Sipponen, Mika; Rahikainen, Jenni; Leskinen, Timo; Pihlajaniemi, Ville; Mattinen, Maija-Liisa; Lange, Heiko; Crestini, Claudia; Österberg, Monika

Structural changes of lignin in biorefinery pretreatments and consequences to enzyme-lignin interactions

Published in:
Nordic Pulp & Paper Research Journal

DOI:
10.3183/npprj-2017-32-04_p550-571_sipponen

Published: 01/01/2017

Document Version
Publisher's PDF, also known as Version of record

Structural changes of lignin in biorefinery pretreatments and consequences to enzyme-lignin interactions

Mika Henrikki Sipponen, Jenni Rahikainen, Timo Leskinen, Ville Pihlajaniemi, Maija-Liisa Mattinen, Heiko Lange, Claudia Crestini, Monika Österberg

KEYWORDS: Lignin, Structure, Biomass, Processing, Enzymatic, Saccharification, Hydrolysis, Non-productive, Adsorption

ABSTRACT: The main target of a biorefinery pretreatment process is to break down the lignin-reinforced plant cell wall structure prior to enzymatic hydrolysis of polysaccharides to fermentable sugars. Various physico-chemical alterations occur in lignin during the biomass pretreatment, but effects of those structural changes on subsequent enzymatic hydrolysis have remained ambiguous. We review the reinforcing and detrimental lignin-enzyme interactions and their underlying mechanisms, and use this structure-function information to assess critical features of current and emerging pretreatment technologies. Our perspective is that truly multidisciplinary research is needed to develop pretreatments that render lignin non-inhibiting to enzymes and with high potential for further valorisation.

ADDRESSES OF THE AUTHORS: Mika Henrikki Sipponen (mika.sipponen@aalto.fi), **Timo Leskinen** (timo.leskinen@aalto.fi), **Maija-Liisa Mattinen** (maija-liisa.mattinen@aalto.fi), **Monika Österberg** (monika.osterberg@aalto.fi) Bioproduct Chemistry, Department of Bioproducts and Biosystems, Aalto University, P.O. Box 16300, FI-00076 Aalto, Espoo, Finland. **Jenni Rahikainen** (jenni.rahikainen@vtt.fi), **Ville Pihlajaniemi** (ville.pihlajaniemi@vtt.fi) VTT Technical Research Centre of Finland Ltd, P.O. Box 1000, FI-02044 VTT, Espoo, Finland. **Heiko Lange** (heiko.lange@uniroma2.it), **Claudia Crestini** (crestini@stc.uniroma2.it) Department of Chemical Sciences and Technologies, University of Rome Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy.
Corresponding author: Mika Henrikki Sipponen

Introduction

Lignins are methoxylated polyphenols that strengthen and protect plant cell walls against decomposition. Lignocellulosic biorefineries relying on enzymatic hydrolysis of carbohydrates, in the following simply referred to as 'biorefinery', require a feedstock pretreatment step to overcome this constraint from lignin complicating the saccharification process (Galbe, Zacchi 2012). Compared to structurally heterogeneous and hot-water extractable hemicelluloses (Borrega et al. 2013), cellulose consist of highly ordered regions in fibres that retard hydrolysis. Unwanted impacts of lignin on the action of cellulases include non-productive adsorption, denaturation, inhibition, and the mere physical constraint to their substrates (Pan 2008). Although lignin has been deemed responsible for the majority of the detrimental effects observed in saccharification (Zeng et al. 2014), some pretreatments produce well hydrolysable substrates without separating lignin (Wyman et al. 2005). In most cases however, structural differences in the lignin component affect the performance of the saccharification process, and also determine the economic viability of utilizing this lignin for the generation of energy or value-added chemicals (Narron et al. 2016; Ragauskas et al. 2014; Rinaldi et al. 2016). A comprehensive assessment of new and emerging pretreatment processes is thus of pivotal interest.

Two recent reviews presented applied strategies to overcome the detrimental lignin-cellulase interactions (Liu et al. 2016; Saini et al. 2016). Recent years have seen also more general reviews focusing on process perspectives of lignocellulose pretreatments (Behera et al. 2014; Galbe, Zacchi 2012; Jönsson, Martín 2016). Generally, all earlier literature suggests that lignin has a major impact on pretreatment, saccharification, and fermentation of lignocellulosic feedstocks to chemicals. Nevertheless, views regarding the consequences of structurally altered and re-localized lignin within pretreated substrates and its interplay with cellulolytic enzymes are scattered and in part conflictive. For instance, intriguing evidence has accumulated concerning the hydrolysis-enhancing impacts of lignin (Cheng et al. 2014; Lai et al. 2014, 2016; Leskinen et al. 2017; Li et al. 2016; Lin et al. 2015; Müller et al. 2015; Rodríguez-Zúñiga et al. 2015; Zhou et al. 2013a; Zong et al. 2016), but their underlying mechanisms need further elucidation.

This paper reviews recent literature on enzyme-lignin interactions as well as the structural changes of lignin in leading and emerging pretreatment processes, and links the two phenomena to analyse impacts on the enzymatic saccharification. We point out areas in which structural information of lignin is missing and that hence deserve future research in this aspect. As an outcome, the reader may find insight into the question of how to critically assess and select pretreatment technologies that can be integrated with biochemical lignocellulose utilization, and help regarding how to predict what kind of lignin arises as a co-product stream.

Overview of structural constitution of native lignin

As a phenolic polymer, lignin is structurally distinct from the other main plant polymers, cellulose and hemicellulose (Lapierre, 2010). Lignification occurs *via* free radical polymerization processes which produce irregularly inter-linked polymers from methoxylated monolignols (*Fig 1*). The proportions of the three main monolignols *p*-coumaryl alcohol (H), coniferyl alcohol (G) and sinapyl alcohol (S) vary according to botanical origin (Faix 1991; Freudenberg, Neish 1968). Typical classification recognizes three main types of lignins, G in softwood, GS in hardwood, and HGS in grasses, based on the monolignols indicated above (Faix, 1991; Lapierre, 2010).

Examples of Norway spruce milled wood lignin (MWL) structures are given in *Fig 1*. The oligomers and polymers resulting from lignification consist of carbon-carbon and ether linkages both in linear and cyclic arrangements. The most abundant bond type is the non-cyclic β-*O*-4' linkage (β-ether, structure A in *Fig 1*) that comprises approximately half of all bonds in native lignin, with higher relative abundance in hardwood lignins (Chakar, Ragauskas 2004). Compared to thermochemically more resistant β-β', 5-5', and 4-*O*-5' interunit bonding motifs the importance of α-ethers and β-ethers is obvious, since these thermochemically labile linkages determine reactivity of lignin in pretreatments.

The association of lignin with other cell wall components is an issue that requires further elucidation. It is generally accepted, albeit still not fully proven, that lignin besides being physically associated with other components, is covalently linked to certain polysaccharides in plant cell walls, forming so-called lignin-carbohydrate complexes (LCCs) (Iiyama et al. 1994; Jacquet et al. 1995; Watanabe et al. 1989). When taken together, the average abundance of lignin-carbohydrate linkages such as benzyl ether and phenyl glycoside linkages has been quantified at 4−9 per 100 aromatic motifs in lignin isolated from pine, birch and poplar wood (Balakshin et al. 2011; Yuan et al. 2011).

While the quantity and quality of lignin-carbohydrate interactions still need elucidation from analytical and application viewpoints, structural characteristics of lignin have received major updates in recent years. The first aspect concerns the molar mass distribution of isolated lignins, which is commonly analysed by size-exclusion chromatography (SEC) (Asikkala et al. 2012; Baumberger et al. 2007; Salanti et al. 2012). SEC-based approaches are constantly revisited, in terms of sample derivatization (Salanti et al. 2011, Asikkala et al. 2012, Leskinen et al 2015a), in terms of using aggregate-breaking solvents (Sjöholm et al. 1999, Zoia et al. 2011), in terms of advanced detector technology (Fredheim et al. 2002), in terms of hardware standardisation and data treatment (Lange et al. 2016a), or just in terms of overall process efficiency (Sulaeva et al. 2017). Alternative methods for the molecular size determination of lignins, such as MALDI-based attempts (Richel et al. 2012) or flow field-flow fractionation (Sulaeva et al. 2015) did not resolve the fundamental analytical challenges involved with lignin SEC. Additionally, the molecular weight of lignin in the form in which it exists in cell walls is potentially inaccessible with any of the currently available analytical methods in spite of recent trials using whole biomass samples (Zoia et al. 2011, Leskinen et al 2015a).

Nevertheless, it has been shown that milled wood lignin (MWL) that is considered structurally close to native lignin in plants in terms of structural motifs as such, consists of linear oligomers rather than branched polymers (Crestini et al. 2011). These oligomeric chains of lignin may be linked *via* relatively few labile α-ether linkages to carbohydrates or form networked or branched structures (Leskinen et al. 2015a). In the former case, SEC analysis may overestimate molecular weight of a lignin molecule due to the associated carbohydrate fragment. The second aspect concerns the extent of "condensed" linkages from the 3 and 5 positions of the aromatic ring, which give rise to branched molecular structures. The proportion of these condensed structures in native lignin is now understood to be very low (Crestini et al. 2011; Lundquist, Parkås 2011). The third aspect concerns the thermal characteristics of lignins and their effect on impeding saccharification as well as the potential value exhibited by the lignin side-stream. In addition to chemical changes, thermally induced reorganization of lignin occurs during pretreatments at temperatures typically exceeding 170ºC (Li et al. 2014a; Selig et al. 2007). Lignins follow the behaviour typically expected for amorphous polymers: the glass transition at elevated temperatures occurs over a broad temperature region depending on the severity of prior thermochemical treatment (Ko et al. 2015). Nevertheless, correlating thermal properties of lignins with selected structural features still remains unsettled (Lange et al. 2016b; Sadeghifar, Argyropoulos 2015; Sevastyanova et al. 2014).

Model systems for studying enzyme-lignin interactions

Research on the inhibitory effects of lignin is challenging *in situ* mainly due to the lack of methods that could distinguish and compare the different inhibitory mechanisms that occur during the actual hydrolysis process. Nevertheless, several systems that employ isolated lignin, hydrolysis residue lignin (Li et al. 2014a; Pan 2008; Rahikainen et al. 2013b; Yu et al. 2014), artificial lignin dehydrogenative polymers (DHPs) (Grabber et al. 1997), lignin derivatives (Pan 2008), and cell wall model materials (Grabber 2005) have been developed to enable mechanistic research on the inhibitory role of lignin. Additionally, although not fully mimicking the complex cell wall structure, constructed thin films enable the use of surface sensitive characterization techniques for tracking interactions of enzymes with cellulose and lignin (Fritz et al. 2015; Hoeger et al. 2012; Martín-Sampedro et al. 2013; Rahikainen et al. 2013b; Sammond et al. 2014). It is needless to emphasize, however, that the main challenge is that the choice of lignin and the method used for constructing the model affects the results.

Over the decades of research, isolation methods for 'analytical' lignin preparations have been established and widely used in fundamental studies regarding lignin reactivity and valorisation. Mechanical particle size reduction, usually by ball milling, is the initial step in these procedures, which yield MWL (Björkman 1956), acidolysis lignin (AL) (Pepper et al. 1959), cellulolytic enzyme lignin (CEL) (Chang et al. 1975), and enzymatic mild acidolysis lignin (EMAL) (Guerra et al. 2006). Isolation of CEL lignin involves an enzymatic hydrolysis stage after ball-milling to remove cell wall carbohydrates, whereas MWL lignin is directly solubilized from the ball-milled biomass. The EMAL protocol is a combination of MWL and CEL isolation involving all three steps: ball milling, enzymatic hydrolysis and lignin extraction in mildly acidic conditions. The CEL method was recently

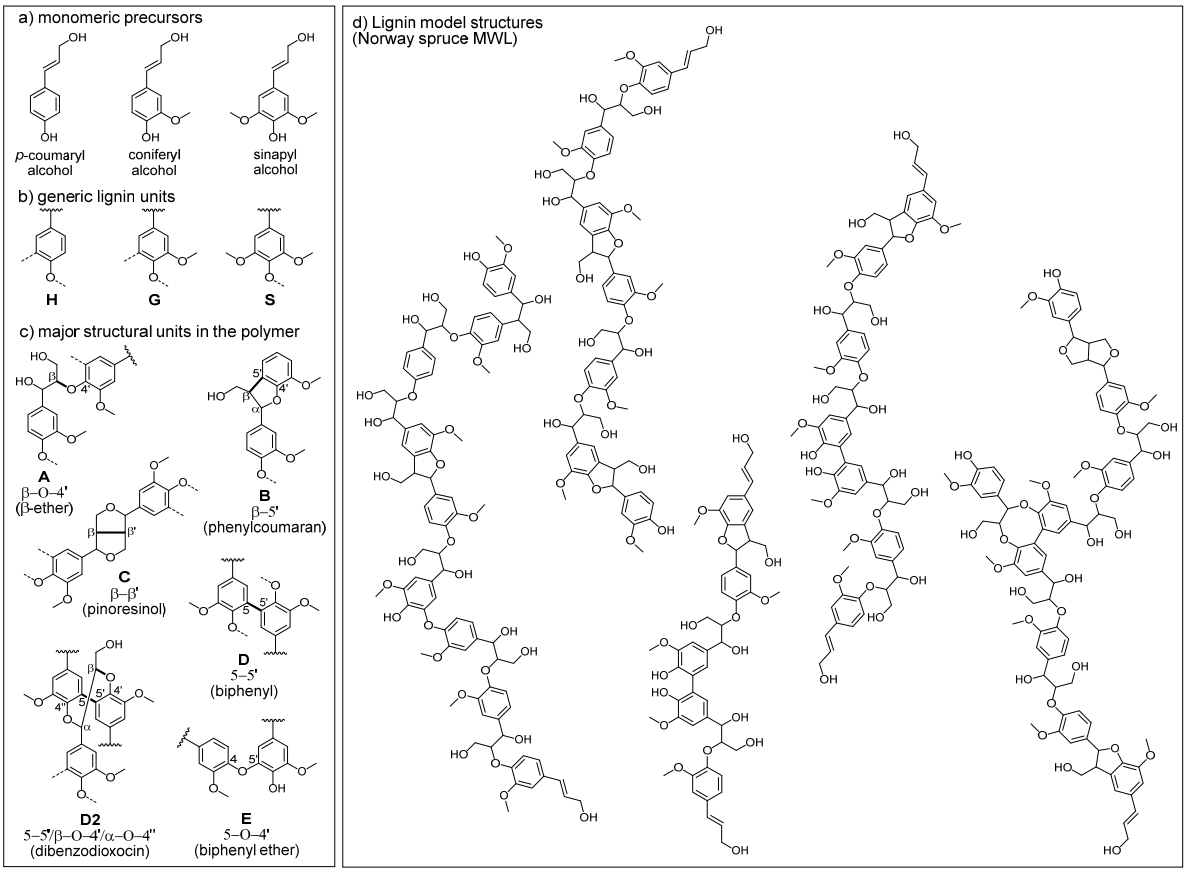

Fig 1 – Monomeric lignin precursors, generic lignin units, typical interunit linkages, and structural examples of softwood milled wood lignin

modified to a regenerated CEL (RCEL) method by dissolution and regeneration of wood prior to enzymatic hydrolysis and extraction of lignin (Capanema et al. 2014). The main benefit of the RCEL preparation is its higher lignin yield compared to those from the other procedures.

A common approach to study the inhibitory effect of lignin in enzymatic hydrolysis is to supplement pure cellulosic substrates such as microcrystalline cellulose with isolated lignins (Chernoglazov et al. 1988; Kumar et al. 2012; Zhang et al. 2016). Hydrolysis residue lignin is commonly applied in the experiments due to the anticipated chemical and morphological similarity with the lignin present in process conditions (Rahikainen et al. 2011, 2013b). The extent of lignin-derived inhibition in this type of model system is dependent on the lignin used but also on the accessibility and specific surface area of the cellulosic substrate (Kumar et al. 2012). Biomass reconstruction was more recently introduced to study the role of lignin in biomass hydrolysis (Jung et al. 2010; Leskinen et al. 2017; Yu et al. 2014). Reconstructed samples can be prepared by acidification induced precipitation of lignin from alkaline solution onto pulp fibres. Another approach is provided by solvent mediated deposition, followed by slow evaporation (Leskinen et al. 2017). The use of reconstructed biomass enables assessing enzyme inhibition arising both from enzyme adsorption and physical blocking of carbohydrate surfaces (Yu et al. 2014), but may not fully represent the complex recalcitrance of real plant cell walls.

Recent advances in understanding lignin-enzyme interactions

Enzymatic hydrolysis of cellulose is a process requiring a physical contact between glycoside hydrolases and their substrates, which is obstructed by lignins in various ways. Illustration of three different inhibitory mechanisms of lignin is shown in *Fig 2*. Insoluble lignin may block enzyme access to carbohydrate surfaces (*Fig 2A*) or it may irreversibly bind enzymes and prevent enzyme access to their substrate (*Fig 2B*). The fate of the catalytic activity of adsorbed cellulases is under debate, as reports exist on lignin-bound cellulases retaining most of the activity (Rodrigues et al. 2012), whereas there is also evidence suggesting gradual inactivation due to protein unfolding (Rahikainen et al. 2011). Soluble lignin-derived compounds such as vanillin (Li et al. 2014a; Qin et al. 2016; Ximenes et al. 2010) may also act as enzyme inhibitors (*Fig 2C*).

The attractive and repulsive interactions between lignin and cellulases

Enzymes, as proteins in general, are amphiphilic molecules and thus have a tendency to assembly at solid-liquid interfaces. The reversibility of the protein binding process depends on the surface properties of a protein, but also on its structural stability (Haynes, Norde 1995).

Structurally stable proteins may adsorb reversibly but the protein binding to a solid surface is often accompanied by several conformational changes in the folded structure,

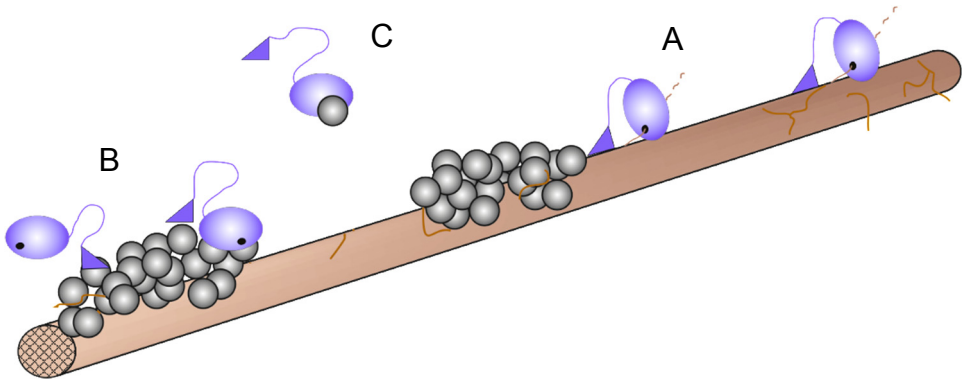

Fig 2 – Three main inhibitory mechanisms of lignin towards cellulases: Physical blocking of mobility and/or hindering accessibility to substrates (A); Irreversible binding to lignin (B); inhibition by soluble lignin fragments (C). The figure is adopted from Rahikainen (2013) with permission from VTT.

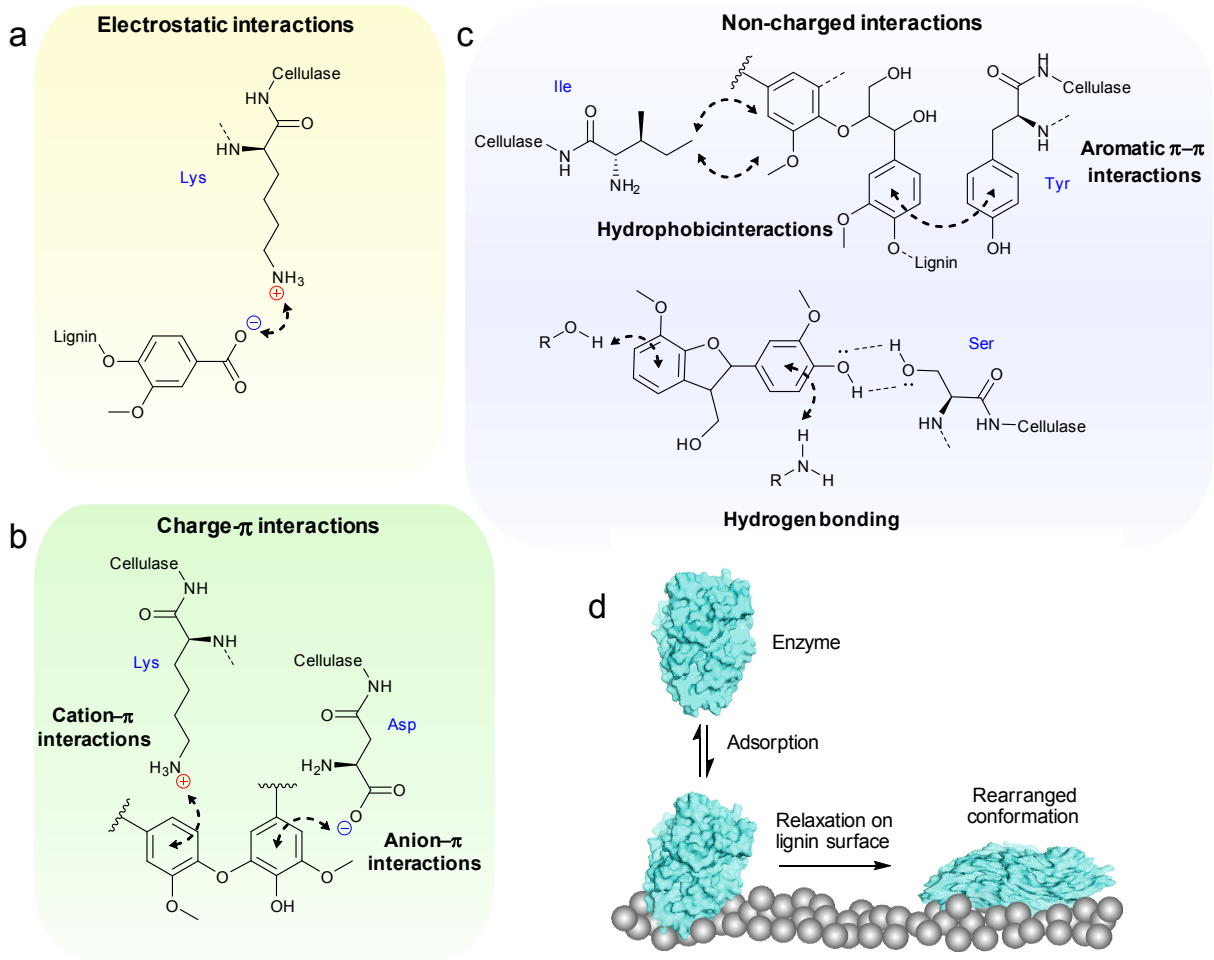

Fig 3 – The main sequence of attractive lignin-enzyme interactions (a-c) that lead to adsorption, structural rearrangements, and irreversible binding of enzymes on lignin surface (d). Indicated amino acid residues of cellulases are examples of possible structures.

which generates stronger interactions with the surface and leads to irreversible binding (Haynes, Norde 1994; McGuire et al. 1995). The negative impact of lignin on hydrolytic enzymes is intensified at elevated reaction temperatures (Börjesson et al. 2007a; Viikari et al. 2007) due to stronger associative interactions (Rahikainen et al. 2011), which lead to unfolding and irreversible enzyme adsorption to lignin (Rahikainen et al. 2013c). It seems logical that the underlying forces are due to hydrophobic interactions and alterations in hydration degree (Leikin et al. 1994) that increase protein-protein and protein-lignin association as temperature increases. Higher temperature may also increase the mobility of the protein molecule, which can lead to temporary exposure of structures with affinity for lignin, and thereby to stronger interaction. General enzyme denaturation events favoured by higher temperatures additionally account for activity loss independent of lignin presence. Structurally rigid thermostable enzymes are thus suggested to better withstand the presence of softwood lignin and harsher

conditions compared to enzymes from a mesophilic organism such as *Trichoderma reesei* (Rahikainen et al. 2013c; Viikari et al. 2007), meaning that benefits of thermostable enzymes could be harnessed at typical reaction temperatures of 45−50 °C.

The irreversible enzyme binding process likely involves several interdependent enzyme-lignin interactions as depicted in *Fig 3*. Enzymes carry positively and negatively charged groups and non-polar chemical moieties exposed at their surfaces, which can interact with lignin's oppositely charged functional groups. Due to their long-range effect (Penna et al. 2014) attractive electrostatic interactions have been argumented as the main driving force for enzymes onto lignin surfaces (Nakagame et al. 2011b; Norde 1996; Rahikainen et al. 2013a), while other researchers have emphasized the effect of hydrophobic interactions (Qin et al. 2014; Sun et al. 2016). Once the enzyme is at the proximity of the lignin surface, charged groups of enzymes may increase affinity to lignin *via* anion-π (Wang, Wang, 2013) and cation-π (Dougherty 1996) interactions, while eventually non-charged interactions such as hydrophobic interactions, hydrogen bonding, including hydrogen-π bonding between aromatic rings (lignin or aromatic amino acids) and OH-groups (Suzuki et al. 1992) and hydrogen-π bonding from amino groups (Rodham et al. 1993) and aromatic units of lignin, can stabilize irreversible binding and unfolding of the enzyme (*Fig 3*). It is noted that the relative magnitudes of these three main types of enzyme-lignin interactions have not been determined, and even if similar enzymes were used, variations can be expected depending on the type of biomass and applied pretreatment. Only a few recent works have attempted to assess the combined effect of the several factors behind lignin-enzyme interactions (Kellock et al. 2017; Sun et al. 2016). A challenge of further research is to differentiate between the simultaneous forces and their possible specific surface patterns, and to provide a lucid mechanism for the irreversible adsorption and gradual inactivation of cellulases on lignin surfaces.

Electrostatic interactions

Attractive electrostatic interactions under hydrolysis conditions are based on the negative charge of lignin that remains in the solids after pretreatment, and the positive net charge of enzymes or individual surface-exposed cationic amino acid residues. Cellulolytic enzymes of *T. reesei* are used in commercial cellulose preparations. The isoelectric point (pI) values of the major endoglucanases and cellobiohydrolases of *T. reesei* vary between 4.3 and 6.2 (*Table* 1) meaning that at pH 5 of enzymatic saccharification reaction some of the cellulases are negatively and some positively charged. Accordingly, cellulases with a cationic net charge have been found to preferentially adsorb on lignin-containing surfaces at pH 5 (Kellock et al. 2017; Ko et al. 2015; Nakagame et al. 2011b; Rahikainen et al. 2013b). Despite the dipole moment of enzymes due to non-uniform distribution of charged amino acid side chains (Fröhlich, 1975), and presently incompletely understood contribution of anion-π interactions (Wang, Wang, 2013) between enzymes and lignin, increasing the negative charge of lignin through pretreatments is beneficial to enzymatic saccharification (Kumar et al. 2011; Nakagame et al. 2011a; Sun et al. 2016; Yang, Pan 2016), suggesting that repulsive electrostatic interactions can outplay the effect of the attractive ones. A recent study (Nordwald et al. 2014) and some patents (Cascao-Pereira et al. 2010; Lavigne et al. 2012; Scott et al. 2010) have also shown that engineering of enzymes with stronger anionic charge is a viable strategy to reduce unproductive enzyme adsorption to lignin. For instance, amino acid mutations that increased negative charge on a CBM of a chimeric *Talaromyces emersonii* cellobiohydrolase were found to direct enzyme binding to cellulose instead of lignin (Strobel et al. 2016). Electrostatic repulsion between lignin and cellulases was increased also by carrying out enzymatic saccharification at elevated pH of 5.5−6 (Lou et al. 2013), albeit at the expense of reduced hydrolysis rates.

Hydrophobic and aromatic interactions

Hydrophobicity of lignin is a common term that is used when non-productive enzyme adsorption on lignocellulosic materials is being discussed. Despite many lignins being quite obviously more hydrophobic than cellulose, they are still in the grey region between the distinctly hydrophilic and hydrophobic materials. In fact,

Table 1 – Characteristics of monocomponent enzymes widely employed in total hydrolysis research. Data is from Kellock *et al.* (2017) if not otherwise stated.

Enzyme	Microbial origin	Abbreviation	Domain architecture	Mw (kDa)	pI[b]
cellobiohydrolase I	*Trichoderma reesei*	*Tr*Cel7A	GH7-CBM1	55.8[a]	3.6-4.3
cellobiohydrolase II	*Trichoderma reesei*	*Tr*Cel6A	GH6-CBM1	56.7[a]	5.4-6.2
endoglucanase I	*Trichoderma reesei*	*Tr*Cel7B	GH7-CBM1	51.9[a]	4.5-4.9, 4.7
endoglucanase II	*Trichoderma reesei*	*Tr*Cel5A	GH5-CBM1	48.2[a]	5.6
β-glucosidase	*Aspergillus niger*	*An*Cel3A	GH3	115.6	4.2
xylanase II	*Trichoderma reesei*	*Tr*Xyn11	GH11	20.8[a]	9.4

[a] Várnai et al. (2013).
[b] pI of the major isoform is underlined

water contact angles of 50–60° have been measured on lignin surfaces (Rahikainen et al. 2013b). Classical hydrophobic effects arise from the desire of non-polar moieties or entire molecules to associate in order to separate from water. Aromatic rings and methoxyl groups are the obvious hydrophobic parts of lignins, but at the molecular level these are scattered around hydrophilic aliphatic and phenolic OH groups. Well hydrated surfaces prevent protein adsorption and hydrophobic ones promote it (Penna et al. 2014; Vogler 2012), eventually causing denaturation of enzymes on lignin surfaces (Börjesson et al. 2007b). However, non-catalytic soy bean proteins exhibited a higher extent of adsorption on organosolv lignin than on synthetic truly hydrophobic surfaces (Salas et al. 2013). Therefore, surface roughness or other supramolecular features may also play a role in the hydrophobicity-mediated adsorption.

True aromatic interactions such as π-π stacking (Hunter, Sanders 1990), cation-π (Dougherty 1996), and anion-π (Wang, Wang, 2013) interactions between lignin and enzymes are likely involved in unproductive enzyme binding, since mutations of aromatic amino acids to non-aromatic amino acids in enzymes (Rahikainen et al. 2013a) and peptides (Yamaguchi et al. 2016) reduce their affinity to lignin. Comparison of π-π interactions between enzymes and lignins with different proportions of free and etherified phenolic hydroxyl groups might bring useful information of the importance of aryl ether cleavage that occurs during pretreatments. Aromatic amino acids are not the only amino acids with hydrophobic character, and surface hydrophobicity in general has been suggested to correlate with the extent of lignin-binding (Sammond et al. 2014). However, some contradictory results have been recently reported; overall surface hydrophobicity of cellulases and a xylanase was not explicitly found to correlate with the extent of lignin-binding (Kellock et al. 2017).

Aromatic side-chains on the flat face of cellulose binding modules (CBMs) of cellulases are highly important for their adsorption on lignin (Palonen et al. 2004; Rahikainen et al. 2013a; Strobel et al. 2015). The major *T. reesei* cellulases possess a family 1 CBM, which is a small, wedge-shaped domain folded of ca. 40 amino acids (Kraulis et al. 1989). Mutagenesis studies (Rahikainen et al. 2013a; Strobel et al. 2016) and molecular dynamics modelling (Vermaas et al. 2015) have shown that conserved aromatic amino acids residues in CBMs that are important for affinity to cellulose are also mediating enzyme binding to lignin (Börjesson et al. 2007a; Palonen et al. 2004). This may suggest that π-π interactions are involved in binding of cellulases to lignin, since the surface exposed aromatic amino acid residues of CBMs can readily stack with lignin's aromatic rings. Some CBM and linker mutations have been found to selectively reduce adsorption on lignin while preserving affinity to cellulose (Strobel et al. 2015, 2016). Another approach to circumvent adsorption of cellulases on lignin is to incorporate a cellulase mixture with a complex-forming sacrificial hydrophobic protein. Zong *et al.* (2016) pre-conditioned a mixture of *T. reesei* cellulases with hydrophobin and achieved 33% and 41% increases in enzymatic conversion of pretreated corn stover and Avicel cellulose compared to that obtained with cellulase alone. Though not offering an economically feasible solution to the problem, it demonstrates that hydrophobic surfaces do play a key role in enzyme-lignin binding.

Hydrogen bonding

Hydrogen bonds are abundant and thus cannot be neglected in lignin-enzyme interactions. Most information related to hydrogen bonding functionalities is found about the abundance of phenolic and aliphatic OH groups in correlation to enzyme adsorption or saccharification performance (Huang et al. 2016; Pan 2008; Sewalt et al. 1997; Sun et al. 2016; Yang, Pan 2016; Yu et al. 2014). Accumulated results suggest that phenolic OH groups bind enzymes *via* hydrogen bonding, unlike aliphatic OH groups that reduce enzyme adsorption (Huang et al. 2016; Sun et al. 2016; Yu et al. 2014). After pretreatment, the residual amounts of these two types of OH groups are often intercorrelated, and the formation of phenolic OH groups may be accompanied with formation of condensed lignin in pretreatment, which has also been affiliated to enzyme inhibition (Sun et al. 2016). However, detrimental effects can be reversed by blocking of phenolic OH groups.

Chelation of phenolic and carboxylic OH groups with Ca^{2+} and Mg^{2+} ions reduces hydrogen bonding between lignin and cellulases and also improves saccharification (Akimkulova et al. 2016; Liu et al. 2010). This chelation phenomenon also increases hydrophobicity of lignin, and can be used to precipitate lignin from alkaline solutions (Sipponen et al. 2016) and so to improve fermentability of lignocellulosic hydrolysates (Palmqvist, Hahn-Hägerdal 2000). Hydroxypropylation blocks covalently the phenolic OH groups while generating new aliphatic OH groups in lignin, and consequently reduces its inhibitory effect on cellulases (Pan 2008; Sewalt et al. 1997). Because the replacement of phenolic OH groups with hydroxypropyl ether groups does not reduce the total number of hydroxyls, these results indicate that aliphatic OH groups may be less detrimental for enzyme binding *via* hydrogen bonds. Another mechanism which has not been elucidated yet is the increased steric hindrance from hydroxypropylation to π-π interactions between the aromatic ring and the enzyme. It is noted that also the spatial arrangement of the lignin molecule influences its surface properties, and hence further work is needed to determine the extent of enzyme binding to materials with known quantities of surface-accessible phenolic OH groups (Sipponen et al. 2014a). Furthermore, hydrogen bonding between the multitude of possibly participating functionalities and particularly the role of carbonyl functionalities, formed by acidolysis and having a strong hydrogen bond acceptor capability, are topics that need to be addressed.

Effects of lignin source on enzyme-lignin interactions

Only minor inhibitory effects were observed when residual lignin, isolated after enzymatic hydrolysis of pretreated wheat straw, softwood, and hardwood, were mixed with cellulose during enzymatic hydrolysis (Rahikainen et al. 2013b; Yu et al. 2014). Identical extents of inhibition to enzymatic hydrolysis of maize cell walls were obtained with H-, G-, and S-type DHPs as lignin models (Grabber

et al. 1997). Hydrothermal pretreatments (Rahikainen et al. 2013b; Sun et al. 2016), as well as acidic and mild alkaline pretreatments (Yu et al. 2014) increase the inhibitory effect of lignins irrespective of their botanical origin; the increasing inhibitory effect is further shown to correlate with increasing pretreatment severity (Sun et al. 2016). Furthermore, although native-type lignins of different botanical origin do not differ in their inhibitory effect as such, they do show differences in their reactions under given pretreatment conditions. It is known that softwood requires pretreatment conditions that are more severe compared to those applied to hardwood or herbaceous feedstocks (Rahikainen et al. 2013b). Due to their lower methoxyl content, softwood lignins are generally more prone under these relatively severe pretreatment conditions to formation of inhibitory and recalcitrant condensed structures. However, condensed phenolic groups occur also in lignins in pretreated hardwood (Sun et al. 2016; Yu et al. 2014), and compared to softwoods, hardwoods and grasses contain more pentosans (Scheller, Ulvskov 2010) that generate lignin-type inhibitory polymerization products from furfural under acidic pretreatment conditions. Side-by-side comparisons of acid-catalyzed pretreatments of different types of biomass is needed to shed light on the underlying reasons causing non-productive enzyme adsorption.

The role of surface distribution of lignin and hemicelluloses

Due to the well demonstrated non-productive adsorption of enzymes on lignin, the degree of surface coverage by lignin of a solid substrate that is being hydrolysed is crucial. As has been demonstrated (Sipponen et al. 2014b), the area of lignocellulosic material covered by lignin can decrease to a half of its original level in hydrothermal pretreatment of wheat straw, and this effect can be correlated to increased enzymatic conversion of cellulose to glucose. Nevertheless, removal or redistribution of lignin (Chundawat et al. 2011; Donohoe et al. 2008) and hemicelluloses also simultaneously increases the total volume of enzyme-accessible micropores within the substrate, a factor that correlates with hydrolysability (Torr et al. 2016). While pretreatments generally have a major effect on lignin surface area and chemistry, only minor changes have been observed in surface-displayed phenolic OH groups during enzymatic hydrolysis, regardless of the degree of saccharification (Pihlajaniemi et al. 2016a). State of the art imaging techniques and surface sensitive chemical analysis procedures should be taken into wider use to analyse the surface distribution of lignin.

Presence of lignin as a saccharification-enhancing factor

Several fairly recent studies report positive effects on enzymatic saccharification yields in the presence of supplemented lignins or after partial delignification of pretreated substrates (Lai et al. 2014, 2016; Leskinen et al. 2017; Li et al. 2016). It is now understood that it is important to maintain a low content of hemicellulose or lignin in the pretreated biomass to avoid collapse of the porous cellulose matrix (Pihlajaniemi et al. 2016b; Zhang et al. 2016). One of the proposed direct positive effects is the ability of lignin to facilitate the oxidative cleavage of cellulose by lytic polysaccharide monoxygenases (LPMOs) (Müller et al. 2015; Rodríguez-Zúñiga et al. 2015). Besides lignins originating from the pretreatment processes, positive effects of lignosulfonates (Zhou et al. 2013a) and other lignin derivatives (Cheng et al. 2014; Lin et al. 2015; Nakagame et al. 2011b) have been shown. Intriguingly, organosolv lignin from hardwood enhanced hydrolysis of various substrates, while negative effects were observed for the corresponding softwood lignin (Lai et al. 2014). The positive effect was rationalized by weak and reversible adsorption of cellulases, while the negative effects arose from strong irreversible binding onto softwood lignin. Later work from the same group (Lai et al. 2016) and from the authors of this review (Leskinen et al. 2017) have demonstrated similar affinity-related dualistic effects between solvent extractable and residual hardwood lignin fractions.

The macromolecular architecture and consequently the accessibility of certain functional groups of insoluble lignin fractions play important roles on the inhibiting effect of lignin. Low molecular weight and hydrophilic/amphipathic fractions of lignin (Leskinen et al. 2017) may act in similar fashion as some nonionic surfactants that increase saccharification yields (Eckard et al. 2013). Lignin fractions that possess weak interactions with enzymes reduce irreversible binding of enzymes on specific cellulose surfaces. Especially endoglucanases would rationally benefit from the presence of some lignin that facilitates their desorption from cellulose surfaces (Li et al. 2016). Overall, these phenomena demonstrate well the complexity of enzyme interactions with lignified substrates, and call for detailed understanding of enzyme interaction with chemically divergent lignin structures.

Structural changes in lignin during common lignocellulose pretreatments

The native structure of lignin is severely modified during lignocellulose pretreatments. *Table 2* lists established structural changes in lignin that may have either detrimental or beneficial impact on saccharification. In general, detrimental effects can be related to increased adsorption of enzymes on lignin. Despite generally showing inverse correlation to saccharification, lignin content exhibits poor correlation to hydrolyzability after dilute acid (DA) and autohydrolysis (Sipponen et al. 2014b) pretreatments because of the associated major morphological and structural changes in lignin and hemicelluloses. Direct chemical changes in lignin that are well documented for their impeding effect on saccharification include formation of condensed units (Huang et al. 2016; Sun et al. 2016; Yu et al. 2014), free phenolic OH groups (Huang et al. 2016; Pan 2008; Sewalt et al. 1997; Sun et al. 2016; Yang, Pan 2016), and water-soluble phenolic degradation products (Li et al. 2014b; Qin et al. 2016; Ximenes et al. 2010). It is noted that many changes in lignin occurring in pretreatments are intercorrelated; formation of carboxylic groups is accompanied with the cleavage of β-ethers, loss of aliphatic OH groups, and formation of free phenolic OH groups as well as condensed lignin units.

Table 2 – Changes in lignin structure caused by pretreatment and their positive (+) and negative (-) effects on enzymatic saccharification of pretreated biomass.

Pretreatment effect	Effect on saccharification	Lignin sources
Reduction of lignin content	**(+)** (Ding et al. 2012; Siqueira et al. 2013; Wallace et al. 2016) **(+/-)** (Zhang et al. 2016) [a]	Corn stover, sugarcane bagasse, *Eucalyptus globulus*
Formation of condensed units	**(-)** (Huang et al. 2016; Sun et al. 2016; Yu et al. 2014)	*E. globulus*, red maple, loblolly pine, mixed hardwood, aspen, bamboo
Formation of phenolic OH	**(-)** (Guo et al. 2014; Huang et al. 2016; Pan 2008; Sewalt et al. 1997; Sun et al. 2016; Yang, Pan 2016)	Technical lignins, aspen, poplar, pine, bamboo, mixed hardwood, barley straw
Elimination of aliphatic OH	**(-)** (Guo et al. 2014; Yu et al. 2014) [b]	*E. globulus*, red maple, loblolly pine, mixed hardwood
Formation of COOH	**(+)** (Guo et al. 2014; Nakagame et al. 2011a; Sun et al. 2016; Yang, Pan 2016)	Aspen, corn stover, poplar, lodgepole pine,
Sulphonation	**(+)** (Yang, Pan 2016) **(-)** (Pareek et al. 2013)	poplar, lodgepole pine, Norway spruce, black cottonwood
Alkoxylation of aliphatic side chains	**(+)** (Lancefield et al. 2017)	Beech
Formation of resinous degradation products	**(-)** (Kumar et al. 2013; Rasmussen et al. 2017)	Wheat straw
Increased hydrophobicity	**(-)** (Huang et al. 2016; Yang, Pan 2016)	Poplar, lodgepole pine, bamboo
Reduced surface coverage by lignin	**(+)** (Kim et al. 2016; Sipponen et al. 2014b)	Wheat straw, several wood and grass species

[a]: A low amount of lignin may have some beneficial effects to enzymatic hydrolysis of associated cellulose, as discussed in above
[b]: Inversely correlated to the formation of condensed lignin units.

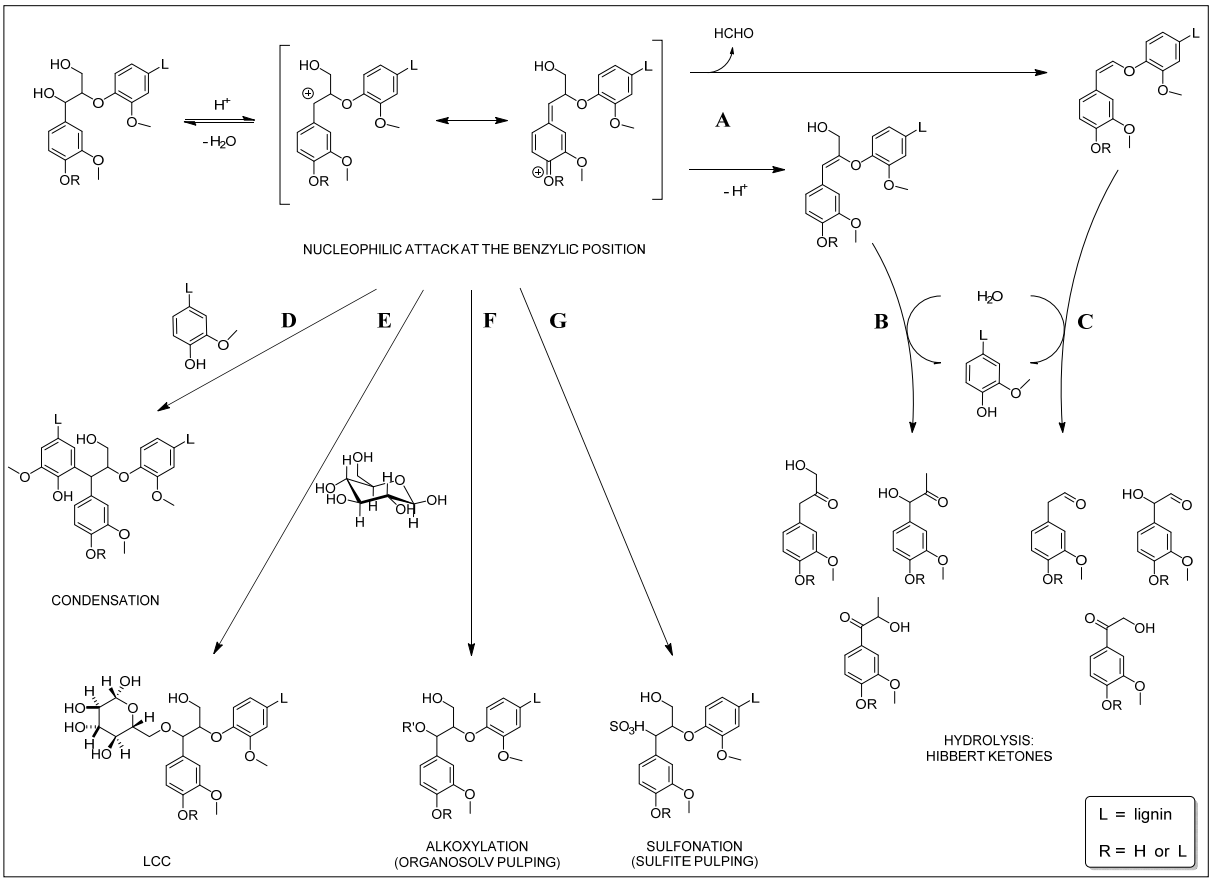

Fig 4 – Key steps in acid pretreatment processes. A−C: generation and hydrolysis of aryl enol ethers; D: condensation; E: LCC formation; F: alkoxylation of aliphatic chain during organosolv pulping; G: main reaction under sulphite pulping

Chemical modification of lignin occurs in the presence of nucleophilic chemicals during pretreatments. Most of these reactions take place at the aliphatic chain and beneficially block the lignin condensation reactions. Obvious examples are alkoxylation (Bauer et al. 2012; Kubo, Kadla 2004; Lancefield et al. 2017) and sulphonation (Lou et al. 2013; Zhou et al. 2013b) at the C-α position, and reactions with so-called carbocation scavengers supplemented to autohydrolysis pretreatments (Li et al. 2007; Pielhop et al. 2016; Wayman, Lora 1978). Acidic hydrothermal processes form resinous degradation products, termed pseudo-lignin or humins, which resemble lignin in terms of structural features(Sannigrahi et al. 2011; Van Zandvoort et al. 2013) and in their detrimental adsorption of cellulases (Sannigrahi et al. 2011). Finally, it has been established that the proportion of the surface-occupied by lignin is more influential to enzyme performance than the mere bulk lignin content as such (Kim et al. 2016; Sipponen 2015).

Reaction routes of acidic pretreatments

The broad use of acidic pretreatments (autohydrolysis, steam-explosion, dilute acid etc.) can be rationalized by the structural acidity of lignocellulosic biomass. The prevailing lignin-modifying reaction under acidic conditions is the generation of a benzylic carbocation followed by the cleavage of the β-aryl ether bond (Lundquist, Lundgren, 1972) or nucleophilic attack at the benzylic position, which defines the fate of the lignin side chain (Gierer, 1985). *Fig 4* shows possible lignin side chain modifications occurring during acid-catalyzed pretreatments. Quinone methide formation represents the key initial step in aliphatic side chain modification and fragmentation reactions (Gierer 1985). Protonation of the benzylic hydroxyl followed by the loss of water generates the corresponding benzyl cation. The protonated quinone methide intermediate can be easily generated either with free phenolic end groups or with etherified. C-4 position (shown). Fragmentation of the side chain results from the proton abstraction and eventual loss of formaldehyde that yield aryl enol ethers (*Fig 4 Route A*). These species are not stable under acidic conditions and undergo hydrolysis to Hibbert ketones and phenyl acetic aldehydes (*Fig 4 Routes B−C*). Released formaldehyde (Lundquist, Ericsson, 1970) can, in turn, undergo condensation reactions *via* hydroxymethylation of the aromatic ring to generate diphenylmethane motifs, or alternatively form cyclic acetal structures with unmodified side chains (Shuai et al. 2016a). The main reaction pathway for the benzylic cation under acidic pathway is the nucleophilic addition to the C-α of the lignin side chain (Wayman, Lora 1978). When the nucleophile is a phenolic fragment or lignin terminal unit, α-5' condensed motifs are formed (*Fig 4 Route D*). Sugars present in solution can also undergo nucleophilic attack at the C-α carbon, yielding LCC structures, albeit in low amount *(Fig 4 Route E)*.

Reactions routes of alkaline pretreatments

Alkaline biomass pretreatments take inspiration from technologies established in the pulp & paper industry (Jiang et al. 2016; Pihlajaniemi et al. 2016c; Qing et al. 2016; Yang et al. 2016), and also lay the basis for the oxidative upgrading of lignin (Lange et al. 2013; Zakzeski et al. 2010). The key step for lignin degradation under alkaline conditions is the generation of quinone methides from phenolate anions shown in *Fig 5* (Dimmel, 1985; Dimmel, Gellerstedt, 2010; Dimmel, Schuller, 1986; Dimmel, Shepard, 1982; Dyall, Winstein, 1972; Gierer, 1985; Gierer, Lindeberg, 1980; Landucci, 1981; Ralph, Adams, 1983; Suckling, 1988). The deprotonated intermediate can lose formaldehyde from the side chain and generate aryl enol ethers, which are stable under alkaline conditions. Therefore such substructures are present in isolated soda lignin, or, according to the different processing conditions, can in turn undergo transformation into Hibbert ketones and contemporary fragmentation of the side chain (*Fig 5, route A*) (Gierer 1985). This constitutes the main reaction pathway during soda pulping. The quinone methide intermediate can also undergo nucleophilic addition at the C-α position. Consequently, phenolate anion nucleophiles from monomeric fragments or terminal moieties of lignin oligomers induce the formation of condensed α-5’ bonded units (*Fig 5 Route B*). Lignin-carbohydrate bonds can be generated when sugar residues undergo nucleophilic addition to the benzylic position (*Fig 5* route C) (Gierer, Wännström 1984). In the case of nonphenolic β-aryl ether bonds an intermediate epoxide is formed. Nucleophilic opening of this structure is followed by side chain fragmentation (*Fig 5, Route E*).

Hydrosulphide ion is the active nucleophile in kraft pulping (Chakar, Ragauskas 2004; Gierer 1980). It gives rise to an episulfide intermediate which undergoes redox reactions with the generation of cinnamyl alcohols (Gierer 1985). Accompanying complex redox processes give rise to fragments carrying one or two carbons in the side chain (*Fig 5 route D*) (Majtnerová, Gellerstedt 2006). Sulphur radicals have been speculated to favour the oxidative repolymerization of lignin fragments *via* formation of condensed 5-5’ and 4-*O*-5’ linkages (Froass et al. 1998; Majtnerová, Gellerstedt 2006). Lignins isolated from alkaline kraft or soda cooking liquors show a significantly reduced amount of aryl ether bonds (Chakar, Ragauskas 2004; Crestini et al. 2017). In addition, some methoxyl groups are converted to OH groups in demethylation reactions (Crestini et al. 2017; Gierer 1985). The aliphatic OH groups are largely depleted and only a few unmodified aliphatic side chains survive (Crestini et al. 2017; Chakar, Ragauskas 2004). Consequently, the lignin backbone is mainly constituted of condensed aromatic units arising from condensation and oxidative re-polymerization of lignin-derived fragments.

Several ammonia based pretreatment technologies have been developed. Ammonia fibre expansion (AFEX) uses gaseous ammonia to alter the biomass structure without removal of lignin (Balan et al. 2009). Ammonia recycled percolation (ARP) uses aqueous ammonia in a percolation mode and dissolves mainly lignin (Wu, Lee 1997). Extractive ammonia (EA) differs from ARP in that it uses anhydrous ammonia and converts crystalline cellulose to the allomorph III which itself facilitates saccharification (da Costa Sousa et al. 2016), as also suggested with AFEX substrates (Gao et al. 2013).

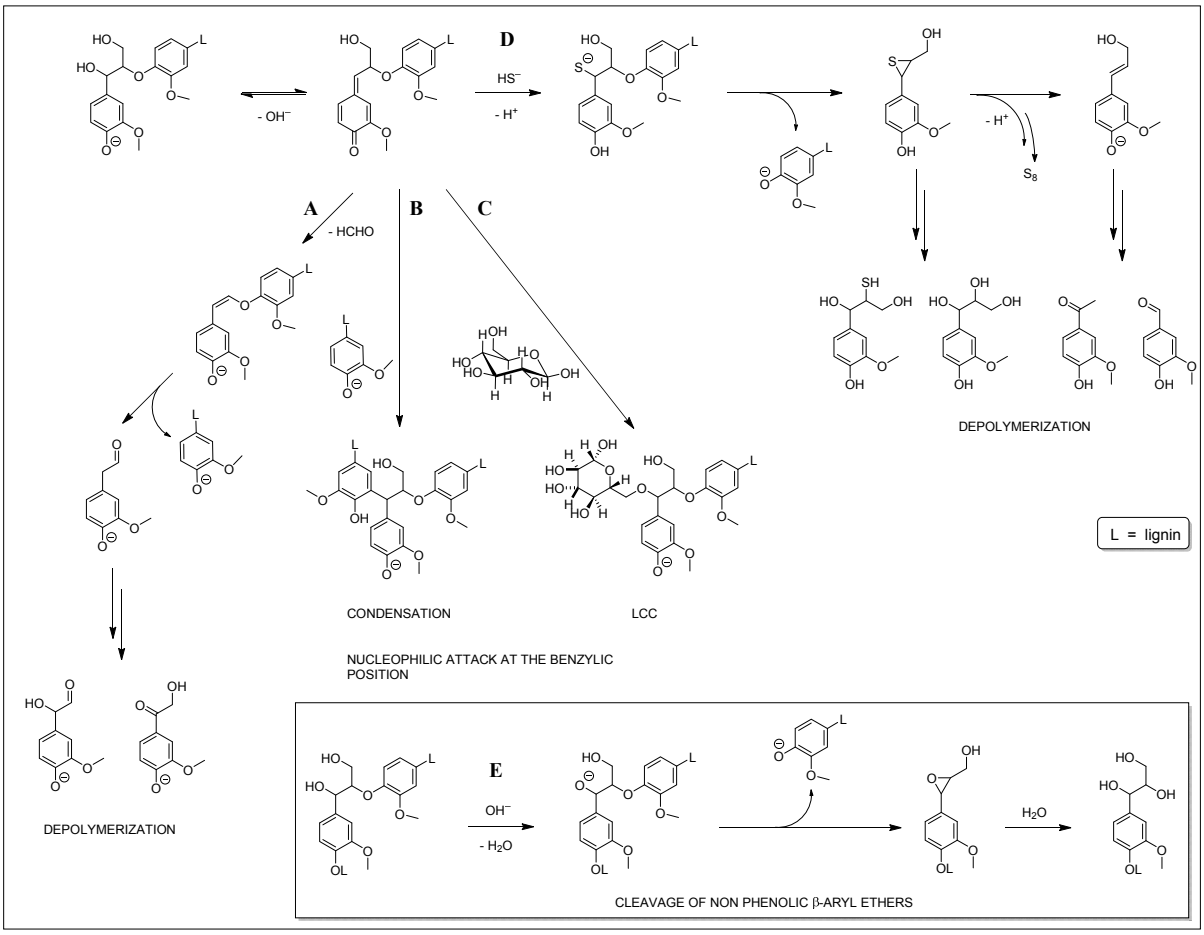

Fig 5 – Key reactions in alkaline pulping leading to depolymerization (A), formation of condensed units (B), LCCs (C), and fragmentation of the side chain (D). Cleavage of non-phenolic β-ethers (E) proceeds several magnitudes slower compared to units with a free phenolic group.

Treatment of cellulolytic enzyme lignin with aqueous ammonia caused a detrimental increase in the adsorption affinity and binding strength of cellulases (Yoo et al. 2017). Further work is needed to clarify whether this elevated affinity to cellulase binding is caused by the documented generation of lignin-derived amides and other nitrogeneous products (Bouxin et al. 2014; Chundawat et al. 2010). AFEX pretreatment of corn stover released inhibitory substances to hydrolytic enzymes, including < 3kDa water-soluble phenolic and Maillard products (Humpula et al. 2014). However, very little is known about the structure of insoluble lignin that remains in the pretreated solids after AFEX treatment.

Pretreatments using novel delignification solvents

Various types of ionic liquids (ILs) can rather selectively dissolve cellulose, hemicellulose, and lignin, or all of these simultaneously (Brandt et al. 2013; Elgharbawy et al. 2016; Leskinen et al. 2014). The ability of ILs to convert cellulose to an amorphous form is highly effective in making lignocellulose substrates more amenable to enzymatic saccharification (Elgharbawy et al. 2016; Tadesse, Luque 2011), while good solvent properties of ILs and IL-co-solvent mixtures allow extensive delignification of biomass. Treatment processes can be realised under a wide temperature range from 80 to 170 °C (Kim et al. 2011; Ma et al. 2016; Sathitsuksanoh et al. 2014; Varanasi et al. 2012; Wen et al. 2014), which also determines the extent of lignin modifying reactions. *Table 3* summarizes lignin transformations in an imidazolium based IL [Emim][OAc] that depolymerizes labile β-ether linkages (Brandt et al. 2013; Leskinen et al. 2014; Wen et al. 2014), reduces the number of aliphatic OH groups (Wen et al. 2014), and increases the frequency of condensed linkages (Kim et al. 2011; Torr et al. 2012; Wen et al. 2014) and phenolic OH groups (Kim et al. 2011; Wen et al. 2014). Protic ILs that can be recycled by distillation hold potential for development of less expensive processes (Achinivu et al. 2014). However, the choice of IL and process conditions is important. A recent study showed that triethylammonium hydrogensulfate, an acidic protic IL, increased the proportion of condensed linkages more than neutral or basic ILs during depolymerization of kraft lignin (Dutta et al. 2017). Stability of the ILs, their complete recovery from biomass (Brandt et al. 2013), and the intolerance of cellulases to ILs need amelioration (Wahlström, Suurnäkki 2015). Deep eutectic solvents (DESs) are generally more affordable and environmentally friendly ionic pretreatment solvents compared to ILs (Procentese et al. 2015). However, DESs are hitherto less efficient than ILs in improving saccharification yields (Wahlström et al. 2016; Xia et al. 2014), and likewise to ILs inactivate cellulases (Lehmann et al. 2014). Acid-catalyzed cleavage of lignin β-ether linkages may occur at elevated temperatures in the

presence of DESs with relatively minor extent of side-chain fragmentation (Alvarez-Vasco et al. 2016), but this point needs further confirmation as new DESs are being tested. In summary, the research with DESs is in its infancy, and in addition to, or instead of, bulk lignocellulose pretreatment, DESs may better serve as selective extraction solvents, e.g. for phenolic compounds (Loow et al. 2017).

Fractionation of lignocellulose using aqueous γ-valerolactone (GVL) has been recently developed (Fang, Sixta 2015; Luterbacher et al. 2014). In addition to its ability to dissolve lignin, GVL promotes hydrolysis of polysaccharides by reducing the activation energy of glycosidic bond cleavage during pretreatment (Mellmer et al. 2014a, b). GVL pretreatment of hardwood was effective at relatively low temperature of 120 °C unlike pretreatments with aqueous tetrahydrofuran (THF), ethanol, dilute acid, or dilute alkali (Shuai et al. 2016b). Scarce information is available on GVL lignins, but very slight increases in phenolic OH (Lê et al. 2016) and drops in aliphatic OH contents (Lê et al. 2016; Zhou et al. 2016) suggest that β-ethers are cleaved only moderately.

Lignocellulose pretreatment in aqueous THF effectively dissolves lignin and restricts its condensation in the solid fraction, thus avoiding formation of detrimental structures to enzymes used in saccharification (Smith et al. 2016). Lignin solutions in THF have been used to produce spherical lignin nanoparticle colloids (Lievonen et al. 2015) and here might be an opportunity for process integration. In contrast to water-miscible THF, 2-methyltetrahydro-furan and water phase separate – a fact that facilitates solvent recycling. By using this biphasic system in the presence of oxalic acid, hydrolysis of hemicelluloses, dissolution of lignin, and their separation from insoluble cellulose fraction was demonstrated (Grande et al. 2015; vom Stein et al. 2011). A common feature of all of these emerging delignification solvent pretreatments is that initial process development has predominated over structural studies of lignin. As these processes develop towards commercial maturity, more detailed characterization of lignin will be needed.

Summing up pretreatment effects on lignin

The effect of lignin on enzymatic saccharification after pretreatment boils down to the residual amount of lignin and surface area occupied by lignin, as well as its chemical modification that has ultimate effects on its interaction with enzymes used for enzymatic biorefinery attempts. All of these together determine the quantity and nature of enzyme-lignin interactions. *Table 3* summarizes changes in functionalities of lignin from various biomass types as a result of different types of pretreatments. The beneficial or detrimental effects indicated by upward and downward pointing arrows are based on the relationship between structural characteristics and their impact on saccharification as listed in *Table 2* above. Cleavage of aryl ether bonds of lignin is achieved by alkaline delignification processes as well as acidic processes including autohydrolysis and DA treatments, and similarly, condensed lignin is formed in both cases. Overall it seems that the most favourable pretreatments cause a high level of lignin removal and increase anionic charge of the residual lignin.

Table 3 - Structural changes of lignin due to industrial and emerging pretreatments. Arrows indicate increase ↑, decrease ↓, or no change ↔, and arrows in parentheses indicate minor changes found in some studies. References are given in the annexed version of the table.

Pretreatment	Lignin content	Condensed linkages	Phenolic OH	Aliphatic OH	COOH
DA and acid-catalyzed steam-EX	↑	↑	↑	↓	↑
Autohydrolysis and autocatalytic steam-EX	↑↓	↑	↑	↓	↑
Acid sulphite (SPORL)	↓	↑	↑	↓	↑
Ethanol-water organosolv	↓	↑	↑	↓	↑
Organic acid organosolv	↓	↑	↑	↓	↑
Aqueous alkali pretreatment	↓	↑ (↓)	↑	↓	↑
AFEX	↔	↓	↔	↔	↔
ARP	↓	↑	↑	↓	↑
Extractive ammonia (EA)	↓	↔	↔	↔	↑
Ionic liquid (IL: [Emim][OAc])	↑↓	↑↔	↑	↓	↔
Deep eutectic solvents (DESs)	↓	↔	↑	↔	?
Gamma-valerolactone (GVL)	↓	?	↑	↓	?
Tetrahydrofuran (THF)	↓	?	↑	?	?
2-MTHF/oxalic acid	↓	?	↑	↓	?

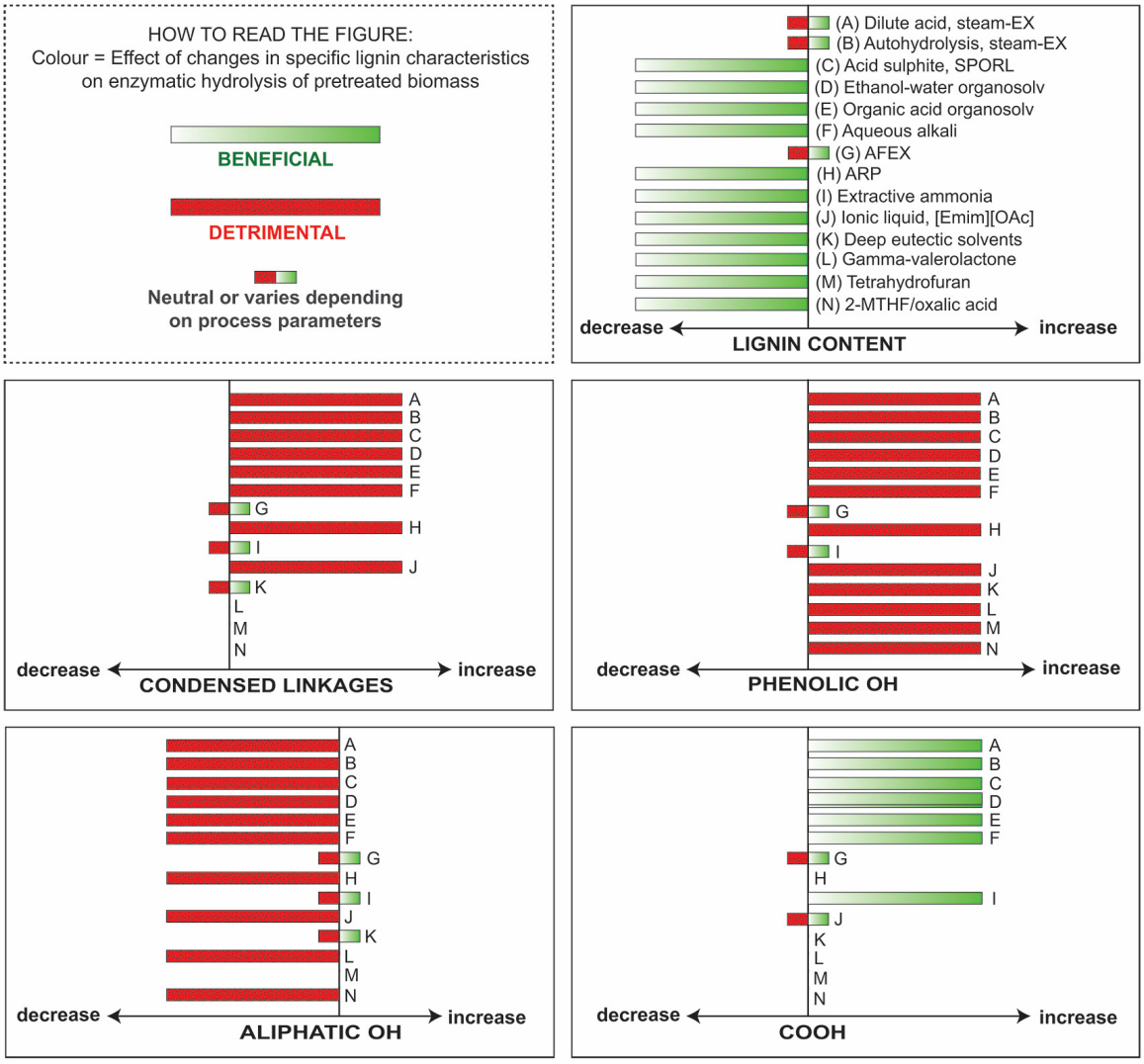

Fig 6 – Effect of different pretreatment types on specific lignin characteristics (lignin content, condensed linkages, phenolic OH, aliphatic OG, and carboxylic groups) The beneficial or detrimental effect on enzymatic saccharification that is associated with increase or decrease in these characteristics is illustrated with green gradient or red patterned bars, respectively. The order of pretreatments, letter coded from A to N, is similar to that shown in *Table 3*.

A central aim of this review was to elucidate the interplay between structural changes in lignin and observed effects on enzymatic saccharification. *Table 2* above summarized literature on effects of various structural changes on saccharification, while *Table 3* earmarked the selected most comprehensively documented changes (lignin content, condensed linkages, and aliphatic, phenolic and carboxylic hydroxyl functionalities) to 14 common pretreatments. As a synthesis of these two tables, *Fig 6* presents a simplified bar chart showing changes in the five categories of lignin characteristics and the associated effects on enzymatic saccharification. Qualitative changes in lignin characteristics are indicated with bars pointing left (decrease) or right (increase), while the colour of the bar describes the associated effect on enzymatic saccharification. It is noted that the figure does not attempt to provide quantitative information, but nevertheless may serve as a useful resource to assess rather complex and interdependent structure-function relationship in this area.

There are two categories in which positive effectS on enzymatic saccharification can be realized. Decreasing lignin content is the most obvious one, with exceptions of autohydrolysis and dilute acid pretreatments, which may increase lignin content, and AFEX that does not cause alterations in this parameter. Increasing carboxylic acid content appears to occur in eight of the fourteen pretreatments evaluated, improving saccharification. As discussed in more detail in the preceding sections, many of the chemical changes are intercorrelated. Predominant increase in the extent of condensed linkages and phenolic hydroxyl content is associated with loss of aliphatic hydroxyls during degradation of lignin either in acid or base catalyzed reactions; each of these have a detrimental effect on enzymatic saccharification. Some non-existing bars in *Fig 6* indicate lack of data or unestablished understanding especially with most recent solvent-based pretreatments.

Perspective and recommendations on future directions

It is clear that more research is needed to fill the blank spaces in the effects that various emerging pretreatments have on lignin, both structurally and topographically with consequences to saccharification. Side-by-side

comparison of softwood, hardwood and grass biomass could help generalize the effects of pretreatments on enzyme-lignin interactions as much as it is possible crossing various lignin origins. Enzymatic saccharification assays should be conducted using "reasonably low" cellulase dosages because excess amount of enzymes can cover lignin and hide detrimental non-productive adsorptive loss of activity (Shen et al. 2016). Viable cellulosic bioethanol production likely requires cellulase dosages below 5 FPU/g dry substrate, and it is this range where greatest benefits from process optimization or utilization of lignin blocking additives can be seen (Eckard et al. 2013; Leskinen et al. 2015b). Better understanding of the interactions that drive enzyme binding to lignin would call for more studies that employ well characterized lignins and pure enzymes with engineered structures that would allow monitoring of binding properties caused by targeted modification of eventually identified binding hotspots. Process economics is the most influential factor in industrial biorefinery operation, and presently this favours adaptation of acidic and hydrothermal pretreatments, which are generally prone to render lignin more condensed and poorly extractable. Solving this dilemma asks for multi-disciplinary research in the areas of plant biotechnology (Van Acker et al. 2014; Eudes et al. 2015; Wilkerson et al. 2014) enzyme engineering, process development, and fundamental research on lignin-enzyme interactions.

Acknowledgements

MHS acknowledges funding from the Academy of Finland (postdoctoral grant 296547).

References

Achinivu, E.C., Howard, R.M., Li, G., Gracz, H. and Henderson, W.A. Lignin extraction from biomass with protic ionic liquids. (2014): Green Chem., 16(3), 1114–1119.

Van Acker, R., Leplé, J.-C., Aerts, D., Storme, V., Goeminne, G., Ivens, B., Légée, F., Lapierre, C., Piens, K., Van Montagu, M.C.E., Santoro, N., Foster, C. E., Ralph, J., Soetaert, W., Pilate, G. and Boerjan, W. (2014): Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase. Proc. Natl. Acad. Sci. U. S. A., 111(2), 845–50.

Akimkulova, A., Zhou, Y., Zhao, X. and Liu, D. (2016): Improving the enzymatic hydrolysis of dilute acid pretreated wheat straw by metal ion blocking of non-productive cellulase adsorption on lignin. Bioresour. Technol., 208, 110–116.

Alvarez-Vasco, C., Ma, R., Quintero, M., Guo, M., Geleynse, S., Ramasamy, K.K., Wolcott, M. and Zhang, X. (2016): Unique low-molecular-weight lignin with high purity extracted from wood by deep eutectic solvents (DES): a source of lignin for valorization. Green Chem., 18(19), 5133–5141.

Asikkala, J., Tamminen, T. and Argyropoulos, D.S. (2012): Accurate and Reproducible Determination of Lignin Molar Mass by Acetobromination. J. Agric. Food Chem., 60(36), 8968–8973.

Balakshin, M., Capanema, E., Gracz, H., Chang, H. and Jameel, H. (2011): Quantification of lignin-carbohydrate linkages with high-resolution NMR spectroscopy. Planta, 233(6), 1097–1110.

Balan, V., Bals, B., Chundawat, S.P.S., Marshall, D. and Dale, B.E. (2009): Lignocellulosic Biomass Pretreatment Using AFEX. Biofuels - Methods Protoc., Jonathan R. Mielenz, ed., Humana Press, 2009.

Bauer, S., Sorek, H., Mitchell, V.D., Ibáñez, A.B. and Wemmer, D.E. (2012): Characterization of Miscanthus giganteus lignin isolated by ethanol organosolv process under reflux condition. J. Agric. Food Chem., 60(33), 8203–8212.

Baumberger, S., Abaecherli, A., Fasching, M., Gellerstedt, G., Gosselink, R., Hortling, B., Li, J., Saake, B. and De Jong, E. (2007): Molar mass determination of lignins by size-exclusion chromatography: Towards standardisation of the method. Holzforschung, 61(4), 459–468.

Behera, S., Arora, R., Nandhagopal, N. and Kumar, S. (2014): Importance of chemical pretreatment for bioconversion of lignocellulosic biomass. Renew. Sustain. Energy Rev., 36, 91–106.

Björkman, A. (1956): Studies on finely divided wood. Part 1. Extraction of lignin with neutral solvents. Svensk. Papperst, 59(13), 477–485.

Borrega, M., Tolonen, L.K., Bardot, F., Testova, L. and Sixta, H. (2013): Potential of hot water extraction of birch wood to produce high-purity dissolving pulp after alkaline pulping. Bioresour. Technol. 135, 665–671.

Bouxin, F.P., David Jackson, S. and Jarvis, M.C. (2014): Isolation of high quality lignin as a by-product from ammonia percolation pretreatment of poplar wood. Bioresour. Technol., 162, 236–242.

Brandt, A., Gräsvik, J., Hallett, J. P. and Welton, T. (2013): Deconstruction of lignocellulosic biomass with ionic liquids. Green Chem., 15(3), 550–583.

Börjesson, J., Engqvist, M., Sipos, B. and Tjerneld, F. (2007a): Effect of poly(ethylene glycol) on enzymatic hydrolysis and adsorption of cellulase enzymes to pretreated lignocellulose. Enzyme Microb. Technol., 41(1–2), 186–195.

Börjesson, J., Peterson, R. and Tjerneld, F. (2007b): Enhanced enzymatic conversion of softwood lignocellulose by poly(ethylene glycol) addition. Enzyme Microb. Technol., 40(4), 754–762.

Capanema, E., Balakshin, M., Katahira, R., Chang, H. and Jameel, H. (2014): How Well Do MWL and CEL Preparations Represent the Whole Hardwood Lignin? J. Wood Chem. Technol., 35(1), 17–26.

Cascao-Pereira, L., Kaper, T., Kelemen, B. R. and Liu, A. (2010): Compositions and methods comprising cellulase variants with reduced affinity to non-cellulosic materials. WO 2009149202 A3.

Chakar, F.S. and Ragauskas, A.J. (2004): Review of current and future softwood kraft lignin process chemistry. Ind. Crops Prod., 20(2), 131–141.

Chang, H., Cowling, E.B. and Brown, W. (1975): Comparative studies on cellulolytic enzyme lignin and milled wood lignin of sweetgum and spruce. Holzforschung, 29(5), 153–159.

Cheng, N., Yamamoto, Y., Koda, K., Tamai, Y. and Uraki, Y. (2014): Amphipathic lignin derivatives to accelerate simultaneous saccharification and fermentation of unbleached softwood pulp for bioethanol production. Bioresour. Technol., 173, 104–109.

Chernoglazov, V., Ermolova, O. and Klyosov, A. (1988): Adsorption of high-purity endo-1,4-beta-glucanases from Trichoderma reesei on components of lignocellulosic materials: cellulose, lignin, and xylan. Enzyme Microb. Technol., 10, 503–507.

Chundawat, S.P.S., Donohoe, B.S., da Costa Sousa, L., Elder, T., Agarwal, U.P., Lu, F., Ralph, J., Himmel, M.E., Balan, V. and Dale, B.E. (2011): Multi-scale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. Energy Environ. Sci., 4(3), 973.

Chundawat, S.P.S., Vismeh, R., Sharma, L. N., Humpula, J.F., da Costa Sousa, L., Chambliss, C. K., Jones, A.D., Balan, V. and Dale, B.E. (2010): Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. Bioresour. Technol., 101(21), 8429–8438.

da Costa Sousa, L., Foston, M., Bokade, V., Azarpira, A., Lu, F., Ragauskas, A.J., Ralph, J., Dale, B. and Balan, V. (2016): Isolation and characterization of new lignin streams derived from extractive-ammonia (EA) pretreatment. Green Chem., 18(15), 4205–4215.

Crestini, C., Melone, F., Sette, M. and Saladino, R. (2011): Milled wood lignin: A linear oligomer. Biomacromolecules, 12(11), 3928–3935.

Crestini, C., Lange, H., Sette, M. and Argyropoulos, D.S. (2017): on the Structure of Softwood Kraft Lignin, Green Chem., 19, 4104–4121.

Dimmel, D., Gellerstedt, G. (2010): Chemistry of Alkaline Pulping. In: Heitner, C., Dimmel, D., Schmidt, J.A. (Eds.), Lignin and Lignans. Advances in Chemistry. CRC Press, Boca Raton, FL, pp. 349–391.

Dimmel, D. (1985): Electron transfer reactions in pulping systems (I): Theory and applicability to anthraquinone pulping. J. Wood Chem. Technol. 5(1), 1–15.

Dimmel, D. and Schuller, L.F. (1986): Structural/Reactivity Studies (II): Reactions of Lignin Model Compounds with Pulping Additives. J. Wood Chem. Technol., 6(4), 565–590.

Dimmel, D. and Shepard, D. (1982): Regioselective Alkylation of Anthrahydroquinone and Anthrone in Water with Quinonemethides and Other Alkylating Agents. J, Org. Chem., 47(1),s 22–29.

Ding, S.Y., Liu, Y.S., Zeng, Y., Himmel, M.E., Baker, J.O. and Bayer, E.A. (2012): How does plant cell wall nanoscale architecture correlate with enzymatic digestibility? Science, 338(6110), 1055–1060.

Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E. and Vinzant, T.B. (2008): Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnol. Bioeng., 101(5), 913–925.

Dougherty, D. A. (1996): Cation-pi interactions in chemistry and biology: a new view of benzene, Phe, Tyr, and Trp. Science, 271(5246), 163–168.

Dutta, T., Isern, N.G., Sun, J., Wang, E., Hull, S., Cort, J.R., Simmons, B.A. and Singh, S. (2017): Survey of Lignin-Structure Changes and Depolymerization during Ionic Liquid Pretreatment. ACS Sustain. Chem. Eng. 10.1021/acssuschemeng.7b02123.

Dyall, L.K. and Winstein, S. (1972): Nuclear Magnetic Resonance Spectra and Characterization of Some Quinone Methides. J. Am. Chem. Soc., 94(7), 2196–2199.

Eckard, A., Muthukumarappan, K. and Gibbons, W. (2013): A Review of the Role of Amphiphiles in Biomass to Ethanol Conversion. Appl. Sci., 3(2), 396–419.

Elgharbawy, A.A., Alam, M.Z., Moniruzzaman, M. and Goto, M. (2016): Ionic liquid pretreatment as emerging approaches for enhanced enzymatic hydrolysis of lignocellulosic biomass. Biochem. Eng. J., 109, 252–267.

Eudes, A., Sathitsuksanoh, N., Baidoo, E.E.K., George, A., Liang, Y., Yang, F., Singh, S., Keasling, J.D., Simmons, B.A. and Loqué, D. (2015): Expression of a bacterial 3-dehydroshikimate dehydratase reduces lignin content and improves biomass saccharification efficiency. Plant Biotechnol. J., 13(9), 1241–1250.

Faix, O. (1991): Classification of Lignins from Different Botanical Origins by FT-IR Spectroscopy. Holzforschung, 45(s1), 21–28.

Fang, W. and Sixta, H. (2015): Advanced Biorefinery based on the Fractionation of Biomass in γ-Valerolactone and Water. ChemSusChem, 8, 73–76.

Fredheim, G.E., Braaten, S.M. and Christensen, B.E. (2002): Molecular weight determination of lignosulfonates by size-exclusion chromatography and multi-angle laser light scattering. J. Chromatogr. A., 942(1-2), 191-199.

Freudenberg, K. and Neish, A.C. (1968): Constitution and Biosynthesis of Lignin. Springer-Verlag Berlin Heidelberg.

Fritz, C., Ferrer, A., Salas, C., Jameel, H. and Rojas, O.J. (2015): Interactions between Cellulolytic Enzymes with Native, Autohydrolysis, and Technical Lignins and the Effect of a Polysorbate Amphiphile in Reducing Nonproductive Binding. Biomacromolecules, 16(12), 3878–3888.

Froass, P., Ragauskas, A. and Jiang, J. (1998): Nuclear magnetic resonance studies. 4. Analysis of residual lignin after kraft pulping. Ind. Eng. Chem. Res., 37(8), 3388–3394.

Fröhlich, H. (1975): The extraordinary dielectric properties of biological materials and the action of enzymes. Proc. Nat. Acad. Sci. USA, 72(11), 4211-4215.

Galbe, M. and Zacchi, G. (2012): Pretreatment: The key to efficient utilization of lignocellulosic materials. Biomass and Bioenergy, 46, 70–78.

Gao, D., Chundawat, S.P.S., Sethi, A., Balan, V., Gnanakaran, S. and Dale, B.E. (2013): Increased enzyme binding to substrate is not necessary for more efficient cellulose hydrolysis. Proc. Natl. Acad. Sci. U.S.A., 110(27), 10922–10927.

Gierer, J. (1980): Chemical aspects of kraft pulping. Wood Sci. Technol., 14(4), 241–266.

Gierer J. and Lindeberg, O. (1980): Reactions of Lignin during Sulfate Pulping. Part XIX.'Isolation and Identification of New Dimers from a Spent Sulfate Liquor. Acta Chem. Scand. B34, 161-170.

Gierer, J. (1985): Chemistry of delignification - Part 1: General concept and reactions during pulping. Wood Sci. Technol., 19(4), 289–312.

Gierer, J. and Wännström, S. (1984): Formation of alkali-stable C-C-bonds between lignin and carbohydrate fragments during kraft pulping. Holzforschung, 38(4), 181–184.

Grabber, J.H. (2005): How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci., 45(3), 820–831.

Grabber, J.H., Ralph, J. and Hatfield, R.D. (1997): p-Hydroxyphenyl , Guaiacyl , and Syringyl Lignins Have Similar Inhibitory Effects on Wall Degradability. J. Agric Food Chem, 45(7), 2530–2532.

Grande, P.M., Viell, J., Theyssen, N., Marquardt, W., Domínguez de María, P. and Leitner, W. (2015): Fractionation of lignocellulosic biomass using the OrganoCat process. Green Chem., 17(6), 3533–3539.

Guerra, A., Filpponen, I., Lucia, L.A. and Argyropoulos, D.S. (2006): Comparative evaluation of three lignin isolation protocols for various wood species. J. Agric. Food Chem., 54, 9696–9705.

Guo, F., Shi, W., Sun, W., Li, X., Wang, F., Zhao, J. and Qu, Y. (2014): Differences in the adsorption of enzymes onto lignins from diverse types of lignocellulosic biomass and the underlying mechanism. Biotechnol. Biofuels, 7(1), 38.

Haynes, C.A. and Norde, W. (1994): Globular proteins at solid-liquid interfaces. Colloids Surfaces B Biointerfaces, 2, 517–566.

Haynes, C.A. and Norde, W. (1995): Structures and Stabilities of Adsorbed Proteins. J. Colloid Interface Sci., 169(2), 313–328.

Hoeger, I.C., Filpponen, I., Martin-Sampedro, R., Johansson, L.S., Österberg, M., Laine, J., Kelley, S. and Rojas, O.J. (2012): Bicomponent lignocellulose thin films to study the role of surface lignin in cellulolytic reactions. Biomacromolecules, 13(10), 3228–3240.

Huang, C., He, J., Min, D., Lai, C. and Yong, Q. (2016): Understanding the Nonproductive Enzyme Adsorption and Physicochemical Properties of Residual Lignins in Moso Bamboo Pretreated with Sulfuric Acid and Kraft Pulping. Appl. Biochem. Biotechnol., 180(8) 1508–1523.

Humpula, J.F., Uppugundla, N., Vismeh, R., Sousa, L., Chundawat, S.P.S., Jones, A.D., Balan, V., Dale, B.E. and Cheh, A.M. (2014): Probing the nature of AFEX-pretreated corn stover derived decomposition products that inhibit cellulase activity. Bioresour. Technol., 152, 38–45.

Hunter, C.A. and Sanders, J.K.M. (1990): The nature of π-π interactions. J. Am. Chem. Soc., 112(14), 5525–5534.

Iiyama, K., Lam, T. and Stone, B.A. (1994): Covalent Cross-Links in the Cell Wall. Plant Physiol., 104(2), 315–320.

Jacquet, G., Pollet, B., Lapierre, C., Mhamdi, F. and Rolando, C. (1995): New Ether-Linked Ferulic Acid-Coniferyl Alcohol Dimers Identified in Grass Straws. J. Agric. Food Chem., 43, 2746–2751.

Jiang, B., Wang, W., Gu, F., Cao, T. and Jin, Y. (2016): Comparison of the substrate enzymatic digestibility and lignin structure of wheat straw stems and leaves pretreated by green liquor. Bioresour. Technol., 199, 181–187.

Jung, S., Foston, M., Sullards, M.C. and Ragauskas, A.J. (2010): Surface characterization of dilute acid pretreated populus deltoides by ToF-SIMS. Energy and Fuels, 24(2), 1347–1357.

Jönsson, L.J. and Martín, C. (2016): Pretreatment of lignocellulose: Formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol., 199, 103–112.

Kellock, M., Rahikainen, J., Marjamaa, K. and Kruus, K. (2017): Lignin-derived inhibition of monocomponent cellulases and a xylanase in the hydrolysis of lignocellulosics. Bioresour. Technol., 232, 183–191.

Kim, J.S., Lee, Y.Y. and Kim, T.H. (2016): A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. Bioresour. Technol., 199, 42–48.

Kim, J.Y., Shin, E.J., Eom, I.Y., Won, K., Kim, Y.H., Choi, D., Choi, I.G. and Choi, J.W. (2011): Structural features of lignin macromolecules extracted with ionic liquid from poplar wood. Bioresour. Technol., 102(19), 9020–9025.

Ko, J.K., Ximenes, E., Kim, Y. and Ladisch, M.R. (2015): Adsorption of enzyme onto lignins of liquid hot water pretreated hardwoods. Biotechnol. Bioeng., 112(3), 447–456.

Kraulis, P.J., Clare, G.M., Nilges, M., Jones, T.A., Pettersson, G., Knowles, J. and Gronenborn, A.M. (1989): Determination of the Three-Dimensional Solution Structure of the C-Terminal Domain of Cellobiohydrolase I from Trichoderma reesei. A Study Using Nuclear Magnetic Resonance and Hybrid Distance Geometry-Dynamical Simulated Annealing. Biochem. 28, 7241–7257.

Kubo, S. and Kadla, J.F. (2004): Poly(ethylene oxide)/organosolv lignin blends: Relationship between thermal properties, chemical structure, and blend behavior. Macromolecules, 37(18), 6904–6911.

Kumar, L., Arantes, V., Chandra, R. and Saddler, J. (2012): The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. Bioresour. Technol., 103(1), 201–8.

Kumar, L., Chandra, R. and Saddler, J. (2011): Influence of steam pretreatment severity on post-treatments used to enhance the enzymatic hydrolysis of pretreated softwoods at low enzyme loadings. Biotechnol. Bioeng., 108(10), 2300–2311.

Kumar, R., Hu, F., Sannigrahi, P., Jung, S., Ragauskas, A.J. and Wyman, C.E. (2013): Carbohydrate derived-pseudo-lignin can retard cellulose biological conversion. Biotechnol. Bioeng., 110(3), 737–753.

Lai, C., Tu, M., Shi, Z., Zheng, K., Olmos, L.G. and Yu, S. (2014): Contrasting effects of hardwood and softwood organosolv lignins on enzymatic hydrolysis of lignocellulose. Bioresour. Technol., 163, 320–327.

Lai, C., Tu, M., Yong, Q. and Yu, S. (2016): Disparate roles of solvent extractable lignin and residual bulk lignin in enzymatic hydrolysis of pretreated sweetgum. RSC Adv., 5, 97966–97974.

Lancefield, C.S., Panovic, I., Deuss, P.J., Barta, K. and Westwood, N.J. (2017): Pre-treatment of Lignocellulosic Feedstocks Using Biorenewable Alcohols: Towards Complete Biomass Valorisation. Green Chem., 19(1), 202–214.

Landucci, L. (1981): formation of carbon-linked anthrone-lignin and anthrahydroquinone-lignin adducts. J. Wood Chem. Technol. 1(1), 61–74.

Lange, H., Decina, S. and Crestini, C. (2013): Oxidative upgrade of lignin - Recent routes reviewed. Eur. Polym. J., 49(6), 1151–1173.

Lange, H., Rulli, F. and Crestini, C. (2016a): Gel Permeation Chromatography in Determining Molecular Weights of Lignins: Critical Aspects Revisited for Improved Utility in the Development of Novel Materials. ACS Sustain. Chem. Eng., 4(10), 5167–5180.

Lange, H., Schiffels, P., Sette, M., Sevastyanova, O. and Crestini, C. (2016b): Fractional Precipitation of Wheat Straw Organosolv Lignin –Macroscopic Properties and Structural Insights. ACS Sustain. Chem. Eng., 4(10), 5136–5151.

Lapierre, C. (2010): Determining lignin structure by chemical degradations. In: Heitner, C., Dimmel, D., Schmidt, J.A. (Eds.), Lignin and Lignans. Advances in Chemistry. CRC Press, Boca Raton, FL, pp. 11–48.

Lavigne, J., Scott, B., Whissel, M. and Tomashek, J. (2012): Family 6 cellulase with decreased inactivation by lignin. US8110389 B2.

Lê, H. Q., Ma, Y., Borrega, M. and Sixta, H. (2016): Wood biorefinery based on γ-valerolactone/water fractionation. Green Chem., 18, 5466–5476.

Lehmann, C., Bocola, M., Streit, W.R., Martinez, R. and Schwaneberg, U. (2014): Ionic liquid and deep eutectic solvent-activated CelA2 variants generated by directed evolution. Appl. Microbiol. Biotechnol., 98(12), 5775–5785.

Leikin, S., Rau, D.C. and Parsegian, V.A. (1994): Direct measurement of forces between self-assembled proteins: Temperature-dependent exponential forces between collagen triple helices. Proc. Natl. Acad. Sci. U.S.A. 72(11), 4211–4215.

Leskinen, T., Kelley, S.S. and Argyropoulos, D.S. (2015a): Determination of molecular weight distributions in native and pretreated wood. Carbohydr. Polym., 119, 44–52.

Leskinen, T., Kelley, S.S. and Argyropoulos, D.S. (2017): E-beam irradiation & steam explosion as biomass pretreatment, and the complex role of lignin in substrate recalcitrance. Biomass and Bioenergy, 103, 21–28.

Leskinen, T., King, A.W. and Argyropoulos, D.S. (2014): Fractionation of lignocellulosic materials with ionic liquids. Prod. Biofuels Chem. with Ion. Liq., Zhen Fang, J. Richard L. Smith, and Q. Xinhua, eds., Springer; Netherlands, 145–168.

Leskinen, T., Salas, C., Kelley, S.S. and Argyropoulos, D.S. (2015b): Wood Extractives Promote Cellulase Activity on Cellulosic Substrates. Biomacromolecules, 16(10), 3226–3234.

Li, H., Pu, Y., Kumar, R., Ragauskas, A.J. and Wyman, C.E. (2014a): Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose hydrolysis, and underlying mechanisms. Biotechnol. Bioeng., 111(3), 485–492.

Li, J., Henriksson, G. and Gellerstedt, G. (2007): Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. Bioresour. Technol., 98(16), 3061–3068.

Li, Y., Qi, B., Luo, J. and Wan, Y. (2016): Effect of alkali lignins with different molecular weights from alkali pretreated rice straw hydrolyzate on enzymatic hydrolysis. Bioresour. Technol., 200, 272–278.

Li, Y., Qi, B. and Wan, Y. (2014b): Inhibitory effect of vanillin on cellulase activity in hydrolysis of cellulosic biomass. Bioresour. Technol., 167, 324–330.

Lievonen, M., Valle-Delgado, J.J., Mattinen, M.-L., Hult, E.-L., Lintinen, K., Kostiainen, M., Paananen, A., Szilvay, G.R., Setälä, H. and Österberg, M. (2015): Simple process for lignin nanoparticle preparation. Green Chem., 18, 1416–1422.

Lin, X., Qiu, X., Yuan, L., Li, Z., Lou, H., Zhou, M. and Yang, D. (2015): Lignin-based polyoxyethylene ether enhanced enzymatic hydrolysis of lignocelluloses by dispersing cellulase aggregates. Bioresour. Technol., 185, 165–170.

Liu, H., Sun, J., Leu, S.-Y. and Chen, S. (2016): Toward a fundamental understanding of cellulase-lignin interactions in the whole slurry enzymatic saccharification process. Biofuels, Bioprod. Biorefining, 10(5), 648–663.

Liu, H., Zhu, J.Y. and Fu, S.Y. (2010): Effects of lignin-metal complexation on enzymatic hydrolysis of cellulose. J. Agric. Food Chem., 58(12), 7233–7238.

Loow, Y., New, E.K., Yang, G.H., Ang, L.Y., Foo, L.Y.W. and Wu, T.Y. (2017): Potential use of deep eutectic solvents to facilitate lignocellulosic biomass utilization and conversion. Cellulose, 24(9), 3591–3618.

Lou, H., Zhu, J. Y., Lan, T. Q., Lai, H. and Qiu, X. (2013): PH-induced lignin surface modification to reduce nonspecific cellulase binding and enhance enzymatic saccharification of lignocelluloses. ChemSusChem, 6(5), 919–927.

Lundquist, K. and Ericsson, L. (1970): Acid Degradation of lignin III. Formation of formaldehyde. Acta Chem. Scand., 24, 3681–3686.

Lundquist, K. and Lundgren, R. (1972): Acid Degradation of lignin Part VII. The Cleavage of ether bonds. Acta Chem. Scand., 26, 2005–2023.

Lundquist, K. and Parkås, J. (2011): Different types of phenolic units in lignins. BioResources, 6(2), 920–926.

Luterbacher, J.S., Rand, J.M., Alonso, D.M., Han, J., Youngquist, J.T., Maravelias, C.T., Pfleger, B.F. and Dumesic, J.A. (2014): Nonenzymatic Sugar Production from Biomass Using Biomass-Derived γ-Valerolactone. Science 343(6168), 277–280.

Ma, H.H., Zhang, B.X., Zhang, P., Li, S., Gao, Y.F. and Hu, X.M. (2016): An efficient process for lignin extraction and enzymatic hydrolysis of corn stalk by pyrrolidonium ionic liquids. Fuel Process. Technol., 148, 138–145.

Majtnerová, A. and Gellerstedt, G. (2006): Radical coupling - A major obstacle to delignification in kraft pulping. Nord. Pulp Pap. Res. J., 21(1), 129–134.

Martín-Sampedro, R., Rahikainen, J.L., Johansson, L.S., Marjamaa, K., Laine, J., Kruus, K. and Rojas, O.J. (2013): Preferential adsorption and activity of monocomponent cellulases on lignocellulose thin films with varying lignin content. Biomacromolecules, 14(4), 1231–1239.

McGuire, J., Wahlgren, M.C. and Arnebrant, T. (1995): Structural Stability Effects on the Adsorption and Dodecyltrimethylammonium Bromide-Mediated Elutability of Bacteriophage T4 Lysozyme at Silica Surfaces. J. Colloid Interface Sci., 170(1), 182–192.

Mellmer, M. a., Martin Alonso, D., Luterbacher, J.S., Gallo, J.M.R. and Dumesic, J.A. (2014a): Effects of γ-valerolactone in hydrolysis of lignocellulosic biomass to monosaccharides. Green Chem., 16(11), 4659–4662.

Mellmer, M.A., Sener, C., Gallo, J.M.R., Luterbacher, J.S., Alonso, D. M. and Dumesic, J.A. (2014b): Solvent effects in acid-catalyzed biomass conversion reactions. Angew. Chemie - Int. Ed., 53(44), 11872–11875.

Müller, G., Várnai, A., Johansen, K.S., Eijsink, V.G.H. and Horn, S.J. (2015): Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. Biotechnol. Biofuels, 8(1), 187.

Nakagame, S., Chandra, R.P., Kadla, J.F. and Saddler, J.N. (2011a): Enhancing the enzymatic hydrolysis of lignocellulosic biomass by increasing the carboxylic acid content of the associated lignin. Biotechnol. Bioeng., 108(3), 538–548.

Nakagame, S., Chandra, R.P., Kadla, J.F. and Saddler, J.N. (2011b): The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. Bioresour. Technol., 102(6), 4507–17.

Narron, R.H., Kim, H., Chang, H.M., Jameel, H. and Park, S. (2016): Biomass pretreatments capable of enabling lignin valorization in a biorefinery process. Curr. Opin. Biotechnol., 38, 39–46.

Norde, W. (1996): Driving forces for protein adsorption at solid surfaces. Macromol. Symp., 103(1), 5–18.

Nordwald, E.M., Brunecky, R., Himmel, M.E., Beckham, G.T. and Kaar, J.L. (2014): Charge engineering of cellulases improves ionic liquid tolerance and reduces lignin inhibition. Biotechnol. Bioeng., 111(8), 1541–1549.

Palmqvist, E. and Hahn-Hägerdal, B. (2000): Fermentation of lignocellulosic hydrolysates. I: Inhibition and detoxification. Bioresour. Technol., 74(1), 17–24.

Palonen, H., Tjerneld, F., Zacchi, G. and Tenkanen, M. (2004): Adsorption of Trichoderma reesei CBH I and EG II and their catalytic domains on steam pretreated softwood and isolated lignin. J. Biotechnol., 107(1), 65–72.

Pan, X. (2008): Role of fuctional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose. J. biobased Mater. bioenergy, 2, 25–32.

Pareek, N., Gillgren, T. and Jönsson, L.J. (2013): Adsorption of proteins involved in hydrolysis of lignocellulose on lignins and hemicelluloses. Bioresour. Technol., 148, 70–77.

Penna, M.J., Mijajlovic, M. and Biggs, M.J. (2014): Molecular-level understanding of protein adsorption at the interface between water and a strongly interacting uncharged solid surface. J. Am. Chem. Soc., 136(14), 5323–5331.

Pepper, J.M., Baylis, P.E.T. and Adler, E. (1959): The isolation and properties of lignins obtained by the acidolysis of spruce and aspen woods in dioxane-water medium. Can. J. Chem. 37, 1241–1248.

Pielhop, T., Larrazábal, G.O. and Rudolf von Rohr, P. (2016): Autohydrolysis pretreatment of softwood – enhancement by phenolic additives and the effects of other compounds. Green Chem., 18, 5239–5247.

Pihlajaniemi, V., Sipponen, M.H., Kallioinen, A., Nyyssölä, A. and Laakso, S. (2016a): Rate-constraining changes in surface properties, porosity and hydrolysis kinetics of lignocellulose in the course of enzymatic saccharification. Biotechnol. Biofuels, 9(1), 18.

Pihlajaniemi, V., Sipponen, M.H., Liimatainen, H., Sirviö, J.A., Nyyssölä, A. and Laakso, S. (2016b): Weighing the factors behind enzymatic hydrolyzability of pretreated lignocellulose. Green Chem., 18, 1295–1305.

Pihlajaniemi, V., Sipponen, M.H., Pastinen, O., Nyyssölä, A. and Laakso, S. (2016c): The effect of direct and counter-current flow-through delignification on enzymatic hydrolysis of wheat straw, and flow limits due to compressibility. Biotechnol. Bioeng., 113(12), 2605–2613.

Procentese, A., Johnson, E., Orr, V., Garruto Campanile, A., Wood, J. A., Marzocchella, A. and Rehmann, L. (2015): Deep eutectic solvent pretreatment and subsequent saccharification of corncob. Bioresour. Technol., 192, 31–36.

Qin, L., Li, W.-C., Liu, L., Zhu, J.-Q., Li, X., Li, B.-Z. and Yuan, Y.-J. (2016): Inhibition of lignin-derived phenolic compounds to cellulase. Biotechnol. Biofuels, 9(1), 70.

Qing, Q., Zhou, L., Guo, Q., Huang, M., He, Y., Wang, L. and Zhang, Y. (2016): A combined sodium phosphate and sodium sulfide pretreatment for enhanced enzymatic digestibility and delignification of corn stover. Bioresour. Technol., 218, 209–216.

Ragauskas, A.J., Beckham, G.T., Biddy, M.J., Chandra, R., Chen, F., Davis, M.F., Davison, B.H., Dixon, R.A., Gilna, P., Keller, M., Langan, P., Naskar, A.K., Saddler, J.N., Tschaplinski, T.J., Tuskan, G.A. and Wyman, C.E. (2014): Lignin valorization: improving lignin processing in the biorefinery. Science, 344(6185), 1246843.

Rahikainen, J. (2013): Cellulase-lignin interactions in the enzymatic hydrolysis of lignocellulose. VTT Science 41.

Rahikainen, J.L., Evans, J.D., Mikander, S., Kalliola, A., Puranen, T., Tamminen, T., Marjamaa, K. and Kruus, K. (2013a): Cellulase-lignin interactions-the role of carbohydrate-binding module and pH in non-productive binding. Enzyme Microb. Technol., 53(5), 315–21.

Rahikainen, J.L., Martin-Sampedro, R., Heikkinen, H., Rovio, S., Marjamaa, K., Tamminen, T., Rojas, O.J. and Kruus, K. (2013b): Inhibitory effect of lignin during cellulose bioconversion: The effect of lignin chemistry on non-productive enzyme adsorption. Bioresour. Technol., 133, 270–278.

Rahikainen, J.L., Moilanen, U., Nurmi-Rantala, S., Lappas, A., Koivula, A., Viikari, L. and Kruus, K. (2013c): Effect of temperature on lignin-derived inhibition studied with three structurally different cellobiohydrolases. Bioresour. Technol., 146, 118–25.

Rahikainen, J., Mikander, S., Marjamaa, K., Tamminen, T., Lappas, A., Viikari, L. and Kruus, K. (2011): Inhibition of enzymatic hydrolysis by residual lignins from softwood-study of enzyme binding and inactivation on lignin-rich surface. Biotechnol. Bioeng., 108(12), 2823–2834.

Ralph, J. and Adams, B.R. (1983): Determination of the Conformation and Isomeric Composition of Lignin Model Quinone Methides By NMR. J. Wood Chem. Technol., 3(2), 183-194.

Rasmussen, H., Tanner, D., Sorensen, H.R. and Meyer, A.S. (2017): New degradation compounds from lignocellulosic biomass pretreatment: Routes for formation of potent oligophenolic enzyme inhibitors. Green Chem., 19(1), 464–473.

Richel, A. Vanderghem, C., Simon, M., Wathelet, B and Paquot, M. (2012): Evaluation of Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry for Second-Generation Lignin Analysis. Anal. Chem. Insights, 7, 79-89.

Rinaldi, R., Jastrzebski, R., Clough, M.T., Ralph, J., Kennema, M., Bruijnincx, P.C.A. and Weckhuysen, B.M. (2016): Paving the Way for Lignin Valorisation: Recent Advances in Bioengineering, Biorefining and Catalysis. Angew. Chemie Int. Ed., 55(29), 8164–8215.

Rodham, D.A., Suzuki, S., Suenram, R.D., Lovas, F.J., Dasgupta, S., Goddard III, W.A. and Blake, G.A. (1993): Hydrogen bonding in the benzene-ammonia dimer. Nature, 362, 735–737.

Rodrigues, A.C., Leitão, A.F., Moreira, S., Felby, C. and Gama, M. (2012): Recycling of cellulases in lignocellulosic hydrolysates using alkaline elution. Bioresour. Technol., 110, 526–533.

Rodríguez-Zúñiga, U.F., Cannella, D., Giordano, R. de C., Giordano, R. de L. C., Jørgensen, H. and Felby, C. (2015): Lignocellulose pretreatment technologies affect the level of enzymatic cellulose oxidation by LPMO. Green Chem., 17(5), 2896–2903.

Sadeghifar, H. and Argyropoulos, D.S. (2015): Correlations of the antioxidant properties of softwood kraft lignin fractions with the thermal stability of its blends with polyethylene. ACS Sustain. Chem. Eng., 3(2), 349–356.

Saini, J.K., Patel, A.K., Adsul, M. and Singhania, R.R. (2016): Cellulase adsorption on lignin: A roadblock for economic hydrolysis of biomass. Renew. Energy, 98, 29–42.

Salanti, A., Zoia, L., Tolppa, E. and Orlandi, M. (2012): Chromatographic Detection of Lignin−Carbohydrate Complexes in Annual Plants by Derivatization in Ionic Liquid. Biomacromolecules, 13(2), 445-454.

Salas, C., Rojas, O. J., Lucia, L. a, Hubbe, M. a and Genzer, J. (2013): On the surface interactions of proteins with lignin. ACS Appl. Mater. Interfaces, 5(1), 199–206.

Sammond, D.W., Yarbrough, J.M., Mansfield, E., Bomble, Y.J., Hobdey, S.E., Decker, S.R., Taylor, L.E., Resch, M.G., Bozell, J.J., Himmel, M.E., Vinzant, T.B. and Crowley, M.F. (2014): Predicting enzyme adsorption to lignin films by calculating enzyme surface hydrophobicity. J. Biol. Chem., 289(30), 20960–20969.

Sannigrahi, P., Kim, D.H., Jung, S. and Ragauskas, A. (2011): Pseudo-lignin and pretreatment chemistry. Energy Environ. Sci., 4(4), 1306.

Sathitsuksanoh, N., Holtman, K.M., Yelle, D.J., Morgan, T., Stavila, V., Pelton, J., Blanch, H., Simmons, B.A. and George, A. (2014): Lignin fate and characterization during ionic liquid biomass pretreatment for renewable chemicals and fuels production. Green Chem., 16(3), 1236.

Scheller, H.V. and Ulvskov, P. (2010): Hemicelluloses. Annu. Rev. Plant Biol., 61(1), 263–289.

Scott, B. R., StPierre, P., Lavigne, J., Masri, N., White, T. C. and Tomashek, J. J. (2010): Novel lignin-resistant cellulase enzymes. EP 2401370 A1.

Selig, M.J., Viamajala, S., Decker, S.R., Tucker, M.P., Himmel, M.E. and Vinzant, T.B. (2007): Deposition of lignin droplets produced during dilute acid pretreatment of maize stems retards enzymatic hydrolysis of cellulose. Biotechnol. Prog., 23(6), 1333–1339.

Sevastyanova, O., Helander, M., Chowdhury, S., Lange, H., Wedin, H., Zhang, L., Ek, M., Kadla, J.F., Crestini, C. and Lindström, M.E. (2014): Tailoring the molecular and thermo-mechanical properties of kraft lignin by ultrafiltration. J. Appl. Polym. Sci., 131(18), 9505–9515.

Sewalt, V.J.H., Glasser, W.G. and Beauchemin, K.A. (1997): Lignin Impact on Fiber Degradation . 3 . Reversal of Inhibition of Enzymatic Hydrolysis by Chemical Modification of Lignin and by additives. J. Agric. Food Chem., 45, 1823–1828.

Shen, X.J., Wang, B., Huang, P.L., Wen, J.L. and Sun, R.C. (2016): Effects of aluminum chloride-catalyzed hydrothermal pretreatment on the structural characteristics of lignin and enzymatic hydrolysis. Bioresour. Technol., 206, 57–64.

Shuai, L., Amiri, M.T., Questell-santiago, Y.M., Héroguel, F., Li, Y., Kim, H., Meilan, R., Chapple, C., Ralph, J. and Luterbacher, J.S. (2016a): Formaldehyde stabilization facilitates lignin monomer production during biomass depolymerization. Science, 354(6310), 1–6.

Shuai, L., Questell-Santiago, Y.M. and Luterbacher, J.S. (2016b): A mild biomass pretreatment using γ-valerolactone for concentrated sugar production. Green Chem., 18, 937–943.

Sipponen, M.H. (2015): Effect of lignin structure on enzymatic hydrolysis of plant residues. Aalto University.

Sipponen, M.H., Pihlajaniemi, V., Littunen, K., Pastinen, O. and Laakso, S. (2014a): Determination of surface-accessible acidic hydroxyls and surface area of lignin by cationic dye adsorption. Bioresour. Technol., 169, 80–87.

Sipponen, M.H., Pihlajaniemi, V., Pastinen, O. and Laakso, S. (2014b): Reduction of surface area of lignin improves enzymatic hydrolysis of cellulose from hydrothermally pretreated wheat straw. Rsc Adv., 4(69), 36591–36596.

Sipponen, M.H., Rojas, O.J., Pihlajaniemi, V., Lintinen, K.S. and Österberg, M. (2016): Calcium chelation of lignin from pulping spent liquor for water-resistant slow-release urea fertilizer systems. ACS Sustain. Chem. Eng., 5(1), 1054–1061.

Siqueira, G., Várnai, A., Ferraz, A. and Milagres, A.M.F. (2013): Enhancement of cellulose hydrolysis in sugarcane bagasse by the selective removal of lignin with sodium chlorite. Appl. Energy, 102, 399–402.

Sjöholm, E., Gustafsson, K. and Colmsjö, A. (1999): Size exclusion chromatography of Lignins using lithium chloride/N,N-dimethylacetamide as mobile phase. II. Dissolved and residual pine kraft lignins. J. Liq. Chrom. & Rel. Technol., 22(18), 2837–2854.

Smith, M.D., Mostofian, B., Cheng, X., Petridis, L., Cai, C.M., Wyman, C.E. and Smith, J.C. (2016): Cosolvent pretreatment in cellulosic biofuel production: effect of tetrahydrofuran-water on lignin structure and dynamics. Green Chem., 18(5), 1268–1277.

vom Stein, T., Grande, P.M., Kayser, H., Sibilla, F., Leitner, W. and Domínguez de María, P. (2011): From biomass to feedstock: one-step fractionation of lignocellulose components by the selective organic acid-catalyzed depolymerization of hemicellulose in a biphasic system. Green Chem., 13(7), 1772.

Strobel, K.L., Pfeiffer, K.A., Blanch, H.W. and Clark, D.S. (2015): Structural insights into the affinity of Cel7A carbohydrate-binding module for lignin. J. Biol. Chem., 290(37), 22818–22826.

Strobel, K.L., Pfeiffer, K.A., Blanch, H.W. and Clark, D.S. (2016): Engineering Cel7A carbohydrate binding module and linker for reduced lignin inhibition. Biotechnol. Bioeng., 113(6), 1369–1374.

Suckling, I.D. (1988): enhanced cleavage of b-aryl ether bonds in lignin model compounds during sulphite-anthraquinone pulping. J. Wood Chem. Technol., 8(1), 43-71.

Sulaeva, I. Sumerskii, I., Bacher, M., Zinovyev, G., Henniges, U., Rosenau, T. and Potthast, A. (2015): Comparing different approaches to measure molar mass of lignin: SEC, DOSY and AsFlFFF. 249th American Chemical Society National Meeting & Exposition, Denver, CO, March 22, 2015.

Sulaeva, I., Zinovyev, G., Plankeele, J., Sumerskii, I., Rosenau, T. and Potthast, A. (2017): Fast Track to Molar-Mass Distributions of Technical Lignins. ChemSusChem, 10(3), 629-635.

Sun, S., Huang, Y., Sun, R. and Tu, M. (2016): The strong association of condensed phenolic moieties in isolated lignins with their inhibition of enzymatic hydrolysis. Green Chem., 18, 4276–4286.

Suzuki, S., Green, P.G., Bumgarner, R.E., Dasgupta, S., Goddard III, W.A. and Blake, G.A. (1992): Benzene forms hydrogen bonds with water. Science, 257(5072), 942–945.

Tadesse, H. and Luque, R. (2011): Advances on biomass pretreatment using ionic liquids: An overview. Energy Environ. Sci., 4(10), 3913.

Torr, K.M., Love, K.T., Çetinkol, Ö.P., Donaldson, L.A., George, A., Holmes, B.M. and Simmons, B.A. (2012): The impact of ionic liquid pretreatment on the chemistry and enzymatic digestibility of Pinus radiata compression wood. Green Chem., 14(3), 778.

Torr, K.M., Love, K.T., Simmons, B.A. and Hill, S.J. (2016): Structural features affecting the enzymatic digestibility of pine wood pretreated with ionic liquids. Biotechnol. Bioeng., 113(3), 540–549.

Varanasi, P., Singh, P., Arora, R., Adams, P.D., Auer, M., Simmons, B.A. and Singh, S. (2012): Understanding changes in lignin of Panicum virgatum and Eucalyptus globulus as a function of ionic liquid pretreatment. Bioresour. Technol., 126, 156–161.

Várnai, A., Siika-Aho, M. and Viikari, L. (2013): Carbohydrate-binding modules (CBMs) revisited: reduced amount of water counterbalances the need for CBMs. Biotechnol. Biofuels, 6(1), 30.

Vermaas, J.V., Petridis, L., Qi, X., Schulz, R., Lindner, B. and Smith, J. C. (2015): Mechanism of Lignin Inhibition of Enzymatic Biomass Deconstruction. Biotechnol. Biofuels, (2015), 1–16.

Viikari, L., Alapuranen, M., Puranen, T., Vehmaanperä, J. and Siika-Aho, M. (2007): Thermostable enzymes in lignocellulose hydrolysis. Adv. Biochem. Eng. Biotechnol., 108, 121–45.

Vogler, E.A. (2012): Protein adsorption in three dimensions. Biomaterials, 33(5), 1201–1237.

Wahlstrom, R.M. and Suurnakki, A. (2015): Enzymatic hydrolysis of lignocellulosic polysaccharides in the presence of ionic liquids. Green Chem., 17(2), 694–714.

Wahlström, R., Hiltunen, J., Pitaluga de Souza Nascente Sirkka, M., Vuoti, S. and Kruus, K. (2016): Comparison of three deep eutectic solvents and 1-ethyl-3-methylimidazolium acetate in the pretreatment of lignocellulose: effect on enzyme stability, lignocellulose digestibility and one-pot hydrolysis. RSC Adv., 6(72), 68100–68110.

Wallace, J., Brienzo, M., García-Aparicio, M.P. and Görgens, J.F. (2016): Lignin enrichment and enzyme deactivation as the root cause of enzymatic hydrolysis slowdown of steam pretreated sugarcane bagasse. New Biotechnol., 33(3), 361–371.

Wang, D. and Wang, M. (2013): Anion-π interactions: Generality, Binding Strength, and Structure. J. Am. Chem. Soc. 135(2), 892–897.

Watanabe, T., Ohnishi, J., Yamasaki, Y., Kaizu, S. and Koshijima, T. (1989): Binding-site analysis of the ether linkages between lignin and hemicelluloses in lignin-carbohydrate complexes by DDQ-oxidation. Agric. Biol. Chem., 53(8), 2233–2252.

Wayman, M. and Lora, J.H. (1978): Aspen autohydrolysis: The effects of 2-naphthol and other aromatic compounds. Tappi, 61(6), 55–57.

Wen, J.L., Sun, S.L., Yuan, T.Q., Xu, F. and Sun, R.C. (2014): Understanding the chemical and structural transformations of lignin macromolecule during torrefaction. Appl. Energy, 121, 1–9.

Wilkerson, C.G., Mansfield, S.D., Lu, F., Withers, S., Park, J.-Y., Karlen, S.D., Gonzales-Vigil, E., Padmakshan, D., Unda, F., Rencoret, J. and Ralph, J. (2014): Monolignol ferulate transferase introduces chemically labile linkages into the lignin backbone. Science, 344(6179), 90–3.

Wu, Z. and Lee, Y.Y. (1997): Ammonia recycled percolation as a complementary pretreatment to the dilute-acid process. Appl. Biochem. Biotechnol., 63–65, 21–34.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R. and Lee, Y.Y. (2005): Coordinated development of leading biomass pretreatment technologies. Bioresour. Technol. 96(18), 1959–1966.

Xia, S., Baker, G.A, Li, H., Ravula, S. and Zhao, H. (2014): Aqueous Ionic Liquids and Deep Eutectic Solvents for Cellulosic Biomass Pretreatment and Saccharification. RSC Adv., 4(21), 10586–10596.

Ximenes, E., Kim, Y., Mosier, N., Dien, B. and Ladisch, M. (2010): Inhibition of cellulases by phenols. Enzyme Microb. Technol., 46, 170–176.

Yamaguchi, A., Isozaki, K., Nakamura, M., Takaya, H. and Watanabe, T. (2016): Discovery of 12-mer peptides that bind to wood lignin. Sci. Rep., 6, 21833.

Yang, H., Xie, Y., Zheng, X., Pu, Y., Huang, F., Meng, X., Wu, W., Ragauskas, A. and Yao, L. (2016): Comparative study of lignin characteristics from wheat straw obtained by soda-AQ and kraft pretreatment and effect on the following enzymatic hydrolysis process. Bioresour. Technol., 207, 361–369.

Yang, Q. and Pan, X. (2016): Correlation between lignin physicochemical properties and inhibition to enzymatic hydrolysis of cellulose. Biotechnol. Bioeng., 113(6), 1213–1224.

Yoo, C.G., Li, M., Meng, X., Pu, Y. and Ragauskas, A.J. (2017): Effects of organosolv and ammonia pretreatments on lignin properties and its inhibition for enzymatic hydrolysis. Green Chem., 19, 2006–2016.

Yu, Z., Gwak, K.S., Treasure, T., Jameel, H., Chang, H.M. and Park, S. (2014): Effect of lignin chemistry on the enzymatic hydrolysis of woody biomass. ChemSusChem, 7(7), 1942–1950.

Yuan, T.Q., Sun, S. N., Xu, F. and Sun, R.C. (2011): Characterization of lignin structures and lignin-carbohydrate complex (LCC) linkages by quantitative 13C and 2D HSQC NMR spectroscopy. J. Agric. Food Chem., 59(19), 10604–10614.

Van Zandvoort, I., Wang, Y., Rasrendra, C.B., Van Eck, E.R.H., Bruijnincx, P.C.A., Heeres, H.J. and Weckhuysen, B.M. (2013): Formation, molecular structure, and morphology of humins in biomass conversion: Influence of feedstock and processing conditions. ChemSusChem, 6(9), 1745–1758.

Zakzeski, J., Bruijnincx, P.C.A., Jongerius, A.L. and Weckhuysen, B.M. (2010): The Catalytic Valorization of Lignin for the Production of Renewable Chemicals.Chem. Rev., 110(6), 3552–3599.

Zeng, Y., Zhao, S., Yang, S. and Ding, S.Y. (2014): Lignin plays a negative role in the biochemical process for producing lignocellulosic biofuels. Curr. Opin. Biotechnol., 27, 98–45.

Zhang, L., Zhang, L., Zhou, T., Wu, Y. and Xu, F. (2016): The dual effects of lignin content on enzymatic hydrolysis using film composed of cellulose and lignin as a structure model. Bioresour. Technol., 200, 761–769.

Zhou, H., Lou, H., Yang, D., Zhu, J.Y. and Qiu, X. (2013a): Lignosulfonate to enhance enzymatic saccharification of lignocelluloses: Role of molecular weight and substrate lignin. Ind. Eng. Chem. Res., 52(25), 8464–8470.

Zhou, H., Zhang, R., Zhan, W., Wang, L., Guo, L. and Liu, Y. (2016): High biomass loadings of 40 wt% for efficient fractionation in biorefineries with an aqueous solvent system without adding adscititious catalyst. Green Chem., 18, 6108–6114.

Zhou, H., Zhu, J.Y., Luo, X., Leu, S., Wu, X., Gleisner, R., Dien, B.S., Hector, R.E., Yang, D., Qiu, X., Horn, E. and Negron, J. (2013b): Bioconversion of Beetle-Killed Lodgepole Pine Using SPORL: Process Scale-up Design, Lignin Coproduct, and High Solids Fermentation without Detoxification. Ind. Eng. Chem. Res., 52(45), 16057–16065.

Zoia, L., King, A.W.T. and Argyropoulos, D.S. (2011): Molecular Weight Distributions and Linkages in Lignocellulosic Materials Derivatized from Ionic Liquid Media. J. Agric. Food Chem., 59(3), 829–838.

Zong, Z., He, R., Fu, H., Zhao, T., Chen, S., Shao, X., Zhang, D. and Cai, W. (2016): Pretreating cellulases with hydrophobins for improving bioconversion of cellulose: an experimental and computational study. Green Chem., 18, 6666–6674.

Manuscript received July 1, 2017
Accepted November 11, 2017

Appendix

Table 4 Structural changes from industrial and emerging pretreatments on lignin and their effects on enzymatic saccharification of the pretreated solid fraction.

Pretreatment	Lignin content	Condensed linkages	Phenolic OH	Aliphatic OH	COOH
DA and acid-catalyzed steam-EX	↑[1–8]	↑[4–10]	↑[4,6,7,9,10]	↓[4,6,7,9,10]	↑[4,10] ↔[7]
Autohydrolysis and autocatalytic steam-EX	↑[11–19] ↓[20,21]	↑[11,14,17,19,20,22–26]	↑[11,14,20,23–25]	↓[11,20,24,25,27]	↑[11,19,23–25]
Acid sulphite (SPORL)	↓[5,28–32]	↑[5]	↑*a (ether cleavage)*	↓*a* (sulfonation)	↑[19,33]
Ethanol-water organosolv	↓[34–37]	↑[37–40] ↔[35]	↑[38–41]	↓[37–41]	↑[37–41]
Organic acid organosolv	↓[42–44]	↑[43,45] ↔[44]	↑[43–45]	↓[43–45]	↑[44] ↓[43]
Aqueous alkali pretreatment	↓[16,46–51]	↑[52,53] ↓[51,54]	↑[52,54]	↓[52–54]	↑[54]
Ammonia fiber expansion (AFEX)	↔[8,55–58]	↓[8]	↔*a*	↔*a*	↔*a*
Ammonia recycled percolation (ARP)	↓[46,55,59–61]	↑[61]	↑*a*	↓*a*	↑*a*
Extractive ammonia (EA)	↓[62,63]	↔[62,63]	↔[62,63]	↔[62,63]	↑[62]
Ionic liquid (IL: [Emim][OAc])	↑[64] ↓[8,65–67]	↑[64,66,68] ↔[65]	↑[66,68]	↓[68]	↔[68]
Deep eutectic solvents (DESs)	↑[69] ↓[70]	?	?	?	?
Gamma-valerolactone (GVL)	↓[71–74]	?	↑*72b*	↓*72b*, [73]	?
Tetrahydrofuran (THF)	↓[74–78]	?	↑*a*	?	?
2-MTHF/oxalic acid	↓[79,80]	?	↑*a*	↓*a*	?

a: Symbols without reference indicate the view of the authors of this review. *b*: Compared to the values of MWL in ref. [81] *c*: In the presence of NaOH.

1. **B. Yang and C. E. Wyman,** *Biotechnol. Bioeng.*, 2006, **94**, 611–617.

2. **J. B. Kristensen, L. G. Thygesen, C. Felby, H. Jørgensen and T. Elder,** *Biotechnol. Biofuels*, 2008, **1**, 5.

3. **P. Sannigrahi, D. H. Kim, S. Jung and A. Ragauskas**, *Energy Environ. Sci.*, 2011, **4**, 1306.

4. **R. Samuel, Y. Pu, B. Raman and A. J. Ragauskas,** *Appl. Biochem. Biotechnol.*, 2010, **162**, 62–74.

5. **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.

6. **P. Sannigrahi, A. J. Ragauskas and S. J. Miller**, *BioEnergy Res.*, 2008, **1**, 205–214.

7. **S. Cao, Y. Pu, M. Studer, C. Wyman and A. J. Ragauskas**, *RSC Adv.*, 2012, **2**, 10925–10936.

8. **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,** *Front. Energy Res.*, 2015, **2**, 1–12.

9. **Y. Li, Z. Sun, X. Ge and J. Zhang**, *Biotechnol. Biofuels*, 2016, **9**, 20.

10. **C. Huang, J. He, D. Min, C. Lai and Q. Yong,** *Appl. Biochem. Biotechnol.*, 2016.

11. **J. Li, G. Gellerstedt and K. Toven,** *Bioresour. Technol.*, 2009, **100**, 2556–2561.

12. **U. Holopainen-Mantila, K. Marjamaa, Z. Merali, A. Käsper, P. de Bot, A. S. Jääskeläinen, K. Waldron, K. Kruus and T. Tamminen,** *Bioresour. Technol.*, 2013, **138**, 156–162.

13. **M. H. Sipponen, V. Pihlajaniemi, O. Pastinen and S. Laakso**, *Rsc Adv.*, 2014, **4**, 36591–36596.

14. **F. Araya, E. Troncoso, R. T. Mendonça and J. Freer,** *Biotechnol. Bioeng.*, 2015, **112**, 1783–1791.

15. **V. Pihlajaniemi, M. H. Sipponen, O. Pastinen, I. Lehtomäki and S. Laakso,** *Green Chem.*, 2015, **17**, 1683–1691.

16. **V. Pihlajaniemi, M. H. Sipponen, H. Liimatainen, J. A. Sirviö, A. Nyyssölä and S. Laakso**, *Green Chem.*, 2016, **18**, 1295–1305.

17. **T. Pielhop, G. O. Larrazábal and P. Rudolf von Rohr,** *Green Chem.*, 2016.

18. **M. Monschein and B. Nidetzky,** *Bioresour. Technol.*, 2016, **200**, 287–296.

19. **R. P. Chandra, Q. Chu, J. Hu, N. Zhong, M. Lin, J. S. Lee and J. Saddler,** *Bioresour. Technol.*, 2016, **199**, 135–141.

20. **J. Li, G. Henriksson and G. Gellerstedt**, *Bioresour. Technol.*, 2007, **98**, 3061–3068.

21. **W. Jin, L. Chen, M. Hu, D. Sun, A. Li, Y. Li, Z. Hu, S. Zhou, Y. Tu, T. Xia, Y. Wang, G. Xie, Y. Li, B. Bai and L. Peng,** *Appl. Energy*, 2016, **175**, 82–90.

22. **M. Wayman and J. H. Lora,** *Tappi*, 1978, **61**, 55–57.

23. **M. Tanahashi**, *Wood Res.*, 1990, **77**, 49–117.

24. **J. Li and G. Gellerstedt,** *Ind. Crops Prod.*, 2008, **27**, 175–181.

25. **R. Samuel, S. Cao, B. K. Das, F. Hu, Y. Pu and A. J. Ragauskas,** *RSC Adv.*, 2013, **3**, 5305.

26. **M. H. Sipponen,** Aalto University, 2015.

27. **Z. Yu, K. S. Gwak, T. Treasure, H. Jameel, H. M. Chang and S. Park,** *ChemSusChem*, 2014, **7**, 1942–1950.

28. **J. Y. Zhu, X. J. Pan, G. S. Wang and R. Gleisner,** *Biotechnol. Prog.*, 2009, **25**, 1086–1093.

29. **J. Y. Zhu, X. J. Pan, G. S. Wang and R. Gleisner,** *Bioresour. Technol.*, 2009, **100**, 2411–2418.

30. **X. Li, X. Luo, K. Li, J. Y. Zhu, J. D. Fougere and K. Clarke,** *Appl. Biochem. Biotechnol.*, 2012, **168**, 1556–1567.

31. **L. Yang, J. Cao, J. Mao and Y. Jin,** *Ind. Crops Prod.*, 2013, **43**, 711–717.

32. **L. Bu, Y. Xing, H. Yu, Y. Gao and J. Jiang**, *Biotechnol. Biofuels*, 2012, **5**, 87.

33. **L. Tan, W. Sun, X. Li, J. Zhao, Y. Qu, Y. M. Choo and S. K. Loh,** *Biotechnol. J.*, 2015, **10**, 915–925.

34. **C. S. Lancefield, I. Panovic, P. J. Deuss, K. Barta and N. J. Westwood,** *Green Chem.*, 2017, **19**, 202–214.

35. **S. Bauer, H. Sorek, V. D. Mitchell, A. B. Ibáñez and D. E. Wemmer,** *J. Agric. Food Chem.*, 2012, **60**, 8203–8212.

36. **S. K. Jang, H. Y. Kim, H. S. Jeong, J. Y. Kim, H. Yeo and I. G. Choi,** *Renew. Energy*, 2016, **87**, 599–606.

37. **M. Yáñez-S, B. Matsuhiro, C. Nuñez, S. Pan, C. A. Hubbell, P. Sannigrahi and A. J. Ragauskas,** *Polym. Degrad. Stab.*, 2014, **110**, 184–194.

38. **R. El Hage, N. Brosse, P. Sannigrahi and A. Ragauskas,** *Polym. Degrad. Stab.*, 2010, **95**, 997–1003.

39. **R. El Hage, N. Brosse, L. Chrusciel, C. Sanchez, P. Sannigrahi and A. Ragauskas,** *Polym. Degrad. Stab.*, 2009, **94**, 1632–1638.

40. **P. Sannigrahi, A. J. Ragauskas and S. J. Miller,** *Energy and Fuels*, 2010, **24**, 683–689.

41. **A. Berlin and M. Balakshin,** *Industrial Lignins: Analysis, Properties, and Applications*, Elsevier, 2014.

42. **H. Chen, J. Zhao, T. Hu, X. Zhao and D. Liu,** *Appl. Energy*, 2015, **150**, 224–232.

' **52**, 15702–15712.

55. **R. Kumar, G. Mago, V. Balan and C. E. Wyman,** *Bioresour. Technol.*, 2009, **100**, 3948–3962.

56. **S. P. S. Chundawat, B. S. Donohoe, L. da Costa Sousa, T. Elder, U. P. Agarwal, F. Lu, J. Ralph, M. E. Himmel, V. Balan and B. E. Dale,** *Energy Environ. Sci.*, 2011, **4**, 973.

57. **D. Gao, C. Haarmeyer, V. Balan, T. a Whitehead, B. E. Dale and S. P. Chundawat,** *Biotechnol. Biofuels*, 2014, **7**, 175.

58. **J. A. Perez-Pimienta, C. A. Flores-Gómez, H. A. Ruiz, N. Sathitsuksanoh, V. Balan, L. da Costa Sousa, B. E. Dale, S. Singh and B. A. Simmons,** *Bioresour. Technol.*, 2016, **211**, 216–223.

59. **T. H. Kim, J. S. Kim, C. Sunwoo and Y. Y. Lee,** *Bioresour. Technol.*, 2003, **90**, 39–47.

60. **T. H. Kim and Y. Y. Lee,** *Bioresour. Technol.*, 2005, **96**, 2007–2013.

61. **F. P. Bouxin, S. David Jackson and M. C. Jarvis**, *Bioresour. Technol.*, 2014, **162**, 236–242.

62. **L. da Costa Sousa, M. Foston, V. Bokade, A. Azarpira, F. Lu, A. J. Ragauskas, J. Ralph, B. Dale and V. Balan,** *Green Chem.*, 2016, **18**, 4205–4215.

63. **L. da Costa Sousa, M. Jin, S. P. S. Chundawat, V. Bokade, X. Tang, A. Azarpira, F. Lu, U. Avci, J. Humpula, N. Uppugundla, C. Gunawan, S. Pattathil, A. M. Cheh, N. Kothari, R. Kumar, J. Ralph, M. G. Hahn, C. E. Wyman, S. Singh, B. A. Simmons, B. E. Dale and V. Balan,** *Energy Environ. Sci.*, 2016, **9**, 1215–1223.

64. **K. M. Torr, K. T. Love, Ö. P. Çetinkol, L. A. Donaldson, A. George, B. M. Holmes and B. A. Simmons,** *Green Chem.*, 2012, **14**, 778.

65. **N. Sathitsuksanoh, K. M. Holtman, D. J. Yelle, T. Morgan, V. Stavila, J. Pelton, H. Blanch, B. a. Simmons and A. George,** *Green Chem.*, 2014, **16**, 1236.

66. **J. Y. Kim, E. J. Shin, I. Y.** Eom, K. Won, Y. H. Kim, D. Choi, I. G. Choi and J. W. Choi, *Bioresour. Technol.*, 2011, **102**, 9020–9025.

67. **P. Varanasi, P. Singh, R. Arora, P. D. Adams, M. Auer, B. A. Simmons and S. Singh,** *Bioresour. Technol.*, 2012, **126**, 156–161.

68. **J. L. Wen, S. L. Sun, T. Q. Yuan, F. Xu and R. C. Sun,** *Appl. Energy*, 2014, **121**, 1–9.

69. **G. C. Xu, J. C. Ding, R. Z. Han, J. J. Dong and Y. Ni,** *Bioresour. Technol.*, 2016, **203**, 364–369.

70. **A. Procentese, E. Johnson, V. Orr, A. Garruto Campanile, J. A. Wood, A. Marzocchella and L. Rehmann,** *Bioresour. Technol.*, 2015, **192**, 31–36.

71. **W. Fang and H. Sixta,** *ChemSusChem*, 2015, **8**, 73–76.

72. **H. Q. Lê, Y. Ma, M. Borrega and H. Sixta,** *Green Chem.*, 2016, **DOI: 10.10**.

73. **H. Zhou, R. Zhang, W. Zhan, L. Wang, L. Guo and Y. Liu,** *Green Chem.*, 2016, **DOI: 10.10**.

74. **L. Shuai, Y. M. Questell-Santiago and J. S. Luterbacher,** *Green Chem.*, 2016, **18**, 937–943.

75. **C. M. Cai, T. Zhang, R. Kumar and C. E. Wyman,** *Green Chem.*, 2013, **15**, 3140.

76. **T. Y. Nguyen, C. M. Cai, R. Kumar and C. E. Wyman,** *ChemSusChem*, 2015, **8**, 1716–1725.

77. **T. Y. Nguyen, C. M.-Z. Cai, O. Osman, R. Kumar and C. E. Wyman,** *Green Chem.*, 2016, **18**, 1581–1589.

78. **M. D. Smith, B. Mostofian, X. Cheng, L. Petridis, C. M. Cai, C. E. Wyman and J. C. Smith,** *Green Chem.*, 2016, **18**, 1268–1277.

79. **T. vom Stein, P. M. Grande, H. Kayser, F. Sibilla, W. Leitner and P. Domínguez de María,** *Green Chem.*, 2011, **13**, 1772.

80. **P. M. Grande, J. Viell, N. Theyssen, W. Marquardt, P. Domínguez de María and W. Leitner,** *Green Chem.*, 2015, **17**, 3533–3539.

81. **E. A. Capanema, M. Y. Balakshin and J. F. Kadla,** *J. Agric. Food Chem.*, 2005, **53**, 9639–9649.