Electronic Supplementary Material (ESI) for Green Chemistry.


**Supplementary Document**

# Synergistic Enzymatic and Microbial Conversion of Lignin for Lipid

Cheng Zhao, Shangxian Xie, Yunqiao Pu, Rui Zhang, Fang Huang, Arthur J. Ragauskasand

Joshua S. Yuan

## Table of Content

## 1. Detailed Material and Methods

### 1.1. Bacteria strain

*Rhodococcus opacus* PD630 (DSM-44193) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig Germany). The strain was recovered in 5 mL Trypticase Soy Broth (TSB) at 28 °C for 2 days. The culture medium was then mixed with 1 mL glycerol, dispensed to 1.5 mL tubes, and chilled in liquid nitrogen for storage. The strain was stored in -80 °C.

### 1.2. Medium preparation

Two types of media were used for the fermentation. The Rhodococcus Minimal (RM) medium was prepared as follows. 1.4 g $(NH_4)_2SO_4$ and 1.0 g $MgSO_4 \cdot 7H2O$ were added into 962 mL dd$H_2O$, and then sterilized by autoclave at 121°C for 20 min. The solution was then cooled down to room temperature, and then added 1mL 15 g/L sterile $CaCl_2 \cdot 2H_2O$, 1.0 mL sterile trace element solution, 1.0 mL sterile stock A solution, and 35.2 mL sterile 1.0 M phosphate buffer. The solution is then finalized to 1000 mL.

For lignin fermentation medium, 0.5 g kraft lignin, 0.14 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$ were added into about 90 mL dd$H_2O$. The pH was adjusted to 12.5 to dissolve the lignin. After the lignin was completely dissolved, the solution was neutralized with HCl to pH 7.0 and adjusted to the total volume of 96.2 mL followed by autoclaved.. The solution was further cooled down to room temperature and added 0.1 mL 15g/L sterile $CaCl_2 \cdot 2H_2O$, 0.1 mL sterile trace element solution, 0.1 mL sterile stock A solution, and 3.52 mL sterile 1.0 M phosphate buffer. The solution was finalized at 100 mL.

The trace element solution was made of 0.5 g $FeSO4 \cdot 7\ H_2O$; 0.4 g $ZnSO4 \cdot 7\ H_2O$; 0.02 g $MnSO4 \cdot H_2O$; 0.015 g $H_3BO_3$; 0.01 g $NiCl_2 \cdot 6H_2O$; 0.25 g EDTA; 0.05 g $CoCl_2 \cdot 6H_2O$; 0.005 g

$CuCl_2 \cdot 2H_2O$, and $ddH_2O$ to make up to 1 L. The solution was then sterilized by filtration through a 0.22 μm filter.

The stock A solution was made by mixing 2.0 g $NaMoO_2 \cdot 2H_2O$, 5.0 g FeNa·EDTA, and $ddH_2O$ to 1L, and further sterilized by filtration through a 0.22 μm filter. The 1 M phosphate buffer was made by adding 113 g $K_2HPO_4$, 47 g $KH_2PO_4$ and ddH2O to make up to 1 L, and further sterilized by filtration. Both the stock A solution and the phosphate buffer were prepared freshly.

1.3. Lignin fermentation

A single colony of *Rhodococcus opacus* PD630 was inoculated into 20 mL Tryptic Soy Broth (TSB) medium, and the cultivated at 28 °C to $OD_{600}$ 1.5. 20 mL. The cells were centrifuged and washed twice with equal volume of RM minimum medium and then re-suspended by 20 mL RM minimum medium. 1 mL re-suspended cells were added to 100 mL of lignin fermentation medium. The fermentation solution was cultivated at 28 °C with shaking speed of 200 rpm for 144 hours.

1.4. Lignin concentration analysis by Prussian Blue assay

The pH for the sample was adjusted to 12.5 with 10 M NaOH to completely dissolve the lignin. In order to completely dissolve lignin, the lignin sample was mixed at speed of 180 rpm for 1 h. The total volume was adjusted to 100 mL by adding RM minimum medium. The samples were further diluted to an optimal concentration using $ddH_2O$ to adjust the final absorbance at 700 nm to be within the range of 0.7-1.5. Approximately 1.5mL of the diluted samples was transferred into 2 mL tubes. 100 μL of 8 mM $K_3Fe(CN)_6$ was added into the tube and followed by the immediate addition of 100 μL 0.1 M $FeCl_3$. The samples were mixed thoroughly by shaking the tube for 5 minutes. The samples were transferred to 1 cm cuvette to

obtain the absorbance at 700 nm by spectrophotometer, using the dd$H_2O$ sample as blank control. Standard curve was established with the same reagents and known concentration of lignin. All experiments were carried out in triplicate.

1.5. Cell concentration determination by serial diluted and plate (SDP) assay

To determine the number of living cell, 100 μL of fermentation culture was serial diluted and plated for $10^4$ dilution. The numbers of colonies were counted from the plates and converted to colony forming unit/mL (CFU/mL).

1.6. Total lipid extraction

In order to determine the total lipid produced by *R. Opacus* PD630, 100 mL fermentation culture was centrifuged at 2500 g for 5 min, and the supernatant was collected. The pellet was re-suspended with 100 mL 0.9% NaCl solution, and was centrifuged at 2500 g for 5 min. The supernatant was collected and combined with previous one. Repeat twice the resuspension and centrifugation step. The collected 300 mL supernatant was centrifuged at 10,000 g for 30 min to pellet cells. The pelleted cells were lyophilized for 24 h. 3 mL chloroform: methanol (2:1) solution was added to cells to homogenize the cells for 3 h, followed by 3000 rpm centrifugation to make pellets. The supernatant was transfer to the freshly weighted tubes. Subsequently, 0.6 mL distilled water was added. After phase separation, the upper phase was discarded. The organic phase containing total lipid was rinsed with chloroform:methanol:water (3:48:47). The upper phase was removed and the organic phase was dried down under $N_2$ stream and the lipid was weighted.

1.7. Lignin analysis by gel permeation chromatography (GPC)

Specifically, lignin was acetylated in a mixture of acetic anhydride/ pyridine (1:1 v/v, 2.0 mL) for 24 h at room temperature. The reaction mixture was diluted with ethanol (30 mL) and stirred

for 30 min and then concentrated under lower pressure. The addition and removal of ethanol was repeated to remove trace acetic acid and pyridine from the samples. The samples were then dissolved in chloroform and added dropwise into diethyl ether to precipitate the samples followed by centrifugation. After air drying, the acetylated samples were dried for 24 h in a vacuum oven at 40°C prior to GPC analysis. Tetrahydrofuran was used as the mobile phase in GPC analysis and the flow rate was 1.0 mL/min. The molecular weights of the derivatized lignin samples were acquired by using a calibration curve established with standard narrow polystyrene samples.

1.8. Lignin analysis nuclear magnetic resonance (NMR)

All the NMR experiments were carried out at a Bruker Avance 400-MHz NMR spectrometer. HSQC spectra were acquired using deuterated dimethyl sulfoxide (500 μL) as solvent for lignin samples (~ 60 - 100 mg) at 45 ºC with the following acquisition conditions: 11-ppm spectra width in F2 (1H) dimension with 2048 data points (232.7-ms acquisition time), 220-ppm spectra width in F1 (13C) dimension with 256 data points (5.8-ms acquisition time); a 1.5-s pulse delay; a $^{1}J_{C\text{-}H}$ of 145 Hz; and 96 scans. The central solvent peak ($\delta_C$ 39.5 ppm; $\delta_H$ 2.5 ppm) was used for chemical shift calibration. For quantitative $^{31}$P NMR analysis, lignin (ca. 20 mg) was dissolved in a solvent of pyridine/$CDCl_3$ (1.6/1.0 v/v, 500 μL) and derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. The spectrum was acquired using an inverse-gated decoupling pulse sequence (Waltz-16), a 90° pulse, and a 25-s pulse delay. N-hydroxy-5-norbornene-2,3-dicarboximide was used as the internal standard. 128 scans were accumulated for each sample. NMR data were processed using the TopSpin 2.1 software (Bruker BioSpin) and MestreNova (Mestre Labs) software packages.

## 2. Supplementary Figures

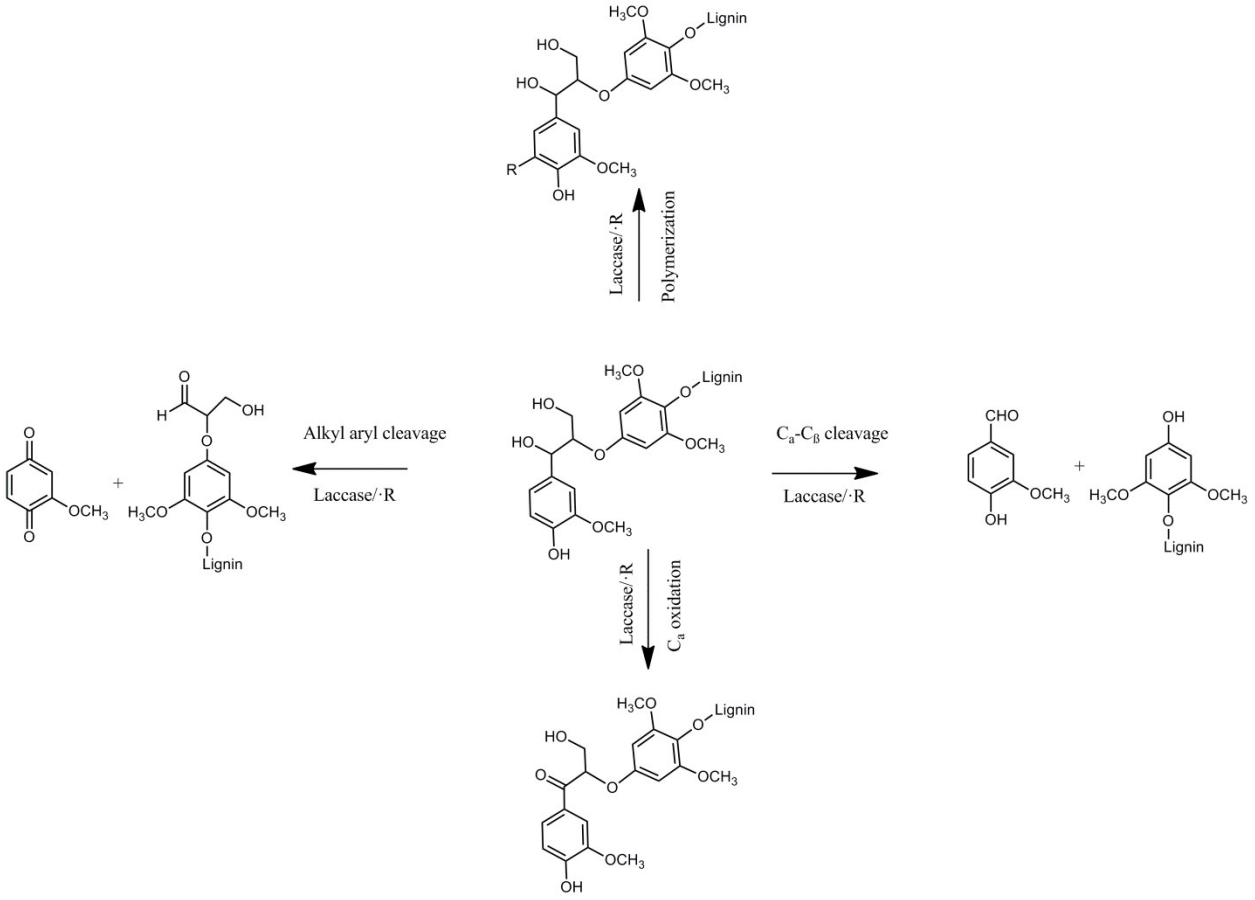


**Figure S1**. The role of electron mediators in facilitating laccase-based oxidation of lignin. Laccase could both polymerize and depolymerize lignin via redox reactions. The initial oxidative attack to lignin by laccase was targeted at the phenolic structures, which generated the phenoxyl radicals (•R). The released phenolic fragments could serve as a reaction mediator for further lignin oxidization. On one side, the phenoxyl radicals could degrade the non-phenolic lignin structure through $C_\alpha$-$C_\beta$ cleavage, $C_\alpha$ oxidation, alkyl aryl cleavage and even aromatic ring cleavage. On the other hand, the radicals could also lead to the oxidation reaction to polymerize aromatic compound monomers.

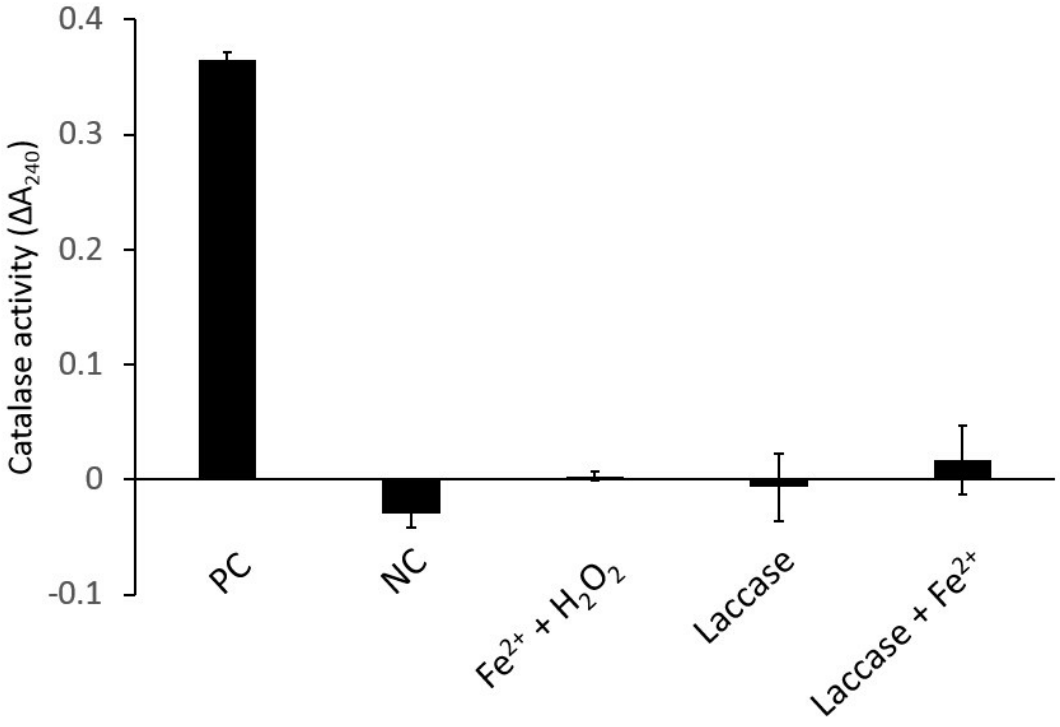


**Figure S2**. The catalase activity of *R. opacus* PD630 supernatant during lignin fermentation under different treatment conditions. The catalase activity was measured by reading the absorbance changes at 240 nm after mixing of 1 μL supernatant with 300 μL 0.036% (w/w) $H_2O_2$. PC, positive control containing 2 units catalase (Sigma, C1345); NC, negative control, which is the supernatant of lignin medium without fermentation; $Fe^{2+}$+$H_2O_2$, the supernatant of *R. opacus* PD630 after lignin fermentation with 0.2 mM $FeSO_4$ and 0.067 mM $H_2O_2$ treatment; Laccase, the supernatant of *R. opacus* PD630 after lignin fermentation with 1 U/mL laccase treatment; Laccase+ $Fe^{2+}$, the supernatant of *R. opacus* PD630 after lignin fermentation with 1 U/mL laccase and 0.2 mM $FeSO_4$ treatment.

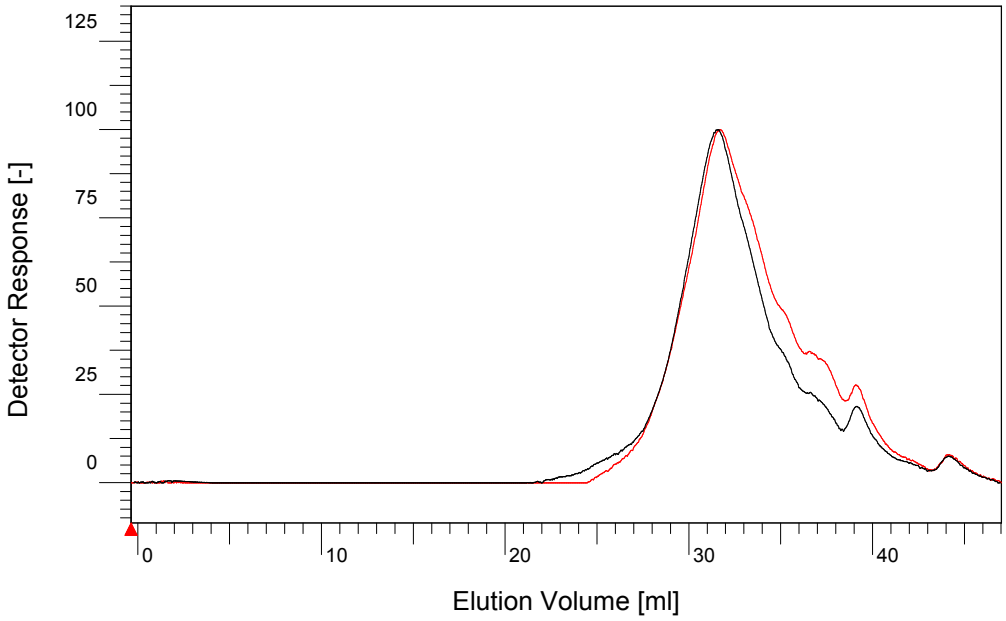


**Figure S3**. Gel permeation chromatography (GPC) of control kraft lignin and laccase-only treated lignin. Black line: control lignin; red line: laccase-treated lignin. The UV detection was at 270 nm.

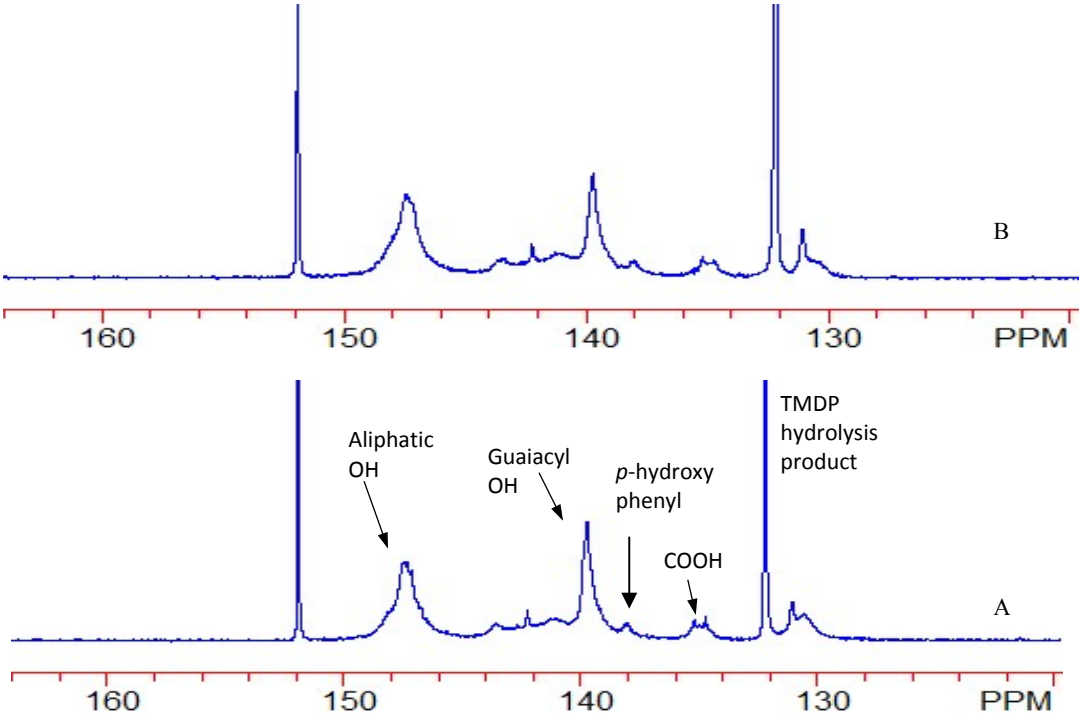


**Figure S4**. Quantitative $^{31}P$ NMR spectra of untreated control kraft lignin and laccase-only treated lignin, derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP). A: control lignin; B: lignin treated with only laccase. The peak at 151.9 ppm is the internal standard, N-hydroxy-5-norbornene-2,3-dicarboximide.

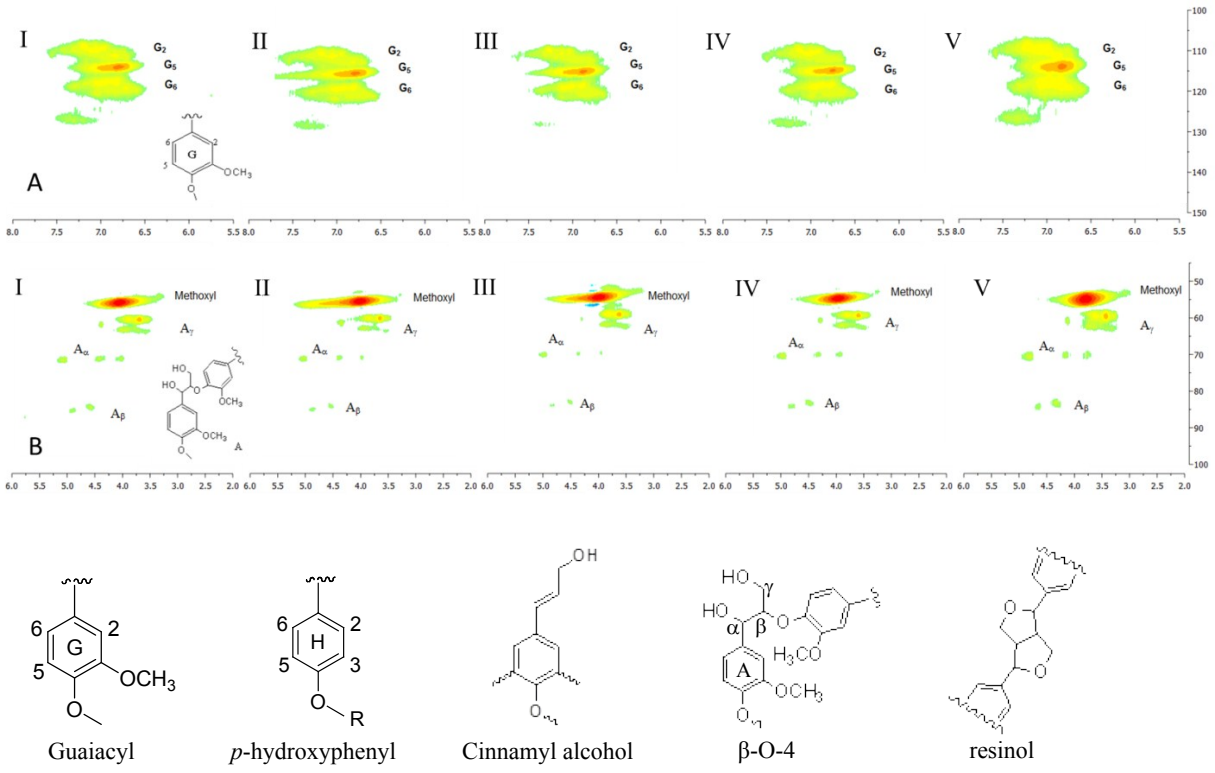


**Figure S5**. HSQC analysis of lignin after different treatments. A. The aromatic region of HSQC NMR spectra of lignin obtained after treatments: (I) no cell, (II) cell only, (III) laccase, (IV) laccase and $Fe^{2+}$, and (V) laccase and Fenton reagent ($Fe^{2+}$+$H_2O_2$). B, Aliphatic region of HSQC NMR spectra of lignin obtained after treatments: (I) no cell, (II) cell only, (III) laccase, (IV) laccase and $Fe^{2+}$, and (V) laccase and Fenton reagent. The diagnostic signals in guaiacyl units for a softwood lignin were well observed at $\delta_C/\delta_H$ of 110.8/7.06 ($G_2$), 115.1/6.78 ($G_5$), and 119.8/6.81 ($G_6$) ppm, with the existence of *p*-hydroxyphenyl (H) unit (i.e., its $C_{2/6}/H_{2/6}$ correlation signal $\delta_C/\delta_H$ around 128.1/7.20 ppm). In the aliphatic region, the interunit linkage of β-O-4 was evident with its signals at 71.2/4.81 ($C_\alpha/H_\alpha$, $A_\alpha$), 84.2/4.32 ($C_\beta/H_\beta$, $A_\beta$), and 59.8/3.48 ppm ($C_\gamma/H_\gamma$, $A_\gamma$), respectively. Signals for resinol subunits were also observed with its C/H correlations around $\delta c/\delta_H$ 85.0/4.66 ($C_\alpha/H_\alpha$), 3.06/53.9 ($C_\beta/H_\beta$), and 70.9/3.78,4.15 ($C_\gamma/H_\gamma$) ppm.

End group cinnamyl alcohol was observed with its Cγ/Hγ correlation signal at $\delta c/\delta_H$ 61.6/4.13 ppm.