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Structural Characterization and Comparison of Switchgrass Ball-milled Lignin Before and After Dilute Acid Pretreatment

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Abstract To reduce the recalcitrance and enhance enzymatic activity, dilute H_2SO_4 pretreatment was carried out on Alamo switchgrass (*Panicum virgatum*). Ball-milled lignin was isolated from switchgrass before and after pretreatment. Its structure was characterized by ^{13}C, HSQC, and ^{31}P NMR spectroscopy. It was confirmed that ball-milled switchgrass lignin is of HGS type with a considerable amount of *p*-coumarate and felurate esters of lignin. The major ball-milled lignin interunit was the β-*O*-4 linkage, and a minor amount of phenylcoumarin, resinol, and spirodienone units were also present. As a result of the acid pretreatment, there was 36% decrease of β-*O*-4 linkage observed. In addition to these changes, the S/G ratio decreases from 0.80 to 0.53.

Keywords Pretreatment · Switchgrass · Ball-milled lignin · HSQC · ^{13}C and ^{31}P NMR spectroscopy

Introduction

Energy security and climate change concerns associated with nonrenewable fossil fuels has led to a resurgence of interest in sustainable biofuels [1–7]. The bioethanol industry generated 9.0 billion gallons bioethanol from renewable resources such as food-derived

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carbohydrates including sugar cane and corn starch in USA with Brazil being the next largest producer yielding 6.5 billion gallons ethanol in 2008 (http://www.ethanolrfa.org/industry/statistics/). As the need for biofuels transitions from an environmental additive to an alternative fuel, the societal costs of ethanol production from food-based sugars is drawing increasing concerns due to limited net energy production and the competition between food and fuel production [8, 9]. Recent studies have suggested that based on the continuing improvements in plant productivity and conversion technologies cellulosic biomass could contribute approximately 30% of US fuels in an environmentally compatible manner [10, 11]. Hence, the next generation of cellulosic-based biofuels will be derived primarily from agricultural and forestry residuals and dedicated energy crops [12].

The main components of lignocellulosis are cellulose, hemicellulose, and lignin, and these biopolymers can be converted to biofuels employing either a thermo-chemical or biological approaches [13]. Both approaches have their own unique strength and challenges, and research is ongoing to improve the efficiencies and decrease capital/operating costs of these technologies. The biological approach is dependent on deconstructing plant polysaccharides to simple monosaccharides which are subsequently fermented to ethanol or other simple alcohols. Using this approach, lignin is a byproduct that is frequently used to address the thermal requirements of producing biofuels, and excess lignin is used as a solid fuel for the production of heat and electricity for the growing biopower market [14, 15].

Although the cost of biomass is low, releasing fermentable sugars from these materials remains challenging. The major steps involved in the production of ethanol from lignocellulosics are (1) pretreatment; (2) enzymatic deconstruction to release monomeric sugars; and (3) fermentation. The latter two steps have been combined into one step referred to simultaneous saccharification and fermentation which has the benefit of reducing some of the capital costs of cellulosic ethanol [16, 17]. The pretreatment stage is required to reduce the recalcitrance of lignocelluloses and remains the most intensive operating/operating cost component of cellulosic ethanol production [6, 14, 18]. Several pretreatment technologies are under investigation; for example, dilute acid, flow-through ammonia fiber explosion, ammonia percolation, lime, steam explosion, and organic solvent pretreatment [12, 16, 19]. However, all these processes have some drawbacks including severe reaction conditions, generation of fermentation inhibitors, energy costs, and large capital investment. As recently summarized by Wyman, although our understanding of pretreatment technologies has advanced significantly over the past decade, additional fundamental knowledge is needed concerning how these treatments change plant cell wall structure and recalcitrance [20]. This knowledge will catalyze developments in engineering low recalcitrance biomass and/or enhanced pretreatment technologies.

The selection of bioresources for biofuel production is highly dependent on regional factors including soil properties, weather, and regional farm practices, but for many parts of the USA and Europe, switchgrass is being favorable reviewed. This selection as a potential renewable energy crop is due to its high net energy production (13.5–17.9 Mg ha^{-1} or 6–8 short tons ac^{-1}), low production costs, low nutrient requirements, low ash content, efficient water utilization, large range of geographic adaptation, and exceptional resistance to naturally occurring pests and diseases [21–24]. In addition to producing biofuels from switchgrass, additional benefits include that it is a perennial crop and also yields ~10% protein [25]. Despite the widespread interest and application of switchgrass, the detailed chemical structural features of its lignin have not been fully explored. The goal of this study was to isolate, characterize, and compare the structural changes in switchgrass ball-milled lignin (BML) before and after pretreatment.

Materials and Methods

Materials

All chemicals used in this study were purchased either from VWR international or Aldrich and used as received except 1,4-dioxane, which was distilled over $NaBH_4$ prior to use. The starting lowland cultivar Alamo switchgrass used in this study was harvested from Ardmore, Oklahama on November 2007, ground to pass a 20-mesh screen and received from National Renewable Energy Laboratory (NREL).

Acid Pretreatment

Dilute sulfuric acid pretreatment was carried out at NREL following the procedure developed by Schell et al. [19]. In brief, the pretreatment pilot scale system consists of acid supply tanks, a biomass mixer, high temperature, high pressure reactor system, and a flash tank. The pretreatment procedure was performed at 190 °C, 0.05 g sulfuric acid/g dry mass with 25% total solids loading. The residence time was 1 min and the mass recovery was 80~90%. The pretreated material was filtered and stored at <0 °C until use. The frozen pretreated switchgrass was thawed to room temperature, filtered through a Buchner funnel, and washed with deionized water until the effluent was pH-neutral. The washed switchgrass was air-dried overnight and then soxhlet extracted with a benzene/ethanol (2:1, *v*/*v*) mixture for 24 h followed by an additional 24 h ethanol extraction. The extracted residue was then air-dried overnight.

Klason Lignin Content

Klason lignin and acid soluble lignin contents were analyzed by a modified literature procedure [26, 27]. In brief, the extracted switchgrass sample was treated with 72 wt.% sulfuric acid for 1 h at 30 °C and then diluted to 3 wt.% sulfuric acid using deionized water and subsequently autoclaved at 121 °C for 1 h. The resulting solution was cooled to room temperature, and the precipitate was filtered, dried, and weighed to get the Klason lignin content. The filtrate was used for the detection of sugar composition by high-performance anion exchange chromatography with pulsed amperometric detection, and acid soluble lignin was measured by ultraviolet spectroscopy. The results were shown in Table 1.

Ball-Milled Lignin Isolation

BML was isolated from soxhlet-extracted untreated and pretreated switchgrass following literature methods [28–30]. In brief, the dried switchgrass was rotary ball-milled in a

Table 1 Composition of switchgrass before and after pretreatment.

Switchgrass	Sugar (%)					Lignin (%)	
	Glucan	Xylan	Mannan	Galactan	Arabinan	Klason	Acid soluble
Untreated	45.6	26.1	0.5	1.1	3.1	20.9	1.8
Pretreated	54.8	7.2	0.2	0.2	0.4	31.4	0.7

Standard error±0.4%

porcelain jar with ceramic balls for 7 days. The powdered material was extracted twice with dioxane–water (96:4, *v*/*v*; 10 mL/g switchgrass) for 48 h each treatment. The extracted mixture was centrifuged and the supernatant was collected. The collected dioxane–water solution was roto-evaporated to reduce the volume to less than 30 mL at 40 °C under reduced pressure. The mixture was then freeze-dried, and the crude lignin was collected. The crude lignin was dissolved in acetic acid–water (90:10, *v*/*v*) (50 mg/mL) and precipitated in deionized water (1.00 g/220.00 mL), centrifuged, and freeze-dried**.** For further purification, the solid product was dissolved in minimum quantity of 1,2-dichloroethane–ethanol mixture (2:1, *v*/*v*) and precipitated in diethyl ether (200 mL), centrifuged, washed with petroleum ether, dried under vacuum at 40 °C for overnight, and stored in a desiccator over P_2O_5 for analysis. The ball-milled lignin isolated from untreated and pretreated switchgrass were 8.9 and 3.4 wt.% of Klason lignin, respectively. The yield of isolated ball-milled lignin is consistent with literature results [31, 32]. It is also reported that the recovery of lignin after a chemical treatment may reduce the yield of ball-milled lignin, and this is in agreement with our results [33, 34].

NMR Characterization

All 1D ^{13}C and ^{31}P NMR spectra were acquired using a Bruker Avance/DMX 400 MHz spectrometer. Quantitative ^{13}C NMR was operated at frequency of 100.59 MHz for the ^{13}C nucleus using DMSO-d_6 as solvent at 323°K with an inverse-gated decoupling sequence, 90° pulse angle, and 12-s pulse delay; 12,288 scans were collected for each sample. 2D heteronuclear single quantum coherence (HSQC) correlation NMR spectra were recorded in a Bruker DRX 500 spectrometer. The HSQC analysis was performed using a standard Bruker pulse sequence with a 90° pulse, 0.11 s acquisition time, a 1.5-s pulse delay, a $^{1}J_{C-H}$ of 145 Hz and acquisition of 256 data points. ^{31}P NMR spectra were acquired after in situ derivatization of the ball-milled lignin samples with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) [35]. Cyclohexanol was used as internal standard. The conditions for ^{31}P NMR spectra were as follows: a 90° pulse angle, 25-s pulse delay, and 256 transients at room temperature [36, 37]. The lignin functional group analysis associated with ^{13}C and ^{31}P NMR measurements had a typical standard deviation of 3.0% and 1.2%, respectively [37].

Size Exclusion Chromatography Analysis

Before size exclusion chromatography analysis, the ball-milled lignin samples were acetylated according to slightly modified published procedures [38, 39]. In brief, the dried lignin (15 mg) was dissolved a 1:1 (*v*/*v*) mixture of acetic anhydride/pyridine mixture (2.00 mL) and stirred at room temperature overnight. Anhydrous ethanol (25 mL) was then added, and after 30 min, the solvent was removed by rotary evaporation. The residue was repeatedly diluted with ethanol and evaporated under reduced pressure until all traces of acetic acid and pyridine was removed from the product. The residue was dissolved in minimum quantity of chloroform (2 mL) and precipitated with diethyl ether. The precipitate was centrifuged, washed with diethyl ether (×3), and dried under vacuum for overnight. The lignin acetate was then dissolved in tetrahydrofuran (THF; 0.5 mg/ml) and filtered through a 0.45-μm membrane filter. SEC analysis was carried out using an HP 1090 liquid chromatography system with an ultraviolet detector on a three-column sequence of Waters™ Styragel columns (HR1, HR3, and HR4). THF was used as eluent, and the flow rate was 0.8 ml/min. Polystyrene standards were used for calibration. Agilent ChemStation

A.10.01 and GPC software A.02.01 were used to collect data and determine molecular weight profiles.

Results

Quantitative ^{13}C NMR Analysis

Structural elucidation for the untreated and pretreated switchgrass ball-milled lignin was carried out by quantitative ^{13}C NMR spectra. The spectra data are presented in Fig. 1, and peak assignments based on literature values as summarized in Table 2 [28, 40–44]. The presence of spirodieneone units is confirmed by the peak in the region δ 183–181 ppm. It was reported that grass lignin has ester linkages with coumaric acid and ferulic acid in which their carbonyl peaks was observed at δ 168–166 ppm [45–47]. For estimation of different lignin moieties from the quantitative spectra, the integral of δ 162–103 ppm region was set as a reference of six aromatic carbons after subtracting the integration values for vinylic carbons in the ferulate and coumarate which was overlapped in the aromatic region [39, 43, 45].

It was observed that the dilute acid pretreatment resulted in considerable changes in recovered ball-milled lignin structure. The presence of carbohydrates in the isolated ball-milled lignin was detected by the small resonance at δ 102 ppm, and this is consistent with literature results [41]. The peak at δ 160 ppm confirmed the presence of *p*-hydroxy phenyl (H) units. The characterization of guaiacyl (G) and syringyl (S) units in the switchgrass ball-milled lignin was identified by the peaks in the regions δ 125–110 and δ 109–103 ppm, respectively.

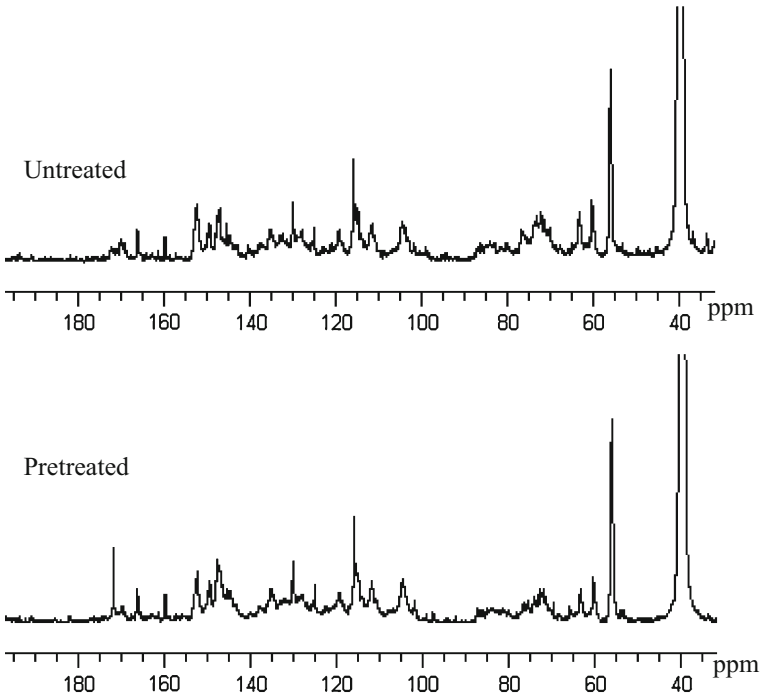

Fig. 1 Quantitative ^{31}C NMR spectra of ball-milled lignins isolated from switchgrass

Table 2 Signal assignment and contents for the quantitative ^{13}C NMR spectra of ball-milled lignins from switchgrass [29, 41–45].

Chemical shift range (ppm)	Assignments	Amount/Ar	
		Untreated	Pretreated
183–181	C═*O* in spirodienone unit	0.02	0.03
173–168	C═*O* in aliphatic COOR	0.22	0.23
168–166	C═*O* in conjugated COOR	0.17	0.18
162–158	C_4 in H unit	0.07	0.09
155–142	C_3/C_4 in G units, C_3/C_5 in S units, C_α in cinnamate	1.71	1.91
140–123	C_1 in G, S, and H units, C_4 in S, $C_{2/6}$ in H	2.17	2.10
123–118	C_6 in G units	0.44	0.46
117–113	C_5 in G, $C_{3/5}$ in H, C_β in cinnamate	0.85	0.84
113–110	C_2 in G units	0.44	0.45
109–103	C_2/C_6 in S units	0.70	0.48
90–78	C_β in β-*O*-4, C_α in β-5 and β–β	0.74	0.54
65–62	C_γ in β-5 and β-*O*-4 with C_α═O in G and S units	0.35	0.20
61–57	C_γ in β-*O*-4 without C_α═*O*	0.39	0.25
58–57	Methoxyl	0.99	0.96
53–51	C_β in β–β and C_β in β-5 structures	0.10	0.07

S syringyl unit, *G* guaiacyl unit, *H* p-hydroxyphenyl

Higuchi et al. has reported that grass lignins are composed of syringyl, guaiacyl, and *p*-hydroxyl phenyl units and considerable proportion of *p*-coumaric and ferulic acid ester [46]. The H/G/S ratio was estimated from the integration value of δ 158–162 for H, δ 113–110 ppm region for G units and half of the integral of δ 103–109 region for S units, respectively [39, 41]. The H/G/S ratio was found to be 8:51:41 and 11:58:31 for the untreated and pretreated switchgrass, respectively. It was noticed that after pretreatment the S/G ratio for recovered BML decreases from 0.80 to 0.53. The amount of β–β and β-5 bonds per C_6 was calculated by the integration of C_β resonances at δ 51.0–53.8 ppm. It was observed the content of β–β and β-5 bonds decreased after pretreatment. The quantification of β-*O*-4 bonds in ball-milled lignin structure without an α-carbonyl group was calculated based on the C_γ resonance peak at δ 60.5 ppm. There was 36% decrease in this linkage after pretreatment.

HSQC ^{13}C–^{1}H Correlation NMR Analysis

HSQC NMR technique is a powerful tool for the detailed understanding of lignin structure. The ball-milled lignin HSQC spectra are summarized Figs. 2 and 3 illustrating the aliphatic side chain and aromatic ^{13}C–^{1}H correlations. The cross peaks were assigned by comparing with the literature data [31, 32, 44] and presented in Table 3. In the side chain region of lignin, the cross signal for methoxyl and β-*O*-4 substructures were the most prominent ones. The C–H correlations in β-*O*-4 substructures (A in Fig. 4) were observed for α-, β-, and γ-C positions at $\delta c/\delta_H$ 71.5/4.8, 84.8/4.3, 60.2/3.6 ppm respectively. The presence of phenyl coumaran substructures (B in Fig. 4) was confirmed by C–H correlations for α-, β-, and γ-C positions at δ_c/δ_H 87.1/5.5, 53.2/3.5, and 62.8/3.8 ppm. Resinol (β–β) unit was also confirmed by its C/H correlations at $\delta c/\delta_H$ 84.7/4.6, 53.6/3.1, and 71.0/(3.8, 4.2) ppm for C_α/H_α, C_β/H_β, and C_γ/H_γ, respectively. Traces of spirodienone unit was observed by its

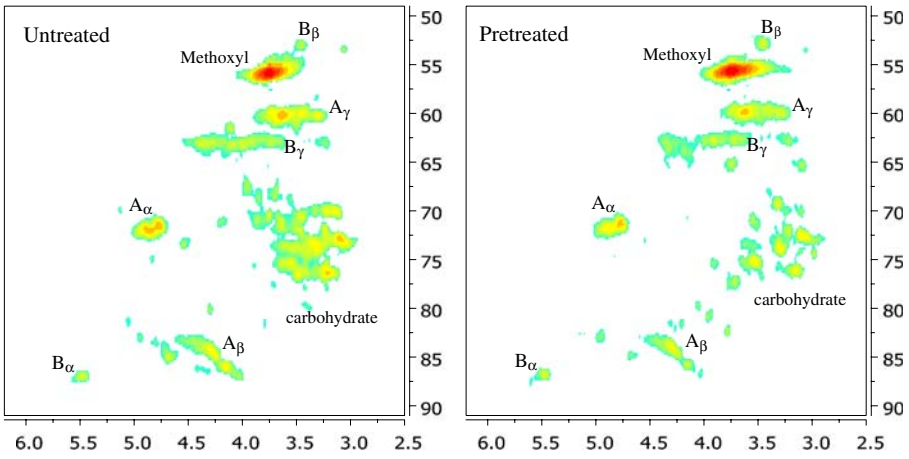

Fig. 2 Aliphatic regions of $^{13}C/^{1}H$ HSQC NMR spectra of ball-milled lignins isolated from switchgrass samples

respective C_α/H_α, $C_{\alpha'}/H_{\alpha'}$, and $C_{\beta'}/H_{\beta'}$ correlations at $\delta c/\delta_H$ 81.4/5.1, 84.7/4.7, and 79.8/4.1, and this was consistent with the aforementioned 1D ^{13}C NMR results.

The main cross signals in the aromatic region correspond to the substituted phenyl rings of lignin units. The signals for syringyl (S) and guaiacyl (G) units appeared separately. The S unit showed a major cross peaks for the $C_{2,6}/H_{2,6}$ correlation at $\delta c/\delta_H$ 104.3/6.7 ppm. The G units showed different correlations for C_2H_2, C_5H_5, and C_6H_6 as G_2, G_5, and G_6 (Fig. 3), respectively. A considerable amount of *p*-hydroxyphenyl (H) units was observed from $C_{2,6}/H_{2,6}$ correlation signals at $\delta c/\delta_H$ 130.0/7.5 ppm. The HSQC analysis confirmed the ^{13}C NMR results that the switchgrass lignin was mainly composed of guaiacyl and syringyl lignin units containing considerable amounts of *p*-hydroxyphenyl units. In addition, the HSQC spectra clearly revealed the presence of cinnamate unit (Fig. 3 and Table 3), which arose from *p*-coumarate and ferulate. The grass lignin has been widely reported containing

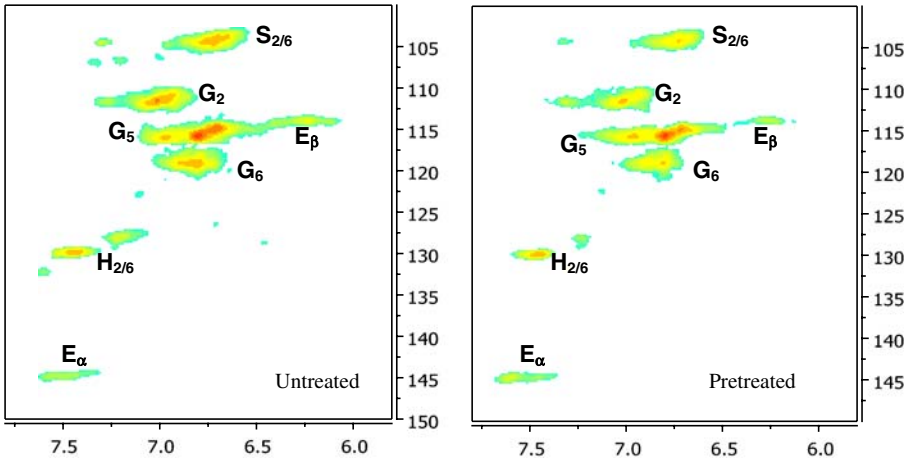

Fig. 3 Aromatic regions of $^{13}C/^{1}H$ HSQC NMR spectra of ball-milled lignins isolated from untreated and pretreated switchgrass samples

Table 3 Assignment of ^{13}C–^{1}H correlation signals in the HSQC spectrum of switchgrass ball-milled lignin [32, 45, 48].

δ_c/δ_H (ppm)	Assignment
53.2/3.5	C_β/H_β in phenylcoumarin substructure (B)
53.6/3.1	C_β/H_β in resinol (β–β) substructure
55.7/3.8	C/H in methoxyl group
60.2/3.6	C_γ/H_γ in β-O-4 substructure (A)
62.8/3.8	C_β/H_β in phenylcoumarin substructure (B)
71.5/4.8	C_α/H_α in β-O-4 linkage
84.8/4.3	C_β/H_β in β-O-4 linkage
81.4/5.1	C_β/H_β in spirodienone substructure(C)
84.7/4.7	C_α/H_α in spirodienone substructure(C)
87.1/5.5	C_α/H_α in phenylcoumarin substructure (B)
104.3/6.7	$C_{2,6}/H_{2,6}$ in etherified syringyl units (S)
105.5/7.3	$C_{2,6}/H_{2,6}$ in oxidized $C_\alpha = O$ (S′)
113.7/6.3	C_β/H_β in cinnamate unit (E)
111.4/7.0	C_2/H_2 in guaiacyl units (G)
115.4/6.77	C_5/H_5 in guaiacyl units (G)
119.3/6.82	C_6/H_6 in guaiacyl units (G)
130.0/7.5	$C_{2,6}/H_{2,6}$ in p-hydroxyphenyl units
144.7/7.5	C_α/H_α in cinnamate unit (E)

A β-*O*-4 ether linkage; *B* β-5/α-*O*-4 phenylcoumararan; *C* spirodienone; *G* guaiacyl unit; *S* syringyl unit; *S′* oxidized syringyl with C_α=O; *E* cinnamate

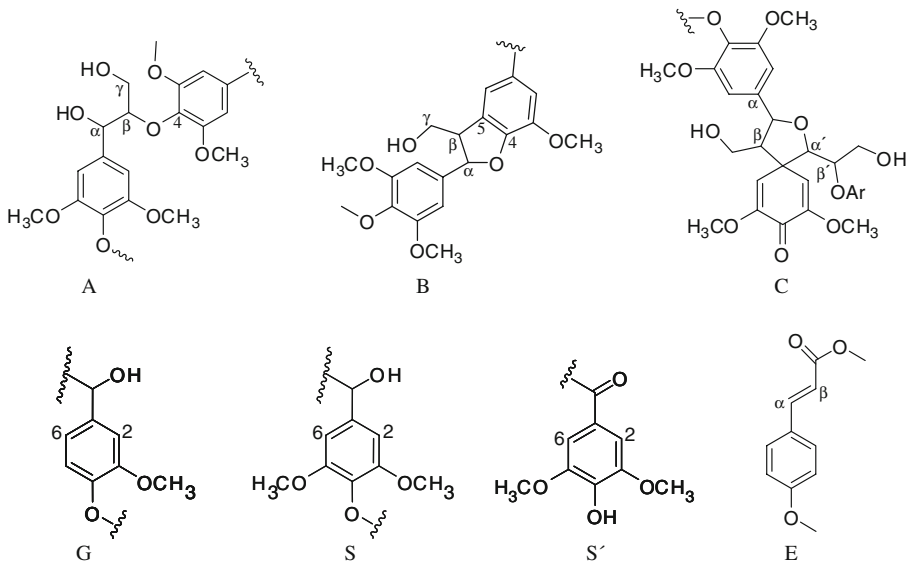

Fig. 4 Structures of identified ball-milled lignin units. (*A* β-*O*-4 ether linkage; *B* β-5/α-*O*-4 phenylcoumararan; *C* spirodienone; *G* guaiacyl unit; *S* syringyl unit; *S′* oxidized syringyl with C_α=O; *E* cinnamate)

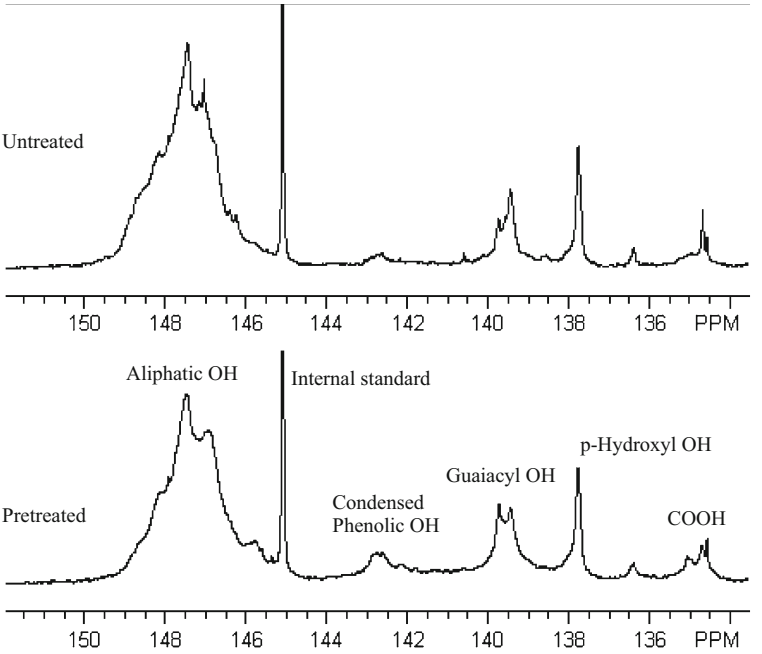

Fig. 5 ^{31}P NMR spectra of ball-milled lignins isolated from pretreated and untreated switchgrass

p-coumarate and ferulate units [48]. Figure 3 demonstrated that the pretreated ball-milled lignin had a decreased signal intensity of S unit. However, there is no dramatic change observed regarding the types of interunit bonding patterns present in the switchgrass ball-milled lignin after pretreatment.

^{31}P NMR Analysis

Quantitative ^{13}P NMR analysis is a valuable tool for estimating the amounts and distribution of various hydroxyl groups in lignin. The ball-milled lignin isolated from switchgrass was derivatized by TMDP and subsequently analyzed by quantitative ^{13}P NMR techniques following literature methods [37, 49–51] as summarized in Fig. 5 and Table 4.

^{31} P NMR results clearly indicated the major hydroxyl groups in switchgrass ball-milled lignin were from aliphatic regions. This was in agreement with the report by Hallac et al. that the dominant OH group in the ball-milled lignin isolated from a perennial shrub *Buddleja davidii* was at aliphatic sites [39]. *B. davidii* lignin has been reported to have very low carboxylic hydroxyl content (i.e., 0.03 mmol/g lignin) [39], while the untreated switchgrass ball-milled lignin had a considerable amount of carboxylic hydroxyl groups

Table 4 Hydroxyl group contents in switchgrass ball-milled lignin calculated from quantitative ^{31}P NMR spectra (mmol/g).

	Aliphatic OH	Condensed phenolic OH	Guaiacyl phenolic OH	*p*-Hydroxy phenyl	Carboxylic OH
Untreated	3.88	0.20	0.48	0.32	0.29
Pretreated	2.83	0.35	0.57	0.33	0.33

Table 5 Molecular weights of acetylated ball-milled lignin from switchgrass.

Samples	Mn (g/mol)	Mw (g/mol)	Polydispersity index
Untreated	2.94×10^3	5.00×10^3	1.70
Pretreated	2.35×10^3	4.16×10^3	1.77

Standard error<±3%

Mn number-average molecular weight; *Mw* weight-average molecular weight

(i.e., 0.29 mmol/g). It was observed that after pretreatment, there was a significant increase in guaiacyl and condensed phenolic units.

SEC Analysis

Lignin samples are only slightly soluble in tetrahydrofuran, a common solvent used for size exclusion chromatography. Under these circumstances, ball-milled lignin isolated from untreated and pretreated switchgrass was acetylated using acetic anhydride/pyridine following literature methods [38, 39]. The number-average molecular weight (Mn) and the weight-average molecular weight (Mw) were measured by size exclusion chromatography and summarized in Table 5. The observed molecular weight of our ball-milled switchgrass lignin is comparable to those values reported for wheat straw ball-milled lignin (i.e., Mw 1,400–2,190) [52, 53]. Compared to untreated switchgrass ball-milled lignin, the pretreated switchgrass ball-milled lignin had a slightly lower molecular weight; this could be due to the short pretreatment time and the less chances of condensation.

Discussion

The results of chemical analysis of the switchgrass samples before and after pretreatment indicated that the Klason lignin content increased due to the pretreatment. Li et al. reported

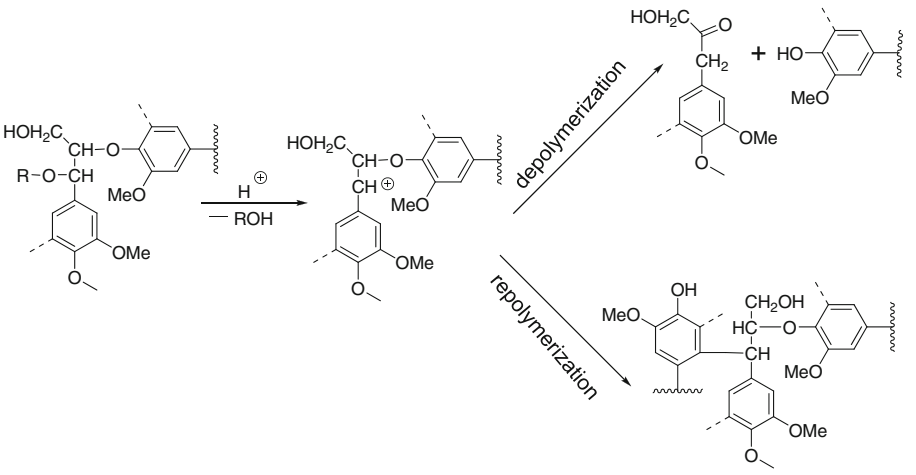

Fig. 6 Representative reaction denoting the competition between depolymerization of a β-*O*-4 structure and repolymerization involving a lignin structure with a reactive aromatic carbon [54]

that during pretreatment, condensation of polysaccharide degradation products took place. Non-lignin material also contributed to in the Klason lignin content and a similar mechanism may contribute, in part, to the Klason lignin content reported here [54]. After analyzing the quantitative ^{13}C NMR spectral data (Table 2), it was observed that there was 36% decrease of β-*O*-4 linkage after pretreatment. A decrease of β–β and β-5 linkages were also observed in the switchgrass ball-milled lignin after pretreatment. In addition, S/G ratio decreases from 0.80 to 0.53. ^{31}P NMR spectral data indicated there was an increase in guaiacyl phenolic OH group as well as an increase in condensed phenolic OH group. Li et al. previously reported the possible reactions in lignin structure under acidic conditions without an added nucleophile were fragmentation by acidolysis of β-*O*-4 linkages and a repolymerization by acid catalyzed condensation between a reactive aromatic carbon and a carbonium ion at C_α of the side chain (Fig. 6) [54].

While analyzing the SEC data before and after pretreatment, it was found that there was a slight decrease in molecular weights. As discussed by Li et al. [54], pretreatment can cause both polymerization and depolymerization of lignin, and in this study, due to the short residence time, the latter effect dominates.

Conclusion

The structure of ball-milled lignin isolated from untreated and pretreated switchgrass has been elucidated by 1D ^{13}C and ^{31}P NMR as well as 2D HSQC NMR. It was observed that switchgrass ball-milled lignin was an HGS type and incorporated with cinnamate esters. The analysis indicated that the major ball-milled lignin interunit was the β-*O*-4 ether linkage, and minor amounts of phenylcoumarin, resinol, and spirodienone units were also present. The major changes in ball-milled lignin structure due to dilute H_2SO_4 pretreatment were β-*O*-4 ether bond cleavage and decrease in syringyl units.

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