Effect of Biological Pretreatment with White-rot Fungus *Trametes hirsuta* C7784 on Lignin Structure in *Carex meyeriana* Kunth

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Carex meyeriana Kunth was subjected to biological pretreatment with the white-rot fungus *Trametes hirsuta* C7784, and the structural changes of the lignin were investigated. Results showed that there was a slight decrease in carbohydrate content after pretreatment for 3 weeks, but a noticeable decrease in lignin and carbohydrate contents after 6 weeks. Detail structural analysis of the isolated lignin from the samples revealed that *Carex meyeriana* Kunth lignin consisted mainly of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units with minor *p*-coumarate and ferulate units. The predominant lignin interunits were *β-O-4´* ether linkages, followed by phenylcoumaran, together with a lower portion of resinol and tricin substructures. After pretreatment for 6 weeks, the contents of *β-β´* and *β*-5´ linkages notably decreased, suggesting that the fungus preferentially attacked these linkages. The pretreatment led to an increase of S/G ratio from 0.64 in the control to 0.83 in the sample pretreated for 6 weeks. The comprehensive understanding of the structural changes of lignin offers new insights into the biological degradation of *Carex meyeriana* Kunth by white-rot fungus.

Keywords: White-rot fungus; Biological pretreatment; Lignin structure; Milled wood lignin

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INTRODUCTION

Currently, in response to the depletion and the serious environmental concern of fossil fuel usage, the development and utilization of lignocelluloses for the production of biofuels has become an urgent global priority (Jordan *et al.* 2012; Liu *et al.* 2012; Yang and Wyman 2007). The conversion of lignocelluloses to bioethanol involves the hydrolysis of carbohydrates to free sugars, and then the fermentation of these sugars into ethanol. Enzymatic hydrolysis is a key step in ethanol production from low-cost ligno-cellulosic materials (Pu *et al.* 2007; Zhao *et al.* 2008). However, the natural recalcitrance of lignocelluloses including the lignin and hemicelluloses contents, the structure of lignin, cellulose crystallinity, porosity of the cellulose, *etc*., are key factors inhibiting the enzymatic conversion of this raw material into fermentable sugars (Huang *et al.* 2011; Sun and Cheng 2002). Generally, it is perceived that the lower lignin content a plant biomass has, the higher the bioavailability of the substrate for bioethanol generation (Pu *et al.* 2013). Besides lignin content, lignin composition, its chemical structures, and lignin-carbohydrate complex (LCC) linkages presented in biomass, *etc*., are key factors impacting its digestibility for fermentable sugars productions (Studer *et al.* 2011). Therefore, pretreatment of lignocelluloses is of vital importance to improve the

enzymatic hydrolysis of carbohydrates. Some chemical and physico-chemical pretreatment processes, such as acid pretreatment, alkaline pretreatment, steam explosion, and ammonia fiber explosion, have been developed and widely used; however, these processes generally require high temperature and operating pressure (Eggeman and Elander 2005; Huang *et al.* 2011; Teymouri *et al.* 2005). The chemical approaches of pretreatment are often limited by the lack of selectivity and specificity. Although they are effective in most cases, the structural units of the substance are damaged, and undesired compounds are usually formed.

Biological pretreatment using microorganisms such as brown-, white- and soft-rot fungi has been employed to degrade lignin and carbohydrates in lignocelluloses. White-rot fungi, which can degrade lignin, are usually used in biological pretreatment of lignocelluloses (Bugg *et al.* 2011; Hakala *et al.* 2005). Different white-rot fungi vary greatly with respect to the relative rates at which they degrade lignin and carbohydrates in lignocelluloses (Leonowicz *et al.* 1999). Selective (or preferential) white-rot fungi decay hemicelluloses and lignin first, resulting in defibrillation through dissolution of the middle lamella. In contrast, non-selective (or simultaneous) white-rot fungi remove lignin and structural carbohydrates at a similar rate, resulting in homogeneous cell wall decay (Pandey and Pitman 2003). In the pretreatment, a mixture of enzymatic complexes secreted by fungus, mainly peroxidases and laccase, are capable of opening the complex structure of lignin by cleaving the chemical bonds between units (Lee *et al.* 2007). Biological pretreatment can enhance the enzymatic hydrolysis of lignocelluloses and has the advantages of mild conditions and low energy consumption (Amirta *et al.* 2006; Sun and Cheng 2002). It allows for loosening the structure of lignocelluloses without utilizing and releasing environmentally harsh chemicals. In fact, the biological pretreatment has been used to pretreat wood in the pulping process to save energy as well as to improve the pulp quality (Lei *et al.* 2012; Masarin *et al.* 2009; Vicentim *et al.* 2009). In addition, the utilization of biological pretreatment for the production of biofuels has received increasing attention nowadays. It has been reported that the pretreatment of wheat straw with *Pleurotus ostreatus* for 5 weeks caused 35% of the raw material to be converted into reducing sugars (Hatakka 1983); similar results have also been obtained in the pretreatment with *Phanerochaete sordida* (Shafizadeh and Bradbury 2003) and *Pycnoporus cinnabarinus* (Okano *et al.* 2005) for 4 weeks. After pretreatment by *Irpex lacteus* CD2 for 20 days, the highest saccharification ratio of corn stover reached 66.4% (Xu *et al.* 2010). In the pretreatment of cornstalks, the maximum hydrolysis yield of glucan of 82% was obtained with a versatile lignin-degrading fungus, *Irpex lacteus,* for 28 days (Du *et al.* 2011). The fungus pretreatment of cornstalk with *Phanerochaete chrysosporium* for enzymatic saccharification led to an increase of maximum enzymatic saccharification by 20.3% over the control (Zhao *et al.* 2012). The white-rot fungus *Trametes velutina* D10149 was use to pretreat poplar (Yang *et al.* 2013). The biological pretreatment for 16 weeks induced an increase of the digestibility of cellulose of 19.5%. The white-rot fungus *Phlebia* sp. MG-60 was found as a good producer of ethanol from lignocelluloses (Kamei *et al.* 2012b). Kamei *et al.* (2012a) proposed a process of unified aerobic delignification and anaerobic saccharification and fermentation of wood by white-rot fungus *Phlebia* sp. MG-60, in which the ethanol yield of 43.9% was achieved after 20 d.

However, there is a little information available on the decay of lignocelluloses by the white-rot fungus *Trametes hirsuta* C7784, and the structural modification of lignin during the fungus degradation has not been reported. Therefore, the aim of the present

study was to explore the consequences of the lignin degradation during the biological treatment of *Carex meyeriana* Kunth, a perennial herb plant with short renewable period and wide distribution in the northeast of China (Mao *et al*. 2011). To acquire in-depth understanding of the biological treatment process, milled wood lignin (MWL) was extracted from the untreated and biodegraded *C. meyeriana*, and the lignin obtained was characterized by sugar component, molecular weight, Fourier transform infrared (FTIR) and heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy. The knowledge of the biodegradation of *C. meyeriana* will be helpful in developing an efficient pretreatment process to overcome the recalcitrance of the lignocellulose in order to obtain fermentable sugars

EXPERIMENTAL

Materials

Carex meyeriana Kunth

Carex meyeriana Kunth, a perennial herb plant, was collected from a farm in Heilongjiang province, China. It was dried under sunshine and ground with a plant crusher to a sample size of 0.18 to 0.25 mm. The ground samples were dewaxed with tolubene-ethanol (2:1, v/v) for 6 h (note: the wax yield was 8.82% of the initial dried material) and oven-dried at 50 °C for 16 h before use.

Microorganism and inoculums preparation

White rot fungus, *T. hirsuta* C7784, was isolated from a live hardwood in Guangdong province, China. The fungus was maintained on 2% (w/v) malt extract agar (MEA) plates at 4 °C in a laboratory. Then the fungus was activated on 100 mL basic medium (g/L: glucose 20, yeast extract 5, K_2HPO_4 1, $MgSO_4$ 0.5, VB_1 0.01), and the mycelial pellets were shaken on a rotary shaker at 28 °C with a speed of 150 rpm for 5 days. After the addition of 100 mL distilled water, the pellets were blended for 30 s at 5000 rpm to obtain homogenate inoculums.

Biological pretreatment of Carex meyeriana

A total of 10 g dewaxed *Carex meyeriana* and 25 mL distilled water were placed in a 250 mL Erlenmeyer flask. The sterilization of the samples was carried out in an autoclave at 121 °C for 20 min., and then 5 mL fungus homogenate was inoculated into the Erlenmeyer flask. The strain cultures were conducted at 28 °C for 3 and 6 weeks, respectively.

Isolation of MWL

MWL of the pretreated and untreated samples was prepared according to the method proposed by Björkman (1954). The ground plant samples were ball-milled in a vibration ball mill for 6 h. Subsequently, the materials were extracted in dioxane/water (96/4, v:v) with a solid-to-liquid ratio of 1:10 (g/mL) for 12 h, and this extraction was repeated four times. The combined extraction liquors were concentrated to ~50 mL by rotary evaporation. The crude lignin was dissolved in acetic acid-water (9/1, v/v) and precipitated into water. The precipitated lignin was dissolved in 1,2-dichloroethane/ ethanol (10 mL; 2:1,v/v), and precipitated into ether (200 mL). Then the lignin was

freeze-dried before analysis. The lignin isolated from the control and the samples pretreated for 3 and 6 weeks were labeled as L0, L1, and L2, respectively.

Analytical Methods

The chemical composition of the specimens was determined according to the National Renewable Energy Laboratory procedure (Sluiter *et al.* 2008). All standard chemicals, such as the monosaccharide used as an internal standard, were chromatographic grade, and other chemical agents were analytical grade.

FTIR spectra were recorded on a FTIR microscope (Thermo Scientific IN 10) under liquid nitrogen condition with a sample holder. Thirty-two scans were taken of each sample, and the data were collected from 4000 to 700 cm^{-1} at a resolution of 2 cm^{-1} in the transmittance mode.

The molecular weights of the lignin fractions were determined by gel permeation chromatography (GPC, Agilent 1200, USA) with a refraction index detector on a PL-gel 10 μm Mixed-B 7.5 mm ID column, calibrated with PL polystyrene standards (peak average molecular weights of 435,500, 66,000, 9200, and 1320 g mol^{-1}, Polymer Laboratories Ltd.). Samples of 2 mg were dissolved in 1 mL tetrahydrofuran, and 20 μL of this solution were injected. The column separation was operated at ambient temperature and eluted with tetrahydrofuran at a flow rate of 1 mL/min.

HSQC experiment was also obtained on a Bruker AVIII 400 MHz spectrometer after 128 scans with 25 mg sample dissolved in 0.5 mL d_6-DMSO. The spectral widths were 2200 and 15400 Hz for the ^{1}H- and ^{13}C-dimensions, respectively. The number of collected complex points was 1024 for ^{1}H-dimension with a relaxation delay of 1.5 s. The number of scan was 128, and 256 time increments were always recorded in ^{13}C-dimension. The $^{1}J_{C\text{-}H}$ used was 146 Hz. Prior to Fourier transformation, the data matrixes were zero filled up to 1024 points in the ^{13}C-dimension.

Derivatization followed by reductive cleavage (DFRC) was performed according to the developed protocol (Lu and Ralph 1997a, b, 1998). The degraded monomers from lignin samples were dissolved in methylene chloride and determined by GC-MS analysis (Agilent 7890A/5975C) under the following conditions: column 0.25 mm × 30 m HP-5MS; He carrier gas; injector 250 °C, initial column temperature 160 °C, kept for 1 min. and ramped at 10 °C/min to 300 °C, held 5 min.; flame ionization detector (FID), and 300 °C.

RESULTS AND DISCUSSION

Chemical Composition of *Carex meyeriana* Kunth

To investigate the effect of the fungus *T. hirsuta* C7784 on the degradation of lignocellulose, *Carex meyeriana* was subjected to pretreatment for relative long periods of 3 and 6 weeks, respectively. This is because previous studies have shown that a short-time bio-treatment yielded little effect on lignocelluloses (Hakala *et al.* 2004; Yu *et al.* 2010). Table 1 illustrates the chemical composition changes of the pretreated sample as compared to the untreated *C. meyeriana*. For the sample pretreated for 3 weeks, there was no major changes in the chemical composition as evidenced by a insignificant decrease in cellulose and hemicellulose contents. This observation was in agreement with a previous study in which the chemical components of corn stalk were not significantly change after the pretreatment with fungus *Irpex lacteus* CD2 for 15 days (Yu *et al.* 2010). After the

biological treatment for 6 weeks, the lignin content of the sample decreased as compared to the untreated sample. In the pretreatment process, approximately 12% of the lignin was removed. However, during the degradation process, the contents of cellulose and hemicellulose decreased by approximately 22% and 13%, respectively. This suggested that the white-rot fungal partially degraded lignin but also induced the degradation of cellulose and hemicelluloses. This is mainly because the degradation of lignocellulose was governed by a variety of factors including the characteristics of the substrate and the enzyme system of the fungus (Sharma and Arora 2010a, b). Lignin and hemicellulose are entangled with one another, and these entangled materials bounded cellulose microfibrils according to the Kerr-Goring cell wall model (Kerr and Goring 1975). The simultaneous removal of carbohydrates during the pretreatment was also attributed to the cellulase and hemicellulase produced by the white-rot fungus in addition to ligninolytic enzymes (laccase, manganese peroxidase, and lignin peroxidase) (Arora *et al.* 2011). It should be noted that there were also some carbohydrates degraded in the current study, thus further optimization of the fungus pretreatment is needed to improve the selectivity.

Table 1. Chemical Composition of *Carex meyeriana* before and after the Fungal Pretreatment

Solid Sample [a]	Chemical Composition (%)		
	Cellulose	Hemicellulose	Lignin [b]
S0	32.2	31.2	21.2
S1	31.3	27.3	21.2
S2	25.0	16.0	18.7

[a] S0, S1, and S2 correspond to the control and the samples pretreated for 3 and 6 weeks.
[b] Lignin presents for the sum of acid soluble lignin and acid insoluble lignin.

Chemical Composition of Milled Wood Lignin

MWL is considered a lignin preparation that is most representative of the native lignin in lignocellulose despite its low yield and some possible modification. In this work, the MWL isolated from the untreated and the pretreated samples were comprehensively analyzed to obtain insights into the structural modifications during the biological pretreatment. Sugar analysis of the isolated lignin was performed and the results are listed in Table 2. In all cases, glucose and xylose were the predominant sugars followed by arabinose and galcose.

Table 2. Sugar Composition of the Isolated Lignin from *Carex meyeriana* before and after the Fungus Pretreatment

Lignin Sample *	Sugar Composition (%)							
	Ara	Gal	Glc	Xyl	Man	Glc A	Gal A	Total
L0	0.19	0.16	1.04	0.69	0.03	0.00	0.04	2.15
L1	0.38	0.17	0.59	1.73	0.03	0.04	0.04	2.98
L2	0.20	0.12	0.40	0.94	0.02	0.02	0.02	1.72

* L0, L1, and L2 correspond to the lignin extracted from control and the samples pretreated for 3 and 6 weeks.

In addition, negligible amounts of glucuronic and galacturonic acids were also detected. These results suggested that arabinoxylan was the main sugar impurity in lignin which was linked with arabinose side chains of xylan by ester/ether bonds in grass (Buranov and Mazza 2008). Overall, the sugar content of the lignin isolated was below 3%, suggesting that the isolated lignin contained a relatively low amount of impurities.

Molecular Weight Analysis

The weight-average and number-average molecular weights, as well as the polydispersity, of the lignin specimens were estimated by the curves of gel permeation chromatography (GPC). The results are given in Table 3. Since the curves were calibrated by standards of polystyrene, the data for the molecular weight cannot be considered absolute values but can be used for comparison. There was a slight increase of the weight average molecular weight from 2770 g/mol in L0 to 3010 g/mol in L1, but a noticeable decrease to 2230 g/mol in L2. Since the lignin fragment was removed in the intial stage, the extracted lignin L1 had a relatively high average molecular weight, whereas the whole lignin was degraded for a longer pretreatment period, resulting in a decrease of the average molecular weight. The low value of the polydispersity of all the lignin preparations (below 2.0) displayed a narrow molecular weight-average distribution.

Table 3. Weight (M_w) and Number Average (M_n) Molecular Weights and Polydispersity (M_w/M_n) of the Isolated Lignin from *Carex meyeriana* before and after the Fungus Pretreatment

Lignin Sample *	Mw (g/mol)	Mn (g/mol)	Polydispersity
L0	2770	1580	1.76
L1	3010	1630	1.84
L2	2230	1200	1.85
* Corresponding to the labels in Table 2			

FTIR Spectral Analysis

Figure 1 illustrates the FTIR spectra of the lignin isolated from the pretreated samples as compared to the untreated *Carex meyeriana*; the IR transmittance peaks are assigned to functional according to a literature report (Faix 1991). Clearly, the functional groups of lignin changed only slightly after pretreatment for 3 weeks, whereas the changes became more noticable after 6 weeks of pretreatment. Although this method did not provide quantitative insights into the functions groups, it yielded characteristic signals corresponding to lignin functional groups.

All lignin samples presented a broad band at 3379 cm^{-1} that was attributed to HO– stretching. The peaks at 2924 and 2852 cm^{-1} are assigned to CH stretching in CH_2 and CH_3, respectively. The peak at 1653 cm^{-1} is assigned to the C=O stretching in conjugated *p*-substituted aryl ketones, whereas the signal at 1730 cm^{-1} is assigned to the C=O stretching in carbonyl, unconjugated ketones, and esters; this signal notably decreased in the sample after pretreatment for 6 weeks. This phenomenon was different from the results previously reported by Yang *et al.* (2010), who found that there was an increase of both the un-conjugated and conjugated C=O groups after fungus pretreatment.

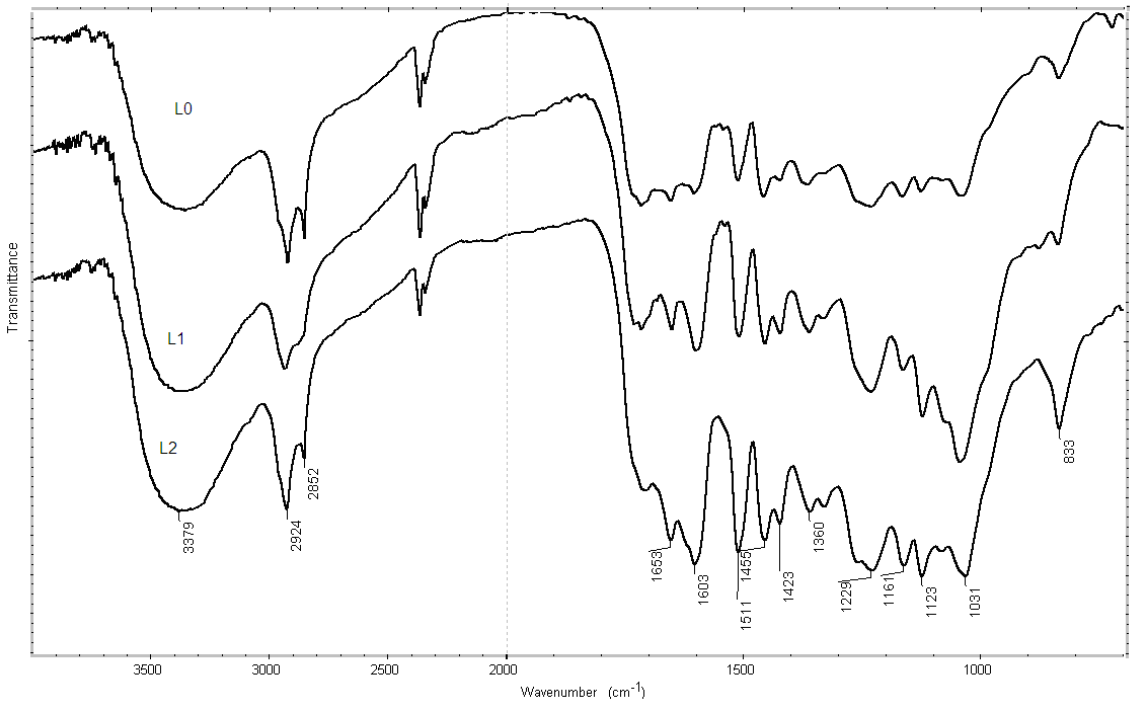

Fig. 1. FTIR spectra of the isolated lignin from *Carex meyeriana* before and after fungus pretreatment

Table 4. Assignment of Main $^{13}C–^{1}H$ Correlation Signals in HSQC Spectra of Lignin from *Carex meyeriana*

Label	δ_C/δ_H (ppm)
-OCH3	55.7/3.43
A_γ	60.2/3.72
I_γ	61.3/4.09
A_γ'	62.7/3.83-4.30
B_γ	62.8/3.67
C_γ	71.0/3.81 and 4.17
A_α/A_α'	71.8/4.86
$A_{\beta(G)}'$	83.6/4.27
$A_{\beta(S)}$	86.4/4.11
B_α	87.3/5.43
F_8	94.5/6.56
F_6	98.8/6.23
F_3	104.1/7.31
$S_{2,6}$	103.9/6.60
$S_{2,6'}$	105.5/6.90 and 106.0/7.02
FA_2	110.8/7.20
G2	111.1/6.90
PCA_β and FA_β	113.5/6.25
$PCA_{3,5}$	115.0/6.77
G_5/G_6	114.0/6.89 and 119.5/6.79
FA_6	123.4/7.10
$H_{2,6}$	127.8,7.23
$PCA_{2,6}$	131.0/7.49
PCA_α and FA_α	144.6/7.41

The signal at 1511 cm^{-1} corresponds to the aromatic skeletal vibrations; the signal at 1460 cm^{-1} corresponds to the C-H deformations, as well as asymmetrical in $–CH_3$ and $–CH_2–$; and the signal at 1423 cm^{-1} corresponds to aromatic ring vibrations. This indicated that the core structures of lignin were not broken after the pretreatment. The signal at 1360 cm^{-1} corresponds to the aliphatic C-H stretching in CH_3. The peak at 1161 cm^{-1}, corresponding to C=O in ester group (conjugated), gave typical signals of phenylpropane structures of lignin. This was also evidenced by the signal at 833 cm^{-1}, which was assigned to the C-H out of plane in positions of 2 and 6 of sryingial (S) lignin units and all positions of H units. The signal at 1320, 1229, and 1123 cm^{-1} are assigned to S lignin units, whereas guaiacyl (G) lignin units absorb at 1267 cm^{-1}. The intensity of the signals of G and S units changed after the pretreatment, indicating the change of the relative ratio of the lignin units, which was quantified by the data shown in Table 4.

HSQC Spectral Analysis

Considering the significant effects of the biological pretreatment on *Carex meyeriana*, further structural analysis of the sample was focused on the sample pretreated for 6 weeks as compared to the untreated control. HSQC NMR spectroscopy is a powerful technique to indentify the primary structures, structural units and inter-unit linkages of lignin (Lewis *et al.* 2006). HSQC spectra of the lignin isolated from the pretreated and the untreated samples were recorded (as shown in Fig. 2), the identified substructures are illustrated in Fig. 3, and the cross-signals were assigned as illustrated in Table 4 according to reported literature sources (Abdelkafi *et al.* 2011; del Rio *et al.* 2012; Villaverde *et al.* 2009).

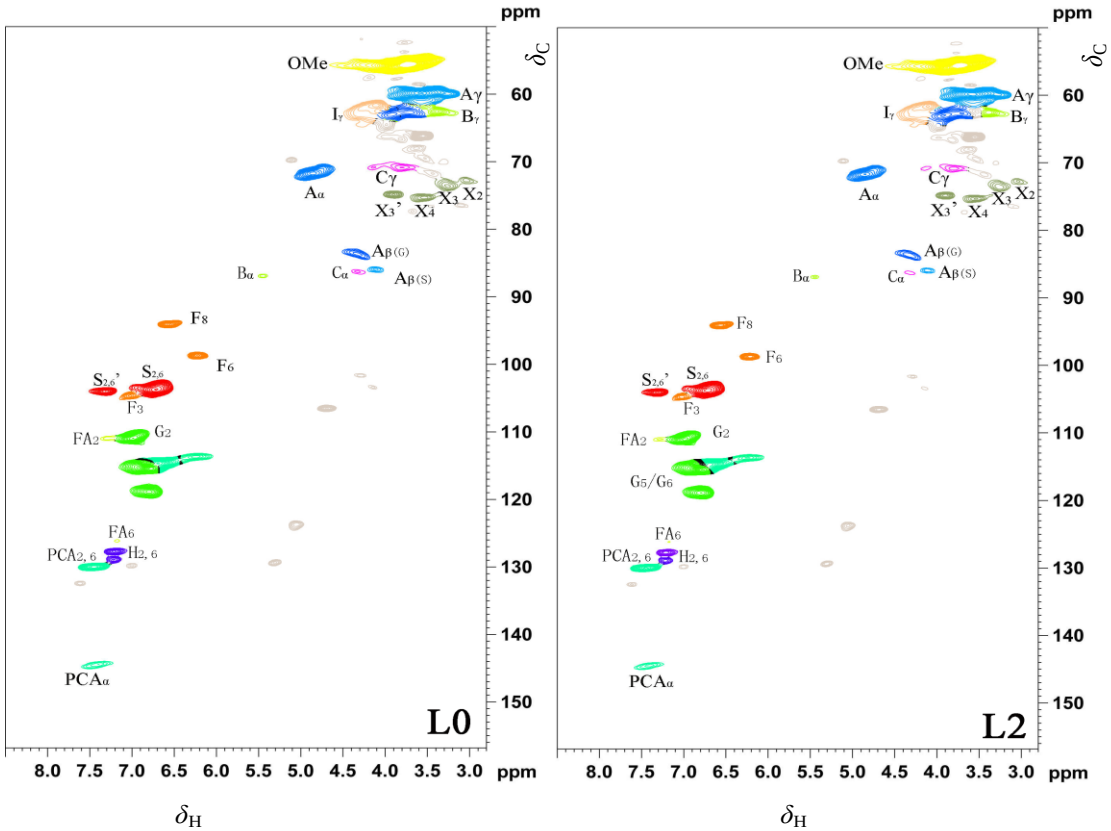

Fig. 2. HSQC spectra of the isolated lignin from *Carex meyeriana* before and after fungal pretreatment

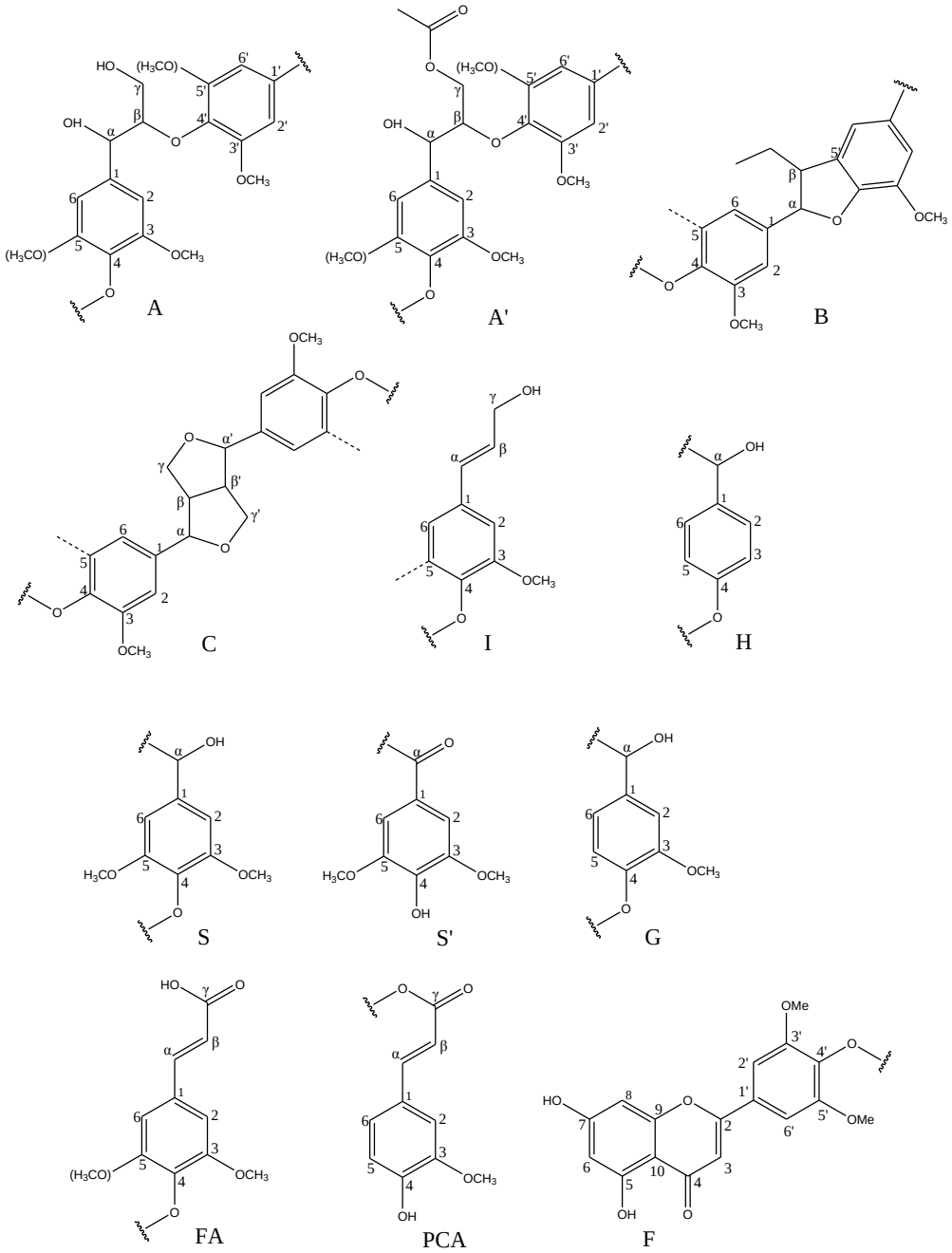

Fig. 3. Main substructures of lignin involving different side-chain linkages and aromatic units identified by HSQC: (A) *β-O-4′* linkages; (A′) *β-O-4′* linkages with a carbonyl group at C_{γ}; (B) phenylcoumaran structures formed by *β-5′/α-O-4′* linkages; (C) resinol structures formed by *β-β′/α-O-γ′/γ-O-α′* linkages; (G) guaiacyl unit; (S) syringyl unit; (S′) oxidized syringyl unit with a carbonyl group at C_{α} (phenolic); (H) *p*-hydroxyphenyl unit; (I) cinnamyl alcohol end-groups; (F) 5,7,4'-trihydroxy-3',5'-dimethoxyflavone; (FA) ferulates; (PCA) *p*-coumarates

In the side chain region, *β-O-4´* substructures (**A**) were observed with their C_{α}-H_{α} and C_{γ}-H_{γ} correlations at 71.8/4.86 and 60.2/3.72, with C_{β}-H_{β} correlations at 83.6/4.27 and 86.4/4.11 in G and S type units. The *β-O-4′* linkages with a carbonyl group at C_{γ} were identified by the C_{γ}-H_{γ} correlations at 62.7/3.83-4.30. In addition to *β-O-4´* substructures, other linkages were also identified. The signals for phenylcoumaran (**B**, *β-5′/α-O-4′*

linkages) were observed with their C_α-H_α and C_γ-H_γ correlations at 87.3/5.43 and 62.8/3.67, respectively. Resinol substructures (**C,** *β-β´/α-O-γ´/γ-O-α´* linkages) were identified by their double C_γ-H_γ correlations at 71.0/3.81 and 4.17. Tricin substructures (**F**) were identified by their correlations at C_3-H_3, C_6-H_6, and C_8-H_8 at 104.1/7.31, 98.8/6.23, and 94.5/6.56, respectively. In addition, the signals at 72.6/3.16，73.8/3.40，75.4/3.58 are assigned to C_2-H_2, C_3-H_3, and C_4-H_4 in the associated xylan, respectively.

The main correlations in the aromatic region corresponded to the aromatic rings of the lignin units. S units showed a prominent signal for $C_{2,6}$-$H_{2,6}$ correlation at 103.9/6.60, and S units with *α*-oxidized structure were observed by $C_{2,6}$-$H_{2,6}$ correlations at 105.5/6.90 and 106.0/7.02. G units show different correlations for C_2-H_2 (111.1/6.90), C_5-H_5 (111.4/6.89), and C_6-H_6 (119.5/6.79). The presence of *p*-hydroxyphenyl lignin units (H) (*i.e.*, phenylcoumaran) were evidenced by the $C_{2,6}$-$H_{2,6}$ correlations at 127.8/7.23. As typical spectra from grass lignin, signals corresponding to *p*-coumarates (PCA) and ferulates (FA) were also observed. The correlations at 144.6/7.41 and 113.5/6.25 were assigned to the C_α-H_α and C_β-H_β correlations from *p*-coumarates (PCA) and ferulates (FA). In addition, PCA was also evidenced by the $C_{2,6}$-$H_{2,6}$ and $C_{3,5}$-$H_{3,5}$ correlations at 131.0/7.49 and 115.0/6.77, whereas FA shows C_2-H_2 and C_6-H_6 correlations at 110.8/7.20 and 123.4/7.10, respectively. The signals at 128.5/6.44 and 61.30/4.09 were tentatively assigned to C_α-H_α and C_γ-H_γ in cinnamyl alcohol end-groups.

In order to estimate the relative abundance of the main lignin side chains, semi-quantitative analysis of the signals of the spectra were performed according to Wen *et al.* (2013). The abundance of lignin side-chains involved in the main substructure found in the isolated lignin is summarized in Table 5.

In all cases, the *β-O-4´* substructures were the predominant ones that corresponded to 58 per 100 aromatic (Ar) units. After the treatment, the contents of *β-β´* and *β*-5´ linkages notably decreased from 4.2 per 100 Ar units to 0.7 per 100 Ar units, and from 6.9 per 100 Ar units to 3.8 per 100 Ar units, respectively. This suggested that the fungus preferentially attacked *β-β´* and *β*-5´linkages. In a previous study on the chemical structural alterations between corn stover lignin undegraded and degraded by *I. lacteus* CD2, a singnificant decrease of the amount of *β-β´* and *β*-5´linkages was also reported (Yang *et al.* 2010).

The S/G ratio calculated by intergrations from the HSQC spectra, as well as estimated by DFRC, is given in Table 5. DFRC is technique that selectively cleaves *β-O-4´* linkages in lignin and provides phenylpropane (C_3-C_6) type monomers for further identification by GC-MS (Ehara *et al.* 2002; Moon *et al.* 2011).

The S/G ratio results from the NMR presents the ratio of all S and G units in the isolated lignin (condensed and non-condensed), while the S/G ratio results from DFRC presents the ratio of S and G units in non-condensed lignin. As can be seen, *Carex meyeriana* showed predominant G units over S units. With respect to all lignin units, the S/G ratio increased from 0.64 in the control to 0.83 for the sample pretreated for 6 weeks. However, the S/G ratio for the non-condensed etherified lignin units showed a slight decrease from 0.70 for the original sample to 0.62 for the sample pretreated for 6 weeks. This indicated that the S units in the non-condensed etherified lignin were degraded after the pretreatment. The reason for this was that a certain amount of $–OCH_3$ groups were removed from the non-condensed S units to form G units, similar to the case in corn stover degraded by white-rot fungus *Irpex lacteu*s CD2 (Yang *et al.* 2010) .

Table 5. Relative Abundance of Main Inter-Unit Linkages and S/G Ratio of Lignin from *Carex meyeriana* before and after the Fungus Pretreatment

Lignin Sample *	Main Inter-unit Linkages (per 100 Ar)			S/G ratio	
	β-O-4'	*β*-*β*'	*β*-5'	NMR	DFRC
L0	58	4.2	6.9	0.64	0.70
L2	58	0.71	3.8	0.83	0.62
* Corresponding to the labels in Table 2					

It is suggested that there are three type of reactions involved in the degradation of lignin by white-rot fungus: (1) oxidative cleavage of the propyl side chains between *α*- and *β*-carbons to form benzoic acids; (2) cleavage of *β*-aryl ether bonds and modification of side chain of lignin; and (3) oxidative ring opening of aromatic nuclei (Chen and Chang 1985; Yang *et al.* 2010). From the spectral analysis above, it can be concluded that during the biological degradation by white rot fungus *T. hirsuta* C7784, a certain amount of methoxy groups were removed and the main carbon-carbon linkages including *β*-*β*´ and *β*-5´ ones were significantly destroyed. This was in agreement with the results reported by Yang *et al.* (2010), who studied the structure characteristic of lignin in corn stover degraded by white-rot fungs *Irpex lacteus* CD2. The fungal pretreatment caused the changes in the chemical components of *Carex meyeriana* as well as the modifications of the structure of lignin, and is a potential approach to overcome the recalcitrance of the plant cell wall. Further enzymatic hydrolysis of the pretreated sample for the production of glucose, as well as subsequent ethanol production from saccharification, is currently being investigated in our laboratory.

CONCLUSIONS

1. The pretreatment of *Carex meyeriana* Kunth with *T. hirsuta* C7784 for 6 weeks resulted in the removal of 12% of lignin. The lignin with low molecular weight was degraded in the initial stage, whereas the whole lignin was degraded into polymer with lower molecular weight when the treatment was prolonged a longer period.
2. The core substructures of lignin, which were mainly composed of *β*-*O*-4´ ether linkages, followed by phenylcoumaran together with a lower proportion of resinol and tricin groups, were not substantially broken after the pretreatment.
3. After pretreatment for 6 weeks, a certain amount of methoxy groups were removed and the main carbon-carbon linkages, including *β*-*β*´ and *β*-5´, were significantly destroyed. With respect to all lignin units, the G units are more easily degraded than the S units during the biological pretreatment process.

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