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Structural and dynamic changes of lignin in *Eucalyptus* cell walls during successive alkaline ethanol treatments

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ABSTRACT

A sequential treatment of *Eucalyptus* with 70% ethanol containing 0.4, 1.0, 2.0, 3.0, and 5.0% NaOH at 80 °C for 2 h, was used to isolate the lignins in the present study. The lignin fractions obtained were subjected to comprehensive composition analysis and structural characterization. Additionally, the lignin distribution and topochemical changes within morphologically distinct cell wall regions were investigated by confocal Raman microscopy. The most noteworthy features of the lignin fractions were the almost absence of bound polysaccharides, and the degradation of lignin resulted from a comparatively high concentration of NaOH. The lignin fractions turned out to be mainly composed of β-*O*-4′ linkages combined with low amounts of *β*–*β*′ and β-5′ linkages. Confocal Raman microscopy was combined to assist monitor the course of delignification during the successive alkaline treatment processes visually, and the Raman spectra analysis revealed that the dissolution rate of lignin in the secondary wall was higher than that in cell corner middle lamella regions during this process. Data from this study potentially enhances the understanding of the mechanisms of alkaline treatment acting on *Eucalyptus* and facilitate the value-added utilization of the lignin fractions in future biorefineries.

1. Introduction

Lignin, the third most plentiful phenolic polymer as well as an essential component of lignocellulosic biomass, has been used in certain application areas such as polymeric materials, biofuels, and also has an important effect on the pulp production (Rencoret et al., 2008; Tejado et al., 2007). It is well known that lignin, which is composed of three basic structural phenylpropane units named syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H), is a highly heterogeneous macromolecule in structure (Zeng et al., 2013). The monolignols lacking of regular and ordered repeating units produce a three-dimensional amorphous lignin polymer by both ether (β-*O*-4′, α-*O*-4′, and 4-*O*-5′) and C—C (*β*–*β*′, β-5′, and β-1′) interunit linkages (Buranov and Mazza, 2008). The inherent complexity and heterogeneity of lignin make it difficult to effectively isolate from lignocellulosic biomass. *Eucalyptus grandis* × *Eucalyptus urophylla* wood, as a promising hardwood plant, has received much attention, especially in the pulp and papermaking industry. Understanding the lignin structure and the delignification process of *Eucalyptus* is beneficial to optimize the biorefinery process and maximize the utilization of underlying values of *Eucalyptus*. Therefore, it is necessary to develop a conducive and economical isolation method for extracting lignin.

It is well-known that alkaline treatment is a widespread and cost-effective method for isolating lignin. In addition, mild alkaline treatment does not seem to cause dramatic chemical modification on the structure of the solubilized lignin beyond the saponification of ester and ether linkages between lignin and hemicelluloses. During the alkaline treatment process, alkali may break some alkali-labile linkages between lignin units, or between lignin and polysaccharides, and effectively reduce the enormous molecular size of lignin (Sun et al., 2012; Yuan et al., 2011). Additionally, lignin usually has a good solubility in ethanol solution (Sun et al., 2012). Therefore, in this study, the lignin fractions were isolated from the dewaxed *E. grandis* × *E. urophylla* by successive alkaline ethanol treatments with the increasing alkali concentrations. The information of lignin yield, purity, dissociation mechanisms, and structural characteristics during the alkaline ethanol treatments is beneficial to dig out the potential value of lignin obtained for availability, renewability, and value-added products, such as sustainable construction materials for replacing petroleum-based products (Doherty et al., 2011; Zhang et al., 2011; Zhao and Wilkins,

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2003). Therefore, the structural features of lignin fractions should be clarified.

In order to better understand the potential course of delignification during the alkaline ethanol treatment process, the spatial distribution changes of lignin in morphologically distinct cell wall regions should be investigated on a subcellular level. Confocal Raman microscopy (CRM) has been widely used to elucidate the spatial distribution and topochemical changes of plant cell wall components in situ, and the chemical imaging of plant cell walls obtained from CRM can provide a nondestructive visualization of spatially resolved chemical information (Gierlinger et al., 2012; Griffiths, 2009; Schmidt et al., 2009). The information of the spatial distribution changes of the predominant components in plant cell walls obtained by CRM during the treatment process would greatly enhance the understanding of the mechanisms and be helpful to efficiently optimize the alkaline treatment process.

The aim of this study was to investigate the detailed information of the structural features of the *Eucalyptus* lignin fractions obtained by successive alkaline ethanol treatments with the increasing alkaline concentrations, and assist monitor the course of delignification during this process visually by combining the microscopic imaging techniques. The structural and dynamic changes of the lignin fractions obtained were investigated by high-performance anion-exchange chromatography (HPAEC), gel permeation chromatography (GPC), Fourier transform infrared (FT-IR), quantitative two-dimensional heteronuclear single-quantum coherence (2D-HSQC) spectroscopy, and CRM.

2. Methods

2.1. Materials

The raw material used in the study was *E. grandis* × *E. urophylla* wood (5 years old) obtained from Guangxi province, China. The dried raw material was chipped into small pieces and then ground, and the particles were further screened for selecting the particles (40–60 mesh) with relatively uniform size. In addition, to avoid the possible effects of extractives on the composition analysis, the biomass (40–60 mesh) was extracted with toluene-ethanol (2:1, v/v) for 6 h in a Soxhlet apparatus, which generally removed the wax and other free-bonded and low molecules of lignin. Then the particles were dried for 16 h in an oven at 60 °C. The composition of the dewaxed material was cellulose 42.4%, hemicelluloses 21.0%, acid-insoluble lignin 27.5%, and acid-soluble lignin 4.1%, which was analysed according to the method published by Sluiter et al. (2008).

2.2. Alkaline ethanol treatment

The scheme for successive alkaline ethanol extractions of lignin fractions from *Eucalyptus* is shown in Fig. 1. The dewaxed powder (20 g) was successively treated with 70% ethanol containing 0.4, 1.0, 2.0, 3.0, and 5.0% NaOH at 80 °C for 2 h under a solid to liquid ratio of 1:20 (g/mL). After filtration, the residues in each step were washed thoroughly with distilled water and then dried in an oven at 60 °C for 16 h. The combined filtrates in each step were neutralized to pH 5.5 with hydrochloric acid and then concentrated to about 30 mL. Subsequently, the concentrated filtrates were poured into 3 volumes of 95% ethanol with constant stirring, and the hemicellulosic pellets were obtained by filtering, washing with 70% ethanol, and then freeze-dried. Then the filtrate was concentrated to about 30 mL, and the pH was adjusted to 1.5–2.0 with hydrochloric acid. The precipitate was centrifuged and then freeze-dried to obtain lignin fractions. These lignin fractions obtained from the sequential treatments with 70% ethanol containing 0.4, 1.0, 2.0, 3.0, and 5.0% NaOH at 80 °C for 2 h were labeled as L1, L2, L3, L4, and L5, respectively.

For Raman microscopy, *Eucalyptus* sample was cut into small blocks (approximately 1 cm × 0.5 cm × 2 cm), and then preserved in glycerol–ethanol mixtures (1:1, v/v). 8 μm thickness cross sections were prepared by a sliding microtome. Subsequently, the sections were successively soaked in 70% ethanol containing 0.4, 1.0, 2.0, 3.0, and 5.0% NaOH at 80 °C for 2 h. After treatment for the designated time, the reactors were cooled down immediately by water. After washed thoroughly with ultrapure water, the samples were stored in ultrapure water at 4 °C for further analysis.

2.3. Characterization of lignin

The associated polysaccharides in the five lignin fractions obtained and the weight-average (M_w) and number-average (M_n) molecular weights of the lignin fractions were determined by HPAEC and GPC, respectively, which were based on the procedures described in a previous literature (Sun et al., 2013). The FT-IR spectra of the lignin fractions were obtained by a thermo scientific nicolet iN10 FT-IR microscope (Thermo Nicolet Corporation, Madison, WI, USA) equipped with a liquid nitrogen cooled MCT detector. The dried samples were ground, and the spectra were recorded in the range of 4000–600 cm^{-1} at 4 cm^{-1} resolution with 128 scans per sample. The solution-state NMR spectra of the lignin fractions were acquired on a Bruker AVIII 400 MHz spectrometer at 25 °C as previously reported (Sun et al., 2013).

2.4. Confocal Raman microscopy

Before Raman detection, the untreated and alkaline ethanol treated *Eucalyptus* cross sections were placed on a clean glass slide with a drop of water, and then covered with a coverslip (0.17 mm thickness). The Raman spectra were obtained on a LabRam Xplora confocal Raman microscope (Horiba Jobin Yvon, Longjumeau, France) equipped with a confocal microscope (Olympus BX51, Tokyo, Japan) and a motorized x and y stage. An air-cooled back-illuminated CCD behind the spectrograph was used to detect the Raman light. An MPlan 100 × oil immersion microscope objective (Olympus, NA = 1.40) and a linear-polarized laser (λ = 532 nm) focused with a diffraction-limited spot size (0.61 λ/NA) were used

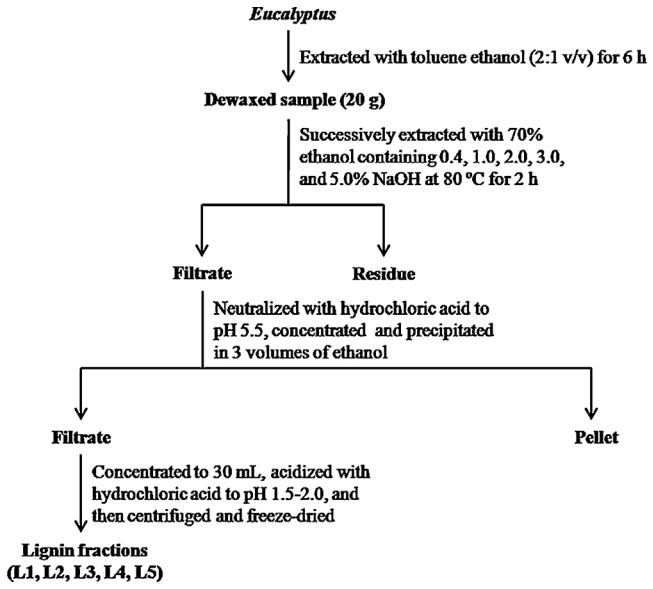

Fig. 1. Scheme for successive alkali ethanol extractions of lignin fractions from *Eucalyptus*.