ABSTRACT

FRITZ, CONSUELO. Lignocellulosic Materials and their Interactions with Proteins and Surface Active Molecules. (Under the direction of Dr. Orlando Rojas and Dr. Hasan Jameel)

Lignocellulosic biomass is a highly valuable resource for the development of value-added biobased chemicals and other materials. However, the efficient utilization of this biomass regards on understanding the different interactions among other components. For example, proteins interactions are relevant in several applications, where enzymatic hydrolysis is needed to obtain different products, especially sugars. Moreover, the heterogeneity and complex chemical and physical structure of this biomass have been identified as the major drawbacks during lignocellulose bioconversion. On the other hand, surface active molecules have been used during bioconversion processes; however, the fundamental interactions with lignocellulose are not fully known. Therefore, this work systematically investigates the interactions among proteins, surfactant, and the most abundant biopolymers in nature, namely cellulose and lignin.

Pretreatment of biomass is performed in order to deconstruct the cell wall and open the structure for subsequent stages. Several different pretreatments have been investigated but they are usually done at high temperature and pressure. A novel approach using complex systems for green biomass pretreatment is investigated under atmospheric pressure and low temperature. It is hypothesized this system can overcome the complex structure of wood by taking advantage of the hydrophobic interactions between the mainly components (lignin, surfactants, oil, proteins). A ternary diagram is built and an appropriated microemulsion formulation is analyzed and used. The effects of this pretreatment on woody material are analyzed by crystallinity, thermogravimetric analysis, and spectroscopy techniques. Sugar
products are quantified after enzymatic hydrolysis. Overall, microemulsions affect the chemical-physical properties of biomass in a larger extent compared with aqueous systems.

Moreover, the non-productive interactions between lignin and enzymes are monitored in situ and in real time using sensitive surface techniques, as quartz crystal microbalance and surface plasmon resonance. Other interactions are also investigated, such as proteins-cellulose, surfactant-cellulose, surfactant-lignin, proteins-surfactant-cellulose, and proteins-surfactant-lignin. As a result, surfactant binds to lignin mainly due to hydrophobic interactions. Remarkably, the enzyme affinity towards lignin is reduced by using surface active molecules without affecting enzyme digestibility.

Understanding self-aggregation and colloidal properties of lignin in aqueous conditions is relevant since there is a growing interest to use kraft lignin in other applications rather than burning it. Here, the colloidal stability of lignin in solution is tested under the addition of surfactants and salts by monitoring changes in size and rheological properties. It is demonstrated the disruptive effect of salt, whereas the interaction with a non-ionic surfactant can improve stability. These results may open new opportunities on applications where a control over aggregation is needed, for example, filtration and separation of lignin from wastewater and lignin nanoparticles formation.
Lignocellulosic Materials and their Interactions with Proteins and Surface Active Molecules

by

Consuelo Fritz

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APPROVED BY:

_______________________________  ________________________________
Dr. Orlando J. Rojas              Dr. Hasan Jameel
Co-Chair of Advisory Committee    Co-Chair of Advisory Committee

_______________________________  ________________________________
Dr. Carlos L. Salas Araujo        Dr. Mari S. Chinn
Member of Advisory Committee      Member of Advisory Committee
DEDICATION

This work is dedicated to my family, especially to my husband. You all gave me your love and support whenever I needed and taught me to fight hard to achieve my goals. Thank you, I love you!!
BIOGRAPHY

I was born and grew up in the capital city of Chile, Santiago. I did my undergraduate studies at Universidad de Chile where I received the bachelor degree of Wood Sciences in 2009. After graduation I worked for the Universidad de Chile as a researcher and researcher assistant in several projects related to bioenergy from biomass, assessment of environmental services, and pruning recycling. In 2010 I was granted with a Fulbright and Becas Chile scholarships to study a doctoral program. In 2012 I moved to Raleigh, North Carolina to start my Ph.D. in Forest Biomaterials under the direction of Dr. Orlando Rojas and Dr. Hasan Jameel.
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I want to take this opportunity to express my gratitude to people who has been very important during these 4 years not only in the academic area but also in my personal life. All of you have impacted my life in such a positive manner and I cannot fail to name you since your support made me continue to this moment.

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**Figure S5.** Representative QCM-D curves (top) for adsorption of: a) cellulases (5 mg/ml) and b) monocomponent CBH-I (1 mg/ml) onto CNF and CNF coated with polyoxyethylene (20) sorbitan monooleate at 6 xCMC, pH 5.5, and 40 °C. AFM images at 5x5 μm before c) and after enzymes treatment d). Zeta potential value for CNF, Ctec2, and CBH-I was -32.9, -9.6, and -7.1 mV, respectively at pH 5.5. For surfactant adsorption the number of layer obtained by dividing the adsorption density at the solid/liquid interface by that at air/liquid interface was found to be 0.2. 106

**Figure S6.** a) Representative QCM-D profiles showing enzyme binding and hydrolysis of NFLC with and without the presence of non-ionic surfactant polyoxyethylene (20) sorbitan monooleate at 6 xCMC, pH 5.5, and 40 °C. AFM images of NFLC b) before and c) after enzymatic hydrolysis. Images at 5 x 5 μm. 107

**Figure S7.** Effect of different surfactants on the aggregation behavior of aqueous lignin solutions (0.05% wt lignin, pH 10.5, 70 °C) as a function of time, with and without salt addition. The inset pictures show the samples after 120 h incubation at 70 °C. In the case of cationic surfactant, lignin precipitation occurred as indicated by the sharp decrease in turbidity. 134

**Figure S8.** Turbidity for lignins as a function of time at various NaCl concentrations and non-ionic surfactant (POE20 at 6×cmc) at pH 10.5 and 70 °C. Aggregation was significant for (A, B) HWK and (C, D) SWK2 samples but not for (E, F) SWK1. The inset in E and F show the low turbidity values obtained for this lignin sample. The rate of aggregation was calculated from the slope between 30 to 120 min. 135

**Figure S9.** Effect of electrolyte and surfactant on surface tension of the different lignin aqueous solutions after incubation for 30 min at 70 °C. The open symbols correspond to the surface tension measured after cooling down at 25 °C, whereas the closed symbols are the values determined at 70 °C. 136

**Figure S10.** Final turbidity of black liquor samples after equilibration (24 h at 70 °C) in the presence of different salts and non-ionic surfactant concentrations: POE20 (up triangle), NaCl (down triangle), Ca²⁺ (open circle), and a mixture of POE and Ca²⁺ (solid circle). A control sample was included (square). The concentrations used are shown in the plot. The pH was kept at 12. 137
List of Publications and Contributions

This dissertation includes the following publications:


Publications not included in this dissertation:


xviii
1 INTRODUCTION

1.1 Motivation

The development of valued-added product from lignocellulosic biomass is increasing; however, its complex chemical composition is a major barrier. The main reason for this is the presence of cross-link between cellulose and hemicellulose with lignin via ester and ether linkages, which originates biomass recalcitrance (1). Therefore, it is important to understand the chemistry of biomass in order to deconstruct the material into components that can be chemically or catalytically converted into biomass-derived fuels, chemicals, composites, or reactive intermediates.

For example, many efforts have been developed to minimize the interactions between lignin and enzymes in order to increase hydrolysis efficiency for producing fermentable sugars. First, it is necessary to fully understand how lignin affects the enzymatic hydrolysis, and then, it is necessary to decrease that effect. Surfactants can improve hydrolysis of lignocellulose material because they can interact with lignin to avoid enzyme-lignin interactions (2). Although there are several reports about the effects of surfactants on cellulose conversion, their mechanism on enzymatic hydrolysis of lignocellulosic material needs to be fully understood.

Impregnation, diffusion, and solubilization are key parameters during processes involving lignocelluloses. These factors allow uniform and complete distribution of chemicals into the cell wall structure (3). Surfactants and complex systems can be potentially used for solubilization and transportation of chemical components into the cell wall. An interesting
finding is that surfactants help to remove hydrophobic components of the cell walls as well as lignin and hemicelluloses that pose physical and chemical constraints for effective enzymatic action (4). Microemulsions effective and efficiently overcome the complex capillary structure of woody biomass by taking advantage of the low surface tension. Therefore, penetration efficiencies were found higher than pure water at room temperature and atmospheric pressure (5, 6). On the other hand, among the different technologies used for biomass pretreatment, alkaline solutions have been applied under relative low temperatures and pressures (7). Therefore, a microemulsion in the presence of alkaline chemicals is explored as a new and green pretreatment.

Moreover, over 70 million tons of lignin are produced annually worldwide, mainly as by product from the kraft pulping process but only 5% is used in commercial applications (8). Understanding the aggregation behavior of lignins and derivatives is relevant to their applications, especially towards value-added products. The colloidal stability of lignin has been studied since the 1940s (9-14) and there is evidence to consider it as a polyelectrolyte in alkaline media (15). Self-association of lignin is mainly controlled by temperature, pH, ionic strength, and molecular weight, which affect the balance between the repulsive and attractive forces. Lignin precipitation can cause problems in a system and re-dissolution may be possible by increasing hydroxide ions but this action can be detrimental in industrial processes (16). Surfactants affect the colloidal stability by steric stabilization (17, 18) or bridging (18) mechanisms. Consequently, investigating the colloidal behavior of lignin, especially kraft lignin, and highlighting their differences and effects of aqueous dispersion conditions are critical to pursue high value products from a biorefinery process.
1.2 Goals

The primary goal of this project is to understand the interactions among lignin, cellulose, and proteins and the effect of surface active molecules on these interactions. First, a complex system was chosen to investigate the effect of complex systems on biomass conversion by taking advantage of their ability to penetrate lignocellulosic biomass and assist in the transport of active chemicals during pretreatment. Different conditions were tested on hardwood biomass. Then, a more fundamental approach was utilized to investigate the interactions on model surfaces with surface active molecules and proteins. For this, model lignin and nanocellulose thin films were developed. QCM and SPR were chosen to monitor these biomolecular interactions in order to elucidate non-productive interactions and reducing them with the aim of surfactants. Thermodynamic and kinetic parameters were derived from QCM analysis for adsorption and hydrolysis. Finally, the colloidal behavior of lignin was studied since its extent of application is greatly influenced by its colloidal properties. Overall, this project is contributing with comprehensive knowledge to develop an efficient biorefinery industry.

1.3 Overview of this thesis

This dissertation focuses on the study of the interactions among lignocellulosic biomass, proteins, and surface active molecules in order to increase the knowledge and understanding in a biorefinery based on this renewable material.
Chapter 2 provides a comprehensive review about lignocellulosic material, its composition, and the interactions that take place among the different components, as well as the pretreatment technologies used to produce relevant value-added products in a biorefinery. Also, it includes a briefly description of the main techniques used to characterize these interactions and other important information, such as quartz crystal microbalance, surface plasmon resonance, dynamic light scattering, and electrophoretic mobility. A description is also provided about the isolation procedures for lignin and nanocellulose in order to produce thin films. The critical features of surfactants/oil/water complex system is reviewed as well as its utilization as a treatment for woody material.

Chapter 3 exhibits a new chemical pretreatment based on surfactants/oil/water system to disrupt the chemical structure of woody biomass in order to obtain monomeric sugars. The effect of this complex system or microemulsion on chemical structure and physicochemical properties of wood is investigated. This system was able to penetrate the biomass, altered its chemical composition thus making the cellulose more accessible to enzymes.

Chapter 4 investigates the interactions among lignin, cellulose, cellulases, and surface active molecules. First, the interaction between lignin and cellulases is confirmed to be non-productive, where the driving forces are hydrophobic in nature. The surface active molecules are able to bind to lignin through hydrophobic interactions, which reduces the affinity of cellulases for lignin in surfactant-coated lignin films. No negative effect was found for the enzymatic hydrolysis of nanocellulose when it is coated by surfactants.
Chapter 5 presents an investigation on the colloidal behavior of lignin under alkaline conditions. Lignin behaves as a polyelectrolyte in aqueous medium and its further utilization depends on understanding its behavior. Salts, temperature, and surface active molecules are able to affect lignin aggregation. The effects of these factors are investigated, where salts are found to be the worst in maintaining colloidal stability while surfactants can be beneficial at certain level in terms of stability. Therefore, the present study investigates the colloidal behavior of different types of kraft lignins and unveils the effect of aqueous dispersion conditions, extensive also to respective submicron lignin particles and lignin in so-called black liquors.

Chapter 6 summarizes all the main conclusions above and provides recommendations for future work.
2 LIGNOCELLULOSES, PROTEINS, AND SURFACE ACTIVE MOLECULES: A REVIEW.

2.1 Lignocellulosic biomass, its recalcitrance, and colloidal aspects

Lignocellulosic materials, which include wood and non-woody (agricultural) biomass, are renewable resources that can be used for the production of a wide variety of value-added bio-products, including biofuels, chemical, pulp and paper, enzymes, nanomaterials, composites, among others. However, their use is restricted by the complex association that exists among the three main components of the cell wall, cellulose, hemicellulose and lignin (1).

The plant cell wall is built up by different layers and its composition and distribution is illustrated in Figure 2.1. Consisting of primary wall, three secondary walls (S1, S2, and S3), and a lumen. The middle lamella is located between the cells and its function is to maintain the cells together. Primary wall is main constituted by cellulose, hemicellulose, pectin and proteins, and embedded lignin. The structural features of this material is briefly reviewed in the next sections.
Figure 2.1. Composition of the cell wall. The cell wall is divided in primary and secondary wall. Middle lamella is between cells. A magnification of a cellulose microfibrils is presented and hemicelluloses and lignin are located between them (19).

The major difference between woody and non-woody biomass are they chemical and physical compositions. Woody biomass contains more lignin than non-woody because it is designed to be resistant to biological or microbial degradation, which makes it more recalcitrance. However, Figure 2.2 shows a basic chemical composition of lignocellulosic material in general.

Figure 2.2. General chemical composition of lignocellulosic biomass (20)
2.1.1 Carbohydrates and plant cell walls components

Cellulose is the most abundant component in plant cell walls (21) and it is a linear homopolysaccharide that consists of glucose units linked by β-(1,4) glycosidic bonds, which repeating units is named cellobiose (Figure 2.3). Its degree of polymerization (DP) is approximately 10,000 for wood cellulose and 15,000 for cotton (22). Due to this linearity, adjacent cellulose chains form a structure called elementary fibrils which contains both crystalline and amorphous regions. The lattice forces are responsible for maintaining the crystalline regions due to inter and intramolecular hydrogen bonding (22). Due to this strong structure, cellulose exhibits a high tensile strength and is insoluble in most solvents. The crystalline structure of cellulose has been extensively studied and four different polymorphs has been identified: Iα, Iβ, II, III, IVI, IVII (19, 22, 23), which have different physical properties (21).

Hemicellulose is a heteropolysaccharide formed by a wide variety of components, pentoses, hexoses, and uronic acids (24). It is more related to cellulose than lignin and this association gives a great stability to the aggregate due to hydrogen bonds and van der Waal forces with cellulose (22). The hemicellulose content of softwoods has a higher content of glucomannans and galactomannans (20 wt%) and low content of xylans (5-10 wt%); in contrast, hardwood presents a higher proportion of glucuronoxylans (15-30 wt%) and low content of glucomannans (2-5 wt%) (22). Hemicellulose plays an important role during pulping since its removal caused larger pores to be produced in the alkali-treated fiber walls, thereby facilitating the removal of lignin (25).
2.1.2 Lignin and lignin-carbohydrates complexes

Lignin is defined as a large group of aromatic phenolic polymers, resulting from the oxidative combinational coupling of 4-hydroxyphenylpropanoids, which are called monolignols (Figure 2.4a). Depending on the number of methoxy groups (OCH$_3$) attached to the phenolic unit, it is possible to distinguish p-coumaryl alcohol, none group; coniferyl alcohol (guayacil unit), one group; and sinapyl alcohol (syringyl unit), with two methoxy groups (Figure 2.4b). Lignin concentration is high in the middle lamella and low in the secondary wall, whereas the highest proportion is found in the later. Softwood contains 26-32 wt% lignin, while hardwoods contains lower amount, 20-28 wt%. (22). Softwoods are composed of coniferyl alcohol (lignin type G), whereas hardwood contains both coniferyl and synapyl (lignin type GS). The carbohydrate conversion has been found to be beneficial at high S/G ratio (27). Generally, the lignin type S would tend to form a more linear chain with less cross-linking than G-rich lignin because this last type has one methoxy group on the C3 position leading the C5 free to participate in branching reactions (28). As a result of this branching, softwood lignin exhibits highly stable β-5 and 5-5 linkages (Table 2.1) (29). In other words, hardwood lignins are more suitable to chemical conversion than softwood because guaiacyl lignin is likely to be spread and distributed, which restricts the swelling of the cellulosic fibers and reduce surface
area for enzymatic hydrolysis (30). Lignin distribution and composition probably constrains the access of cellulases to cellulose. In fact, Ramos et al. (30) suggested that guaiacyl lignin restricts fiber swelling more than syringyl type lignin. In addition to lignin content, the cell wall thickness and microfiber orientation also contribute to the carbohydrate degrading enzymes ability to get cellulose and hemicellulose (31). Furthermore, there are covalent linkages between lignin and polysaccharides given the existence of lignin-carbohydrate complexes (LCC) (22). Therefore, there is an unfavorable correlation between the amount of lignin and the successful hydrolysis of woody biomass, especially in softwood species (32).

**Figure 2.4.** Lignin units. Monolignols, M, or primary lignin monomers (a), lignin polymer units, P, (b), and major structural units in the polymer (c) (33)
Table 2.1. Common linkages between monolignols in lignin (34).

<table>
<thead>
<tr>
<th>Bond</th>
<th>Percentage of total bond linkages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Softwood</td>
</tr>
<tr>
<td>β-O-4</td>
<td>50</td>
</tr>
<tr>
<td>α-O-4</td>
<td>2-8</td>
</tr>
<tr>
<td>β-5</td>
<td>9-12</td>
</tr>
<tr>
<td>5-5</td>
<td>10-11</td>
</tr>
<tr>
<td>4-O-5</td>
<td>4</td>
</tr>
<tr>
<td>β-1</td>
<td>7</td>
</tr>
<tr>
<td>β-β</td>
<td>2</td>
</tr>
</tbody>
</table>

2.2 Enzymes for lignocellulose

Due to the heterogeneity of lignocellulosic biomass, a synergistic action of different enzymes is needed for its degradation, such as cellulases, hemicellulases, and lacasses and peroxidases. These enzymes are able to degrade cellulose, hemicellulose, and lignin substrates, respectively. The major structural features that restrict the enzymatic degradation of cellulose (low reaction rate and low conversion yields) include: degree of water swelling (35); specific surface area (36); pore size (36, 37); degree of polymerization (36); crystallinity (1, 35, 36, 38); molecular arrangement and content of other associated macromolecules, such as lignin (39); and the capillary structure of cellulosic fibers, which make the lignocellulosic material highly recalcitrance to microbial degradation (1). Crystallinity and capillary structure of cellulose have been recognized as the most important structural features since the hydrolysis of cellulose is determined by the accessibility of enzymes to the cellulose surface (1, 35, 36, 38, 40).
2.2.1 Cellulases

Cellulases represent a group of hydrolytic and oxidative enzymes which degrade cellulose in nature. Typically, cellulases are produced by fungi and bacteria (41) and are able to catalyze the cleavage of β-1,4 glycosidic bonds on cellulase. A classical enzyme system based on *Trichoderma* cellulases includes: (i) endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as celloextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) β-glucosidases or β-D-glucoside glucohydrolases (cellobiases) (EC 3.2.1.21), and their physical properties as shown in Table 2.2. A simplify illustration of the enzymatic hydrolysis of cellulose is presented in Figure 2.5. Endoglucanases start the degradation by randomly cleaving the amorphous site of cellulose chain, reducing the degree of polymerization, and generating oligosaccharides (41). Cellobiohydrolases attack the reducing and non-reducing ends of cellulose chain, releasing mainly cellobiose (42). Finally, cellobiases hydrolyze cellobiose to glucose.

Table 2.2. Physical properties of *Trichoderma reesei* cellulases (43, 44)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total number of aminoacids</th>
<th>Molecular weight (kDa)</th>
<th>Isoelectric point</th>
<th>Position of the CBM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBHI (Cel7A)</td>
<td>497</td>
<td>59-68</td>
<td>3.5-4.2</td>
<td>C</td>
</tr>
<tr>
<td>CBHII (Cel6A)</td>
<td>447</td>
<td>50-58</td>
<td>5.1-6.3</td>
<td>N</td>
</tr>
<tr>
<td>EGI (Cel7B)</td>
<td>437</td>
<td>50-55</td>
<td>3.9-6.0</td>
<td>C</td>
</tr>
<tr>
<td>EGII (Cel5A)</td>
<td>397</td>
<td>48</td>
<td>4.2-5.5</td>
<td>N</td>
</tr>
<tr>
<td>EGIII (Cel12A)</td>
<td>218</td>
<td>25</td>
<td>6.8-7.5</td>
<td>No</td>
</tr>
<tr>
<td>EGIV (Cel61A)</td>
<td>225</td>
<td>23</td>
<td>7.4-8.7</td>
<td>No</td>
</tr>
<tr>
<td>EGV (Cel45A)</td>
<td>713</td>
<td>75</td>
<td>4.8</td>
<td>C</td>
</tr>
<tr>
<td>β-GI (Cel3A)</td>
<td>700</td>
<td>114</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C means C-terminal and N, N-terminal
Figure 2.5. Simplify mechanism of enzymatic hydrolysis of cellulose by cellulases. (a) Initial cellulose consisting of crystalline and amorphous regions. (b) Partially hydrolyzed cellulose. (c) Outer solution containing cellobiose (disaccharide) as a major intermediate product, together with minor amounts of higher oligosaccharides and glucose. (d) Final glucose monomers. The open circles represent anhydroglucose residues in cellulose and oligosaccharides; the solid circles represent reducing ends of cellulose and oligosaccharides or glucose (45).

2.2.2 Hemicellulases and lacasse-peroxidases

Hemicellulases correspond to a groups of enzymes which hydrolyze different hemicellulosic components. Xylanases and mannanases are responsible for degrading xylan and mannan, respectively. Two types of enzymes catalyzed the degradation: depolymerized
enzymes, which are involve in the cleaving of the polymer backbone; and accessories enzymes that act on the polymer branches. In the case of xylan, the formers are endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8), which releases oligosaccharides, and β-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37), which hydrolyzed these oligosaccharides into xylose. In the case of xylan the depolymerized enzymes are endo-1,4-β-mannanases (EC 3.2.1.78) and exoacting β-mannosidases (EC 3.2.1.25).

Lacasses (EC 1.10.3.2, blue-copper enzymes), lignin peroxidases (EC 1.11.1.14), and manganese peroxidases (EC 1.11.1.13) catalyzed reactions in a similar way (46). Lacasses and peroxidases oxidize phenolic (and non-phenolic compounds in the case of lacasses (47)) which results in the formation of highly reactive phenoxy radicals on the substrate, undergoing polymerization and depolymerization reactions (48).

This study will be focus on cellulases, therefore, the following section describe the interactions of cellulases with the different lignocellulosic components.

2.3 Cellulases interactions with lignocellulose

The interaction and adsorption of cellulases among the different components in the lignocellulosic biomass have diverse behavior. These enzymes are able to interact with cellulose hydrolyzing it into oligomers and monomers. However, lignin represents a major obstacle for this purpose.
2.3.1 Cellulase-cellulose interactions

Among the different cellulases, some preferentially hydrolyze amorphous regions, whereas others are able to attack highly ordered crystalline regions. However, all cellulases shared a modular structure composed of a catalytic domain (CD) linked to a cellulose binding module (CBM) (49), which is responsible for a highly binding to cellulose surface (50, 51), especially at hydrophobic faces of cellulose (52, 53). Remarkably, the CBM has been even observed to decrease the specific activity of the adsorbed enzyme by presumably preventing the hydrolysis of cellulose through unproductive binding (54). Recent studies have been focused in the effect of this CBM on the hydrolysis of cellulose (55-57) without consensus if its presence is improving or reducing the sugars release.

2.3.2 Cellulase-lignin interactions

The cellulase-lignin interaction is the most relevant type of interaction since it deals with a hydrophobic residual component from the pretreatment of the biomass, lignin, which presence and surface distribution is detrimental during enzymatic hydrolysis of lignocellulosic biomass (44, 56, 58-60). The mechanism of lignin inhibition is mainly due to a physical or steric hindrance of the cellulose (61) or through a reversible/irreversible adsorption of cellulases into lignin instead of cellulose (62-64). Hydrophobic (2, 39, 65), electrostatic (39), and hydrogen bonds (66) have been suggested as the main interactions forces that contribute to the enzymes affinity towards lignin. These forces have been measured using atomic force microscopy (AFM) (67), indicating that adhesion forces between kraft lignin and cellulase were on average 45% higher than forces between hydroxypropyl cellulose and cellulase.
Moreover, using modified AFM tips to represent hydrophobic, H-bonding, and charge-charge interactions, it was found that forces between hydrophobic tips and cellulase were on average 43% and 13% higher than forces between cellulase with tips exhibiting OH and COOH groups, respectively. Therefore, hydrophobic interactions seem to govern the cellulases non-productive binding to lignin.

### 2.3.2.1 Hydrophobic interactions

Hydrophobic interactions are favorable entropic contributions associated to changes in water structuring; as a result, hydrophobic molecules tend to aggregate in order to minimize the surface area exposed to water. This type of interaction has been recognized as the primary driving force on proteins adsorption (68). It is expected that the proteins absorb in a large extent onto hydrophobic surfaces. The amino acids exposed on the surface of T. reesei enzymes have different hydrophobic character (43, 44, 69) (Table 2.2) and therefore the cellulases may bind differently to the hydrophobic surface of lignin. Lignin is considered hydrophobic, thus causing a nonspecific or nonproductive interaction with cellulases (63). The hydrophobic interactions between lignin and enzymes have been discussed by Palonen et al. (64) who found that the carbohydrate binding module (CBM) of Trichoderma reesei enzymes plays a significant role in the non-productive adsorption on lignin. In the case of Cel7A enzyme onto lignin, it was found a faster and larger adsorption extent compared to the Cel7A without CBM, evidencing that hydrophobic interactions are a key driver in the adsorption process (59). However, that interaction was found to be pH-dependent for Cel45A (58), suggesting that electrostatic interactions also play a role during adsorption.
2.3.2.2 Electrostatic interactions

Electrostatic interactions between molecules/polymers can be either attractive or repulsive depending on the signs of the charge. The presence of charged (COOH, OH) or partially charged (CO) functional groups on both lignin and enzyme surface could also mediate ionic-type lignin-enzyme interactions (62). The dissociation or association of the surface groups is one of the major mechanisms causing a surface charge. If both the protein and polymer surface have the same charge, they repulse each other. If they possess opposite charges, they adsorb. pH not only affect substrate surface charge, but also cellulases charge (69). Usually, enzymatic hydrolysis is carried out at pH 4.8, at which some cellulases carry negative charges whereas others are in fact oppositely charged, inducing attraction with lignin (39). In contrast, enzymes and lignin become negatively charged at high pH and thus electrostatic repulsion reduces their affinity (70). The main acidic groups that exist on the surface of lignin are carboxylic acids (71, 72), which display different isoelectric points and thus affect to different degree the interactions, depending on the pH of the medium. The amount of carboxylic groups present in the substrate varies depending on the plant species and the method used in its isolation. In milled wood lignin from aspen and pine these groups are measured to be 0.28 and 0.30 mmol per gram of lignin, respectively, whereas from technical hardwood and softwood these increase to 0.41 and 0.47, respectively (73).

2.3.2.3 Hydrogen bonds

Hydrogen bonding occurs through hydroxyl groups present in lignins and enzymes (62, 63, 66). Among these groups, the phenolic hydroxyls are directly responsible for enzyme
adsorption (63, 66, 74), while aliphatic and carboxylic hydroxyl groups have been indicated to contribute to lignin-enzyme ionic interactions (62, 75). Pan (66) verified the block effect on enzyme digestibility by esterified the lignin’s phenolic hydroxyls groups (hydroxypropylation) and found that the inhibitory effect was decreased. Among the phenols, tannic acid is a major inhibitor and deactivator for cellulases, especially for β-glucosidases (74). Despite the evidence accumulated, however, the detailed nature of these interactions still needs further examination.

2.4 Lignin colloidal behavior in solutions

The colloidal stability of lignin has been studied since the 1940s (9-14). It is known that KL in alkaline conditions starts to coagulate at elevated temperatures due to the decrease in dissociation of phenolic groups, sometimes to levels below the threshold of solvency (16). Moreover, KL starts to coagulate and precipitate at high concentration of monovalent metal ion salts in neutral (9) and alkaline (76, 77) conditions. Lignin contains weakly acidic groups, namely phenolic and carboxyl groups. It has been found that the pKa value of these groups is correlated to the temperature, ion strength and the solvent used (78). The apparent pKa of kraft lignin has also been found to be a function of the molecular weight of lignin (79, 80). There is evidence to consider lignin as a polyelectrolyte in alkaline media (15), exhibiting a marked critical coagulation concentration (ccc) of added electrolytes (10). Moreover, the colloidal behavior of KL can be described by the DLVO theory (81, 82), which establishes that the interaction forces are determined as the contributions from the van de Waals and the double layer interactions. If the attractive forces, van de Waals and other hydrophobic forces,
dominate the system, then aggregation is promoted. In contrast, if repulsive electrostatic forces between the lignin molecules dominate, then colloidal stability is provided. However, the structural characteristics of lignin, such as its functional groups (80), and the conditions of the solution, such as temperature, ionic strength, and pH affect a stable lignin colloidal dispersion (14).

The mechanism of lignin precipitation in the alkaline region can be regarded as a protonation of ionized acid groups on the lignin molecule (83), especially because of the carboxylic groups added during pulping (84), which lead to an increased lignin surface charge that results in a low negative zeta potential. The zeta potential (\(\zeta\), see section 34, Figure 2.12 for more information) could be used as an indicator to predict stability and precipitation. Stability is reached through electrostatic interactions between the lignin molecules. When the pH is decreased to near or close the pKa of the acidic groups, the hydrogen ions interact with the negatively-charged lignin and neutralize the charges on the molecular surface. The repulsive forces are reduced and, eventually, the precipitation of lignin occurs. Moreover, ions have strong influence on the stability of lignin in solution (10, 16), especially those belonging to the so-called Hofmeister or lyotropic series (85).

Self-association of KL is complex due to its chemical and physical heterogeneity. A hypothesis of the formation and growth of lignin particles has been proposed by Norgren et al. (85) and shown in Figure 2.6. Nucleation of KL is the mechanism responsible for starting the formation of, first seedpoints or nuclei, and then large particles. At this point, low molecular weight lignin fractions absorb onto the particles surfaces, leading them to grow in size and number. Finally, the growing lignin particles aggregate in a fractal cluster structure. Density
of these fractal structures vary depending on the rate of aggregation, defining by the Brownian motion. For this, two limited regimes of kinetics have been identified in general for aggregation of colloidal particles (86-90): (i) rapid diffusion-limited cluster-cluster aggregation (DLCA) and (ii) slow reaction-limited cluster-cluster aggregation (RLCA). The first is referred as the result of negligible repulsive forces between the colloidal particles and causing particle to stick upon contact and form loosely jointed and highly disordered structures. In the case of RLCA, several collisions are possible before the particles aggregates since the sticking probability is much lower due to a substantial repulsive force between the particles.

![Figure 2.6](image.png)

**Figure 2.6.** Schematic representation of the modes of aggregation in kraft lignin (KL) solutions forming a macromolecular KL and finally a fractal KL cluster (85).

### 2.5 Monitoring interactions at solid-liquid interfaces

Monitoring the interactions at solid liquid interfaces can be done by using surface sensitive techniques such as quartz crystal microbalance (QCM) and surface plasmon resonance (SPR). These sensitive techniques allow to monitor in-situ and in real-time the adsorption behavior of different molecules or polymers onto model thin surfaces. In the case
of cellulose-enzyme-lignin interactions, these methods have been used to elucidate the mechanism behind enzymatic hydrolysis and nonproductive binding between enzyme and lignin (56, 91-95). Hence, the next sections briefly describe these two techniques that will be used in this study.

2.5.1 Principle of Quartz crystal microbalance (QCM)

A Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) consists of a thin plate of a piezoelectric quartz crystal inserted between a pair of electrodes which measures changes in resonance frequency and dissipation, due to adsorption on crystal surface. An alternating electric field is applied across the quartz crystal through upper and lower metal electrodes covering the quartz surface and the quartz crystal starts to oscillate at a fundamental resonance frequency, 5 MHz with odd overtones of 15, 25, 35, 45, 55, and 75 MHz (Figure 2.7). Measuring multiple overtones allows the analysis of vertical variations in the layer due to a decreasing detection range from the sensor surface with increasing overtone number. An increase in mass bound to the quartz surface causes the crystal’s oscillation frequency to decrease. In contrast, frequency increases as the mass decreases, which means removal of layers. A change in the adsorbed mass ($\Delta m$) can be evaluated from the frequency using Sauerbrey (96) model (Eq. 1).

$$\Delta m = -\frac{C\Delta f}{n}$$

Eq. 1

where C represents a device specific constant (0.177 mg m$^{-2}$ Hz$^{-1}$), $\Delta f$ the change in oscillation frequency of the crystal, and $n$ is the overtone used. This equation is strictly valid for thin and
rigid layers in air. As the film thickness increases, the damping of the oscillating crystal also increases due to viscous dissipation in the film. For this reason, the frequency change, even when measured in air, is a consequence of both the mass change and the viscoelastic properties of the film (97). The model taking into account the viscoelastic properties of the adsorbed film was developed by Johannsmann et al. (97) considering this layer as an electrical component with characteristic impedance. A simplify description of the equations was proposed by Naderi and Claesson (98) (Eq. 2).

\[ m^* = -\frac{\sqrt{\rho_q \mu_q} \Delta f}{2f_0 f} \]  

Eq. 2

where \( \rho_q \) is the specific density, \( \mu_q \) is the elastic shear modulus for quartz, \( f_0 \) the fundamental frequency of the crystal in air, \( \Delta f \) the frequency response, and \( f \) the resonant frequency of the crystal in the solution.

**Figure 2.7.** Illustration of the QCM chamber (left) and the concept to measure changes in frequency.

QCM-D also gives information about the viscoelastic properties of the adsorbed layers. For this, the energy dissipation is measured as the ratio of energy dissipated to the energy
stored by the system, providing information about the flexibility of the adsorbed film. For example, it is possible to differentiate the changes caused by swelling of the film from a change caused by the adsorption of other substances. By turning off the driving voltage for a short moment and monitoring the decay of the oscillation, the dissipation at several frequencies is collected and calculated according to Eq. 3.

\[
D = \frac{E_{\text{lost}}}{2\pi E_{\text{stored}}}
\]

Eq. 3

where \( E_{\text{lost}} \) is the dissipated energy and \( E_{\text{stored}} \) is the total energy stored in the oscillator. A large value of \( \Delta D \) signifies a large energy dissipative power of the adsorbed layer, and this is most often observed for thick and nonrigid layers.

The changes in energy dissipation (\( \Delta D \)) can be plotted as a function of the frequency change (\( \Delta f \)) during adsorption stage in order to obtain information about structural transitions occurring as the adsorbed layer grows in mass, \textit{i.e.}, changes in polymer conformations as the layer builds up (99-101).

2.5.2 Principle of Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) is a label-free detection method which has emerged during the last two decades as a suitable and reliable platform for biomolecular interactions in \textit{situ} and in real-time (102). This surface-sensitive spectroscopy technique can be used to characterize ultrathin layers at gold, silver, and copper surfaces.
SPR device can detect refractive index (RI) changes smaller than \(10^{-5}\) with a time resolution of a few seconds. Because it senses with an evanescent wave, an SPR sensor responds to the RI of its analyte only to a depth of \(~200\) nm from the sensor surface. This allows SPR sensors to be applied to the detection of adsorption onto the sensor surface from liquid solutions for several applications (102). Surface plasmon resonance occurs when a photon of incident light (monochromatic p-polarized light) hits a metal surface (gold surface). At a certain angle of incidence, a portion of the light energy couples through the metal coating with the electrons in the metal surface layer, which then move due to excitation (plasmon), and they propagate parallel to the metal surface. Detection is thus accomplished by measuring the changes in the reflected light obtained on a detector, Figure 2.8a. In addition, the amount of surface concentration can be quantified by monitoring the reflected light intensity or tracking the resonance angle shifts.

**Figure 2.8.** Concept of a surface plasmon resonance (SPR) instrument. a) Illustration of the geometry. b) Spectrum of the reflected before and after refractive index changes. c) Response signal caused by the changes in refractive index.
A plot of reflected intensity versus the angle of incidence shows a minimum corresponding to the excitation of surface plasmons at the gold-solution interface. The value of the minimum angle shifts with changes in the refractive index of the interfacial region near the surface of the gold. Therefore, the change in reflectivity of the sensor to adsorbed mass is expressed in resonance units or angle shift. The thickness of the adsorbed layer is calculated using Eq. 4 (103).

\[ d = \frac{l_d \Delta \theta}{2m (\eta_a - \eta_o)} \]  
Eq. 4

where \( d \) is the thickness, \( l_d \) is the characteristic evanescent electromagnetic field decay (240 nm), \( m \) is the sensitivity factor for the sensor obtained by calculating the slope of a \( \Delta \theta \) calibration curves for a series of solutions at different concentrations and known refractive indices, \( \eta_a \) and \( \eta_o \) are the refractive indices of the adsorbed specie (proteins, surfactants) and bulk solutions, respectively. Finally, the surface excess concentration \( (\Gamma) \) can be determined by using Eq. 5.

\[ \Gamma = \rho d \]  
Eq. 5

where \( \rho \) is the bulk density of the adsorbed species.

2.5.3 Preparation of model thin films surfaces

Thin films of cellulose and lignin will be used in this study as model substrates to monitor their interactions with cellulases. Therefore, this section contains a briefly description of some methods for isolation of these biopolymers from lignocellulosic biomass.
2.5.3.1. Isolation of lignin

Isolation of lignin from the lignocellulose material partially changes its native structure; however, the following methods are considered as the ones who affect it in a less extent (34).

**From wood and pulp fibers.** Different methods have been proposed though the following are the most studied. Using ball milling before the extraction of the milled wood with 96% dioxane produces an isolated lignin defined as milled wood lignin (MWL) (104) with Klason lignin content ~25%. Furthermore, by treating the finely ground wood with cellulolytic and hemicellulolytic enzymes prior to dioxane extraction produces a cellulolytic enzyme lignin (CEL) with higher Klason lignin content than the former method (105). Another procedure known as EMAL involves the use of mild enzymatic hydrolysis of milled wood followed by mild acid hydrolysis (106).

**From spent kraft pulping liquors.** Due to the cleavage of the β-O-4 ether linkages during the pulping process, this isolated lignin contains more carboxylic groups than the native lignin (84), thus exhibits a higher surface charge than MWL (34). Acidification is the most used method for precipitate the lignin from black liquor (107). Ultrafiltration is another way to isolate lignin from kraft liquors (108).

2.5.3.2. Nanofibrillated cellulose

Nanofibrillated cellulose or cellulose nanofibrils (NFC) corresponds to nanosized cellulose fibrils containing both amorphous and crystalline regions and have a high aspect ratio (4-20 nm wide, 500-2000 nm length) (109). NFC is usually prepared by mechanical methods.
via high shearing followed by homogenization at high pressure. The equipment commonly used to produce NFC includes high pressure homogenizers, refiners, grinders, cryo-crushers, and microfluidizer (110). This nanomaterial has a high amount of hydroxyl group on the surface that makes it very appealing for chemical modification and for studying interactions with proteins, especially cellulases, lignin, and other components of interest (56, 92, 111-113).

2.6 Strategies to prevent nonproductive interactions between enzymes and lignocellulosic materials

Reducing nonproductive interaction between lignin and enzymes has been studied in the past decade in order to obtain higher sugars yields by improving enzyme efficiency (2, 114-116). To overcome this interaction, high enzymes dosages can be used; however, this is not economically feasible. As previously discussed, by increasing the pH of the enzymatic hydrolysis medium both substrate and proteins change their surface charge. It has been proposed that the optimum pH for enzymatic saccharification of lignocellulose using a second generation cellulases should be performed between 5.2 and 6.2 (70, 117). By doing this, the lignin becomes more negatively charged, increasing its hydrophilicity and enzymes also experience an increase in the charge, resulting in a coulombic repulsion (70), thus reducing nonproductive interaction (59, 70, 117).

Covalent and noncovalent modification of lignin have been also studied to reduce enzyme affinity. Covalent modification has been achieved by sulfonation (117, 118) where the addition of sulfonic or carboxyl groups increases the lignin hydrophilicity, thus reducing enzyme affinity. Surfactants, proteins, and polymers have been reported to effectively increase
enzymatic hydrolysis by non-covalent modification of lignin. Bovine serum albumin (BSA) has been utilized to reduce enzyme affinity without affecting activity \(119\). Surfactants on the other hand, reduce the affinity between enzymes and lignin mainly due to the hydrophobic interactions \(2, 120-122\) 1,2,3,4, and hydrogen bonds \(120\) that take place between the lignin and surfactants. Non-ionic has been proven to be the most effective for improving enzymatic hydrolysis of lignocellulosic biomass \(2, 123-125\). To understand the effect of these surfactants on enzymatic hydrolysis of lignocellulose, different mechanisms have been proposed: (1) the surfactant changes the structure of the substrate, making the cellulose more susceptible to enzymatic attack \(121, 122\); (2) surfactants increase enzyme stability \(2, 122\); and (3) surfactants affect enzyme-substrate interactions because they improve wettability of the substrate and reduce irreversible enzyme adsorption \(2, 121, 122\). Despite the utilization of surfactant to enhance enzymatic saccharification, the mechanism of adsorption/desorption onto cellulose and lignin surfaces and its effect on enzyme activity is not yet documented.

### 2.7 Lignocellulosic pretreatments

A critical step during the bioconversion of biomass into biofuels or biopolymers is the pretreatment since it has a large impact on the efficiency of the whole process. The aim of pretreatment is to disrupt recalcitrant structures of cellulosic biomass to make cellulose more accessible to the enzymes. There are several key aspects that determine the efficiency and effectiveness of pretreatments on the subsequent enzymatic degradation of lignocellulosic biomass: cellulose crystallinity \(126\), accessible surface area \(36\), lignin \(32, 39, 44\), and hemicellulose \(127\) content, and minimization of inhibitors\(128\). Low hemicellulose and
lignin contents in the pretreated material will increase the efficiency of cellulose hydrolysis (127). The effect of these parameters on lignocellulose structure are summarized in Table 2.3. It is possible to distinguish different categories of pretreatments (129, 130): physical, chemical, physical-chemical, biological, and electrical, or a combination of these. During the pretreatment, the extent of removal of lignin and hemicellulose depends on the pretreatment conditions and severity. For example, acidic chemical pretreatment removes most of hemicellulose. The lignin is condensed when pretreating temperature reaches above 170 °C. On the contrary, the ammonia fiber explosion (AFEX) pretreatment does not significantly remove hemicellulose.

**Table 2.3.** Effects of different pretreatments on lignocellulose structure (128-132).

<table>
<thead>
<tr>
<th>Category</th>
<th>Pretreatment</th>
<th>Changes crystallinity</th>
<th>Increases accessible surface area</th>
<th>Removes hemicellulose</th>
<th>Removes lignin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical</td>
<td>Mechanical</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Steam explosion</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Liquid hot water</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>AFEX (ammonia-based)</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>CO₂ explosion</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Oxidative</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Wet oxidation</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Microwave</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td>Physico-chemical</td>
<td>Acid</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Alkali</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Ozonolysis</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Organosolv</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td></td>
<td>Ionic liquids</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
<tr>
<td>Biological</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
<td>☻</td>
</tr>
</tbody>
</table>

☻ = major effect, ☻ = moderate effect, ☻ = no effect, in blank = not determined.
2.8 Surfactants/oil/water (SOW) systems as a pretreatment for lignocelluloses

2.8.1 Surfactants and their properties

Surfactant is an abbreviation for surface active molecule, which is characterized for its tendency to adsorb at interfaces. The driving force for adsorbing at an interface is to lower the free energy of the boundary between two phases (oil and water).

Surfactants are amphiphilic molecules that consists of two parts: one that is soluble in a specific fluid (lyophilic or polar part) and one insoluble (lyophobic or non-polar part), as shown in Figure 2.9a. The non-polar part may be branched or linear and it is usually a chain of hydrocarbon units (17). The degree of chain branching, the position of the polar group, and the length of the chain are important parameters for the physicochemical properties of surfactants. The polar section can be charged positively, cationic surfactant; negatively, anionic surfactant; or non-charged, non-ionic surfactant, some examples are shown in Figure 2.9b. There is another category, zwitterionic surfactants which contains both an anionic and cationic charged under normal conditions.
Figure 2.9. Surface active molecules. a) Schematic illustration of surfactants. b) Examples of an anionic, cationic, and non-ionic surfactants: sodium dodecyl sulfate, cetrimonium bromide, and polyethylene sorbitan monooleate, respectively from top to bottom.

Adsorption and association are particular properties of surfactants as illustrated in Figure 2.10. As stated, surfactants adsorbed at interfaces, which can be any of these five: solid-vapor, solid-liquid, solid-solid, liquid-vapor, and liquid-liquid. When the interface is covered by surfactant, the surface tension or the amount of work required to expand the interface is reduced. The degree of surfactant concentration at that boundary depends on its structure and also on the nature of the phases. In this document, the discussion is restricted to interfaces involving a liquid phase. The adsorption of surfactant at the solid-liquid interface it is strongly influence by: (1) the nature of the structural groups on the solid surface, whether the surface contains highly charged sites or essentially nonpolar groupings and the nature of the atoms of which these sites or groupings are constituted; (2) the molecular structure of the surfactant being adsorbed, whether it is ionic or nonionic, and whether the hydrophobic group is long or short, straight-chain or branched, aliphatic, or aromatic; and (3) the environment of the aqueous
phase, its pH, its electrolyte content, the presence of any additives such as short-chain polar solutes (alcohol, urea, etc.), and its temperature. Together these factors determine the mechanism by which adsorption occurs, and the efficiency and effectiveness of adsorption. Therefore, the choice of a “good” surfactant will depend on the application. There is a limit for lowering the energy and in normal cases that limit is reached when the molecules start to form micelles in bulk solution. The formation of these micelles starts at a concentration known as the critical micelle concentration (CMC), which is another fundamental property of surface active molecules. This micellization occurs when the monomers or unimers tend to form aggregates. It can be viewed as an alternative mechanism to adsorption at the interfaces for removing hydrophobic groups from contact with water thus reducing the free energy of the system. Surfactant molecules behave very different when present in micelles than as free monomers in solution, as can be seen in the next chapters of this dissertation. For a detailed review on mechanism and kinetics see Zhang and Somasundaran (133).

![Diagram of surfactant adsorption and association](image)

**Figure 2.10.** Adsorption and association mechanism of a surfactant.
2.8.2 Microemulsions

Microemulsion systems have been described by Winsor (134) and they refer to a thermodynamically stable dispersions of two immiscible liquids (oil and water) displaying optical transparency and having very unique properties such as super-solubilizing ability for both, polar and nonpolar fluids. In order to form a microemulsion, a surfactant or surfactant mixture is required to decrease to ultralow values the interfacial tension between oil and water and promote the spontaneous mixing of the otherwise immiscible phases. The nature of the interactions between oil and water are repulsive forces, the presence of the surfactant molecules changes the balance between the forces towards the attractive forces. Mixing of the phases occurs spontaneously, due to the low surface tension, and leads to systems that are thermodynamically stable. Microemulsions can be categorized depending on the phase that is dispersed and the one that is continuous. If water is the continuous phase, then thin drops of oil are dispersed (oil-in-water, O/W), whereas the oil as continuous phase induces the formation of thin water drops (water-in-oil, W/O). A special case is found when neither water nor oil are the continuous phase originating a bicontinuous microemulsion. In order to formulate an adequate microemulsion, ternary or phases diagrams were proposed by Winsor (134).

As previously discussed, surfactant, especially nonionic surfactants, would help to enhance enzymatic hydrolysis yield (2, 124) by removing hydrophobic components of the cell walls, as lignin and hemicelluloses, that pose physical and chemical constraints for enzymatic action (4). Recently, thermodinamically stable, spontaneously-formed microemulsions have been prepared from two immiscible liquids stabilized by surfactants and have been utilized for
wood pretreatment. They have been shown to overcome the physical and chemical heterogeneities that prevent effective fluid penetration in biomass (5). As a pretreatment for lignocellulosic material, this new methodology has been used in combination with ionic liquids resulting in changes in cellulose crystallinity and thermal stability, and increased in glucos yield. (135, 136). However, a better understanding of the effect of this new pretreatment on lignocelluloses structures is needed as well as the utilization of environmentally-friendly active components.

### 2.9 Principle of light scattering and its utilization

Light may be scattered whenever it passes through a medium that is ‘polarizable’ or has a dielectric constant different from unity. The light interacts with the electrons bound in the material which re-radiates the light as scattered light. Rayleigh and Mie theories are the basic concepts on this field; however, explaining these theories is beyond the scope of this dissertation and will not be discussed (for more information see (137). Dynamic light scattering and electrophoresis are two applications based on light scattering which are used to characterize solutions and they are briefly discussed on the next sections.

*Dynamic light scattering* (DLS), also refers as Photon Correlation Spectroscopy (PCS) or Quasi-Elastic light Scattering (QELS), is a non-invasive technique for measuring the size distribution of particles in suspensions based on Brownian motion. A schematic representation of the DLS theory is presented in Figure 2.11. A sample solution is irradiated with a laser beam, then part of the incoming light is scattered as a consequence of the interaction of light with the electric field of a particle. A detector measures the intensity of this scattered light
either at 90 or 173 degrees, then a correlator processes the signal (Figure 2.11a). The correlator compares the scattering intensity at successive time intervals to derive the rate at which the intensity is varying and finally a software determines the size distribution (Figure 2.11b).

**Figure 2.11.** Dynamic Light Scattering (DLS) theory. a) Schematic experimental set-up. b) Correlation functions as function of time and their interpreted size for small and large size samples.

Brownian motion is the random movement of particles in a suspension due to collisions caused by the atoms or molecules in the solvent that surround them. DLS measures the speed of particles undergoing Brownian motion, which is influenced by particle size, sample viscosity, and temperature. The smaller the particle is, the more rapid the motion becomes; whereas, the larger, the slower. As temperature increases, the Brownian motion becomes faster. Velocity of the Brownian motion is measured by the translational diffusion coefficient D,
which can be converted into particle size by the Stokes-Einstein equation $d_H = kT/(3\pi\mu D)$, where $d_H$ is the hydrodynamic diameter, $k$ is Boltzmann’s constant, $T$ is absolute temperature, and $\mu$ is viscosity. The hydrodynamic diameter (Figure 2.11c) is defined as the diameter of a hard sphere that diffuses at the same speed as the particle or molecule being measured. It will depend not only on the size of the particle, but also on any structure on its surface as well as ions present in the medium. In the case of adsorbing polymers, they can affect the diffusion speed changing the apparent particle size. For example, if the polymer is projecting out into the medium, the diffusion speed will be reduced more than if it is lying flat on the particle surface. Ions affect the particle diffusion speed by changing the thickness of the electrical double layer called Debye length $\kappa^{-1}$, which measures the rate for the mean potential decay. A low concentration of ions will produce an extended double layer around the particle, reducing the diffusion speed resulting in a large apparent particle size.

The measured hydrodynamic diameter is in fact an average of the hydrodynamic diameter of the individual aggregates, weighted by their scattered light intensities ($G^{(2)}(\tau)$). The correlation function that is measured in a normal light scattering measurement arises from the multiplication of the intensity of scattered light by itself, the so-called homodyne or self-beat signal. The diffusion process is actually quantified by the first-order auto-correlation function or electric field correlation function ($g^{(1)}(\tau)$) which predicts the degree of correlation in the signal as a function of $\tau$ the correlation time. Overall, the correlation function can be modeled as $G^{(2)}(\tau) = A(1+B|g^{(1)}(\tau)|^2)$, where $A$ and $B$ are constants and $g^{(1)}(\tau) = e^{-q\tau D}$, with $q$ as scattering vector, where the factor $D$ is entered to the Stokes-Einstein equation. Usually, two methods are used to fit the correlation functions and obtain the $D$ parameter: cumulant and distribution.
analysis. In this dissertation research, the former is used to determine particle size, which is define by the International Standard on Dynamic Light Scattering ISO13321 (1996) and ISO 22412 (2008). This analysis only gives a mean particle size, z-average, and an estimate of the width of the distribution, polydispersity index.

**Surface charge, zeta potential and electrophoretic mobility.** A particle suspended in an ionic medium arises a surface charge where any measurement done on the particle is considered as a joint system. This potential plays an important role in governing the stability of a colloidal system and may be significant in reactivity and transport phenomena, although, steric forces may also contribute to this stability, as will be discuss in Chapter 5. The suspended particles originate electrostatic forces due to the overlap of ionic double layer at the adjacent surfaces. The liquid surrounding the particle is divided in two regions: an inner region, called Stern layer, where the ions are strongly bound and an outer or diffuse region, where they are less firmly attached, as shown in Figure 2.12a. Within the diffuse layer there is a notional boundary where the ions and particles form a stable entity; however, when a particle moves, the ions within the boundary move with it, but any ions beyond the boundary do not travel with the particle. This boundary is called the surface of hydrodynamic shear or slipping plane. The potential that exists at this boundary is called zeta potential (ζ), which can be used as an indication of the stability of the system. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there is no tendency to flocculate. Although microelectrophoresis is the best method of determining the zeta potential of cellulosic material (138), laser Doppler electrophoresis or simply electrophoresis is the most used (83, 139, 140). Electrophoresis is the movement of a charged particle relative to the liquid

37
it is suspended in under the influence of an applied electric field. The measurement can be used to determine the sign of the charges on the particles and also their electrophoretic mobility, which is related to zeta potential through the Henry equation. The electrophoresis system constitutes a cell with electrodes at either end to which a potential is applied. Particles move towards the electrode of opposite charge, their velocity is measured and expressed in unit field strength as their mobility (Figure 2.12b). A laser beam is passed through a sample undergoing electrophoresis and light is scattered at an angle of 17°. The scattered light from the moving particles is frequency shifted and this shift in frequency is measured by an interferometer. The scattered light is combined with the reference beam producing a fluctuating intensity signal where its rate is proportional to the speed of the particles. A digital signal processor is used to extract the characteristic frequencies in the scattered light. A refinement of the system involves modulating one of the laser beams with an oscillating mirror. This gives an unequivocal measure of the sign of the zeta potential.

The most important factor that affect zeta potential is pH, in fact, a ζ value is meaningless without reporting pH. Varying pH of the aqueous phase influences two mechanism: functional groups dissociation and ions adsorption. For example, if alkali is added to a particle suspension with a negative ζ then the particles will tend to acquire a more negative charge; in contrast, if acid is added the suspension will reach a point where the negative charge is neutralized and probably acquire a positive charge. In the case of lignocellulosic suspensions, pH plays a role in terms of the degree of ionization of the different acidic groups present in wood (83, 84). Proteins become more negatively charged with increasing ionic strength due to decreasing the thickness of electrical double layer (141).
Figure 2.12. Schematic representation of a) electrical double layer at the surface of a negatively charged particle suspended in an ionic medium: the potential difference is expressed as a function of distance from the surface of a particle, where it is possible to recognize the surface and zeta potentials and b) folded capillary cells utilized during zeta potential measurements.
3 BIOCHEMICAL CONVERSION OF BIOMASS FACILITATED BY MICROEMULSION PRETREATMENTS

3.1 Abstract

Here we introduce microemulsion systems for green biomass pretreatment that overcomes the complex structure of wood under atmospheric pressure and low temperature. This novel approach takes advantage of the hydrophobic interactions between the components of the system, mainly, proteins, lignin and the microemulsion (oil phase and surfactant hydrophobe). A lignin removal efficiency of up to ~20 wt% was achieved by using microemulsions containing NH$_4$OH or NaOH in the aqueous phase. Cell wall heteropolysaccharides were most extensively recovered by the pretreatment (90 wt% glucan extraction, with a xylan recovery of ~46%). As a result, 53% glucan conversion was achieved with alkaline microemulsions (70 ºC, 12 h) upon enzymatic hydrolysis. The measured high sugar yield indicates an effective biochemical conversion of woody biomass by the proposed, new sugar biorefinery platform.

3.2 Introduction

Besides its availability, lignocellulosic biomass is a suitable source of fermentable sugars that does not compete with food sources (142). However, its biochemical conversion is challenged by the inherent complex and recalcitrant structure of woody biomass. Therefore, different pretreatment strategies have been proposed to overcome these barriers and to obtain value-added products from lignocellulose. Among the different technologies used for biomass
pretreatment, alkaline solutions have been applied under relative low temperatures and pressures (7). Impregnation and diffusion into the cell wall structure are critical steps for uniform and complete distribution of chemicals (3). This is because the porous structure of woody biomass and the high capillary forces at the air-water-solid interfaces impairs mass transport of chemicals. To address these issues, surfactants have been utilized in pretreatments and to enhance the enzymatic hydrolysis of biomass (2, 143). An interesting finding is that surfactants help to remove hydrophobic components of the cell walls as well as lignin and hemicelluloses that pose physical and chemical constraints for effective enzymatic action (4). In fact, lignin has been shown to cause non-productive interactions with cellulases (2, 39, 56, 59, 60, 65, 91, 113) and therefore passivation of lignin is a plausible strategy to reduce the effect of undesired interactions. In such cases, non-ionic surfactants have been found to be useful for the enhancement of the enzymatic hydrolysis yield (2, 124).

Recently, thermodynamically stable, spontaneously-formed microemulsions have been prepared from two immiscible liquids stabilized by surfactants and have been utilized for wood pretreatment. They have been shown to overcome the physical and chemical heterogeneities that prevent effective fluid penetration in biomass (5). Here, we hypothesize that microemulsions can be used as advanced pretreatment that will lead to an effective distribution of chemicals in the cell wall and can also supply surface-active molecules to facilitate subsequent bioprocessing steps. Specifically, ammonium hydroxide and sodium hydroxide were tested as active components of the aqueous phase in the oil-in-water (O/W) microemulsions, which were applied at low temperature and atmospheric pressures to disrupt the lignocellulosic structure and to make it accessible to cellulolytic enzymes.
3.3 Experimental

3.3.1 Materials

Lignocellulosic biomass from yellow poplar (*Liriodendron tulipifera* L.) was used in this study. The material was air-dried and then milled using a Wiley mill with a 2-mm sieve. After this, the biomass was screened and the particles between 40 and 20 mesh were collected and used for the pretreatment. A mixture of enzymes (endoglucanases, exoglucanases, and beta-glucosidases) under commercial name of Cellic® CTec 2 was kindly provided by Novozymes. Cellulase activity was 140 FPU/ml as determined by the filter paper method (144). Alkyl polyglucoside surfactant (C8–C10) was supplied by Henkel KGaA, under trade name Glucopon 225 K; polyoxyethylene (20) sorbitan monooleate (Tween 80), benzene, and ethanol were obtained from Sigma Aldrich. n-pentanol was used as a co-surfactant and supplied by Acros Organics. Oil, R- (+)-limonene, was purchased from Fluka. Sulfuric acid and sodium acetate were purchased from Fisher Scientific. Components of the microemulsion aqueous phase were ammonium hydroxide (NH₄OH) and sodium hydroxide (NaOH), both obtained from Sigma Aldrich.

3.3.2 Pseudo-ternary phase diagrams and microemulsion formulation

The phase behavior of surfactant-oil-water systems (SOW) was investigated by identifying the type and number of phases obtained at equilibrium following the titration method, as previously reported (134). In short, aqueous surfactant solutions of different concentrations were titrated with the oil phase. After equilibration the number and type of phases were identified and plotted in a composition (pseudo-ternary) diagram. A mixture of
non-ionic surfactants was used in the microemulsion formulations. This was based on previous observations that nonionic surface active molecules enhance enzymatic hydrolysis of lignocellulose (2). The hydrophilic, non-ionic surfactants, alkyl polyglucoside and polyoxyethylene sorbitan monooleate, present different molecular size and hydrophilic-lipophilic balance (HLB values) and were chosen in order to attain a high packing density at the interface, thus reducing the interfacial tension to ultra-low values, as required in the target application. The alkyl polyglucoside surfactant was found to improve the penetration of the microemulsions in hardwood (6) while a co-surfactant (n-pentanol) was added to the mixture to prevent the formation of liquid crystals and also to reduce the water/oil interfacial tension.

Aqueous solutions of ammonium and sodium hydroxide have been widely used to pretreat both woody and non-woody biomass in order to improve enzymatic hydrolysis yield (7, 145-147). Here, these “active” chemicals were incorporated in the aqueous phase of the microemulsions in order to improve the delivery inside the biomass and thus to enhance enzymatic hydrolysis.

The surface tension, viscosity, drop size, and transmittance of the microemulsion were determined. The surface tension was measured using a 312 Electrobalance from Cahn Instruments Inc. (Cerritos, CA, USA) equipped with a platinum Wilhelmy plate. For the viscosity measurements, an AR2000 rheometer from TA Instruments (New Castle, DE, USA) was used. The drop size of the microemulsions at different temperatures was measured by dynamic light scattering using a Nano-ZS Zetasizer (ZS90 Malvern Instruments Ltd, Malvern, UK). The transmittance of the microemulsion at 25 and 70 °C was determined with a UV-Vis spectrophotometer at 650 nm.
3.3.3 Biomass pretreatment

Pre-screened wood particles were immersed in give O/W microemulsion system (ME) at a solid-to-liquid ratio of 1:15 by weight. This pretreatment was performed in an incubator shaker under controlled temperature and continuous agitation, 70°C and 150 rpm, respectively. Impregnation time was fixed at 6 and 12 h for pretreatments based on microemulsions containing NH₄OH and NaOH, respectively. The respective aqueous solutions (in the absence of surfactants, co-surfactant, and oil) were used under the same conditions and referred thereafter as AQ (for alkaline solution treatment). After pretreatment, the wood solid was separated by filtration (Büchner funnel), washed with distilled water to remove free chemicals, and air-dried for several days until constant weight. It is worth noting that temperatures above the cloud point of the surfactants can destabilize the O/W microemulsion systems. In the present case such temperature limit was set to 70 °C.

3.3.4 FTIR analysis

Fourier transform-infrared spectroscopic was used to determine the chemical changes in pretreated biomass using the standard KBr pellet technique (1 wt% of sample). The pellets were made under vacuum and compression of 10 t. Each spectrum was recorded with 64 scans in the frequency range of 4000 to 400 cm⁻¹ with a resolution of 4 cm⁻¹. All the curves were baseline-corrected.
3.3.5 Compositional analysis

In order to evaluate the effect of the microemulsion pretreatments, compositional analyses were performed. First, the extractives were removed by Soxhlet extraction with benzene:ethanol (2:1, 24 cycles). Following, 100 mg of extractive-free material was reacted with 1.5 ml of sulfuric acid (72% H₂SO₄) at 25 °C for 2 h with stirring every 20 min. After this, the slurry was diluted and autoclaved at 121 °C for 1.5 h, cooled down overnight, and filtered through a fine crucible for gravimetric determination of acid-insoluble lignin (Klason lignin). The filtrated was used to determine acid-soluble lignin by UV-VIS spectroscopy at 205 nm (Lambda XLS, PerkinElmer, Inc), and sugar content (glucose, xylose, galactose, arabinose, and mannose) by HPLC, as previously reported (148). After the microemulsion pretreatment, the samples were filtrated and the % solid yield, glucan and xylan recovery as well as lignin removal were calculated by using the equations Eq. 6-Eq. 9:

\[
\text{Solid yield (\%)} = \frac{\text{weight after pretreatment (g)}}{\text{weight before pretreatment (g)}} \times 100 \quad \text{Eq. 6}
\]

\[
\text{Lignin removal (\%)} = \frac{\text{lignin in raw biomass (g)} - \text{lignin in pretreated biomass (g)}}{\text{lignin in raw biomass (g)}} \times 100 \quad \text{Eq. 7}
\]

\[
\text{Glucan recovery (\%)} = \frac{\text{glucan in pretreated biomass (g)}}{\text{glucan in raw biomass (g)}} \times 100 \quad \text{Eq. 8}
\]

\[
\text{Xylan recovery (\%)} = \frac{\text{xylan in pretreated biomass (g)}}{\text{xylan in raw biomass (g)}} \times 100 \quad \text{Eq. 9}
\]
3.3.6 XRD analysis

X-ray diffractograms were acquired with a XRD instrument operating at 40kV and 44 mA, at angles between 5° and 40° and wavelength of 0.154 nm, using a goniometer scan range of 0.6°/min (0.1° and 10 sec). All the measurements were conducted at atmospheric pressure. Apparent crystallinity indices were calculated using the empirical index of Segal, Eq. 10 (149):

\[
CrI (\%) = \frac{I_{002} - I_{am}}{I_{002}} \times 100
\]

where \(I_{002}\) is the intensity of the (002) peak at about 2\(\theta\)=22.5° and \(I_{am}\) is the intensity at about 2\(\theta\)=18°. The XRD data were normalized by scaling between 0 and 1 using the maximum and minimum values before index determination.

3.3.7 Thermal analysis

The thermal stability of the pretreated-biomass was analyzed by thermogravimetric (TGA) analysis and respective derivative (DTGA). The weight loss was recorded within the range of 40 to 700 °C, with a heating rate of 10 °C/min. Nitrogen gas was used as a purge gas for pyrolysis at a flow rate of 40 ml/min. The curves were normalized before analysis.

3.3.8 Enzymatic hydrolysis

Enzymatic saccharification was performed with the multicomponent enzyme system (Cellic Ctec2) in an incubator shaker with continuous agitation (180 rpm) at 50 °C and during 72 h. Briefly, one oven-dried gram of biomass was immersed in 20 ml of acetate buffer (100 mM and pH 5.5). The cellulase dosage was set at 20 FPU/g of biomass substrate. After
hydrolysis, the respective vial was immersed in boiling water for 5 min to denature the enzymes. An aliquot was taken from the liquid, centrifuged at 10,000 rpm for 10 min and the monomeric sugars content, glucose, xylose, galactose, arabinose and mannose, were measured by high-performance liquid chromatography (HPLC). The experiments were ran by duplicate. After enzymatic hydrolysis, the glucan conversion was calculated according to Eq. 11. The gain in glucan conversion was calculated as the ratio of glucan conversion from microemulsion pretreatment over its corresponding aqueous control in order to express the impact of using a microemulsion pretreatment.

\[
\text{Glucan conversion (\%) = } \frac{\text{glucose (mg)} \times \frac{162}{180} + \text{cellulose (mg)} \times \frac{324}{342}}{\text{glucan (mg) in raw biomass}} \times 100
\]  
Eq. 11

3.4 Results and discussion

3.4.1 Pseudo-ternary phase diagrams and microemulsion formulation

An equilibrium Surfactant-Oil-Water (SOW) pseudo-ternary diagram (Figure 3.1) was obtained and used to formulate a suitable microemulsion for biomass pretreatment. The diagram comprises the composition zone corresponding to (single-phase) oil-in-water microemulsions (ME) and the two-phase neighboring zones (2Φ), in which the SOW system separates with time into two phases. The ME region extends to oil (limonene) and surfactant mixture concentrations of >50 and >10 wt%. All microemulsions belonging to this region were optically clear and presented a yellowish color, typical of the nonionic surfactants used (Figure 3.1). As the concentration of limonene increased to values between 35 and 50%, the
microemulsions were not able to hold the oil phase and separated into two phases, one in the bottom consisting of an O/W microemulsion and the other, upper phase, containing excess oil (Figure 3.1).

**Figure 3.1.** Pseudo-ternary phase diagram for the system Surfactant-Limonene-Water prepared at 25 °C. The ME region represents the single-phase, microemulsion region, whereas 2Φ corresponds to two-phase systems. The composition used in this study is indicated by the square symbol located in ME zone. The surfactant mixture comprises two non-ionic surfactants and a co-surfactant (1:1: ratio by weight).

Pretreatments were applied according to two criteria: (1) use of pretreatment fluid consisting of a single-phase, microemulsion system and, (2) minimize the surfactant concentration. The composition indicated by a square symbol in the pseudo-ternary diagram shown in Figure 3.1 was chosen, which corresponds to a ratio by weight of the three
components in the surfactant mixture (alkyl polyglucoside:polyoxyethylene sorbitan monooleate:n-pentanol) of 5:3:2. This composition ensured the largest single-phase composition region in the ternary diagram while keeping a low surfactant concentration (data from preliminary tests, not included).

The alkaline species for pretreatment (NH₄OH and NaOH) were incorporated in the aqueous phase of the microemulsions at 5 or 10% (v/v) (the surfactant mixture and limonene concentration were kept at 13.5 and 5%, respectively). Thereafter, the microemulsions containing NH₄OH and NaOH are indicated as “ME NH₄OH” or “ME NaOH”, respectively. The concentration of the alkaline specie and pretreatment time are indicated as tags to the respective system; for example, “ME NaOH 5.12” refers to a microemulsion pretreatment using sodium hydroxide at 5% (v/v) and applied during 12 h.

The microemulsions presented low surface tensions, with an average value of 25.5 ±0.2 and 25.2 ±0.3 mN/m at 25 and 70 °C, respectively. The dynamic viscosity was higher at 25 °C compared to that at 70 °C. The maximum difference was found at a shear rate of 200 s⁻¹ where the value of the apparent viscosity decreased from 3.1 to 1.5 mPa·s. The viscosity of limonene and water were ~0.94 and 1.04 mPa·s at 25 °C, respectively (5). The changes in viscosity with temperature are attributed to changes in drop size or disperse phase structure as well as structural transition to bicontinuous phases (150). Therefore, the characteristic size of the “oil” phase in the emulsion was measured as a function of temperature. The z-average drop size diameter for the microemulsion system was ~20 nm at 25 °C. As the temperature increased, the drop size reached a maximum of ~196 nm (70 °C). This increase in drop size with temperature corresponds to the transition of the surfactant to a less hydrophilic condition (due
to desolvation), which leads to micro-phase separation or aggregation (151). Despite this increase in drop size with temperature, the microemulsion system remained stable, with a low viscosity and size polydispersity (below 0.3 nm at 70 °C). The stability of the system was followed by transmittance measurements at 650 nm (note that the % transmittance at 25 and 70 °C was very similar, at 91 ± 0.2 and 89.3 ± 0.9, respectively).

3.4.2 Biomass chemical composition

The effect of the O/W microemulsion in the compositional changes of yellow poplar upon treatment is presented in Table 3.1 based on moisture-free solids before and after pretreatment. Figure S1 includes this information expressed as % relative to the initial woody.

Table 3.1. Compositional (% based on moisture-free solids) of biomass (yellow poplar) before and after treatment with ME NH4OH and ME NaOH. Mass balance (%) represents the sum of carbohydrates, lignin, and extractives. Solid yield (%) indicates the residual mass after pretreatment. Aqueous controls are also included.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Carbohydrates (%)</th>
<th>Lignin (%)</th>
<th>Extr (%)</th>
<th>Mass balance (%)</th>
<th>Solid yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>44.5</td>
<td>16.1</td>
<td>0.7</td>
<td>4.7</td>
<td>23.2</td>
</tr>
<tr>
<td>NH4OH</td>
<td>46.6</td>
<td>17.1</td>
<td>0.9</td>
<td>5.1</td>
<td>22.4</td>
</tr>
<tr>
<td>AQ.10.6</td>
<td>44.8</td>
<td>16.8</td>
<td>0.8</td>
<td>5.1</td>
<td>22.5</td>
</tr>
<tr>
<td>ME.5.12</td>
<td>42.5</td>
<td>15.4</td>
<td>1.4</td>
<td>4.0</td>
<td>22.3</td>
</tr>
<tr>
<td>AQ.5.12</td>
<td>43.5</td>
<td>15.9</td>
<td>2.2</td>
<td>4.7</td>
<td>23.0</td>
</tr>
<tr>
<td>ME.10.12</td>
<td>43.1</td>
<td>14.4</td>
<td>1.4</td>
<td>3.0</td>
<td>21.1</td>
</tr>
<tr>
<td>AQ.10.12</td>
<td>43.3</td>
<td>14.7</td>
<td>1.8</td>
<td>4.0</td>
<td>23.2</td>
</tr>
<tr>
<td>NaOH</td>
<td>56.1</td>
<td>10.1</td>
<td>1.1</td>
<td>3.6</td>
<td>26.2</td>
</tr>
<tr>
<td>AQ.5.12</td>
<td>54.5</td>
<td>9.6</td>
<td>1.1</td>
<td>3.3</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Glu=glucan, X=xylan, A=arabinose, M=mannose, Ins=insoluble, Sol=soluble, Extr=extractives
(*) A+M means the content of arabinose plus mannose
The total carbohydrate content on untreated yellow poplar was 66 wt%, which included 45 wt% of cellulose (glucan content) and 22 wt% of hemicelluloses (xylan, galactan, mannose, and arabinose). The total lignin content was 25% wt that contained 23% of acid-insoluble and 2% acid-soluble lignin. These values are in agreement with previous studies (152, 153). After microemulsion pretreatment with NH$_4$OH at 10% (v/v), for 6 h (ME NH$_4$OH 10.6) the carbohydrate content increased to 70%; however, it was reduced to 62% at the longer pretreatment time (ME NH$_4$OH 10.12). Ammonium hydroxide concentration did not affect significantly the carbohydrate content after 12 h pretreatment, 63 and 62% of carbohydrates were determined after treatment with ME NH$_4$OH at 5 and 10% (v/v), respectively. In the case of sodium hydroxide microemulsion pretreatment, the carbohydrate content increased to 71%. The effect of microemulsion pretreatment on extractives and lignin content is discussed in detail below. The most significant observation is a reduction in acid-insoluble lignin and extractives.

It has been shown that wood impregnation with microemulsions facilitates solubilization of cell wall components after full immersion (5). For this reason, the solid yield (%) after pretreatment was calculated using Eq. 6. The amount of wood components solubilized upon pretreatment with ME NH$_4$OH was slightly higher than the respective aqueous solution (AQ NH$_4$OH). It was found that the pretreatment time had a greater effect on solubilization than NH$_4$OH concentration (the solubilized material increased from 4.2 to 6.4 wt% as the treatment time was changed from 6 to 12 h). In contrast, sodium hydroxide produced the highest solubilization; in fact, not only extractives were removed but also lignin and carbohydrates, as shown by the compositional analyses (Table 3.1). A slight increase in
solubilization was noted by application of ME NaOH pretreatment (27 wt% biomass solubilization) compared to 25 wt% in the case of aqueous solution (AQ NaOH). These findings are supported by the fact that the hydrophobic components of wood, such as extractives, are removed by the surfactant and oil phase of the microemulsion. Such changes indicated that the extractive content decreased from 2.3 wt% (untreated material) to 0.6 wt% (ME NH$_4$OH 10.12) after pretreatment (compositional analysis in Table 3.1). The presence of extractives limits fluid impregnation in wood, possibly due to their effect in occluding pit openings (154); thus, it can be expected that extractive removal will facilitate chemical transport.

The total lignin removed (%) was determined upon pretreatment (Eq. 7, Table S1). A total lignin removal of 7 wt% was achieved by using a microemulsions containing 10% NH$_4$OH and 12 h treatment (ME NH$_4$OH 10.12). Interestingly, a higher total lignin removal (14 wt%) was found upon application of ME NaOH 5.12. The acid-insoluble lignin removal (%) after pretreatment was also determined (Table S1). ME NH$_4$OH pretreatment removed more lignin than the respective treatment with the aqueous solutions (AQ NH$_4$OH), for all conditions (Table S1). The maximum acid-insoluble lignin removal was 15 wt% for ME NH$_4$OH 10.12. Soluble lignin increased in all the conditions applied. In the case of NaOH systems, the acid-insoluble lignin removal was almost the same for the microemulsion and the aqueous pretreatment, 18 and 19 wt%, respectively. Overall, the microemulsion pretreatments removed more lignin than the aqueous systems. Thus, O/W microemulsions are expected to enhance the penetration and transportation of chemicals through the cell wall (5). This effect, associated with the presence of a nonpolar oil and a surfactant, occurs via hydrophobic
interactions (2) that make the lignin more soluble and extractable by the aqueous systems (4) and reduces or prevents lignin re-deposition on the surface of the fiber (143). Taking these observations together, it was expected that microemulsion pretreatment can enhance sugar yields after enzymatic hydrolysis, a point that is addressed in the next sections. Moreover, alkali pretreatment is known for solubilizing hemicellulose as well as swelling the cell walls, thus improving enzyme accessibility (142). In this way, the recovery of glucan and xylan was calculated after pretreatment using Eq. 8 and the results are presented in Table S1. Both pretreatments, based on NH₄OH and NaOH, removed glucan from the biomass, as was observed in Figure S1. Application of ME NH₄OH 10.6, ME NH₄OH 5.12, and ME NH₄OH 10.12 produced a glucan recovery of 100, 89 and 91%, respectively, which indicates a negative effect under excessive pretreatment time. Pretreatment with ME NaOH for 12 h produced a glucan recovery of 92%. On the other hand, xylan recovery values were 100 and 83% for ME NH₄OH 10.6 and ME NH₄OH 10.12, respectively. Finally, ME NaOH 5.12 removed xylans by 54%. These results can be related to lignin-carbohydrate complexes, LCC, that tend to be solubilized under alkali pretreatment (147).

3.4.3 Effect of microemulsion pretreatment on the physicochemical properties of wood

The physiochemical properties of the material before and after pretreatment were analyzed by using FTIR (Figure 3.2), XRD (Figure 3.3), and TGA (Figure 3.4). The FTIR spectra of the biomass before and after pretreatment are included in (Figure 3.2). The bands at 1734 and 1240 cm⁻¹ (bands 1 and 5) corresponds to the stretching vibration of C=O and C= of the ester units present in hemicellulose (155, 156) and carbonyls that exist in the side chains of lignin (157). The intensity reduction observed in these bands is indicative of the dissolution...
of both hemicelluloses and lignin. This is explained by the cleavage of C=O from lignin and hemicelluloses caused by ammonium and sodium hydroxide. The signal corresponding to C=O stretching vibration in conjugated carbonyl of lignin shifted slightly from 1640 to 1650 cm\(^{-1}\) (band 2). Moreover, another peak related to residual lignin was detected at 1590 cm\(^{-1}\) (band 3), corresponding to the aromatic skeletal vibration. The aromatic skeletal vibration combined with C-H in plane deformation was detected at 1424 cm\(^{-1}\) associated to cellulose (band 4). After pretreatments this peak was still visible and can be attributed to a well-preserved cellulose. The band at 1160 cm\(^{-1}\) (band 6) remains unaltered and is assigned to the C-O-C ring vibrational stretching of \(\beta\)-(1,4)-glycosidic linkages in cellulose and hemicelluloses. Another band that remained unchanged was detected at 890 cm\(^{-1}\) (band 9), associated to the \(\beta\)-glycosidic linkages of cellulose and hemicellulose and the C-H deformation of cellulose. The intensity of this peak was slightly higher after application of ME NH\(_4\)OH compared to ME NaOH. The C-OH stretching vibration of the cellulose and hemicellulose was detected at around 1060 cm\(^{-1}\) (band 7). After pretreatment the intensity of this band decreased, indicating a partial removal of both components, which is consistent with the compositional analysis (Figure S1). The band at 1030 cm\(^{-1}\) (band 8) disappeared after microemulsion and aqueous pretreatments. This peak has been associated with C-O, C=C and C-C-O vibrational stretching of glycosidic linkages among carbohydrates, which may indicate the dissolution or removal of cellulose and hemicelluloses. However, some authors relate this band to both cellulose and lignin and to the aromatic C-H in-plane deformation plus the C-O deformation in primary alcohols. Overall, the FTIR results are in qualitative
agreement with the compositional analyses that indicated that lignin, hemicelluloses and cellulose were partially removed after the given pretreatment.

![FTIR spectra](image)

**Figure 3.2.** Fourier transform infrared spectra of yellow poplar before (untreated) and after different pretreatment conditions, as indicated.

The X-ray diffraction (XRD) patterns of the samples before and after treatment are shown in Figure 3.3. The NH₄OH microemulsion at 12 h and corresponding aqueous system were selected for this purpose since the FTIR analysis (Figure 3.2) showed that ME NH₄OH at 6 h did not produce significant changes. As such, Figure 3.3 compares the effect of ME NH₄OH 10.12, AQ NH₄OH 10.12, ME NaOH 5.12, and AQ NaOH 5.12. The relative apparent crystallinity (Eq. 10) for the untreated material was 63%. After pretreatment with NH₄OH, the
crystallinity increased to 78 and to 66% for the microemulsion and the aqueous systems, respectively. On the other hand, the use of NaOH increased the crystallinity up to 75 and 76% for the microemulsion and aqueous systems, respectively. The observed increase in crystallinity can be attributed to the removal of lignin and hemicelluloses during the pretreatment (162-164), as was determined by changes in the compositional analysis and by disappearance of the FTIR bands at 1734, 1240, and 1030 cm\(^{-1}\).

![XRD profiles for yellow poplar before (untreated) and after respective pretreatment, as indicated.](image)

**Figure 3.3.** XRD profiles for yellow poplar before (untreated) and after respective pretreatment, as indicated.

The thermogravimetric and derivative analyses (TGA, wt% and DTGA, wt%/°C) for biomass before and after treatment with ME NH\(_4\)OH 10.12, AQ NH\(_4\)OH 10.12, ME NaOH 5.12, and AQ NaOH 5.12 are shown in Figure 3.4. In general, the decomposition of the three main components of the cell wall has been establish at 220-315 °C for hemicellulose, 315-400 °C for cellulose, and 100-900 °C for lignin but at low mass loss rate (165, 166). The main
degradation region of yellow poplar started at 200 °C, with a maximum rate of mass loss of 1.4 wt%/°C at 339 °C that levels off at 380 °C (mass-loss rate of 0.08 wt%/°C) and yielded 8% residual solids. Similar trend was found for NH₄OH pretreatments. The main (pyrolysis) region for the pretreated lignocellulosic biomass started at 200 °C and finished at 380 °C; however, the maximum thermal mass loss peak shifted: to 353 °C (rate of 1.3 wt%/°C) and 343 °C (rate of 1.5 wt%/°C) for ME NH₄OH 10.12 and AQ NH₄OH 10.12, respectively. At temperatures over 380 °C the mass loss rate was less than 0.08 wt%/°C and the residual solids were low, at 13 and 8% for the ME and AQ pretreatment, respectively. These results may indicate a greater resistant to thermal decomposition, especially for ME NH₄OH 10.12 compared with NaOH. This can be explained as a combination of several properties, such as a higher crystallinity degree compared with the untreated material (Figure 3.3); the presence of a band associated to carbonyl ester groups, which disappeared after NaOH pretreatment, as revealed by FTIR (Figure 3.2) (167); and the chemical composition as discuss below. In the case of NaOH-pretreated biomass, the thermal degradation was faster and started at slightly lower temperature, 190 °C. The maximum mass loss rate was observed at 303 °C (1.3 wt%/°C) and at 300 °C (1.4 wt%/°C) for ME and AQ pretreatments, respectively. Over 350 °C the weight loss rate was less than 0.12 wt%/°C and the residual solids were low, at 12%. Besides these pyrolysis results and in order to identified and separate the thermal decomposition for the three main components (168), the combustion (in air) of the biomass with and without pretreatments was tested and shown in Figure S2. The 44% mass loss determined at 273 °C for ME NaOH-pretreated biomass could be associated to the decomposition of hemicellulose (200-315 °C) while a mass loss of 50-65% for untreated and ME NH₄OH-pretreated biomass would arise
from the decomposition of cellulose (315-400 °C), and the mass loss of ~95% at temperatures >400 °C can be associated to decomposition of lignin (168). These results indicate a decrease in the thermal stability of the material pretreated with both NaOH systems since it caused a disruption of hydrogen and chemical bonds in noncrystalline regions, thus decreasing the cohesion in the fibers (135). Moreover, during pyrolysis the material before pretreatment exhibited a shoulder at ~290 °C, which is attributed to hemicelluloses (169). This peak weakened significantly after the pretreatment with both NH₄OH systems and disappeared after ME and AQ NaOH pretreatments. According to Table S1 up to 54% hemicelluloses were removed by using ME NaOH; the FTIR analysis (Figure 3.2) revealed that the peaks associated to hemicelluloses were partially removed (peak at 1734 and 1240 cm⁻¹, and 1590 cm⁻¹).

![Figure 3.4](image.jpg)

**Figure 3.4.** Thermogravimetric analysis (TGA) reported as normalized mass % change for yellow poplar before and after NH₄OH- and NaOH-based pretreatments, as indicated. The profiles in the inset represent the first derivative of the thermograms (DTGA).
3.4.4 Effect of microemulsion pretreatment on enzymatic saccharification

Glucan conversion was determined (Eq. 11) after 72 h enzymatic hydrolysis using the multicomponent cellulases system. Pretreatment with the oil-in-water microemulsion had a positive impact on enzymatic hydrolysis. The results in Figure 3.5 are shown in terms of conversion gains in systems pretreated with the microemulsions (ME NH₄OH 10.6, ME NH₄OH 10.12, and ME NaOH 5.12) relative the respective aqueous system (AQ). Besides the changes in chemical composition, ME NH₄OH 10.12, and ME NaOH 5.12 caused significant effects in biomass properties, whereas ME NH₄OH 10.6 produced a high glucan recovery. The glucan conversion increased (with respect to the respective control) by 2, 14 and 11% when ME NH₄OH 10.6, ME NH₄OH 10.12, and ME NaOH 5.12 were used, respectively. The changes in microemulsion viscosity and drop size affected positively the sugar yield. At 70 °C the microemulsion viscosity was reduced and the drop size increased compared with the values obtained at 25 °C. The glucan conversion calculated at 25 °C (data not shown) resulting in similar conversion values for ME and AQ systems, 12%. As Carrillo et al., (5) concluded, the viscosity is a critical property for fluid penetration and consequently the effectiveness of the pretreatment in terms of glucan conversion. These observations can be also explained by the changes in the hydrophilic–lipophilic balance of the nonionic surfactants: at high temperatures the system becomes less hydrophilic due to the dehydration of polar groups (151). It was found that in the case of pretreatment with ME NH₄OH the incubation time did not affect the sugar conversion yield. The ME NH₄OH 10.12 pretreatment removed more insoluble lignin than ME NH₄OH 10.6 (Table S1). However, a similar glucan conversion was achieved (35 and 36%, respectively). In contrast, NaOH microemulsion pretreatment improved cellulose accessibility.
to enzymes, increasing the glucan conversion by up to 53%. These results can be attributed to lignin removal and the changes in physicochemical characteristics of the biomass after pretreatment, as was discussed. Lignin acts as an inhibitor, causing non-productive interactions with enzymes (2, 39, 56, 59, 60, 65, 91, 113), thereby decreasing the hydrolysis rate (60). Hydrophobic (2, 39, 64, 65) and electrostatic (39) interactions are relevant factors in this regard. On the other hand, during pretreatment surfactants bind to lignin via hydrophobic interactions (121, 122), thus reducing protein adsorption on the substrate. Moreover, alkaline pretreatment is known for cleaving C-O-C bonds in lignin, as well as ether and ester bonds between lignin and cellulose (147), which inhibit enzymatic attack. In the case of NH₄OH, the chemical composition was not significantly affected (Figure 3.2 and Figure 3.4): the maximum acid-insoluble lignin removal was 15% (Table S1), equivalent to 7.3% of the total lignin removed; this indicates that cellulose was likely protected by the microemulsion. In contrast, microemulsion pretreatment with NaOH removed 18% of lignin (Figure 3.2), equivalent to 14% of the total lignin removed; it altered lignin’s functional groups (Figure 3.2), and reduced the thermal stability of the biomass (Figure 3.2 and Figure 3.4).
Besides the removal of lignin, lignocellulosic pretreatment helps to reduce the crystallinity of the substrate and thus increases sugar yield (170, 171). The glucan conversion was improved even if the crystallinity was increased. Thus, the increased bioconversion upon pretreatment is ascribed to the removal of lignin and hemicelluloses (162). Similar trend was found by Yu et al., (164) who determined that crystallinity increased with lignin removal. Note that that the crystallinity index does not provide a clear indication of biomass digestibility (38). The measured increase in glucan conversion when using ME NH₄OH 10.12 and ME NaOH 5.12 by 14 and 11%, respectively, can make an economic impact in the feasibility of the process, specifically in terms of the net present value (NPV). The total carbohydrate content before enzymatic hydrolysis and the resulting monomeric sugars relate directly with pretreatment and ethanol yield (172). It has been found, for example, that a carbohydrate

Figure 3.5. Glucan conversion increment (%) relative to aqueous pretreatment (after 72 h enzymatic hydrolysis, multicomponent cellulases 20 FPU/g biomass). The glucan conversion obtained from untreated and pretreated yellow poplar with ME NH₄OH 10.6, AQ NH₄OH 10.6, ME NH₄OH 10.12, AQ NH₄OH 10.12, ME NaOH 5.12, and AQ NaOH 5.12 were 5, 35, 34, 36, 32, 53, and 47%, respectively.
content above 66% is necessary to achieve a desirable internal rate of return of 12% (172), which together produce a higher NPV in hardwood-based biorefineries (173). Therefore, this new proposed technology can be applied for biomass conversion in order to achieve a high carbohydrate content that can increase the benefits and profitability of the process.

3.5 Conclusions

Surfactant-limonene-water microemulsion systems were used to pretreat lignocellulosic biomass at low temperature and atmospheric pressure and enhanced the enzymatic hydrolysis of the substrate. The alkaline species in the aqueous phase of the microemulsion, ammonium hydroxide and sodium hydroxide, produced important changes in apparent crystallinity, lignin and hemicelluloses content, as revealed by TGA, XRD, and FTIR, analyses. Compared to microemulsions containing NH₄OH, lignin removal and consequently glucan conversion was higher by application of NaOH-based microemulsions.
3.6 Supporting information

**Figure S1.** Chemical composition (% based on initial wood mass) for yellow poplar samples before (untreated) and after microemulsion (ME) pretreatment (ME NH$_4$OH and ME NaOH systems). Percent carbohydrates, glucan, xylan, galactan, and arabinose and mannose (Ara+Man), acid-insoluble and acid-soluble lignin as well as extractives are shown. The chemical composition for treatments with corresponding aqueous (AQ) systems is included.

**Table S1.** Lignin removal (%) and glucan and xylan recoveries (%) from yellow poplar after pretreatment with ME NH$_4$OH and ME NaOH.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lignin removal (%)</th>
<th>Glucan recovery (%)</th>
<th>Xylan recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insoluble</td>
<td>Soluble</td>
<td>Total</td>
</tr>
<tr>
<td>NH$_4$OH</td>
<td>ME.10.6</td>
<td>7.5</td>
<td>(+78)</td>
</tr>
<tr>
<td></td>
<td>AQ.10.6</td>
<td>3.3</td>
<td>(+80)</td>
</tr>
<tr>
<td></td>
<td>ME.5.12</td>
<td>10.5</td>
<td>(+76)</td>
</tr>
<tr>
<td></td>
<td>AQ.5.12</td>
<td>7.2</td>
<td>(+63)</td>
</tr>
<tr>
<td></td>
<td>ME.10.12</td>
<td>15.0</td>
<td>(+77)</td>
</tr>
<tr>
<td></td>
<td>AQ.10.12</td>
<td>6.0</td>
<td>(+66)</td>
</tr>
<tr>
<td>NaOH</td>
<td>ME.5.12</td>
<td>18.0</td>
<td>(+35)</td>
</tr>
<tr>
<td></td>
<td>AQ.5.12</td>
<td>19.1</td>
<td>(+29)</td>
</tr>
</tbody>
</table>

(+ ) indicates increased % in soluble lignin as obtained after respective pretreatment.
Figure S2. Thermal decomposition in air of yellow poplar before (untreated) and after NH₄OH- and NaOH-microemulsion based pretreatments, as indicated.
4 INTERACTIONS BETWEEN CELLULOLYTIC ENZYMES WITH NATIVE, AUTOHYDROLYSIS AND TECHNICAL LIGNINS AND THE EFFECT OF A POLYSORBATE AMPHIPHILE IN REDUCING NON-PRODUCTIVE BINDING

4.1 Abstract

Understanding enzyme-substrate interactions is critical in designing strategies for bioconversion of lignocellulosic biomass. In this study we monitored molecular events, in-situ and in real time, including the adsorption and desorption of cellulolytic enzymes on lignin and cellulose by using quartz crystal microgravimetry and surface plasmon resonance. The effect of a non-ionic surface active molecule was also elucidated. Three lignin substrates relevant to the sugar platform in biorefinery efforts were considered, namely, hardwood autohydrolysis cellulolytic (HWAH), hardwood native cellulolytic (MPCEL), and non-wood native cellulolytic (WSCEL) lignin. In addition, Kraft lignins derived from softwoods (SWK) and hardwoods (HWK) were used as references. The results indicated a high affinity between the lignins with both, monocomponent and multicomponent enzymes. More importantly, the addition of non-ionic surfactants at concentrations above their critical micelle concentration reduced remarkably (by over 90%) the non-productive interactions between the cellulolytic enzymes and the lignins. This effect was hypothesized to be a consequence of the balance of hydrophobic and hydrogen bonding interactions. Moreover, the reduction of surface roughness and increased wettability of lignin surfaces upon surfactant treatment contributed to a lower affinity with the enzymes. Conformational changes of cellulases were observed upon their adsorption on lignin carrying pre-adsorbed surfactant. Weak electrostatic interactions were
determined in aqueous media at pH between 4.8 and 5.5 for the native cellulolytic lignins (MPCEL and WSCEL), whereby a ~20% reduction in the enzyme affinity was observed. This was mainly explained by electrostatic interactions (osmotic pressure effects) between charged lignins and cellulases. Noteworthy, adsorption of non-ionic surfactants onto cellulose, in the form cellulose nanofibrils, did not affect its hydrolytic conversion. Overall, our results highlight the benefit of nonionic surfactant pre-treatment to reduce non-productive enzyme binding while maintaining the reactivity of the cellulosic substrate.

4.2 Introduction

It has been shown that during the hydrolysis process of lignocellulosic biomass enzymes adsorb on the substrate and also undergo desorption and re-adsorption cycles (174). The enzyme adsorption is affected by various factors, such as the presence of lignin (39, 64), type of pretreatment method used (60, 65, 175), as well as lignocellulose surface area and pore volume (174). The kinetics of cellulase adsorption on cellulose is considerably different from that on lignin (65). The lignin-rich components in biomass cause a non-productive interaction with cellulolytic enzymes (2, 39, 56, 59, 60, 65, 91, 113), thereby decreasing the hydrolysis rate (60). The most common types of interactions reported to occur between lignin and enzymes include hydrophobic (2, 39, 65), electrostatic (39), and hydrogen bonding (66). They explain some of the fundamental reasons for observed changes in affinity of proteins in aqueous media (176). Specifically, hydrophobic effects are favorable owing to entropic contributions associated to changes in water structuring; as a result, hydrophobic molecules tend to aggregate in order to minimize the surface area exposed to water. The hydrophobic
interactions between lignin and enzymes have been discussed by Palonen et al. who found that the carbohydrate binding module (CBM) of *Trichoderma reesei* enzymes plays a significant role in the non-productive adsorption on lignin (64). The amino acids exposed on the surface of *T. reesei* enzymes have hydrophobic character (69) and therefore the enzyme may bind to the hydrophobic surface of lignin.

The electrostatic interactions were studied by Nakagame et al., who determined that at pH 4.8 some cellulases carry negative charges whereas others are in fact oppositely charged (39). It was concluded that the positively charged proteins are preferentially adsorbed onto lignin, which display an anionic character at the same pH. In contrast, enzymes and lignin become negatively charged at high pH and thus electrostatic repulsion reduces their affinity (70). Moreover, the main acidic groups that exist on the surface of lignocellulosic fibers are associated to the uronic acid in xylans and the carboxylic acid in lignin (71, 72), both of which display different isoelectric points and thus affect to different degree the interactions, depending on the pH of the medium. The amount of carboxylic groups present in the substrate varies depending on the plant species and the method used in its isolation. In milled wood lignin from aspen and pine these groups are measured to be 0.28 and 0.30 mmol per gram of lignin, respectively, whereas from technical hardwood and softwood these increase to 0.41 and 0.47, respectively (73).

Hydrogen bonding occurs through hydroxyl groups present in lignins and enzymes (62, 63, 66). Among these groups, the phenolic hydroxyls are directly responsible for enzyme adsorption (63, 66), while aliphatic and carboxylic hydroxyl groups have been indicated to
contribute to lignin-enzyme ionic interactions (62). Despite the evidence accumulated, however, the detailed nature of these interactions still needs further examination.

Surfactants are known to reduce the affinity between enzymes and lignin, mainly by affecting the hydrophobic effects (2, 114, 120-122, 124) and also by bonding (H-bonds) with lignin (120). Among the amphiphile molecules, non-ionic surfactants have been proven to be most effective to enhance enzymatic hydrolysis of lignocellulosic biomass (2, 123, 124, 177, 178). Proposed mechanisms for the effect of surfactant molecules on bioconversion include their ability to (1) open the structure of the lignocellulose substrate, making cellulose more accessible to enzymes (122), (2) improve enzyme stability (122, 177, 179, 180), and (3) change enzyme-substrate interactions by blocking the lignin and reducing its affinity with enzymes (122). Direct evidence of these mechanisms have been elusive, however, understanding the molecular role of surfactants and lignin surfaces remain highly relevant not only for enhancing sugar yields during lignocellulosic hydrolysis but also for considering possible enzyme recycling options.

Polyoxyethylene (20) sorbitan monooleate, a nonionic surfactant, has been recognized to improve enzymatic hydrolysis (2, 122); however, mechanistics aspects related to its interaction with lignin substrates are only known to a limited extent. In order to demonstrate how non-ionic surfactants reduce non-productive binding between lignin and enzymes, we carried out studies in real time and in-situ by using surface-sensitive methods such as quartz crystal microbalance with dissipation monitoring (QCM-D) and surface plasmon resonance (SPR). The role of pH in the binding between lignin and enzymes was also elucidated. Five different lignin substrates were used, including native, enzymatically-produced,
autohydrolysis and reference (technical) lignins. In addition, a cellulose substrate in the form of cellulose nanofibrils (CNF) was used to evaluate the adsorption of the non-ionic surfactant onto such hydrophilic material and its impact on enzymatic digestibility.

4.3 Experimental

4.3.1 Materials.

All the chemicals were used as received without purification. Milli-Q water (18 Ω) was used in all the experiments. Polyoxyethylene (20) sorbitan monooleate surfactant (trade name Tween 80), acetic anhydride (certified ACS), polystyrene (Mw 230,000), polyethylenimine (Mw 750,000), and toluene were purchased from Sigma Aldrich. 1,4 dioxane (certified ACS) was obtained from Acros. Sulfuric acid, hydrogen peroxide (30%v/v), sodium acetate, and acetic acid were purchased from Fisher Scientific. A commercial mixture of enzymes (endoglucanases, cellobiohydrolases, and beta-glucosidases), under the trade name Cellic® CTec2, and a monocomponent enzyme, cellobiohydrolase I (CBH-I) from Trichoderma reesei, were kindly provided by Novozymes and the Technical Research Centre of Finland (VTT), respectively. The cellulase activity was 140 FPU/ml, as determined by the filter paper method (144).

4.3.2 Lignin and cellulose substrates.

A low-ash softwood Kraft lignin (SWK) and a hardwood Kraft lignin (HWK) with high ash content were kindly donated by Domtar Inc. (Plymouth Pulp Mill, NC) and Suzano Pulp Mill (Brazil), respectively. Hardwood autohydrolysis cellulytic lignin (HWAH) was
prepared in our lab after autohydrolysis pretreatment of biomass (180 °C for 1 h). Native cellulolytic lignin from a hardwood (maple) (MPCEL) and non-wood biomass (wheat straw) (WSCEL) were also prepared by using planetary ball-milling for 10 and 4 hours, respectively. These latter lignin samples were extracted with 96% dioxane and isolated by enzymatic hydrolysis by using Ctec2 and Htec (9:1 ratio), also from Novozymes (5% solids content, acetate buffer 50 mM, pH 4.8, 10 FPU/g o.d. fiber, 96 h, 50 °C). The lignin samples were purified by washing with milliQ-water (Millipore) and centrifugation (Beckman Centrifuge Model J-21C, 3000 rpm) several times. After the purification step, the lignin samples were freeze-dried prior to use. Additionally, cellulose nanofibrils (CNF) were produced by microfluidization of refined, fully bleached birch Kraft fibers that were supplied by International Paper (Riegelwood, North Carolina).

### 4.3.3 Lignin composition and characterization.

The insoluble and soluble components of the lignins were determined by acid hydrolysis. Briefly, 100 mg of the given lignin source were reacted with 1.5 ml of sulfuric acid (72%v/v H₂SO₄) at 25 °C for 2 h with stirring every 20 min. The dispersion was then diluted and autoclaved at 121 °C for 1.5 h, cooled down overnight, and filtered through a fine crucible for gravimetric determination of acid-insoluble lignin (Klason lignin). The supernatant was used to determine acid-soluble lignin by UV-VIS spectroscopy at 205 nm (Lambda XLS, PerkinElmer, Inc). After neutralization of this supernatant, sugar analyses were performed in a HPLC unit, as previously reported. Hydroxyl groups content and molecular weight determinations were carried out by ³¹P NMR and gel permeation chromatography (GPC) following the procedures published elsewhere.
4.3.4 Lignin surface charge and size of associated structures.

The zeta potential of lignins and size of surfactant micelles were determined using a Zetasizer Nano-ZS (Malvern Instrument Ltd). The zeta potential of the commercial mixture of enzymes and lignin at different pH values were measured in acetate buffer solution (50 mM) and used to evaluate the possible role of electrostatic interactions. Commercial enzymes were diluted to 5 mg/ml in 50 mM acetate buffer and pH of 4.8 and 5.5. Buffer solutions at different pH levels were used with a lignin concentration of 0.03% (w/v). Each lignin solution was mixed during 1 hour at 50 °C and 200 rpm using an incubator shaker, and allowed to settle for 1 h prior to zeta potential measurement. The change in the size of the micelle upon interaction with lignin was determined in aqueous media. For this, lignin samples were dissolved in 0.1 mg/ml non-ionic surfactant solution to a final concentration of 0.03% (w/v) using a sonication bath for 15 min to allow dispersion, then an aliquot was centrifuged at 10000 rpm during 10 minutes. The supernatant was used to determine the size of the associated structures.

4.3.5 Thin film substrates.

Gold-coated QCM-D sensors were cleaned with piranha solution (70% v/v H₂SO₄ + 30% v/v H₂O₂ (30%)) for 10 min, rinsed with abundant milli-Q water, dried with nitrogen, and followed by UV/ozone treatment for 10 min. Silica wafers were also used after treatment with NaOH solution (1 M) during 20 seconds followed by with milli-Q water rinsing and drying with nitrogen. Finally, they were subjected to UV/ozone for 5 min. For thin lignin film preparation, each sample was dissolved in 1,4-dioxane overnight (0.5 wt%). The respective lignin solution was let to settle and a given supernatant volume was used for spin coating.
Polystyrene (PS) was used as an intermediate layer to hold the lignin film on the gold-coated sensor or silica substrates. PS was dissolved in toluene (0.5 wt%) and spin-coated onto the cleaned surfaces using 2000 rpm and an acceleration of 1785 rps$^{-1}$. Then they were dried in an oven at 80 °C for 30 min. After this, four layers of the dissolved lignin were spin-coated on the PS-coated surfaces, using a speed of 2000 rpm and acceleration of 1785 rps$^{-1}$. Cellulose thin films were prepared dispersing CNF in milli-Q water (0.1 wt% solids content), followed by dispersion for 10 min at 25% amplitude in the tip sonicator and centrifuged at 10,000 rpm for 30 min. CNF in the supernatant was spin-coated (3,000 rpm for 30 s) on gold sensors carrying pre-adsorbed layer of polyethylenimine (PEI) (15 min adsorption using 500 ppm solution, washed, and dried with nitrogen gas), and dried in oven at 80 °C for 30 min. These thin films were stored in a desiccator until use.

4.3.6 Thin film characterization.

AFM imaging was performed to assess the morphology, roughness and material distribution of the films. The different dispersions dried on silica surfaces were mounted on aluminum sample holders and examined with a Dimension 3000 scanning probe microscope from Veeco Metrology Group. Scanning was performed in tapping mode in air using silicon cantilevers (NSC15/AIBS) delivered by Olympus AC160TS. The drive frequency of the cantilever was about 275–325 kHz (nominal resonance of 300 kHz). The scanned areas were imaged. No image processing except flattening was made. Images were taken with a feedback loop to keep the amplitude of oscillation constant and the response of the feedback loop was measured. The response was used to measure how far the scanner was moved in Z in order to keep the amplitude of oscillation constant. Film thickness was measured using a variable angle
spectroscopic ellipsometry (VASE) (J. A. Woollam, Co., Inc.) with a wide spectral range capability of 190-1100 nm. The thickness was evaluated from the experimentally measured ellipsometric angles Ψ and Δ using the supplied software as the angle of incidence was varied between 65° and 70° between 400 and 800 nm. Water contact angles (WCA) of the thin films were measured by using a contact angle goniometer SEO Phoenix 300 (Korea), via the sessile drop method with a drop volume of 20 μl at ambient conditions. WCA were calculated using Image J software. All measurements were done in triplicate. Silica wafers and PS-coated surfaces exhibited a WCA and roughness of 8±1° and 0.92 nm, and 86±3° and 0.38 nm, respectively. The characterization of the different lignin thin films is included in Table 4.1.

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Roughness (nm)</th>
<th>Thickness (nm)</th>
<th>WCA (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td>10</td>
<td>24.6 ± 5.5</td>
<td>63 ± 1</td>
</tr>
<tr>
<td>HWK</td>
<td>2.4</td>
<td>14.3 ± 2.4</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>HWAH</td>
<td>3.4</td>
<td>8.9 ± 3</td>
<td>60 ± 2</td>
</tr>
<tr>
<td>MPCEL</td>
<td>0.53</td>
<td>21.4 ± 0.02</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>WSCEL</td>
<td>0.55</td>
<td>23.5 ± 0.03</td>
<td>70 ± 3</td>
</tr>
</tbody>
</table>

### 4.3.7 Adsorption experiments.

Adsorption and desorption phenomena were followed by using a quartz crystal microbalance (QCM) with dissipation monitoring (Model E4,Q-Sense AB, Göteborg, Sweden) operated in continuous mode at 25 °C (40°C in the case of cellulose model surfaces) and constant flow rate of 100 µl/min. Commercial cellulases (Cellic® CTec2 from Novozymes) were diluted at 5 mg/ml in the acetate buffer at 50 mM, whereas the monocomponent CBH-I
was used at 1 mg/ml at pH 5.5. Concentrations below and above the critical micelle concentration (CMC) of polyoxyethylene (20) sorbitan monooleate were used. The CMC was 0.012 mM, as quoted by the supplier and concentrations used are reported as multiple units of the CMC: 0, 0.1 xCMC (~0.001 mg/ml), 0.8 xCMC (~0.013 mg/ml), 3 xCMC (~0.05 mg/ml), 6 xCMC (~0.1 mg/ml), and 19 xCMC (~0.3 mg/ml). The lignin films were stabilized in buffer prior to the adsorption experiments; then, the different surfactant solutions were added until equilibrium. The surfactant excess was rinsed with buffer solution until the QCM signal reached a plateau. In binding experiments, once the thin films were primed with the adsorbed nonionic surfactant, the enzymes were injected and the shift in frequency was monitored. After the enzymes reached the maximum binding capacity, acetate buffer was used to remove the unbounded enzymes. The adsorbed mass before and after rinsing was calculated from the shift in QCM frequency according to the Johannsmann model using Eq. 12 (97, 98):

\[
m^* = -\frac{\sqrt{\rho \mu} \Delta f}{2 f_0 \cdot f}
\]

Eq. 12

All measurements were recorded at a fundamental resonance frequency of 5 MHz and its overtones corresponding to 15, 25, 35, 55, and 75 MHz. The third, fifth, and seventh were used for data processing. The experiments were run at least for triplicated and an analysis of variance (ANOVA) was performed to test the differences for the irreversible adsorbed mass of cellulases onto lignin surfaces under the given surfactant concentrations. Representative QCM-D curves for the adsorption of enzymes onto lignin pre-treated with surfactant can be found as Supporting Information (Figure S4-Figure S6).
Adsorption was also monitored by using surface plasmon resonance (SPR, Navi 200, Oy BioNavis Ltd., Tampere, Finland); a description of this technique, as it applies to lignin films, can be found in our earlier publication (183). The experimental conditions were similar to those used for QCM-D adsorption experiments, except for the flow rate (15 µl/min) (184). The thickness of the adsorbed surfactant layer was calculated using Eq. 13.

\[
    d = \frac{l_d \Delta \theta}{2m (\eta_a - \eta_o)}
\]

Eq. 13

where \(d\) is the thickness, \(l_d\) is the evanescent electromagnetic field decay (240 nm) (103), \(m\) is the sensitivity factor (109.95°/RIU) (183), \(\eta_a\) and \(\eta_o\) are the refractive index of the surfactant and buffer solutions (1.472 and 1.334), respectively.

The extent of binding and hydrolysis were determined by fitting the QCM data to exponential decay (Eq. 14) and Boltzmann-sigmoidal (Eq. 15) equations (185). The QCM \(\Delta f\) (Hz) shift of the third overtone was used.

\[
    \Delta f = M_{max} (1 - e^{-\frac{t}{\tau}})
\]

Eq. 14

\[
    \Delta f = A + \frac{B - A}{1 + e^{(V_{50} - t)/C}}
\]

Eq. 15

The adsorption parameters \(M_{max}\) and \(1/\tau\) represent the maximum adsorption capacity (determined from the minimum QCM frequency shift in Hz) and the adsorption rate (min\(^{-1}\)), respectively. Hydrolytic parameters \(A\), \(B\), \(V_{50}\), and \(1/C\) represent the frequency (Hz) at which hydrolysis starts and ceases, the time for maximum conversion, and the hydrolysis rate, respectively.
respectively. All these parameters, except $A$, were obtained by minimizing the sum of the squared error between experimental and computed values from QCM data before rinsing.

It was found that for CNF the enzyme binding highly correlated to the Boltzmann-sigmoidal equation (Eq. 16) (92):

$$\Delta f = A + \frac{M_{max} - A}{1 + e^{(\bar{W}_{50} - t)/\tau}}$$  \hspace{1cm} \text{Eq. 16}$$

The non-ionic surfactant adsorption isotherms were fit to empirical functions such as the Langmuir or the one-step models (186), which assume that surfactant molecules interact with the solid substrate forming at equilibrium a solloid or hemi-micelle:

$$\text{surface site + monomer} \leftrightarrow \text{hemi-micelle}$$

where the equilibrium constant is $k = a_{hm}/a_s a$ with $a_{hm}$, $a_s$, and $a$ are the activities of adsorbed hemi-micelle, surface site, and surfactant monomer in solution, respectively. At low concentrations $a$ is equal to the surfactant concentration, $C$. Thus, for $C < \text{CMC}$, $a_{hm}$ and $a_s$ are the concentration of adsorbed hemi-micelle and unoccupied surface areas, respectively. Through mass action law the activities can be converted to adsorption density ($\Gamma$) by using Eq. 17 (186):

$$kC^n = \frac{\Gamma}{\Gamma_\infty - \Gamma}$$ \hspace{1cm} \text{Eq. 17}$$

where $\Gamma_\infty$ is the maximum adsorption density at high solution concentrations and $n$ is the surfactant aggregation number. Furthermore, the thermodynamic surface free energy of
micellization ($\Delta G^0_m$) and aggregation ($\Delta G^0_{sa}$) were calculated according to equations Eq. 18 and Eq. 19, respectively.

\[
\Delta G^0_m = -RT \ln \text{cmc}
\]  

\[
\Delta G^0_{sa} = -(RT \ln k)/n
\]  

where $R$ and $T$ are gas constant and absolute temperature, respectively. The fitting parameters $\Gamma_\infty$, $k$, and $n$ were obtained by minimizing the sum of the squared error between experimental and computed data.

### 4.4 Results and discussion

#### 4.4.1 Lignins and thin films.

The chemical characteristics of the lignins used for the preparation of thin films are shown in Table 4.2, which includes the acid-insoluble (AIL, equivalent to Klason lignin) and acid-soluble (ASL) fraction content, carbohydrates and hydroxyl group concentration ($^{31}$P-NMR) as well as molecular mass.

**Table 4.2.** Chemical characteristics of the lignins used in this study

<table>
<thead>
<tr>
<th>Lignin</th>
<th>AIL wt%</th>
<th>ASL wt%</th>
<th>Carbohydrates wt%</th>
<th>Mw</th>
<th>Mn</th>
<th>Hydroxyl group content (mmol/g lignin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Aliphatic OH</td>
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</tr>
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<td>8</td>
<td>12</td>
<td>9383</td>
<td>4041</td>
<td>8.2</td>
</tr>
</tbody>
</table>

C= condensed structure, N-C= non-condensed structure
Based on the nature of the lignin samples they are regarded as suitable precursor of models to study the interaction with enzymes and non-ionic surfactants. Lignins, as those considered here are of great importance not only to study interactions with enzymes but also with different molecules relevant to fiber processing, mainly in biorefinery platforms. Kraft pulping generates phenolic units on fibers due to the cleavage of aryl-ether linkages of lignin (187). In contrast, autohydrolysis pretreatment mainly removes hemicelluloses (169), leaving the other components less affected, mainly lignin and cellulose (169, 188). In the case of lignin, the severity of this type of pretreatment caused its recondensation and relocation on the solid substrate during the process (169, 189). β-O-4 linkages and ester bonds were acid-catalyzed, which resulted in a more condensed lignin (39, 132) with a high molecular weight and more phenolic hydroxyl groups (190). Samuel et al. (191) pretreated poplar at 200 °C for 30 min and determined a reduction of 43% in aliphatic hydroxyl groups and an increase of 130 and 96% in the total phenolic and carboxylic groups, respectively. Therefore, the autohydrolysis pretreatment can cause a significant effect on lignin. However, we note that the conditions used in this work were comparatively milder (180 °C for 1 h). On the other hand, the cellulolytic lignin, which undergoes minor structural changes, has been considered as representative of the whole lignin present in lignocellulosic biomass (192).

The AFM images of the different thin lignin films (Figure 4.1) indicate complete and uniform surface coverage. High magnification images (second and third columns in Figure 4.1) reveal the arrangement of lignin after spin-coating on the solid support. As the solvent evaporated, lignin self-associated to create distinctive features (spherical, nanosized particles), likely stabilized by hydrogen bonding and van der Waals forces (193). Section analyses of
these images (Table S2) reveal a dense structure for SWK. Characteristic properties of these lignin films are presented in Table 4.1. The root-mean-square (RMS) roughness was determined from the images in Figure 4.1 (5x5 μm). SWK thin lignin film showed the highest roughness, 10 nm, whereas those for HWK and HWAH were 2.4 and 3.4 nm, respectively. The native cellulolytic lignins exhibited the lowest values, 0.53 and 0.55 nm for MPCEL and WSCEL, respectively. The thickness of these thin lignin films was determined by ellipsometry and noted to be in the range between 9 and 25 nm. Based on the ellipsometric model used and the fitted data, the refractive indices of the lignins was determined to be ~1.60 at 630 nm, which indicate compact and dense films (193). The wettability of the films was also determined by WCA values included in Table 4.1. All the lignin exhibited similar values, between 60 and 79 degrees; the native cellulolytic lignins showed the highest WCA.
Figure 4.1. AFM tapping mode images of the lignin thin films used in this study (columns from left to right correspond to scan sizes of 5 x 5 μm, 1 x 1 μm, and 500 x 500 nm). The samples included the following lignins (from top to bottom): SWK, HWK, HWAH, MPCEL, and WSCEL. The height scale corresponds to Z values between 0 and 90 nm, as indicated by the bar.

The stability of the lignin films supported on silica was tested by immersion in deionized water and 1 M NaOH solution for 3 h. It was noted that immersion in water did not
affect the wettability of these lignin films, indicating strong attachment to the solid support. However, a slight decrease in roughness was observed, possibly due to hydration or desorption of loosely attached lignin molecules. Immersion in the alkali solution caused partial lignin removal from the solid support.

### 4.4.2 Effect of pH on lignin surface charge.

Electrostatic interactions affect non-productive binding between lignin and enzymes (39); therefore, the surface charge, assessed by zeta potential measurements, was determined for the lignins dispersed in aqueous media at given pH. The zeta potential became more negative as the pH increased (Figure 4.2) due to the deprotonation of carboxyl groups (83, 84). Similar observations applied to lignin residues after pretreatment with dilute acid and sulfite to overcome recalcitrance of lignocellulose (SPORL) (70). A linear regression indicated that pH has a strong effect on the surface charge of HWAH lignin; however, it showed the lowest zeta potential values, probably because of the reduction in the number density of –COOH groups after autohydrolysis pretreatment. Native lignins exhibited intermedium values, whereas the kraft lignins (SWK and HWK) presented a high surface charge due to the large number of carboxylic groups generated during the kraft pulping process, (84) which can more easily ionize in water (83).

Enzymatic hydrolysis of biomass with *Trichoderma reesei* cellulases similar to those used in this work indicated an optimal pH range for hydrolysis of 5.5 to 6.2, while pH=4.8 has been used for pure cellulosic substrates (117). In this study, two pH levels, 4.8 and 5.5, were selected to study the affinity of enzymes with lignin, as discussed in the next section.
Figure 4.2. pH-induced charging of lignin (as measured by the zeta potential, $\zeta$) for the five different lignins dispersed in aqueous acetate buffer (50 mM) at different solution pH. A linear fit to the equation $Z = k \cdot pH + Z_0$ yielded $k$ values of $-4.06$, $-3.60$, $-8.36$, $-4.08$, and $-5.60$ for SWH, HWK, HWAH, MPCEL, and WSCEL lignins, respectively.

4.4.3 Enzyme adsorption on lignin thin films.

The non-productive adsorption of cellulases on lignin was investigated. The isolated lignins used in this study are expected to differ from un-extracted or native lignin present in the biomass. In fact, any fractionation method would unavoidably affect the characteristics and properties of the isolated lignin. Keeping this in mind, electrostatic forces are expected to influence unproductive interactions between enzymes and lignin (39, 65, 70). QCM adsorption profiles for cellulases adsorbing onto lignin substrates at two pH levels, 4.8 and 5.5, are shown in Figure 4.3. The binding parameters were determined and included as Supporting
Information (Table S3). At pH 4.8 the maximum binding was higher for SWK and HWK (46 Hz or 8.2 mg/m² and 51 Hz or 8.6 mg/m², respectively); however, the adsorption rate was similar for the five lignin types. After rinsing with background aqueous solution and upon reaching equilibrium, the amount of irreversibly adsorbed enzymes was calculated: the native cellulolytic lignins, MPCEL and WSCEL, adsorbed enzymes to a lesser extent compared to the other samples: 4.4±0.2 and 4.7±0.1 mg/m², respectively whereas for SWK, HWK, and HWAH the corresponding values were 6.5±0.2, 6.8±0.1, and 5.7±0.1 mg/m². The same pattern was observed at pH 5.5. However, as the pH increased the maximum amount of adsorbed enzymes was lower. The most notorious effect was observed for the adsorption rate, which decreased significantly by changing the pH of the medium from 4.8 to 5.5 (Table S3). This reduction in the rate of adsorption is due to the increased negative charge of both lignin (Figure 4.2) and enzymes. In fact, the zeta potential of the enzyme mixture diluted in acetate buffer exhibited values of -6.4 and -9.6 mV at pH 4.8 and 5.5, respectively. The higher negative values for both lignin and enzymes explain increased electrostatic repulsion. The enzyme irreversibly adsorbed at pH 5.5 was calculated to be 6.3±1.6, 6.5±0.5, 5.5±0.1, 3.3±0.1, and 3.8±0.1 mg/m² for SWK, HWK, HWAH, MPCEL, and WSCEL lignins, respectively. Although electrostatic repulsion by the increased osmotic pressure is likely to have a major role, the difference in the amount of enzymes adsorbed irreversibly on SWH, HWK and HWAH substrates at pH 4.8 and 5.5 was not significant (<5%). In contrast, the reduction of irreversibly adsorbed enzymes onto native cellulolytic lignins was ~20%, suggesting that a weak electrostatic interaction existed between these lignin and the cellulases. The similar
adsorbed amount measured here for the commercial cellulases suggests that hydrophobic interactions are a driving factor that control the affinity to lignin.

Correlations among the different hydroxyl groups in lignin and the maximum frequency upon enzyme adsorption are plotted in Figure S3. Phenolic hydroxyl groups have been related to increased enzyme affinity for lignin (63, 66). In this work, MPCEL, SWK, and HWK lignins contained more phenolic hydroxyl groups than HWAH and WSCEL (Table 4.2). This fact partially explains the higher amount of bound enzyme at the two pH levels tested for SWK, HWK, HWAH, and WSCEL lignins, with the exception of the MPCEL sample (Figure S3b). In contrast, a reduction in the amount of absorbed enzymes was observed as the carboxylic group content increased (Figure S3c), which can favor a reduction in non-productive interactions (194), for example, by increasing the negative charge of lignin (83, 84, 194). Lignin not only acts as a physical barrier but, due to its nature and pretreatment method used for isolation, it is also a chemically heterogeneous macromolecule that takes part in non-specific interactions with the enzymes (66).

Figure 4.3. Representative shifts in QCM frequency upon adsorption of cellulases from aqueous media (5 mg/ml) onto lignin films at pH= 4.8 (a) and 5.5 (b): SWK (◇), HWK (○),
HWAH (△), MPCEL (□), and WSCEL (+). Baseline upon enzyme injection and rinsing with 50 mM acetate buffer at pH 4.8 and pH 5.5 are indicated by solid and dotted arrows, respectively.

4.4.4 Mechanism of surfactant adsorption onto lignin.

Hydrophobic interactions between the alkyl chains and the hydrophobic sites of the surface have been ascribed as main driving force for surfactant adsorption (195). Figure 4.4 shows the adsorption isotherms for the polysorbate surfactant onto the thin lignin films before (total adsorption, Figure 4.4a) and after (irreversible adsorption, Figure 4.4b) rinsing. As expected, the surfactant adsorbed mass increased as the concentration increased. Upon rinsing the adsorbed mass decreased by ~50% and a plateau was reached for surfactant concentrations above the CMC. A sharp increase in adsorption at concentration close to the CMC is due to the lateral interactions between surfactant monomers which start to associate (196). Using Gibbs adsorption isotherms, the maximum surface excess (Γ∞,air/liquid) for the surfactant at the air-liquid interface was found to be 29.3×10⁻⁷ mol/m², similar to values reported in previous work (197). Significantly lower adsorbed amounts were determined after rinsing, indicating the removal of loosely-bound molecules and yielding an irreversibly adsorbed surfactant layer. These findings suggest the complexity of surfactant adsorption onto lignin surfaces, mainly due to their heterogeneous structural features.
Figure 4.4. Adsorbed mass isotherms for different lignin substrates upon adsorption of polyoxyethylene (20) sorbitan monooleate at given concentrations (as units of the CMC). Data presented correspond to adsorbed excess before (a) and after (b) rinsing. Note that partial substrate removal occurred for HWK lignin in experiments with surfactant concentrations above the CMC (see QCM-D profiles in Figure S4); therefore, no rinsing was applied in this case and the surfactant adsorption data showed for HWK Figure (b) is not as relevant.

The QCM frequency data obtained upon surfactant adsorption before rinsing was fitted to the kinetic models introduced before for the maximum adsorption capacity (Eq. 14), and the one-step model (Eq. 17). The results for the first model are presented in Table S4 whereas those for the one-step model are shown in Table 4.3. Both models described satisfactorily the maximum adsorption of surfactant onto lignin (high correlation values except for SWK lignin). As the surfactant concentration increased, the binding rate ($1/\tau$) calculated by the exponential decay model increased. The highest maximum binding ($M_{max}$) was found for HWK, while the highest binding rate corresponded to MPCEL lignin. Remarkably, a high total adsorption generally correlated with a high total hydroxyl content (Table 4.2), which is expected to contribute to hydrogen bonding with the surfactant polar groups. In the one-step model the surfactant adsorption is described as reactions between unoccupied sites and surfactant molecules to form hemi-micelles (186). Furthermore, the standard free energy for surface
micellization (Eq. 18) was found to be 28 kJ/mol, whereas the standard free energy of aggregates (Eq. 19) adsorbed onto the different lignin substrates yielded lower values (Table 4.3), indicating that surface aggregation was energetically favored. Maximum adsorption density, $\Gamma_\infty$ ($10^{-7}$ mol/m$^2$) values were similar for all the lignins (Table 4.3). A value of average aggregation number parameter $n > 1$ suggests surface micellization, whereas a value $< 1$ suggests that the each adsorbed molecule occupies more than one site. Interestingly, in the case of HWK lignin $n > 1$, indicating surface micellization. This would explain partial removal of lignin molecules upon surfactant adsorption and rinsing (note Figure S4b,c,d where the surfactant adsorption is followed by a sharp increase in frequency due to the presence of surfactant). A total lignin removal of 19±2% was calculated by QCM measurements in air for the sensor with HWK lignin before and after surfactant adsorption. Surfactants can solubilize lignin by hydrophobic interactions (4). The micelle size of polyoxyethylene (20) sorbitan monooleate in water is 10.3 ± 0.4 nm at 25 °C, as determined by dynamic light scattering. It was found that in the presence of HWK lignin the surfactant tended to form aggregates with lignin, with sizes up to 128 ± 4 nm and lignin solubility increased up to 25% in the aqueous medium.
Table 4.3. Fitting parameters for the one-step model upon adsorption of polyoxyethylene (20) sorbitan monooleate onto different lignins before rinsing.

<table>
<thead>
<tr>
<th>Lignin</th>
<th>$\Gamma_\infty \times 10^{-7}$ (mol/m$^2$)</th>
<th>k</th>
<th>N</th>
<th>$R^2$</th>
<th>$-\Delta G_{sa}^0$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td>50.7</td>
<td>3.7</td>
<td>0.14</td>
<td>0.71</td>
<td>23.6</td>
</tr>
<tr>
<td>HWK</td>
<td>37.2</td>
<td>4.0x10$^5$</td>
<td>1.7</td>
<td>0.99</td>
<td>25.5</td>
</tr>
<tr>
<td>HWAH</td>
<td>27.7</td>
<td>3.6x10$^4$</td>
<td>0.9</td>
<td>0.98</td>
<td>29.9</td>
</tr>
<tr>
<td>MPCEL</td>
<td>23.9</td>
<td>7.0x10$^3$</td>
<td>1.0</td>
<td>0.98</td>
<td>33.4</td>
</tr>
<tr>
<td>WSCEL</td>
<td>30.9</td>
<td>3.2x10$^3$</td>
<td>0.7</td>
<td>0.99</td>
<td>30.5</td>
</tr>
</tbody>
</table>

$\Gamma_\infty$ (10$^{-7}$ mol/m$^2$) corresponds to the maximum adsorption density; k is the equilibrium constant; n is the aggregation number; $R^2$ corresponds to the coefficient of determination for the model; and $\Delta G_{sa}^0$ (kJ/mol) is the standard free energy of surface aggregation.

The changes in morphology and wettability of lignin films after adsorption of polyoxyethylene (20) sorbitan monooleate (6xCMC) are shown in Figure 4.5. Compared to the values of the original films of SWK, HWK, and HWAH, the surface roughness was reduced upon surfactant adsorption (Table 4.1). In contrast, the roughness increased for MPCEL and WSCEL, suggesting the possibility of steric hindrance effects, which correlates with lower enzyme accessibility. As revealed by AFM images, the morphology of the lignin surface changed after surfactant adsorption, which can be associated to the formation of surfactant aggregates since polyoxyethylene (20) sorbitan monooleate forms half-micelles or hemi-micelles on hydrophobic surfaces (198, 199). Imaging of the micelles was challenging due to the high roughness of the surfaces. The mechanism of micellar break up leading to the formation of hemi-micelles on hydrophobic surfaces may occur by the contribution of various effects: hydrophobic lateral interactions and hydrophobic interactions between the hydrocarbon chains and the surface (133), as well as hydrogen bonding (120, 133). Surfactant adsorption at 6xCMC was monitored by SPR, which allows to determine the thickness of the adsorbed layer by using Eq. 13. The results indicated a layer thickness between 2.3-4.3 nm at
maximum adsorption (before rinsing) and 0.9-3 nm for the molecules irreversibly bounded (measured after rinsing) onto the lignin surfaces (Table S5). At close-packed coverage, the surfactant molecules would be arrange in an end-to-end configuration with a molecule distance \( \sim 5 \text{ nm} \) (200). Surfactant layer of 6.7 nm has been reported on hydrophobic surfaces (WCA 110°) by using the surface force apparatus,(200) whereas on surfaces with a WCA \( \sim 71° \) the thickness was found to be \( \sim 1.2 \text{ nm} \) for polyoxyethylene (20) sorbitan monooleate by using a dual slab waveguide interferometer (201). A rough estimation of the apparent number of layers on the solid can be determined dividing the maximum surfactant adsorption density at the solid/liquid interface \( (\Gamma_\infty) \) (Table 4.3) by the maximum adsorption density at the air/liquid interface \( (\Gamma_{\infty,\text{air/liquid}}) \) (133), which gives values close to 1 (before rinsing) and <1 (after rinsing) surfactant adsorption. Moreover, the number of molecules per nm\(^2\) and the aggregation number \( n \) were both <1 at irreversible adsorption (Figure 4.5). Therefore, the results in this study suggest the presence of a monolayer or a disorganized or patchy bilayer structure since the thickness values obtained in this study after rinsing are comparable to those obtained with similar WCA values (201). The low thickness values compare to the end-to-end conformation and can be related to the chemical structure of the surfactant used, which possess a double-carbon bond in the aliphatic chain that would force the molecule to stand at an angle <90°(200). Moreover, the WCA values decreased after surfactant adsorption, indicating that the surfactant head groups form the outmost layer.
Figure 4.5. AFM tapping mode height (first row) and phase (second row) images at 1x1 μm of different lignins carrying adsorbed polysorbate surfactant (adsorption from aqueous medium at 6 xCMC concentration followed by rinsing). (Note: for HWK lignin the image was taken before rinsing). The number of molecules per nm² calculated by the Gibbs adsorption isotherm was 0.7, 2.0, 0.8, 0.5, and 0.7 for SWK, HWK, HWAH, MPCEL, and WSCEL, respectively. This indicates a maximum packing of 1.8 molecules/nm² at the air-liquid interface. The aggregation number n obtained by using the one-step model was 0.3, 0.24, and 0.7 for SWK, MPCEL, and WSCEL, respectively (a poor correlation existed for the other lignin samples). The height and phase scale correspond to Z values between 0 and 10 nm, and 0 and 40° (0-20° in the case of MPCEL and WSCEL), respectively.

4.4.5 Surfactant blocking mechanism for enzymes exposed to lignins.

From previous results (Figure 4.3), a slight decrease in the amount of enzymes adsorbed on lignin occurs with increased pH, explained by electrostatic repulsion. Therefore, a pH of 5.5 was selected for the aqueous medium to study the effect of surfactant on the reduction of enzyme affinity with lignin, especially at surfactant concentrations above the CMC. The maximum binding was determined according to Eq. 14 for both surfactant and enzymes (Table S4). As the surfactant concentration increased, the surfactant total adsorbed mass and adsorption rate increased, whereas the opposite behavior was observed for the enzymes. The
total enzyme adsorption decreased significantly and the presence of the surfactant layer slowed down enzyme adsorption on the lignin surfaces, especially at concentrations above the CMC. The relative mass of irreversibly adsorbed surfactant and commercial enzyme after rinsing is presented in Table S6 (in the case of HWK lignin the enzymes were added without rinsing since the surfactant caused partial lignin removal). There was a small amount of adsorbed surfactant at 0.1 xCMC (free surfactant in solution) on the lignin surface (data not shown) and the molecules were removed easily by rinsing; thus, it was not possible to calculate the amount of surfactant irreversibly adsorbed on SWK, HWK, and HWAH films. In the case of SWK and HWK films and for surfactant concentration close to the CMC (but still at sub-micellar concentrations) the reduction of enzyme affinity was less than 20%. Remarkably, for the autohydrolysis and native cellulytic lignins the surfactant prevented enzyme adsorption by more than 80%. At sub-micellar concentrations, the surfactant molecules attach to the hydrophobic surfaces, possibly with the hydrocarbon chains aligned parallel to the surface (198). In fact, the unsaturated carbon-carbon double bond in the alkyl chain of polyoxyethylene (20) sorbitan monooleate favors the molecule to adopt a parallel configuration at the surface (200), whereas lateral interactions between monomers take place at concentrations close to the CMC (196). Finally, at concentrations above the CMC, the molecules become decreasingly tilted with respect to the surface, and have a greater tendency to form aggregates (198). It is found that the affinity of polyoxyethylene (20) sorbitan monooleate with lignin increases with concentration, for example, increasing concentration from 0.85 to 20 xCMC (Figure 4.4). As the irreversibly surfactant adsorbed mass increased, the affinity of cellulases with lignin decreased. Using the ANOVA statistical analysis (α-level 5%) a significant difference in the
amount of enzymes irreversibly adsorbed was determined (for surfactant concentration below and above the CMC), for all the lignins except for MPCEL. A surfactant concentration close to the CMC was effective in reducing the enzyme affinity. Concentrations above the CMC showed no significant difference in the amount of enzymes irreversible adsorbed. Moreover, the effect of surfactant (6 xCMC) on the reduction of lignin affinity with monocomponent CBH-I enzyme was investigated (QCM) and the results for binding parameters are shown in Table S7. This monocomponent enzyme exhibited higher affinity for the technical and pretreated lignins (SWK, HWK and HWAH) compared to the native cellulolytic lignins. Interestingly, the surfactant prevented the monocomponent adsorption for all the lignins: except for the case of SWK substrates, negligible or no adsorption was determined on lignins primed by the surfactant. This is explained by the blocking of the hydrophobic sites by the adsorbed surfactant, thus preventing non-productive interaction with the CBH-I that contains a carbohydrate binding domain as primary site for hydrophobic interactions with lignin (59, 64, 120).

The deactivation or inhibitory effect of lignin toward cellulases was studied by enzymatic hydrolysis and SDS-PAGE analyses (data not shown) and by using the fluid containing residual free enzymes that were extracted from the QCM module during the respective experiment. A low glucan conversion was observed upon incubation of microcrystalline cellulose and cellobiose with the fluid recovered, which is expected to be due to the large amount of cellulases adsorbed onto the lignin substrates (Tables S2 and S6). SDS-PAGE gels revealed bands associated with free enzymes contained in the fluid recovered after adsorption on lignin but they displayed much lower intensity compared to that from fresh
enzyme solutions. This indicates the negative effect of enzyme affinity with lignin. In contrast, the reduction of enzymes adsorption upon surface treatment with the polysorbate surfactant, yielded a microcrystalline cellulose glucan conversion similar to that of fresh enzymes. Moreover, the bands in the SDS-PAGE gels displayed similar intensity to those from the fresh enzymes. Monocomponent enzymes, such as cellobiohydrolases (CBH), have a catalytic domain (CD) connected by a linker with a carbohydrate-binding module (CBM) (202). CBM facilitates the non-productive adsorption onto lignin (59) through hydrophobic interactions (120). β-glucosidase exhibits larger hydrophobic path regions compared to endoglucanases and cellobiohydrolases (203), which have been found to be responsible for a high binding on lignin, mainly because of hydrophobic interactions (203, 204). Phenolic compounds have been reported to inactivate β-glucosidase to a large extent (74, 205). Therefore, there is evidence to support that the hydrophobic interactions with the enzymes are greatly reduced by blocking lignin sites with surfactant. By using the residual free enzymes extracted from the QCM module, it was observed that once lignin was pre-treated with a non-ionic surfactant, the glucan conversion from cellobiose increased. Although, this conversion did not reach the same level as that observed for fresh enzymes, this negative effect was not notorious when the substrate was microcrystalline cellulose, as explained above, indicating that free cellulases were still catalytically active.

4.4.6 **Effects of polysorbate surfactant on cellulose bioconversion.**

Cellulose nanofibrils (CNF) were used to test the effect on enzymatic hydrolysis of surfactant treatment. Polyoxyethylene (20) sorbitan monooleate (6xCMC) was adsorbed onto CNF films before enzyme addition (Figure S5). Interestingly, the surfactant adsorbed on CNF
to a lower extent compared to that on lignin (Figure 4.4b), $5.1 \pm 0.9 \times 10^{-7}$ mol/m$^2$ (=0.7±0.1 mg/m$^2$) but more irreversibly (rinsing was not effective for surfactant removal). Enzyme affinity of CBH-I was higher for cellulose (Table S8) than for lignin (Table S7) but a different behavior was found for commercial cellulases, where a higher maximum adsorption was found on SWK and HWK lignins compared to that on pure cellulose (Table S3 and Table S8). The combination of electrostatic (Table S3) and hydrophobic interactions, mainly caused by the presence of endoglucanases (64, 120) and $\beta$-glucosidases (203, 204) in the commercial cellulases mixture, may contribute to the non-productive affinity of cellulases with lignin. Fitting the enzyme binding data (Eqs. 3 and 5) for substrates consisting of neat cellulose film and that after surfactant treatment (Table S8), a decrease in the total cellulase adsorption ($M_{max}$) and adsorption rate (1/$\tau$) was observed: from 36 to 29 (Hz) and from 2.7 to 2.1 (min$^{-1}$), respectively in the case of cellulases mixtures. For CBH-I the maximum adsorption decreased from 43 to 40 (Hz) but the adsorption rate was similar. However, the hydrolytic parameters (Eq. 15) were similar (Table 4.4) without and with pre-adsorbed surfactant. Therefore, there is indication that the polysorbate amphiphile does not affect negatively the biocatalytic process. The affinity of non-ionic surfactants with cellulose has been found to be higher than for other hydrophilic surfaces (206). Based on the QCM dissipation data (not reported), the presence of the non-ionic surfactant during the application of enzyme increased the swelling of the cellulose film by ~43%. Probably, this swelling prevents enzyme deactivation by facilitating desorption (121). Both the ethylene oxide head group and the aliphatic tails contribute cooperatively to the adsorption on cellulose at low concentrations; at higher concentration, lateral attraction becomes dominant (206). Surfactant adsorption data onto CNF at three
concentrations (above the CMC) was fitted to the one-step model (Eq. 17, Table S9); the results indicated an aggregation number n>1, which suggests micellization (207). Moreover, the number of molecules per area was found to be <0.4 molecules/nm² and the apparent number of layers <1. Also low dissipation values (Figure 4.6b inset) were measured and the layer thickness was found to be <0.5 nm, as determined by SPR (Table S5). Hydrogen bonding between the surfactant polar groups and the hydroxyl groups of the surface may contribute to such results.

Table 4.4. Hydrolytic parameters (Eq. 15) for cellulases (Ctec2, 5 mg/ml) and monocomponent CBH-I (1 mg/ml) onto cellulose nanofibrils (CNF) used in QCM experiments at pH 5.5 and 40 °C.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Surfactant (xCMC)</th>
<th>Hydrolytic parameters</th>
<th>B</th>
<th>V₅₀</th>
<th>1/C</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>B</td>
<td>V₅₀</td>
<td>1/C</td>
<td>R²</td>
</tr>
<tr>
<td>Ctec2</td>
<td>0</td>
<td>78.4±5.8</td>
<td>1.3±0.2</td>
<td>1.6±0.3</td>
<td>0.9904</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>75±25</td>
<td>1.3±0.2</td>
<td>1.5±0.4</td>
<td>0.9885</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>78±8.3</td>
<td>1.2±0.2</td>
<td>1.5±0.3</td>
<td>0.9883</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>68±7.4</td>
<td>1.4±0.4</td>
<td>1.4±0.3</td>
<td>0.9896</td>
<td></td>
</tr>
<tr>
<td>CBH-I</td>
<td>0</td>
<td>49.22±2.2</td>
<td>78.1±0</td>
<td>0.02±0</td>
<td>0.9527</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>49.2±6.1</td>
<td>82±3</td>
<td>0.02±0</td>
<td>0.9860</td>
<td></td>
</tr>
</tbody>
</table>

B corresponds to the frequency at which hydrolysis ends, V₅₀ is the time for maximum conversion, and 1/C is the hydrolysis rate.

In order to demonstrate the blocking mechanism of the non-ionic surfactant onto lignocellulosic material, nanofibrillated lignocellulosic biomass was utilized to prepare thin films. For this, softwood kraft pulp (~14% lignin, ~78% cellulose) was fibrillated and the different characteristics of these films are shown in Table S10. Enzyme adsorption and hydrolysis was monitored by QCM-D on NFLC and surfactant-coated NFLC and the profiles are shown in Figure S6. Enzymes bond to NFLC surface and hydrolyzed the cellulose at a rate of 2.9 (1/τ) and 0.3 (1/C), respectively (Table S11). Non-ionic surfactant also bond to NFLC.
at similar extent and rate than on lignin films. Interestingly, enzymes adsorption was reduced under the presence of a surfactant layer onto the NFLC film, suggesting that the lignin sites were covered by the surfactant thus reducing enzyme affinity to lignin. Although this reduction, the enzyme adsorption rate \( (1/\tau) \) increased and the hydrolysis increased in extent \( (B) \) and rate \( (1/C) \) compared with the non-coated NFLC film. These results demonstrated the positive effects of non-ionic surfactant on enzymatic hydrolysis of lignocellulosic biomass.

### 4.4.7 Conformation of the adsorbed layers.

The changes in the viscoelastic properties of the adsorbed layers were followed in the QCM experiments. The dissipation factor or total energy dissipation \( (\Delta D) \) is the ratio of the energy dissipated to the energy stored at the interface. Large dissipation values are obtained when the adsorbed molecules are loosely adsorbed or a soft, low density layer accumulates on the surface. By plotting the changes in total energy dissipation \( (\Delta D) \) versus the changes in frequency \( (\Delta f) \), it was possible to describe qualitatively the macromolecule conformation upon layer build up (see Figure 4.6). A small slope in the \( \Delta D-\Delta f \) profiles indicates that the adsorbed layer does not greatly undergo conformational changes. This was observed in Figure 4.6a for the adsorption of cellulases on lignin films (the profiles exhibited a straight and small slope, suggesting a flat conformation of the molecule on the surface). In contrast, a steeper slope, with a slight curvature was observed for the cellulases adsorbed on lignin films pretreated with the polysorbate amphiphile (Figure 4.6b), suggesting conformational changes of the proteins on the surface upon adsorption. It is thus plausible that the non-ionic surfactant prevents the adsorption of enzymes through their hydrophobic domains \( (59) \); therefore, the enzymes tend to adopt an end-to-end, instead of a flat conformation. Similar response was obtained onto
CNF substrates; however, here the enzymes needed to overcome the pre-adsorbed surfactant layer by changing more significantly their conformation, as revealed by a sharp slope in the respective profiles. For all the substrates, the surfactant layer exhibited a small slope and dissipation values upon adsorption (less than 1 unit) (see inset in Figure 4.6b), which indicate that a rigid layer covered the surface.

Although the main purpose of this work was to study the binding mechanism of enzymes on lignin surfaces in the presence of a pre-adsorbed non-ionic surfactant, the results are also useful in elucidating the potential for enzyme recycling. The surfactants can make the substrate more accessible for enzymatic hydrolysis (122) and also facilitate recycling under conditions relevant to their application (208).

**Figure 4.6.** Changes in dissipation versus frequency upon adsorption of cellulases (5 mg/ml, pH 5.5) without (a) and with (b) polysorbate surfactant (6 xCMC) used to pretreat thin films of SWK (◇), HWK (○), HWAH (△), MPCEL (□) and WSCEL (+) lignin, and CNF (●). The inset in b) shows the conformation of surfactant layer onto the different thin films to highlight the low dissipation values that suggest a rigid and flat adsorbed layer.
4.5 Conclusions

The interactions between lignin, a nonionic surfactant and a commercial multicomponent cellulolytic enzyme mixture were studied. Electrostatic interactions were found to have a minor effect on the affinity of enzymes with the lignins studied, with the exception of native lignins, which exhibited a ~20% reduction in enzyme affinity by inducing lignin charging at pH 5.5. A dominant effect of hydrophobic interactions was found by application of the non-ionic surfactant that reduced the non-productive adsorption of cellulases onto lignin by up to 63, 92, 93, 100 and 100% for SWK, HWK, HWAH, MPCEL, and WSCEL, respectively. The kinetics of surfactant adsorption followed a one-step model and surface aggregation onto lignin was found to be an energetically favorable and reversible process. The low adsorbed thickness values suggest surfactant adsorption as a monolayer or patchy layers. The extent of adsorption of the non-ionic surfactant onto the lignins increased with surfactant concentration above the critical micelle concentration (CMC), leading to better protection toward non-productive binding. Compared to the case of lignin substrates, the polysorbate amphiphile adsorbed to a lower degree but irreversibly on cellulose; however, cellulase digestibility in surfactant-treated cellulose was not affected negatively.
### 4.6 Supporting information

**Table S2.** Section analysis on lignin films from AFM images before and after surfactant adsorption (6xCMC).

<table>
<thead>
<tr>
<th></th>
<th>Before adsorption experiment</th>
<th>After surfactant adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td><img src="image1" alt="SWK before adsorption" /></td>
<td><img src="image2" alt="SWK after adsorption" /></td>
</tr>
<tr>
<td>HWK</td>
<td><img src="image3" alt="HWK before adsorption" /></td>
<td><img src="image4" alt="HWK after adsorption" /></td>
</tr>
<tr>
<td>HWAH</td>
<td><img src="image5" alt="HWAH before adsorption" /></td>
<td><img src="image6" alt="HWAH after adsorption" /></td>
</tr>
<tr>
<td>MPCEL</td>
<td><img src="image7" alt="MPCEL before adsorption" /></td>
<td><img src="image8" alt="MPCEL after adsorption" /></td>
</tr>
<tr>
<td>WSCEL</td>
<td><img src="image9" alt="WSCEL before adsorption" /></td>
<td><img src="image10" alt="WSCEL after adsorption" /></td>
</tr>
</tbody>
</table>
Table S3. Fitting parameters from QCM-D profiles obtained at 25 °C and describing maximum enzyme (Ctec2, 5 mg/ml) binding through exponential decay Eq. 14 on lignin films at two pH levels.

<table>
<thead>
<tr>
<th>Lignin</th>
<th>pH</th>
<th>-M&lt;sub&gt;max&lt;/sub&gt; (Hz)</th>
<th>1/τ (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td>4.8</td>
<td>46.42±5.06</td>
<td>1.66±0.23</td>
<td>0.9865</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>44.83±1.69</td>
<td>0.86±0.16</td>
<td>0.9868</td>
</tr>
<tr>
<td>HWK</td>
<td>4.8</td>
<td>51.31±0.12</td>
<td>1.63±0.37</td>
<td>0.9742</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>48.26±1.06</td>
<td>0.98±0.23</td>
<td>0.9988</td>
</tr>
<tr>
<td>HWAH</td>
<td>4.8</td>
<td>40.85±0.14</td>
<td>1.00±0.37</td>
<td>0.9863</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>36.56±1.26</td>
<td>0.73±0.07</td>
<td>0.9951</td>
</tr>
<tr>
<td>MPCEL</td>
<td>4.8</td>
<td>34.92±0.44</td>
<td>1.26±0.19</td>
<td>0.9878</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>26.20±0.41</td>
<td>0.41±0.01</td>
<td>0.9878</td>
</tr>
<tr>
<td>WSCEL</td>
<td>4.8</td>
<td>34.4±7.51</td>
<td>1.51±0.30</td>
<td>0.9561</td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>28.14±0.18</td>
<td>0.53±0.03</td>
<td>0.9870</td>
</tr>
</tbody>
</table>

Table S4. Fitting parameters from QCM-D profiles obtained at 25 °C and describing surfactant and enzyme (Ctec2, 5 mg/ml) maximum binding through exponential decay Eq. 14 on lignin films at pH 5.5.

<table>
<thead>
<tr>
<th>Lignin substrate</th>
<th>Surfactant (xCMC)</th>
<th>-M&lt;sub&gt;max&lt;/sub&gt; (Hz)</th>
<th>1/τ (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>R²</th>
<th>-M&lt;sub&gt;max&lt;/sub&gt; (Hz)</th>
<th>1/τ (min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td>0.1</td>
<td>nd</td>
<td>nd</td>
<td>5.3±1.5</td>
<td>2.5±1.0</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>18.71±5.98</td>
<td>0.13±0.04</td>
<td>0.99</td>
<td>35.7±0.96</td>
<td>0.39±0.03</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.45±0.71</td>
<td>0.40±0.3</td>
<td>0.99</td>
<td>37.26±0.57</td>
<td>0.41±0.07</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>21.12±2.92</td>
<td>0.41±0.05</td>
<td>0.97</td>
<td>29.87±2.33</td>
<td>0.22±0.10</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>20.74±0.90</td>
<td>2.37±0.01</td>
<td>0.98</td>
<td>24.83±1.42</td>
<td>0.48±0.01</td>
<td>0.98</td>
</tr>
<tr>
<td>HWK</td>
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<td>nd</td>
<td>nd</td>
<td>47.4±1.0</td>
<td>1.4±0.4</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>5.0±3.4</td>
<td>0.55±0.5</td>
<td>0.99</td>
<td>40.23±1.03</td>
<td>0.7±0.07</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14.0±1.4</td>
<td>2.05±0.5</td>
<td>0.99</td>
<td>15.8±3.0</td>
<td>0.25±0.13</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>23.2±2.5</td>
<td>1.03±0.07</td>
<td>0.99</td>
<td>14.5±5.1</td>
<td>0.55±0.13</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>26.2±0.5</td>
<td>1.53±0.05</td>
<td>0.99</td>
<td>13.8±3.8</td>
<td>0.49±0.14</td>
<td>0.95</td>
</tr>
<tr>
<td>HWAH</td>
<td>0.1</td>
<td>1.2±0.1</td>
<td>0.05±0.1</td>
<td>0.98</td>
<td>37.8±1.5</td>
<td>0.16±0.1</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>20.8±0.85</td>
<td>0.48±0.12</td>
<td>0.99</td>
<td>9.37±0.81</td>
<td>0.27±0.02</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
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<td>15.8±1.04</td>
<td>0.70±0.12</td>
<td>0.99</td>
<td>7.14±0.87</td>
<td>0.47±0.03</td>
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<tr>
<td></td>
<td>6</td>
<td>15.4±4.25</td>
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<td>8.58±0.42</td>
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<tr>
<td></td>
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<td>19.6±1.06</td>
<td>1.93±0.05</td>
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<td>6.5±0.54</td>
<td>0.47±0.1</td>
<td>0.97</td>
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<tr>
<td>MPCEL</td>
<td>0.1</td>
<td>5.74±0.02</td>
<td>0.11±0.01</td>
<td>0.99</td>
<td>22.1±0.8</td>
<td>0.16±0.01</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>15.7±0.16</td>
<td>0.78±0.14</td>
<td>0.99</td>
<td>6.0±0.24</td>
<td>0.38±0.00</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.7±0.07</td>
<td>1.03±0.26</td>
<td>0.99</td>
<td>3.7±0.74</td>
<td>0.65±0.13</td>
<td>0.98</td>
</tr>
<tr>
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<td>4.9±0.46</td>
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<td>0.98</td>
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<tr>
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<td>15.6±0.42</td>
<td>4.28±0.56</td>
<td>0.95</td>
<td>4.3±0.15</td>
<td>0.56±0.21</td>
<td>0.98</td>
</tr>
<tr>
<td>WSCEL</td>
<td>0.1</td>
<td>5.3±0.22</td>
<td>0.11±0.01</td>
<td>0.99</td>
<td>29.0±0.70</td>
<td>0.16±0.01</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>14.6±0.09</td>
<td>0.70±0.01</td>
<td>0.99</td>
<td>7.5±0.19</td>
<td>0.37±0.03</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.4±0.01</td>
<td>1.27±0.71</td>
<td>0.99</td>
<td>3.6±0.13</td>
<td>0.87±0.18</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>20.1±0.44</td>
<td>1.45±0.23</td>
<td>0.98</td>
<td>4.8±0.05</td>
<td>0.73±0.19</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
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<td>20.8±2.77</td>
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<td>0.96</td>
<td>3.19±0.16</td>
<td>1.14±0.02</td>
<td>0.97</td>
</tr>
</tbody>
</table>

(nd): surfactant adsorption was not detected
Table S5. Surfactant (6xCMC) layer thickness before and after rinsing monitored by SPR onto CNF and lignin thin films at pH 5.5 and 25 °C.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Before rinsing (nm)</th>
<th>After rinsing (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNF</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>SWK</td>
<td>4.3±0.3</td>
<td>3.0±1.0</td>
</tr>
<tr>
<td>HWK</td>
<td>3.0±0.3</td>
<td></td>
</tr>
<tr>
<td>HWAH</td>
<td>2.3±0.1</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>MPCEL</td>
<td>3.3±0.3</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>WSCEL</td>
<td>2.4±0.9</td>
<td>0.9±0.3</td>
</tr>
</tbody>
</table>

Table S6. Relative irreversible adsorbed mass (mg/m2) of polyoxyethylene (20) sorbitan monooleate and cellulases (Ctec2) onto lignin substrates obtained from QCM experiments. The number of surfactant molecules per nm² and the % irreversible binding are included. For cellulases (5 mg/ml, pH 5.5) the reduction (%) in the irreversible affinity is shown.

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Surfactant</th>
<th>Cellulases</th>
<th>Reduction in irreversible binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>xCMC</td>
<td>Adsorbed mass (mg/m²)</td>
<td>Molecules/ nm²</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SWK</td>
<td>0</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>2.0 ± 0.4</td>
<td>0.9±0.20</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.4 ± 0.2</td>
<td>0.64±0.09</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.5 ± 0.5</td>
<td>0.69±0.23</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>2.1 ± 0.1</td>
<td>0.97±0.05</td>
</tr>
<tr>
<td>HWK</td>
<td>0</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.4 ± 0.2</td>
<td>0.2±0.09</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.48 ± 0.2 (a)</td>
<td>1.14±0.09 (a)</td>
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<td></td>
<td>6</td>
<td>4.14 ± 0.1 (a)</td>
<td>1.9±0.05 (a)</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>4.58 ± 0.1 (a)</td>
<td>2.11±0.05 (a)</td>
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<td>Nd</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>nd</td>
<td>Nd</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>1.8 ± 0.2</td>
<td>0.83±0.09</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.4 ± 0.3</td>
<td>0.64±0.14</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.7 ± 0.2</td>
<td>0.78±0.09</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>1.8 ± 0</td>
<td>0.83±0</td>
</tr>
<tr>
<td>MPCEL</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.69 ± 0</td>
<td>0.32±0</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>1.6 ± 0</td>
<td>0.74±0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.8 ± 0.1</td>
<td>0.83±0.05</td>
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</table>
Table S6 Continued

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Surfactant (xCMC)</th>
<th>$M_{\text{max}}$ (Hz)</th>
<th>$1/\tau$ (min⁻¹)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSCEL</td>
<td>0</td>
<td>17±1</td>
<td>2.2±0.9</td>
<td>0.9890</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8.1±0.9</td>
<td>1.5±0.1</td>
<td>0.9590</td>
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<td>HWK</td>
<td>0</td>
<td>16.4±0.3</td>
<td>1.2±0.03</td>
<td>0.9842</td>
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<tr>
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<td>6</td>
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<td>Nd</td>
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<td></td>
<td>6</td>
<td>Nd</td>
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</table>

(1) These values correspond to the maximum adsorbed mass of surfactant before rinsing and their respective molecules per nm².
(2) Reduction based on the irreversible amount of adsorbed cellulases on pure lignin films at pH 5.5 (no surfactant addition).
(nd): Adsorbed amount below limit of detection

Table S7. Binding parameters describing the maximum adsorption for monocomponent enzyme CBH-I (1 mg/ml) through exponential decay Eq. 14 on lignin films used in QCM-D experiments at pH 5.5 and 25 °C.

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Surfactant (xCMC)</th>
<th>$M_{\text{max}}$ (Hz)</th>
<th>$1/\tau$ (min⁻¹)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK</td>
<td>0</td>
<td>17±1</td>
<td>2.2±0.9</td>
<td>0.9890</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>8.1±0.9</td>
<td>1.5±0.1</td>
<td>0.9590</td>
</tr>
<tr>
<td>HWK</td>
<td>0</td>
<td>16.4±0.3</td>
<td>1.2±0.03</td>
<td>0.9842</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HWAH</td>
<td>0</td>
<td>16.03±0.6</td>
<td>1.2±0.05</td>
<td>0.9796</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Nd</td>
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<td></td>
</tr>
<tr>
<td>MPCEL</td>
<td>0</td>
<td>3.7±0.3</td>
<td>0.9±0.3</td>
<td>0.9920</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSCEL</td>
<td>0</td>
<td>2.2±0</td>
<td>0.8±0.2</td>
<td>0.9726</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Nd</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(nd): CBH-I adsorption was not detected on these lignin surfaces primed with surfactant
Table S8. Adsorbed mass (mg/m²) on cellulose nanofibrils (CNF) and fitted binding parameters for cellulases (Ctec2, 5mg/ml) and monocomponent CBH-I (1 mg/ml) according to models shown in Eq. 14 and Eq. 16 from QCM-D experiments at pH 5.5 and 40 °C.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Surfactant (xCMC)</th>
<th>Enzyme adsorption (mg/m²) and binding parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-M_{max} (mg/m²)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctec2</td>
<td>0</td>
<td>6.5±0.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.9±0.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.9±0.2</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>5.4±0.3</td>
</tr>
<tr>
<td>CBH-I</td>
<td>0</td>
<td>7.8±0.4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>7.3±0.6</td>
</tr>
</tbody>
</table>

(a) Calculated from exponential model according to Eq. 14
(b) Calculated from Boltzmann-sigmoidal model according to Eq. 16

Table S9. Surfactant adsorption (Γx10⁻⁷ mol/m²), molecules per area, and fitted parameters (maximum adsorption density, Γ∞; equilibrium constant, k; and aggregation number, n) from one-step model (eq 6) including the standard free energy of micellization (∆G_{sa}) (Eq 8) onto cellulose surface (CNF) from QCM-D experiments at pH 5.5 and 40 °C.

<table>
<thead>
<tr>
<th>Surfactant (xCMC)</th>
<th>Γ (10⁻⁷ mol/m²)</th>
<th>Molecules/nm²</th>
<th>Γ∞ (10⁻⁷ mol/m²)</th>
<th>k</th>
<th>n</th>
<th>R²</th>
<th>-∆G_{sa} (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.3±0.8</td>
<td>0.2±0.05</td>
<td>5.88</td>
<td>1.05x10¹⁰</td>
<td>2.24</td>
<td>0.9999</td>
<td>25.52</td>
</tr>
<tr>
<td>6</td>
<td>5.06±0.9</td>
<td>0.31±0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>5.8±0.8</td>
<td>0.4±0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure S3. Maximum adsorption frequency (Hz) for the enzyme mixture (Ctec2) at pH 4.8 (□) and 5.5 (■) associated with the different hydroxyl groups found in the lignin substrates: aliphatic hydroxyl (a), total phenolic (b), carboxylic (c), and total hydroxyl (d) groups.
Figure S4. Representative QCM-D curves for adsorption of polyoxyethylene (20) sorbitan monooleate at 0.8 (a), 3 (b), 6 (c), and 19 (d) xCMC, followed by cellulases addition (5 mg/ml, pH 5.5, 25 °C, 50 mM) onto lignin substrates SWK, (◇), HWK (○), HWAH (△), MPCEL (□), and WSCEL (+). Surfactant and enzyme injection are marked by the solid arrows whereas the rinsing step is shown with dotted arrows.
Figure S5. Representative QCM-D curves (top) for adsorption of: a) cellulases (5 mg/ml) and b) monocomponent CBH-I (1 mg/ml) onto CNF and CNF coated with polyoxyethylene (20) sorbitan monooleate at 6 xCMC, pH 5.5, and 40 °C. AFM images at 5x5 µm before c) and after enzymes treatment d). Zeta potential value for CNF, Ctec2, and CBH-I was -32.9, -9.6, and -7.1 mV, respectively at pH 5.5. For surfactant adsorption the number of layer obtained by dividing the adsorption density at the solid/liquid interface by that at air/liquid interface was found to be 0.2.

Table S10. Characteristics of nanofibrillated lignocellulosic (NFLC) initial material and thin film.

<table>
<thead>
<tr>
<th>Material:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignin content %</td>
<td>14.3</td>
</tr>
<tr>
<td>Cellulose content %</td>
<td>78.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thin film:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Roughness (nm)</td>
<td>5.6±0.02</td>
</tr>
<tr>
<td>Thickness (nm)</td>
<td>4.3±0.05</td>
</tr>
<tr>
<td>Water contact angle (°)</td>
<td>24±2</td>
</tr>
</tbody>
</table>
Figure S6. a) Representative QCM-D profiles showing enzyme binding and hydrolysis of NFLC with and without the presence of non-ionic surfactant polyoxyethylene (20) sorbitan monooleate at 6 xCMC, pH 5.5, and 40 °C. AFM images of NFLC b) before and c) after enzymatic hydrolysis. Images at 5 x 5 μm.

Table S11. Fitted binding and hydrolytic parameters for enzymes and surfactant onto nanofibrillated lignocellulosic material (NFLC)

<table>
<thead>
<tr>
<th></th>
<th>Binding parameters</th>
<th>Hydrolytic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-M_{\text{max}}) (Hz)</td>
<td>(\text{R}^2)</td>
</tr>
<tr>
<td>Enzymes on NFLC</td>
<td>15.31</td>
<td>0.99</td>
</tr>
<tr>
<td>Surfactant on NFLC</td>
<td>10.8</td>
<td>0.97</td>
</tr>
<tr>
<td>Enzymes on surfactant coated NFLC</td>
<td>6.13</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>(1/\tau) (min(^{-1}))</td>
<td>(W_{50}) (min)</td>
</tr>
<tr>
<td>Enzymes on NFLC</td>
<td>2.9</td>
<td>0.31</td>
</tr>
<tr>
<td>Surfactant on NFLC</td>
<td>0.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Enzymes on surfactant coated NFLC</td>
<td>4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

(a) Calculated from exponential model according to Eq. 14
(b) Calculated from Boltzmann-sigmoidal model according to Eq. 16
5 ELECTROLYTE AND SURFACTANT CONTROL OVER SELF-ASSOCIATION AND AGGREGATION OF KRAFT LIGNINS IN ALKALINE SOLUTION AND STABILIZATION OF SUB-MICRON LIGNIN PARTICLES

5.1 Abstract

Understanding the aggregation behavior of lignins is relevant to their application, especially towards value-added products. Similar to the case of polyelectrolytes in solution, the colloidal behavior of lignin is mainly controlled by its molecular mass and surface charge as well as temperature, pH and ionic strength of the medium. Here we report on the self-association of kraft lignin derived from hardwood and softwood under mild temperature and pH conditions and in the presence of various salts and surfactants. Monovalent salt ions increase lignin aggregation while non-ionic surfactants enhances the colloidal stability of the system by steric effects, depending on the concentration. The combination of salt and surfactant enable control of the colloidal aggregation and the rheological properties of the aqueous dispersions. While pH affects the colloidal stability of lignin in solution, it plays a minor effect on submicron lignin particles dispersed in water, indicating contributions beyond those from carboxylic or phenolic groups. Filtration and separation of lignin from aqueous media (processing streams, wastewater, etc.), as well as applications that require regulation of lignin stability can be addressed suitably by application of the simple methods presented here.
5.2 Introduction

Lignin is a biomacromolecule that is nowadays attracting renewed interest in several fields (209-213). Annually, over 70 million tons of lignin are produced worldwide, mainly as by product from the kraft pulping process but only 5% is used in commercial applications (8). Understanding kraft lignin (KL) colloidal behavior is needed to guarantee suitable uses. During the kraft pulping process the aryl-ether bonds in lignin are cleaved (22) and the residual KL contains more acidic groups (84). Lignin is soluble in a wide range of solvents such as aliphatic alcohols and acids, methyl and ethyl acetate, acetone, chloroform, dioxane, pyridine, dimethyl sulfide, and tetrahydrofuran. However, KL can only be dissolved in alkaline conditions (140) and its phase behavior is controlled by temperature, pH, and ionic strength (14). Self-associative behavior of KL has been widely studied (10, 11, 14, 85, 214, 215) and is considered as that of typical polyelectrolytes in aqueous media (15). This has been relevant to our recent reports on the synthesis of supracolloidal lignin particles (213) and their interactions with proteins (59, 91, 183). Here, dispersion interactions (van der Waals forces) and hydrogen bonding are affecting the colloidal stability in aqueous dispersions. Aggregation is favorable if these effects are dominant; conversely, a stable colloidal suspension is afforded if strong repulsive electrostatic forces exist between the aggregates. Usually, van de Waals forces are attractive (216) and mostly independent of pH and ionic strength (14). This in contrast to double layer electrostatic interactions, which are repulsive (216) and highly affected by these variables (14). At low pH the charge of the particle is low; therefore, it is expected that lignin association in solution results in flocculation. Nyman et al. (214) found that in the presence of salt and high pH, lignin behaves as a stable colloid and, only at high electrolyte concentration
lignin flocculate by decreasing the thickness of the electrical double layer. Moreover, Norgren et al. (14) studied the aggregation of softwood kraft lignin and concluded that pOH, electrolyte concentration, the valence of the counterions and temperature are key parameters to regulate related effects. Lignin precipitation can cause problems in given systems and re-dissolution may be possible by increasing the concentration of hydroxide ions but, on the other hand, this can be detrimental in many industrial processes. (14) Ions such as Ca\(^{2+}\) or Mg\(^{2+}\) have strong influence on the stability of lignin in solution (10, 14), especially those belonging to the so-called Hofmeister or lyotropic series (85). In a salt-rich polyelectrolyte system, the electrostatic repulsion between the charged entities is strongly screened and this can be very unfavorable for the stability of the polyelectrolyte solution. Conversely, surfactants can enhance the colloidal stability through a steric stabilization (17). Among the different types of surfactants, and compared to anionic surfactants, non-ionic ones adsorb more strongly on hydrophobic surfaces due to the absence of electrostatic repulsion (17). Overall, understanding the colloidal aggregation of KL is crucial as a prerequisite for successful control over aggregative phenomena and for improving its utilization. Therefore, the present study investigates the colloidal behavior of three types of kraft lignins and unveils the effect of aqueous dispersion conditions, extensive also to respective submicron lignin particles and lignin in so-called black liquors.
5.3 Materials and methods

5.3.1 Materials

Three lignin samples were used: a low sulfonate alkali lignin (SWK1, provided by Sigma-Aldrich, CAS 8068-05-1), a low-ash softwood kraft lignin (SWK2, donated by Domtar Inc. Plymouth Pulp Mill, NC), and a hardwood kraft lignin (HWK) with high ash content (donated by Suzano Pulp Mill, Brazil). The inorganic salts and surfactants were used without further purification.

5.3.2 Lignin characterization

The lignin samples were purified by washing with milliQ-water (Millipore) and centrifugation (Beckmann Centrifuge Model J-21C, 3000 rpm) several times. After the purification step, the lignin samples were freeze-dried prior to use. The insoluble and soluble components of the lignins were determined by acid hydrolysis. Briefly, 100 mg of the given lignin source were reacted with 1.5 ml of sulfuric acid (72% v/v H₂SO₄) at 25 °C for 2 h with stirring every 20 min. The dispersion was then diluted and autoclaved at 121 °C for 1.5 h, cooled down overnight, filtered through a fine crucible, and then dried for gravimetric determination of acid-insoluble lignin (Klason lignin). The supernatant was used to determine acid-soluble lignin by UV-VIS spectroscopy at 205 nm (Lambda XLS, PerkinElmer, Inc). After neutralization of this supernatant, sugar analyses were performed in a HPLC unit, as previously reported (148). Hydroxyl groups content and molecular weight determinations were carried out by ³¹P-NMR and gel permeation chromatography (GPC) following the procedures published elsewhere (181, 182).
5.3.3 Preparations of lignin dispersions and colloidal characterization

The different lignin dispersions were prepared by dissolution in 10 mM NaOH under magnetic stirring for 3 h after which water was added to obtain the given lignin concentration. The dispersions were allowed to dissolve overnight. The pH was adjusted to 10.5 unless noticed otherwise with 1 M NaOH or 1 M HCl. During the experiments, the samples were incubated at 25 or 70 °C in a temperature-controlled oven.

The turbidity of the different aqueous dispersions was determined using an Orion AQ4500 turbidimeter at 25 °C. The zeta potential of lignin dispersions and the average diameter of aggregates were determined using a Zetasizer Nano-ZS (Malvern Instrument Ltd) at 25 or 70 °C. The surface tension was measured using a DCA-312 Electrobalance from Cahn Instruments Inc. (Cerritos, CA, USA) equipped with a platinum Wilhelmy plate. Measurements were performed at 25 and 70 °C using a thermostatic vessel connected to an external bath/circulator heating system.

5.3.4 Rheological analysis

Rheological tests were performed in an AR2000 rheometer from TA Instruments (New Castle, DE, USA). The measurement system was equipped with a 40-mm-diameter parallel plate geometry. The apparent viscosity $\eta$ (mPa) and shear stress $\tau$ (Pa) were recorded as a function of the shear rate $\dot{\gamma}$ from 1 to 200 s$^{-1}$ using a 200 μm gap. Temperature was kept at 25 °C controlled by a Peltier heating system. Prior to test, the samples were equilibrated at 25 °C for 2 minutes and an average of three repetitions is reported. The data was fitted to the Power-
Law (Eq. 20) (217), Carreau-Yasuda (Eq. 21) (218, 219), and Herschel-Bulkley (Eq. 22) (220, 221) models:

\[ \eta(\dot{\gamma}) = k\dot{\gamma}^{(n-1)} \]  
Eq. 20

\[ \eta(\dot{\gamma}) = \eta_o + (\eta_o - \eta_\infty)\left[1 + (\dot{\gamma} \lambda)^a\right]^{-\frac{1}{a}} \]  
Eq. 21

\[ \tau(\dot{\gamma}) = \tau_H + K_H^{(n)} \]  
Eq. 22

where \( k \) is a consistency index and \( n \) is the flow behavior index. \( \eta_0 \) is the viscosity (mPa) at zero shear rate and \( \eta_\infty \) is the viscosity (mPa) at infinite shear rate. \( \lambda \) corresponds to a constant with \( 1/\lambda \) the critical shear rate at which viscosity begins to decrease. \( \alpha \) represents the width of the transition between \( \eta_0 \) and the power law region. \( K_H \) is the consistency coefficient (Pa s\(^n\)) and a large value may indicate a strong structure. \( \tau_H \) is the yield stress (Pa), which correlates with stability of the system; \( n \) is the flow index.

5.3.5 Stability and aggregation

Two systems were tested with regards to stability: submicron lignin particles and lignin precipitation from black liquors. In the first case lignin particles, SWK1, SWK2 and HWK were used to produce particles following the recently introduced dialysis method of Lievonen et al (139). The aggregate size was monitored over time using dynamic light scattering (DLS). In the second case hardwood lignin was aggregated and precipitated from black liquor. For this, white birch (Betula papyrifera) was digested (16% AA, 25% sulfidity, 160 °C with 4:1
liquid-to-solid ratio) for an H-factor = 800 (kappa value of 20) and centrifuged to remove suspended solids. The turbidity of the black liquor (BL) samples was monitored by using an Orion AQ4500 turbidimeter. The lignin content was determined by UV-Vis spectrophotometer at 280 nm with an extinction coefficient of 20 (g⁻¹Lcm⁻¹) (222). A Whatman Nuclepore membrane (1 µm pore size) was used in a Millipore filtration unit under constant pressure (690 KPa) in order to determine the mass filtrated over time after BL samples were incubated with salts and surfactant.

5.4 Results and discussion

The aggregation behavior of lignin in solution is affected by the nature of the lignin (its source) and the method used in its isolation. For instance, the kraft pulping process introduces phenolic hydroxyl and carboxyl groups in the lignin structure (84). The lignin samples used in this study consisted of kraft lignins and their main characteristics are shown in Table 5.1 for the phenolic hydroxyl groups, molecular weight and zeta potential. Among the lignin samples studied, SWK1 shows the lowest value. In fact, SWK2 and HWK lignins dissolved relatively well in tetrahydrofuran (solvent used for molecular weight determination), whereas SWK1 did not dissolve in THF. Particularly, the phenolic groups were found higher for SWK2 and HWK than for SWK1, suggesting that the difference in solubility can be partially explained due to the phenolic groups content. Phenol components are moderate soluble in water due to their ability to form hydrogen bonds with water; however, the phenyl group is non-polar. Non-polar substances are more likely to be soluble in nonpolar solvents, whereas polar solutes are more likely to be soluble in polar solvents (223). KL exhibits high negative values of zeta potential.
as the pH increases (83), with an isoelectric pH (IEP) close to unity (83). The lignin ionization mechanism can be explained by a simplified model described by Dong et al. (83) where the ionization of acid groups, especially the carboxylic groups added during pulping (84), lead to an increased lignin surface charge that, in turn, results in a low negative zeta potential. Monovalent, divalent and trivalent salts affect the zeta potential. Calcium and sodium chloride reduce the magnitude of the zeta potential but they do not cause a shift of the IEP because of the negligible adsorption or affinity of Na⁺ or Ca²⁺ ions with lignin (83). In contrast, zinc sulfate and aluminum chloride cause charge reversal and a shift in IEP at high pH (83), Lignin molecular weight is affected by the isolation method used and its source (wood specie). As Table 5.1 shows, the samples have a broad molecular weight distribution with high polydispersity index (PdI).

### Table 5.1. Chemical characterization of different kraft lignin samples according to sugar analysis, lignin determination, hydroxyl groups by PNMR, GPC, and zeta potential.

<table>
<thead>
<tr>
<th>Kraft Lignin</th>
<th>Sugars, %</th>
<th>Lignin(1), %</th>
<th>Aliphatic OH, mmol/g</th>
<th>Phenolic OH, mmol/g</th>
<th>Total OH, mmol/g</th>
<th>COOH, mmol/g</th>
<th>Mw, g/mol</th>
<th>PdI</th>
<th>ζ(2), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWK1</td>
<td>nd</td>
<td>90 (8)</td>
<td>1.4a</td>
<td>1.6a</td>
<td>3.4a</td>
<td>0.5a</td>
<td>10,000b</td>
<td>nd</td>
<td>-41</td>
</tr>
<tr>
<td>SWK2</td>
<td>&lt;1</td>
<td>94 (5)</td>
<td>2.7</td>
<td>5.4</td>
<td>8.1</td>
<td>0.7</td>
<td>4,470</td>
<td>4.2</td>
<td>-37</td>
</tr>
<tr>
<td>HWK</td>
<td>1.2</td>
<td>85 (6)</td>
<td>1.8</td>
<td>5.1</td>
<td>6.9</td>
<td>0.5</td>
<td>3,240</td>
<td>3.2</td>
<td>-32</td>
</tr>
</tbody>
</table>

(1) Acid insoluble % (Acid soluble %)
(2) Zeta potential measured on aqueous lignin solutions (0.1 wt%) at pH 10.5 and 25 °C
(nd)=not determined
aData obtained from Lievonen et al. (139)
bData provided by Sigma-Aldrich
5.4.1 Temperature- and salt-induced aggregation

The colloidal stability of lignin solutions is controlled by pH, ionic strength, molecular weight (85), temperature (16, 85), and lignin concentration (13). An increase in temperature from 25 to 70 °C promoted the aggregation of the tested lignins, with the exception of SWK1, the commercial alkali lignin (Figure 5.1a). In this figure the increase in turbidity is taken as indication of lignin aggregation in solution. For the softwood kraft lignin (SWK2), there is a tendency to form aggregates even at room temperature, to an extent that is beyond the limit of the turbidity measurement (at 0.3% lignin concentration the turbidity was over the instrumental range). There is a threshold limit concentration (~0.3%) where temperature (70 °C) promotes aggregation and finally causes precipitation of the lignin in solution. In the case of hardwood kraft lignin (HWK) and above 0.20% lignin concentration, there is a sharp increase in turbidity, which is associated to the formation of heat-induced aggregates.

Figure 5.1. (a) Effect of lignin concentration and temperature on the aggregation behavior in aqueous solution monitored by turbidity measurements at 25 °C and after 30 min incubation at 70 °C. (b) The effect of electrolyte (1 M NaCl) on the aggregation behavior of the lignin solutions after incubation at 70 °C for 30 min is also shown. The open symbols correspond to solutions containing lignin and NaCl. The samples included alkali lignin (SWK1), softwood
kraft lignin (SWK2), and hardwood kraft lignin (HWK). Note the broken turbidity axis used to display some high values that were recorded.

The presence of electrolytes affects the stability of the system. In fact, the electrostatic repulsion between the charged units is strongly screened, and the ions can cause an increase in formation of intra- and intermolecular ionic complexes with the counterion and act as a bridge between two or more charged groups on the lignin polyelectrolyte (16). According to the turbidity measurements shown in Figure 5.1b, the addition of 1 M NaCl affect the aggregation behavior of the various lignin samples, except for SWK1. In this latter case, salt did not induce aggregation (the values of turbidity were below 5 turbidity units). For the other two samples, the turbidity values at 0.1% lignin concentration significantly increased, especially for SWK2 where the turbidity value was beyond the instrumental range at 0.3% lignin concentration. Therefore, the stability of the lignin in aqueous solution is affected by the presence of ions in solution.

The aggregation kinetic was monitored at various ionic strengths (see “Modes of aggregation and kinetics” section). Norgren and Edlund (77) studied the precipitation of kraft lignin in alkaline medium in the presence of various ions of the Hofmeister or lyotropic series and concluded that among the cations the lowest stability was observed in the presence of cesium, sodium, and potassium; whereas among the anions, chlorine and bromine were the worst in enabling the stability. Some explanations for these ion-specific interactions have been related to their differences in adsorption/desorption at interfaces and perturbations of water structuring (77).
5.4.2 Lignin association with surface active molecules.

It can be anticipated that surfactants and polyelectrolytes affect the aggregation behavior of lignin in solution and thus the colloidal stability of the system. Particularly, surfactants can be effective towards the colloidal stabilization via steric interactions (17, 18). In order to assert the effect of different types of surfactants, a screening study was carried out that revealed that non-ionic polyoxyethylene glycol sorbitan monooleate (indicated thereafter as “POE20”) increased the stability of neat lignin dispersion, in the presence or absence of electrolytes (Figure S7). In contrast, a cationic surfactant (cetrimonium bromide, CTAB) caused precipitation of lignin due to strong electrostatic interactions. The effect of nonionic surfactants can be rationalized by the fact that in absence of electrostatic repulsion they adsorb more strongly on hydrophobic surfaces (17) and could also partake in hydrophobic interactions with lignin (2). Therefore, the effect of the POE20 non-ionic surfactant on the dispersion behavior of lignin was further elucidated (Figure 5.2). It is plausible that the association of polyoxyethylene-type surfactants induced by heating could interfere with the destabilization of the system; however, our tests in the presence of lignin in solution indicated turbidity values below 8 units. In other words, the incubation with POE20 surfactant at high temperature (70 °C, 30 min) was in fact effective in reducing the turbidity, indicating a better dispersion. Interestingly, the most notorious effect was found for SWK2, where the turbidity decreased from 2800 to 2 units at 0.2% lignin concentration. In order to verify these results, dynamic light scattering was used to determine the average size of the aggregates in aqueous media (Figure 5.2d).
Lignin association has been indicated to involve expanded random coils in alkaline media that can adopt a rod-like conformation in the case of low molecular weight fractions. A more spherical shape is adopted by the higher molecular weight lignin fraction, as studied by size-exclusion chromatography (12). On the other hand, it has been postulated that lignin can be considered as a fractal system, with guaiacyl units given chaotically branched fractal structures, while guaiacyl-syringyl units yield star-like structures (224). In the case of SWK1 it was not possible to determine the size of the aggregates since the cumulant method did not provide good correlation functions, which may indicate a loosely aggregate structure. For SWK2 and HWK lignins, an increase in size was observed with the increased concentration (neat lignin solution), with values above 750 nm mainly due to the intermolecular interactions between neighboring molecules. For hardwood lignin (HWK), the addition of surfactant at concentrations above the critical micelle concentration (cmc) not only decreased the turbidity (Figure 5.2c) but also reduced the aggregate sizes, indicating a steric stabilization of this system where the adsorbed surfactant prevents lignin association. Unfortunately, no good correlation functions were obtained from SWK2 in the presence of surfactant, indicating a more disorganized structure, which is in agreement with the drastic decrease in turbidity. Lindström (18) studied the flocculation of kraft lignin solutions with a high molecular weight polyethylene oxide (POE), and concluded that the phenolic groups can act as the primary adsorption sites for this type of molecule, which would explain the interaction with SWK2 and HWK.
Figure 5.2. The effect of non-ionic surfactant POE20 at 40×cmc (red symbols) on the aggregation of different lignin aqueous solutions after 30 min at 70 °C as measured by (a, b, and c) turbidity and (d) dynamic light scattering (DLS). Note the low values in the y-axis for SWK1, indicating no aggregation or presence of large aggregates (<5 units) (cmc = surfactant critical micelle concentration).
5.4.3 Modes of aggregation and kinetics

In this study it has been demonstrated that KL aggregates over time, especially in the presence of salt, whereas the addition of a non-ionic surfactant reduces aggregation and brings a colloidal stabilizing effect. The kinetics of aggregation was studied at various ionic strengths and using a low lignin concentration (0.1%) and the stability ratio (W-ratio) was calculated from the slope of the turbidity plots (Figure S8). The dimensionless W-ratio (225) factors the aggregation rate coefficient in the fast regime, and that at the conditions of interest. Thus, it represents the ratio of rapid or diffusion-controlled aggregation (in the absence of repulsive forces) to the slow aggregation. The stability ratio is close to unity in the fast regime, increases in the slow regime, and becomes very large when the suspension is stable. Therefore, with increasing repulsion the value of W increases. At high ionic strength, aggregation is promoted as revealed by the high turbidity values for SWK2 and HWK lignin samples. From the turbidity plots it can be seen that there is a sharp change in slope at high NaCl concentration and, in the interval between 1.2-1.5 M, the curves overlap. This region can be recognized as a rapid aggregation or diffusion-limited aggregation, whereas at salt concentrations below 1.0 M the behavior corresponds to a slow regime. Based on this observation, the W-ratios were determined for SWK2 and HWK as shown in Figure 5.3. Regions of significant colloidal stability (W≈1), low stability (W>1), and instability (W<1) are clearly observable. In all the cases, the critical coagulation concentration (ccc), which separates the fast from the slow regime, was located at an electrolyte concentration of 1.2 M NaCl, which is in agreement with another study (85). Interestingly, at NaCl concentrations from 0.5 to 1 M, the stability curve shows a change in the slope. Higher slopes were observed in the presence of the surfactant,
especially for HWK lignin where, for example, the addition of a non-ionic surfactant improved the stability at low electrolyte concentration (<0.8 M NaCl). The presence of a simple electrolyte induces a reduction of the electrostatic double layer thickness, whereas it has been hypothesized that polyethylene oxide forms interparticle bridging. Thus, electrostatic repulsion is expected to decrease at smaller interparticle distances and consequently the amount of electrolyte needed to cause flocculation should be less than in the absence of the polymer (18). However, such behavior was not observed at this point, suggesting that no bridging occurred; therefore, the addition of non-ionic surfactant at $6 \times \text{cmc}$ is mainly responsible for steric stabilization.

![Figure 5.3](image)

**Figure 5.3.** Stability ratio $W$ based on turbidity measurements of (a) lignin solutions and (b) non-ionic surfactant-lignin solutions (POE20 at $6 \times \text{cmc}$) as a function of NaCl concentration at pH 10.5 and 70 °C.

The stability of lignin solutions also depends on its chemical and physical heterogeneity (85). An experiment was performed to test the ability of a salt/surfactant system to flocculate and precipitate lignin. For this, the stability of the low molecular weight HWK lignin was tested for turbidity over 48 h at different sodium chloride and non-ionic surfactant
concentrations, as shown in Figure 5.4. At 0.1% HWK lignin concentration all conditions induce aggregation up to certain level; at the highest salt and surfactant concentrations the lignin precipitated out. A somewhat different behavior was observed at a slightly higher lignin concentration, 0.3%, where aggregation was faster and more extensive. In this case, besides the conditions that caused precipitation, as previously mentioned, lignin precipitated at 0.8 M NaCl and POE20 6×cmc. Bridging flocculation by the non-ionic surfactant at high concentration seems to favor the precipitation of lignin in the system probably as a result of the reduced electrical double layer that reduces the inter-particle distance. Overall, the addition of non-ionic surfactant improves the stability of the systems up to certain level, then a synergistic effect with salt promotes aggregation and precipitation.

Figure 5.4. Turbidity measurements of HWK lignin as a function of time, sodium chloride and surfactant concentrations at pH 10.5 and 70 °C. Two lignin concentrations, 0.1 and 0.3%, were studied in order to highlight differences in aggregation behavior. In (a) the systems aggregated over time and lignin precipitated at 0.5M NaCl + 40×cmc, 0.75M NaCl + 40×cmc, 1M NaCl + 6×cmc, and 1M NaCl + 40×cmc conditions. In contrast, most of the systems shown in (b) are aggregated or the aggregation extent was higher than that in (a) and lignin precipitation occurred at 0.5M NaCl + 40×cmc, 0.75M NaCl + 6×cmc, 0.75M NaCl + 40×cmc, 1M NaCl + 6×cmc, and 1M NaCl + 40×cmc. Note the broken turbidity axis at 100 (a) and 9000 (b) turbidity units.
5.4.4 Rheology

Since most of the samples experienced aggregation in a relatively short period, it was of interest to evaluate the aggregation behavior through viscosity measurements of samples incubated at 70 °C for 30 min. The plots of apparent viscosity as a function of the shear rate are shown in Figure 5.5. It is clear that the apparent viscosity decreases as the shear rate increases, for all salt concentrations, indicating non-Newtonian, shear thinning behavior. The three different models used to fit the data, Power-Law, Carreau-Yasuda, and Herschel-Bulkley, yielded the parameters and correlation coefficients given in Tables S1, S2, and S3. Power law and Carreau-Yasuda models help to explain the zero-shear viscosity. As the lignin concentration increased, the viscosity at 25 °C (no incubation) was slightly affected; however, after 30 min at 70 °C, the viscosity increased as lignin content increased. A possible explanation is that in the absence of incubation the formed clusters (if any) become more compact, therefore lowering the viscosity. Lindstrom (18) concluded that heating causes intramolecular lignin chain rearrangements in alkaline solution due to disruption of hydrogen bonds. As temperature and time increases, these clusters become in contact, creating larger aggregates that lead to a viscosity increase (aggregate immobilization). In the absence of electrolytes, or without a counter ion, the negative charge produces strong intermolecular repulsion and thus a more expanded molecule. This may explain the high viscosity of lignin without salt/surfactant. Electrolyte (NaCl) addition to the solutions decreases the extent of the electrical double layer around lignin. In general, the viscosity at zero-shear rate of HWK increased by the addition of salt (Table S1), whereas for SWK2 and SWK1 it decreased (Tables S2 and S3). The addition of cations reduces repulsion and molecule expansion, decreasing
network structure, which probably explains the reduction in viscosity in the case of SWK1 and SWK2 solutions. The non-ionic surfactant decreased the viscosity in all cases maybe due to the steric stabilization of lignin.

Figure 5.5 shows the shear stress as a function of shear rates (right frames). It can be seen that above 15 s\(^{-1}\) shear rate the shear stress exhibits a yield value and undergoes a linear increase that is explained by the Herschel-Bulkley model. This model predicts a higher apparent viscosity when \(K_H\) is higher, which means the system has a strong structure upon shear, whereas \(\tau_H\) (yield stress) explains the stability of the system, the higher the value, the stronger the stability of the system. The presence of salt and non-ionic surfactant affect the organizational structure of aggregates at low shear rates by decreasing the shear stress, which would indicate a more organized structure. The higher \(K_H\) and \(\tau_H\) values were obtained for HWK lignin, suggesting a strong and compact structure in agreement with the high viscosity and well-defined sizes (Figure 5.2).

From the Carreau-Yasuda model, the zero-shear viscosity was determined and plotted against the volume fraction in order to better display the concentration dependence, as shown in Figure 5.6. Data for lignin concentrations higher than 0.3 wt% are not presented because of precipitation. The data was fitted with a power-law equation were the exponent value for HWK lignin in water was 0.38. In salt (1 M NaCl), the exponent value was 0.54 for HWK and 0.47 for SWK1, indicating a denser packing in saline solutions. No good fitting was observed for systems containing non-ionic surfactant and lignin, suggesting that aggregation was prevented due to steric stabilization.
Figure 5.5. Apparent viscosity (profiles on the left) and shear stress (profiles on the right) of the different alkaline lignin solutions as a function of shear rates (open circles) and in the presence of sodium chloride (1 M, solid circles) and non-ionic surfactant ($40 \times \text{cmc}$, triangle...
Figure 5.6. Zero-shear viscosity $\eta_0$ of hardwood kraft (HWK), softwood kraft (SWK2), and softwood kraft (SWK1) lignin solutions as a function of the volume fraction. The data was fitted to the power law equation $y=ax^b$, and the values for a, b, and $R^2$ were 3.2, 0.4 and 0.99; 10.9, 0.54, and 0.92 for HWK in water and saline solutions, respectively. Attempts to fit SWK1 gave values of 2.64, 0.47 and 0.78 for solutions with electrolyte and 5.25, 0.56, and 0.63 for solutions of the non-ionic surfactant. The data fit for SWK2 was very poor.

The above results coincide with the trend of decreasing surface tension at the water-air interface, as shown in Figure S9. Low surface tension is a desired property in several applications were lignin can be used, such as in emulsions (209, 213), electrospun (210) and carbon fibers (211), among others. Lignin can be considered as a surface active polymer in aqueous media since it produces, depending on the conditions, a reduction in water’s surface tension (226). All the samples exhibited surface tension values lower than water and decreased with the increased lignin concentration. It has been suggested that the increased hydrophilic groups of lignin decreases its surface activity and increase its solubility by reducing the surface excess density (227). The surface tension was higher for SWK1 at 70 °C probably due to the low hydroxyl groups (Table 5.1). The lowest surface tension was found for samples containing the non-ionic surfactant even after the solution was cooled down at room temperature (note
that POE20 solution at 40×cmc exhibited 30 mN/m). In the case of saline solutions at 70 °C, NaCl affects aggregation on HWK and SWK2, which might explain the similar values of surface activity for the aqueous and saline lignin solutions, due to the ability of ions to cause perturbations in water structure (77). No correlation was found between the surface tension of the solutions and molecular weight of lignin.

5.4.5 Relevance of colloidal stability and aggregation

The colloidal behavior of lignin in solution is not only of great importance from an environmental viewpoint, for example, in wastewater treatment, but in many other areas. Furthermore, lignin aggregation significantly impacts the developing and preparation of advanced materials. In order to further inquire into this subject, the stability of lignin nanoparticles and black liquor was tested by addition of salt and non-ionic surfactant. The surface charge provided by phenolic hydroxyl and carboxylic groups on lignin is expected to facilitate the electrostatic double layer which favor (nano)particle stabilization (139) but it can be challenged by addition of salt and surfactants. For this, the stability of submicron lignin particles was studied and the particle size over a period of 10 days is shown in Figure 5.7. In pure water the initial average size (day 0) was ~285 and ~460 nm for SWK2 and HWK, respectively. We note that no reliable size data was possible for SWK1, which indicate nanoparticle formation with ill-defined shapes. The initial zeta potential values of the particles were ~-60, ~-55, and ~-49 mV for HWK, SWK1, and SWK2, respectively. Similar values have been previously reported (139). The surface potential of lignin in solution can be affected by pH since the thickness of the electrostatic double layer decreases at low values; however, pH was found to have a minor effect on the tested sub-micron lignin particles dispersed in water.
This may indicate that the carboxylic and phenolic groups play a minor role in aggregation of these particles. Different particle aggregation behavior was found by the addition of salt. Aggregation was observed upon increasing NaCl concentration from 0.5 to 1 M. The most stable system was SWK2 at 0.5 M NaCl, with no significant changes on particle size after 10 days. HWK particles were the most affected since no reliable data was collected from the size measurements, indicating the presence of aggregates with large or not well-defined shapes. These results are in agreement with the DLVO theory (81, 82) that predicts a decrease in the double layer repulsion between particles because of the salt addition, thus aggregation is promoted as the van der Waals forces become dominant. Interestingly, lignin particles coated by the non-ionic surfactant maintained their stability and size, a verification that particle aggregation did not occur. The concentration of the non-ionic surfactant seems to have minor effect on average particle size. The steric stabilization provided by the surfactant may open new opportunities for lignin in colloidal and nanoscience fields (139).
Figure 5.7. Stability of lignin nanoparticles monitored by dynamic light scattering (DLS) in water, electrolyte and surfactant solutions at two pH levels: 10.5 (profiles on the left) and 7 (profiles on the right). Sodium chloride was used at 0.5 and 1M. Surfactant (POE20) was used
at a concentration close to the $cmc (=cmc)$ and at higher concentrations ($40\times cmc \text{ or } >cmc$, as noted). Note that for HWK the addition of salt affected the aggregate shape and no reliable data was obtained from DLS analysis.

In order to examine the effects of salt and surfactant on the stability of industrial streams and the potential for lignin isolation, a black liquor was tested under similar conditions as those used in the particle systems. After some investigations to determine the appropriate conditions for aggregation (Figure S10), the results of mass filtered over time are shown in Figure 5.8. It is worth mentioning that filtration of neat black liquor sample was faster (completed in less than 50 seconds) compared to the other conditions studied and no cake was formed on the filtration surface. Similar result was obtained for black liquor equilibrated with non-ionic surfactant at $2 \text{ gL}^{-1}$. In contrast, the synergistic effect of salt (Ca ions) and surfactant promoted colloid aggregation leading to the formation of a lignin cake upon filtration at high lignin concentration in alkaline conditions. Usually, precipitation of lignin is carried out by acidification (228) and ultrafiltration (229, 230). Therefore, these results may bring new opportunities to separate and fractionate lignin from pulping mill wastewater avoiding environmental and corrosive problems due to the decrease in pH value, as well as facilitating filtration steps by increasing aggregate size. Further studies are needed in order to investigate the properties and purity of the precipitated lignin as well as to optimize conditions that will lead to get high lignin recovery yields.
Figure 5.8. Mass of lignin from hardwood black liquor samples, BL (~14gL⁻¹ lignin), retained on a Fann filter paper (47 mm diameter, 1 μm pore size) as a function of time in the presence of salt or surfactant: BL+POE20 2gL⁻¹ (up triangle), BL+Ca²⁺ 4gL⁻¹ (circle), BL+POE20 0.6gL⁻¹+Ca²⁺ 4gL⁻¹ (square), and BL+POE20 2gL⁻¹+Ca²⁺ 4gL⁻¹ (down triangle). In all the cases the pH was 12. The inset photos correspond to the residual lignin deposited over the Fann filter paper after 14 ml BL dispersion was filtered at constant pressure of 100 psig, and room temperature. Note that in the presence of non-ionic surfactant no lignin cake was formed.

5.5 Conclusions

The colloidal aggregation of three kraft lignins was studied by evaluation of turbidity, dynamic light scattering, and rheology. Lignin tended to self-aggregate over time, especially at high temperatures (70 °C). On the other hand, the presence of a non-ionic surfactant improved lignin colloidal stability due to steric stabilization effects. Compared to the case of SWK1, sodium chloride induces faster and to a larger extent the aggregation of HWK and
SWK2. According to the results, salt screened the electrostatic repulsions, causing the formation of intra and inter molecular complexes. In fact, rheology measurements indicate a disruptive effect by electrolytes involving the swelling of the aggregates and the weakening of their structure. By taking advantage of aggregation, the filtration of hardwood black liquor was improved, whereas colloidal stability was achieved for submicron lignin particles.

5.6 Supporting information
Figure S7. Effect of different surfactants on the aggregation behavior of aqueous lignin solutions (0.05% wt lignin, pH 10.5, 70 °C) for HWK and SWK1 as a function of time, with and without salt addition. The inset pictures show the samples after 120 h incubation at 70 °C. In the case of cationic surfactant, lignin precipitation occurred as indicated by the sharp decrease in turbidity.
Figure S8. Turbidity for lignins as a function of time at various NaCl concentrations and non-ionic surfactant (POE20 at $6 \times \text{cmc}$) at pH 10.5 and 70 °C. Aggregation was significant for (A, B) HWK and (C, D) SWK2 samples but not for (E, F) SWK1. The inset in E and F show the
low turbidity values obtained for this lignin sample. The rate of aggregation was calculated from the slope between 30 to 120 min.

**Figure S9.** Effect of electrolyte and surfactant on surface tension of the different lignin aqueous solutions after incubation for 30 min at 70 °C. The open symbols correspond to the surface tension measured after cooling down at 25 °C, whereas the closed symbols are the values determined at 70 °C.
Figure S10. Final turbidity of black liquor samples after equilibration (24 h at 70 °C) in the presence of different salts and non-ionic surfactant concentrations: POE20 (up triangle), NaCl (down triangle), Ca²⁺ (open circle), and a mixture of POE and Ca²⁺ (solid circle). A control sample was included (square). The concentrations used are shown in the plot. The pH was kept at 12.
Table S12. Fitted parameters for given rheology models for HWK lignin solutions at different lignin concentrations (%), with and without the addition of sodium chloride and a non-ionic surfactant, POE20 (40×cmc).

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Table S13. Fitted parameters for given rheology models for SWK2 lignin solutions at different lignin concentrations (%), with and without the addition of sodium chloride and a non-ionic surfactant, POE20 (40×cmc).

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**Table S14.** Fitted parameters for given rheology models for SWK1 lignin solutions at different lignin concentrations (%), with and without the addition of sodium chloride and a non-ionic surfactant, POE20 (40×cmc).

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6 CONCLUSIONS AND FUTURE WORK

The interactions among lignocelluloses, proteins, and surface active molecules were investigated, elucidated and demonstrated in this dissertation.

A surfactant-limonene-water complex microemulsion system was successfully developed and applied as a biomass pretreatment at low temperature and atmospheric pressure. This pretreatment was able to overcome the complex capillary structure of wood mainly due to the lower surface tension. The alkaline species in the aqueous phase of the microemulsion, such as ammonium hydroxide and sodium hydroxide, produced important changes in lignin and hemicelluloses content and in the physicochemical properties of biomass. However, cellulose was less affected than lignin and hemicellulose, indicating that this pretreatment can be utilized in processes that aim is to obtain cellulose nanofibers or monomeric and oligomeric sugars.

Moreover, the interactions between lignin, a nonionic surfactant and commercial multicomponent and monocomponent cellulolytic enzymes were further elucidated. Among these interactions, hydrophobic and electrostatic were found to be the driving forces responsible for causing non-productive interaction between lignin and enzymes. A non-ionic surfactant irreversible bound to lignin following the kinetic one-step model, due to mainly hydrophobic interactions. These results highlighted that the surfactant coated-lignin exhibited less affinity to enzymes. This reduction in affinity also depends on the type of lignin, the treatment for isolating it, as well as the solutions conditions, such as pH, ionic strength, and temperature. Although irreversible surfactant adsorption was also detected on cellulose
surfaces, cellulases digestibility was not affected, indicating the positive effects of a non-ionic surfactant on enzymatic hydrolysis.

The behavior of kraft lignin in aqueous solutions was further investigated under the addition of salts and surfactants. It was found that lignin tended to self-aggregate over time, especially at high temperature. The type of surfactant and its concentration have a significant impact on the colloidal stability of lignin solutions. According to the results, salt screened the electrostatic repulsions, causing the formation of intra and inter molecular complexes. These results are relevant to applications in industrial processes were fractionation and separation of lignin are needed. In fact, by taking advantage of the synergistic effect between salt and non-ionic surfactant the filtration of hardwood black liquor was improved at alkaline conditions. The steric stabilization mechanism of the non-ionic surfactant was tested on lignin nanoparticles dispersion, demonstrating that it is possible to control the aggregation by simply using the methods developed in this work.

In this dissertation the proposed microemulsion pretreatment was successfully applied; however, an optimization and evaluation of the process is needed to make it more attractive for industrial applications. For instance, the system can be applied in a biorefinery not only for obtaining sugars, as presented here, but also for other chemicals and value-added products, as fibers or nanofibers. Impregnation of woody biomass for preserving it is another relevant application that could be explored using a complex system as presented. Besides the advantages discussed in this work, the complex system contains a renewable oil and the chemicals have the potential to be recycled.
The development of model surfaces for studying the fundamental interactions among proteins, cellulose, lignin, and surface active molecules could be further expanded to applications where functionalization of them are relevant, for example in nanocellulose and proteins interactions for developing biocompatible materials. The promising features of this nanocellulose includes biocompatibility, low toxicity, and biodegradability, among others. This material has a potential to be used in biomedical applications by taking advantage of its interactions with proteins, for instance.

On the other hand, understanding the interactions of lignin will allow controlling its colloidal behavior and as a consequence its self-aggregation in solution. Controlling these phenomena could increase the efficient use of lignin, for instance, on lignin separation from wastewater and preparation of controlled-shape particles. Lignin nanoparticles could be used in several applications, such as coatings, dispersants, glue, composites, Pickering emulsions, drug delivery, and antimicrobial materials.
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