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Journal

Green Chemistry, 20(10)

ISSN

1463-9262 1463-9270

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Publication Date

2018

DOI

10.1039/C8GC00353J

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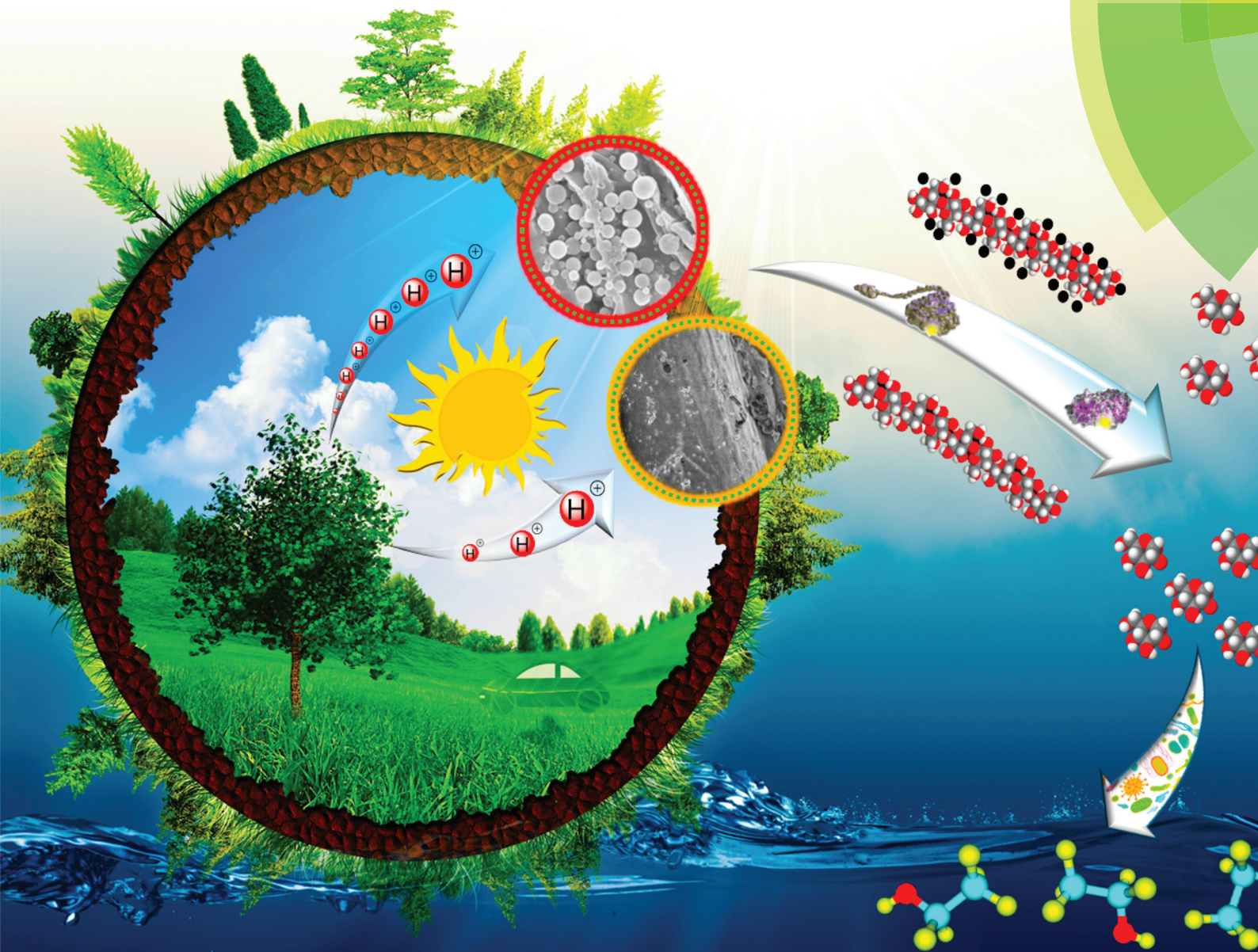


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ISSN 1463-9262



CRITICAL REVIEW

Arthur J. Ragauskas *et al.*





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

CRITICAL REVIEW



Cite this: *Green Chem.*, 2018, **20**, 2192

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

Somnath D. Shinde,  ^{†a} Xianzhi Meng,  ^{†a} Rajeev Kumar  ^{b,c,d} and Arthur J. Ragauskas  ^{*a,c,d,e,f}

The formation of lignin-like structures by the degradation primarily of plant polysaccharides has been observed after the 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 pseudo-lignins 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 the polymerization/condensation reactions from key intermediates such as 3,8-dihydroxy-2-methylchromone and 1,2,4-benzenetriol derived from furfural (FF) and 5-hydroxymethylfurfural (5-HMF), respectively. Furthermore, 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 the 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 biofuel synthesis through a 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 the conversion of biomass to biofuels, the second part focuses on the chemistry and mechanism of pseudo-lignin formation, the third part emphasizes on the 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 the practical consideration regarding the design of the processes for the prevention of pseudo-lignin formation.

Received 31st January 2018,
Accepted 17th March 2018

DOI: 10.1039/c8gc00353j

rscl.li/greenchem

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 the natural recalcitrance of biomass, which refers to 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 the 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. 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 pretreat-

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ments. 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 in 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 unable 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 structure was preferentially produced during high severity low-pH biomass pretreatment.¹⁵ This type of lignin-like structure could be formed either through substitution reactions on lignin aromatic rings or acid-catalyzed condensation reactions on 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 an aromatic material that yields a positive K-lignin value.¹⁷

It is well known that lignin plays a negative role in the conversion process of biomass to biofuels.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-hydroxymethylfurfural (5-HMF), and levulinic acid, which can be further degraded into carbon-enriched aromatic struc-

tures.^{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 severity and the pseudo-lignin deposited on the surface of cellulose reduces cellulose accessibility and make less cellulase available for action on cellulose through unproductive binding.²³ Hu *et al.* further revealed that pseudo-lignin is more detrimental to the enzymatic hydrolysis of cellulose than dilute acid pretreated lignin.²⁴ As a result, dilute acid pretreatment should be performed under 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 under high severity pretreatment conditions.²⁵ The flowthrough pretreatment technique can also suppress the pseudo-lignin formation due to its ability to constantly remove 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 the conversion of biomass to biofuels, the second part focuses on the chemistry and mechanism of pseudo-lignin formation, the third part emphasizes on the different analytical techniques used for pseudo-lignin characterization and recalcitrance elucidation, the fourth part illustrates the recalcitrant behaviour of pseudo-lignin, and the final section deals with the practical consideration regarding the design of the processes for the prevention of pseudo-lignin formation.



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ORNL/UTK in 2015. His research interests are improving the utilization of lignocellulosic biomass by understanding its characteristics and creating sustainable chemical solutions essential for converting lignocellulosic biomass resources to bio-materials, bio-fuels, and bio-chemicals.

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 the lignin across the plant cell wall.²² Although low pH thermo-chemical pretreatments, such as dilute sulfuric acid, are ineffective in terms of the physical removal of lignin, 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 the lignin content during dilute acid pretreatment for softwood, hardwood, and herbaceous feedstocks. Cao *et al.* reported that dilute acid pretreated poplar at 170 °C over a 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 °C.²⁸ Similarly, the 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 under similar conditions.²⁹ For example, nearly 36% of lignin was removed from poplar in a flowthrough dilute acid pretreatment at 180 °C while the lignin content in the batch pretreated poplar remained at the same level compared to that in the 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 that of 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 the partial solubilization of cellulose were 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 The results revealed that the 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 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 the *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 the migration of lignins from the cell wall to the bulk liquid phase followed by re-deposition onto the surface of the 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 pre-



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Arthur J. Ragauskas

Arthur Ragauskas held the first Fulbright Chair in Alternative Energy and is a Fellow of AAAS, IAWS and TAPPI. In 2014, he assumed a Governor's Chair for Biorefining based in the University of Tennessee's Department of Chemical and Biomolecular Engineering, with a complementary appointment in the UT Institute of Agriculture and serves in the US Energy and Environmental Sciences Directorate, Biosciences Division,

at ORNL. His research program is directed at understanding and exploiting innovative sustainable bioresources. He is the recipient of the TAPPI Gunnar Nicholson Gold Medal Award, the ACS Affordable Green Chemistry award and AIChE Green Processing Award.

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

Substrate	Pretreatment conditions	Klason lignin before pretreatment (%)	Klason lignin after pretreatment (%)	Ref.
Hardwood				
<i>Populus</i>	160 °C, 0.1 M H ₂ SO ₄ , 2.5 min	~28	~35	81
	160 °C, 0.1 M H ₂ SO ₄ , 5 min	~28	~31	81
	160 °C, 0.1 M H ₂ SO ₄ , 10 min	~28	~36	81
	160 °C, 0.1 M H ₂ SO ₄ , 60 min	~28	~44	81
	170 °C, 0.5% H ₂ SO ₄ , 0.3 min	24.6	26.0	82
	170 °C, 0.5% H ₂ SO ₄ , 1.1 min	24.6	25.0	82
	170 °C, 0.5% H ₂ SO ₄ , 5.4 min	24.6	25.8	82
	170 °C, 0.5% H ₂ SO ₄ , 8.5 min	24.6	24.4	82
	170 °C, 0.5% H ₂ SO ₄ , 27 min	24.6	25.6	82
Softwood				
Spruce	180 °C, 1% H ₂ SO ₄ , 30 min	29.0	48.5	83
Pine	160 °C, 1.2% H ₂ SO ₄ , 20 min	29.9	34.4	84
Douglas fir HL ^a	180 °C, 2.2% H ₂ SO ₄ , 20 min	27.5	40.0	85
Douglas fir HE ^b	180 °C, 2.2% H ₂ SO ₄ , 20 min	32.9	40.2	85
Douglas fir SL ^c	180 °C, 2.2% H ₂ SO ₄ , 20 min	24.6	34.8	85
Douglas fir SE ^d	180 °C, 2.2% H ₂ SO ₄ , 20 min	30.9	35.8	85
Loblolly pine	180 °C, 1% H ₂ SO ₄ , 30 min	27.3	35.7	86
Herbaceous				
Corn stover	160 °C, 1% H ₂ SO ₄ , 5 min	19.3	16.1	28
	160 °C, 0.5% H ₂ SO ₄ , 20 min	17.2	26.8	87
Switchgrass	160 °C, 1.2% H ₂ SO ₄ , 20 min	17.8	26.8	88
	120 °C, 1% H ₂ SO ₄ , 60 min	20.5	48.4	89
	160 °C, 1% H ₂ SO ₄ , 2 min	31.2	48.5	90
	160 °C, 1% H ₂ SO ₄ , 5 min	31.2	60.8	90
	160 °C, 1% H ₂ SO ₄ , 10 min	31.2	44.3	90
Miscanthus	170 °C, 1% H ₂ SO ₄ , 30 min	21.7	22.5	91
	160 °C, 1% H ₂ SO ₄ , 10 min	22.1	29.0	92

^a HL: heartwood latewood. ^b HE: heartwood earlywood. ^c SL: sapwood latewood. ^d SE: sapwood earlywood.

treated holocellulose also has spherical structures and tends to be deposited on cell surfaces.¹⁵ The size of the 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 the 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 the generation of pseudo-lignin from wheat straw, and their study elucidated that higher severity induced the accumulation of more pseudo-lignin within the temperature range of 170–200 °C.²⁶ Kumagai *et al.* also confirmed the formation of pseudo-lignin in 200 °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 a reaction temperature of 170 °C actually showed no pseudo-lignin, and the lignin-derived compounds, including lignin oligomers and phenolic monomers accounted for ~80% of the CIPL while carbohydrate degradation products

such as FF and 5-HMF only accounted for ~10% of the CIPL formed during the cooling process.³⁶ This is consistent with another study that reported that only a trace amount of pseudo-lignin is detected from the hot water pretreatment of holocellulose at 170 °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 upgrading of biomass can also be fulfilled *via* either torrefaction, carbonization, or pyrolysis where biomass is thermally degraded.³⁹ Torrefaction of Norway spruce performed under mild (260 °C for 8 min) and intermediate conditions (310 °C for 8 min) was found to result in increased K-lignin content which was mainly attributed to the pseudo-lignin formation.⁴⁰ The hydrothermal carbonization of the loblolly pine at above 200 °C generated hydrochars with only polyphenolic structures with up to 98% of K-lignin and it was claimed to contain significant amounts 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 The structural characterization of isolated pseudo-lignin from dilute acid pretreated poplar holocellulose by FTIR and ¹³C NMR revealed that this material was predominantly comprised of carbonyl, carboxylic, and aromatic structures.¹⁵ Due to the heterogeneity of the reaction media and the complexity of biomass components, the exact mechanisms leading to the formation of pseudo-lignin at elevated temperatures is still under investigation.^{14,42,43} A hypothesized reaction pathway for pseudo-lignin formation is shown in Fig. 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 compounds 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 a major aromatic product in the acid degradation of xylose.⁴⁶ In addition, Luijckx *et al.* reported that 1,2,4-benzenetriol (BTO) can be formed in the hydrothermal treatment of carbohydrates *via* the rearrangement reactions of HMF by hydrolysis of the furan ring of HMF followed by intramolecular ring-closing and dehydration.^{47,48} Pseudo-lignin can then be formed *via* the 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* the oxidative polymer-

ization of BTO in the 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 components⁵² 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 the literature for the assessment of biomass and pseudo-lignin structures.

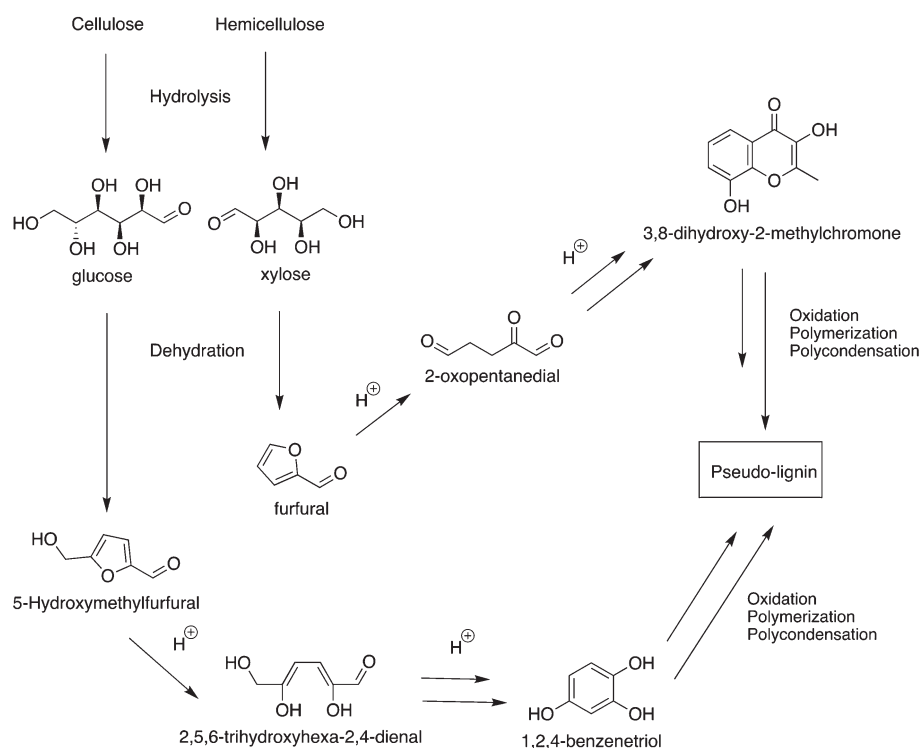


Fig. 1 Hypothesized reaction pathways for pseudo-lignin formation.¹⁵

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

Method/parameter	Properties explored/determined	Ref.
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 and biomass porosity	15, 17, 20, 23, 53, 81 and 97–104
X-ray photoelectron spectroscopy (XPS)	Quantitative elemental compositions, the bonding states of atoms, the locations of atoms in the samples, surface compositions, and chemical modifications	37
Fourier-transform infrared spectroscopy (FTIR)	Functional groups and structural change identification	15 and 17
Small angle neutron scattering (SANS)	Morphological changes across the molecular to submicrometer length scales	90 and 104–109
Nuclear magnetic resonance (NMR)	Chemical structural elucidation	15 and 17
Gel permeation chromatography (GPC)	Molecular-weight distribution and the degree of polymerization of glucan, xylan, lignin, pseudo-lignin and their oligomers	5, 15, 17 and 110–112
Crystallinity index	Cellulose crystallinity	3, 5, 57, 82, 104, 107, 109 and 113–118
Protein adsorption	Protein binding capacity of pseudo-lignin	23

Scanning electron microscopy (SEM)

SEM has been a widely applied technique for the ultrastructural imaging of biomass to elucidate the 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 a slightly rough exterior surface and resembled lignin-balls in appearance. The frequency of the 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 (Fig. 2).²³ Cellulose mixed with xylan or xylose showed prominent evidence of droplets in comparison with 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 Fig. 2e and e' was entirely made up of pseudo-lignin spheres as were the solids prepared at higher severity shown in Fig. 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 the 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 an 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 a 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 tendency to deposit as droplets onto the surface of the cell wall after the thermochemical pretreatment of biomass.^{7,32,54} Pretreatment conditions above the glass transition temperature of lignin result in the coalescence of lignin in the inner region of the cell wall. This coalesced 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 the enzymatic digestibility of carbohydrates as it can improve the accessibility of the majority of cellulose and hemicellulose microfibrils by opening the structure of the cell wall matrix⁵ 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 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 poly-aromatic 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 the surface chemistry, composition, and chemical modifications of fiber and the pulp surface.^{62,63} Ma *et al.* used XPS to trace pseudo-

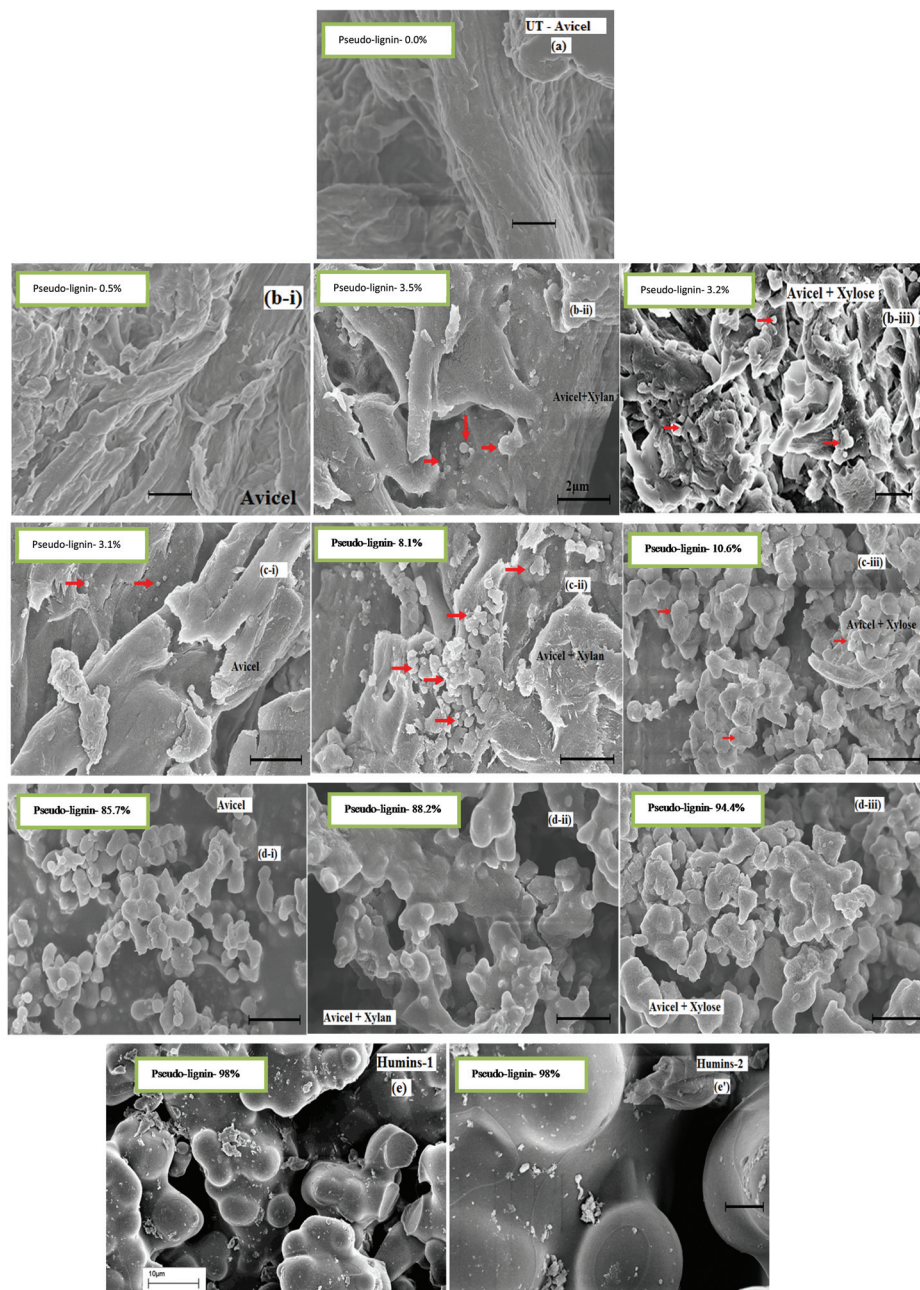


Fig. 2 SEM images of (a) untreated Avicel cellulose (magnification 20kx) and of pretreated solids at (b) CSF 2.66 (magnification 20kx), (c) 2.95 (magnification 20kx), and (d) 3.56 (magnification 20kx). (e) and (e') xylose derived-pseudo-lignin at 5kx and 20kx magnifications. Marker (i) designates the solids from the pretreatment of Avicel cellulose alone, (ii) from the 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 ref. 23.

lignin formation and elaborated the structure of the isolated pseudo-lignin during the hydrothermal pretreatment of holocellulose (Fig. 3).³⁷ The chemical shifts of carbon (C1s) in cellulosic fibers can be deconvoluted into four categories C₁ (C–C/C–H), C₂ (C–O), C₃ (C=O/O–C–O), and C₄ (O–C=O).^{37,64} Lignin and its extractives are mainly responsible for the C₁ (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 C₄ to C₁.³⁷ The chemical shifts of the oxygen (O1s) peaks generally involve O₁ (C=O) and O₂ (C–O/C–O–C), where O₁ is mainly allocated to the oxygen in lignin, whereas O₂ originates primarily from the oxygen in carbohydrates.³⁷ In fact, O₂ chemical shifts are also found in lignin. However, most of the hydroxyls and ethers in lignin are associated with the benzene ring structure. A larger polarizability of the aromatic substituent results in a lower bonding energy of the oxygen core level

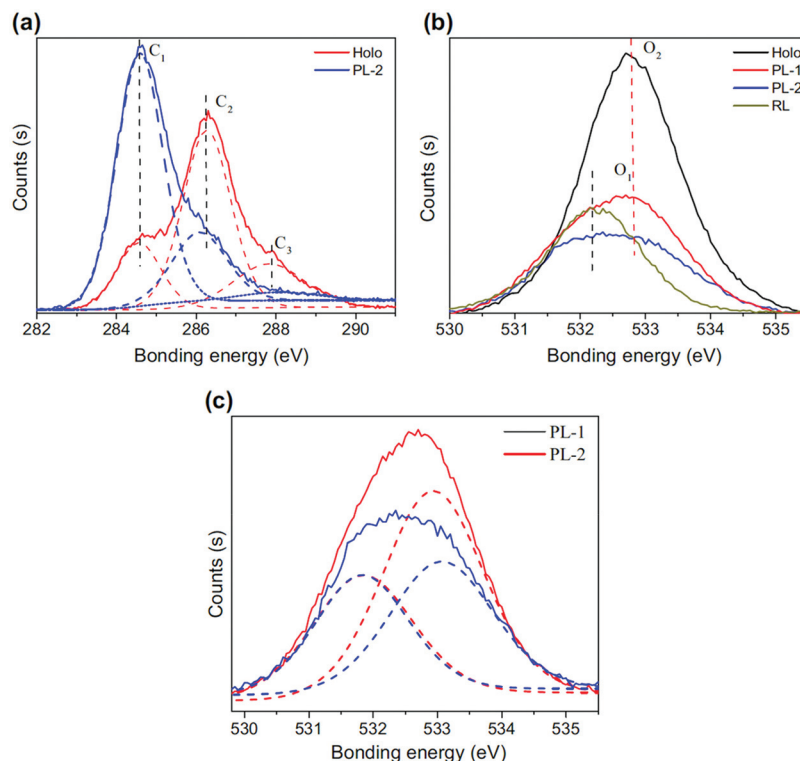


Fig. 3 High-resolution scans of C1s and O1s peaks. (a) C 1s. (b) O 1s. (c) Deconvoluted peaks of the oxygen of pseudo-lignin. Holo: holocellulose; PL-1: hydrothermal pretreated holocellulose at 170 °C, 150 min; PL-2: hydrothermal pretreated holocellulose at 170 °C, 240 min; RL: reference lignin. Reproduced with permission from ref. 37.

electron.^{37,66} This effect leads the C–O and C–O–C in lignin to the lower binding energy, and thus makes them assigned to O₁.^{37,66} As a result, XPS is promising for the quantitative estimation of the 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 C₁ (C–C/C–H) concentration and a decrease in the O/C ratio on the both exterior and interior surfaces of the hydrothermally pretreated bamboo chips.⁶⁹ With an extended pretreatment time, an increasing amount of lignin/pseudo-lignin was deposited on the exterior surface of the bamboo chips. Furthermore, the 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 the droplets on the surface of hydrothermally pretreated holocellulose.³⁷ C₁ increased from 8.9 to 23.0% along with an increase in K-lignin from 1.0 to 6.2%. In theory, the degradation of polysaccharides has negligible effects on their relative content of C, O, and O/C. However, the pseudo-lignin produced from repolymerization of the polysaccharide degradation products^{14,30} and/or polymerization with lignin^{14,30} cause a drastic increase of C₁, and a decrease in C₂, C₃, and O₂ 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

The 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 the pretreated α -cellulose and holocellulose of poplar (Fig. 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 the aromatic ring, whereas the bands in the 1320–1000 cm⁻¹ region correspond to the C–O stretching (in alcohols, ethers, or carboxylic acids) (Table 3).¹⁵ The strong and broad hydroxyl stretching peaks at 3238 cm⁻¹ indicate the presence of hydrogen-bonding in isolated pseudo-lignin. These observations indicate 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 the C–H out-of-plane bending suggests that the benzene rings of pseudo-lignin are 1,3,5-trisubstituted.¹⁵

In another study, Kumar *et al.* observed similar findings when comparing the FTIR spectra of untreated Avicel cellulose, solids prepared from different severity pretreatments of cell-

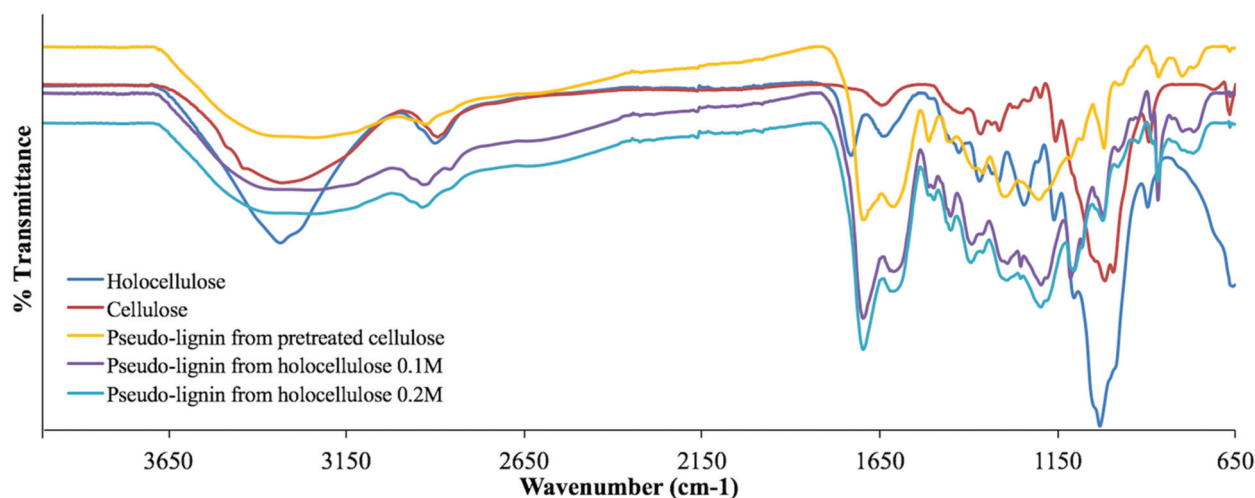


Fig. 4 FTIR spectra of cellulose, holocellulose, and pseudo-lignin extracted from dilute acid pretreated α -cellulose (180 °C, 0.1 M H_2SO_4 , and 40 min), pretreated holocellulose A (180 °C, 0.1 M H_2SO_4 , and 40 min) and pretreated holocellulose B (180 °C, 0.2 M H_2SO_4 , and 60 min). Reproduced with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

Table 3 Peak assignments for the 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

ulose alone and mixed with xylan/xylose, and xylose-derived pseudo-lignin.²³ The FTIR spectra indicated that the solids prepared at a combined severity factor (CSF) of 3.56 had strong peaks associated with the carbonyl and aromatic stretching regions while the Avicel cellulose structure stayed intact under all conditions until a CSF of 3.56.²³ Maohua *et al.* studied the 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 the 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 a broader hydroxyl stretching peak at ~3300 cm⁻¹ in comparison with the 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 the structural similarities and differences. The ¹³C 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 the C=O in ketones and the C=O in aldehydes, respectively, whereas the peaks centred at 178–172 ppm correspond to the C=O in carboxylic acids. The ¹³C 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. The 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. The molecular weight (MW) of pseudo-lignin isolated from the dilute acid and the hydrothermal pretreatment of different resources are shown in Fig. 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 weights (M_w) of milled poplar lignin

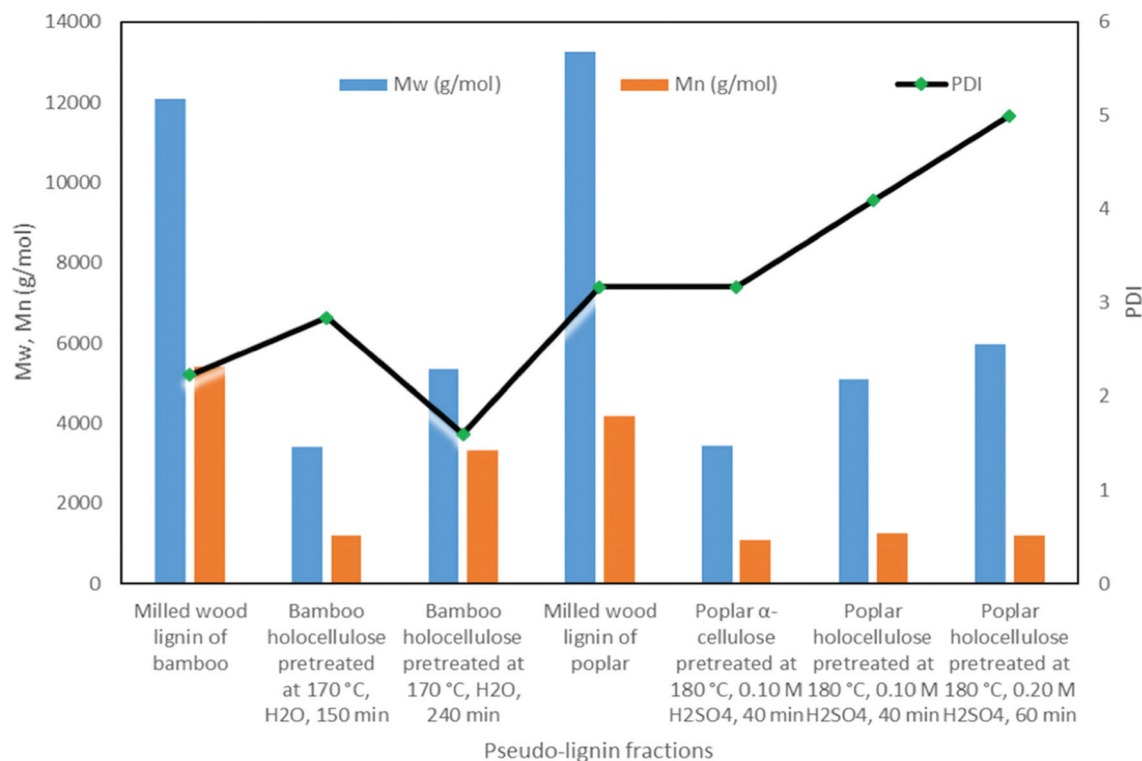


Fig. 5 Weight average (M_w) and number average (M_n) molecular weight distribution and polydispersity index (PDI) of pseudo-lignin isolated from dilute acid and hydrothermal pretreated cellulosic substrates.^{15,37,70,71}

and pseudo-lignin derived from dilute acid pretreated poplar holocellulose at 180 °C were found to be 13 250 g mol⁻¹ and 5050 g mol⁻¹, respectively,^{15,70} whereas the M_w s of milled bamboo lignin and pseudo-lignin isolated from the hydrothermal pretreatment at 170 °C were found to be 12 090 g mol⁻¹ and 5340 g mol⁻¹, respectively.^{37,71} However, as the pretreatment severity was increased, there was an increase in the 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 Fig. 5 indicated that repolymerization reactions occurred during the pretreatment; therefore aromatic rings can be alkylated significantly to produce heavy condensation compounds.³⁷ However, a lower value of the polydispersity index (PDI) for pseudo-lignin isolated from hydrothermal pretreated bamboo holocellulose at 170 °C and 240 min suggested a moderate molecular weight distribution of pseudo-lignin polymers in comparison with pseudo-lignin isolated at 170 °C, 150 min. The MW of the pseudo-lignin derived from dilute acid pretreated holocellulose was found larger than that of the pseudo-lignin extracted from pretreated α-cellulose. In addition, with an increase in the severity of dilute acid pretreatment of poplar holocellulose, the PDI of pseudo-lignin increased which suggested a broad molecular weight distribution. As pseudo-lignin is formed *via* polycondensation of the 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 the pseudo-lignin's hydrophobic nature enables unproductive binding with enzymes, and 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 the 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 24 h of hydrolysis) with no visible 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 with 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 *et 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 the nature of hydrolysis resources. For example, the current recalcitrance study of lignin is usually achieved by physically mixing lignin resources with the lignocellulosic samples, and thus the cellulose accessible surface area of the lignocellulosic samples also plays an important role besides lignin structures. It is well known that lignin has a tendency to unproductively bind to cellulases due to its hydrophobic structural features, including hydrogen bonding, methoxy groups, and polyaromatic structures.^{20,73,74} The methoxy and polyaromaticity structural features of pseudo-lignin and its water insolubility suggest that pseudo-lignin is hydrophobic in nature and thus contributes to the non-productive binding of lignin to cellulases.²⁴ Furthermore, 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 hemicellulose (xylan)-derived pseudo-lignin. Protein adsorption experiments confirmed that pseudo-lignin binds to the enzymes unproductively.²³ The impact of pseudo-lignin inhibition is high at low enzyme loadings suggesting that for commercial applications it is necessary to avoid the formation of pseudo-lignin during the biomass pretreatment. Given the interest in lignin valorization, especially for cellulosic ethanol operations, researchers need to 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 the presence of acids, reducing the pretreatment severity is an obvious way to reduce or avoid the formation of pseudo-lignin. However, as the pretreatment severity decreases, the biological conversion of pretreated solids to sugar drops substantially due to plant recalcitrance. Flowthrough 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 flowthrough pretreatments, respectively.⁷⁵ More importantly, its ability to constantly remove hemicellulose oligomers into the aqueous phase effectively restricts the presence of sugar dehydration and condensation products. Almost complete xylan removal was achieved at the reaction severity of $\log R_0 > 4.5$ and $\log R_0 > 4.2$ for water-only and dilute acid flowthrough operation, respectively.⁷⁵ As a result, flowthrough pretreatment can reduce pseudo-lignin formation through the 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 with the ~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 the introduction of dimethyl sulfoxide (DMSO) into the acidic medium could effectively suppress HMF degradation which is one of the key intermediates during the pseudo-lignin formation.⁷⁷ A recent study modified a series of dilute acid pretreatments 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 the HMF–DMSO interaction over that of the C1– H_2O interaction can protect the cleavage of the 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 has exceptional pseudo-lignin suppression properties, 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 on the 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 pretreatment 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 this pretreatment provides another effective way to reduce pseudo-lignin formation and increase sugar yields at commercially viable low enzyme loadings.⁸⁰

Conclusions and future perspective

The increased K-lignin content of acid and hydrothermal-based biomass pretreatments under severe pretreatment conditions are mainly products of the polycondensation reactions of the 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 the enzymatic hydrolysis of cellulose through reduced cellulose accessibility and/or cellulase effectiveness by its non-productive binding to enzymes. 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.

Conflicts of interest

There are no conflicts to declare.

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 the 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 are thankful for the support of U.S. DOE (Department of Energy) EERE (Energy Efficiency and Renewable Energy) and BETO (Bioenergy Technology Office) (DE-EE0007104). 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, make any warranty, expressed or implied, or assume any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represent that its use would not infringe privately owned rights.

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