Modified Hydrotropic Pretreatment of Eucalyptus under Alkali and Acid Conditions for Lignin Removal and Enhancing Enzymatic Hydrolysis

Chao Chao Xu, Hongyan Mou,* Ming Lei, Jin Huang, Weiying Li, and Hongquan Huang

Hydrotropic pretreatment is an environmentally friendly technology that can be applied for lignin isolation from lignocellulosic biomass to enhance enzymatic hydrolysis. In this study, conventional hydrotropic pretreatment was modified with additional alkali and acid to investigate the lignin removal mechanism. Lignin recovered from the hydrotropic solution was analyzed by Heteronuclear Single Quantum Coherence-Nuclear Magnetic Resonance (HSQC-NMR). It was found that the amounts of β-O-4 linkages in the different types of hydrotropic lignin were more than 50%. Alkali-hydrotropic lignin particularly contained 74.9% of β-O-4 linkages. Also, the cleavage of α-O-4 formed more phenolic groups in alkali-hydrotropic lignin. The addition of acid in the hydrotropic treatment could reinforce the broken β-O-4 bond. Modified hydrotropic pretreatments more easily displaced the lignin from the fiber surface and improved the enzymatic accessibility of the pretreated substrates. Considering other impact factors including the fiber structure and total lignin content, enzymatic hydrolysis was heavily influenced and facilitated by the acid-hydrotropic method.

Keywords: Alkali-hydrotropic pretreatment; Acid-hydrotropic pretreatment; Enzymatic hydrolysis; Hydrotropic lignin; HSQC-NMR

Contact information: State Key Laboratory of Pulp and Papermaking, School of Light Industry and Engineering, South China University of Technology, Guangzhou, China, P.R.-510640;
** Corresponding author: fehymou@scut.edu.cn*

INTRODUCTION

Hydrotropic technology was a novel, environment-friendly method for lignin separation from lignocellulosic biomass. The advantages of hydrotropic pretreatment include high lignin removal efficiency, simple lignin recovery, and the recyclability of the hydrotropic agent. In previous work, sodium xylenesulphonate (SXS) was suggested to be the best agent when considering delignification for enhancing enzymatic saccharification of lignocellulosic biomass (Mou *et al.* 2013a). For both wood and nonwood biomass, hemicelluloses degradation usually happened simultaneously with the lignin extraction (Mou *et al.* 2013b). Relying on the feedstock materials and expected properties of the product, the treatment conditions of the hydrotropic technology was recommended to be carried out at 150 ºC to 170 ºC with 30 to 40% (w/v) SXS (Procter 1971; Mou and Wu 2016). At 150 ºC, over 70% of lignin could be extracted from Nordic birch chips by 30% SXS after a period of 12 h (Korpinen and Fardim 2006). Prolonged treatment time and higher temperatures were able to improve the delignification efficiency of the hydrotropic treatment. With the addition of H_2O_2 or acid, the treatment time could be reduced; meanwhile the delignification efficiency could be improved (Gabov *et al.* 2014; Mou *et al.* 2014a). According to a study by Gabov *et al.* (2014), after using 36% SXS for the treatment

of birch at 170 ºC for 2 h with the addition of H_2O_2, the residual lignin of the pretreated birch was reduced to 1.4%. Generally, hydrotropic treatment of lignocellulosic biomass was conducted at high temperatures with a high dosage of the hydrotropic agent.

In the authors' previous work, it was indicated that the addition of a small amount of formic acid into the hydrotropic pretreatment could reinforce the removal of hemicelluloses and lignin from the fiber cell wall, thus enhancing the enzyme accessibility of lignocellulosic biomass (Mou *et al.* 2014a, 2016, 2017). Applying the peracetic acid method prior to the hydrotropic process could reduce hydrotrope consumption and simultaneously enhance the delignification efficiency (Mou *et al.* 2014b). However, detailed information about the delignification mechanism and chemical properties of hydrotropic lignin is seldom mentioned. There are only a handful of publications referring to the properties of hydrotropic lignin. For instance, Zoldners and Surna (1969) claim that hydrotropic lignin is more condensed than the native lignin. The degree of degradation and the reactivity of the hydrotropic lignin were significantly effected by the treatment conditions (Gabov *et al.* 2014). Some researchers are devoted to utilizing hydrotropic lignin, eventually establishing that hydrotropic lignin is a valuable bio-based polymer that could potentially be used to produce biochemicals (Gromov and Pormale 1961; Kalninsh *et al.* 1962; Telysheva *et al.* 1966). Recently, Gabov *et al.* (2017) used hydrotropic birch lignin to make antimicrobial cellulose-lignin beads that showed great potential as an antibacterial agent against *S. aureus*. Hydrotropic lignin has potential to be used in the preparation of bio-based composites and chemicals.

Eucalyptus is one of the most important sources of raw material for pulping and papermaking in China. In this work, alkali is first used for modifying the hydrotropic pretreatment to enhance the digestibility of eucalyptus by comparison with conventional hydrotropic and acid-hydrotropic methods. Analyzing the chemical properties of lignin isolated from eucalyptus by different hydrotropic treatments could supply more information to explain the lignin removal mechanism of hydrotropic technology. Moreover, after a variety of hydrotropic pretreatments, changes in the fiber structure and chemical compositions on the fiber surface and cell wall were detected. How the different hydrotropic pretreatments influence cellulose conversion into glucose yield was also comprehensively investigated.

EXPERIMENTAL

Materials

Eucalyptus chips were collected from the Chen Ming pulp mill (Zhanjiang, China). The chemical components of the lignocellulosic biomass were calculated according to the method of the National Renewable Energy Laboratory (NREL). The chemicals were purchased from commercial sources. Sodium xylene sulphonate (Sigma-Aldrich, Shanghai, China) and cellulase mixture obtained from Genencor (Shanghai, China). Other chemicals were purchased from Guangzhou chemical company (Guangzhou, China). In this work, all experiments were conducted in duplicate and the results were averaged.

Hydrotropic pretreatments

Hydrotropic pretreatment was performed in a digester with 30% (w/v) of the hydrotrope agent (sodium xylansulphonate with a purity of 90%) at 160 ºC for 1.5 h with an addition of 2.5% (w/w basis on the O.D. raw material) formic acid and 5% (w/w basis

on the O.D. raw material) sodium hydroxide into the hydrotrope solution. The resulting pH values of the modified hydrotropic pretreatments changed from 7.3 to 3.8 and 12.2. The heating rate was 3 ºC/min and the liquid to wood ratio was 8:1. Conventional hydrotropic pretreatments were conducted at 160 ºC with dwelling times of 1.5 and 3 h, separately, for comparison. The treatment conditions are summarized in Table 1.

Table 1. Treatment Conditions for a Variety of Pretreatments

Pretreatments	Hydrotrope dosage (% w/v)	Treatment temperature (ºC)	Treatment time (h)	pH of fresh solution
Hydrotropic pretreatment (S1)	30	160	1.5	7.3
Hydrotropic pretreatment (S2)	30	160	3	7.3
Acid-modified hydrotropic pretreatment (SF)	30	160	1.5	3.8
Alkali-modified hydrotropic pretreatment (SN)	30	160	1.5	12.2

Lignin separation

After pretreatments, the spent solution was recovered for lignin precipitation. The separation methods were reported in the authors' previous work (Mou *et al.* 2018). The isolated lignin fractions were purified by an addition of concentrated sulfuric acid according to the method in previous literature (Toledano *et al.* 2010).

Methods

X-ray diffraction (XRD) analysis

The crystallinity of the pretreated substrates was analyzed with an X-ray diffractometer (D8 ADVANCE, Bruker, Karlsruhe, Germany). The scattering angle (2θ) ranged from 50º to 60º with a scan rate of 4º min^{-1}. The crystallinity index (CrI) calculation was indicated by Segal *et al.* (1959).

X-ray photoelectron spectroscopy (XPS) analysis

After pretreatments, the sample's surface chemical components were detected using an X-ray photoelectron spectroscope (Kratos Axis Ulra DLD, Shimadzu, Japan). The sample preparation and analysis method was described elsewhere (Mou *et al.* 2013a, 2013b). The surface coverage by lignin (Slig) and carbohydrates (Scar) of samples was calculated based on the oxygen-to-carbon (O/C) ratios observed from the XPS spectra, as described by Ström and Carlsson (1992).

Glucose yield analysis

The untreated and pretreated substrates were hydrolyzed by the commercial cellulase mixture at a dosage of 20 FPU/g (dry matter) at 50 ºC at a pH of 5.0, for 2, 6, 24, and 48 h. The consistency was 2% (w/v) in a 10 mL sodium acetate buffer (0.1 M). Upon completion, the hydrolyzate was centrifuged at 35000 g for 30 min. The supernatant was

filtrated using a 0.25-μm membrane that was used for glucose determination by ion chromatography (ICS-900; Thermo Fisher Scientific, Waltham, MA, USA).

Protein adsorption analysis

Enzyme adsorption onto the pretreated samples was carried out with 0.5 mL cellulase at a pH of 4.8 under ambient temperature for 90 min. Before the addition of cellulase, excessive bovine serum albumin (BSA; 0.2 $mg.mL^{-1}$) was reacted with the pretreated samples for 60 min. The supernatant was separated *via* centrifuging at 35,000 g for 20 min. The amount of free protein in the solution was determined according to the method described by Bradford (1976). Samples lacking enzymes were used as reference. The cellulase adsorption capacity was calculated based on the following equation (Mou *et al.* 2016b, 2017):

$$\text{Cellulase adsorption capacity}(\%) = \frac{(\text{Total protein} - \text{Free protein})}{\text{Total protein}} \times 100 \quad (1)$$

Molecular weight analysis of lignin

The molecular weight was determined by gel permeation chromatography (GPC; Waters, MA, USA), the mobile phase was determined by tetrahydrofuran (THF), and the sample preparation was described elsewhere (Mou *et al.* 2018). All results presented in this study were averaged.

Surface charge and phenolic groups determination

The surface charge of the different hydrotropic lignin recovered from the spent solution was determined according to Rahikainen *et al*. (2013). The calculation method was described in the work of Li *et al.* (2016b). The utilized method for the phenolic and total acid groups was described by Goldschmid (1954) and Wu *et al.* (1993).

^{13}C-^{1}H HSQC NMR analysis

The chemical structure of recovered lignin was analyzed using the ^{13}C-^{1}H HSQC NMR method. Around 80 mg lignin was dissolved in 1 mL of DMSO-D6 and subsequently detected by a Bruker ADVANCE III HD 600 nuclear magnetic resonance spectroscopy (NMR; Bruker, Beijing, China). The experiments used Bruker's "hsqcetgpsi2" pulse program with spectral widths of 5,000 Hz (from 10 to 0 ppm) and 2,0843 Hz (from 165 to 0 ppm) for the ^{1}H and ^{13}C dimensions. The spectra analysis was recognized according to reported literature (Ralph *et al.* 1999, 2004)

RESULTS AND DISCUSSION

Major Chemical Compositions of Eucalyptus Before and After a Variety of Hydrotropic Pretreatments

The modified hydrotropic method at different pH condition was applied for the treatment of eucalyptus chips. The modified hydrotropic method at different pH condition was applied for the treatment of eucalyptus chips. After a variety of pretreatments, the major chemical compositions of the pretreated substrates were analyzed and is shown in Table 2.

When compared with the reference sample, the glucose content in all pretreated substrates increased. Simultaneously, lignin was removed to different degrees during the variety of pretreatments. For samples treated by the hydrotropic method for 1.5 h (EuS1), and the pretreated eucalyptus with the conventional hydrotropic method for a longer time (EuS2), there was a reduction of lignin, and the hemicellulose started to degrade as well. Meanwhile, the cellulose was retained well in the pretreated samples. In comparison with the conventional hydrotropic pretreatment, the reduction of lignin and hemicelluloses was heavily influenced by an acid-modified hydrotropic pretreatment. It was speculated that the linkage of lignin-carbohydrates was severely broken during the acid-modified hydrotropic pretreatment. As a result, the cellulose content in the EuSF sample was over 74%. However, the lignin removal efficiency of the alkali-modified hydrotropic pretreatment was not remarkable, while the cellulose content in the EuSN sample was slightly higher than that in the ES1sample.

Table 2. Major Chemical Components of Pretreated Eucalyptus (Eu) Samples

Samples	Glucose (%)	Acid Soluble Lignin (%)	Acid Insoluble Lignin (%)	Extractives (%)	Ash (%)	Hemicelluloses (%)
Euref	40.01	2.34	24.66	5.64	4.88	29.51
EuS1	51.86	1.25	14.12	5.62	1.68	28.2
EuS2	69.33	1.01	13.64	5.49	1.28	17.85
EuSN	56.64	1.34	20.34	2.58	1.08	24.74
EuSF	74.73	0.79	10.62	4.19	1.78	11.5

According to the results in Table 2, the mild acid condition was beneficial for lignin removal from the fiber cell wall. This was established in previous work (Mou *et al.* 2014a). Compared with the conventional hydrotropic pretreatment at similar conditions (EuS2), an alkali-hydrotropic pretreatment promoted hemicellulose degradation instead of lignin separation. The decrease in extractives was observed in the modified hydrotropic pretreated substrates, which was typical for an alkali-modified hydrotropic pretreatment (EuSN). The ash content in the pretreated substrates was reduced during the variety of pretreatment processes. Concerning the hydrotropic mechanism, it is believed that hydrotropic agent molecules can penetrate the cell wall and dissolve the lignin into water (Raman and Gaikar 2003). Lignin removal, shown in Table 2, could provide support to prove this speculation.

Surface Chemical Composition of Eucalyptus Before and After Different Hydrotropic Pretreatments

After the pretreatments, the changes in the surface chemical compositions of the pretreated substrates were determined *via* XPS. The oxygen to carbon (O/C) ratio was observed from the C1s spectra of XPS. The surface coverage by lignin and carbohydrates were calculated from the O/C ratio of the extracted samples. The results are given in Table 3. The carbon ratio observed from the XPS spectra was mainly a lignin contribution because the extractives were completely removed. After the pretreatments, the lignin surface coverage was reduced, while the carbohydrate surface coverage increased, which indicated that the surface lignin was removed, leading to the exposure of carbohydrates on the fiber surface. In comparison with the reference samples, the surface coverage by carbohydrates of EuSN and EuSF was increased from 68.2% to 94.8% and 97.8%,

respectively. The influence of alkali-hydrotropic and acidic-hydrotropic pretreatments on the reduction of surface coverage by lignin was more remarkable than the conventional hydrotropic pretreatment. The surface analysis depth of the biomass for XPS was approximately 10 nm. The reduction of the lignin distribution on the surface of fiber was prone to improving the initial enzymatic hydrolysis rate, which has been illustrated in a previous study (Mou *et al*. 2013a).

Table 3. Surface Coverage by Lignin and Carbohydrates of Pretreated Eucalyptus Samples

Samples	C (%)	O (%)	O/C	Surface Coverage by Lignin (%)	Surface Coverage by Carbohydrates (%)
Euref	52.06	34.94	0.67	31.77	68.23
EuS1	38.99	51.01	0.76	13.13	86.87
EuS2	39.01	50.99	0.77	12.71	87.29
EuSN	40.08	49.84	0.80	5.17	94.83
EuSF	40.98	50.02	0.82	2.15	97.85

Crystallinity of Eucalyptus Before and After Pretreatments

Crystallinity (CrI) could indicate the enzymatic accessibility of lignocellulose as one of the important factors to enzymatic hydrolysis efficiency. It was demonstrated that the crystallinity of lignocellulose had a negative correlation with the enzymatic hydrolysis rate (Hall *et al.* 2010). The CrI degree observed from the XRD spectra (Fig. 1) is summarized in Table 4. As shown in Table 4, the increase in the CrI degree was caused by removal of the amorphous substances during pretreatment, such as hemicelluloses and lignin (Mou *et al.* 2014b).

According to the results in Table 4, after different types of hydrotropic pretreatments, the crystallinity of the pretreated samples increased noticeably. However, the CrI degree of EuSN was lower than that of EuSF and EuS2. After prolonged treatment time, the CrI degree was increased (EuS2 > EuS1) because of the lignin and hemicelluloses removal (Table 2). As presented in Fig. 1 of the XRD spectra, the lignocellulose structure changed considerably after the different types of hydrotropic pretreatments.

Table 4. Crystallinity of Different Pretreated Eucalyptus Samples

Samples	CrI (%)
Euref	49.48
EuS1	65.43
EuS2	74.36
EuSN	69.91
EuSF	72.97

Enzymatic Hydrolysis of Pretreated Samples

After the pretreatments, the pretreated substrates were hydrolyzed by cellulase subsequently. In Fig. 1, the yield of cellulose conversion into glucose was presented. After a variety of pretreatments, the enzymatic hydrolysis efficiency of the pretreated substrates was dramatically improved compared with the reference sample. Because the lignin was removed from both the fiber cell wall and the surface after pretreatments (Table 2 and Table 3), the pretreated substrates were more prone to hydrolysis.

As shown in Fig 1, in the initial stage of enzymatic hydrolysis (before 3 h), the cellulose conversion rate of EuSF and EuS2 was faster than EuSN and EuS1. However, the cellulose hydrolyzed rate of EuSN was close to EuS1. The difference in the initial hydrolysis rate was correlated to the change of the crystallinity of the pretreated substrates (Hall *et al.* 2010). Moreover, lignin content was reduced significantly from EuSF and EuS2, which could improve the enzymatic digestibility of lignocellulose too (Table 2). When the enzymatic hydrolysis was conducted for longer than 3 h, the glucose yield of EuSF was higher than the other samples. Particularly, after 48 h of hydrolysis, the glucose yield of EuSF and EuSN showed an obvious increase, as indicated in Fig. 2. Similar results were found in the authors' previous work, which proposed a relation to the reduction of the surface coverage by lignin (Mou *et al.* 2013a). A decrease of surface coverage by lignin can also prove the improvement in the enzymatic digestibility of the pretreated substrates. For further investigation on the influence of different hydrotropic pretreatments on enzymatic hydrolysis of lignocellulose, the enzyme adsorption capacity of pretreated samples was determined as a supplement.

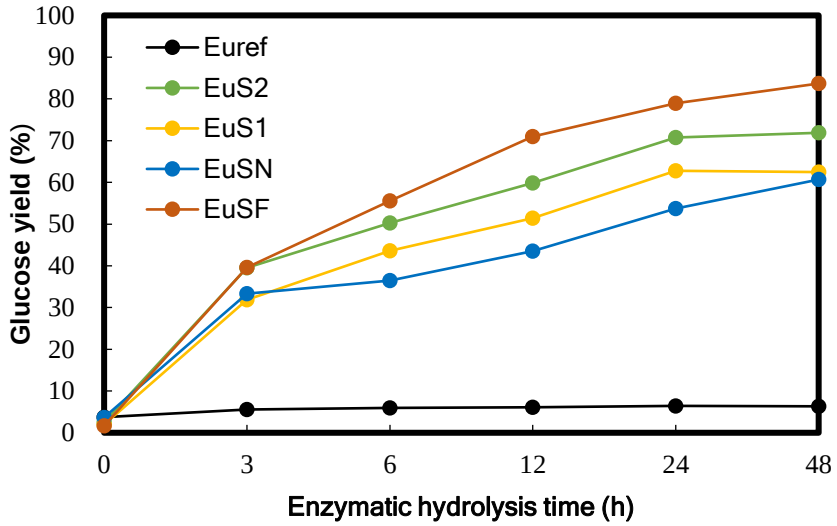

Fig. 1. Glucose yield of samples after different pretreatments

Enzyme Adsorption Capacity of Pretreated Samples

Enzyme adsorption capacity can reveal the enzymatic accessibility of pretreated substrates (Mou *et al.* 2016). Thereby, the cellulase adsorption capacity of the pretreated substrates was detected and presented in Fig. 2.

After a variety of pretreatments, the cellulase adsorption capacity of the pretreated samples was increased for all samples. In comparison with the conventional hydrotropic pretreatment (EuS1, EuS2), after the alkali-hydrotropic and acid-hydrotropic pretreatments, the cellulase adsorption capacity of the pretreated substrates (EuSF and EuSN) was typically facilitated. This is because the adsorption of cellulase on pretreated lignocellulosic biomass was a prerequisite for enzymatic hydrolysis reaction (Jäger *et al.* 2010). Therefore, in theory, the enzymatic hydrolysis efficiency of EuSN and EuSF may have an obvious enhancement when compared with the reference. Considering multiple influence factors, such as lignin content, crystallinity, surface coverage by lignin, and enzymatic adsorption capacity, the maximum glucose yield was undoubtedly observed from the substrates after the acid-modified hydrotropic (EuSF) pretreatment (Fig. 1).

Combined with the results in Fig. 2, the enhancement of enzyme adsorption capacity of EuSN could help to explain why the glucose yield of EuSN kept increasing after 48 h of hydrolysis. The optimum condition of the alkali-hydrotropic pretreatment needs further study. Considering on the recyclable of hydrotrope and high lignin removal efficiency, hydrotropic pretreatment is potentially used for the reinforcement of the enzymatic hydrolysis in commercial production process.

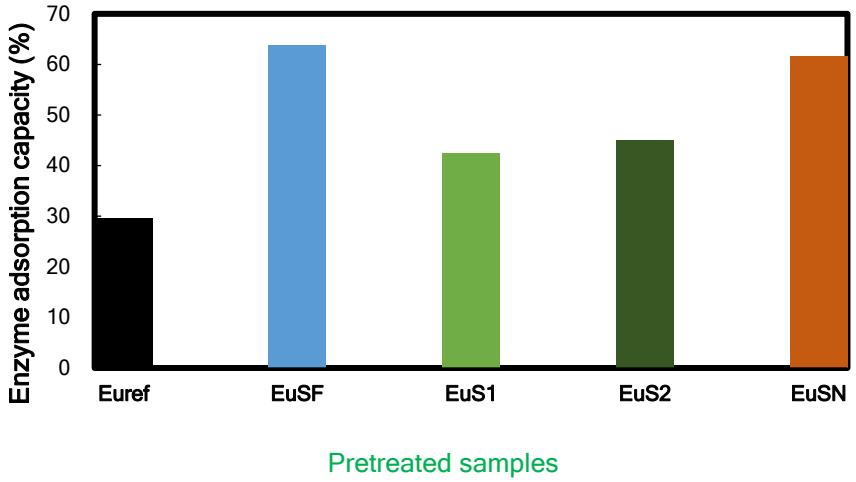

Fig. 2. Cellulase adsorption capacity of pretreated samples

Chemical Properties of Lignin Recovered from Different Hydrotropic Solutions

Other than the investigation on the pretreated substrates after hydrotropic pretreatment, the chemical properties of lignin precipitated from the conventional hydrotropic solution (3 h), acid-hydrotropic solution, and alkali-hydrotropic solution were analyzed by GPC and ^{13}C-^{1}H HSQC NMR. Besides the molecular weight, the functional groups and surface charge were detected and summarized in Table 5 as well.

According to the results given in Table 5, it can be seen that lignin depolymerization led to a lower molecular weight during pretreatments. The molecular weight of the conventional hydrotropic lignin (Lig-sxs) after treatment for 3 h was approximately 9851 Da. This value is higher than the hydrotropic lignin of birch (Gabov *et al.* 2014), which indicated that hydrotropic treatment conditions had an influence on the lignin molecular weight.

The molecular weight of alkali-hydrotropic lignin (Lig-SN) was lower than that of acid-hydrotropic lignin (Lig-SF) and Lig-sxs. The polydispersity index (PDI) value of Lig-SN was clearly higher than that of Lig-SF and Lig-sxs. Alkaline conditions could degrade lignin into smaller fragments through cleavage of the β-ether bond that contributed to the lignin removal (Cao *et al.* 2012). Furthermore, additional alkali during the hydrotropic pretreatment resulted in a higher uneven distribution of the molecular weight.

Lig-sxs contained more surface charge than Lig-SF and Lig-SN, as shown in Table 5. By using chemical titration and ultraviolet (UV) determination methods, the amount of total acid group and phenolic group of lignin were observed and presented in Table 5 as well.

Table 5. The Chemical Properties of Precipitated Lignin

Samples	Mw (Da)	Mn (Da)	PDI	Surface Charge	Total Acid Group Amount 10^{-6} (mol/mg)	Phenolic Group Amount 10^{-6} (mol/mg)
Lig-sxs	9851	3219	3.1	-0.98	5.73	4.90
Lig-SN	7497	2053	3.7	-0.50	12.5	11.8
Lig-SF	9558	2636	3.6	-0.64	6.00	3.79

The amount of total acid groups in Lig-SN was higher than Lig-SF and Lig-sxs. Moreover, the Lig-SN sample contained more phenolic groups than Lig-SF and Lig-sxs lignin. The higher phenolic hydroxyl group content in the Lig-SN lignin was caused by the cleavage of aryl-ether bonds (Leschinsky *et al.* 2008). Subsequently, more detailed chemical information of lignin analyzed by ^{13}C-^{1}H (HSQC) NMR was presented.

HSQC-NMR Analysis of Lignin Recovered from Different Hydrotropic Solutions

In Fig. 3, useful information about various linkages between structural units present in lignin was provided *via* NMR spectra. From the side-chain region in the 2D-HSQC NMR, the β-aryl ether bond (β-O-4', I), phenylcoumaran (α-O-4' and β-5, II), resinol (β-β and α-O-γ, III), and spirodienones (β-1 and α-O-α) linkages were observed in the HSQC-NMR spectra of Fig. 3, and the binding types are shown in Fig. 4. The aromatic region of lignin is usually located at δH ≈ 6.0 to 8.0 ppm and δC ≈ 100 to approximately 150 ppm. The aromatic region shown in Fig. 3 revealed that the precipitated lignin mainly contained guaiacyl (G), syringyl (S), and hydroxyl phenyl (H) units. The hydroxyl linked to Cα on the side chain of phenylpropane was easily oxidized. Therefore, the chemical shift in the oxidized hydrogen atom on the carbon of the aromatic ring was easily observed in the HSQC-NMR spectrogram. Because NMR analysis is a semi-quantitative method. In this study, the benzene ring was taken as the internal standard. Based on the hydrogen atoms in the aromatic ring, the proportion of chemical bond linkages per 100 structural units could be determined. In addition, the integral results of all side chain structures could be obtained with 100 aromatic rings as reference also.

As shown in Fig. 3, the β-aryl ether bond (β-O-4') and resinol (β-β and α-O-γ) in precipitated lignin were retained relatively well. The β-O-4' linkage was the major bond for the three types of lignin samples. The amount of β-O-4' in Lig-sxs and Lig-SN was 64.2% and 74.9%, respectively. Only 58.3% of β-O-4' linkages were contained in the Lig-SF sample, which indicated that the acid-hydrotropic lignin was more degraded than the conventional hydrotropic lignin sample. The chemical structure of MWL (Milled Wood Lignin) was close to the native lignin in wood. As illustrated in publications before, the β-O-4' bond in MWL lignin of eucalyptus totals 85% to 90% of all detectable side chains (Zhang *et al.* 2010). In comparison with the linkage information of MWL and the eucalyptus lignin reported by Zakzeski *et al.* 2010, the cleavage of β-O-4' bonds in three types of hydrotropic lignin could be further proven. Furthermore, the β-aryl ether linkages retained better by alkali-hydrotropic pretreatment than acid-hydrotropic and conventional hydrotropic methods. However, spirodienones (β-1 and α-O-α), presented in the three types of lignin samples, was low in quantity.

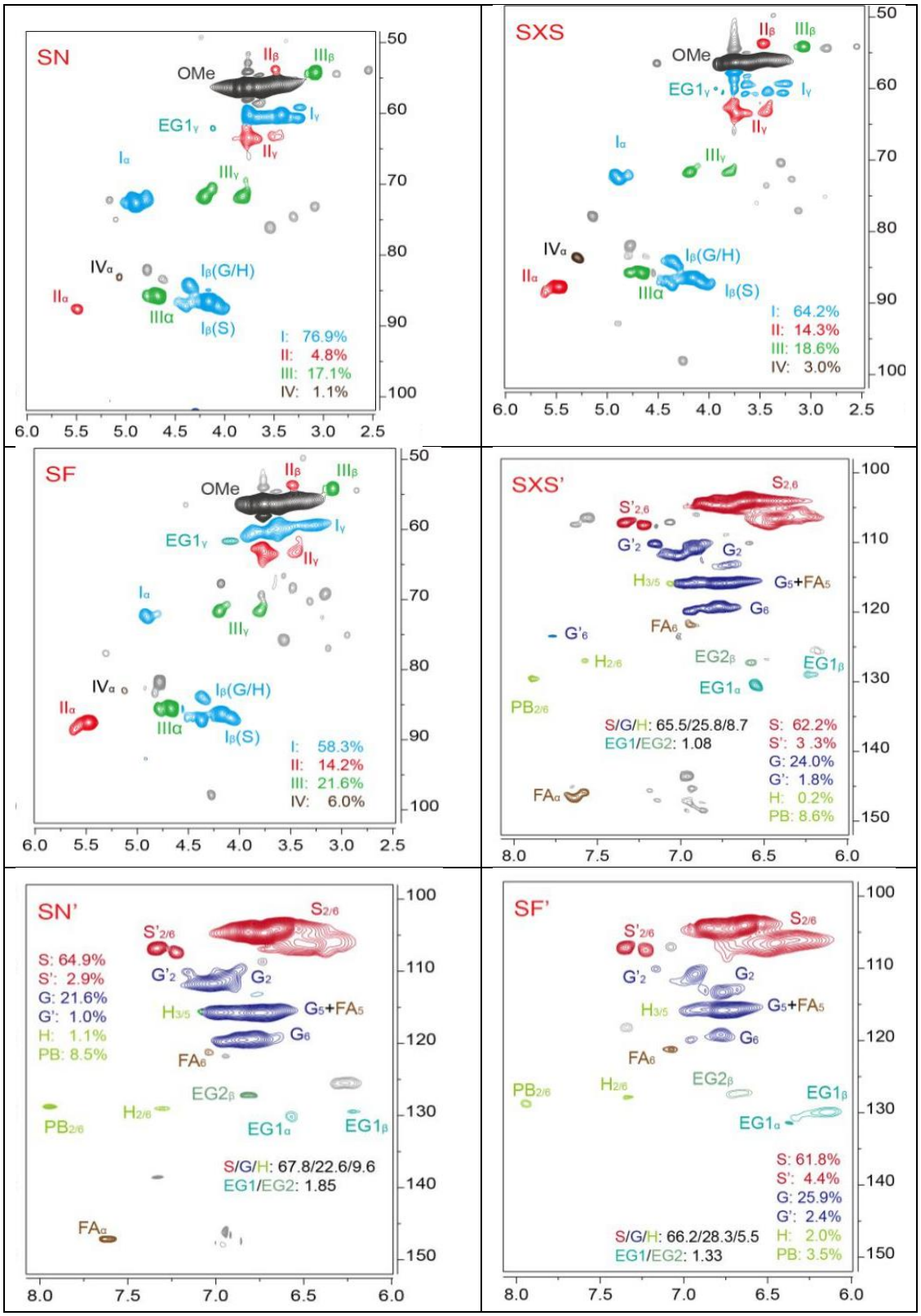

Fig. 3. 2D NMR spectra revealing lignin interunit distributions and unit compositions. Partial short-range ^{13}C-^{1}H (HSQC) spectra (side chain regions and aromatic regions) with color-coded structures is given

The amount of phenylcoumaran (α-O-4'and β-5) and resinol (β-β and α-O-γ, III) in Lig-sxs and Lig-SF samples was more than that in Lig-SN. This could explain why the phenolic group amount in Lig-SN was higher than that of Lig-sxs and Lig-SF. At the alkali condition, the cleavage of the α-O-4' bond could form new phenolic-hydroxyl groups (Leschinsky *et al.* 2008). The pH of the hydrotropic and acid-hydrotropic solution was 7.3 and 3.8, respectively (Table 1). Under neutral and acidic conditions, the main reaction was homolytic cleavage of the β-O-4' bond through a quinone methide intermediate followed by radical coupling and a radical exchange reaction (Li *et al.* 2000; Leschinsky *et al.* 2008). The former path led to the formation of β-β and β-5 structures, which indicate that the lignin degradation severely happened during the acid modification of hydrotropic lignin (Gabov *et al.* 2014). Furthermore, the condensation reactions of lignin occurred in the three different hydrotropic pretreatments. As indicated before, the condensation reaction could prevent β-ether bond cleavage and lower the amount of degradation products (Sixta *et al.* 1992; Li *et al.* 2000).

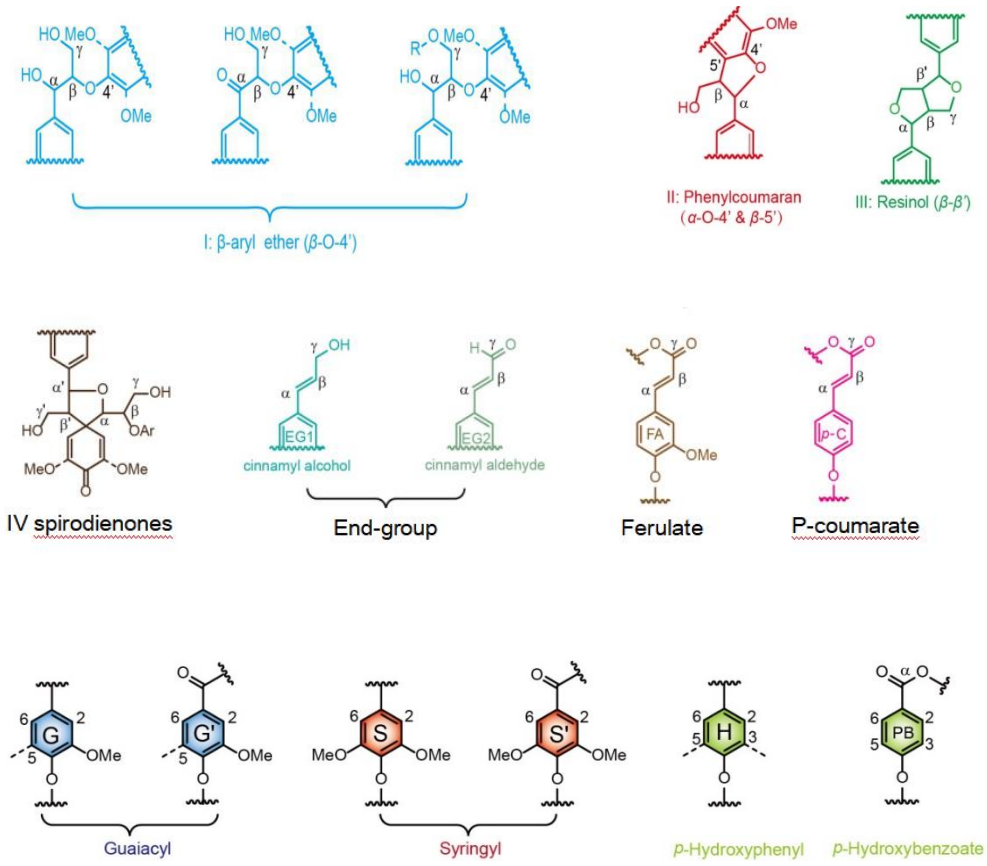

Fig. 4. Main structures present in three types of hydrotropic lignins: (I) β-aryl ether linkages; (II) phenylcoumaran structures formed by α-O-4' and β-5' linkages; (III) resinol structures formed by β-β' and α-O-γ' linkages; (IV) spirodienone structures formed by β-1' and α-O-α' linkages; (EG1) cinnamyl alcohol end-groups; (EG2) cinnamyl aldehyde end-groups; (S) syringyl units; (S') Cα-oxidized syringyl units; (G) guaiacyl units; (G') Cα-oxidized guaiacyl units; (H) p-hydroxyphenyl unit; (PB) p-hydroxybenzoate substructures; (p-CA) p-coumarates; and (FA) ferulates

According to the integral results of the aromatic region of the HSQC spectra, three types of lignin showed differences in the ratio of the structure units. The ratio of S/G/H in Lig-sxs was 65.5:25.8:8.7, the ratio of S/G/H in Lig-SN was 67.8:22.6:9.6, and the ratio of S/G/H in Lig-SF was 66.2:28.3:5.5. The S units of Lig-SN were clearly more than that of Lig-sxs and Lig-SF because the S units were easily released from the plant cell walls under the alkaline condition (Li *et al.* 2016a). Additionally, a little amount of the end group in the side chain of lignin (EG1and EG2) was detected in all lignin samples. This probably because the recovered lignin was long chain structures rather than oligomers having lower molecular weight.

CONCLUSIONS

1. Hydrotropic pretreatment as an efficient and environmentally benign technology was modified by alkali and acid addition for improvement of the enzymatic digestibility of eucalyptus.
2. Lignin removal from the fiber cell wall by an acid-hydrotropic treatment was most prominent. However, lignin on the fiber surface was displaced more noticeably by alkali and acid hydrotropic pretreatment than the conventional hydrotropic pretreatment. As a result, the enzyme absorption capacity of the pretreated substrates was considerably improved by the modified hydrotropic pretreatments.
3. The difference in the chemical properties of lignin recovered from the spent solution was observed, which could help to understand the fractionation mechanism of different hydrotropic pretreatments. An alkali-hydrotropic pretreatment could supply the lignin containing higher amounts of phenolic groups. However, the maximum glucose yield was observed from the acid hydrotropic pretreatment. Thus, according to the research purpose, different hydrotropic pretreatment conditions could be considered.

ACKNOWLEDGMENTS

This study was financially supported by the Chinese National Natural Science Foundation (31600469), the PhD Start-up Fund of the Natural Science Foundation of Guangdong Province, China (2017A030332343), and the State Key Laboratory of Pulp and Paper Engineering (201711).

REFERENCES CITED

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Anal. Biochem.* 72(1-2), 248-254. DOI: 10.1016/0003-2697(76)90527-3

Cao, W., Sun, C., Liu, R., Yin, R., and Wu, X. (2012). "Comparison of the effects of five pretreatment methods on enhancing the enzymatic digestibility and ethanol production from sweet sorghum bagasse," *Bioresource Technol.* 111, 215-221. DOI: 10.1016/j.biortech.2012.02.034

Gabov, K., Gosselink, R. J. A., and Smeds, A. I. (2014). "Characterization of lignin extracted from birch wood by a modified hydrotropic process," *J. Agr. Food Chem.* 62(44), 10759-10767. DOI: 10.1021/jf5037728

Gabov, K., Oja, T., Deguchi, T., Fallarero, A., and Fardim, P. (2017). "Preparation, characterization and antimicrobial application of hybrid cellulose-lignin beads," *Cellulose* 24(2), 641-658. DOI: 10.1007/s10570-016-1172-y

Goldschmid, O. (1954). "Determination of phenolic hydroxyl content of lignin preparations by ultraviolet spectrophotometry," *Anal. Chem.* 26(9), 1421-1423. DOI: 10.1021/ac60093a009

Gromov, V., and Pormale, M. (1961). "Hydrotropic and alkaline pulping of deciduous woods with simultaneous hydrogenation of lignin. II. Fractionation of the products of hydrogenation of lignin and separation of phenols," *Latv. PSR Zinat. Akad. Vestis 1*. 4, 85-92.

Hall, M., Bansal, P., Lee, J. H., Realff, M. J., and Bommarius, A. S. (2010). "Cellulose crystallinity-a key predictor of the enzymatic hydrolysis rate," *FEBS J.* 277(6), 1571-1582. DOI: 10.1111/j.1742-4658.2010.07585.x

Jäger, G., Wu, Z., Garschhammer, K., Engel, P., Klement, T., Rinaldi, R., Spiess, A. C., and Büchs, J. (2010). "Practical screening of purified cellobiohydrolases and endoglucanases with α-cellulose and specification of hydrodynamics," *Biotechnol. Biofuels* 3(1), 18-30. DOI: 10.1186/1754-6834-3-18

Kalninsh, A. I., Surna, J. A., and Zoldners, J. A. (1962). "The utilization of hydrotropic lignin for the preparation of molding powders," *Lesokhoz. Probl. Khim. Drev., Akad. Nauk Latv. SSR 1962*, 24, 65-70.

Korpinen, R., and Fardim, P. (2006). "Characterisation of sawdust-like wood materials," in: *Proceedings of the 9th European Workshop on Lignocellulosics and Pulp*, Vienna, Austria, pp. 27-30.

Leschinsky, M., Zuckerstätter, G., and Weber, H. K. (2008). "Effect of autohydrolysis of *Eucalyptus globulus* wood on lignin structure. Part 1: Comparison of different lignin fractions formed during water prehydrolysis," *Holzforschung* 62(6), 645-652. DOI: 10.1515/HF.2008.117

Li, H. Y., Wang, C. Z., Chen, X., Cao, X. F., Sun, S. N., and Sun, R. C. (2016a). "Structural elucidation of eucalyptus lignin and its dynamic changes in the cell walls during an integrated process of ionic liquids and successive alkali treatments," *Bioresource Technol.* 222, 175-181. DOI: 10.1016/j.biortech.2016.09.111

Li, S., Lundquist, K., and Westermark, U. (2000). "Cleavage of arylglycerol β-aryl ethers under neutral and acid conditions," *Nord. Pulp Pap. Res. J.* 15(4), 292-299. DOI: 10.3183/NPPRJ-2000-15-04-p292-299

Li, Y., Qi, B., Luo, J., and Wan, Y. (2016b). "Effect of alkali lignins with different molecular weights from alkali pretreated rice straw hydrolyzate on enzymatic hydrolysis," *Bioresource Technol.* 200, 272-278. DOI: 10.1016/j.biortech.2015.10.038

Mou, H. Y., Orblin, E., Kruus, K., and Fardim, P. (2013a). "Topochemical pretreatment of wood biomass to enhance enzymatic hydrolysis of polysaccharides to sugars," *Bioresource Technol.* 142, 540-545. DOI: 10.1016/j.biortech.2013.05.046

Mou, H. Y., Heikkilä, E., and Fardim, P. (2013b). "Topochemistry of alkaline, alkaline peroxide and hydrotropic pretreatments of common reed to enhance enzymatic hydrolysis efficiency," *Bioresource Technol.* 150, 36-41. DOI: 10.1016/j.biortech.2013.09.093

Mou, H. Y., Heikkilä, E., and Fardim, P. (2014a). "Topochemistry of environmentally friendly pretreatments to enhance enzymatic hydrolysis of sugar cane bagasse to fermentable sugar," *J. Agr. Food Chem.* 62(16), 3619-3625. DOI: 10.1021/jf500582w

Mou, H. Y., Li, B., and Fardim, P. (2014b) "Pretreatment of corn stover with modified hydrotropic method to enhance enzymatic hydrolysis," *Energ. Fuel.* 28(7), 4288-4293. DOI: 10.1021/ef5001634

Mou, H. Y., and Wu, S. (2016). "Comparison of organosolv and hydrotropic pretreatments of eucalyptus for enhancement of enzymatic hydrolysis," *Bioresource Technol.* 220, 637-640. DOI: 10.1016/j.biortech.2016.08.072

Mou, H. Y., Fardim, P., and Wu, S. (2016). "Chapter 13-A novel green biomass fractionation technology: Hydrotropic pretreatment," in: *Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery*, S. I. Mussatto (ed.), Elsevier, Amsterdam, Netherlands, pp. 281-313. DOI: 10.1016/B978-0-12-802323-5.00013-X

Mou, H. Y., and Wu, S. (2017). "Comparison of hydrothermal, hydrotropic and organosolv pretreatment for improving the enzymatic digestibility bamboo," *Cellulose* 24(1), 1-10. DOI: 10.1007/s10570-016-1117-5

Mou, H. Y., Wu, S., He, M., Liu, H., Huang, Q., and Xu, C. (2018). "Study of the difference between enzyme adsorption onto hydrotropic and alkali lignin separated from Eucalyptus and Bamboo," *BioResources* 13(1), 1441-1456. DOI: 10.15376/biores.13.1.1441-1456

Procter, A. R. (1971). "Review of hydrotropic pulping," *Pulp Pap. Mag. Can.* 72(8), 67-74.

Rahikainen, J. L., Evans, J. D., Mikander, S., Kalliola, A., Puranen, T., Tamminen, T., Marjamaa, K., and Kruus, K. (2013). "Cellulase-lignin interactions – The role of carbohydrate-binding module and pH in non-productive binding," *Enzyme Microb. Tech.* 53(5), 315-321. DOI: 10.1016/j.enzmictec.2013.07.003

Ralph, J., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P. F., Marita, J. M., Hatfield, R. D., Ralph, S. A., Christensen, J. H., *et al.* (2004). "Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids," *Phytochem. Rev.* 3(1-2), 29–60. DOI: https://doi.org/10.1023/B:PHYT.0000047809.65444.a4

Ralph, J., Marita, J. M., Ralph, S. A., Hatfield, R. D., Lu, F., Ede, R. M., Peng, J., Quideau, S., Helm, R. F., Grabber, J. H., *et al.* (1999). "Solution-state NMR of lignin," in: *Advances in Lignocellulosics Characterization*, D. S. Argyropoulos (ed.), TAPPI Press, Atlanta, USA, pp. 55-108.

Raman, G., and Gaikar, V. G. (2003). "Hydrotropic solubilization of boswellic acids from *Boswellia serrata* resin," *Langmuir* 19(19), 8026-8032. DOI: 10.1021/la034611r

Segal, L., Creely, J. J., Martin, A. E., and Conrad, C. M. (1959). "An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer," *Textile Res. J.* 29(10), 786-794. DOI: 10.1177/004051755902901003

Ström, G., and Carlsson, J. (1992). "Wettability of kraft pulps – Effects of surface composition and oxygen plasma treatment," *J. Adhes. Sci. Technol.* 6(6), 745-761. DOI: 10.1163/156856192X01088

Telysheva, G. M., Sergeeva, V. N., and Mozheiko, L. N. (1966). "Alkaline oxidative decomposition of hydrotropic and sulfuric acid lignins," *Latv. PSR Zinat. Akad. Vestis, Kim. Ser.* 6, 720−728.

Toledano, A., García, A., Mondragon, I., and Labidi, J. (2010). "Lignin separation and fractionation by ultrafiltration," *Sep. Purif. Technol.* 71(1), 38-43. DOI: 10.1016/j.seppur.2009.10.024

Wu, B., Zhao, J., and Du, J. (1993). "Determination of phenolic hydroxyl group of lignin by differential ultraviolet spectrophotometry," *Journal of Northeast Forestry University* 21(2), 56-65.

Zakzeski, J., Bruijnincx, P. C. A., and Jongerius, A. L. (2010). "The catalytic valorization of lignin for the production of renewable chemicals," *Chemical Reviews* 110(6), 3552-3599. DOI:10.1021/cr900354u

Zhang, A., Lu, F., and Liu, C. (2010). "Isolation and characterization of lignins from *Eucalyptus tereticornis* (12ABL)," *J. Agr. Food Chem.* 58(21), 11287-11293. DOI: 10.1021/jf103354x

Zoldners, J. A., and Surna, J. A. (1969). "Comparison of the reactivities of hydrotropic, alkali, and dioxane lignins from aspen," *Khim. Drev.* 3, 123-127.

Article submitted: July 4, 2018; Peer review completed: September 9, 2018; Revised version received and accepted: September 21, 2018; Published: September 28, 2018.
DOI: 10.15376/biores.13.4.8578-8592