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Effect of organosolv lignin and extractable lignin on enzymatic hydrolysis of lignocelluloses

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Effects of organosolv lignin and extractable lignin on enzymatic hydrolysis of lignocelluloses

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Abstract

Biofuels, derived from lignocelluloses, is an attractive supplement to fuel produced from non-renewable resources. Various pretreatment methods have been developed to overcome the recalcitrance of lignocelluloses to provide highly digestible substrates for enzymatic hydrolysis. Organosolv pretreatment with ethanol is a promising method for increasing cellulose accessibility, releasing sugars from hemicellulose, and recovering relatively pure lignin as a by-product. The use of other short-chain aliphatic alcohols in the organosolv pretreatment, such as methanol and propanol, was considered to have high-efficiency delignification. The following step of biochemical conversion is enzymatic hydrolysis, by which cellulose and hemicellulose are converted into fermentable sugars. The high cost of enzymes is one of the major bottlenecks in biochemical conversion.

In this work, methanol, ethanol, and propanol organosolv lignins from poplar, eucalyptus, aspen, Loblolly pine, and kenaf have been evaluated for their effects on enzymatic hydrolysis of lignocelluloses. Two dimension heteronuclear single quantum coherence spectroscopy (2D-HSQC), heteronuclear single quantum coherence-total correlated spectroscopy (HSQC-TOCSY), and heteronuclear multiple bond correlation spectroscopy (HMBC) have been used to characterize the structure changes of lignins before and after organosolv pretreatment. The spectra of HSQC, HSQC-TOCSY, and HMBC revealed that the alkylation (e.g., methylation, ethylation and propylation) of hydroxyl groups took place not only at C_α, but also at C_β and C_γ, and potentially also at the phenolic hydroxyl group of lignin. These results showed that propanol organosolv lignins (POLs) from poplar and aspen had higher stimulatory effects than the ethanol and methanol organosolv lignins (EOLs and MOLs) on the enzymatic hydrolysis of Avicel. The alkylation degree will affect the hydrophobicity of resulting organosolv lignins, which in turn will control

their positive or negative effect on the enzymatic hydrolysis. Methanol, ethanol and propanol organosolv lignins from poplar and aspen could increase the enzymatic hydrolysis of Avicel by 2-7% while organosolv lignin from eucalyptus and pine decreased the yield of the enzymatic hydrolysis of Avicel by 5-20%. The strong inhibition of eucalyptus and pine lignin on enzymatic hydrolysis might be controlled by their high hydrophobicity.

Extractable lignins, extracted from pretreated biomass by solvent (methanol, ethanol, propanol), have been accessed for their effect on the enzymatic hydrolysis of lignocelluloses as well in this study. The results showed that extractable lignins from aspen and pine decreased the yield of enzymatic hydrolysis of Avicel. The organosolv lignins from aspen were stimulatory on the enzymatic hydrolysis and the organosolv lignins from pine were less inhibitory than the extractable lignins. The hydrophobicity, enzyme binding strength, zeta potential, molecular weight, and HSQC NMR spectra of extractable lignins and organosolv lignins have been determined and compared. HSQC spectra showed similar chemical structure for the organosolv lignins and extractable lignins. It was observed that extractable lignins showed higher hydrophobicity and binding strength than organosolv lignins. In addition, it was observed that 72 h final yield of pretreated aspen and pine was decreased by 3-17%, when the pretreated substrates were washed with organic solvents. This indicates that the solvent washing is not necessary for the organosolv pretreatment.

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Chapter 1: Introduction and research objectives

1.1 Background

According to recent reports of the United Nations, the world population has reached 7.8 billion in 2020 and is expected to increase to 9.8 billion by 2050. With the population growth, the total world energy consumption is estimated to increase to 815 quadrillions Btu by 2040, especially in the sector of electricity, transportation, and industry[1]. However, the high dependence of energy resources on fossil fuels faces high pressure due to its non-renewable nature. Furthermore, as NASA reported in February 2020, the global carbon dioxide level has risen to a massive number of 413 ppm. Shortage and environmental concerns such as the greenhouse gas emissions related to non-renewable resources requires a transfer of the society from non-renewable energy to a renewable energy[2].

Biomass-derived biofuel is an attractive alternative to fuel production from non-renewable resources while mitigating CO_2 emission. Although the first generation of biofuels, such as corn or wheat ethanol, plays a major role in energy supplies worldwide. The second generation of biofuels from lignocellulosic biomass was reported to decrease land and water competition, normally used from food production[3, 4]. Simultaneously, the use of the second generation of biofuels decreases around 27% of greenhouse gas emissions than non-biofuel energy consumption. Therefore, such biofuels attract constant attention worldwide[5], while the interest in sustainable energy promotes the development of the lignocellulosic biofuel industry. However, to compete with the conventional non-renewable sources, the biochemical conversion should be cost-effective. The raw materials and enzymes are the main factor regulating the overall biofuel production and its large-scale application [6, 7]. In this dissertation, the focus is on the pretreatment of the materials

and the enzymatic hydrolysis aiming to lower the conversion cost. Before the introduction of pretreatment, the basic knowledge of lignocellulose chemical composition is needed.

Lignocellulosic biomass, including municipal waste residues, agricultural residues, forestry and industrial solid waste, is a environmentally friendly, economic. It contains 55-65% 75% of carbohydrates which could be converted to biofuel and biofuel products (e.g., ethanol, butanol, furfural, and lipids[8]. The most practical resources for biofuel production are agricultural residues, hardwood, and softwood. Cellulose, hemicellulose, and lignin constitute lignocellulosic biomass.

Regarding the concept of biorefinery, the utilization of hemicellulose and the lignins attracted increased attention[9]. Cellulose constitutes 40-50% dry weight of lignocellulosic biomass. It is made of highly crystalline glucose chain, making it resistant to the enzyme and chemical attack. Hemicelluloses are amorphous polymers composed of hexoses, pentoses, and some sugar acids. They constitute 20-30% dry weight of biomass. Lignin, which is composed of phenylpropanoid alcohol units, is a great aromatic resource globally. It is an amorphous polymer and plays a significant role in cementing the cell together, ensuring the structural recalcitrance of plant cells. These polymers interact with each other, and their relative composition varies with the type, species, source, and even growth conditions[10]. Due to this complex composition and tight interactions, lignocellulosic biomass is rigid to enzymatic digestion[11]. In order to access to the biofuel and related products, biochemical conversions including pretreatment and enzymatic hydrolysis are conducted. As aforementioned, pretreatment is the first step of biochemical conversion.

The pretreatment is a process of biomass pre-hydrolysis applied before any biochemical conversion. It is an essential step since it allows breaking down the hetero-matrix of plant cell

walls and renders the sugars to be more accessible[12]. Various pretreatment methods, including hot water, dilute acid, steam, and organosolv pretreatment, have been developed to release the sugars and make the biomass more susceptible to the cellulases attack[13, 14]. The solid fraction of pretreatment is designated as pretreated substrates, and the liquid fraction is called the prehydrolysates. Simultaneously, sugar monomers or oligomers from hemicellulose are either released in the prehydrolysate or further degraded into some microbial-inhibitory compounds. The pretreated lignin can be deposited in the pretreated substrates, depolymerized, dissolved in the prehydrolysate, or further degraded into inhibitors in the prehydrolysate[15]. Based on the synergistic effect of temperature, duration, and pH of the original solution before reaction, a severity factor was proposed to evaluate the severity of the various pretreatment methods[16]. Moreover, the pretreatment efficiency could be assessed by the sugars released from the hemicellulose and the carbohydrates from the pretreated substrates.

Among different pretreatment methods, organosolv pretreatment can enhance the biomass hydrolyzability with high hemicellulose sugar recovery and good lignins purity[17, 18]. It is also one of the most feasible and high-efficiency approaches. The biomass is pretreated with an organic solvent with an acid catalyst at high temperature (100-250 °C) in this process. It could separate cellulose and dissolve hemicellulose and depolymerized lignin. Methanol, ethanol, propanol and organic acids (e.g., salicylic acids and oxalic acids) are commonly applied in the organosolv pretreatment[19]. Organosolv pretreatment is very effective for delignification regardless of the materials size or lignin-condensed biomass as the softwoods[20]. Since the cost of organic solvents in the pretreatment is one of the most important disadvantages of this method, the distillation of solvent from prehydrolysate is always conducted for solvent recovery and reuse to reduce the cost efficiently[21].

The saccharification of cellulose into glucose is critical for the subsequent biofuel production. Comparing to the chemical process, enzymatic saccharification of pretreated substrates does not require complicated operating stages, the energy consumption is low, and the yield and selectivity are relatively high[22]. Enzymatic hydrolysis is the saccharification of cellulose and hemicellulose with a series of hydrolysis enzymes. Cost-effective enzymatic saccharification with high yield is the focus of the industrial biorefineries. The high cost of cellulase enzyme is the most crucial barrier for efficient use and, thus, for applying the enzymatic hydrolysis. Both substrates influence the cellulase enzymes efficiency in the enzymatic saccharification and enzyme-related factors[23, 24]. A certain amount of lignin remains in the pretreated substrates and significantly limits cellulose accessibility. Therefore, cellulase enzyme, lignin and cellulose are all essential factors in the enzymatic hydrolysis[25, 26].

1.2 Compositions in lignocellulosic biomass

High total carbohydrates make biomass a promising resource for biofuel; however, the complicated compositions and structure of biomass also make the utilization challenges. It is necessary to understand its composition and structure to utilize the lignocellulosic biomass cost-effectively. Cellulose, hemicellulose, and lignin constitutes lignocellulosic biomass. Hemicellulose and lignin form a strong network connect through hydrogen bonds and cement the highly linear and crystalline cellulose fibers. This three-dimensional structure of biomass is significantly recalcitrant for biochemical conversion, which cellulose also reinforces the lignin–carbohydrate complexes (LCC)[27].

The ratio of these three major components varies in different lignocellulosic biomass species (e.g., hardwood, softwood, and herbaceous biomass). For example, herbaceous biomass is

considered hemicellulose-rich, while hardwood is mainly composed of cellulose[15, 28]. The sugar constitution of hemicellulose is different in hardwood, softwood, and herbals[29]. The amount of cellulose, hemicellulose, and lignin varies because of its living conditions, light exposure, and the stage of growth[30].

Extractives are another type of composition which constitutes no more than 10% dry weight of the lignocellulosic biomass. It is a non-structural constituent of wood and acts as a natural defense for the microorganism. The composition of extractives is complex, containing more than 100 types of compounds, including fatty acids, alkanes, aldehydes, and esters[31]. During the pretreatment, hydrophobic nature of extractives prevents water-soluble molecules from the surface of the raw materials. This may affect the pretreatment efficiency and reduce the following enzymatic hydrolysis[32]. Some compounds from the extractives are reported to be detrimental to the subsequent fermentation process[33]. However, some hardwood extractives were stimulatory on the enzymatic saccharification. As a confusing component (always been combined in the acid insoluble lignin) in chemical composition analysis of biomass, solvent extraction is usually conducted as the first step in protocols to get rid of extractives.

1.2.1 Cellulose

Cellulose constitutes 40-50% dry weight of lignocelluloses. In most woody plants, cellulose is the major component. It is a D-glucopyranose chain connected with commonly 5,000 to 10,000 glucose units, but up to 15,000 units in cotton and up to 20,000 units in some algae-produced cellulose[34]. In ultrastructural arrangements within native cellulose, cellulose fibers have a linear and homogeneous structure. These polysaccharides are connected through covalent bonds, hydrogen bonds, and Waals force to form microfibrils. 30-200 microfibers are tightly bundled

together to form cellulose fibers with high tensile strength[35, 36]. This glucose-cellulose chain-macro cellulose structure (Fig. 1) makes the structure and rigidity of the biomass cell. These cellulose natures make it highly stable to physical, chemical, and biological attacks, including enzyme degradation[37].

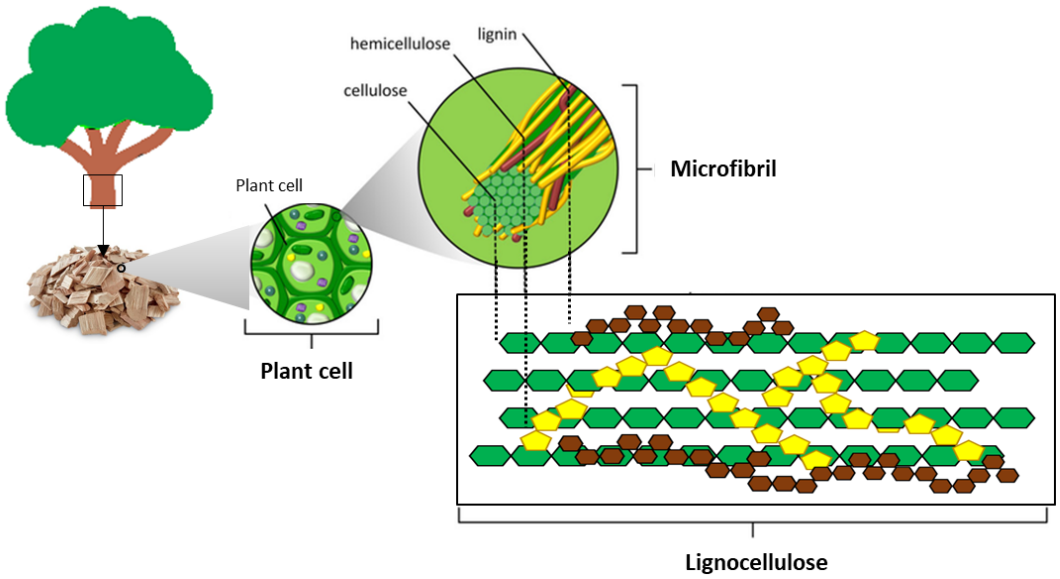


Fig. 1 Structure of lignocelluloses

Cellulose is highly crystalline and contains both crystalline and amorphous regions. Among these regions, the amorphous region is the starting point of the hydrolysis[38]. However, there is no apparent distinction between these two regions, which increases cellulose complexity [39]. There are three hydroxyl groups in each glucopyranose unit. They determine the hydrophilicity and reactivity of cellulose[40]. And based on the reaction of hydroxyl groups in cellulose modification, it may also influence the electrostatic interaction between cellulose and cellulase enzymes[41]. This cohesive structure of cellulose made the utilization of cellulose difficult. Therefore it will be essential to fractionate cellulose from hemicellulose and lignin with the method of pretreatment.

1.2.2 Hemicellulose

Hemicelluloses constitute 20-35% dry weight of biomass. In most plants like hardwood and herbals, xylan is the primary molecule in the hemicellulose skeleton. In hemicellulose from the softwood, the major chain is composed of the galactoglucomannans. It is the D-glucopyranose and D-mannopyranose units with the branch of galactopyranoses[31]. And the hardwood is usually more acetylated than the softwood. In some cases, there are also acetylated ferulate in the hemicellulose sidechain. Molecular weight and degree of polymerization of hemicellulose is much lower than that of cellulose[42]. Hemicellulose is connected with cellulose by hydrogen bonds, which makes the backbone of plant cells.

Hemicellulose is sensitive to heat and chemical treatment. Monomer sugars (e.g., xylose) could easily be released in autohydrolysis, dilute acid, and other pretreatment methods[43]. This nature is sometimes promising and challenging at the same time. On the one hand, pretreatment is widely used in processing and producing xylose products[44]. On the other hand, the pentoses and hexoses dissolve in the prehydrolysate will be released into hydrolysates and further degraded[45, 46]. Because of the bright prospect of biorefinery, one novel organosolv pretreatment with Maleic Acid has been developed. This approach could selectively release hemicellulose from biomass and convert the xylose into valuable products[47].

1.2.3 Lignin

Lignin has the most complicated structure among the three major components. It plays a critical role as a filler and binder together with hemicellulose surrounding the cellulose macro fibers through ester and ether bonds to give further structural rigidity and hold plant fibers based

on the structure of carbohydrates complex[48]. The lignin samples obtained by different separation methods generally have a molecular weight of 1000-20000 g/mol[49]. Since the structure of lignin is amorphous and lacks order, the natural degree of polymerization is difficult to be measured after fractionation[50].

Lignin is a polymerized macro-molecule consists of Sinapyl alcohol, Coniferyl alcohol, and *p*-Coumaryl alcohol. These building blocks are derived from the corresponding phenylpropanoid alcohol precursors (Fig. 2)[51]. The existence and ratio of subunits vary in different types of biomass. Softwood is mainly composed of guaiacyl subunits. Hardwood contains both guaiacyl and syringyl lignin, while herbaceous plants constitute guaiacyl, *p*-hydroxyphenyl, and syringyl subunits[52].

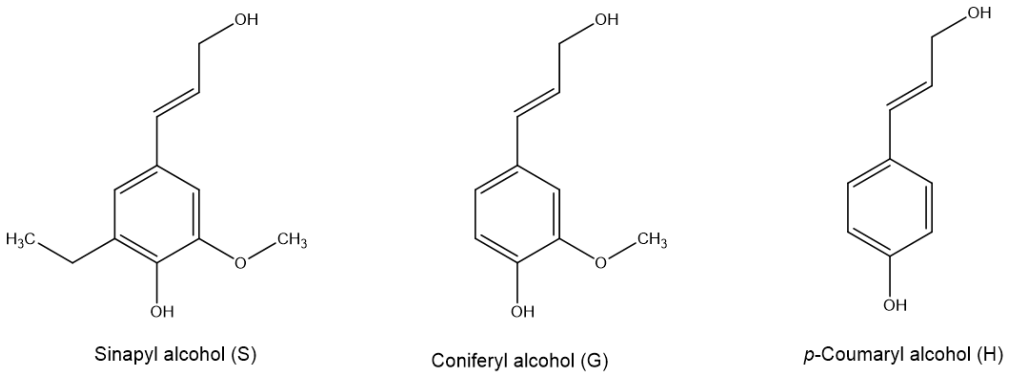


Fig. 2 Phenylpropanoid alcohol precursors of lignin.

Three basic lignin units are connected by various chemical bonds to form the three-dimensional structure of lignin. These three units are connected by specific linkages such as β-O-4, β-β, and β-5 linkages (Fig. 3). Among them, β-O-4 linkages constitute more than 50% of subunits connection[53, 54]. These chemical bonds connect different lignin monomer structural units into a highly branched, random, hydrophobic polymer. Lapierre et al. reported that hardwood is reported to have more than 60% of the ether bonds and less branched than softwood[55], so this

could be why hardwood is more accessible for different biochemical conversion. They also mentioned that around one-third of the G units linked with β-O-4 linkages are terminal (i.e., with a free hydroxyl group), while only less than 10% of S units are similar in hardwood.

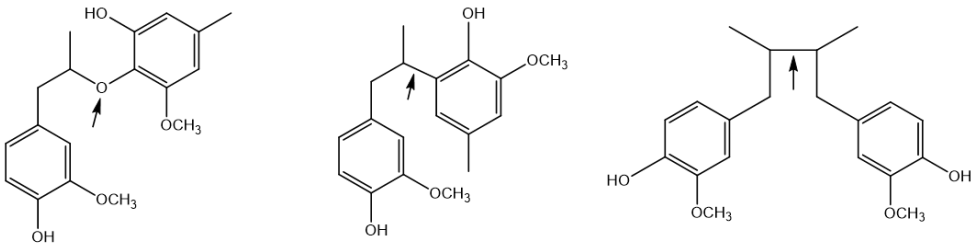


Fig. 3 Representative linkages of lignin

Previously, a large amount of lignin has been generated in different biorefinery industries but not well utilized. Every year, more than 50 million tons of lignin can be generated from paper-pulp plants as by-products[56], but most lignin is burned for heat and electricity and not used efficiently. It is still not a sustainable and highly efficient way. The low efficient utilization of lignin is mainly due to its high polymerization, complex structure, large molecular weight, and large structural differences. As a great resource for natural aromatics, economical lignin utilization is the trend of biorefinery. Because of the rich-phenol structure, lignin could be utilized as an antioxidant[57]. Different degradation methods (e.g., catalytic degradation, hydroprocessing, gasification, pyrolysis) have been developed to convert lignin into valuable aromatic monomers[58]. Although native lignin works well with these methods, lignin after pretreatment, even the high-quality organosolv lignins[59], was reported to have a relatively low yield with different catalytic degradation methods[60].

1.3 Biochemical conversion of lignocellulosic biomass

There are five major processes for the biochemical conversion of the lignocelluloses: pretreatment (release carbohydrates and reduce the recalcitrance of the materials); enzymatic hydrolysis of cellulose (saccharification of polysaccharides into monomer sugars like glucose and xylose); separation of lignin and lignin residue (lignin precipitation or extraction); microbial fermentation of carbohydrates (convert mono-sugars into ethanol or other biofuels. It is significant to have a cost-effective conversion process in the industry world. The major route for biochemical conversion is the degradation of cellulose and hemicellulose polymers to sugar monomers; and the fermentation of mono-sugars into biofuels i.e., ethanol. As regards to the recalcitrance of lignocelluloses, pretreatment is the base for these conversions to improve the cellulose accessibility for the subsequent enzymatic hydrolysis. This dissertation mainly focuses on the first two steps of biochemical conversion: pretreatment and enzymatic hydrolysis to improve biochemical conversion efficiency.

1.4 Pretreatment and chemical pretreatment

If the lignocelluloses are directly subjected to enzymatic hydrolysis, the saccharification efficiency is low. This is because of its complicated structure mentioned above. Therefore, pretreatment technology is essential to destroy the recalcitrance of lignocellulosic raw materials. Since a hemicellulose matrix and lignin well protect the native cellulose, an effective pretreatment of biomass is needed. It should rupture the structure of lignocellulosic materials by releasing hemicellulose, depolymerizing and dissolving the lignin, and releasing carbohydrates. The goal of pretreatment is to prepare biomass to facilitate bioconversion. The following requirements are usually listed to assess the overall efficiency of pretreatment[61-63]:

- High recovery of all carbohydrates.
- Reasonable recovery of lignins.
- High glucose yield of the substrates.
- Minimize degradation of major products before any detoxification.
- Low energy demand and low overall operational cost.

Chemical pretreatment is the most widely-applied pretreatment method. Chemical pretreatment, including acid, alkali, and organosolv pretreatment, is currently the leading pretreatment technology in biorefinery. In the pretreatment, chemicals i.e., organosolv, acid, and alkaline will be utilized to improve the cellulose accessibility. Lignin-carbohydrates complex (LCC) cements the cellulose chains, which blocks the microorganisms from the cellulose. Due to the chemicals in the pretreatment, a good cooking process can be achieved at a milder pretreatment temperature. After chemical pretreatment, the lignocellulosic biomass becomes more accessible. However, the pretreatment liquid generated during the pretreatment process is often discarded, which causes pollution to the environment. So the fermentation of the prehydrolysate and the control of its toxicity also became a research focus.

1.4.1 Organosolv pretreatment

Organosolv pretreatment is a typical chemical pretreatment involving solvent fractionation, by which high delignification efficiency and complete hemicellulose removal can be achieved[18]. Organosolv and acid catalyst (e.g., H_2SO_4 or HCl) are needed in the organosolv pretreatment to cleave the internal bonds in LCC[64]. Short-chain aliphatic alcohols, including ethanol, methanol, and polyols such as ethylene glycol, triethylene glycol, and other solvents, such as acetone, dioxane, and phenol. Organosolv pretreatment could significantly remove the lignin and partially

the hemicellulose of different types of lignocellulosic biomass by hydrolyzing 1) the internal lignin bonds, 2) chemical bonds in LCC, and 3) bonds between cellulose and hemicellulose, depending on the pretreatment conditions[65-67]. Most of the organic solvents are good for the delignification and removal of hemicellulose. Although organic solvents do not favor cellulose decrystallization, most of them could effectively increase cellulose accessibility[68].

The delignification, including depolymerization and dissolution of lignin, is the most critical reaction of the organosolv pretreatment. With the increase in temperature and pressure, hydrogen ions are released from hemicellulose in the initial stage of the organosolv pretreatment. They will act as catalysts, facilitating the breaking of C_α and C_α linkage of lignin, significantly, β-O-4 linkage cleavage (55-60%, Fig. 4 depolymerization)[69]. Simultaneously, smaller lignin fragments are produced and dissolved in the prehydrolysates. The concentration of the organic solvent and the acid catalyst are critical factors of the process of delignification. With the acid catalyst, the lignin cleaves into smaller molecules through ether bonds and dissolves in the organosolv[70]. Simultaneously, the carbocation intermediates would be attacked by nucleophilic molecules i.e., aromatic ring, and generate more condensed molecules by the formation of C-C bonds[71, 72] (Fig. 4 repolymerization).

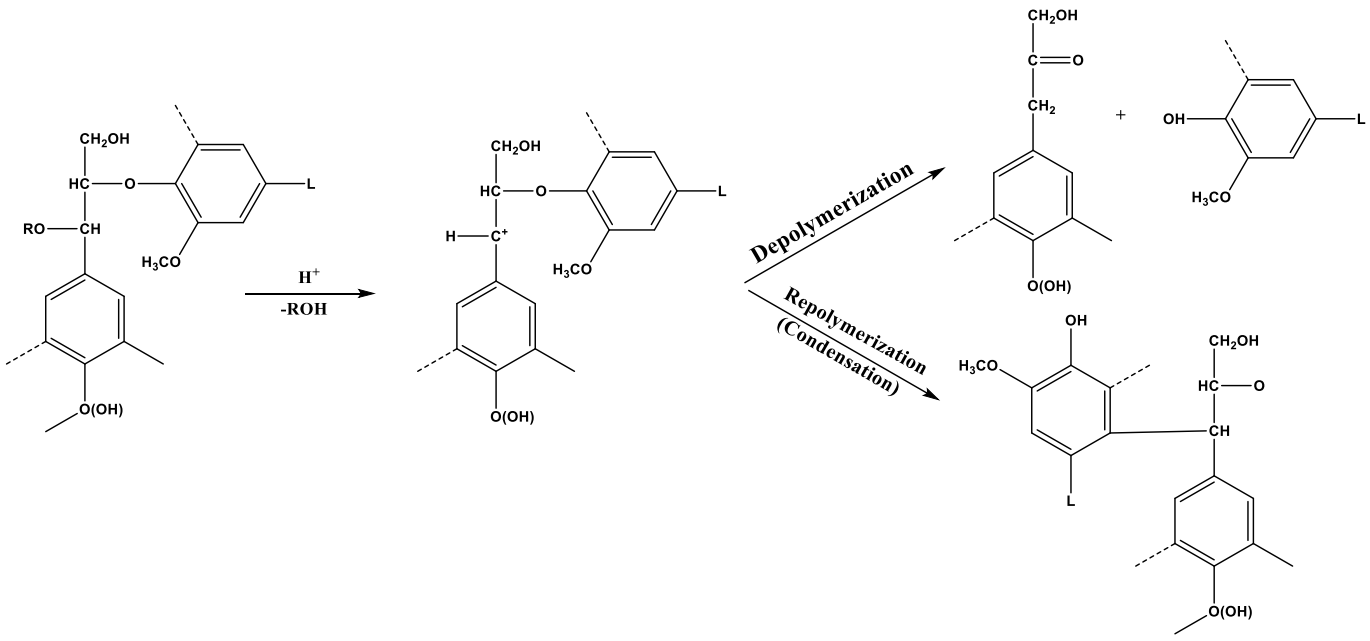


Fig. 4 Depolymerization and repolymerization of pretreatment

Condensation (repolymerization) is a significant side reaction in the depolymerization of lignin. As shown in Fig. 4, the carbocation intermediate not only depolymerizes into smaller fragments. It can also be attacked by nucleophilic atoms, e.g., the carbon in aromatic rings, and forms a condensed lignin structure. Condensed lignins are considered as the potential cause of high non-specific adsorption of cellulase enzyme[73]. To note, the C-C bonds themselves are not only less susceptible to catalytic degradation, but they could also trap some of the internal β-O-4 linkages and makes them inaccessible[69]. Therefore, decreasing the lignin condensation in the pretreatment is significant for both utilization of carbohydrates in biomass and the utilization of lignin. Few reactions were observed on the aliphatic side chain of lignin in acetone and dioxane organosolv pretreatment[74], α-ethylation during ethanol organosolv pretreatment was reported and proved to be beneficial for the reduction of lignin condensation (Fig. 5)[75] and the weakening of the inhibition of the lignin in the enzymatic hydrolysis[76].

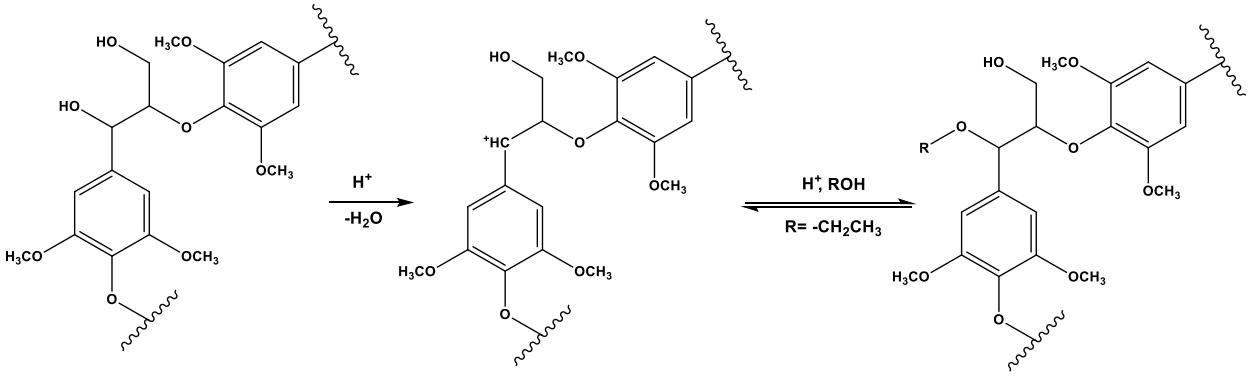


Fig. 5 Potential α-ethylation of lignin in ethanol organosolv pretreatment

Although delignification is significant in the organosolv pretreatment, there are several reactions involving cellulose and hemicellulose[77, 78]. These processes could restrain the amorphous region of the cellulose, thus improving cellulose accessibility[66, 79].

Some new solvents are introduced to increase the profit and application of organosolv pretreatment. For example, a novel organosolv pretreatment with maleic acid has been developed to selectively release hemicellulose from biomass and convert the xylose into valuable products[47]. Organosolv pretreatment process using the water-γ-valerolactone (GVL) system was reported to be good at lignin dissolution and the increment of proton activity in lignin depolymerization[80, 81]. It is a popular and safe solvent and fuel and could be derived from biomass raw material [82].

1.5 Lignins after fractionation

According to different methods of fractionation, the most common lignins after fractionation like pretreatment are organosolv lignin, extractable lignins, lignin sulfonate and milled wood lignin.

1.5.1 Milled wood lignin

Milled wood lignin is lignin separated by physical grinding and solvent extraction at room temperature. A typical method is ball-milling the raw material for more than 24 h and extraction of the resulting solid fraction using aqueous dioxane to get MWL from the material[83]. Björkman first proposed this method in 1954, so it is also called Björkman lignin. MWL is a typical isolated lignin which is close to the native lignin in the biomass[84, 85]. From the view of the structure, although the native lignin has a three-dimensional structure, MWL is a linear oligomer determined by QQ-HSQC [86].

1.5.2 Lignin sulfonates

The lignin sulfonate obtained can be divided into the following basic lignin sulfonate and acid lignin sulfonate according to different pH values of cooking liquid[87]. Lignosulfonates are by-products of the delignification in sulfite pulping. In the lab-scale, lignosulfonates could be obtained in sulfite pretreatment[88]. Lignosulfonates have extensive ranges of molecular mass, in which 10^3–10^6 da has been reported in the pulping process[89]. Meanwhile, softwood lignosulfonates were reported to have a higher molecular weight than hardwoods[90]. Lignosulfonates are similar to acid lignin from sulfuric acid treatment but have more sulfur groups; therefore, they are more hydrophilic have a higher degree of sulfonation than that of Kraft lignin[91]. Since the lignin sulfonates are rich in sulfonic acid groups, they have adequate water solubility and reactivity. Therefore they could be used to process water reducers, additives, etc.[92]

1.5.3 Organosolv lignins

Organosolv lignin is precipitated from diluted organic solvents collected from organosolv pretreatment. At the lab scale, organosolv lignin from organosolv pretreatment with aqueous ethanol is first hydrolyzed and dissolved in the solvent phase (Fig. 6). Organosolv pretreatment is a easy resource of high purity, high quality, and low molecular weight technical lignins[18, 93].

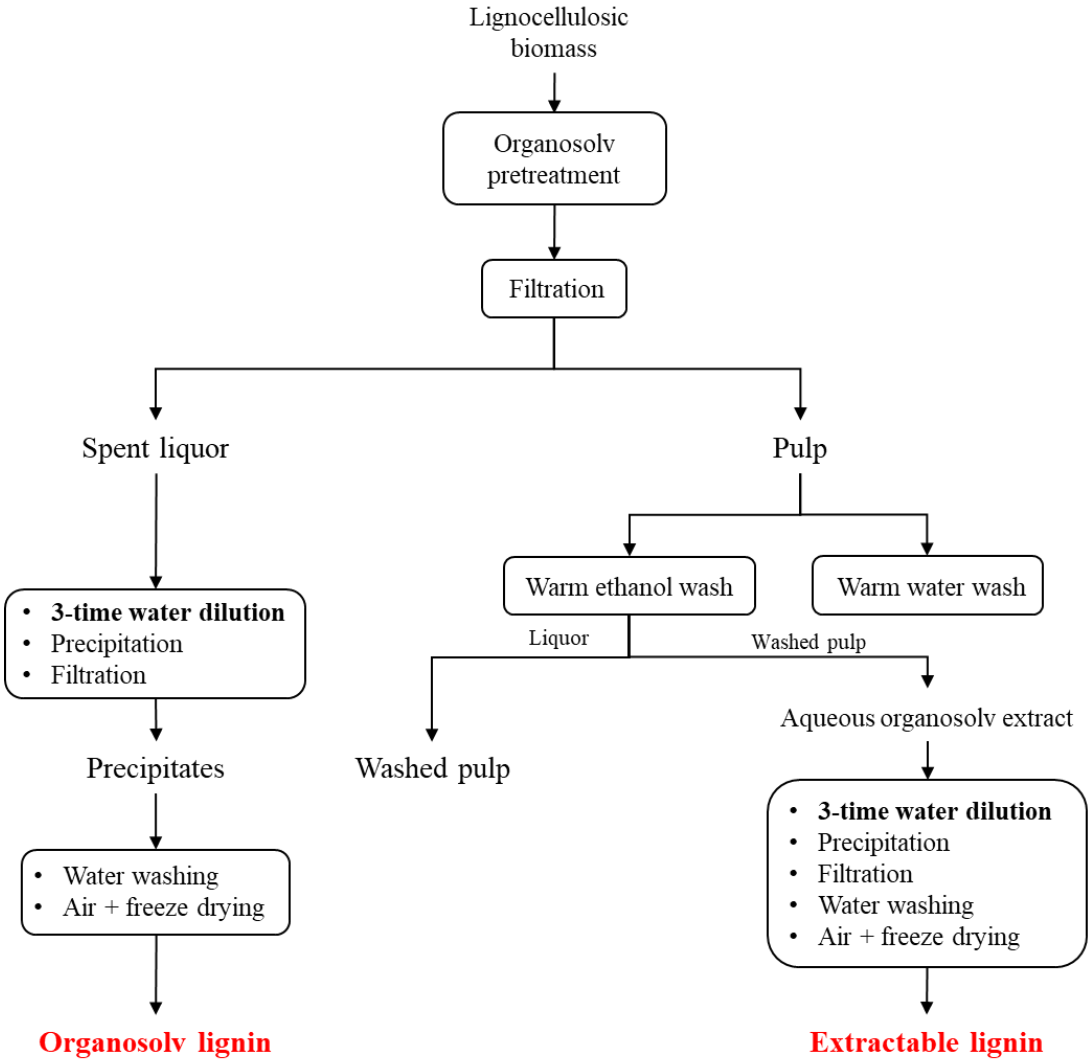


Fig. 6 Preparation of organosolv lignin and extractable lignin in organosolv pretreatment

Organosolv lignins from ethanol pretreatment of hardwood could stimulate the enzymatic hydrolysis[94, 95]. Ethanol organosolv lignins from hardwood (other than eucalyptus), collected from organosolv pretreatment below 190 °C, could facilitate the enzymatic hydrolysis due to less

lignin condensation repressed by ethylation[76]. Organosolv lignin from ethanol pretreated softwood (e.g., pine), which is considered detrimental to enzymatic hydrolysis[94], could also stimulate the enzymatic hydrolysis when the pretreatment temperature is above 190 °C.

It was previously shown that organosolv lignin is a good resource of sulfur-free lignin[96] and suitable for combustion because it has less ash, unlike other technical lignins[97]. It could be an appropriate solution in the production of phenol-formaldehyde resin[98]. Furthermore, it could be applied as an antioxidant, improved antioxidant activity obtained from organosolv lignins with low molecular weight and more aromatic alcohol groups[99]. Besides, organosolv lignins have been proved to be useful as antimicrobials and sunscreen products[100]. Organosolv lignins could be converted into polyols and used as platform chemicals or surfactants[101, 102].

1.5.4 Extractable lignins

Extractable lignins are obtained from the aqueous solvent extract of organosolv pretreated biomass (Fig. 6). Sawdust extractable lignin could increase the final yield of enzymatic hydrolysis. This is because this lignin hampered the process lignin adsorbs enzymes. Extractable lignin was considered as a part of organosolv lignins collected from the pretreatment before a distinguishing effect was found between these two types of lignins[17]. They were collected from different fractions of pretreatment. For example, a 5.3% increase was reported with the addition of extractable lignins in enzymatic hydrolysis of Avicel, while 11.5% enhancement was reported with ethanol organosolv lignins from sweetgum under similar pretreatment conditions[103]. It was also reported that organosolv lignins and extractable lignins from the same batch are chemically similar based on the analysis of ^{13}C NMR spectra.

1.6 Chemical structure of lignins

Functional groups would determine the physical properties such as hydrophobicity in lignin[104] and could be tested by further spectral analysis, including Fourier-transform infrared spectroscopy (FTIR), ultraviolet-visible spectroscopy (UV-vis), and nuclear magnetic resonance spectroscopy (NMR). Carboxyl groups (-COOH), methoxy groups (-OCH_3), phenolic hydroxyl groups (Ph-CH_2OH), phenolic hydroxyl groups (Ph-OH), and carbonyl groups (C=O) are common functional groups of lignins. Acylation, sulfonation, oxidation, and condensation were also often analyzed during the separation process. Since the extraction of any type is prone to alter the original lignin structure, it is difficult to understand the natural lignin structure[105].

1.6.1 Determination of functional groups of lignins by FTIR

Surface functional groups of lignin is commonly determined by FTIR. In the infrared spectrum, the absorption peaks for syringyl, methoxy group, aromatic ring, carbonyl group conjugated with the aromatic ring methyl group, methylene group are listed in Table 3[106, 107]. Quantitative and qualitative analysis of FTIR spectrum both work for the characteristic absorption peak position of the infrared absorption spectrum to infer its chemical reaction principle.

Table 1 FTIR absorption bands (cm^{-1}) assignment

Absorption wavelength (cm^{-1})	Assignment
3440	O-H stretching
2950	C-H stretching of methyl, methylene or methane group
1730	C=O stretching
1606	Aromatic ring
1463	Aromatic methyl group

1435	Aromatic ring
1375	C-H stretch in methyl group
1330	Syringyl ring with C-O stretching
1245	C-O stretching in S units
1180	C-O stretching in ester groups
1130	Aromatic C-H in S unit
1045	Aromatic C-H in G unit

1.6.2 NMR spectroscopic analysis of organosolv lignins

Nuclear magnetic resonance technology is an important technology to study the chemical structure of lignin. 1D ^{1}H, ^{13}C, ^{31}P NMR, and 2D HSQC, HSQC-TOCSY, and HMBC NMR analysis are commonly introduced to elucidate chemical structures of organosolv lignin from different biomass resources about its primary units, linkages, and functional groups (Table 4).

Signals of chemical shift and peak splitting of protons shows the structure of lignin in the proton spectrum (^{1}H-NMR, Table 5). Quantitative ^{1}H-NMR could be conducted with *p*-nitrobenzaldehyde as an internal standard. The chemical shift of ^{1}H-NMR usually ranges in 0-12 ppm, which may cause signal overlaps.

Table 2 NMR analysis of organosolv lignins

Biomass	Organosolv	Pretreatment condition	^{1}H	^{13}C	^{31}P	HSQC	HMBC	Reference
Corn Stover	Acetone	Acetosolv pulping from the plant				+		[108]
Corncob	Butanol	H_2O/n-butanol fractionation				+		[109]
Wheat straw	Ethanol	60 wt% aqueous ethanol, 190 °C, 1 h with H_2SO_4			+	+		[110]
Wheat straw	Acetone	50% wt% aqueous acetone, 140 °C, 120 min with H_2SO_4				+		[111]
Bamboo	Organic acid	84% aqueous organic acid (70:30 v/v formic acid/acetic acid), 90 °C, 3 h	+					[112]
Switchgrass	Ethanol	65% (v/v) aqueous ethanol, solid to liquid ratio 1:8, 180 °C, for 60 min with 0.9% (w/w) H_2SO_4			+			[113]
Buddleja davidii	Ethanol	65% (v/v) aqueous ethanol, 195 °C, 1 h with 1.50% (w/w) H_2SO_4		+	+			[114]
Spruce	Ethanol	60 wt% aqueous ethanol, 190 °C, 1 h with H_2SO_4			+	+		[110]
Loblolly pine	Ethanol	75% (v/v) aqueous ethanol, solid to liquid ratio 1:7, 170, 180, 190 and 200 °C, 1 h with 1.0 % (w/w) H_2SO_4	+	+		+	+	[76, 94]
Pine	Ethanol	65% (v/v) aqueous ethanol, solid to liquid ratio 1:8, 180 °C, for 1 h with 0.9% (w/w) H_2SO_4			+			[113]
Sweetgum	Ethanol	25/75 % (v/v) aqueous ethanol, solid to liquid ratio 1:7, 160, 170, 180 and 190 °C, 1 h with 1.0 % (w/w) H_2SO_4	+	+		+	+	[76, 94]
Poplar	Ethanol	60 % (v/v) aqueous ethanol, 180 °C, 20 min with 1.25 % (w/w) H_2SO_4			+	+		[115]
Hybrid poplar	Methanol	80%(v/v) aqueous methanol, 220 °C, 30 min				+		[116]
Hybrid poplar	Propanol	80%(v/v) aqueous propanol, 220 °C, 30 min				+		[116]
Hybrid poplar	Butanol	80%(v/v) aqueous butanol, 220 °C, 30 min				+		[116]
Populus trichocarpa deltoides	Ethanol	60 % (v/v) aqueous ethanol/GVL, 180 °C, 1 h with 1.25 % (w/w) H_2SO_4		+	+	+		[117]

Populus trichocarpa deltoides	GVL	60 % (v/v) aqueous ethanol/GVL, 180 °C, 1 h with 1.25 % (w/w) H_2SO_4	+	+	+	[117]
Aspen	Ethanol	65 % (v/v) aqueous ethanol, solid to liquid ratio 1:7, 160 °C, 1 h with 1.0 % (w/w) H_2SO_4			+	[95]
Black willow	Ethanol	65 % (v/v) aqueous ethanol, solid to liquid ratio 1:7, 160 °C, 1 h with 1.0 % (w/w) H_2SO_4			+	[95]
Cottonwood	Ethanol	65 % (v/v) aqueous ethanol, solid to liquid ratio 1:7, 160 °C, 1 h with 1.0 % (w/w) H_2SO_4			+	[95]
Eucalyptus	Ethanol	65 % (v/v) aqueous ethanol, solid to liquid ratio 1:7, 170 °C, 1 h with 15 mM H_2SO_4		+	+	[118]
Eucalyptus	Ethanol	65 % (v/v) aqueous ethanol, solid to liquid ratio 1:7, 160 °C, 1 h with 1.0 % (w/w) H_2SO_4			+	[95]
Eucalyptus	Ethanol	65 % (v/v) aqueous ethanol, solid to liquid ratio 1:7, 170 °C, 1 h with 15 mM H_2SO_4		+	+	[118]
Eucalyptus	1,4-butanediol	65 % (v/v) aqueous 1,4-butanediol, solid to liquid ratio 1:7, 170 °C, 1 h with 15 mM H_2SO_4		+	+	[118]
Eucalyptus	GVL	80 % (v/v) aqueous GVL, solid to liquid ratio 1:10, 120 °C, 30-60 min, with 20-100 mM H_2SO_4	+	+	+	[119]

Table 3 The assignment the signals of ^{1}H NMR spectrum

δ (ppm)	Assignment
9.4-8.5	Unsubstituted phenolic
8.5-7.9	Substituted phenolic
7.9-6.3	Aromatic
6.3-4.0	Aliphatic (H_α and H_β)
4.0-3.5	Methoxy and H_γ
2.9-2.8	H_β and β-1

^{13}C NMR has much broader spectra than ^{1}H NMR (i.e., 0-180 ppm) and, therefore, usually contains more information. Both qualitative and quantitative analyses could be conducted with ^{13}C NMR because of the settled number of benzene ring carbons. Table 6 summarizes the assignments for chemical shifts of woody biomass using deuterated dimethyl sulfoxide (DMSO) as solvent.

Table 4 The assignment the signals of ^{13}C NMR spectrum

δ (ppm)	Assignment
175-168	Carboxylic acid or ester
132.7-130.8	PB
155-140	Aromatic C-O
140-125	Aromatic C-C
125-102	Aromatic C-H
157-151	$S_{3,5}$ with C_4OR
150-145	G_3, $S_{3,5}$ with C_4OH
90-58	Alk-O-
90-77	Alk-O-Ar, α-O-Alk
77-65	γ-O-Alk, secondary OH

61.3-58	β-O-4
58-54	CH_3O
54-53	β-β, β-5

2D-HSQC NMR is widely conducted to characterize the lignin structure by determining the hydrocarbon (C-H) correlations. 2D NMR spectra are more efficient in chemical structure analysis of lignins because they provide more information than 1D ^{1}H, ^{13}C spectra, and could cause less signal overlaps in spectra. HSQC could measure β-O-4, β-β, β-5 from the side chain regions like, and the $S_{2,6}$, $G_{2,6}$, $G_{3,5}$, and *p*-hydroxybenzoic acid (PB) signals from the aromatic regions. 2D NMR could show information of functional groups such as methoxy group and ethoxy group introduced by organosolv pretreatment[76, 116]. Sometimes HMBC and HSQC-TOCSY will also be analyzed based on HSQC spectra to elucidate more details about the chemical structure of lignins[120]. However, it will be difficult for quantitative analysis when a spectral overlap occurs, so it is more efficient when only the relative amount of inter-unit linkage is based on the integral of total linkages. As shown in Table 3, HSQC is the most commonly used 2D NMR spectrum for structural identification and estimation of the relative amount of S/G/H ratios and chemical bonds between lignins (β-O-4, β-β, β-5) as well. Table 6 summarizes assignments of chemical shifts of typical inter-unit linkages and subunits in hardwood and softwood lignin structure (DMSO as solvent).

^{31}P NMR can be applied to elucidate the content of different hydroxyl groups in the lignin, like the phenolic hydroxyl groups and alcoholic hydroxyl groups. These structures affect the chemical properties of lignin and the bindings of cellulase[121]. In the laboratory scale, multistep derivatizations of lignins are needed for the analysis of ^{31}P NMR spectra[122]. ^{31}P NMR is more

effective in determining the presence and amount of hydroxyl groups in lignin with a broader spectrum.

1.7 Cellulase and enzymatic hydrolysis

Before converting carbohydrates into biofuel, lignocellulosic biomass must first degrade carbohydrates into fermentable sugars such as glucose and xylose by enzymatic saccharification before they can be further utilized by microorganisms. Enzymatic hydrolysis is the most promising method to obtain biomass glucose[15]. During the hydrolysis of cellulose, the effect of a single enzyme is limited, and the synergistic effect of several enzymes can significantly improve the efficiency of cellulose saccharification. So a series of cellulase enzymes will be applied to cooperate and complete enzymatic hydrolysis. Generally, hemicellulose and lignin are considered to be inhibitory to the enzymatic saccharification of cellulose, resulting from the reduction of enzyme availability or accessibility. Three main factors are restricting the utilization efficiency of cellulase in cellulose hydrolysis: 1) physical barrier of hemicellulose and lignin wrapping on cellulose; 2) complicated structure of lignin-carbohydrate-complex (LCC); 3) non-productive adsorption of cellulase on lignin. Therefore, lignin and pretreated substrates containing lignin and hemicellulose are both important factors for enzymatic hydrolysis. The following discussion will discuss three of the most significant factors influencing the enzymatic hydrolysis: cellulase enzyme, substrates, and lignin.

Trichoderma reesei (*T. reesei*) is a fungus of enzyme producer, which secrets different cellulases and hemicellulases. Among these enzymes, *T. reesei* cellulases are among the most well-studied cellulase systems[123]. There are three major components included in the cellulase system: endoglucanases (EGs: Cel5A, Cel7B, etc.), cellobiohydrolases (CBHs: Cel6A, Cel7A), which are

also known as exoglucanase, and β-glucosidases (BGs: Cel3A). These three components cooperate in cellulose degradation (Fig. 7): EGs cleave internal β-1,4-D-glycoside bonds in the cellulose polymer randomly liberating single cellulose microfibers and free chain ends; CBHs degrade cellulose by hydrolyzing the 1,4-β-D-glycosidic bonds from the ends non-reducing (EC 3.2.1.91) or reducing (EC 3.2.1.176) ends of cellulose with the release of cellobiose; after this, EGs degrade amorphous region of cellulose facilitating degradation of CBHs at free ends in the crystalline region; BGs hydrolyze cellobiose from CBHs and EGs to free glucose molecules, which also reduce the product inhibition on cellobiohydrolases[26, 124, 125]. The whole process of degradation did not happen in sequence but occurs simultaneously.

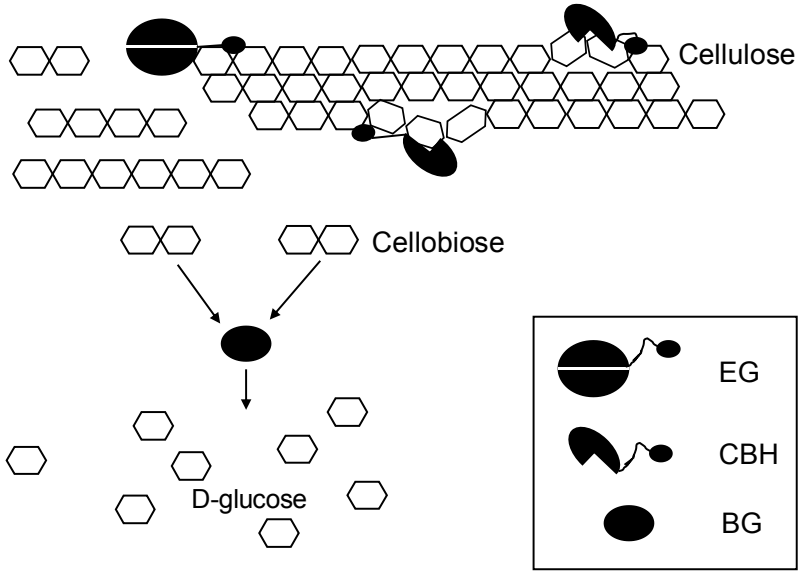


Fig. 7 Cooperation of cellulases in enzymatic hydrolysis

Most of these cellulase enzymes have a two-domain structure, consisting of a catalytic domain (CDs) and a cellulose-binding module (CBMs) connected by a linker[126]. CBMs directionally binds to cellulose, and CDs work as a catalyst to degrade cellulose in different ways. It has been proved that inhibition of lignin lies more in CBMs than CDs[127]. Furthermore, CBMs

in Cel7A and Cel5A was proved to have a high affinity on lignins and contribute to the cellulase binding on isolated lignins and pretreated substrates[128].

1.8 Lignin in the enzymatic hydrolysis

Lignin was reported to be the major detrimental factor of enzymatic hydrolysis[129] and associated with the adsorption of cellulase on lignin. Therefore, lignin, cellulose, and hemicellulose are all important factors affecting the efficiency of enzymatic saccharification. The following discussion will be about the interaction and the inhibitory and stimulatory effects of the lignin on the enzymatic hydrolysis.

1.8.1 Interaction between lignins and cellulase enzymes

The most significant interactions between lignins and cellulase enzymes are hydrophobic interaction, electrostatic interaction, and hydrogen bonds[130]. Determination of physio-chemical properties of lignins, including hydrophobicity, zeta potential, and chemical structures, describes these interactions and is crucial for the binding of enzymes on lignins[131].

Hydrophobic interaction: Hydrophobic interaction is one of the main reasons for the non-specific adsorption of enzymes to lignin. In an aqueous environment, hydrophobic groups in protein molecules aggregate to reduce contact with the surrounding water, so proteins will preferentially adsorb on hydrophobic solid surfaces[132]. Moreover, when the hydrophobicity of the protein and substrate surface increases, the adsorption between the protein and substrates also increases[133]. Therefore, adsorption affinity between enzymes and lignin will be determined by the lignin hydrophobicity.

The hydrophobicity of the enzyme is related to its CBM. Adsorption affinity of CBM to substrates depends on the type and arrangement of amino acid residues. According to the previous study, specific amino acids in some CBMs can increase the hydrophobicity of cellulase and promote the adsorption of cellulase to cellulose and lignins[134]. For example, the hydrophobic cellulose-binding surface of the CBM family interacts with a lignin-rich surface. Cel7A (CBH I) CBM has four tyrosine residues. Three of them form the flat surface of CBM, which determines the adsorption affinity on crystalline cellulose. Cel7B (EG I) CBM has five aromatic amino acid residues (four tyrosine and one tryptophan). Compared with CBM of Cel7A, Cel7B contains tryptophan, which makes it more hydrophobic. Thus, Cel7B (EGI) also binds more tightly to lignin.

Lignin has a higher contact angle than cellulose, and it is more hydrophobic[135]. Simultaneously, the average adhesion between the hydrophobic end of lignin and the enzyme is higher than the interaction between cellulose and enzyme, so the hydrophobic interaction between lignin and enzyme is stronger, it is easier to bind with cellulase enzymes. Nakagame et al. evaluated the characteristics of lignin from corn stover, poplar, and pine after pretreatment. They found that the pretreated corn stover had the highest cellulose saccharification rate. The lignin within the pretreated corn stover had low hydrophobicity[136]. Experiments showed that the hydrophobicity of lignin plays an important role in the non-specific adsorption of cellulase on lignin and is related to the low efficiency of lignocellulose hydrolysis.

The functional groups of lignin will affect the hydrophobicity of lignin and therefore influence the inhibition of lignins on enzymatic saccharification. For instance, Berlin compared ethanol organosolv dissolved lignin with enzymatic residual lignin and found that it was found that ethanol organosolv dissolved lignin had a greater inhibition[137]. Since less aliphatic hydroxyl groups and carboxyl groups suggested a stronger hydrophobicity, ethanol organosolv dissolved

lignin contained more of phenolic hydroxyl groups, 46% less aliphatic hydroxyl, and 67% less carboxylic groups than enzymatic residual lignin. Thus ethanol dissolved lignin with higher hydrophobicity led to a higher inhibition on enzymatic hydrolysis.

Electrostatic interaction: Electrostatic interaction is another major reason for the bindings of enzymes to lignin. Generally, there are -COOH, $-NH_2$, and other charged functional groups on the polymer surface, such as enzyme molecules and lignin. The sum of the charges of these surface functional groups is the net charge on the polymer. The direct electrostatic attraction or repulsion of lignin and cellulase can be evaluated and characterized by zeta potential. Cellulase enzymes have different charges under different pH. When pH > pI, the net charge of the cellulase component is negative; when pH < pI, the net charge of the cellulase component is positive[138]. According to electrostatic interaction between different charged molecules, if cellulase and lignin molecules have opposite charges, they will attract each other. While if they are both positively charged or negatively charged, they will repel each other. The more they charged, the stronger repulsive force there will be. Generally, the pH value of the cellulase hydrolysis is 4.8-5.0. Under this condition, there are negatively charged cellulases include EG I (pI 3.9, 4.5, 4.7), EG II (pI 4.2), CBH I (pI 3.6, 3.9), and BG (pI 4.0). Positively charged cellulases include BG I (pI 8), EG III (pI 6.8, 7.4), EG II (pI 5), and CBH II (pI 5.2, 5.9)[139]. While alkali lignin, cellulolytic enzyme lignin, lignin sulfonates, and ethanol organosolv lignin are all negative charged[94, 140]. Therefore, compared with negatively charged cellulase, positively charged CBH II, EG II, EG III, and BGare easily to be adsorbed on negatively charged lignin.

In recent years, researchers have discovered that the electrostatic repulsion of cellulase and lignin is related to acid groups (sulfonate and carboxylic groups) in lignin and will be changed under different pH values of hydrolysis. They can reduce the non-specific adsorption of cellulase

by increasing the electrostatic repulsion between enzyme and lignin[141]. Therefore, the modification of enzymes or lignin with charged functional groups or the method of increasing the pH value of enzyme hydrolysis (> 4.8) could be feasible strategies to improve the hydrolysis efficiency of various lignocellulosic materials[142].

Hydrogen bonds: At present, few studies have clarified the relationship between the hydrogen bonding and binding of cellulase on lignins. Studies have shown that the formation of hydrogen bonds between cellulase and lignin is because of the phenolic hydroxyl groups and aliphatic hydroxyl groups in the lignin structure[131, 143]. However, the formation of hydrogen bonding could not be the only factor that affects cellulase hydrolysis. For example, comparing with lignin from steam explosion pretreatment, organosolv pretreated lignin contains more phenolic hydroxyl groups. The blocking of the phenolic hydroxyl group was beneficial for reducing hydrogen bonds between lignins and amino acids of cellulase enzyme[144]. However, alkylation of the lignin from the organosolv pretreatment promoted the cellulose hydrolysis process regardless of the high ratio of phenolic hydroxyl groups[76]. Therefore, hydrogen bonds could be one factor to consider to explain lignin inhibition on enzymatic hydrolysis.

1.8.2 Inhibitory effect of lignins in the enzymatic hydrolysis

In recent years, the cellulase binding on lignin and its influence on hydrolysis has attracted extensive attention from researchers. However, many studies have shown that the presence of lignin inhibits the lignocellulose saccharification. The issues about: how does lignin affect enzymes, what is the relationship between lignin inhibition and its physical and chemical properties, and how to control lignin inhibition effectively are still not fully understood. The mechanism of lignin inhibition on cellulase is very complicated. Different sources of lignin have

different inhibitory effects on the enzymatic hydrolysis process. Based on this, additives like surfactant or extra proteins have been added to enzymatic saccharification to reduce the lignin-cellulase interaction and improve the enzyme activity. Furthermore, some lignins proved beneficial to the enzymatic hydrolysis, like ethanol organosolv lignins and lignosulfonates, which were also discussed. The presence of lignin is always considered detrimental for the enzymatic hydrolysis[145]. The inhibition of lignin will be enhanced with higher lignin content[146]. The lignin inhibition mechanism can be divided into physical barriers, inhibition from lignin-derived molecules, and binding.

Physical blocking: In lignocellulosic raw materials, lignin and hemicellulose are connected by chemical bonds to form a lignin-carbohydrate complex (LCC). It entangles with cellulose and hinders the adsorption of cellulose to cellulase. As a physical barrier, lignin prevents the cellulase enzymes from approaching cellulose macromolecules. This decreases the accessibility of cellulose to the cellulolytic enzymes[130]. Although researchers insisted that lignin inhibition from the binding of cellulase is more significant. Djajadi et al. reported that hydrothermally pretreated grass with a higher apparent lignin surface had low enzyme digestibility. These results proved that the detrimental lignin in enzymatic saccharification acts more like a physical barrier[147].

Inhibition from lignin-derived molecules: The lignin degradation products, including vanillin, catechol, and ferulic acid, has been proved to have competitive or non-competitive inhibitory effects on cellulase enzymes[148]. In the cellulase system, lignin-derived small molecules cause enzyme inactivation. Depending on the type of enzymes and the type of phenolic compounds, the inhibition mechanism from these lignin-derived-phenols on the cellulose saccharification process is different[149].

Non-specific adsorptions: Besides the physical barrier from lignin, non-specific adsorptions of cellulases to lignin has been considered as a significant factor that limits the enzymatic hydrolysis of pretreated biomass[150, 151]. The hydrophobic and electrostatic nature of lignin is potentially important for the ineffective adsorption of enzyme proteins, which reduces the enzyme activity and the rate or yield of the saccharification of biomass[73, 151]. The free energy change for adsorption-induced denaturation is only at the level of a few hydrogen bonds[152]. When proteins interact with substrates or lignins, they may be conformationally altered or denatured[153]. Non-specific adsorption of cellulases on lignins is a principal reason leading to cellulase denaturation in the enzymatic saccharification of the whole slurry[154]. Lignins from pretreated steam spruce were observed to cause severe enzyme denaturation and significant enzyme activity loss by non-specific adsorptions[26].

The non-specific adsorption of enzymes and lignin can be regulated by hydrophobic interaction, electrostatic repulsion, or hydrogen bonding interactions[151]. However, due to the structural complexity of lignocellulose substrates and lignin, it is difficult to determine which force is the dominant driving force to increase the non-productive adsorption of cellulase. It may also be the synergistic effect of several forces determines the binding capacity of cellulase on lignin.

1.8.3 Stimulatory effect of lignins in the enzymatic hydrolysis

The stimulatory effect of lignosulfonates has been discovered in enzymatic saccharification of SPORL pretreated biomass. Lignosulfonates potentially worked as polyelectrolytes or anionic surfactants and generated lignosulfonate-cellulase complexes due to the opposite charges in both molecules[140]. Such complexes reduced the non-specific adsorption binding of cellulase on residual lignins, such improvement offsetting the negative effect while the yield of the hydrolysis

was increased proportionally to the amount of lignosulfonate. Furthermore, the enhancement effect could be increased by increasing the pH of the media from 5.0 to 5.5[142]. Interestingly, some lignosulfonates and ethanol organosolv lignin were stimulatory to enzymatic hydrolysis[94, 155].

In addition, these lignins with a stimulatory effect could enhance the enzymatic saccharification of pure cellulose (filter paper or Avicel)[94, 156]. Thus, it is suggested that these two technical lignins could decrease the non-specific adsorption on residual lignin and improve the efficiency of the enzyme. Since there will always be non-specific adsorption bindings of cellulase on lignin because of the hydrophobic nature of lignin, the stimulatory effect of organosolv lignins will explain that the enhancement of lignin addition offsets its detrimental effect. The enhancement of lignin could be explained as follows: 1) They may reduce the irreversible non-specific adsorption binding of cellulase on the cellulose chains as the hardwood lignin extractives[157]. This mechanism could be partially supported by the improved activity of free enzyme when organosolv lignin is added during hydrolysis[95]. 2) 2) They may work as surfactants affecting the hydrophobic aggregation and denaturation of cellulase enzymes[158]. 3) They may prevent the cellulose from the product inhibition, especially at the initial state of hydrolysis[159].

1.9 Substrate-related factors in the enzymatic hydrolysis

Besides enzymes and lignins, the properties of the substrate are also significant in enzymatic hydrolysis. After pretreatment, the residual hemicellulose and the cellulose are two significant aspects to be considered for the properties of substrates.

1.9.1 Cellulose in enzymatic hydrolysis

There are three major factors of cellulose that are well accepted to have a great effect on the enzymatic hydrolysis of cellulose: 1) Crystallinity (affected mainly by hydrogen bonding strength and electrostatic interaction); 2) degree of polymerization (DP); 3) accessible surface area and specific area (particle size). Pretreatment could increase the accessibility of the substrates in enzymatic hydrolysis. The changes in pretreatment are relatively complicated but mainly changes these three factors of substrates: under high-temperature and high-pressure conditions of pretreatment, cellulose will be more or less degraded, and in this way, DP of cellulose in the substrate will decrease, more reducing ends will be exposed, and the crystallinity will also be changed, which will facilitate the adsorption of cellulase.

Crystallinity: Based on the complicated ultrastructural arrangements of cellulose, the rapid initial hydrolysis in enzymatic digestion of substrates was due to the saccharification of its amorphous region. The hydrolysis will then slow down or even could not finish when it comes to crystalline constituents[160]. Due to the removal of hemicellulose and amorphous cellulose in dilute acid pretreatment, the crystallinity of cellulose in substrates does not decrease but rises, and the results of enzymatic hydrolysis show that the materials pretreated can also reach a higher level[161]. Other pretreatment technologies such as the alkaline method, ionic liquid, and organic solvent pretreatment can destroy the highly crystalline cellulose area and improve the hydrolysis rate and efficiency of cellulose[11]. These results indicate that the destruction of crystallinity is sometimes beneficial but may not be crucial for the change of cellulase hydrolysis efficiency.

Degree of polymerization (DP): DP of cellulose in biomass is a significant factor as a substrate-related factor in enzymatic hydrolysis because of the mechanism of cellulase hydrolysis enzyme[162]. Cellulose appeared to be recalcitrant when its DP is above a certain range[163].

Enzymatic hydrolysis will also change the DP of cellulose but may not change its size distribution[164].

Accessible surface area: The pretreatment methods currently studied can effectively degrade hemicellulose or lignin and increase the accessible surface area of cellulose by the generation of pores[165]. In enzymatic hydrolysis, cellulase first diffuses and then adsorbs to cellulose molecules for hydrolysis, so the accessible surface area of the substrate is a key factor affecting the efficiency of enzyme hydrolysis. In addition, some scholars have found that increasing the external specific surface area by grinding of the material is stimulatory to the enzymatic hydrolysis of cellulose[71]. In fact, under the same external specific area, the density and porosity of the material have a great impact on the efficiency of enzymatic hydrolysis[166]. Accessible surface area could be tested by Bennet-Emmit-Teller (BET) method, but its accuracy will be limited by the requirement of dry raw materials and the size difference between nitrogen molecules and cellulase enzymes[167]. A better solution to the measurement will be the solute exclusion technique. This method determines the accessible area by different dextran molecules in pores and cavities in the substrates[168]. Since the adsorption of cellulase conducted the hydrolysis on substrates, a specific area, which is mainly determined by particle size, may govern the hydrolysis rate because it indicates the number of binding sites. But this phenomenon will only occur for certain types of substrates except for pure cellulose like microcrystalline cellulose or cotton[169]. So the fiber size or the specific area is probably not a limiting factor for the digestibility of the substrate.

Cellulose accessibility and other factors: Other influential characteristics of cellulose include pore volume and cellulose accessibility, which are sometimes related to change of accessible surface area. Low efficiency of hydrolysis was reported to be potentially caused by

insufficient pore volume[170]. But the problem with the pore volume is that its measurement will be greatly affected by the probe being applied. Specific accessibility of cellulose could be determined by two methods: direct red dye or water; dextran molecules as in solute exclusion. It was reported to be linearly correlated to the hydrolysis yield[171, 172]. The accuracy of this factor will also be affected by the accessible surface area, and the measurement probe molecule applied. The clear point for an accurate test will be when the probes are used, their molecular size similar to that of cellulase at approximately 51 Å, which is the cellulase enzyme size[173].

1.9.2 Hemicellulose in enzymatic hydrolysis

Meng et al. studied through the technologies of de-hemicellulose and delignification in the pretreatment. It is pointed out that the presence of hemicellulose has a stronger inhibition on enzymatic hydrolysis during pretreatment and enzymatic hydrolysis compared with lignin[166]. Dilute acid pretreatment above 160 °C was reported to be effective because of hemicellulose decomposition[14]. Therefore, it is believed that the removal of hemicellulose should also be significant as the removal of lignin in pretreatment technologies.

The influence of hemicellulose on cellulase hydrolysis is mainly manifested in three aspects. Firstly, hemicellulose can hinder cellulase contact and cellulose through a physical barrier similar to lignins[174]. Residual xylan is an important physical barrier to cellulose, which inhibits the following enzymatic hydrolysis and usually be removed by extra post-pretreatment[175]. The initial hydrolysis rate of enzymatic hydrolysis was limited by residual xylan[103, 176]. Secondly, since hemicellulose will be degraded during the pretreatment process, a certain amount of free xylan will be generated in the prehydrolysate. This part of xylan also has a strong inhibition on enzymatic hydrolysis. Xylan could decrease exoglucanase activity because cellulose and xylan are

both the substrates for this enzyme[177]. Zhang et al. added xylan to the enzymatic saccharification system of microcrystalline cellulose and found that the yield of hydrolysis was greatly reduced. Furthermore, results showed that with the introduction of xylan, cellobiose from CBH decreased in the enzymatic hydrolysis system[175]. The indication will be that xylan can inhibit the activity of both endoglucanase and exoglucanase (CBH) in the cellulase system. Thirdly, other degradation compounds from hemicellulose in the substrate may also inhibit enzymatic hydrolysis.

1.10 Research objectives

The lack of cost-efficient biorefinery approaches limits the effective biochemical conversion of biomass due to the recalcitrance of lignocelluloses[145, 178]. Pretreatment and enzymatic hydrolysis are two of the most important and costly processes in biochemical conversion. Therefore, they are the focus of biorefinery-related research[179]. Lignins, pretreated substrates, and cellulase enzymes are three of the most important factors influencing the hydrolysis rate and yield in enzymatic hydrolysis[10, 136, 180]. Among them, organosolv lignins are proved to have high purity, high quality (less condensed), and the lowest molecular weight among different technical lignins[18, 93]. Hence, it is necessary to further study the effect of the organosolv lignin on the enzymatic hydrolysis to improve organosolv pretreatment and subsequent enzymatic hydrolysis.

Lai et al. reported that ethanol organosolv lignin from sweetgum could increase the enzymatic hydrolysis yield of Avicel and pretreated softwood and hardwood[94]. Meunier and Penner extracted lignin from dilute acid pretreated switchgrass residues and studied the effect of its content on the cellulose saccharification[181]. The results showed an increase in lignin content from 10 to 40 %, the cellulose saccharification rate of 60 h was only reduced by 3%. Therefore, it

is of great significance to understand the mechanism of different lignins behaviors on enzymatic hydrolysis to achieve the economic biochemical conversion of biomass.

The electrostatic interactions, hydrophobic interactions, and hydrogen bonding between cellulase and lignin are the main factors that affect the hydrolysis efficiency of the enzyme. Qin et al. used atomic force microscopy (AFM) to study cellulase and lignin[139]. They found that the interaction between cellulase and Kraft lignin is strong due to its hydrophobic nature, which is also well accepted to be the main force between cellulase and lignin. Sammond et al. used real-time quartz crystal microbalances to measure the enzyme and lignin adsorption. They also used Rosetta to estimate the surface hydrophobicity and showed the hydrophobic interaction between enzyme and lignin is related to the characteristics of the enzyme and lignin surface, such as hydrophobic groups[182]. Electrostatic interaction is another important factor that affects the adsorption of enzymes and lignins. T[142][143][137][139][140][147][155][161][163][164]he electrostatic interaction between cellulase and lignin is related to the pH value during the enzymatic hydrolysis. As the pH value increases, the free enzyme content in the supernatant increases[142]. However, the hydrophobic interaction may interfere with the electrostatic interaction between cellulase and lignin, but there is no report on the synergistic effects on the enzymatic hydrolysis.

The content of functional groups of lignins could explain the interactions between cellulase and lignins as well. Nakagame et al. extracted protease-pretreated lignin and enzymatically hydrolyzed lignin from three different lignocellulosic materials. It was suggested that the inhibition of lignin on the enzymatic hydrolysis is related to the content of carboxyl groups[141]. The higher the carboxyl group content, the stronger the non-specific adsorption of the cellulase on the lignin will be. Therefore the enzymatic hydrolysis is not favored. Pan et al. found that the phenolic hydroxyl groups play an important role in the non-specific adsorption of enzymes on lignins[144].

Sun et al. found that the eugenol group and the guaiacyl group of eucalyptus would undergo a condensation reaction during hot water pretreatment, causing the increase in the hydrophobicity of lignin and thus, an inhibition on the enzymatic hydrolysis[131].

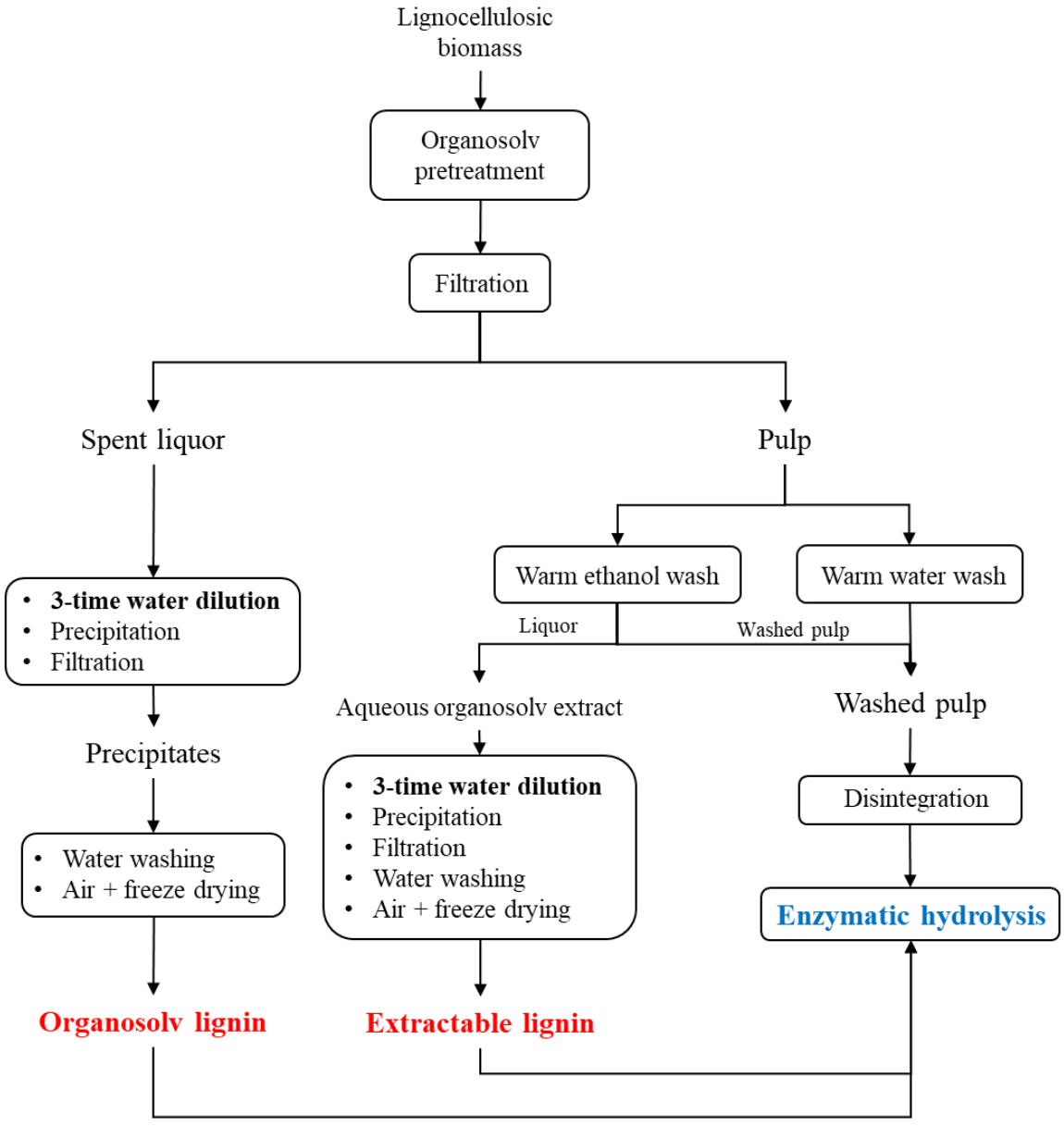


Fig. 8 Flowchart of pretreatment and enzymatic hydrolysis of lignocellulosic biomass

This dissertation will discuss the effect of organosolv and extractable lignins on the enzymatic hydrolysis of lignocelluloses. Organosolv lignins and extractable lignins were collected

through the pretreatment of two types of hardwood, i.e., aspen and poplar, and one softwood, pine, with methanol, ethanol, and propanol as shown in Fig.8. The properties of these lignins were characterized to clarify the interactions between lignins and cellulase enzymes. Hence, the adsorption strength of lignin-cellulase, hydrophobicity, electrostatic interactions, and the structural characteristics of lignins were all studied in detail. For the substrates, the effect of the solvent washing on enzyme digestibility of pretreated substrates was investigated. The solvent effect was also evaluated by the cellulose accessibility and water retention value of pretreated substrates.

The overall objective of this dissertation is to better understand chemical and physical properties change of lignin (structure heterogeneity and resource variability) during the fractionation of organosolv pretreatment to improve the glucose yield of enzymatic hydrolysis by:

1. To study the effect of methanol, ethanol, and propanol organosolv lignins from poplar and eucalyptus on enzymatic hydrolysis of lignocellulose;
2. To study the effect of ethanol organosolv lignins from kenaf on enzymatic hydrolysis of Avicel;
3. To study the effects of ethanol washing and ethanol extractable lignins from aspen on enzyme digestibility of lignocellulose;
4. To compare the effect of organosolv and extractable lignins from aspen and pine on enzymatic hydrolysis of Avicel and their differences in the chemical structure and physical properties.

Chapter 2: Effect of organosolv lignins from poplar and eucalyptus on enzymatic hydrolysis of lignocelluloses

2.1 Abstract

Organosolv pretreatment with ethanol is a promising method for increasing cellulose accessibility, releasing sugars from hemicellulose, and recovering relatively pure lignin as a by-product. The use of other short-chain aliphatic alcohols in the organosolv pretreatment, such as methanol and propanol, was considered to have high-efficiency delignification. The subsequent step of biochemical conversion is enzymatic hydrolysis, by which cellulose and hemicellulose are converted into fermentable sugars. In this work, the effect of methanol, ethanol, and propanol organosolv lignins from poplar and eucalyptus has been assessed, and organosolv lignins from poplar could improve the yield of enzymatic hydrolysis of lignocelluloses, while methanol, ethanol, and propanol organosolv lignins from eucalyptus inhibited the enzymatic hydrolysis of Avicel. The spectra of HSQC, HSQC-TOCSY, and HMBC revealed that the alkylation (e.g., methylation, ethylation, and propylation) of hydroxyl groups took place not only at Cα, but also at Cβ and Cγ, and potentially also at the phenolic hydroxyl group of lignin. In addition, the hydrophobicity and the quantification of 13C and HSQC signals of lignins explain the distinct effect of organosolv lignins from poplar and eucalyptus.

2.2 Introduction

Although lignin is widely accepted as an inhibitory factor in enzymatic hydrolysis[183, 184], ethanol organosolv lignins obtained from organosolv pretreatment under certain conditions are stimulatory to the enzymatic hydrolysis of pretreated biomass and can even enhance enzymatic

saccharification of pure cellulose[76]. Ethanol organosolv pretreatment is the most common organosolv pretreatment originated from industrial organosolv pulping[185]. In most acid-catalyzed pretreatment, including ethanol organosolv pretreatment, fractionation of lignins mainly occurred due to the decomposition of aryl-ether bonds like β-O-4 α-O-4 linkages, although condensation due to the formation of C-C bonds occurred simultaneously[186, 187]. This explains the higher condensation level and β-β, β-5 linkage ratio in technical lignins than in mill wood lignin from raw materials[69]. Lignin condensation can be suppressed by the addition of capping agents in the pretreatment. The methoxylation of α- and γ-hydroxyl groups in the lignin C_9 structure with formaldehyde, resulting in 1,3-dioxane/acetal formation, was reported to fractionate the less condensed lignin[188]. In addition, lignin condensation can be suppressed by the ethanol organosolv pretreatment as well. The alkylation of α-hydroxyl groups, which possibly mitigated the lignin condensation, was reported in the ethanol organosolv pretreatment[76].

Application of more hydrophobic solvents such as propanol, butanol, and pentanol in the organosolv pretreatment showed high delignification efficiency[189]. Propanol showed similar solid recovery, sugar recovery, and yield of pretreated substrates as ethanol[190]. Other short-chain aliphatic alcohols, such as methanol, are frequently applied for enzymatic hydrolysis owing to their low toxicity and easy access to recovery[191]. However, studies on the effect of methanol and propanol organosolv lignins on enzymatic hydrolysis and their characterization are scarce. Hence, alkylation of lignin by methanol and propanol during organosolv pretreatment should be investigated to identify more solvents that can act as condensation capping agents. It has been elucidated that the effect of ethanol organosolv lignins can be explained by their physical properties such as hydrophobicity, zeta potential, particle size, and molecular weight[131, 192]. The

chemical structures of lignin such as primary units, functional groups, condensation levels, and linkages of units can be determined by analy^{zing} ^{13}C and 2D-HSQC NMR spectra[76, 131].

In this chapter, the effect of methanol, ethanol, propanol organosolv lignins from hybrid poplar and eucalyptus was investigated on enzymatic hydrolysis of Avicel and the corresponding pretreated substrates. The effects of different enzyme loadings and lignin loadings on enzymatic hydrolysis of Avicel were also determined to optimize the stimulatory effect of organosolv lignins. The hydrophobicity of organosolv lignins was analyzed to explain the hydrophobic interaction between cellulase and lignins. The results of FTIR analysis of organosolv lignins from poplar and eucalyptus were conducted were compared to that of the untreated biomass to understand the changes in the surface functional groups after the organosolv pretreatment. Furthermore, compared to the mill wood lignin from untreated poplar, NMR spectra revealed potential alkylation reactions of lignin that were not reported before were revealed previously. The signals of ^{13}C and HSQC NMR spectra of methanol, ethanol, and propanol organosolv lignins were quantified to determine the chemical structure changes during the organosolv pretreatment. Relationships between the lignin alkylation ratio, condensation level, and their effect on enzymatic hydrolysis were indicated by quantifying NMR signals.

2.3 Materials and methods

2.3.1 Cellulase enzymes and biomass

Hybrid poplar (*Populus deltoids x Populus nigra*) wood chips (moisture content 5.9%, chip size 4 mm × 1 mm) were obtained from INL Bioenergy Feedstock Library. Forest Products Laboratory collected eucalyptus wood chips (moisture content 11.7%, chopped by Waring pulverizer to the size of 10 mm × 10 mm × 0.3 mm (L × W × H)) at Auburn University.

Sulfuric acid (H_2SO_4, 96% purity), 99.9 atom % D dimethyl sulfoxide-d_6 (DMSO-d_6), and Avicel PH-101 were purchased Sigma Aldrich. Certified ACS grade methanol, certified ACS grade 1,4-dioxane, and certified grade propanol were obtained from Fisher Chemical. Ethanol (100% Reagent alcohol) was purchased from Fisherbrand. Certified ACS grade sodium hydroxide (NaOH), Bengal Rose (95% purity) was obtained from Fisher. Novozymes North America, Inc provided commercial cellulase (Cellic@ CTec2, filter paper enzyme activity: 91 FPU/ml) used in this dissertation.

2.3.2 Organosolv pretreatment

The poplar or eucalyptus wood chips (100 g, dry weight) were soaked in 700 mL 65% methanol/ethanol/propanol water solution with 1 % (w/w, sulfuric acid/dry biomass) of sulfuric acid overnight before the hydrothermal process of organosolv pretreatment. The mixture was transferred into a 2-L stainless steel Par batch reactor with a stirrer and a PID controller (Parr 4848, USA) and heated to 160°C. After 1 h hydrothermal cooking, the reactor was water quenched to room temperature to terminate the reaction. The solid and the liquid fraction were separated from the reacted slurry by vacuum filtration using Whatman No.1 filter paper. The spent liquor (liquid part) was kept for organosolv lignin precipitation. The pretreated substrates (solid part) were washed and kept for enzymatic hydrolysis and analysis.

Organosolv lignins were collected by adding 3-fold volume of water into the spent liquor of pretreatment. The pH of the resulting mixture was adjusted to 4 by addition of NaOH. The precipitates were filtrated, washed by warm water 3 times, and collected as organosolv lignins. Organosolv lignins from methanol, ethanol, propanol pretreated poplar, and eucalyptus was designated as MOL-HP, EOL-HP, POL-HP, MOL-EU, EOL-EU, POL-EU. After filtration and

water wash, organosolv lignins were first air-dried in the fume hood and then freeze-dried for enzymatic hydrolysis and analysis.

The pretreated poplar or eucalyptus was washed for further enzymatic hydrolysis with the following steps. The fractionated solid part (from 100 g dry biomass) from pretreatment was first washed by 3 times (233 ml each wash, 700 ml in total) of 60°C 65% methanol, ethanol, or propanol mixing with the hot solvent aqueous solution for 10 min. The resulting slurry was filtrated with a vacuum, and the collected solids were homogenized with 60°C water (same amount as solvent) in a blender for 30 s, and then the liquor was filtrated. The water washing steps were repeated 3 times after 3 times of ethanol washing to get the organosolv pretreated substrates. The washed substrates were collected and stored at 4°C for the subsequent enzymatic hydrolysis. The methanol, ethanol, and propanol pretreated substrates from poplar after solvent and water wash were named MPHP, EPHP, PPHP. The methanol, ethanol, and propanol pretreated substrates from eucalyptus after solvent and water wash were named MPEU, EPEU, and PPEU.

2.3.3 Enzymatic hydrolysis of Avicel and pretreated substrates with lignins

Enzymatic hydrolysis of Avicel (98.72% glucan), organosolv pretreated biomass (MPHP, EPHP, PPHP, MPEU, EPEU, PPEU) were carried out in 250 mL Erlenmeyer flasks with stoppers at 50 ℃, 150 rpm in 50 mM sodium citrate buffer (pH 4.8) with 2% glucan (w/v) for 72 h. The enzyme loading for all hydrolysis was chosen to be 5 FPU/g glucan, except during the enzymatic hydrolysis with different loading in section 2.3.1. The enzyme loading was set to be 2.5, 5, 7.5, 10, and 15 FPU in the test. To study the effect of organosolv lignins on enzymatic hydrolysis, 4 g/L of different organosolv lignins were added to the enzymatic hydrolysis 1 h before adding cellulase at room temperature. To determine the effect of organosolv lignins on enzymatic hydrolysis, lignin

concentration was set to be 1, 2, 4, 8 g/L in section 2.3.1 and 2.3.2. Samples from the enzymatic hydrolysis were taken at 0, 3, 6, 12, 24, 48, 72 h, and centrifuged for 5 min at 10000 rpm to collect supernatants. The concentration of glucose in the supernatant was determined by HPLC. All experiments were carried out in duplicates. The enzymatic hydrolysis yield was calculated by the correlated glucan from the released glucose divided by the theoretical glucan into the substrates.

2.3.4 High-performance liquid chromatography analysis and Fourier transform infrared spectroscopy analysis.

The concentration of the sugars from chemical composition analysis was analyzed by a High-performance liquid chromatography (HPLC) system (Agilent 1260 Infinity) with a Hi-Plex Pb guard column (5 × 3 mm, Bio-Rad), a Hi-Plex Pb column (300 × 7.8 mm, Bio-Rad) and a RID detector. DI-water was used as mobile phase with a flow rate of 0.6 $mL \cdot min^{-1}$. The detector and oven temperatures were set to be 45°C and 80°C, respectively. The concentration of glucose from enzymatic hydrolysis was analyzed by an HPLC system (Agilent 1260 Infinity) with a Hi-Plex H guard column (5 × 3 mm, Agilent), a Hi-Plex H column (300 × 7.8 mm, Agilent), and a RID detector. 5 mM H_2SO_4 in aqueous solution was used as mobile phase with a flow rate of 0.6 $mL \cdot min^{-1}$. The detector and oven temperatures were both set to be 45°C.

The Fourier transform infrared spectroscopy (FTIR) spectrometer (Perkin Elmer Spectrum Two FTIR system), kindly provided by Dr. Hairong Guan from the Department of Chemistry, University of Cincinnati, was employed to examine the changes of chemical structures of the organosolv pretreated lignin compared to the mill wood lignin. Attenuated total reflectance (ATR) technique was used for the FTIR sampling. The scanning wavenumber was set from 500 to 4000 cm^{-1}, and the interval was 0.50 cm^{-1}.

2.3.5 Determination of hydrophobicity of organosolv lignins

The hydrophobicity of lignins was quantified by Rose Bengal assay[193]. Increasing concentrations of the lignins (0.2-4.0 g/L) were mixed with 40 mg/L Rose Bengal in citrate buffer (50 mM, pH 4.8) and incubated at 50 °C, 150 rpm for 2 h. The free Rose Bengal dye in the supernatant and the lignins were separated by centrifugation of 10000 rpm, 5 min. The free dye concentration was determined by UV-Vis spectrometer at 543 nm. The partitioning quotient (PQ) was calculated by the following equation:

$$PQ = \frac{\text{amount of Rose Bengal bound on lignin surface}}{\text{amount of Rose Bengal disperse in the medium}}$$

PQ was plotted against the substrate content. The slopes of the linear trendline were considered as the surface hydrophobicity of lignin (L/g).

2.3.6 Mill wood lignin isolation from poplar

The mill wood lignin from poplar was collected based on a method reported previously[131]. Grounded poplar powder was extracted with reflux ethanol for at least 6 h to remove the extractives. The air-dried wood powder was ball-milled with 1 min pulse every 2 min milling for 24 h. 20 g ball-milled poplar was extracted with 400 ml 96% (v/v) 1,4-dioxane-water solution at 25 °C for 24 h without exposure to light. After 24 h, the solid was filtered and washed with dioxane solution for 3 times. The liquid part was concentrated to around 50 mL with rotary evaporation. The concentrated solution was added into 10-fold volume of acidic water (pH 2.0), and the mill wood lignins were precipitated. After washing, mill wood lignin from untreated poplar (MWL-HP) was freeze-dried and kept in a desiccator for further hydrolysis addition and characterization.

2.3.7 NMR spectroscopic analysis of lignins

NMR spectroscopy analysis of all organosolv from poplar and eucalyptus was conducted on a Bruker Avance III HD Ascend 700 MHz spectrometer. 60 mg of freeze-dried lignin sample was dissolved in 0.5 ml of DMSO-d_6 to collect 1D ^{1}H and quantitative ^{13}C spectra, and 2D HSQC, HSQC-TOCSY, HMBC spectra. The central DMSO solvent peak was used as an internal reference for all samples (δ_C 39.520, δ_H 2.500 ppm). 10.5 μs pulse angle, 1.95 s acquisition time, 2 s relaxation delay with a total of 32 scans per sample was conducted as the operating condition for ^{1}H spectra. For quantitative ^{13}C spectra, 9.85 μs pulse angle, 0.5 s acquisition time, 18 s relaxation delay with 768 scans per sample was conducted as the operating condition. The total acquisition time was 4 h. Bruker pulse program "hsqcetgpsisp2" was applied for HSQC and HSQC -TOCSY spectra. The ^{1}H dimension (F_2) was acquired from 12 to 0 ppm with 1024 complex points; the ^{13}C dimension (F_1) was obtained from 160 to 0 ppm with 200 complex points and 100 increments. Acquisition time for HSQC is 60.8 ms for ^{1}H, and 3.55 ms for ^{13}C was applied, and the total acquisition time was 2 h. The total acquisition time was 2-4 h. Bruker pulse program "hmbcgplpndqf" was applied for HMBC spectra. The ^{1}H dimension (F_2) was acquired from 12 to 0 ppm with 2048 complex points; the ^{13}C dimension (F_1) was obtained from 160 to 0 ppm with 128 complex points. Acquisition time of 72.6 ms for ^{1}H and 1.51 ms for ^{13}C was applied, and the total acquisition time was 2 h.

2.4 Results and discussion

2.4.1 Effect of organosolv lignins from poplar on enzymatic hydrolysis of lignocelluloses

The effect of organosolv lignins from poplar (MOL-HP, EOL-HP, POL-HP) on enzymatic hydrolysis of Avicel was determined (Fig. 9). Compared to the enzymatic hydrolysis of Avicel,

MOL-HP, EOL-HP, and POL-HP were all stimulatory to the 72 h final yield of the Avicel enzymatic hydrolysis. MOL-HP promoted the 72 h yield of enzymatic hydrolysis of Avicel slightly from 62.02% by 0.91%, while EOL-HP and POL-HP increased the final yield by 3.10% and 7.33% to 65.11% and 69.34%, respectively. The potential mechanism behind the stimulatory effect of these three organosolv lignins on the hydrolysis of Avicel is that they may serve as a surfactant, which reduces the irreversible non-specific adsorption hydrophobic binding between cellulases on the cellulose chain[157, 158]. The initial 3 h hydrolysis rate and final yield for Avicel with lignin addition were summarized in Table 8. During the initial stage of the hydrolysis reaction, MOL-HP, EOL-HP, and POL-HP promoted the hydrolysis rates from 3.83 g/L/h to 3.87, 3.90, and 4.09 g/L/h, respectively. In the initial state of enzymatic hydrolysis of Avicel, these lignins may prevent the cellulose from product inhibition in the system and therefore increase the initial hydrolysis rates[159]. Similar increase of initial hydrolysis rate by ethanol organosolv lignins was also observed with EOL lignin from black willow, aspen, and sweetgum[94, 95]. The potential mechanism behind the stimulatory effect may involve reducing cellulase aggregation similar to that caused by surfactants[101, 194].

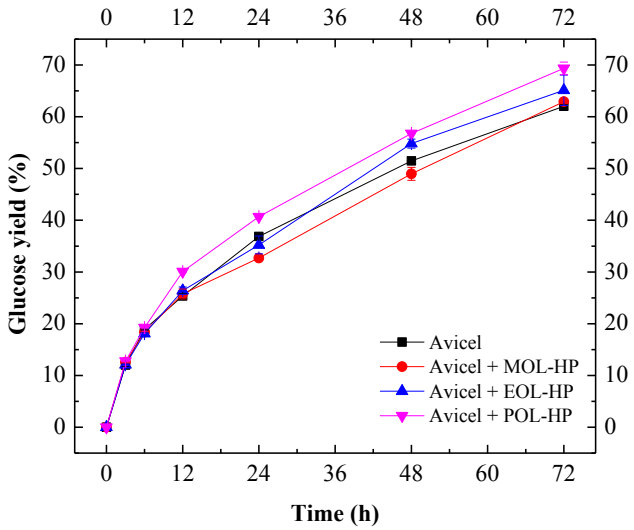


Fig. 9 Effect organosolv lignins from poplar on enzymatic hydrolysis of Avicel

Table 5 Initial hydrolysis rates and final glucose yield of hydrolysis of Avicel

Lignin addition	Initial hydrolysis rate (g/L/h)	72 h hydrolysis yield (%)
No addition	3.83 ± 0.02	55.28 ± 1.41
MOL-HP	3.87 ± 0.03	56.54 ± 0.86
EOL-HP	3.90 ± 0.09	59.32 ± 0.11
POL-HP	4.09 ± 0.03	61.00 ± 0.08

To optimize the stimulatory effect of the organosolv lignins, the effect of ethanol organosolv lignins from poplar (EOL-HP) on enzymatic hydrolysis of Avicel was determined using different concentrations of lignin and enzyme loadings. The effect of EOL-HP on enzymatic hydrolysis of Avicel was observed with 5.0 FPU of enzyme loading and 1, 2, 4, 8 g/L of EOL-HP addition (Fig. 10). Interestingly, 1 g/L EOL-HP slightly inhibited final hydrolysis yield of Avicel from 55.28% to 54.99%, while 2, 4, and 8 g/L EOL-HP promoted the yield of Avicel hydrolysis from 55.28% to 58.93%, 59.48%, and 60.84% by 3.65%, 4.20%, 5.56%, respectively. The stimulatory effect of EOL-HP in Avicel hydrolysis was the highest with 8 g/L EOL-HP. Hence, the addition of a high concentration of EOL-HP may increase the stimulatory effect of organosolv lignins. Similar promotion of stimulatory effect with higher amounts of lignin was reported by Lai etc., using EOL from sweetgum[94]. The increase in 72 h final yield upon the addition of 4 g/L EOL-HP with different enzyme loadings (2.5, 5, 7.5, 10, 15 FPU) to Avicel has been summarized Fig. 11. EOL-HP increased the final yield of Avicel hydrolysis with 2.5, 5, 7.5, 10, 15 FPU of enzyme loading by 3.99%, 4.21%, 5.89%, 7.30%, and 6.44%, respectively. The final yield was promoted from 73.28% to 80.57% with 10 FPU and promoted from 79.56% to 86.00% with 15

FPU. The stimulatory effect was the highest with 10 FPU. This indicated that the stimulatory effect of 4 g/L EOL-HP increased with higher loading of the enzyme to hydrolysis yield of 80%, although this promotional effect did not increase when more enzyme was loaded. Therefore, the stimulatory effect of organosolv lignin addition increased when more lignins and enzymes were loaded.

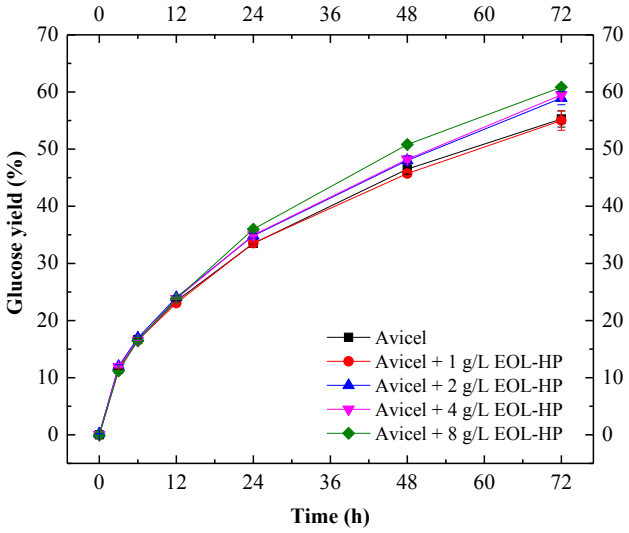


Fig. 10 Effect of different concentrations of EOL-HP on enzymatic hydrolysis of Avicel

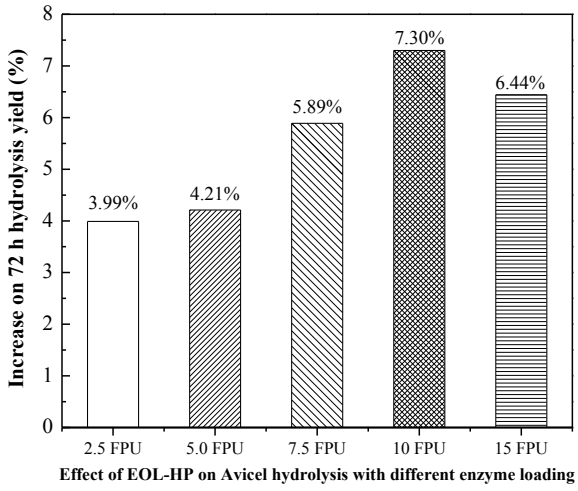


Fig. 11 Effect of 4 g/L EOL-HP on 72 h final yield of Avicel with different enzyme loading

We performed the enzymatic hydrolysis of ethanol pretreated hybrid poplar (Fig. 12). The final hydrolysis yield of pretreated biomass was in the range of 15.42-25.82%. This low digestibility of the organosolv pretreated substrates can be related to the oven-dry method of the raw materials[195]. The effect of poplar organosolv lignins (MOL-HP, EOL-HP, POL-HP) on the enzymatic hydrolysis of ethanol pretreated hybrid poplar was observed (EPHP, Fig. 13). These three organosolv lignins stimulated the 72 h final yield of the enzymatic hydrolysis of EPHP from 15.42% to 17.18%, 18.31%, and 20.30%, respectively. The final yield was increased by 1.26%, 4.04%, and 5.73%, respectively. Interestingly, the propanol organosolv lignin showed a stronger stimulatory effect not only for Avicel but also for the ethanol pretreated poplar than ethanol organosolv lignin and methanol organosolv lignin. This might be explained by the characterizations of lignin in the subsequent sections.

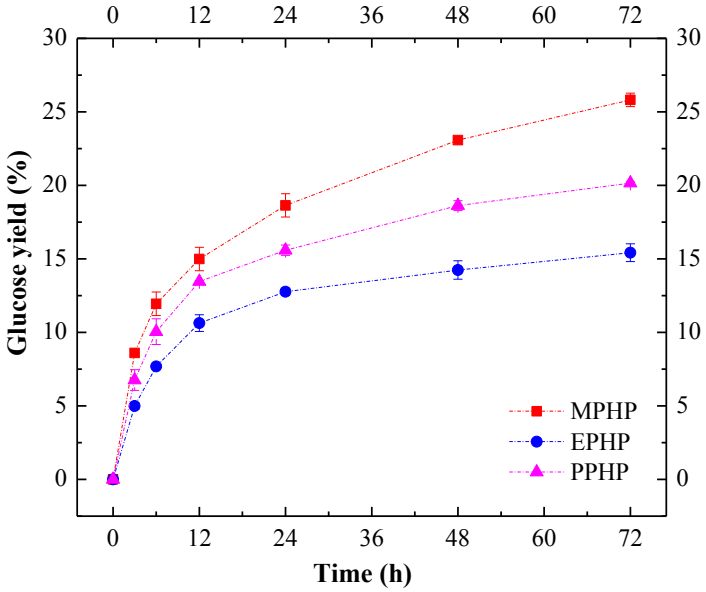


Fig. 12 Enzymatic hydrolysis organosolv pretreated poplar

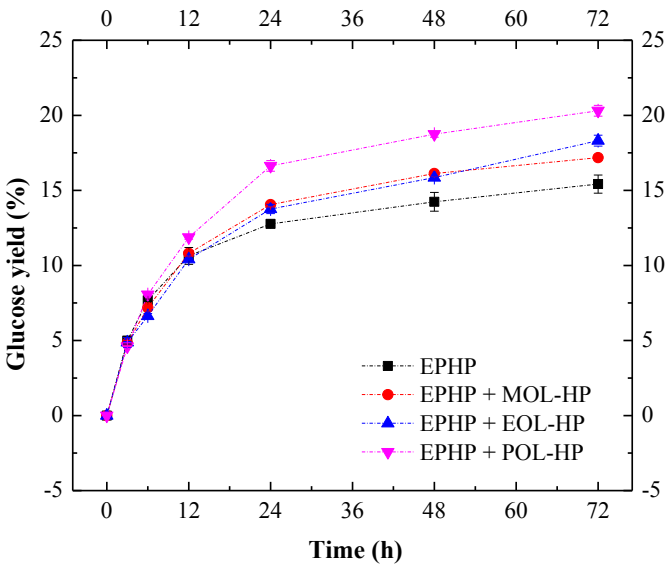


Fig. 13 Effect of organosolv lignins from poplar on enzymatic hydrolysis of ethanol pretreated poplar

2.4.2 Effect of organosolv lignins from eucalyptus on enzymatic hydrolysis of lignocelluloses

The effect of organosolv lignins from eucalyptus (MOL-EU, EOL-EU, POL-EU) on the enzymatic hydrolysis of Avicel was determined after adding them to the hydrolysis (Fig. 14). The initial 3 h hydrolysis rate and 72 h glucose yield for Avicel with lignin addition have been summarized in Table 8. MOL-EU significantly decreased the final yield of enzymatic hydrolysis of Avicel from 49.81% to 29.02% by 20.97%, while EOL-EU decreased the final yield from 49.81% to 39.18% by 10.63%. Interestingly, POL-EU only decreased the final yield of Avicel hydrolysis by 1.56%, from 49.81% to 47.26%. Although MOL-EU, EOL-EU, POL-EU all inhibited the final glucose yield of Avicel enzymatic hydrolysis, they did so to different extents. Although eucalyptus is classified as a hardwood, it is similar to softwood with respect to chemical composition[196]. Thus, the effects of organosolv lignins from eucalyptus may have been distinct from those of

poplar. During the initial stage of the hydrolysis reaction, MOL-EU, EOL-EU, and POL-EU promoted the hydrolysis rates from 3.36 g/L/h to 2.34, 2.93, and 3.24 g/L/h, respectively (Table 9).

The enzyme digestibility of organosolv pretreated eucalyptus was investigated. The 72 h hydrolysis yield of MPEU, EPEU, and PPEU was 56.24%, 46.56%, and 38.56% (Fig. 15). As all the wood chips of eucalyptus were air dried, they were more susceptible to enzyme digestion than the organosolv pretreated hybrid poplar in the previous section. The effect of eucalyptus organosolv lignins was observed on enzymatic hydrolysis of ethanol pretreated eucalyptus (EPEU, Fig. 16). Interestingly, EOL-EU and POL-EU turned to be positive on the final glucose yield of the pretreated eucalyptus. POL-EU increased the final yield of enzymatic hydrolysis from 46.56% to 55.59% by 9.03%, EOL-EU increased the final yield to 48.28% by 1.72%. MOL-EU only slightly reduced the final yield of enzymatic hydrolysis to 44.30% by 2.26%. Similar results for distinct effect of eucalyptus organosolv lignin on enzymatic hydrolysis of Avicel and pretreated biomass were reported before by Huang etc.,[95]

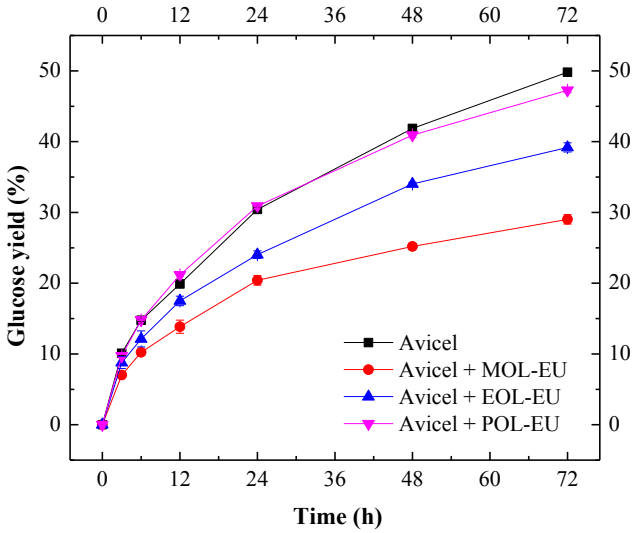


Fig. 14 Effect of organosolv lignins from eucalyptus on enzymatic hydrolysis of Avicel

Table 6 Initial hydrolysis rates and final glucose yield of hydrolysis of Avicel

Lignin concentration added	Initial hydrolysis rate (g/L/h)	72 h hydrolysis yield (%)
No addition	3.36 ± 0.11	49.81 ± 0.06
MOL-EU	2.34 ± 0.02	29.02 ± 0.66
EOL-EU	2.93 ± 0.28	39.18 ± 0.65
POL-EU	3.24 ± 0.08	47.26 ± 0.34

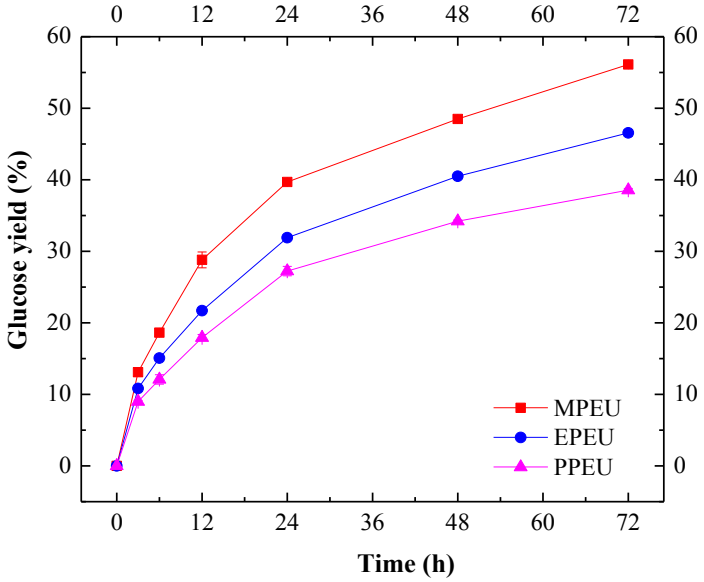


Fig. 15 Enzymatic hydrolysis of organosolv pretreated eucalyptus

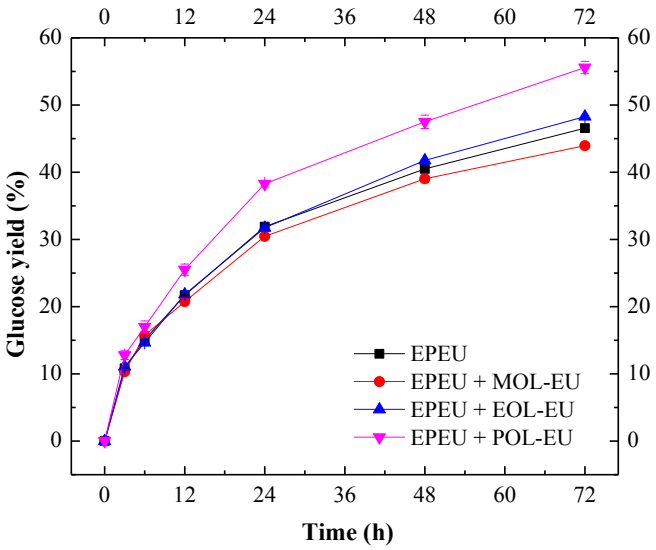


Fig. 16 Effect of organosolv lignins from poplar on enzymatic hydrolysis of ethanol pretreated eucalyptus

The effect of POL-EU (1, 2, 4, 8 g/L) on enzymatic hydrolysis of Avicel was observed (Fig. 17). 1 g/L POL-EU slightly promoted the final yield of Avicel from 49.81% to 50.69%, while 2, 4, and 8 g/L POL-EU decreased the Avicel hydrolysis from 49.81% to 48.69%, 47.26% and 43.23% by 1.12%, 2.55%, 6.58%, respectively. The inhibition of 1, 2, 4, 8 g/L POL-EU in Avicel hydrolysis was the highest with 8 g/L POL-EU. Similar to the stimulatory effect of EOL-HP in the previous section, the inhibition of organosolv lignins from eucalyptus was exacerbated when a higher concentration of lignin was added.

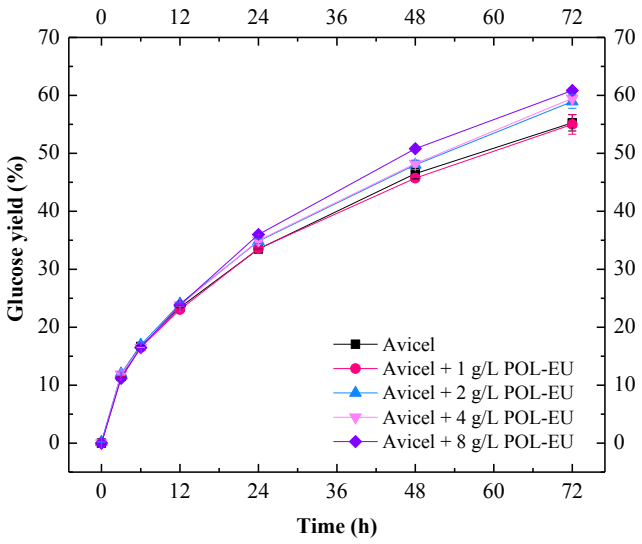


Fig. 17 Effect of different concentrations of POL-EU on enzymatic hydrolysis of Avicel

2.4.3 Hydrophobicity of lignins

Lignin surface hydrophobicity was reported to be one significant factor regulating the effect of lignins on enzymatic hydrolysis[197, 198]. Because lignin with higher hydrophobicity can adsorb more enzymes by non-specific adsorption hydrophobic interaction. To elucidate the hydrophobic interaction of organosolv lignins to the cellulase enzyme, the surface hydrophobicity of the organosolv lignins from poplar (MOL-HP, EOL-HP, POL-HP) and eucalyptus (MOL-EU, EOL- EU, POL- EU) was determined (Table 10). Among organosolv lignins from poplar, MOL-HP showed the highest hydrophobicity (0.19 L/g), which was higher than that of EOL-HP (0.16 L/g) and POL-HP (0.07 L/g). This may explain the stronger stimulatory effect of POL-HP than EOL-HP and MOL-HP on enzymatic hydrolysis. Similar to those of organosolv lignins from eucalyptus, MOL-EU showed the highest hydrophobicity (1.76 L/g), which was higher than those of EOL-EU (1.49 L/g) and POL-EU (1.27 L/g). In addition, hydrophobicity of propanol organosolv lignin from eucalyptus was more than 18 times as that of poplar, hydrophobicity of

methanol organosolv lignin from eucalyptus was almost 10 times as that of poplar. This may be the reason for significant inhibition of organosolv lignins from eucalyptus, especially MOL-EU. The higher surface hydrophobicity of lignins may result in stronger interaction between lignin and the aromatic amino acid region in the productive binding site of cellulase[127]. This explains the robust enzymatic inhibition hydrolysis by lignin[199]. The surface hydrophobicity of methanol, ethanol, and propanol organosolv lignins from poplar and eucalyptus can explain the distinguishing effect of these lignins on enzymatic hydrolysis.

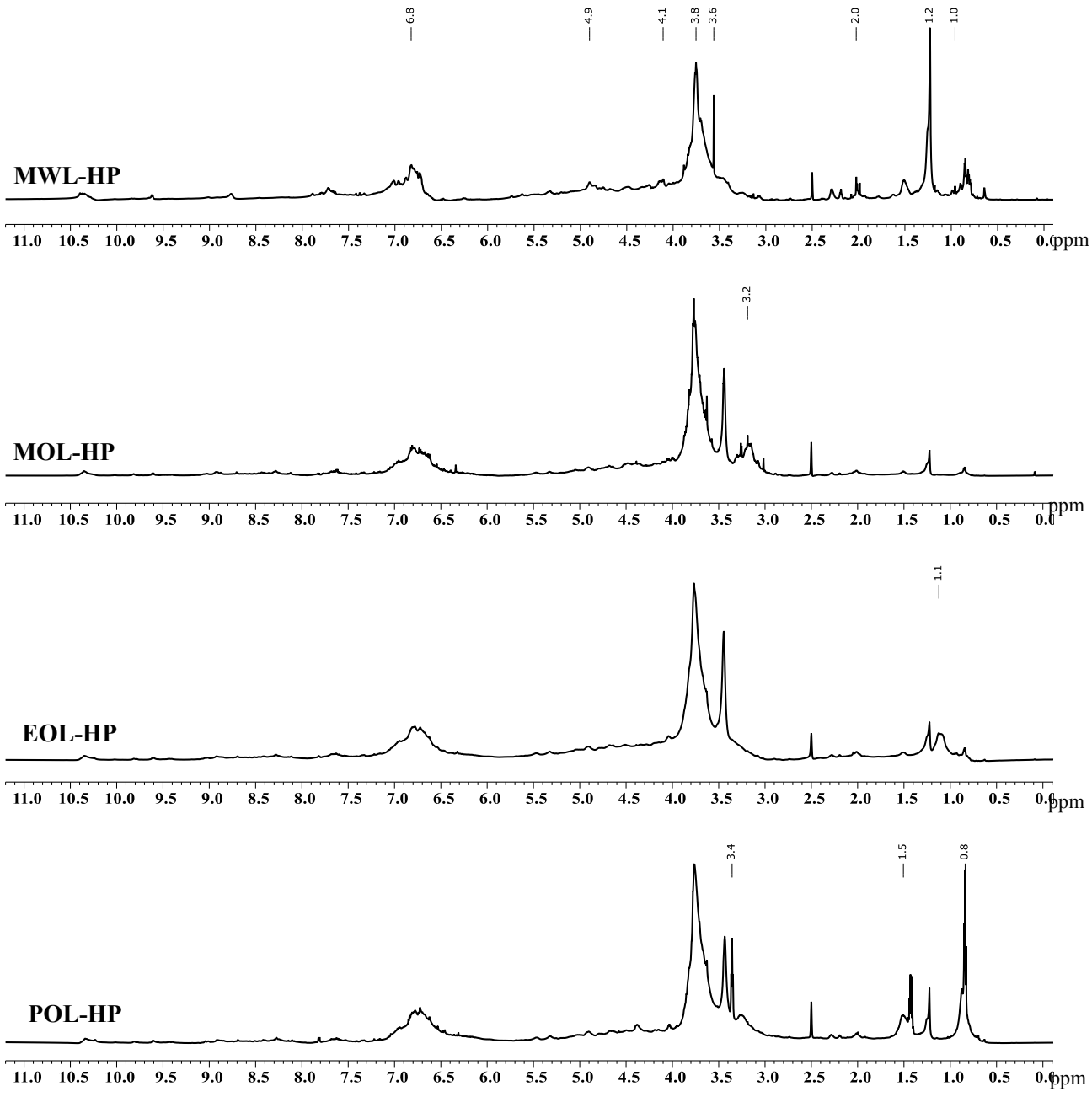
Table 7 Hydrophobicity of organosolv lignins from poplar and eucalyptus

Lignin	Hydrophobicity (L/g)
MOL-HP	0.19
EOL-HP	0.16
POL-HP	0.07
MOL-EU	1.76
EOL-EU	1.49
POL-EU	1.27

2.4.4 NMR spectra illustrate potential reactions of lignin in organosolv pretreatment

To investigate the reason underlying the distinct effect of organosolv lignins based on their chemical structures, we analyzed their ^{1}H (Fig. 18), quantitative ^{13}C (Fig. 19), and 2D HSQC, HSQC-TOCSY, HMBC NMR spectra (Fig. 20-22). The ^{1}H NMR spectra of organosolv lignin was first analyzed. Compared to that of MWL-HP, a new peak with the chemical shift of 3.2 ppm was observed in ^{1}H NMR spectra of MOL-HP. This peak represents the proton in the methoxy group (-OC$\underline{H}_3$) on the side chain of lignin. This indicated the methylation of lignin after the methanol organosolv pretreatment. One new peak appeared at 1.1 ppm of ^{1}H NMR spectra of EOL-HP, representing the proton in the ethoxy group (-OCH$_2$C$\underline{H}_3$). This indicated the ethylation reaction of lignin. There were three new peaks appeared at 3.4 (-OC$\underline{H}_2$CH$_2$CH$_3$), 1.5 (-OCH$_2$C$\underline{H}_2$CH$_3$), and

0.8 ppm (-$OCH_2CH_2C\underline{H}_3$) of ^{1}H NMR spectra of POL-HP, representing the proton in the propoxy group. Similar new peaks representing methylation, ethylation, and propylation of eucalyptus lignins were observed in ^{1}H NMR spectra of MOL-EU, EOL-EU, and POL-EU as well.



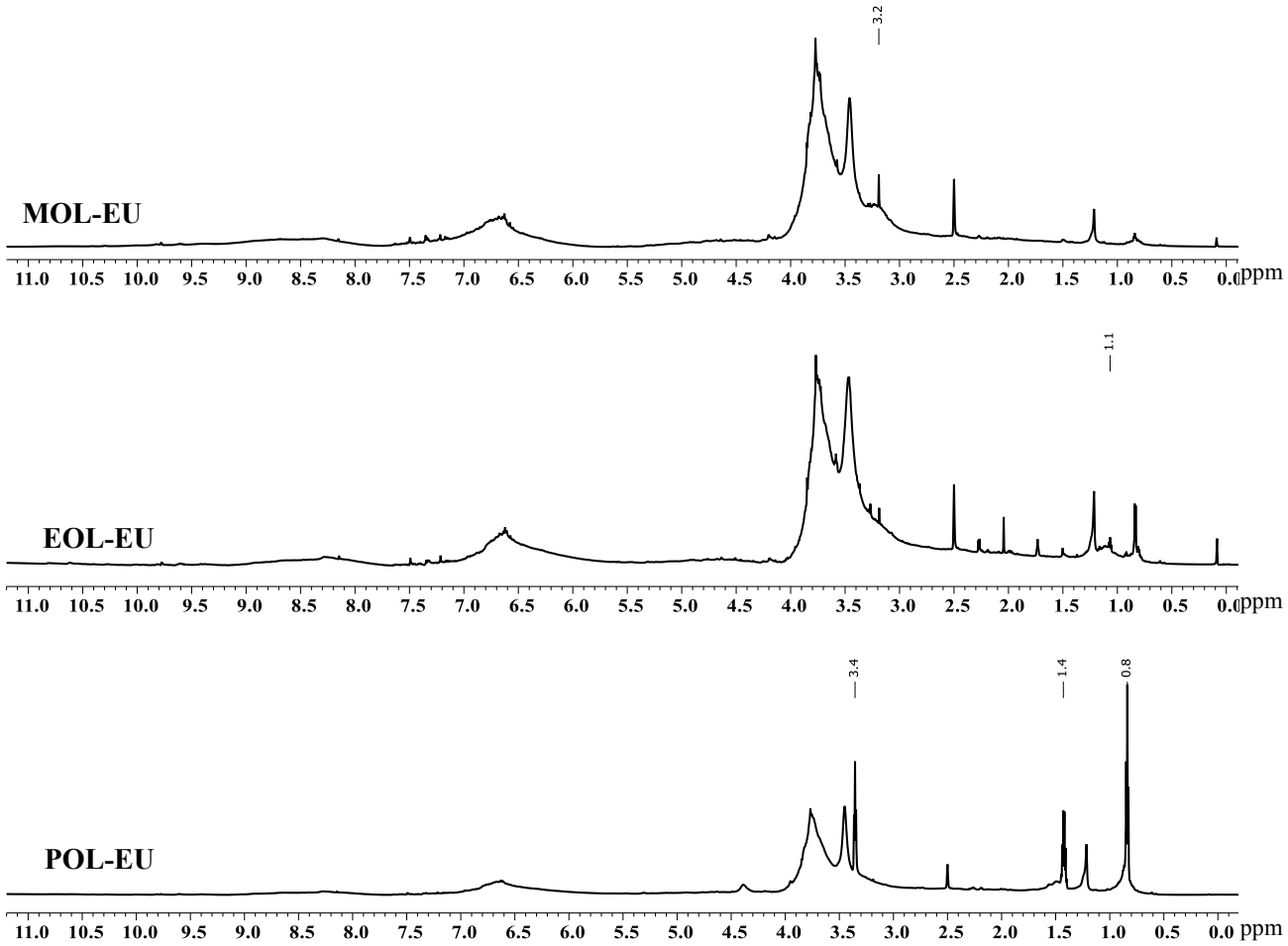
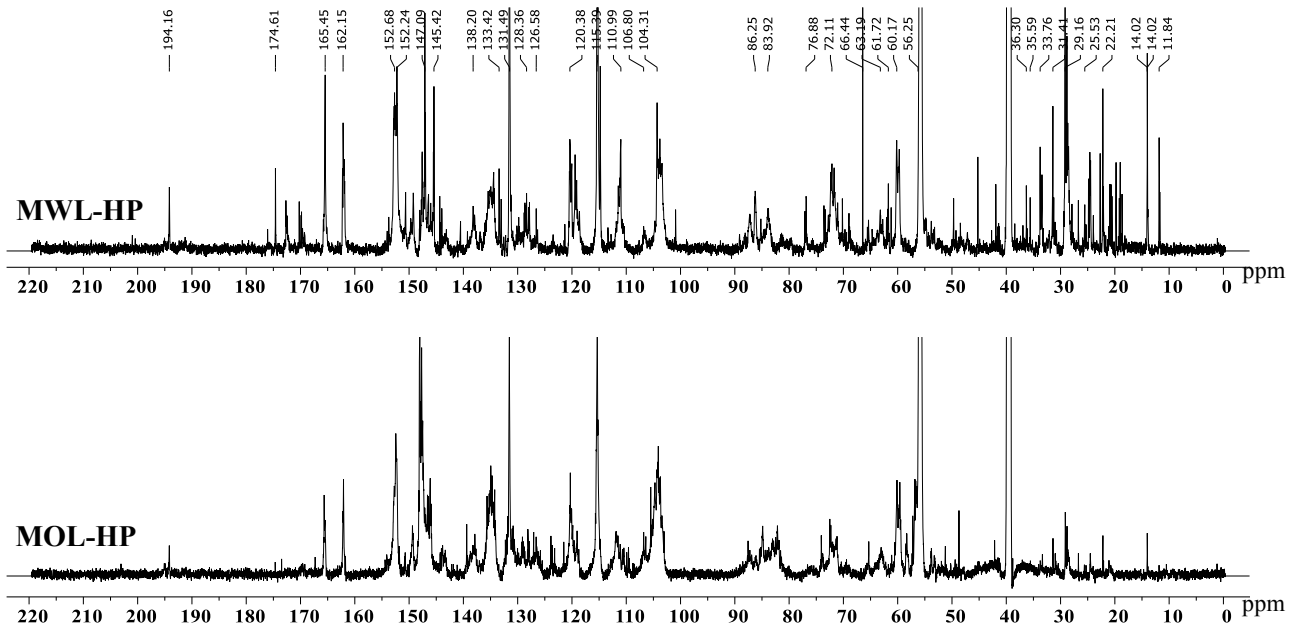


Fig. 18 ^{1}H spectra of organosolv lignin from poplar and eucalyptus

As regards the ^{13}C spectra of organosolv lignins, three new peaks appeared representing carbons in methyl peak (-$OCH_2CH_2\underline{C}H_3$) at 10.5 ppm, in methylene group ($OCH_2\underline{C}H_2CH_3$) at 25.7 ppm, and another corresponding methylene group ($O\underline{C}H_2CH_2CH_3$) at 71.1 ppm in POL-HP, compared to the ^{13}C spectrum of MWL-HP (Fig. 19). Interestingly, eight, instead of three, new peaks were observed in the HSQC spectra (Fig. 20), compared to the HSQC spectrum of MWL-HP. These new peaks of POL-HP were observed at δ_C/δ_H: 63/3.4, δ_C/δ_H: 71/3.3, δ_C/δ_H:68/3.5, δ_C/δ_H: 66/4.0, 26/1.4, δ_C/δ_H: 23/1.5, δ_C/δ_H: 11/0.8, δ_C/δ_H: 10/0.9. This might indicate the propylation reaction at different positions of lignins. To further elucidate the propylation reaction, the spectra

of HSQC-TOCSY of POL-HP were analyzed to show the through-bond C-H correlations between a carbon to all other coupled protons. Several new peaks appeared in the HSQC-TOCSY spectrum of POL-HP compared to its HSQC spectrum (Fig. 20), which had a similar carbon chemical shift as the new peaks in HSQC. There are four peaks appeared at δ_C/δ_H: 63/3.4 (-O$\underline{CH}_2CH_2CH_3$), 26/1.4 (-OCH$_2\underline{CH}_2CH_3$), 11/0.8 (-OCH$_2$CH$_2\underline{CH}_3$) in HSQC, the corresponding through-bond correlation of protons to the carbons in propyl group were discovered at δ_C/δ_H 63/1.4 (-O$\underline{C}H_2C\underline{H}_2CH_3$), 63/0.8 (-O$\underline{C}H_2CH_2C\underline{H}_3$); 26/3.4 (-OC$\underline{H}_2\underline{C}H_2CH_3$), 26/0.8 (-OCH$_2\underline{C}H_2C\underline{H}_3$); 11/3.4 (-OC$\underline{H}_2CH_2\underline{C}H_3$), 11/1.4 (-OCH$_2C\underline{H}_2\underline{C}H_3$) in the HSQC-TOCSY spectrum of POL-HP. These results might indicate the propylation on the C_α of β-O-4 lignin based on the chemical shift and the relative integration. Similarly, there are four peaks appeared at δ_C/δ_H: 71/3.3 (-O$\underline{CH}_2CH_2CH_3$), 23/1.5 (-OCH$_2\underline{CH}_2CH_3$), 10/0.9 (-OCH$_2$CH$_2\underline{CH}_3$) in HSQC, the corresponding through-bond correlation of protons to the carbons were discovered in the HSQC-TOCSY spectrum of POL-HP: δ_C/δ_H 71/1.5 (-O$\underline{C}H_2C\underline{H}_2CH_3$), 71/0.9 (-O$\underline{C}H_2CH_2C\underline{H}_3$); 23/3.3 (-OC$\underline{H}_2\underline{C}H_2CH_3$), 23/0.9 (-OCH$_2\underline{C}H_2C\underline{H}_3$); 10/3.3 (-OC$\underline{H}_2CH_2\underline{C}H_3$), 10/1.5 (-OCH$_2C\underline{H}_2\underline{C}H_3$). These results might indicate the propylation on the C_β of β-O-4 lignin. Two more sets of new peaks were discovered in HSQC and HSQC-TOCSY spectra and were distinguished by their chemical shifts and the integration (Table 11). New C-H peaks of POL-HP at δ_C/δ_H: 68/3.5 (-O$\underline{CH}_2CH_2CH_3$), 23/1.5 (-OCH$_2\underline{CH}_2CH_3$), 11/0.8 (-OCH$_2$CH$_2\underline{CH}_3$) in HSQC showed the propoxy group on C_γ of β-O-4 lignin. The corresponding through-bond correlation of protons and carbons in the HSQC-TOCSY spectrum were observed at δ_C/δ_H 68/1.5 (-O$\underline{C}H_2C\underline{H}_2CH_3$), 68/0.8 (-O$\underline{C}H_2CH_2C\underline{H}_3$); 23/3.5 (-OC$\underline{H}_2\underline{C}H_2CH_3$), 23/0.8 (-OCH$_2\underline{C}H_2C\underline{H}_3$); 11/3.5 (-OC$\underline{H}_2CH_2\underline{C}H_3$), 11/1.5 (-OCH$_2C\underline{H}_2\underline{C}H_3$). New C-H peaks of POL-HP at δ_C/δ_H: 66/4.0 (-O$\underline{CH}_2CH_2CH_3$), 22/1.5 (-OCH$_2\underline{CH}_2CH_3$), 10/0.9 (-OCH$_2$CH$_2\underline{CH}_3$) in HSQC showed the propoxy group on the benzene ring of β-O-4 lignin. The corresponding through-bond

correlation of protons and carbons in the HSQC-TOCSY spectrum were observed at δ_C/δ_H 66/1.5 (-O<u>C</u>H$_2$C<u>H</u>$_2$CH$_3$), 66/0.9 (-O<u>C</u>H$_2$CH$_2$C<u>H</u>$_3$); 22/4.0 (-OC<u>H</u>$_2$<u>C</u>H$_2$CH$_3$), 22/0.9 (-OCH$_2$<u>C</u>H$_2$C<u>H</u>$_3$); 10/4.0 (-OC<u>H</u>$_2$CH$_2$<u>C</u>H$_3$), 10/1.5 (-OCH$_2$C<u>H</u>$_2$<u>C</u>H$_3$). Wu etc. reported the etherification of C_α with propoxy group from propanol organosolv pretreatment of poplar by identifying a new peak in 2D-HSQC spectrum with the assignment of δ_C/δ_H: 68/3.28[116]. A similar new peak of propoxy group (δ_C/δ_H: 68-71/3.3-3.5) was discovered in HSQC spectra of POL-HP, representing the propylation at C_β and C_γ. Although the corresponding peaks in the HSQC-TOCSY spectrum of POL-HP are not well separated because of condensation, better separation of propyl group on C_β and C_γ in the HSQC-TOCSY spectrum of POL-EU (Fig. 20). Therefore, propylation of hydroxyl group might not only occurred on C_α, but on C_β, C_γ, and aromatic ring in propanol organosolv pretreatment of both poplar and eucalyptus.



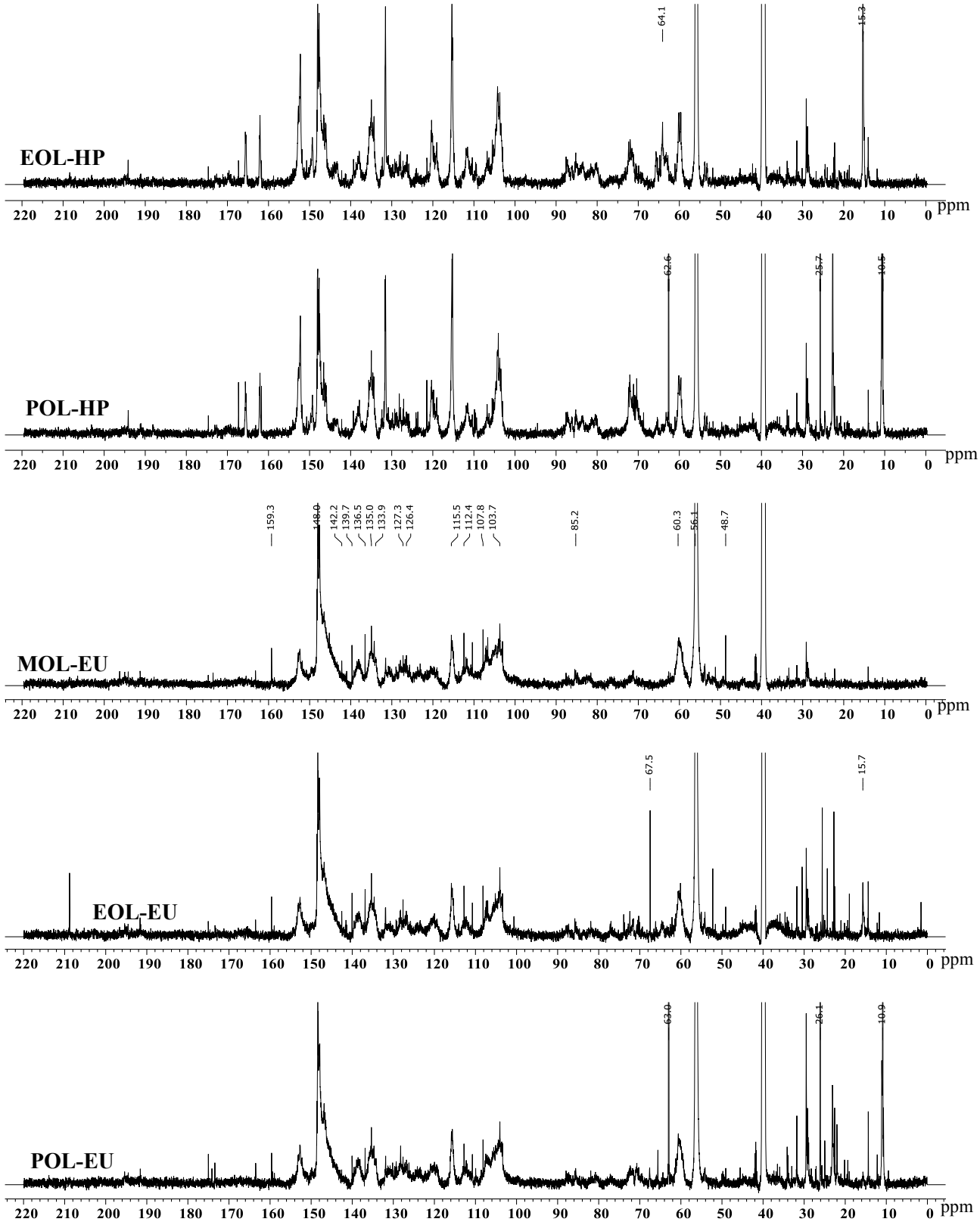
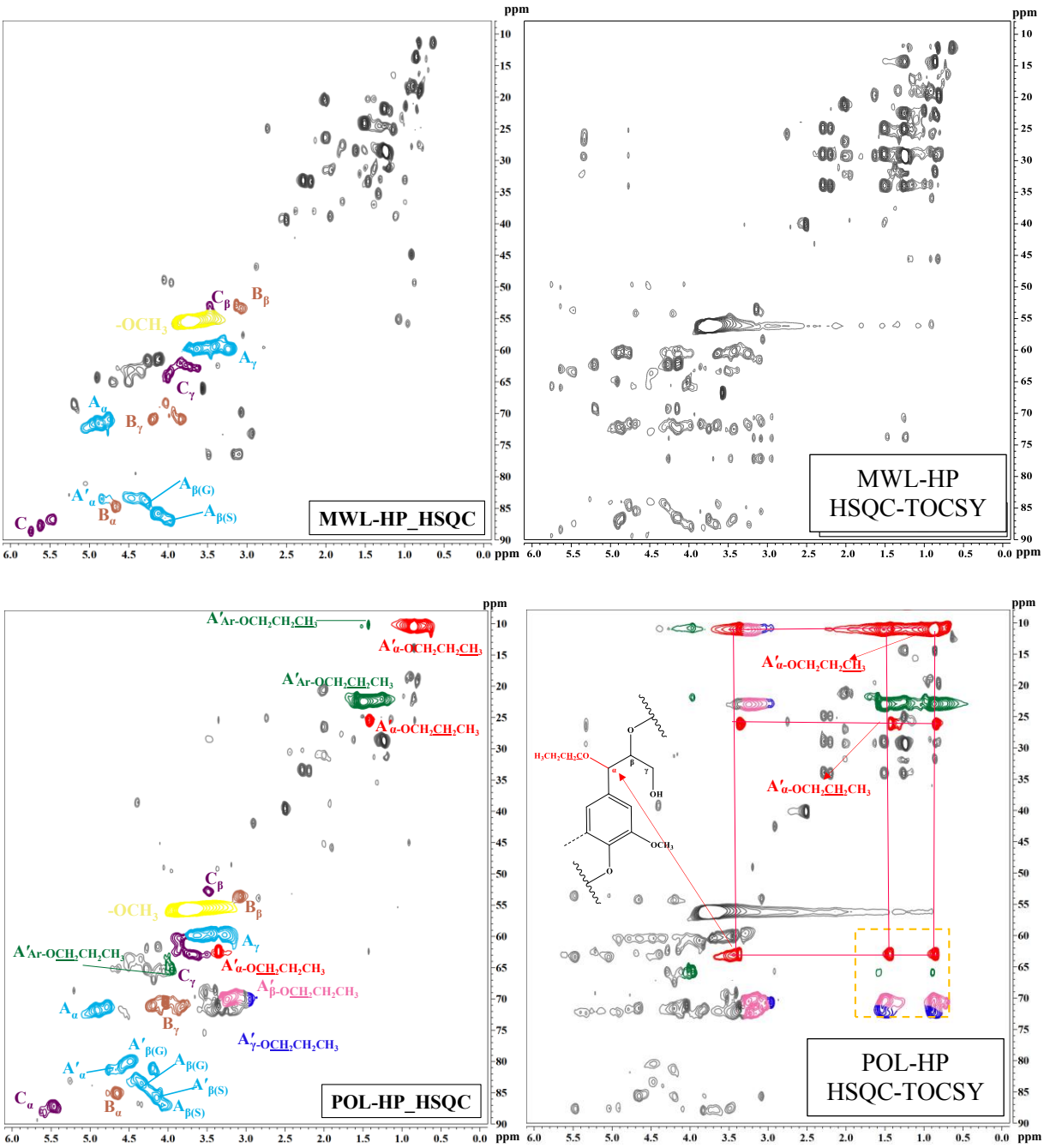


Fig. 19 Quantitative ^{13}C spectra of organosolv lignin from poplar and eucalyptus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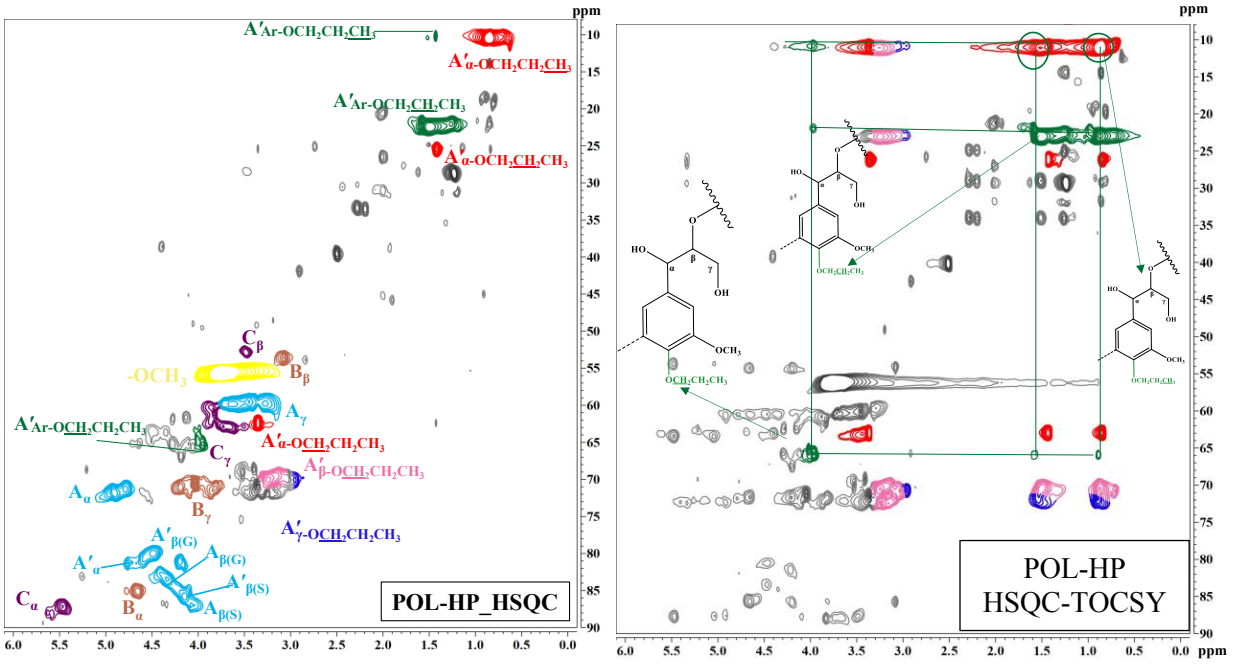
MWL-HP
HSQC-TOCSY
POL-HP_HSQC
POL-HP
HSQC-TOCSY
ppm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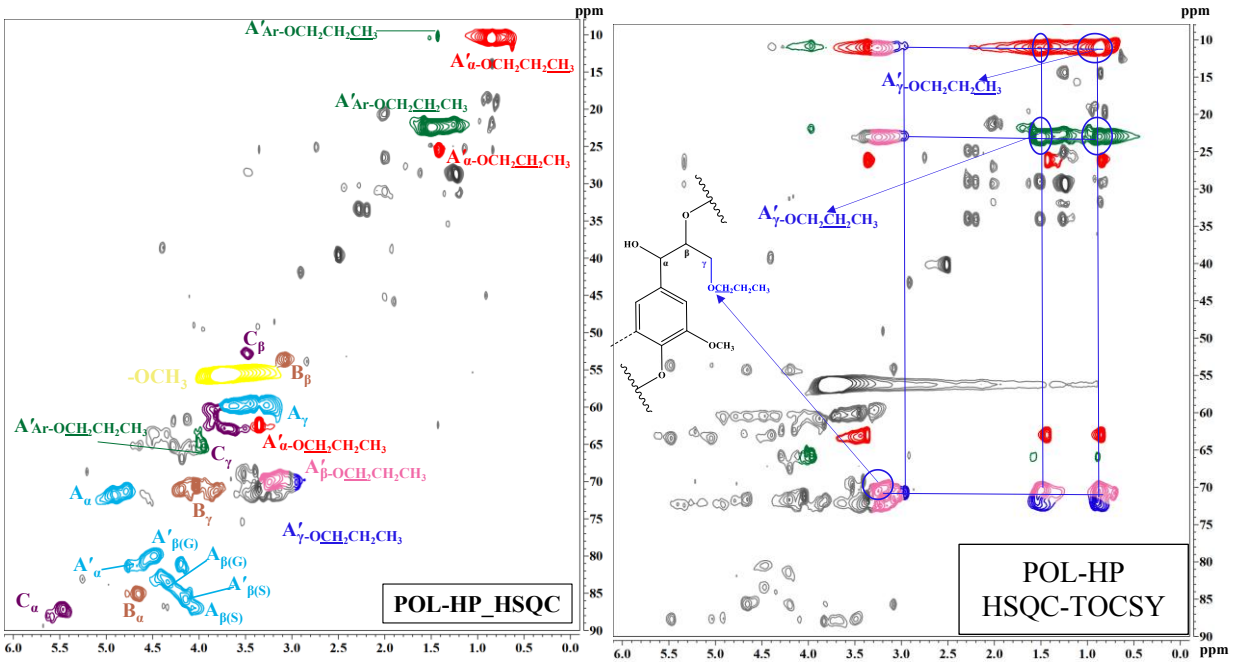
POL-HP
HSQC-TOCSY
ppm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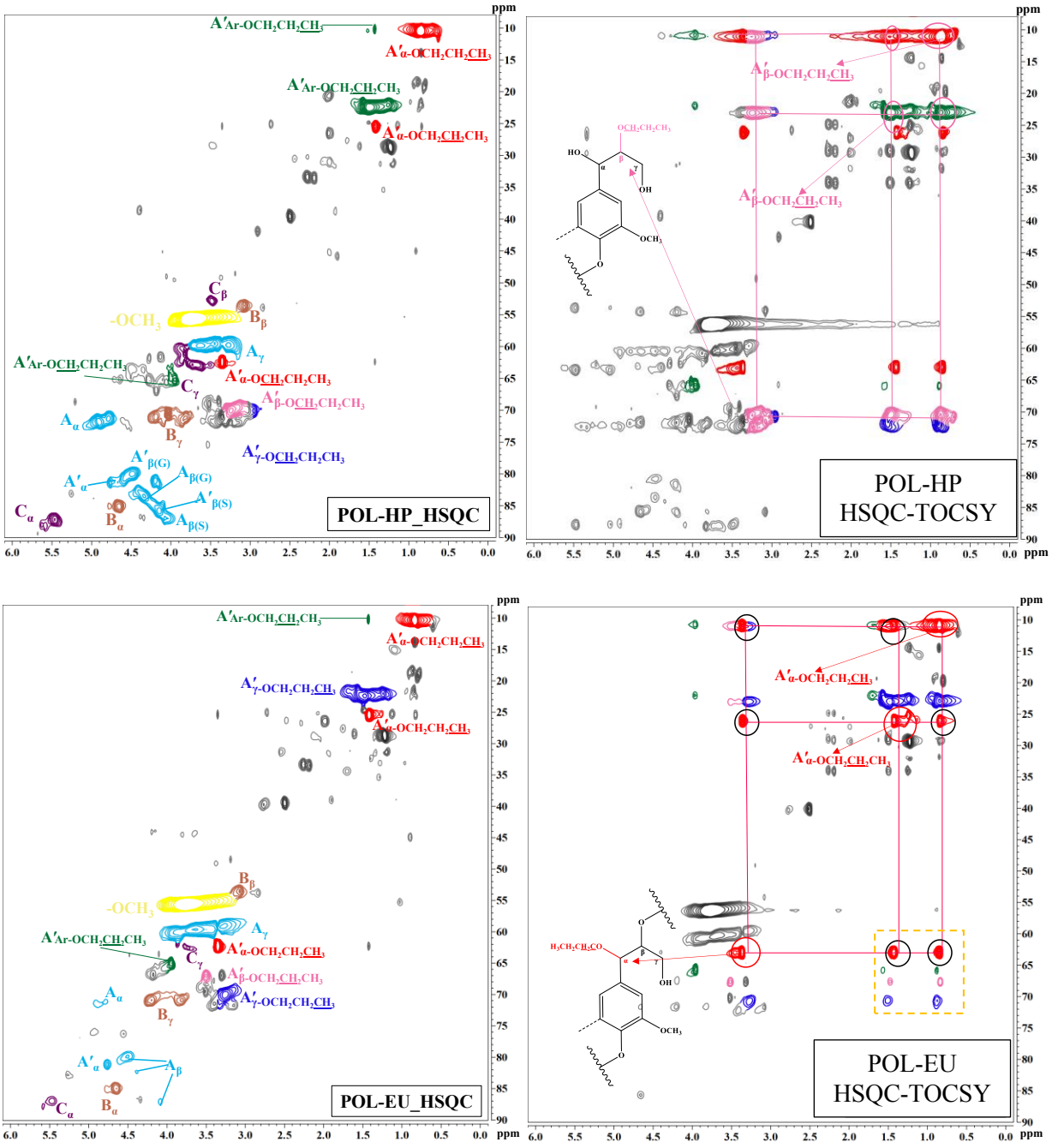
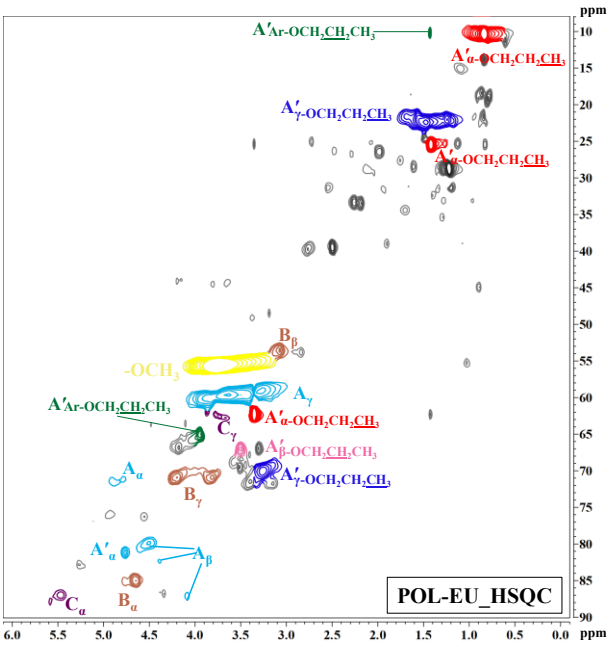
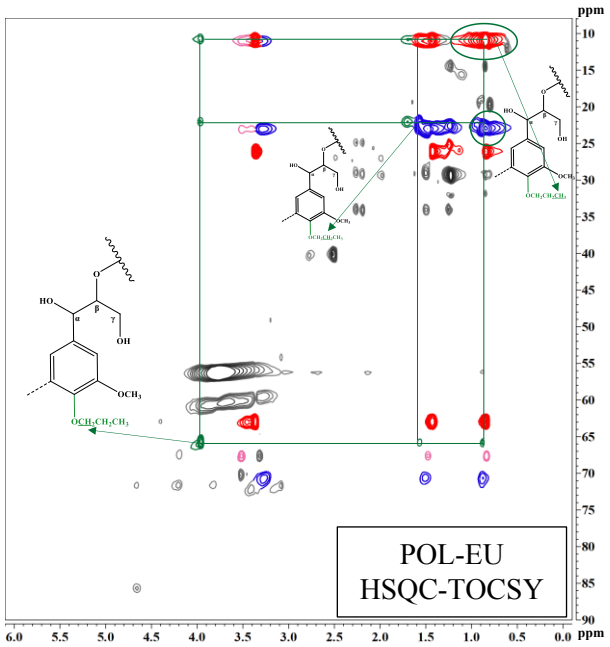
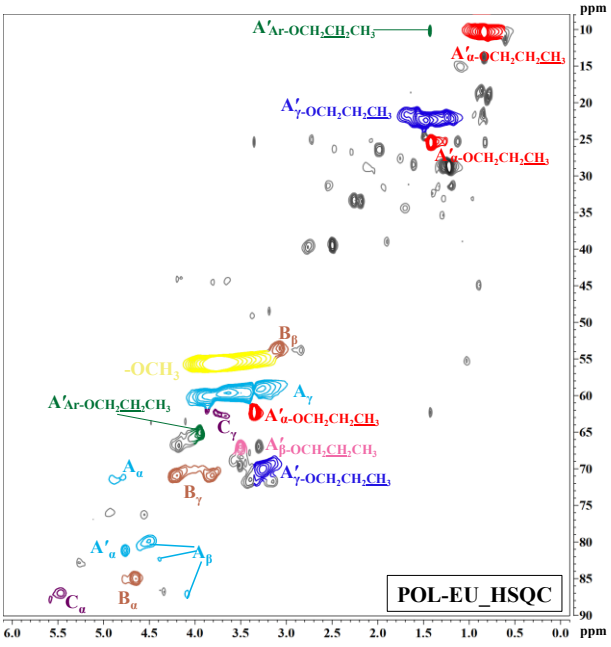
POL-HP
HSQC-TOCSY
ppm


POL-HP_HSQC
POL-HP
HSQC-TOCSY
POL-EU_HSQC
POL-EU
HSQC-TOCSY
ppm


A′Ar-OCH2CH2CH3
A′α-OCH2CH2CH3
A′γ-OCH2CH2CH3
A′α-OCH2CH2CH3
Bβ
-OCH3
Aγ
A′Ar-OCH2CH2CH3
A′α-OCH2CH2CH3
Cγ
A′β-OCH2CH2CH3
A′γ-OCH2CH2CH3
Aα
Bγ
A′α
Aβ
Cα
Bα
POL-EU_HSQC
ppm


POL-EU
HSQC-TOCSY
ppm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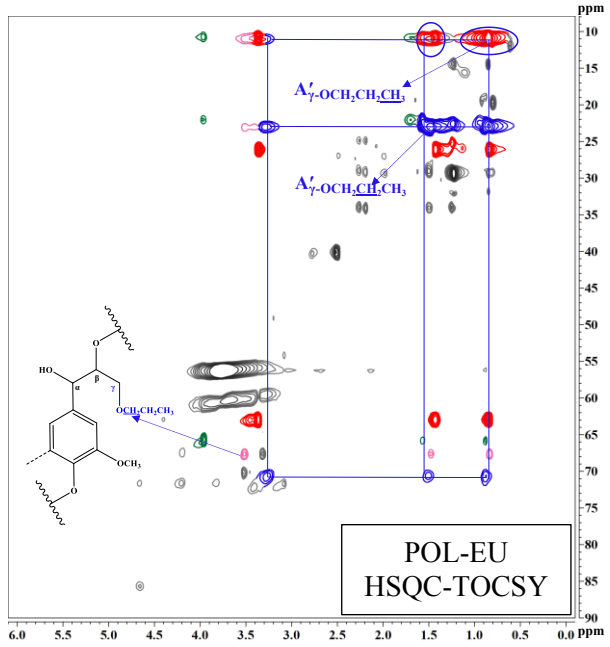
A′α-OCH2CH2CH3
A′γ-OCH2CH2CH3
A′α-OCH2CH2CH3
Bβ
-OCH3
Aγ
A′Ar-OCH2CH2CH3
A′α-OCH2CH2CH3
Cγ
A′β-OCH2CH2CH3
A′γ-OCH2CH2CH3
Aα
Bγ
A′α
Aβ
Cα
Bα
POL-EU_HSQC
ppm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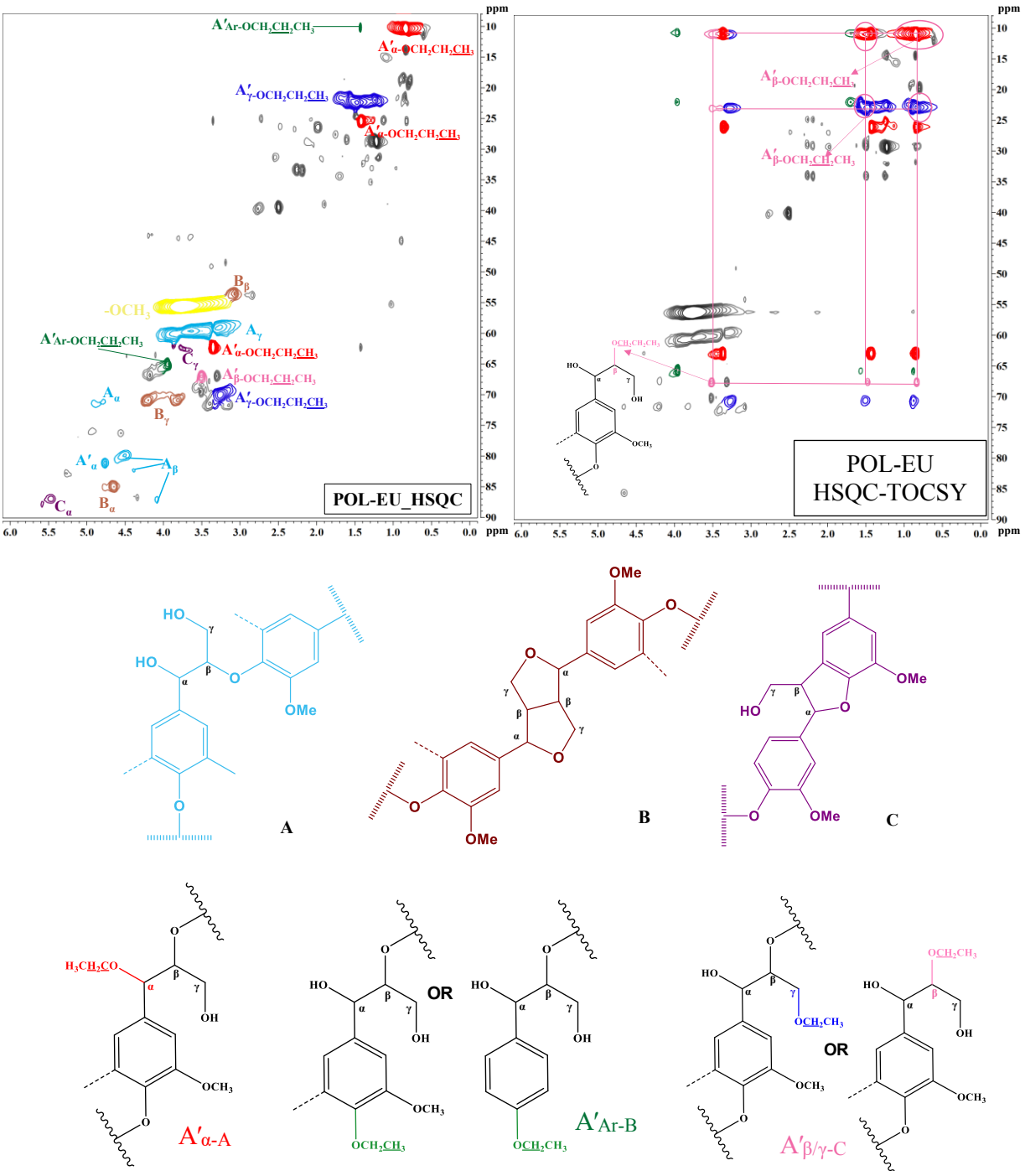
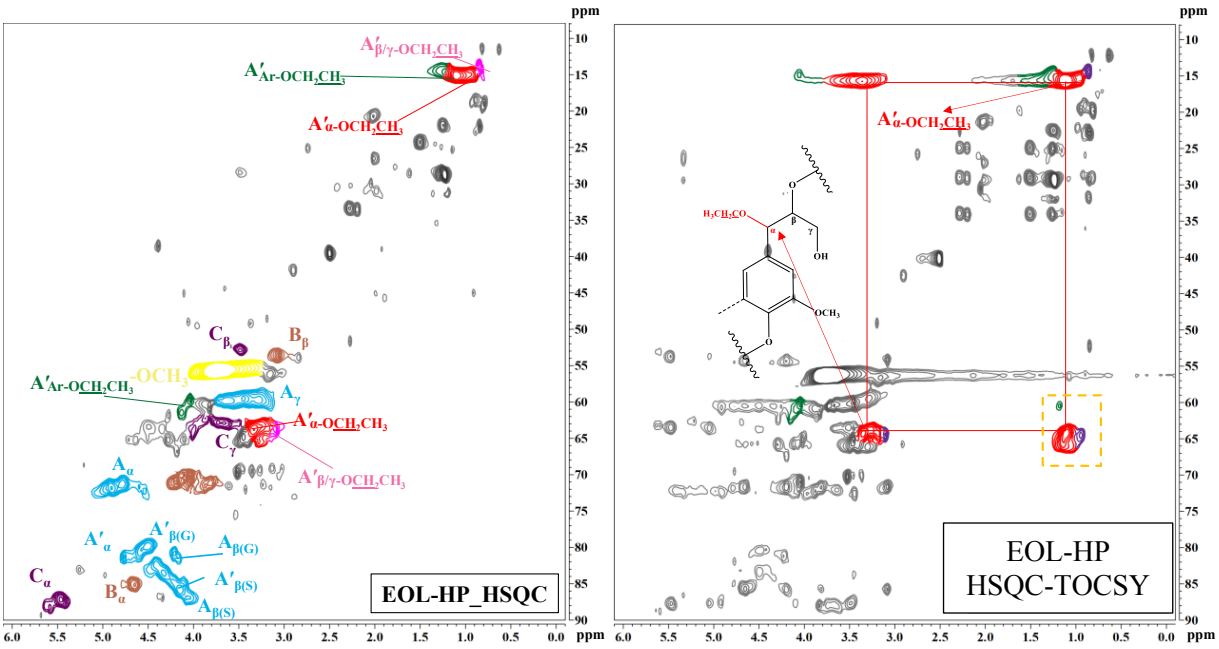
A′γ-OCH2CH2CH3
POL-EU
HSQC-TOCSY
ppm

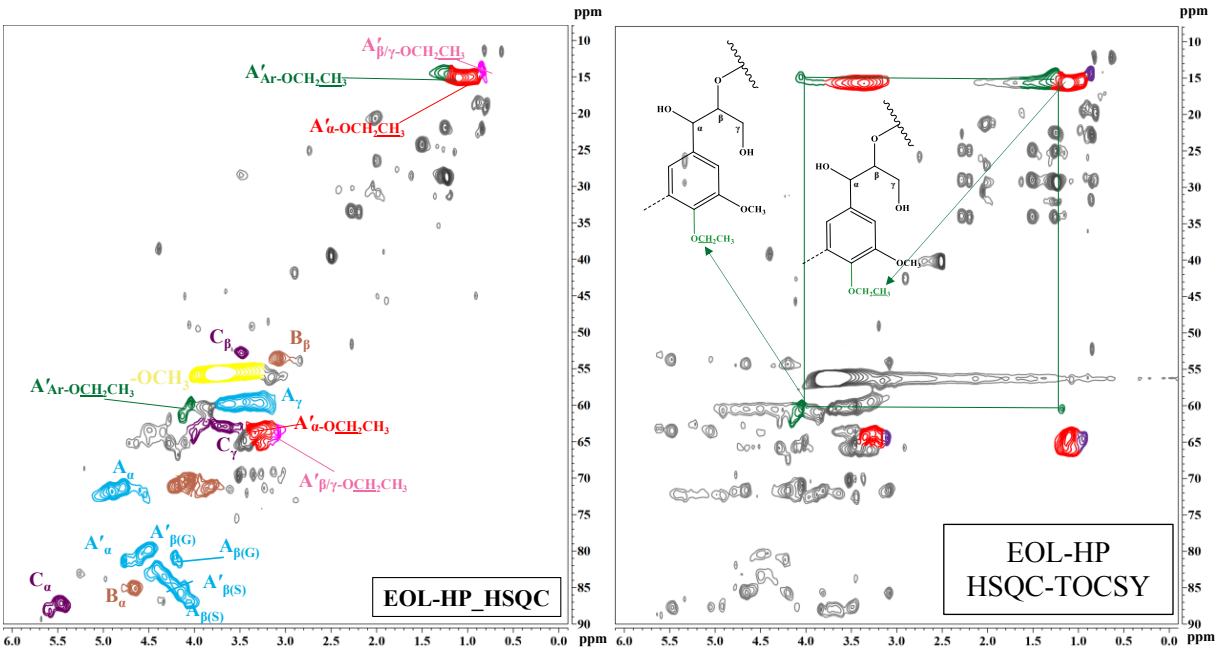


(A: β-aryl-ether units (β-O-4); B: resinol substructures (β-β); C: phenyl-coumaran substructures (β-5);)

Fig. 20 2D NMR spectra illustrate potential propylation of lignin

^{13}C spectra showed a methyl peak (-$OCH_2\underline{C}H_3$) at 15.3 ppm and a corresponding methylene group (-$O\underline{C}H_2CH_3$) at 64.1 ppm in both EOL-HP and EOL-EU. So it is possible that an ethoxy group was introduced to C_α through etherification of the alcohol on the benzene ring as they analyzed the HSQC-TOCSY and HSQC NMR spectrum of EOL from *Miscanthus giganteus*[74]. Three sets of new peaks were found in HSQC, HSQC-TOCSY spectra of EOL-HP, and EOL-EU (Fig. 21). The assignments for C-H single-bond and C-H through-bond correlations were listed in Table 11. The only difference is that it is difficult to distinguish the new peak at δ_C/δ_H 66/3.3, 15/1.0 between ethylation of C_β or C_γ. Therefore, similar to the propylation for POL-HP and POL-EU, ethylation of hydroxyl group not only occurred on C_α, but also on $C_{\beta/\gamma}$, and aromatic ring in ethanol organosolv pretreatment of both poplar and eucalyptus.

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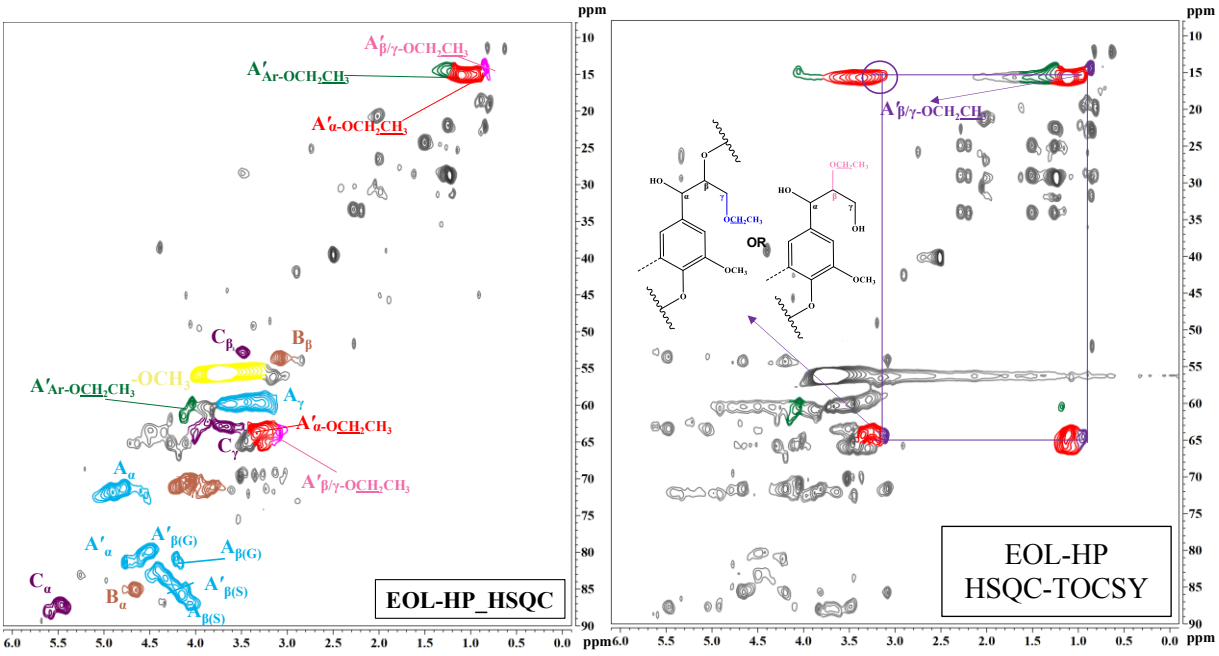
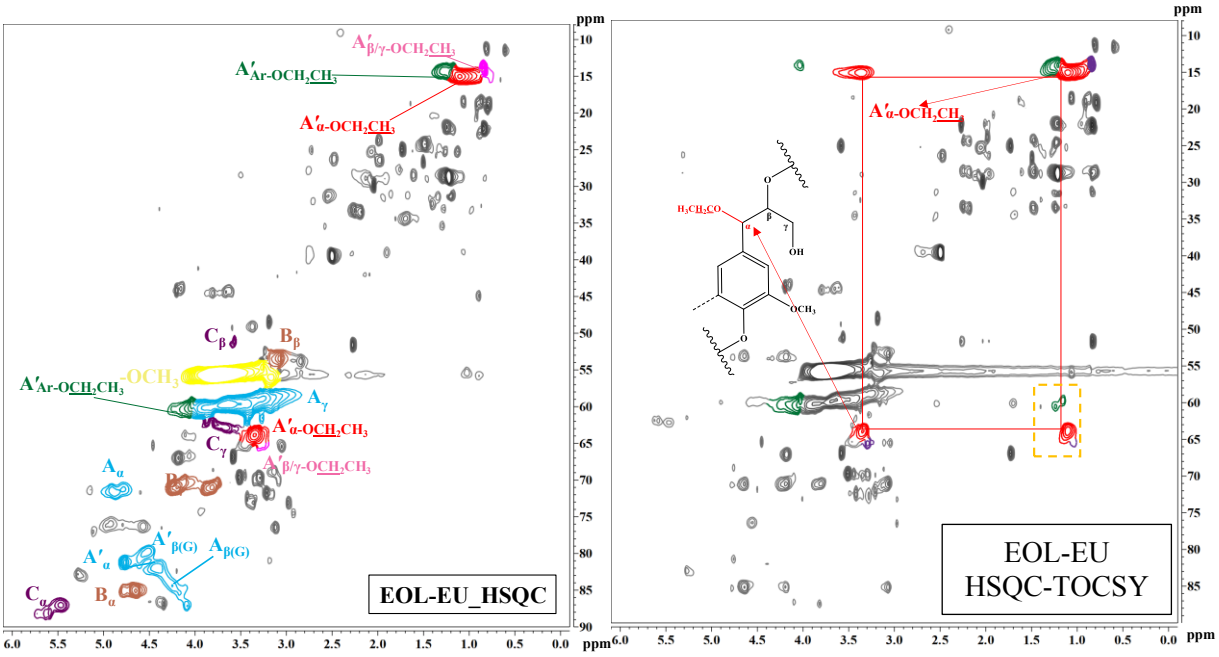
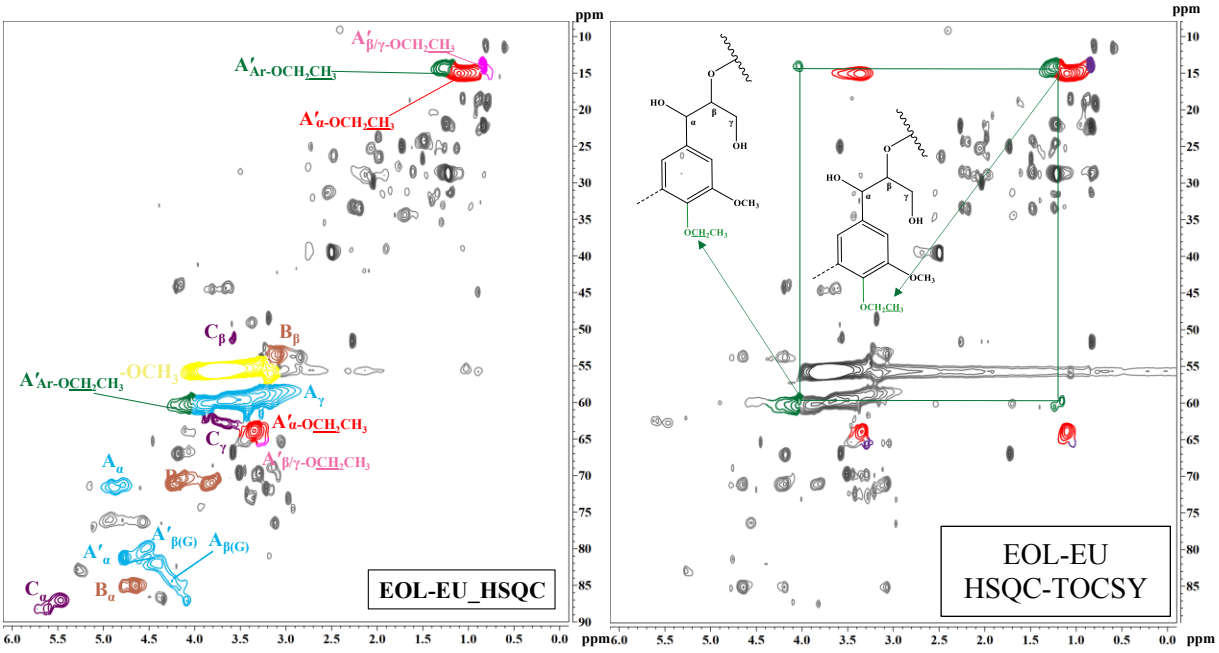
HSQC-TOCSY


EOL-HP_HSQC
EOL-HP
HSQC-TOCSY
OR


EOL-EU_HSQC
EOL-EU
HSQC-TOCSY


EOL-EU_HSQC
EOL-EU
HSQC-TOCSY

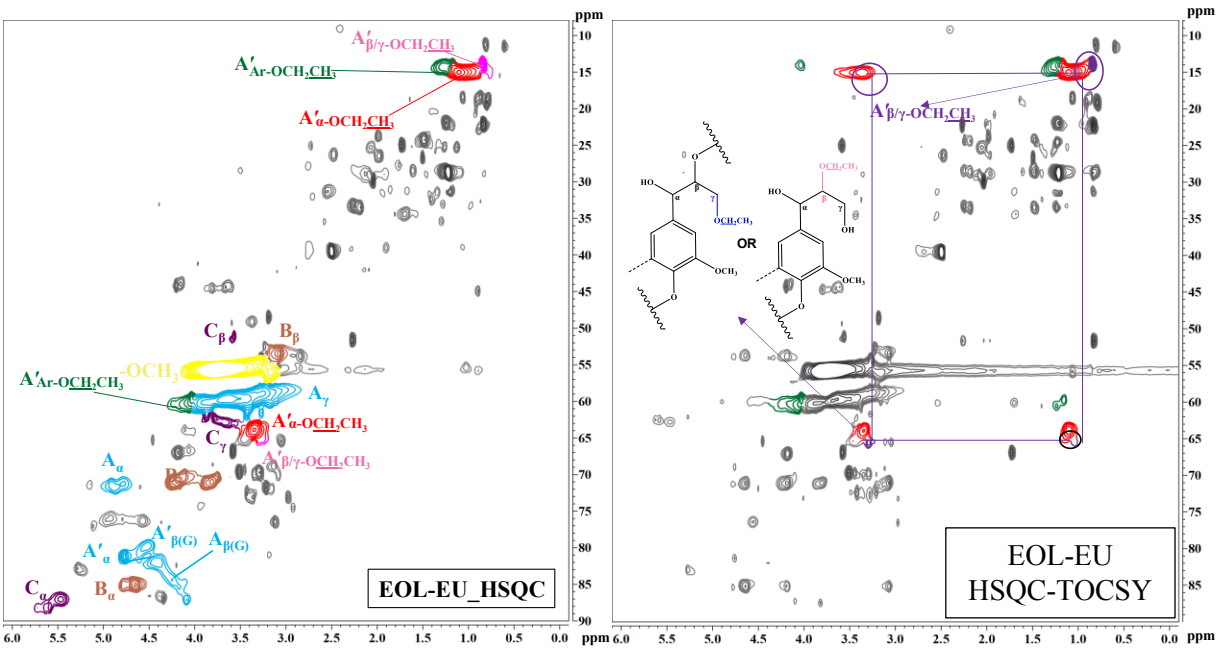


Fig. 21 2D NMR spectra illustrate potential ethylation of lignin

Table 8 C-H correlation assignment in HSQC-TOCSY spectra of methylation, ethylation and propylation of lignin

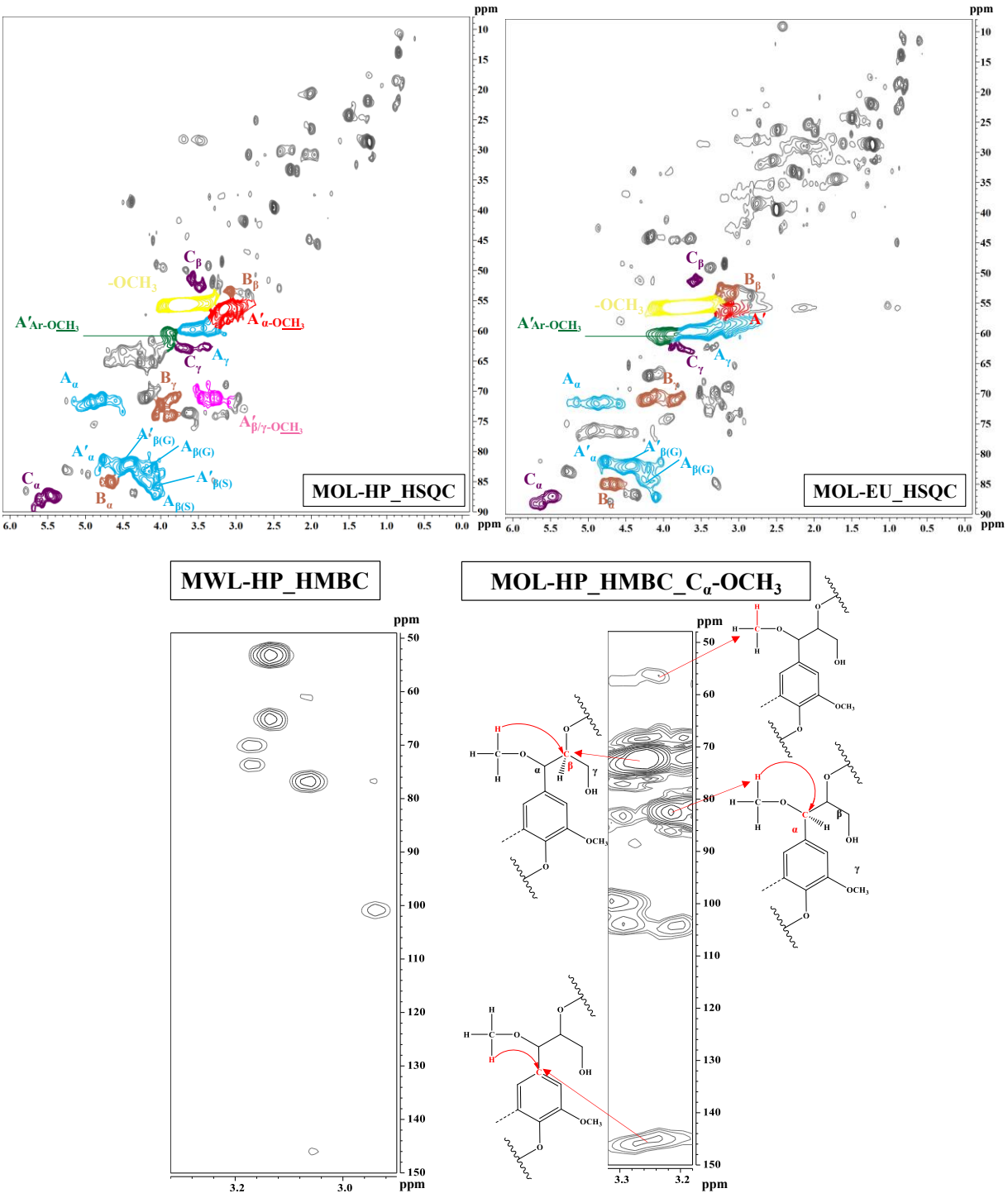
Label	δ_C/δ_H (ppm)	Assignments
A′$_\alpha$-O<u>CH</u>$_2$CH$_2$CH$_3$	63/3.4	**-O<u>CH</u>$_2$CH$_2$CH$_3$ at C$_\alpha$** in *β-O*-4 substructures
A′$_\alpha$-O<u>C</u>H$_2$C<u>H</u>$_2$CH$_3$	63/1.4	-O<u>C</u>H$_2$C<u>H</u>$_2$CH$_3$
A′$_\alpha$-O<u>C</u>H$_2$CH$_2$C<u>H</u>$_3$	63/0.8	-O<u>C</u>H$_2$CH$_2$C<u>H</u>$_3$
A′$_\alpha$-OCH$_2$<u>CH</u>$_2$CH$_3$	26/1.4	**-OCH$_2$<u>CH</u>$_2$CH$_3$**
A′$_\alpha$-OC<u>H</u>$_2$<u>C</u>H$_2$CH$_3$	26/3.4	-OC<u>H</u>$_2$<u>C</u>H$_2$CH$_3$
A′$_\alpha$-OCH$_2$<u>C</u>H$_2$C<u>H</u>$_3$	26/0.8	-OCH$_2$<u>C</u>H$_2$C<u>H</u>$_3$
A′$_\alpha$-OCH$_2$CH$_2$<u>CH</u>$_3$	11/0.8	**-OCH$_2$CH$_2$<u>CH</u>$_3$**
A′$_\alpha$-OC<u>H</u>$_2$CH$_2$<u>C</u>H$_3$	11/3.4	-OC<u>H</u>$_2$CH$_2$<u>C</u>H$_3$
A′$_\alpha$-OCH$_2$C<u>H</u>$_2$<u>C</u>H$_3$	11/1.4	-OCH$_2$C<u>H</u>$_2$<u>C</u>H$_3$
A′$_\beta$-O<u>CH</u>$_2$CH$_2$CH$_3$	71/3.3	**-O<u>CH</u>$_2$CH$_2$CH$_3$ at C$_\beta$** in *β-O*-4 substructures
A′$_\beta$-O<u>C</u>H$_2$C<u>H</u>$_2$CH$_3$	71/1.5	- O<u>C</u>H$_2$C<u>H</u>$_2$CH$_3$
A′$_\beta$-O<u>C</u>H$_2$CH$_2$C<u>H</u>$_3$	71/0.9	-O<u>C</u>H$_2$CH$_2$C<u>H</u>$_3$
A′$_\beta$-OCH$_2$<u>CH</u>$_2$CH$_3$	23/1.5	-OCH$_2$<u>CH</u>$_2$CH$_3$

A′$_{\beta}$-OC$\underline{H_2}$C$\underline{H_2}$CH$_3$	23/3.3	-OC$\underline{H_2}$C$\underline{H_2}$CH$_3$
A′$_{\beta}$-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$	23/0.9	-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$
A′$_{\beta}$-OCH$_2$CH$_2$C$\underline{H_3}$	10/0.9	-OCH$_2$CH$_2$C$\underline{H_3}$
A′$_{\beta}$-OC$\underline{H_2}$CH$_2$C$\underline{H_3}$	10/3.3	-OC$\underline{H_2}$CH$_2$C$\underline{H_3}$
A′$_{\beta}$-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$	10/1.5	-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$
A′$_{\gamma}$-O$\underline{CH_2}$CH$_2$CH$_3$	68/3.5	**-O$\underline{CH_2}$CH$_2$CH$_3$ at C$_{\gamma}$** in *β-O*-4 substructures
A′$_{\gamma}$-O$\underline{C}$H$_2$C$\underline{H_2}$CH$_3$	68/1.5	- O$\underline{C}$H$_2$C$\underline{H_2}$CH$_3$
A′$_{\gamma}$-O$\underline{C}$H$_2$CH$_2$C$\underline{H_3}$	68/0.8	-O$\underline{C}$H$_2$CH$_2$C$\underline{H_3}$
A′$_{\gamma}$-OCH$_2$C$\underline{H_2}$CH$_3$	23/1.5	-OCH$_2$C$\underline{H_2}$CH$_3$
A′$_{\gamma}$-OC$\underline{H_2}$C$\underline{H_2}$CH$_3$	23/3.5	-OC$\underline{H_2}$C$\underline{H_2}$CH$_3$
A′$_{\gamma}$-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$	23/0.8	-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$
A′$_{\gamma}$-OCH$_2$CH$_2$C$\underline{H_3}$	11/0.8	-OCH$_2$CH$_2$C$\underline{H_3}$
A′$_{\gamma}$-OC$\underline{H_2}$CH$_2$C$\underline{H_3}$	11/3.5	-OC$\underline{H_2}$CH$_2$C$\underline{H_3}$
A′$_{\gamma}$-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$	11/1.5	-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$
A′$_{Ar}$-O$\underline{CH_2}$CH$_2$CH$_3$	66/4.0	**-O$\underline{CH_2}$CH$_2$CH$_3$ at aromatic ring** in *β-O*-4 substructures
A′$_{Ar}$-O$\underline{C}$H$_2$C$\underline{H_2}$CH$_3$	66/1.5	-O$\underline{C}$H$_2$C$\underline{H_2}$CH$_3$
A′$_{Ar}$-O$\underline{C}$H$_2$CH$_2$C$\underline{H_3}$	66/0.9	-O$\underline{C}$H$_2$CH$_2$C$\underline{H_3}$
A′$_{Ar}$-OCH$_2$C$\underline{H_2}$CH$_3$	23/1.5	-OCH$_2$C$\underline{H_2}$CH$_3$
A′$_{Ar}$-OC$\underline{H_2}$C$\underline{H_2}$CH$_3$	23/4.0	-OC$\underline{H_2}$C$\underline{H_2}$CH$_3$
A′$_{Ar}$-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$	23/0.9	-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$
A′$_{Ar}$-OCH$_2$CH$_2$C$\underline{H_3}$	10/0.9	-OCH$_2$CH$_2$C$\underline{H_3}$
A′$_{Ar}$-OC$\underline{H_2}$CH$_2$C$\underline{H_3}$	10/4.0	-OC$\underline{H_2}$CH$_2$C$\underline{H_3}$
A′$_{Ar}$-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$	10/1.5	-OCH$_2$C$\underline{H_2}$C$\underline{H_3}$
A′$_{\alpha}$-OCH$_2$C$\underline{H_3}$	15/1.1	**-OCH$_2$C$\underline{H_3}$ at C$_{\alpha}$** *β-O*-4 substructures
A′$_{\alpha}$-OC$\underline{H_2}$CH$_3$	15/3.4	-OC$\underline{H_2}$CH$_3$
A′$_{\alpha}$-O$\underline{CH_2}$CH$_3$	64/3.4	-O$\underline{CH_2}$CH$_3$
A′$_{\alpha}$-O$\underline{C}$H$_2$C$\underline{H_3}$	64/1.1	-O$\underline{C}$H$_2$C$\underline{H_3}$
A′$_{\beta/\gamma}$-OCH$_2$C$\underline{H_3}$	15/1.0	**-OCH$_2$C$\underline{H_3}$ at C$_{\beta/\gamma}$** *β-O*-4 substructures
A′$_{\beta/\gamma}$-OC$\underline{H_2}$CH$_3$	15/3.3	-OC$\underline{H_2}$CH$_3$

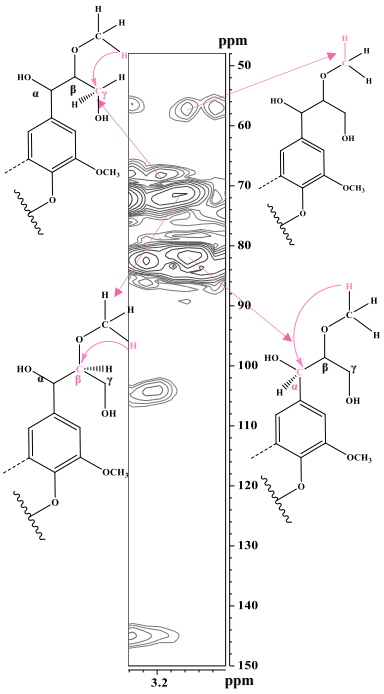
$A'_{\beta/\gamma}$-O$\underline{C}H_2CH_3$	66/3.3	-O$\underline{C}H_2CH_3$
$A'_{\beta/\gamma}$-O$\underline{C}H_2C\underline{H}_3$	66/1.0	-O$\underline{C}H_2C\underline{H}_3$
A'_{Ar}-OCH$_2\underline{C}H_3$	15/1.2	**-OCH$_2\underline{C}H_3$ at aromatic ring** *β-O*-4 substructures
A'_{Ar}-OC$\underline{H}_2\underline{C}H_3$	15/4.0	-OC$\underline{H}_2\underline{C}H_3$
A'_{Ar}-O$\underline{C}H_2CH_3$	60/4.0	-O$\underline{C}H_2CH_3$
A'_{Ar}-O$\underline{C}H_2C\underline{H}_3$	60/1.2	-O$\underline{C}H_2C\underline{H}_3$
A'_{α}-O$\underline{C}H_3$	55/3.1	**-O$\underline{C}H_3$ at C$_\alpha$** *β-O*-4 substructures
$A'_{\beta/\gamma}$ -O$\underline{C}H_3$	71/3.5	**-O$\underline{C}H_3$ at C$_\beta$** *β-O*-4 substructures
$A'_{\beta/\gamma}$ -O$\underline{C}H_3$	71/3.3	**-O$\underline{C}H_3$ at C$_\gamma$** *β-O*-4 substructures
A'_{Ar}-O$\underline{C}H_3$	59/3.9	**-O$\underline{C}H_3$ at aromatic ring** *β-O*-4 substructures

Compared to the spectra of MWL-HP, no new peak appeared in the ^{13}C NMR spectrum of MOL-HP. However, four new peaks were observed in the HSQC spectrum of MOL-HP at δ_H/δ_C 55/3.1, 71/3.3, 71/3.5, 59/3.9, and two new peaks were observed in the HSQC spectrum of MOL-EU at δ_H/δ_C 55/3.1, 59/3.9 (Fig. 22). Since there was no new through-bond correlation for the methylated lignin, no new peak was observed in the HSQC-TOCSY spectrum of MOL-HP. The multi-bond correlation of MOL-HP was analyzed by the HMBC NMR spectrum. Regarding the protons that had chemical shifts at 3.9, 3.5, 3.3, and 3.1 ppm, several multi-bond C-H correlations of these four protons were observed in HMBC in Fig. 22. HMBC of MOL-HP showed multi-bond C-H correlations for methoxylation of C_α, C_β, C_γ, and aromatic ring. HMBC of MOL-EU only showed multi-bond correlations for methoxylation of C_α, C_β, and C_γ. Hence, the methylation of C_α, C_β, C_γ and aromatic ring of MOL-HP was confirmed by HSQC and HMBC spectrum, while the only methylation of C_α of MOL-EU was confirmed by HSQC and HMBC spectrum. In summary, a comprehensive analysis of NMR spectra showed potential alkylation of C_α, C_β, C_γ, and aromatic ring in methanol, ethanol, and propanol organosolv pretreatment for both poplar and eucalyptus

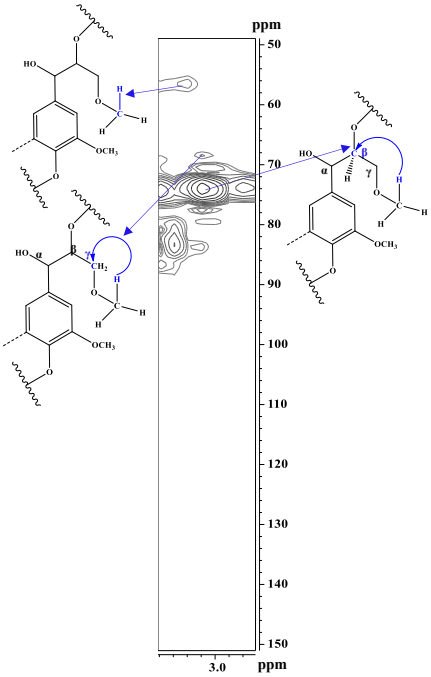
lignins (Fig. 23). The methylation reaction of lignin in organosolv pretreatment was previously indicated based on FTIR spectroscopy and HSQC NMR analysis by Sun etc. as well[116, 189].



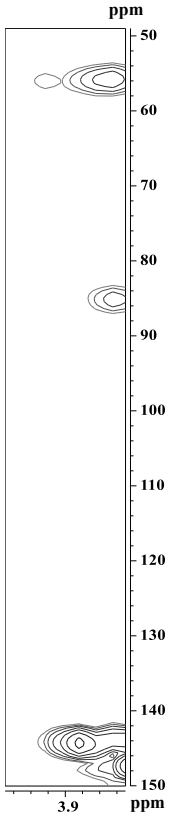
MOL-HP_HMBC_C_β-OCH_3



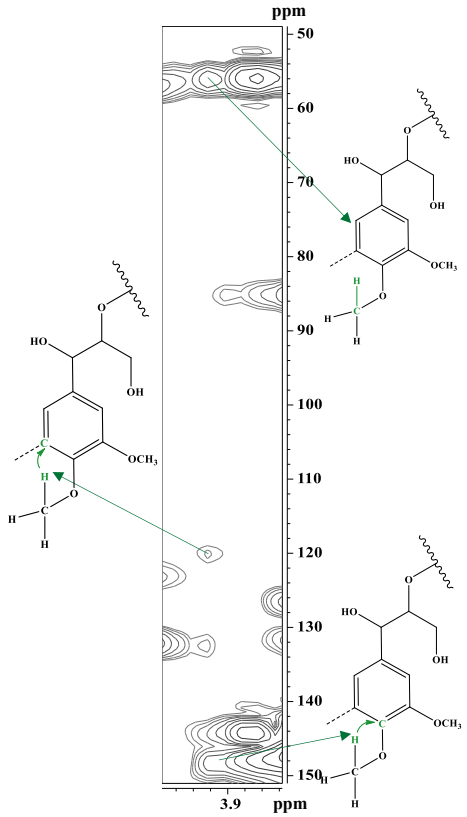
MOL-HP_HMBC_C_γ-OCH_3



MWL-HP_HMBC



MOL-HP_HMBC_C_{AR}-OCH_3



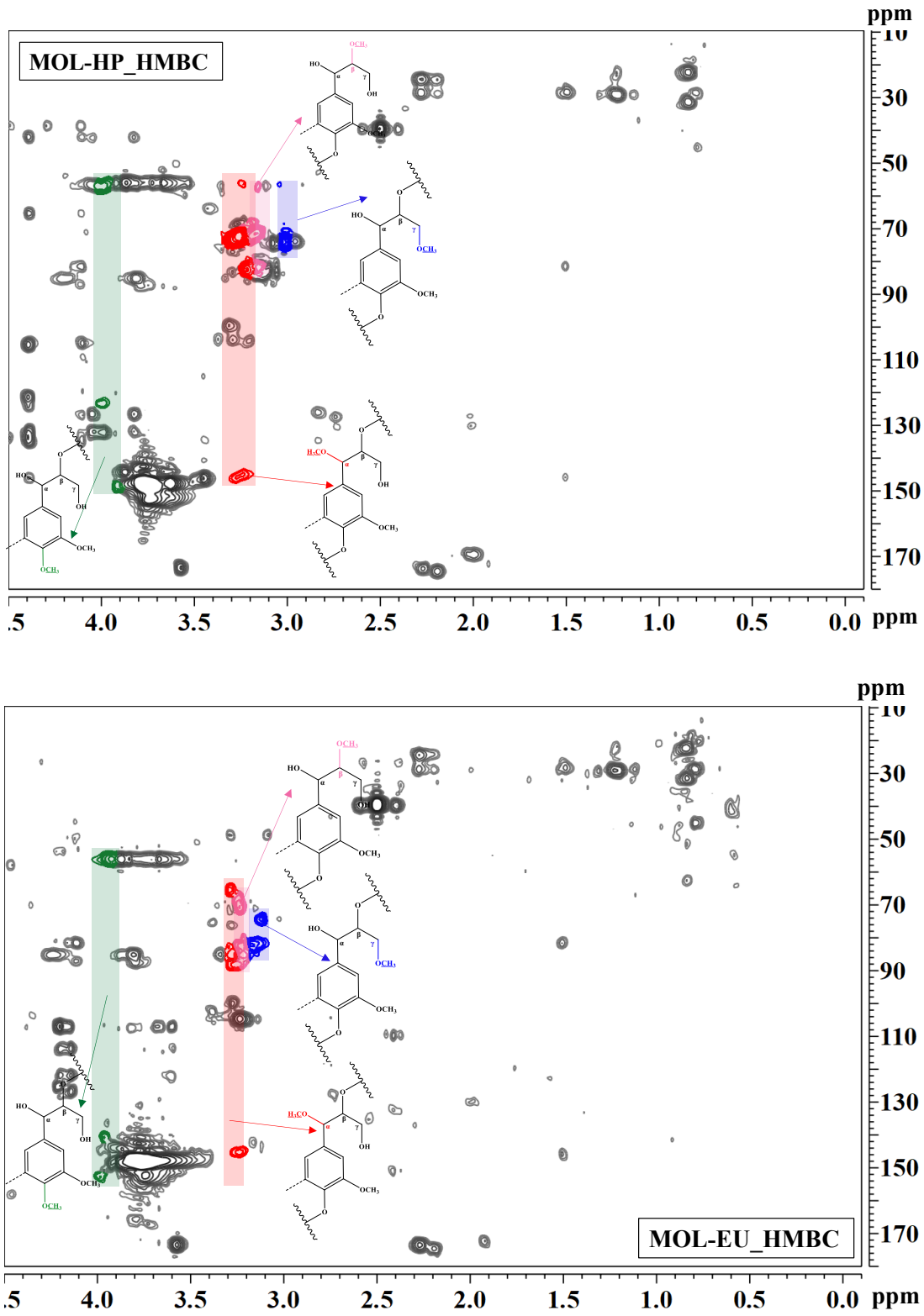


Fig. 22 2D NMR spectra illustrate potential methoxylation of lignin

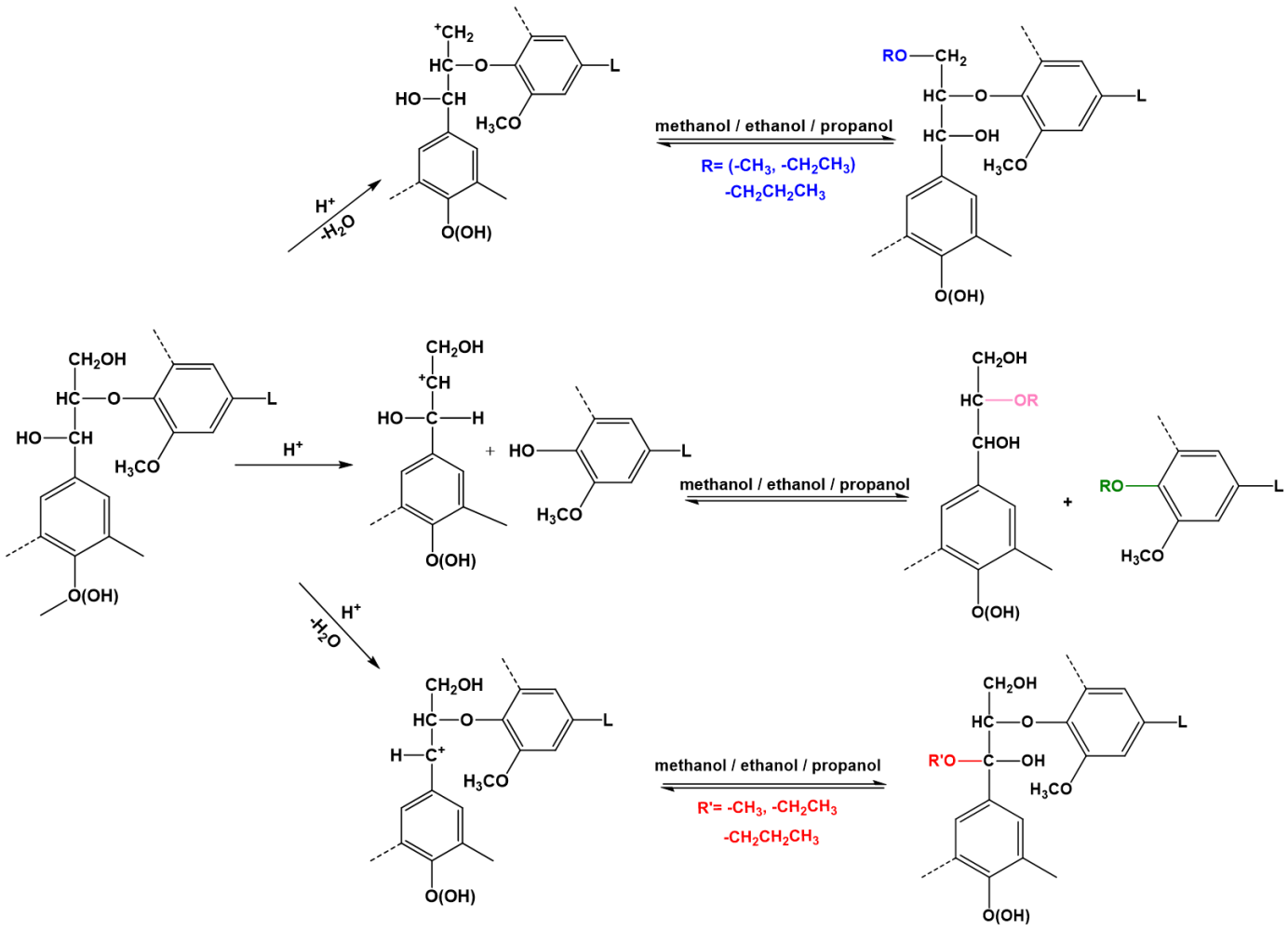


Fig. 23 Potential reactions of lignin in methanol, ethanol and propanol organosolv pretreatment

2.4.5 Quantification of NMR signals of organosolv lignins

The quantification of the signals of quantitative ^{13}C NMR (results expressed per Ar) of MWL-HP, MOL-HP, EOL-HP, POL-HP, MOL-EU, EOL-EU, POL-EU has been summarized in Table 12. Quantification of alkylation of lignins differed in the organosolv lignins. For organosolv lignins from poplar, total alkylation and C_γ alkylation were highest in POL-HP, which were higher than those of EOL-HP; and the lowest alkylation was observed in MOL-HP. As POLs exerted the strongest stimulatory effect on enzymatic hydrolysis of lignocelluloses, the effects of propanol organosolv lignins may be due to the higher degree of alkylation. The underlying mechanism is

possibly related to the suppression of lignin condensation during the pretreatment, as indicated in ethanol organosolv pretreatment[76]. The blocking of the phenolic hydroxyl group was beneficial for reducing hydrogen bonds between lignins and amino acids of cellulase enzyme[144, 200]. This could explain why the propanol organosolv lignins were the most stimulatory lignins among all lignins.

Table 9 The quantitative analysis of the signals of quantitative ^{13}C spectra

δ (ppm)	Assignment	MWL−HP	MOL-HP	EOL-HP	POL-HP	MOL-EU	EOL-EU	POL-EU
132.7-130.8	PB	0.45	0.42	0.37	0.38	0.10	0.12	0.13
155-140	Aromatic C-O	1.84	1.96	2.13	2.02	2.53	2.68	2.54
140-125	Aromatic C-C	1.61	1.70	1.61	1.66	1.34	1.34	1.42
125-102	Aromatic C-H	2.54	2.34	2.28	2.32	2.25	2.10	2.16
90-58	Alk-O-	2.25	1.86	2.00	2.12	1.10	1.27	1.58
77-65	γ-O-Alk, secondary OH	0.85	0.51	0.58	0.80	0.22	0.31	0.38
61.3-58	β-O-4	0.44	0.44	0.38	0.38	0.51	0.48	0.47
58-54	$-OCH_3$	1.63	2.01	1.74	1.78	1.89	2.09	1.97
15.9-15	$-OCH_2\underline{C}H_3$	0	0	0.26	0	0	0.12	0
67.5-64	$-O\underline{C}H_2CH_3$	0	0	0.23	0	0	0.12	0
11-10	$-OCH_2CH_2\underline{C}H_3$	0	0	0	0.48	0	0	0.47
26.1-25	$-OCH_2\underline{C}H_2CH_3$	0	0	0	0.14	0	0	0.27
63-62	$-O\underline{C}H_2CH_2CH_3$	0	0	0	0.18	0	0	0.33

The quantitative analysis of the lignin fraction by integration was summarized in Table 13. The inter-unit linkage of β-aryl-ether (β-O-4, A), resinol (β-β, B), phenylcoumaran (β-5, C) were identified by their cross-peaks at δ_H/δ_C 71.95/4.85 (A_α), 83.03/4.34 ($A_{\beta(G)}$), 85.76/4.14 ($A_{\beta(S)}$), 60.26/3.60 (A_γ), 85.41/4.66 (B_α), 53.87/3.08 (B_β), 71.68/3.81-4.17 (B_γ), 87.50/5.45 (C_α), 53.23/3.47 (C_β) and 63.17/3.66 (C_γ), respectively. HSQC quantitative analysis showed that *p*-hydroxybenzoate (PB) units in POL-HP (13.1%) were highest than that in EOL-HP (12.6%),

MOL-HP (12.5%). As PB was reported to be related to the stimulatory effect of lignins[95], the ability to distinguish the effect of MOL-HP, EOL-HP, and POL-HP on Avicel hydrolysis may be related to the ratio of PB units in their lignin structure. The β-β, β-5 linkages ratio in MOL-HP (19.5%, 38.1%) was higher than those in EOL-HP (17.1%, 29.5%) and POL-HP (16.8%, 26.5%). Higher β-β and the β-5 ratio was reported to be related to higher condensation, more non-specific adsorption binding, and more inhibition on enzymatic hydrolysis[201]. Hence, it can be indicated that the stronger stimulatory effect of organosolv lignin might be due to the suppressed lignin condensation caused by the alkylation.

Table 10 Quantification analysis of the HSQC signals

Lignins	S/G	β-O-4 (%)	β-β (%)	β-5 (%)	PB (%)
MWL-HP	1.02	69.1	15.1	15.8	33.7
MOL-HP	1.74	42.4	19.5	38.1	15.1
EOL-HP	1.81	53.5	17.1	29.5	16.2
POL-HP	1.87	56.3	16.8	26.9	16.4
MOL-EU	2.16	22.8	47.4	29.8	0
EOL-EU	2.05	26.2	46.2	27.7	0
POL-EU	1.62	30.4	45.7	23.9	0

2.4.6 FTIR analysis of lignins

The assignments of FT-IR absorption bands of the lignins were listed in Table 14. Fig. 24 shows the FT-IR spectra of methanol, ethanol and propanol organosolv lignins from poplar and eucalyptus. Absorption due to the C-H stretching of methyl, methylene, or methane group at 2945 cm^{-1} is higher in EOL-HP and POL-HP, which is lower in MOL-HP and lowest in MWL-HP. As regards to the organosolv lignins from eucalyptus, the absorption of C-H stretching of methyl was also lower in MOL-EU than that in EOL-EU and POL-EU. These results indicated the introduction of alkyl groups in the organosolv lignins from both poplar and eucalyptus. A strong peak observed

at 1460 cm^{-1} in spectrum (a) than that in MWL-HP, indicative of more aromatic methyl group vibration in organosolv lignins of poplar. The increase in the signal of C-H stretching of aromatic methyl group vibration might correlate with the alkylation of lignin on aromatic ring with methanol, ethanol and propanol organosolv pretreatment. Obviously, there was a remarkable increase of aromatic C-H syringyl (S) unit, as observed in spectrum (a) at 1115 cm^{-1} and 1125 cm^{-1} in spectrum (b). This correlated with the relative ratio increase of S units in the organosolv lignins after organosolv pretreatment of both poplar and eucalyptus.

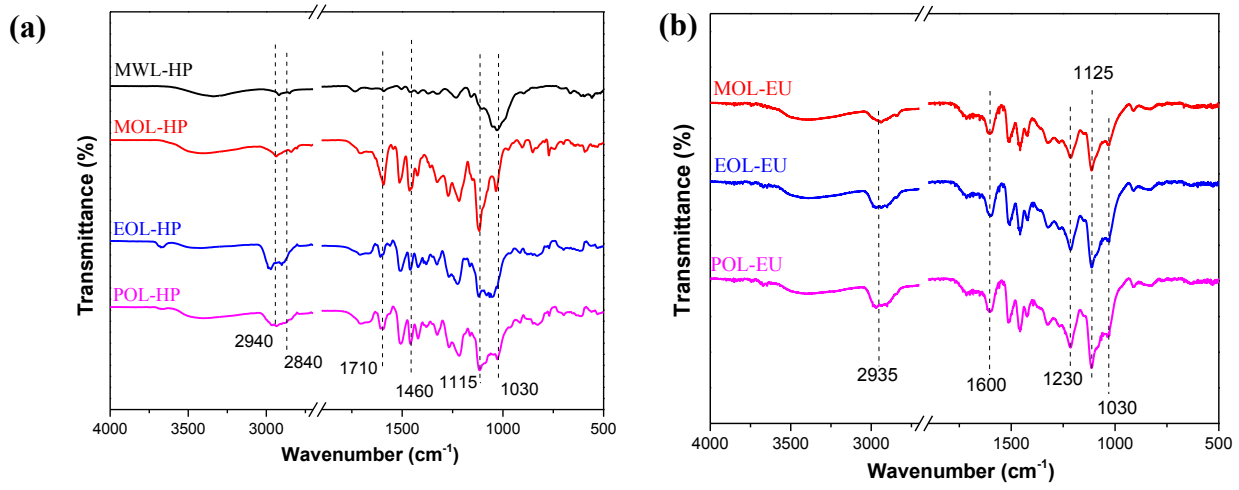


Fig. 24 FTIR analysis of organosolv lignins from (a) poplar and (b) eucalyptus

2.5 Conclusions

The stimulatory effect of organosolv lignins from poplar (POL-HP, EOL-HP, MOL-HP) was observed on enzymatic hydrolysis of Avicel and organosolv pretreated poplar. Among them, POL-HP has the strongest stimulatory effect, probably due to its lower hydrophobicity. On the contrary, organosolv lignins from eucalyptus (POL-EU, EOL-EU, MOL-EU) inhibited the enzymatic hydrolysis of Avicel and had much higher hydrophobicity than that of organosolv lignins from poplar. Both stimulatory effect and inhibitory effect of organosolv lignins could be

exaggerated with a higher lignin concentration. The spectra of HSQC, HSQC-TOCSY, and HMBC revealed that the alkylation (e.g., methylation, ethylation, and propylation) of hydroxyl groups took place not only at C_α, but also at C_β and C_γ, and potentially also at the phenolic hydroxyl group of lignin. The stronger stimulatory effect of organosolv lignin can be explained by repressing lignin condensation caused by a higher level of alkylation.

Chapter 3: Effect of organosolv lignins from herbaceous kenaf on enzymatic hydrolysis of Avicel

3.1 Abstract

Biomass biofuel is an attractive alternative to transportation fuels from non-renewable resources. Various pretreatment methods have been developed to overcome the recalcitrance of lignocelluloses and get highly digestible substrates for enzymatic hydrolysis. In this work, ethanol organosolv pretreatment was introduced to herbaceous kenaf, and the effect of ethanol organosolv lignins on the enzymatic hydrolysis has been assessed. The ethanol organosolv kenaf lignins could improve the yield of the enzymatic hydrolysis of Avicel. The inhibition of isolated lignins from ethanol organosolv pretreatment, dilute acid pretreatment, and untreated kenaf on the enzymatic hydrolysis of Avicel was shown. The lignin hydrophobicity could explain the stimulatory effect of EOL lignin from kenaf. In addition, the spectra of HSQC, HSQC-TOCSY, and HMBC revealed that the alkylation (e.g., methylation, ethylation, and propylation) of hydroxyl groups took place not only at C_α, but also at C_β and C_γ, and potentially also at the phenolic hydroxyl group of lignin.

3.2 Introduction

The resources of biomass include forestry residues, industrial wastes, municipal solid waste, agriculture residues, and dedicated energy solid[202]. Forestry residues like kenaf are among the most cheap and rich renewable biomass resources for biofuel production[203]. Kenaf core has received considerable attention as a renewable resource because of its fast growth and low lignin content[204]. It also attracts attention because of its capacity for metal and CO_2 adsorption[205]. However, the current utilization of kenaf is limited. One main reason is the lack of economic and

efficient pretreatment technology to deconstruct these lignocellulosic materials[206]. To enhance the bioconversion efficiency, various pretreatment methods, including water, acid, and alkaline pretreatment of kenaf, have been reported to improve hydrolyzability[207]. Ethanol was widely applied in the organosolv pretreatment because it is one of the major biomass refinery products and its low toxicity, low cost, and proper boiling point to recover. The ethanol organosolv pretreatment mechanism was also reported to be the alkylation of α-hydroxyl groups[76]. So it is meaningful to determine the effect of ethanol organosolv lignin from kenaf on enzymatic hydrolysis of Avicel and investigate the reaction mechanism of kenaf lignin in ethanol organosolv pretreatment. Determination of physical properties, including hydrophobicity, ^{13}C, and 2D-HSQC NMR spectra of lignins ethanol organosolv lignins chemical structures about lignin units, functional groups, condensation levels, and their linkages could be conducted to explain its effect on enzymatic hydrolysis[131, 192].

In this chapter, the effect of ethanol organosolv lignins from kenaf was investigated on enzymatic hydrolysis of Avicel comparing to that on mill wood lignin from untreated kenaf. Ethanol pretreated kenaf and diluted acid kenaf. The hydrophobicity and FTIR of organosolv lignins and isolated lignins from kenaf have been assessed. Furthermore, comparing to the mill wood lignin from untreated kenaf, ethanol pretreated kenaf, and diluted acid kenaf, potential alkylation reactions of kenaf lignin that were analyzed by 1D-NMR including ^{1}H and ^{13}C spectra and 2D-NMR HSQC, HSQC-TOCSY, and HMBC spectra. Signals of ^{13}C and 2D-HSQC NMR spectra of kenaf lignins were analyzed for the chemical structure change during the organosolv pretreatment.

3.3 Materials and methods

3.3.1 Cellulase enzymes and biomass

Kenaf (*Hibiscus cannabinus*) was obtained from INL Bioenergy Feedstock Library. The information of chemical reagents including ethanol, sulfuric acid, sodium hydroxide for organosolv pretreatment, the Avicel, and cellulase enzymes for the enzymatic hydrolysis, Bengal Rose for lignin hydrophobicity, 99.9 atom % D DMSO-d_6 for NMR analysis was mentioned in section 2.2.1. Coomassie Protein Assay kit for enzyme distribution was obtained from Thermo Scientific.

3.3.2 Dilute acid and organosolv pretreatment

The dilute acid pretreatment of kenaf (50 g, dry weight) was soaked in 1000 mL 1 % (w/w, sulfuric acid/dry biomass) of sulfuric acid overnight before the hydrothermal process of dilute acid pretreatment. The mixture was transferred into a 2-L stainless steel Par batch reactor with a stirrer and a PID controller and heated to 170°C. After 1 h hydrothermal cooking, the reactor was water quenched to room temperature to terminate the reaction. The solid and the liquid fraction were separated from the reacted slurry by vacuum filtration using Whatman No.1 filter paper. The collected solids were homogenized with 60°C water in a blender for 30 s, and then the liquor was filtrated. The water washing steps were repeated for 3 times and kept for further enzymatic hydrolysis.

The kenaf (80 g, dry weight) were soaked in 1600 mL 75% (v/v) ethanol/water solution with 1 % (w/w, sulfuric acid/dry biomass) of sulfuric acid overnight before the organosolv pretreatment. The mixture was transferred into a 2-L stainless steel Par batch reactor with a stirrer and a PID controller and heated to 170°C. After 1 h pretreatment, the reactor was quenched to room temperature by tap water. The solid and the liquid fraction were separated from the reacted

slurry by vacuum filtration. The spent liquor (liquid part) was kept for organosolv lignin precipitation. Organosolv lignins from ethanol pretreated kenaf were designated as EOL-KE. The pretreated substrates (solid part) were washed and kept for enzymatic hydrolysis and analysis. Ethanol pretreated kenaf and dilute acid pretreated kenaf after wash were designated as EPKE and DAKE.

3.3.3 Enzymatic hydrolysis of lignocelluloses

Enzymatic hydrolysis of Avicel (98.72% glucan) and biomass (untreated kenaf, EPKE, DAKE) were carried out in 250 mL Erlenmeyer flasks with stoppers at 50 ℃, 150 rpm in 50 mM sodium citrate buffer (pH 4.8) with 2% glucan (w/v) for 72 h. The enzyme loading for all hydrolysis was chosen to be 5 FPU/g glucan. To study the effect of isolated lignins from kenaf on enzymatic hydrolysis, 4 g/L of isolated lignins were added to the enzymatic hydrolysis 1 h before the addition of cellulase at room temperature. The concentration of glucose in the supernatant was determined by HPLC. All experiments were carried out in duplicates.

3.3.4 HPLC analysis and FTIR analysis

The concentration of glucose from enzymatic hydrolysis was analyzed by an HPLC system (Agilent 1260 Infinity) with a Hi-Plex H guard column (5 × 3 mm, Agilent), a Hi-Plex H column (300 × 7.8 mm, Agilent), and a RID detector. 5 mM H_2SO_4 in aqueous solution was used as mobile phase with a flow rate of 0.6 $mL \cdot min^{-1}$. The detector and oven temperatures were both set to be 45°C. The FTIR spectrometer (Perkin Elmer Spectrum Two FTIR system) was employed to examine the changes of chemical structures of the organosolv pretreated lignin compared to the

mill wood lignin. ATR technique was used for FTIR sampling. The scanning wavenumber was set from 500 to 4000 cm^{-1}, and the interval was 0.50 cm^{-1}.

3.3.5 Determination of hydrophobicity of lignins

Increasing concentrations of the lignins (0.2-4.0 g/L) were mixed with 40 mg/L Rose Bengal in citrate buffer (50 mM, pH 4.8) and incubated at 50 ℃, 150 rpm for 2 h. The free Rose Bengal dye in the supernatant and the lignins were separated by centrifugation of 10000 rpm, 5 min. The free dye concentration was determined by UV-Vis spectrometer at 543 nm, showing the difference between the initial dye to calculate the adsorbed dye on the lignins. The calculation of hydrophobicity of lignins was determined as section 2.2.5.

3.3.6 Mill wood lignin isolation from kenaf

Grounded untreated kenaf or pretreated kenaf powder was extracted with reflux ethanol for at least 6 h to remove the extractives. The air-dried wood powder was ball-milled with 1 min pulse every 2 min milling for 24 h. 20 g ball-milled poplar was extracted with 400 ml 96% (v/v) 1,4-dioxane-water solution at 25 °C for 24 h without exposure to light. After 24 h, solid was filtered and washed with dioxane solution for 3 times. The liquid part was concentrated to around 50 mL with a rotary evaporation. The concentrated solution was added into 10-fold volume of acidic water (pH 2.0), and the mill wood lignins was precipitated. After washing, mill wood lignin from untreated kenaf (MWL-KE), dilute acid pretreated kenaf (MWL-AK), ethanol pretreated kenaf (MWL-EK) were freeze-dried and kept in a desiccator for further hydrolysis addition and characterization.

3.3.7 NMR spectroscopic analysis of lignins

NMR spectroscopy analysis of all organosolv from poplar and eucalyptus was conducted on a Bruker Avance III HD Ascend 700 MHz spectrometer. 60 mg of freeze-dried lignin sample was dissolved in 0.5 ml of DMSO-d_6 to collect 1D ^{1}H and quantitative ^{13}C spectra, and 2D HSQC, HSQC-TOCSY, HMBC spectra. 10.5 μs pulse angle, 1.95 s acquisition time, 2 s relaxation delay with a total of 32 scans per sample was conducted as the operating condition for ^{1}H spectra. For quantitative ^{13}C spectra, 9.85 μs pulse angle, 0.5 s acquisition time, 18 s relaxation delay with a total of 768 scans per sample was conducted as the operating condition. The ^{1}H dimension (F_2) was acquired from 12 to 0 ppm with 1024 complex points; the ^{13}C dimension (F_1) was obtained from 160 to 0 ppm with 200 complex points and 100 increments. The acquisition time for HSQC is 60.8 ms for ^{1}H, and 3.55 ms for ^{13}C was applied. The total acquisition time was 2-4 h. For HMBC spectra. The ^{1}H dimension (F_2) was acquired from 12 to 0 ppm with 2048 complex points; the ^{13}C dimension (F_1) was obtained from 160 to 0 ppm with 128 complex points. Acquisition time of 72.6 ms for ^{1}H and 1.51 ms for ^{13}C was applied.

3.4 Results and discussion

3.4.1 Effect of ethanol organosolv lignin from kenaf on enzymatic hydrolysis of Avicel

The effect of ethanol organosolv lignins (EOL-KE) on enzymatic hydrolysis was compared on the enzymatic hydrolysis of Avicel with the addition of mill wood lignins from untreated kenaf (MWL-KE), ethanol pretreated kenaf (MWL-EK), and dilute acid pretreated kenaf (MWL-AK) (Fig. 25). Ethanol organosolv lignin from kenaf increased the 72 h final yield of Avicel from 54.41% to 63.10% by 8.69%, while the other isolated lignins from kenaf inhibited the final yield in different levels. A similar promotional effect was observed with isolated lignin addition in

enzymatic hydrolysis with alkali lignins from rice straw[208, 209]. MWL-AK significantly decreased the hydrolysis yield to 39.48% by 14.95%. This correlates with the high inhibition of dilute acid lignin from poplar on enzymatic hydrolysis[210]. MWL-EK and MWL-KE decreased the final yield of Avicel by 2.26% and 1.95% to 52.14% and 52.46%. Residual lignin extracted from ethanol pretreated substrates of Loblolly pine was more condensed than the ethanol organosolv lignin of pine[71]. This could be the reason of the distinguishing effect of MWL-EK than EOL-KE. The digestibility of ethanol pretreated kenaf (9.61%) and dilute acid pretreated kenaf (32.44%) was showed in Fig. 26. The final yield was of ethanol organosolv pretreated kenaf was only around 10%. This low digestibility of the organosolv pretreated substrates could be related to the oven-dry method of the materials[195].

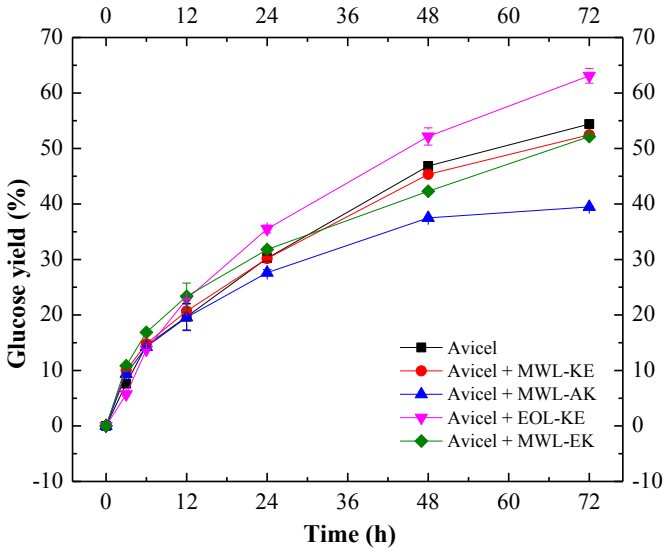


Fig. 25 Effect of isolated lignins from kenaf on enzymatic hydrolysis of Avicel

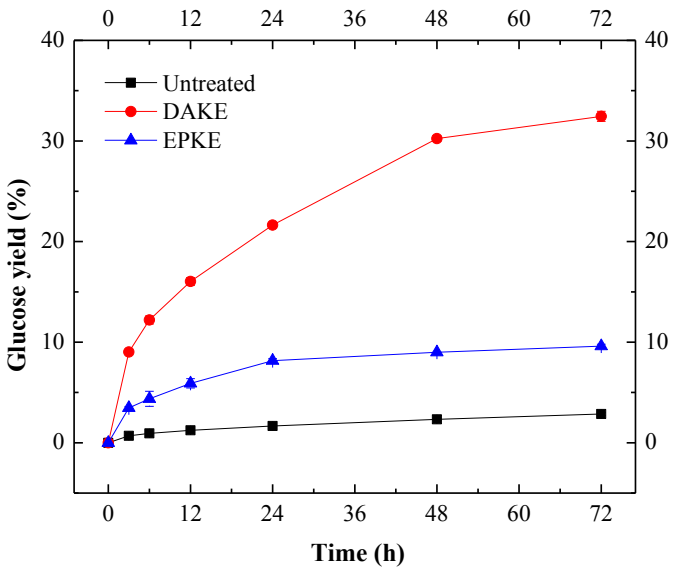


Fig. 26 Enzymatic hydrolysis of untreated kenaf, ethanol organosolv pretreated kenaf and dilute acid pretreated kenaf

The concentration of free cellulase enzyme were tested during the final glucose yield of Avicel (Fig. 27). The results showed that the addition of EOL–KE increased the free cellulase enzymes in solution, but MWL-AK, MWL-EK, and MWL-KE reduced the free cellulase enzymes significantly in solution. The addition of EOL–EOK increased the free enzyme percentage from 63.78% (Avicel at 72 h) to 75.57% at 72 h for the enzymatic hydrolysis of Avicel. The increase of free cellulase enzymes by 11.79% could be the potential reason for the positive effects of ethanol organosolv lignins from kenaf. On the contrary, the addition of MWL-AK, MWL-EK, and MWL-KE decreased the free enzymes from 63.78% to 30.29%, 50.48%, and 53.64% at 72 h, respectively. This indicated that the positive effects of EOL-EOK possibly decrease the non-specific adsorption binding between enzyme and cellulose, which increased final yield. Simultaneously, the negative effects of MWL-AK, MWL-EK, and MWL-KE reduced the free cellulase enzymes due to the increase of the non-specific adsorption binding between enzyme and isolated lignins from untreated and pretreated kenaf substrates.

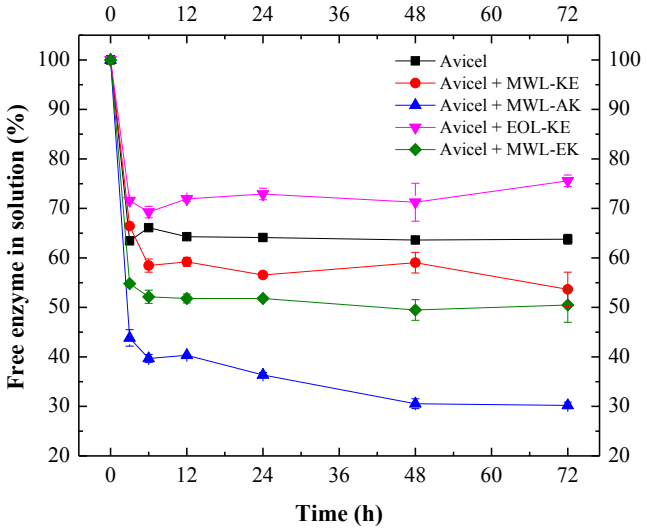


Fig. 27 Effect of isolated lignins from kenaf on enzyme distribution during enzymatic hydrolysis of Avicel

3.4.2 Hydrophobicity of isolated lignins from kenaf

Lignin surface hydrophobicity was reported to be one most significant factors influencing the effect of lignins on enzymatic hydrolysis because of the hydrophobic interactions between lignins and aromatic and methoxy regions of cellulase enzymes[121]. The hydrophobicity of the EOL-KE and MWL-EK from kenaf were 1.25 and 1.96 L/g, respectively (Table 15). However, the hydrophobicity of MWL-AK (5.46 L/g) and MWL-KE (3.89 L/g) was much higher than that of EOL-KE and MWL-EK. So the hydrophobicity may be only a good indicator for lignin inhibition within the same type of lignocellulose and similar pretreatment methods.

Table 11 Hydrophobicity of isolated lignins from kenaf

Lignin	Hydrophobicity (L/g)
EOL-KE	1.25
MWL−KE	3.89
MWL-EK	1.96
MWL-AK	5.46

3.4.3 FTIR analysis of isolated lignins from kenaf

[4] The assignment of FTIR absorption bands was summarized in Table 16. Fig. 29 illustrates the FT-IR spectra of ethanol organosolv lignins from kenaf comparing to other isolated lignins from kenaf. As shown in the figure, the absorption of CH stretching of methyl, methylene, or methane group at 2945 cm^{-1} and the absorption of C=O stretch in unconjugated ketone and a carboxyl group at 1732 cm^{-1} are both higher in the mill wood lignin than the lignin after pretreatment. Obviously, there was a remarkable increase of aromatic C-H syringyl (S) units observed in the spectrum at 1135 cm^{-1}.

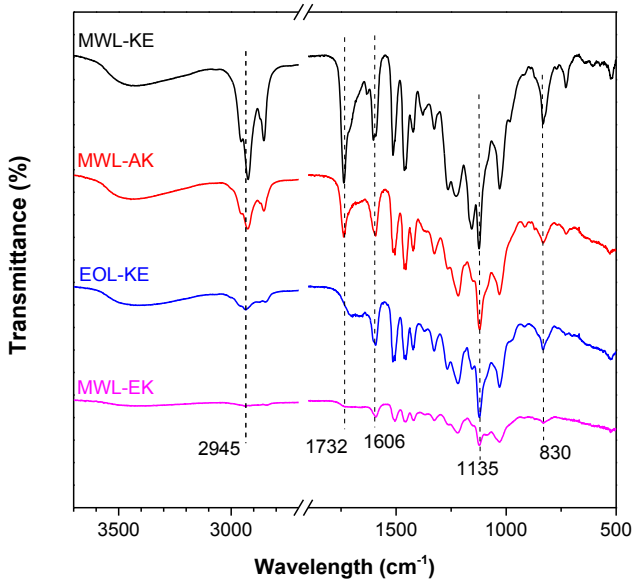
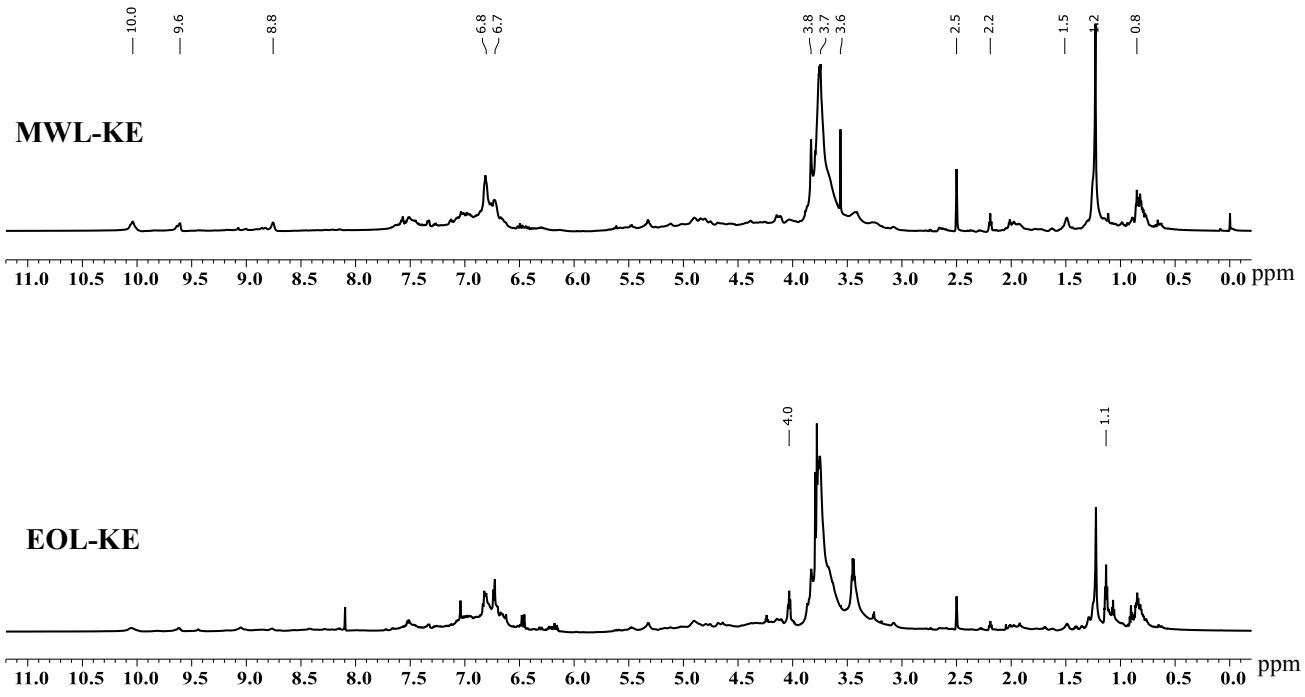


Fig. 28 FTIR analysis of lignins from kenaf

3.4.4 NMR analysis of isolated lignins from kenaf

Compared to the spectrum of MWL-KE, ^{1}H spectra showed protons in a methyl peak (-$OC\underline{H}_2CH_3$) at 4.0 ppm and a corresponding methylene group (-$OCH_2C\underline{H}_3$) at 1.1 ppm in EOL-KE (Fig. 29). ^{13}C spectra showed a methyl peak (-OCH_2CH_3) at 15.2 ppm and a corresponding

methylene group (-OCH_2CH_3) at 64.7 ppm in EOL-KE (Fig. 30). Similarly, a new peak was observed in HSQC correlation with a methylene group at δ_H/δ_C 63.79/3.34 ppm (Fig. 31). So it is possible that an ethoxy group was introduced to C_α through etherification of the alcohol on the benzene ring as they analyzed the TOSY-HSQC NMR spectrum of EOL from *Miscanthus giganteus*[74]. In NMR spectra of ^{13}C and HSQC of MWL-EK, the same new peaks were also investigated. This correlated to the previous report from ethanol organosolv lignin, and residual lignin from ethanol pretreated Loblolly pine[71].



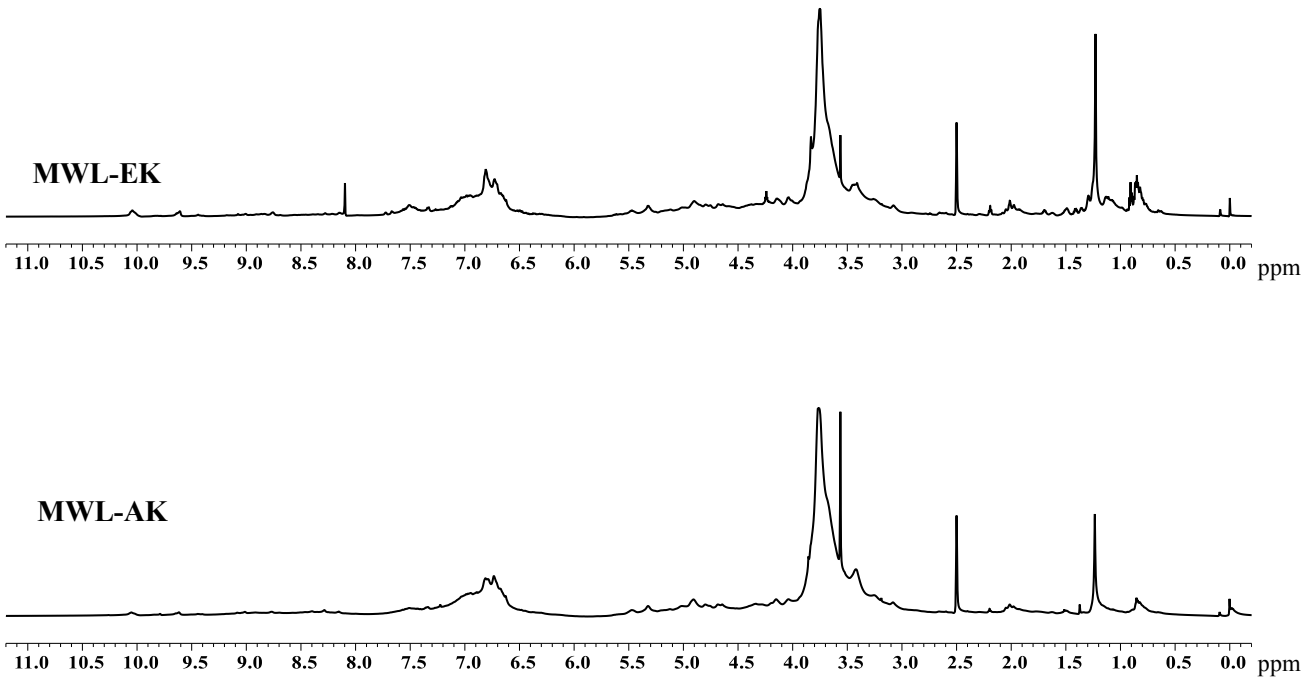
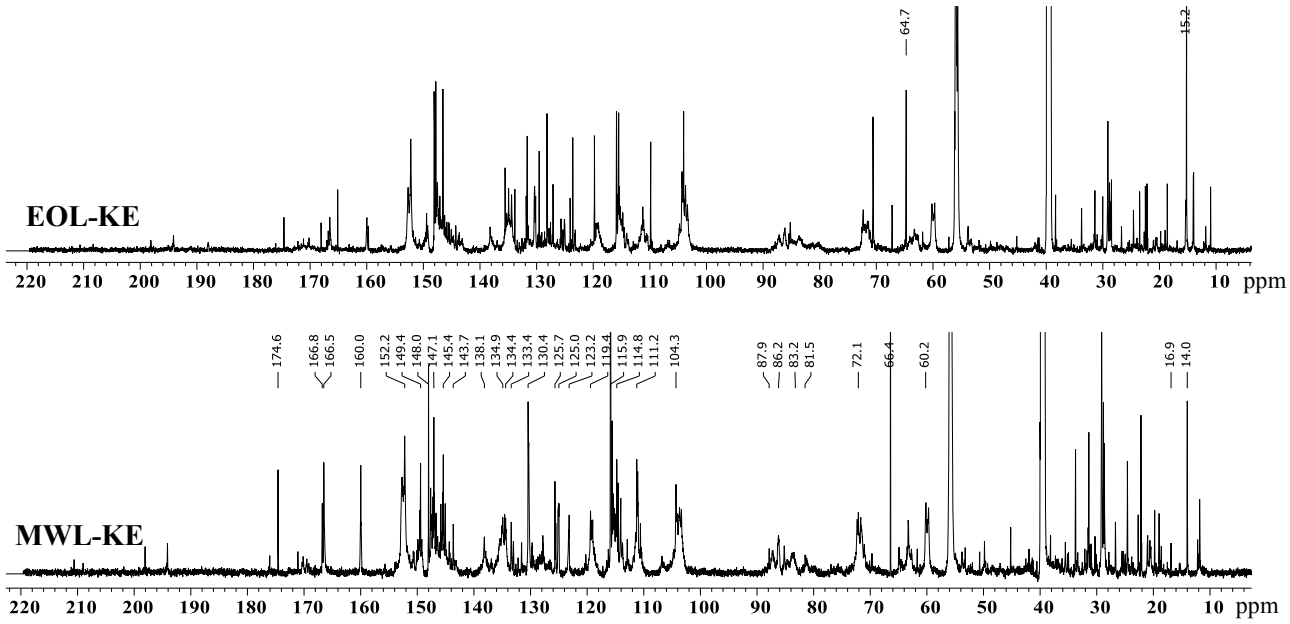


Fig. 29 [1]H spectra of isolated lignins from kenaf



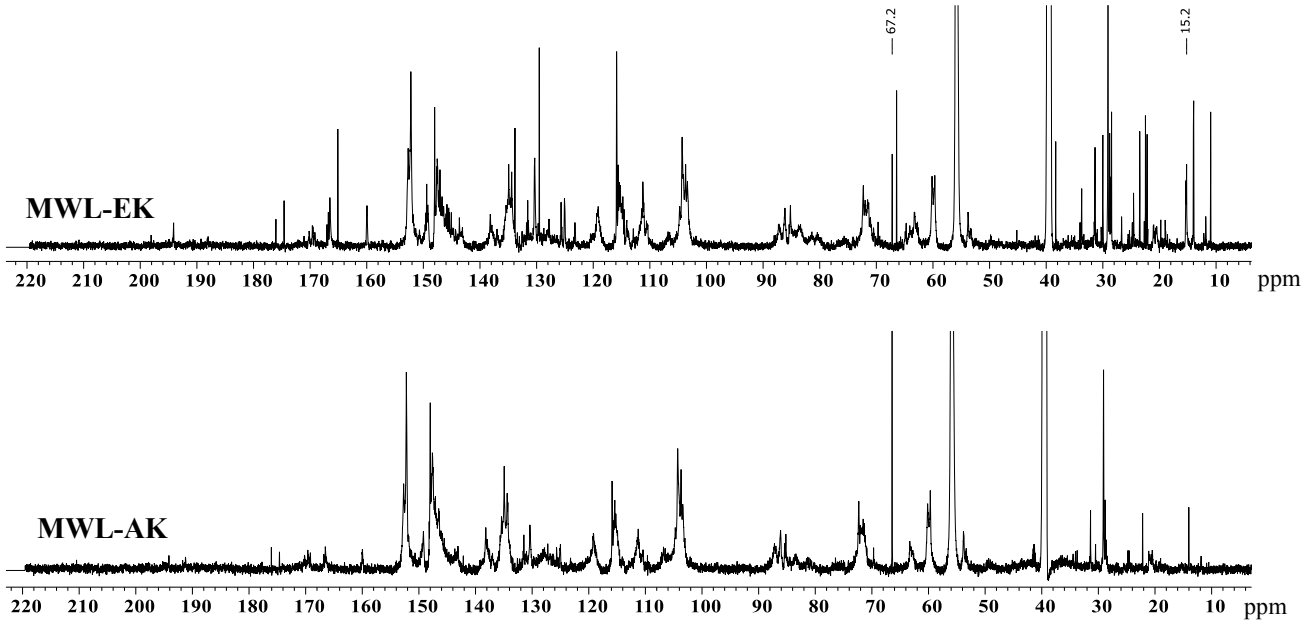
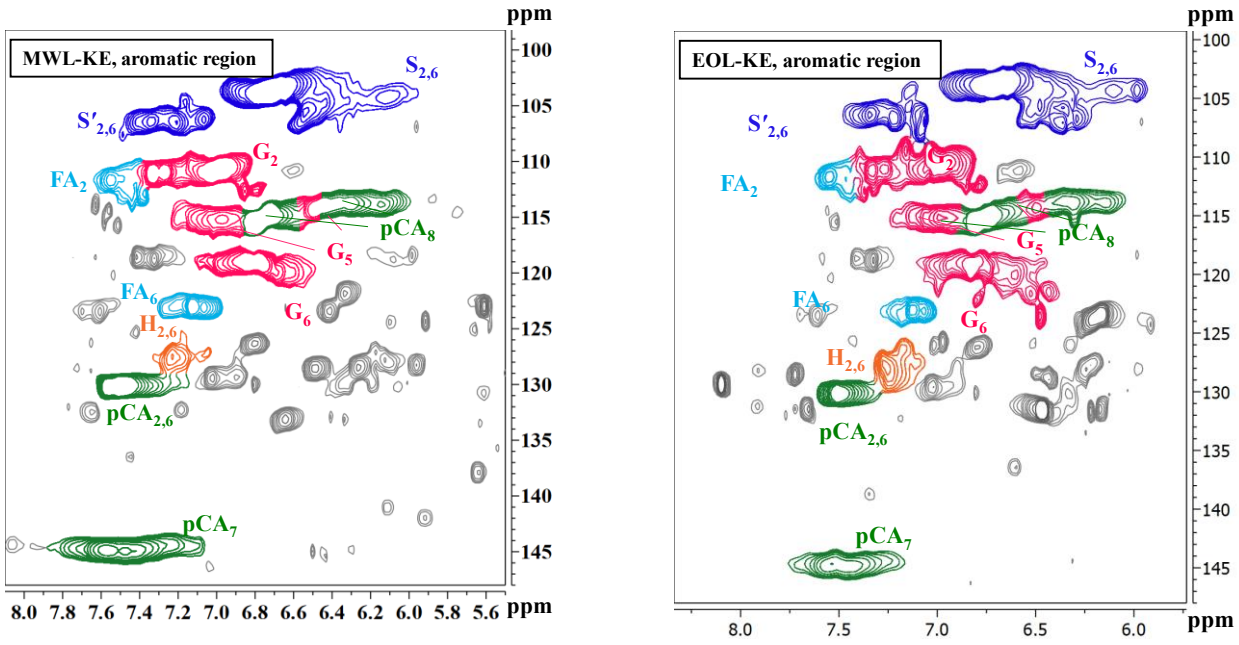
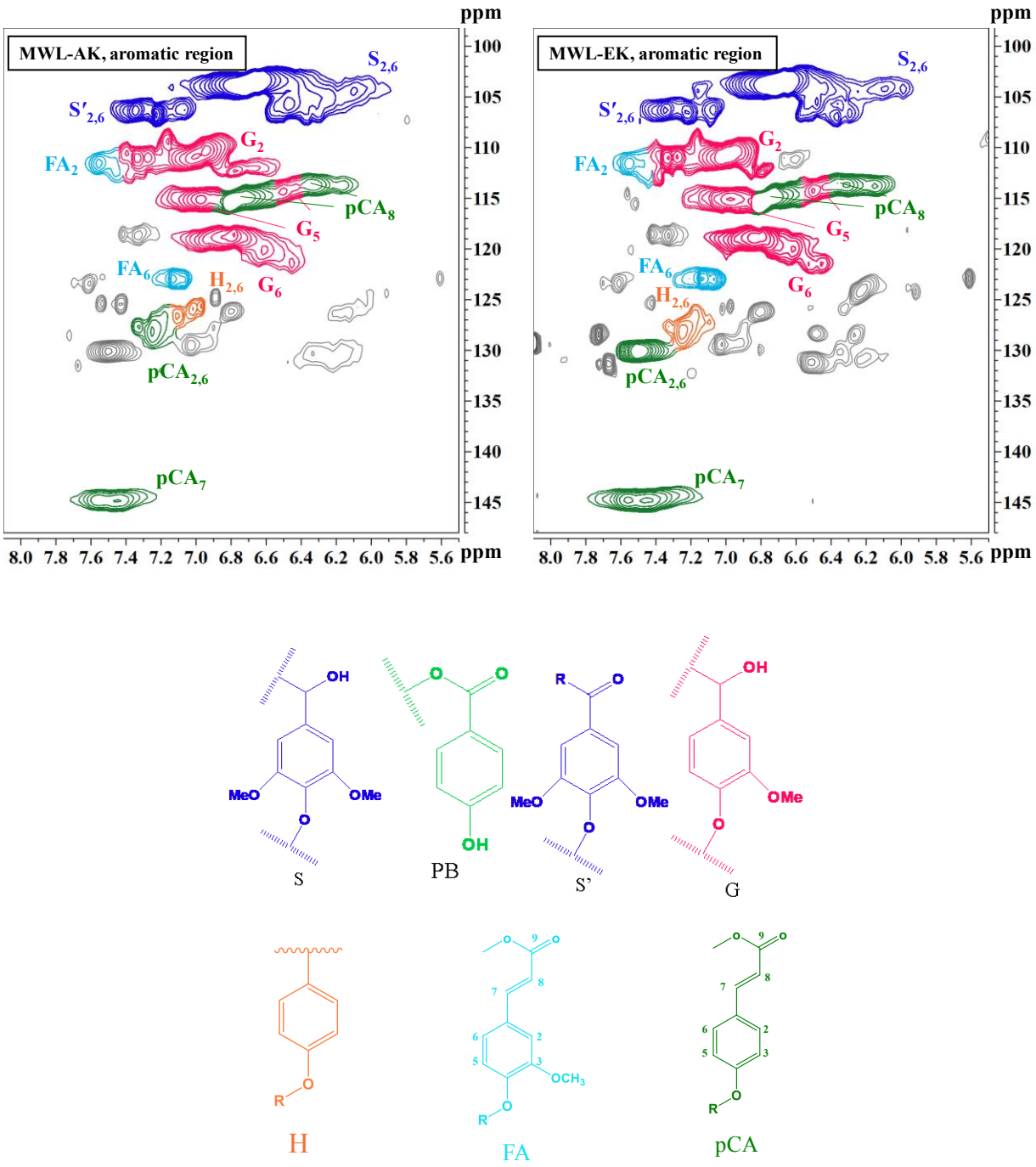


Fig. 30 Quantitative ^{13}C spectra of isolated lignins from kenaf

Similar spectra were observed for MWL-KE, EOL-KE, MWL-EK, and MWL-AK in the aromatic region of HSQC NMR spectra (Fig. 31). This indicates that the ethylation reaction had a limited effect on the NMR spectra of the aromatic region. On the side-chain region, the inter-unit linkage of β-aryl-ether (β-O-4, A), resinol (β-β, B), phenylcoumaran (β-5, C) were identified by their cross-peaks at δH/δC 71.95/4.85 (A_α), 83.03/4.34 ($A_{\beta(G)}$), 85.76/4.14 ($A_{\beta(S)}$), 60.26/3.60 (A_γ), 85.41/4.66 (B_α), 53.87/3.08 (B_β), 71.68/3.81-4.17 (B_γ), 87.50/5.45 (C_α), 53.23/3.47 (C_β) and 63.17/3.66 (C_γ), respectively. Three new peaks were investigated at δ_H/δ_C 65.1/3.55, 62.2/3.45, and 66.5/4.05 in HSQC NMR spectra in Fig. 32. These three peaks indicated with the introduction of the ethoxy group at C_α, $C_{\beta/\gamma,}$ and benzene ring alcohol in β-aryl-ether structure. As shown in Fig. 31, the peak of ethoxy group (δ_C/δ_H: 65.1/3.55) at C_α could correlate with δ_C/δ_H 66.3/1.0 of multi-bond correlation of proton in methyl group and carbon in methylene group (-O$\underline{C}H_2C\underline{H}_3$); δ_C/δ_H 15.1/3.55 of multi-bond correlation of proton in methylene group and carbon in methyl group (-OC$\underline{H}_2\underline{C}H_3$) in HSQC-TOCSY spectrum. The peak of ethoxy group (δ_C/δ_H: 62.2/3.45) at Cβ/γ could

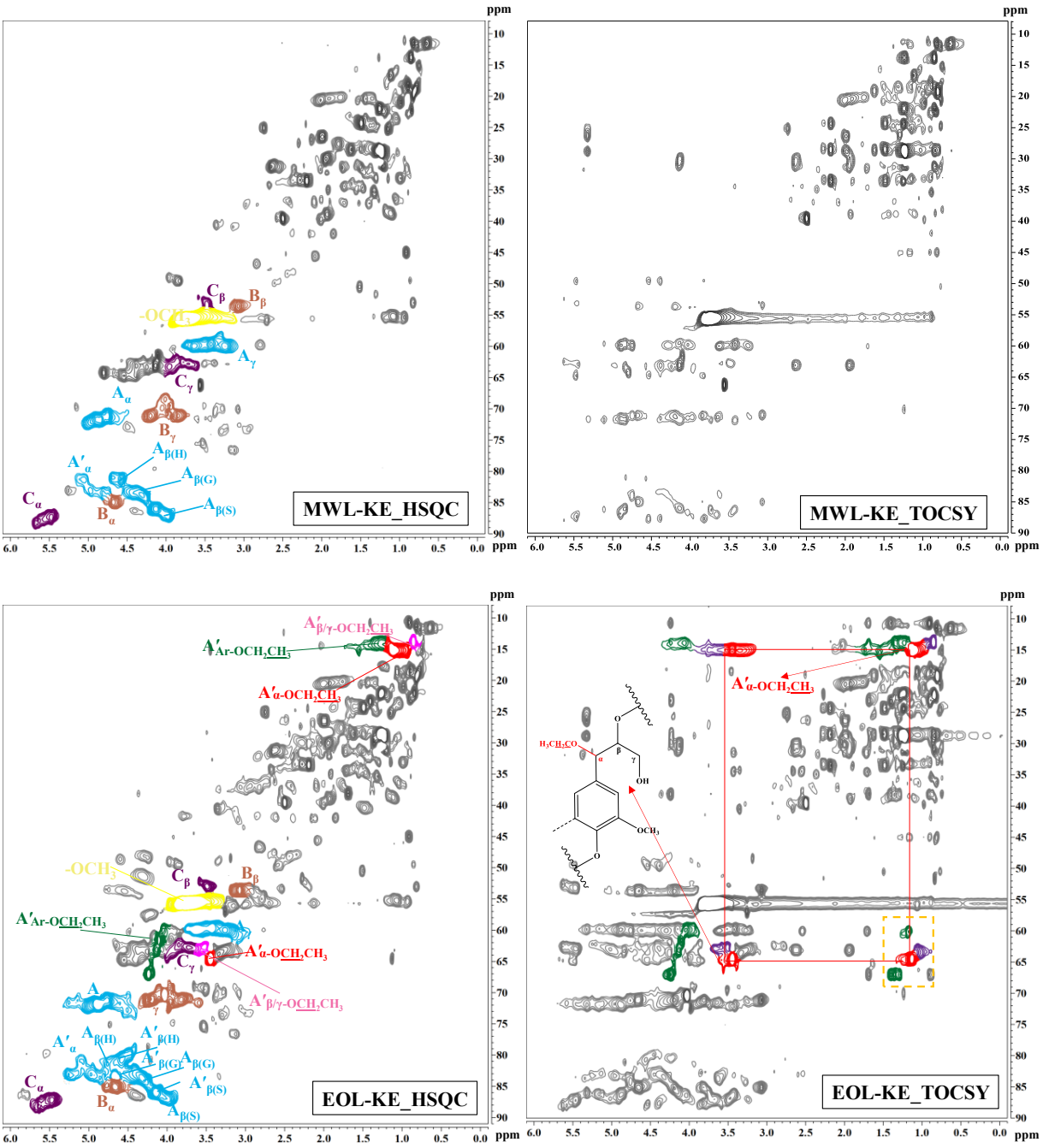
correlate with δ_C/δ_H 62.2/1.0 of multi-bond correlation of proton in methyl group and carbon in methylene group (-O<u>C</u>H$_2$C<u>H</u>$_3$); δH/δC 15.1/3.45 of multi-bond correlation of proton in methylene group and carbon in methyl group (-OC<u>H</u>$_2$<u>C</u>H$_3$) in HSQC-TOCSY spectrum. Peak of ethoxy group (δ_C/δ_H: 66.5/4.05) at benzene ring alcohol could correlate with δ_C/δ_H 66.5/1.0 of multi-bond correlation of proton in methyl group and carbon in methylene group (-O<u>C</u>H$_2$C<u>H</u>$_3$); δH/δC 15.1/4.05 of multi-bond correlation of proton in methylene group and carbon in methyl group (-OC<u>H</u>$_2$<u>C</u>H$_3$) in HSQC-TOCSY spectrum. For protons with chemical shifts at 4.05, 3.55, 3.45 ppm, three-bond correlations of three nearby carbon were observed in HMBC in Fig. 32. This confirmed the introduction of ethoxy groups on C_α, $C_{\beta/\gamma}$, and benzene ring alcohol. Similar results of new peak introduction with analysis HSQC, HSQC-TOCSY, and HMBC together were shown by Bauer[74].

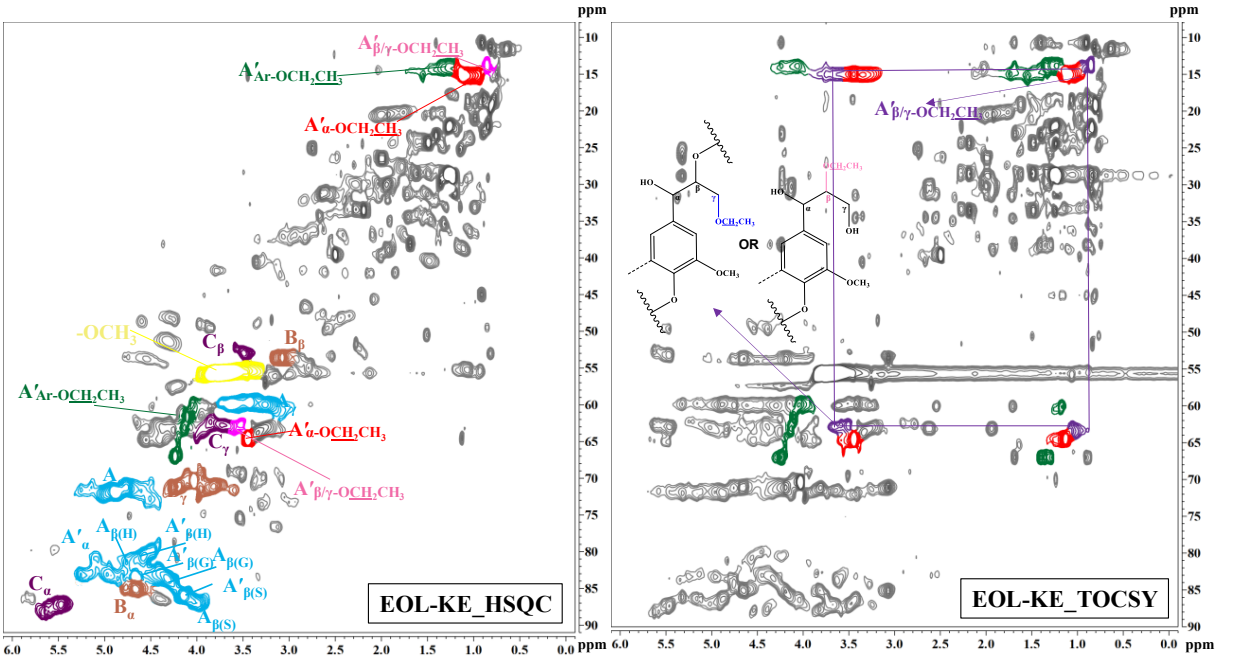


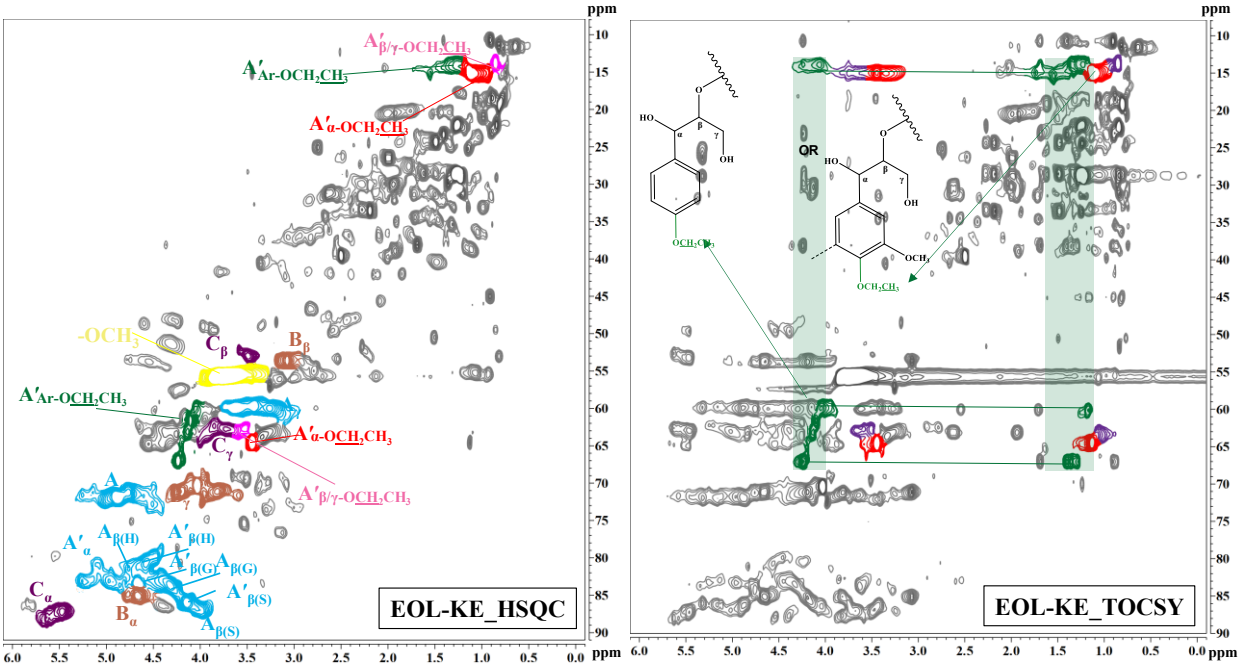


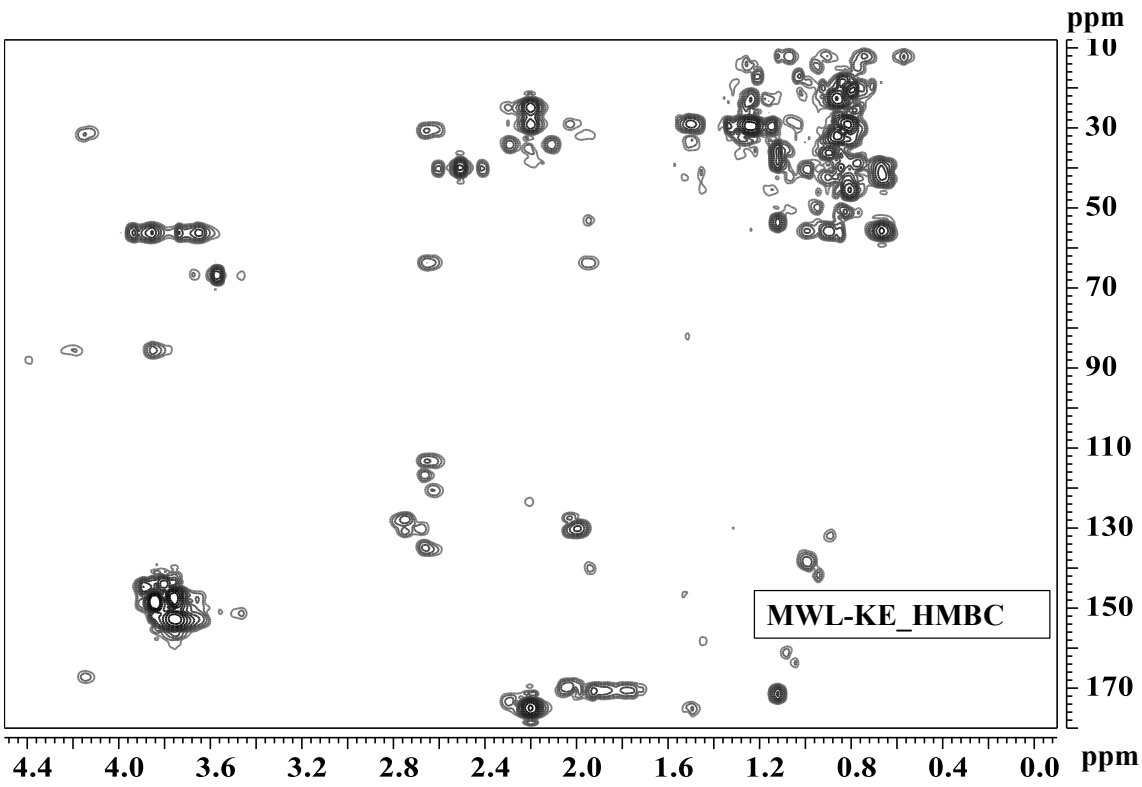
(G: guaiacyl units; S: syringyl units; S': oxidized syringyl units bearing a carbonyl at C_α; PB: *p*-hydroxybenzoate units; H: *p*-Hydroxyphenly, pCA: *p*-Coumarate; FA: Ferulate;)

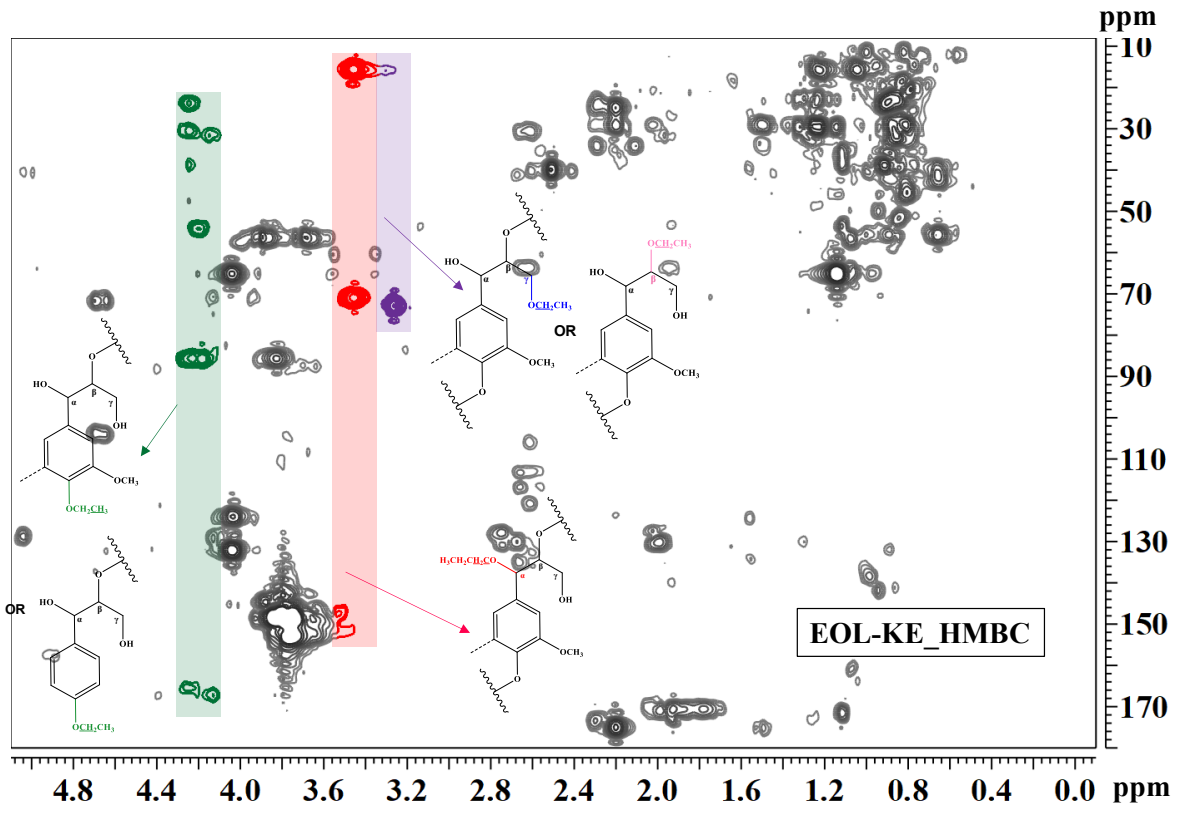
Fig. 31 The signals of aromatic region of 2D HSQC of lignins from kenaf


MWL-KE_HSQC
MWL-KE_TOCSY
EOL-KE_HSQC
EOL-KE_TOCSY
ppm


EOL-KE_HSQC
EOL-KE_TOCSY
OR
ppm


EOL-KE_HSQC
EOL-KE_TOCSY
QR
ppm


ppm
10
30
50
70
90
110
130
150
170
MWL-KE_HMBC
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
ppm


ppm
10
30
50
70
90
110
130
150
170
OR
OR
EOL-KE_HMBC
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
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ppm

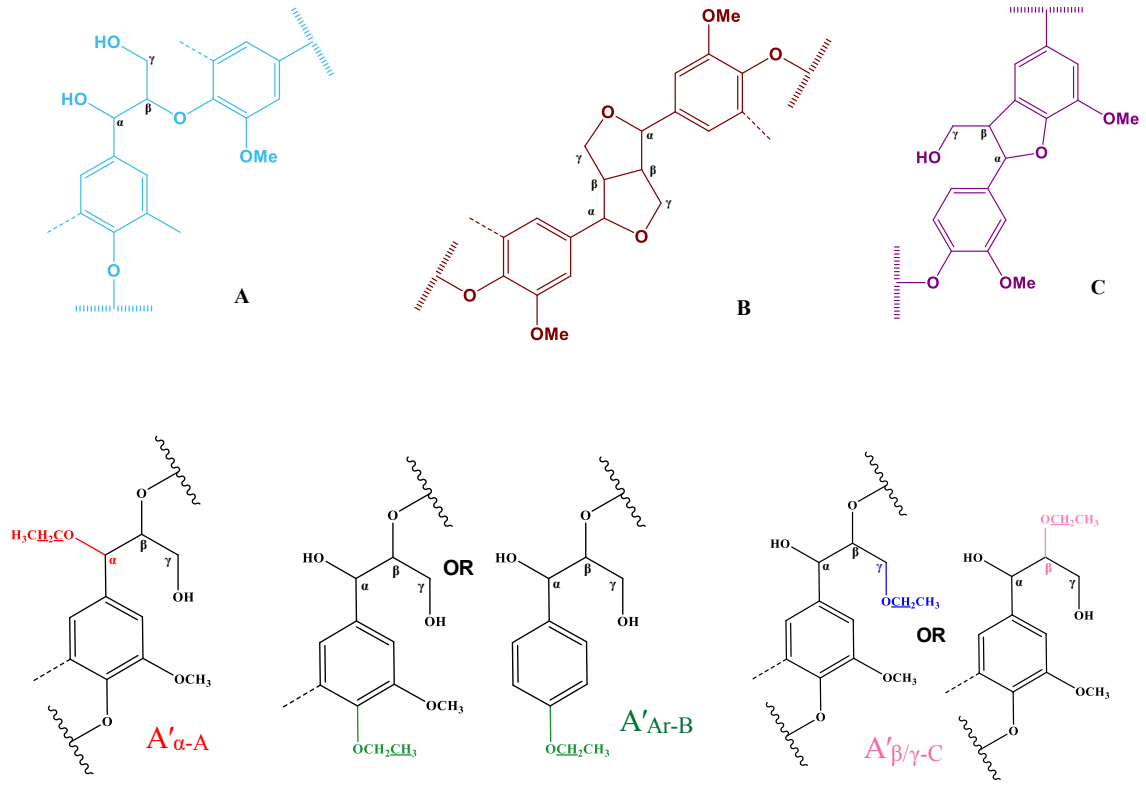


Fig. 32 Potential ethoxylation of kenaf lignins in ethanol organosolv pretreatment based on 2D HSQC, HMBC and HSQC-TOCSY spectra

The quantification of the signals of quantitative ^{13}C and HSQC (results expressed per Ar) of MWL-KE, EOL-KE, MWL-EK, MWL-AK was also summarized in Table 16 and 17. Besides introducing the ethoxy group in the EOL-KE and MWL-EK, the quantification of alkylation of lignins was different in these three lignins. Although the degree of alkylation, β-β, β-5 linkages ratio was similar in EOL-KE (2.13, 21.3%, 17.3%) and MWL-EK (2.38, 20.1%, 16.2%). The relative reaction ratio on C_α, $C_{\beta/\gamma}$, and the aromatic ring was different in EOL-KE and MWL-EK (Table 19). EOL-KE had a similar ethylation reaction ratio on C_α (49.1%) and aromatic ring (47.2%), while the ratio of ethylated benzene structure in MWL-EK (66.7%) was twice as much as that of the ethylated C_α (30.2%). EOL-KE increased the hydrolysis yield (+8.69%), but MWL-

EK had a slight inhibition (-1.95%). It could be indicated that the stimulatory effect of lignin might be related to the higher ethylated ratio of the hydroxyl group on C_α but not on the aromatic ring of lignin.

Table 12 The quantitative analysis of the signals of quantitative ^{13}C spectra

δ (ppm)	Assignment	MWL−KE	EOL-KE	MWL-EK	MWL-AK
175-168	Carboxylic acid or ester	0.18	0.21	0.20	0.19
132.7-130.8	PB	0.06	0.20	0.15	0.12
155-140	Aromatic C-O	2.03	1.99	2.14	2.33
140-125	Aromatic C-C	1.48	1.63	1.61	1.56
125-102	Aromatic C-H	2.61	2.51	2.37	2.24
90-58	Alk-O-	2.02	2.13	2.38	2.05
90-77	Alk-O-Ar, α-O-Alk	0.62	0.71	0.82	0.62
77-65	γ-O-Alk, secondary OH	0.72	0.66	0.81	0.82
61.3-58	β-O-4	0.38	0.37	0.41	0.41
58-54	CH_3O	1.61	1.74	1.87	2.18
67.5-64	$-OCH_2\underline{C}H_3$	0	0.19	0.15	0
15.9-15	$-O\underline{C}H_2CH_3$	0	0.18	0.12	0

Table 13 Quantification analysis of the HSQC signals

Lignins	S (%)	G (%)	S/G	H (%)	β-O-4 (%)	β-β (%)	β-5 (%)
MWL-KE	38.3	60.8	0.63	0.9	70.6	10.9	18.5
EOL-KE	51.0	46.4	1.10	2.6	61.4	21.3	17.3
MWL-EK	52.4	46.0	1.14	1.6	63.6	20.1	16.2
MWL-AK	46.0	38.3	1.57	1.7	62.9	21.3	15.7

Table 14 Ratio of ethylation reaction on different positions of EOL-KE and MWL-EK based on the relative integration of HSQC NMR spectra

Lignins	C_α (%)	$C_{\beta/\gamma}$ (%)	Aromatic ring (%)
EOL-KE	49.1	3.7	47.2
MWL-EK	30.2	3.1	66.7

3.5 Conclusions

The stimulatory effect of ethanol organosolv lignin from kenaf (EOL-KE) on the enzymatic hydrolysis of Avicel was investigated compared to the inhibition of mill wood lignin from untreated (MWL-KE), ethanol pretreated (MWL-EK), and dilute acid pretreated kenaf substrates (MWL-AK). The spectra of HSQC, HSQC-TOCSY, and HMBC revealed that the alkylation (e.g., methylation, ethylation, and propylation) of hydroxyl groups took place not only at C_α, but also at C_β and C_γ, and potentially also at the phenolic hydroxyl group of lignin. More alkylation reaction on the aliphatic hydroxyl group might be the reason for the stimulatory effect of EOL-KE. In addition, EOL-KE was less hydrophobic than the other isolated lignin from kenaf and can release more free enzymes during the enzymatic hydrolysis.

Chapter 4: Effect of extractable lignin from aspen on enzymatic hydrolysis of lignocelluloses

4.1 Abstract

Organosolv pretreatment with aqueous ethanol is one of the most efficient methods to increase cellulose accessibility, release sugars from hemicellulose, and produce lignins of high purity. Some organosolv lignin from hardwood could improve the yield of enzymatic hydrolysis. Extractable lignins, extracted from pretreated aspen by ethanol, have been accessed for their effect on the enzymatic hydrolysis of lignocelluloses in this study. All extractable lignins were detrimental to the enzymatic hydrolysis of Avicel, but some of them turned to have a positive effect on the enzymatic hydrolysis of pretreated aspen. The hydrophobicity, enzyme binding strength, zeta potential, molecular weight of extractable lignins have been determined. It was observed that extractable lignins extracted with the same solvent as pretreatment showed the highest hydrophobicity and binding strength and the highest inhibition on the enzymatic hydrolysis. In addition, it was observed that final yield and the cellulose accessibility of pretreated aspen were decreased by 8-17% when the pretreated substrates were washed with ethanol.

4.2 Introduction

Ethanol organosolv lignin (EOL) from organosolv pretreatment of sweetgum, cottonwood, black willow, and aspen is all reported to enhance the enzymatic hydrolysis Avicel and organosolv pretreated hardwood substrates[94, 95]. Solvent extractable lignins, which was washed out from the organosolv pretreated substrates, were combined with precipitated lignins from spent liquor as organosolv lignins in the lab scale[17]. However, 5.3% promotion was reported with the addition

of extractable lignins in enzymatic hydrolysis of Avicel, while 11.5% enhancement was reported with EOL from sweetgum from similar pretreatment condition[103]. The stronger stimulatory effect of EOL than extractable lignins from the same type of hardwood indicated the potential difference between these two types of lignins from the organosolv pretreatment. It also mentioned that organosolv lignins and extractable lignins from the same batch were chemically similar based on the analysis of the ^{13}C spectrum. So, it is not clear how physical properties contribute to the effect of chemically-similar lignins in enzymatic hydrolysis. The elucidation of this phenomenon is essential to distinguish the effect of organosolv lignin and extractable lignins during the fractionation of organosolv pretreatment, considering the structural heterogeneity and resource variability of lignins. On the other hand, lignin is well-accepted to be inhibitory to the enzymatic hydrolysis[145], so solvent washing was always conducted to remove the unwilling residuals like extractable lignins[211]. It is possible that the washing of extractable lignins of the pretreated substrate with solvent could increase the cellulose accessibility of substrates and therefore improve the enzymatic hydrolysis yield. The verification of the effect of solvent washing on the digestibility of substrates is meaningful to control the cost of the whole bioconversion process and promote the hydrolysis yield.

In this chapter, the effect of the extractable lignin of aspen from different ethanol washing concentrations was investigated on enzymatic hydrolysis of Avicel and pretreated aspen, compared to the EOL-AS (ethanol organosolv lignins from aspen). The effect of ethanol washing of pretreated aspen was investigated as well. The enzyme adsorption on extractable lignins and pretreated aspen after extraction was carried out to explain the interaction between cellulase and lignins. The physical properties, including hydrophobicity, zeta potential, and molecular weight of

extractable lignins, were examined. The cellulose accessibility and water retention value of substrates were determined.

4.3 Materials and methods

4.3.1 Cellulase enzymes and biomass

Aspen (*Populus tremuloides*) wood chips (moisture content 11.0%, chip size 5 mm × 2 mm) was AlfaPet small animal aspen bedding purchased from Walmart. The information of chemical reagents including methanol, ethanol, sulfuric acid, and sodium hydroxide for organosolv pretreatment, the Avicel, and cellulase enzymes for the enzymatic hydrolysis, Bengal Rose for lignin hydrophobicity, Coomassie Protein Assay kit for enzyme adsorption was mentioned in section 3.2.1. Cellulase C2730 (protein content: 41 mg/ml) applied in the test of enzyme adsorption was obtained from Sigma Aldrich. Direct red dye (DR28, 95% purity) for cellulose accessibility was obtained from Fisher.

4.3.2 Organosolv pretreatment

The aspen (100 g, dry weight) were soaked in 700 mL 65% methanol/ethanol/propanol water solution with 1 % (w/w, sulfuric acid/dry biomass) of sulfuric acid overnight before the hydrothermal process of organosolv pretreatment. The mixture was transferred into a 2-L stainless steel Par batch reactor with a stirrer and a PID controller (Parr 4848, USA) and heated to 160°C. After 1 h hydrothermal cooking, the reactor was water quenched to room temperature to terminate the reaction. The solid and the liquid fraction were separated from the reacted slurry by vacuum filtration using Whatman No.1 filter paper. Organosolv lignins from ethanol pretreated aspen were designated as EOL-AS. For extractable lignins from ethanol pretreated aspen, different ethanol

concentrations (25%, 50%, 65%, 75%, and 100%) were applied to wash the pretreated aspen after pretreatment to extract the organosolv extractable lignins. For instance, of 25% ethanol extractable lignins, the ethanol pretreated aspen (from 100 g dry weight wood chip pretreatment) was mixed with 233 ml of 25% ethanol solution at 60 °C for 10 min and filtrated. Two more times of 25% ethanol wash was conducted as the previous step, and all the resulting liquid (700 ml in total) was combined. Then 3-fold volume of water into the washing ethanol and the pH of the mixture was adjusted to 4 to precipitate the lignins. The extractable lignin was collected by vacuum filtration and 3 times of warm water washing. It was designated as EL-AS(E25). Similarly, extractable lignins were also collected by the extraction of unwashed ethanol pretreated aspen with 50%, 65%, 75%, and 100% ethanol, respectively, and were designated as EL-AS(E50), EL-AS(E65), EL-AS(E75) and EL-AS(E100).

The pretreated aspen was washed for further enzymatic hydrolysis with the following steps. The fractionated solid part (from 100 g dry biomass) from pretreatment was first washed by 3 times (233 ml each wash, 700 ml in total) of 60°C ethanol of different concentrations (25%, 50%, 65%, 75%, 100%) by mixing with hot ethanol solution for 10 min. The resulting slurry was filtrated with vacuum, and the collected solids were homogenized with 60°C water (same amount as ethanol) in a blender for 30 s, and then the liquor was filtrated. The water washing steps were repeated for 3 times after 3 times of ethanol washing to get the organosolv pretreated aspen. The washed substrates were collected and stored at 4°C for the following enzymatic hydrolysis. The pretreated aspen substrates after washing were designated as EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100, and the pretreated aspen with just water washed for three times was named as EPAS-H_2O.

4.3.3 Enzymatic hydrolysis of Avicel and pretreated substrates with lignins

Enzymatic hydrolysis of Avicel and pretreated substrates (EPAS-W, EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100) with lignins were carried out in 250 mL Erlenmeyer flasks with stoppers at 50 ℃, 150 rpm in 50 mM sodium citrate buffer (pH 4.8) with 2% glucan (w/v) for 72 h. The enzyme loading for all hydrolysis was chosen to be 5 FPU/g glucan. To study the effect of extractable lignins from aspen on enzymatic hydrolysis, 4 g/L of lignins were added to the enzymatic hydrolysis 1 h prior to the addition of cellulase at room temperature. The concentration of glucose in the supernatant was determined by HPLC. All experiments were carried out in duplicates.

4.3.4 Scanning electron microscope analysis, HPLC analysis, and FTIR analysis

Scanning electron microscope (SEM) analysis of ethanol pretreated aspen after ethanol extraction (EPAS-H_2O, EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100) was carried out by a JEOL 7000F SEM. The operating tension was set to be 5kV, 300ns, 50 pA, and the images were taken at the magnification of approximately 60000×. The substrates sample was first dispersed in DI-water (~0.5%) as a suspension, which was dropped (two droplets) onto the SEM sample plate, and then air-dried in a fume hood. Before the analysis, samples were coated 20 s for a thin layer of gold be PELCO SC-6 sputter coater.

The concentration of glucose from enzymatic hydrolysis was analyzed by an HPLC system (Agilent 1260 Infinity) with a Hi-Plex H guard column (5 × 3 mm, Agilent), a Hi-Plex H column (300 × 7.8 mm, Agilent), and a RID detector. 5 mM H_2SO_4 in aqueous solution was used as mobile phase with a flow rate of 0.6 $mL \cdot min^{-1}$. The detector and oven temperatures were both set to be 45°C. The FTIR spectrometer (Perkin Elmer Spectrum Two FTIR system) was employed to

examine the changes of chemical structures of the organosolv pretreated lignin compared to the mill wood lignin. ATR technique was used for FTIR sampling. The scanning wavenumber was set from 500 to 4000 cm^{-1}, and the interval was 0.50 cm^{-1}.

4.3.5 Enzyme adsorption isotherms of lignins

Enzyme adsorption on lignins was conducted with 1% (w/v) of lignins or glucan in the substrates at 4 °C and 150 rpm for 3 h. Different initial concentrations of cellulase C2730 (six points between 0.01-0.4 mg protein/mL) were applied with 50 mM citrate buffer. After the adsorption equilibrium at 3 h, the samples were centrifuged for 10,000 rpm for 5 min. Bradford assay was used for the determination of the free enzyme in the supernatant[212]. The adsorbed enzyme was the difference between the initial protein amount and the free protein amount in the sample solution. Langmuir adsorption isotherm (Equation. 1) was employed as the fitting model of the cellulase adsorption, in which the factors are: Γ, the amount of protein adsorbed on the lignins (mg/g lignin); C, the amount of free protein in the supernatant (mg/mL); Γ_{max}, the maximum adsorption capacity of protein on lignins (mg/g lignin); K is the Langmuir affinity constant (mL/mg protein).

$$\Gamma = \frac{\Gamma_{max}\mathrm{KC}}{1+\mathrm{KC}} \quad (1)$$

The distribution coefficient (R, L/g) was calculated from Eq. 2

$$R = \Gamma_{max} \times \mathrm{K} \quad (2)$$

The fitting of Langmuir isotherm was conducted by non-linear regression in origin (OriginLab, Massachusetts, USA).

4.3.6 Determination of zeta potential, molecular weight and hydrophobicity of lignins

The zeta potentials of the organosolv lignins from aspen were measured by a Zetasizer (Nano-ZS, Marvern Instrument Ltd, Worcestershire, U.K.). 2 mg of lignin was dispersed in 2 ml of 50 mM citrate buffer using ultrasound for the test. The analysis of the measurement was conducted by Zetasizer software. All measurement of hydrophobicity and zeta potential was carried out in duplicates.

Molecular weight of organosolv lignins from aspen was determined by Gel Permeation Chromatography (GPC) using an HPLC system (Agilent 1100) with three Styragel column (4.6 mm × 300 mm, HR5E, HR4, HR2, Waters, Milford, MA) in tandem, and an ultraviolet detector (U.V.) at 240 nm. Tetrahydrofuran (THF) was used as mobile phase with a flow rate of 1.0 $mL \cdot min^{-1}$. The oven temperatures were set to be ambient temperature. The injection volume is 20 μL with 2 g/L lignin in THF.

Increasing concentrations of the lignins (0.2-4.0 g/L) were mixed with 40 mg/L Rose Bengal in citrate buffer (50 mM, pH 4.8) and incubated at 50 °C, 150 rpm for 2 h. The free Rose Bengal dye in the supernatant and the lignins were separated by centrifugation of 10000 rpm, 5 min. The free dye concentration was determined by UV-Vis spectrometer at 543 nm, showing the difference between the initial dye to calculate the adsorbed dye on the lignins. The calculation of hydrophobicity of lignins was conducted as section 2.2.5.

4.3.7 Determination of water retention value and cellulose accessibility of pretreated substrates

The determination of water retention value (WRV) was modified from previous methods[213]. 0.5 g (dry weight) of pretreated substrate was suspended in 500 mL deionized water and disintegrated for 10,000 revolutions at 200 rpm for 50 min in a disintegrator. The resultant

suspension was vacuum filtered with a 0.45 um nylon membrane. The filter cake was collected and added into ~5 ml of deionized water to make a suspension for 2 h. The suspension was then transferred into a 100 um nylon screen bag placing in a 50 ml centrifuge tube with enough space for water centrifuged out. Then the suspension was centrifuged with 3000 rcf for 30 min. The centrifuged cellulosic samples were weighed before and after oven drying at 105 °C for the determination of WRV. WRV of the substrate could be calculated from the percentage of the amount of water retained after centrifuge divided by the dry weight of the substrate. All experiments were carried out in duplicates.

To evaluate cellulose accessibility in Avicel and pretreated substrates, direct red dye (DR28) adsorption was determined by previously described[214]. The adsorption of the dye was conducted at 1% (w/v) lignocellulosic substrate with a direct red solution of six different concentrations within the range of 0-4.0 g/L at 50 °C, 150 rpm for 24 h. After equilibrium, the samples were collected and centrifuged at 10000 rpm for 10 min. Supernatant was analyzed by UV-vis spectrometer at 498 nm for the free dye concentration. The concentration of the adsorbed dye was plotted against the free dye. Langmuir adsorption isotherm was applied for the fitting and to assess the maximum adsorption capacity of direct red dye, which showed the cellulose accessibility of the substrates.

4.4 Results and discussion

4.4.1 Effect of extractable lignins on enzymatic hydrolysis of lignocelluloses

The characteristic effect of EOL-AS and five ethanol extractable aspen lignins (EL-AS(E25), EL-AS(E50), EL-AS(E65), EL-AS(E75), EL-AS(E100)) was compared on the enzymatic hydrolysis of Avicel (Fig. 33). The effect of EOL and extractable lignins on Avicel

hydrolysis was different. EOL-AS promoted the 72 h yield of enzymatic hydrolysis of Avicel, and all the extractable lignins showed inhibitory effect (Table 21). With EOL-AS, the 72 h yield of enzymatic hydrolysis was increased from 58.13 to 61.03%, while EL-AS(E25), EL-AS(E50), EL-AS(E), EL-AS(E75), EL-AS(E100) decreased the final yield to 55.16, 49.23, 45.63, 48.45 and 46.25%, respectively. The promotion effect of EOL-AS (+2.10%) agreed well with the previous finding by Huang that it could promote the 72 h yield of Avicel hydrolysis by 5.04%[95]. Lignin was proved to affect both substrates and cellulase in the enzymatic hydrolysis[184]. Different results were reported about the effect of extractable lignins on enzymatic hydrolysis. The addition of EOL-AS in Avicel hydrolysis slightly increased the final yield of Avicel from 58.13% to 60.23%. However, the 72 h glucose yield of Avicel was decreased from 58.13% to 55.16%, 49.23%, 45.63%, 48.45%, 46.25%, respectively by the addition of EL-AS(E25), EL-AS(E50), EL-AS(E65), EL-AS(E75), EL-AS(E100). All extractable lignins showed inhibition on Avicel hydrolysis (EL-AS(E25): -3.77%, EL-AS(E50): -9.70%, EL-AS(E75): -10.48%, EL-AS(E100): -12.44%), especially EL-AS(E65) (-13.30%). A decrease of 12.44% was investigated in the hydrolysis of Avicel by addition of 4 g/L EL-AS(E100) in this study, while Lai et al. observed 5.3% promotion of addition of 4 g/L solvent extractable lignins (extracted by 95% ethanol) from sweetgum in enzymatic hydrolysis of Avicel[176]. The difference between extractable lignins on enzymatic hydrolysis may be related to the different wood applied.

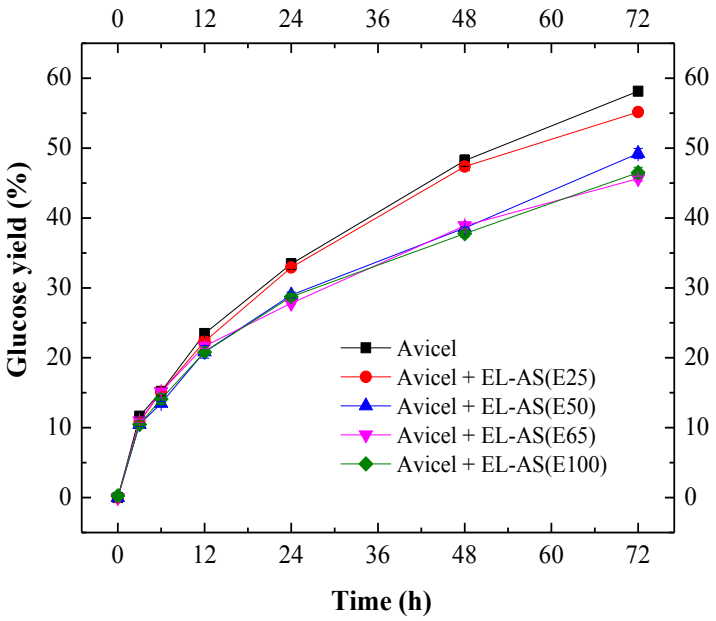


Fig. 33 Effect of extractable lignins on enzymatic hydrolysis of Avicel

Enzymatic hydrolysis of EPAS-E65 was also investigated with EOL-AS, EL-AS(E25), EL-AS(E50), EL-AS(E65), EL-AS(E75), EL-AS(E100) (Fig. 34). The addition of EOL-AS, EL-AS(E25), and EL-AS(E50) promoted the 72 h final yield of Avicel from 58.67% to 69.05%, 67.81%, and 61.05%, respectively. While the addition of EL-AS(E65), EL-AS(E75), EL-AS(E100) decreased the glucose yield of Avicel from 58.67% to 56.92%, 58.51%, and 57.69%. Like the hydrolysis of Avicel, the strongest inhibition in the final yield of EPAS was also investigated with EL-AS(E65) (-1.75%). And the strongest stimulatory effect was investigated with the addition of EOL-AS (+10.37%). Unexpectedly, compared to the hydrolysis of Avicel, the inhibitory effect of the addition of extractable lignins was abated. The stimulatory effect of EOL-AS was intensified in the enzymatic hydrolysis of EPAS. A similar difference between the lignin effect on pure cellulose and lignocellulosic substrates was reported by Lou with lignosulfonate and Lai with EOL from sweetgum[94, 155].

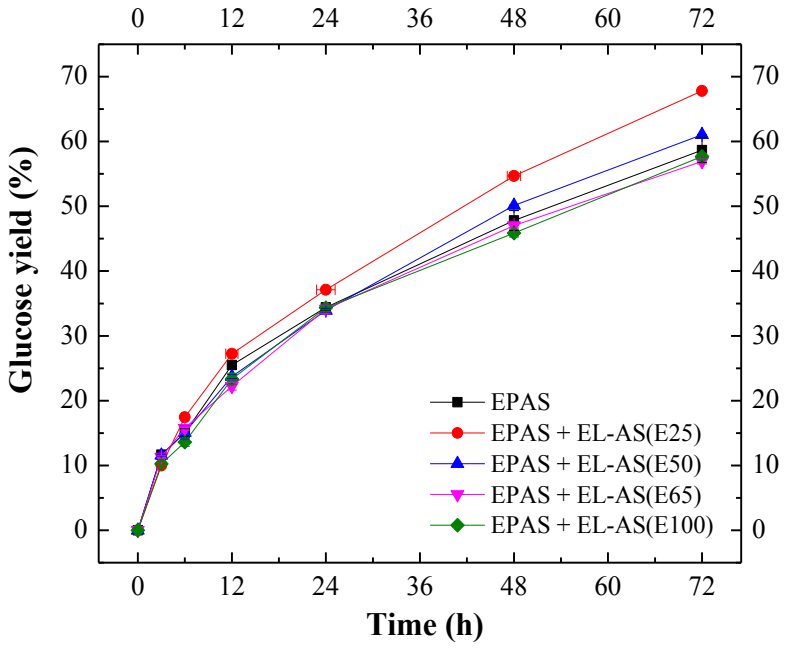


Fig. 34 Effect of extractable lignins on enzymatic hydrolysis of ethanol pretreated aspen

On the other hand, the abated inhibition of EL-AS(E65), EL-AS(E75), and EL-AS(E100) on EPAS than on Avicel may also be related to the increase in accessible cellulose. Lignin was reported to have no effect on enzyme adsorption when the substrates were highly cellulose-accessible[215]. To test the difference between cellulose accessibility of Avicel and EPAS, the cellulose accessibility of these two materials was evaluated by the maximum adsorption of direct red dye (DR28) (Table 20). The results showed that Avicel has much lower cellulose accessibility (37.27 mg/g) than OPAS (130.10 mg/g). This explains the abated inhibition of some lignins (EL-AS(E65), EL-AS(E75), EL-AS(E100)) on enzymatic hydrolysis of EPAS than that of Avicel. Li et al. revealed similar competitive relationships between cellulose, cellulase, and hydrolysis inhibitors like some lignin[216]. More available cellulose could attract more productive cellulase, therefore decrease the inhibition from the same number of inhibitors when the cellulase amount is unchanged. Kumar et al. reported that the less accessible the cellulose of the substrates is, the more

significant role the isolated lignin would play by non-specific adsorption binding[217]. They also mentioned the cellulose accessibility of oven-dried Avicel was much less than the never-dried pretreated substrates. This was also correlated with the difference between the cellulose accessibility of Avicel and EPAS.

Table 15 Cellulose accessibility of Avicel and EPAS

Substrates	Γ_{max}/DR28 (mg/g)
Avicel	37.27
EPAS	130.10

The initial hydrolysis rates of enzymatic hydrolysis with lignins were listed in Table 20. Compared to the initial hydrolysis rate of Avicel (3.88 g/L/h), it was increased to 4.07 g/L/h with the addition of EOL-AS but was decreased to 3.60 g/L/h, 3.45 g/L/h, 3.61 g/L/h, 3.54 g/L/h, 3.39 g/L/h with the addition of EL-AS(E25), EL-AS(E50), EL-AS(E65), EL-AS(E75) and EL-AS(E100). The initial hydrolysis rate of EPAS-E65 was increased from 3.89 g/L/h to 4.41 g/L/h with EOL-AS. But the yield was decreased to 3.32 g/L/h, 3.85 g/L/h, 3.77 g/L/h, 3.55 g/L/h, 3.30 g/L/h with the addition of EL-AS(E25), EL-AS(E50), EL-AS(E65), EL-AS(E75), and EL-AS(E100). A similar increase of the initial hydrolysis rate by EOL-AS was observed with EOL lignin from sweetgum[94].

Table 16 Initial hydrolysis rates and 72 h glucose yield of enzymatic hydrolysis of Avicel and pretreated substrates

Lignin	Initial hydrolysis rate with Avicel (g/L/h)	Initial hydrolysis rate with EPAS (g/L/h)	72 h hydrolysis yield with Avicel (%)	72 h hydrolysis yield with EPAS (%)
No lignin	3.88 ± 0.04	3.89 ± 0.03	58.13 ± 0.12	58.67 ± 1.93
EOL-AS	4.07 ± 0.06	4.41 ± 0.03	60.23 ± 0.44	69.05 ± 0.56
EL-AS(E25)	3.60 ± 0.02	3.32 ± 0.08	55.16 ± 0.44	67.81 ± 0.25

EL-AS(E50)	3.45 ± 0.04	3.85 ± 0.04	49.23 ± 0.73	61.05 ± 0.03
EL-AS(E65)	3.61 ± 0.04	3.77 ± 0.07	45.63 ± 0.35	56.92 ± 0.38
EL-AS(E75)	3.54 ± 0.01	3.55 ± 0.17	48.45 ± 0.14	58.51 ± 0.87
EL-AS(E100)	3.39 ± 0.03	3.30 ± 0.13	46.25 ± 0.77	57.69 ± 0.44

4.4.2 Hydrophobicity and zeta potential analysis and molecular weight of lignins

The non-specific adsorption is commonly accepted to be related to the physical properties [218, 219]. The effect of EOL lignins on enzymatic hydrolysis was reported to be governed collectively by lignin surface hydrophobicity (inhibition related) and zeta potentials (stimulation related)[95]. Molecular weight was also reported to be related to the lignin-cellulase adsorption[220]. To examine the driving force of the non-specific adsorption of cellulase on lignins, zeta potentials, hydrophobicity, and molecular weight of EOL-AS and extractable lignins were studied (Table 22). Except EL-AS(E100) (-10.38 mV), EOL-AS (-9.99 mV) has higher zeta potential than EL-AS(E25) (-5.57 mV), EL-AS(E50) (-8.68 mV), EL-AS(E65) (-9.06 mV), EL-AS(E75) (-9.31 mV). EL-AS(E) (1.09 L/g) showed six to seven folds higher hydrophobicity than EOL-AS (0.16 L/g). Besides EL-AS(E65), which showed the highest hydrophobicity, all extractable lignins (EL-AS(E25): 0.72 L/g, EL-AS(E50): 0.79 L/g, EL-AS(E75): 0.90 L/g, EL-AS(E100): 0.97 L/g) showed at least three times higher hydrophobicity than EOL-AS.

Table 17 Hydrophobicity, zeta potential and molecular weight of lignins

Lignin	Hydrophobicity (L/g)	Zeta potential (mV)	M_w *(g/mol)*
EOL-AS	0.16 ± 0.02	-9.99 ± 0.12	3477
EL-AS(E25)	0.72 ± 0.01	-5.57 ± 0.27	1869
EL-AS(E50)	0.79 ± 0.11	-8.68 ± 0.80	3043
EL-AS(E65)	1.09 ± 0.06	-9.06 ± 0.68	4005

EL-AS(E75)	0.90 ± 0.12	-9.31 ± 0.68	3984
EL-AS(E100)	0.97 ± 0.10	-10.38 ± 0.19	3018

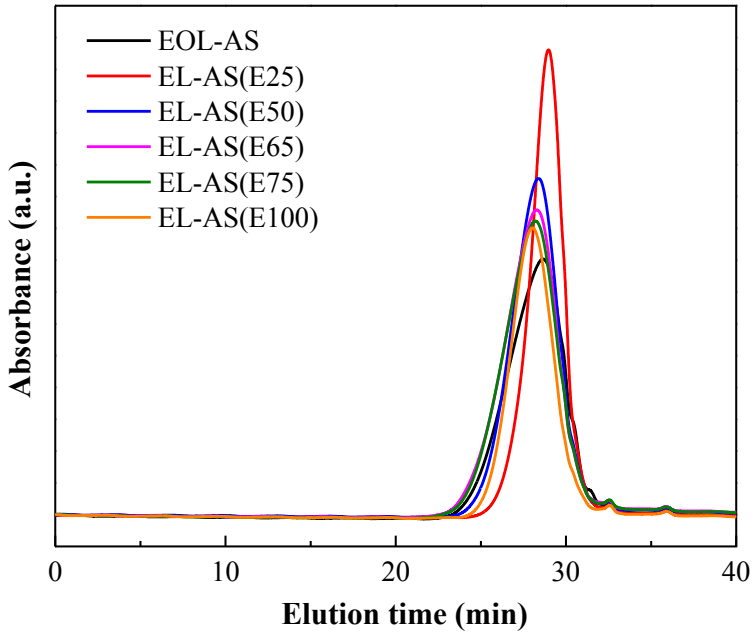


Fig. 35 GPC chromatogram of extractable lignins and EOL-AS

The molecular weight of lignins was also investigated by GPC. The molecular weight of extractable lignins was listed in Table 22, and their molecular weight distribution was presented by distinguishing elution time in GPC, as shown in Fig. 35. Extractable lignins had similar molecular weight distribution. The EL-AS(E65 (4005 g/mol) has a larger molecular weight than that of EOL-AS (3477 g/mol). For extractable lignins from aspen, the concentration of ethanol, which was closer to 65%, extracted out larger molecular weight lignins. The molecular weight of lignins did not strongly correlate to the 72 h yield of enzymatic hydrolysis. There were no consistent correlations between the molecular weight of lignins and their effect on enzymatic hydrolysis for the previous study of lignins. Lou et al. reported a negative correlation between the molecular weight of poplar lignosulfonate and their enhancement of final hydrolysis yield[155]. The

smaller was the lignin molecule weight, the more stimulatory it would be in the enzymatic hydrolysis. However, the stimulatory effect of alkali lignin from rice straws was reported, and the enhancement for hydrolysis increased with increasing molecular weights of alkali lignins[209]. As discussed above, the molecular weight could be a factor that contributes to hydrophobicity and surface charge, while it might not be a dominant factor for all cases. Therefore, the correlations between the molecular weight of isolated lignins and their effect on enzymatic hydrolysis need to be further elucidated. It may be especially difficult to find when the source and the method to get the lignins were different.

4.4.3 Enzyme adsorption of lignins from aspen

To quantify the non-specific adsorption of cellulase on lignins, cellulase adsorption on EOL and extractable lignins were investigated and fitted with the Langmuir model (Fig. 36, Table 23). The adsorption capacity of extractable lignins (EL-AS(E25): $\Gamma_{max} = 8.648$ mg/g, EL-AS(E50): $\Gamma_{max} = 15.906$ mg/g, EL-AS(E65): $\Gamma_{max} = 14.294$ mg/g, EL-AS(E75): $\Gamma_{max} = 12.832$ mg/g, EL-AS(E100): $\Gamma_{max} = 13.221$ mg/g) were two to four times of that of EOL-AS (4.158 mg/g). This indicates that extractable lignins may have more binding sites of cellulase on the lignin surface than the EOL-AS. Langmuir constant (K) shows the affinity of cellulase on the lignins[221]. EOL-AS (5.831 mL/mg) had similar affinity with cellulase as EL-AS(E25) (5.576 mL/mg) and EL-AS(E50) (4.989 mL/mg), while EL-AS(E65) (9.911 mL/mg), EL-AS(E75) (10.416 mL/mg) and EL-AS(E100) (10.943 mL/mg) had higher affinity than them. This explained why the three extractable lignin had a negative effect on the enzymatic hydrolysis of lignocellulosic substrates and had a higher inhibition than EOL-AS, EL-AS(E25), and EL-AS(E50) in Avicel hydrolysis. The affinity of cellulase on lignins may be related to the hydrophobicity of lignins. The distribution

coefficient (R) is the factor that represents the binding strength of lignins with cellulase enzymes[222]. Based on their difference in adsorption capacity and affinity, all extractable lignins (EL-AS(E25): 0.048 L/g, EL-AS(E50): 0.079 L/g, EL-AS(E65): 0.142 L/g, EL-AS(E75): 0.134 L/g, EL-AS(E100): 0.139 L/g) had much higher binding strength than EOL-AS (0.024 L/g). Especially, the binding strength of EL-AS(E65) was five folds higher than that of EOL-AS. In general, extractable lignins may cause more non-specific adsorption of cellulase than EOL-AS. Extractable lignins had much higher distribution coefficient (0.048-0.142 L/g) than the previous report solvent extractable lignins from sweetgum (0.007 L/g). This revealed the reason extractable lignins, in this case, could not stimulate the hydrolysis[176].

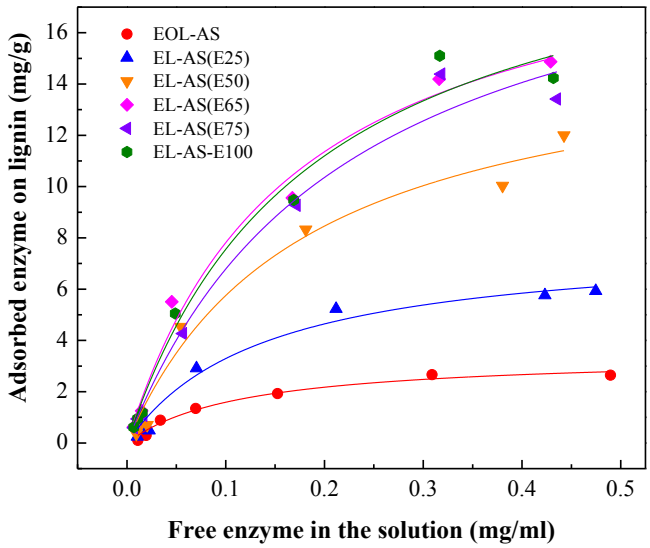


Fig. 36 Cellulase adsorption on extractable lignins from aspen

Table 18 Enzyme adsorption parameters based on Langmuir isotherm

Lignin	Γ_{max} (mg/g)	K (ml/mg)	R (L/g)
EOL-AS	3.443	8.577	0.030
EL-AS(E25)	7.876	7.183	0.057
EL-AS(E50)	15.990	5.616	0.090
EL-AS(E65)	20.807	6.011	0.125

EL-AS(E75)	21.881	4.493	0.098
EL-AS(E100)	21.639	5.361	0.116

4.4.4 FTIR analysis of lignins from aspen

Fig. 37 illustrates the FT-IR spectra of EOL and extractable lignins from aspen. As can be seen, the absorption of C.H. stretching of methyl, methylene, or methane group at 2945 cm^{-1} is slightly higher in EL-AS(E100). The absorption of C=O stretch in unconjugated ketone and a carboxyl group at 1732 cm^{-1} and aromatic C-H syringyl (S) unit at 1115 cm^{-1} is slightly higher in EL-AS(E25). But overall, the FTIR absorbance of EOL and extractable lignins from aspen has no huge difference compared to each other. This was probably due to the same reaction condition they came from.

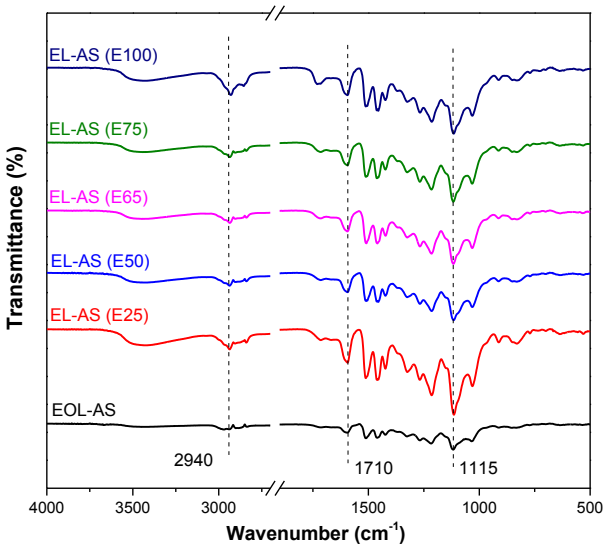


Fig. 37 FTIR analysis of EOL and extractable lignins from aspen

4.4.5 Effect of ethanol washing on enzymatic hydrolysis of ethanol pretreated aspen

To examine the enzyme digestibility of the washed substrates, the enzymatic hydrolysis of ethanol washed aspen (EPAS-H_2O, EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100) was

investigated (Fig. 38). 72 h final yield was 76.01%, 74.75%, 67.98%, 63.55%, 59.79%, and 59.55%, respectively for EPAS-H_2O, EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100. With the washing of 25%, 50%, 65%, 75%, 100% ethanol, the final yield was decreased by 1.25%, 8.03%, 12.46%, 16.21%, and 16.45% compared to the water washed substrates (EPAS-H_2O). Ethanol wash decreased the enzyme digestibility of the organosolv pretreated aspen, final hydrolysis yield was lower when the higher concentration of ethanol wash was applied. Similar reduction caused by ethanol washing was reported in the enzymatic hydrolysis of ethanol pretreated sweetgum[103]. However, eliminating the promotional effect of extractable lignin aspen was excluded because of the negative effect of extractable lignins on enzyme hydrolysis yield. Therefore, there should be a deeper reason for the reduction of digestibility by ethanol washing. It was important that the reason for the reduction should be related to the interaction between polysaccharides and lignins because this effect was not observed with ethanol pretreated pure cellulose[103].

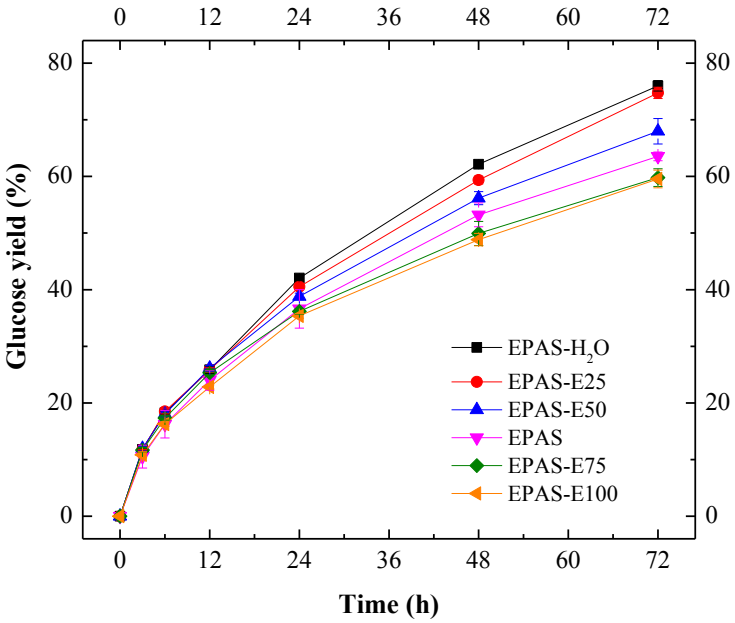


Fig. 38 Effect of ethanol washing on enzyme digestibility of ethanol pretreated aspen

The initial hydrolysis rates of the substrates were listed in Table 24. Initial hydrolysis rates of EPAS (3.50 g/L/h) hand EPAS-E100 (3.61g/L/h) were 10% lower than that of the other substrates (EPAS-H_2O: 3.93 g/L/h, EPAS-E25: 3.91 g/L/h, EPAS-E50: 3.98 g/L/h, EPAS-E75: 3.89 g/L/h). This may be due to the higher xylan content of EPAS (2.82%) and EPAS-E100 (2.61%) comparing to other substrates (EPAS-H_2O: 2.40%, EPAS-E25: 2.44%, EPAS-E50: 2.54%, EPAS-E75: 2.70%). The significant effect of residual xylan on the initial rate of hydrolysis was previously reported by Lai and Li[103, 223].

Table 19 Initial hydrolysis rates and 72 h glucose yield of enzymatic hydrolysis of pretreated substrates

Substrates	Initial hydrolysis rate (g/L/h)	72 h hydrolysis yield (%)
EPAS-H_2O	3.93 ± 0.05	76.01 ± 0.93
EPAS-E25	3.91 ± 0.03	74.75 ± 0.99
EPAS-E50	3.98 ± 0.15	67.98 ± 2.24
EPAS	3.50 ± 0.67	63.55 ± 0.77
EPAS-E75	3.89 ± 0.11	59.79 ± 1.55
EPAS-E100	3.61 ± 0.18	59.55 ± 1.56

4.4.6 Cellulose accessibility and water retention value (WRV) of pretreated aspen

Substrates-related enzymatic hydrolysis factors are important in the hydrolysis rate and hydrolysis yield of enzymatic hydrolysis[224]. Cellulose accessibility played a more important role in enzymatic hydrolysis than lignin content in the view of cellulase and cellulose interaction[217]. To explain the effect of ethanol wash, the direct red adsorption on EPAS-H_2O, EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100 was determined to show the cellulose accessibility of

substrates (Table 25). The water washed substrates EPAS-W (60.27 mg/g) had the highest cellulose accessibility, and the 100% ethanol washed substrates EPAS-E100 showed the lowest cellulose accessibility (43.43 mg/g). The cellulose accessibility of the 25% (59.77 mg/g), 50% (54.90 mg/g), 65% (49.63 mg/g), 75% ethanol (48.84 mg/g) extracted substrates was higher than that of EPAS-E100 and lower than that of EPAS-H_2O. This indicated that ethanol washing of pretreated substrates decreased the cellulose accessibility. And the higher concentration of ethanol was applied in the washing process, the lower cellulose accessibility was observed. Strong linear correlations were inspected between the cellulose accessibility and the 72 h hydrolysis yield of these substrates ($y = 0.8895x - 6.7379, R^2 = 0.931$). It indicated that cellulose accessibility had a significant effect on the enzyme digestibility of pretreated aspen. This correlated with the previous findings that cellulose accessibility played a more important role than lignin content in enzyme digestibility of pretreated biomass[214, 225].

Another substrate-related factor, water retention value (WVR), which was first introduced to show the extent of fibrillation of micro and nanofibrils in the pulp and paper industry, also influences the accessibility of substrates to enzymes[171]. To further explain the effect of ethanol washing on enzyme digestibility of ethanol pretreated aspen, the WRV of EPAS-H_2O, EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100 was first determined (Table 25). EPAS-E25 (148.3%) had the lowest water retention value of the substrates, and EPAS-E75 (169.8%) had the highest WRV. WRV of EPAS, EPAS-E50, EPAS-E65, and EPAS-E100 was 152.1%, 153.6%, 160.0% and 153.4%. WRV of pretreated substrates did not show a specific trend with a higher concentration of washing ethanol. Furthermore, no correlation was found between the WRV and hydrolysis results of substrates. It may reveal that WRV is not a crucial factor influencing the enzyme digestibility of substrates with ethanol washing.

Table 20 WRV and cellulose accessibility of pretreated substrates

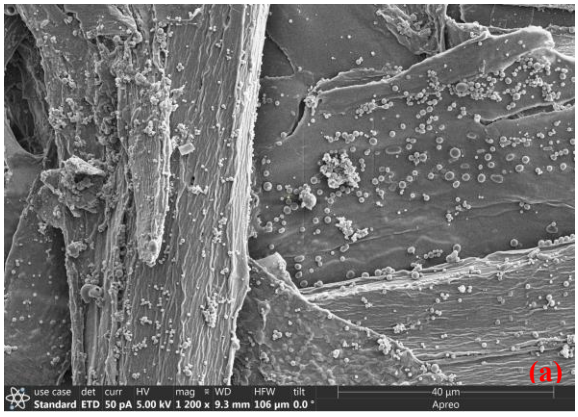
Substrates	WRV (%)	Γ_{max} /DR28 (mg/g)
EPAS-H_2O	152.1±4.4%	60.27
EPAS-E25	148.3±2.2%	59.77
EPAS-E50	153.6±0.3%	54.90
EPAS	160.0±3.6%	49.63
EPAS-E75	169.8±2.8%	48.84
EPAS-E100	153.4±1.6%	43.43

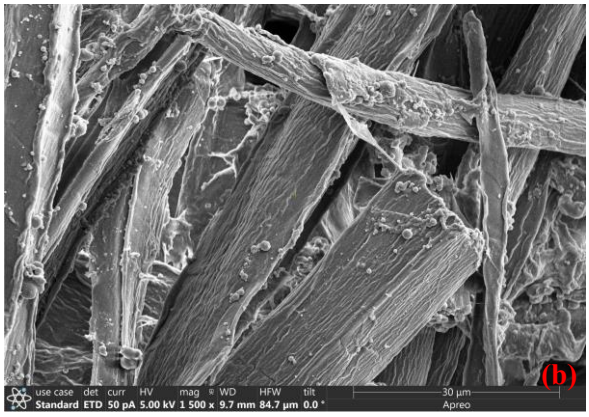
4.4.7 Effect of ethanol washing on SEM image of pretreated substrates

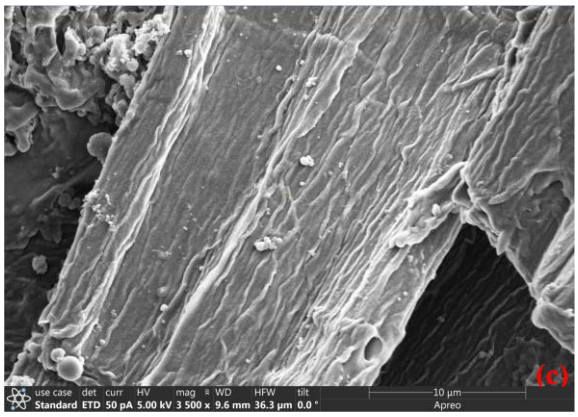
To examine the effect of ethanol washing on the surface morphology, their surface morphology was tested with 10-50 μm (Fig. 39). Lignin droplets (size 0.5-1.9 μm, Fig. 39 (a), (g)) were observed on the water washed substrates EPAS-H_2O. Similar spherical droplets of lignin ranging 0.5-2.0 μm from organosolv pulping were reported by Xu etc.[226] With the washing of ethanol, some of the lignin droplets were washed out (Fig. 39(b), (c)). Especially for substrates EPAS and EPAS-E75 (Fig. 39(d), (e)), almost all of the lignin precipitates were removed. Xu etc., also found that 42% ethanol is the threshold of the exponential increase of lignin turbidity. This correlates with the dramatic droplets decrease with 50% of ethanol washing or higher (Fig. 39(c)-(e)). The deposit of lignin droplets from organosolv pretreatment was also revealed to be related to increased pore volume and surface area, hence promoting the enzyme conversion of the pretreated biomass[227]. This may be the potential reason of the digestibility reduction of pretreated substrates after ethanol washing.

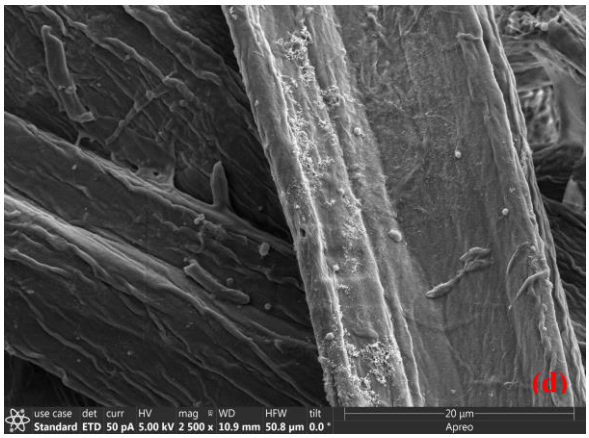
With 100% ethanol washing, there were some morphology alterations observed (Fig. 39(f)), which seems to be potential fibers re-bonding[228]. This may also be indicated by the sudden decrease of WRV when the concentration of washing ethanol increased from 75% (WRV: 169.8%)

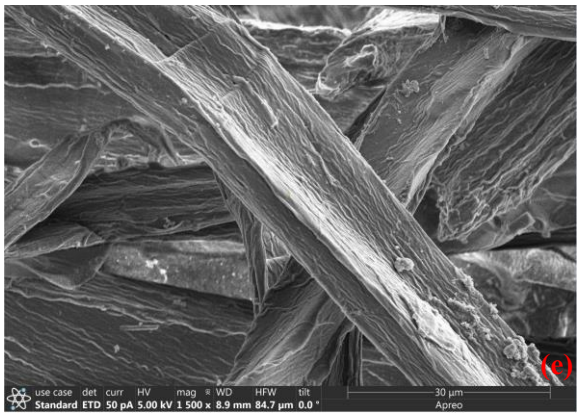
to 100% (WRV: 153.4%). This may explain the low digestibility of EPAS-E100 comparing to the other substrates.

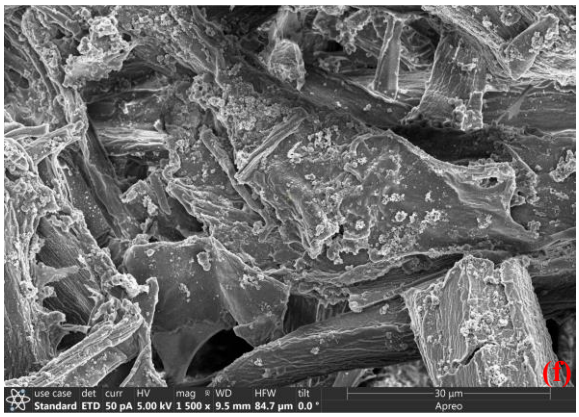












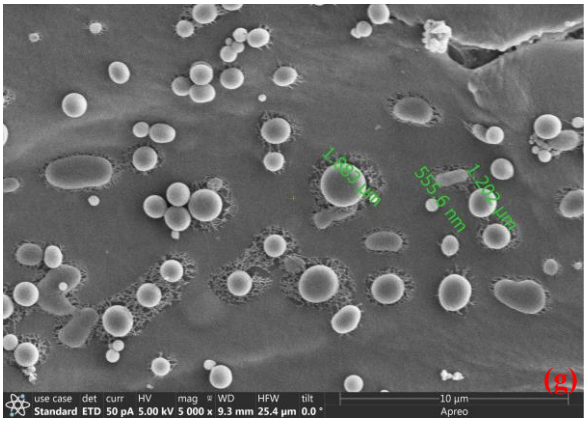


Fig. 39 Effect of ethanol washing on SEM image of ethanol pretreated aspen: (a) EPAS-W, (b) EPAS-E25, (c) EPAS-E50, (d) EPAS, (e) EPAS-E75, (f) EPAS-E100, (g) lignin droplets on EPAS-W

4.4.8 Chemical composition of extractable lignins and pretreated substrates

The chemical composition of both lignins and substrates was proved to be a potential factor influencing the enzymatic hydrolysis[25]. Therefore, the chemical composition analysis was conducted with extractable lignins and pretreated aspen after washing different ethanol concentrations. As showed in Table 26, the chemical compositions of extractable lignins were listed. All extractable lignins were mainly composed of more than 90% of Klason lignin and less than 5% of acid-soluble lignins (ASL). They all had a similar composition, except the high ratio of Klason lignin (99.01%) EOL-AS had. The chemical composition of EOL-AS and extractable lignins may be not correlated to their effect on enzymatic hydrolysis.

Table 21 Effect of ethanol extraction on chemical composition of substrates

Lignin	Klason lignin (%)	ASL (%)	Glucan (%)	Xylan (%)	Galactan (%)	Arabinan (%)	Mannan (%)
EOL-AS	99.01 ± 1.70	1.89 ± 0.03	0.02 ± 0.00	0.16 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.94 ± 0.09
EL-AS(E25)	95.67 ± 3.33	2.13 ± 0.41	0.14 ± 0.03	0.55 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	1.46 ± 0.37

EL-AS(E50)	95.44 ± 2.87	2.01 ± 0.22	0.04 ± 0.01	0.20 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.96 ± 0.04
EL-AS(E65)	93.33 ± 0.31	2.82 ± 0.04	0.06 ± 0.02	0.21 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.04 ± 0.00
EL-AS(E75)	94.38 ± 2.98	3.58 ± 0.34	0.09 ± 0.00	0.16 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	1.01 ± 0.04
EL-AS(E100)	93.96 ± 0.69	3.01 ± 0.07	0.14 ± 0.00	0.12 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.12 ± 0.14

To further explain the effect of ethanol washing on enzyme digestibility of ethanol pretreated aspen, the chemical composition of EPAS-H_2O, EPAS-E25, EPAS-E50, EPAS, EPAS-E75, EPAS-E100 was also determined (Table 27). With ethanol washing, ethanol extractives of substrates were decreased from 7.84% (EPAS-H_2O) to 3.38% (EPAS-E25), 2.15% (EPAS-E50), 1.03% (EPAS), 0.83% (EPAS-E75), and 1.70% (EPAS-E100), respectively. This may be one potential reason for the decrease of enzyme digestibility after ethanol extraction because some extractive molecules from hardwood were proved to be beneficial to the enzymatic hydrolysis[157].

Content of glucan, and acid soluble lignin (ASL) remained similar with ethanol washing. Except slight increase of EPAS-E25, Klason lignin of other ethanol washed substrates (EPAS-E50: 6.36%, EPAS: 4.52%, EPAS-E75: 4.40%, EPAS-E100: 6.77%) decreased 3-5% comparing to 9.41% Klason lignin in EPAS. Hemicellulose content is an important factor influencing the enzymatic hydrolysis[146]. There was 16.45% difference between the final yield of substrates with and without ethanol washing. But the composition of xylan in all substrates was within the range of 2.40% to 2.82%. Therefore, the content of xylan was possibly not the crucial factor influencing the yield of the enzyme digestibility of the substrates.

Table 22 Effect of ethanol wash on chemical composition of substrates

Substrates	Ethanol extractives (%)	Glucan (%)	Xylan (%)	Galactan (%)	Arabinan (%)	Mannan (%)	Klason lignin (%)	ASL (%)
EPAS-H_2O	7.84 ± 1.47	74.67 ± 3.00	2.40 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	9.41 ± 4.42	1.24 ± 0.05
EPAS-E25	3.38 ± 1.47	72.15 ± 3.00	2.44 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	11.10 ± 0.22	0.97 ± 0.00

EPAS-E50	2.15 ± 1.07	77.52 ± 0.31	2.54 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.36 ± 2.02	1.22 ± 0.25
EPAS	1.03 ± 1.07	83.26 ± 0.62	2.82 ± 0.07	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.52 ± 1.36	1.50 ± 0.08
EPAS-E75	0.83 ± 0.53	80.94 ± 0.17	2.70 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	4.40 ± 1.64	1.49 ± 0.00
EPAS-E100	1.70 ± 0.58	81.74 ± 0.05	2.61 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	6.77 ± 0.21	1.47 ± 0.00

4.5 Conclusion

Ethanol extractable lignins, washed using the same solvent as in the organosolv pretreatment, mostly inhibit the enzymatic hydrolysis of lignocelluloses. The inhibition and stimulation of lignin on enzymatic hydrolysis may be controlled by lignin hydrophobicity and zeta potential together. A higher concentration of ethanol washing reduced final yield and cellulose accessibility of organosolv pretreated biomass.

Chapter 5: Comparison of organosolv lignin and extractable lignin on enzymatic hydrolysis of lignocelluloses

5.1 Abstract

Organosolv pretreatment with aqueous ethanol is one of the most efficient methods to increase cellulose accessibility, release sugars from hemicellulose, and produce lignins of high purity. Pretreatment with similar short-chain aliphatic alcohol like methanol and propanol was also reported to have high delignification efficiency. Some organosolv lignin from hardwood could improve the yield of enzymatic hydrolysis. Extractable lignins, extracted from pretreated biomass by solvent (methanol, ethanol, and propanol) have been accessed for their effect on the enzymatic hydrolysis of lignocelluloses and compared with organosolv lignins in this study. The extractable lignins reduced the yield of enzymatic hydrolysis of Avicel, while the organosolv lignins from aspen could improve the yield of the hydrolysis. The organosolv lignin from pine showed less inhibition than the extractable lignins from pretreated pine. The hydrophobicity, enzyme binding strength, and HSQC NMR spectra of extractable lignins and organosolv lignins have been determined and compared. HSQC spectra showed a similar chemical structure for the organosolv lignins and extractable lignins. It was observed that extractable lignins showed higher hydrophobicity and binding strength than organosolv lignins. In addition, it was observed that final yield of pretreated aspen and pine was decreased by 3-17% when the pretreated substrates were washed with organic solvents. This indicates that solvent washing is not necessary for the organosolv pretreatment.

5.2 Introduction

818.8 million acres of the U.S. land area is covered by forest, which counts for more than 35 percent of the area. Across the whole country, nearly 58 percent of the growing stock on the forest land is softwoods, such as Loblolly pine and Lodged pine[229]. However, in the biorefinery, bioconversion of softwood is challenging due to its high recalcitrance. One of the important reasons for its recalcitrance is its high lignin amount and resistant lignin structure[230]. Organosolv pretreatment of softwood was considered one of the most efficient methods to fractionate cellulose, hemicellulose, and lignin[231]. The effect of isolated lignins on enzymatic hydrolysis was also different based on the types of biomass. For example, Nakagame, etc., reported that organosolv lignins from hardwood and herbals were stimulatory or only had negligible effect on enzymatic hydrolysis of Avicel, while the organosolv lignin from softwood was detrimental to the enzymatic hydrolysis[136]. Lai etc. reported that ethanol organosolv lignins from sweetgum were stimulatory for enzymatic hydrolysis, while the ethanol organosolv softwood lignins with similar pretreatment conditions from pine were detrimental for the enzymatic hydrolysis[94]. Based on the effect of organosolv and extractable lignins from poplar and aspen from the previous two chapters, it is meaningful to determine the effect of methanol, ethanol, and propanol organosolv lignins and extractable lignins from softwood and compare it with that of hardwood. This leads to an extensive understanding of the effect of organosolv lignins on enzymatic hydrolysis of lignocellulosic biomass.

In this chapter, the effect of organosolv lignins and extractable lignins from hardwood (aspen) and softwood (Loblolly pine) was investigated on enzymatic hydrolysis. After the washing of 65% methanol, ethanol, and propanol, the enzyme digestibility of methanol, ethanol, and propanol pretreated aspen and pine was determined. The hydrophobicity and enzyme adsorption

of organosolv lignins and extractable lignins was carried out to determine the interaction between lignins and cellulase. Quantitative ^{13}C and 2D-HSQC NMR analysis of organosolv and extractable lignins from aspen and pine were conducted to examine the chemical structure.

5.3 Material and methods

5.3.1 Cellulase enzymes and biomass

Aspen (*Populus tremuloides*) wood chips (moisture content 9.8%, chip size 5 mm × 2 mm) was Kaytee small animal aspen bedding purchased from Amazon. Loblolly Pine (*Pinus taeda*) wood chips (moisture content 9.5%, chip size 10 mm × 3 mm) were obtained from INL Bioenergy Feedstock Library.

The information of chemical reagents including methanol, ethanol, propanol, sulfuric acid, and sodium hydroxide for organosolv pretreatment, the Avicel, and cellulase enzymes for the enzymatic hydrolysis, Bengal Rose for lignin hydrophobicity, Coomassie Protein Assay kit for enzyme adsorption.

5.3.2 Organosolv pretreatment

The aspen or pine wood chips (100 g, dry weight) were soaked in 700 mL 65% methanol/ethanol/propanol water solution with 1 % (w/w, sulfuric acid/dry biomass) of sulfuric acid overnight before the hydrothermal process of organosolv pretreatment. The mixture was transferred into a 2-L stainless steel Par batch reactor with a stirrer and a PID controller (Parr 4848, USA) and heated to 160°C for aspen was set to be 160 °C, 170 °C for pine. After 1 h hydrothermal cooking, the reactor was water quenched to room temperature to terminate the reaction. The solid and the liquid fraction were separated from the reacted slurry by vacuum filtration using Whatman

No.1 filter paper. The spent liquor (liquid part) was kept for organosolv lignin precipitation. The pretreated substrates (solid part) were washed and kept for enzymatic hydrolysis and analysis.

The organosolv lignins from methanol, ethanol, and propanol pretreated aspen were designated as MOL-AS, EOL-AS, POL-AS, respectively. The organosolv lignins from methanol, ethanol, and propanol pretreated aspen were designated as MOL-LP, EOL-LP, POL-LP, respectively. The corresponding extractable lignins were named as EL-AS(M), EL-AS(E), EL-AS(P), EL-LP(M), EL-LP(E), and EL-LP(P), respectively. The water washed methanol, ethanol, propanol pretreated aspen was named MPAS-H_2O, EPAS- H_2O, PPAS-H_2O. The water washed methanol, ethanol, propanol pretreated pine was named MPLP-H_2O, EPLP-H_2O, PPLP-H_2O. The 65% methanol washed methanol pretreated aspen and pine after water wash was named MPAS and MPLP. The 65% ethanol washed methanol pretreated aspen and pine after water wash was named EPAS and EPLP. The 65% propanol washed propanol pretreated aspen and pine after water wash was named PPAS and PPLP.

5.3.3 Enzymatic hydrolysis of Avicel and pretreated substrates with lignins

Enzymatic hydrolysis of Avicel and pretreated substrates with lignins were carried out in 250 mL Erlenmeyer flasks with stoppers at 50 ℃, 150 rpm in 50 mM sodium citrate buffer (pH 4.8) with 2% glucan (w/v) for 72 h. The enzyme loading for all hydrolysis was chosen to be 5 FPU/g glucan. 4 g/L of lignins were added to the enzymatic hydrolysis 1 h prior to the addition of cellulase at room temperature to determine the effect of different organosolv lignins and extractable lignins. All experiments were carried out in duplicates.

5.3.4 HPLC analysis and FTIR analysis

The concentration of glucose from enzymatic hydrolysis was analyzed by an HPLC system (Agilent 1260 Infinity) with a Hi-Plex H guard column (5 × 3 mm, Agilent), a Hi-Plex H column (300 × 7.8 mm, Agilent) and a RID detector. 5 mM H_2SO4 in aqueous solution was used as mobile phase with a flow rate of 0.6 mL·min^{-1}. The detector and oven temperatures were both set to be 45°C.

The FTIR spectrometer (Perkin Elmer Spectrum Two FTIR system) was employed to examine the changes of chemical structures of the organosolv pretreated lignin compared to the mill wood lignin. ATR technique was used for the FTIR sampling. The scanning wavenumber was set from 500 to 4000 cm^{-1}, and the interval was 0.50 cm^{-1}.

5.3.5 Determination of hydrophobicity and enzyme adsorption isotherms of lignins

Increasing concentrations of the lignins (0.2-4.0 g/L) were mixed with 40 mg/L Rose Bengal in citrate buffer (50 mM, pH 4.8) and incubated at 50 °C, 150 rpm for 2 h. The free Rose Bengal dye in the supernatant and the lignins were separated by centrifugation of 10000 rpm, 5 min. The free dye concentration was determined by UV-Vis spectrometer at 543 nm, showing the difference between the initial dye to calculate the adsorbed dye on the lignins.

Enzyme adsorption on lignins was conducted with 1% (w/v) of lignins or glucan in the substrates at 4 °C and 150 rpm for 3 h. Different initial concentrations of cellulase C2730 (six points between 0.01-0.4 mg protein/mL) were applied with 50 mM citrate buffer. After the adsorption equilibrium at 3 h, the samples were centrifuged for 10,000 rpm for 5 min. The adsorbed enzyme was the difference between the initial protein amount and the free protein amount in the sample solution.

5.3.6 NMR spectroscopic analysis of lignins

NMR spectroscopy analysis of all organosolv from poplar and eucalyptus was conducted on a Bruker Avance III HD Ascend 700 MHz spectrometer. 60 mg of freeze-dried lignin sample was dissolved in 0.5 ml of DMSO-d_6 to collect ^{1}H and quantitative ^{13}C spectra and 2D HSQC spectra. 10.5 μs pulse angle, 1.95 s acquisition time, 2 s relaxation delay with a total of 32 scans per sample was conducted as the operating condition for ^{1}H spectra. For quantitative ^{13}C spectra, 9.85 μs pulse angle, 0.5 s acquisition time, 18 s relaxation delay with a total of 768 scans per sample was conducted as the operating condition. Bruker pulse program "hsqcetgpsisp2" was applied for HSQC spectra. The ^{1}H dimension (F_2) was acquired from 12 to 0 ppm with 1024 complex points; the ^{13}C dimension (F_1) was obtained from 160 to 0 ppm with 200 complex points and 100 increments. Acquisition time for HSQC is 60.8 ms for ^{1}H, and 3.55 ms for ^{13}C was applied, and the total acquisition time was 2 h.

5.4 Results and discussion

5.4.1 Effect of organosolv lignins and extractable lignins on enzymatic hydrolysis of Avicel

The characteristic effect of methanol, ethanol, and propanol organosolv lignins from aspen (MOL-AS, EOL-AS, POL-AS) and pine (MOL-LP, EOL-LP, POL-LP, Fig. 40) was compared on the enzymatic hydrolysis of Avicel. For organosolv lignins from aspen, POL-AS and EOL-AS promoted the final hydrolysis yield from 52.34% by 5.83% and 2.52%, while MOL-AS decreased the 72 h yield of enzymatic hydrolysis of Avicel by 1.45% (Fig. 40). A similar stimulatory effect of EOL-AS was reported by Huang etc.[95] Propanol organosolv lignins had the strongest stimulatory effect on enzymatic hydrolysis, which was higher than that of ethanol and methanol organosolv lignins. However, MOL-AS was a little detrimental on final glucose yield of Avicel.

This indicates the effect of organosolv lignin on enzymatic hydrolysis may also depend on the species of wood. On the other hand, methanol, ethanol, and propanol organosolv lignins from pine was inhibitory on enzymatic hydrolysis of Avicel. 72 h yield of enzymatic hydrolysis was decreased from 52.34% by 0.75%, 4.57%, and 3.39% by MOL-LP, EOL-LP, and POL-LP, respectively. A similar stimulatory effect of EOL-LP on enzymatic hydrolysis of Avicel was reported previously[94]. Unlike the trend of the effect organosolv lignin from hardwood on hydrolysis, methanol organosolv lignin from pine did not have the strongest detrimental effect. EOL-LP had the strongest inhibition, while MOL-LP had the lowest inhibitory effect but not POL-LP. This shows the effect of different solvents on organosolv lignins was distinguish between hardwood and softwood.

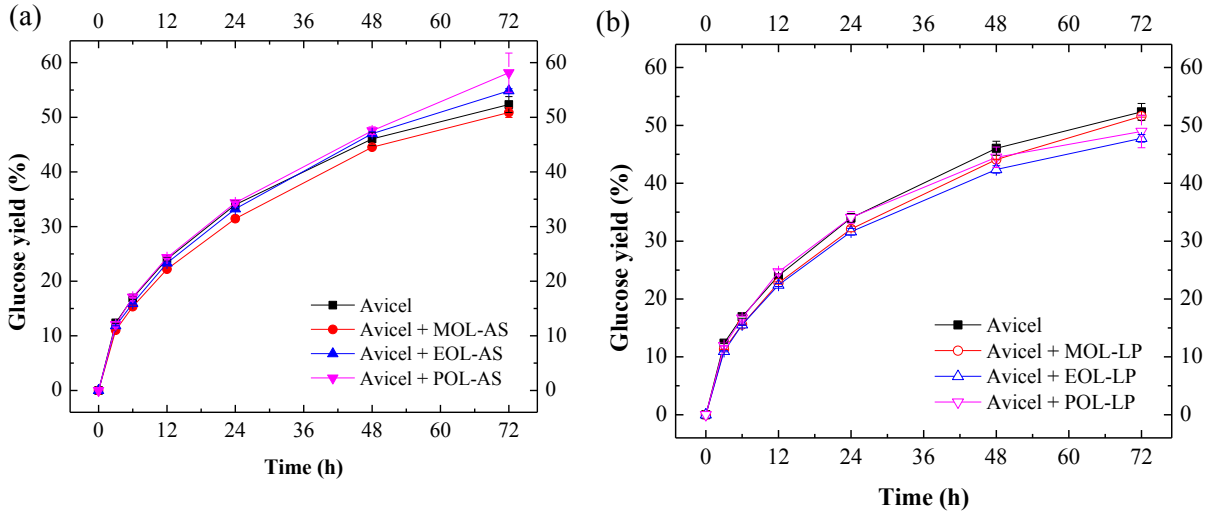


Fig. 40 Effect of organosolv lignins from aspen (a) and pine (b) on enzymatic hydrolysis of Avicel

For the effect of extractable lignins from organosolv pretreated aspen and pine, they were all detrimental to the enzymatic hydrolysis of Avicel (Fig. 41). EL-AS(M), EL-AS(E), and EL-

AS(P) decreased the final yield from 50.02% by 6.05%, 7.31%, and 7.83% to 42.18%, 42.71%, and 43.96%, respectively. While EL-LP(M), EL-LP(E), and EL-LP(P) decreased the final yield of enzymatic hydrolysis of Avicel from 55.66% by 19.39%, 21.67%, and 19.59% to 36.27%, 33.99%, and 36.06%, respectively. The trend of the effect of extractable lignins from methanol, ethanol, and propanol pretreated aspen and pine was the same as that of organosolv lignins on enzymatic hydrolysis of Avicel, which extractable lignins from methanol pretreated substrates had the strongest inhibitory effect than from ethanol pretreated substrates. And EL-AS(P) was the least detrimental. A similar trend showed between organosolv lignins and extractable lignins from pine. EL-LP(M) had the lowest inhibition on Avicel hydrolysis, which was lower than EL-LP(P). And EL-LP(E) had the strongest detrimental effect. This result could correlate because both water extractives and ethanol extractives from pinewood were detrimental to enzymatic hydrolysis[232]. No matter for organosolv lignins from hardwood or softwood, extractable lignins were more inhibitory in the enzymatic hydrolysis of Avicel than that of the corresponding organosolv lignins. This confirmed the indication from chapter 4. Furthermore, the addition of organosolv lignins and extractable lignins from both woods decreased the initial hydrolysis rates of Avicel.

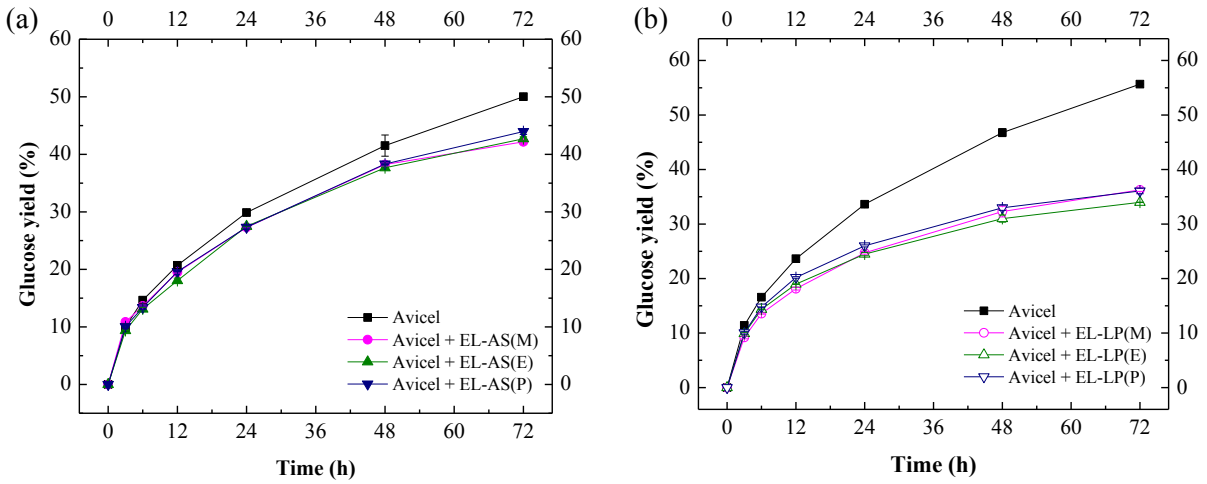


Fig. 41 Effect of extractable lignins from aspen (a) and pine (b) on enzymatic hydrolysis of Avicel

Table 23 Initial hydrolysis rates and 72 h glucose yield of enzymatic hydrolysis of Avicel with organosolv and extractable lignins

Lignins addition	Initial hydrolysis rate (g/L/h)	72 h hydrolysis yield (%)
No addition	4.12 ± 0.02	52.34 ± 1.46
MOL-AS	3.68 ± 0.00	50.88 ± 0.91
EOL-AS	3.95 ± 0.03	54.86 ± 0.39
POL-AS	4.02 ± 0.15	58.16 ± 0.84
MOL-LP	3.76 ± 0.02	51.59 ± 0.17
EOL-LP	3.66 ± 0.03	47.76 ± 0.66
POL-LP	3.97 ± 0.02	50.88 ± 2.81
EL-AS(M)	3.62 ± 0.04	42.18 ± 0.24
EL-AS(E)	3.12 ± 0.02	42.71 ± 0.33
EL-AS(P)	3.35 ± 0.03	43.96 ± 0.51
EL-LP(M)	3.07 ± 0.09	36.27 ± 0.28
EL-LP(E)	3.30 ± 0.08	33.99 ± 0.05
EL-LP(P)	3.35 ± 0.07	36.06 ± 0.50

5.4.2 Enzyme adsorption and hydrophobicity of organosolv and extractable lignins

To quantify the binding strength of cellulase on lignins, cellulase adsorption on organosolv lignins and extractable lignins was investigated and fitted with the Langmuir model (Table 29). Γ_{max} showed the adsorption capacity of cellulase on lignins. The adsorption capacity (Γ_{max}) of extractable lignins (EL-AS(M): 10.56 mg/g, EL-AS(E): 7.487 mg/g, EL-AS(P): 7.593 mg/g, EL-LP(M): 8.475 mg/g, EL-LP(E): 9.413 mg/g, EL-LP(P): 8.874 mg/g) were higher than the capacity of all organosolv lignins (MOL-AS: 4.058 mg/g, EOL-AS: 4.428 mg/g, POL-AS: 3.718 mg/g,

MOL-LP: 7.868 mg/g, EOL-LP: 7.192 mg/g, POL-LP: 6.975 mg/g). This indicates that extractable lignins had more binding sites of cellulase on the lignin surface than that of organosolv lignins[221]. Langmuir constant (*K*) shows the affinity of cellulase on the lignins. Extractable lignins from pine (EL-LP(M): 11.14 mL/mg, EL-LP(E): 21.90 mL/mg, EL-LP(P): 14.83 mL/mg) had higher cellulase affinity (*K*) than that of organosolv lignins (MOL-LP: 4.781 mL/mg, EOL-LP: 9.968 mL/mg, POL-LP: 7.993 mL/mg), while organosolv lignins from aspen (MOL-AS: 10.29 mL/mg, EOL-AS: 6.024 mL/mg, POL-AS: 5.993 mL/mg) had similar affinity with cellulase to that of extractable lignins (EL-AS(M): 6.202 mL/mg, EL-AS(E): 9.527 mL/mg, EL-AS(P): 7.280 mL/mg).

Distribution coefficient (*R*) was reported to be a good indicator of the binding strength of cellulase on the lignins, so the distribution coefficient of organosolv lignins and extractable lignins[95]. Extractable lignins from aspen (EL-AS(M): 0.065 L/g, EL-AS(E)-E65: 0.071 L/g, EX-POL-AS: 0.055 L/g) had higher cellulase binding strength than that of organosolv lignins organosolv lignins from aspen (MOL-AS: 0.042 L/g, EOL-AS: 0.027 L/g, POL-AS: 0.022 L/g). Extractable lignins from pine (EL-LP(M): 0.094 L/g, EL-LP(E): 0.206 L/g, EL-LP(P): 0.132 L/g) also had higher cellulase binding strength than that of organosolv lignins (MOL-LP: 0.038 L/g, EOL-LP: 0.072 L/g, POL-LP: 0.056 L/g). This explained why the extractable lignin had a stronger negative effect on the enzymatic hydrolysis of Avicel than that of the corresponding organosolv lignins. The binding strength of extractable lignins from pine was much higher than extractable lignins from aspen and organosolv lignins from aspen and pine. This may be the reason for their strong inhibition (decreased around 20%).

Table 24 Enzyme adsorption parameters based on Langmuir isotherm

Cellulases	Γ_{max} (mg/g)	K (mL/mg)	R (L/g)

Cellulase on MOL-AS	6.100	4.080	0.025
Cellulase on EOL-AS	3.884	5.883	0.023
Cellulase on POL-AS	2.408	8.154	0.020
Cellulase on MOL-LP	6.183	6.511	0.040
Cellulase on EOL-LP	6.290	12.29	0.077
Cellulase on POL-LP	5.365	12.51	0.067
Cellulase on EL-AS(M)	8.812	8.312	0.073
Cellulase on EL-AS(E)	7.115	9.586	0.068
Cellulase on EL-AS(P)	7.593	7.280	0.055
Cellulase on EL-LP(M)	7.937	10.80	0.086
Cellulase on EL-LP(E)	9.498	21.80	0.207
Cellulase on EL-LP(P)	8.709	14.88	0.130

Hydrophobicity of lignins indicates the non-specific adsorption of cellulase enzymes on lignins[95]. Surface hydrophobicity of organosolv and extractable lignin from aspen and pine was determined (Table 30). Extractable lignins from aspen (EL-AS(M): 1.08 L/g, EL-AS(E): 0.81 L/g, EL-AS(P): 0.55 L/g) had higher surface hydrophobicity than that of organosolv lignins organosolv lignins from aspen (MOL-AS: 0.31 L/g, EOL-AS: 0.16 L/g, POL-AS: 0.07 L/g). Extractable lignins from pine (EL-LP(M): 1.64 L/g, EL-LP(E): 2.25 L/g, EL-LP(P): 2.00 L/g) also had higher hydrophobicity than that of organosolv lignins (MOL-LP: 0.41 L/g, EOL-LP: 0.53 L/g, POL-LP: 0.62 L/g). This explained why the extractable lignins from aspen and pine both had a stronger negative effect on the enzymatic hydrolysis of Avicel than that of the corresponding organosolv lignins. The hydrophobicity of extractable lignins from pine was much higher than extractable lignins from aspen and organosolv lignins from aspen and pine. This may also be the reason for their strong inhibition in enzymatic hydrolysis of Avicel.

Table 25 Hydrophobicity of organosolv lignins and extractable lignins from aspen and pine

Lignin	Hydrophobicity (L/g)
MOL-AS	0.31 ± 0.02
EOL-AS	0.16 ± 0.02
POL-AS	0.07 ± 0.01

MOL-LP	0.41 ± 0.04
EOL-LP	0.53 ± 0.01
POL-LP	0.62 ± 0.04
EL-AS(M)	1.08 ± 0.05
EL-AS(E)	0.81 ± 0.01
EL-AS(P)	0.55 ± 0.08
EL-LP(M)	1.64 ± 0.13
EL-LP(E)	2.25 ± 0.07
EL-LP(P)	2.00 ± 0.20

5.4.3 Effect of solvent washing on the enzyme digestibility of pretreated substrates

Since extractable lignins from softwood were much more detrimental than that of hardwood. It would be meaningful to determine the effect of solvent washing on the digestibility of pretreated substrates for hardwood and softwood. To test the effect of solvent washing on the enzyme digestibility of organosolv pretreated substrates, the enzymatic hydrolysis of methanol, ethanol and propanol pretreated hardwood and softwood substrates with water wash was compared to that of solvent washed substrates. The effect of solvent washing on enzyme digestibility was conducted and summarized in Fig. 42. Methanol extraction decreased the methanol pretreated aspen and pine from 74.83% and 27.45% by 12.11% and 7.21%. Ethanol extraction decreased the ethanol pretreated aspen and pine from 67.38% and 48.22% by 12.37% and 3.65%. propanol washing decreased the propanol pretreated aspen and pine from 67.18% and 71.57% by 9.68% and 12.26%. The digestibility of organosolv pretreated substrates was all decreased with solvent extraction and the water washed substrates all had higher enzyme digestibility. This confirmed the results from the effect of ethanol washing of ethanol pretreated aspen in chapter 4 that regardless of how strong the inhibition of extractable lignin was, solvent washing decreased the enzyme digestibility of pretreated substrates. From the view of substrates digestibility, water wash is the better process and solvent washing solvent extraction is not necessary for organosolv pretreatment.

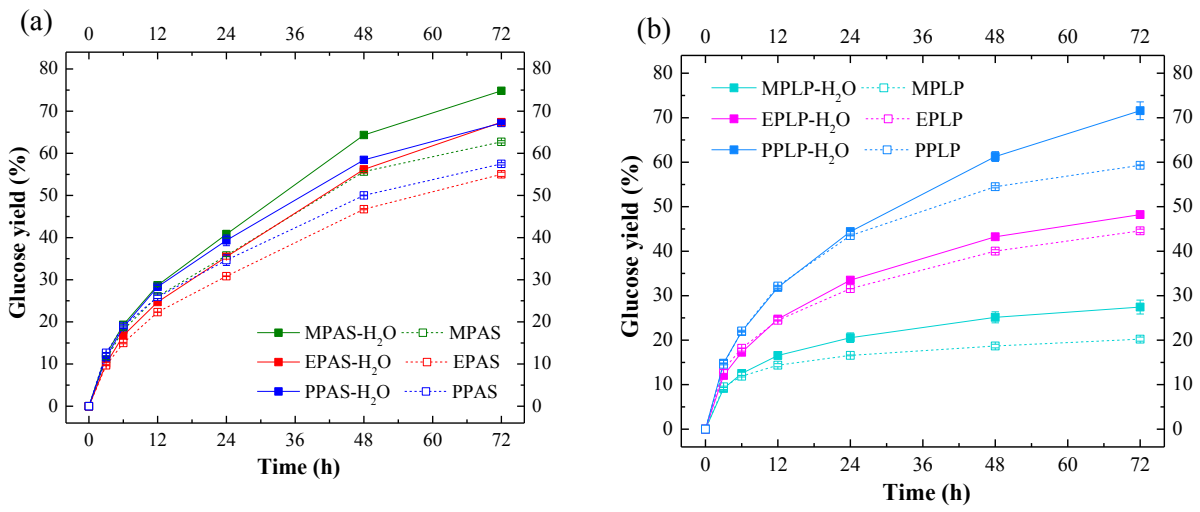
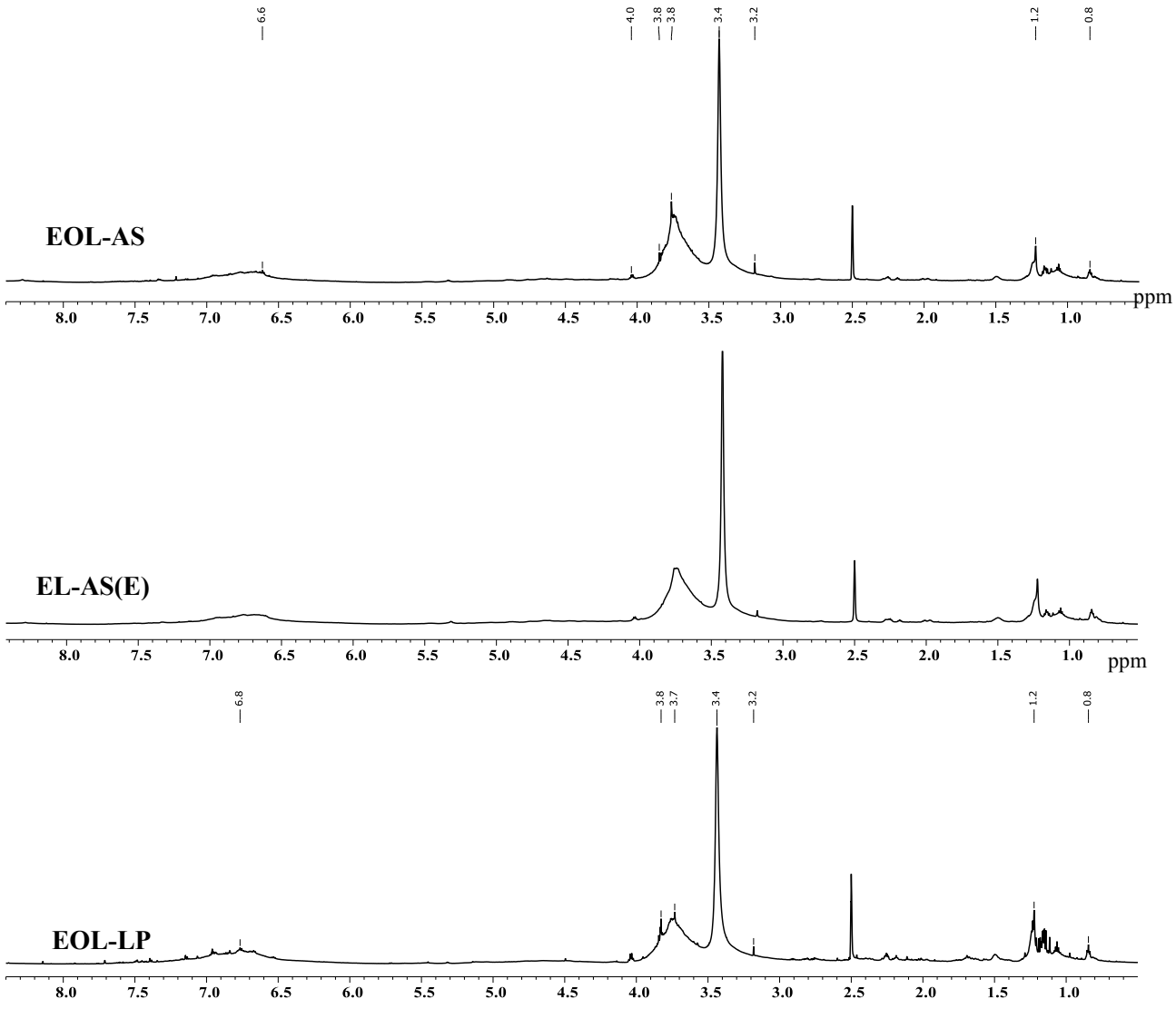


Fig. 42 Effect solvent washing on enzyme digestibility of pretreated aspen (a) and pine (b)

5.4.4 NMR analysis of organosolv lignins and extractable lignins from aspen and pine

To examine the potential reason for the distinctive effect of EOL and extractable lignin based on their chemical structures, ^{1}H, quantitative ^{13}C NMR, and 2D HSQC were conducted to characterize EOL-AS, EOL-LP, EL-AS(E), and EL-LP(E). Since the EOL and extractable lignins were generated from the same reaction system, no major chemical structure changes were discovered in the ^{1}H NMR spectra (Fig. 44). No major chemical structure changes were discovered in the ^{13}C NMR spectra (Fig. 45 Table 30). Similar results were reported from the ethanol extractable lignin and EOL from sweetgum with ^{13}C NMR[103]. Carboxylic acid modification of lignin increased the carboxylic acid content of lignin and enhanced the enzymatic hydrolysis[141]. Higher carboxylic acid content was always related to higher electrostatic repulsion, lower non-specific adsorption, and less detrimental effect on enzymatic hydrolysis. Meanwhile, less non-etherified $S_{3,5}$ and G_3 (δ 150.0-145.0 ppm) and CH_3O (δ 58.0-54.0) was observed in the E65 than in EOL-AS. This may indicate less phenolic hydroxyl groups in the EL-AS(E65). Although phenolic hydroxyl groups were proved to be related to the inhibition of lignins, this effect may not

be predominant, so the decrease of phenolic hydroxyl groups did not abate the inhibition of EL-AS(E65)[144]. The degree of condensation of EOL-AS and EL-AS(E65) was reported to be the same with EOL-AS and EL-AS(E65) [233] [234] [228] [230] [231] [238] [245] [251] [253] [254] [261] [261] [261][261]. Therefore, more information about the condensation should be considered with other quantification and spectrum.



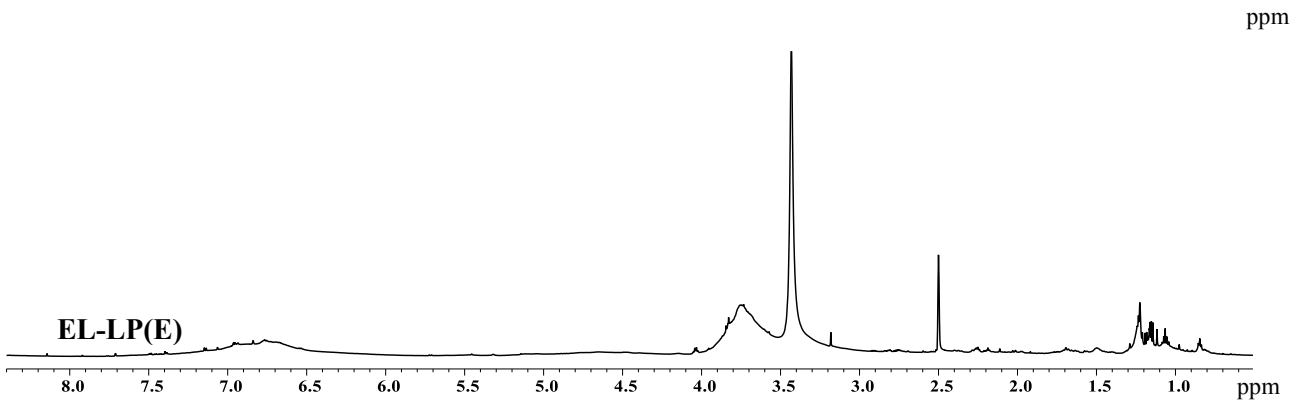
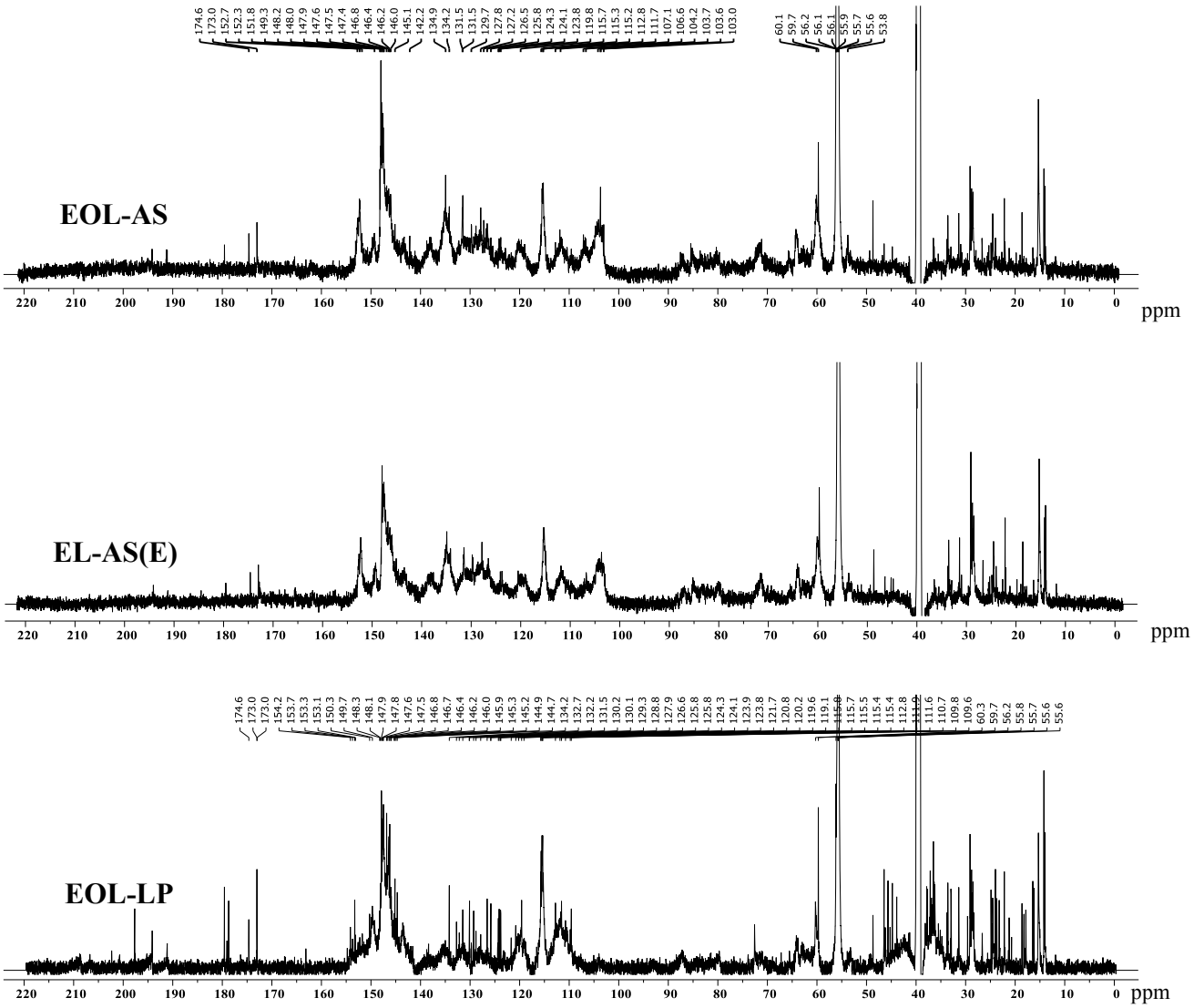


Fig. 43 ^{1}H spectra of EOL-AS, EL-AS(E), EOL-LP and EL-LP(E).



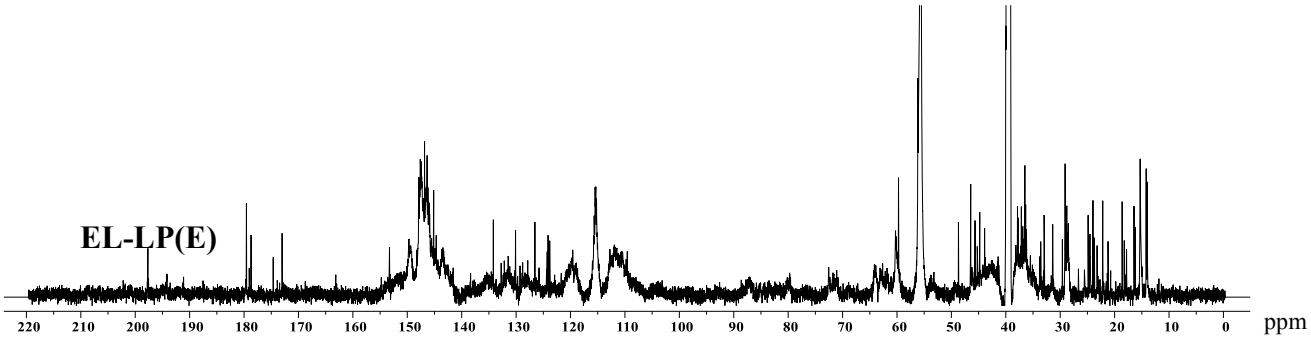


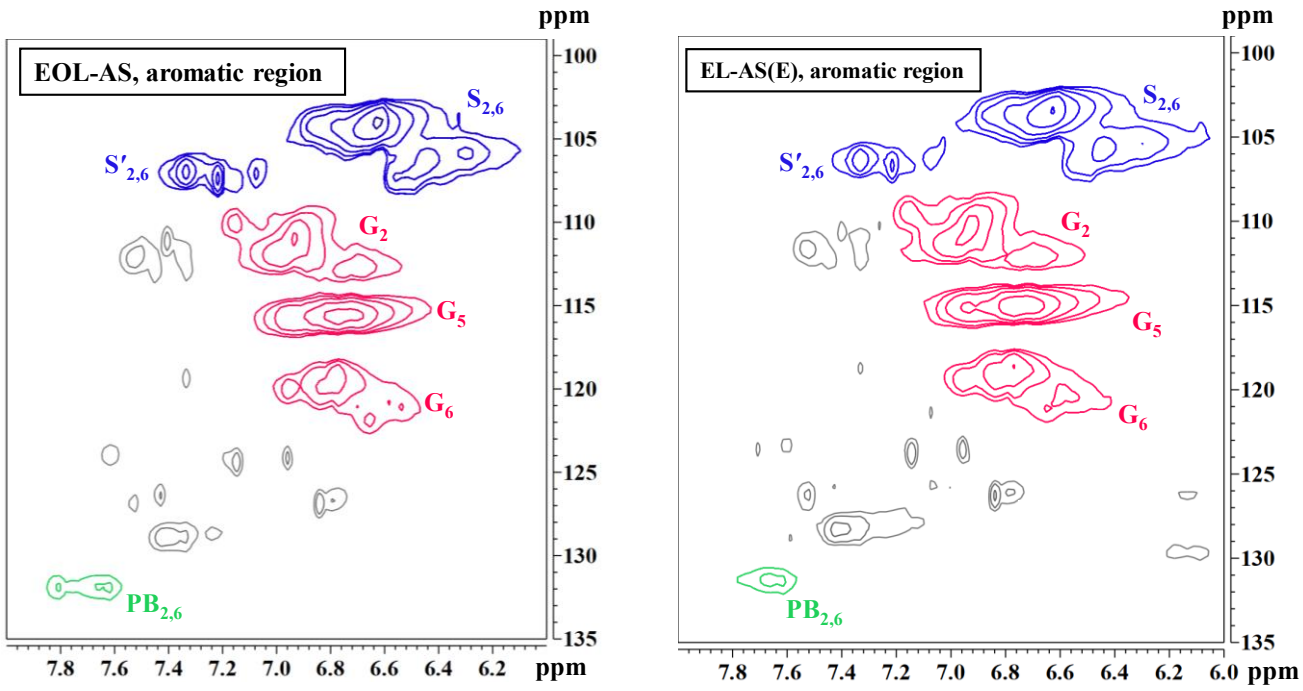
Fig. 44 Quantitative ^{13}C spectra of EOL-AS, EL-AS(E), EOL-LP and EL-LP(E).

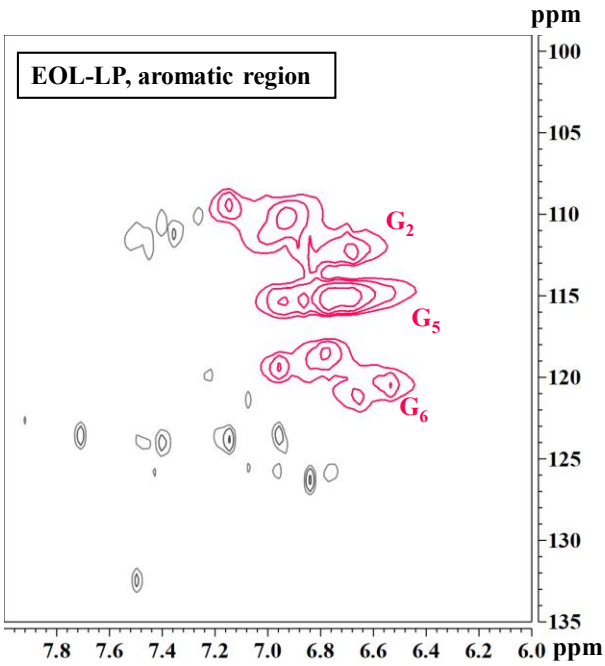
Table 26 The quantitative analysis of the signals of quantitative ^{13}C spectra

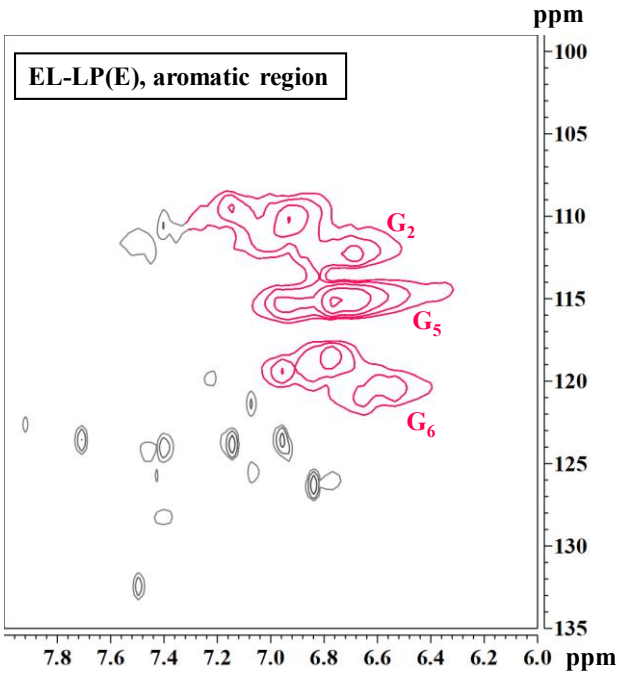
δ (ppm)	Assignment	EOL-AS	EL-AS(E)	EOL-LP	EL-LP(E)
175.0-168.0	Carboxylic acid or ester	0.20	0.19	0.16	0.16
155.0-140.0	Aromatic C-O	2.08	2.04	2.77	2.77
140.0-124.0	Aromatic C-C	1.98	2.02	1.09	1.05
124.0-102.0	Aromatic C-H	1.94	1.94	2.14	2.19
132.7-130.8	PB	0.26	0.26	0	0
157.0-151.0	$S_{3,5}$ with C_4OR	0.41	0.40	0.39	0.29
150.0-145.0	G_3, $S_{3,5}$ with C_4OH	1.24	1.18	1.78	1.79

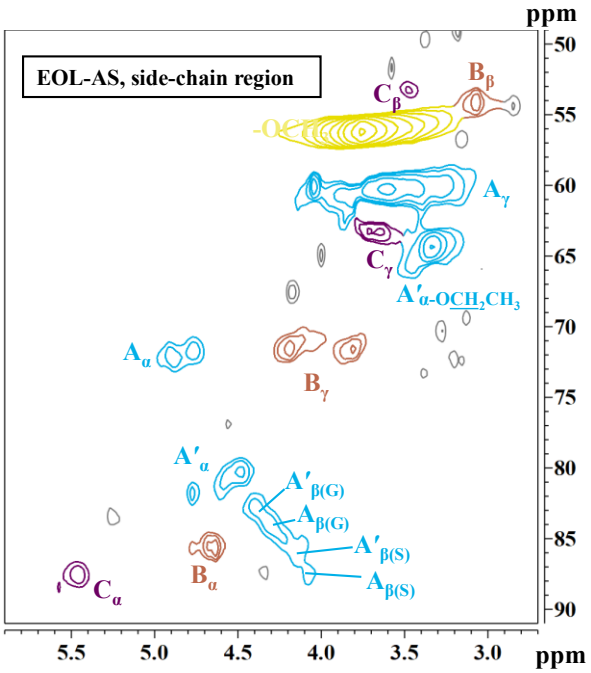
2D HSQC were conducted to characterize EOL-AS and EL-AS(E65. With 65% ethanol wash, although the β-β, β-5 (δ 54-53 ppm) linkage observed to be unchanged in quantitative ^{13}C, the β-O-4 (δ 61.3-58.0 ppm) linkage amount was decreasing. More details were revealed in the 2D HSQC spectra and quantitative analysis of the lignin fraction by integration (Figure 27, Table 23 and Table 24). The inter-unit linkage of β-aryl-ether (β-O-4, A), resinol (β-β, B), phenylcoumaran (β-5, C), were identified by their cross peaks at δ_H/δ_C 71.95/4.85 (A_α), 83.03/4.34 ($A_{\beta(G)}$), 85.76/4.14 ($A_{\beta(S)}$), 60.26/3.60 (A_γ), 85.41/4.66 (B_α), 53.87/3.08 (B_β), 71.68/3.81-4.17 (B_γ), 87.50/5.45 (C_α), 53.23/3.47 (C_β) and 63.17/3.66 (C_γ), respectively. HSQC quantitative analysis showed that the β-O-4 linkages ratio in EL-AS(E) (73.4%) was similar to that in EOL-AS (78%). β-β and β-5 linkages ratio in EL-AS(E65 (18.5%, 8.1%) was also similar to that in EOL-AS (15.5%,

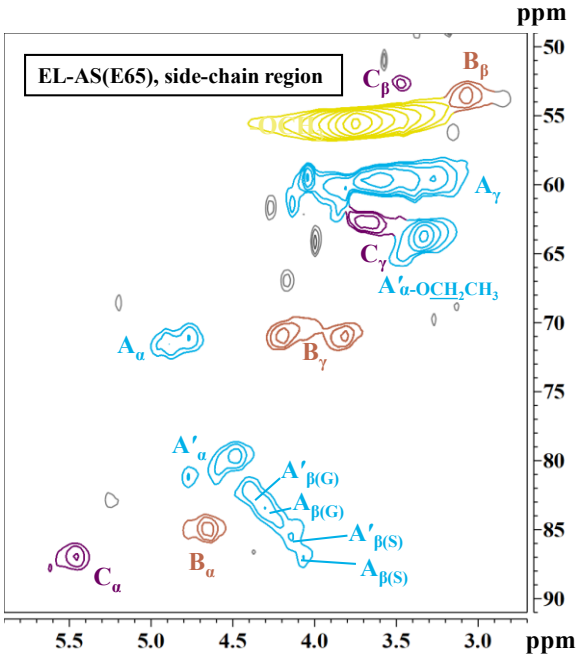
6.4%). Higher β-β and β-5 both reported to be related to higher condensation, more non-specific adsorption and more inhibition[234, 235]. Recently, β-5 linkages were reported to have positive correlation with enzyme adsorption, which indicated higher non-specific adsorption of cellulase[201]. Therefore, detrimental effect of extractable lignins may be only related to their difference in physical properties like hydrophobicity. Similar β-O-4 (EOL-LP: 61.5%, EL-LP(E): 59.5%) and β-β linkages ratio (EOL-LP: 13.8%, EL-LP(E): 16.7%) were also investigated in pine lignin. Therefore, no matter for hardwood and softwood, solvent extractable lignins were similar to the organosolv lignins from spent liquor. The reason for low catalytic degradation yield of organosolv lignins is the high ratio of C-C bonds like β-β and β-5, which conserve higher bond energy[236]. So when the yield of total lignin amount matters, solvent washing was still meaningful to combine the solvent extracts and the spent liquor as recommended in the tradition lab scale organosolv pretreatment process[17].

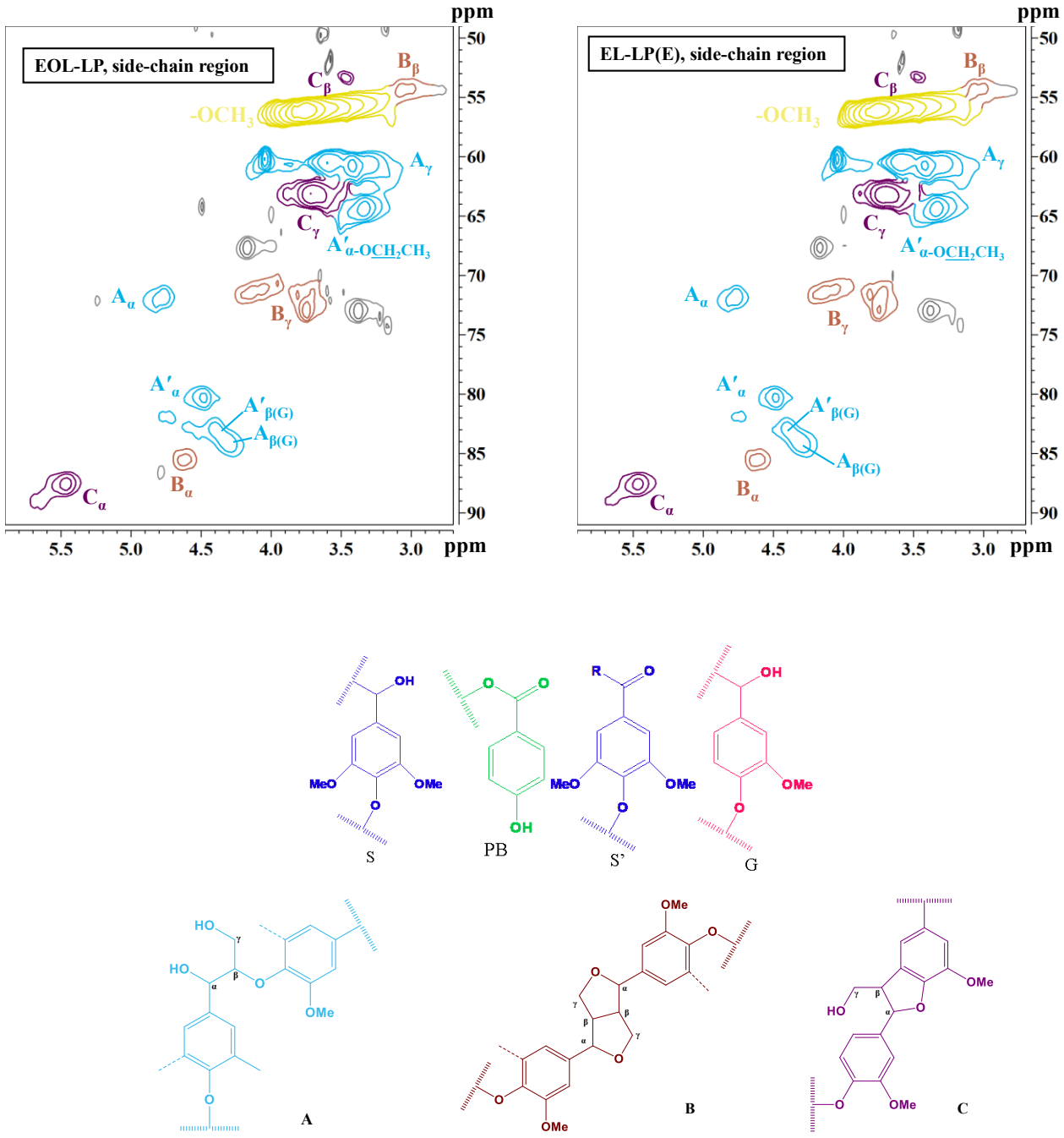



EOL-LP, aromatic region
ppm
G2
G5
G6
7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 ppm


EL-LP(E), aromatic region
ppm
G2
G5
G6
7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 ppm


EOL-AS, side-chain region
ppm
Bβ
Cβ
Aγ
Cγ
A′α-OCH2CH3
Aα
Bγ
A′α
A′β(G)
Aβ(G)
A′β(S)
Aβ(S)
Bα
Cα
5.5 5.0 4.5 4.0 3.5 3.0 ppm


EL-AS(E65), side-chain region
ppm
Bβ
Cβ
Aγ
Cγ
A′α-OCH2CH3
Aα
Bγ
A′α
A′β(G)
Aβ(G)
A′β(S)
Aβ(S)
Bα
Cα
5.5 5.0 4.5 4.0 3.5 3.0 ppm



(G: guaiacyl units; S: syringyl units; S': oxidized syringyl units bearing a carbonyl at C_α; PB: *p*-hydroxybenzoate units; A: β-aryl-ether units (β-O-4); B: resinol substructures (β-β); C: phenyl-coumaran substructures (β-5).)

Fig. 45 2D-HSQC spectra of lignins

Table 27 Quantification analysis of the HSQC signals

Lignins	S/G	β-O-4 (%)	β-β (%)	β-5 (%)	PB (%)
EOL-AS	0.50	78.0	15.5	6.4	3.1
EL-AS(E)	0.39	73.4	18.5	8.1	2.3
EOL-LP	0.01	61.5	13.8	24.8	ND
EL-LP(E)	0.01	59.5	16.7	23.9	ND

5.4.5 SEM images of EOL and ethanol extractable lignins from aspen and pine

To examine the effect of ethanol extraction on the surface morphology of lignins, the SEM images of EOL-AS, EL-AS(E), EOL-LP, EL-LP(E) were collected with 5-20 μm (Fig. 46). No huge difference was discovered between aspen and pine and between EOL lignins and extractable lignins.

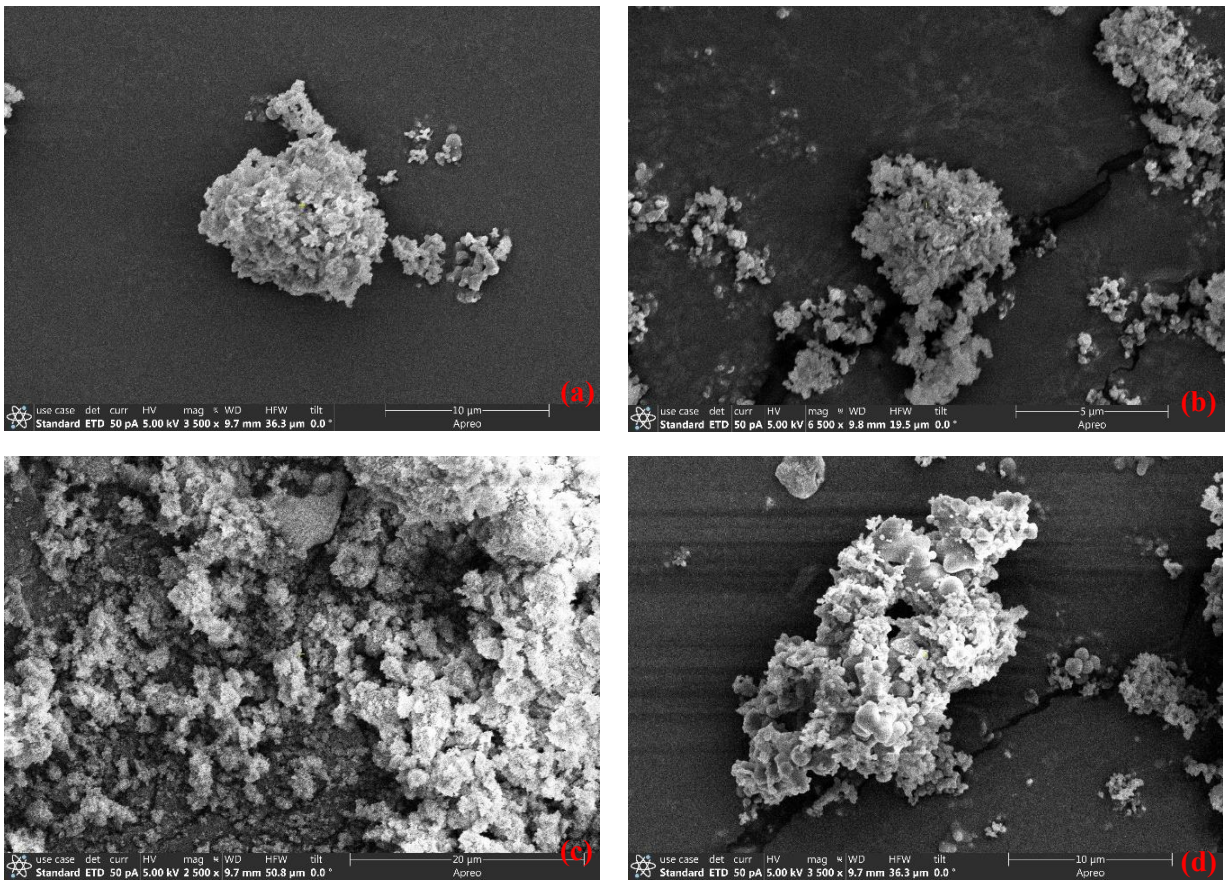


Fig. 46 Scanning electron microscopy (SEM) images of EOL lignins and ethanol extractable lignins from aspen and Loblolly pine: ethanol organosolv aspen lignin (EOL-AS, a), EL-AS(E) (b), EOL-LP (c) and EL-LP(E) (d).

5.4.6 FTIR analysis of EOL and ethanol extractable lignins from aspen and pine

Fig. 47 illustrates the FT-IR spectra of EOL and extractable lignins from aspen and pine. As can be seen, the FTIR absorbance of EOL and extractable lignins from aspen has no huge difference when compared to each other. This was probably due to the same reaction condition they came from.

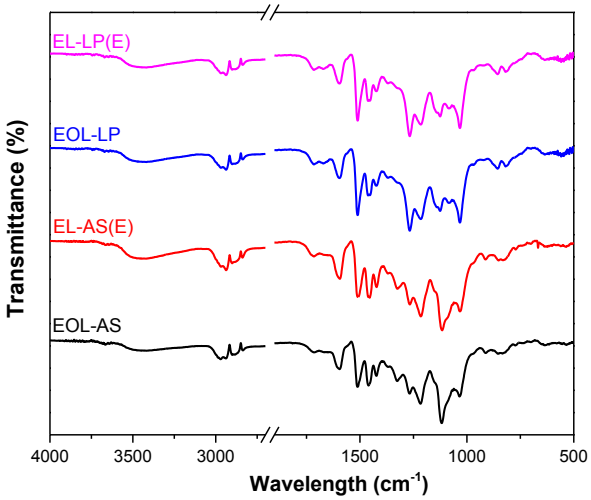


Fig. 47 FTIR analysis of EOL lignins and ethanol extractable lignins from aspen and pine

5.4.7 Chemical composition analysis of organosolv pretreated aspen and pine

To further explain the effect of pretreatment of different solvents and effect of solvent washing on enzyme digestibility of pretreated substrates, the chemical composition of methanol, ethanol, and propanol organosolv pretreatment with or without solvent washing was determined (Table 33). With solvent washing, extractives of substrates decreased from 14-27% to 1-6%. This

may be one potential reason for the decrease of enzyme digestibility after ethanol extraction because some extractive molecules from hardwood were proved to be beneficial to the enzymatic hydrolysis[157]. The glucan and xylan of substrates after solvent washing were higher in substrates (MPAS, EPAS, PPAS, MPLP, EPLP, PPLP). Interestingly, for both aspen and pine, glucan (major component of cellulose) was the highest in propanol pretreated substrates (PPAS: 91.20%, PPLP: 88.86%) compared to the other organosolv pretreated substrates after solvent washing (MPAS: 82.69%, EPAS:82.68%, MPLP: 81.37%, EPLP:76.52%). While xylan (major component of hemicellulose) and acid insoluble lignin (AIL) were lowest in in propanol pretreated substrates (PPAS: xylan: 5.76%, AIL:2.84%; PPLP: xylan: 2.17%, AIL:6.08%;) than other pretreated substrates (MPAS: xylan: 5.96%, AIL:7.40%; EPAS: xylan: 6.27%, AIL:4.77%; MPLP: xylan: 4.98%, AIL:7.12%; EPLP: xylan: 2.28%, AIL:8.85%;), when the acid soluble lignin (ASL) was similarly 1-2%. This indicates that propanol organosolv pretreatment could be considered an efficient pretreatment process from the review of cellulose recovery, delignification, and hemicellulose removal.

Table 28 Chemical composition analysis of the organosolv pretreated aspen and pine

Substrates	Glucan	Xylan	Galactan	Arabinan	Mannan	ASL	AIL	Extractive	**SUM**
MPAS-H_2O	61.18 ± 0.03	3.30 ± 0.15	0.00	0.00	1.96 ± 0.55	0.61 ± 0.00	4.57 ± 0.25	27.51	99.13
MPAS	82.69 ± 0.80	5.96 ± 0.04	0.00	0.00	4.79 ± 0.43	1.06 ± 0.00	7.40 ± 0.48	5.01	103.90
EPAS-H_2O	59.13 ± 2.17	4.02 ± 0.01	0.00	0.00	2.85 ± 0.45	0.39 ± 0.00	4.62 ± 0.49	23.18	94.18
EPAS	82.68 ± 0.05	6.27 ± 0.04	0.00	0.00	3.39 ± 0.15	0.69 ± 0.01	4.77 ± 0.36	2.16	99.96
PPAS-H_2O	72.23 ± 0.62	2.86 ± 0.13	0.00	0.00	3.56 ± 0.32	0.90 ± 0.01	4.18 ± 1.35	18.66	102.39
PPAS	91.20 ± 1.44	5.76 ± 0.22	0.00	0.00	5.30 ± 0.18	1.25 ± 0.02	2.84 ± 0.07	1.62	102.99
MPLP-H_2O	61.67 ± 0.52	2.13 ± 0.87	0.00	0.00	3.34 ± 0.69	1.36 ± 0.00	14.22 ± 0.73	14.09	96.81
MPLP	81.37 ± 0.78	4.98 ± 0.59	0.00	0.00	4.05 ± 0.68	2.19 ± 0.00	7.12 ± 0.17	3.77	103.46
EPLP-H_2O	58.89 ± 0.93	2.65 ± 0.85	0.00	0.00	3.44 ± 0.23	0.68 ± 0.01	10.05 ± 0.69	25.83	101.54
EPLP	76.52 ± 0.29	2.28 ± 0.17	0.00	0.00	3.17 ± 0.02	0.94 ± 0.04	8.85 ± 0.71	6.20	97.97
PPLP-H_2O	58.11 ± 0.80	1.34 ± 0.26	0.00	0.00	6.85 ± 0.22	0.75 ± 0.07	13.82 ± 0.71	19.43	100.29
PPLP	88.86 ± 0.05	2.17 ± 0.18	0.00	0.00	3.79 ± 0.13	1.80 ± 0.01	6.08 ± 0.00	2.79	102.50

5.5 Conclusions

Extractable lignin showed greater inhibition than corresponding organosolv lignin toward enzymatic hydrolysis of Avicel. Extractable lignin had higher hydrophobicity and enzyme adsorption binding strength than organosolv lignin. But they had similar chemical structures based on FTIR and NMR analysis. Solvent washing reduced final hydrolysis yield of organosolv pretreated biomass. The results showed that propanol organosolv pretreatment could be more efficient in cellulose recovery, hemicellulose removal, and lignin removal of pretreated biomass. In addition, methanol was not a good solvent for organosolv pretreatment of softwood like such as pine. From the point of view of chemical composition analysis of organosolv pretreated substrates, propanol organosolv pretreatment could be considered efficient because of high cellulose recovery, delignification, and hemicellulose removal.

Chapter 6: Future work and acknowledgement

6.1 Summary

In this work, the effect of organosolv lignin from hardwood poplar, eucalyptus, aspen; softwood Loblolly pine; and herbaceous kenaf has been assessed on the enzymatic hydrolysis of Avicel and pretreated biomass. The effect of extractable lignins from aspen and pine on enzymatic hydrolysis of lignocelluloses was investigated and compared to the organosolv lignins. Their distinguishing effect on enzymatic hydrolysis was explained by the chemical structure characterization by NMR analysis and physical property characterization by determining hydrophobicity, enzyme adsorption, and zeta potential.

methanol, ethanol, and propanol organosolv lignins from poplar, eucalyptus, aspen, Loblolly pine, and kenaf have been evaluated for their effects on enzymatic hydrolysis of lignocelluloses. Two-dimension heteronuclear single quantum coherence spectroscopy HSQC, HSQC-TOCSY, and HMBC have been used to characterize the structural changes of lignins before and after organosolv pretreatment. The spectra of HSQC, HSQC-TOCSY, and HMBC revealed that the alkylation of hydroxyl groups took place not only at C_α, but also at C_β and C_γ, and potentially also at the phenolic hydroxyl group of lignin. These results showed that propanol organosolv lignins (POLs) from poplar and aspen had higher stimulatory effects than the ethanol and methanol organosolv lignins (EOLs and MOLs) on the enzymatic hydrolysis of Avicel. The alkylation degree will affect the hydrophobicity of resulting organosolv lignins, which in turn will control their positive or negative effect on the enzymatic hydrolysis.

Extractable lignins, extracted from pretreated biomass by solvent (methanol, ethanol, propanol), have been accessed for their effect on the enzymatic hydrolysis of lignocelluloses as well in this study. The results showed that extractable lignins from aspen and pine had detrimental effects on

the enzymatic hydrolysis of Avicel. The hydrophobicity, enzyme binding strength, zeta potential, molecular weight, and HSQC NMR spectra of extractable lignins and organosolv lignins have been determined and compared. HSQC spectra showed a similar chemical structure for the organosolv lignins and extractable lignins. It was observed that extractable lignins showed higher hydrophobicity and binding strength than organosolv lignins. In addition, it was observed that 72 h hydrolysis yield of pretreated aspen and pine was decreased by 3-17% when the pretreated substrates were washed with organic solvents.

Since these four chapters are closely connected to each other but conducted with three hardwoods (poplar, eucalyptus, and aspen), one softwood (Loblolly pine), and one herbal lignocellulose (kenaf), some indications could also be drawn across four chapters. Propanol organosolv lignins from poplar, eucalyptus, and aspen had the strongest stimulatory effect on enzymatic hydrolysis. Among these organosolv lignins from hardwood, MOL, EOL, and POL from poplar had the strongest stimulatory effect. This could be correlated to their highest PB ratio and lowest hydrophobicity[95]. This shows the important role played by PB in lignin structure and surface hydrophobicity of organosolv lignins. Overall, solvent washing after the organosolv pretreatment may not be necessary to sacrifice the substrate digestibility. Propanol organosolv pretreatment can be a good pretreatment solvent for both hardwood and softwood regarding cellulose recovery, delignification, and hemicellulose removal.

6.2 Future Work

1) New alkylation mechanisms were reported in this work in methanol and propanol organosolv pretreatment. Propanol lignins were more stimulatory than methanol and ethanol organosolv lignins in enzymatic hydrolysis. However, methanol is not a common solvent to use

because of its toxicity and flammability recently. Furthermore, it is difficult to recover propanol from the aqueous solution because propanol (97 °C) had similar boiling points to that of water (100 °C)[237]. Therefore, methanol and propanol could be good in lab research for the elucidation of lignin structure change in organosolv pretreatment but may be difficult to be applied in the industry. So safer and more accessible organic solvent like GVL should be applied and studied.

2) It is expected that the lignin from the pretreatment could be more degradable, and the substrates could have better digestibility. Organosolv pretreatment is good at the release of carbohydrates, but lignin condensation is still high in organosolv pretreatment even for propanol compared to mill wood lignin of untreated biomass. So the organosolv lignin was always proved to be less susceptible to catalytic degradation. The possible reason is that more C-C bonds, which conserve higher bond energy, were created by the repolymerization during the pretreatment[236]. Lignin condensation was also reported to be a potential reason for inhibitory in enzymatic hydrolysis[238]. To prevent the lignin condensation, propanol works better than ethanol, but some condensation capping agent*s* such as formaldehyde or phenols like 2-naphthol could be added to reduce the lignin condensation further while maintain or even promote the efficiency of pretreatment[188, 239]. Furthermore, the high efficiency of the 'lignin-first' approach to isolate lignin was getting more and more popular better to utilize lignin before the condensation in the fractionation. Some heterogeneous catalyst systems were directly applied with solid biomass under hydrogen or protection gas, mild high temperature, high pressure to degrade lignin macromolecules before fractionation[240].

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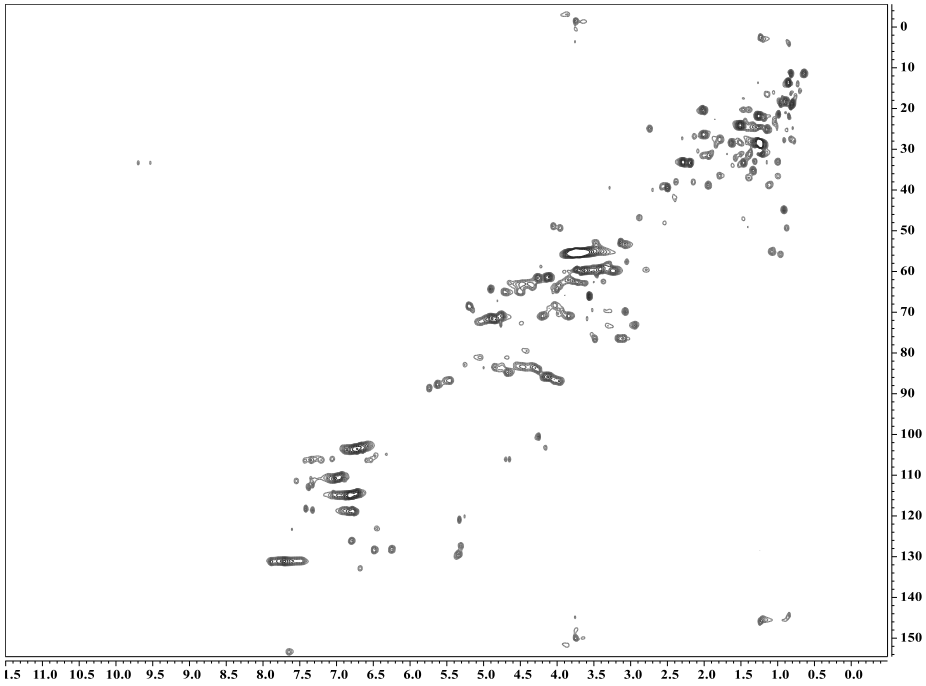
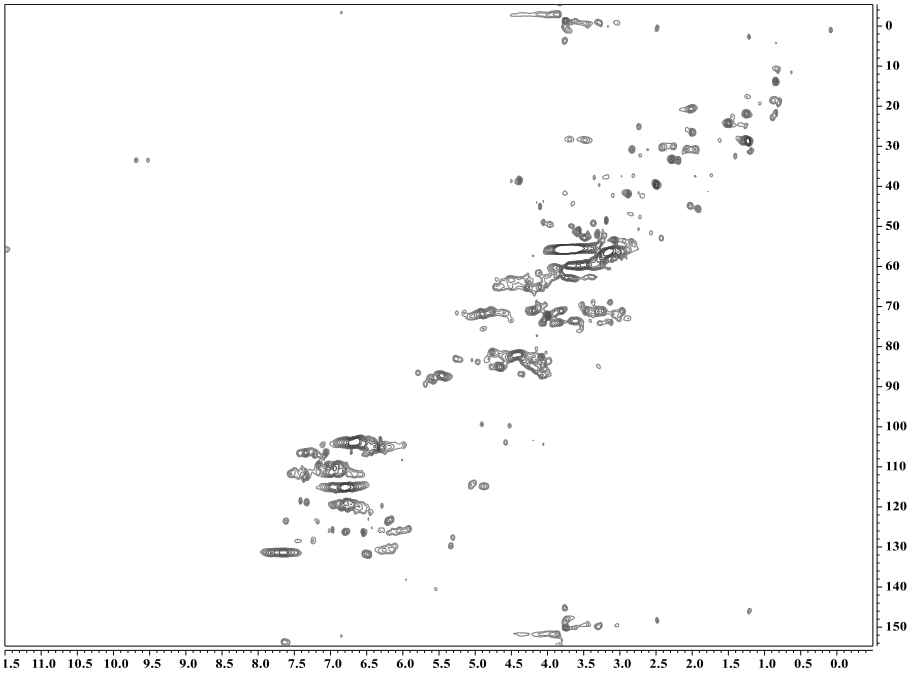
Appendices

Full spectrum of 2D-NMR spectra of lignins

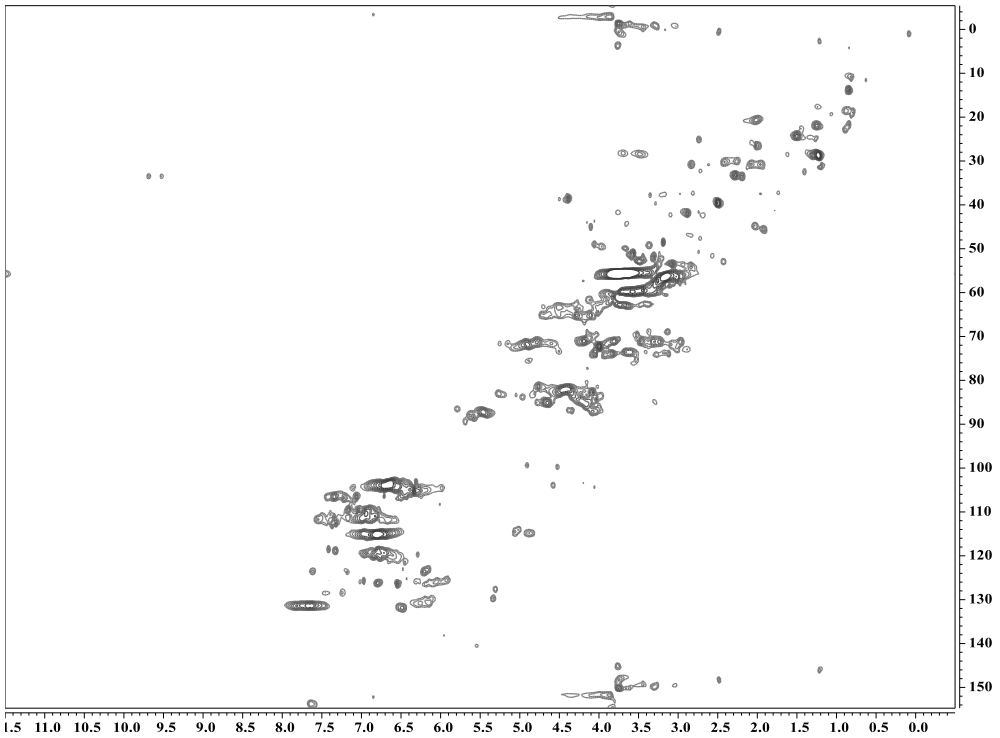
1. **MWL-HP_HSQC** (mill wood lignin from hybrid poplar)



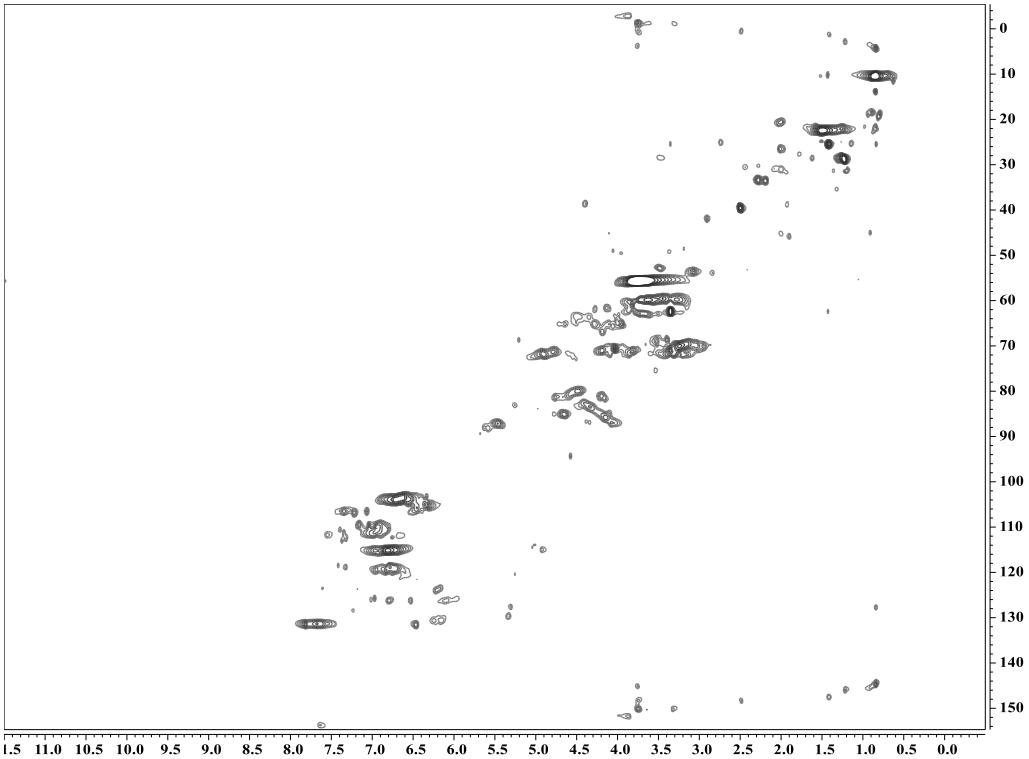
2. **MOL-HP_HSQC** (methanol organosolv lignin from hybrid poplar pretreated at 65% methanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



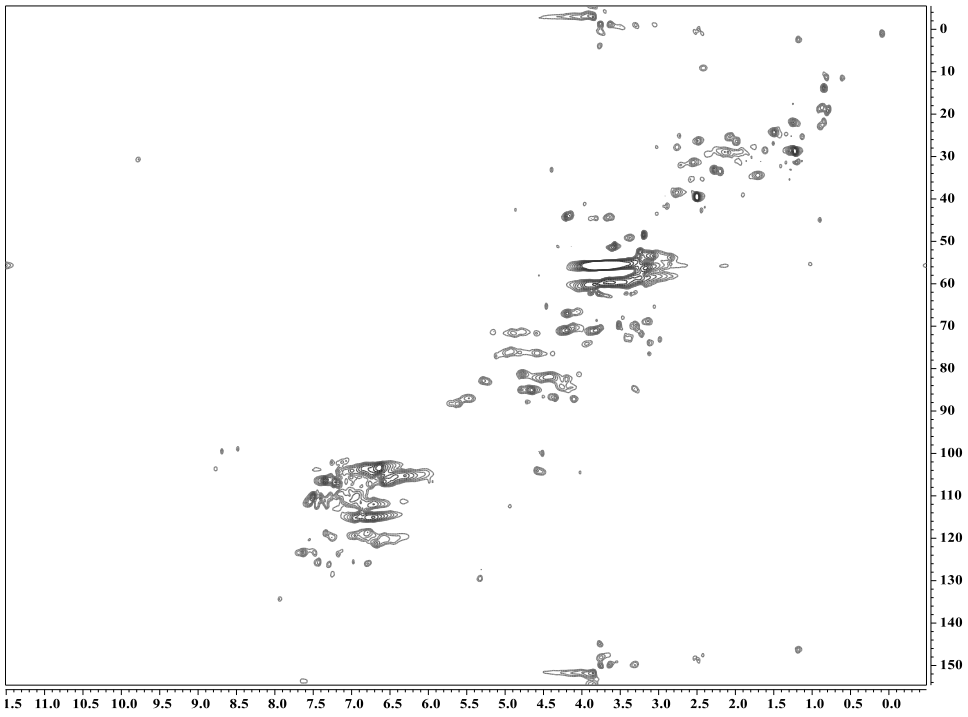
3. **EOL-HP_HSQC** (ethanol organosolv lignin from hybrid poplar pretreated at 65% ethanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



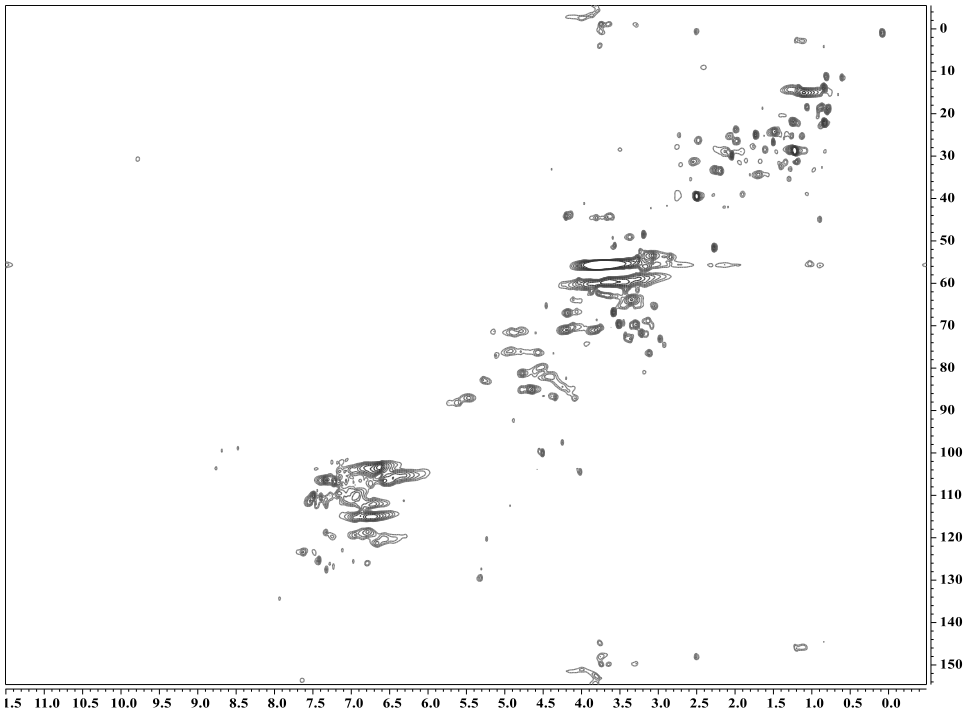
4. **POL-HP_HSQC** (propanol organosolv lignin from hybrid poplar pretreated at 65% propanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



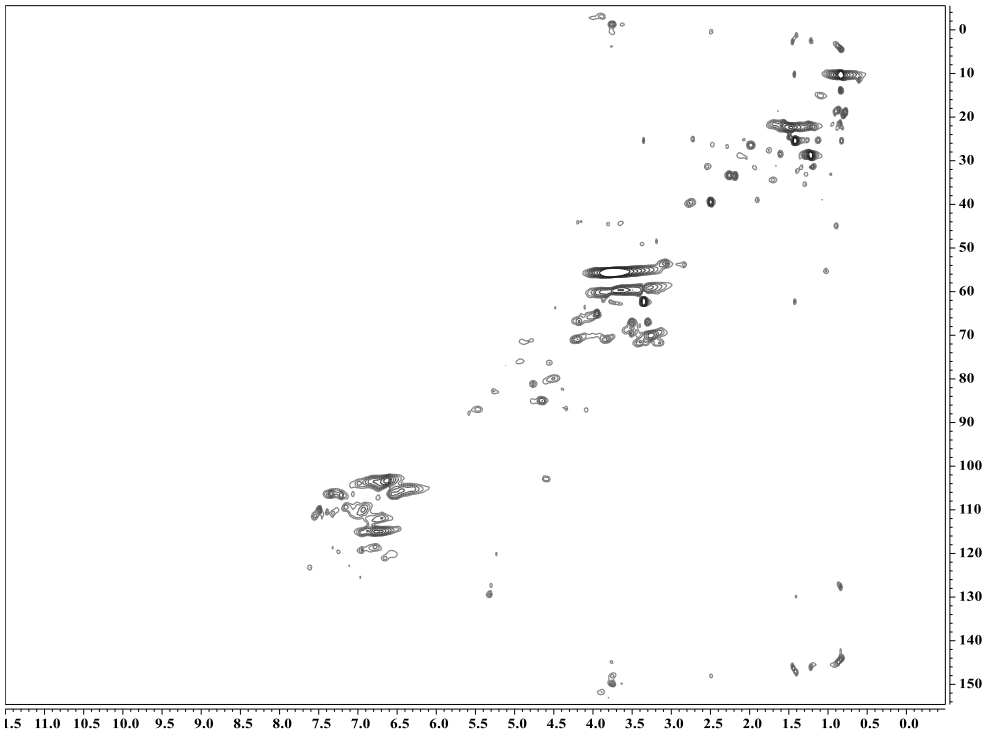
5. **MOL-EU_HSQC** (methanol organosolv lignin from eucalyptus pretreated at 65% methanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



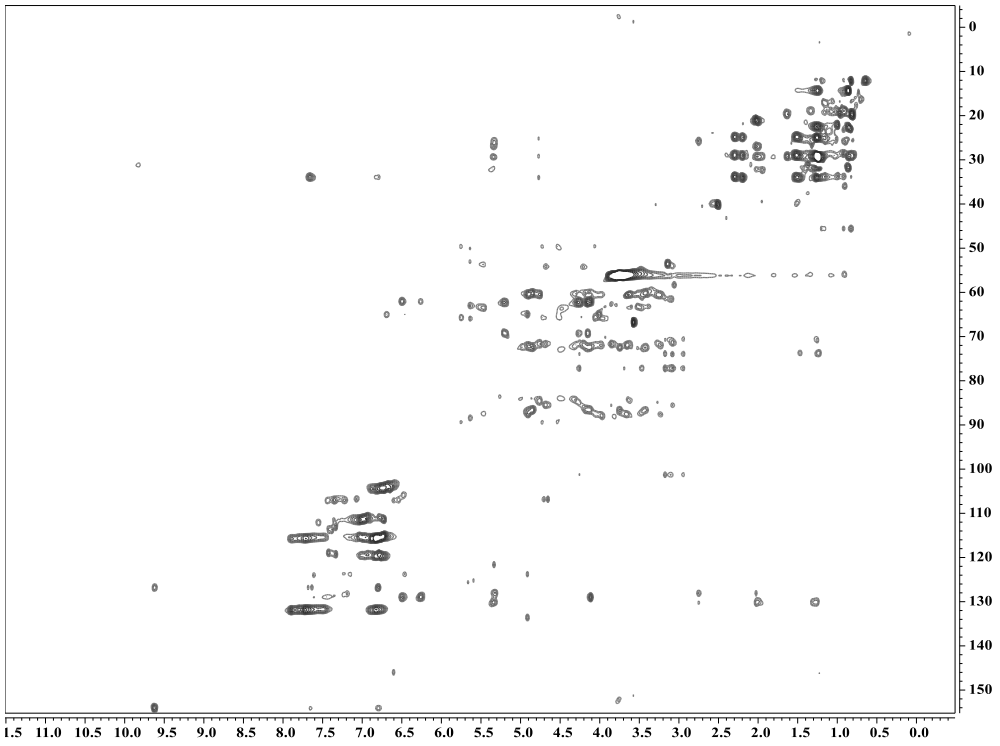
6. **EOL-EU_HSQC** (ethanol organosolv lignin from eucalyptus pretreated at 65% ethanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



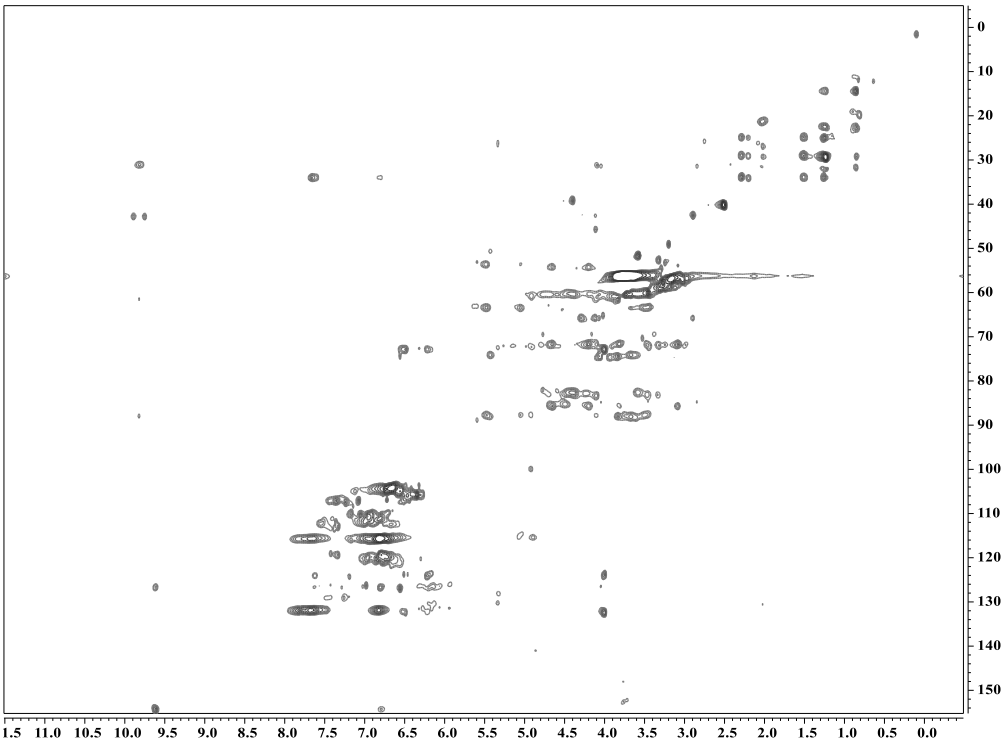
7. **POL-EU_HSQC** (propanol organosolv lignin from eucalyptus pretreated at 65% propanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



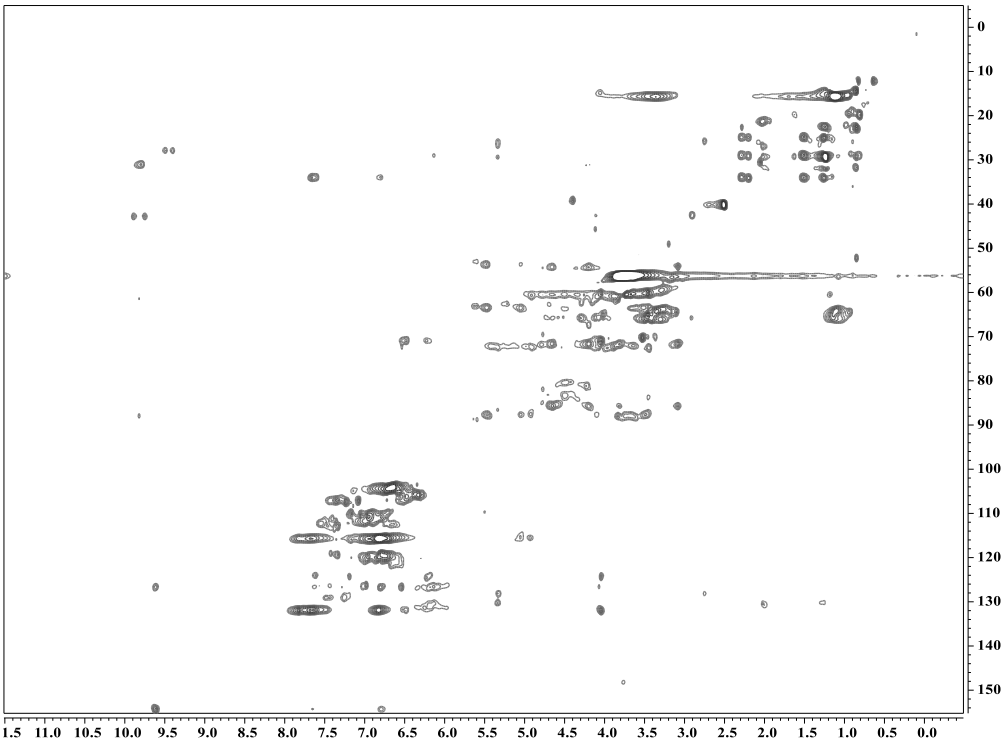
8. MWL-HP_HSQC-TOCSY (mill wood lignin from hybrid poplar)



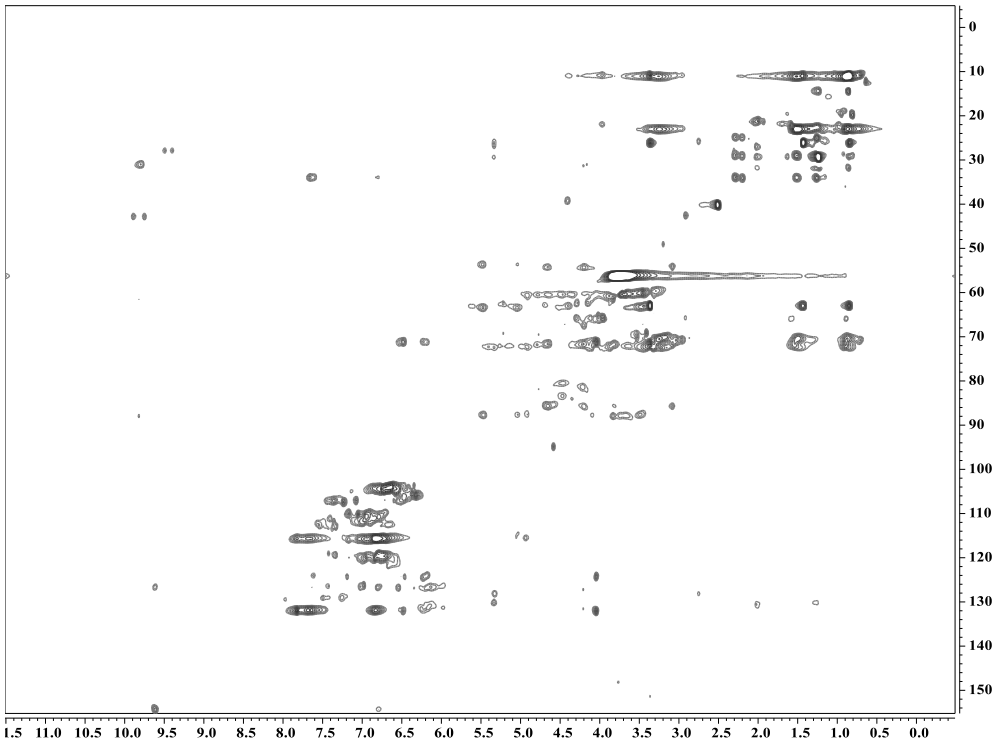
9. **MOL-HP_HSQC-TOCSY** (methanol organosolv lignin from hybrid poplar pretreated at 65% methanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



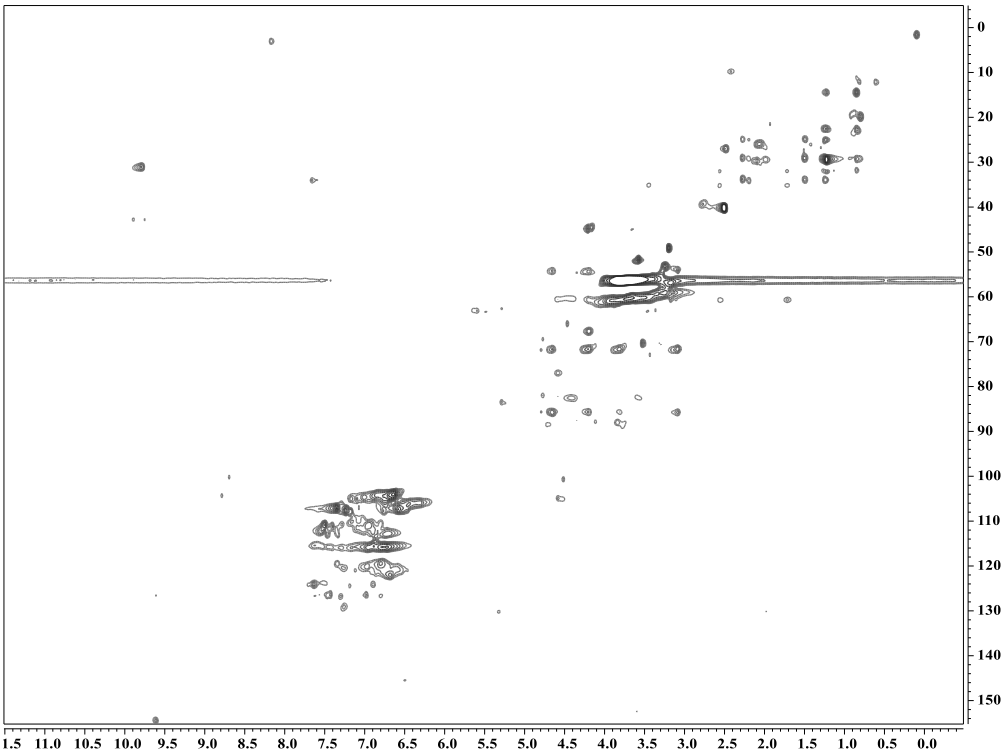
10. **EOL-HP_HSQC-TOCSY** (ethanol organosolv lignin from hybrid poplar pretreated at 65% ethanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



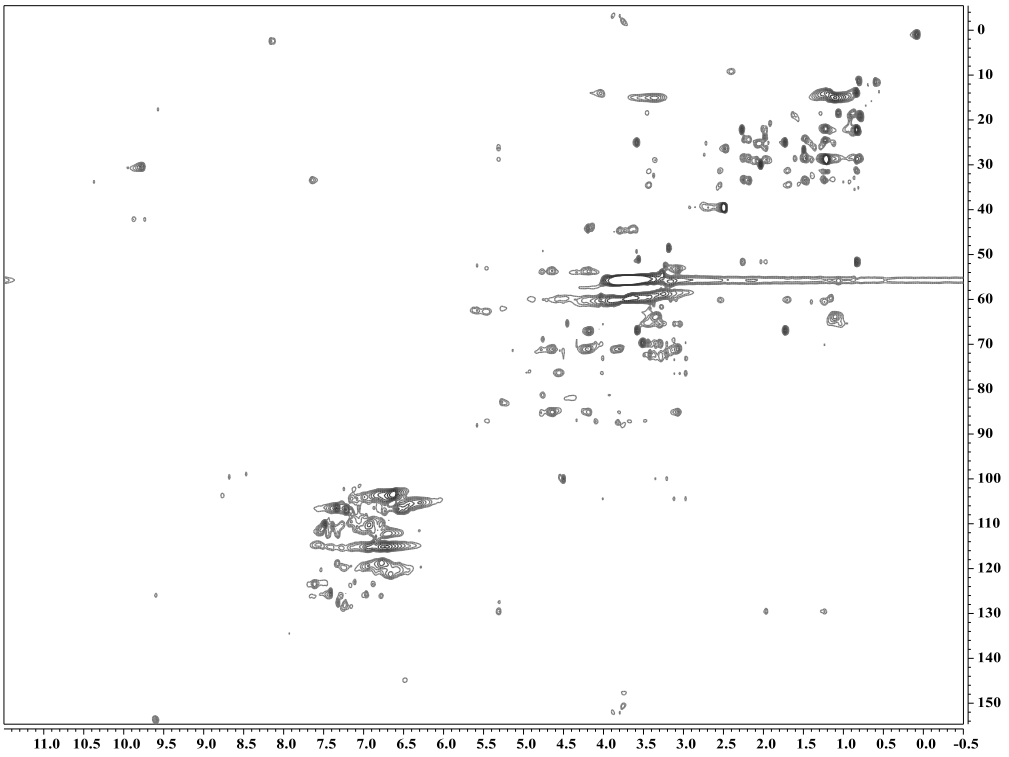
11. **POL-HP_HSQC-TOCSY** (propanol organosolv lignin from hybrid poplar pretreated at 65% propanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



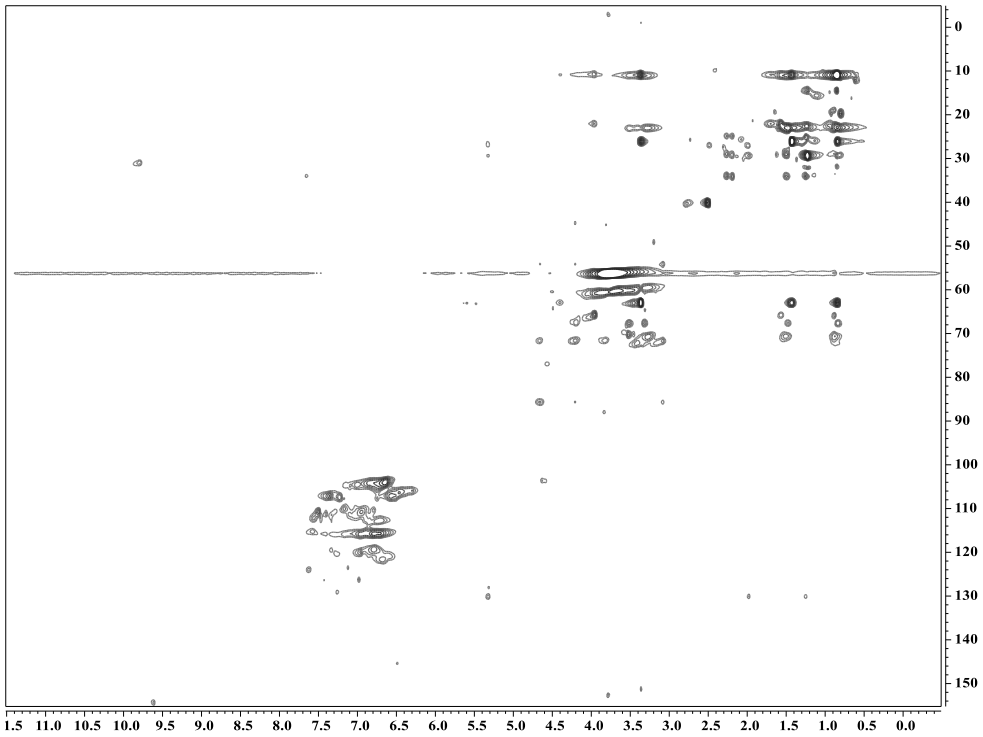
12. **MOL-EU_HSQC-TOCSY** (methanol organosolv lignin from eucalyptus pretreated at 65% methanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



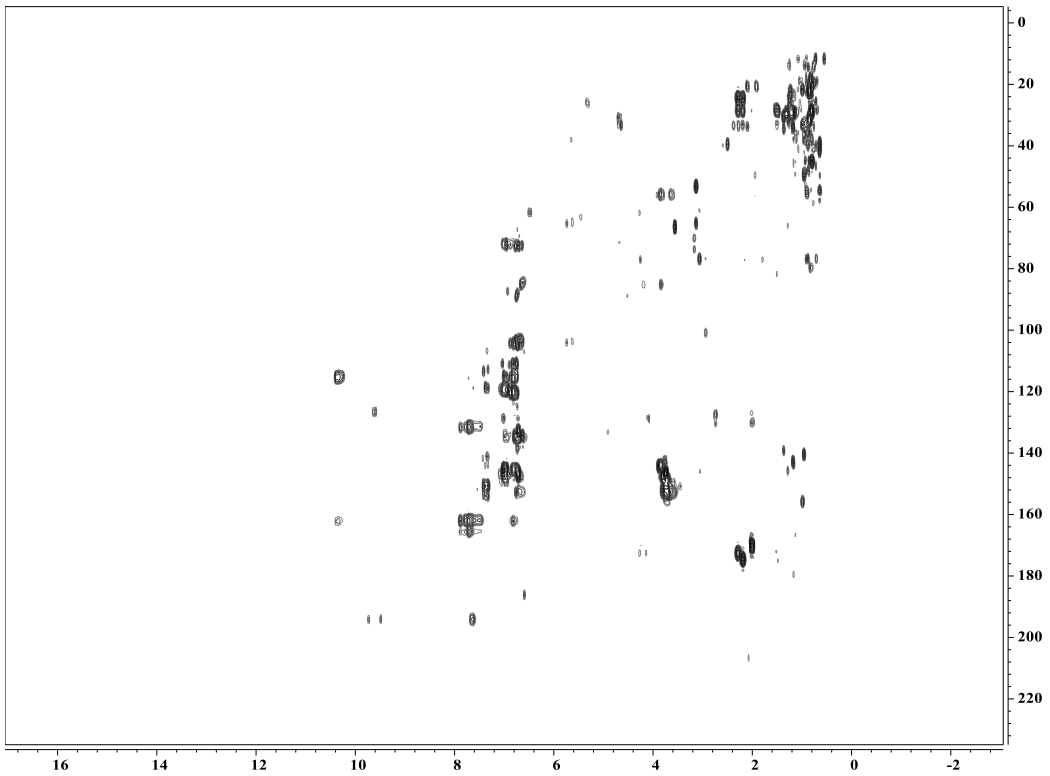
13. **EOL-EU_HSQC-TOCSY** (ethanol organosolv lignin from eucalyptus pretreated at 65% ethanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



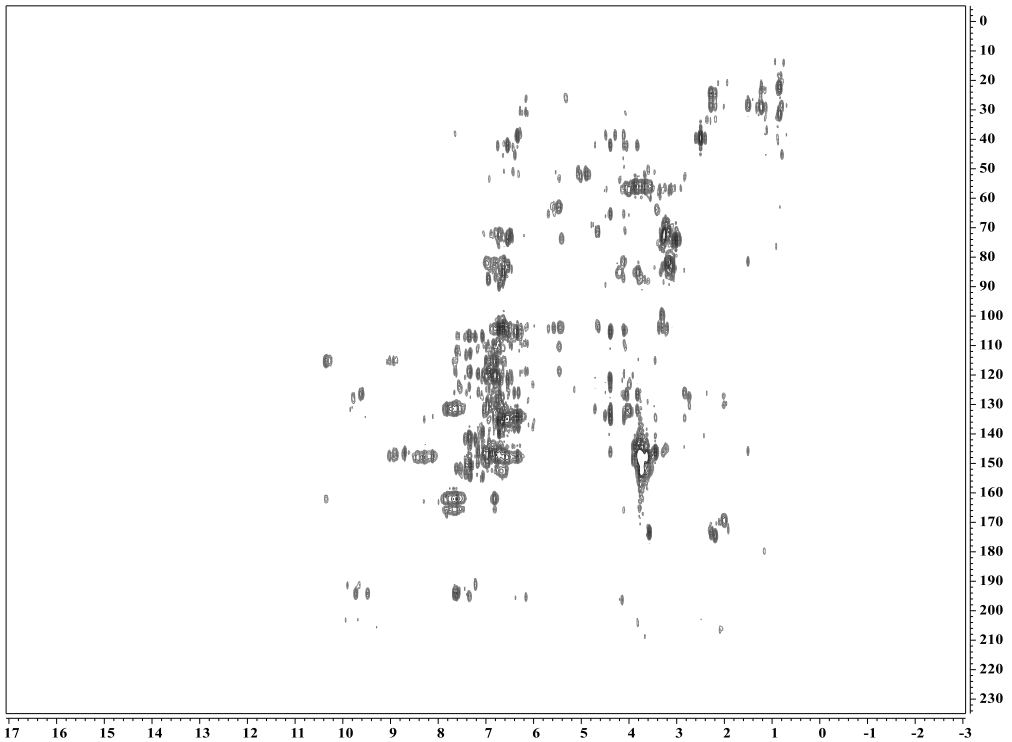
14. **POL-EU_HSQC-TOCSY** (propanol organosolv lignin from eucalyptus pretreated at 65% propanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



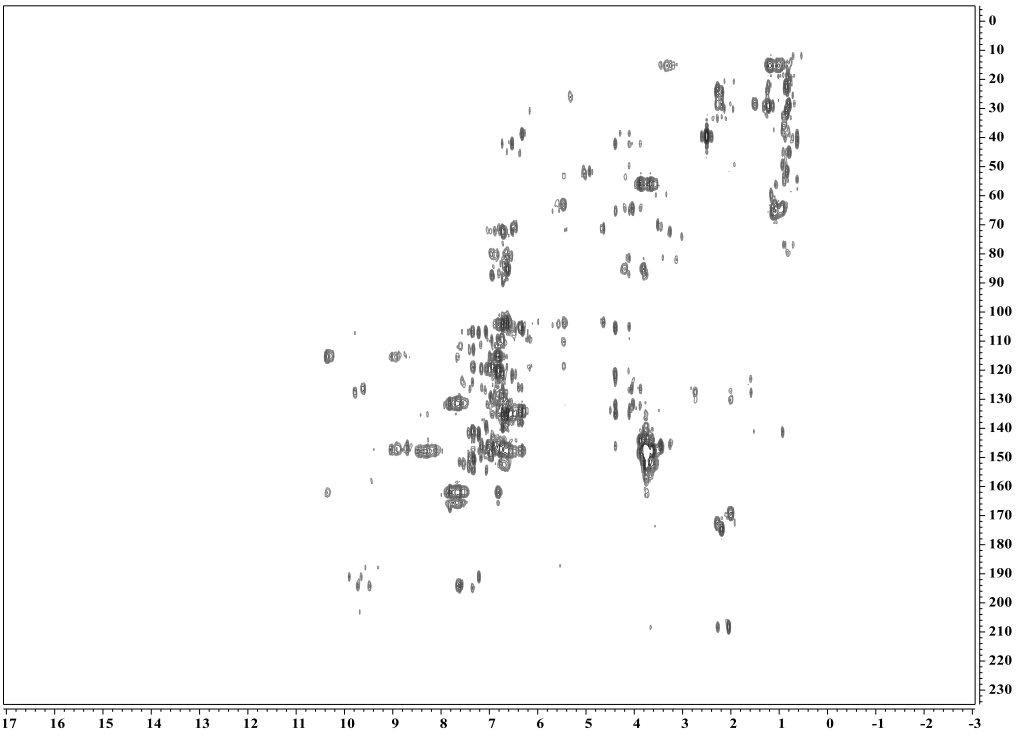
15. MWL-HP_HMBC (mill wood lignin from hybrid poplar)



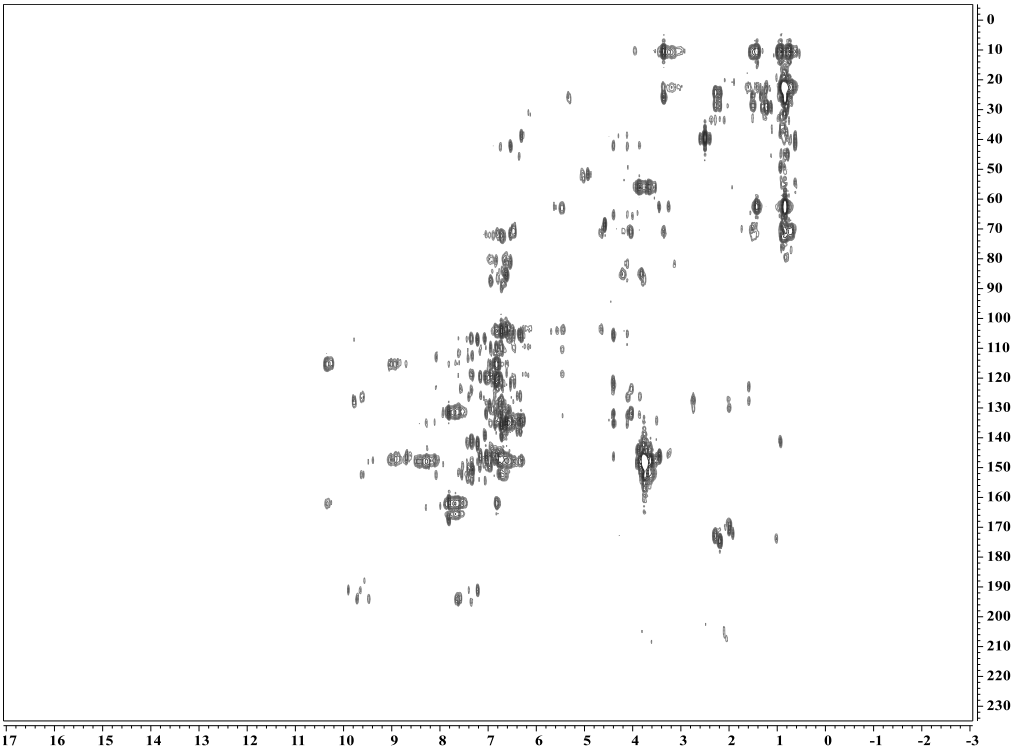
16. **MOL-HP_HMBC** (methanol organosolv lignin from hybrid poplar pretreated at 65% methanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



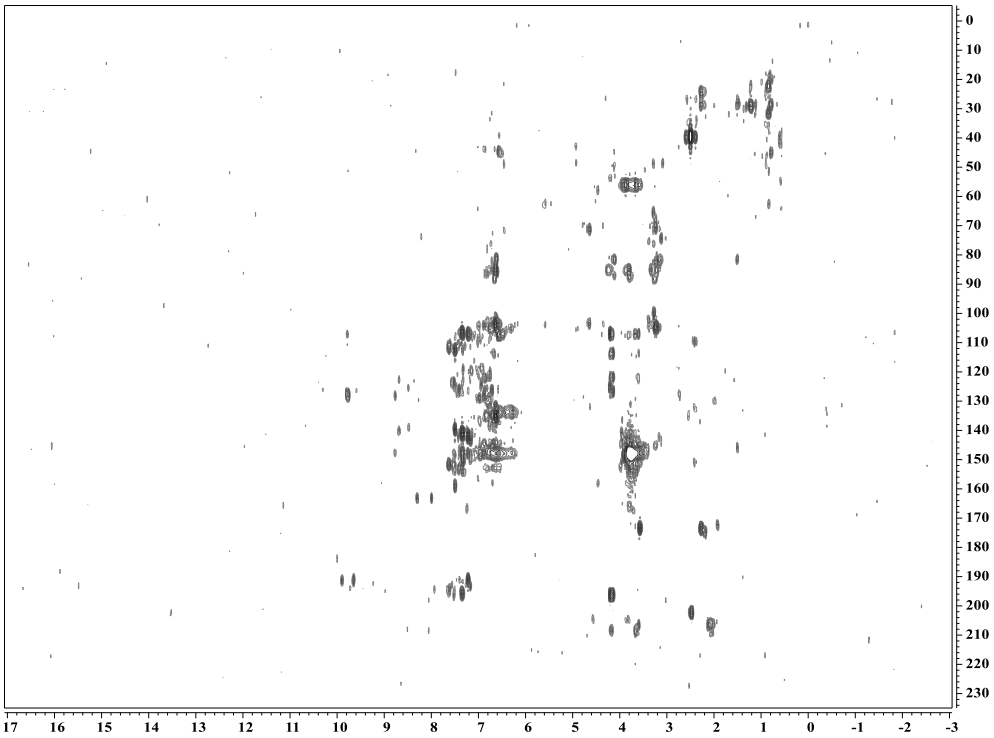
17. **EOL-HP_HMBC** (ethanol organosolv lignin from hybrid poplar pretreated at 65% ethanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



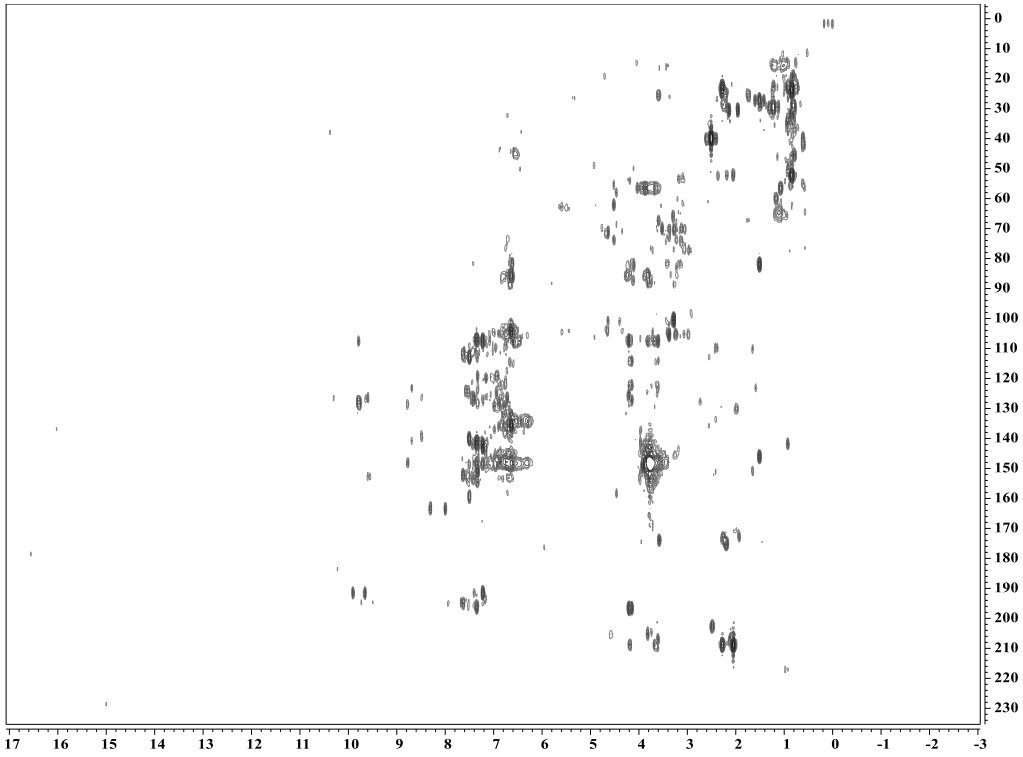
18. **POL-HP_HMBC** (propanol organosolv lignin from hybrid poplar pretreated at 65% propanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 160 °C, 1 h)



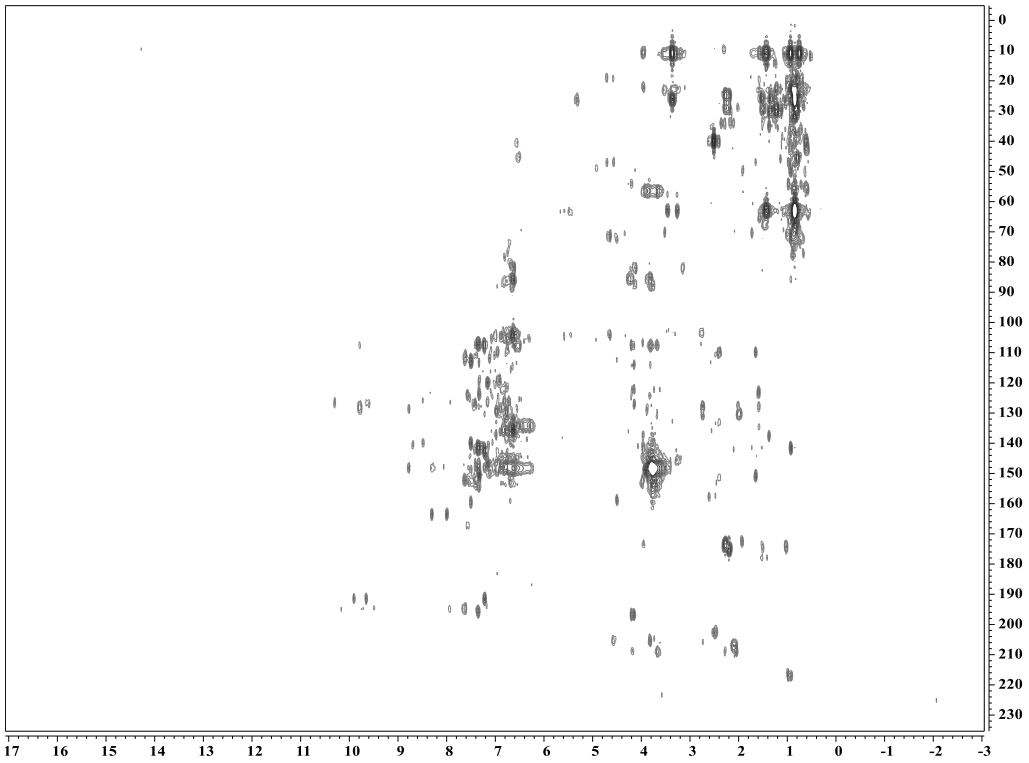
19. **MOL-EU_HMBC** (methanol organosolv lignin from eucalyptus pretreated at 65% methanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



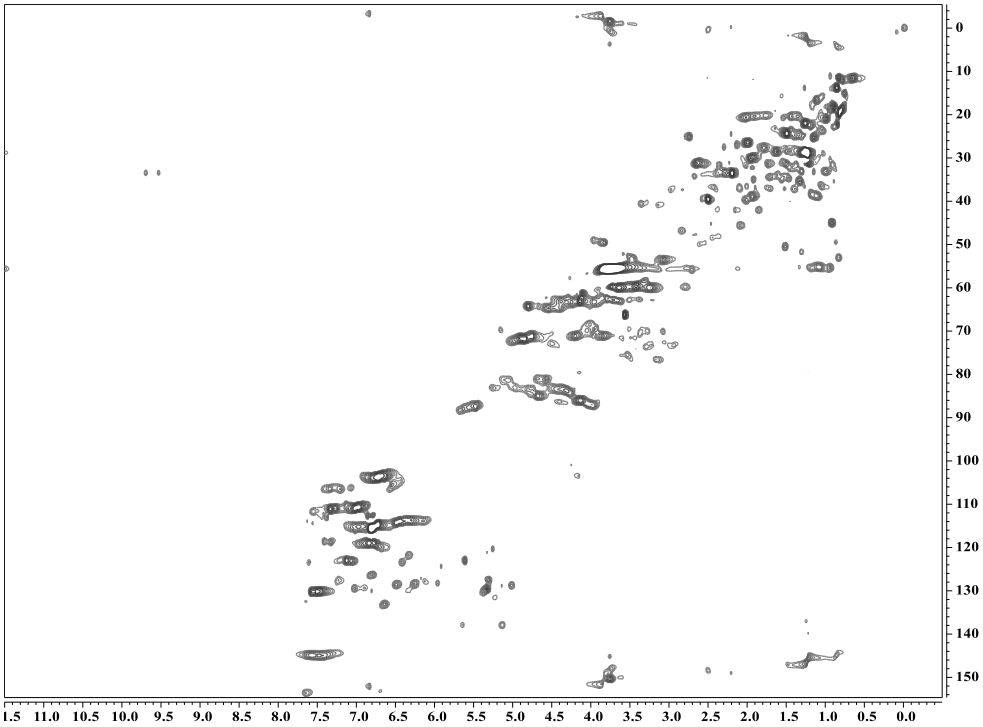
20. **EOL-EU_HSQC-TOCSY** (ethanol organosolv lignin from eucalyptus pretreated at 65% ethanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



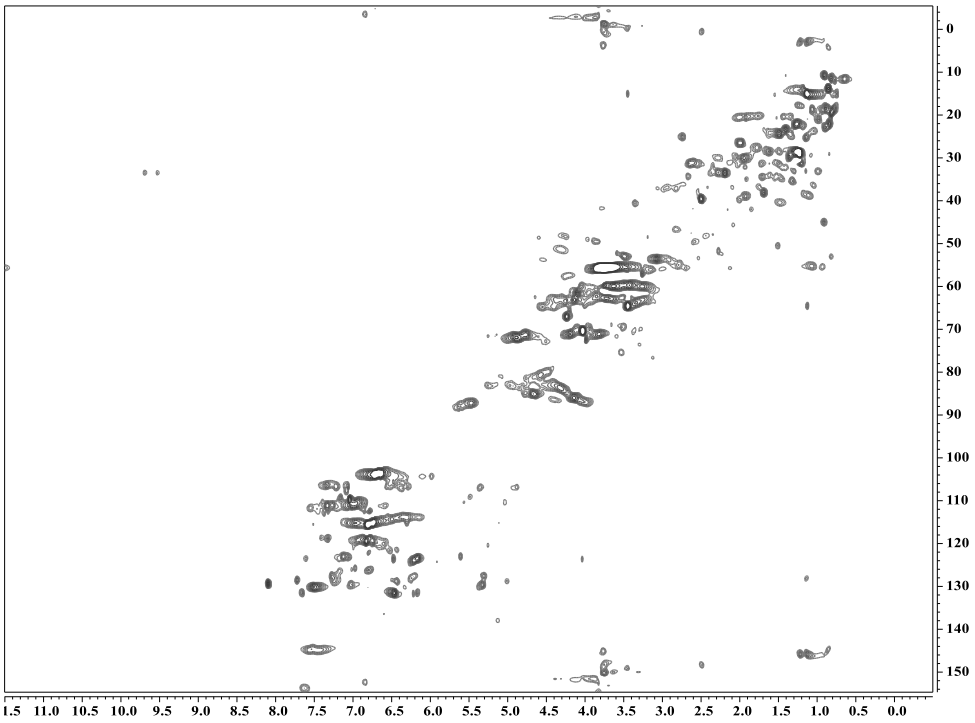
21. **POL-EU_HSQC-TOCSY** (propanol organosolv lignin from eucalyptus pretreated at 65% propanol, 1% sulfuric acid, 100 g dry weight, 1:7 solid : liquid, 170 °C, 1 h)



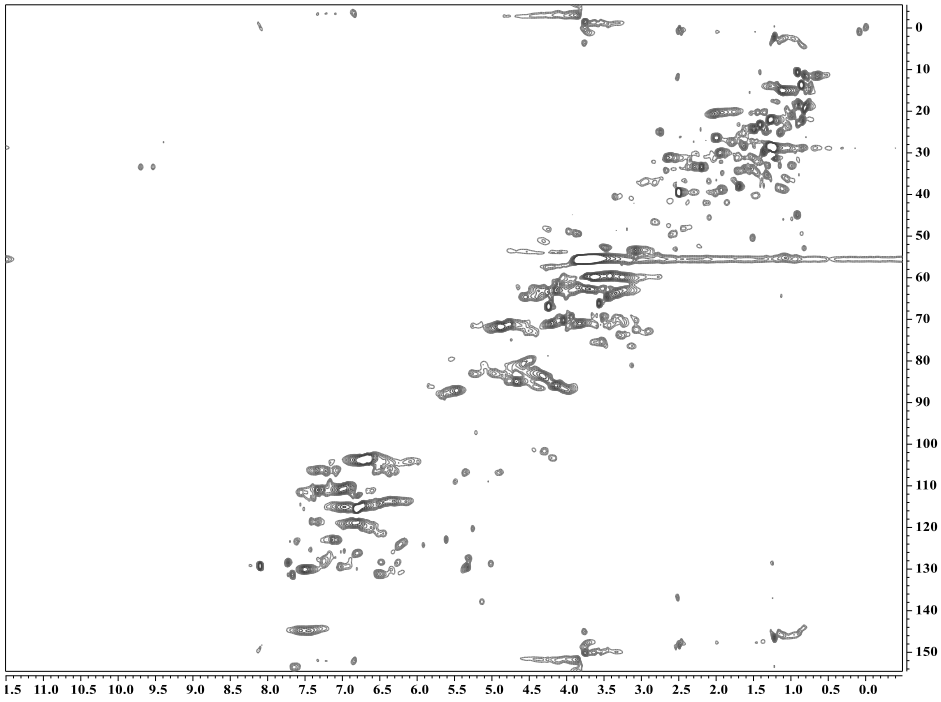
22. MWL-KE_HSQC (mill wood lignin from herbaceous kenaf)



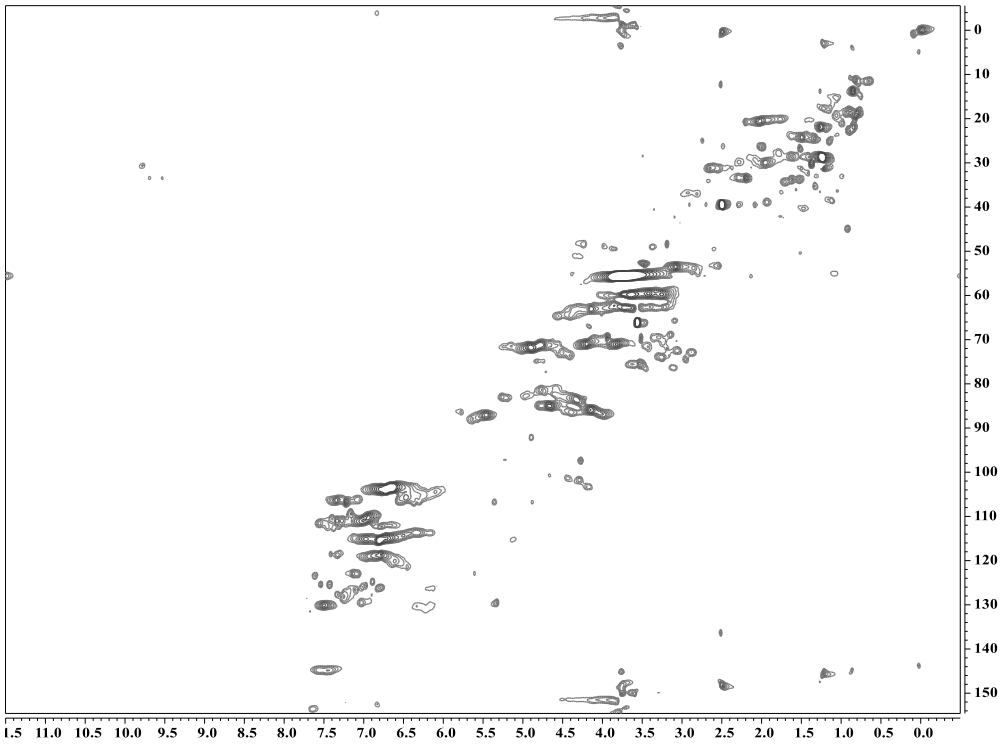
23. **EOL-KE_HSQC** (ethanol organosolv lignin from herbaceous kenaf pretreated at 75% ethanol, 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



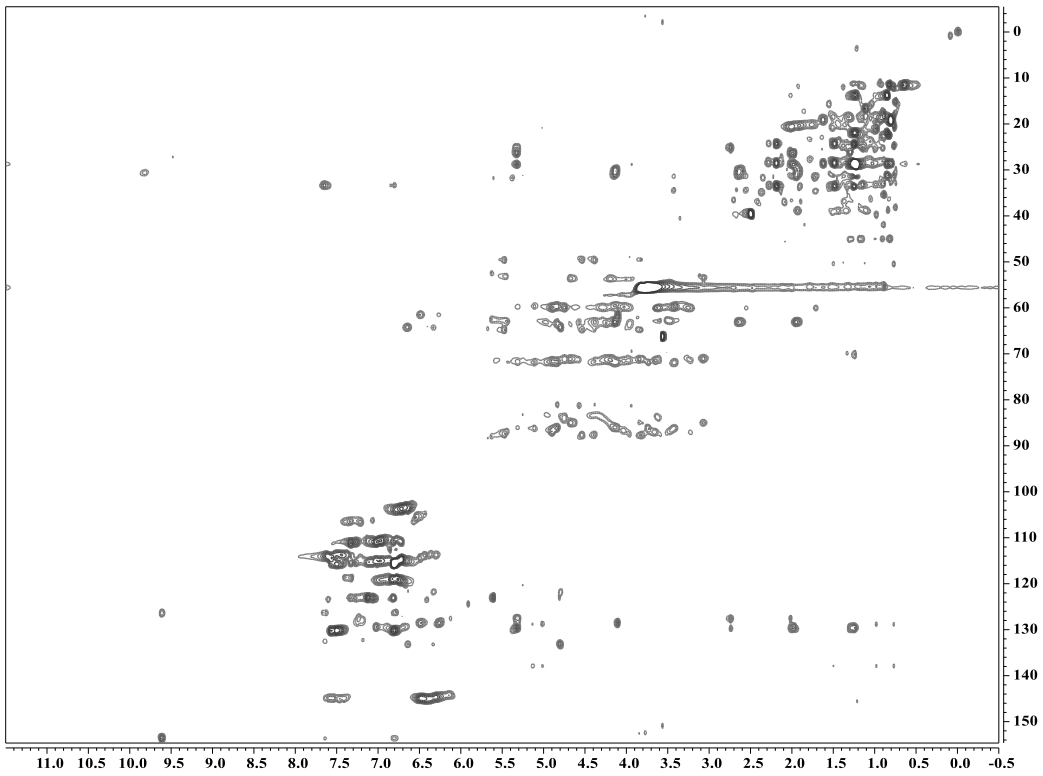
24. **MWL-EK_HSQC** (mill wood lignin from ethanol pretreated kenaf pretreated at 75% ethanol, 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



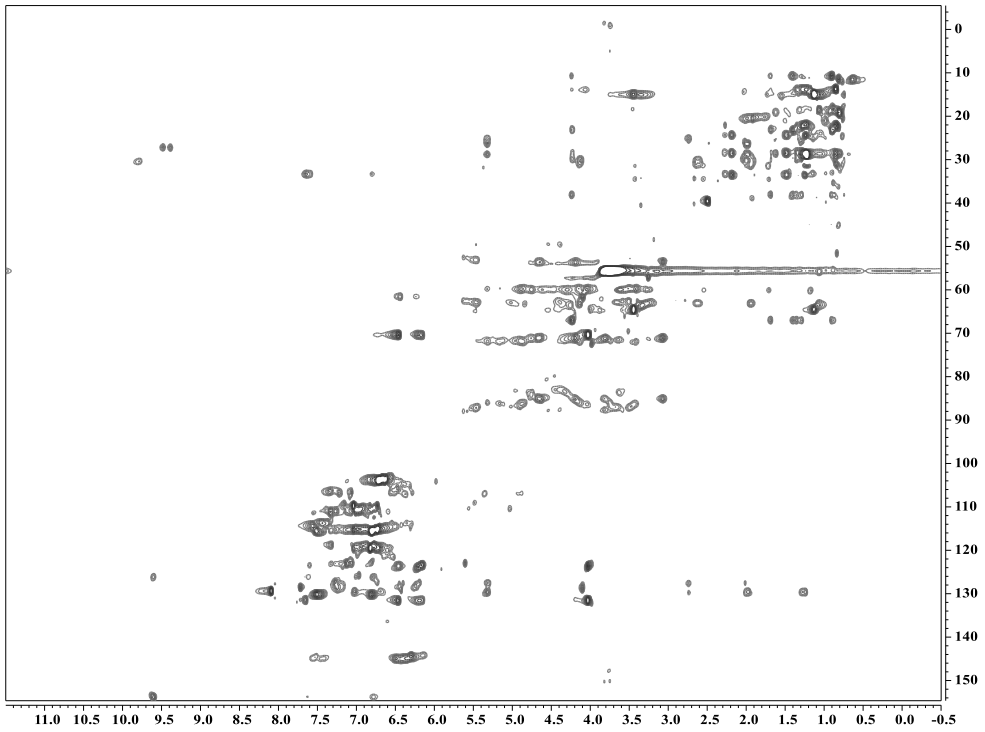
25. **MWL-AK_HSQC** (mill wood lignin from acid pretreated kenaf pretreated at 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



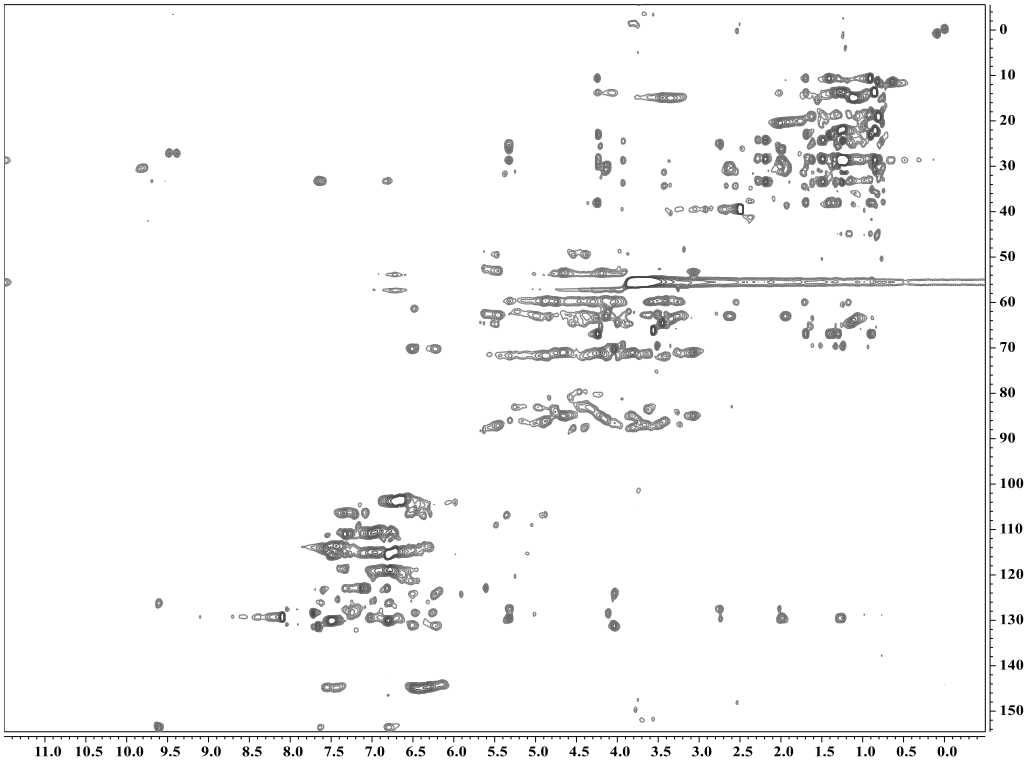
26. MWL-KE_HSQC-TOCSY (mill wood lignin from herbaceous kenaf)



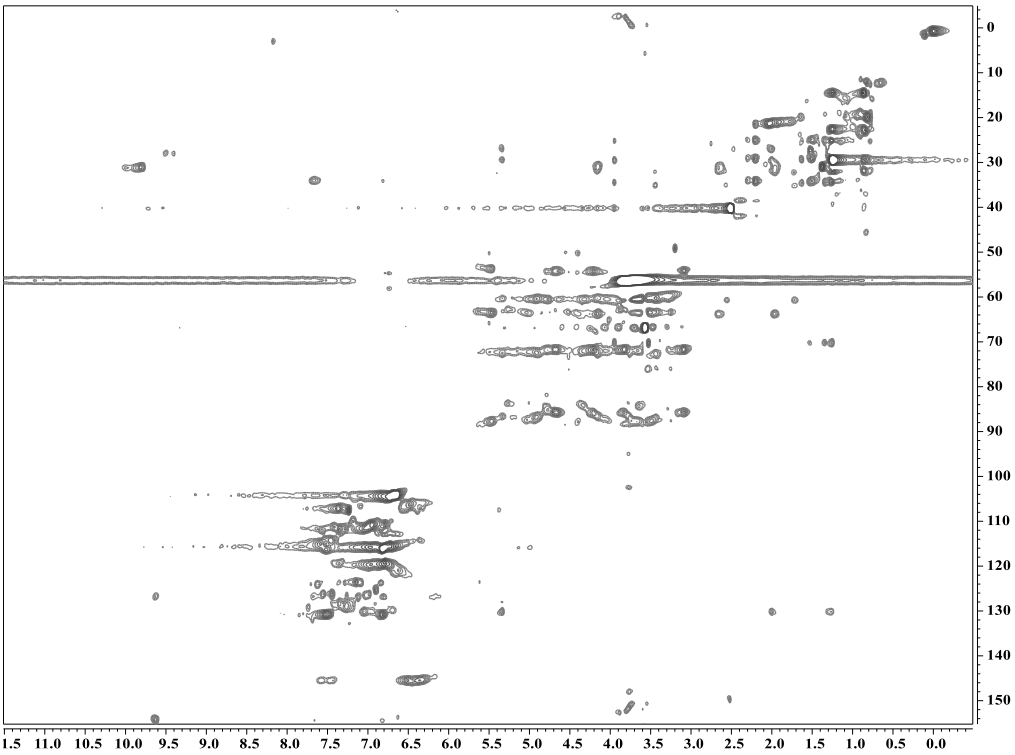
27. **EOL-KE_HSQC-TOCSY** (ethanol organosolv lignin from herbaceous kenaf pretreated at 75% ethanol, 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



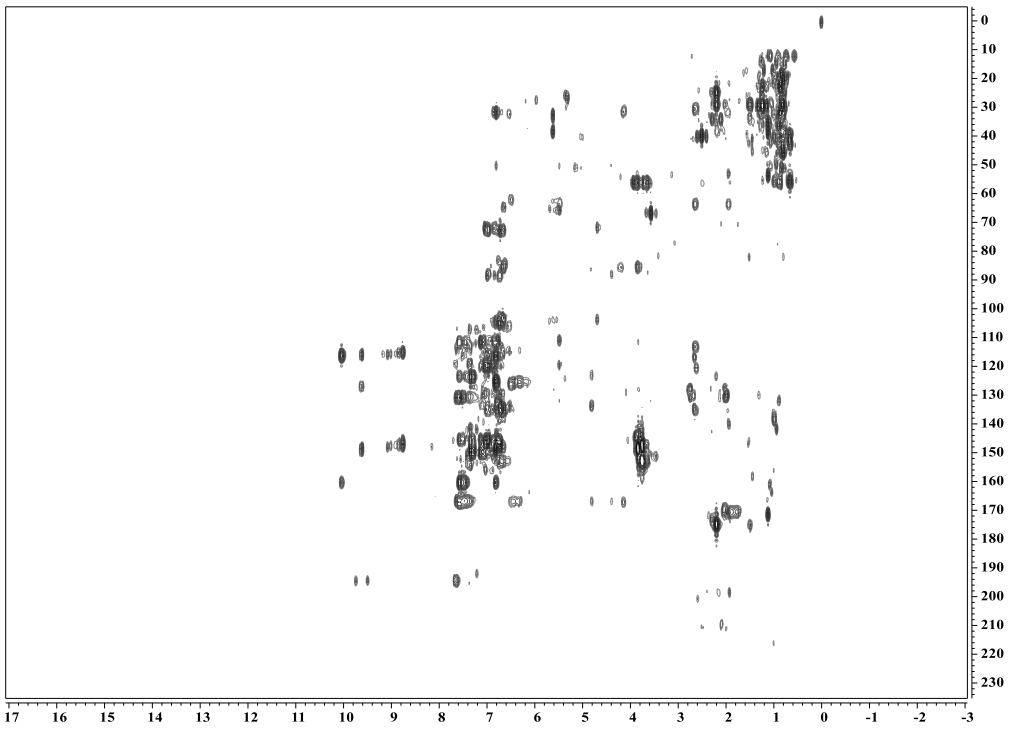
28. **MWL-EK_HSQC-TOCSY** (mill wood lignin from ethanol pretreated kenaf pretreated at 75% ethanol, 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



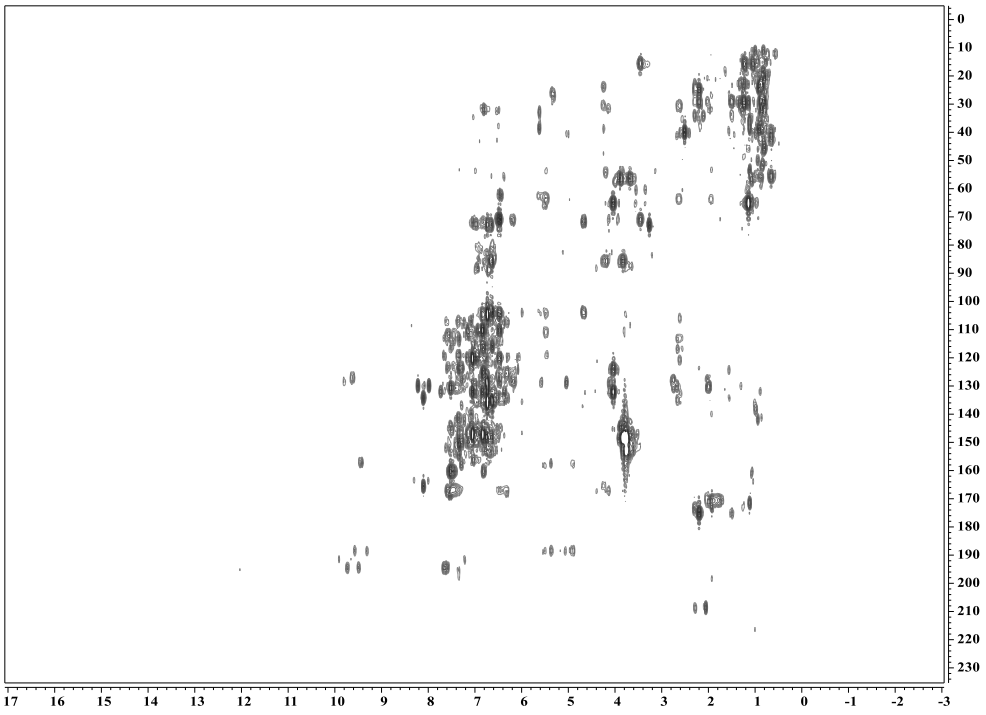
29. **MWL-AK_HSQC-TOCSY** (mill wood lignin from acid pretreated kenaf pretreated at 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



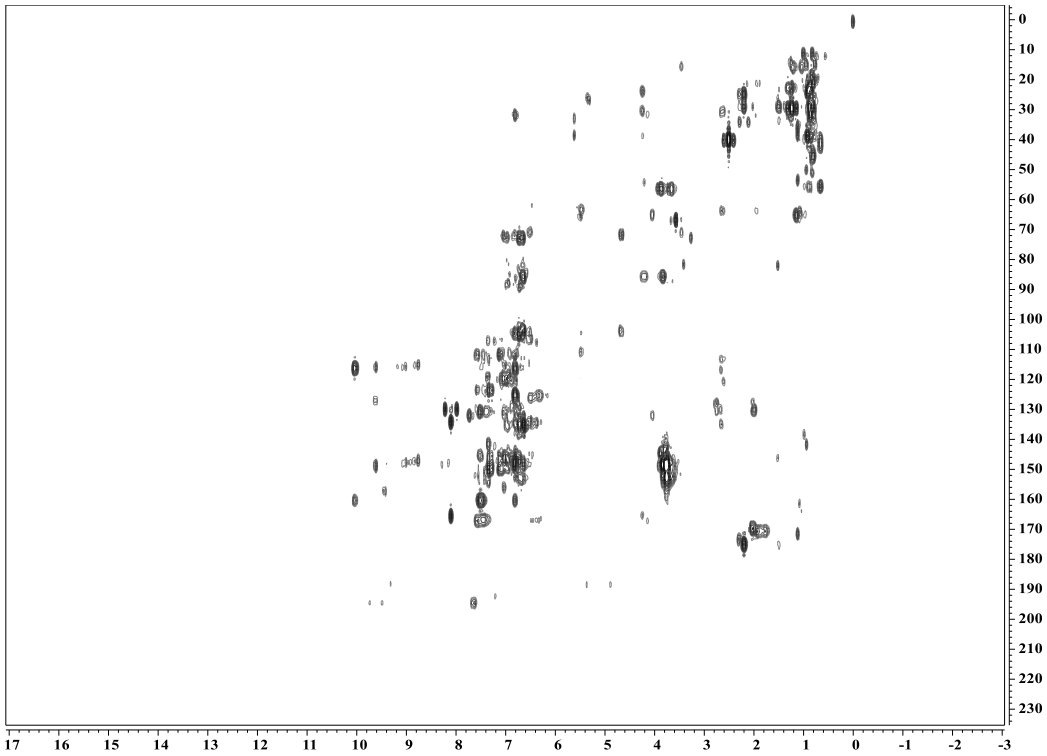
30. MWL-KE_HMBC (mill wood lignin from herbaceous kenaf)



31. **EOL-KE_HMBC** (ethanol organosolv lignin from herbaceous kenaf pretreated at 75% ethanol, 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



32. **MWL-EK_HMBC** (mill wood lignin from ethanol pretreated kenaf pretreated at 75% ethanol, 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)



33. **MWL-AK_HMBC** (mill wood lignin from acid pretreated kenaf pretreated at 1% sulfuric acid, 80 g dry weight, 1:20 solid : liquid, 170 °C, 1 h)