# ELUCIDATION OF THE STRUCTURE OF LIGNIN AND LIGNIN CARBOHYDRATE COMPLEX OF GRAMINEAE

LAN YAO, HAITAO YANG, JIAJUE XU, NENG ZHANG, YING CHEN and YIMIN XIE

*Hubei Provincial Key Laboratory of Green Materials for Light Industry, Hubei University of Technology, Wuhan, China*

✉ *Corresponding author: Haitao Yang, pphtyang1979@aliyun.com; Yimin Xie, ppymxie@scut.edu.cn*

*Received* September 3, 2013

In order to analyze the structure of lignin and lignin-carbohydrate complex of gramineae, $^{13}C$ labeled coniferin was injected into living rice stalk. Milled wood lignin (MWL) and lignin carbohydrate complex (LCC) were extracted from rice stalk by the Björkman method. From the analysis of FT-IR and $^{13}C$-NMR spectra of MWL and LCC, it was found that the main linkage between lignin and polysaccharide was the benzyl ether bond. It was also possible that they were connected by an ester bond and an acetal bond. The main lignin structures were β-O-4, β-β, β-5 and β-1, with minor coniferyl alcohol. Then, LCC was treated by cellulase and hemicellulase to get enzyme degraded LCC (EDLCC). The parts dissolved and not dissolved in water were called EDLCC-WS and EDLCC-IS, respectively. From FT-IR and $^{13}C$-NMR analyses of EDLCC-WS and EDLCC-IS, it was concluded that the linkages between lignin and carbohydrate included a benzyl ether bond, an ester bond and an acetal bond.

***Keywords***: lignin, lignin-carbohydrate complex, 13C-NMR, isotopic tracer

## INTRODUCTION

Second-generation bioethanol is produced from lignocellulosic biomass, such as agriculture wastes, forest wastes and energy crops. China produces millions of tons of agriculture wastes every year, including 22 million tons of corn stover. It is feasible and important to make use of agriculture wastes to produce bioethanol.[1-2] As one of the main components of lignocellulosic biomass, lignin is connected tightly with cellulose and hemicelluloses to form the lignin-carbohydrate complex (LCC). It has a great effect on the enzymatic hydrolysis as it shields cellulose from enzymatic hydrolysis and fermentation.[3-4]

A lot of research has been focused on the structural character of lignin and LCC.[5-7] To analyze the structure of lignin and LCC more accurately, it is feasible to use non-destructive analytical methods such as NMR.[8-10] From NMR analysis, important results have been found: for example that acetylated 4-O-methylglucuronoxylan is the main carbohydrate associated with lignins; acetyl groups frequently acylate the C2 and C3 positions in poplar wood; uronic acid residue is attached to the γ position of the side chain in the ester lignin-carbohydrate linkages in pine; lignin structure (S/G ratio) and lignin content affect the levels of LCCs for transgenic hybrid poplars.

Although $^{13}C$-NMR has been widely used in studying lignin structure, it is difficult to analyze the overlapping signals from lignin and carbohydrates. $^{13}C$-NMR isotopic tracer method combined with $^{13}C$-NMR determination is a useful non-destructive analytical method. The chemical shift of carbon atoms of side chain with $^{13}C$ labeling could be observed clearly from $^{13}C$-NMR to elucidate linkages of lignin and LCC. $^{13}C$ isotope-labeled technology has been used to reveal the chemical bonds between cellulose and dehydrogenation polymer, lignin and xylan of wheat straw. It was found that the linkage between lignin and cellulose was a benzyl ether bond. β-O-4 units were linked with cellulose at the α-position through benzyl ether bonds and acetal bonds. LC bonds between lignin and xylan were at C2 by

γ-ester bonds and C5 position by benzyl ether bonds of xylan.[11-14]

In this study, $^{13}C$ labeled lignin predecessor, i.e. coniferin-[side chain-α-13C] was injected into rice stalk to label the side chain of lignin. Combined with $^{13}C$-NMR spectrum, the linkages at α position of lignin side chain were obtained to study the structure of lignin and lignin carbohydrate complex by FT-IR and $^{13}C$-NMR.

## EXPERIMENTAL

### Method of administration of lignin precursor to rice stalk

A special cultivar of rice plant (*Oryza sativa L.*) planted in the field in March in 2012 was chosen. A mixed solution containing coniferin-[side chain-α-$^{13}C$] (100 mg/20 mL) and coniferin (100 mg/20 mL) was injected into the 2nd, 3rd, and 4th sections of the internodes (starting from the root) of the rice straw within 30 days. After the injection, the wheat straw was allowed to grow for 20 days.

### Preparation of rice stalk powder

After the rice plants matured, the internode parts that had been administered coniferin-[side chain-α-$^{13}C$] and coniferin were collected. They were milled (60~80 mesh), after being air-dried and extracted thoroughly with ethanol-benzen (1/2, v/v) and hot water.

### Preparation of milled wood lignin (MWL) and lignin-carbohydrate complex (LCC)

The rice stalk powder was dried for three weeks *in vacuo* over phosphorus oxide. Afterwards, it was ground for 72 h in a vibration ball mill with water cooling. Then, it was extracted for three times with water-dioxane (4/96, v/v). The MWL and LCC were purified according to the Björkman method.[11,15]

### Preparation of enzyme-degraded LCC residue

LCC (5.0 g) was suspended in an enzyme solution containing 0.4% cellulase (Onozuka RS, 1.036 g, Yakult Pharmaceutical Ind. Co., Nishinomiya, Japan) and 0.4% hemicellulase (from *Aspergillus niger*, 0.01-0.1 U/mg, Sigma) in 0.05 M sodium citrate buffer (pH 4.5). A few drops of toluene were added as preservative. The mixture was agitated gently on a shaker for 48 h at 50 °C. After centrifugation and washing with distilled water, water soluble and insoluble parts were drawn.

#### *Isolation of water soluble part*

The solution of enzyme-degraded LCC was treated by adsorption chromatography on a Toyopearl HW-40F (2.0 cm × 60.0 cm) column. First, distilled water was used as mobile phase. When the water-soluble parts were completely eluted, 50% dioxane solution was selected as the mobile phase. The fragments containing abundant low molecular lignin-carbohydrate complexes were eluted and determined according to the method described by Yang.[11] EDLCC-WS-Intact (0.1407 g) and EDLCC-WS-[α-$^{13}C$] (0.125 g) were obtained from rice stalk-intact and rice straw-[α-$^{13}C$], respectively.

#### *Isolation of water insoluble part*

After being washed with distilled water and air dried, EDLCC-IS-Intact (0.163 g) and EDLCC-IS-[α-$^{13}C$] (0.140 g) were obtained from rice stalk-intact and rice straw-[α-$^{13}C$], respectively.

### FT-IR determination

Infrared spectra were determined using an FT-IR 710 infrared spectrophotometer (Nicolet, Madison, WI). Dried samples of 1-2 mg were milled into powder with a diameter less than 1 mm. A certain amount of the powder was dispersed in spectroscopic grade KBr and subsequently pressed into disks using 10 tons of pressure for 1 min. A total of 100 scans with a 2 $cm^{-1}$ resolution were signal averaged and stored; the wavenumber range scanned was 4000-500 $cm^{-1}$.

### CP/MAS $^{13}C$-NMR determination

The CP-MAS $^{13}C$ NMR spectra were recorded on a VARIAN Infinityplus-400 (Bruker Instrument, Inc., Billerica, MA) equipped with CP-MAS accessories. Dipolar decoupling was systematically used during the acquisition sequence. The samples were spun at a rate of 7 kHz at room temperature, the accumulation of 4331 scans was used to obtain a satisfactory signal to noise ratio. The optimal contact time was 50.0 μs, spectral width 25.0 kHz, acquisition time 3.0 ms.

### $^{13}C$ NMR spectroscopy

Both water-soluble and water-insoluble samples (100 mg) were dissolved in DMSO-d6 and put in a ϕ 5 mm probe tube. The $^{13}C$ NMR spectra were recorded on a BRUKER Advance-600 NMR spectrometer at 150MHz at 50 °C. The pulse angle was 90° with a 1.75 s pulse delay time. A total of 20,000 scans were done.

## RESULTS AND DISCUSSION

### Effect of injection of lignin precursor on rice stalk

He *et al.*[16-17] found that after injection of coniferin-[aromatic ring-2-3H] into living rice, a large amount of radioactivity was transferred to the guaiacyl of lignin. However, it is possible to cause an abnormal metabolism to the plant by using the $^{13}C$ tracer method, due to the high content of lignin precursor. So, it is necessary to study the effect of coniferin injection on rice stalk. CP/MS $^{13}C$-NMR spectra of rice stalk-intact, rice stalk-coniferin-control and rice stalk-coniferin- [α-$^{13}C$]

are shown in Fig. 1. Comparing the three spectra, no difference is remarked regarding the aliphatic regions (δ0-110 ppm) and the aromatic regions (δ110-160 ppm). The signals between 60 and 110 ppm are assigned to cellulose and those at 21 and 173 ppm to the acetyl groups from hemicelluloses. The small shoulder at 56 ppm belongs to the methoxyl group.[18] Due to the overlapping of the signals from lignin and carbohydrates, the signals of lignin are difficult to find. It could be concluded that the coniferin injection did not interfere with the normal metabolism of rice stalk, which is in agreement with the research results obtained earlier.

**FT-IR determination**

***FT-IR determination of MWL***

The FT-IR spectra of MWL-intact and MWL-[α-$^{13}$C] are presented in Fig. 2. The signals at 1600 cm$^{-1}$, 1513 cm$^{-1}$ and 1423 cm$^{-1}$ assigned to the vibration of the aromatic ring are easy to note.[13] From the intensity of these signals, it can be concluded that most of the sample is lignin. Besides, the signal at 1716 cm$^{-1}$ belongs to non-conjugated C=O stretching vibration.[19] The signal at 1655 cm$^{-1}$ is characteristic of conjugated C=O stretching vibration. The signals at 2940 cm$^{-1}$, 1461 cm$^{-1}$, 1126 cm$^{-1}$ and 834 cm$^{-1}$ are assigned to the C-H stretching vibration of methyl, methylene, methoxyl of aromatic ring. The signals at 1358 cm$^{-1}$ and 1240 cm$^{-1}$ are characteristic of the vibration of hydroxyl and C-O from the aromatic ring. The signal of C-O from ether or primary alcohol is located at 1045 cm$^{-1}$. Comparing the two spectra, it may found that they are quite similar. $^{13}$C-NMR is necessary to further investigate on the difference of the two MWLs.

***FT-IR determination of LCC***

The FT-IR spectra of LCC-intact and LCC-[α-$^{13}$C] are shown in Fig. 3. In the spectra, the signals assigned to the vibration of the aromatic ring at 1515 cm$^{-1}$, 1516 cm$^{-1}$, 1421 cm$^{-1}$ and 1420 cm$^{-1}$ are easy to note. The signals at 3387 cm$^{-1}$ and 3389 cm$^{-1}$ correspond to hydroxyl. The vibrations of alcohol C-O are located at 1077 cm$^{-1}$, 1076 cm$^{-1}$, 1043 cm$^{-1}$ and 1044 cm$^{-1}$. From the analysis, it could be concluded that both lignin and carbohydrate were present in the sample. Besides, the signals from the stretching vibration of C=O of xylan at 1730 cm$^{-1}$ and 1729 cm$^{-1}$ also proved the existence of carbohydrate. $^{13}$C-NMR analysis is necessary to study how lignin is connected with the carbohydrate.

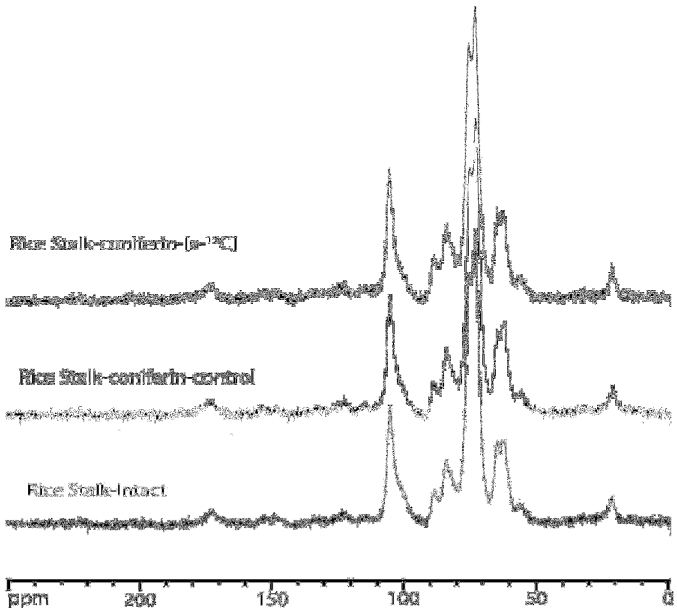


Figure 1: CP/MAS $^{13}$C-NMR of rice stalk injected with coniferin-[α-$^{13}$C], unlabeled coniferin and intact rice stalk

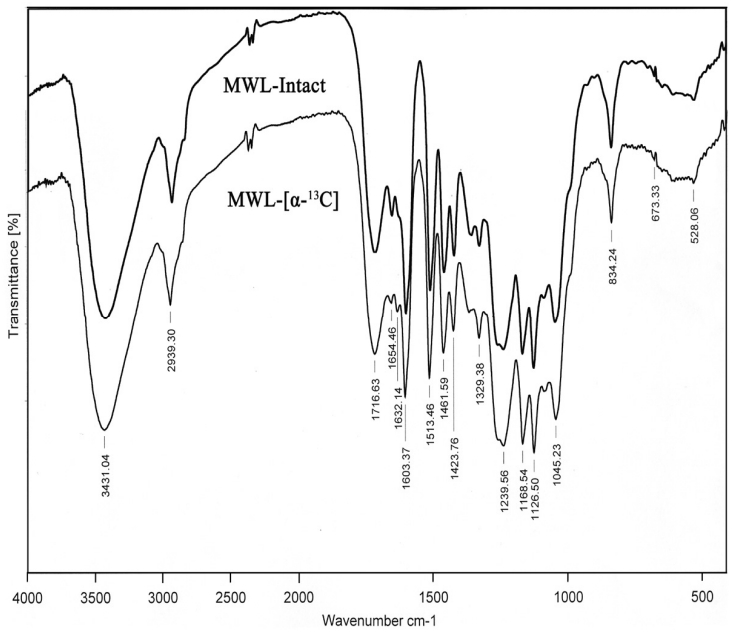


Figure 2: FT-IR spectra of MWL from rice stalk

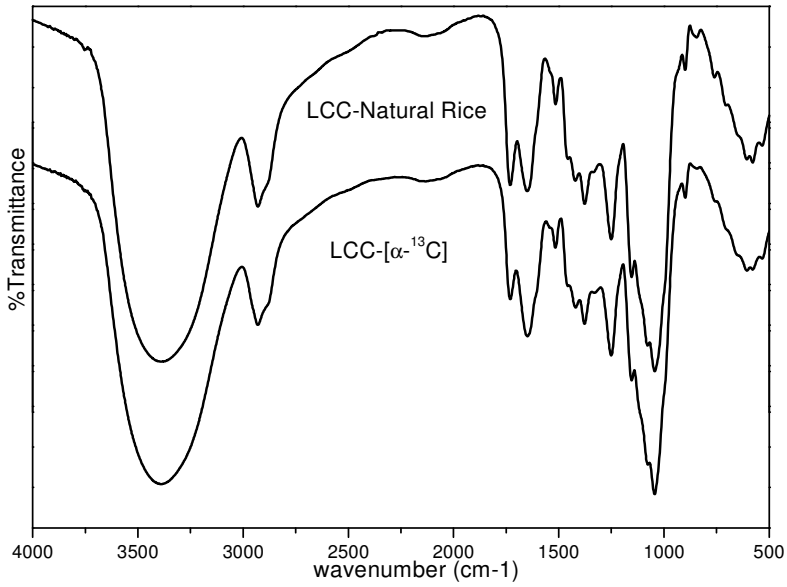


Figure 3: FT-IR spectra of LCCs from rice stalk

***FT-IR determination of EDLCC-IS***

The FT-IR spectra of EDLCC-intact and EDLCC-[α-$^{13}$C] are presented in Fig. 4. By comparing the two spectra with that of LCC, it may be remarked that the intensity of the signals assigned to the vibration of the aromatic ring (1600 cm$^{-1}$, 1515 cm$^{-1}$, 1425 cm$^{-1}$) is stronger after enzymolysis. This is due to the overlapping of the signals belonging to lignin and carbohydrate before enzyme hydrolysis. The vibration signals at 1460 cm$^{-1}$ from the methyl of the aromatic ring and at 1330 cm$^{-1}$ from syringyl also lead to the same conclusion. The disappearance of the signal at 1720 cm$^{-1}$ assigned to the non-conjugated C=O stretching

vibration of xylan is also due to the enzymolysis of LCC. However, the strong signals at 1167 $cm^{-1}$ from the C-O-C vibration of cellulose and hemicelluloses, and at 1035 $cm^{-1}$ from the C-O vibration of polysaccharides demonstrate the existence of carbohydrates in EDLCC-IS.

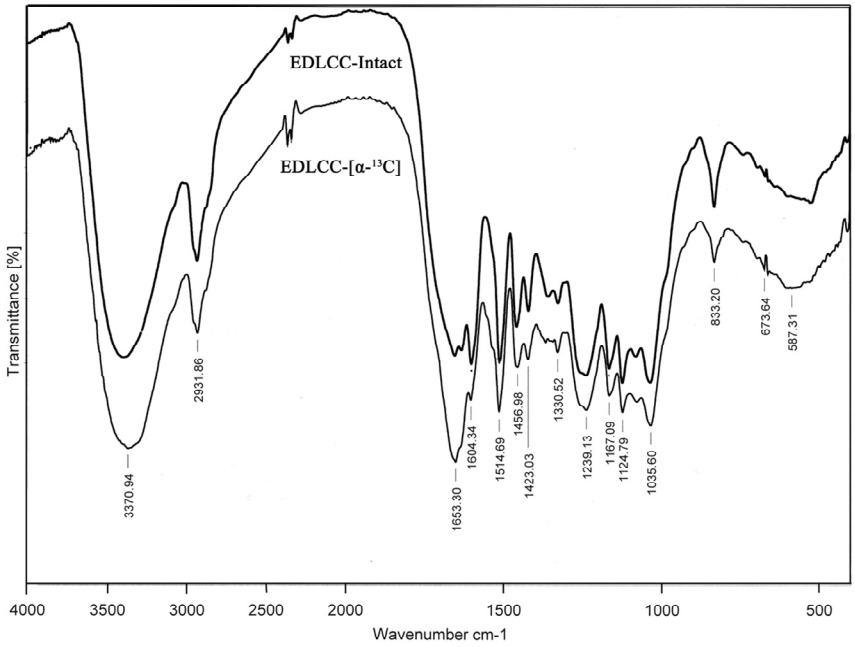


Figure 4: FT-IR spectra of EDLCC-IS from rice stalk

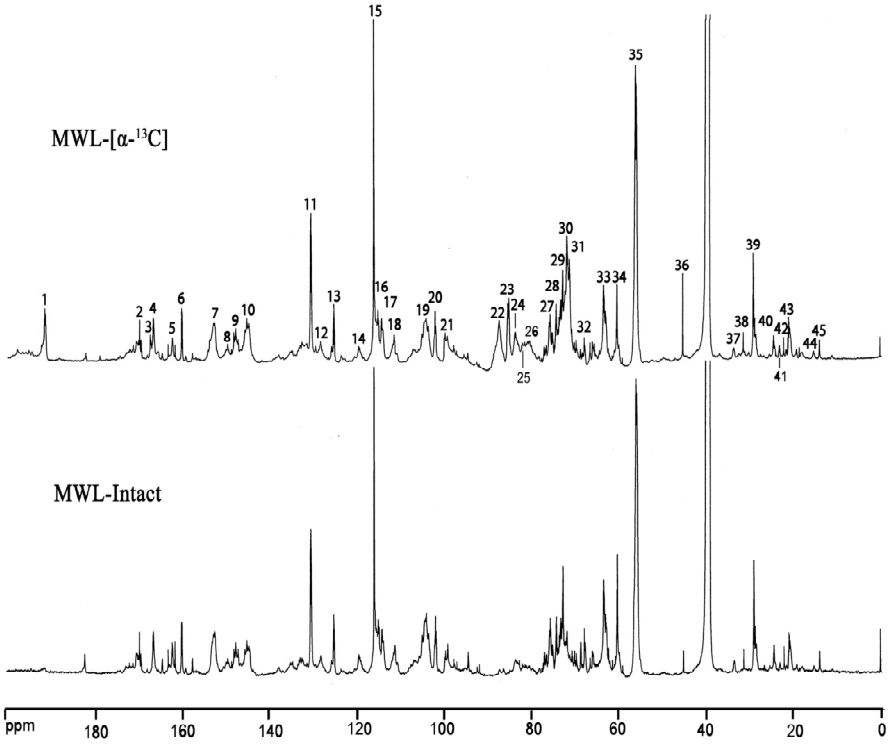


Figure 5: $^{13}$C-NMR spectra of MWL prepared from rice stalk

**$^{13}$C-NMR determination of MWL**

$^{13}$C-NMR spectra of MWL-intact and MWL-[α-$^{13}$C] are shown in Fig. 5. Signal No. 1 (191.4 ppm) belongs to α-CHO.[20] No. 22 (87.4 ppm) and No. 23 (85.4 ppm) are assigned to intensified α-C of β-5 and β-β. A weak signal at 81.9 ppm (No. 25) from α-C connected with polysaccharide by a benzyl ether bond proves the existence of LCC. The signal from α-C of β-O-4 (with α-aryl ether linkage) is located at 80.5 ppm. Signals No. 28 (74.3 ppm), No. 29 (72.8 ppm), No. 30 (71.8 ppm) and No. 31 (71.3 ppm) are all assigned to α-C of β-O-4, with the first from *threo* β-O-4, while the other three from *erythro* β-O-4.[11] Comparing with the MWL-intact, it may be found that the signals No. 29, No. 30 and No. 31 were all intensified in MWL-[α-$^{13}$C]. It could be concluded that the main lignin substructure in the sample is β-O-4.

**$^{13}$C-NMR determination of LCC**

***$^{13}$C-NMR determination of EDLCC-IS***

$^{13}$C-NMR spectra of EDLCC-IS and EDLCC-IS-[α-$^{13}$C] are given in Fig. 6. The weak signal of No. 1 (191.6 ppm) is assigned to α-CHO. No. 19 (87 ppm) and No. 20 (85 ppm) correspond to intensified α-C of β-5[21] and β-β.[20] A weak signal of No. 21 from α-C connected with polysaccharide by a benzyl ether bond proves the existence of LCC. The strong signals of No. 22 (72.5 ppm) and No. 23 (71.7 ppm) are assigned to α-C of β-O-4 and C-α connected with polysaccharide by an ester bond.

***$^{13}$C-NMR determination of EDLCC-WS***

$^{13}$C-NMR spectra of EDLCC-WS and EDLCC-WS-[α-$^{13}$C] are shown in Fig. 7. The weak signal at 192 ppm (No. 1) of α-CHO proves the low content of vanillin in the sample. Signals No. 2 (168.4 ppm) and No. 3 (166.7 ppm) correspond to ferulic acid with -COO-. The signals from C3, C4 and C5 of guaiacyl and syringyl are located at 153-144 ppm.[22] The intensified signal at 130.3 ppm (No. 9) is assigned to α-C of coniferyl alcohol.[23] The intensified strong signal proves a large amount of coniferyl alcohol in EDLCC-WS. Signals No. 10-No. 13 (110-129 ppm) belong to C2, C3, C5 and C6 of the aromatic ring.

Comparing the two spectra, it may be noted that the intensified signal at 102 ppm corresponds to α-C connected with polysaccharide by an acetal bond.[24] The intensified signals at 85-88 ppm are assigned to α-C from β-5 and β-β. The intensity of the two signals proves the low content of the two substructures. Signal No. 19 at 80.1-82.6 ppm belongs to α-C connected with polysaccharide by a benzyl ether bond. Signals No. 20 (72.5 ppm), No. 23 (63.8 ppm) and No. 24 (60.4 ppm) are assigned to α-C of β-O-4(*erythro*), α-C of β-1 and γ-C of β-O-4, respectively. The weak signal at 29 ppm (No. 28) is from methylene with $C_5$-$CH_2$-$C_5$ structure. From the analysis of the $^{13}$C-NMR spectra, it can be concluded that the main structure of lignin from EDLCC-WS includes β-O-4, β-5 and β-β, with minor vanillin and LCC connected by an acetal bond and a benzyl ether bond.

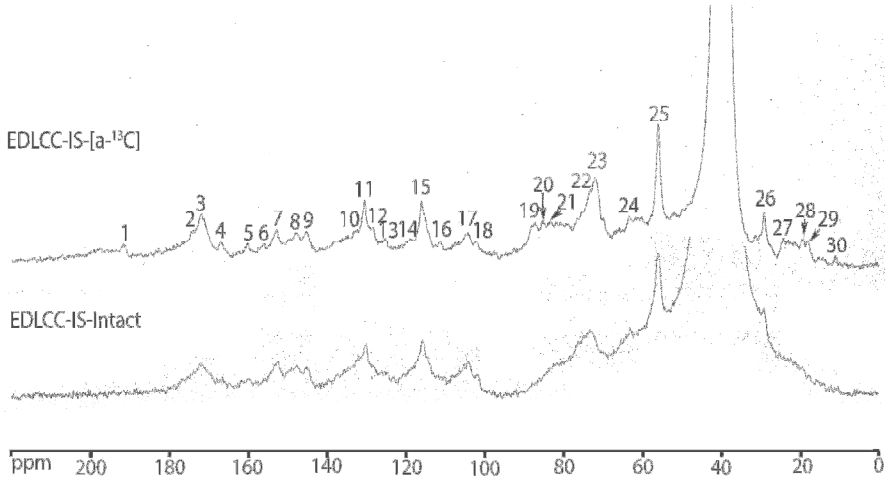


Figure 6: $^{13}$C-NMR spectra of EDLCC-IS

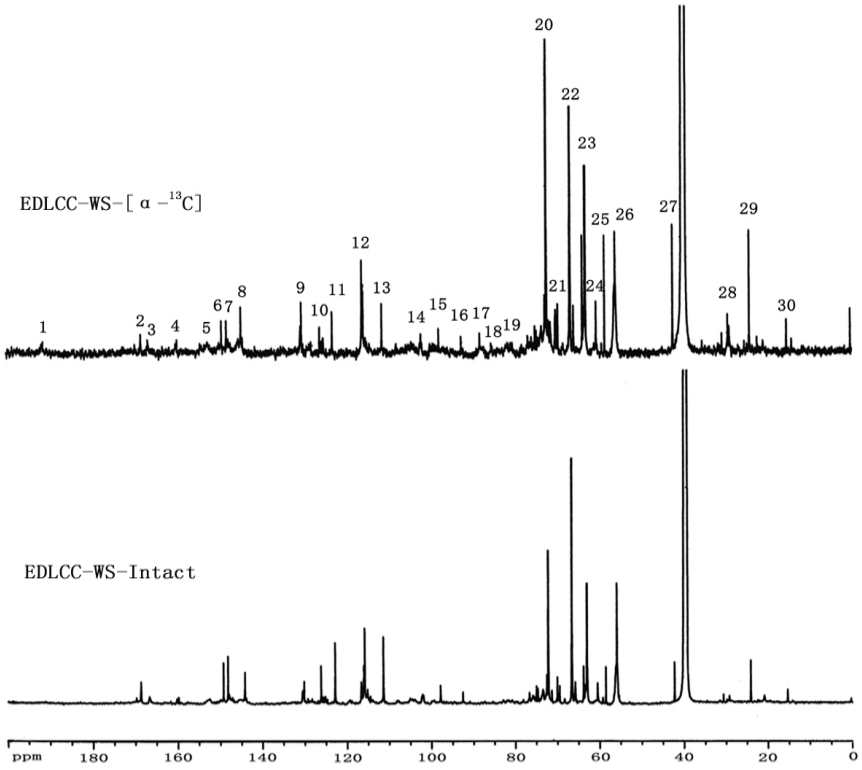


Figure 7: $^{13}$C-NMR spectra of EDLCC-WS

## CONCLUSION

(1) The injection of exogenous carbon source ($^{13}$C isotope) did not interfere with the lignification of rice stalk.

(2) Milled wood lignin and lignin carbohydrate complex were extracted from $^{13}$C labeled rice stalk. From the analysis of FT-IR and $^{13}$C-NMR spectra, it was found that the main linkages between lignin and polysaccharide were a benzyl ether bond, an ether bond and an acetal bond, while the main substructures of lignin were β-O-4, β-β, β-5 and β-1, with minor coniferol.

(3) Carbohydrate was removed from LCC by cellulase and hemicellulase treatment to get EDLCC-WS and EDLCC-IS. From the analysis of FT-IR and $^{13}$C-NMR spectra, it was found that part of lignin and carbohydrate from natural rice stalk was connected by a benzyl ether bond, an ether bond and an acetal bond. Thus, it was proven again that the main substructures of lignin were β-O-4, β-β, β-5 and β-1, with minor coniferyl.

***ACKNOWLEDGEMENTS***: This work was supported by NSFC (No. 30901140, 31370574) of China and State Key Laboratory of Microbial Technology of Shandong University (No. M 2010-01 and M 2012-07) and China Scholarship Council (No. 2011842330).

## REFERENCES

[1] L.Yan, J. Yue, J. Zhao, J. Q. Dong, X. Z. Li *et al*., *Bioresour. Technol*., **101**, 8755 (2010).

[2] X. Fang, Y. Shen, J. Zhao, X. M. Bao, Y. B. Qu, *Bioresour. Technol*., **101**, 4814 (2010).

[3] M. E. Himmel, S. Y. Ding, D. K. Johnson. W. S. Adney, M. R. Nimlos *et al*., *Science*, **315**, 804 (2007).

[4] J. J. Zeng, G. L. Helms, X. Gao, *J. Agric. Food Chem*., **61**, 10848 (2013).

[5] J. J. Zeng, Z. H. Tong, L. T. Wang, J. Y. Zhu, L. Ingram, *Bioresour. Technol*., **154**, 274 (2014).

[6] A. U. Buranov, G. Mazza, *Ind. Crop. Prod*., **28**, 237 (2008).

[7] Y. Uraki, Y. Usukura, T. Kishimoto, M. Ubukata, *Holzforschung*, **60**, 659 (2006).

[8] T. Q. Yuan, S. N. Sun, F. Xu, R. C. Sun, *J. Agric. Food Chem*., **59**, 10604 (2011).

[9] M. Y. Balakshin, E. A. Capanema, H. M. Chang, *Holzforschung*, **61**, 1 (2007).

[10] D. Y. Min, C. M. Yang, V. Chiang, H. Jameel, H. M. Chang, *Fuel*, **116**, 56 (2014).

[11] H. T. Yang, Y. M. Xie, L. Yao, S. J. Wang, *Cellulose*

*Chem. Technol.*, **41**, 361 (2007).
[12] H. T Yang, X. Zheng, L. Yao, Y. M. Xie, *CIESC Journal*, **64**, 1069 (2013).
[13] H. T. Yang, X. Zheng, L. Yao, Y. M. Xie, *Cellulose Chem. Technol.*, **47**, 143 (2013).
[14] H. T. Yang, X. Zheng, L. Yao, Y. M. Xie, *BioResources*, **9**, 176 (2014).
[15] A. Björkman, *Svensk Papperstidn.*, **59**, 477 (1956).
[16] L. He and N. Terashima, *Mokuzai Gakkaishi*, **35**, 116 (1989).
[17] L. He and N. Terashima, *Mokuzai Gakkaishi*, **35**, 123 (1989).
[18] P. Sannigrahi, D. H. Kim, S. Jung, A. Ragauskas, *Energ. Environ. Sci.*, **4**, 1306 (2011).
[19] J. Dorado, J. A. Field, G. Almendros, R. Sierra-Alvarez, *Appl. Microbiol. Biot.*, **57**, 205 (2001).
[20] E. A. Capanema, M. Y. Balakshin, J. F. Kadla, *J. Agric. Food Chem.*, **52**, 1850 (2004).
[21] C. M. Popescu, P. T. Larsson, C. Vasile, *Carbohyd. Polym.*, **83**, 808 (2011).
[22] B. L. Xue, M. F. Li, F. Xu, R. C. Sun, G. Jones, *Ind. Crop. Prod.*, **36**, 209 (2012).
[23] R. Gu and Y. M. Xie, *Paper Sci. Technol.*, **20**, 1 (2001).
[24] Y. M. Xie, S. Yasuda, H. Wu, H. Liu, *J. Wood Sci.*, **46**, 130 (2000).