Diol Pretreatment to Fractionate a Reactive Lignin in Lignocellulosic Biomass Biorefinery

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Abstract

Lignin-first fractionation has become a new biorefinery target to obtain valuable monomers toward the complete utilization of lignocellulosic biomass; but increasing delignification through conventional pretreatment approach often affects the structural integrity of the dissociated lignin. We discovered a new reactive lignin with great solvent solubility and preserved β-O-4 linkages from eucalypts after a modified organosolv pretreatment using 1,4-butanndiol (1,4-BDO). Unlike conventional organosolv pretreatment using ethanol, lignin precipitation was not observed in 1,4-BDO pretreatment. Meanwhile, 2D HSQC NMR analysis revealed that the residual lignin obtained from 1,4-BDO pretreated eucalypts contained a relatively higher amount of β-O-4 interunit linkages, indicating a higher integrity than ethanol pretreated lignin. This result agreed with the lower content of phenolic hydroxyl groups in dissolved lignin via ^{31}P NMR analysis. With increasing pretreatment severity, the amount of aliphatic hydroxyl groups decreased in ethanol pretreated lignin while it remained at a higher level in 1,4-BDO pretreated lignin. These results suggested that 1,4-BDO quenched the benzyl carbocation intermediate and formed ether linkage with a hydroxyl tail at the α position of the lignin. Solubility tests revealed that grafting aliphatic hydroxyl groups on 1,4-BDO lignin increased its dissolution. This phenomenon was further demonstrated for four different diols with similar structures. In addition, more than 90% cellulose conversion was obtained for all the diol pretreated eucalyptus after 48 h of enzymatic hydrolysis with cellulase at a loading of 7.5 FPU per grams of glucan. Diol pretreatment thus offers a promising reaction pathway to coincide with three pillars of biorefinery for lignin fractionation, lignin structural integrity, and cellulose hydrolysis.

Keywords: diol pretreatment; lignin fractionation; NMR; lignin solubility; β-O-4; hydroxyl groups

1. Introduction

Developing biorefinery technologies to harvest fuels and chemicals from lignocellulosic biomass is a promising strategy to reduce the reliance on nonrenewable petroleum refinery products.[1] Biomass pretreatment is one of the most commonly used techniques to break down plant cell wall and facilitate carbohydrate utilization in the biorefinery. Conventional pretreatment approaches (*e.g.*, steam explosion and dilute acid/alkaline processes) have been carried out at elevated temperature with various chemicals to remove or redistribute hemicellulose and lignin. However, the required harsh reaction conditions can lead into undesired irreversible lignin degradation and condensation reactions (Fig.1),[2-5] especially when the biomass feedstock is a mixture of different plant species and the pretreatment conditions cannot be well-monitored or optimized. Lignin degradation normally occurs with the cleavage of ether linkages, resulting in formation of new phenolic hydroxyl groups which negatively impact the digestibility of the pretreated substrate by reinforcing the unproductive interactions between lignin and enzymes.[6, 7] On the other hand, lignin condensation is carried out with the formation of carbon-carbon (C-C) linkages, which increases the hydrophobicity of the residual lignin and leads to the precipitation of dissolved lignin on the surface of pretreated substrate and subsequently impacts its digestibility by effecting the accessible surface area of the activated cellulose.[8] Compared with native lignin, pretreated lignin could have limited amount of ether linkages and high amounts of C-C linkages,[9] which is detrimental to lignin-to-aromatic conversion processes. This lignin condensation reaction also reduces the yield of fermentable sugars and lignin monomers in the downstream processes.[10-12]

As lignin ether linkages are directly related to lignin valorization opportunities, several innovative strategies have been recently disclosed to produce "reactive lignin" (Fig. 1, right column**)**. In terms of pretreatment processes, ammonia,[13] ionic liquid (IL),[14-16] γ-valerolactone (GVL)[17, 18] and mild organosolv pretreatments (<120°C)[19] have been introduced to promote biomass fractionation under "mild" conditions. These mild pretreatment strategies aim to limit the formation of reactive intermediates and hence preserve the ether linkages in lignin. Besides modifying the pretreatment medium, catalysts or functional reagents have also been applied to quench or trap the reactive intermediates such as C_α benzylic carbocation, C_2-aldehyde-substitited phenolics, and Hibbert's ketones which are prone to repolymerization.[20] Formaldehyde-assisted lignin fractionation encourages the formation of stable acetals (*i.e.*, 1,3-

dioxane rings) between lignin's C_α-OH and C_γ-OH, which actively preserves ether linkages and blocks the formation of C_α benzylic carbocation.[21] Peroxidation of C_α-OH to C_α=O weakens the β-O-4 ether linkage and avoids lignin condensation.[22] *In situ* trapping of Hibbert's ketones through reaction with alcohols, hydrogenation, or decarbonylation also suppress lignin condensation.[23, 24] Nonetheless, few studies have addressed the conflicts between maintaining the structural integrity of pretreated lignin and increasing the extent of delignification thus improving enzymatic digestibility of pretreated biomass. Deuss et al. (2017) investigated 27 types of lignins and reported the 'trading' between lignin yield and amounts of ether linkages, and there results indicated that lignin fractionation still significantly relied on the cleavage of ether linkage.[25] Side-reactions such as grafting of functional groups (*e.g.*, formaldehyde and GVL processes) on cellulose can interfere with the productive binding of cellulase to cellulose.[21, 26-28] An efficient process to withdraw lignin from the plant cell wall without causing significant lignin condensation is in demand and needs to be investigated.

This study aims to explore the potential of an innovative pretreatment reagent (*i.e.*, diols) to isolate lignin without sacrificing its integrity. Organosolv pretreatment has been shown to be a promising strategy to fractionate lignin and enhance enzymatic saccharification.[29-31] During a typical acid catalyzed organosolv pretreatment, the hydroxyl groups from the solvent could carry out a nucleophilic reaction with the C_α benzylic carbocation, which minimizes lignin degradation and condensation reactions.[30, 32] However, the extent of delignification in organosolv pretreatment still relies on the effective cleavage of ether linkages (*i.e.,* lignin degradation), which conflict with the beneficial effects of nucleophilic reaction. To address this particular concern, it is hypothesized that 1,4-butandiol (1,4-BDO) can cease this conflict and fractionate reactive lignin in a modified organosolv pretreatment. Systematic experiments on organosolv pulping with diols (*e.g.,* 1,3-BDO and 1,4-BDO) were carried out by Kishimoto et al.[33-35] 1,4-BDO is a very effective delignification solvent for both hardwoods and softwoods even without acid catalyst at 200 °C and 220 °C, respectively.[34] Homolytic cleavage of ether linkage in lignin can be achieved via quinone methide intermediates under the pulping conditions, which eliminate the formation of Hibbert's ketones.[33] Meanwhile, Hildebrand solubility theory suggested that 1,4-BDO has a smaller relative energy difference (RED) with lignin than other alcohols, implying a better lignin dissolution capability of this organosolv pretreatment.[35, 36] In this study, we prepared various types of lignin from dilute acid (DA), ethanol (EtOH), 1,4-BDO

and other diols pretreated Eucalyptus and the composition and structure of the obtained lignin were investigated via chemical composition analysis, gel permeation chromatography (GPC), and NMR analyses. The potential and mechanisms of diol pretreatment to fractionate reactive lignin were clarified.

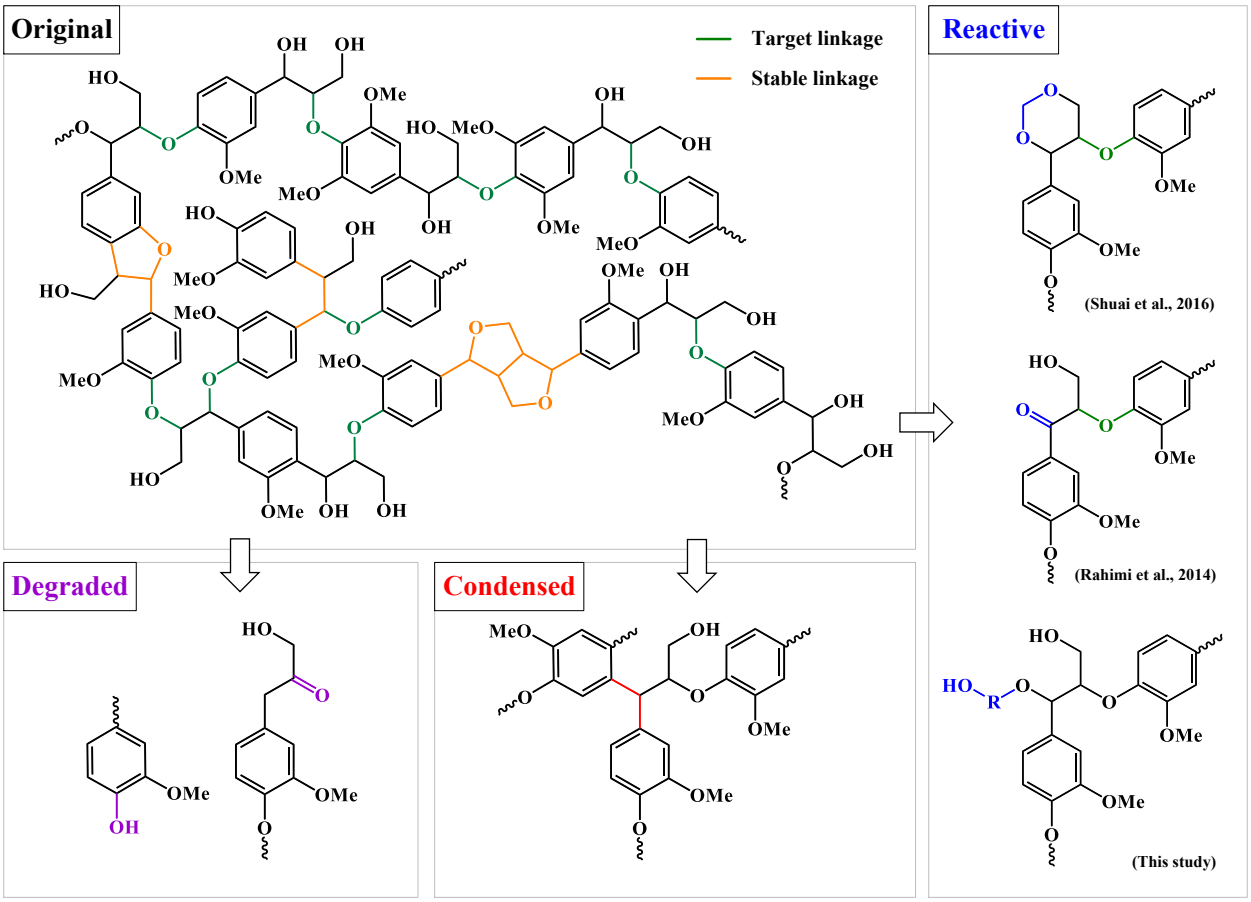


Fig. 1 Types of linkages (original, degraded, condensed, and active) in lignin structure before and after pretreatment.

2. Materials and methods

2.1.Materials

Eucalyptus chips were provided by Leizhou forestry bureau (Guangdong, China). Pretreatment solvents and other chemicals were purchased from J&K Acros Organics (Beijing, China). Commercial cellulase Cellic Ctec2 was generously provided by Novozymes Investment Co. Ltd. (Beijing, China) and Pronase was obtained from Sigma Chemical Company (USA).

2.2.Pretreatment

Eucalyptus chips were manually debarked, Wiley-milled, screened (mesh size < 2 mm), and Soxhlet-extracted with ethanol/toluene (1:2, v/v) for 24 h. The extractives-free sawdust was then aqueous organosolv (e.g. EtOH and 1,4-BDO) pretreated with sulfuric acid as a catalyst using a custom-built, eight-vessel (500 ml each) rotating digester made by Xian Yang Tong Da Light Industry Equipment Co. Ltd. (Shanxi, China). A 50.00 g (oven-dried weight) batch of wood powder was mixed with 15 mM to 35 mM sulfuric acid and 65%-85% (v/v) organosolv reagents at a liquid-to-solid ratio of 7:1. The digester was heated to 170 ± 3 °C at a rate of 3 °C per min and maintained at the temperature for 60 min. After pretreatment, the substrate and spent liquors were then separated using a nylon cloth. The substrate was washed three times (75 mL each) with warm (60 °C) aqueous organosolv with the same concentration as the pretreatment liquor. The spent liquor, the organosolv washes were then combined and then mixed with three volumes of water to precipitate the dissolved lignin. The substrate was then washed three times with water at 60 °C, and the washes were discarded.

2.3.Chemical compositional analysis

The chemical composition of eucalyptus before and after pretreatment was determined according to the procedures established by the National Renewable Energy Laboratory (NERL).[37] The hydrolyzed monomeric sugar units were quantified via high-performance liquid chromatography (HPLC, Shimadzu) equipped with CHO-782 Transgenomic column and acid insoluble lignin content was calculated gravimetrically.

2.4.Enzymatic hydrolysis

Enzymatic hydrolysis was conducted at 2% (w/v) solid loading in acetate buffer (50 mM, pH 4.8) with an enzyme loading of 7.5 FPU/g glucan in a 500 mL Erlenmeyer flask. The mixture was incubated in a rotary shaker at 50 °C and 150 rpm for 72 h. A 1.00 ml sampling of supernatant was collected at 3, 6, 9, 24, 48, 72 h, quenched by submersion in a boiling water batch for 10 min followed by immediately frozen to -20 ºC until sugar analysis on high-performance liquid chromatography (HPLC, Shimadzu) equipped with CHO-782 Transgenomic column.

2.5.Preparation of organosolv soluble lignin and residual lignin

Seven different lignin samples were prepared from the DA, EtOH and 1,4-BDO pretreated eucalyptus based on the experimental procedure presented in Fig. 2. The pretreatment conditions were fixed at 15 mM sulfuric acid and 65% organosolv and sample separation procedure was described in Section 2.2.

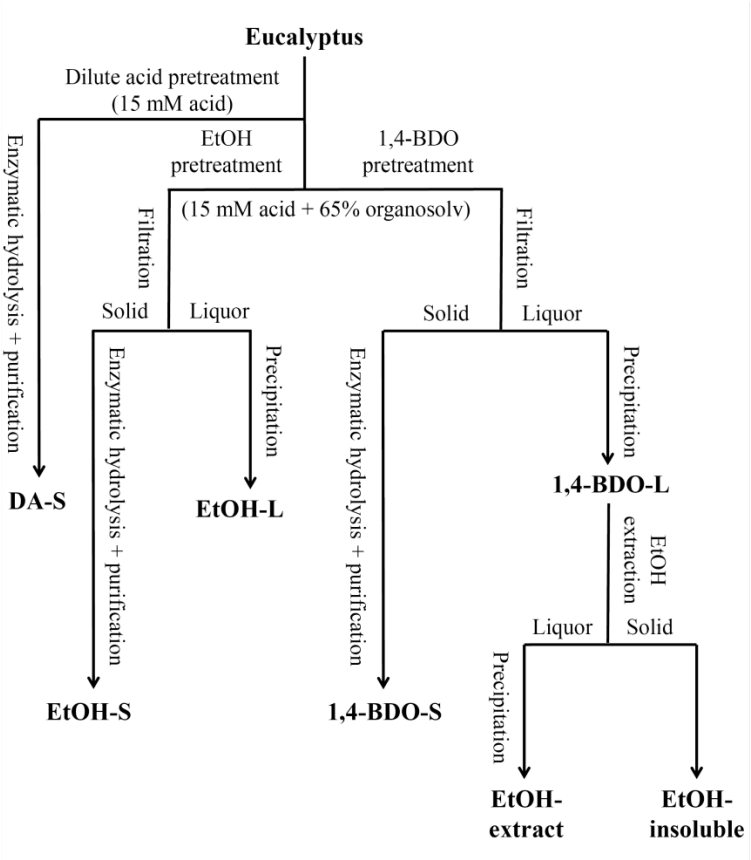


Fig. 2 Procedure of lignin fractionation/solubility experiments.

Residual lignins (DA-S, EtOH-S and 1,4-BDO-S) were prepared and purified according to a modified procedure described by Meng et al. (2017).[38] The pretreated eucalyptus was hydrolyzed by cellulase at 2% (w/v) solid loading in 50mM sodium citrate buffer (pH = 4.8), 50 °C, 150 rpm for 72 h in a shaker. Enzyme loading was set at 40 FPU/g substrate to remove most of the carbohydrates in the pretreated substrate. After hydrolysis, the lignin residues were

recovered by centrifugation and rinsed three times with distilled water. The crude lignin was then incubated overnight at 37 °C in 50 mM phosphate buffer (pH=7) with 1 U/mL Pronase. After filtration, the solid residues were extracted twice with 96% (v/v) dioxane/water mixture at room temperature for 48 h. The extracts were combined, rotary evaporated, and freeze-dried to obtain residual lignin for further analysis.

Organosolv soluble lignins (i.e., EtOH-L and 1,4 BDO-L) were isolated from the liquid product according to Pan et al. (2005).[30] Three volumes of water were added into the pretreatment spent liquor and the precipitated lignin was rinsed with water to remove soluble impurities. The purified lignin was freeze-dried under vacuum for 24 h and stored in desiccators before analysis. To characterize the unique feature of the 1,4-BDO-L lignin, a 0.10 g lignin sample was extracted with 10 mL ethanol for 24 h. The extract was rotary-evaporated and freeze-dried to obtain EtOH-extract lignin, and the solid residue after the ethanol extraction was freeze-dried to obtain EtOH-insoluble lignin. Organosolv soluble lignin from the other pretreatments in this work was prepared as described above.

2.6. Gas chromatography and mass spectrometry (GC-MS)

Organosolv soluble lignins (i.e., EtOH-L and 1,4 BDO-L) were dissolved in THF and analyzed by GC-MS using an Agilent 7890B series GC equipped with a HP5-MS capillary column and an Agilent 5977A series mass spectroscopy detector. The following operating conditions were used: injection temperature at 250°C, a column temperature program of 50°C (1 min), 15°C/min to 300°C and 300°C (7 min), and a detection temperature of 290°C.

2.7. Size-exclusion chromatography (SEC)

The weight-averaged molecular weight (Mw) and number-averaged molecular weight (Mn) of the lignin samples were measured by SEC as described previously.[39] Briefly, lignin samples were dissolved in N,N-dimethylacetamide with addition of 0.11 M LiCl solution, filtered through a 0.45 mm filter and placed in a 2 mL autosampler vial prior to GPC analysis. The molecular weights were estimated by size-exclusion separation performed on the HPLC equipped with Agilent PLgel MIXED-B column.

2.8.Nuclear magnetic resonance (NMR) analysis

Two-dimensional heteronuclear single quantum coherence (2D HSQC) nuclear magnetic resonance (NMR) analyses were conducted to determine the molecular-scale structure of pretreated lignin samples.[40] For each test, approximately 50 mg lignin samples were dissolved in 0.5 mL DMSO-d_6. The 2D HSQC ^{1}H-^{13}C experiments were performed using a JEOL ECZR 500 MHz spectrometer equipped with a 5 mm ROYAL probe. A JEOL standard pulse sequence was applied under the following parameters: spectral width of 11 ppm in F2 (^{1}H) with 1024 data points and 190 ppm in F1 (^{13}C) with 256 data points; 64 scans and 1.5 s interscan delay. JEOL's Delta 5.0 software was used for volume integration of contours in HSQC spectra.

For the quantification of hydroxyl groups in the lignin samples, phosphorylation of each sample was performed with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) in a solvent of pyridine/$CDCl_3$ (1.6/1.0, v/v) as described in the previous study of Meng et al. (2018).[41] In detail, the stock solution was prepared with pyridine/$CDCl_3$ (500 μL) and 5 mg·mL^{-1} internal standard (cyclohexanol). Approximately 20.0 mg of lignin sample was dissolved in the stock solution, and phosphorylation was conducted by adding 100 μL of the phosphorylating reagent TMDP. Quantitative ^{31}P NMR spectra were acquired on the JEOL ECZR 500 MHz spectrometer using an inverse-gated decoupling pulse sequence, 90° pulse, 25 s pulse delay with 32 scans. All chemical shifts reported are relative to internal standard at 145 ppm. The quantitative calculation of the hydroxyl groups was based on the amount of the internal standard.

2.9.Calculation of relative energy differences and solubility tests

Hansen solubility parameters of various alcohols were calculated to clarify the solvent power dissociating the lignin.[31, 35] Thermodynamics requires that the free energy of mixing must be zero or negative for the solution process to occur. Hildebrand and Scott pointed out that solubility parameter should be considered as a free energy parameter.[35] Solubility parameters (δ) are derived from the energy required to convert a liquid to a gas, which directly reflects the degree of dispersion forces, permanent dipole-permanent dipole forces, and hydrogen bonding hold to molecules of the liquid. It can be calculated by the following equation:

$$\delta^2 = \delta_D^2 + \delta_P^2 + \delta_H^2 \quad (1)$$

where δ_D is the dispersion component calculated using a homomorphic method; δ_P is the polar component; and δ_H is the hydrogen bonding component with the latter parameters determined from best reflecting empirical evidence. The ability of solvents to dissolve or swell polymer can be predicted by the relative energy difference (RED) as follows:[42]

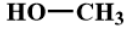
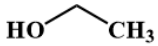
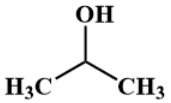
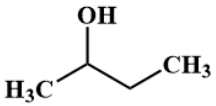
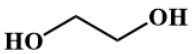
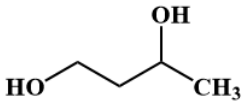
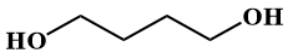
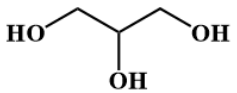
$$(R_a)^2 = 4 \cdot (\delta_{D2} - \delta_{D1})^2 + (\delta_{P2} - \delta_{P1})^2 + (\delta_{H2} - \delta_{H1})^2 \quad (2)$$

$$RED = R_a / R_o \quad (3)$$

where R_a is an empirically parameter developed by Skaarup. It represents the difference between the Hansen solubility parameters for a solvent (1) and a polymer (2). Smaller RED numbers indicate higher affinity between the solvent and the polymer, indicating higher degree of dissolution of the polymer in the solvent. By plotting δ_D, δ_P, and δ_H of the studied solvent over the same parameters of the solute. The point falling into a boundary sphere with critical distance to the origin was considered a good solvent.[42]

Solubility tests on 1,4-BDO-L lignin were carried out with three types of solvents, including (i) monohydric alcohols, *i.e.*, methanol (MeOH), EtOH, 2-Propanol (2-ProOH), 2-Butanol (2-BuOH); (ii) diols, *i.e.*, ethylene glycol (EG), 1,3-propanediol (1,3-PG), 1,3-butanediol (1,3-BDO), 1,4-butanediol (1,4-BDO); (iii) glycerol; and (iv) tetrahydrofuran (THF).

Table 1 Structure and Hansen solubility parameters ($MPa^{1/2}$) of selected organic solvents

Solvent	Structure	Dispersion component (δ_D)	Polar component (δ_P)	Hydrogen bonding component (δ_H)	Relative energy difference (RED)
Lignin	See Fig.1	21.9	14.1	16.9	0
Methanol (MeOH)		15.1	12.3	22.3	1.08
Ethanol (EtOH)		15.8	8.8	19.4	0.99
2-Propanol (2-ProOH)		15.8	6.1	16.4	1.07
2-Butanol (2-BuOH)		15.8	5.7	14.5	1.10
Ethylene Glycol (EG)		17	11	26	1.00
1,3-Propanediol (1,3-PG)		16.8	13.5	23.2	0.88
1,3-Butanediol (1,3-BDO)		16.6	10	21.5	0.89
1,4-Butanediol (1,4-BDO)		16.6	15.3	21.7	0.85
Glycerol		17.4	12.1	29.3	1.13
Tetrahydrofuran (THF)		16.8	5.7	8	1.16

3. Results and discussion

3.1.Optimization of pretreatment conditions

Hansen solubility parameters of various alcohols to lignin are presented in Table 1. EtOH and 1,4-BDO showed the lowest REDs among the selected monohydric alcohols and diols, respectively. Thus, they were selected as the solvent for biomass pretreatment and lignin valorization, and DA pretreatment was conducted as a control. The selected hardwood species eucalyptus is a widely used and well-studied woody material as a bioenergy feedstock. It consists of ~42% cellulose, ~21% hemicellulose, and ~25% Klason lignin. The optimal organosolv processing condition for eucalyptus in recovering cellulose, hemicellulose, and lignin was defined as 170 °C, 60 min, 15 mM H_2SO_4 and 65% EtOH.[29, 30] This condition was applied in our pretreatment experiments and followed by more server conditions to fractionate more lignin for valorization.

The chemical compositions of untreated, DA, EtOH, and 1,4-BDO pretreated substrates were presented in Fig. 3a through Fig. 3c, respectively. Hemicelluloses in the eucalyptus were completely removed after DA pretreatment, leaving only cellulose and lignin in the substrates (Fig. 3a). Lignin removal was very limited during DA pretreatment as expected. Lignin was subjected to depolymerization reactions during DA pretreatment, but the solubility of depolymerized lignin was limited in dilute acid.[35, 43] In the EtOH pretreatment, delignification was improved but still limited when acid charge was at 15 mM. The content of lignin residues was reduced to 4.6% when acid and EtOH doses were 20 mM and 65%, respectively (Fig. 3b). However, increasing the concentration of acid (25 mM) and/or EtOH (85%) both resulted in an increase in the content of residual lignin in the substrates up to ~7.5%, indicating that lignin may be condensed and/or precipitated on the surface of the substrate under these conditions. Similar results were also obtained for the 1,4-BDO pretreatment (Fig. 3c) where the delignification was quite limited when acid charge was at 15 mM. Klason lignin content was reduced to approximately 1.6% when acid and solvent charges increased to 20 mM and 65%, respectively. Unlike EtOH pretreatment, Klason lignin content in the pretreatment residues was not increased at higher pretreatment severity in 1,4-BDO pretreatment and were all less than 2% even when acid charge and solvent concentration were increased to 35 mM and 85%, respectively. As condensation reactions can be carried out through the formation of resonance stabilized benzyl carbocation during organosolv pretreatment,[30] our results

suggest that lignin condensation was either alleviated or the condensed lignin (with changed chemical properties after pretreatment) could still be dissolved in 1,4-BDO. As optimal pretreatment conditions are usually biomass-specific, the capability of 1,4-BDO in keeping lignin in the liquor can be beneficial to handle complex feedstocks with different amounts and types of lignin in the feedstock. The following section provides more detailed analysis on the change of lignin physicochemical structures during biomass pretreatment.

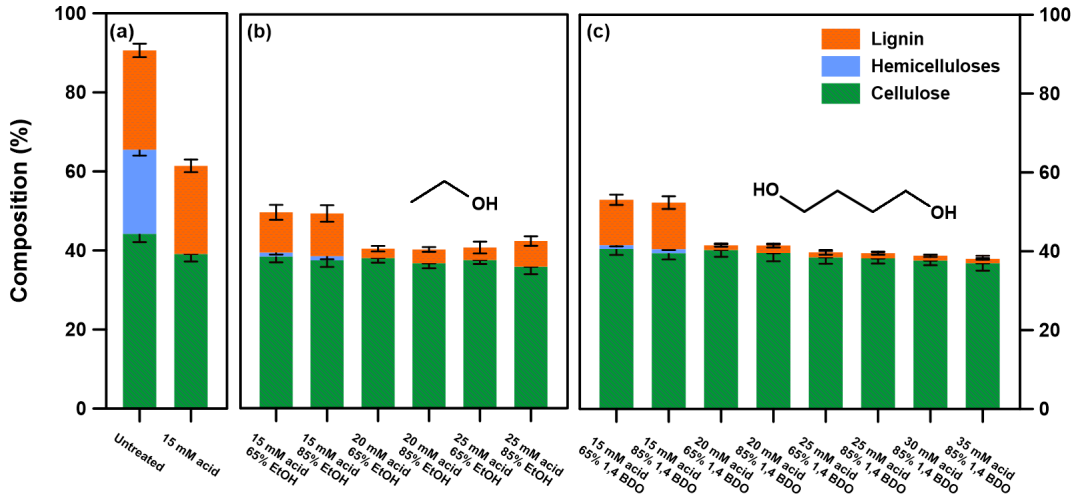


Fig. 3 Chemical compositions of untreated, DA, EtOH and 1,4-BDO pretreated eucalyptus.

3.2.Comparison of physicochemical properties of EtOH and 1,4-BDO lignins

Fig. S1 showed the GC-MS of EtOH and 1,4-BDO soluble lignins (15 mM, 65% organosolv). The lignin samples were identified by comparison with retention times and mass spectra of authentic samples. Acidolysis products such as Hibbert's ketones were found to be formed in Fig. S1, while stilbene structures from homolytic cleavage of ether linkages were not observed. Pretreatment conditions used in this study are milder than high boiling solvent (HBS) pulping conditions[34] and only lignin monomers can be readily detected by GC-MS, so lignin samples were further analyzed by 2D HSQC NMR.

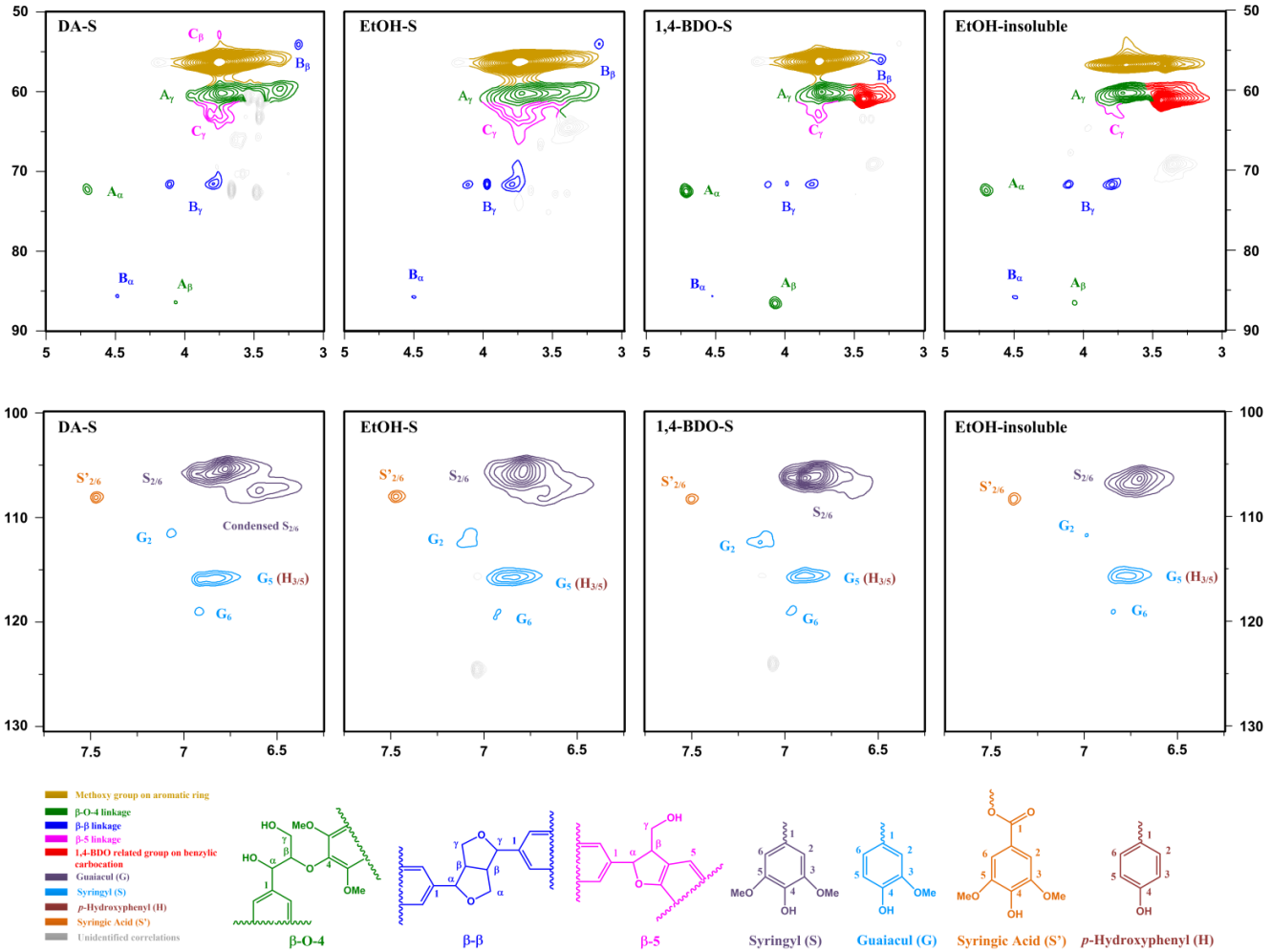


Fig. 4 2D HSQC NMR spectra from pretreated lignin. Abbreviation was depicted in Fig. 2.

The representative 2D HSQC NMR spectra of DA, EtOH and 1,4-BDO pretreated lignin residues and their corresponding soluble lignins (15 mM, 65% organosolv) are summarized in Fig. 4, and Fig. S2, respectively. In the aliphatic regions of the HSQC NMR spectra of pretreated lignin residues, β-O-4, β-5, and β–β were the dominant interunit linkages (Fig. 4, upper row). A unique signal was discovered in the HSQC spectra of 1,4-BDO pretreated lignin residues and soluble lignin (marked in red, Fig. 4 and Fig. S2), which indicated the 1,4-BDO related functional group on the α position of β-O-4 linkage (Fig. 1, the bottom of right column). It has been reported that alcohols can act as a nucleophile and react with benzylic carbocation to form α esterified lignin.[32, 34] Deuss et al.[23] reported that diols could capture Hibbert's ketones and form a dioxane type of structure to prevent lignin condensation, but this type of structure was not observed in our work due to the fact that 7 members ring is not energetically favorable. The aromatic regions of the spectra are presented in the bottom row of Fig. 4. The pretreated lignin demonstrated its major aromatic compositions consisting of S and G units for hardwood species.[8] Condensed lignin was not observed in organosolv pretreated lignin, indicating that both EtOH and 1,4-BDO alleviated lignin condensation. The inability to directly observe EtOH related α esterified signal in aliphatic regions of HSQC NMR spectra was possibly due to overlapping of signals from other interunit linkages of the lignin (Fig. S2).[19] In addition, most of the interunit linkages were absent in the NMR spectra of soluble lignin samples, implying significant degradation of lignins during organosolv pretreatments (Fig. S2).[44] In order to understand the structure/functionality of 1,4-BDO-L lignin, EtOH extraction was conducted as described in Section 2,5 (bottom right of Fig. 2). One interesting phenomenon found in our study was that 1,4-BDO-L lignin was partially dissolved in EtOH, and β-O-4 linkages were still observed in the EtOH insolubilized fraction (Fig. 4). It is indicated that some lignin can dissolve in 1,4-BDO and preserve β-O-4 linkages in 1,4-BDO pretreatment.

The relative distributions of different lignin subunits and inter-unit linkages in each lignin residues were summarized in Table 2. The values were calculated from HSQC NMR spectra by integrating the peak volumes of $S_{2/6}$ and G_2 at δ_C/δ_H 104.0/6.68 ppm and δ_C/δ_H 111.0/6.96 ppm, respectively. The peaks at δ_C/δ_H 71.4/4.81 ppm, δ_C/δ_H 86.9/5.47 ppm, and δ_C/δ_H 85.1/4.65 ppm represented the correlation of α position of β-aryl ether (β-O-4), phenylcoumaran (β-5), and resinols (β–β),

respectively. The S/G ratios of lignin subunits were lower in organosolv lignin residue (3.6 and 4.4) than that in DA-S lignin (10). Approximately 10% of β-5 and 2% of β-β were detected in all the lignin samples, indicating that organosolv did not impact these inter-unit linkages in comparison with DA lignin. Compared with original lignin from Eucalyptus,[45] cleavage of β-O-4 linkages were confirmed in all lignin residues from the three pretreatments. The content of β-O-4 linkages over total lignin aromatic subunits in EtOH-S was only 4%, while 1,4-BDO-S and EtOH-insoluble was 22% and 17%, respectively. The structure of lignin obtained from 1,4-BDO pretreatment had higher integrity than EtOH pretreated lignin. Acid-catalyzed cleavage of β-O-4 interunit linkages has been recognized as the major mechanism of lignin depolymerization and dissolution in acid catalyzed organosolv pretreatments,[31] but it should be noted that over-cleavage of β-O-4 linkages to yield more dissolved lignin can damage the lignin structure for valorization under harsh pretreatment conditions.

Table 2 Quantitative information for lignin subunits and inter-unit linkages in pretreated lignin.

	DA-S	EtOH-S	1,4-BDO-S	EtOH-insoluble
Lignin subunits				
Syringyl (S)	90%	81%	78%	90%
Guaiacyl (G)	10%	19%	22%	10%
S/G	9.0	4.4	3.6	9.0
Inter-unit linkages				
β-O-4	9%	4%	**22%**	**17%**
β-5	10%	10%	11%	10%
β-β	2%	2%	2%	2%

Abbreviation was depicted in Fig. 2

The ^{31}P NMR technique was conducted to characterize and quantify the functional OH groups in the fractionated lignin samples (Fig. 5a to Fig. 5c). The results for different lignins were colored coded throughout the three subfigures as follows (up to bottom): the solid residues of DA (DA-S), EtOH (EtOH-S), and 1,4-BDO (1,4-BDO-S); the dissolved lignin from EtOH (EtOH-L), 1,4-BDO (1,4-BDO-L); EtOH extractable 1,4-BDO-L (EtOH-extract); and EtOH insoluble 1,4-BDO-L samples (EtOH-insoluble). The peaks shown in the NMR spectra (from left to right in Fig. 5a) represent the aliphatic OH (i); cyclohexanol as an internal standard (ii); C_5 substituted OH (iii); guaiacyl phenolic OH (iv); and carboxyl OH (v), respectively.

Fig. 5b showed the molecular weight of the seven lignin samples measured by SEC. The content of each OH group was calculated by integrating the area of phosphorylated hydroxyl groups with respect to the known concentration of the internal standard (cyclohexanol), and the results were presented with the weighted average molecular weight in Fig. 5c. Compared with DA-S, a decrease in aliphatic OH was observed in both the EtOH-L and EtOH-S, suggesting that the α position of β-O-4 had been transformed into an ether linkage. This reaction pathway is supported by the relatively higher amount of aliphatic OH in 1,4-BDO-L. The soluble lignins (EtOH-L and 1,4-BDO-L) contained higher amounts of phenolic OH groups than the insoluble lignins (EtOH-S and 1,4-BDO-S), which can be attributed to cleavage of β-O-4 linkages, allowing low molecular weight lignin to be solubilized. It should be emphasized that EtOH-S and EtOH-L both contained significantly more phenolics than 1,4-BDO-L and 1,4-BDO-S, which supported our observation in the HSQC NMR analysis that EtOH lignin had less β-O-4 linkage than 1,4-BDO lignin.

Hydroxyl groups of soluble lignin at different pretreatment severities were also quantified and presented in Fig. S3. The amount of phenolic groups increased with the increasing pretreatment severity. New phenolic OH groups were formed by cleavage of β-O-4 linkage, which depends on the increased doses of acids and organosolv reagents as well as the reaction severity of the pretreatment.[6] Meanwhile, formation of phenolic OH was more substantial in EtOH pretreatment than in 1,4-BDO pretreatment under similar pretreatment conditions. A decrease in aliphatic OH was observed with the increasing acid charge, which can be attribute to the loss of γ-methylol group as formaldehyde and OH groups on $C_{\alpha,\beta}$ or the whole side chain to form stilbene types of structures.[33, 46, 47]

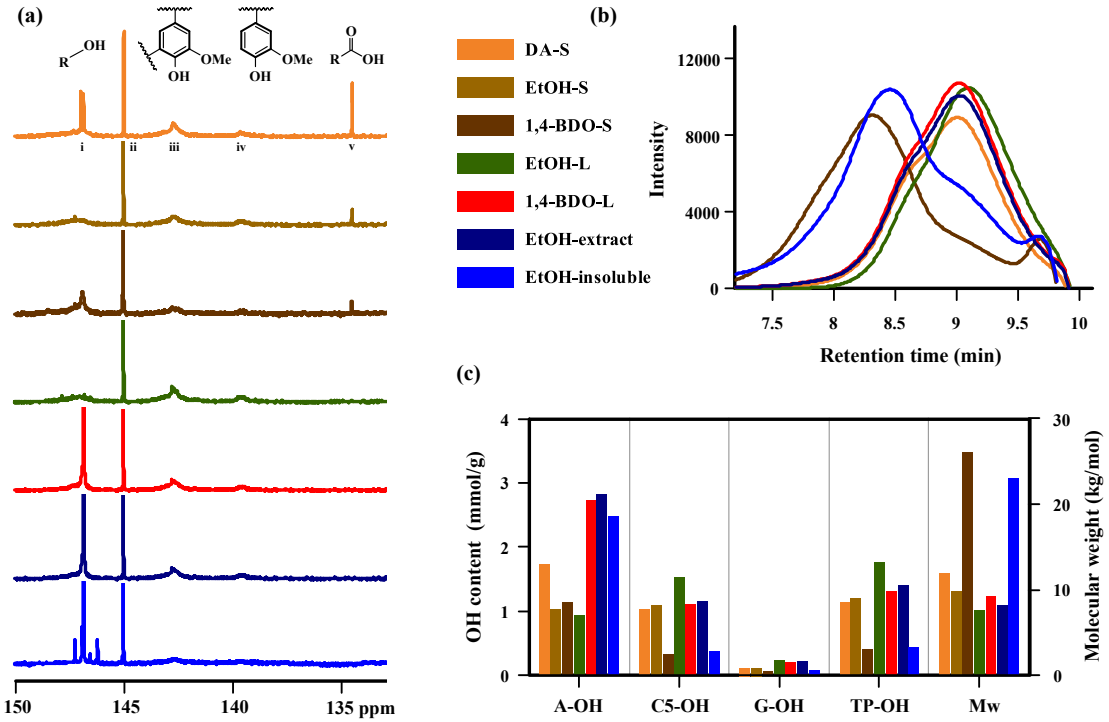


Fig. 5 Chemical characteristics pretreated lignin samples after DA (15 mM acid); EtOH; and 1,4-BDO (15 mM acid and 65% organosolv). (a) ^{31}P NMR spectra; (b) SEC spectra; and (c) overall comparisons. S: residue lignin; L: organosolv soluble lignin; EtOH-extract and EtOH-insoluble lignin was harvested from 1,4-BDO-L lignin as detailed in Fig.2 and the text.

Functional OH groups and molecular weight of EtOH extraction and EtOH insoluble lignin (bottom right of Fig. 2) was also analyzed by the ^{31}P NMR technique and SEC (Fig. 5). EtOH-insoluble lignin had larger molecular weight and lower amount of phenolic OH groups than EtOH-extract and EtOH-L, and its property is very similar with 1,4-BDO-S. Meanwhile, EtOH-insoluble lignin had more aliphatic OH groups than EtOH-L. The results indicated that aliphatic OH groups can facilitate dissolution of lignin in 1,4-BDO. As new phenolic OH groups were generated by cleavage of β-O-4 linkages of lignin, results obtained here are in consistent with the analysis of 2D HSQC that this type of lignin (EtOH-insoluble) can dissolve in 1,4-BDO and preserve β-O-4 linkages in 1,4-BDO pretreatment. Dissociation of lignin without breaking the β-O-4 linkages is a new biorefinery target aiming at completely utilization of building block chemicals (*i.e.*, cellulose and lignin valorization).[3] During 1,4-BDO pretreatment, 1,4-BDO quenched C_α benzylic carbocation and formed α-etherified β-O-4 linkages on lignin. As 1,4-BDO contains two OH groups, the

unreacted hydroxyl group (*i.e.* hydroxyl tail) at the α position of on 1,4-BDO lignin would increase the amount of aliphatic OH groups on lignin after pretreatment (OH on lignin decreased in EtOH pretreatment). This reaction offers 1,4-BDO pretreatment an alternative pathway to increase the solubility of lignin without significantly breaking the β-O-4 linkages, hence implying a potential of higher yield of lignin monomer in the downstream processes. Further investigation on process optimization is expected to promote this pathway and to obtain more reactive lignin.

3.3.Solubility of 1,4-BDO-L lignin

Solubility tests and extra pretreatments were conducted to investigate whether the beneficial effects on lignin dissolution may occur in other organic solvents (Fig. 6). 1,4-BDO-L lignin was completely dissolved in all the tested diols (*i.e.*, EG, 1,3-PG, 1,3-BDO) and THF, but various amounts of precipitate occurred in monohydric alcohols. The solubility of 1,4-BDO-L lignin in MeOH, EtOH, 2-ProOH, 2-BuOH and glycerol were 81%, 87%, 75%, 73% and 61%, respectively. Schuerch (1952) confirmed similar solubility of seven lignins by establishing the first sets of Hansen solubility parameters.[36] Skaarup (1967) defined the empirical weighting factor "4" in Equation (2),[42] as dispersion component δ_D was considered as a more sensitive parameter than δ_P and δ_H. The δ_D of diols is closer to lignin than that of monohydric alcohols, and hence smaller REDs were calculated as well as solubility than other alcohols. Meanwhile, δ_H of lignin is smaller than diols, implying that the solubility of 1,4-BDO-L lignin can be increased by grafting OH group on the lignin.

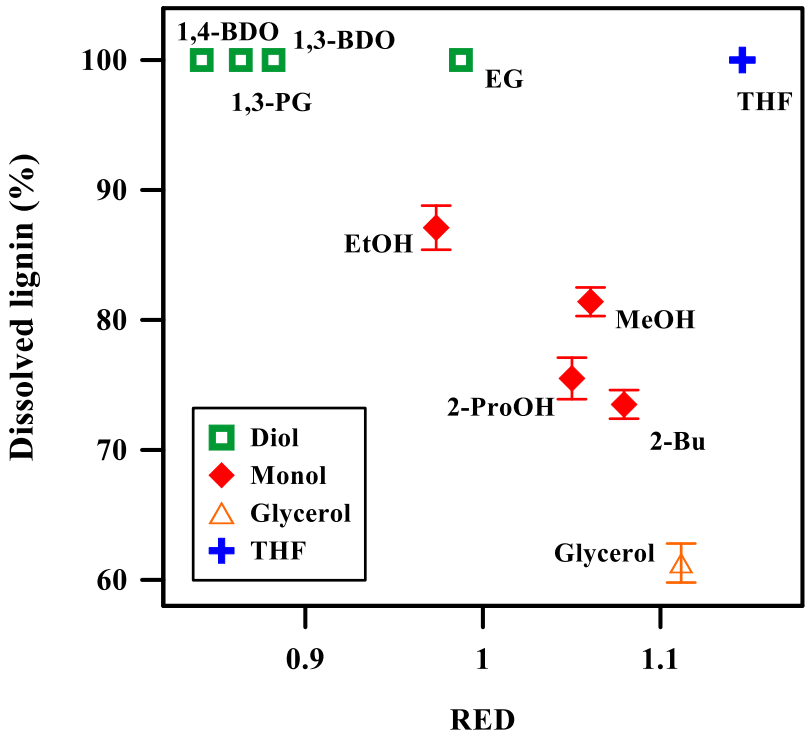


Fig. 6 1,4-BDO pretreated lignin solubility as a function of various alcohols and THF with different relative energy difference (REDs) between lignin and solvents.

3.4.Diol pretreatment

Based on the solubility tests, we hypothesized that all diols (*e.g.* EG, 1,3-PG, 1,3-BDO) can create similar beneficial effects as 1,4-BDO during organosolv pretreatment and the related experiments were designed and performed to validate it. Fig. 7 showed the levels of lignin dissolution in organosolv pretreatment at two severities, *i.e.*, 20mM 65% and 25mM 85%, respectively. As expected, diols removed more lignin than monohydric alcohols (in order, methanol, ethanol, 2-propanol, 2-BuOH and glycerol) and the differences were more significant with the increase of pretreatment severity. Residual lignin contents of 2-ProOH, 2-BuOH and glycerol pretreatment were more than 60% at elevated pretreatment severity (25 mM acid, 85% organosolv). As β-O-4 linkages were not observed in the HSQC spectra of soluble lignin, the amount of phenolic OH groups were used to estimate the structure integrity of lignin. The amount of phenolic OH groups of lignin from diols pretreated eucalyptus was approximately 20% higher than MeOH and EtOH lignins. 2-ProOH, 2-Bu and glycerol pretreated samples contained almost same amount of phenolic OH groups (index of preferable structural integrity), but the yield of dissolved lignin was

much lower than all the other solvents. The trends of molecular weights of dissolved lignin in different solvents are consistent with functional group measurements (from ^{31}P NMR analysis). Both M_w and M_n of lignin from diols pretreated eucalyptus were larger than the ones from monohydric alcohols. All the diols soluble lignin (*i.e.*, EG, 1,2-PG, 1,3-PG, 1,3-BDO) showed similar features as 1,4-BDO-L .

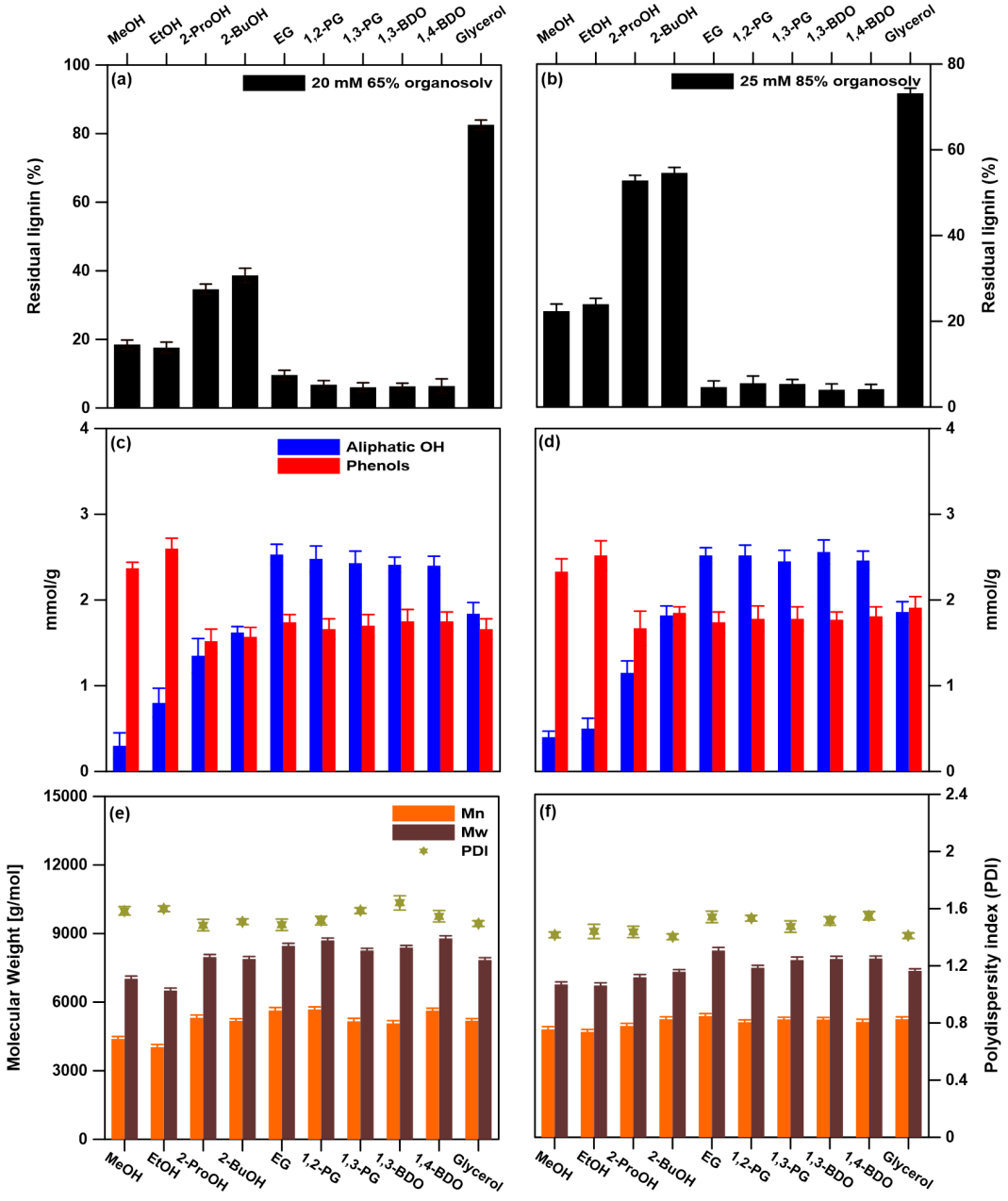


Fig. 7 Effects of organosolv pretreatments on isolated lignin yields and lignin structure integrity among different solvents. (a) and (b) percentage of residual lignin after organosolv pretreatments at two severities; (c) and (d) hydroxyl group contents of organosolv soluble lignin; (e) and (f) Molecular weights and polydispersity index of organosolv soluble lignin**.**

3.5.Enzymatic hydrolysis

Enzymatic hydrolysis experiments were carried out to evaluate the potential improvements or impacts of diols pretreatment on sugar release. The digestibilities of the pretreated eucalyptus were demonstrated in Fig. 8a to Fig. 8c. At a relatively low enzyme dose (7.5 FPU/g glucan) cellulose conversion (%) increased rapidly in the first 12 h of hydrolysis and reached a plateau after 24 h operation. At lower pretreatment severity (20 mM acid, 65% organosolv), cellulose conversions of MeOH and EtOH pretreated eucalyptus were more than 90% when the residual lignin content was around 20% (Fig. 8a). However, cellulose conversion of MeOH and EtOH pretreated eucalyptus reduced to 80% when pretreatment severity increased to 25 mM, 85% organosolv due to the increase of lignin content (>30%) in the residue. This situation was even worse in 2-ProOH and 2-Bu pretreated eucalyptus as the cellulose conversion of organosolv pretreatment with 2-ProOH and 2-Bu were less than 40% at 25 mM, 85% organosolv (Fig. 8b). The 48 h cellulose conversions of glycerol pretreated eucalyptus were lower than 20% at both pretreatment severities due to its high lignin content (over 80%). On the other hand, the cellulose conversions of diol pretreated eucalyptus were all higher than 90%, as lignin content in pretreated residue was both below 10% at the two pretreatment severities (Fig. 8c). In addition, cellulose conversion after 9 h enzymatic hydrolysis was almost proportional to the residual lignin contents when comparing the digestibility of all the organosolv pretreated substrates (Fig. 8d).

Lignin inhibits the access of the enzymes to cellulose by forming a physical barrier to restrict cellulose accessibility to enzymes, competing for the enzymes with cellulose by non-productive adsorption, and even deactivating the enzymes.[48, 49] Organosolv pretreatment is effective to remove lignin and eliminate lignin inhibition on enzymatic hydrolysis. Residual lignin after pretreatment can still pose non-productive adsorption via hydrophobic interaction, electrostatic interactions and hydrogen bond.[7] Hydrophobic interaction is expected to play a dominant role in the adsorption of cellulase on lignin, which has been widely proposed.[6] During organosolv pretreatment, lignin interunit linkages such as β-O-4 ether bonds could be cleaved by pretreatment chemicals, which would result in lignin depolymerization, while lignin condensation also occurred.[3030] New carbon-carbon bonds could be

formed and C_α-OH served as a leaving group during lignin condensation, which increased hydrophobicity of lignin. Besides high lignin content, condensed lignin existed in organosolv pretreatment with MeOH, EtOH, 2-ProOH, 2-Bu and glycerol at higher pretreatment severity was also responsible for the lower cellulose conversion of enzymatic hydrolysis.

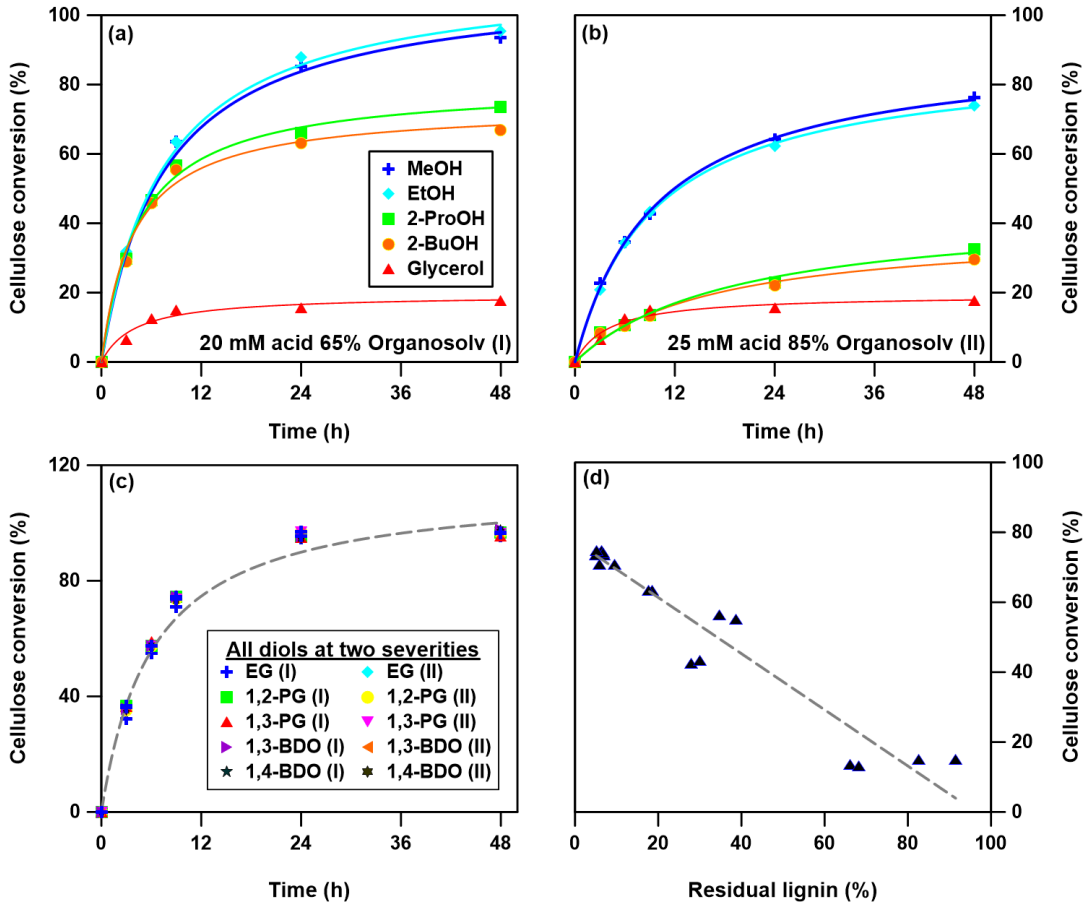


Fig. 8 Enzymatic digestibilities of organosolv pretreated eucalyptus. (a), (b) and (c) Cellulose conversion in enzymatic hydrolysis of organosolv pretreated eucalyptus over time, (d) Correlations between residual lignin and cellulose conversion at 9 h.

4. Conclusion

Diols pretreatment offered a reaction pathway to fractionate a new reactive lignin in biorefinery. During acid catalyzed conventional organosolv pretreatment, cleavage of α-ether linkage of lignin forms a resonance stabilized benzyl carbocation, resulting in either lignin condensation reaction or cleavage of β-O-4 linkages. The cleavage of β-O-4 linkage increases the solubility of lignin but affect the structural integrity of dissolved lignin, which may further reduce the yield of lignin monomer in

the downstream process. Diols acted as nucleophiles quenching the resonance stabilized benzyl carbocation and formed α-etherified lignin with hydroxyl tail. The hydroxyl tails (i.e. aliphatic hydroxyl group) improve the dissolution of lignin in diols, preserving the β-O-4 linkage in lignin residue for future lignin valorization. In the future, strategies need to be raised up for selectively accelerating α-esterification during diol pretreatment. In addition, due to the high solubility of the pretreated lignin without the need of sophisticated process optimization, this pretreatment technology may be applicable treating complex lignocellulosic biomass such as urban wastes or food processing residues.

Conflicts of interest

There are no conflicts to declare

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