm i.d., 9 μm, Aminex HPX-87P column and a 30 mm × 4.6 mm i.d. guard column of the same material (Bio-Rad, Hercules, CA) was used to separate and quantitate individual sugars. Water was used as the mobile phase at an isocratic flow rate of 0.6 mL/min to separate sugars, and the temperature of the column was maintained at 85 °C during the elution. The chemical composition of biomass was determined in duplicate.

Cellulase Enzymes and Enzymatic Hydrolysis. Commercial cellulase preparation (Novozym 22C) was obtained from Novozymes (Franklinton, NC). The enzyme activity of the Novozym 22C (100 FPU/mL) was determined using Whatman #1 filter paper as the substrate, and the β-glucosidase activity (343 IU/mL) of Novozym 22C was determined using *p*-nitrophenyl-β-D-glucoside (PNPG) as a substrate. Multifect Pectinase (~1660 IU/mL, 24 mg/mL protein) and Multifect Xylanase (~25200 IU/mL, 13.4 mg/mL protein, according to Dien et al.[30]) were used as accessory enzymes (Genencor International, Palo Alto, CA) for the enzymatic hydrolysis of pretreated substrates.[30] Cellulase from *Trichoderma reesei* ATCC 26921 was obtained from Sigma-Aldrich (St. Louis, MO) for cellulase adsorption isotherm determination. The enzymatic hydrolysis was carried out in duplicate.

Enzymatic hydrolysis of OPLP and OPSG was carried out in 100 mL of 50 mM sodium citrate buffer (pH 4.8) at a 2% consistency (w/w glucan) as previously described.[31] In brief, the reaction mixture of pretreated biomass and enzyme was incubated at 45 °C with shaking at 150 rpm. The cellulase loading was 10 or 20 FPU/g glucan. No extra β-glucosidase enzyme was added into the reaction due to the sufficient β-glucosidase activity in the Novozym 22C. Samples were taken from the reaction at various time intervals (2, 5, 8, 12, 24, 48, and 72 h) and centrifuged to remove the insoluble material (solid phase). The glucose and xylose contents were measured by HPLC with the same Aminex HPX-87P column. The glucose or xylose yield (%) of the substrates was calculated from the released glucose or xylose content, as a percentage of the theoretical sugars available in the pretreated substrates. The initial hydrolysis rate (r) was calculated based on the released sugars in the first 5 or 6 h of enzymatic hydrolysis of substrates $[r = (C_t - C_0)/t]$, where C_0 and C_t and are the sugar concentrations at 0 and t h, respectively. For the pectinase and xylanase supplementation, the enzymatic hydrolysis was carried out with 10 FPU/g glucan of Novozym 22C, and Multifect Pectinase or Multifect Xylanase was added into the reaction mixture based on the protein content at 3.6 and 2.7 mg/g glucan as previously described.[30] The protein content in the supernatant was measured by the Bradford assay using bovine serum albumin (BSA) as the protein standard.

Yeast Strain, Culture Medium, and SSF Process. *Saccharomyces cerevisiae* (Baker's yeast) was grown in a YPG liquid medium composed of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose at 30 °C. SSF was performed (with 10 or 20 FPU/g glucan of Novozyme 22C) in 50 mM citrate buffer with pretreated OPLP and OPSG substrates (2% w/w glucan) at 37 °C and 180 rpm for 96 h. The initial yeast innoculation was 2.0 g/L. Samples were taken and

analyzed by HPLC for sugars and ethanol determination with a 300 mm × 7.8 mm i.d., 9 μm, Aminex HPX-87H column and a 30 mm × 4.6 mm i.d. guard column of the same material (Bio-Rad). Ethyl xyloside in SSF amples was analyzed by an Ultra Performance LC Systems (Waters, Milford, MA) coupled with a quadrupole time-of-flight mass spectrometer with electrospray ionization (ESI) in positive ESI-MS operated by the Masslynx software (V4.1). The sample was directly injected into the ESI source at a flow rate of 50 μL/min with a mobile phase of 100% acetonitrile or 50% acetonitrile in water. The ion source voltages were set at 3 KV for positive and negative ion mode acquisitions, respectively. The sampling cone was set at 37 V, and the extraction cone was at 3 V. In both modes, the source and desolvation temperatures were maintained at 120 and 225 °C, respectively, with the desolvation gas flow at 200 L/h.

Cellulases Adsorption Isotherm. Cellulase adsorption on organosolv pretreated substrates was performed at 4 °C in 50 mM citrate buffer (pH 4.8) as described previously.[31] Cellulase C2730 (from *T. reesei* ATCC 26921, protein content 44 mg/mL, Sigma-Aldrich) was used to determine the adsorption isotherm. Novozym 22C was not used due to the high β-glucosidases in this cellulase mixture. A range of enzyme concentrations from 0.01 to 2.0 mg/mL were added in 50 mM citrate buffer (pH 4.8) with the substrates suspended at 2% consistency (based on glucan). The mixture was incubated at 4 °C for 2 h to reach equilibrium. The protein content in the supernatant was determined for the free cellulases by Bradford assay using BSA as the protein standard. The adsorbed cellulase was calculated by taking the difference between the initial cellulase content and the free cellulase content in the supernatant. The classical Langmuir adsorption isotherm was applied to the cellulases adsorption on OPLP and OPSG in solution. In this case, the surface concentration of adsorbed enzymes (Γ) was given by the equation

$$\Gamma = \frac{\Gamma_{max} KC}{1 + KC}$$

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 protein concentration in the bulk solution (mg/mL).

RESULTS AND DISCUSSION

Chemical Composition of OPLP (*P. taeda*) and OPSG (*L. styraciflua*). The chemical compositions of untreated loblolly pine (softwood) and sweetgum (hardwood) are compared in Table 1. The carbohydrates (cellulose and hemicellulose) represented approximately 63.3 wt % in loblolly pine and 63.4 wt % in sweetgum. The total lignin content was higher in softwood (28.7 wt %) than that in hardwood (25.8 wt %). These results were in agreement with previous reports on the composition of pine and sweetgum.[32−35] After organosolv pretreatment, the lignin content in OPLP substrate was 18.6%, which was twice that of OPSG substrate (9.0%). The solid yields were 53.8 (loblolly pine) and 58.7% (sweetgum). The carbohydrate portion of the OPLP and OPSG substrates was composed mainly of glucan with similar content, but the xylan content (9.7%) was much higher in hardwood OPSG substrate than that in softwood OPLP substrate (2.9%). The mannan content was reduced from 12.2 to 4.5% in OPLP.

SEM Image Analysis. SEM can reveal surface morphology of plant cell wall of pretreated biomass.[36−38] The SEM images of raw biomass and pretreated biomass (Figure 1A−D) indicated that the bound fibers in biomass were liberated or separated after organosolv pretreatment. Organosolv pretreatment is a process similar to pulping, by which the biomass is reduced to a fibrous mass. It ruptures the bonds between fibers within biomass. Furthermore, we observed a considerable amount of spherical droplets covering the cell wall surface in OPLP (Figure 1C). On the contrary, very few droplets could be seen from the cell wall surface of OPSG (Figure 1D). The diameter of these droplets was from 0.2 to 10 μm. Previously, these droplets on plant cell wall of pretreated biomass were found to contain lignin by FTIR and NMR analysis.[37] These lignin droplets tended to coalesce together probably due to the hydrophobicity of lignin. The lignin droplets redispersed on the surface of plant cell wall depending on the pretreatment methods. Dilute acid pretreatment produced numerous lignin droplets on the surface of pretreated corn stover,[37] while a limited amount of lignin droplets displayed on substrates from the ethanol organosolv process. This occurred probably because in the organosolv pretreatment, a considerable amount of lignin was depolymerized and dissolved in aqueous ethanol. Consequently, this dissolved lignin was filtrated into prehydrolysate, while a small amount of dissolved lignin in substrate was reprecipitated when washed with water. Fewer lignin droplets on OPSG could be caused by the lower lignin in raw biomass and hardwood lignin being easier to break down. For the structural changes, we observed the rupture of fibers and cracks on the surface fibers in OPLP but not in OPSG. The surface of OPSG was much smoother than that of OPLP. After enzymatic hydrolysis (72 h), residues of OPLP and OPSG were also examined by SEM (Figure 1E,F). More packed and uniform lignin was shown in OPSG residues (Figure 1F), and more unhydrolyzed big particles (20−50 μm) were shown in OPLP residues (Figure 1E).

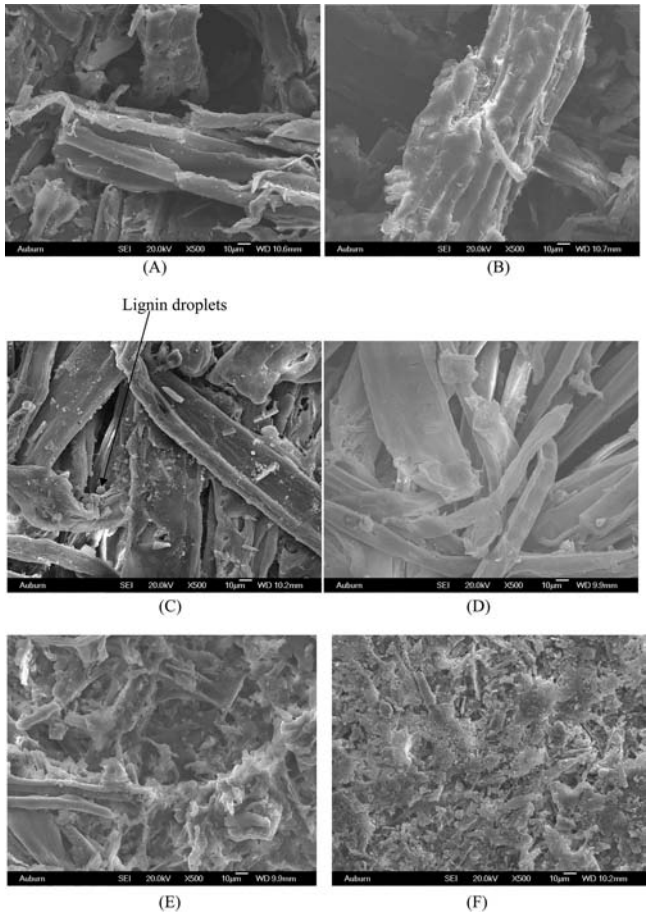

Figure 1. SEM images of the pretreated biomass OPLP (A) and OPSG (B) and enzymatic hydrolyzed OPLP substrate (C) and OPSG (D) after 72 h with 10 FPU per gram of glucan.

Cellulase Adsorption Isotherms on OPLP and OPSG Substrates. Cellulase adsorption isotherms have been used to characterize the cellulase enzymes affinity to the substrates and the enzymatic hydrolysis of substrates.[10,31] The adsorption of cellulase enzymes on OPLP and OPSG substrates was fitted using nonlinear regression (Figure 2). From the adsorption

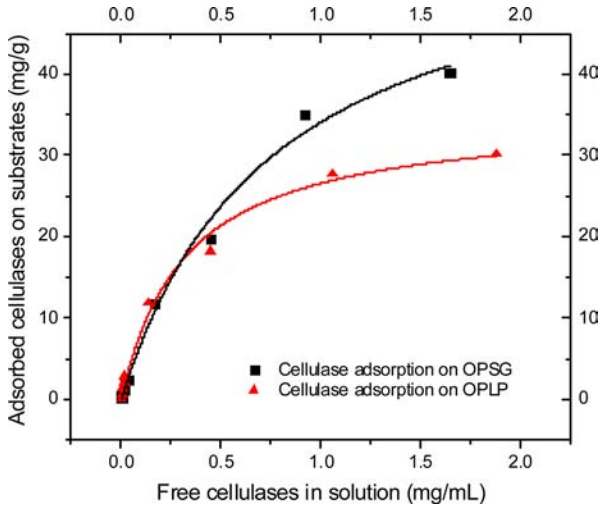

Figure 2. Cellulase adsorption isotherms on OPLP and OPSG substrates at 4 °C.

isotherm, it was apparent that the cellulase enzymes showed higher affinity to OPLP than that to OPSG based on the slope of adsorption curve (distribution coefficient). In the case of cellulase adsorption to OPLP substrate, the Langmuir constant (K) was 3.11 mL/mg, and the maximum amount of adsorbed cellulase enzymes (Γ_{max}) was 35.09 mg/g. In the case of cellulase adsorption to OPSG substrate, the Langmuir constant was 1.31 mL/mg, and the maximum amount of adsorbed cellulase enzymes was 60.19 mg/g. It was very interesting to find that the Langmuir constant of cellulase on OPLP substrate was 3-fold higher than that on OPSG substrate (Table 2),

Table 2. Langmuir Constant from Cellulase Adsorption on OPLP and OPSG Substrates

cellulases	Γ_{max} (mg/g)	K (mL/mg)	R (L/g)
cellulases on OPLP	35.09	3.11	0.11
cellulases on OPSG	60.19	1.31	0.08
celluclast on EPLP[a]	87.69	3.48	0.31
celluclast on SELP[a]	101.05	1.45	0.15

[a]From our previous data[10] EPLP-ethanol pretreated lodgepole pine and SELP-steam exploded lodgepole pine.

which was anticipated to affect the initial hydrolysis rates significantly in the OPLP and OPSG substrates. The distribution coefficient (R) is another useful constant from Langmuir adsorption isotherm that can be used to estimate the relative affinity of cellulase enzymes on substrates.[6,31,39] The distribution coefficient can be expressed as

$$R = \Gamma_{max} \times K$$

Our results indicated that the distribution coefficient was higher for the adsorption of cellulase on softwood OPLP substrate (0.11 L/g) than that on hardwood OPSG substrate (0.08 L/g). These coefficients were in a similar range as those reported in the previous work.[6] It was interesting that the lignin content (19%) in OPLP substrate was much higher than that in OPSG substrate (9.0%), but the relative affinity (based on distribution coefficient) was found to be higher on OPLP substrate. It indicated that lignin probably did not play a significant role in the initial adsorption of cellulase on substrates.

Effects of Residual Xylan and Lignin on Enzymatic Hydrolysis of OPLP and OPSG Substrates. The effects of residual xylan and lignin on enzymatic hydrolysis of OPLP and OPSG substrates using Novozym 22C were first examined (Figure 3). The glucan-to-glucose yield reached 60% on OPLP

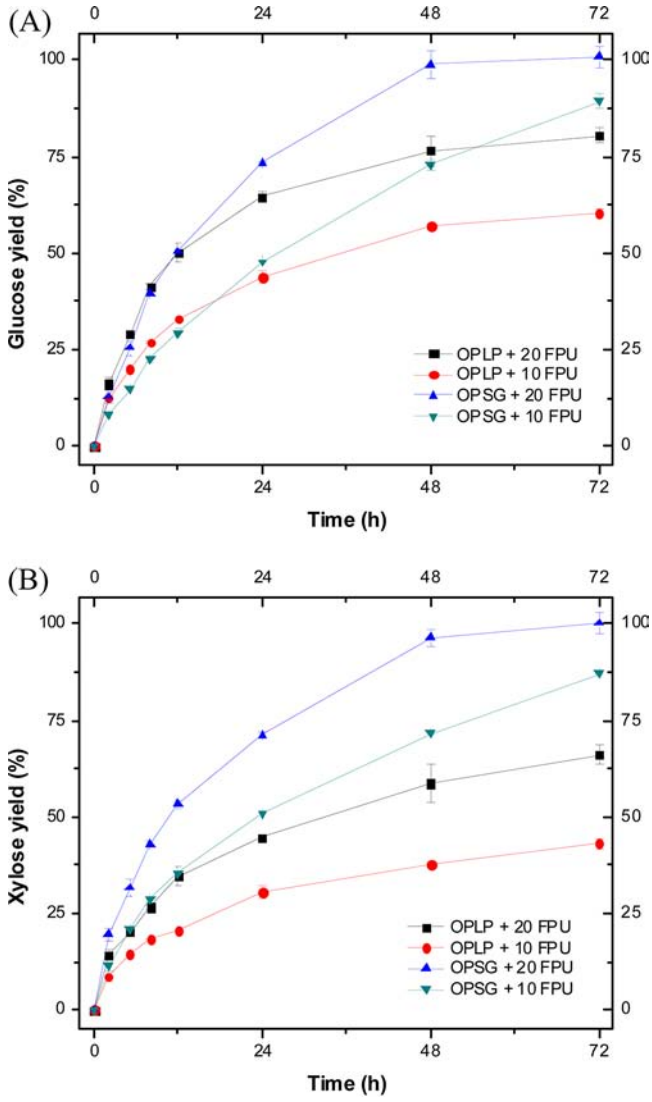

Figure 3. Effect of enzyme loading on the enzymatic hydrolysis of glucan (A) and xylan (B) in OPLP and OPSG substrates.

and 89% on OPSG under the 10 FPU of enzyme loading per gram of glucan. When we increased the enzyme loading to 20 FPU, the glucose yield of OPLP increased significantly to 81%, and the OPSG substrate was almost completely hydrolyzed. The hydrolyzability of OPSG substrate was found to be much better as compared to the OPLP substrate, probably because of higher residual lignin content (19%) in the softwood OPLP substrate (Table 1). However, when we examined the glucan hydrolysis curve, the hydrolysis yield of OPLP was higher than

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that of OPSG in the initial phase (12 h). The initial hydrolysis rate (1.00 or 1.45 g/L/h) of glucan was higher in OPLP than that (0.68 or 1.19 g/L/h) in OPSG under 10 or 20 FPU, although the amount of residual lignin was higher in OPLP. This indicated that residual lignin likely did not play a significant role in the initial hydrolysis of organosolv pretreated biomass. Interestingly, we observed that the residual xylan in OPSG substrate (9.7%) was 3-fold higher than that in OPLP substrate (2.9%), which could limit the initial enzymatic hydrolysis. It suggested that the residual xylan in pretreated substrate affected the initial hydrolysis rate more than the residual lignin. As for the xylose yield of enzymatic hydrolysis of OPLP and OPSG substrates (Figure 3B), the softwood OPLP substrates showed lower xylose yield (43%) than that in hardwood OPSG substrate (87%) after 72 h under 10 FPU of enzyme loading. Increasing the enzyme loading to 20 FPU resulted in higher xylose yields in OPLP substrate (66%) and OPSG substrate (~100%). The higher amount of residual lignin in OPLP substrate could be the main reason for its lower xylose yield. The initial hydrolysis rate of xylan was also lower in OPLP than that in OPSG. In the experiment that follows, we further explored the roles of residual xylan and lignin on the enzymatic hydrolysis of OPLP and OPSG substrates with the supplementation of pectinase and xylanase.

Effects of Supplementing Pectinase and Xylanase on Enzymatic Hydrolysis of OPLP and OPSG Substrates. The effects of supplementing pectinase and xylanase, respectively, on enzymatic hydrolysis of OPLP and OPSG substrates were evaluated with the enzyme loading of 10 FPU (Figure 4). Pectinase supplementation has been found to increase the glucan hydrolysis yields considerably for various pretreated substrates.[8,40] In the case of supplementing pectinase (3.65 mg/g glucan) for enzymatic hydrolysis of OPLP and OPSG, the glucose yield increased from 60 to 68% for OPLP substrate after 72 h and from 89 to 100% for OPSG substrate (Figure 4A). The xylose yield increased from 43 to 48% for OPLP substrate and from 87 to 100% for OPSG substrate (Figure 4B). The pattern for initial hydrolysis rate did not change. The initial glucan hydrolysis rate was still higher in OPLP substrate.

In the case of supplementing xylanase (2.7 mg/g glucan), the glucose yield increased from 60 to 67% for OPLP substrate after 72 h and from 89 to ~100% for OPSG substrate (Figure 5A). Moreover, the initial hydrolysis rates of glucan were improved differently on the OPLP and OPSG substrates. The initial hydrolysis rate of glucan in OPSG substrate was increased from 0.68 to 1.02 g/L/h, and the initial hydrolysis rate of glucan in OPLP substrate remained almost the same at ~1.04 g/L/h. It indicated that xylanase removed xylan effects on the initial hydrolysis rate in hardwood by hydrolyzing the xylan or the xylooligomers present in the solution. The hardwood substrate (OPSG) could be hydrolyzed much quicker than softwood substrate (OPLP) after removing xylan, and the enzymatic hydrolysis of both substrates was still limited due to the presence of lignin.

Effects of Residual Xylan on Ethanol Yields and Ethyl Xyloside Production in the SSF Process. The ethanol yields of OPLP and OPSG substrates in the SSF process have also been compared under enzyme loadings of 10 and 20 FPU (Figure 6A). The same pattern was observed in terms of the initial ethanol production rates and the final ethanol yields. OPSG substrates showed higher ethanol yields (8.8 and 10.3 g/L) than OPLP substrates (7.8 and 9.8 g/L), respectively, after

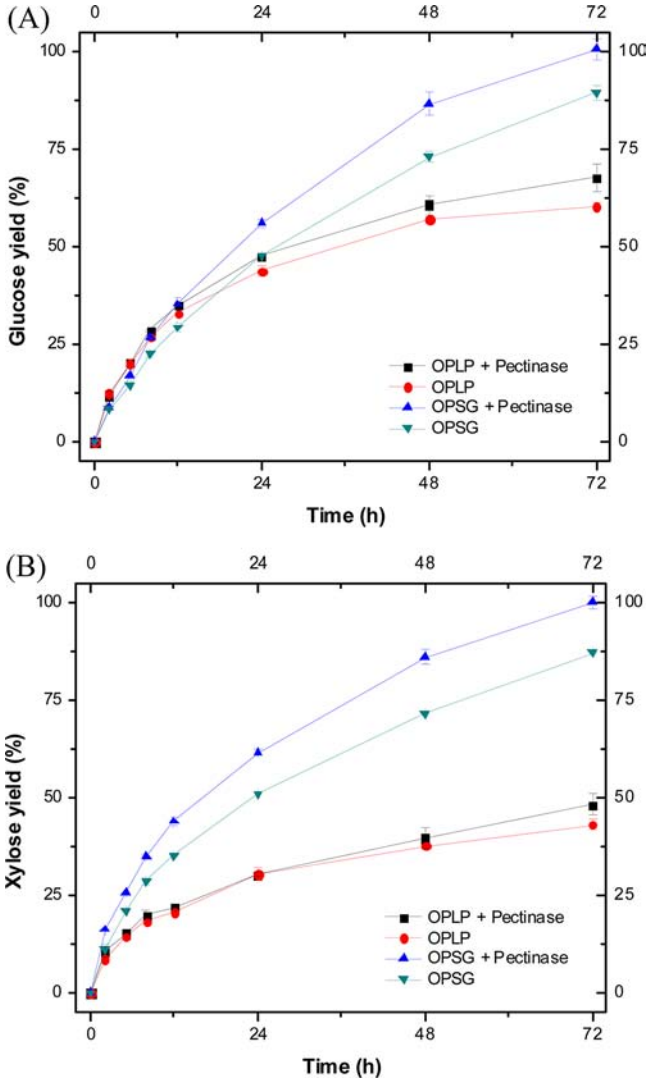

Figure 4. Effect of pectinase on the enzymatic hydrolysis of glucan (A) and xylan (B) in OPLP and OPSG substrates.

96 h for both enzyme loadings. However, the initial ethanol production rates with OPLP substrate were 0.47 and 0.81 g/L/h at 10 and 20 FPU, respectively. The initial ethanol production rates with OPSG substrate were 0.31 and 0.62 g/L/h at 10 and 20 FPU, respectively. It suggested that higher residual xylan content in OPSG substrate resulted in lower initial ethanol production rates, and higher residual lignin content in OPLP substrate resulted in lower final ethanol yields. Meanwhile, we observed that it took about 58 h for the ethanol yield from OPSG in SSF to catch up with that from OPLP substrate (10 FPU). In the enzymatic hydrolysis with the same enzyme loading, it took about 17 h for the glucose yield of OPSG substrate to catch up with that in OPLP substrate. This probably was caused by the different glucan hydrolysis rate under different temperatures (37 vs 45 °C). When enzyme loading was increased to 20 FPU, it took ~20 h for OPSG to reach the same ethanol yield as OPLP and then exceeded the ethanol yield in OPLP until the end of the SSF process. Interestingly, we also found that the xylose concentration increased steadily in the first 48 h and then decreased until 96 h in the SSF process under 20 FPU (Figure 6B). However, the xylose concentration kept stable after 48 h under 10 FPU. This

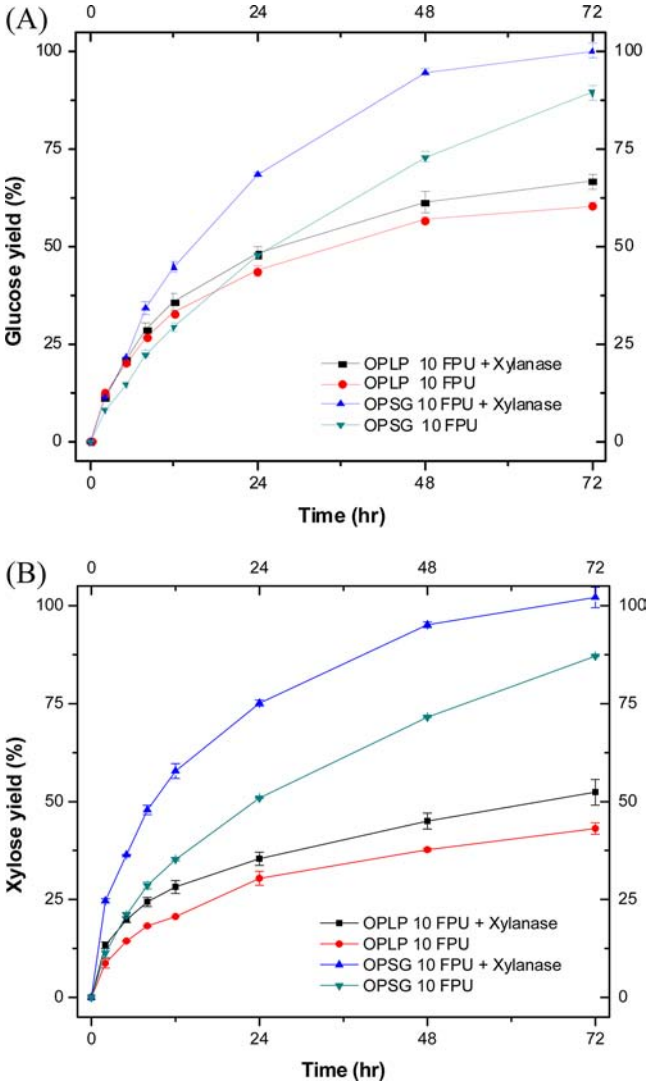

Figure 5. Effect of xylanase on the enzymatic hydrolysis of glucan (A) and xylan (B) in OPLP and OPSG substrates.

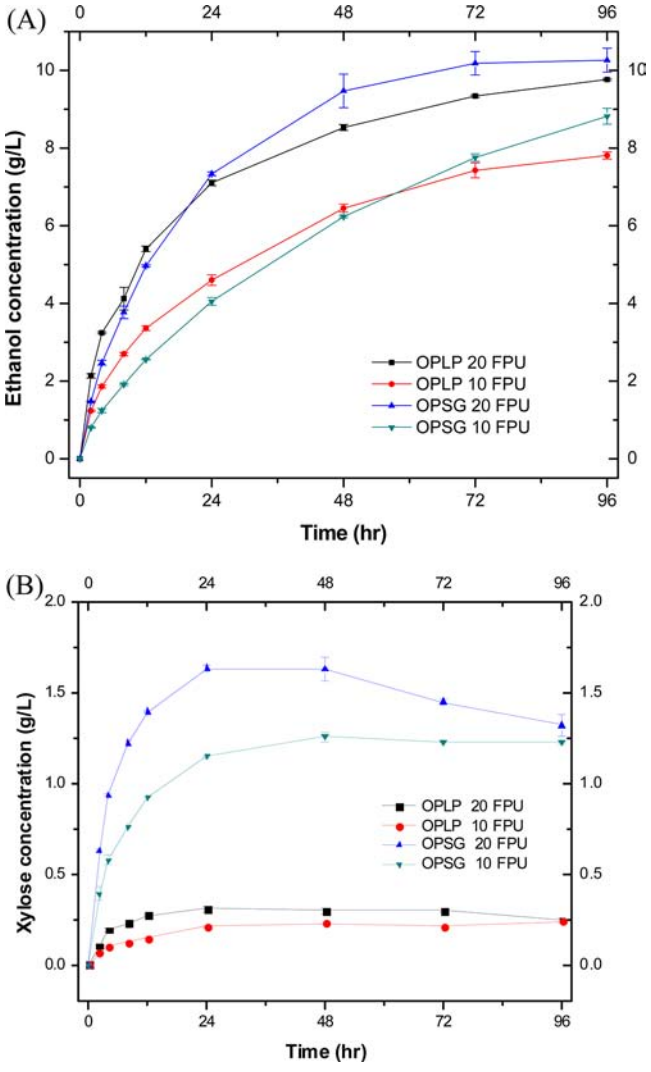

Figure 6. Effects of enzyme loading on ethanol yield (A) and xylose yield (B) in the SSF process.

indicated that released xylose under high enzyme loading could be converted to other products in the SSF process.

HPLC and LC/MS were used to analyze potential products between xylose and ethanol in the SSF process. HPLC chromatograms (Figure 7) indicated that the xylose concentration decreased after 48 h, and another compound (ethyl xyloside) correspondingly increased. This agreed well with the previous report in SSCF of paper sludge.[41] The mass spectra results showed that a new compound with m/z 179.09 ($[M + H]^+$) was produced after 48 h. A composition analysis of this compound in the Masslynx software gave a formula of $C_7H_{14}O_5$ (ppm-3.9), which was exactly the chemical formula of ethyl xyloside. The ethyl xyloside concentration of OPSG in SSF with 20 FPU/g glucan at 96 h was 2.25 ± 0.21 g/L.

Langmuir Adsorption Isotherm and Initial Hydrolysis Rates of Glucan. The Langmuir adsorption isotherm often has been used to characterize affinity of cellulase enzymes for substrates and enzyme accessibility to cellulose.[10,42] Here, we found a good linear correlation between the Langmuir adsorption isotherm (distribution coefficient) and the initial hydrolysis rate of glucan (R^2 = 0.91). Previously, others have tried to build a correlation between the amount of adsorbed enzyme and the initial hydrolysis rate or pore size and the initial hydrolysate rate.[43,44] Factors such as surface area, crystallinity, and lignin content have also been investigated for their potential correlation with the initial hydrolysis rate.[45] However, few studies have been focused on the relationship between distribution coefficient and the initial hydrolysis rate of glucan. R represents a combination of maximum adsorbed enzyme (Γ_{max}) and Langmuir constant (K). This indicates that the initial hydrolysis rate is not only related to the adsorbed enzymes but also their affinity to the substrates.

Effects of the Amount of Residual Xylan and Lignin on Enzymatic Hydrolysis. We also observed that the amount of residual xylan in substrates affected the initial hydrolysis rate, and the amount of lignin affected the final hydrolysis yield because xylan effects could be removed gradually by hydrolysis of xylan. Effects of xylan and lignin removal on enzymatic hydrolysis of lignocellulose have been explored on different substrates in the past decades.[24,46−48] Xylan and lignin have been suggested as two important physical barriers to enzymatic hydrolysis of cellulose and even inhibit cellulase activity.[48] Accessory enzymes (xylanolytic activities) are often required in cellulases mixture to improve the cellulose hydrolysis when xylan content is high.[47] Little research has distinguished the

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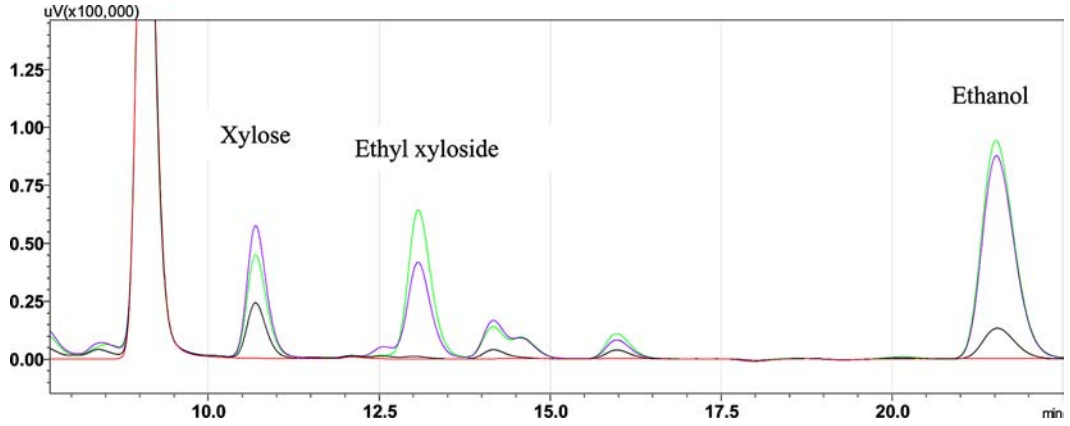

Figure 7. Ethyl xyloside production in the SSF process at 0 (red), 2 (black), 48 (purple), and 96 h (green).

potential different roles of xylan and lignin in limiting enzymatic hydrolysis.[49] On the basis of the cross point of glucose yield (Figure 3A) on softwood and hardwood substrates, we could distinguish two phases in hydrolysis. The initial hydrolysis phase is controlled by residual xylan, and the second hydrolysis phase is governed by residual lignin. The lignin droplets on the plant cell wall do not seem to be affecting the initial hydrolysis rate of cellulose (glucan); probably, the bulk lignin plays a greater role in limiting enzymatic hydrolysis in the second stage. We calculated that approximately 4−5% of residual xylan is the critical point for the phase change. To quantitate the correlation between the initial hydrolysis rate and the xylan content, in the preliminary result, we compared the results from organosolv pretreated lodgepole pine (EPLP), loblolly pine (OPLP), sweetgum (OPSG), and hybrid polar (EPHP) under 20 FPU enzymatic hydrolysis. We found that the linear correlation (Pearson's r = −0.80) between the amount of residual xylan and the initial hydrolysis rate was strong (Figure 8A). The linear correlation between the amount of residual lignin and the final hydrolysis yield was also strong (Pearson's r = −0.78, Figure 8B). These correlations indicated the distinct roles of residual xylan on the initial hydrolysis rate and residual lignin on the final hydrolysis yield. However, the residual lignin and xylan in OPSG and OPLP are probably structurally and compositionally different. The residual lignin in OPSG is syringyl-guaiacyl lignin, and one in OPLP is guaiacyl lignin. In addition, the phenolic hydroxyl groups, carbonyl groups, and methoxyl groups in two types of residual lignin could be different as well. As for residual xylan, hardwood does not contain an arabinose side chain, while softwood does contain a small amount of arabinose as indicated in Table 1. To address this issue, we will further investigate the effect of residual lignin and xylan on hydrolysis in the future work by designing new experiments to pretreat hardwood at various conditions and prepare a number of substrates with different lignin and xylan contents.

Ethyl Xyloside Production in the SSF Process. In the SSF process, residual xylan was also found to affect the fermentation step. The xylose concentration unexpectedly decreased after 48 h. One possible reason is that xylose or xylan reacts with ethanol to produce ethyl xyloside. A similar observation has been reported in a simultaneous saccharification and cofermentation of paper sludge.[41] This is a reversible reaction; a significant amount of xylose or xylan is converted into ethyl xyloside in the high consistency hydrolysis (Figure 7). Because of the consumption of ethanol in this potential enzymatic catalyzed reaction, the formation of ethyl xyloside will considerably reduce the final ethanol yield if released xylose cannot be consumed by the microorganism. Therefore, xylose fermenting microorganism development is critical to improve total ethanol or other alcohol yields from glucan and xylan.

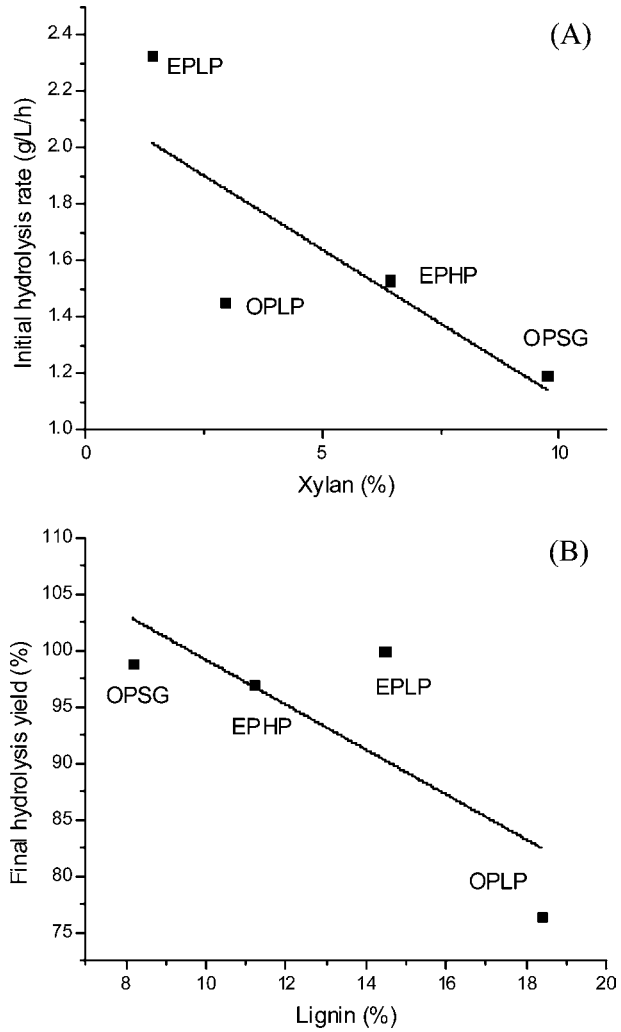

Figure 8. Correlation between the amount of residual xylan and the initial hydrolysis rates (A) and between the amount of residual lignin and the final hydrolysis yields of glucan (B). Organosolv pretreated lodgepole pine (EPLP), loblolly pine (OPLP), sweetgum (OPSG), and hybrid polar (EPHP).

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In summary, enzymatic hydrolysis of organsolv pretreated softwood and hardwood showed totally different hydrolysis curve patterns under the same enzyme loading. The initial hydrolsis rate of OPLP substrate was much faster than that of OPSG substrate, probably due to the high xylan content 9.7% in OPSG substrate. However, the final hydrolysis yield of glucan from OPSG was 29% higher than that from OPLP substrate probably due to the higher lignin content and lower enzyme accessbility (related to pore size, crystallinity, and degree of polymerization) in OPLP substrate. Consequently, the enzymatic hydrolysis of organsolv pretreated substrates could be divided into two phases. The first phase is mainly controlled by residual xylan, and the second phase is dominated by residual lignin. The phase changing point is located in the range of 4−5% xylan. We also found that the initial hydrolsis rate of glucan was correlated very well with the distribution coefficient from the Langmuir adsorption isotherm. In addition, we observed that released xylose in the SSF process can be converted into xyloside by potential enzymatic catalysis of xylose and ethanol. This indicates that xylose must be used in the SSF process or removed before fermentation. Otherwise, it will negatively affect the final ethanol yield. Future work will focus on the correlation of residual xylan and initial hydrolysis rate with various woody biomass and visulization of residual xylan on enzymatic hydrolysis.

■ ASSOCIATED CONTENT

Supporting Information

Correlation between the distribution coefficient (R) and the initial hydrolysis rates of glucan (Figure S1) and mass spectra of ethyl xyloside in the SSF process (Figure S2). 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.

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