Recent advances in understanding the pseudo-lignin formation in lignocellulosic biorefinery

Journal:	*Green Chemistry*
Manuscript ID	GC-CRV-01-2018-000353.R1
Article Type:	Critical Review
Date Submitted by the Author:	16-Mar-2018
Complete List of Authors:	Shinde, Somnath; University of Tennessee Knoxville, Chemical and Biomolecular Engineering Meng, Xianzhi; University of Tennessee, Kumar, Rajeev; University of California, Center for Environmental Research and Technology Ragauskas, Arthur; University of Tennessee,

Green Chemistry

Review ARTICLE

Recent advances in understanding the pseudo-lignin formation in lignocellulosic biorefinery

Somnath. D. Shinde,[a] Xianzhi Meng,[a] Rajeev Kumar, [b,c,d] and Arthur J. Ragauskas*[a,,c,d,e,f]

[a.] *Department of Chemical and Biomolecular Engineering, The University of Tennessee, Knoxville, TN 37996-2200, USA*
[b.] *Center for Environmental Research and Technology, Bourns College of Engineering, 1084 Columbia Avenue, Riverside, California 92507; telephone: 95 1-78 1-5668; fax: 95 1-781-5790.*
[c.] *BioEnergy Science Center (BESC), Oak Ridge National Laboratory (ORNL), Oak Ridge, TN 37831, USA*
[d.] *Center for BioEnergy Innovation (CBI), Oak Ridge National Laboratory (ORNL), Oak Ridge, TN 37831, USA*
[e.] *Joint Institute for Biological Sciences, Biosciences Division, Oak Ridge National Laboratory (ORNL), Oak Ridge, TN 37831, USA*
[f.] *Department of Forestry, Wildlife and Fisheries, Center for Renewable Carbon, The University of Tennessee, Institute of Agriculture, Knoxville, TN 37996-2200, USA*
* email: aragausk@utk.edu

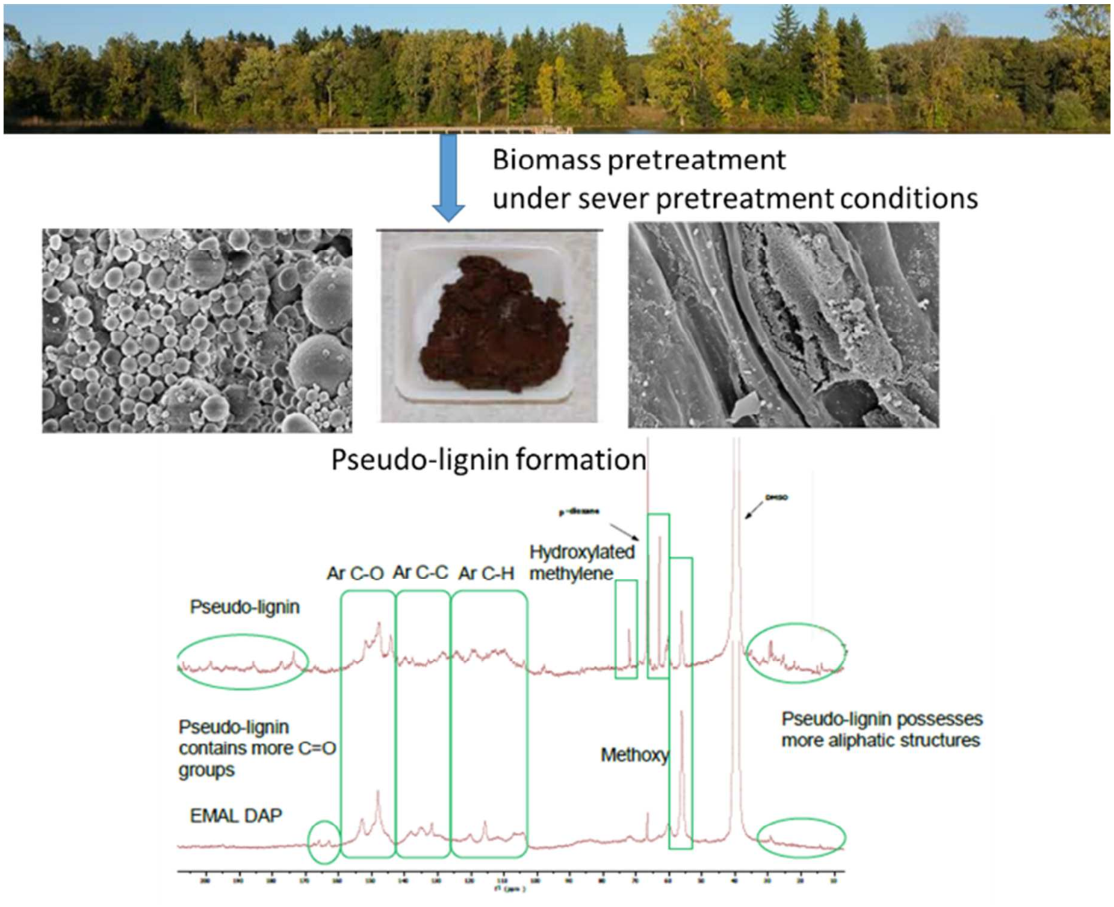
Note: This manuscript has been authored, in part, by UT-Battelle, LLC under contract no. DE-AC05-00OR22725 with the U.S. Department of Energy. The publisher, by accepting the article for publication, acknowledges that the United States Government retains a non-exclusive, paid-up, irrevocable, world-wide license to publish or reproduce the published form of this manuscript, or allow others to do so, for United States Government purposes. The Department of Energy will provide public access to these results of federally sponsored research in Accordance with the DOE Public Access Plan (http://energy.gov/downloads/doe-public-access-plan).

Abstract

The formation of lignin-like structures by the degradation primarily of plant polysaccharides has been observed after severe thermochemical acidic pretreatment of lignocellulosic biomass. These structures were found to be deposited as droplets and microspheres on the surface of solid biomass residue and/or in liquid effluent. These structures showed lignin like properties and yielded a positive Klason lignin (K-lignin) value, and are termed as pseudo-lignin and/or humins. Pseudo-lignin is an aromatic material containing hydroxyl and carbonyl functional groups, which contributes to K-lignin values but is not derived thereof. Pseudo-lignin arises from polymerization/condensation reactions from key intermediates such as 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol derived from furfural (FF) and 5-hydroxymethyl furfural (5-HMF), respectively. Further, pseudo-lignin retards the biological conversion of pretreated biomass through unproductive binding to enzymes/microbes and a physical hindrance to enzymes and microbes by blocking the active cellulose surface binding sites. This necessitates a fundamental understanding of pseudo-lignin structure and its effect on biomass recalcitrance. This review examines the pseudo-lignin formation during acidic and hydrothermal biomass pretreatments and the cooling process after pretreatment, which are applied to biomass for biofuels synthesis through the biochemical route. The review article is divided into five parts: the first part gives the background information on pseudo-lignin formation during different pretreatment processes for biomass to biofuels, the second part focuses on the chemistry and mechanism of pseudo-lignin formation, the third part emphases on different analytical techniques used for pseudo-lignin characterization and recalcitrance elucidation, the fourth part illustrates the recalcitrance behaviour of pseudo-lignin, and the fifth part deals with practical consideration regarding the design of processes for prevention of pseudo-lignin formation.

Keywords: Lignocellulosic biomass, pretreatment, pseudo-lignin, recalcitrance, fuel

Graphical abstract



Introduction

Lignocellulosic biomass has been recognized as one of the most promising low-cost, renewable sources to produce biofuels in response to environmental and long-term energy security needs.[1] However, due to natural recalcitrance of biomass, which refers to the inherent characteristics of lignocellulosics due to the complex structure that protects its carbohydrates from degradation by enzymes and/or microbes, pretreatment must be applied to biomass to alter the physicochemical structures of plant cell walls to remove and/or redistribute cell wall components, and ultimately increase the accessible surface area of carbohydrates to cellulolytic enzymes.[2-4] Among many pretreatment techniques that have been developed in the past two decades, pretreatment applying dilute sulfuric acid remains one of the most commonly used and effective pretreatment methods in the course of the bioconversion process.[5] A tremendous amount of effort has been made to understand the exact impact of acid pretreatment on the structure of lignocellulosic biomass. Up to date, it is quite clear that acid pretreatment is capable of solubilizing hemicellulose, restructuring and redistributing lignin, and increasing cellulose accessibility.[6-8]

In recent years, a large fraction of the literature has focused on understanding the behaviour of lignin during acid pretreatments. For example, lignin has been shown to depolymerize via homolytic and acidolytic cleavage reactions.[9] Several publications have reported that the relative percentage of acid insoluble lignin, i.e. Klason lignin (K-lignin) is higher in the acid pretreated material than that of the starting untreated material.[3, 10-13] Li *et al.* reported that ~50% of the K-lignin extracted from hydrothermal pretreated aspen was actual lignin, and the remaining lignin-like material was not able to be distinguished from the lignin due to the fact that natural lignin is not the only organic source that is insoluble in sulfuric acid during a typical K-lignin analysis.[14] Later studies confirmed that this type of lignin-like structures were preferentially produced during high severity low-pH biomass pretreatment.[15]

This type of lignin-like structures could be formed either through substitution reactions on lignin aromatic rings or acid catalyzed condensation reactions dehydrated/fragmented polysaccharides. Several studies later confirmed that only the carbohydrate fraction is large enough to be the source of this lignin-like structure, referred to as pseudo-lignin.[16, 17] Coupled with compositional analysis and physicochemical structural analysis, pseudo-lignin can be generally defined as aromatic material that yields a positive K-lignin value.[17]

It is well known that lignin plays a negative role in the conversion of biomass to biofuels process.[18] It can physically limit polysaccharide accessibility and unproductively bind to cellulase (and other) enzymes during enzymatic hydrolysis.[19, 20] Thus, a fundamental understanding of the chemistry associated with pseudo-lignin is important from a biomass to fuels and chemicals via biochemical route perspective. Carbohydrate monomers released during acid pretreatment can be degraded into compounds such as furfural (FF), 5-hydroxymethyl furfural (5-HMF), and levulinic acid, which can be further degraded to carbon enriched aromatic structures.[17, 21] These aromatic structures are considered as key intermediates in the formation of pseudo-lignin.[22] Kumar *et al.* investigated the effects of carbohydrate derived pseudo-lignin on cellulose enzymatic hydrolysis and found that xylan/xylose are prone to undergo degradation even at low severities and the pseudo-lignin deposited on the surface of cellulose reduces cellulose accessibility and make less cellulase available for action on cellulose through unproductively binding.[23] Fan *et al.* further revealed that pseudo-lignin is more detrimental to enzymatic hydrolysis of cellulose than dilute acid pretreated lignin.[24] As a result, dilute acid pretreatment should be performed at less severe conditions to avoid the formation of pseudo-lignin. Hu and Ragauskas demonstrated that using a water-DMSO mixture in dilute acid pretreatment can significantly reduce the formation of pseudo-lignin at high severity pretreatment conditions.[25] Flowthrough pretreatment technique can also suppress the pseudo-lignin formation due to its ability to constantly removing dissolved sugar residues and lignin into the aqueous phase and from the reactor.[26]

This review paper discusses recent advances in the understanding of the molecular mechanisms of the pseudo-lignin formation during biomass pretreatment processes. It is divided into five parts: the first part gives the background information on pseudo-lignin formation during different pretreatment processes for biomass to biofuels, the second part focuses on the chemistry and mechanism of pseudo-lignin formation, the third part emphases on different analytical techniques used for pseudo-lignin characterization and recalcitrance elucidation, the fourth part illustrates the recalcitrance behaviour of pseudo-lignin, and the final section deals with practical consideration regarding the design of processes for prevention of pseudo-lignin formation.

Pseudo-lignin formation during biomass pretreatment processes

Lignin, a three-dimensional heterogeneous polyphenolic polymer, is the most abundant aromatic biopolymer on earth. It provides rigidity to the plants and significantly contributes to biomass recalcitrance.[1] In general, biomass pretreatment is necessary prior to its biological conversion to often remove or at least redistribute lignin across the plant cell wall.[22] Although low pH thermo-chemical pretreatments, such as dilute sulfuric acid, are ineffective in terms of lignin physical removal, they still remain as one of the most promising pretreatment methods with respect to industrial implementation.[27] During acid pretreatment, lignin fragmentation is expected causing a slight delignification and the extent of lignin removal depends strongly on the pretreatment severity. Table 1 highlights the changes in lignin content during dilute acid pretreatment for softwood, hardwood, and herbaceous feedstocks. Cao *et al.* reported that dilute acid pretreated poplar at 170 ^{o}C over the reaction time of 0.3 to 27 min had the same level of K-lignin content (24.4-26.0%) as the untreated poplar (24.6%). Avci *et al.* reported a lignin reduction of 15.5% in dilute acid pretreated corn stover at 160 ^{o}C.[28]

Similarly, flowthrough dilute acid pretreatment is also capable of removing substantial amounts of lignin from lignocellulosic biomass, indeed more lignin could be solubilized in a flowthrough pretreatment than in a batch pretreatment performed at similar conditions.[29] For example, nearly 36% of lignin was removed from poplar in a flow-through dilute acid pretreatment at 180 ^{o}C while the lignin content in batch pretreated poplar remained at the same level compared to untreated sample.[29] On the other hand, the majority of the literature results reported that the percentage of K-lignin content of acid pretreated biomass to be much higher than the untreated material as shown in Table 1. This phenomenon has been reported for hardwood, softwood, and herbaceous energy crops. The significant removal of hemicellulose and partial solubilization of cellulose was frequently attributed to the lignin percentage increase; however, a few studies have hypothesized that the repolymerization of polysaccharide degradation products such as FF and 5-HMF also contributed to the increase of lignin content after pretreatment.[14, 30] To confirm the contributions of polysaccharides to the formation of a lignin-like structure and to eliminate the possibility of lignin repolymerization, dilute acid pretreatment was performed on holocellulose which is a mixture of cellulose and hemicellulose.[17] Results revealed that K-lignin content increased with increasing pretreatment severity and the pretreated holocellulose comprised around 95% K-lignin prepared under the most severe conditions with a combined severity of 3.74.[17] Since the starting material had a very small amount of K-lignin, this new type of lignin-like structure termed as pseudo-lignin was directly formed from polysaccharides.

Lignin has the potential to exist in formations such as globular droplets through strong intermolecular forces responsible for holding these lignin globules together as evidenced by image analysis of *in vitro* enzymatic polymerization of coniferyl alcohol.[31] It has been reported that spherical lignin droplets could be formed on the surface of high-temperature dilute acid pretreated lignocellulosic biomass as a result of migration of lignins from the cell wall to the bulk liquid phase followed by redeposition onto the surface of plant cell wall.[7] It was believed that these droplets were derived at least in part from lignin present in the plant cell wall as confirmed by FTIR, NMR, antibody labelling, and cytochemical staining.[32] Similarly, the formed pseudo-lignin in dilute acid pretreated holocellulose also has spherical structures and tends to be deposited on cell surfaces.[15] The size of pseudo-lignin droplets is in a range of 0.3 to 8.0 μm and the droplets exhibit a slightly rough exterior.[17]

While the formation of pseudo-lignin has been reported during dilute acid pretreatment of biomass or holocellulose, it is likely to occur during all kinds of pretreatments accomplished at a low pH and high temperature conditions such as hot water pretreatment and steam explosion. Two recent studies reported that the formation of pseudo-lignin was also observed in hydrothermal pretreated straw and bagasse.[33, 34] Sipponen and co-workers studied the impact of hot water pretreatment severity on generation of pseudo-lignin from wheat straw, and their study elucidated that higher severity induced accumulation of more pseudo-lignin within the temperature range of 170-200 ^{o}C.[26] Kumagai *et al.* also confirmed the formation of pseudo-lignin in 200 ^{o}C hot compressed water pretreated *Hinoki Cypress* by atomic force microscopy.[35] Furthermore, it has been shown that pseudo-lignin can also be formed during the cooling process after pretreatment, termed cooling-induced pseudo-lignin (CIPL).[36] A recent study found that hot water pretreated *Populus* collected isothermally at reaction temperature 170 ^{o}C actually showed no pseudo-lignin, and lignin-derived compounds including lignin oligomers and phenolic monomers accounted for ~80% of CIPL while carbohydrate degradation products such as FF and 5-HMF only accounted from ~10% of CIPL formed during cooling process.[36] This is consistent with another study that reported only trace amount of pseudo-lignin is detected from hot water pretreatment of holocellulose at 170 ^{o}C for less than 90 min.[37] In addition, Bauer *et al.* also reported that increasing severity in steam explosion pretreatment also led to the formation of pseudo-lignin for late harvested hay.[38] The upgrade of biomass can also be fulfilled via either torrefaction, carbonization, or pyrolysis where biomass is thermally degraded.[39] Torrefaction of Norway spruce performed at mild (260 ^{o}C for 8 min) and intermediate conditions (310 ^{o}C for 8 min) was found to result in increased K-lignin content which was mainly be attributed to pseudo-lignin formation.[40] Hydrothermal carbonization of loblolly pine at above 200 ^{o}C generated hydrochars with only polyphenolic structure with up to 98% of K-lignin and it was claimed to contain significant amount of pseudo-lignin.[41]

Chemistry and mechanism of pseudo-lignin formation

Unlocking the mechanisms of pseudo-lignin formation during acid pretreatment requires a deep fundamental understanding of the physicochemical structure of pseudo-lignin. It has been reported that high temperature, low pH and the presence of oxygen are crucial conditions for the formation of pseudo- lignin.[25] Structural characterization of isolated pseudo-lignin from dilute acid pretreated poplar holocellulose by FTIR and ^{13}C NMR revealed that this material was predominantly comprised of carbonyl, carboxylic, and aromatic structures.[15] Due to the heterogeneity of the reaction media and complexity of biomass components, the exact mechanisms leading to the formation of pseudo-lignin at elevated temperature is still under investigation.[14, 42, 43] A hypothesized reaction pathway for pseudo-lignin formation is shown in Figure 1. FF and 5-HMF are mainly formed from 5 and 6-carbon sugars such as xylose and glucose, respectively, via acid catalyzed dehydration reactions in the thermochemical pretreatment of lignocellulose.[44, 45] These compound can be further converted to other aromatics which might be key intermediates in the formation of pseudo-lignin.[15] For example, a benzenoid derivative named 3,8-dihydroxy-2-methylchromone was found to be major aromatic product in acid degradation of xylose.[46] In addition, Luijkx *et al.* reported that 1,2,4-benzenetriol (BTO) can be formed in hydrothermal treatment of carbohydrates via rearrangement reactions of HMF by hydrolysis of the furan ring of HMF followed by intramolecular ring closing and dehydration.[47, 48] Pseudo-lignin can be then formed via polymerization or polycondensation of these key intermediates. For example, HMF and its ring products such as BTO could polymerize to form a three-dimensional polymer via aromatic electrophilic substitution.[49] Polyphenol compounds are also possible pseudo-lignin precursors as a poly(hydrophenylene) like structure could be formed via oxidative polymerization of BTO in presence of oxygen.[50] This is supported by the study from Ma *et al.* who reported that pseudo-lignin isolated from hydrothermal pretreated bamboo had more alicyclic structures and hydroxyl substituted groups than the lignin control, and pseudo-lignin was further modified from exhibiting more aliphatic structures to being rich in aromatic structures with increased pretreatment severity.[51] However, it has not been unequivocally proven that these intermediates and reactions give rise to pseudo-lignin formation and the hypothesized mechanisms need further studies.

Analytical techniques for pseudo-lignin characterization and recalcitrance elucidation

The composition and amount of pseudo-lignin generated varies significantly depending on the type of biomass and pretreatment conditions used. Different analytical techniques have been utilized to characterize the structure of biomass components[52] and the application of these methodologies to pseudo-lignin characterization has rapidly developed. For characterization purposes, researchers have produced and isolated pseudo-lignin from dilute acid pretreated lignin-free materials such as α-cellulose, holocellulose[15, 23, 37], and cellulose mixed with xylan or xylose[23]. Table 2 illustrates the different characterization methods used in literature for assessment of biomass and pseudo-lignin structure.

Scanning electron microscopy (SEM)

SEM has been a widely applied technique for ultrastructural imaging of biomass to elucidate morphological changes after pretreatment. Under severe acid[17, 23] and hydrothermal pretreatment conditions[36, 37], the presence of pseudo-lignin on the surface of the pretreated material was observed in the size range of ~0.3 to 8.0 µm as spherical balls or droplets. Sannigrahi *et al.* reported the first direct evidence for the presence of pseudo-lignin.[17] They observed the presence of discrete spherical balls or droplets on the surface of acid treated poplar holocellulose. These droplets exhibited slightly rough exterior surface and resembled lignin-balls in appearance. The frequency of appearance of these droplets was found to increase with increased pretreatment severity. Kumar *et al.* observed a similar trend of occurrence of these spherical droplets when Avicel cellulose was used alone or mixed with beech wood xylan or xylose and underwent a dilute acid pretreatment at severities ranging from 2.66 to 3.56 (Figure 2).[23] Cellulose mixed with xylan or xylose showed prominent evidence of droplets in comparison to cellulose alone at similar pretreatment severities. The occurrence of such droplets was not widespread for severities less than 2.95, suggesting that these conditions were not severe enough to form significant amounts of solid degradation products. The xylose-derived pseudo-lignin sample shown in Figure 2e and e' was entirely made up of pseudo-lignin spheres as were the solids prepared at higher severity shown in Figure 2c and d.[23]

Pseudo-lignin has also been observed in the effluent liquid stream of acidic pretreatments as microspheres.[53] These microspheres were reported for high–pressure water pretreatment of corn stem rind and were divided into two categories based on their location as free microspheres or adsorbed microspheres. The density of microspheres increased with increase in pretreatment temperature with the maximum formation of free microspheres reported at 180 °C. Zhuang *et al.* observed a progressive deposition of spherical droplets of CIPL on the surface of the treated poplar wood during the cooling process.[36] However, treated wood which was collected isothermally at the temperature of the pretreatment showed quite smooth surface with no signs of pseudo-lignin deposition on the surface.

The presence of pseudo-lignin spheres is complicated by the well known phenomena that lignin has a tendacy to deposit as droplets on to the surface of the cell wall after thermochemical pretreatment of biomass.[7, 32, 54] Pretreatment conditions above the glass transition temperature of lignin results in the coalescence of lignin in the inner region of the cell wall. This coalescened larger molten lignin bodies move within the cell wall which can redeposit onto the cell wall surface.[55] Re-localization of lignin is generally beneficial for enzymatic digestibility of carbohydrates as it can improve the the accessibility of the majority of cellulose and hemicellulose microfibrils by opening the structure of the cell wall matrix[5] which is important for dilute acid and hydrothermal pretreatments since they generally lead to an insignificant delignification.[56-58] On the other hand, the re-localized lignin may "enwrap" or partly cover the cellulose fibers and therefore block the access of enzymes, and ultimately reduce the enzymatic hydrolysis efficiency.[59] It is important to further characterize these surface droplets using FTIR, NMR, and other relevant analysis to confirm the presence of pseudo-lignin structures in the pretreated biomass.

X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), has frequently been used to characterize the surface chemistry and degree of disorder of the polyaromatic stacks.[60, 61] XPS is also used to determine the quantitative elemental compositions, the bonding states of atoms as well as the locations of atoms in the samples.[37] XPS has been used extensively to study surface chemistry, composition, and chemical modifications of fiber and pulp surface.[62, 63] Ma *et al.* used XPS to trace pseudo-lignin formation and elaborated the structure of the isolated pseudo-lignin during the hydrothermal pretreatment of holocellulose (Figure 3).[37] The chemical shifts for carbon (C1s) in cellulosic fibers can be deconvoluted into four categories C_1 (C–C/C–H), C_2 (C–O), C_3 (C=O/O–C–O), and C_4 (O–C=O).[37, 64] Lignin and extractives are mainly responsible for C_1 (C–C or C–H) chemical shifts.[37, 64, 65] The C1s peak centres around the bonding energy of 284 eV with a gradual decrease from C_4 to C_1.[37] The chemical shifts for oxygen (O1s) peaks generally involve O_1 (C=O) and O_2 (C–O/C-O-C), where O_1 is mainly allocated to oxygen in lignin, whereas O_2 originates primarily from oxygen in carbohydrates.[37] In fact, O_2 chemical shifts are also found in lignin. However, most of hydroxyls and ethers in lignin are associated to benzene ring structure. Larger polarizability of the aromatic substituent results in a lower bonding energy of the oxygen core level electron.[37, 66] This effect leads C–O and C–O–C in lignin to the lower binding energy and thus makes them assigning to O_1.[37, 66] As a result, XPS is promising for quantitative estimation of lignin content of the biomass by quantifying elemental compositions and the oxygen–carbon (O/C) ratio.[37, 67, 68] A high O/C ratio reflects higher cellulose and/ or hemicellulose content, while a low O/C suggests the presence of more lignin. Ma *et al.* reported an increase of C_1 (C-C/C-H) concentration and a decrease in O/C ratio on the both exterior and interior surface of the hydrothermally pretreated bamboo chips.[69] With extended pretreatment time, an increasing amount of lignin/pseudo-lignin deposited on the exterior surface of bamboo chips. Furthermore, O/C was found to decrease to a low level that approached lignin values.[69]

Ma *et al.* observed a gradual increase in the deposition of droplets on the surface of hydrothermally pretreated of holocellulose.[37] C_1 increased from 8.9 to 23.0 % along with an increase in K-lignin from 1.0 to 6.2 %. In theory, degradation of polysaccharides has negligible effects on its relative content of C, O, and O/C. However, the pseudo-lignin produced from repolymerization of polysaccharides degradation products[14, 30] and/or polymerization with lignin[14, 30] cause a drastic increase of C_1, a decrease in C_2, $C_{3,}$ and O_2 which led to a lower O/C.[37] Accordingly, more alicyclic structures and hydroxyl groups might be involved in pseudo-lignin.[37]

FTIR spectroscopic analysis

FTIR spectra of pseudo-lignin provides further evidence on the chemical transformations taking place during severe acid and hydrothermal pretreatments.[17, 23, 53] Hu *et al.* compared the FTIR spectra of α-cellulose, holocellulose, and pseudo-lignin extracted from pretreated α-cellulose and holocellulose of poplar (Figure 4).[15] From the FTIR spectra, they observed that pseudo-lignin is composed of hydroxyl, carbonyl, and aromatic structures. The strong bands at 1697 cm^{-1} and 1611 cm^{-1} together with the band at 1512 cm^{-1} can be attributed to C=O (carbonyl and/ or carboxylic) conjugated with aromatic ring, whereas the bands in the 1320–1000 cm^{-1} region correspond to C–O stretching (in alcohols, ethers, or carboxylic acids) (Table 3).[15] The strong and broad hydroxyl stretching peaks at 3238 cm^{-1} indicated the presence of hydrogen-bonding in isolated pseudo-lignin. These observations indicated that under severe acid pretreatment conditions, carbohydrates undergo dehydration, rearrangement, aromatization and condensation reactions to form pseudo-lignin. In addition, the peak at 867 cm^{-1} arising from C–H out-of-plane bending suggests the benzene rings of pseudo-lignin are 1,3,5-trisubstituted.[15]

In another study, Kumar *et al.* observed similar findings when comparing FTIR spectra of untreated Avicel cellulose, solids prepared from different severity pretreatment for cellulose alone and mixed with xylan/xylose, and xylose derived pseudo-lignin.[23] FTIR spectra indicated that solids prepared at combined severity factor (CSF) of 3.56 had strong peaks associated with the carbonyl and aromatic stretching regions while Avicel cellulose structure stayed intact at all conditions until a CSF of 3.56.[23] Maohua *et al.* studied changes in the prevalence of the main functional groups of the solid surface of corn stem rind treated under high pressure water pretreatment. With an increase in pretreatment severity, a gradual decrease of the peak intensity at 1740 cm^{-1} (assigned to the acetyl group in hemicelluloses or the carbonyl on the carboxyl group) indicated a reduction in the content of hemicellulose.[53] Hu *et al.* compared the FTIR spectra of pseudo-lignin and dilute acid pretreated lignin.[24] Pseudo-lignin showed broader hydroxyl stretching peak at ~3300 cm^{-1} in comparison to dilute acid pretreated lignin. Furthermore, both samples indicated aromatic absorptions at ~1600 and 1500 cm^{-1} albeit with different intensities. This suggested the presence of different aromatic structures and differing aromatic substitution patterns between pseudo-lignin and dilute acid-pretreated lignin.[24]

NMR spectroscopic analysis

NMR characterization of lignin and pseudo-lignin structures further helps to get insight into structural similarities and differences. ^{13}C NMR spectra confirmed the predominant presence of polyphenolic, lignin-like, materials with carbonyl, carboxylic, methoxyl, aromatic, and aliphatic structures in pseudo-lignin isolated from α-cellulose and poplar holocellulose.[15, 17] The peaks centred at 208–205 ppm and 203–185 ppm can be attributed to C=O in ketones and C=O in aldehydes, respectively. Whereas the peaks centred at 178– 172 ppm correspond to C=O in carboxylic acids. The ^{13}C NMR spectra of isolated pseudo-lignin also presented common peaks in the aromatic region (δ 155–96 ppm). These signals were not present in the spectra from cellulose and/or xylan from which pseudo-lignin was derived. This again confirmed that pseudo-lignin was a polyphenolic, lignin-like material with aliphatic, aromatic, and carbonyl structures derived from cellulose/hemicellulose fragments released during acid hydrolysis reactions.

Gel permeation chromatography (GPC)

GPC is a widely used technique to assess the molecular weight distribution of lignin. Molecular weight distribution of pseudo-lignin after different pretreatment conditions can provide important information about the fragmentation and recondensation reactions occurring during dilute acid and hydrothermal pretreatment of biomass. Molecular weight (MW) of pseudo-lignin isolated from dilute acid and hydrothermal pretreatment of different resources is shown in Figure 5.[15, 37, 70, 71] The observed MW of pseudo-lignin was found to be lower than that of milled wood lignin.[15] For example, the weight-average molecular weight (M_w) of milled poplar lignin and pseudo-lignin derived from dilute acid pretreated poplar holocellulose at 180 °C were found to be 13250 g/mol and 5050 g/mol, respectively.[15, 70] Whereas the M_w of milled bamboo lignin and pseudo-lignin isolated from hydrothermal pretreatment at 170 ^{o}C were found to be 12090 g/mol and 5340 g/mol, respectively.[37, 71] However, as pretreatment severity was increased, there was an increase in MW of pseudo-lignin observed for both pseudo-lignin derived from bamboo holocellulose as well as from poplar holocellulose. The M_w and number-average molecular weight (M_n) of the pseudo-lignin isolated from bamboo holocellulose as shown in Figure 5 indicated that repolymerization reactions occurred during the pretreatment; therefore aromatic rings can be alkylated significantly to produce heavy condensation compounds.[37] However, lower value of polydispersity index (PDI) for pseudo-lignin isolated from hydrothermal pretreated bamboo holocellulose at 170 °C, 240 min suggested moderate molecular weight distribution of pseudo-lignin polymers in comparison to pseudo-lignin isolated at 170 °C, 150 min. The MW of pseudo-lignin derived from dilute acid pretreated holocellulose was found larger than that of pseudo-lignin extracted from pretreated α-cellulose. In addition, with increase in severity of dilute acid pretreatment of poplar holocellulose, PDI of pseudo-lignin increased which suggested broad molecular weight distribution. As pseudo-lignin is formed via polycondensation of intermediates from sugar degradation products, pseudo-lignin molecular weight distributions in the pretreated materials seem to be influenced by competition between fragmentation and condensation reactions which is contingent on the pretreatment severity and biomass composition.

Recalcitrance from pseudo-lignin

There is no doubt that pseudo-lignin's hydrophobic nature enables unproductive binding with enzymes, therefore it plays a negative role in enzymatic hydrolysis. However, the exact effect of pseudo-lignin on enzymatic hydrolysis compared to dilute acid-pretreated lignin is still under debate. Hu *et al.* reported inhibition of cellulase enzyme activity by pseudo-lignin with reduced overall conversion yields while dilute acid-pretreated lignin inhibition was observed at the initial stage (before 24h of hydrolysis) with no obvious effect on the overall conversion yield.[24] Pseudo-lignin showed a strong inhibition of 9.5-25.1% on the overall enzymatic conversion yield of cellulose in comparison to a 50/50 mixture of pseudo-lignin and dilute acid pretreated lignin (1.9-6.7%) which suggested that pseudo-lignin might be more detrimental to enzymatic hydrolysis than dilute acid-pretreated lignin. On the other hand, He at al. revealed that bamboo pseudo-lignin actually exerted less inhibitory effects upon enzymes compared to dilute acid pretreated bamboo lignin, which could be due to the pseudo-lignin's lower negative surface charge and hydrophobic nature, and more importantly lower binding strength with cellulases.[72] In conclusion, the effect of pseudo-lignin versus dilute acid pretreated lignin on enzymatic hydrolysis depends on the nature of lignin resources as well as nature of hydrolysis resources. For example, current recalcitrance study of lignin is usually achieved by physically mixing lignin resources with lignocellulosic samples, thus cellulose accessible surface area of the lignocellulosic samples also plays an important role besides lignin structures. It is well known that lignin has tendency to unproductively bind to cellulases due to its hydrophobic structural features including hydrogen bonding, methoxy groups, and polyaromatic structures.[20, 73, 74] Methoxy and polyaromaticity structural features of pseudo-lignin and its water insolubility suggest pseudo-lignin is hydrophobic in nature and thus contributes to the non-productive binding of lignin to cellulases.[24] Further, pseudo-lignin also deposits on the surface of the pretreated solids thus directly decreasing cellulose accessibility by blocking the surface binding sites.[23, 24] Kumar *et al.* studied the influence of hemicellulose derived pseudo-lignin on cellulose conversion at the moderate to low enzyme loadings.[23] A significant inhibition of cellulose hydrolysis was observed for hemicelluloses (xylan) derived pseudo-lignin. Protein adsorption experiments confirmed that pseudo-lignin binds to the enzymes unproductively.[23] Impact of pseudo-lignin inhibition is high at low enzyme loadings suggesting for commercial applications it is necessary to avoid formation of pseudo-lignin during biomass pretreatment. Given the interest in lignin valorization, especially from cellulosic ethanol operations, researchers need be aware of the presence of pseudo-lignin and its possible presence in fermentation residues.

Prevention of pseudo-lignin formation during biomass pretreatment

As pseudo-lignin can only be formed at high pretreatment severity and in presence of acid, reducing the pretreatment severity is an obvious way to reduce or avoid the formation of pseudo-lignin. However, as the pretreatment severity decreases, biological conversion of pretreated solids to sugar drops substantially due to plant recalcitrance. Flow-through pretreatment has recently been shown to dramatically increase lignin removal from about 75.6 to 98.0% and 59.3 to 87.8% for dilute acid and hydrothermal flow-through pretreatments, respectively.[75] More importantly, its ability to constantly remove hemicelluloses oligomers into the aqueous phase effectively restricts the presence of sugar dehydration and condensation products. Almost complete xylan removal was achieved at reaction severity of $\log R_0 > 4.5$ and $\log R_0 > 4.2$ for water-only and dilute acid flow-through operations, respectively.[75] As a result, flow-through pretreatment can reduce pseudo-lignin formation through simultaneous removal of sugar fragments.[75, 76]

Hu and Ragauskas examined the role of oxygen in pseudo-lignin formation as they reported that a dilute acid pretreatment of holocellulose under O_2 at high severity provided treated biomass with ~89% pseudo-lignin in comparison to ~49% of pseudo-lignin for DAP conducted under N_2 and 42% under air. This suggested that N_2 could suppress oxidative reactions but could not suppress other possible reaction pathways responsible for pseudo-lignin formation. Thus, the use of a N_2 atmosphere was not effective in terms of suppressing pseudo-lignin formation although an enriched oxygen atmosphere significantly facilitated additional pseudo-lignin formation. It was also reported that introduction of dimethyl sulfoxide (DMSO) to the acidic medium could effectively suppress HMF degradation which is one of key intermediates during the pseudo-lignin formation.[77] A recent study modified a series of dilute acid pretreatment by using N_2, surfactant Tween-80, or DMSO-water mixture as the reaction medium to test these hypotheses for new methods of suppressing pseudo-lignin formation without significantly reducing the pretreatment severity.[25] The introduction of DMSO significantly reduced the pseudo-lignin content by ~30%. This was attributed to the reduced coordination of HMF with water in the presence of DMSO due to the stronger interaction of DMSO oxygen than water oxygen to be in the first solvation shell of HMF.[78] Also, the hydrophobic nature of the sulphur atom and methyl group of DMSO in the first solvation shell of HMF can push the second water solvation shell farther away from the HMF molecule.[25, 78, 79] Additionally, the prevailing carbonyl carbon atom (C1) of HMF-DMSO interaction over C1-H_2O interaction can protect the cleavage of C1 atom of HMF which eventually leads to form pseudo-lignin.[25, 78]

From a reaction mechanism perspective, the reduction of HMF water coordination could protect the HMF molecule from further reactions to form pseudo-lignin.[25] Although DMSO had exceptional pseudo-lignin suppression property, it did not change the pseudo-lignin molecular weight or any of its structural features significantly. In addition, although DMSO did reduce the inhibition effect of pseudo-lignin to enzymatic deconstruction of cellulose, its pseudo-lignin suppression effect in turn increased the enzymatic digestibility of cellulose after DAP.[25] Although it has not been studied in detail whether Organosolv pretreatments such as using ethanol and tetrahydrofuran (THF) prevents pseudo-lignin formation completely, near theoretical sugar yields in the case of THF-cosolvent pretreatment suggests that these pretreatments provide another effective way to reduce pseudo-lignin formation and increase sugar yields at commercially viable low enzyme loadings.[80]

Conclusions and future perspective

Increased K-lignin content of acid and hydrothermal based biomass pretreatments under severe pretreatment conditions are mainly products of polycondensation reactions of degradation products of plant carbohydrates. These lignin-like structures termed as pseudo-lignin, are produced in significant quantities from cellulose and hemicelluloses when biomass is pretreated with a dilute acid and/or autohydrolysis at high severities. The resulting pseudo-lignin structures can deposit on the surface of pretreated biomass and affect enzymatic hydrolysis of cellulose through reduced cellulose accessibility and/or cellulase effectiveness by its non-productive binding to enzyme. Therefore, it is important to develop pretreatment processes that can effectively avoid sugar degradation and hence pseudo-lignin formation. Understanding the fundamental chemistry associated with pseudo-lignin formation is crucial for the future bioethanol production.

Acknowledgements

This study was partially supported by UT-Battelle, LLC under Contract No. DE-AC05-00OR22725 with the U.S. Department of Energy. This study was supported and performed as part of the BioEnergy Science Center (BESC) and Center for BioEnergy Innovation (CBI). The BioEnergy Science Center and CBI are U.S. Department of Energy Bioenergy Research Centers supported by the Office of Biological and Environmental Research in the DOE Office of Science. In addition, we wish to thank support of U.S. DOE (Department of Energy) EERE (Energy Efficiency and Renewable Energy) BETO (Bioenergy Technology Office) (DE-FOA-0001085). The views and opinions of the authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, expressed or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights.

References

1. A. J. Ragauskas, C. K. Williams, B. H. Davison, G. Britovsek, J. Cairney, C. A. Eckert, W. J. Frederick, Jr., J. P. Hallett, D. J. Leak, C. L. Liotta, J. R. Mielenz, R. Murphy, R. Templer and T. Tschaplinski, *Science (Washington, DC, U. S.)*, 2006, **311**, 484-489.
2. X. Zhao, L. Zhang and D. Liu, *Biofuels, Bioproducts and Biorefining*, 2012, **6**, 465-482.
3. M. Foston and A. J. Ragauskas, *Biomass Bioenergy*, 2010, **34**, 1885-1895.
4. X. Meng and A. J. Ragauskas, *Curr. Opin. Biotechnol.*, 2014, **27**, 150-158.
5. Y. Pu, F. Hu, F. Huang, B. H. Davison and A. J. Ragauskas, *Biotechnol. Biofuels*, 2013, **6**, 15.
6. Y. Zhang, G. Yu, B. Li, X. Mu, H. Peng and H. Wang, *Carbohydrate Polymers*, 2016, **141**, 238-243.
7. M. J. Selig, S. Viamajala, S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant, *Biotechnol. Prog.*, 2007, **23**, 1333-1339.
8. X. Meng, T. Wells, Jr., Q. Sun, F. Huang and A. Ragauskas, *Green Chem.*, 2015, **17**, 4239-4246.
9. L. Zhang, L. Yan, Z. Wang, D. D. Laskar, M. S. Swita, J. R. Cort and B. Yang, *Biotechnology for Biofuels*, 2015, **8**, 203.
10. Q. Sun, M. Foston, X. Meng, D. Sawada, S. V. Pingali, H. M. O'Neill, H. Li, C. E. Wyman, P. Langan, A. J. Ragauskas and R. Kumar, *Biotechnol. Biofuels*, 2014, **7**, 150/151-150/114, 114 pp.
11. Q. Sun, M. Foston, D. Sawada, S. V. Pingali, H. M. O'Neill, H. Li, C. E. Wyman, P. Langan, Y. Pu and A. J. Ragauskas, *Cellulose (Dordrecht, Neth.)*, 2014, **21**, 2419-2431.
12. S. D. Kshirsagar, P. R. Waghmare, P. Chandrakant Loni, S. A. Patil and S. P. Govindwar, *RSC Advances*, 2015, **5**, 46525-46533.
13. S. Chen, Z. Wang, K. Fang, G. Fan, W. Peng and J. Song, *BioResources*, 2014, **9**, 7636-7652, 7617 pp.

14. J. Li, G. Henriksson and G. Gellerstedt, *Bioresour. Technol.*, 2007, **98**, 3061-3068.
15. F. Hu, S. Jung and A. Ragauskas, *Bioresour. Technol.*, 2012, **117**, 7-12.
16. C. Sievers, T. Marzialetti, T. J. C. Hoskins, M. B. Valenzuela Olarte, P. K. Agrawal and C. W. Jones, *Bioresour. Technol.*, 2009, **100**, 4758-4765.
17. P. Sannigrahi, D. H. Kim, S. Jung and A. Ragauskas, *Energy Environ. Sci.*, 2011, **4**, 1306-1310.
18. Y. Zeng, S. Zhao, S. Yang and S.-Y. Ding, *Curr Opin Biotechnol*, 2014, **27**.
19. X. Meng, M. Foston, J. Leisen, J. DeMartini, C. E. Wyman and A. J. Ragauskas, *Bioresour. Technol.*, 2013, **144**, 467-476.
20. B. Yang and C. E. Wyman, *Biotechnol. Bioeng.*, 2006, **94**, 611-617.
21. X. Qian, M. R. Nimlos, D. K. Johnson and M. E. Himmel, *Appl. Biochem. Biotechnol.*, 2005, **121-124**, 989-997.
22. F. Hu and A. Ragauskas, *BioEnergy Res.*, 2012, **5**, 1043-1066.
23. R. Kumar, F. Hu, P. Sannigrahi, S. Jung, A. J. Ragauskas and C. E. Wyman, *Biotechnol. Bioeng.*, 2013, **110**, 737-753.
24. F. Hu, S. Jung and A. Ragauskas, *ACS Sustainable Chem. Eng.*, 2013, **1**, 62-65.
25. F. Hu and A. Ragauskas, *RSC Adv.*, 2014, **4**, 4317-4323.
26. M. H. Sipponen, V. Pihlajaniemi, S. Sipponen, O. Pastinen and S. Laakso, *RSC Adv.*, 2014, **4**, 23177-23184.
27. L. J. Jönsson and C. Martín, *Bioresource Technology*, 2016, **199**, 103-112.
28. A. Avci, B. C. Saha, G. J. Kennedy and M. A. Cotta, *Bioresource Technology*, 2013, **142**, 312-319.
29. S. Bhagia, H. Li, X. Gao, R. Kumar and C. E. Wyman, *Biotechnology for Biofuels*, 2016, **9**, 245.
30. J. Li, G. Henriksson and G. Gellerstedt, *Appl. Biochem. Biotechnol.*, 2005, **125**, 175-188.
31. K. RadotiĆ, M. MiĆIĆ and M. JeremiĆ, *Annals of the New York Academy of Sciences*, 2005, **1048**, 215-229.
32. B. S. Donohoe, S. R. Decker, M. P. Tucker, M. E. Himmel and T. B. Vinzant, *Biotechnol. Bioeng.*, 2008, **101**, 913-925.
33. D. M. de Carvalho, J. H. de Queiroz and J. L. Colodette, *BioResources*, 2017, **12**, 3088-3107.
34. D. M. d. Carvalho, O. Sevastyanova, L. S. Penna, B. P. d. Silva, M. E. Lindström and J. L. Colodette, *Industrial Crops and Products*, 2015, **73**, 118-126.
35. A. Kumagai, S.-H. Lee and T. Endo, *Biotechnol. Bioeng.*, 2016, **113**, 1441-1447.
36. J. Zhuang, X. Wang, J. Xu, Z. Wang and M. Qin, *Wood Sci. Technol.*, 2017, **51**, 165-174.
37. X. Ma, X. Yang, X. Zheng, L. Chen, L. Huang, S. Cao and H. Akinosho, *Cellulose (Dordrecht, Neth.)*, 2015, **22**, 1687-1696.
38. A. Bauer, J. Lizasoain, F. Theuretzbacher, J. W. Agger, M. Rincon, S. Menardo, M. K. Saylor, R. Enguidanos, P. J. Nielsen, A. Potthast, T. Zweckmair, A. Gronauer and S. J. Horn, *Bioresour. Technol.*, 2014, **166**, 403-410.
39. S.-W. Du, W.-H. Chen and J. A. Lucas, *Bioresource Technology*, 2014, **161**, 333-339.
40. M. Normark, L. Pommer, J. Graesvik, M. Hedenstroem, A. Gorzsas, S. Winestrand and L. J. Joensson, *BioEnergy Res.*, 2016, **9**, 355-368.
41. Q. Wu, L. Huang, S. Yu, S. Liu, C. Xie and A. J. Ragauskas, *Energy (Oxford, U. K.)*, 2017, **133**, 171-178.
42. M. J. Negro, P. Manzanares, J. M. Oliva, I. Ballesteros and M. Ballesteros, *Biomass Bioenergy*, 2003, **25**, 301-308.
43. Q. A. Nguyen, M. P. Tucker, F. A. Keller and F. P. Eddy, *Appl. Biochem. Biotechnol.*, 2000, **84-86**, 561-576.
44. M. T. Guarnieri, M. Ann Franden, C. W. Johnson and G. T. Beckham, *Metabolic Engineering Communications*, 2017, **4**, 22-28.
45. H. Li, X. Chen, J. Ren, H. Deng, F. Peng and R. Sun, *Biotechnology for Biofuels*, 2015, **8**, 127.
46. T. Popoff and O. Theander, *Carbohyd. Res.*, 1972, **22**, 135-149.
47. G. C. A. Luijkx, F. van Rantwijk and H. van Bekkum, *Carbohydr. Res.*, 1993, **242**, 131-139.
48. G. C. A. Luijkx, W. van der Horst, S. O. A. Koskinen, F. van Rantwijk and H. van Bekkum, *J. Anal. Appl. Pyrolysis*, 1994, **28**, 245-254.
49. A. Chuntanapum and Y. Matsumura, *Industrial & Engineering Chemistry Research*, 2010, **49**, 4055-4062.
50. K. Yamamoto, T. Asada, H. Nishide and E. Tsuchida, *Bull. Chem. Soc. Jpn.*, 1990, **63**, 1211-1216.
51. X. Ma, X. Zheng, L. Lin, L. Chen, S. Survase, L. Huang and S. Cao, *Wood Sci. Technol.*, 2015, **49**, 945-955.
52. M. Foston and A. J. Ragauskas, *Ind. Biotechnol.*, 2012, **8**, 191-208.
53. M. Yang, J. Xing, Y. Liu, Y. Liu, T. Mu and J. Zhou, *BioResources*, 2015, **10**, 8474-8486.
54. H. Palonen, F. Tjerneld, G. Zacchi and M. Tenkanen, *J Biotechnol*, 2004, **107**.
55. J. D. DeMartini, S. Pattathil, U. Avci, K. Szekalski, K. Mazumder, M. G. Hahn and C. E. Wyman, *Energy Environ Sci*, 2011, **4**.
56. M. H. Studer, J. D. DeMartini, M. F. Davis, R. W. Sykes, B. Davison, M. Keller, G. A. Tuskan and C. E. Wyman, *Proc Natl Acad Sci USA*, 2011, **108**.
57. P. Sannigrahi, A. J. Ragauskas and S. J. Miller, *BioEnergy Res*, 2008, **1**.
58. R. Samuel, Y. Pu, B. Raman and A. J. Ragauskas, *Appl. Biochem. Biotechnol.*, 2010, **162**, 62-74.

59. T. Pielhop, G. O. Larrazabal, M. H. Studer, S. Brethauer, C.-M. Seidel and P. Rudolf von Rohr, *Green Chemistry*, 2015, **17**, 3521-3532.
60. K. Ishimaru, T. Hata, P. Bronsveld, D. Meier and Y. Imamura, *J. Mater. Sci.*, 2007, **42**, 122-129.
61. D. Kocaefe, X. Huang, Y. Kocaefe and Y. Boluk, *Surf. Interface Anal.*, 2013, **45**, 639-649.
62. A. Toth, O. Faix, G. Rachor, I. Bertoti and T. Szekely, *Appl. Surf. Sci.*, 1993, **72**, 209-213.
63. H. Chtourou, B. Riedl and B. V. Kokta, *J. Colloid Interface Sci.*, 1993, **158**, 96-104.
64. P. Nzokou and D. P. Kamdem, *Surf. Interface Anal.*, 2005, **37**, 689-694.
65. L.-S. Johansson, J. M. Campbell, K. Koljonen and P. Stenius, *Appl. Surf. Sci.*, 1999, **144-145**, 92-95.
66. X. Hua, S. Kaliaguine, B. V. Kokta and A. Adnot, *Wood Sci. Technol.*, 1993, **28**, 1-8.
67. W. Shen and I. H. Parker, *Cellulose (Dordrecht, Neth.)*, 1999, **6**, 41-55.
68. J. Zhang, H. Zhang and J. Zhang, *Cellulose (Dordrecht, Neth.)*, 2014, **21**, 569-579.
69. X. J. Ma, S. L. Cao, L. Lin, X. L. Luo, L. H. Chen and L. L. Huang, *Bioresour. Technol.*, 2013, **136**, 757-760.
70. A. Tolbert, H. Akinosho, R. Khunsupat, A. K. Naskar and A. J. Ragauskas, *Biofuels, Bioprod. Biorefin.*, 2014, **8**, 836-856.
71. M.-F. Li, S.-N. Sun, F. Xu and R.-C. Sun, *Chemical Engineering Journal*, 2012, **179**, 80-89.
72. J. He, C. Huang, C. Lai, C. Huang, X. Li and Q. Yong, *Industrial Crops and Products*, 2018, **113**, 368-375.
73. T. Vu, A. Chaffee and I. Yarovsky, *Mol. Simul.*, 2002, **28**, 981-991.
74. R. Kumar and C. E. Wyman, *Biotechnol. Prog.*, 2009, **25**, 807-819.
75. L. Yan, L. Zhang and B. Yang, *Biotechnology for Biofuels*, 2014, **7**, 76.
76. H. Shen, C. R. Poovaiah, A. Ziebell, T. J. Tschaplinski, S. Pattathil, E. Gjersing, N. L. Engle, R. Katahira, Y. Pu, R. Sykes, F. Chen, A. J. Ragauskas, J. R. Mielenz, M. G. Hahn, M. Davis, C. N. Stewart and R. A. Dixon, *Biotechnology for Biofuels*, 2013, **6**, 71.
77. Y. Roman-Leshkov, J. N. Chheda and J. A. Dumesic, *Science (Washington, DC, U. S.)*, 2006, **312**, 1933-1937.
78. S. H. Mushrif, S. Caratzoulas and D. G. Vlachos, *Phys. Chem. Chem. Phys.*, 2012, **14**, 2637-2644.
79. R. L. Mancera, M. Chalaris, K. Refson and J. Samios, *Phys. Chem. Chem. Phys.*, 2004, **6**, 94-102.
80. T. Y. Nguyen, C. M. Cai, R. Kumar and C. E. Wyman, *ChemSusChem*, 2015, **8**, 1716-1725.
81. M. Foston and A. J. Ragauskas, *Energy Fuels*, 2010, **24**, 5677-5685.
82. S. Cao, Y. Pu, M. Studer, C. L. Wyman and A. J. Ragauskas, *RSC Advances*, 2012, **2**.
83. L. Shuai, Q. Yang, J. Y. Zhu, F. C. Lu, P. J. Weimer, J. Ralph and X. J. Pan, *Bioresour. Technol.*, 2010, **101**, 3106-3114.
84. C. Li, L. Sun, B. A. Simmons and S. Singh, *BioEnergy Res.*, 2013, **6**, 14-23.
85. C. Zhang, X. Lei, C. T. Scott, J. Y. Zhu and K. Li, *BioEnergy Res.*, 2014, **7**, 362-370.
86. F. Huang and A. J. Ragauskas, *Ind Biotechnol*, 2012, **8**.
87. J. D. Mao, K. M. Holtman and D. Franqui-Villanueva, *J. Agric. Food Chem.*, 2010, **58**, 11680-11687.
88. C. Li, B. Knierim, C. Manisseri, R. Arora, H. V. Scheller, M. Auer, K. P. Vogel, B. A. Simmons and S. Singh, *Bioresour Technol*, 2010, **101**.
89. X. Meng, Q. Sun, M. Kosa, F. Huang, Y. Pu and A. J. Ragauskas, *ACS Sustainable Chem. Eng.*, 2016, **4**, 4563-4572.
90. S. V. Pingali, V. S. Urban, W. T. Heller, J. McGaughey, H. O'Neill, M. Foston, D. A. Myles, A. Ragauskas and B. R. Evans, *Biomacromolecules*, 2010, **11**, 2329-2335.
91. Z. Ji, X. Zhang, Z. Ling, R.-C. Sun and F. Xu, *Carbohydrate Polymers*, 2016, **154**, 247-256.
92. N. A. Boakye-Boaten, S. Xiu, A. Shahbazi, L. Wang, R. Li, M. Mims and K. Schimmel, *Bioresource Technology*, 2016, **204**, 98-105.
93. H. B. Sluiter A, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. , *Technical report NREL/TP-510-42618*, 2008.
94. W. Schwald, R. Concin, G. Bonn and O. Bobleter, *Chromatographia*, 1985, **20**, 35-40.
95. Y. Yu, B. Song, Y. Long and H. Wu, *Energy & Fuels*, 2016, **30**, 8787-8789.
96. A. Sluiter, Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., *Journal*, 2008.
97. H. E. Grethlein, *Nat Biotechnol*, 1985, **3**.
98. M. Zeng, N. S. Mosier, C. P. Huang, D. M. Sherman and M. R. Ladisch, *Biotechnol Bioeng*, 2007, **97**.
99. M. Zeng, E. Ximenes, M. R. Ladisch, N. S. Mosier, W. Vermerris, C. P. Huang and D. M. Sherman, *Biotechnol Bioeng*, 2012, **109**.
100. T. C. Hsu, G. L. Guo, W. H. Chen and W. S. Hwang, *Bioresource Technol*, 2010, **101**.
101. W.-H. Chen, Y.-J. Tu and H.-K. Sheen, *Appl Energy*, 2011, **88**.
102. C.-T. Yu, W.-H. Chen, L.-C. Men and W.-S. Hwang, *Ind Crops Prod*, 2009, **29**.
103. W.-H. Chen, Y.-J. Tu and H.-K. Sheen, *Int. J. Energy Res.*, 2010, **34**, 265-274.
104. X. Yuan, L. He, S. Singh, B. A. Simmons and G. Cheng, *Energy & Fuels*, 2017, **31**, 10874-10879.
105. G. W. Lynn, W. Heller, V. Urban, G. D. Wignall, K. Weiss and D. A. A. Myles, *Physica B: Condensed Matter*, 2006, **385-386**, 880-882.
106. J. Ilavsky and P. R. Jemian, *Journal of Applied Crystallography*, 2009, **42**, 347-353.

107. X. Yuan, Y. Duan, L. He, S. Singh, B. Simmons and G. Cheng, *Bioresource Technology*, 2017, **232**, 113-118.
108. S. V. Pingali, H. M. O'Neill, J. McGaughey, V. S. Urban, C. S. Rempe, L. Petridis, J. C. Smith, B. R. Evans and W. T. Heller, *Journal of Biological Chemistry*, 2011, **286**, 32801-32809.
109. S. Singh, G. Cheng, N. Sathitsuksanoh, D. Wu, P. Varanasi, A. George, V. Balan, X. Gao, R. Kumar, B. E. Dale, C. E. Wyman and B. A. Simmons, *Frontiers in Energy Research*, 2015, **2**.
110. C. A. Hubbell and A. J. Ragauskas, *Bioresour. Technol.*, 2010, **101**, 7410-7415.
111. J. M. Martínez, J. Reguant, M. Á. Montero, D. Montané, J. Salvadó and X. Farriol, *Ind Eng Chem Res*, 2007, **36**.
112. B. Yang and C. E. Wyman, *Bioresour Technol*, 2008, **99**.
113. L. Segal, J. J. Creely, A. E. Martin and C. M. Conrad, *Text Res J*, 1959, **29**.
114. R. Kumar, G. Mago, V. Balan and C. E. Wyman, *Bioresour. Technol.*, 2009, **100**, 3948-3962.
115. R. Samuel, Y. Pu, M. Foston and A. J. Ragauskas, *Biofuels*, 2010, **1**.
116. L. P. Xiao, Z. J. Sun, Z. J. Shi, F. Xu and R. C. Sun, *Bioresources*, 2011, **6**.
117. J. M. Lee, H. Jameel and R. A. Venditti, *Bioresource Technol*, 2010, **101**.
118. S. Park, J. O. Baker, M. E. Himmel, P. A. Parilla and D. K. Johnson, *Biotechnol Biofuels*, 2010, **3**.

Figure captions

Figure 1. Hypothesized reaction pathways for pseudo-lignin formation.[15]

Figure 2. SEM images of (a) untreated Avicel cellulose (magnification 20k×) and of pretreated solids at (b) CSF 2.66 (magnification 20k×), (c) 2.95 (magnification 20k×), and (d) 3.56 (magnification 20k×). (e) and (e') xylose derived-pseudo-lignin at 5k× and 20k× magnifications. Marker (i) designates solids from pretreatment of Avicel cellulose alone, (ii) from pretreatment of cellulose mixed with xylan, and (iii) cellulose mixed with xylose. For example, Figure notation 2c-iii is for pretreated solids prepared at CSF 2.95 of cellulose mixed with xylose. Scale bar length = 2 μm, unless otherwise noted. Reproduced with permission from reference 23.

Figure 3. High resolution scans of C1s and O1s peaks. (a). C1s. (b). O1s. (c). deconvoluted peaks of oxygen of pseudo lignin. Holo: holocellulose; PL-1: Hydrothermal pretreated holocellulose at 170 ^{o}C, 150 min; PL-2: Hydrothermal pretreated holocellulose at 170 ^{o}C, 240 min; RL: Reference lignin. Reproducted with permission from reference 37.

Figure 4. FTIR spectra of cellulose, holocellulose, pseudo-lignin extracted from dilute acid pretreated α-cellulose (180 °C, 0.1 M H_2SO_4, 40 min), pretreated holocellulose A (180 °C, 0.1 M H_2SO_4, 40 min) and pretreated holocellulose B (180 °C, 0.2 M H_2SO_4, 60 min). Reproduced with permission from reference 15.

Figure 5. Weight average (M_w) and number average (M_n) molecular weight distribution and polydispersity index (PDI) of pseudo-lignin isolated from dilute acid and hydrothermal pretreated cellulosic substrates.[15, 37 70, 71]

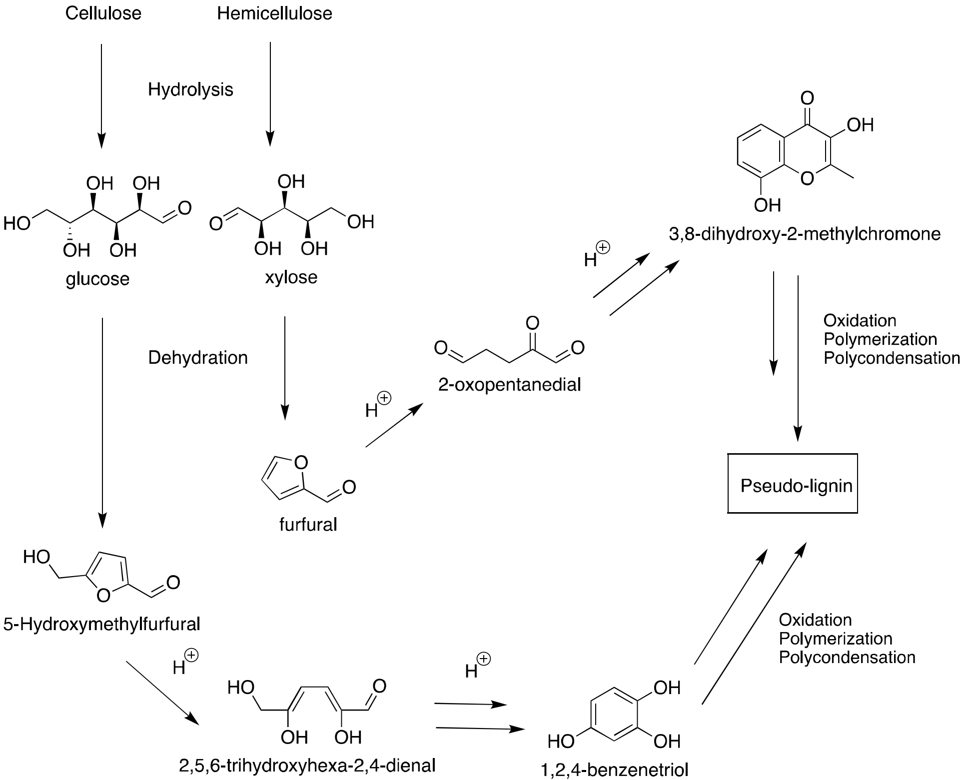


Figure 1. Hypothesized reaction pathways for pseudo-lignin formation.[15]

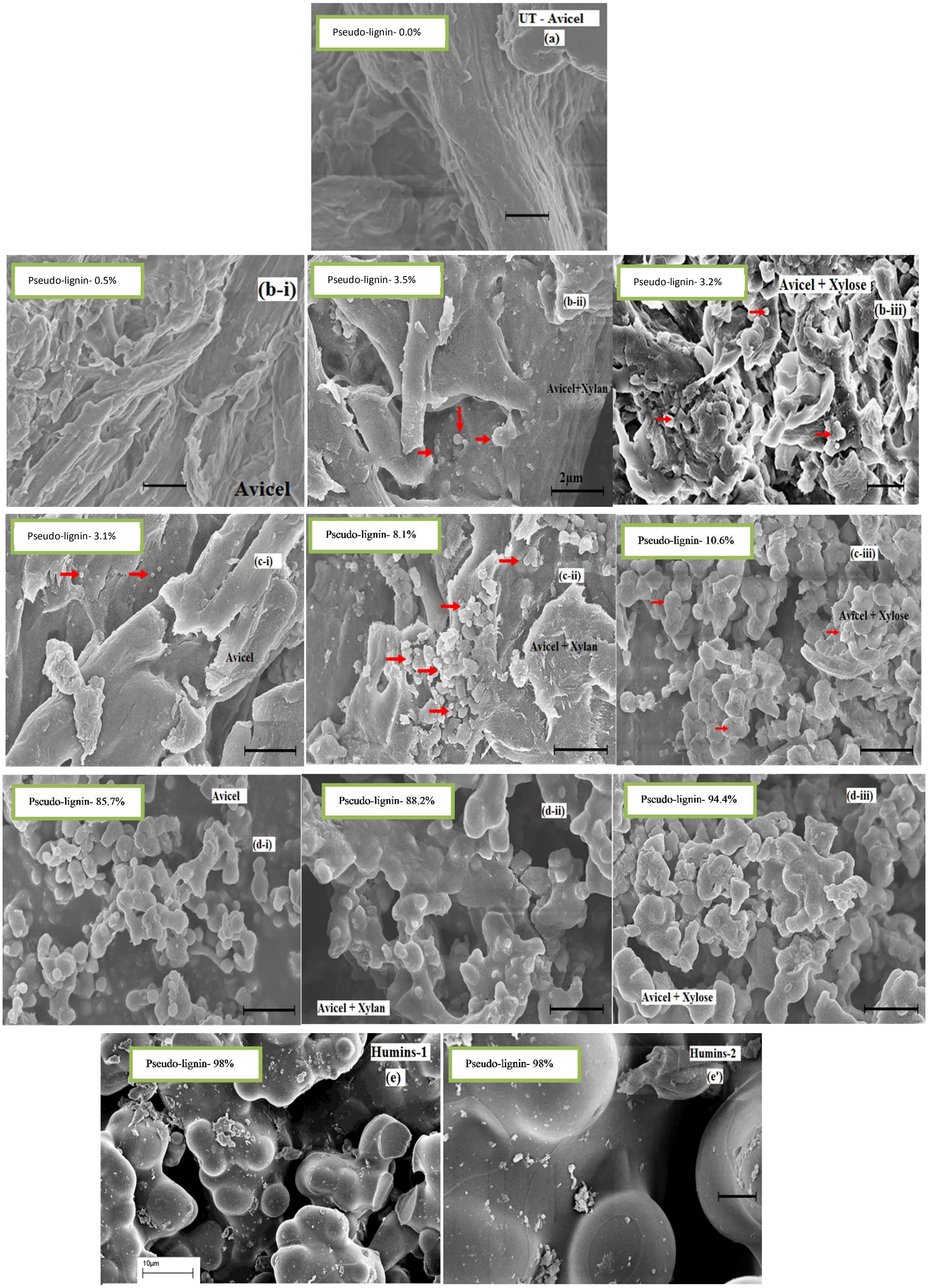

Pseudo-lignin- 0.0%
UT - Avicel
(a)
Pseudo-lignin- 0.5%
(b-i)
Avicel
Pseudo-lignin- 3.5%
(b-ii)
Avicel+Xylan
2µm
Pseudo-lignin- 3.2%
Avicel + Xylose
(b-iii)
Pseudo-lignin- 3.1%
(c-i)
Avicel
Pseudo-lignin- 8.1%
(c-ii)
Avicel + Xylan
Pseudo-lignin- 10.6%
(c-iii)
Avicel + Xylose
Pseudo-lignin- 85.7%
Avicel
(d-i)
Pseudo-lignin- 88.2%
(d-ii)
Avicel + Xylan
Pseudo-lignin- 94.4%
(d-iii)
Avicel + Xylose
Pseudo-lignin- 98%
Humins-1
(e)
10µm
Pseudo-lignin- 98%
Humins-2
(e')

Figure 2. SEM images of (a) untreated Avicel cellulose (magnification 20k×) and of pretreated solids at (b) CSF 2.66 (magnification 20k×), (c) 2.95 (magnification 20k×), and (d) 3.56 (magnification 20k×). (e) and (e') xylose derived-pseudo-lignin at 5k× and 20k× magnifications. Marker (i) designates solids from pretreatment of Avicel cellulose alone, (ii) from pretreatment of cellulose mixed with xylan, and (iii) cellulose mixed with xylose. For example, Figure notation 2c-iii is for pretreated solids prepared at CSF 2.95 of cellulose mixed with xylose. Scale bar length = 2 μm, unless otherwise noted. Reproduced with permission from reference 23.

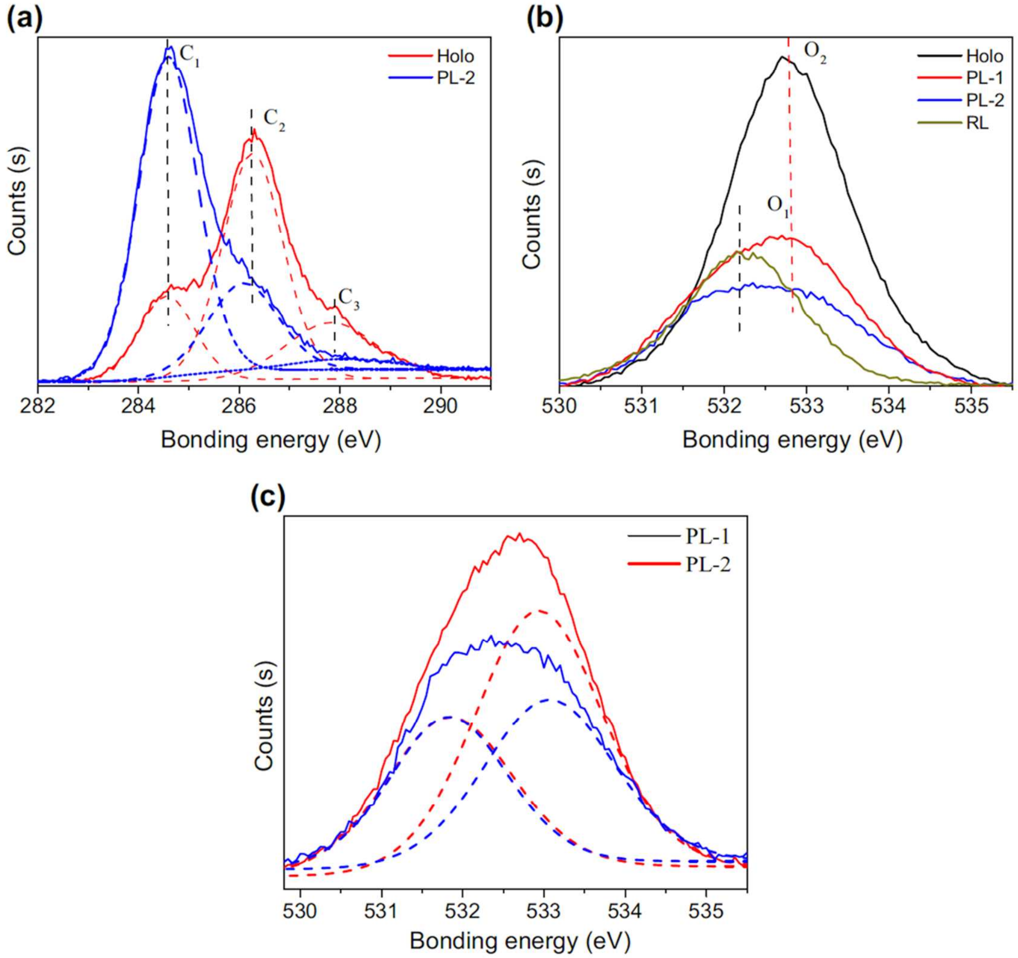


Figure 3. High resolution scans of C1s and O1s peaks. (a). C1s. (b). O1s. (c). deconvoluted peaks of oxygen of pseudo lignin. Holo: holocellulose; PL-1: Hydrothermal pretreated holocellulose at 170 °C, 150 min; PL-2: Hydrothermal pretreated holocellulose at 170 °C, 240 min; RL: Reference lignin. Reproducted with permission from reference 37.

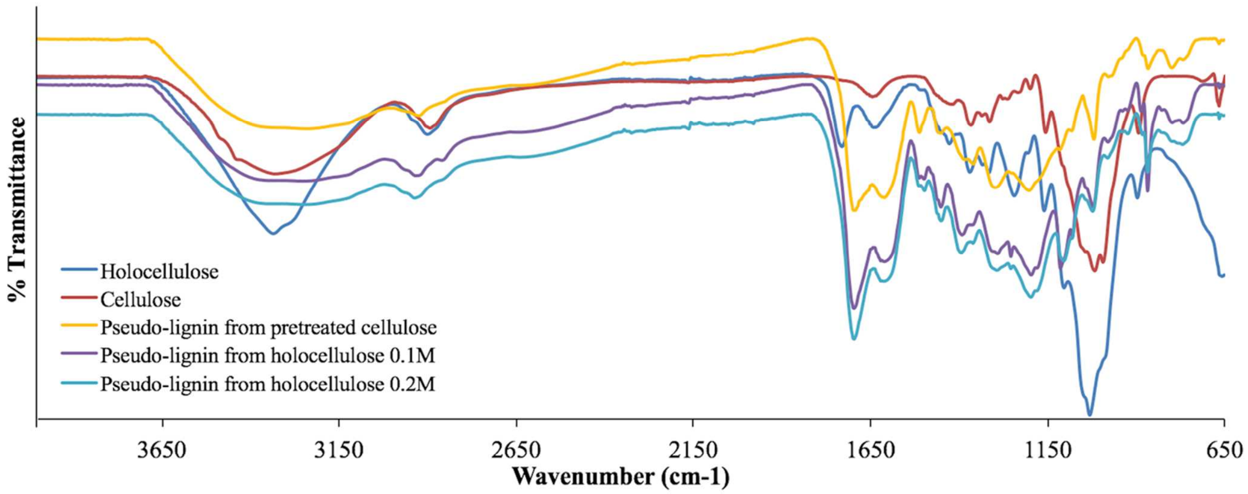


Figure 4. FTIR spectra of cellulose, holocellulose, pseudo-lignin extracted from dilute acid pretreated α-cellulose (180 °C, 0.1 M H_2SO_4, 40 min), pretreated holocellulose A (180 °C, 0.1 M H_2SO_4, 40 min) and pretreated holocellulose B (180 °C, 0.2 M H_2SO_4, 60 min). Reproduced with permission from reference 15.

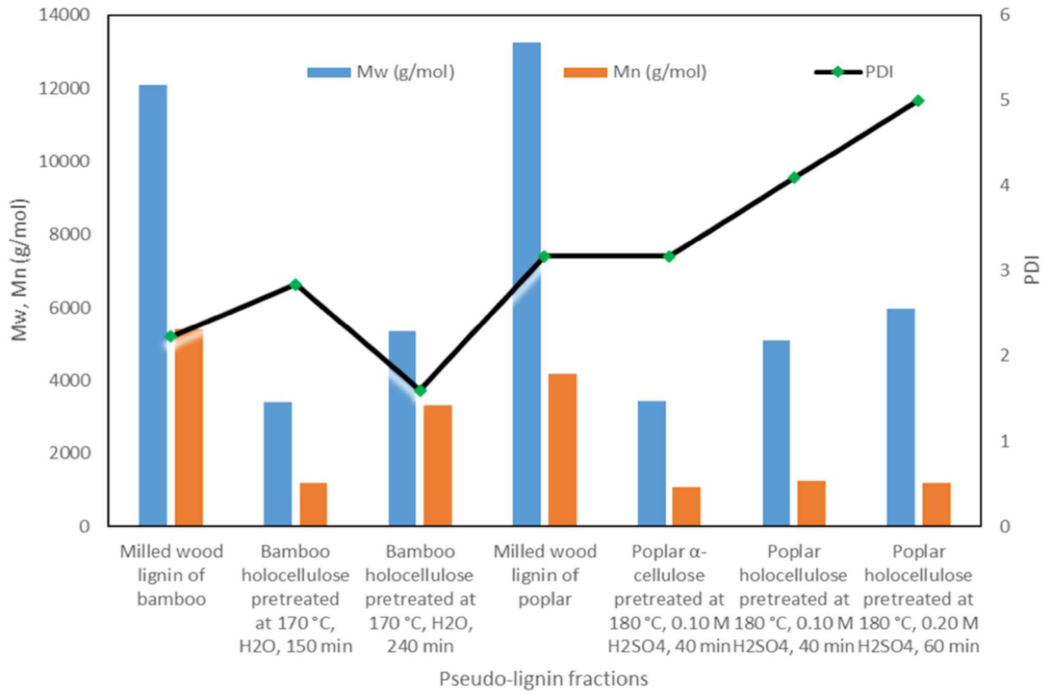


Figure 5. Weight average (M_w) and number average (M_n) molecular weight distribution and polydispersity index (PDI) of pseudo-lignin isolated from dilute acid and hydrothermal pretreated cellulosic substrates.[15, 37 70, 71]

Table 1. Klason lignin content (wt%, dry basis) before and after dilute acid pretreatment for hardwood, softwood, and herbaceous feedstocks.

Substrate	Pretreatment conditions	Klason lignin before pretreatment (%)	Klason lignin after pretreatment (%)	Reference
Hardwood				
Populus	160 °C, 0.1 M H_2SO_4, 2.5 min	~28	~35	[81]
	160 °C, 0.1 M H_2SO_4, 5 min	~28	~31	[81]
	160 °C, 0.1 M H_2SO_4, 10 min	~28	~36	[81]
	160 °C, 0.1 M H_2SO_4, 60 min	~28	~44	[81]
	170 °C, 0.5% H_2SO_4, 0.3 min	24.6	26.0	[82]
	170 °C, 0.5% H_2SO_4, 1.1 min	24.6	25.0	[82]
	170 °C, 0.5% H_2SO_4, 5.4 min	24.6	25.8	[82]
	170 °C, 0.5% H_2SO_4, 8.5 min	24.6	24.4	[82]
	170 °C, 0.5% H_2SO_4, 27 min	24.6	25.6	[82]
Softwood				
Spruce	180 °C, 1% H_2SO_4, 30 min	29.0	48.5	[83]
Pine	160 °C, 1.2% H_2SO_4, 20 min	29.9	34.4	[84]
Douglas fir HL[1]	180 °C, 2.2% H_2SO_4, 20 min	27.5	40.0	[85]
Douglas fir HE[2]	180 °C, 2.2% H_2SO_4, 20 min	32.9	40.2	[85]
Douglas fir SL[3]	180 °C, 2.2% H_2SO_4, 20 min	24.6	34.8	[85]
Douglas fir SE[4]	180 °C, 2.2% H_2SO_4, 20 min	30.9	35.8	[85]
Loblolly pine	180 °C, 1% H_2SO_4, 30 min	27.3	35.7	[86]
Herbaceous				
Corn stover	160 °C, 1% H_2SO_4, 5 min	19.3	16.1	[28]
	160 °C, 0.5% H_2SO_4, 20 min	17.2	26.8	[87]
Switchgrass	160 °C, 1.2%	17.8	26.8	[88]

	H_2SO_4, 20 min			
	120 °C, 1% H_2SO_4, 60 min	20.5	48.4	[89]
	160 °C, 1% H_2SO_4, 2 min	31.2	48.5	[90]
	160 °C, 1% H_2SO_4, 5 min	31.2	60.8	[90]
	160 °C, 1% H_2SO_4, 10 min	31.2	44.3	[90]
Miscanthus	170 °C, 1% H_2SO_4, 30 min	21.7	22.5	[91]
	160 °C, 1% H_2SO_4, 10 min	22.1	29.0	[92]

[1]HL: heartwood latewood; [2]HE: heartwood earlywood; [3]SL: sapwood latewood; [4]SE: sapwood earlywood

Table 2 Different characterization methods for assessment of biomass and pseudo-lignin structure and its recalcitrance

Method/ Parameter	**Properties explored/determined**	**References**
Biomass compositional analysis	Carbohydrates and lignin content	[93]
Oligomeric sugar analysis	Oligomeric and monomeric sugar content	[94-96]
Scanning electron microscopy (SEM) and/or transmission electron microscopy (TEM)	Ultrastructural morphology of biomass, biomass porosity	[15, 17, 20, 23, 53, 81, 97-104]
X-ray photoelectron spectroscopy (XPS)	Quantitative elemental compositions, the bonding states of atoms, the locations of atoms in the samples, surface composition, and chemical modifications	[37]
Fourier-transform infrared spectroscopy (FTIR)	Functional groups and structural changes identification	[15, 17]
Small angle neutron scattering (SANS)	Morphological changes across molecular to submicrometer length scales	[90, 104-109]
Nuclear magnetic resonance (NMR)	Chemical structural elucidation	[15, 17]
Gel permeation chromatography (GPC)	Molecular weight distribution and degree of polymerization of glucan, xylan, lignin, pseudo-lignin and their oligomers	[5, 15, 17,5, 110-112]
Crystallinity index	Cellulose crystallinity	[3, 5, 57, 82, 104, 107, 109, 113-118]
Protein adsorption	Protein binding capacity of pseudo-lignin	[23]

Table 3 Peak assignments for FTIR spectra of pseudo-lignin.[15, 24]

Wavenumber (cm^{-1})	Assignment
3238	O-H stretching in alcohols, phenols or carboxylic acids
2923	Aliphatic C-H stretching
1697	C=O stretching in carboxylic acids, conjugated aldehydes or ketones
1611, 1512	Aromatic C=C stretching (in ring)
1360	Aliphatic C–H rocking
1299, 1203, 1020	C–O stretching in alcohols, ethers, or carboxylic acids
867, 800	Aromatic C–H out-of-plane bending