Recalcitrance of Wood to Biochemical Conversion - Feedstock Properties, Pretreatment, Saccharification, and Fermentability

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Logic will get you from A to B. Imagination will take you everywhere.

– Albert Einstein

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Abstract

Lignocellulose is an inexpensive and abundant renewable resource that can be used to produce advanced biofuels, green chemicals, and other bio-based products. Pretreatment and efficient enzymatic saccharification are essential features of bioconversion of lignocellulosic biomass. The aims of the research were to achieve a better understanding of the recalcitrance of woody biomass to bioconversion, to explore different pretreatment techniques that can be used to decrease the recalcitrance of the biomass and improve the digestibility of the cellulose, and to investigate by-products of acid pretreatment that cause enzymes and microorganisms to work less efficiently.

The recalcitrance of wood from aspen, birch, and spruce was investigated before and after acid pretreatment. Before pretreatment, birch exhibited the highest recalcitrance, which was attributed to structural factors. After pretreatment, spruce showed the highest recalcitrance, which was attributed to chemical factors, such as high lignin content. Deacetylation of hybrid aspen *in planta* by a CE5 acetyl xylan esterase decreased the recalcitrance, and the glucose yield of enzymatic saccharification of non-pretreated wood increased with 27%.

Pretreatment options based on ionic liquids and steam explosion were further explored. The effects of the anionic constituents of a series of imidazolium-based ionic liquids on pretreatment of aspen and spruce were investigated. $[HSO_4]^-$ was efficient only for aspen, which was attributed to acid degradation of xylan. $[MeCO_2]^-$ was efficient for both aspen and spruce, which was attributed to its capability to create a disordered cell wall structure rather than to removal of lignin and hemicellulose. A comparison was made between using sulfuric acid and sulfur dioxide for pretreatment of spruce. Although sulfur dioxide resulted in a pretreatment liquid that was more inhibitory to both enzymes and yeast, it was still superior to pretreatment with sulfuric acid, a phenomenon that was attributed to the particle size of the pretreated material.

In a comparison of microbial inhibitors in pretreatment liquids from steam explosion of spruce, formaldehyde was found to be the most important inhibitor of yeast. Enzyme inhibition by catalytically non-productive adsorption to lignins and pseudo-lignin was investigated using quantitative proteomics. The results indicate that protein adsorption to pseudo-lignin can be as extensive as adsorption to real lignin.

List of Abbreviations

ASL	Acid-soluble lignin
BET	Brunauer–Emmett–Teller
CBHs	Cellobiohydrolases
CBM	Carbohydrate-binding module
CMC	Carboxy-methyl cellulose
Conc.	Concentration
CrI	Crystallinity index
DAD	Diode array detector
DAP	Dilute-acid pretreatment
DB	Direct Blue dye
DNS	Dinitrosalicylic acid
DO	Direct Orange dye
DP	Degree of polymerization
EGs	Endoglucanases
EnzHR	Enzymatic hydrolysis residue
FPU	Filter paper unit
FTIR	Fourier-transform infra-red spectroscopy
HMF	5-Hydroxymethylfurfural
HMW	High molecular weight
HPAEC	High-performance anion-exchange chromatography
HPLC	High-performance liquid chromatography
HTP	Hydrothermal pretreatment
ILs	Ionic liquids
LPMOs	Lytic polysaccharide monooxygenases
NMR	Nuclear magnetic resonance spectroscopy
Py-GC/MS	Pyrolysis-gas chromatography/mass spectrometry
RID	Refractive index detector
SEM	Scanning electron microscopy
S:G ratio	Syringyl:guaiacyl ratio
wt	Weight
XRD	X-ray diffraction

List of Publications

Paper I

Wang Z, Winestrand S, Gillgren T and Jönsson LJ (2018). Chemical and structural factors influencing enzymatic saccharification of wood from aspen, birch and spruce. *Biomass Bioenerg.* 109, 125-34.

Paper II

Wang Z[†], Pawar PM[†], Derba-Maceluch M, Hedenström M, Chong S-L, Tenkanen M, Mellerowicz E and Jönsson LJ. Bioprocessing properties of hybrid aspen expressing a Carbohydrate Esterase Family 5 acetyl xylan esterase under control of a wood-specific promoter. Manuscript.

Paper III

Wang Z, Gräsvik J, Jönsson LJ and Winestrand S (2017). Comparison of $[HSO_4]^-$, $[Cl]^-$ and $[MeCO_2]^-$ as anions in the pretreatment of aspen and spruce with imidazolium-based ionic liquid. *BMC Biotechnol.* 17, 82.

Paper IV

Wang Z, Wu G and Jönsson LJ (2018). Effects of impregnation of softwood with sulfuric acid and sulfur dioxide on chemical and physical characteristics, enzymatic digestibility, and fermentability. *Bioresour. Technol.* 247, 200–8.

Paper V

Martín C, Wu G, Wang Z, Stagge S, Jönsson LJ. Formation of microbial inhibitors in steam-explosion pretreatment of softwood impregnated with sulfuric acid and sulfur dioxide. Manuscript.

Paper VI

Wang Z and Jönsson LJ. Comparison of catalytically non-productive adsorption of fungal proteins to lignins and pseudo-lignin using isobaric mass tagging. Manuscript.

[†]Joint first authors

Enkel sammanfattning på svenska

Lignocellulosa från skogs- och jordbruk kan användas för framställning av avancerade biodrivmedel, gröna kemikalier och biobaserade material. En ökad användning av lignocellulosa kan minska beroendet av råolja och ge positiva miljöeffekter.

Lignocellulosa består huvudsakligen av cellulosa, hemicellulosa och lignin. Cellulosan och hemicellulosan kan brytas ned till socker, som kan utgöra ett mellanled i produktionen av gröna kemikalier och biodrivmedel. Ett av de mest studerade sätten att använda ved och andra former av lignocellulosa är att göra en termokemisk förbehandling, vanligtvis inom intervallet 120°C-210°C, följt av en enzymatisk försockring av cellulosan. Lignocellulosa från olika växter uppvisar olika mottaglighet mot förbehandling och enzymatisk försockring, men orsaken till det har varit dåligt känd.

Ved från asp, björk och gran undersöktes med avseende på mottagligheten för förbehandling och enzymatisk försockring. Både kemiska och strukturella faktorer befanns vara väsentliga för hur mottagliga olika sorters ved är för enzymatisk försockring. Vidare har aspar som uttrycker höga halter av enzymet acetylxylanesteras undersökts. Acetylxylanesteraset minskade aspvedens acetyleringsgrad med i genomsnitt 13%, vilket resulterade i att veden blev lättare att försockra med hjälp av enzymer och i minskad bildning av ättiksyra, vilket är en fördel i samband med mikrobiell förjäsning av frigjorda sockerarter.

Möjligheten att använda joniska vätskor för förbehandling av ved har också studerats och speciellt hur anjonen hos den joniska vätskan påverkar förbehandlingens effektivitet. En annan förbehandlingsmetod som studerades var ångexplosion med tillsats av svavelsyra eller svaveldioxid. Resultaten visar tydligt orsakerna till varför svaveldioxid fungerar bättre vid förbehandling av barrved än vad svavelsyra gör.

I samband med ångexplosionen bildas även substanser som hämmar de mikroorganismer som används i påföljande förjäsningssteg. Av de substanser som studerades var formaldehyd det viktigaste hämmande ämnet, något som förbisetts i tidigare forskning inom området. Även substanser som hämmar de enzymer som används vid försockring av cellulosa studerades och speciellt hur enzymernas katalytiska aktivitet störs av lignin och pseudo-lignin, något som tidigare varit dåligt känt. Pseudo-lignin uppvisar vissa ytliga likheter med lignin, men bildas från kolhydrater i samband med förbehandlingen. Kunskaper om de bakomliggande orsakerna till att processer fungerar mer eller mindre bra kan användas för att utveckla nya tekniska lösningar och mer effektiva konverteringsmetoder.

1. Biorefining

The global energy supply is facing major challenges in coming decades. The population of the world has been predicted to increase from the current level of around 7.6 billion to 12.3 billion in 2100 (Gerland et al., 2014). The demand for energy in industry, households, and transportation sector is set to increase. In 2015, the world energy consumption was 575 quadrillion British thermal units (Btu) and it has been predicted to increase with 28% to 736 quadrillion Btu in 2040 (IEA, 2017). With petroleum and coal still being the main sources of energy, increased emissions of greenhouse gases could lead to anthropogenic climate change.

IEA (2017) has predicted that renewable energy will be a fast-growing energy source and provide around 125 quadrillion Btu in 2040. Sustainable production of liquid biofuels from renewable feedstocks can contribute to environmentally friendly energy consumption. Liquid biofuels can be produced through biorefining of biomass. Feedstocks for biorefining could be lignocellulosic residues from agriculture and forestry, algae, and organic waste (Dahlquist, 2013).

Multiple products can be produced from the different constituents of lignocellulosic biomass (Cherubini, 2010). This is the purpose of biorefining. Analogous to a traditional oil refinery, a biorefinery refers to a facility that utilizes biochemical and/or thermochemical processes to produce fuels, chemicals, and materials from biomass (Cherubini, 2010). Thermochemical processes include combustion, pyrolysis, hydrothermal liquefaction, and gasification. In biochemical processes, enzymes and/or microorganisms are utilized in some of the conversion steps. A biorefinery could produce a mix of biofuels, green chemicals, bioplastics, and energy (Cherubini, 2010). Examples of current industrial wood biorefineries include Domsjö Fabriker in Örnsköldsvik in northern Sweden and Borregaard in Sarpsborg in south-east Norway.

2. Lignocellulosic feedstocks

Lignocellulosic feedstocks for biorefining include residues from agriculture and forestry. With regard to forest trees, the most abundant part of the woody tissue is the so-called secondary xylem. Woody materials can generally be divided into two groups, softwood and hardwood. The designations come from the medieval timber trade, where the wood from gymnosperms, which has a uniform texture, was considered easy to work with and was named softwood (Philipson et al., 1971). Wood from dicotyledons, which have more varied cell types, was considered to be difficult to work with and was named hardwood (Philipson et al., 1971). These terms are still used today. However, the terms could be regarded as misnomers as a softwood (e.g. yew wood) can be harder than a hardwood (e.g. balsa wood), and vice versa (Butterfield and Meylan, 1980).

Wood cells usually have an elongated structure that extends along the stem of the tree. Depending on the orientation of the cells, they can be longitudinal, i.e. oriented along the stem, or transverse, i.e. oriented across the stem. Most cell types, including tracheids, vessel elements, and fibers, that run along the stem can easily be identified after cutting in the transverse direction. Some parenchyma cells, such as rays spreading across the stem, are easy to identify after cutting in the longitudinal direction.

Softwood usually has a more homogeneous structure than hardwood, as the main (90-95%) cell type in softwood is the tracheid, which has a function with respect to both physical support and water transportation (Butterfield and Meylan, 1980; Sjöström et al., 1993). Some thin-walled parenchyma cells are found in the transverse direction.

As it contains a larger variety of cell types, hardwood exhibits a more complex structure than softwood. Vessels, which consist of several vessel elements joined together, have a function in water transportation, and long thick-walled fibers provide physical support (Butterfield and Meylan, 1980). In a broad sense, the term fiber refers to all kind of wood cells, but in a narrow sense it refers to support cells in hardwood. Both libriform cells and fiber tracheids are hardwood support cells that, in a narrow sense, can be referred to as fibers (Sjöström et al., 1993). Depending on species and age, the length and the cell wall thickness varies for different types of fibers, and the diameters of lumina vary with the vessels. Axial (vertical) parenchyma and ray (radial) parenchyma cells are abundant in hardwood and occupy a large volume. Fig. 1 shows some of the different wood cells in species of softwood (Norway spruce) and hardwood (hybrid aspen).

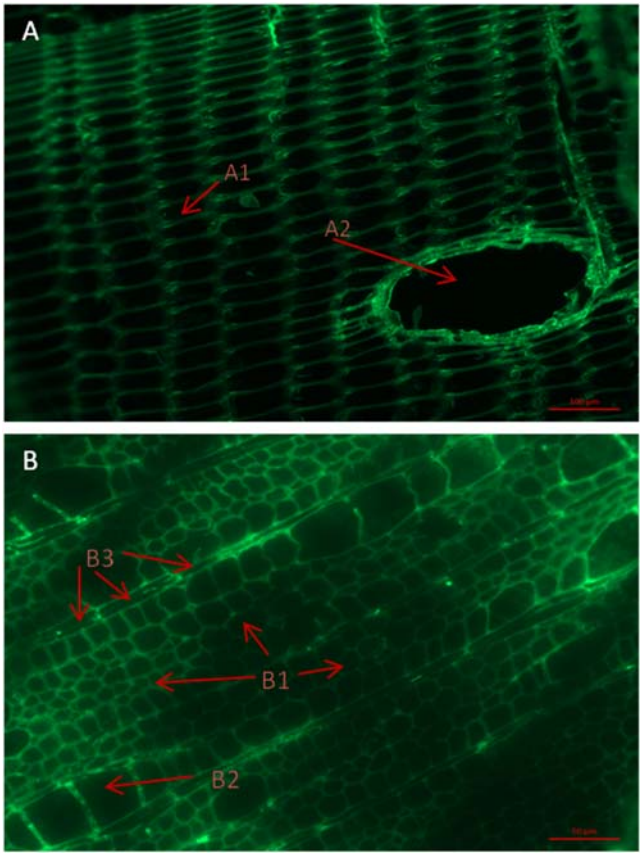


Fig. 1. Fluorescence microscopy showing lignin autofluorescence and examples of different types of wood cells (transverse surface) in softwood (A, red bar 100 μm) and hardwood (B, red bar 50 μm). Softwood (A) has a more homogeneous structure and most of the cells are tracheids (A1). A resin channel is also visible (A2). Hardwood (B) has a more heterogeneous distribution of cell types. The image shows fibers (B1) of varied shape and size, a vessel with intervessel plates between several vessel elements (B2), and ray parenchyma cells (B3).

2.1 Wood and cell wall structure

Despite the differences with respect to the types of wood cells, the general structure of the cell wall is the same for both softwood and hardwood. Cell walls typically consist of a multilayer structure, which can be divided into the primary wall and the secondary wall on basis of the time of formation (Fig. 2A). The primary wall is assembled first, and, in some cells, it could be the only wood layer (e.g. in some ray parenchyma cells). The secondary wall is typically much thicker than the primary wall. The secondary cell wall can be further divided into sub-layers S1 (outer), S2 (middle), and S3 (inner), in order from the primary wall to the lumen (Fig. 2A) (Mellerowicz and Gorshkova, 2012).

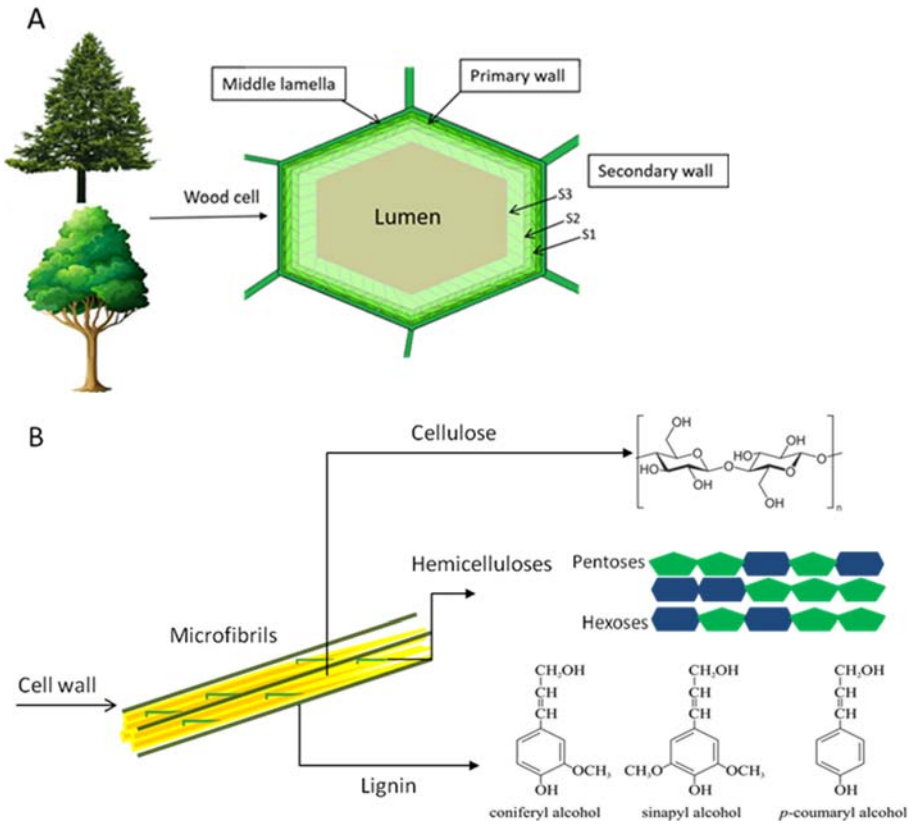


Fig. 2 Multilayer structure of wood cell walls (A), and major constituents of wood (B).

Apart from water, the cell wall consists of three major constituents, namely cellulose, hemicelluloses, and lignin (Fig. 2B). In addition there are minor constituents, such as wood extractives and inorganic material (sometimes referred to as ash). The cellulose is usually formed as long microfibrils that are closely associated with hemicelluloses and lignin in a matrix structure. For each layer of the cell wall, the direction of the microfibrils and the chemical composition can be different (Butterfield and Meylan, 1980). The structure of the primary wall is characterized by irregularly arranged microfibrils. The primary wall is rich in lignin and contains relatively little cellulose and hemicellulose. In the secondary wall, the S1 and S3 sub-layers are thin and are built up by microfibrils oriented in nearly transverse direction. S2 is the thickest sub-layer, and the microfibrils in S2 run in a small angle to the longitudinal direction. In the direction from S1 to S3, the contents of cellulose and hemicelluloses increase and the content of lignin decreases (McMillan, 1994; Agarwal, 2006). Besides the common multilayer structure of the cell wall, some wood cells can have a warty layer that covers the S3 layer towards the lumen. The warty layer is believed to have a high content of lignin, amorphous

cellulose, and hemicellulose (Agarwal, 2006). Outside the primary wall, the middle lamella joins the individual wood cells together. The middle lamella has a high content of lignin, low contents of cellulose and hemicelluloses, and a high content of pectin (Agarwal, 2006).

2.2 Cellulose

Cellulose, which accounts for 40-45% of the dry mass of wood, is the most abundant organic polymer in nature (Klemm, 2005; Sjöström et al., 1993). It is a homo-polysaccharide that consists of β-D-glucopyranosyl units linked together by β-1,4 glucosidic bonds (Fig. 2B) (Sjöström et al., 1993). Every second glucopyranosyl ring is rotated 180° in the plane to accommodate the preferred bond angles of the acetal oxygen bridges (Klemm, 2005). Native cellulose has a linear structure and consists of long chains (Sjöström et al., 1993). The degree of polymerization (DP), which is used to define the length of cellulose chains, refers to the (average) number of glucopyranosyl units in the chains. The DP value differs depending on plant species and preparation methods (Klemm, 2005). In cotton and plant fibers, the DP value can reach 10 000, and for wood pulp the values are in the range of 300-1 700 (Klemm, 2005; Jørgensen et al., 2007).

With intra- and inter-molecular hydrogen bonds, cellulose molecules are aggregated together in microfibrils and exhibit high chain stiffness and poor solubility in most solvents (Jørgensen et al, 2007; Hu and Ragauskas, 2012). Cellulose has both highly ordered (crystalline) regions and disordered (amorphous) regions that together form a fringed fibrillar structure (Fink and Philipp, 1985). Native cellulose (Cellulose I) has two co-existing crystalline allomorphs, Iα and Iβ, that can exist not only in the same cellulose sample but also alongside each other in the mircrofibrils (Atalla and Vanderhart, 1984; Sugiyama et al., 1991a). Studies with NMR spectroscopy and X-ray diffraction (XRD) have determined that Iα has triclinic unit cells and Iβ has monoclinic unit cells (Sugiyama et al., 1991a; Finkenstadt and Millane, 1998). Iα can be converted to Iβ by annealing (Horii et al., 1987a). The relative proportions of cellulose Iα and Iβ differ depending on the biological origins of the cellulose. For example, the cell walls of algae and some bacterial cellulose have more Iα, whereas cotton, wood, and ramie fibers have more Iβ (Horii et al., 1987b; Sugiyama et al., 1991b).

2.3 Hemicelluloses

Hemicelluloses are heterogeneous polysaccharides that account for 20-30% of the dry mass of wood (Sjöström et al., 1993). Hemicelluloses have a branched structure with a DP value that is typically below 200 (Sjöström et al., 1993; Jørgensen et al., 2007). The constituents of hemicelluloses are different monosaccharide units, such as D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, and L-rhamnose, as well as some uronic acids (Fig. 2B) (Sjöström et

al., 1993). Hemicelluloses are classified according to the main sugar unit in the backbone of the polymer, e.g. as xylan (β-1,4-linked xylose residues) or as mannan (β-1,4-linked mannose residues). Hemicelluloses are often acetylated (Hu and Ragauskas, 2012). In xylan, 40-70% of the xylopyranosyl units can be acetylated (Pawar et al., 2013). There are considerable differences with respect to the chemical composition, such as the ratio of different monosaccharide units, of hemicelluloses from different types of lignocellulosic biomass (Sjöström et al., 1993). In softwoods, such as pine and spruce, galacto-glucomannans and arabino-glucuronoxylans are the most abundant and second most abundant types of hemicellulose (Willför et al., 2005a). However, 4-O-methyl-glucuronoxylans and glucomannans are the most abundant and second most abundant types of hemicellulose in hardwoods, such as aspen, birch, beech, and oak (Willför et al., 2005b).

2.4 Lignin

Lignin is an amorphous, complex and cross-linked aromatic biopolymer that is polymerized from 4-hydroxyphenylpropanoid units (Halpin, 2004; Ralph et al., 2004). The three phenylpropanoids that predominantly contribute to the polymerization of lignin are the monolignols *p*-coumaryl alcohol (precursor of *p*-hydroxyphenyl or H units), coniferyl alcohol (precursor of guaiacyl or G units), and sinapyl alcohol (precursor of syringyl or S units) (Fig. 2B) (Ragauskas, 2013). The initial step of lignin polymerization is an enzyme-catalyzed oxidation that produces monolignol free radicals. The radicals subsequently couple to each other and generate dilignols and further on protolignin (Ragauskas, 2013). However, dimerization of monolignol radicals and cross-coupling to protolignin is not well understood (Hatfield and Vermerris, 2001; Boerjan et al., 2003; Bonawitz et al., 2014).

The abundance of the lignin units varies depending on the plant species. Generally, both hardwood and softwood contain only small fractions of H units. Softwood lignin predominantly consists of G units (Ragauskas, 2013). Hardwood lignin is composed of considerable proportions of both S and G units, but the S:G ratio depends on the wood species and can vary also in different trees from the same species. The different distribution of S and G units in, for example, birch also depends on the cell type (e.g. fiber or vessel) and the spatial position in the cell wall (e.g. if it is the S2 sub-layer or the middle lamella) (Saka and Goring, 1988).

The most common inter-unit linkage of lignin is the β-O-4 (β-aryl ether) linkage, which is also the most easy to cleave (Ragauskas, 2013). Other inter-unit linkages, such as β-5, β-β, 5-5, 4-O-5, and β-1, are more resistant to degradation (Boerjan et al., 2003). The proportions of the different inter-unit linkages of lignin depend on the biological origins of the wood. Softwood lignin,

which has more G units, has more β-5, 5-5, and 4-O-5 linkages than lignins with more S units, such as hardwood lignin (Boerjan et al., 2003; Ralph et al., 2004).

2.5 Extractives and ash

Besides the three main constituents, wood also consists of a large number of low-molecular-weight substances that can be extracted using water or organic solvents. These substances, which are known as extractives, generally account for less than 10% of the dry-weight of wood (Sjöström et al., 1993). The composition of the extractives is highly dependent on the wood species, and even differs in different parts of the same tree, e.g. branches, stem, bark, needles, and roots (Sjöström et al., 1993).

Extractives can be divided into three main groups, viz. (*i*) terpenoids and steroids, (*ii*) fats and waxes, and (*iii*) phenolic constituents (e.g. stilbenes, lignans, flavonoids and tannins)(Sjöström, 1993). Terpenoids and steroids are located in the resin canals of both hardwood and softwood and they protect the wood from attacks by microbes and insects. Fats are common in the parenchyma cells of softwood and hardwood. Fats and waxes are involved in the energy metabolism of wood cells, and are also utilized commercially (tall oil biodiesel, soft soap). A large variety of phenolic constituents are found in the heartwood and in the bark. They have fungicidal properties and protect the trees from microbiological attack.

Wood also contains a small fraction of inorganic constituents that are classified as ash (Sjöström, 1993). The ash content of wood is normally less than 1% (dry wt). However, the fraction of ash is different for different parts of the tree. The ash content in leaves, needles, and bark can be much higher than that of wood. Ash includes anions, such as carbonates, silicates, and phosphates, and cations, such as calcium, potassium, magnesium, iron, and manganese.

3. Bioconversion of lignocellulose

Bioconversion of lignocellulose refers to a process for production of advanced biofuels and bio-based chemicals and materials in which lignocellulosic materials are used as the feedstock and enzymes and/or microorganisms are involved in some of the process steps. Feedstocks based on starch or sucrose can also be used to produce biofuels and other bio-based chemicals, but lignocellulosic biomass is more recalcitrant to bioconversion. As lignocellulosic feedstocks are resistant to conversion by enzymatic saccharification and fermentation, it is essential to include a pretreatment step prior to enzymatic saccharification (Galbe and Zacchi, 2012; Limayem and Ricke, 2012). A schematic bioconversion process is shown in Fig. 3. It includes mechanical size fractionation, thermochemical pretreatment, enzymatic saccharification, and microbial fermentation. There are two main processing strategies, namely separate hydrolysis and fermentation (SHF) and

simultaneous saccharification and fermentation (SSF). Consolidated bioprocessing (CBP) of lignocellulose to bioethanol is gaining more attention. CBP refers to a process where production and secretion of saccharolytic enzymes, hydrolysis of hemicellulose and cellulose, and fermentation of hexose sugars and pentose sugars are combined in one reactor (van Zyl et al., 2007).

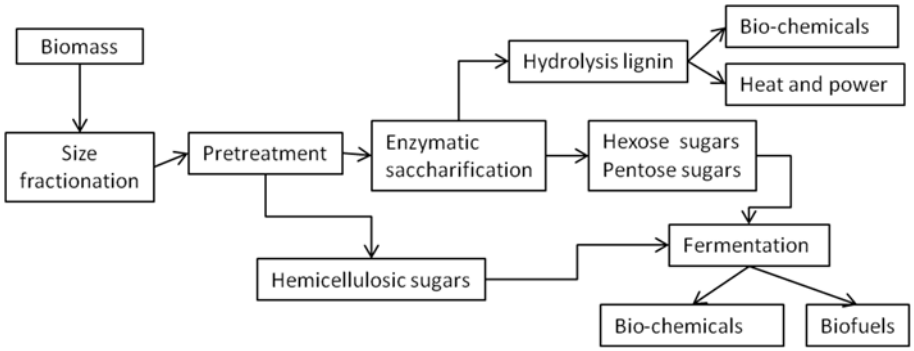


Fig. 3 Bioconversion of woody biomass.

3.1 Pretreatment

After the mechanical process (cutting, chipping, and milling), the lignocellulose is typically pretreated through a thermochemical pretreatment that targets the hemicellulose or the lignin and improves the accessibility of the cellulose to cellulolytic enzymes. Depending on the chemical used and the intended target, pretreatment can be divided into five main types. These include mild alkaline pretreatment, chemical pulping processes, oxidative pretreatment, acidic hydrothermal pretreatment, and pretreatment with alternative solvents (Jönsson and Martin, 2016).

Mild alkaline pretreatment is typically used to remove lignin, and it also causes some solubilization of hemicelluloses (Jönsson and Martin, 2016). The most commonly used types of alkali are sodium hydroxide and potassium hydroxide (Jönsson and Martin, 2016). Alkaline solutions with a certain temperature can break the bonds between lignin and polysaccharides and partly dissolve the lignin (Galbe and Zacchi, 2012). It is considered suitable mainly for lignocellulosic materials that have relatively low recalcitrance, such as agricultural residues and herbaceous crops (Galbe and Zacchi, 2012).

Chemical pulping aims at solubilizing the lignin and to some extent the hemicelluloses. It includes different techniques, such as Kraft pulping, sulfite pulping, soda pulping, and organosolv pulping (Jönsson and Martin, 2016). Kraft pulping and sulfite pulping are typically applied for woody materials, whereas soda pulping is typically used for non-woody plants (Jönsson and Martin, 2016).

Oxidative pretreatment include alkaline peroxide pretreatment, ozonolysis, and wet oxidation. In the pretreatment, oxidants are used to reduce the crystallinity of the cellulose and disrupt links between carbohydrates and lignin (Galbe and Zacchi, 2012; Jönsson and Martin, 2016).

Pretreatment under acidic conditions is the major method used in industry and it aims at removing the hemicellulose by hydrolysis (Fig. 4). Pretreatment under acidic conditions can be performed in many different ways and the conditions used can be very different (Fig. 4). Processes based on concentrated acid, such as concentrated sulfuric acid and CHAP (concentrated hydrochloric acid process) more serve as alternatives to pretreatment and enzymatic hydrolysis. The terminology differs somewhat, but acidic pretreatment methods based on heated water and steam can generally be summarized as hydrothermal pretreatment (HTP). They are acidic regardless of whether acid from an external source is added or not, because during the heating the hemicelluloses will be degraded resulting in formation of carboxylic acids, for example acetic acid and uronic acids (Garrote et al., 2008). Low concentrations of acids or acid-generating chemicals, for example sulfuric acid or sulfur dioxide, can be added. This approach includes methods sometimes referred to as dilute-acid pretreatment (DAP) to distinguish them from processes based on concentrated sulfuric acid or hydrochloric acid. DAP is typically performed with less than 4 wt% of a mineral acid, such as hydrochloric acid, nitric acid, phosphoric acid, peracetic acid, and, most commonly, sulfuric acid. The temperature is typically in the range of 120-210 °C (Yang and Wyman, 2008; Hu and Ragauskas, 2012), which requires the use of pressurized reactor vessels. HTP sometimes refers to processes where the temperature is in the range 160-260°C and in which high pressure (corresponding to 0.69-4.83 MPa) is used for maintaining the water as liquid (Sun and Cheng, 2002). HTP also includes steam explosion pretreatment.

Steam explosion can be carried out in batch mode or in continuous mode. The bach system is commonly used in laboratory scale. The (impregnated) biomass is put into the steam explosion reactor, which is pressurized and heated with steam (Jedvert et al., 2012). The continuous system is used more in industrial or demonstration scale. The impregnated biomass is loaded into a presteaming bin, and discharged into a plug screw feeder which squeezes out the moisture and condenses the material. With a cooking screw, the condensed material is moved inside the reactor. The pressure of the reactor can be controlled manually and the retention time can be adjusted by the rotation speed of the cooking screw. After treatment, the material is transferred into a cyclone, and the material is further disrupted through the sudden drop in pressure (Fang et al., 2011).

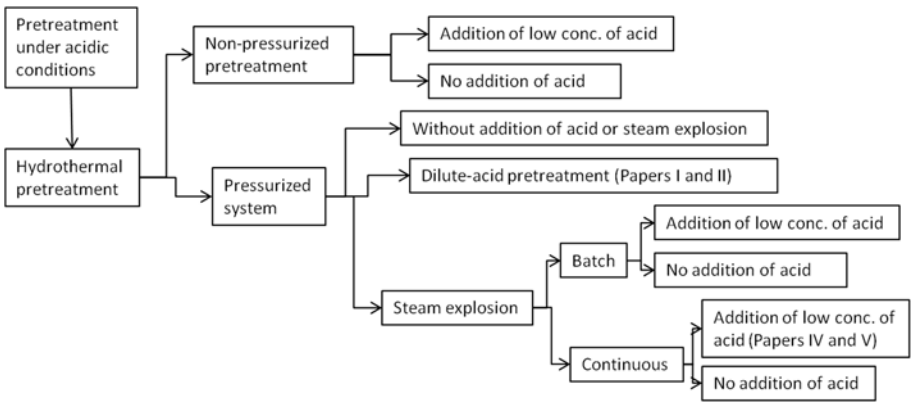


Fig. 4 Acid-based pretreatment. The type of pretreatment used in the studies described in Papers I, II, IV, and V are indicated.

HTP with addition of acid is suitable for most lignocellulosic biomass including softwood, hardwood, and agricultural residues. By optimizing the severity so that it fits the feedstock, high recoveries of hemicellulosic sugars in the pretreatment liquid and enhanced enzymatic convertibility of the cellulose in the solid phase can be achieved (Jönsson and Martin, 2016). The severity of pretreatment under acidic conditions is often compared using the combined severity (CS) (Chum et al., 1990):

$$CS = \log\{t \exp[(T-T_{ref})/14.7]\} - pH$$

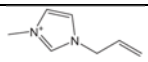
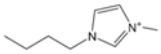
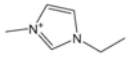
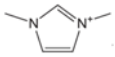
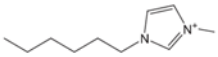
...where t is pretreatment time (min), T is the temperature (°C), and T_{ref} is 100°C.

There will be formation of inhibitory by-products when the hemicelluloses, and perhaps small parts of the cellulose and the lignin, are degraded (Jönsson and Martin, 2016). Moreover, under harsh conditions, pseudo-lignin can form from degradation of polysaccharides (Sannigrahi et al., 2011). Although pseudo-lignin is derived from polysaccharides, it is determined as Klason lignin in compositional analysis. There will be further discussion about by-products in Sections 5 and 6.

Pretreatment using alternative solvents, for example ionic liquids (ILs), is an emerging technology. ILs are non-volatile and green solvents that have potential to dissolve lignocellulosic materials under mild conditions (Brandt et al., 2013; George et al., 2015; Sun et al., 2016a). The most studied ILs used for pretreatment of lignocellulose is imidazolium-based ILs (Karatzos et al., 2012; Brandt et al., 2013). Table 1 shows imidazolium-based ionic liquids commonly used for pretreatment of biomass. The capability of an IL to dissolve or partly dissolve wood constituents is strongly linked to the functionality of the anion (Shafiei et al., 2013; Bahrani et al., 2015; Li and Xu, 2017). IL-pretreated cellulose can be regenerated by addition of an anti-solvent. The regenerated

cellulose will exhibit an amorphous structure and improved enzymatic digestibility (Kilpeläinen et al., 2007; Dadi et al., 2007).

Table 1 Commonly used imidazolium-based ionic liquids for pretreatment of biomass.

Common cations			
Name	Abbreviation	Structure	Ref. (examples)
1-Allyl-3-methylimidazolium	Amim/ [C=C_2C_1im]		Gräsvik et al., 2014; Wang et al., 2011
1-Butyl-3-methylimidazolium	Bmim/ [C_4C_1im]		Paper III; Li and Xu, 2017; Shafiei et al., 2013
1-Ethyl-3-methylimidazolium	Emim/ [C_2C_1im]		Bian et al., 2014; Torr et al., 2012; Sun et al., 2009
1,3-Dimethyl-imidazolium	Mmim/ [C_1C_1im]		Bahrani et al., 2015; Zhi et al., 2012
1-Hexyl-3-methylimidazolium	Hmim/ [C_6C_1im]		Wang et al., 2017; Lynam et al., 2012
Common anions			
Name	Abbreviation	Structure	Ref. (examples)
Chloride	[Cl]	Cl^-	Paper III; Gräsvik et al., 2014; Gremos et al., 2011
Acetate	OAc/ [$MeCO_2$]		Paper III; Bian et al., 2014; Shafiei et al., 2013;
Hydrogen sulfate	[HSO_4]		Paper III; Chen et al., 2014; Brandt et al., 2011

Brandt et al. (2013) proposed two approaches for pretreatment with ILs, namely the Dissolution Process and the Ionosolv Process. In a Dissolution Process, the IL has a basic anion, such as $[MeCO_2]^-$, and has the capacity to completely dissolve the lignocellulosic feedstock. In an Ionosolv Process, the IL has an acidic anion, such as $[HSO_4]^-$, and only partially dissolves the lignocellulosic feedstock. The difference also involves the effects on the cellulose. A clear correlation has been observed between the basicity of the anion of the IL and its capability to dissolve cellulose (Brandt et al., 2013). A possible explanation could be interactions between basic anions and the hydroxyl groups of the cellulose (Brandt et al., 2013). On the other hand, both

hemicelluloses and lignin could be partly dissolved through acid-catalyzed hydrolysis in an Ionosolv Process (De Gregorio et al., 2016). The IL, the acidity, and the presence of water are sufficient for promoting cleavage of covalent bonds in hemicelluloses and lignin (De Gregorio et al., 2016).

Apart from the functionality, the costs of the ILs and their recyclability are relevant issues for industrial implementation of processes based on IL (Li et al., 2010; George et al., 2015; Sun et al., 2016a). With respect to recyclability, an Ionosolv Process could be an easier option than a Dissolution process as it has better tolerance to moisture and better stability (Cao and Yu, 2014; Brandt et al., 2013; George et al., 2015). Therefore, a less expensive acidic anion, such as $[HSO_4]^-$, would have advantages with regard to reducing the costs and improving the possibilities to recycle the IL.

3.2 Enzymatic saccharification

Enzymatic saccharification of the cellulose and part of the hemicelluloses into monosaccharide sugars has potential advantages, such as high selectivity, high yields, mild conditions, and low energy costs. It is a synergetic multi-step reaction with complex enzyme preparations that can include both cellulolytic and hemicellulolytic enzymes (Ragauskas, 2013; Malgas et al., 2017). These enzymes are mostly derived from aerobic or anaerobic bacteria and fungi found in nature.

Saccharification of cellulose by cellulases generally requires three steps: (*i*) the cellulolytic enzymes are adsorbed onto the surface of the celluose; (*ii)* the enzymes get access to the cellulose; (*iii*) three main types of cellulases co-operate to produce glucose from cellulose (Arantes and Saddler, 2010). The first two steps can be referred to as amorphogenesis, and would be related to the carbohydrate-binding module (CBM) of enzymes and the accessibility of the cellulose (Arantes and Saddler, 2010 and 2011). Most fungal cellulases and some hemicellulases have CBMs. The CBM can adsorb to an accessible site on the cellulose and form a complex held by specific, non-covalent, thermodynamically favorable bonds (Lynd et al., 2002). CBMs are currently classified into 83 families (CAZy, Carbohydrate Active enZyme database). There are three categories of CBMs: type A, type B, and type C (Gilbert et al., 2013). Type A CBMs are specific for the surfaces of crystalline polysaccharides, and the families CBM-1, CBM-3, CBM-5, and CBM-64 all belong to this type (Gilbert et al., 2013; Armenta et al., 2017). Type B CBMs are specific for internal glycan chains (endo-type). Type C CBMs are specific for the termini of glycan chains (exo-type) (Gilbert et al., 2013). Examples of CBM families belonging to type B include CBM-4, CBM-6, CBM-36, and CBM-80. Type C includes CBM-9, CBM-13, and CBM-66 (Armenta et al., 2017). Besides adsorption and selectivity, CBMs also have a disruptive function that can weakly split the hydrogen bonds of crystalline cellulose without showing any hydrolytic activity

(Wang et al., 2008). With the CBM targeting the surface of the cellulose, the catalytic module of cellulases can catalyze the cleavage of the β-1,4 glucosidic bonds.

The three main cellulase enzymes are endoglucanases (EGs), cellobiohydrolases (CBHs) and β-glucosidases (Bommarius et al., 2014). EGs catalyze the random cleavage of β-1,4 glucosidic bonds of both amorphous and crystalline cellulose chains, thus producing oligosaccharides of various size. The DP value of the cellulose decreases, and more chain ends are created. The resulting oligosaccharides can be further hydrolyzed by CBH, either from the reducing or from the non-reducing ends of the chain, and converted into cellobiose. β-Glucosidases catalyze the hydrolysis of cellobiose to glucose (Lynd et al., 2002; Horn et al., 2012). *Trichoderma reesei* (*Hypocrea jecorina*) cellulases are the most studied. *T. reesei* secretes eight EG isozymes [Cel7B (EGI), Cel5A (EGII), Cel12A (EGIII), Cel61A (EGIV), Cel45A (EGV), Cel74A (EGVI), and Cel61B (EGVII)] and two CBH isozymes [Cel7A (CBHI) and Cel6A (CBHII)] (Nimlos et al., 2007; Bommarius et al., 2014). As the β-glucosidase activity in *T. reesei* cellulase mixtures is very low, *Aspergillus niger* β-glucosidase is commonly added as a supplement.

Some accessory proteins including lytic polysaccharide monooxygenases (LPMOs) and swollenins can also promote enzymatic saccharification of cellulose (Yang et al., 2011; Jager et al., 2011; Guo et al., 2017). LPMOs are copper-dependent enzymes that can oxidative cleave polysaccharides and produce shorter cellulose chains with oxidized ends (Horn et al., 2012; Johansen, 2016). LPMOs are found in some fungi, but also in some bacteria (Johansen, 2016). The reaction requires an oxidant, such as O_2 or H_2O_2 (Bissaro et al., 2017), and an electron donor, which could be a divalent metal ion, ascorbic acid, gallic acid, reduced glutathione, and lignin (Horn et al., 2012). Swollenins have been reported to disrupt the cellulose surface and cause amorphogenesis of cellulose (Gourlay et al., 2015; Guo et al., 2017). However, Eibinger et al. (2016) claimed that swollenins are not auxiliary factors of enzymatic saccharification of cellulose. In a study of swollenin purified from *T. reesei,* Andberg et al. (2015) reported that swollenins have activities that share similarities to both EGs and CBHs.

Hemicellulases include a large number of glycoside hydrolases and carbohydrate esterases, e.g. endo-xylanase, acetyl xylan esterase, β-xylosidase, endo-mannanase, α-L-arabinofuranosidase, α-glucuronidase, ferulic acid esterase, α-galactosidase, and *p*-coumaric acid esterase (Shallom and Shoham, 2003; Meyer et al., 2009). They act in concert to degrade the different bonds in the backbone and side-chains of hemicelluloses.

Some lignin-modifying and pectin-degrading enzymes can also be used to improve the saccharification of lignocellulosic feedstocks (Ragauskas, 2013).

Most lignin-modifying enzymes are secreted by fungi causing white-rot decay of wood, e.g. manganese peroxidase, lignin peroxidase, and laccase (Ikehata et al., 2004; Aro et al., 2005; Sánchez, 2009). These oxidoreductases can catalyze the oxidation of phenolic and non-phenolic moieties in lignin. Enzymes as pectin methyl esterase, pectate lyase, polygalacturonase, and rhamnogalacturonan lyase have the ability to degrade pectin (Ragauskas, 2013).

3.3 Fermentation

The hexose (six-carbon) and pentose (five-carbon) sugars derived from the hydrolysis of cellulose and hemicelluloses can be fermented to desirable products using yeasts, bacteria, and filamentous fungi. The major end-products in industry, bioethanol and biobutanol, are typically produced by fermentation of carbohydrates using yeast and anaerobic bacteria. The most common yeast used for fermentation is *Saccharomyces cerevisiae*, which is especially well suited for ethanol production from glucose (Chandel et al., 2011). The bacterium *Zymomonas mobilis* can metabolize glucose, sucrose, and frucose and produce ethanol (Panesar et al., 2006). Biobutanol has several advantages as biofuel compared to ethanol including higher energy density, higher hydrophobicity, and lower flammability (Dahman, 2012). Butanol can be produced in a bacterial ABE (acetone-butanol-ethanol) fermentation process using clostridia such as *Clostridium acetobutylicum* (Ragauskas, 2013). Moreover, through chemical conversion of sugars, substances such as xylitol, furans, sorbitol, and levulinic acid can be produced. Cellulosic ethanol can also be further converted to green chemicals and bioplastics, e.g. biopolyethylene (Zhang and Yu, 2013).

4. Recalcitrance of woody feedstocks

Pretreatment reduces the resistance of the lignocellulosic biomass to enzymatic saccharification, improves sugar yields, and lowers enzyme costs. The ability of biomass to resist chemical and biological deconstruction is defined as recalcitrance (Himmel et al., 2007). Biomass recalcitrance increases energy requirements, increases the cost and the complexity of the operations in the biorefinery, and reduces the product yields (McCann and Carpita, 2015). The recalcitrance is intimately related to the unique chemical and physical characteristics of the plant cell walls. Many studies have addressed different factors that contribute to the recalcitrance of biomass (Table 2). The multiple and inter-related factors that govern recalcitrance can restrict enzymatic saccharification by impeding the access to the cellulose.

4.1 Cellulose

The crystallinity of cellulose has traditionally been assumed to be an important factor contributing to biomass recalcitrance, especially with regard to the initial hydrolysis rate (Hall et al., 2010; Chundawat et al., 2011; Xu et al., 2012; Li et al., 2017). However, there are also studies reporting no or negative correlation between cellulose crystallinity and enzymatic digestibility (Laureano-Perez et al., 2005; Yu et al., 2011; Nelson et al., 2017). Pu et al. (2006) found that among different cellulose allomorphs cellulose Iβ was more difficult to hydrolyze than cellulose Iα, para-crystalline, and amorphous regions at the initial phase of the hydrolysis (fast hydrolysis rate). However, in a more recent study, Pu et al. (2013) reported that the correlation between crystallinity and cellulose digestibility was not clear. There is strong evidence that although the crystallinity of the biomass increases after acid pretreatment resulting in degradation of the amorphous regions of the cellulose, the pretreated biomass exhibits much faster hydrolysis rates (Kim et al., 2003; Kim and Lee, 2005; Park et al., 2010; Zhao et al., 2010).

As the methods that can be used to modify crystallinity (e.g. ball-milling, gamma irradiation, hydrothermal treatment) may also change other characteristics of the lignocellulose, it is over-simplified to only relate the crystallinity to recalcitrance (Ragauskas, 2013). In studies of enzymatic saccharification of milled loblolly pine, Agarwal et al. (2013) found that modification of the ultrastructure of the wood was more important for the digestibility than the change in cellulose crystallinity.

Another important property of cellulose is the DP value. As cellobiohydrolases are active at the ends of cellulose chains and as β-glucosidases have the ability to hydrolysis cellulose chains with low DP, short cellulose chains are considered easier to hydrolyze (Hall et al., 2010). There are some studies that report that DP was one of the main factors for biomass recalcitrance (Table 2). However, Yang et al. (2011) found that the DP of cellulose has less influence on enzymatic saccharification than other factors, such as cellulose accessibility.

Table 2. Studies addressing recalcitrance factors of different lignocellulosic feedstocks.

Ref.	Material[a]	Recalcitrance factor[b]												
		CrI	DP	LC	S:G	LCC	HC	A	P	PS	SAP	SP	CA	SC
Paper I	Aspen, birch, spruce	Y		Y	Y		Y	Y				Y	Y	
Paper II	GMO hybrid aspen			Y	Y		Y	Y			Y		Y	
Paper III	Aspen, spruce	Y		Y			Y	Y				Y		
Paper IV	Spruce			Y			Y			Y			Y	
Biswal et al., 2018	GMO *Populus*						Y		Y					
Ding et al., 2018	Poplar			Y							Y			
Santos et al., 2018a	Elephant grass											Y		
Santos et al., 2018b	Sugarcane hybrids			Y							Y		Y	
Arévalo et al., 2017	*Eucalyptus globulus*											Y		
Djajadi et al., 2017	Corn stover, *M. giganteus*, wheat straw			Y			Y							Y
Dumitrache et al., 2017	*Populus deltoides*			Y										Y
Edmunds et al., 2017	GMO loblolly pine			Y	Y									
Giummarella and Lawoko, 2017	Birch, spruce					Y								
Ji et al., 2017	Rice straw	Y								Y				
Jiang et al., 2017	Douglas fir	Y	Y							Y		Y		
Leskinen et al., 2017	Birch, pine			Y										
Li et al., 2017	Rice	Y	Y											
Meng et al., 2017	Natural poplar variants		Y	Y	Y								Y	
Nelson et al., 2017	GMO switchgrass			Y			Y							

Pawar et al., 2017b	GMO aspen				Y		Y	Y					
Chen et al., 2016	Wheat straw			Y			Y						
Jiang et al., 2016	Corn stalk	Y	Y								Y		
Rico et al., 2014	Eucalypt			Y									
Sun et al., 2014	Hybrid poplar	Y		Y									Y
DeMartini et al., 2013	Switchgrass, poplar			Y			Y						
Skyba et al., 2013	Hybrid poplars				Y								
Santos et al., 2012	Nine hardwood species			Y	Y								
Wiman et al., 2012	Spruce										Y		
Xu et al., 2012	*Miscanthus*	Y					Y						
Hu et al., 2011	Corn stover, sweet sorghum bagasse, lodgepole pine						Y						
Studer et al., 2011	*Populus trichocarpa*			Y	Y								
Yu et al., 2011	Loblolly pine, Hardwood			Y									
Chandra et al., 2009	Lodgepole pine									Y			Y
Yoshida et al., 2008	*Miscanthus sinensis*	Y		Y									
Davison et al., 2006	Hybrid poplar			Y	Y								

[a]GMO: Genetically modified organism. *M. giganteus*: *Miscanthus giganteus*.

[b]Y indicates inclusion in the study. CrI: cellulose crystallinity. DP: cellulose degree of polymerization. LC: lignin content. S:G: the ratio of syringyl units and guaiacyl units in lignin. LCC: lignin carbohydrate complex. HC: hemicellulose content. A: acetylation. P: pectin. PS: particle size. SAP: specific surface area and porosity, usually with BET analysis. SP: surface properties, usually with SEM analysis. CA: cellulose accessibility, usually with Simons' stain analysis. SC: surface content of lignin, hemicelluloses, or wax, usually with ATR-FTIR (attenuated total reflectance-Fourier transform infrared spectroscopy) analysis.

4.2 Lignin

It is commonly recognized that a high content of lignin and/or hemicellulose will contribute to increased recalcitrance. As one of the major constituents of plant cell walls, lignin provides strength to the cell wall framework and protects cellulose against microbial attack and chemical damage. As could be expected, several studies show that lignin removal results in improved cellulose digestibility (Table 2).

Biomass recalcitrance caused by lignin can be attributed to (*i*) the formation of covalent bonds to hemicelluloses (referred to as LCC; lignin-carbohydrate complexes) and (*ii*) to lignin acting as a physical barrier that prevents access of enzymes to cellulose (Laureano-Perez et al., 2005; Giummarella and Lawoko, 2017). LCC includes benzyl ester bonds formed between lignin and the carboxyl groups of 4-*O*-methyl-D-glucuronic acid residues in xylan (Jørgensen et al., 2007). Factors that may affect the recalcitrance caused by lignin include (*i*) the content of lignin, (*ii*) the distribution of different lignin units (S, G, and H), (*iii*) the distribution of different inter-unit linkages, and (*iv*) different content of phenolic groups (Laureano-Perez et al., 2005). The mechanisms by which lignin protects cellulose from enzymatic saccharification are not fully understood. The structure of the lignin and the content and type of LCC are believed to play important roles for lignin recalcitrance (Chandra et al., 2007; Pawar et al., 2017b). Even though decreased lignin content can improve enzymatic digestion of cellulose, some researchers have concluded that delignification is not always necessary since hemicellulose removal can lead to redistribution of lignin and improval of cellulose accessibility (Yang and Wyman, 2008; Rollin et al., 2010; Leu and Zhu, 2013).

Lignin can also cause irreversible non-productive binding of cellulases resulting in decreased saccharification of cellulose. Studies addressing non-productive binding of enzymes to lignin preparations and the methods used in such studies are summarized in Table 3. Hydrophobic interactions between the CBMs of cellulases and lignin have commonly been suggested to be the main factor responsible for non-productive binding of cellulases to lignin (Palonen et al., 2004; Pareek et al., 2013; Qin et al., 2014; Yang and Pan, 2016;). Besides the CBM, the hydrophobic area on the surface of the catalytic domains of cellulases are also believed to be important for non-productive binding of cellulases to lignin (Sammond et al., 2014).

The adsorption of cellulase on lignin is highly dependent on the functional groups of the lignin, which can be affected by pretreatment methods and biological origins (Berlin et al., 2007; Nakagame et al., 2011b; Pareek et al., 2013). For instance, both Nakagame et al. (2011b) and Pareek et al. (2013) found a negative correlation between the carboxylic acid content of the lignin preparation and the adsorption of cellulases. Nakagame et al. (2011b) reported

that enzymatic hydrolysis residue lignin (EnzHR lignin) from steam-pretreated biomass had higher carboxylic acid content than organosolv-pretreated biomass. Moreover, EnzHR lignin from lodgepole pine had lower content of carboxylic acid groups than poplar and corn stover (Nakagame et al., 2011b). On basis of studies of carboxylation and sulfonation of lignin, Yang and Pan (2016) reported that hydrophilic modification of lignin could significantly reduce the binding of cellulases to lignin for both softwood and hardwood.

Delignification using different methods could be an approach to reduce non-productive binding of cellulases to lignin (Zhu et al., 2009; Zhao et al., 2010). Other methods to alleviate non-productive binding could be addition of other proteins or surfactants (Pan et al., 2005; Yang and Wyman, 2006; Zheng et al., 2008).

4.3 Hemicelluloses

Together with lignin, hemicelluloses also play a role as physical barriers limiting the accessibility of cellulases to cellulose (Öhgren et al., 2007; Yang and Wyman, 2008). The xylan content has been found to affect the sugar recovery in both enzymatic hydrolysis and in pretreatment processes (Bura et al., 2009). Xylanases can increase cellulose conversion during enzymatic hydrolysis (Kumar and Wyman, 2009).

Hemicelluloses are often acetylated. Grohmann et al. (1989) suggested that acetyl groups play an important role in biomass recalcitrance. Deacetylation of plant cell walls through genetic engineering has been linked to improved enzymatic saccharification of cellulose (Pawar et al., 2017 a, b). Acetyl groups may contribute to lignocellulose recalcitrance (*i*) by changing surface hydrophobicity, (*ii*) by inhibiting productive binding of hydrolytic enzymes, and (*iii*) by decreasing cellulose accessibility through steric hindrance (Pan et al., 2006; Ragauskas, 2013).

Compared to lignin, hemicelluloses are easier to remove during pretreatment. Acid pretreatment aiming at removal of hemicelluloses can also cause modification of lignin, thus improving cellulose accessibility in two ways (Yang and Wyman, 2008; Leu and Zhu, 2013). However, pseudo-lignin may form from polysaccharides under harsh pretreatment conditions (Sannigrahi et al., 2011). Although some studies indicate that pseudo-lignin will impair the bioconversion of cellulose (Hu et al., 2012), the mechanisms behind that phenomenon are not clear.

Table 3. Studies addressing non-productive binding of enzymes to lignin preparations.

Ref.	Lignin preparation[a]	Enzymes[b]	Studied methods[c]							
			Sac	PC	LA	EA	SDS	MS	QCM	TMT
Paper VI	EnzHR of SP birch and spruce; OL of beechwood, PL of xylan	Celluclast 1.5L, Novozym 188		Y	Y	Y	Y	Y		Y
Kellock et al., 2017	EMAL of SP spruce and wheat straw	CBHI, CBHII, EGI, EGII, XynII (*T. reesei)*. BGL (*A. niger)*.	Y						Y	
Yao et al., 2017	Three OLs of DAP *Broussonetia papyrifera*	CBHI (*T. longibrachiatum)*		Y	Y					
da Silva et al., 2016	KL of sugarcane and eucalyptus	BGL1 and BGL3 (*T. petrophila)*				Y				
Lu et al., 2016	MWL and EnzHR of untreated and LHW corn stover	Cellulase (*P. oxalicum JU-A10-T)*	Y			Y	Y	Y		
Huang et al., 2016	MWL of DAP and KP bamboo	Celluclast 1.5L	Y	Y	Y					
Sun et al., 2016b	MWL of HP aspen	Cellic CTec2, Celluclast 1.5L	Y	Y	Y					
Yang and Pan 2016	OL of poplar and pine with carboxylation, sulfonation, or hydroxypropylation	Celluclast 1.5L β-glucosidase	Y	Y	Y					
Lai et al., 2015	OL and EnzHR of OP sweetgum	Celluclast 1.5L	Y	Y	Y					
Strobel et al., 2015	EnzHR of DAP *Miscanthus*	CBHI, + CBM mutants (*T. reesei*)	Y	Y	Y					
Yarbrough et al., 2015	OL of SP corn stover	Cellic CTec2	Y	Y		Y	Y			
Sammond et al., 2014	OL of switchgrass	CBHI, Axe1, XynII (*T. reesei*), AbfB, BGL1 (*A. niger*), Xyn1 (*T. lanuginosus)*. E1 (*A. cellulolyticus*)							Y	
Yu et al., 2014	MWL of untreated and EH eucalyptus, maple, pine	Cellulase, xylanase, β-glucosidase	Y	Y	Y					

Pareek et al., 2013	APL of spruce and *Populus*, AL, hydrolytic lignin, OL	Celluclast 1.5L, Novozym 188		Y	Y	Y			
Rahikainen et al., 2013a	EnzHR of SP spruce and wheat straw	EG45A, + CBM mutants (*Melanocarpus albomyces*)	Y	Y	Y		Y		
Rahikainen et al., 2013b	EMAL of ball-milled spruce, wheat straw with and without SP	CBHI (*T. reesei*)	Y						Y
Rahikainen et al., 2013c	EnzHR of HP wheat straw and SP spruce	CBHI, +CBM1/CBM3 (*T. reesei*)	Y				Y		
Zhang et al., 2013	EnzHR of HP spruce	Xylanases (*N. flexuosa*)	Y	Y	Y				
Zheng et al., 2013	EnzHR of DAP corn stove, SP corn stover and SP rice straw	Cellulase Accellerase 1000	Y	Y	Y				
Hu et al., 2012	PL and EMAL of hybrid poplar	Cellulase (*T. reesei*), Novozym 188	Y						
Nakagame et al., 2011a	EnzHR of SP Douglas fir	Cellulases Spezyme CP, Novozym 188	Y				Y		
Rahikainen et al., 2011	EnzHR of SP spruce and APL of spruce	Celluclast 1.5L , Novozym 188				Y	Y		

[a]Lignin preparation. APL: acid-pretreated lignin (two steps H_2SO_4). AL: alkali lignin DAP: dilute acid pretreatment. EH: enzymatic hydrolysis. EMAL: enzymatic mild acidolysis lignin. EnzHR: enzymatic hydrolysis residue lignin. HP: hydrothermal pretreatment. KL: kraft lignin KP: kraft pulped. LHW: liquid hot water pretreatment. MWL: milled wood lignin. OL: organosolv lignin. OP: organosolv pretreatment. PL: pseudo-lignin. SP: steam pretreatment.

[b]Enzymes. Abfb: arabinofuranosidase. Axe: acetyl xylan esterase. BGL: β-glucosidase. CBH: cellobiohydrolase. CBM: carbohydrate binding module. E: endocellulase. EG: endoglucanase. Xyn: endoxylanase.

[c]Methods. Sac: enzymatic saccharification of substrates with additional lignin preparations. PC: protein content assay. LA: Langmuir adsorption isotherm with protein content assay. EA: enzyme activity assays, usually including cellobiohydrolase, endoglucanase, β-glucosidase, and xylanase activity. SDS: sodium dodecyl sulfate gel electrophoresis. MS: mass spectroscopical analysis of proteins in bands from SDS-PAGE. QCM: quartz crystal microbalance with dissipation monitoring, used to study protein adsorption onto a thin lignin films. TMT: Tandem mass tags and LC-MS/MS analysis.

4.4 Structure

Compared to the chemical composition and the crystallinity of cellulose, the structure of the lignocellulosic material *per se* has been little investigated with regard to its effect on recalcitrance (Table 2). Structural factors include specific surface area, particle size, porosity, and cellulose accessibility. The accessibility of cellulose is a critical factor for the efficiency of enzymatic saccharification of cellulose. The particle size can be decreased by chipping and milling, and higher hydrolysis rate and cellulose conversion was observed for smaller particle size of sawdust slurry (Dasari and Berson, 2007). The reduction of the particle size could increase the specific surface area of the biomass, but there could also be inherent differences between different types of biomass. In addition to the particle size, the specific surface area is also related to the pore volume, which is affected by the size of the lumen and the number of substrate pores. Through the pores on the cell wall, the enzymes can bind to the cellulose, and thus the accessible surface area increases with the specific surface area (Luo and Zhu, 2011). However, based on the size of enzymes, the pore size is also important for increasing the accessible surface area. Only the pores with a size larger than the size of the enzymes could contribute to both specific surface area and accessible surface area.

5. Enzyme inhibition

Besides the recalcitrance caused by the chemical and physical properties of the lignocellulosic biomass, water-soluble products produced in the pretreatment can also decrease the efficiency of the conversion process. Monosaccharides, disaccharides, and hemicellulose-derived oligosaccharides have been proposed to have an inhibitory effect on enzymatic saccharification by binding to the active sites of cellulases (Qing et al., 2010; Kumar and Wyman, 2014; Ko et al., 2015). Kumar and Wyman (2014) reported that mannan polysaccharides have strong inhibitory effects on cellulases. Based on comparisons of the inhibitory effects of xylose, xylan, and xylooligomers on the enzymatic saccharification of Avicel (microcrystalline cellulose), Qing et al. (2010) and Kim et al. (2011) reported that xylooligomers were stronger inhibitors than the other two substances.

Besides soluble sugars, phenolic compounds have been found to have a strong inhibitory effect on enzymes involved in saccharification reactions (Kim et al., 2011; Ximenes et al., 2011; Malgas et al., 2016). Kim et al. (2011) reported that phenolic compounds caused much stronger inhibition of cellulases than soluble sugars, organic acids, and furans. Malgas et al. (2016) reported that lignin derivatives were stronger inhibitors of mannanolytic enzymes than organic acids, furans, and monosaccharides. Ximenes et al. (2011) reported that 4 mg of vanillin could reduce the rate of cellulose hydrolysis by 1 mg of endo- and exo- cellulases by 50%.

6. Microbial inhibition

By-products formed during pretreatment cause inhibition not only of enzymes but also of the fermenting microorganisms. Microbial inhibitors have traditionally been separated into three groups, namely aromatic compounds, furan aldehydes, and aliphatic carboxylic acids (Jönsson et al., 2013; Ko et al., 2015).

Aromatic compounds can be further divided into phenolics and non-phenolics. A large number of phenolic compounds can be formed from the splitting of β-O-4 ether bonds or other linkages of lignin and also from some extractives that contain phenolic groups (Jönsson and Martin, 2016). Common phenolic compounds derived from lignin include 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, vanillin, coniferyl aldehyde, ferulic acid, and Hibbert's ketones (Mitchell et al., 2014). Gallic acid from extractives has also been reported (Du et al., 2010; Mitchell et al., 2014). Some aromatic constituents are non-phenolic compounds, such as benzoic acid, benzyl alcohol, cinnamic acid, and *p*- and *o*-toluic acid (Du et al., 2010; Kim et al., 2013; Mitchell et al., 2014). The different functional groups of these aromatic compounds determine their inhibitory effects (Larsson et al., 2000; Jönsson et al., 2013; Jönsson and Martin, 2016).

Furan aldehydes, including furfural and HMF (5-hydroxymethylfurfural), are formed when the hemicellulose is degraded. Dehydration of pentoses and hexoses results in the formation of furfural and HMF, respectively (Jönsson and Martin, 2016). The molar toxicity of furan aldehydes is low compared to that of many phenolic compounds, but the concentration of furan aldehydes can be high especially if severe pretreatment conditions were used (Jönsson and Martin, 2016).

Commonly occurring aliphatic carboxylic acids include acetic acid, formic acid, and levulinic acid. Acetic acid is formed by hydrolysis or saponification of the acetyl groups of hemicelluloses (Jönsson and Martin, 2016). Levulinic acid and formic acid are further degradation products from furan aldehydes (Danon et al., 2013). Aliphatic acids are commonly the most abundant by-products in the pretreatment liquid, especially when hardwood or agricultural residues serve as feedstocks. However, the molar toxicity of the aliphatic carboxylic acids is low. Larsson et al. (1999) reported that around 100 mM of aliphatic carboxylic acids were needed to detect inhibition of *S. cerevisiae*.

Other inhibitors including benzoquinones and small aliphatic aldehydes, such as formaldehyde, can have strong toxic effects on yeast, although the concentrations in the pretreatment liquid are relatively low (Cavka et al., 2015; Stagge et al., 2015). Cavka et al. (2015) reported that the inhibitory effects of formaldehyde on yeast started at 1 mM, while Larsson et al. (2000) showed that 0.19 mM of benzoquinone completely inhibited the growth of yeast.

7. Aim of study

The aims of this study were to understand the recalcitrance of wood to biochemical conversion, to investigate how different pretreatment techniques can be used to decrease the recalcitrance of lignocellulosic biomass and improve the digestibility of the cellulose, and to explore how inhibition phenomena associated with acid pretreatment cause enzymes and microorganisms to work less efficiently.

The specific objectives were:

(I) To understand the recalcitrance of softwood and hardwood with respect to both chemical and structural factors, and with and without acid pretreatment.

(II) Investigate how deacetylation of hybrid aspen through expression of a Carbohydrate Esterase Family 5 (CE5) acetyl xylan esterase affects recalcitrance.

(III) Explore the effects of different anionic constituents of ionic liquids on the pretreatment of hardwood and softwood.

(IV) Investigate how the conditions used for pretreatment by steam explosion affect the recalcitrance of softwood to enzymatic saccharification and microbial fermentation.

(V) Understand microbial inhibition caused by by-products of steam-pretreatment of softwood.

(VI) Investigate catalytically non-productive binding to lignin and pseudo-lignin of individual isozymes in commercial cellulase preparations using quantitative proteomics.

8. Methods

8.1 Compositional analysis of lignocellulosic biomass

Quantification of the chemical composition of lignocellulosic biomass is an important step in the evaluation of feedstocks, pretreatment methods, and enzymatic saccharification.

8.1.1 Compositional analysis based on sulfuric acid

The chemical composition of lignocellulosic biomass with respect to carbohydrates (arabinan, galactan, glucan, xylan, and mannan) and lignin can be determined by a two-step treatment with sulfuric acid (Tappi standard T 249 protocol). To achieve more accurate results, an extraction step is required prior to the treatments with sulfuric acid. Soxhlet extraction is widely utilized, and a mixture of solvents of varying polarity can be used to extract both lipophilic and hydrophilic low-molecular weight substances. The solvents are typically chosen based on the biomass, and the temperature used depends on the boiling points of the solvents. The amount of extractives can be measured gravimetrically after the evaporation of the solvents. The first acidic step is based on treatment with 72% sulfuric acid at 30°C for 1 h (Sluiter et al., 2012). In the second step, the sulfuric acid is diluted to 4% with water and the suspension is autoclaved at 121°C for 1 h. Through this two-step treatment, hemicelluloses and cellulose should be degraded to monomeric sugars. The portion of the lignin that is dissolved in the sulfuric acid is classified as ASL (acid-soluble lignin), while the acid-resistant lignin is defined as Klason lignin. The ASL content is determined by using UV/Vis spectrophotometry based on the absorption of phenolic carbonyl structures at 240 nm. The Klason lignin content is measured gravimetrically as the solid residue obtained after solubilization of polysaccharides and ASL.

The content of monomeric sugars can be analyzed using high-performance liquid chromatography (HPLC) with a refractive index detector (RID), as described by Sluiter et al. (2012). A more modern approach is quantitation of the monosaccharides using high-performance anion-exchange chromatography (HPAEC) with an electrochemical detector.

8.1.2 Cellulose composition

The cellulose assay according to Updegraff is a rapid method for determination of the part of glucan that is categorized as cellulose (Updegraff, 1969). Extractive-free biomass is treated with the Updegraff reagent [an acetic acid:nitric acid:water mixture in the ratio 8:1:2 (v/v)] at 100°C for 30 min to remove hemicelluloses. The solid residue is then hydrolyzed by using 72% sulfuric acid at 30°C for 1 h and the released glucose is determined by using an anthrone-based assay with spectrophotometric quantitation at 620 nm.

8.1.3 Hemicellulose composition

The hemicellulose composition can be analyzed by using gas chromatography for determination of sugars derivatized through trimethylsilylation (TMS) (Gandla et al., 2015). Extractive-free biomass is methanolysed at 85°C for 24 h with HCl/MeOH. Then, the residue is silylated with Sylon HTP [HMDS:TMCS:Pyridine, 3:1:9 (v/v)] at 80°C for 20 min. Silylated monosaccharides and uronic acids, including arabinose, rhamnose, fucose, xylose, mannose, galactose, glucose, glucuronic acid, and galacturonic acid, are then analyzed using gas chromatography.

The acetyl groups in hemicelluloses can be measured as released acetic acid after hydrolysis of the ester linkages through saponification (Gille et al., 2011). The analysis of acetic acid can be performed either by using HPLC or HPAEC (Section 8.3).

8.1.4 Pyrolysis-gas chromatography/mass spectrometry

Pyrolysis-gas chromatography combined with mass spectrometry (Py-GC/MS) is a rapid and sensitive method for analysis of lignocellulosic biomass (Meier et al., 1992). A substance is heated to temperatures above 500°C under a stream of nitrogen, helium, or argon gas. The pyrolysis degrades the biomass to individual fragments that provide information about the origin of the substance. The fragments are then separated by using gas chromatography and identified by using mass spectrometry (Kusch, 2012).

Although Py-GC/MS does not provide much information about the carbohydrates, it is a useful method for characterization of lignin. Py-GC/MS will determine the relative ratios of lignin units of the guaiacyl type, the syringyl type, and the *p*-hydroxyphenyl type. The ratio of carbohydrate and lignin (C:L) and the ratio of lignin units (S:G:H) will be provided by Py-GC/MS analysis. Moreover, as only degradation products from phenylpropanoids are recognized as lignin by Py-GC/MS, it is a useful method to detect pseudo-lignin formation, as pseudo-lignin will be accounted for as carbohydrate. Thus, by combining Py-GC/MS and sulfuric-acid-based compositional analysis, the pseudo-lignin content can be estimated.

8.1.5 Fourier-transform infrared spectroscopy

Infrared spectroscopy is a technique that measures the infrared absorption or emission spectrum of the functional groups of a solid, a liquid, or a gas over a wide spectral range (Griffiths and de Haseth, 2007). The mathematical process Fourier transform is typically used to convert the raw data of the infrared absorption spectrum. FTIR can be used for qualitative and quantitative compositional analysis of lignocellulosic biomass with simple sample preparation and fast and precise analysis (Tucker et al., 2001; Hames et al., 2003; Liebmann et al., 2010).

For lignocellulosic biomass, the vibrations at wavenumbers between 800 and 1800 cm^{-1} are frequently related to functional groups of hemicelluloses, cellulose, and, especially, lignin. The aromatic ring vibrations of lignin units exhibit differences at certain wavenumbers (signals at 1510 cm^{-1} are more from G units, whereas signals at 1595 cm^{-1} are more from S units), which provides information about the characteristics of the lignin (Hergert, 1971; Faix, 1991; Faix and Böttcher, 1992). On the other hand, FTIR provides only limited information about the carbohydrates. However, the intensity ratio 1429/897 can be used to calculate the Total Crystalline Index (TCI), i.e. the infrared crystallinity ratio (Nelson and O'Connor, 1964). The band at around 1429 cm^{-1} (CH_2 symmetric bending) is assigned to the crystalline structure, whereas the band at 897 cm^{-1} (C1 group frequency) is associated with the amorphous region (Nelson and O'Connor, 1964). Besides TCI, the ratio 1372/2900 has been proposed as an empirical crystallinity index (crystalline:amorphous cellulose) - the Lateral Order Index (LOI) (Nelson and O'Connor, 1964).

8.2 Structural analysis of lignocellulosic biomass

Besides the chemical compositional analysis, variations in cellulose structure, cellulose accessibility, surface property, and cell wall structure are essential for characterization of feedstock properties and bioconversion steps.

8.2.1 Crystallinity

Cellulose crystallinity is an important factor for assessing the structure of cellulose. Crystallinity has been widely studied using different methods including X-ray diffraction (XRD), solid-state ^{13}C CP-MAS NMR, and FTIR spectroscopy (Garvey et al., 2005; Newman, 1999; Larsson et al., 1997; Zuckerstätter et al., 2009; Nelson and O'Connor, 1964). Solid-state ^{13}C CP-MAS NMR and FTIR spectroscopy provide only relative values for crystalline and non-crystalline regions of the cellulose, while XRD provides more detailed information regarding crystallinity.

As the structure of cellulose is a mixture of amorphous and different crystalline regions (according to the fringed fibrillar model), the degree of crystallinity and crystallite size dimensions are equally important for investigation of cellulose structure. XRD can be used to analyze the crystallinity and the crystallite size. Four individual crystalline peaks (101, 10$\bar{1}$, 002, and 040) and a broad peak at around 21.5° assigned to the amorphous contribution could be extracted by a curve-fitting process using spectra from XRD analysis of woody materials (Fig. 5).

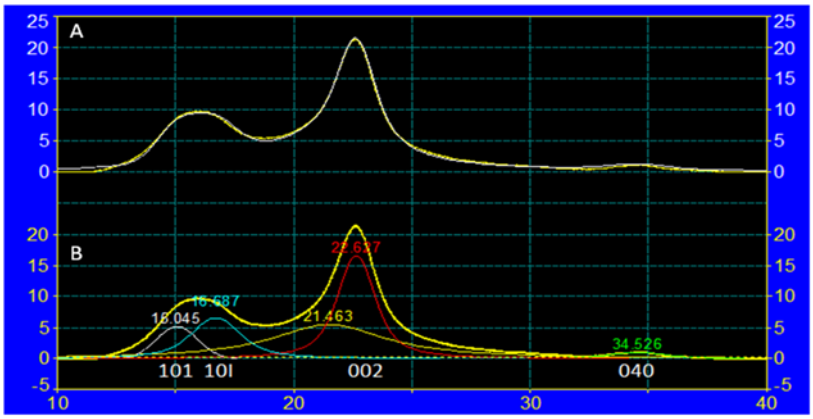


Fig. 5 XRD analysis for measuring the crystallinity of a wood sample. A: Spectrum from the XRD analysis. B: Curve-fitting process with Gaussian function for the cellulose crystalline peaks (101: 15.045; 10$\bar{I}$: 16.687; 002: 22.627; and 040: 34.526) and for the amorphous region (21.463).

The crystallinity index (CrI) is calculated from the ratio of the area of all crystalline peaks and the total area (Garvey et al., 2005). The crystallite size is calculated using the Scherrer equation based on the width of the crystalline peak at half height. The d-spacings (lattice spacing in one of the crystalline planes) can be calculated based on the Bragg equation.

$$D(hkl)=K\lambda/B(hkl)\cos\theta \qquad \text{(Scherrer equation)}$$

$$d(hkl)= \lambda/2\sin\theta \qquad \text{(Bragg equation)}$$

Here, (hkl) is the lattice plane, D(hkl) is the size of the crystallite, d(hkl) is the lattice spacing, K is the Scherrer constant (0.89), λ is the X-ray wavelength, B(hkl) is the full-width half maximum of the measured hkl reflection, and θ is the Bragg angle.

The Iα and Iβ allomorph that is dominant in the cellulose can be analyzed according to the d-space [d(hkl)] (Wada et al., 1995) with the Z-discriminate function.

$$Z = 1693d1 - 902d2 - 549 \qquad \text{Z-discriminate function;}$$

Here, d1 is the d-spacing of peak 101, and d2 is the d-spacing of peak 10$\bar{I}$. While Iα-rich cellulose is associated with $Z>0$, $Z<0$ indicates Iβ-dominant crystalline cellulose (Wada et al., 2001).

8.2.2 Surface properties

Scanning electron microscopy (SEM) is a form of electron microscopy in which a focused electron beam is scanning a specimen and creates an image of its surface. The electrons of the beam can interact with the atoms at different depth of the specimen and create emission of secondary electrons. By collecting

signals from the secondary electrons, information about the surface topography and the chemical composition of the specimen can be attained (Stokes, 2008). Therefore, the surface of the specimens must be electrically conductive. A coating layer with metals, such as gold, gold/palladium alloy, platinum, or iridium, is usually required (Suzuki, 2002).

SEM studies of woody materials have created a better understanding of the wood structure in three dimensions (Butterfield and Meylan, 1980; Stokes, 2008). With alternative resolutions, both the general structure and details on the surface of the cell wall can be studied by using SEM (Fig. 6). SEM has been widely used to observe structural changes of cell walls after using different pretreatment methods (Donohoe et al., 2011; Karimi and Taherzadeh, 2016). For example, Selig et al. (2009) observed the rough surface texture of alkaline-peroxide-pretreated corn stover compared to non-pretreated samples. Rezende et al. (2011) found exposed fiber strips in acid-pretreated sugarcane bagasse and collapsed and porous cell walls in alkaline-pretreated samples. Donohoe et al. (2008) found lignin-rich particles on the cell surface after DAP. These structural changes of the cell wall can be related to cellulose accessibility and the efficiency of the following enzymatic saccharification.

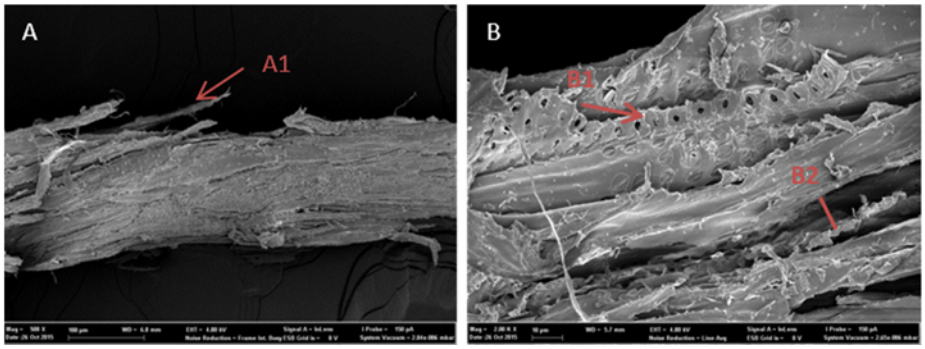


Fig. 6 SEM images providing an overview of a piece of aspen wood (A, magnification 500 ×), and detailed surface characters (B, magnification 2000 ×). A: Aspen wood cells gathered together as a lose structure with some dispersed fibers (A1). B: Pits. (B1) indicates the exposed cell lumen of a broken wood cell, and (B2) the wide gap between fibers confirming the lose structure of aspen wood.

8.2.3 Cell wall structure

Fluorescence microscopy is a technique that generates images based on the fluorescence or the phosphorescence of the specimen. A light source with an excitation filter generates specific wavelengths that are absorbed by the fluorophores of the specimen. This causes the emission of light with a longer wavelength. Through an emission filter, the fluorescence of the specimen can be recorded by the detector.

Lignin is the predominant fluorophore in plant cell walls. Lignin can be excited under both UV and visible light (Castellan and Davidson, 1994; Olmstead and Gray, 1997; Albinsson et al., 1999). The distribution of lignin in woody materials can be analyzed based on autofluorescence (De Micco and Aronne, 2007; Donaldson et al., 2010; Rahikainen et al., 2011). Within the broad fluorescence emission range, spectra are acquired by excitation at 420–480 nm and emission at 500-550 nm (Rahikainen et al., 2011; Donaldson and Radotic, 2013). With fluorescence microscopy, the general distribution of lignin in the lignocellulosic material can be observed. Thus, basic wood cell properties (such as lumen size and cell wall thickness) of different wood species can be determined. Moreover, pretreatment methods aiming at transformation of lignin can be evaluated. Fig. 7 shows lignin autofluorescence observed by using fluorescence microscopy.

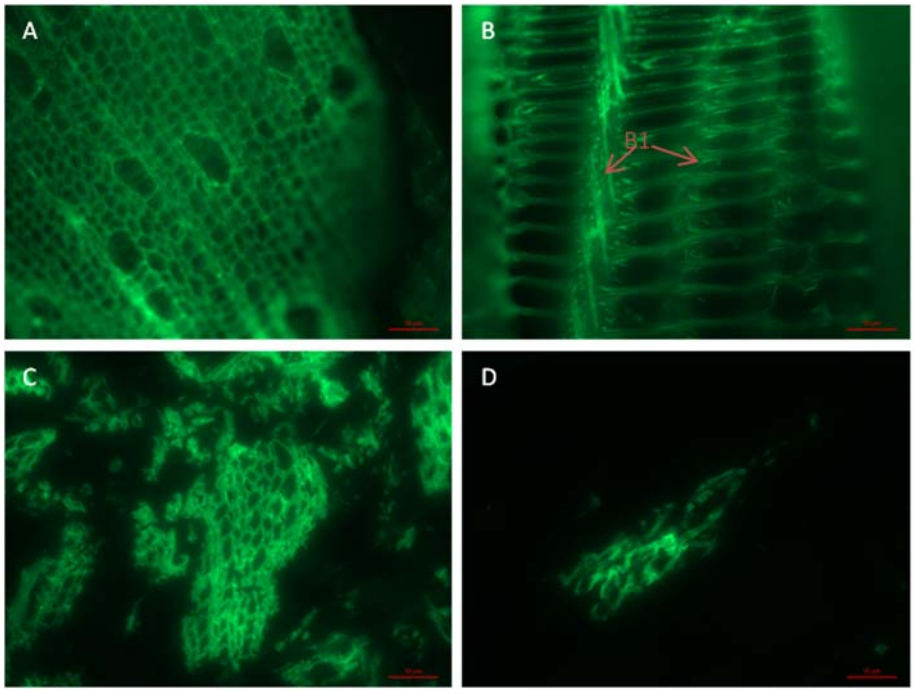


Fig. 7 Fluorescence microscopy images based on lignin autofluorescence. The images, which have the same magnification (red bar 50 μm), show aspen (A), spruce (B), acid-pretreated aspen (C), and acid-pretreated spruce (D). Before pretreatment, aspen and spruce show different cell wall structure and the strong signal at the middle lamella indicates a high lignin content (B1). After acid pretreatment, aspen was fragmented into small pieces and showed a distorted structure (C). With acid pretreatment at higher temperature, the structure of the spruce wood collapsed (D).

8.2.4 Surface area and porosity

Simons' staining is a method based on solute exclusion that can be used to test the accessibility of cellulose to enzymes. Two dyes are employed, namely Direct Blue (DB, Pontamine Fast Sky Blue 6BX) and Direct Orange (DO, Pontamine Fast Sky Orange 6RN). DB has a molecular mass of 993 Da (Yu et al., 1995). The high-molecular weight (HMW) fraction of DO is prepared by filtering a solution of DO through a 100 kDa ultrafiltration membrane. Simons' staining can be performed according to the modified procedure described by Chandra et al. (2008). Using that method, a series of solutions of the two dyes (DB and HMW DO) is incubated with the biomass sample for 6 h. Then, the supernatant is collected and analyzed spectrophotometrically at 455 nm and 624 nm.

The concentration of dyes in the supernatant can be calculated from Lambert-Beer's law using the following two equations (Esteghlalian et al., 2001):

$$A_{455} = \varepsilon O/455 \times L \times CO + \varepsilon B/455 \times L \times CB$$

$$A_{624} = \varepsilon O/624 \times L \times CO + \varepsilon B/624 \times L \times CB$$

....where A is the absorption of the mixture, L is the path length of the cuvette (usually 1 cm), ε is the extinction coefficient (the values used in this study were $\varepsilon O/455 = 35.62$, $\varepsilon B/455 = 2.59$, $\varepsilon O/624 = 0.19$, $\varepsilon B/624 = 15.62$ L g^{-1} cm^{-1}), and CO and CB are the concentrations of the orange dye and the blue dye, respectively. The differences between the concentrations of added dyes and the concentrations of dyes in the supernatant show the concentration of adsorbed dyes. Adsorption of dyes gives a rough indication of the pore size and the accessible surface area of cellulose.

The Brunauer–Emmett–Teller (BET) method can be used to assess the specific surface area and the surface porosity of a solid material. BET analysis is based on probing gases that adsorb on the surface of the material, which allows quantification of the specific surface area (Brunauer et al., 1938). The gas molecules should not react chemically with the material, and nitrogen gas is most commonly employed. Prior to analysis, a degassing process needs to be carried out to remove contaminants that are adsorbed on the surface of samples.

8.3 Quantification of by-products in the pretreatment liquid

Analysis of pretreatment by-products including aromatic compounds, furan aldehydes and aliphatic acids can be performed with an HPLC system equipped with an RID or/and a UV/VIS detector (preferrably a DAD; diode array detector) (Persson et al., 2002; Sluiter et al., 2012). An HPAEC system

equipped with a conductivity detector can also be used for analysis of the small aliphatic carboxylic acids. These detection methods are based on the retention times, and, for DAD, the absorption spectra of the analytes.

A more general overview of the by-products can be performed by measuring the Total Aromatic Content (TAC) and the Total Carboxylic Acid Content (TCAC). TAC is a spectrophotometrical estimation of the content of furan aldehydes and phenolic compounds, which both absorb at 280 nm. TCAC is determined by titration of the pretreatment liquid with an aqueous solution of sodium hydroxide. After steam explosion with sulfuric acid or sulfur dioxide, the pH of the pretreatment liquids is often ~2. Most carboxylic acids have pK_a values of 4-5 and by raising the pH from ~2 to close to neutral an estimate of the content of carboxylic acids is obtained.

8.4 Protein assay

8.4.1 Total protein content

Chromogenic methods to determine the total protein content are based on the absorbance of products formed from interactions between proteins and organic molecules. A calibration curve based on the absorbance of a series of protein solutions of known concentration is required. The absorption can be based on reactions involving different chemical groups including peptide bonds, aromatic side-groups, basic side-groups, and aggregated proteins. The commonly used UV-visible methods are based on different chemical groups, e.g. the Biuret protein assay based on peptide bonds reacting with copper ions under alkaline conditions, the Lowry protein assay that combines the Biuret assay with oxidation of aromatic residues in proteins, and the Bradford assay that is based on the Coomassie Brilliant Blue dye binding to basic and aromatic amino-acid residues (Ninfa et al., 2009).

The Bradford assay (Bradford, 1976) is the most widely used method, as it is relatively sensitive and can be performed fast. The method is based on the absorbance shift of the dye Coomassie Brilliant Blue G-250 from 465 to 595 nm due to binding to proteins in acidic solution through electrostatic interactions and van der Waals forces. Under acid conditions, the unbound dye stays in cationic and neutral form (red/green, absorbance 465 nm). After binding to proteins, the dye transforms to the anionic form (blue, absorbance 595 nm). By measuring the increased absorbance at 595 nm, the amount of protein present in the sample can be determined.

8.4.2 Enzyme activity assays

The filter paper assay (FPA) is the most commonly used measurement of total cellulase activity. It is an established method by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). The filter paper unit (FPU) refers to the amount of enzyme that releases 2 mg glucose from 50 mg of

Whatman No. 1. filter paper in 60 min (Adney and Baker, 1996). The reducing sugar concentration is usually measured by the dinitrosalicylic acid (DNS) reagent in a colorimetric method in which glucose or xylose are used as the calibration standard. The principle for DNS is that the sugar can reduce the nitro form of DNS to the amino form, thereby changing the color from bright yellow to reddish brown (Ghose, 1987). Based on the spectrophotometric method, the amount of sugars can be calculated from the color change at 540 nm.

Endoglucanase activity can be measured with amorphous cellulose (carboxymethylcellulose, CMC) as substrate (also referred to as CMCase activity). β-Glucosidase activity is measured using cellobiose (cellobiase assay) or 4-nitrophenyl-β-D-glucopyranoside (pNPG) as substrate (Ghose, 1987). The DNS colorimetric method is used for determination of reducing sugar generated from the CMC assay and the cellobiase assay. For the pNPG method, the liberated 4-nitrophenol was measured by absorption at 400 nm. The xylanase activity assay is based on using wood xylan as substrate, and the reducing sugar that is released can be measured using the DNS method.

For different substrates, the required temperature, pH, and time may differ in different assays. Even for the same assay, results presented in different studies may be difficult to compare as enzyme activities are sensitive to slight variations in temperature and pH. Moreover, as both glucose and cellobiose are reducing sugars that can react with DNS, cellobiase activity may be difficult to discern from the background (Ghorai et al., 2010).

8.4.3 Proteomics

Proteomics refer to large-scale analysis of proteins and can be focused on the structure, expression, or function of proteins (Anderson and Anderson, 1998). Expression proteomics is defined as the quantitative study of proteins expressed under different conditions. Technologies as gel electrophoresis and multidimensional liquid chromatography linked with mass spectrometry are commonly used for protein separation and mass spectrometric analysis.

Gel electrophoresis can be performed as native PAGE, as SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under denaturing conditions, and as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The enzymes are separated in the gel and, depending on the technique used, the mobility depends on the size, the conformation, and the charge of the protein (Ninfa et al., 2009). The proteins that are separated in the gel can then be identified by using mass spectrometry after digestion of proteins to peptides through proteolytic cleavage. The proteolysis can be performed *in situ* in the gel or after cutting out a piece of gel and extracting the protein.

Another method used in quantitative proteomics is isobaric mass tagging. The protein mixture is first labeled isotopically, and then digested as labeled peptides. Multidimensional liquid chromatography combined with mass spectrometry is used to separate, identify, and quantitate the labeled peptides (Weston and Hood, 2004).

9. Results and Discussion

9.1 Feedstock properties and recalcitrance (Papers I & II)

Efficient enzymatic saccharification of cellulose (and perhaps some hemicelluloses) to fermentable sugars is a fundamental challenge in bioconversion of lignocellulosic feedstocks. Without high sugar yields, the yields of the fermentation products will also be low, and it is unlikely that the process will be economically viable. Therefore, feedstock properties associated with recalcitrance to enzymatic saccharification have been widely studied (Table 2). However, as many studies in the area included only one lignocellulosic material and as the methods used to evaluate recalcitrance and the basic effects of pretreatment on the properties of the biomass were limited, it is difficult to compare results and arrive at a comprehensive view of the main causes of lignocellulose recalcitrance. Therefore, we compared the recalcitrance of both hardwood and softwood species, and characterized the materials with respect to both chemical composition and structural properties (Paper I). Two species of hardwood, aspen and birch, and one species of softwood, spruce, were compared. Two species of hardwood were studied, as they have more heterogeneous wood cells than softwood, and a more varied lignin composition. The study was different from other studies in the field because of the set of analytical techniques used, the equal emphasis on structural and chemical factors, the comparison of different types of wood species, and the set of different enzymatic cocktails that were used.

Analytical non-exhaustive enzymatic digestion of untreated aspen, birch, and spruce showed that aspen provided the highest sugar yield and birch the lowest. The difference in glucose yield between birch and aspen was as high as one order of magnitude (Paper I, Table 5). This result was unexpected, as saccharification of one of the hardwood species (birch) was more similar to that of a softwood species than to another species of hardwood, especially as softwood is typically regarded as highly recalcitrant compared to hardwood (Galbe and Zacchi, 2007). As expected, chemical analysis including compositional analysis using a modification of the NREL protocol (Sluiter et al., 2012), Py-GC/MS, and FTIR, showed that aspen and birch were much more similar to each other than to spruce. Moreover, XRD analysis of crystallinity indicated that the highest values for CrI and crystallite size were found in either aspen or spruce. These results indicated that chemical composition and

cellulose crystallinity were not the main factors behind the differences in recalcitrance. However, analysis with Simons' staining for determination of cellulose accessibility indicated that aspen had the highest cellulose accessibility and birch the lowest. This result indicated that structural factors were more important than chemical differences. Observation of surface properties using SEM and imaging of lignin distribution by fluorescence microscopy confirmed that birch had a more dense and integrated structure than aspen. Therefore, the work presented in Paper I shows that structural factors are closely linked to the efficiency of enzymatic saccharification of untreated woody biomass.

The comparison of the recalcitrance of aspen, birch, and spruce further showed that dilute-acid pretreatment efficiently improved the digestibility of all three wood species (Paper I). The increased crystallite sizes of the three pretreated wood species confirmed that high crystallinity does not impede cellulose digestibility, which agrees with previous studies, such as Pu et al. (2013) and Nelson et al. (2017). The improvement of digestibility was more significant for birch, as pretreated birch and pretreated aspen exhibited similarly high sugar yield compared to pretreated spruce. The efficiency of the acid pretreatment can be attributed to removal of hemicelluloses and to fragmentation of the cell wall structure. The most abundant hemicellulosic sugar in the pretreatment liquids was xylose from birch.

The study of hybrid aspen expressing fungal acetyl xylan esterase (Paper II) provided a better understanding of the importance of xylan and acetylation for the recalcitrance of hardwood to enzymatic saccharification. On average, the transgenic aspen wood showed a 13% decrease in xylan acetylation (Paper II, Fig. 2), and a 5% decrease in xylan content (Paper II, Table 2), but no general changes in the contents of cellulose and lignin (Paper II, Table 1). BET analysis and Simons' staining showed that the transgenic aspen had, on average, 11% larger specific surface area (Paper II, Table 4) and 10% increased dye adsorption (Paper II, Fig. 7). The transgenic aspen exhibited a 27% increase in glucose yield after enzymatic saccharification of non-pretreated wood, and a 3% increase in glucose yield after acid pretreatment and enzymatic saccharification (Paper II, Fig. 6). The increased specific surface area and cellulose accessibility of the transgenic aspen could be the consequence of decreased xylan content and changes of interactions between cellulose and xylan caused by decreased acetylation. It is noteworthy in this context that Busse-Wicher et al. (2014) found that acetylation of xylan supported a two-fold helical screw of the xylan backbone and stabilized xylan-cellulose interactions.

9.2 Reducing recalcitrance with pretreatment (Papers III & IV)

Pretreatment using IL is an emerging technology that may become increasingly interesting in the future provided that fundamental issues related to the moisture content of the biomass, the cost of the ILs, and the recycling of the ILs are addressed. The most widely studied ILs for biomass pretreatment are imidazolium-based. A previous study had indicated that the efficiency of pretreatment with imidazolium-based ILs was very different depending on whether the pretreated biomass was hardwood or softwood (Gräsvik et al., 2014), but without providing an explanation. Therefore, pretreatment of wood from aspen and spruce with 1-butyl-3-methylimidazolium-based ($[C_4C_1im]$) ILs with $[HSO_4]^-$, $[Cl]^-$, or $[MeCO_2]^-$ as anionic constituents was studied to address this issue (Paper III). To further investigate the impact of the ILs on the constituents of the wood, torrefied biomass was included. Torrefaction decreases the influence of hemicelluloses and increases the influence of Klason lignin.

Pretreatment with $[MeCO_2]^-$ was most efficient for all types of wood. A thorough analysis of the composition and the mass balance showed that IL pretreatment with $[MeCO_2]^-$ as anion was not that different with respect to changing the chemical composition of the pretreated biomass compared to the other two anions. Instead, analysis of the cell wall structure by using fluorescence microscopy and SEM (Paper III, Fig. 1d and Fig. S2) indicated that using $[MeCO_2]^-$ as anion resulted in a clearly disordered cell wall structure. Obviously, the change in cell wall structure contributed to the higher susceptibility to enzymatic saccharification. This result is unexpected considering that the scientific literature in the area typically attributes pretreatment effects of ILs to their capability to remove lignin and/or hemicelluloses. For instance, Karatzos et al. (2012) found that EmimOAc was efficient for pretreatment of sugarcane bagasse, and attributed that to the capacity of the IL to delignify the biomass. Zhang et al. (2012) reported that improved glucan digestibility of sugarcane bagasse after pretreatment with Bmim[Cl] was due to delignification and removal of xylan. Brandt et al. (2011) reported that Bmim$[HSO_4]$ pretreatment improved the glucose yield from *Miscanthus* by efficient removal of hemicelluloses. Again, the results point towards an underestimation of the importance of structural factors in relation to chemical factors.

Compared to pretreatment with $[C_4C_1im][Cl]$ and $[C_4C_1im][MeCO_2]$, pretreatment with $[C_4C_1im][HSO_4]$ removed more xylan from the woody materials (Paper III, Table 2). The investigation shows that this effect was important for efficient saccharification of aspen, whereas it was not important for spruce or for torrefied samples. Evidently, the acidity of $[HSO_4]^-$ caused extensive hydrolysis of xylan, which then facilitated pretreatment of the xylan-

rich hardwood, but this was not relevant for low-xylan feedstocks, such as softwood and torrefied wood.

Although IL-based pretreatment is an interesting development in the field, the state-of-the-art method for pretreatment of woody biomass is hydrothermal pretreatment under acidic conditions. This is especially relevant in an industrial context and for larger pilot plants and demonstration facilities. Steam-exploded material was prepared in the Biorefinery Demo Plant (BDP) at Domsjö Development Area in Örnsköldsvik, Sweden. The BDP has reactors for continuous steam explosion of biomass. For softwood, acidic impregnation chemicals, such as SA (sulfuric acid) or SD (sulfur dioxide), should be added during the pretreatment. Although it is known from previous studies that SD is advantageous for pretreatment of softwood, the reasons for this were not clear before the study presented in Paper IV.

The study in Paper IV differed from previous investigations by using continuous steam explosion and by using similar combined severities for pretreatments with SA and SD at three different temperatures (195°C, 205°C, and 215°C). Thus, if differences between pretreatment with SA and SD at the same temperature were observed, they could be attributed to different pretreatment chemistry rather than to different combined severity. Furthermore, the analytical tool-box used in the study presented in Paper IV was more extensive than in previous studies, and included not only convertibility, inhibition of both enzymes and microorganisms, and chemical characterization, but also physical characterization of the pretreated material.

The sulfur content in the washed pretreated material was five to six times higher in SD-pretreated material than in SA-pretreated material (Paper IV, Table 1). This difference can be attributed to sulfonation of lignin by bisulfite formed from SD through formation of sulfurous acid. As could be expected, the results suggest that the sulfonation reaction proceeded faster at higher temperatures.

The study presented in Paper IV showed a clear positive linear correlation between the fraction of fine-sized particles and the enzymatic digestibility of the pretreated spruce. This explained the superior performance of SD pretreatment compared to SA pretreatment. Delignification through SD pretreatment with concomitant hydrolysis of amorphous regions of cellulose would contribute to fractionation and increased formation of dust and fine particles in the pretreated spruce, and higher temperatures would result in more fractionation and formation of dust and fine-sized particles. The ASL content was found to be a key to understanding the effects of SD pretreatment. When more lignin was degraded, more ASL was formed. Regression analysis pointed towards a positive linear correlation between the ASL content and the fractions of dust and fine particles. Partial removal of lignin would contribute

to the decrease in the particle size and therefore improve enzymatic digestibility.

After acid pretreatment, the remaining solid mainly consists of lignin and cellulose. Lignin can affect enzymatic saccharification of cellulose in three ways: (*i*) Blocking the access of cellulolytic enzymes to the cellulose. (*ii*) Causing non-productive binding of enzymes. (*iii*) Inhibition of enzymes by water-soluble aromatic compounds derived from lignin (Table 1; Table 2; Kim et al., 2013; Malgas et al., 2016). After removal of hemicelluloses through acid pretreatment, the first effect may not be that relevant anymore. Catalytically non-productive binding of enzymes to lignin will reduce the efficiency of the enzymatic saccharification and at least partially prevent recycling of the enzymes.

To elucidate the effects of different pretreatment conditions on formation of inhibitory by-products, the inhibitory effects of the six steam-pretreatment liquids on enzymatic hydrolysis of Avicel were compared (Paper IV). The results showed that the inhibitory effects of the SD pretreatment liquids were higher than those of the SA pretreatment liquids, and that the inhibitory effect increased with increasing pretreatment temperature. The chemical analysis had shown that SD pretreatment caused delignification and that higher temperatures accelerated that reaction. As a consequence of that, phenolic substances were most abundant in the SD pretreatment liquid from the highest temperature (215°C)(SD-H). Determination of TAC (Total Aromatic Content) also showed that SD-H had the highest value (Paper IV, Table 2). It is reasonable to relate the high content of aromatics to the inhibition of enzymatic saccharification of Avicel. The inhibitory effects of phenolic substances on cellulolytic and other hydrolytic enzymes are supported by several studies (Ximenes et al., 2011; Cavka and Jönsson, 2013; Zhai et al., 2016; Malgas et al. 2016).

Besides having an inhibitory effect on the enzymes used for saccharification, by-products from acid pretreatment also have an inhibitory effect on microorganisms. The set of six steam pretreatment liquids were also used for investigation of the inhibitory effects on *S. cerevisiae* yeast (Paper IV). The results showed that SD pretreatment liquids were more inhibitory than SA pretreatment liquids. The result seems to contradict previous reports (Tengborg et al., 1998; Martín et al., 2002). However, both studies (Tengborg et al., 1998; Martín et al., 2002) were based on conventional batch steam pretreatment, whereas the study presented in Paper IV was based on an advanced continuous steam pretreatment process that removed almost all hemicellulose from the softwood.

9.3 Inhibition of microbial and enzymatic biocatalysts (Papers V & VI)

Microbial inhibitors in the steam pretreatment liquids from Norway spruce were further analyzed in the study presented in Paper V. This work especially addresses the lack of knowledge on formation and inhibitory effects of formaldehyde and *p*-benzoquinone. The concentrations of 10 individual inhibitors in the pretreatment liquids were determined: formaldehyde, furfural, 5-hydroxymethylfurfural (HMF), acetic acid, formic acid, levulinic acid, vanillin, coniferyl aldehyde, *p*-hydroxybenzaldehyde, and *p*-benzoquinone (Fig. 8). In addition, the total concentration of phenolics was measured using the Folin-Ciocalteu assay. While furfural and HMF are predominant among furanic compounds, and acetic acid and formic acid are predominant among carboxylic acids, it is difficult to point at quantitatively dominant compounds among the phenols. Furthermore, the biomass species will determine which phenolic compounds that are formed. While syringyl compounds will be abundant in hardwood hydrolysates, guaiacyl compounds will be predominant in softwood hydrolysates. Therefore, group analysis of phenols is typically a better way to estimate their abundance than analysis of a few selected phenolic compounds. The results of the quantitation of inhibitors showed that the concentrations of inhibitors were generally higher in SD-pretreated material than in SA-pretreated material (Paper V). This explains the previous report (Paper IV) that SD-pretreated material had higher toxicity than SA-pretreated material.

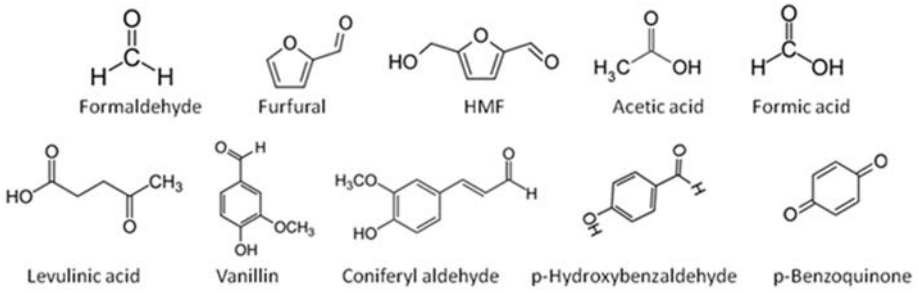


Fig. 8 Structure of 10 individual inhibitors detected in the steam pretreatment liquids.

The inhibitory effects of the quantitated inhibitors were compared with each other (Paper V), except for *p*-benzoquinone, which was detected only in very low concentrations. Toxicity rankings based on molar toxicity showed that coniferyl aldehyde and formaldehyde exhibited the strongest molar toxicity. Coniferyl aldehyde had much higher toxicity than the two other phenols studied, namely vanillin and *p*-hydroxybenzaldehyde. The result shows that the

toxicity of the phenolic constituents is related to the exact composition of the total phenolic content. It is probable that the toxicity of the total phenolics would be somewhere in between that of *p*-hydroxybenzaldehyde and vanillin (relatively low inhibition) and that of coniferyl aldehyde (relatively high inhibition).

When the actual concentrations of individual inhibitors in the pretreatment liquids were taken into account, formaldehyde was the most important inhibitor (Paper V). Furan aldehydes (furfural and HMF) and aliphatic acids such as acetic acid are present in high concentrations in pretreatment liquids and are easy to quantitate using HPLC. Furan aldehydes and acetic acid have received a lot of attention during the past decades. However, the work presented in Paper V shows that it is not always the inhibitors that are present in the highest concentrations and the ones that are easiest to analyze that are most significant with respect to inhibition.

The work presented in Paper IV showed the inhibitory effects on enzymes of water-soluble substances in the pretreatment liquids from steam-exploded softwood. As many other studies of enzyme inhibition of substances in pretreated liquids, the results point towards aromatic compounds being most significant. Aromatic substances, such as lignin and pseudo-lignin, are also present in the solid fraction. The investigation presented in Paper VI addressed the problem with catalytically non-productive binding of individual isozymes to lignin and pseudo-lignin. This investigation was based on studies of complex systems in which different enzymes and isozymes compete for the adsorption sites on lignin and pseudo-lignin. The lignin preparations studied were EnzHR lignin from softwood (Norway spruce) and hardwood (birch), organosolv lignin from hardwood (beech), and pseudo-lignin prepared from birch xylan. The investigation differs from many other studies in the field through the detailed comparison of protein adsorption to lignin and pseudo-lignin, and through the use of both conventional methodology and quantitative proteomics to analyze the adsorption phenomena.

The comparison of lignin preparations and pseudo-lignin indicated that catalytically non-productive adsorption of hydrolytic enzymes was similar or even more extensive for pseudo-lignin compared to the hardwood lignin preparations, i.e. EnzHR lignin from birch and organosolv lignin from beech. However, protein adsorption to EnzHR lignin from spruce was more extensive than adsorption to the other lignins and to pseudo-lignin. The high protein adsorption affinity of EnzHR lignin from softwood could be related to its high surface area, as revealed by BET analysis. This explanation could not apply to pseudo-lignin, which retained the low surface area of the starting material. The mechanisms behind protein adsorption to pseudo-lignin need to be addressed further in future research. It is a difficulty in research in this area that the properties of the lignin preparations, and most probably also the properties of

pseudo-lignin preparations, are dependent not only on the biological origins of the material but also on the preparation methods.

Analysis using quantitative proteomics based on TMTsixplex tagging of peptides revealed detailed information about individual isoenzymes involved in the degradation of cellulose and xylan. The data, which are further supported by measurements of adsorption of enzyme activities and analysis of SDS-PAGE gels stained with SYPRO Ruby, suggest that catalytically non-productive adsorption of some enzymes, such as the *T. reesei* CBM-containing enzymes CBHI, EGII, and EGIV, shows little or no dependence on the properties of the lignin preparations. Adsorption of some other enzymes, such as *T. reesei* xylanases and xylosidase, seemed to be more dependent on the properties of the lignin and pseudo-lignin preparations. Hydrophobic interactions between the CBMs of fungal cellulases and lignin have been proposed to be the main reason for catalytically non-productive binding of cellulases to lignin (Palonen et al., 2004; Pareek et al., 2013; Qin et al., 2014; Yang and Pan, 2016). The results presented in Paper VI agree with the notion that CBMs play an important role in this context.

10. Conclusions and further perspectives

Overcoming the recalcitrance is an essential step in bioconversion of lignocellulosic feedstocks. A better understanding of the different factors that affect recalcitrance is helpful for utilization of the feedstock in the most efficient way. Among factors that affect recalcitrance, the cell wall structure has often been ignored. Changes in the chemical composition, such as lignin content, hemicellulose content, and acetyl content, can affect the cell wall structure, which might be the underlying reason why the recalcitrance is different. Even though the chemical composition of different types of biomass might be similar, the cell wall structure can still be very different. Therefore, it is advantageous to analyze not only the chemical composition but also structural features when the recalcitrance of different types of lignocellulosic feedstocks is compared.

It is commonly recognized that pretreatment is required to reduce the recalcitrance of lignocellulosic feedstocks. The techniques of pretreatment are continuously developing. Pretreatment with ILs as green solvents draws attention due to the possibility to use mild reaction conditions. The pretreatment efficiency of ILs was significantly affected by the anionic constituent, but that was related to the properties of the feedstock. Furthermore, the results presented in the thesis strongly suggest that the effects of IL pretreatment on biomass structure have been neglected in comparison to the effects on chemical composition. A detailed analysis of the mass balance indicated that a large fraction of the biomass could be lost during IL pretreatment, which might pose a problem with respect to processes that are highly yield sensitive. Although using acetate as anion worked well for improving the cellulose digestibility of the studied lignocellulosic biomass, the sensitivity to moisture is a difficulty that makes the pretreatment difficult to perform in a practical way. Further research and development is needed to make pretreatment with ILs industrially feasible.

Hydrothermal pretreatment under acidic conditions is by far a more mature technology than IL pretreatment, and industrial implementation has already commenced. However, there are many different ways to carry out hydrothermal pretreatment, and the underlying reasons behind why some ways are better than others are sometimes poorly understood. The research presented in the thesis shows that continuous steam pretreatment with acid catalyst very efficiently removed the hemicellulosic content from softwood, typically regarded as the most challenging lignocellulosic feedstock, and greatly improved the digestibility of the cellulose. The investigation showed fundamental differences between using SA and SD as the acid catalyst. Even if it has been known previously that SD is superior for pretreatment of softwood, the underlying reasons were not well understood. Future work in this area can

address similar questions for other types of feedstocks, such as hardwood and agricultural residues.

Acid pretreatment technologies have many advantages, but will also result in formation of substances that inhibit cellulases and fermenting microorganisms, such as yeast. A comparison of fractions from the steam-pretreatment of spruce showed that formaldehyde was most significant among the water-soluble substances that inhibit yeast. Despite decades of research efforts in area, typically with focus on inhibitors such as furan aldehydes and aliphatic carboxylic acids, the important role played by formaldehyde has been unknown up to now. As a consequence of that finding, future studies of detoxification techniques and adaption or engineering of microorganisms for improved resistance to inhibitors would need to take formaldehyde into account besides more well-known inhibitors.

It is a well-known phenomenon that lignin in the solid phase of pretreated biomass impairs enzymatic saccharification of cellulose through catalytically non-productive binding of enzymes. However, pretreated biomass typically also contains pseudo-lignin. Using both conventional methodology and quantitative proteomics, we showed that pseudo-lignin cause non-productive adsorption of hydrolytic enzymes and other proteins on a level that was similar and sometimes even higher than that of lignin preprations from hardwood. Protein adsorption to lignin prepared from softwood was more extensive than adsorption to lignin preparations from hardwood and to pseudo-lignin. Softwood requires more severe pretreatment conditions than hardwood and agricultural residues, which means that pretreated softwood will also contain more pseudo-lignin. The results explain two of the reasons why bioconversion of softwood is more challenging than conversion of other feedstocks. The results also suggest that the adsorption to lignin of some proteins is more dependent on the lignin quality than the adsorption of other proteins. This phenomenon requires further attention in future studies. A better understanding of protein properties associated with strong adsorption to lignin may be helpful for engineering enzymes that show little or minimal non-productive adsorption and that exhibit improved efficiency for saccharification of pretreated biomass that is rich in lignin and pseudo-lignin.

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