Characterization of Lignins from Sugarcane Bagasse Pretreated with Green Liquor Combined with Ethanol and Hydrogen Peroxide

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Sugarcane bagasse was pretreated by green liquor combined with ethanol (GL-Ethanol) and green liquor combined with H_2O_2 (GL-H_2O_2). After 72 h of enzymatic hydrolysis, the glucose yields of sugarcane bagasse pretreated with GL-Ethanol and GL-H_2O_2 were 97.7% and 41.7%, respectively. The reason that GL-Ethanol was more effective than GL-H_2O_2 has not been elucidated clearly. In this study, the chemical composition of the sugarcane bagasse and chemical structure of the isolated lignins after these two pretreatment methods were characterized to investigate their correlation with the enzymatic hydrolysis of sugarcane bagasse. The removal of lignins with GL-Ethanol pretreatment was much higher than that of GL-H_2O_2. In addition, the decomposition of cellulose was lower in the case of GL-Ethanol than in that of GL-H_2O_2. According to Fourier transform infrared spectroscopy (FT-IR) and ^{1}H-nuclear magnetic resonance (NMR) studies, the ester bonds (belonging to lignin-carbohydrate complex) could be broken during GL-Ethanol treatment. It was also found that the molecular weight of lignins obtained from GL-Ethanol was lower than that of lignins from GL-H_2O_2.

Keywords: Sugarcane bagasse; GL-Ethanol; GL-H_2O_2; Lignin; Structure

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INTRODUCTION

Converting biomass-derived wastes to high value-added products is one of the most challenging topics in the world. Such wastes provide new resources without competing with food production. Sugar cane bagasse is a fibrous residue remaining after crushing the sugarcane to get juice and has been applied in sugar industries or bio-ethanol production. Approximately 54 million dry tons of bagasse are produced per year throughout the world (Cerqueira *et al.* 2007). The most common use of sugarcane bagasse is for energy production by combustion (Ramjeawon 2008). Currently, a large amount of sugarcane bagasse is used for pulping (Lima *et al.* 2003), activated carbon production (Girgis *et al.* 1994), and furfural production (Mesa *et al.* 2014). However, with continuing improvements in sugar factories, more and more sugar bagasse will be used in other applications. Sugarcane bagasse is composed of approximately 50% cellulose, 25% hemicellulose, and 25% lignin (Su *et al.* 2015). It provides a promising lignin cellulosic-biomass feedstock that can be hydrolyzed to sugar by chemical or bio-chemical methods. Then, these sugars can be fermented to alcohol and other bio-chemicals (Nigam and Singh 2011).

There are many pretreatment methods being studied to separate the lignin from the lignocellulosic waste to improve the enzymatic digestibility of cellulose. They can be divided into chemical (*e.g.*, acid, alkali, and alkaline peroxide), physicochemical (such as steam explosion and ammonia fiber explosion), biological, and organosolv (*e.g.*, ethanol) pretreatments (Mosier *et al.* 2005; Mesa *et al.* 2011). Green liquor (GL) is a mixture of sodium carbonate and sodium hydroxide from pulp mills (Jin *et al.* 2010). To date, GL pretreatment has been studied as one of the alkaline pretreatment approaches with moderate alkaline conditions to produce bioethanol from biomass (Gu *et al.* 2013). It has shown effective performance in improving enzymatic digestibility during the pretreatment, as it tends to selectively remove lignin and leave the cellulose and hemicellulose (Wu *et al.* 2012). A significant advantage of GL pretreatment is that no toxic byproducts such as furfural and acetic acid (from hemicellulose degradation) are produced to affect the further fermentation step and cause corrosion in the equipment (Gu *et al.* 2013).

However, compared with organosolv pretreatment, the waste water in GL pretreatment is more difficult to recycle. To reduce the process cost, integrating bio-ethanol production with a pulp mill is an interesting possibility. Compared with auto-catalyzed organosolv pretreatment, GL pretreatment combined with ethanol has been viewed as a potential method to increase the cellulose accessibility, retaining polysaccharides as much as possible for further enzymatic hydrolysis (Yu *et al.* 2014, 2015). Yu *et al.* (2014) concluded that ethanol-GL pretreatment could improve the enzymatic hydrolyzability of furfural residues (FRs). Under optimal conditions (*i.e.*, 1.0 mL GL/g dry substrate and 140 °C), approximately 42.7% lignin was removed and the glucose yield increased from 69% to 85.9%.

Alkaline hydrogen peroxide is a typical environment-friendly agent for dissolving lignin (Mou *et al.* 2013) and leaves no residue in biomass. In addition, alkaline hydrogen peroxide pretreatment can lead to high glucose yield under moderate temperature and pressure (Rabelo *et al.* 2011). The oxidative action of H_2O_2-derived radicals is thought to depolymerize lignin by attacking only the aliphatic part of lignin and fragmenting the lignin macrostructure into a variety of low-molecular weight compounds (Gould 1984). Therefore, it is possible to obtain low-molecular weight lignin fragments under these conditions. These depolymerized lignins could be transformed to high-value chemicals and fuels.

In our previous study, GL combined with ethanol and H_2O_2 was used to pretreat furfural residues (Yu *et al.* 2013). The lignin can be effectively removed during the pretreatment. Yu *et al.* (2015) also compared GL-Ethanol and GL-H_2O_2 pretreatments of sugarcane bagasse to improve the overall sugar yield. The glucose yield (97.7%) after 72 h of hydrolysis of sugarcane bagasse from GL-Ethanol pretreatment was higher than that from GL-H_2O_2 pretreatment (41.7%).

In this study, the chemical composition of the pretreated bagasse and chemical structure of isolated lignins under various pretreatment conditions (*e.g.*, GL-Ethanol and GL-H_2O_2) were compared. The lignins recovered from the separated liquor were characterized by elemental analysis, FT-IR, GPC, and solution-state ^{1}H-NMR. A good understanding of the delignification mechanism during pretreatment is beneficial for the further improvement of pretreatment. To our knowledge, no study investigating the differences in the chemical structure of lignins from sugarcane bagasse during GL-Ethanol and GL-H_2O_2 pretreatment has been reported.

EXPERIMENTAL

Raw Materials

Sugarcane bagasse was kindly provided by a sugar mill (Guang Xi, China) and air dried. The bagasse was ground by a horizontal pulverizer and sieved to retain particles with diameters of 0.425 mm. Then, the bagasse was stored in a sealed bag at room temperature before pretreatment. The moisture content of the collected bagasse was 9.68%. Green liquor (GL) was supplied from Chenming Group (Shandong, China). The main component of GL is sodium carbonate, with a concentration of 75.2 ± 0.25 g/L (Yu *et al.* 2014).

Methods

Pretreatment with combined green liquor and ethanol

Ten grams of bagasse was placed into a polytetrafluoroethylene (PTFE) pretreatment reactor with a volume of 200 mL. For each experiment, 100 mL of a 50:50 ethanol and water mixture (v/v) containing the desired amount of GL (1.0 mL/g dry substrate) and 1% anthraquinone was mixed well with the sugarcane bagasse. The effect of EDTA on enhancing delignification was to lessen the consumption of peroxide by reducing the inhibition of metal ions (Yu *et al.* 2013).

Various pretreatment temperatures (*e.g.*, 100, 130, 140, and 160 °C) were evaluated. After sealing, the PTFE reactor was loaded into a suitable stainless tank. Then, the system (PTFE reactor and stainless tank) was fixed in a chamber that could be rotated at different speeds. After 3 h of pretreatment, the system was rapidly cooled using tap water. The solid residues were collected by filtration with quantitative paper and washed with 200 mL of ethanol-water mixture (50:50%, v/v), followed by distilled water until obtaining a neutral pH. The separated liquor after pretreatment was stored in plastic bottles before the recovery of lignin.

Pretreatment with combined green liquor and hydrogen peroxide

This pretreatment was based on published work (Yu *et al.* 2015). The sugarcane bagasse was slurried in water (5%, w/v) containing green liquor (2 mL/g based on dry substrate) and different amounts of H_2O_2 (0.1 g H_2O_2/g, 0.3 g H_2O_2/g, and 0.6 g H_2O_2/g based on dry substrate) and 1% ethylenediaminetetraacetic acid (EDTA, 98%, Sinopharm chemical reagent Beijing Co. Ltd., China) to reduce the degradation of H_2O_2. The pretreatment was carried out in a polytetrafluoroethylene (PTFE) reactor for 3 h at 80 °C. At the end of pretreatment, the insoluble residues were collected by filtration and washed with sufficient distilled water until the pH became neutral.

Some washed samples after GL-Ethanol and GL-H_2O_2 treatment were dried in an oven at 103 ± 2 °C until absolutely dry to evaluate the yield. The solid yield was calculated using the following equation:

$$Solid\ yield\ (\%) = \frac{Mass\ of\ dry\ pretreated\ solid\ (g)}{Mss\ of\ dry\ untreated\ solid\ (g)} \times 100\% \quad (1)$$

The compositional analysis of GL-Ethanol- and GL-H_2O_2-pretreated bagasse was conducted according to the method developed by the National Renewable Energy Laboratory (Sluiter *et al.* 2008).

Extraction of black liquor lignin

The lignin in the separated liquor was recovered by lowering the solution pH (Mancera *et al.* 2010). The pH was dropped to 2 by gradually adding 2 M HCl. Precipitated solid was washed with dilute acid (0.01 M HCl) to remove sugars, followed by centrifugation. The washed lignin residues were dried at 50 °C for 24 h. No additional purification step was performed. The lignin recovery yield was calculated based on the dry weight of lignin removed during pretreatment.

Characterization of lignin

The C, H, O, N, and S contents of isolated lignins were determined using a CHNS analyzer (Thermo FLASH2000, USA). The C and H contents were measured after combustion and quantification of CO_2 and H_2O by a thermal conductivity detector (USA). Oxygen was measured after pyrolysis by quantification of CO.

The FT-IR analyses were carried out on a FT-IR spectrophotometer (Nicolet-750, USA) to obtain structural information. Pellets were prepared by mixing 10-mg samples in 150 mg of KBr. The range of measurement was from 4000 to 400 cm^{-1} with 4-cm^{-1} steps.

The GPC analysis was used to determine the molecular weight of lignin. Analyses were carried out on Waters liquid chromatographic system (Waters 2695e, USA) equipped with a UV detector with a mobile phase of tetrahydrofuran (THF) (0.6 mL/min). Separation was performed on Tskgel α-3000 and Tskgel α-2500 (Tosoh Bioscience Shanghai Co., Ltd., China) column. The samples were dissolved in THF at a concentration of 0.2% and filtered through a 0.22-μm PTFE membrane syringe filter. Ten-microliter samples of solution were injected. Size exclusion chromatography-multiangle laser light scattering (SEC-MALLS, Wyatt) was performed using a HPLC pump (Wyatt, USA) equipped with an online degasser, column (PLgel Mixed-D, Agilent), and two detectors: refractometer (Optilab Wyatt, USA) and multiangle laser light scattering detector (DAWN HELEOS-Ⅱ Wyatt, USA). The mobile phase was THF (HPLC-grade). The flow rate was 0.5 mL/min, and the injection volume was 200 μL (concentration 2 mg/mL). The sample parameter dn/dc (mg/L) was set at 0.185.

The solution-state ^{1}H-NMR spectra were obtained on a Bruker spectrometer (Germany) operating in the Fourier Transform (FT) mode at 74.5 MHz. The lignin sample (25 mg for ^{1}H-NMR) was dissolved in 1 mL of DMSO-d_6 (99.8%). Tetramethylsilane was used as an internal standard.

RESULTS AND DISCUSSION

Chemical Composition of Pretreated Sugarcane Bagasse

The composition of bagasse under different temperatures during GL-Ethanol pretreatment is shown in Table 1. The untreated bagasse was found to comprise 43.6% glucan, 26.4% Klason lignin, and 21.5% xylan, accounting for more than 90% of the bagasse. Approximately 3.6% ash was detected in the untreated sample.

After GL-Ethanol pretreatment, the solid yields decreased with increasing temperature, being in the range of 81.85% to 69.6%. The reduction of solid yield was primarily a result of the removal of lignin, as well as glucan decomposition.

The ash content (0.8% to 2.0%) based on the original weight of raw bagasse varied with pretreatment temperature because of the inorganic content variation in sugarcane bagasse and green liquor. Compared with the ash content of the raw bagasse (3.6%), the

ash content of the treated samples decreased slightly, which could be ascribed to the wash process of solid residues during the pretreatment.

Table 1. Effect of GL-Ethanol Pretreatment Temperature on Chemical Composition of Sugarcane Bagasse

	Samples	Composition (%)				Solid Yield (%)
		Klason Lignin (%)	Glucan (%)	Xylan (%)	Ash (%)	
GL-Ethanol GL:1 mL/g DS	Untreated	26.4±0.5	43.6±0.2	21.5±0.6	3.6±0.04	100
	100 °C	17.41±0.13	52.90±0.36	23.6±0.16	0.78±0.25	81.85
	130 °C	11.27±0.27	54.76±0.65	24.70±0.16	1.42±0.08	77.57
	140 °C	10.2±0.15	58.1±0.63	24.0±0.57	0.9±0.04	73.1
	160 °C	10.89±0.54	56.07±0.23	27.59±0.36	2.01±0.15	69.6

DS, dry substrate. All values are based on the oven-dried weight of samples.

Approximately 46.0% and 66.9% lignin were removed after GL-Ethanol pretreatment with 1.0 mL/g DS at 100 °C and 130 °C, respectively, as shown in Table 2. Yu *et al.* (2015) reported that approximately 71.8% lignin was dissolved with 1.0 mL GL/g of DS and incubation at 140 °C for 3 h. However, after pretreatment at 160 °C, the lignin removal in bagasse did not increase any more, suggesting that condensation of lignin fragments had already begun, which would inhibit the delignification process; the results were in agreement with a previous report by Timilsena *et al.* (2013). In addition, the content of lignin in the solid residue increased from 10.2% to 10.9%. This is because of the high degradation of glucan, approximately 10.2%. An effective pretreatment should not only maximize the removal of lignin, but also minimize the degradation of glucan.

Yu *et al.* (2015) studied the GL-H_2O_2 pretreatment of sugarcane bagasse. Compared with GL-Ethanol pretreatment, the solid yields did not show a significant change with increasing H_2O_2 loading, and lignin was the main component removed during pretreatment. Approximately 35.6% lignin was degraded into liquid when the H_2O_2 loading was at a low level of 0.1 g/g H_2O_2. The decomposition of cellulose (6.29%) was higher than 5% at 0.6 g/g DS H_2O_2 loading, suggesting that cellulose was not stable at high H_2O_2 loadings. The removal rate of lignin did not increase dramatically with increasing H_2O_2 loading. The main degradation reactions during GL-Ethanol pretreatment were the cleavage of α-aryl ether and α-alkyl ether (Gierer 1985). However, delignification during alkaline hydrogen peroxide was caused by oxidation of phenolic aromatic rings, quinones, and side chains with C=O and α-β olefin aldehyde type (Gierer 1986).

Table 2. Effect of Pretreatment Conditions on Lignin Removal and Degradation of Glucan

	Lignin Type	Lignin removal (%)	Decomposition of Glucan (%)
GL-Ethanol	100 °C	46.02±0.13	0.7±0.36
	130 °C	66.89±0.27	2.6±0.65
	140 °C	71.74±0.10	2.6±0.70
	160 °C	71.21±0.54	10.2±0.23
GL-H_2O_2	0.1 g H_2O_2/g DS	35.6±0.34	1.0±0.11
	0.3 g H_2O_2/g DS	37.3±0.21	3.6±0.28
	0.6 g H_2O_2/g DS	38.6±0.46	6.3±0.37

Lignin removal (%) = [Klason lignin (%) in untreated sample - (Klason lignin (%) in pretreated sample) × solid yield (%)] × 100/ (Klason lignin (%) in untreated sample).

Because ether linkages are the main linkages in lignin, accounting for more than two thirds of the linkages, the removal rate of lignin of GL-Ethanol pretreatment was much higher than that of GL-H_2O_2 one. Increased enzymatic hydrolysis of biomass was observed with increasing lignin removal (Soares and Gouveia 2013; Zeng *et al.* 2014). Thus, GL-Ethanol was proved to be a better pretreatment than GL-H_2O_2 pretreatment for improving the overall sugar yield.

Elemental Analysis

The lignin recovery yield and elemental analysis of lignins extracted from black liquor are shown in Table 3. With increasing temperature and H_2O_2 loading, the lignin recovery decreased. This suggests that some large-molecular weight lignin fragments were degraded to small-molecular weight lignin fragments, which were difficult to precipitate by adjusting the pH to 2.

Table 3. Recovery Yield and Elemental Analysis of Lignins from Different Pretreatment Liquor

	Lignin Type	Lignin recovery yield (%)	C (%)	H (%)	O (%)
GL-Ethanol	100 °C	54.32	58.05	4.54	36.57
	140 °C	37.51	58.92	4.64	35.43
	160 °C	33.55	59.18	4.70	34.96
GL-H_2O_2	0.1 g H_2O_2/g DS	40.00	52.42	4.49	41.48
	0.6 g H_2O_2/g DS	22.94	47.52	4.16	46.21

Lignin recovery yield (%) = (Mass of recovered lignin (g))×100/(Mass of lignin removed during pretreatment (g))

After GL-Ethanol pretreatment, the elemental analysis showed carbon content ranged from 58.05% and 59.18% and the hydrogen content in the range of 4.54% to 4.70%. The carbon content increased, while the oxygen content decreased slightly, indicating that lignins dissolved in black liquor underwent condensation reactions with increasing temperature under the GL-Ethanol pretreatment. However, the GL-H_2O_2 system favored the degradation of lignin, corresponding to the changes in carbon and oxygen contents.

FT-IR

The functional groups and structural fragments of the GL-Ethanol and GL-H_2O_2 lignins were characterized by FT-IR in the range of 800 to 3900 cm^{-1} (Fig. 1). The spectra profiles of the absorption of vibration were similar in isolated lignins, indicating that the "core" of the structure of lignin did not change greatly under different pretreatment conditions. The absorption bands of vibration of lignins were assigned based on reports in the literature.

The wide peak at 3440 cm^{-1} can be attributed to O-H stretching adsorption by phenolic and aliphatic hydroxyl structures. The peak at 1702 cm^{-1} is associated with the carboxylic acids aromatic ring. The intensity of this peak decreased with increasing temperature and H_2O_2 loading, probably because of the destruction of C=O during pretreatment. The aromatic skeleton features in lignin were observed at 1600, 1510, and 1460 cm^{-1} (Bu *et al.* 2011). Interestingly, with increasing severity of delignification, both in GL-Ethanol and GL-H_2O_2 pretreatment, the intensity of these peaks showed a downward trend. This indicated that the aromatic skeletons of the lignins were partly broken during pretreatment. The peak at 1328 cm^{-1} can be assigned to C-O stretching in syringyl units (Long *et al.* 2013). The intensity of the band at 1328 cm^{-1} decreased with increasing pretreatment temperature and H_2O_2 loading, showing the decrease of the syringyl type units in isolated lignins. A small band at 1162 cm^{-1} was due to the retention of ester bonds during the GL-Ethanol and GL-H_2O_2 pretreatments (Sahoo *et al.* 2011). As the temperature increased in GL-Ethanol pretreatment, the intensity of this peak decreased, probably because of the cleavage of bonds between lignin and polysaccharides during GL-Ethanol pretreatment, which could disrupt the barriers for enzymes to access the cellulose and improve enzymatic hydrolysis (Li *et al.* 2014). The peak that occurred at 1031 cm^{-1} is associated with C-O and C-C stretching, which were broken down during GL-Ethanol pretreatment. The peak at 981 cm^{-1} corresponds to CH=CH bending (Kline *et al.* 2010). Interestingly, this signal was only detected in lignin isolated under 100 °C from GL-Ethanol pretreatment. It may be related to bleaching above 100 °C during GL-Ethanol pretreatment and the breaking of unconjugated and/or conjugated C=C during GL-H_2O_2 pretreatment. Finally, peaks at 916 and 831 cm^{-1} are related to C-H bending of syringyl units. With increasing temperature and H_2O_2 loading, lignins underwent a decrease in the S type unit and cleavage of unsaturated carbon bonds. However, the ester bonds (belonging to lignin-carbohydrate complex) could be broken down only during GL-Ethanol pretreatment, which is important for the improvement of enzymatic hydrolysis.

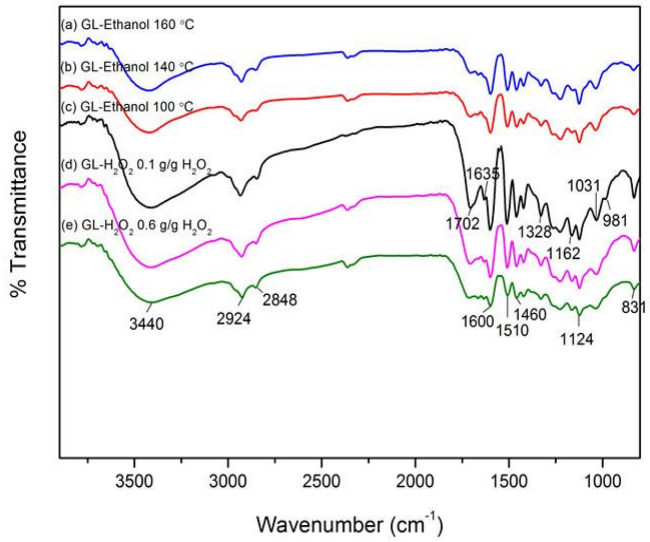

Fig. 1. FT-IR spectra of GL-Ethanol lignins and GL-H_2O_2 lignins

Molecular Weight Distribution

SEC-MALLS and GPC variations of weight average (M_w) and number average (M_n) molecular weights of lignins isolated from different pretreatment liquor are presented in Fig. 2. When a light scattering detector was used, the M_w values of all the samples were higher than those determined by GPC. The M_w measured by the light scattering detector was not relative to the polystyrene standard. Hence, the absolute M_w determined by the light scattering detector represented a more accurate M_w (Saito *et al.* 2014). For GL-Ethanol pretreatment, the M_w and M_n of lignins decreased slightly, then increased, with increasing temperature, and the polydispersity index (PI=M_w/M_n) decreased with increasing pretreatment temperature. The M_n value could overemphasize the importance of small molecules produced from the pretreatment. As the temperature increased from 100 to 140 °C, the depolymerization of lignin began because ethanol can also degrade lignin fragments (Muurinen *et al.* 2000). This result is in agreement with the FT-IR results.

When temperature was approximately 160 °C, recondensation/repolymerization reactions were predominant in the presence of a strong alkali. As a result, the M_w of lignin increased to 1055 g/mol. The condensation of lignin would inhibit delignification, which is consistent with the removal rate of GL-Ethanol. However, the M_w and M_n of GL-H_2O_2 measured by GPC did not have obvious differences when changing the H_2O_2 loading. The removal of lignin was constant with increasing H_2O_2 loading. According to Fig. 2, the M_w values of GL-H_2O_2 lignins were higher than those of GL-Ethanol ones. These data imply that the severity of GL-Ethanol pretreatment in lignin removal was higher than that of GL-H_2O_2. After GL-Ethanol pretreatment, lignins tended to be degraded to smaller molecular fragments.

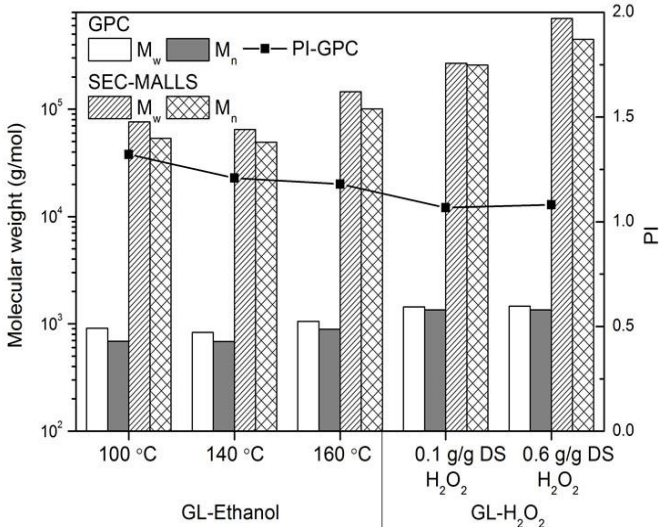

Fig. 2. Molecular weight of lignin determined by GPC and SEC-MALLS. GPC was determined by UV

^{1}H-NMR Study

The ^{1}H-NMR spectra of analyzed lignins are shown in Fig. 3. The integral of all the signals between 0.8 and 1.4 ppm belongs to the aliphatic moiety in lignin, whereas those between 6.7 and 7.2 ppm belong to aromatic protons in guaiacyl and syringyl units (Tejado *et al.* 2007). The sharp signal at 2.5 ppm is due to the protons in DMSO. The portion of the spectra at 3.0 to 4.5 ppm can be attributed to methoxyl protons or methoxyl groups that join two aromatic rings of lignin samples. All the lignins showed high proton percentage in this region.

One of the most striking differences in lignins from different pretreatment methods occurred at 3.3 ppm, representing the associated polysaccharides (H-3 in the 1-4 linked β-D-Xyl residues) (Wang *et al.* 2010). The signals appeared only in the spectrum of lignins from GL-H_2O_2, suggesting that the linkages between lignin and hemicellulose were not completely broken by GL-H_2O_2 pretreatment and that GL-Ethanol pretreatment enhanced the degradation of hemicellulose and cleavage of lignin-carbohydrate complex to improve the enzymatic hydrolysis (Li *et al.* 2014).

The region between 6.0 and 8.5 ppm represents the aromatic region. The proportion of this region in lignins from GL-EtOH was higher than that in lignins from GL-H_2O_2. The other difference was the signal representing =CH-OH at 8.0 to 9.35 ppm. These signals disappeared in lignins after GL-H_2O_2 pretreatment. In addition, with increasing temperature in GL-Ethanol, the intensities of these signals decreased.

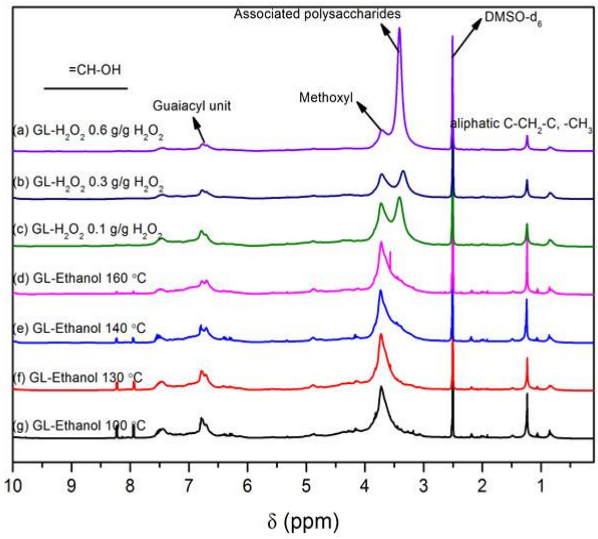

Fig. 3. ^{1}H-NMR spectra of analyzed lignins

CONCLUSIONS

1. Sugarcane bagasse was subjected to GL-Ethanol and GL-H_2O_2 pretreatments. The removal of lignin by GL-Ethanol (71.74%) was higher than that by GL-H_2O_2 (38.6%). The higher removal of lignin from sugarcane bagasse could cause a higher glucose yield.
2. The cleavage of ester bonds during GL-Ethanol pretreatment can disrupt the barrier for enzyme to access cellulose.
3. According to FT-IR, ^{1}H-NMR, and GPC studies, the chemical structure of lignin was considerably more degraded by GL-Ethanol pretreatment compared with GL-H_2O_2.
4. The decomposition of cellulose was easier to control using GL-Ethanol in comparison with GL-H_2O_2. GL-Ethanol may therefore be a more effective pretreatment method for sugarcane bagasse compared with GL-H_2O_2.

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