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Understanding Lignin Fractionation and Characterization from Engineered Switchgrass Treated by an Aqueous Ionic Liquid

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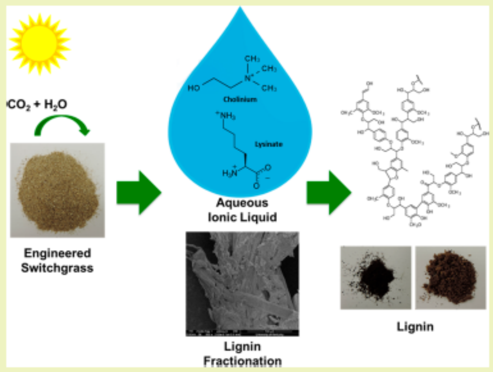
Supporting Information

ABSTRACT: Aqueous ionic liquids (ILs) have received increasing interest because of their high efficacy in fractionating and pretreating lignocellulosic biomass while at the same time mitigating several challenges associated with IL pretreatment such as IL viscosity, gel formation during pretreatment, and the energy consumption and costs associated with IL recycling. This study investigated the fate of lignin, its structural and compositional changes, and the impact of lignin modification on the deconstruction of cell wall compounds during aqueous IL (10% w/w cholinium lysinate) pretreatment of wild-type and engineered switchgrass. The *4CL* genotype resulting from silencing of 4-coumarate:coenzyme A ligase gene (*Pv4CL1*) had a lower lignin content, relatively higher amount of hydroxycinnamates, and higher S/G ratio and appeared to be less recalcitrant to IL pretreatment likely due to the lower degree of lignin branching and more readily lignin solubilization. The results further demonstrated over 80% of lignin dissolution from switchgrass into the liquid fraction under mild conditions while the remaining solids were highly digestible by cellulases. The soluble lignin underwent partial depolymerization to a molecular weight around 500−1000 Da. $^{1}H-^{13}C$ HSQC NMR results demonstrated that the variations in lignin compositions led to different modes of lignin dissolution and depolymerization during pretreatment of engineered switchgrass. These results provide insights into the impact of lignin manipulation on biomass fractionation and lignin depolymerization and lead to possible ways toward developing a more selective and efficient lignin valorization process based on aqueous IL pretreatment technology.



KEYWORDS: *Lignin, Pretreatment, Ionic liquid, Engineered switchgrass*

INTRODUCTION

Despite the recent fluctuation of oil and chemical markets, shifting society's dependence on petroleum-based fuels and chemicals to biomass derived products is important not only to address environmental challenges but also to increase the robustness of our energy security and economic stability.[1,2] Biofuels derived from lignocellulosic biomass are well-suited to address the challenges associated with petroleum liquid fuels. However, truly cost-effective production of biofuels has not been attained with existing technologies and processes.[3,4] Consequently, development of biomass feedstocks with desirable traits for cost-effective conversion is one of the focus areas in biofuels research.[5−7]

Genetic modification of plants has been investigated to enhance the crop yield, drought and pest resistance, and the ease of conversion to biofuels and bioproducts.[8−10] Particularly, manipulations of lignin pathways have drawn extensive attention. Lignin usually consists of three different phenyl-propane units, i.e., *p*-coumaryl, coniferyl, and sinapyl, and plays important roles in plant structural support and resistance against microbial and oxidative stresses.[11] In principle, reduction of lignin content or modification of lignin

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composition, lignin deposition, and lignin–carbohydrates linkage could all possibly lead to reduced feedstock recalcitrance for biochemical conversion.[12,13] Switchgrass (*Panicum virgatum*) is a promising bioenergy crop in North America with high productivity and low energy and nutrient requirements.[14,15] Previous studies have demonstrated that genetic modification of lignin in switchgrass can lead to a reduction of recalcitrance and improvement of bioconversion efficiency.[8,16]

To overcome the recalcitrant nature of lignocellulosic biomass, a pretreatment step is commonly needed prior to the downstream saccharification and fermentation processes in a lignocellulose-based biorefinery. Compared with the other pretreatment approaches (e.g., dilute acid, ammonia, steam explosion, sodium hydroxide), ionic liquid (IL) pretreatment has received increasing interest because of certain ILs' high efficacy in fractionating and pretreating lignocellulosic biomass. Imidazolium-based ILs, such as 1-ethyl-3-methylimidazolium acetate ([C_2C_1Im][OAc]), 1-butyl-3-methylimidazolium chloride ([C_4C_1Im][Cl]), and 1-ethyl-3-methylimidazolium chloride ([C_2C_1Im][Cl]), have been evaluated and proven highly effective in pretreatment for a variety of biomass feedstocks, including corn stover, switchgrass, poplar and pine wood, and municipal solid waste.[17−22]

Some biobased ILs containing ions made of naturally occurring bases and acids from protein, hemicellulose, and lignin have recently emerged and been estimated to be more cost-effective when compared with imidazolium-based ILs.[23−26] Cholinium lysinate, a bioderived and biocompatible IL, has been demonstrated to be effective for biomass pretreatment owing to its efficacy in solubilizing lignin.[23,27,28] The lignin streams derived during pure cholinium-lysinate-pretreated switchgrass, hardwood, and softwood were characterized in a recent study.[29] The recent study also demonstrated that an aqueous IL ([C_2C_1Im][OAc]) can be as effective as pure IL in pretreating plant biomass. Our recent work has demonstrated that aqueous IL pretreatments by 10% cholinium lysinate were as effective as dilute acid and soaking in aqueous ammonia pretreatment in terms of improving enzymatic digestibility of pretreatment switchgrass varieties.[30] Using IL–water mixtures as pretreatment agents could reduce viscosity, eliminate gel formation during pretreatment, and significantly reduce the energy requirements and costs associated with IL recycling.[31] Furthermore, the biocompatibility of certain ILs provides a potential way to upgrade lignin by biological catalysts in an aqueous IL solution.

Pretreatment is a crucial step for making biomass feedstocks more amenable to biological conversion by unlocking polysaccharides for enzymatic hydrolysis and subsequent fermentation. Nevertheless, as suggested by techno-economic analyses, the success of a lignocellulose-based biorefinery largely relies on the utilization of lignin to generate value-added products, such as fuels and chemicals. The fate of lignin and its structural/compositional changes during pretreatment have recently received increasing attention; however, the effect of genetic modification on the fractionation and depolymerization of lignin from engineered plants is not fully understood. This study aims to fractionate and characterize the lignin streams from wild-type and engineered switchgrass species using an aqueous IL. The effects of lignin manipulation on the composition and enzymatic digestibility after pretreatment and enzymatic hydrolysis were investigated and compared with lignin in untreated switchgrass. The molecular weight of the lignin fractions recovered from the liquid and solids streams after pretreatment and enzymatic hydrolysis was determined by gel permeation chromatography (GPC), while the cleavage of interunit lignin linkages was evaluated by $^{1}H-^{13}C$ HSQC NMR and compared with results from lignin in untreated switchgrass. Results from this study provide a better understanding of how lignin engineering of switchgrass influences lignin fractionation and upgrading during conversion processes based on aqueous IL pretreatment technology.

EXPERIMENTAL SECTION

Materials. Wild-type and genetically engineered switchgrass (*Panicum virgatum*) were grown during the year 2015 in a greenhouse at Virginia Polytechnic Institute and State University (Blacksburg, VA). Transgenic RNAi-*4CL* switchgrass plants with silenced 4-coumarate:coenzyme A ligase gene (*Pv4CL1*; denoted as *4CL* thereafter) have reduced lignin content of the cell wall biomass.[9] Transgenic plants with overexpression of an *Arabidopsis* transcription factor AtLOV1 (denoted as *AtLOV1* thereafter) have erected leaf phenotype and increased lignin content.[10] The transgenic plants along with the wild-type controls were clonal propagated and maintained in a greenhouse with temperatures set at 22/28 °C, night/day with a 12–14 h light regime. The plants were grown in Miracle-Gro Potting Mix (Miracle-Gro Lawn Products, Inc., Marysville, OH) in 11 L pots and watered about twice a week. Plant samples (the stems between the second and third internodes above the ground) were collected at the R3 stage.[32] Collected samples were dried at 60 °C for 3 days and then ground by a Wiley mill (Model 4) into a 1 mm size fraction and sieved by a Ro-Tap testing sieve shaker (Model B, W. S. Tyler Industrial Group, Mentor, OH). Cholinium lysinate (>95% purity) was synthesized following a method described elsewhere.[23] The commercial enzymes including cellulase (Cellic CTec2) and hemicellulase (Cellic HTec2) were gifts from Novozymes, North America (Franklinton, NC).

Compositional Analysis. Structural carbohydrates (i.e., glucan and xylan), and acid-soluble and acid-insoluble lignin were determined according to an NREL laboratory analytical procedure.[33] Before compositional analysis, extractable materials in the switchgrass sample were removed by water and ethanol using a Dionex Accelerated Solvent Extractor (ASE) 350 system (Dionex, Sunnyvale, CA). Briefly, an air-dried and extractive-free sample was mixed with 72% (w/w) sulfuric acid at a ratio of 1:10. The mixture was incubated at 30 ± 3 °C for 60 ± 5 min, and stirred every 10 min. After 60 min of hydrolysis, deionized (DI) water was added to the mixture to reach an acid concentration of 4% (w/w), after which the mixture was autoclaved at 121 °C for 1 h. After two-stage acid hydrolysis, acid-soluble lignin was measured using a spectrophotometer at 205 nm;[34] acid-insoluble lignin was obtained by subtracting the ash content from the weight of solid residues. Monomeric sugars (glucose and xylose) were determined by HPLC following a method shown in the Analytical Methods section.

Aqueous Ionic Liquid Pretreatment. Aqueous cholinium lysinate was used for all the pretreatment experiments. Cholinium lysinate (10% w/w) and switchgrass were mixed at a ratio of 9:1 (w/w) in a 20 mL stainless steel (SS316) reactor, capped, and then heated at 140 ± 2 °C in a stirred oil bath for 1 h (pretreatment reactions were conducted under air). After heating, the reactor was removed from the oil bath and quenched in iced water. The mixture was transferred from the reactor to a 50 mL centrifuge tube and centrifuged at 4000 rpm for 10 min to separate the solids and liquid. The solids were washed three times with 150 mL of hot DI water to remove the excess cholinium lysinate. The washed solids were used for further enzymatic hydrolysis. The liquid was titrated using 6 M HCl until the pH reached 1–2, and then stored at 4 °C for 7 days to precipitate lignin. After precipitation, the recovered lignin was washed and freeze-dried. Monomeric sugars in the liquid phase were determined according to NREL laboratory analytical procedure.[33] Briefly, the residual liquid was adjusted to an acid concentration of 4% (w/w) sulfuric acid and was then autoclaved

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Table 1. Compositional Analysis of Untreated and Aqueous IL-Pretreated SwitchgrassCompositional Analysis of Untreated and Aqueous IL-Pretreated Switchgrass[a]

	Raw switchgrass			Pretreated switchgrass		
	WT	*4CL*	*AtLOV1*	WT-IL	*4CL*-IL	*AtLOV1*-IL
Extractives, %	14.6 ± 0.3	18.0 ± 0.2	13.0 ± 0.1	ND	ND	ND
Glucan, %	34.5 ± 0.1	33.5 ± 0.1	36.0 ± 0.4	49.1 ± 0.9	50.3 ± 0.8	49.4 ± 1.8
Xylan, %	21.9 ± 2.4	22.7 ± 0.2	21.3 ± 0.3	22.1 ± 0.4	22.8 ± 0.2	20.6 ± 0.8
Lignin, %	18.6 ± 0.0	16.3 ± 0.3	19.1 ± 0.3	12.5 ± 1.2	8.6 ± 1.2	12.6 ± 1.2
ASL, %	2.3 ± 0.0	3.1 ± 0.0	2.0 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.3 ± 0.1
AIL, %	16.3 ± 0.0	13.1 ± 0.3	17.1 ± 0.3	11.3 ± 1.2	7.4 ± 1.2	11.3 ± 1.2
Ash, %	4.5 ± 0.1	5.3 ± 0.1	4.3 ± 0.1	ND	ND	ND
Solid recovery, %	N/A	N/A	N/A	44.0 ± 2.2	42.7 ± 3.7	51.0 ± 3.0

[a]Based on dry biomass; ASL = acid-soluble lignin; AIL = acid-insoluble lignin; ND = not determined; N/A = not applicable; the composition data were adopted from a previous article. (Part of the composition data was adapted with permission from ref 30. Copyright 2017 Springer.)

at 121 °C for 1 h. Glucose and xylose in the residual liquid were determined by HPLC.

Enzymatic Hydrolysis. Enzymatic hydrolysis of the untreated and pretreated switchgrass followed the NREL laboratory analytical procedure.[35] After pretreatment, the recovered solids were mixed with 50 mM citrate buffer, 0.01 g/L sodium azide, and enzymes (CTec2/HTec2, 9:1, v/v). Two enzyme loadings, 20 mg and 5.25 mg of enzyme protein/g of starting biomass, were tested. The saccharification was performed at 50 °C for 72 h in an orbital shaker (Thermo Forma 435, Thermo Fisher Scientific Inc., Waltham, MA). After hydrolysis, monomeric sugar concentration was determined by HPLC. The residual solids were collected, washed, and freeze-dried for compositional analysis and lignin characterization.

Mass Balance. Mass balances (sugars and lignin) were closed on the liquid and solid streams of fractionated switchgrass after aqueous IL pretreatment and enzymatic hydrolysis. Glucan, xylan, and lignin for mass balances were determined according to an NREL laboratory analytical procedure.[33]

Analytical Methods. The major monomeric sugars (glucose, xylose, and arabinose) in the liquid streams from compositional analysis and enzymatic saccharification were measured by a Dionex HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA, US) instrument equipped with a refractive index detector and Aminex HPX-87H column and guard column assembly, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.4 mL/min and a column temperature of 50 °C.

Lignin Characterization. *Cellulolytic Enzyme Lignin (CEL) Isolation.* The untreated switchgrass, including wild-type (WT) and two transgenic plants (*4CL* and *AtLOV1*), and their precipitated lignin-enriched solids from ionic liquid pretreatment were thoroughly extracted with a mixture of toluene−ethanol (2/1, v/v) in a Soxhlet extractor for 24 h. CEL was isolated from the extracted switchgrass and the lignin-enriched solids according to a published literature procedure (SI, Figure S1).[36,37] In brief, the extractive-free samples were loaded into a 50 mL ZrO_2 grinding jar (including 10 × 10 ball bearings) in a Retsch Ball mill PM 100. The biomass was then ball milled at 580 rpm for 5 min, followed by a 5 min pause; this procedure was repeated for 1.5 h in total. The milled fine cell wall powder was then subjected to enzymatic hydrolysis with a mixture of Cellic CTec2 and HTec2 in acetic acid/sodium acetate buffer (pH 4.8, 50 °C) under continuous agitation at 200 rpm for 48 h. The residue was isolated by centrifugation and was hydrolyzed once more with freshly added enzymes. The residue obtained was rich in lignin and was washed with DI water, centrifuged, and freeze-dried. The lignin-enriched residue was extracted with dioxane−water (96% v/v, 10.0 mL/g biomass) for 24 h. The extracted mixture was centrifuged, and the supernatant was collected. Dioxane extraction was repeated once by adding fresh dioxane−water. The extracts were combined, rotoevaporated to reduce the volume at a temperature of less than 45 °C, and freeze-dried. The obtained lignin samples, designated as CEL, were used for further analysis.

Gel Permeation Chromatographic (GPC) Analysis. The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of lignin were measured by GPC after acetylation as previously described.[38] Briefly, lignin derivatization was conducted on a basis of ~3 mg of lignin in 1 mL of pyridine/acetic anhydride (1:1, v/v) in the dark with magnetic stirring at room temperature for 24 h. The solvent/reagents were removed by coevaporation at 45 °C with ethanol, several times, using a rotatory evaporator until dry. The resulting acetylated lignin was dissolved in tetrahydrofuran (THF), and the solution was filtered through 0.45 μm membrane filter before GPC analysis. Size-exclusion separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc., Santa Clara, CA) equipped with Waters Styragel columns (HR1, HR4, and HR5; Waters Corporation, Milford, MA). A UV detector (270 nm) was used for detection. THF was used as the mobile phase at a flow rate of 1.0 mL/min. Polystyrene narrow standards were used for establishing the calibration curve.

NMR Spectroscopic Analysis. Nuclear magnetic resonance (NMR) spectra of isolated lignin samples were acquired in a Bruker Avance III 400-MHz spectrometer, and spectral processing was carried out using Bruker Topspin 3.5 (Mac) software. A standard Bruker heteronuclear single quantum coherence (HSQC) pulse sequence (hsqcetgpspsi2) was used on a BBFO probe with the following acquisition parameters: spectra width 10 ppm in F2 (^{1}H) dimension with 2048 time of domain (acquisition time 256.1 ms), 210 ppm in F1 (^{13}C) dimension with 256 time of domain (acquisition time 6.1 ms), a 1.5 s delay, a $^1J_{C-H}$ of 145 Hz, and 32 scans. The central DMSO solvent peak (δ_C/δ_H at 39.5/2.49) was used for chemical shifts calibration. The relative abundance of lignin compositional subunits and interunit linkages was estimated using volume integration of contours in HSQC spectra.[36,38,39] For monolignol compositions of S, G, H, *p*-coumarate (*p*CA), and ferulate (FA) measurements, the $S_{2/6}$, G_2, $H_{2/6}$, $pCA_{2/6}$, and FA_2 contours were used with G_2 and FA_2 integrals doubled. The Cα signals were used for contour integration for the estimation of interunit linkages.

Statistical Analysis. All experiments were conducted in triplicate, and the data were presented as means with standard deviations. The statistical analysis was performed by using SAS 9.4 (SAS Institute, Cary, NC) software, with a significance level of $P < 0.05$ for all the data obtained from experiments.

RESULTS AND DISCUSSION

Aqueous IL Pretreatment of Wild-Type and Engineered Switchgrass. Due to the genetic variations in the wild-type and engineered switchgrass, *4CL* and *AtLOV1*, differences in the compositions of the three tested switchgrass lines are expected, both before and after aqueous IL pretreatment. According to the results shown in Table 1, similar levels of glucan and xylan content were observed in the three lines of switchgrass. For the WT and *4CL* switchgrass, the glucan contents were around 34%, only slightly lower than the

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Table 2. Relative Abundance of Lignin Subunits (S/G/H), Hydroxycinnamates Quantified on the Basis of $^{1}H-^{13}C$ HSQC NMR Spectra

		Raw switchgrass			Pretreated switchgrass		
		WT	*4CL*	*AtLOV1*	WT-IL	*4CL*-IL	*AtLOV1*-IL
S/G/H abundance	S%	29.6	38.3	34.8	39.3	58.7	45.2
	G%	66.0	52.8	63.5	59.4	41.3	53.5
	H%	4.3	8.9	1.7	1.3	0.0	1.2
	S/G	0.45	0.73	0.55	0.66	1.42	0.85
Hydroxycinnamates (% of Ar)	*p*CA%	59.7	85.3	57.4	21.3	16.8	24.7
	FA%	17.0	31.0	14.1	14.8	0.0	13.6
	*p*CA/FA	3.51	2.75	4.06	1.44	N/A	1.83

glucan content of *AtLOV1* switchgrass. The xylan contents in the three types of switchgrass were around 21−22%. However, the lignin content in *4CL* switchgrass was significantly lower ($P < 0.05$) than that of WT and *AtLOV1* switchgrass. In contrast, there was no significant difference in lignin content between WT switchgrass and *AtLOV1* switchgrass. These results align well with the corresponding gene modifications to the switchgrass plants, with reduced lignin content for *4CL* as a result of silencing the 4-coumarate:coenzyme A ligase gene,[9] while the overexpression of an *Arabidopsis* transcription factor *AtLOV1* leads to a slight increase in lignin content as compared with WT switchgrass.[10]

Genetic modification to lignin pathways not only causes differences in the composition of untreated switchgrass but could also impact the dissolution and depolymerization of biomass components during a pretreatment process.[40] In this study, aqueous IL, 10% (w/w) cholinium lysinate, was used to pretreat the switchgrass samples. After pretreatment, the composition of the recovered solids was determined (see Table 1). The solid recovery for the three types of switchgrass was different. After aqueous IL pretreatment, approximately 43−44% of the raw switchgrass was recovered for *4CL* and WT switchgrass; however, the solid recovery was 51% for *AtLOV1* switchgrass. The high weight loss for *4CL* switchgrass is a combined effect of solubilization of lignin and xylan, and removal of extractives. Despite a reduction in lignin content for all of the three types of switchgrass, more lignin was removed from *4CL* switchgrass as compared to WT and *AtLOV1* switchgrass. These results demonstrate that aqueous cholinium lysinate is a good solvent for lignin and that *4CL* switchgrass is more susceptible to aqueous IL pretreatment. The reduced lignin content and altered S/G ratio plausibly lead to decreased recalcitrance of the *4CL* switchgrass, thus affecting the lignin dissolution during aqueous IL pretreatment.

Cholinium lysinate is a bioderived IL and has been shown highly effective for lignin solubilization, due to the greater hydrogen bond basicity for the IL with [Lys]-anions as compared with acetate ILs.[23] The solvent property of an IL is the key for biomass deconstruction and lignin depolymerization. Recent studies have demonstrated that, in some cases, an aqueous IL system can be as effective as pure IL for fractionating plant materials and extracting lignin.[31] It was reported that diluting IL [C_2C_1Im][OAc] with water decreases the hydrogen bond basicity (β value) of the IL−water mixture as compared to the β value of pure IL.[31] Nevertheless, within a range of IL concentrations, IL−water mixtures can be as effective as pure IL for solubilizing cellulose microfibrils into individual chains as suggested by both experimental and molecular dynamics simulations.[38,41] Results from this study demonstrate the effectiveness of 10% w/w cholinium lysinate in solubilizing lignin, corroborating previous reports using IL of its pure form or aqueous solution as pretreatment media.

Table 2 lists the relative abundance of lignin subunits (S, G, and H) and hydroxycinnamates (*p*CA and FA) based on 2D NMR results. In general, *4CL* switchgrass had a higher S/G ratio (0.73) than WT and *AtLOV1* switchgrass (0.45 and 0.55, respectively). It is generally believed that biomass with a higher S/G ratio is more digestible by cellulolytic enzymes.[42,43] After pretreatment, the S/G ratios of pretreated switchgrass all increased, especially for *4CL* switchgrass for which the S/G ratio increased from 0.73 to 1.42. The increased S/G ratio can be explained by the preferential removal of G lignin as reported in a previous study which showed that certain ILs can selectively solubilize G lignin.[44,45] Ferulate (FA) and *p*-coumarate (*p*CA) are the hydroxycinnamates particularly with a high amount in untreated switchgrass lignin, which are readily cleaved off from the bulky lignin due to the readily cleavable ester linkages between hydroxycinnamates and bulky lignin.[46,47] Our results consistently showed that both *p*CA and FA units were significantly removed during aqueous IL pretreatment, especially for *4CL* switchgrass for which FA can be barely detected in the pretreated biomass (Table 2). Therefore, the higher content of hydroxycinnamates in untreated *4CL* compared with WT is partially contributing to reduced biomass recalcitrance for pretreatment and saccharification. In addition, previous studies have suggested that untreated switchgrass with lower *p*CA/FA ratio is more digestible.[48] The overall lower *p*CA/FA ratio in untreated *4CL* could be a plausible reason for the reduced recalcitrance of *4CL* switchgrass.

Saccharification of Aqueous IL-Pretreated Switchgrass. Pretreatment with certain ILs has been shown as an efficient way to overcome the recalcitrance of biomass, unlock the lignin−carbohydrate complexes, and improve accessibility of cellulose/hemicellulose to hydrolyzing enzymes.[17,49] However, the effect of an aqueous IL pretreatment on the enzymatic digestibility of engineered switchgrass has not been reported. Figure 1 shows the glucose and xylose yield under different enzyme loadings (low and high enzyme loadings of 5.25 mg of protein/g and 20 mg of protein/g of starting biomass, respectively). Aqueous IL pretreatment greatly improved the sugar yield as compared to untreated switchgrass. At both low and high enzyme loadings, pretreated *4CL* switchgrass gave the highest glucose and xylose yields among the three tested feedstocks. After 72 h of enzymatic hydrolysis, a glucose yield of 98.8% and xylose yield of 63.2% were observed for pretreated *4CL* switchgrass under high enzyme loading. Sugar yields of pretreated WT and *AtLOV1* switchgrass were significantly lower than that of *4CL* switchgrass. The *AtLOV1* switchgrass with upregulated lignin resulted in lower sugar yield than *4CL* switchgrass; however, there was no significant

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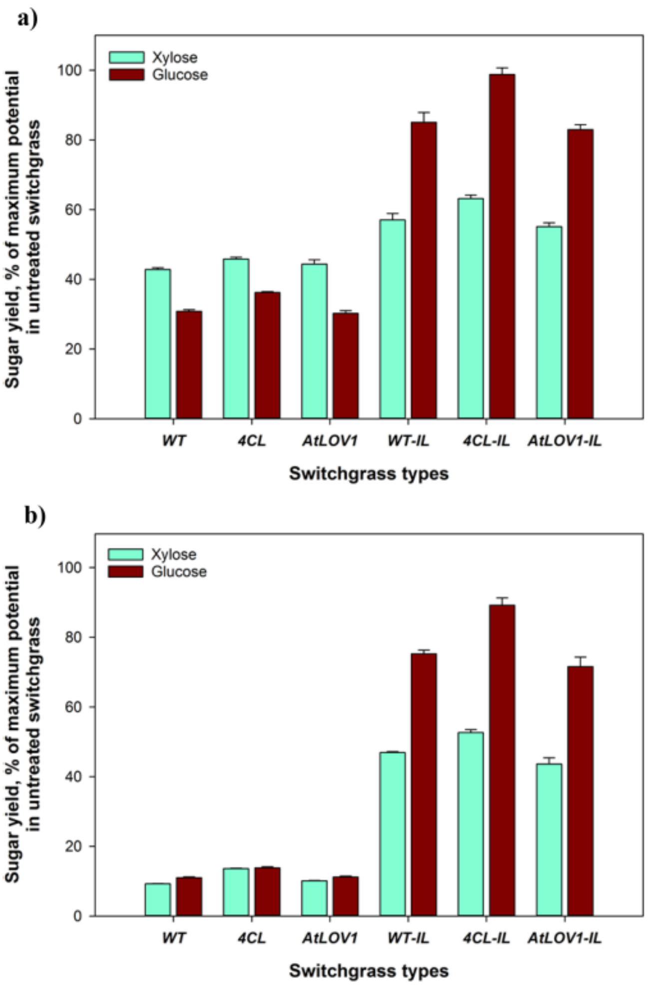

Figure 1. Glucose and xylose yield during enzymatic hydrolysis of untreated and aqueous IL-pretreated switchgrass under (a) high and (b) low enzyme loading. (Adapted with permission from ref 30. Copyright 2017 Springer.)

difference in sugar yield between *AtLOV1* switchgrass and WT switchgrass.

Results indicate that *4CL* switchgrass was more digestible as compared to WT and *AtLOV1* switchgrass, suggesting that downregulation of lignin content, its associated increase in hydroxycinnamates content, and slight increase of the S/G ratio in plants can together lead to reduced cell wall recalcitrance, thereby increasing the sugar yield. This observation is in agreement with previous studies on switchgrass, poplar, *Arabidopsis*, and other biomass feedstocks.[7,40,42,43] Although high enzyme loading led to ∼10−15% higher glucose and xylose yields, from an economics perspective the cost of commercial enzymes is still a significant contributor to biofuel production cost.[50] Thus, a low enzyme loading (5.25 mg of protein/g of starting biomass) was used in this study to establish a baseline for the aqueous IL fractionation process.

Fractionation of Lignin Streams and Mass Balances. In order to elucidate the mass flow of major biomass components during aqueous IL pretreatment and subsequent enzymatic hydrolysis, mass balances for glucan, xylan, and lignin for different types of switchgrass were tracked, the results being illustrated in Figure 2. As a general observation, starting with 100 g (2 g of biomass was used in the actual pretreatment, and 100 g is an extrapolation of the results) of raw WT switchgrass, a large portion of the lignin was solubilized in the liquid stream during aqueous IL pretreatment. However, only a small portion of glucan and xylan was extracted in the pretreatment liquid, the majority being carried by the pretreated solids and hydrolyzed to monomeric sugars during the enzymatic hydrolysis step. Figure 2a demonstrates that a total amount of 28.9 g of glucose and 11.7 g of xylose were recovered from liquid streams for WT switchgrass, which correspond to overall recoveries of 77.8% and 65.7% for glucose and xylose, respectively. For *4CL* switchgrass, the overall recoveries from liquid streams were 92.5% and 71.1% for glucose and xylose, respectively (Figure 2b). The lowest overall glucose (73.6%) and xylose (61.0%) recoveries from liquid streams were observed for *AtLOV1* switchgrass, mainly due to the overall lower sugar yields during enzymatic hydrolysis compared with *4CL* switchgrass. In other words, the overall glucose yield from liquid streams for *4CL* switchgrass was 15% higher than that of WT switchgrass and 19% higher than that of *AtLOV1* switchgrass. The overall xylose yield from liquid streams for *4CL* switchgrass was around 5% and 10% higher than WT and *AtLOV1* switchgrass, respectively. It can be concluded that *4CL* switchgrass is relatively more digestible due to the decreased biomass recalcitrance caused by the low lignin content and increased S/G ratio.

Clearly, the aqueous IL (cholinium lysinate) exhibited great lignin solubilization capabilities, based on the compositional analysis. The fractionation of lignin into liquid and solids streams for different types of switchgrass is illustrated in Figure S2. Approximately 60−65% of lignin was solubilized in the pretreatment liquid stream for WT and *AtLOV1* switchgrass. However, over 80% of lignin was fractionated into the liquid stream for *4CL* switchgrass. In addition to the lower lignin content and higher S/G ratio, fewer branching lignin linkages were observed in *4CL* switchgrass when compared with WT and *AtLOV1* switchgrass from 2D NMR results. Furthermore, the low *p*CA/FA ratio in *4CL* switchgrass can be another contributor to the reduced recalcitrance. Although high contents of hydroxycinnamates may contribute to the lignin solubilization during pretreatment, a recent study suggested that the monolignol ferulate bearing ester bonds are readily cleaved during alkaline pretreatment,[51] high contents of *p*CA and FA in pretreated biomass have been found to be unfavorable to the digestibility of polysaccharides.[52] Therefore, the low lignin content, high amount of *p*CA and FA, and high S/G ratio in untreated *4CL* switchgrass, in combination, make it more susceptible to aqueous IL pretreatment than the WT and *AtLOV1* switchgrass.

Characterization of Lignin Streams. *Gel Permeation Chromatographic Analysis.* To better understand the lignin depolymerization during aqueous IL pretreatment, GPC analysis was conducted to determine the molecular weight (MW) distribution of lignin in both untreated and pretreated (residual solids) switchgrass using a set of polystyrene molecules as standards. GPC chromatograms show that the lignin MWs were significantly reduced after IL pretreatment (Figure 3). As shown in Figure 4, the untreated switchgrass mainly contains relatively high-molecular-weight lignins. For instance, the lignin in *AtLOV1* switchgrass has a number-average molecular weight (M_n) of 5862 g/mol and a weight-average molecular weight (M_w) of 15 236 g/mol, values which are slightly higher than those of WT switchgrass (with M_n and M_w equal to 5630 and 14 458 g/mol, respectively). In

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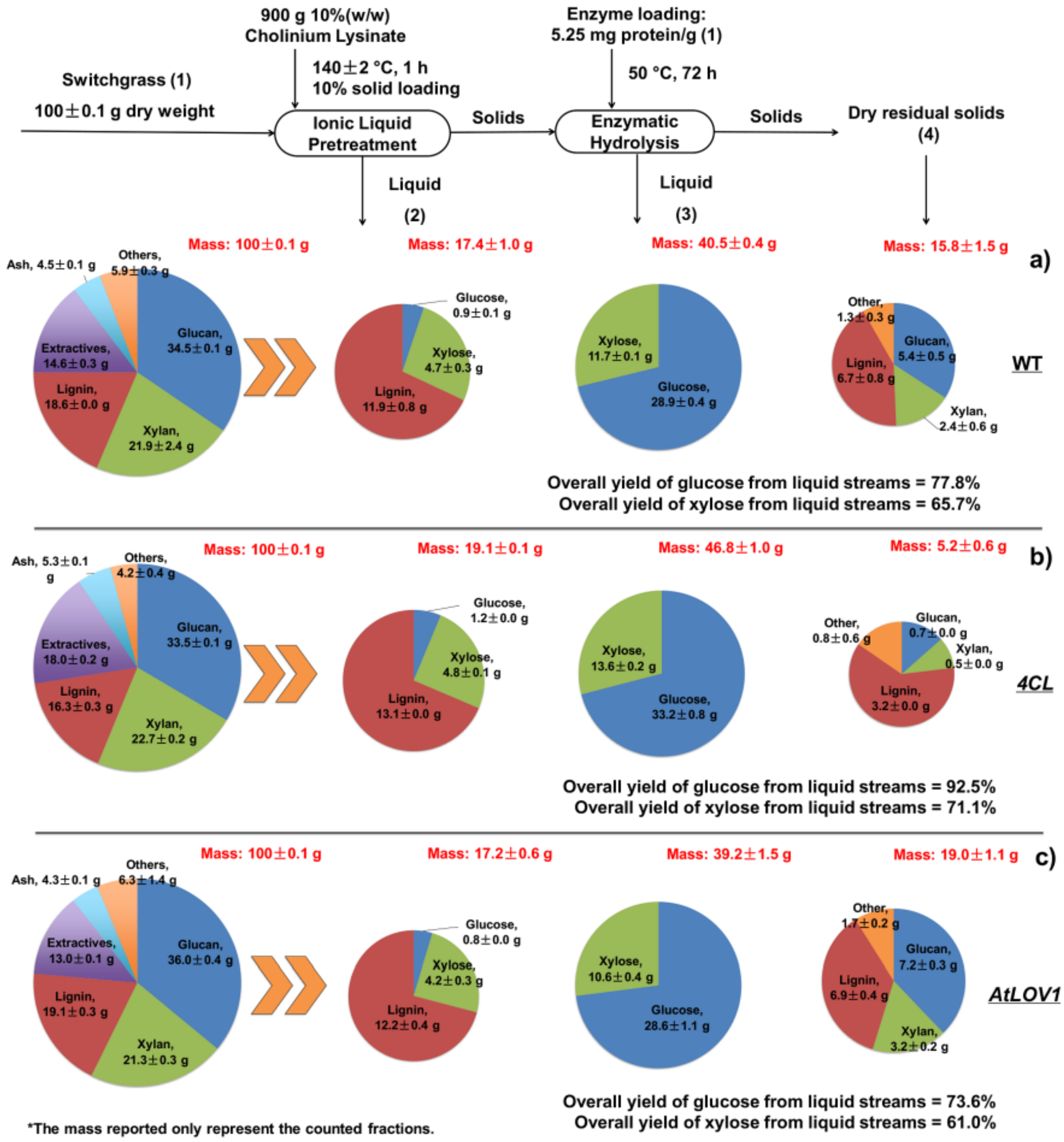

Figure 2. Mass balances for aqueous IL pretreatment and saccharification of different switchgrass genotypes: (a) WT, (b) *4CL*, and (c) *AtLOV1* (Note: 2 g of raw biomass was used in the actual pretreatment in this study, and 100 g of raw biomass in this figure is an extrapolation of the results). (Partially adapted with permission from ref 30. Copyright 2017 Springer.)

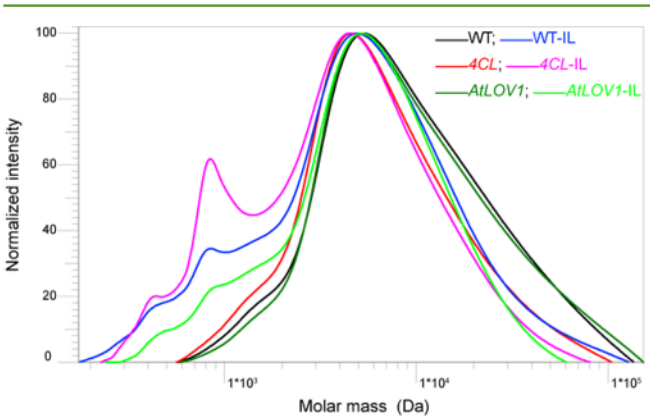

Figure 3. GPC chromatograms of lignin isolated from untreated and aqueous IL-pretreated switchgrass.

comparison, the lignin in *4CL* switchgrass has lower M_n and M_w of 4482 and 9806 g/mol, respectively. Both M_n and M_w were remarkably reduced (52−59% reduction in M_n and 37−54% reduction in M_w) for the lignin in residual solids after aqueous IL pretreatment. Furthermore, the polydispersity index (PDI) of the lignin in pretreated WT switchgrass and *4CL* switchgrass increased compared with that of untreated switchgrass (Table S1 in the SI) indicating a wide span of MW after pretreatment. Taken together, these results suggest that lignin underwent significant depolymerization during the aqueous IL pretreatment, such that the majority of lignin was depolymerized and solubilized in the liquid stream.

Table 3 provides further details of the lignin MW distribution in the liquid streams. Soluble lignin in aqueous IL after pretreatment has an M_w of ~3300 g/mol and M_n of ~1100 g/mol for all three types of switchgrass, with slightly lower M_w and M_n observed for the *4CL* switchgrass sample. After pH

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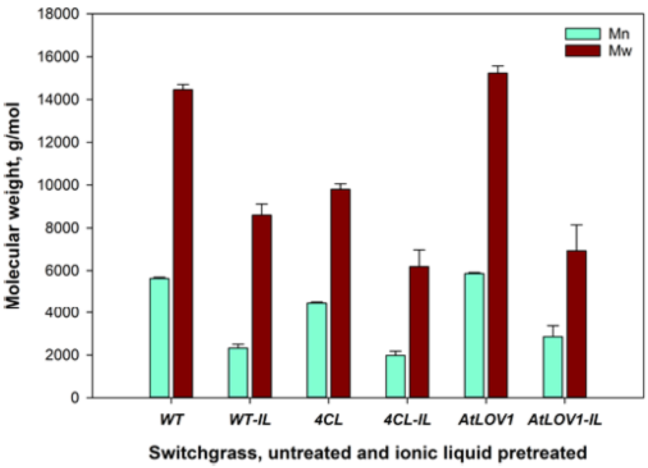

Figure 4. Number-average (M_n) and weight-average (M_w) molecular weights of lignin from untreated and aqueous IL-pretreated switchgrass (solid stream lignin). (Adapted with permission from ref 30. Copyright 2017 Springer.)

adjustment to 1−2, about 30% of the lignin precipitated out from the liquid stream. The precipitated lignin has higher M_w and M_n than the soluble lignin, probably because larger-MW lignin tends to precipitate readily at acidic pH values. Surprisingly, about 70% of the lignin remains dissolved in the liquid phase after pH adjustment, while the solubilized lignin had low MW (<1000 g/mol for M_w, representing low-molecular-weight lignin fractions). The low-molecular-weight lignin fractions in aqueous IL were likely a result of the high extent of lignin depolymerization in the pretreatment medium as compared with the other pretreatment methods such as dilute acid or hot water pretreatment. Given the fact that certain lignin degrading enzymes or microbes can assimilate and convert low-molecular-weight lignin to fuel and chemical molecules,[5,53,54] the low-molecular-weight lignin that accumulated in the liquid phase is potentially suitable for further upgrading via catalytic or biological conversion pathways.

2D HSQC NMR Spectroscopic Analysis. To achieve a better understanding of the changes in lignin chemical structure and transformation during aqueous IL pretreatment, $^1H-^{13}C$ HSQC NMR was applied to the lignins isolated from the untreated switchgrass and IL-pretreated solids. Figure 5 illustrates the 2D NMR spectra of the aromatic regions of lignin structural subunits and the aliphatic regions of lignin interunits and side chains for untreated and IL-pretreated switchgrass. The contour sizes of lignin H, S, and G subunits and hydroxycinnamates were used to quantify and compare the relative abundances between lignins (Table 2). The *4CL* switchgrass had a higher S/G ratio than WT and *AtLOV1* switchgrass. High S/G ratio in lignin has been found to positively correlate with the enzymatic digestibility of some biomass types after pretreatment, mainly because the relatively higher concentration of labile β−O−4′ linkages is beneficial for lignin depolymerization, migration, and removal during pretreatment.[55] In agreement with high S/G, the HSQC spectra of the aliphatic region revealed that lignin in *4CL* was mostly composed of β−O−4′ linkages with only trace amount of β−5′ and β−β' present, whereas the WT and *AtLOV1* lignins had less amounts of β−O−4′ linkages (<90%, Figure 6). The WT and *AtLOV1* lignins were found to contain more β−5′ (∼11%) and β−β' (∼2%), indicating more branched or condensed linkages in the lignin structure. The reduced recalcitrance of *4CL* switchgrass can be partially explained by the relatively higher S/G ratio accompanied by the higher amount of β−O−4′ linkages, in addition to its lower lignin content in comparison to WT and mutant *AtLOV1*.

After pretreatment, the lignin remaining in the biomass had a very different structure when compared to that in the raw biomass. The S/G ratio for all three genotypes of switchgrass was increased (Table 2), which indicates that more G lignin was removed during the aqueous IL pretreatment. Similar results of increased S/G ratio have been reported by other researchers for lignin from IL-pretreated poplar[44] and *Arundo donax Linn.*[45] Mutant *4CL* demonstrated a greater change in S/G ratio, almost double (from 0.73 to 1.42), compared with WT and mutant *AtLOV1*. In addition, *p*CA and FA were removed to a significant degree during the IL pretreatment. The mutant *4CL* switchgrass exhibited a more striking removal of *p*CA and FA than the WT and mutant *AtLOV1*. For instance, the FA was completely depleted, and 80% of *p*CA was removed in the mutant *4CL* switchgrass as revealed by NMR results. A recent study reported that incorporating monolignol ferulate during plant lignification resulted in reduced recalcitrance likely because the monolignol ferulate bearing readily cleavable ester bonds is more susceptible for alkaline pretreatment.[51] This is in accord with our study, given that the aqueous IL, cholinium lysinate, exhibits alkaline properties. Therefore, the higher content of FA, as well as the greater extent of its removal, can be another important factor associated with the reduced recalcitrance and increased saccharification efficiency of *4CL* switchgrass.

Lignin C−C interunit linkages have been historically thought to be more stable than alkyl−aryl ether bonds during most pretreatment.[56] Our results showed that two types of C−C bonds in switchgrass lignin, β−5′ and β−β', exhibited different

Table 3. Molecular Weight Distribution of Liquid Stream Lignin

	Switchgrass type	Weight (% of total liquid stream lignin)	Molecular weight distribution (g/mol)		
			M_w	M_n	PDI
Liquid stream lignin (total)	WT	100	3400	1279	2.66
	4CL	100	3239	1025	3.16
	AtLOV1	100	3318	1092	3.04
Precipitated lignin	WT	29.4	4006	1780	2.25
	4CL	30.0	3852	1869	2.06
	AtLOV1	28.4	3813	1843	2.07
Soluble lignin in liquid stream	WT	70.6	783	507	1.54
	4CL	70.0	964	587	1.64
	AtLOV1	71.6	985	599	1.64

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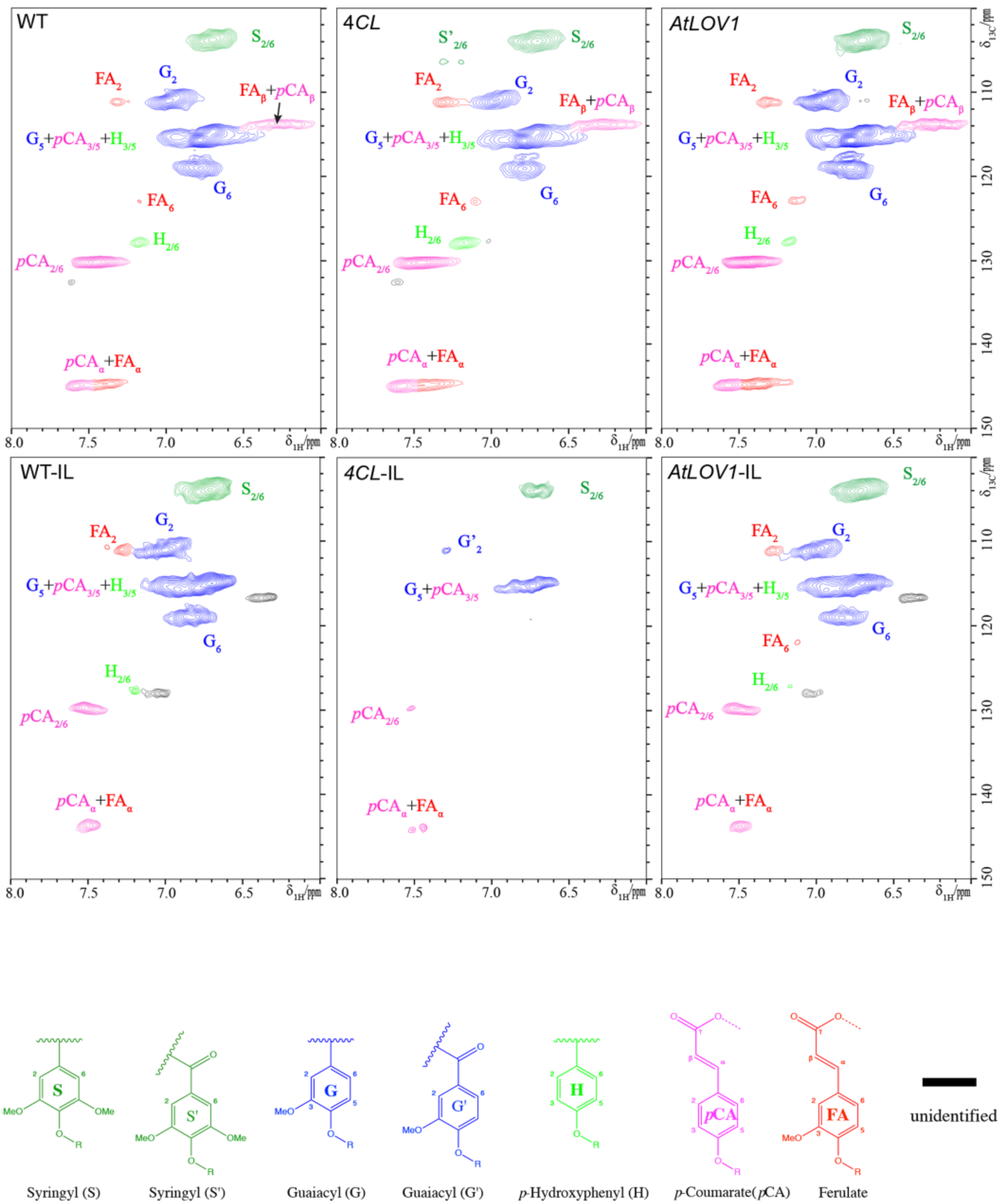

Figure 5. continued

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b)

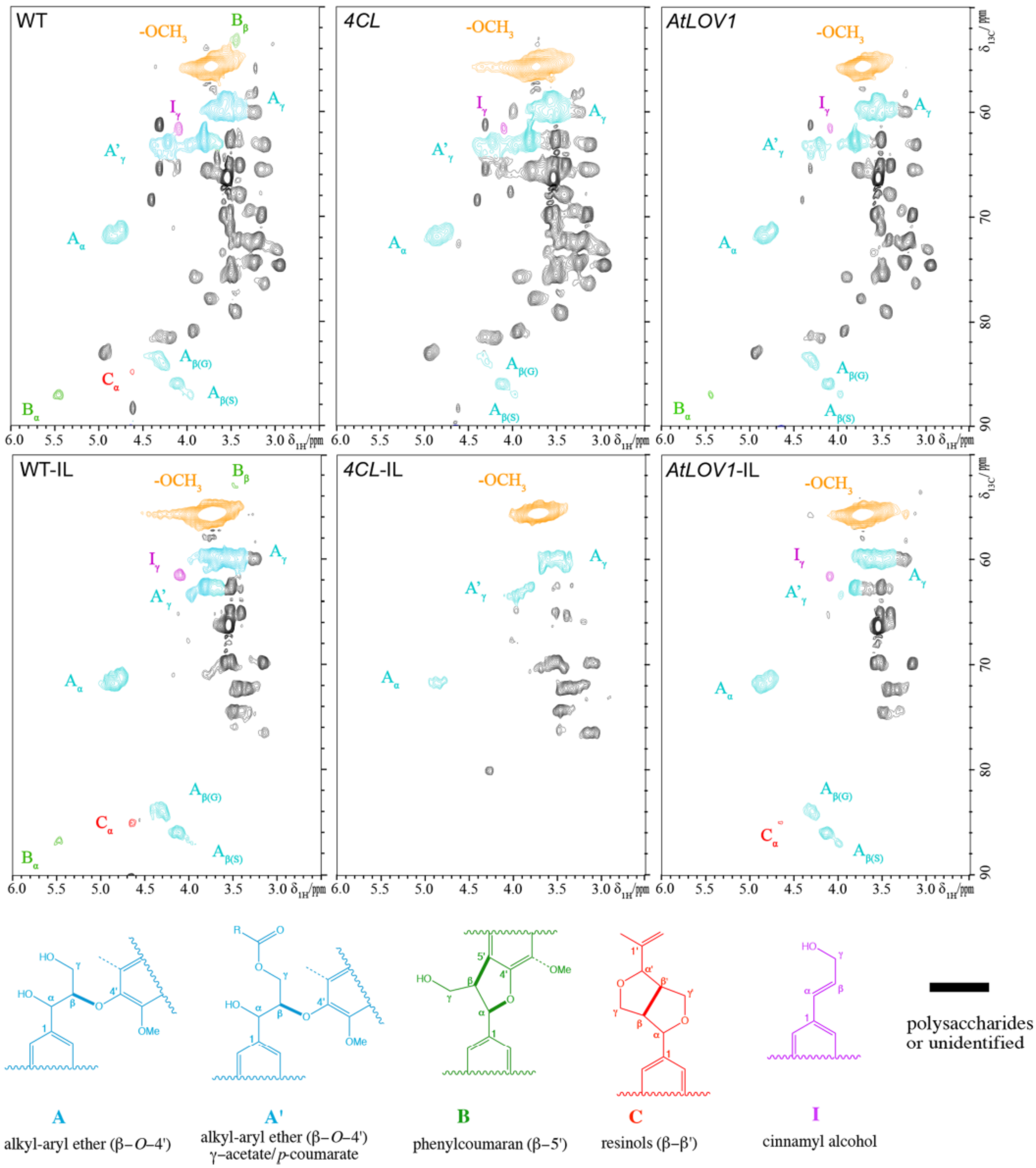

Figure 5. ^{1}H–^{13}C HSQC NMR of (a) aromatic regions of lignin structural subunits and (b) aliphatic regions of lignin interunits and side chains for untreated and aqueous IL-pretreated switchgrass.

trends after IL pretreatment. For instance, the amount of $\beta{-}5'$ linkages was significantly reduced for the three switchgrass types whereas the relative abundance of $\beta{-}\beta'$ linkages in WT and *AtLOV1* switchgrass was slightly increased (Figure 6). The decreased amount of $\beta{-}5'$ and increased amount of $\beta{-}\beta'$ in lignin suggest preferential cleavage of lignin fragments containing $\beta{-}5'$ linkages during aqueous IL pretreatment.

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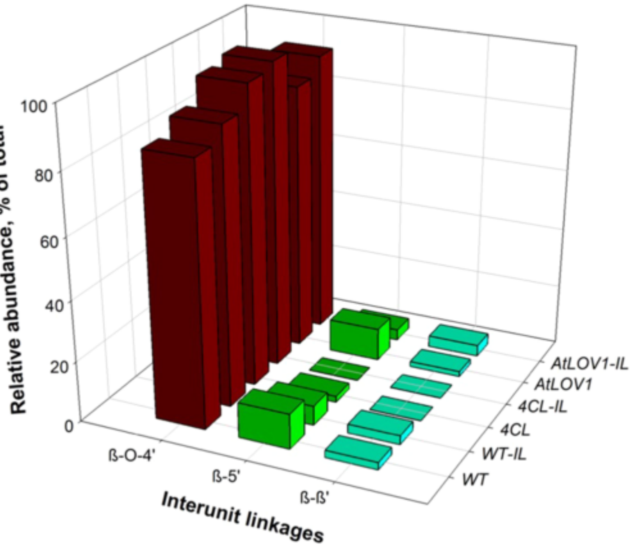

Figure 6. Relative abundance of lignin interunit linkages.

■ CONCLUSIONS

Lignin engineering represents one of the main approaches for the development of biomass feedstocks with desirable traits for cost-effective conversion to biofuels and chemicals. Pretreatment is an essential step to unlock sugars from cellulosic biomass for fermentation and to extract lignin for upgrading. This study investigated the fate of lignin and its structural and compositional changes during aqueous IL pretreatment of wild-type and engineered switchgrass. Results indicate that switchgrass mutant *4CL* was more susceptible to aqueous IL pretreatment and more digestible during enzymatic saccharification due to its lower lignin content, higher S/G ratio, strikingly higher amount of β–O–4′ linkages, and greater pCA and FA amounts as compared with the WT and mutant *AtLOV1* switchgrass. Aqueous IL (10% cholinium lysinate) was highly effective in solubilizing and depolymerizing lignin with decreased molecular weight of lignin in residual solids and a liquid stream containing low-molecular-weight lignin. Results provide insights into the impact of lignin manipulation on biomass fractionation and lignin depolymerization. Furthermore, this study leads to possible directions for developing a more selective and efficient lignin valorization process based on aqueous IL pretreatment technology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.8b00384.

Cellulolytic enzyme lignin (CEL) isolation procedure, fractionation of lignin distributed in liquid and solid streams, and PDI of lignin (solid stream) isolated from untreated and aqueous IL-treated switchgrass (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Obama, B. The irreversible momentum of clean energy. *Science* 2017, *355* (6321), 126–129.

(2) Chu, S.; Majumdar, A. Opportunities and challenges for a sustainable energy future. *Nature* 2012, *488* (7411), 294–303.

(3) Wyman, C. E.; Balan, V.; Dale, B. E.; Elander, R. T.; Falls, M.; Hames, B.; Holtzapple, M. T.; Ladisch, M. R.; Lee, Y. Y.; Mosier, N.; Pallapolu, V. R.; Shi, J.; Thomas, S. R.; Warner, R. E. Comparative data on effects of leading pretreatments and enzyme loadings and formulations on sugar yields from different switchgrass sources. *Bioresour. Technol.* 2011, *102* (24), 11052–11062.

(4) Shi, J.; Qing, Q.; Zhang, T.; Lloyd, T. A.; Wyman, C. E. Aqueous processing of cellulosic biomass for biological and chemical conversion to ethanol and other fuels. In *Fundamentals of Materials for Energy and Environmental Sustainability*; Cahen, D. G. a. D., Ed.; Materials Research Society and Cambridge University Press, 2012.

(5) Ragauskas, A. J.; Beckham, G. T.; Biddy, M. J.; Chandra, R.; Chen, F.; Davis, M. F.; Davison, B. H.; Dixon, R. A.; Gilna, P.; Keller, M.; Langan, P.; Naskar, A. K.; Saddler, J. N.; Tschaplinski, T. J.; Tuskan, G. A.; Wyman, C. E. Lignin Valorization: Improving Lignin Processing in the Biorefinery. *Science* 2014, *344* (6185), 1246843.

(6) Rinaldi, R.; Jastrzebski, R.; Clough, M. T.; Ralph, J.; Kennema, M.; Bruijnincx, P. C.; Weckhuysen, B. M. Paving the way for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. *Angew. Chem., Int. Ed.* 2016, *55* (29), 8164–8215.

(7) Simmons, B. A.; Loque, D.; Ralph, J. Advances in modifying lignin for enhanced biofuel production. *Curr. Opin. Plant Biol.* 2010, *13* (3), 312–319.

(8) Fu, C.; Mielenz, J. R.; Xiao, X.; Ge, Y.; Hamilton, C. Y.; Rodriguez, M.; Chen, F.; Foston, M.; Ragauskas, A.; Bouton, J. Genetic manipulation of lignin reduces recalcitrance and improves

DOI: 10.1021/acssuschemeng.8b00384
ACS Sustainable Chem. Eng. 2018, 6, 6612–6623

ethanol production from switchgrass. *Proc. Natl. Acad. Sci. U. S. A.* 2011, *108* (9), 3803−3808.
(9) Xu, B.; Escamilla-Treviño, L. L.; Sathitsuksanoh, N.; Shen, Z.; Shen, H.; Percival Zhang, Y. H.; Dixon, R. A.; Zhao, B. Silencing of 4-coumarate: coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. *New Phytol.* 2011, *192* (3), 611−625.
(10) Xu, B.; Sathitsuksanoh, N.; Tang, Y.; Udvardi, M. K.; Zhang, J.-Y.; Shen, Z.; Balota, M.; Harich, K.; Zhang, P. Y.-H.; Zhao, B. Overexpression of AtLOV1 in switchgrass alters plant architecture, lignin content, and flowering time. *PLoS One* 2012, *7* (12), e47399.
(11) Davin, L. B.; Lewis, N. G. Lignin primary structures and dirigent sites. *Curr. Opin. Biotechnol.* 2005, *16* (4), 407−415.
(12) Sticklen, M. B. Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. *Nat. Rev. Genet.* 2008, *9* (6), 433−443.
(13) Hisano, H.; Nandakumar, R.; Wang, Z.-Y. Genetic modification of lignin biosynthesis for improved biofuel production. *In Vitro Cell. Dev. Biol.: Plant* 2009, *45* (3), 306−313.
(14) McLaughlin, S. B.; Adams Kszos, L. Development of switchgrass (Panicum virgatum) as a bioenergy feedstock in the United States. *Biomass Bioenergy* 2005, *28* (6), 515−535.
(15) Keshwani, D. R.; Cheng, J. J. Switchgrass for bioethanol and other value-added applications: a review. *Bioresour. Technol.* 2009, *100* (4), 1515−1523.
(16) Shen, H.; Poovaiah, C. R.; Ziebell, A.; Tschaplinski, T. J.; Pattathil, S.; Gjersing, E.; Engle, N. L.; Katahira, R.; Pu, Y.; Sykes, R. Enhanced characteristics of genetically modified switchgrass (Panicum virgatum L.) for high biofuel production. *Biotechnol. Biofuels* 2013, *6* (1), 71.
(17) Shi, J.; Gladden, J. M.; Sathitsuksanoh, N.; Kambam, P.; Sandoval, L.; Mitra, D.; Zhang, S.; George, A.; Singer, S. W.; Simmons, B. A.; Singh, S. One-pot ionic liquid pretreatment and saccharification of switchgrass. *Green Chem.* 2013, *15* (9), 2579−2589.
(18) Montalbo-Lomboy, M.; Grewell, D. Rapid dissolution of switchgrass in 1-butyl-3-methylimidazolium chloride by ultrasonication. *Ultrason. Sonochem.* 2015, *22*, 588−599.
(19) Perez-Pimienta, J. A.; Lopez-Ortega, M. G.; Varanasi, P.; Stavila, V.; Cheng, G.; Singh, S.; Simmons, B. A. Comparison of the impact of ionic liquid pretreatment on recalcitrance of agave bagasse and switchgrass. *Bioresour. Technol.* 2013, *127*, 18−24.
(20) Samayam, I. P.; Schall, C. A. Saccharification of ionic liquid pretreated biomass with commercial enzyme mixtures. *Bioresour. Technol.* 2010, *101* (10), 3561−3566.
(21) Varanasi, P.; Singh, P.; Arora, R.; Adams, P. D.; Auer, M.; Simmons, B. A.; Singh, S. Understanding changes in lignin of Panicum virgatum and Eucalyptus globulus as a function of ionic liquid pretreatment. *Bioresour. Technol.* 2012, *126*, 156−161.
(22) Zhang, J.; Feng, L.; Wang, D.; Zhang, R.; Liu, G.; Cheng, G. Thermogravimetric analysis of lignocellulosic biomass with ionic liquid pretreatment. *Bioresour. Technol.* 2014, *153*, 379−382.
(23) Sun, N.; Parthasarathi, R.; Socha, A. M.; Shi, J.; Zhang, S.; Stavila, V.; Sale, K. L.; Simmons, B. A.; Singh, S. Understanding pretreatment efficacy of four cholinium and imidazolium ionic liquids by chemistry and computation. *Green Chem.* 2014, *16* (5), 2546−2557.
(24) Socha, A. M.; Parthasarathi, R.; Shi, J.; Pattathil, S.; Whyte, D.; Bergeron, M.; George, A.; Tran, K.; Stavila, V.; Venkatachalam, S.; Hahn, M. G.; Simmons, B. A.; Singh, S. Efficient biomass pretreatment using ionic liquids derived from lignin and hemicellulose. *Proc. Natl. Acad. Sci. U. S. A.* 2014, *111* (35), E3587−E3595.
(25) George, A.; Brandt, A.; Tran, K.; Zahari, S.; Klein-Marcuschamer, D.; Sun, N.; Sathitsuksanoh, N.; Shi, J.; Stavila, V.; Parthasarathi, R.; Singh, S.; Holmes, B. M.; Welton, T.; Simmons, B. A.; Hallett, J. P. Design of low-cost ionic liquids for lignocellulosic biomass pretreatment. *Green Chem.* 2015, *17* (3), 1728−1734.
(26) Liu, L. P.; Hu, Y.; Wen, P.; Li, N.; Zong, M. H.; Ou-Yang, B. N.; Wu, H. Evaluating the effects of biocompatible cholinium ionic liquids on microbial lipid production by Trichosporon fermentans. *Biotechnol. Biofuels* 2015, *8*, 119.
(27) Sun, J.; Konda, N. M.; Shi, J.; Parthasarathi, R.; Dutta, T.; Xu, F.; Scown, C. D.; Simmons, B. A.; Singh, S. CO2 enabled process integration for the production of cellulosic ethanol using bionic liquids. *Energy Environ. Sci.* 2016, *9* (9), 2822−2834.
(28) Xu, F.; Sun, J.; Konda, N. M.; Shi, J.; Dutta, T.; Scown, C. D.; Simmons, B. A.; Singh, S. Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol. *Energy Environ. Sci.* 2016, *9* (3), 1042−1049.
(29) Dutta, T.; Papa, G.; Wang, E.; Sun, J.; Isern, N. G.; Cort, J. R.; Simmons, B. A.; Singh, S. Characterization of lignin streams during bionic liquid-based pretreatment from grass, hardwood and softwood. *ACS Sustainable Chem. Eng.* 2018, *6* (3), 3079−3090.
(30) Liu, E.; Das, L.; Zhao, B.; Crocker, M.; Shi, J. Impact of Dilute Sulfuric Acid, Ammonium Hydroxide, and Ionic Liquid Pretreatments on the Fractionation and Characterization of Engineered Switchgrass. *BioEnergy Res.* 2017, *10* (4), 1079−1093.
(31) Shi, J.; Balamurugan, K.; Parthasarathi, R.; Sathitsuksanoh, N.; Zhang, S.; Stavila, V.; Subramanian, V.; Simmons, B. A.; Singh, S. Understanding the role of water during ionic liquid pretreatment of lignocellulose: co-solvent or anti-solvent? *Green Chem.* 2014, *16* (8), 3830−3840.
(32) Hardin, C. F.; Fu, C.; Hisano, H.; Xiao, X.; Shen, H.; Stewart, C. N.; Parrott, W.; Dixon, R. A.; Wang, Z.-Y. Standardization of Switchgrass Sample Collection for Cell Wall and Biomass Trait Analysis. *BioEnergy Res.* 2013, *6* (2), 755−762.
(33) Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D., *Determination of structural carbohydrates and lignin in biomass.* Laboratory Analytical Procedure, Technical Report, Contract DE-AC36-08GO28308; National Renewable Energy Laboratory: Golden, CO, 2008.
(34) Li, C.; Knierim, B.; Manisseri, C.; Arora, R.; Scheller, H. V.; Auer, M.; Vogel, K. P.; Simmons, B. A.; Singh, S. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. *Bioresour. Technol.* 2010, *101* (13), 4900−4906.
(35) Selig, M.; Weiss, N.; Ji, Y. *Enzymatic Saccharification of Lignocellulosic Biomass.* Laboratory Analytical Procedure, Technical Report, NREL/TP-510-42629; National Renewable Energy Laboratory: Golden, CO, 2008.
(36) Yoo, C. G.; Pu, Y.; Li, M.; Ragauskas, A. J. Elucidating Structural Characteristics of Biomass using Solution-State 2D NMR with a Mixture of Deuterated Dimethylsulfoxide and Hexamethylphosphoramide. *ChemSusChem* 2016, *9* (10), 1090−1095.
(37) Hu, Z.; Yeh, T.-F.; Chang, H.-m.; Matsumoto, Y.; Kadla, J. F. Elucidation of the structure of cellulolytic enzyme lignin. *Holzforschung* 2006, *60* (4), 389−397.
(38) Samuel, R.; Pu, Y.; Jiang, N.; Fu, C.; Wang, Z.-Y.; Ragauskas, A. Structural characterization of lignin in wild-type versus COMT down-regulated switchgrass. *Front. Energy Res.* 2014, *1*, 1−9.
(39) del Río, J.C.; Lino, A. G.; Colodette, J. L.; Lima, C. F.; Gutiérrez, A.; Martínez, Á. T.; Lu, F.; Ralph, J.; Rencoret, J. Differences in the chemical structure of the lignins from sugarcane bagasse and straw. *Biomass Bioenergy* 2015, *81*, 322−338.
(40) Shi, J.; Pattathil, S.; Parthasarathi, R.; Anderson, N. A.; Im Kim, J.; Venketachalam, S.; Hahn, M. G.; Chapple, C.; Simmons, B. A.; Singh, S. Impact of engineered lignin composition on biomass recalcitrance and ionic liquid pretreatment efficiency. *Green Chem.* 2016, *18* (18), 4884−4895.
(41) Parthasarathi, R.; Balamurugan, K.; Shi, J.; Subramanian, V.; Simmons, B. A.; Singh, S. Theoretical insights into the role of water in the dissolution of cellulose using IL/water mixed solvent systems. *J. Phys. Chem. B* 2015, *119* (45), 14339−14349.
(42) Studer, M. H.; DeMartini, J. D.; Davis, M. F.; Sykes, R. W.; Davison, B.; Keller, M.; Tuskan, G. A.; Wyman, C. E. Lignin content in natural Populus variants affects sugar release. *Proc. Natl. Acad. Sci. U. S. A.* 2011, *108* (15), 6300−6305.

DOI: 10.1021/acssuschemeng.8b00384
ACS Sustainable Chem. Eng. 2018, 6, 6612−6623

(43) Li, X.; Ximenes, E.; Kim, Y.; Slininger, M.; Meilan, R.; Ladisch, M.; Chapple, C. Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. *Biotechnol. Biofuels* 2010, *3* (1), 27.

(44) Wen, J.-L.; Yuan, T.-Q.; Sun, S.-L.; Xu, F.; Sun, R.-C. Understanding the chemical transformations of lignin during ionic liquid pretreatment. *Green Chem.* 2014, *16* (1), 181−190.

(45) You, T.; Zhang, L.; Guo, S.; Shao, L.; Xu, F. Unraveling the Structural Modifications in Lignin of Arundo donax Linn. during Acid-Enhanced Ionic Liquid Pretreatment. *J. Agric. Food Chem.* 2015, *63* (50), 10747−10756.

(46) Muhammad, N.; Omar, W. N.; Man, Z.; Bustam, M. A.; Rafiq, S.; Uemura, Y. Effect of ionic liquid treatment on pyrolysis products from bamboo. *Ind. Eng. Chem. Res.* 2012, *51* (5), 2280−2289.

(47) Ong, R. G.; Chundawat, S. P. S.; Hodge, D. B.; Keskar, S.; Dale, B. E. Linking plant biology and pretreatment: understanding the structure and organization of the plant cell wall and interactions with cellulosic biofuel production. In *Plants and Bioenergy*; Springer, 2014; pp 231−253.

(48) Gabrielsen, B.; Vogel, K.; Anderson, B.; Ward, J. Alkali-labile cell-wall phenolics and forage quality in switchgrasses selected for differing digestibility. *Crop Sci.* 1990, *30* (6), 1313−1320.

(49) Li, C. L.; Tanjore, D.; He, W.; Wong, J.; Gardner, J. L.; Sale, K. L.; Simmons, B. A.; Singh, S. Scale-up and evaluation of high solid ionic liquid pretreatment and enzymatic hydrolysis of switchgrass. *Biotechnol. Biofuels* 2013, *6*, 154.

(50) Klein-Marcuschamer, D.; Simmons, B. A.; Blanch, H. W. Techno-economic analysis of a lignocellulosic ethanol biorefinery with ionic liquid pre-treatment. *Biofuels, Bioprod. Biorefin.* 2011, *5* (5), 562−569.

(51) Wilkerson, C.; Mansfield, S.; Lu, F.; Withers, S.; Park, J.-Y.; Karlen, S.; Gonzales-Vigil, E.; Padmakshan, D.; Unda, F.; Rencoret, J. Monolignol ferulate transferase introduces chemically labile linkages into the lignin backbone. *Science* 2014, *344* (6179), 90−93.

(52) Ralph, J. Hydroxycinnamates in lignification. *Phytochem. Rev.* 2010, *9* (1), 65−83.

(53) Linger, J. G.; Vardon, D. R.; Guarnieri, M. T.; Karp, E. M.; Hunsinger, G. B.; Franden, M. A.; Johnson, C. W.; Chupka, G.; Strathmann, T. J.; Pienkos, P. T. Lignin valorization through integrated biological funneling and chemical catalysis. *Proc. Natl. Acad. Sci. U. S. A.* 2014, *111* (33), 12013−12018.

(54) Zhao, C.; Xie, S. X.; Pu, Y. Q.; Zhang, R.; Huang, F.; Ragauskas, A. J.; Yuan, J. S. Synergistic enzymatic and microbial lignin conversion. *Green Chem.* 2016, *18* (5), 1306−1312.

(55) Li, M.; Pu, Y.; Ragauskas, A. J. Current understanding of the correlation of lignin structure with biomass recalcitrance. *Front. Chem.* 2016, *4*, 45.

(56) Pu, Y.; Hu, F.; Huang, F.; Ragauskas, A. J. Lignin structural alterations in thermochemical pretreatments with limited delignification. *BioEnergy Res.* 2015, *8* (3), 992−1003.

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