



## Green and efficient conversion strategy of *Eucalyptus* based on mechanochemical pretreatment

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### ABSTRACT

Herein, the mechanochemical process combined diluted acid impregnation and ball-milling treatment was developed to overcome the recalcitrance of lignocellulose. The high-yield fermentable sugars (glucose and xylose) and impregnated enzymatic lignin (IEL) streams have been simultaneously obtained via mechanochemistry and enzymatic hydrolysis process. It was found that the mechanochemical pretreatment significantly facilitated the enzymatic hydrolysis of the treated substrates, releasing high yield (> 90%) of fermentable sugars for further utilization. CP/MAS <sup>13</sup>C NMR and solution-state 2D NMR techniques showed that the crystallinity index (CrI) and degree of polymerization (DP) of cellulose in the treated substrates were both decreased. In addition, detailed structural characterizations of the obtained IEL fractions were achieved by the state-of-the-art NMR and GPC techniques. Results showed that the IEL fractions presented similar structural features as compared to the corresponding native lignin (DEL). The study provides an efficient and clean pathway for the conversion of energy from lignocelluloses; meanwhile, the residual lignin with unchanged structures could serve as ideal feedstock for the liquid fuel, bio-based materials, and chemicals in the current biorefinery process.

### 1. Introduction

Renewable biofuels and platform chemicals originated from lignocellulosic biomass have taken a leading position as the growing emissions of carbon dioxide and depletion of fossil fuels [1,2]. Lignocelluloses provide the largest reserves of renewable, high-energy content carbon materials in the world. In the current biorefining industry, comprehensive utilization of biomass is not only essentially reduces carbon emissions and costs, but also actuates research in the conversion of raw biomass into high value-added commercial products instead of low-value fuel [3,4]. This also promotes lignocellulose as economically reasonable alternatives to fossil fuels [5]. In lignocellulosic biomass, cellulose fibrils show crystalline-like order, which is interacted with intermolecular hydrogen bonds forming cross-linking and sealed by a polymeric matrix of hemicelluloses and lignin [6,7]. Cellulose fibrils contribute to the major the mechanical strength of the cell walls and act as the framework of cell wall. Hemicelluloses are heteropolysaccharides, which are mainly comprised of xylose and glucuronic acid in hardwood species. It can be largely hydrolyzed into soluble mono- and oligosaccharides, which can be further converted into value-added chemicals [8]. Lignin, acted as the vital glue giving

plants their structural integrity, is a complex and heterogeneous aromatic polymer in which the major interunit linkages such as  $\beta$ -O-4,  $\beta$ - $\beta$ ,  $\beta$ -5, and  $\beta$ -1 can be easily observed [9]. Recently, Yue et al. [10] had identified the 4-O-5 linkages in the lignin of softwood via NMR techniques, which proposed the new concept that lignin is rather “U type” linear structure than “highly branched” in conventional theory. However, the sophisticated structure resulted in the recalcitrance of lignocelluloses, which highly restricts the enzymatic saccharification of cellulose [11,12]. Efficient and selective separation approach for the main components of lignocelluloses is conducive to its subsequent high-value of utilization and production in current biorefinery models, such as biochemicals, bioenergy, and biomaterials [1,13,14]. Thus, advisable pretreatment to reduce recalcitrance of lignocelluloses is considered to be a primary precondition for the subsequent cellulose enzymatic saccharification and lignin deconstruction and depolymerization [15,16].

Usually, pretreatment methods such as physical, chemical, and biological pretreatment have been widely applied to various lignocellulosic materials on account of the properties of substrates. However, most of the existing pretreatments processes cannot realize a real sense of economical and eco-friendly technology, because the traditional pretreatments usually are the intricate and time-consuming

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procedures, trouble in solvent stability and recycling [17]. Apart from aforementioned pretreatment methods, mechanochemistry technique is increasingly moving into the mainstream approach due to the feasibility in nowadays industry [17–19]. Mechanical milling, as a green and efficient pretreatment approach provided by a ball milling at ambient conditions [18,20], often requires long time to reduce the crystalline degree of cellulose and only marginally reduces the molecular mass of lignocellulosic components both in academia and industry [21]. In recent years, mechanochemical pretreatment has been used to prepare water-soluble oligosaccharides from cellulose by hydrolysis [22–25], and to facilitate the cleavage of  $\beta$ -O-4 bonds in lignin and model compounds [26–28]. Under the presence of mechanical forces, the depolymerization of acid-impregnated solid biomass completely overcomes the problems posed by the recalcitrance of lignocellulose [29]. In this case, the lignocellulosic substrates could be fully converted into water-soluble products by the acid-impregnation and mechanical force [23–25], however, some limitations, for example, the acid-impregnated stage often involves in the utilization of  $H_2SO_4$  and diethyl ether, will restrict the large-scale utilization of this method to some extent. Moreover, it was found that the solid-state reaction of acid-impregnated substrate was also related to the concentration and type of acid (e.g.  $H_2SO_4$  or HCl). Furthermore, lignin was depolymerized to a lesser extent in the presence of HCl as compared to  $H_2SO_4$  [24]. Considering the lower corrosivity and cost of the acid at the same concentration, HCl is more environmentally-friendly acid than  $H_2SO_4$  in current biorefinery industry. Inspired by these recent researches, an effective strategy was presented to fully convert biomass into individual components (glucose, xylose, and lignin) in a green and efficient approach. The integrated method begins with the *Eucalyptus* wood, which was firstly impregnated with environmentally-friendly and nontoxic diluted acid (i.e., acetic acid and HCl), followed by a solvent-free and short-time ball-milling assisted solid-state reaction, which facilitated simultaneously enzymatic saccharification and fractionation of lignin.

In general, hardwood biomass is more recalcitrant for enzymatic saccharification, meanwhile, its structural and chemical characteristics are related to the enzymatic hydrolysis for biofuel production [30]. The most-used pretreatment methods, including hot water pretreatment [30–32], ammonia pretreatment [33], Fenton oxidation (FO)-hydrothermal treatment [34], and diluted acid pretreatments [35,36], have respective potential and limitation towards hardwoods. In addition, high severity of pretreatment also resulted in the structural changes of lignin, which showed different extents of inhibition on enzymatic hydrolysis of cellulose in hardwood [36]. *Eucalyptus* has become an important hardwood species in the south of China, which is considered to be the crucial raw material in pulping and biorefinery fields. In the present study, *Eucalyptus urophydis* was selected as raw material to investigate and assess the effectiveness of the proposed conversion strategy based on mild acid-impregnation and ball-milling process. The structural features and enzymatic hydrolysis efficiency of the substrates as well as the structural characteristics of the impregnated enzymatic lignin (IEL) are systematically assessed by solid-state CP/MAS  $^{13}C$  NMR spectroscopy, solution-state NMR techniques, HPAEC (high-performance anion-exchange chromatography), 2D-HSQC (2D heteronuclear single quantum coherence) NMR,  $^{31}P$  NMR spectroscopy, and GPC (gel permeation chromatography).

## 2. Materials and methods

### 2.1. Materials

*Eucalyptus urophydis* (4 year-old trees, cellulose 42.2%, hemicelluloses 21.6%, and lignin 23.6%), harvested from Guangxi Province, China, was ground into sawdust (40–60 mesh) by a laboratory mill and dried in an oven at 60 °C. The ground samples were subjected to Soxhlet extraction with ethanol/benzene (1:2, v/v) for 8 h and sealed storage for subsequent experiment. The chemicals, such as acetic acid, sodium

acetate, and hydrochloric acid were purchased from Sigma Chemical Co. (Beijing, China). The commercial enzyme (Cellic@ CTec2, 100 FPU/ml) was kindly provided from Novozymes (Beijing, China).

### 2.2. Wet impregnation of substrates and mechanically-assisted depolymerization

The *Eucalyptus* wood (5 g) was mixed with different diluted acidic solutions (acetic acid-1.0%, 2.5%, 5.0%; hydrochloric acid-0.5%, 1.0%, 2.0%; v/v%) with a solid/liquid ratio of 1:5 (g/mL) in the plastic beaker, the entire impregnation process was carried out at room temperature (25 °C) for 20 h without agitation. The moisture of impregnated-substrate was removed by lyophilization after desired time. The dried impregnated substrates were subjected to mechanical treatment in a planetary ball mill (Fritsch GmbH, Idar-Oberstein, Germany) operating at 450 rpm for desired time (2 h). The ball-milling procedure was set a 10 min interval after every 10 min of milling to prevent excessively high temperature destroying the subtle molecular structure of lignin. To reduce the ball-milling time, 0.5 h and 1 h ball-milling was also performed under the optimized condition (Acetic acid, 2.5%), which was optimized according to the yield of glucose and xylose. In addition, the mechanical treatment of raw material (without impregnation process) was also performed under same condition as a control (labeled as  $S_{BM-2h}$ ,  $S_{BM-1h}$ , and  $S_{BM-0.5h}$ , respectively). Each experiment was conducted in duplicate. All the substrates after mechanochemical treatment were then collected and kept in an air-tight vial prior to saccharification experiments (labeled as  $S_{A1-2h}$ ,  $S_{A2.5-2h}$ ,  $S_{A5-2h}$ ,  $S_{H0.5-2h}$ ,  $S_{H1-2h}$ ,  $S_{H2-2h}$ ,  $S_{A2.5-1h}$ , and  $S_{A2.5-0.5h}$ , respectively. “A1” represents the concentration of acetic acid (1.0%, v/v), “H0.5” represents the concentration of hydrochloric acid (0.5%, v/v), and “2h” represents milling time).

### 2.3. Enzymatic hydrolysis of the pretreated substrates and lignin fractionation

All the ball-milled *Eucalyptus* samples were enzymatically hydrolyzed at a solid substrate concentration of 5.0% (w/v) with 10 FPU/g of substrate by Cellic® CTec2 (Novozymes). All experiments were performed in acetate buffer (pH = 4.8) at 50 °C with a shaker at 150 rpm for 48 h. The reactions were monitored and 200  $\mu$ L supernatant sucked at specific time intervals, followed by deactivating the enzymes of the withdrawn samples, and then centrifugation for 5 min. The released glucose and xylose were analyzed by HPAEC (Dionex, ISC 3000, USA) on a CarboPac PA-100 analytical column. The yields of glucose and xylose via enzymatic hydrolysis were calculated on the basis of the following equation:

$$\text{Glucose yield(\%)} = \left[ \frac{\text{(glucose produced via enzymatic hydrolysis} \times 0.9)}{\text{(glucan in corresponding } Eucalyptus \text{ sample)}} \right] \times 100 \quad (1)$$

$$\text{Xylose yield(\%)} = \left[ \frac{\text{(xylose produced via enzymatic hydrolysis} \times 0.88)}{\text{(xylan in corresponding } Eucalyptus \text{ sample)}} \right] \times 100 \quad (2)$$

Once the desired enzymolysis time had reached, the mixtures were centrifuged and the residual solids (rich-lignin) were washed extensively with acetate buffer (pH = 4.8) and hot water to remove the hydrolyzed carbohydrates, and then freeze-dried. The obtained lignin fractions (Impregnated Enzymatic Lignin, IEL) were weighted to quantitate the yields and named as  $IEL_{A1-2h}$ ,  $IEL_{A2.5-2h}$ ,  $IEL_{A5-2h}$ ,  $IEL_{H0.5-2h}$ ,  $IEL_{H1-2h}$ ,  $IEL_{H2-2h}$ ,  $IEL_{A2.5-1h}$ , and  $IEL_{A2.5-0.5h}$ , respectively. Additionally,  $S_{BM-2h}$ ,  $S_{BM-1h}$ , and  $S_{BM-0.5h}$  without impregnation as control were also hydrolyzed by enzyme under same condition and the residues (Enzymatic Lignin, EL) were labeled as  $EL_{BM-2h}$ ,  $EL_{BM-1h}$ , and  $EL_{BM-0.5h}$ , respectively.

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