# ADAPTING A CHEMI-THERMOMECHANICAL PULPING (CTMP) PROCESS AS A POSSIBLE PRETREATMENT/FRONT-END FOR AN ENZYME-BASED BIOREFINERY

by

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# Abstract

To try to take advantage of existing infrastructure and experience a chemi-thermomechanical pulping (CTMP) process was assessed for its potential as a "front-end" for a biochemical-based bioconversion process. It had been shown that biomass, after mechanical pulping treatment, remained highly recalcitrant to enzymatic hydrolysis. This was largely due to the presence of lignin restricting enzyme accessibility to cellulose. Considering the high costs related to complete delignification, mild chemical treatment such as sulfonation and oxidation under neutral/alkaline conditions were assessed to minimize lignin's inhibitory influence while maximizing the recovery of hemicellulose in the water-insoluble component. Sulfonation and oxidation were able to incorporate acid groups onto the lignin macromolecule, consequently enhancing substrate swelling. This increased enzyme accessibility to the cellulose while reducing non-productive lignin binding *via* increased lignin hydrophilicity.

CTMP-based pretreatment was shown to be effective on agricultural and hardwood substrates. Mild alkali treatment of agricultural residues induced deacetylation of the hemicellulose and partial delignification. This resulted in enhanced enzyme accessibility to the hemicellulose and cellulose and increased enzymatic hydrolysis. Although hardwood lignin was more resistant to delignification, the incorporation of oxygen treatment into the CTMP treatment of the hardwood substrate substantially reduced the negative effects of lignin on enzymatic hydrolysis. As the lignin present in the CTMP treated substrate was enriched in acid groups, this resulted in increased substrate swelling and a decrease in the non-productive binding of enzymes to the lignin (*via* hydrophobic interactions).

Both softwood chips and pellets where pretreated using the adapted CTMP process to provide both a comparison with hardwood and agriculture feedstocks and to assess any differences between pellets and chips. Alkali addition prior to CTMP pulping enhanced lignin sulfonation. This predominantly occurred within the secondary-cell-wall, consequently increasing cellulose accessibility. However, the pretreated softwood chips and pellets remained relatively recalcitrant to enzymatic hydrolysis. Although the reduced particle size of softwood pellets was anticipated to facilitate chemicals and enzyme access, the high temperatures used during pelletisation resulted in lignin condensation. This was indicated by higher molecular weight and lower  $\beta$ -O-4 linkages of pellet-derived lignin, probably contributing to this substrate's higher recalcitrance.

# Lay Summary

The use of biomass to produce biofuels, biomaterials and biochemicals as replacements for exiting petroleum-based products is one potential solution to reducing global greenhouse gas emissions. As the effective deconstruction of biomass is hindered by its recalcitrant nature, a pretreatment step that reduces biomass recalcitrance prior to the enzymatic hydrolysis and deconstruction is typically required. To make use of the existing infrastructure and expertise of the mechanical pulping/newsprint industry, the work assessed the potential of adapting a chemi-thermomechanical pulping (CTMP) process as a "front-end" for a bioconversion process. The proposed pretreatment approach was able to maximize carbohydrate recovery while selectively modify the lignin/hemicellulose, consequently enhancing enzyme-mediated cellulose hydrolysis. Although this approach worked effectively on agricultural and hardwood substrates, softwoods proved to be more recalcitrant with both pellets and chips poorly hydrolyzed.

# Preface

All research work reported in this thesis work was planned and conducted by Jie Wu in the Forest Products Biotechnology/Bioenergy laboratory at the University of British Columbia, Vancouver campus, under supervision of Professor John (Jack) N. Saddler and Dr. Richard P. Chandra.

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# List of Abbreviations

| AFEX                | ammonia fibre/freeze expansion                   |
|---------------------|--|
| AIL                 | acid-insoluble lignin                            |
| AO                  | alkaline oxygen                                  |
| ASL                 | acid-soluble lignin                              |
| BSA                 | bovine serum albumin                             |
| Ca(OH) <sub>2</sub> | calcium hydroxide                                |
| СВН                 | cellobiohydrolase                                |
| CBM                 | cellulose binding modules                        |
| CDCl <sub>3</sub>   | deuterated chloroform                            |
| CELF                | co-solvent enhanced lignocellulose fractionation |
| CO <sub>2</sub>     | carbon dioxide                                   |
| CTMP                | chemi-thermomechanical pulping                   |
| DA                  | dilute acid                                      |
| DES                 | deep eutectic solvents                           |
| DI                  | deionized water                                  |
| DMSO                | dimethyl sulfoxide                               |
| DO                  | direct orange                                    |
| EG                  | endoglucanase                                    |
| EMAL                | enzymatic mild acidolysis lignin                 |
| FQA                 | fibre quality analyzer                           |
| GPC                 | gel permeation chromatography                    |
| GVL                 | γ-valerolactone                                  |
| $H_2O_2$            | hydrogen peroxide                                |
| $H_2SO_3$           | sulfurous acid                                   |
| HBA                 | hydrogen bond acceptors                          |
| HBD                 | hydrogen bond donor                              |
| HMF                 | hydroxymethylfurfural                            |
| HPLC                | high performance liquid chromatography           |
| HSQC                | heteronuclear single-quantum correlation         |
| kDa                 | kilodalton                                       |
|                     |  |
| KMnO <sub>4</sub>   | potassium permanganate                           |

| LCC                             | lignin carbohydrate complex                                       |
|---------------------------------|---|
| LCNF                            | lignin-containing cellulose nanofibril                            |
| LiBr                            | Lithium bromide   |
| LW                              | length weighted   |
| MA                              | maleic acid   |
| MHz                             | megahertz   |
| MP                              | mechanical pulp   |
| Na <sub>2</sub> CO <sub>3</sub> | sodium carbonate  |
| NaHCO <sub>3</sub>              | sodium bicarbonate  |
| NaOH                            | sodium hydroxide  |
| NMR                             | nuclear magnetic resonance  |
| NSSC                            | neutral sulfite semi chemical pulping                             |
| PBS                             | Phosphate-buffered saline   |
| PFI                             | papirindustriens forskningsinstitutt                              |
| РНК                             | pre-hydrolysis kraft  |
| pI                              | isoelectric point   |
| PTFE                            | polytetrafluoroethylene   |
| PTL                             | protease treated lignin   |
| p-TsOH                          | p-toluenesulfonic acid  |
| QM                              | quinone methide   |
| RMP                             | refiner mechanical pulping  |
| SEM                             | scanning electron microscope                                      |
| $SO_2$                          | sulfur dioxide  |
| SPORL                           | sulfite pretreatment to overcome the recalcitrance lignocellulose |
| SSA                             | specific surface area   |
| TEM                             | transmission electron microscopy                                  |
| THF                             | tetrahydrofuran   |
| TMDP                            | 2-chloro- 4,4,5,5-tetramethyl-1,2,3-dioxaphospholane              |
| TMP                             | thermomechanical pulping  |
| UV                              | ultraviolet   |
| WRV                             | water retention value   |
| XPS                             | x-ray photoelectron spectroscopy                                  |

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# 1. Introduction

### 1.1 Background

The ever-growing concerns over increasing greenhouse gas emissions resulting from fossil fuel consumption as well as their diminishing supply has motivated numerous strategies to seek renewable resources as alternatives (Yat et al., 2008). In particular, the development of lignocellulose-derived biofuel is one of the key strategies being pursued to reduce greenhouse gas emission from the transportation sector while enhancing global energy security (Chu and Majumdar, 2012). Lignocellulosic residues (i.e., biomass) have been suggested as an attractive feedstock that could be used as low-carbon intensive substitute for a variety of fossil fuel-based products (Kudakasseril Kurian et al., 2013). Over the past couple of decades, considerable effort has been dedicated to better utilizing biomass by converting the polysaccharides (i.e., cellulose and hemicellulose) and lignin into biofuels, biochemicals and biomaterials via enzyme-mediated bioconversion. This process typically utilizes a mixture of enzymes to breakdown the cellulose and hemicellulose into monomeric sugars for subsequent fermentation process into biofuels and other forms of bioproducts, while the lignin isolated from this process could be utilized as the feedstock for the production of bio-based chemicals and materials (Bornscheuer et al., 2012).

Depending on the location of the refinery, different types of biomass can be used, such as agricultural and forest residues as well as energy crops (Cherubini, 2010). Densified biomass such as pellets have the potential to be utilized globally in a large scale due to their high transportability. However, despite the biomass type they are all quite recalcitrant to bioconversion. Most biomass is designed by nature to have a heterogeneous and complex structure, from its compact arrangement of fibre cells to the molecular organization of the cellulose, hemicellulose and lignin components (Chundawat et al., 2011b). As a result, the accessibility of enzymes to cellulose is significantly restricted by the complex arrangement as well as the presence of hemicellulose and lignin (Chandra and Saddler, 2012). Therefore, typically, it is necessary to employ a pretreatment prior to the enzymatic deconstruction/hydrolysis step, to reduce the recalcitrance of biomass and to enhance enzymatic hydrolysis of the cellulose. Ideally, a pretreatment method should have low capital and operational costs and the capability of processing a wide range of biomass feedstock with minimal effort involved in preparation and handling. The pretreatment should also minimize the degradation of the carbohydrate components and maximize their recovery (Agbor et al., 2011; Chandra et al., 2007).

Steam pretreatment is an attractive pretreatment method due to its limited use of chemicals and its relatively low requirements for energy (Chandra et al., 2007; Jacquet et al., 2015). However, acidic steam pretreatment process conditions typically result in the formation of inhibitory compounds that hinder downstream fermentation processes (Duque et al., 2016). As any novel pretreatment process might involve the construction of new facilities and need a significant amount of capital investment (Alvira et al., 2010), as discussed here, an alternative approach might be to modify and utilize the existing facilities of the mechanical pulping industry.

Thermomechanical pulping (TMP) is a process that resembles steam pretreatment, where steam is incorporated into the mechanical pulping process (Vena, 2005). Due to the decrease in the demand for newsprint, mechanical pulping has lost its traditional newsprint market, forcing the closure of many mills. Thus, the existing infrastructure may be available for potential biorefinery development, using a modified pretreatment front end for biochemical based bioconversion of biomass. The advantage of using mechanical pulping as a pretreatment method is its ability to recover cellulosic substrates in high yield while retaining most of the carbohydrates in the waterinsoluble component. It should also produce a minimum amount of inhibitors that would restrict downstream processes (McDonald et al., 2004; Sandberg et al., 2020). However, to date, modified mechanical pulping techniques have not yet produced substrates that are easily hydrolyzed when low enzyme loadings are used to hydrolyze woody substrates. Despite the fibrillation of fibres during the TMP process that leads to the disruption of cell wall structure, generally, the hemicellulose and lignin are inaccessible, resulting in substrate recalcitrance (Gharehkhani et al., 2015; Kang et al., 2006). Although recent advances in the development of hemicellulase enzymes have shown the possibility of overcoming limited cellulose accessibility induced by the presence of hemicellulose (Hu et al., 2015), lignin remains as a serious issue for the efficient breakdown of cellulose. Previous work has shown that the lignin present in the biomass inhibits cellulose hydrolysis by limiting swelling, consequently restricting cellulose accessibility as well as binding to the cellulolytic enzymes and limiting their activities (del Rio et al., 2011; Kumar et al., 2012a; Nakagame et al., 2010; Rahikainen et al., 2013). Although lignin removal has been shown to be an effective way of enhancing enzyme-mediated cellulose hydrolysis (Mooney et al., 1998), to date, no cost-effective delignification-based methods have been commercialized, primarily due to the cost associated with the use of chemicals (Takada et al., 2020).

Rather than completely removing lignin, one alternative approach has targeted lignin

modification by acid group incorporation. Past work has assessed both sulfonation and oxidation treatments as both chemicals are routinely used by the pulping industry. Alkaline oxidation has been predominantly used to remove residual lignin from unbleached Kraft pulp (Kalliola et al., 2011) while sulfonation is predominantly used to soften the lignin prior to the mechanical pulping of wood chips (Börås and Gatenholm, 1999). As will be described in this thesis, oxidation and sulfonation-based pretreatments have both been used to improve enzymatic hydrolysis without the need for complete lignin removal (Chandra et al., 2016; Chu et al., 2018, 2017a; K. Song et al., 2019). As will be described, acid groups addition enhanced cellulose accessibility by facilitating fibre swelling as well as reducing the non-productive binding of enzymes by increasing the hydrophilicity and negative charge of the lignin (del Rio et al., 2011; Nakagame et al., 2011a).

The focus of the work described here was to investigate the potential of using milder conditions prior to the mechanical pulping step. As will be described, the steaming step that is typically utilized prior to thermomechanical pulping can be used to incorporate chemical treatments, such as sulfonation or oxygen, to improve enzymatic hydrolysis.

# **1.2.** Typical pretreatment methods

## 1.2.1 General summary

Generally speaking, pretreatments can be classified into the four categories of physical, chemical, physio-chemical and biological pretreatment.

The main objective of most physical pretreatments is to reduce the particle size of biomass and increase overall surface area. Some physical pretreatments also lead to a decrease in cellulose crystallinity while reducing the size of biomass, such as milling (e.g. ball milling and hammer milling) methods (Lin et al., 2010). A mechanical pulping/refining-based pretreatment is another example that results in an increase in the surface area of fibres.

Typical chemical pretreatments use acid or alkali under elevated temperatures to alter the structure/composition of biomass. Acid pretreatments (e.g. dilute sulfuric acid and phosphoric acid) generally hope to fractionate the hemicellulose from the biomass into the water-soluble stream, thereby enhancing the accessibility of enzymes to the remaining cellulose component in the water-insoluble component (Auxenfans et al., 2017; Ramos and Pereira Ramos, 2003). One of the challenges of this approach is the toxicity, corrosiveness and the recovery of acids(Sun and

Cheng, 2002). In addition, acidic pretreatment environments have been shown to degrade the cellulose and hemicellulose into furfural and hydroxymethylfurfural(HMF), which are known inhibitors to subsequent fermentation processes (Yu and Christopher, 2017). In contrast, alkali (e.g. NaOH, KOH, CaOH<sub>2</sub> etc.) based pretreatments target the removal of lignin, acetyl groups and uronic acids associated with the hemicellulose (Haghighi Mood et al., 2013). Unlike acid pretreatments, although alkali methods are less likely to dissolve the hemicellulose, they often cause swelling of fibrous cellulose as well as the cleavage of lignin-carbohydrate linkages. This contributes to the fractionation and dissolution of lignin into the alkali solution under elevated temperatures (especially for agricultural biomass) (P. Kumar et al., 2009; Meng Li et al., 2016). The potential limitation of an alkali pretreatment comes from its relatively long residence time and the need to neutralize the pretreatment slurry. This adds to the operating costs. Organosolv pretreatment is another type of chemical pretreatment that uses organic solvents (e.g. ethanol and ethylene glycol and tetrahydrofurfuryl alcohol) to specifically remove lignin by breaking down internal lignin linkages (Borand and Karaosmanoğlu, 2018).

Physio-chemical pretreatments refer to pretreatments that involve the combined input of physical and chemical treatments that can alter the structure and composition of biomass. Steam explosion is a typical physio-chemical method that combines the action of a hydrothermal treatment and an explosive pressure-release (Chandra et al., 2007). Other methods use chemicals such as ammonia (Ammonia Fibre/Freeze Expansion, AFEX), carbon dioxide (CO<sub>2</sub> explosion), oxygen(wet oxidation) to more effectively alter the lignin and hemicellulose, in combination with subsequent explosive treatment that further increase the surface area of the biomass (Haghighi Mood et al., 2013; Kumari and Singh, 2018).

Biological pretreatment processes have been suggested to be more environmentally friendly approaches as they only use microorganisms, without the need of adding any chemicals (Kumari and Singh, 2018; Sindhu et al., 2016). White, brown and soft-rot fungi are typical microorganisms that have been used to alter the composition of biomass(Sindhu et al., 2016). In particular, white rot fungi have been shown to be quite effective as they specifically break down the lignin. However, the main limitations of biological pretreatments are their requirement for long process times, large storage areas as well as continuous monitoring of the microbial growth (Haghighi Mood et al., 2013; Kumar and Sharma, 2017).

#### 1.2.2 Steam pretreatment

As mentioned earlier, steam pretreatment has been widely recognized as an attractive process

(Duque et al., 2016; Jacquet et al., 2015). Originating from the Masonite process, this method involves a high temperature hydrothermal step that targets hemicellulose removal, followed by an explosive decompression that can lead to structural disruption. The main advantages of steam pretreatment are its limited usage of chemicals and relatively low levels of energy (Chandra et al., 2007). Depending on the conditions employed, it results in the recovery most of the cellulose in the water-insoluble fraction and most of the hemicellulose in the water-soluble fraction.

During autohydrolysis (i.e., no addition of acid catalyst), hemicellulose removal is facilitated by the disassociation of acetyl groups attached to the xylan backbone of biomass, particularly for agricultural and hardwood residues (Duque et al., 2016; Leschinsky et al., 2009). However, including acid catalysts when pretreating softwood biomass has proven beneficial, as these substrates lacks acetyl groups on the hemicellulose component (Tooyserkani et al., 2013). The addition of acid catalyst was also found to reduce the need for more severe reaction conditions, resulting in greater hemicellulose recovery (Bura et al., 2003). Sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and sulfur dioxide (SO<sub>2</sub>) are the most commonly used catalysts (at a loading between 0.3% and 3% w/w). In many cases, gaseous SO<sub>2</sub> has proven more attractive, compared to H<sub>2</sub>SO<sub>4</sub>, as it is easier to add and better penetrates into the internal structure of the biomass (Mackie et al., 1985).

However, similar to typical acid pretreatments, the acidic environment during the steam pretreatment process generates compounds which are inhibitory to subsequent fermentation process. This is primarily due to the fractionation/degradation of hemicellulose and lowmolecular-weight lignin. These inhibitors, including acetic acid, furfural, HMF and phenolic compounds, are often produced when the conditions utilized for steam pretreatment are too severe (high temperature, residence time and acid). As a result, a compromise between providing a hydrolysable substrate, good sugar recovery and minimizing the formation of inhibitors often needs to be made when using steam pretreatment (Chandra et al., 2007). To try to better utilize the hemicellulose-derived sugars in the water-soluble fraction, many researchers have used a detoxification step, which increases the operating cost due to the use of chemicals such as activated charcoal, organic solvent, polymeric resins, alkali and reducing agents (Alriksson et al., 2011; Cavka et al., 2011; Mateo et al., 2013; Yu and Christopher, 2017). The removal of hemicellulose also enriches the lignin content in the resulting substrate, which is more obvious in the case of woody, especially softwood biomass. Therefore, it is often necessary to incorporate a post-treatment step aimed at lignin removal/modification prior to the subsequent enzymatic hydrolysis step. For example, using sulfite and oxidizing agents under neutral/alkaline conditions

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(Kumar et al., 2011; Pan et al., 2004; Yang et al., 2002a). However, nearly all of these posttreatment strategies required high chemical loadings, making them economically unattractive.

## 1.2.3 Thermomechanical pulping

#### 1.2.3.1 Mechanical pulping techniques

Thermomechanical refining/pulping is a variation of steam pretreatment and commercial plants have infrastructure that can be readily adapted to a biorefinery approach. Similar to steam pretreatment, traditional thermomechanical pulping processes also employ pressurized steam to soften the lignin and enhance the swelling of fibres, either before or during the mechanical pulping process.

Mechanical pulping of woody biomass can be traced back to 1800s when stone ground wood (SGW) pulping was developed. Later, in the early 1950s, refiner-based mechanical refining was established (refiner mechanical pulping, RMP), which did not involve the use of steam. This early method was designed to directly convert wood chips into fibrous mechanical pulp, using rotating metal discs or plates equipped with various types of grooves and raised bars (Biermann, 1996a; Smook, G, 1989). The typical refining system uses a single disc refiner for both primary and secondary refining (Figure 1). Primary refining was designed to progressively break chips into smaller particles and finally into individual fibres, with the aim of producing single and long fibres and minimums debris. The subsequent, secondary refining converts fibres into fibrils and small fragments using plates with shorter breaker bar sections. The continuous employment of physical forces (i.e., compression and shear forces), on the biomass leads to the delamination of inner cell walls (i.e., internal fibre crushing and delamination) as well as an increase in the surface area of fibres by peeling away layers of microfibrils from the outside of fibres. This results in the formation of "hairy fibrils" (i.e., external fibrillation) (Gharehkhani et al., 2015; Smook, G, 1989), contributing to stronger fibre-to-fibre binding in the subsequent papermaking stage. With the continuous development of multiple disc refiners, in some cases, traditional two stages disc refining can be displaced by a single stage refining process (Lönnberg, 2009). This is beneficial for both energy reduction and improving the homogeneity of the resulting pulp (Gharehkhani et al., 2015).



*Figure 1. The segments of refiner plate for primary refining (a) and secondary refining (b) (Smook, G, 1989).* 

The original purpose of adding a steaming step to the thermomechanical pulping process was to reduce the refining energy required by softening the lignin prior to refining the wood chips. Later work showed that, using temperatures between 135-140 °C can avoid the separation of fibres at the middle lamella that would result in the production of less flexible fibres that are resistant to fibrillation (Biermann, 1996b; Roffael et al., 2001; Smook, G, 1989; Vena, 2005). The resulting fibres within thermomechanical pulps (TMPs) have improved properties compared to refiner mechanical pulp (RMP), including better fibre characteristics (e.g., higher conformability, higher bonding properties, higher strength properties and lower shive/fines content), higher densities as well as better printability.

With the goal of further reducing the energy requirements for mechanical pulping as well as developing higher drainage pulps more suitable for other applications (e.g. food board, printing and writing paper, tissue and sanitary papers), chemi-thermomechanical pulping (CTMP) was developed (Petit-Conil et al., 1994; Smook, G, 1989; Vena, 2005). During the CTMP process wood chips are treated with relatively low amounts of chemicals such as sodium sulfite and alkalis to soften the lignin before or between the refining stages. Typically, the chemical treatment of softwood chips is conducted with 2-4% sodium sulfite, at pH 9-10, 120-135°C for 2-15 minutes (Lönnberg, 2009).Unlike steaming, which results in only temporary softening, the addition of chemicals such as sodium sulfite, results in sulfonation of the lignin matrix (Smook, G, 1989). In comparison to TMP, chemi-thermomechanical pulps (CTMPs) have several superior properties due to lignin sulfonation. These include its higher long-fibre content, improved fibre flexibility, reduced shive and fines content. These features contribute to the enhanced mechanical property of pulp (e.g., tensile strength, tear and burst index). The brightness of the resulting pulp can also be improved with the addition of chemicals, as the alkaline

conditions remove lignin chromophores and extractives that reduces the adsorption coefficients of the pulp (Hirashima and Sumimoto, 1994).

In recent decades, a variety of advances in refiner design have been made, such as the development of multiple disc refiners, conical refiners and cylindrical refiners(Gharehkhani et al., 2015; Lönnberg, 2009). There have also been efforts to utilize mechanical refining techniques to improve the properties of lignocellulosic substrates. In particular, the Bivis extruder/equipment that consists of two identical intermeshing screws that have different zones of pitches was found to be a good candidate in refining the biomass and/or pulp at high consistency (Figure 2) (Sjöberg and Höglund, 2007).



Figure 2. illustration of the Bivis process (Sjöberg and Höglund, 2007).

### 1.2.3.2 Mechanical pulping/refining as pretreatment

As mechanical pulping processes retain the lignin in the solid component, it limits the pulp use to products such as newsprint (Smook, G, 1989). However, due to the decrease in the demand of newsprint, mechanical pulping has lost its traditional market. As this has resulted in the closure or less frequent operation of many pulp mills, these facilities could be repurposed as the "front-end" of potential biorefineries. The advantage of using mechanical pulping as a pretreatment method is its ability to produce cellulosic substrates at high yield while keeping all of the carbohydrate components in the water-insoluble fraction and minimizing inhibitor production.

Past work has shown that mechanical based pretreatments have considerable potential to enhance the cellulose hydrolysis of substrates containing low levels of lignin such as bleached Kraft pulps (Hoeger et al., 2013). It has been shown that mechanical pulping/refining led to extensive fibre disruption while decreasing the crystallinity of the cellulose (Dome et al., 2020). It also increased the total accessible specific surface area via internal delamination, external fibrillation and in some cases, fibre cutting and generation of fines (Barakat et al., 2014; De Assis et al., 2018; Molin and Daniel, 2004). However, biomass, especially woody biomass, has been shown to remain quite recalcitrant to enzymatic hydrolysis after mechanical pretreatment (Boussaid and Saddler, 1999; Mooney et al., 1998) with the poor hydrolysis primarily attributed to the lignin in the substrates. Earlier work has shown that mechanical refining has a very limited impact on the fibrillation of substrate containing high levels of lignin (Hoeger et al., 2013). As discussed earlier, the lignin present in the substrates significantly hinders the swelling and accessibility of cellulose to enzymes, as well as binding enzymes during the enzymatic hydrolysis (Kumar et al., 2012a).

However, a number of studies have evaluated the feasibility of using mechanical refining as a post treatment, in combination with hydrothermal (e.g. Liquid hot water and steam) pretreatment (De Assis et al., 2018; Dou et al., 2016; Gonzalez et al., 2011; Yue et al., 2019) and chemical pretreatment (e.g. green liquor, alkali and acidic sulfite) (Chen et al., 2016; Jones et al., 2013; J Y Zhu et al., 2009) that target the alteration/removal of the hemicellulose and lignin components.

# **1.3. Biomass feedstock**

#### 1.3.1 Biomass recalcitrance

A mentioned previously, biomass recalcitrance is a result of its complex organization governed by physicochemical properties spanning from macroscopic fibre features (plant cells) to the molecular organization of cellulose, hemicelluloses, and lignin.

Cellulose is composed of glucose units linked via repeating  $\beta$ -(1-4) glycosidic bonds and some individual cellulose chains form crystalline sheets that stack together and are stabilized by hydrogen bonding and van der Waals interactions. These cellulose microfibrils further aggregate together to form larger bundles of macrofibrils (Donaldson, 2007). The tight packing of cellulose chains with high crystallinity prevents enzymes from easily breaking it down. Unlike cellulose, hemicellulose is a relatively amorphous, branched polysaccharide composed of different types of monomeric sugars (e.g., glucose, xylose, mannose, arabinose and galactose, glucouronic acid etc.). The composition varies across species and the developmental stage of the plant, as well as and tissue type and location of the cells (Fengel and Wegener, 1989). Hemicellulose can act as a physical barrier around the cellulose fibrils (Oksanen et al., 1997), blocking the cellulase enzymes

from accessing the cellulose (Bura et al., 2009). Lignin is an amorphous aromatic polymer, which primarily consists of three different phenolic alcohol monomers: p-coumaryl alcohol (p-hydroxyphenyl unit), coniferyl alcohol (guaiacyl unit), and sinapyl alcohol (syringyl unit) (Ralph et al., 2004). As will be further discussed, lignin is widely recognized as a major factor that inhibits effective hydrolysis of cellulose.

#### 1.3.2 Types of biomass that might be used in a biorefinery

In general, the potential biomass feedstocks that can be used for enzyme-mediated bioconversion processes can be categorized as either, herbaceous (agricultural), hardwood or softwood substrates.

#### 1.3.2.1 Herbaceous biomass

Herbaceous biomass, including agricultural residues (e.g., corn stover, wheat straw and sugarcane bagasse) and perennial herbaceous grasses (e.g., switchgrass, silvergrass) are typically less recalcitrant to bio-deconstruction. Xylan is the main hemicellulose constituent of herbaceous biomass and often substituted with acetyl groups that restrict the accessibility of xylanase enzymes to the xylan during the bio-deconstruction process (Selig et al., 2009). Although the lignin from herbaceous biomass consists of all three phenolic alcohol monomers, overall, the lignin content is generally lower than that of woody biomass (Fengel and Wegener, 1989), which likely contributes to the lower recalcitrance of these substrates. Within the lignin macromolecule cinnamic acids (e.g. ferulic acids and p-coumaric acids) are found, which increases its hydrophilicity, making it easier to be fractionated during water-based pretreatments (Sun et al., 2002; Takada et al., 2018).

#### 1.3.2.2 Hardwood biomass

Various fast-growing species of hardwoods, such as poplar (*Populus*) and Gum trees (*Eucalyptus*), have been considered as potential energy crops. In general, hardwoods are more recalcitrant to bio-deconstruction than herbaceous biomass. This is partly due to their higher lignin content (23-30%), which primarily consists of guaiacyl and syringyl lignin units, along with minor amounts of p-hydroxyphenyl units. Due to their lower cinnamic acid content and higher molecular weight, the lignin isolated from woody biomass is more hydrophobic than agriculture plant derived biomass (Huang et al., 2017; Takada et al., 2019). However, the

hemicelluloses of hardwoods are similar to those of herbaceous biomass, which includes a xylan backbone and the presence of acetyl groups(Demirbaş, 2005; Fengel and Wegener, 1989).

#### 1.3.2.3 Softwood biomass

Softwoods, including Lodgepole pine (*Pinus* contorta), Norway spruce (*Picea abies*), Western hemlock (*Tsuga heterophylla*) and Douglas-fir (*Pseudotsuga menziesii*) are among British Columbia's most abundant tree species. Softwoods are utilized as commercial commodities (e.g. timber and pulp), particularly in the Nordic countries, Canada and Russia (Unece & Fao, 2014, 2006). In addition, softwoods have the advantage of having a hemicellulose component (i.e., galactoglucomannan) that is primarily composed of hexose sugars (glucose, galactose and mannose) which can be more readily fermented to biofuels by traditional yeasts. However, softwoods are widely recognized to be the most recalcitrant type of biomass due to their fibre structure and high lignin content (26-40%), which is primarily composed of guaiacyl units (Fengel and Wegener, 1989; Rowell et al., 2000). Due to the lack of methoxyl substitutions on the aromatic rings, guaiacyl lignin has a higher tendency to repolymerize and form more-cross-linked lignin during pretreatments. This contributes to its inhibition of enzyme-mediated hydrolysis of cellulose (Ferrer et al., 2008).

## 1.3.2.4 Densified softwood pellets

The ready access to large quantities of biomass has proven to be one of the major limitations to effectively commercializing lignocellulose-based biorefineries (Kudakasseril Kurian et al., 2013). In particular, previous work has indicated that biomass features such as low density and high moisture content can constitute up to 50% of the total cost of biofuel/bio-chemicals production by decreasing the efficiency of the transportation (Kumar et al., 2006). To try to improve the efficiency of feedstock collection, transportation and long-term storage, wood derived biomass has been successfully densified via pelletisation.

To date, most softwood pellets are made by northern, forested countries such as Finland, Canada and Sweden (Thrän et al., 2019). In British Columbia, the production of lumber and pulp generates large quantities of residues that can serve as the raw materials for pellets (Spelter and Toth, 2009; Thrän et al., 2019). Although pellets are almost exclusively used in combustion, the relatively low costs of transportation could make them suitable bioconversion feedstocks.

As well as enhancing feedstock logistics, the production of pellets reduces fibre integrity, due to size-reduction treatment prior to the pelletisation process (Mostafa et al., 2019). This will likely enhance the efficiently and effectively of pretreatment by accelerating the penetration of chemicals. However, recent work has suggested that the high temperatures employed during the pelletisation process and the drying step might enhance the recalcitrance of the pellets to bio-deconstruction (Kumar et al., 2012b; Tang et al., 2018; Whittaker and Shield, 2017).

#### 1.3.3 Estimation of cellulose accessibility

It is recognized that the main cause of biomass recalcitrance to enzyme-mediated biodeconstruction is the lack of cellulase enzyme accessibility to the cellulose (Kumar et al., 2012a). As mentioned before, contributing factors include the presence of lignin, hemicellulose, acetyl groups and lignin-carbohydrate complex (LCC) linkages as well as the hierarchical structure of biomass, which all limit cellulose accessibility (Bura et al., 2009; Chundawat et al., 2011a; Grohmann et al., 1989; Kumar et al., 2012a; Öhgren et al., 2007). Although measuring/estimating cellulose accessibility to enzymes has proven problematic, various methods have been assessed including, water retention value (WRV), the Simons' staining methods, gas adsorption, mercury porosimetry and the use of specific cellulose binding modules (CBMs) (Aïssa et al., 2019; Chandra et al., 2009; Chandra and Saddler, 2012; Novy et al., 2019; Weiss et al., 2018).

The WRV method was initially developed to measure the ability of cellulosic materials (e.g., pulp fibres) to hold water. This can be used to assess the swelling potential of pulps while indirectly evaluating the degree of fibrillation (Gu et al., 2018; Ogiwara and Arai, 1968). It has also been suggested that the WRV can be used as an indication of the recalcitrance of pretreated biomass to enzymatic hydrolysis (Chandra et al., 2009; Weiss et al., 2018). However, the correlation between the WRV and cellulose hydrolysis can be problematic as water is much smaller than enzymes and this value indicates a physical phenomenon that might differ from cellulose accessibility to enzymes. In addition, the presence of relatively hydrophilic components in the pretreated substrates, such as the hemicellulose and oxidized/sulfonated lignin components, would often have a high WRV. However, the lignin and hemicellulose will still act as physical barriers to the enzymatic hydrolysis of the cellulose. The Simons' staining technique was originally developed by Simons in 1950 as a microscopic method to assess changes in mechanical pulp fibres (Simons, 1950). The method mixes the substrate with two types of dyes, the direct blue dye and direct orange dye, with the direct blue dye having a smaller molecular size and weaker affinity for cellulose compared to the direct orange dye (Yu et al., 1995).

Due to the unique properties of the direct blue and orange dyes, Simons' staining has been applied to the cellulolytic hydrolysis as a method to evaluate cellulose accessibility and effectiveness of pretreatment(Chandra et al., 2008, 2009; Hubbe et al., 2019). The advantage of the Simons' staining technique is its ability to assess the interior surface of cellulose and measure the specific surface area (SSA) in a wet state. Other methods, such as electron microscopy, gas adsorption and mercury porosimetry etc., require drying of the substrate, possibly resulting in the collapse of substrate "pores" (Chandra et al., 2009). The Simons' staining method has been improved by isolating the high-molecular-weight fraction of the direct orange dye via ultrafiltration and using it directly as a probe (Chandra et al., 2009; Chandra and Saddler, 2012). The isolated direct orange dye has a size in the range of 4–7nm, which is similar to the diameter of many cellulase enzymes present in the *Trichoderma reesei* cellulase preparation (e.g. CBH I, CBH II) (Divne et al., 1994; Lynd and Zhang, 2002). This method has been found to be effective at estimating cellulose accessibility of enzymes to substrates pretreated under varying conditions (Chandra and Saddler, 2012).

# 1.4. Negative impacts of lignin

## 1.4.1 Physical blocking

With most plant tissues, lignin is located in the middle lamella and plant cell wall, surrounding the cellulose and hemicellulose (Donaldson, 1991). Consequently, it physically blocks the accessibility of the cellulose to cellulase enzymes. The hydrophobic nature of the lignin present in the biomass restricts the swelling of fibres, further restricting cellulose accessibility (Cheng et al., 2010; Eriksson et al., 1991). Several studies have found that, as well as the lignin content, the location of the lignin also plays a significant role in restricting cellulose accessibility (Ju et al., 2013a; Yu et al., 2014). For example, to try and migrate/relocate lignin, hydrothermal pretreatments (e.g., liquid hot water and steam pretreatment) have used high temperatures and pressures. When the pretreatment temperature surpasses lignin's glass-transition point, the lignin becomes fluidized and is able to transition through the cell wall matrix. After pretreatment, this hydrophobic lignin often reforms as spherical droplets upon cooling, minimizing its surface area and exposing more of the cellulose to enzymes (Donaldson et al., 1988; Pu et al., 2015; Selig et al., 2007; Takada et al., 2019; Xiao et al., 2011).

### 1.4.2 Non-productive binding

As well as restricting accessibility to cellulose, lignin binds enzymes and results in non-

productive binding that reduces the efficiency of the hydrolytic enzyme. Previous studies have suggested that non-productive binding is driven by factors such as hydrophobic interactions, electrostatic interactions and hydrogen bonding (Berlin et al., 2006; Eriksson et al., 2002; Li and Zheng, 2017).

#### 1.4.2.1 Hydrophobic interaction

It has been suggested that hydrophobic interactions are the primary mechanism behind nonproductive binding of enzymes to lignin (Palonen et al., 2004b; Rahikainen et al., 2013). Earlier studies have shown that cellulase enzymes have a higher tendency to adsorb to lignin, rather than cellulose, partly because lignin is more hydrophobic than cellulose (due to its aromatic structure) (Norgren et al., 2007, 2006). It was also shown that the hydrophobic surface of lignin interacts with the hydrophobic cellulose binding module as well as the catalytic module (Eriksson et al., 2007; Palonen et al., 2004b). Analyses on lignin structures has suggested that the hydrophobicity of lignin decreases with increasing aliphatic hydroxyl and carboxylic content as well as decreasing amounts of phenolic hydroxyl groups (Huang et al., 2016; Yu et al., 2014). Other work has also suggested that the S/G ratio of lignin has an impact on its tendency to adsorb enzymes (Mi Li et al., 2016). To try to reduce non-productive binding, researchers have incubated pretreated substrates with surfactants to try to block the hydrophobic site of lignin, prior to enzyme hydrolysis. Bovine serum albumin (BSA) (Yang and Wyman, 2006), commercial soy protein (Luo et al., 2019), Tween series (Seo et al., 2011) and lignosulfonates (Wang et al., 2013) have also been assessed.

#### 1.4.2.2 Electrostatic interaction

Previous work has suggested that both the enzymes and lignocellulosic substrates possess charged groups. The association and dissociation of these groups under aqueous phase can impact their electrostatic interactions (Nakagame et al., 2011a), as components with the same and opposite charge repulse and attract each other, respectively. Past work has shown that electrostatic interaction between the two components is heavily influenced by the pH of the reaction media (Li and Zheng, 2017) with most pretreated substrates possessing a negative charge under typical hydrolysis condition ( pH 4.8-5). This is likely due to the dissociation of the carboxylic groups and the ionization of the phenolic hydroxyls in the remaining lignin (Sun et al., 2016). Under the same conditions, the majority of enzyme components (e.g., CBHI, the CBHI core, EGI, and EGII components from *T. reesei*,  $\beta$ -glucosidase from *Aspergillus niger*) from a conventional enzyme

mixture (Celluclast and Novozyme 188), hold a predominantly negative charge as their isoelectric point (pI) is lower than 5. Recent work has suggested that elevating the pH of hydrolysis buffer could enhance electrostatic repulsion between enzymes and negatively charged lignin (Lan et al., 2013; Lou et al., 2013). However, more work needs to be done to better elucidate the mechanisms behind this phenomenon.

# 1.4.2.3 Hydrogen bonding

Although the effect of hydrogen bonding on the non-productive binding has not been investigated in detail, earlier work has suggested that potential interactions driven by hydrogen bonding can occur between cellulase and lignin. This is due to the presence of hydroxyl/carboxylic groups on the lignin interacting with the hydroxyl groups attached to the cellulose (Berlin et al., 2006; Pan, 2008). Previous work has shown that the hydroxypropylation of the phenolic hydroxyls reduced the negative effect of lignin on the hydrolysis reaction (Sewalt et al., 1997). However, subsequent work showed that hydroxypropylation reduced lignin's inhibition on enzyme activity rather than enzyme adsorption (Yang and Pan, 2016).

### 1.4.3 Inhibition of cellulase activity

It has been shown that the water-soluble phenolic compounds derived from lignin are strongly inhibitory to the cellulase enzymes, hindering the efficiency of the enzymatic hydrolysis of the pretreated biomass (Hodge et al., 2008; Ximenes et al., 2010; Zhai et al., 2016). Earlier work has indicated that acid-catalyzed pretreatment of biomass, such as liquid hot water, dilute acid and steam pretreatment, generate lignin-derived compounds that inhibits and/or deactivates the cellulase and hemicellulase enzymes (e.g., endo-glucanase, exo-glucanase,  $\beta$ -glucosidase and  $\beta$  xylosidase) (Kim et al., 2011; Michelin et al., 2016; Ximenes et al., 2011, 2010). These watersoluble compounds include tannic acid, gallic acid hydroxy-cinnamic acid, vanillin, syringaldehyde, trans-cinnamic acid, and hydroxybenzoic acid etc. Previous work by Zhai et al. has shown that the phenolics derived from steam-pretreated softwood were much more inhibitory to cellulose hydrolysis than hardwood derived lignins (Zhai et al., 2018a). It was also shown that phenolics with a smaller molecular size (less than 1 kDa) and a greater carbonyl content were most inhibitory to cellulose hydrolysis (Zhai et al., 2018a). More recent work has suggested that the type of pretreatment also affects the properties of the resulting phenolic compounds, directly impacting enzymatic hydrolysis and fermentation (Chen et al., 2020). For example, it has been shown that AFEX-derived phenolics contain phenolic amide groups, likely leading to their strong

inhibitory effect on enzymatic hydrolysis (Chen et al., 2020).

### 1.4.4 Lignin structural alterations during pretreatment

During most pretreatment processes lignin undergoes depolymerization and repolymerization/condensation reactions, changing its molecular weight and hydrophobicity. In general, lignin has a higher tendency to condense under acidic as compared to alkali conditions. During acid-catalyzed pretreatments, depolymerization (acidolysis) and repolymerization(condensation) of lignin tend to occur simultaneously, resulting in highly heterogeneous lignin structure (Figure 3) (Li et al., 2007). A number of studies have also shown that higher pretreatment severities result in a more condensed lignin structure (Sun et al., 2016) (e.g., decreased ether linkages and aliphatic -OH, increased condensed phenolic -OH, molecular weight). This resulted in more pronounced hydrolysis inhibition and greater binding of cellulase enzymes (Kellock et al., 2019; Ko et al., 2015; Nakagame et al., 2011a). In addition, the guaiacyl lignin subunit, which is the major building block of softwood lignin, is more prone to condensation and resistant to fractionation compared to the syringyl lignin subunit. When pretreating hardwood and agricultural biomass, a number of studies have reported reduced S/G ratio (syringyl/guaiacyl) of the residual lignin, as the syringyl unit was preferentially removed relative to guaiacyl unit (Jiang et al., 2016; Trajano et al., 2013).



*Figure 3.* Depolymerization and repolymerization of lignin during acid-catalyzed pretreatments (*Li et al., 2007*).

# 1.5. Methods targeting lignin removal

As lignin inhibits the effective bio-deconstruction of biomass, the most effective way to solve this issue is completely remove the lignin. This was confirmed in earlier where almost complete

hydrolysis of Kraft pulp and delignified RMP was observed (Mooney et al., 1998). As summarized in the next section, a series of pretreatment methods have been developed to selectively remove lignin from the biomass.

#### 1.5.1 Conventional organosolv pretreatment

Organosolv was originally used as a pulping method, (using organic solvents, e.g., methanol, ethanol, propanol, butanol, etc.) to effectively remove lignin from woody and non-woody biomass and as a potential replacement to the Kraft pulping process (Johansson et al., 1987). However, various challenges such as its high energy requirements, the use/recovery of costly solvents and the potential for explosion prevented it from it evolving into a fully commercialized process (Alvira et al., 2010; Borand and Karaosmanoğlu, 2018).

The organosolv method has been adapted as a pretreatment method, partly due to its ability to produce sulfur-free lignin as a co-product (Pan et al., 2005) with ethanol the typical solvent used under acidic conditions at elevated temperature (100-250°C). During acidic organosolv pretreatment, both the lignin and hemicellulose are solubilized through the cleavage of LCC and lignin-lignin linkages. This results in a water-insoluble, cellulose rich fraction. However, the harsh conditions required for lignin removal also resulted in lignin condensation as well as compromising effective hemicellulose recovery (Pan et al., 2007; Sannigrahi et al., 2010). In addition, the use of an organic solvent to increase lignin solubilisation also necessitated extensive washing after the pretreatment, substantially increasing operational costs (Zhao et al., 2009).

### 1.5.2 Recently developed organosolv technologies

A recently developed method, named Co-Solvent Enhanced Lignocellulose Fractionation (CELF), utilizes a mixture of water and tetrahydrofuran (THF) to fraction lignin from biomass at a temperature of 160-180°C. This results in the production of sugars and co-products such as furfural, HMF, and levulinic acid (Cai et al., 2013; Meng et al., 2018). The tetrahydrofuran (THF) is considered to be a green solvent as it can be produced by biomass-derived products and easily recovered after low-temperature distillation (Hunter et al., 2006). It has also been shown that the lignin isolated after CELF treatment has a lower ether linkage content and non-condensed structural features (e.g., low molecular weight and rich in phenolic hydroxyls). This modified lignin has the potential to be the feedstock for biochemicals and biomaterials (Meng et al., 2018).

Another biomass-derived solvent used in recent organosolv pretreatment technologies is  $\gamma$ -valerolactone (GVL), due to its attractive properties such as low melting point, high boiling and flashpoints and low toxicity and recyclability (Shuai et al., 2016; Zhong et al., 2017). Recent work has shown that GVL/water solvent systems were three times more effective at delignification compared to the THF and ethanol approach (Shuai et al., 2016). It has been reported that 80% delignification of hardwood could be achieved at 120°C under mild acidic environment, resulting in the near-complete recovery of xylose and glycose. The lignin isolated from GVL pretreatment was also shown to have a similar structure to native lignin(i.e., preserving  $\beta$ -ether linkages), likely due to the mild reaction conditions used in the pretreatment process (Luterbacher et al., 2015).

#### 1.5.3 Deep Eutectic Solvents (DES)

Deep Eutectic Solvents (DES) are another promising green solvent that have been increasingly gaining attention. This is mostly due to their low toxicity, recyclability, high biodegradability and the ability to be produced from biomass-derived chemicals. Deep Eutectic Solvents have been shown to depolymerize lignin at mild conditions by cleaving the ether linkages (Tang et al., 2017; Xu et al., 2016). They can be prepared by mixing hydrogen bond donors (HBDs) with hydrogen bond acceptors (HBAs) into a single liquid phase. While separated HBDs and HBAs usually exhibit high melting points, the melting point of the mixture at a specific molar ratio can be reduced dramatically. This is due to the formation of numerous hydrogen bonds between the HBA and HBD that restricts cation–anion electrostatic interactions (Stefanovic et al., 2017; Wagle et al., 2016).

Previous work has shown that DES made from lactic acid (HBD)and betaine (HBA) provides a promising post-treatment approach that is high selective at extracting lignin at 130°C from steampretreated substrates (Tian et al., 2017). Related work has further demonstrated that lactic acid/ betaine based DES removed lignin from corn stover and willow-derived mechanical pulps with good selectivity and high recovery of the carbohydrate components achieved at a temperature of 140°C(Song et al., 2019). However, the lignin fractionated was found to be more condensed and hydrophobic compared to its native form, resulting in a greater tendency to adsorb cellulase enzymes (Song et al., 2020).Other work has shown that DES can be made from lignin-derived phenolics such as 4-hydroxybenzyl alcohol, vanillin, p-coumaric acid (Kim et al., 2018). This work also proposed the concept of a closed-loop biorefinery through the integration of renewable DES produced from lignin-derived phenolic aldehydes (Kim et al., 2019).

## 1.5.4 Acid hydrotropes

Recyclable acid hydrotropes such as p-toluenesulfonic acid (p-TsOH) are promising as it was shown that, when using p-TsOH as the pretreatment solvent, effective lignin fractionation from agricultural and hardwood biomass can be achieved at mild temperatures ( $\leq 80^{\circ}$ C) (Yu et al., 2020). In particular, 80% delignification was achieved with corncobs, wheat straw, and silvergrass within 10 min of pretreatment at 80°C (Yang et al., 2019). Related recent work has shown that 68% and 50% delignification of corn stover and willow can be respectively achieved at temperature of 50°C (Song et al., 2019). It was also suggested that, in addition to effective delignification (85% delignification of birch at 80 °C for 20 min), lignin-containing crystalline cellulose nanofibrils can be produced as a coproduct after p-TsOH pretreatment (Bian et al., 2017).

Maleic acid (MA) has also been shown to be another competitive hydrotrope that is able to remove lignin at mild reaction conditions, resulting in a lignin with less a condensed structure (e.g., higher content of  $\beta$ -O-4 linkage). Maleic acid pretreatment also resulted in the carboxylation of the residual lignin present in the solid fraction (Cai et al., 2020b). This not only benefited cellulose hydrolysis, by reducing lignin's non-productive binding to enzymes, it also facilitated substrate nano-fibrillation. This helped in the production of lignin-containing cellulose nanofibrils (LCNFs) (Cai et al., 2020a, 2020b).

### 1.6. Alternative approach: lignin modification via acid group incorporation

Although lignin removal has been shown to enhance cellulose hydrolysis, there is yet to be a delignification-based pretreatment method that is economically feasible for the bioconversion of biomass. This is mainly due to the cost associated with the use of chemicals (Takada et al., 2020). As an alternative solution, the addition of acid groups to the substrate has been investigated over the past several years. For example, compared to alkaline hydrogen peroxide(delignification method), applying a neutral sulfonation post-treatment to steam-pretreated softwoods resulted in substrates with a 30% higher lignin content but similar hydrolysis yields (Kumar et al., 2011). This, along with later work suggested that lignin modification via acid group incorporation could improve enzymatic hydrolysis of cellulose without the need for complete lignin removal. This

was achieved by increasing cellulose accessibility, facilitating fibre swelling and reducing nonproductive binding and making the lignin more hydrophilic and negatively charged (Nakagame et al., 2011b). With the increase in lignin hydrophilicity there should be less hydrophobic interactions between the lignin and cellulase enzymes. Therefore, less non-productive binding will result. The negative charge on the lignin will also decrease enzyme binding by electrostatic repulsion since the majority of enzyme components are negatively charged in a typical hydrolysis environment (del Rio et al., 2011; Nakagame, 2010). Currently, sulfonation and oxidation are the most common methods used to modify lignin through the incorporation of sulfonic and carboxylic acid groups, respectively. As discussed below, both approaches should be scalable as they are mature technologies currently used by the pulp and paper industry.

#### 1.6.1 Lignin modification via sulfonation

#### 1.6.1.1 Sulfite pulping

Sulfite pulping is a chemical pulping method that was developed in early 1900s. It is one of the most successful examples of using a sulfonation reaction at a commercial scale. Depending on the pH of the cook, sulfite pulping can be classified into the different categories of acidic sulfite pulping and neutral sulfite semi-chemical pulping (Biermann, 1996a).

Acidic sulfite pulping utilizes a mixture of sulfurous acid (H<sub>2</sub>SO<sub>3</sub>) and the bisulfite ion (HSO<sub>3</sub><sup>-</sup>) to attack and solubilize lignin. It can be carried out over a wide range of acidic pHs and it is typically divided into acid sulfite and bisulfite pulping. Acid sulfite pulping refers to pulping using an excess of free sulfurous acid. It is often conducted under pH 1-2, at temperature from 120-135 °C for 4-40 hours (Biermann, 1996a). During sulfite cooking, the interaction between the lignin substructures and acid associated dehydration resulted in the formation of electron-deficient centres (carbonium ions) which are often located on the  $\alpha$ -ether groups (Fengel and Wegener, 1989). This is followed by the addition of hydrogen sulfite/bisulfite ions (HSO<sub>3</sub><sup>-</sup>) to the intermediary carbonium ion (Fengel and Wegener, 1989). The sulfonation of lignin under acidic condition is limited to the  $\alpha$ -C and  $\gamma$ -C positions, whereas the β-aryl ether linkages and the methoxyl groups remain intact (Gratzl and Chen, 2009).

Traditionally, sulfurous acid was used as the only chemical during acidic sulfite pulping with free sulfurous acid combined with lignin to produce relatively insoluble lignosulfonic acid. Subsequently, the sulfonated lignin undergoes cleavage to smaller and more soluble molecular
fragments. It was shown that discoloration of pulp can occur due to the condensation of lignin, which can occur under conditions of high acid concentration and/or high temperature. To try to solve this issue, a calcium base was added to the cooking process, leading to the formation of more soluble lignosulfonic salts (Biermann, 1996b). Due to the relative insolubility of calcium, the traditional calcium acid sulfite cook needed to be conducted at a lower pH of around 1.5. Later work used soluble bases such as sodium, ammonium, and magnesium to replace the use of calcium, allowing the pH increase to 4 (Biermann, 1996a). In contrast, bisulfite pulping is typically carried out under less acidic (pH 3-5), higher temperature (140-160 °C) and shorter residence time (2-4 hour) conditions (Biermann, 1996b). This is mostly due to the use of soluble bases including monovalent bases such as sodium and ammonium. This results in less lignin condensation as they diffuse faster into the biomass. The main advantage of acid sulfite and bisulfite pulping is that it can result in relatively high yields of bright unbleached pulps which can be easily refined and bleached to full brightness. However, the difficulty in recovering the chemicals as well as the environmental concerns regarding the use of sulfite has hindered its growth and continued use.

Neutral Sulfite Semi Chemical pulping (NSSC) has been used in the US as a semi-chemical pulping method since 1926. This process involves relatively mild chemical treatments followed by mechanical refining. The main chemical used for NSSC is sodium sulfite (Na<sub>2</sub>SO<sub>3</sub>), which is buffered with sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) to neutralize the organic acids liberated from the wood during cooking. During NSSC pulping, the reaction of lignin is limited to the phenolic lignin units only. This starts with the deprotonation of phenolic groups, which forms quinone methide (QM) intermediates that could be attacked by hydrogen sulfite and sulfite ions (HSO<sub>3</sub><sup>-</sup>, SO<sub>3</sub><sup>2-</sup>). The resulting  $\alpha$ -sulfonic acid structures undergoes sulfitolytic cleavage of β-aryl ethers (Figure 4). Depending on the pH of the cook, cleavage of methyl-aryl ether linkages can also occur during NSSC pulping.



Figure 4. lignin undergoing neutral sulfonation reaction (Matsushita, 2015).

Typically, NSSC is carried out under pH 7-9, at 160-180°C for 15-60 minutes, with the principal goal of removing of up to 50% of the lignin while preserving the cellulose and hemicellulose components. This results in relatively high pulp yields (65-85%, even up to 92%) compared to chemical pulping processes. Another advantage of NSSC is its low requirement for wood quality and species. Although this process originally targeted hardwoods, later studies showed it could be successfully used on agricultural feedstocks (Keskin and Paprican, 1994; Mohammad et al., 2010; Tarasov et al., 2017). Other advantages include its relatively low consumption of chemicals and low capital investment.

Earlier work has incorporated sulfite treatment into the steam explosion process, with the objective of producing high-yield pulps with reduced energy. This so called "explosion pulp" resulted from the impregnation of wood chips with sulfite at mild condition (in solutions containing 8% Na<sub>2</sub>SO<sub>3</sub> and various concentration of Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub>, ranging from 0 to 2%, at 60 °C for 24 h) prior to steam explosion at around180-210 °C for 1-2 minutes (Kokta and Ahmed, 1998). The pulp was subsequently subject to a mechanical refining step to improve its mechanical properties (Kokta and Ahmed, 1998)(Kokta and Ahmed, 1998). It was found that the addition of sulfite was able to

enhance the mechanical properties of the resulting pulps while decreasing the refining energy as compared to the original process. Chemical impregnation was later used in the Chemithermomechanical pulping process, with the goal of sulfonating the lignin to reduce the energy required for the subsequent mechanical refining process, as well as improving the fibre properties.

#### 1.6.1.2 SPORL pretreatment

Other researchers have tried to repurpose acidic sulfite pulping in the so-called Sulfite Pretreatment to Overcome the Recalcitrance Lignocellulose (SPORL) process. The principle of SPORL pretreatment is to employ acidic sulfite treatment with the purpose of fractionating the majority of hemicellulose component into the water-soluble fraction as well as partial delignification and sulfonation of the residual lignin. The water-insoluble fraction, which is rich in cellulose and sulfonated lignin, was subject to mechanical refining to further enhance the accessibility of cellulose to enzymes. It was suggested that the removal of hemicellulose was more critical to enhancing the enzymatic hydrolysis of cellulose compared to lignin removal (Wang et al., 2009; Zhang et al., 2013). Typical chemical used for SPORL pretreatment are sodium bisulfite (NaHSO<sub>3</sub>) and sulfuric acid ( $H_2SO_4$ ), at various loadings depending on the biomass feedstock. The loading of NaHSO3 for hardwoods and agricultural residues was around 1-7%, whereas softwood biomass tends to required higher loading of NaHSO<sub>3</sub> (8-12%) for the pretreatment to be effective. Loading of  $H_2SO_4$  also varied from 1-5%, with softwoods requiring higher acid loadings, likely due to their lack of acetyl groups facilitating hemicellulose removal and lignin sulfonation. Initially, the cooking conditions for SPROL pretreatment were 180°C for 30min when it was first introduced by Wang et al. in 2009 (Wang et al., 2009). Later work conducted the sulfite cooking at 165 °C for 75 min. This was the same severity as the initial conditions which reduced the generation of HMF by 50% while maintaining the effectiveness of pretreatment for delignification, hemicellulose removal and enhancing cellulose hydrolysis (Zhang et al., 2014). SPORL pretreatment has been shown to generate much less furfural and HMF in the water-soluble fraction compared to traditional acidic pretreatments such as Dilute Acid (DA). This helps simplify the detoxification process. In addition, the soluble lignin forms lignosulfonates that could possibly serve as valuable co-products. The advantages of the SPROL pretreatment include the possibility to use existing infrastructure, high efficiency in enhancing cellulose hydrolysis and the generation of relatively low amounts of fermentation inhibitors. Previous work has shown that Douglas-fir residues could be pretreated by SPORL, followed by simultaneous saccharification and fermentation (SSF) of the whole slurry, without the need for

detoxification (Cheng, 2015).

#### 1.6.1.3 Neutral/alkaline sulfite pretreatment

Sulfonation under neutral/alkaline conditions has been shown to selectively sulfonate lignin while preserving the hemicellulose components. Thus, several past studies employed neutral sulfonation as a post-treatment to further enhance cellulose hydrolysis, using 16% Na<sub>2</sub>SO<sub>3</sub> at a temperature of 160 °C for 1 hour (Chandra et al., 2016; Kumar et al., 2012a, 2011). It was found that neutral sulfonation enhanced cellulose hydrolysis similar to that of a fully-bleached substrate. As earlier work had suggested the use of sulfite treatment to detoxify the pretreatment liquor, more recent work has demonstrated that sulfite post-treatment could simultaneously detoxify the steam-pretreated softwood substrates while increasing its susceptibility to enzymatic hydrolysis. In particular, using extended sulfite treatment at mild condition (8% Na<sub>2</sub>SO<sub>3</sub> and 2% Na<sub>2</sub>CO<sub>3</sub> at 70 °C for 12h) researchers were able to simultaneously detoxify and sulfonate whole slurries of steam-pretreated softwoods (Zhong et al., 2019).

Rather than trying to sulfonate condensed lignin as a post-treatment, recent work investigated the sulfonation of lignin prior to steam pretreatment as it was likely that the condensed lignin structure was more resistant to sulfonation. This worked showed that employing sulfonation prior to steam pretreatment was more effective at lignin removal/sulfonation while enhancing cellulose hydrolysis (Chandra et al., 2016; Chu et al., 2018) and preserving the hemicellulose in the waterinsoluble fraction. The enhanced effectiveness of sulfonation prior to steam pretreatment allowed the use of milder conditions (130-135°C for 20-30 min), which preserved the hemicellulose component in the water-insoluble component. Earlier studies had shown that the residual hemicellulose present in water-insoluble component after pretreatment might hinder cellulose hydrolysis by restricting its accessibility to cellulases (Bura et al., 2009; Öhgren et al., 2007). However, recent advances in hemicellulase and accessory enzymes development have shown promising results, likely alleviating this issue. Previous work has suggested that xylanses could act synergistically with conventional cellulase mixture, hydrolyzing cellulose and hemicellulose simultaneously at high efficiency (Hu et al., 2015, 2011). As well as enhancing hemicellulose recovery, this avoids the loss of sugars in the water-soluble liquid stream that contains the various fermentation inhibitors mentioned in previous sections.

## 1.6.2 Lignin modification via oxidation

#### *1.6.2.1 Alkaline oxygen delignification/bleaching*

Alkaline oxygen treatments that remove residual lignin from un-bleached Kraft pulp have been used in the Kraft pulping process for decades. The Kraft pulping process utilizes alkaline sulfide cooking to remove the majority of lignin from the lignocellulosic feedstock with the goal of obtaining lignin-free pulp with excellent fibre strength and brightness for papers and packing applications. To remove the residual lignin, the pulp needs to be delignified while retaining the  $\alpha$ cellulose and pulp viscosity (Leh et al., 2008). Although earlier work used chlorine dioxide to delignify the pulp, it is not environmentally friendly. Therefore, oxygen has been used as a bleaching agent as it provides benefits both environmentally and economically. By using oxygen instead of other bleach plant oxidizing chemicals such as chlorine dioxide, ozone, or hydrogen peroxide, a tremendous saving in operating costs have also be achieved(van Heiningen et al., 2018). However, the main disadvantage of oxygen delignification compared to delignification by chlorine dioxide is its lower reactivity with lignin and lower delignification/cellulose degradation selectivity. Therefore, when oxygen delignification has been used as an intermediate step between Kraft pulping and bleaching, this normally removes about 50% of the residual lignin after Kraft pulping (Argyropoulos and Suchy, 2001). The optimum temperature employed for pulping is around 100°C with the alkali loading used in various delignification attempts falls in the range of 1-5% (w/w) NaOH. Higher temperatures and alkali loadings can have a negative impact on the yield and prosperities of the resulting pulp, such as reducing the  $\alpha$ -cellulose content and pulp viscosity.

The initial step of the lignin oxidation reaction usually requires the deprotonation of the phenolic groups under alkaline conditions. This allows for the transfer of one electron to oxygen and results in a phenoxyl radical and a superoxide in a solvent cage. The main result of oxidation is the electrophilic addition reaction that occurs on the phenolic groups. The phenoxy radicals are attacked by hydroperoxy radicals at the centres of high electron densities, leading to reactions including demethylation, ring opening and side chain elimination (Figure 5a). Ring opening leads to the formation of catechols and various mono/dicarboxylic acids. These moieties resemble muconic acid moieties. These side chain eliminations result in the formation of other organic acids and para-quinones, thus depolymerizing lignin to form vanillin-like structures (Asgari and Argyropoulos, 1998). Earlier work showed that, during oxygen delignification, the superoxide anion radical preferably attacks at the 3-carbon position where the methoxyl group is attached. This is part of the reason why softwood lignins are more resistant to oxidization (Ji et al., 2009).

The oxidation can also occur on the non-phenolic groups of lignin, with the benzylic oxidation of lignin resulting in the formation of  $\alpha$ -carbonyl groups (Figure 5b). As the carbonyl moieties are readily attacked by oxygen (Gierer, 1986), subsequently, the formation of conjugated acids can occur by the cleavage of the C $\alpha$ -C $\beta$  bond of etherified structures containing a-carbonyl group as well as the formation of benzylic-type carboxyl acids.



*Figure 5.* General reactions of phenolic groups(a) and non-phenolic groups (b) during alkaline oxidation of lignin (Asgari and Argyropoulos, 1998; Gierer, 1986).

Previous work has also suggested that oxidation resulted in a reduction in the phenolic hydroxyl and methoxyl groups, a decrease in molar mass as well as an increase in hydrophilicity (Tamminen and Hortling, 2001). This is due to the incorporation of  $\alpha$ -carbonyl groups and carboxylic acid groups onto the lignin during oxidation (Yang et al., 2003).

One of the limitations of oxygen bleaching is its difficulty in completely delignifying high-kappa Kraft pulps. Earlier work has shown that the residual lignin cannot be removed beyond 75% of the original levels (Fu and Lucia, 2003). These workers suggested that the resistance of lignin to

oxidation could due to numbers of reasons such as the matrix structure of the lignocellulosic material, the crystalline structure of cellulose and the presence of lignin carbohydrate complex (LCC) linkages further impeding the diffusion of oxygen (Fu and Lucia, 2003; Jafari et al., 2014; Lawoko et al., 2004). Other work has also suggested that the reactivity of the residual lignin decreases with the extent of delignification (Rosenau, 2019). Decreased reactivity has also be shown to result from the loss of reactive groups including hydroxyl and carboxyl groups, as well as an increase in condensed phenolics after oxidation of lignin (Rosenau, 2019; Yang et al., 2003). It has been suggested that the p-hydroxy phenyl units are generally inert (unreactive) during oxygen delignification (Lucia et al., 2002; Yang et al., 2003).

### 1.6.2.2 Alkaline oxidation-based pretreatments

When earlier work examined the effect of oxygen delignification on the hydrolysis of a model substrate (pulp) using two waste lignocellulosic materials (primary clarifier sludge, and steam-exploded wood) at mild temperature (80-100°C), it was found that that lignin from the steam-exploded Douglas-fir was more resistant to oxygen delignification compared to the lignin present in a commercial pulp (Draude et al., 2001). When subsequent work tried to optimize the delignification of steam pretreated softwood using alkaline oxygen treatment, the use of 15% NaOH with oxygen at 110°C lead to >84% lignin removal with the more severe conditions (e.g., high NaOH concentration and temperature) resulting in lignin condensation (low lignin removal) and cellulose degradation (Pan et al., 2004). It was also noticed that an increase in temperature accelerated the delignification rate. However, there was a simultaneous increase in the risk of cellulose degradation. Later work used an alkaline oxygen post treatment on green liquor-pretreated hardwood biomass at a milder condition (5% NaOH at 110 °C). This resulted in a significant increase in cellulose accessibility and hydrolysis yields (Koo et al., 2011). This was likely due to the fact that green liquor-pretreated hardwood lignin was more reactive and less condensed compared to the steam-pretreated softwood lignin.

As mentioned earlier, as lignin after steam pretreatment is more condensed and less reactive (Shevchenko et al., 1999), researchers have adapted direct oxygen treatment as a pretreatment method, similar to sulfite treatments. For example, wet oxidation pretreatments have incorporated oxygen treatment into conventional hydrothermal pretreatment such as steam pretreatment (Biswas et al., 2015; Palonen et al., 2004a). Compared to conventional hydrothermal pretreatments, the addition of oxidizing agents such as oxygen and hydrogen peroxide during wet

oxidation facilitated hemicellulose removal and partial delignification (Martín et al., 2007b, 2007a). Thus, the same pretreatment efficiency could be achieved using lower temperatures and residence times (Arvaniti et al., 2012). The addition of oxygen also promoted the exothermic reaction, further reducing the heating/energy required for pretreatment (Ahring et al., 1996). In addition, oxygen addition reduced the formation of fermentation inhibitors such as furfural, HMF and organic acids (Biswas et al., 2015).

When wet oxidation pretreatment is conducted under alkaline conditions, greater levels of hemicellulose can be retained in the water-insoluble component as the lignin is selectively oxidized (Palonen et al., 2004a; Varga et al., 2003). Other work has shown that alkaline oxidation pretreatment destroys the backbones and ester bonds of the hemicellulose (Shi et al., 2012). With the goal of improving the penetration of oxygen, recent work has impregnated hardwood chips with oxygen at a milder temperature (110 °C) for 2 hours, prior to steam pretreatment at higher temperature (195 - 210°C) (Chu et al., 2017b). This approach recovered more than 80% of the original carbohydrate components (cellulose and hemicellulose). It also selectively fractionated (58%) and modified the lignin by incorporating carboxylic acid groups, resulting in a cellulose component that was more susceptible to enzymatic hydrolysis. Recent work has combined the alkaline oxygen treatment with liquid hot water pretreatment to enhance carbohydrates recovery and cellulose hydrolysis. In this case, hardwood pellets were impregnated with oxygen at 60°C and 130°C prior to high temperature water treatment at 210°C (Song et al., 2019). The residual lignin from the pretreatment liquor and hydrolysis residue was shown to have potential as an adsorbent for heavy metal ions (Song et al., 2019).

#### 1.7. Thesis overview

As described within the main body of this thesis, the research looked at adapting a chemithermomechanical pulping (CTMP) process as a potential "front-end" for a biochemical-based bioconversion process. However, previous work had shown that, even after refining, the cellulose within CTMP pulps was still quite recalcitrant to enzymatic hydrolysis. Thus, additional mild chemical treatments such as sulfonation and oxidation were assessed for their potential to enhance hydrolysis and retain as much of the hemicellulose as possible with the cellulose while minimizing any negative effects on the lignin. Although this approach proved to be effective on agricultural and hardwood substrates, softwoods proved to be much more resistant to enzymemediated hydrolysis. Initially, and as described in more detail in research chapter 1, we first assessed if extended refining might enhance subsequent hydrolysis of the cellulose component. However, despite an apparent increase in fibrillation and a hoped-for increase in cellulose accessibility poor hydrolysis was still obtained. As it was possible that enzyme binding to lignin was limiting hydrolysis the more refined mechanical pulps were preincubated with Bovine Serum Albumin (BSA) to try to minimize non-productive enzyme binding prior to hydrolysis. However, the lack of any significant impact suggested that it was lignin's role in restricting substrate swelling that predominated, suggesting that it was this mechanism which hindered the effective hydrolysis of the cellulose in CTMP pulps rather than the enzymes binding to the lignin.

As complete delignification had been shown to be costly, primarily due to the high cost of chemicals and energy, in the work described in research chapter 2 we assessed the potential of modifying the lignin via sulfonation or oxidation. One of the attractions of this approach is that both of these chemicals are routinely used by the pulp and paper sector. As will be described in more detail, similar amounts of sulfonic and carboxylic acid groups could be added to lignin while minimizing delignification. It was apparent that both enzyme accessibility and cellulose hydrolysis were enhanced by sulfonic and carboxylic acid addition. These treatments also decreased enzyme binding to the lignin, likely due to the reduction in phenolic hydroxyl groups and the incorporation of acid groups enhancing lignin hydrophilicity. Consequently, this increased cellulose hydrolysis. We next assessed whether the addition of a mild alkaline pretreatment might further enhance the retention of the hemicellulose with the cellulose in the water-insoluble component. In this way both components could be enzymatically hydrolyzed at the same time. This combined alkali-mechanical pulping pretreatment resulted in good hydrolysis of corn stover using relatively low enzyme loadings.

However, as reported in research chapter 3, it was apparent that both deacetylation and delignification were occurring during this process. Thus, we next wanted to assess which mechanism predominated. As reported in more detail, deacetylation at 80°C, removed more than 20% of the lignin while treatment at room temperature minimized delignification. It was apparent that partial delignification, particularly from the surface of corn stover, also enhanced enzyme accessibility and cellulose hydrolysis. The delignification and deacetylation mechanisms could be further enhanced by supplementing the alkaline solution with sodium sulfite.

It is known that hardwood lignins are more hydrophobic and higher in molecular weight than

agriculture crop derived lignins. This makes them more resistant to alkali-mediated solubilization. Thus, the work described in the research chapter 4 assessed the potential of the modified CTMPbased pretreatment process to pretreat hardwoods by incorporating alkali and oxygen impregnation steps prior to pre-steaming and mechanical pulping. Oxidation was preferentially assessed compared to sulfonation as it is typically considered to be a more environmentally attractive approach. Using this approach, more than 70% of the hemicellulose from hardwood chips could be retained in the water-insoluble component, together with the oxidized lignin enriched in carboxylic acid groups. It was apparent that this treatment improved fibre swelling and increased cellulose accessibility with the substrate readily hydrolyzed when the cellulase mixture was supplemented with xylanases.

Although the thesis work showed that the CTMP-based pretreatment approach could be successfully applied to both agriculture and hardwood derived feedstocks, as detailed in research chapter 5, as anticipated, softwood derived feedstocks were much more recalcitrant. As the most common, globally traded, biomass is softwood pellets, we wanted to assess how the CTMP-based pretreatment process might be modified to enhance the enzyme-mediated hydrolysis of both softwood chips and pellets. The work in this chapter showed that alkali addition prior to CTMP pulping enhanced lignin sulfonation with this occurring predominantly within the secondary-cell-wall lignin. This, consequently, increased cellulose accessibility. However, the softwood substrates, particularly the pellets, remained relatively recalcitrant after the pretreatment. It was anticipated that softwood pellets might be easier to pretreat compared to chips due to this feedstock's reduced particle size and loss of fibre integrity improving accessibility to the chemicals (e.g., sulfite and alkali) and cellulase enzymes. However, it appeared that the high temperature used during pelletisation resulted in lignin condensation, as indicated by the pellet-derived lignin showing a higher molecular weight and containing less native  $\beta$ -O-4 linkages, resulting in less enzyme accessibility.

As described within the thesis, a modified CTMP-based pretreatment process was able to maximize carbohydrate recovery in the water-insoluble component while enhancing enzymatic hydrolysis of both the cellulose and hemicellulose components. However, softwoods proved to be more recalcitrant with lignin proving to be a major reason why both pellets and chips were still poorly hydrolyzed. Ongoing work continues to assess ways of modifying lignin so that softwoods can be more readily hydrolyzed when using lower enzyme loadings.

## 2. Materials and methods

### 2.1 Biomass and chemicals

#### 2.1.1Biomass

Corn stover was provided by Novozymes (Davis, California). Sodium hydroxide, potassium hydroxide and sodium sulfite were purchased from VWR International. Aspen wood chips were obtained from a pulp mill in Western Canada. The chips were screened at ranges between 2.5 × 2.5 cm and 5.0 ×5.0 cm. Softwood (Lodgepole pine and Douglas fir) chips was provided by Canfor. Inc. Softwood pellets (mixture of spruce, pine and fir) was provided by Pinnacle Renewable Energy Group (Prince George, BC). The cellulose-rich delignified pre-hydrolyzed Kraft pulp (PHK) was donated by Fortress Ltd. The protease enzymes, sodium percarbonate, sodium sulfite and anhydrous ether were all purchased from Millipore Sigma (Oakville, Canada).

### 2.1.2 Chemicals

Sodium carbonate, sodium acetate, acetic acid, tetrahydrofuran (THF), pyridine and deuterated chloroform (CDCl<sub>3</sub>) were purchased from Fisher Scientific. Sodium chlorite, Rose Bengal dye were purchased from Acros organics. Sodium sulfite, sodium hydroxide and potassium hydroxide were purchased from VWR international. Copper (II) ethylenediamine (CED), protease, dioxane, hypochlorite acid, sulfuric acid, lithium bromide (BrLi), dimethyl sulfoxide-d<sub>6</sub> (DMSO-d<sub>6</sub>), 2- chloro- 4,4,5,5-tetramethyl-1,2,3-dioxaphospholane (TMDP), chromium (III) acetylacetonate and N-hydroxy-5- norbornene-2,3-dicarboximide were purchased from Sigma Aldrich. Oxygen was received from Praxair Canada Inc. Direct orange 15 was received from Pylam Products. The dye was isolated using the method illustrated in Chandra et al.(Chandra and Saddler, 2012) to fractionate the high molecular weight portion.

#### 2.1.3 Enzymes

Commercial enzymes Celluclast 1.5L (cellulase mixture), Novozyme 188(β-glucosidase), Cellic CTec 3 (cellulase enzyme cocktail) and HTec xylanase (hemicellulase) used in the enzymatic hydrolysis/treatments were received from Novozymes (Novozymes, Bagsvaerd, Denmark). The protein content of these enzymes preparation was measured using the ninhydrin essay, according to Mok et al. (Mok et al., 2015).

## 2.2 Pretreatment methods

#### 2.2.1 Lab-scale mechanical pulping/refining

Mechanical pulping/refining of starting and pretreated biomass was conducted by a commercial twin gear juicer (super angel juicer model 8500) in a total volume of 10 L of water to mimic the primary refining step of the industrial mechanical pulping process. Primary-refined mechanical pulp was then subject to PFI milling for various revolutions according to TAPPI standard T-248 method. In research chapter 1, Aspen and Douglas fir was size-reduced by a hammer mill. Sized-reduced Aspen, Douglas fir and starting corn stover was mechanically refined using the commercial juicer and PFI milling for 5000, 15000 and 45000 revolutions.

In order to remove the fines, mechanical pulps made from corn stover, Aspen and Douglas fir was disintegrated with water and processed through a Bauer-Mcnett fibre fractionator. For this work, only the 100-mesh screen was used to separate the fines from the rest of the fibres.

#### 2.2.2 Deacetylation pretreatments

NaOH-based deacetylation of corn stover was conducted according to Chen et al. (Chen et al., 2012a). Briefly, 50g of oven dried corn stover was impregnated with 4.8% (w/w) NaOH (12:1 liquid: wood ratio, 0.1M) in an 80°C water bath for 3 h. For Na<sub>2</sub>SO<sub>3</sub>/NaOH deacetylation, an additional 3% (w/w) of Na<sub>2</sub>SO<sub>3</sub> was added to the NaOH solution prior to the impregnation, with the 4.8% (w/w) NaOH being replaced by 3% (w/w) Na<sub>2</sub>SO<sub>3</sub> for the sulfite control group.

The KOH-based deacetylation of corn stover used the method described by Jiang & Xu (Jiang and Xu, 2016). Briefly, 50 g (oven-dry basis) of corn stover was impregnated with 7.5% (20:1 liquid: wood ratio, 0.07M) in a 25 °C water bath for 24 h.

The chemically/water treated corn stover samples were subsequently refined at room temperature using a commercial juicer (super angel juicer model 8500) and a total volume of 10 L of water, followed by PFI milling for 2000 revolutions, according to TAPPI standard T-248 method. It was anticipated that the fibre separation and fibrillation resulting from sequential juicer and PFI milling treatment effectively mimicked industrial mechanical pulping at a lab scale.

### 2.2.3 Alkaline oxidation and neutral sulfonation of pretreated Aspen.

The acid wash of the pretreated Aspen mechanical pulp was conducted according to Meng et al.

(Meng et al., 2015). 20g of pretreated and acid-washed mechanical pulp was incubated overnight with either 16% (w/w) sodium sulfite (Na<sub>2</sub>SO<sub>3</sub>) or 32% (w/w) sodium percarbonate (Na<sub>2</sub>CO<sub>3</sub>·1.5  $H_2O_2$ ) at a10% (w/v) solids loading in a 70°C water bath. The substrates were washed with water after the reactions and stored at 4°C for further analyses.

#### 2.2.4 Alkaline-oxygen impregnated mechanical refining

Aspen chips were autoclaved at 121°C for 30 min before impregnation with 15% (w/w) sodium carbonate (4:1 liquid: wood ratio) in the 70°C water bath overnight. The purpose of autoclaving was to remove the inherent air from the chips, which facilitates the penetration of sodium carbonate into the chips (Malkov et al., 2002). Aspen chips were then impregnated with oxygen in the Parr reactor at 100 psig and 110 °C for 2 h prior to steam treatment at either 190 or 130°C for 15 min in a stainless-steel basket. After the steam treatment, the chips were subject to lab-scale mechanical pulping/refining using the twin-gear juicer and PFI milling for 10, 000 revolutions, as described above.

## 2.2.5 Water-based pre-hydrolysis treatment

Pre-hydrolysis treatment was carried out in a lab scale rotating digester (Aurora products Ltd. Savona, BC, Canada). In brief, 250 g (oven-dry basis) of Aspen chips were soaked with water in the cooking vessel at a liquid: wood ratio of 4:1. The cooking vessel was then moved into the digester. The temperature was heated to 170°C within 1.5 hours and stayed at 170°C for another hour. The vessel was then cooled down in the cold water before the pre-hydrolysis liquor was removed, filtered and undergo subsequent mechanical refining.

#### 2.2.6 Neutral/alkaline sulfonation treatments followed by mechanical pulping

Before the impregnation, softwood chips were autoclaved at 121°C for 30 min to remove the inherent air, as described in previous studies (Malkov et al., 2002). Subsequently, softwood chips were impregnated with 8% (w/w) sodium sulfite and various loadings (w/w) of sodium carbonate (0%, 2%, 4%, 6% and 8%) at 4: 1 liquid: wood ratio in the 70 °C water bath overnight. Impregnated chips were steam-pretreated in a stainless basket for the neutral/alkaline sulfonation treatments at 190°C for 15 min.

Alkaline sulfonation of softwood pellets was conducted in a similar way, with softwood pellets impregnated with 8% (w/w) sodium sulfite and 4% sodium carbonate directly at 4: 1 liquid: wood ratio in a 70°C water bath overnight, followed by steam pretreatment at 190°C for 15 min.

Subsequently, the mechanical pulping of neutral/alkaline sulfonated softwood chips and pellets was carried out in a commercial juicer (super angel juicer model 8500), followed by lab-scale PFI refining for 5000 revolutions according to TAPPI standard T-248 method.

## 2.3 Enzymatic hydrolysis

During the enzymatic hydrolysis (in duplicate) at 10% (w/v) solids loading, substrates were mixed with acetate buffer (50 mM, pH 5.0) and enzymes in the 50ml glass septa bottles and moved to a shaking incubator at 50 °C and speed of 180 rpm for 72 h. Enzymatic hydrolysis of the pretreated substrates (in duplicate) at 2% (w/v) solids loading was conducted in the 2ml screwcap tubes with acetate buffer (50 mM, pH 5.0) and enzymes. The tubes were moved to a rotating incubator at 50°C for 48 h. In the case of the hydrolysis with bovine serum albumin (BSA), samples were impregnated with acetate buffer (50 mM, pH 5.0) containing 10 mg/ml of BSA at room temperature overnight prior to the addition of enzymes.

## 2.4 Chemical composition analysis

The chemical composition of the corn stover and isolated lignin were assessed using the TAPPI standard T-22 om-88 method (in triplicate). Briefly, 0.2 g of extractive-free substrate (oven-dry basis) was Wiley milled and mixed prior to stirring with 3 ml of 72% H<sub>2</sub>SO<sub>4</sub> for 2 h. The mixture was diluted with 112 ml of de-ionized water and autoclaved at 121°C for 1 h. The acid-insoluble lignin (AIL) was collected and measured using a 30 ml fritted glass crucible. The acid-soluble lignin (ASL) was analyzed by determining the absorbance at 205 nm. The carbohydrate components of the acid-soluble fraction were measured using a Dionex (Sunnyvale, CA) HPLC (ICS-3000). The acetyl content was determined as the acetic acid present in the acid-soluble fraction according to Jiang and Xu (Jiang and Xu, 2016), using a UV detector installed in an HPLC (ICS-500) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA).

## 2.5 Microscopic analyses

## 2.5.1 Scanning Electron Microscope (SEM)

SEM of mechanical pulps and delignified PHK was conducted using a FEI Quanta 400F (Thermo Fisher Scientific). Freeze-died samples were placed on an SEM stub and gold-coated using a Hummer VI sputtering system, prior to the observation at accelerating voltages between 5 and 15 kV.

#### 2.5.2 Transmission Electron Microscopy (TEM)

Topochemical analysis of pretreated substrates was done by a TEM microscopy (Hitachi H7600) at an acceleration voltage of 80 keV. Prior to the analysis, substrates were dehydrated with ethanol-water mixtures with increasing ethanol concentration up to 99.5%, followed by solvent exchange using acetone. Subsequently, the samples were embedded in the epoxy resin to obtain ultrathin sections of 80nm using a diamond knife mounted on an ultramicrotome. With the purpose of selectively staining the lignin, 1% KMnO<sub>4</sub> solution was added to the section, and the section was then mounted on copper grids for TEM analysis.

## 2.6 Acid group titration

Conductometric titration of acid groups was a modified version of the method developed by Katz et al.(Katz R.P.; Scallan, A.M., 1984). Briefly, 0.15 g (oven-dry basis) of pre-washed material was weighed out and mixed with 15 ml of 0.1 N HCl in the 15mL falcon tube overnight. The sample was then washed by filtration with 250 ml nanopure water through a small Buchner funnel. After the filtration, the sample was transferred to a plastic beaker with the filter paper and re-suspended in 50 mL of 0.001 M NaCl solution containing 200  $\mu$ L of 0.05 N HCl. The mixture was then titrated with 20  $\mu$ L of 0.05N NaOH. The conductivity of pulp suspension was measured by a conductivity meter and recorded after each addition to plot the conductometric titration curve. The total content of carboxylic acid groups was calculated using the following equation:

$$X = C*V/m$$

Where X is the content of carboxylic acid groups, C is the concentration of NaOH, V is the volume of NaOH consumed by weak acids and m is the dry weight of the sample.

## 2.7 Simons' staining technique

Direct orange 15 (DO) dye was used to measure the accessibility of cellulose, according to previous studies by Chandra et al.(Chandra and Saddler, 2012). Briefly, a set of 10mg (oven-dry basis) of material were mixed with PBS buffer and increasing concentration of DO dye in 2mL screw cap tubes overnight. The tubes were then moved to a shaking incubator at 60 °C and speed of 180 rpm overnight. The tubes were subsequently centrifuged and the absorbance of supernatant at 450 nm was measured by a spectrophotometer.

## 2.8 Water retention value (WRV)

WRV was measured (in triplicate) using TAPPI Useful Method-256. In brief, approximately 0.5 g (oven-dry basis) of the pulp was soaked in 50mL water overnight prior to filtration through 200mesh screen in the WRV unit. The resulting pulp pad was then centrifuged at 900g for 30 min at 25 °C. The subsequent sample was weighed and dried in the oven at 105°C overnight. WRV was calculated by the equation:

WRV = (Wet mass – Dry mass) / (Dry mass)

Where Wet mass is the weight of wet sample after the centrifugation and Dry mass is the weight of the dried sample.

## 2.9 Pulp characterizations

#### 2.9.1 Fibre length measurement

The length weighted fibre length was measured by the Fibre Quality Analyzer (FQA, LDA02, OpTest Equipment, Inc., Hawkesbury, ON, Canada) using ISO 16065setting as measurement method. The pulp suspension was diluted prior to the measurement in order to make sure less than 40 fibres were measured per second.

#### 2.9.2 Viscosity measurement

Viscosity of selected substrates was measured by a capillary viscometer (Cannon Ubbelohde Viscometer, Cannon Instrument Co., State College, PA) using TAPPI Standard Method T230 om-08. Prior to viscosity measurements, the lignin in the substrates was removed by sodium chlorite bleaching process according to Kumar et al. (Kumar et al., 2012a). Briefly, 4 g sodium chlorite and 0.5 ml glacial acetic acid was added to 5g of substrate at 15:1 liquor to wood ratio. The reaction was carried out in the fume hood at room temperature for 3 hours. The slurry after reaction was filtered and washed extensively in the Buchner funnel. The delignification process was repeated in order to delignify the substrates.

## 2.10 X-ray photoelectron spectroscopy (XPS)

The substrate was disintegrated and filtered in a Buchner funnel to form a substrate sheet (80  $g/m^2$ ), which was then dried and pressed at 40 psig for 5 min. The XPS measurements were conducted using a Leybold Max 200 X-ray photoelectron spectrometer (Cologne, Germany) with

a monochromated Al Ka X-ray source. The detector position was at an angle of 90 relatives to the sample surface. The analysis measures the oxygen to carbon of the substrate surface at a depth of 5-10 nm, to estimate the relative amount of lignin that is present on the surface. The theoretical surface lignin coverage was calculated from the O/C ratios according to Laine et al. using the following equation:

Surface lignin coverage  $\Phi_{\text{lignin}} = (O/C_{(\text{Sample})} - O/C_{(\text{cellulose})})/(O/C_{(\text{lignin})} - O/C_{(\text{cellulose})})$ 

Where O/C  $_{(sample)}$  is the O/C ratio of the analyzed sample, and O/C  $_{(cellulose)}$  and O/C  $_{(lignin)}$  is the theoretical O/C ratios of pure cellulose (0.83) and lignin (0.33).

## 2.11 Elemental analysis

The elemental analysis of the corn stover substrates (C, H, N, and S) was assessed using a Thermo Flash 2000 Elemental Analyzer. Substrates were oven-dried, Wiley milled and stored in the 1.5 mL centrifuge tube prior to the analysis.

## 2.12 Lignin isolation

## 2.12.1 Protease treated lignin (PTL) isolation

The PTL was isolated from the biomass substrates using the method previously described by Nakagame et al. (Nakagame, 2010). In brief, the pretreated substrates were subjected to enzymatic hydrolysis using the Cellic CTec 3 preparation at 2% (w/v) solids and protein loading of 30mg/ g cellulose at 50 °C for 72 h. The hydrolysis residue was centrifuged, washed and subjected to a second round of enzymatic hydrolysis using the same conditions, followed by centrifugation and washing. This residue was incubated with protease (1 U/mL) in phosphate buffer (50 mM, pH 7) at 37°C for 24 h and subsequently transferred to a 90 °C water bath for 2 h to deactivate the protease. After washing, the protease treated lignin (PTL) was passed through a 40-mesh screen and freeze-dried.

## 2.12.2 Enzymatic mild acidolysis lignin (EMAL) isolation

The EMAL lignin from chapter 2 was extracted from the protease treated lignin (PTL) samples. In brief, the freeze-dried PTL was subject to mild acidolysis in an acidic aqueous dioxane solution (85% Dioxane: 15% water, 0.01M HCl) under reflux at 87°C for 3 h. The mixture was then centrifuged (5000rpm, 5 min) and the supernatant collected and precipitated in anhydrous ether. After the lignin had settled it was filtered through a polyvinylidene fluoride (PVDF) membrane, washed with anhydrous ether and dried in a 40°C vacuum oven.

The EMAL from research chapter 5 was extracted from starting biomass of Lodgepole pine chips and softwood pellets. In brief, extractive-free biomass was ball milled for 48 h, followed by enzymatic hydrolysis using Cellic CTec 3 at 5% (w/v) solids and enzyme loading of 40mg/g cellulose for 72h. The hydrolysis residue was washed thoroughly with Deionized water and freeze-dried. The freeze-dried residue was subjected to mild acidolysis, in an acidic aqueous dioxane solution (85% Dioxane: 15% water, 0.01M HCl) under reflux at 87°C for 3 h. The mixture was then centrifuged and the supernatant was collected, pH neutralized and precipitated in acidic Deionized water (pH 2). The precipitated lignin was collected after centrifugation and freeze-dried for further analysis.

### 2.12.3 Lignin isolation from the deacetylation liquor

The precipitation of lignin from the deacetylation liquor of corn stover was carried out by lowering the pH of the deacetylation liquor to pH 2. The precipitated lignin was collected through centrifugation, washed with deionized water and freeze-dried.

## 2.13 Lignin analyses

#### 2.13.1 Hydrophobicity test of lignin

The hydrophobicity of the protease treated lignin (PTL) samples was estimated by their adoption to Rose Bengal dye, according to Huang et al. (Huang et al., 2017). Briefly, various concentrations of lignin (2–10 g/L) was mixed with Rose Bengal (at a concentration of 40mg/L) in 50 mM citrate buffer (pH 4.8) in 1.5mL screw-cap centrifuge tubes. The tubes were incubated in a rotating incubator at 50 °C for 2 h and centrifuged to collect the supernatant. The content of unbound Rose Bengal dye in the supernatant was determined by measuring its adsorption of light at 543 nm using a UV–Vis spectrometer. The partitioning quotient was calculated as the amount of Rose Bengal bound on surface divided by the amount Rose Bengal in dispersion medium. The surface hydrophobicity of lignin (L/g) can be calculated as the slope of the line when partitioning quotient was plotted against the lignin content

### 2.13.2 Zeta potential measurement of lignin

The Zeta potential values were determined in triplicate using a Zeta-Meter 3.0+ (ZETA-METER,

INC., Staunton, VA). Samples were dispersed in 50 mM Na-acetate buffer (pH 4.8 and pH 6) before measurement.

#### 2.13.3 Adsorption of lignin to advanced enzyme cocktail (Cellic CTec3)

The adsorption of the enzyme presents in the Cellic CTec3 to the isolated protease treated lignin (PTL) samples was performed at 10 °C in 2.0 mL crew-cap centrifuge tubes using 1ml of sodium acetate buffer (50 mM, pH 4.8 and pH 6). Vials containing 1% (w/v) lignin and enzymes (0.5 mg/mL) were incubated for 3 h, followed by centrifugation and the supernatant collected. The protein content of the supernatants was measured by the ninhydrin method according to Mok et al., using BSA as the protein standard (Mok et al., 2015). The amount of enzyme adsorbed onto the lignin was determined as the difference between the initial enzyme loading and the free enzyme present in the supernatant.

## 2.13.4 Gel permeation chromatography (GPC)

Lignin samples isolated from the deacetylation liquor (research chapter 3) were acetylated to allow dissolution in tetrahydrofuran (THF) prior to the analysis of molecular weight using GPC. In brief, 50 mg of lignin was mixed with 3 mL each of pyridine and acetic anhydride (1:1) in round bottom flasks. The mixture was stirred at 70 °C for 1 h and 72 h at room temperature. Ethanol (30 mL) was added to the mixture and the mixture was concentrated in a rotating evaporator under reduced pressure. This procedure was repeated 3 times to allow for complete removal of the pyridine and acetic anhydride. Acetylated lignin was dissolved in chloroform, washed twice with DI water in a separatory funnel and dried over sodium sulfate. The lignin was precipitated in diethyl ether and dried in a 40 °C vacuum oven for 24 h. The acetylated lignin sample was then dissolved in anhydrous THF at a concentration of 5 mg/ml and stored at room temperature for 48 h prior to filtration via 0.45 µm PTFE syringe filters and GPA measurement. GPC measurements were conducted using Agilent 1100 GPC equipment (USA). The eluting solvent, THF was used as the mobile phase, at a flow rate of 0.7 mL/min. In brief, the system injected 100 µL of each lignin solution and separated them into different molecular weights. The samples are then analyzed using a Wyatt Optilab T-Rex refractive index detector (dRI, USA), 785 nm at 35 °C.

The molecular weight of EMAL lignin isolated from softwood biomass (research chapter 5) was analyzed by GPC using an Agilent 1100 equipped with a Wyatt Optilab T-Rex differential refractive index detector (dRI, USA) and poly(styrenesulfonate) as standard and DMSO/LiBr

(0.5% w/v) as eluent at a flow rate of 0.5 ml/min. Prior to analysis, 10 mg of lignin was dissolved in 1 ml DMSO/BrLi (0.5% w/v), left at room temperature for 48 h and filtered through 0.45  $\mu$ m PTFE filters. The data were collected and analyzed using ASTRA 6.0 software.

## 2.13.5<sup>31</sup>P Nuclear magnetic resonance (<sup>31</sup>P NMR)

The types and amount of hydroxyl groups located on the EMAL lignin was analyzed by <sup>31</sup>P NMR using a Bruker Avance 300 MHz spectrometer. Chromium (III) acetylacetonate and N-hydroxy-5-norbornene-2,3-dicarboximide were selected as the respective relaxation reagent and internal standard and they were dissolved in pyridine/ CDCl<sub>3</sub> mixture (1.6:1, v/v) at concentrations of 5.6 and 9.0 mg/mL respectively. 20 mg of dried EMAL lignin was dissolved in 400  $\mu$ L of pyridine/ CDCl<sub>3</sub> mixture, where 100  $\mu$ L of internal standard solution, 40  $\mu$ L of relaxation reagent solution, and 50  $\mu$ L of 2-chloro- 4,4,5,5-tetramethyl-1,2,3-dioxaphospholane (TMDP) were added prior to <sup>31</sup>P NMR analysis. An inverse gated decoupling pulse was used with the following parameters; number of scans 800, relaxation delay 5 s, acquisition time 1.4 s, pulse length 6  $\mu$ s, and 90° pulse width.

# 2.13.6 Two-dimensional heteronuclear single-quantum correlation nuclear magnetic resonance (2D HSQC NMR)

2D HSQC NMR of lignin isolated from the deacetylation liquor (research chapter 3) was first dissolved in DMSO- $d_6$ , prior to 2D <sup>1</sup>H–<sup>13</sup>C HSQC NMR analysis, using a Bruker Avance III 400-MHz spectrometer. The structure of the EMAL isolated from softwood biomass (research chapter 5) was dissolved in DMSO- $d_6$  analyzed by 2D <sup>1</sup>H-<sup>13</sup>C HSQC NMR using a Bruker Avance III 600 MHz spectrometer. After the acquisition of the HSQC spectra, the relative abundance of the lignin compositional subunits and inter-unit linkages was assessed using volume integration of cross peak contours.

# **3.** Research chapter 1: the impacts of mechanical pulping/refining on substrate accessibility to cellulase enzymes

## 3.1 Introduction

As mentioned in previous sections, it has been shown that mechanical pulping/refining alone has only a limited impact on the enzymatic hydrolysis of biomass. This is primarily thought to be because lignin hinders the fibrillation of fibres. However, there is yet to be a study that demonstrated the impacts of excessively refining on different types of biomass. This would more fully illustrate the limitation of single-step mechanical refining as a front end for biochemicalbased biorefinery.

Therefore, the initial research pretreated various types of biomass using a mechanical refining/pulping process, with the refining intensities increased from a mild to an aggressive extent. As described earlier, disc plates that are used during the primary and secondary refining stages are typically constructed differently to serve the need of fibre separation and fibrillation, respectively. To mimic the industrial pulping process at a lab scale, mechanical attrition using a commercial twin gear juicer (super angel juicer model 8500) equipped with rotating gears followed by a PFI milling step were used to represent the primary and secondary refining steps. Mechanical refining using a twin-gear juicer had already been demonstrated in previous work (Chu et al., 2017a). With the purpose of producing mechanical pulps from various source of biomass, Douglas fir, Aspen and corn stover were selected as the representatives of softwood, hardwood and agriculture biomass. To evaluate the effects of mechanical refining on lignin-free substrate, a delignified pre-hydrolyzed Kraft pulp (PHK) was also selected and subject to PFI milling.

Fibrillation of fibres using PFI milling has been demonstrated by numerous studies, where enhanced fibre swelling and external fibrillation were observed for cellulosic substrates and chemically pretreated biomass (Fougere et al., 2015; Jones et al., 2013; Liu et al., 2016). However, very limited increase in fibrillation and enzymatic digestibility was observed with substrates contained high amounts of unmodified lignin (Hoeger et al., 2013). It could be possible that the limited impacts of mechanical pulping were in part due to insufficient refining intensities. Therefore, in this study we extended the refining intensities of PFI milling from zero to 5000, 15000 and 45000 revolutions. This was done prior to the estimation of the extent of pulp fibrillation and susceptibility to enzymatic hydrolysis. This was done to assess the effects of refining on substrate accessibilities from different levels of intensity. It was hypothesized that, even though the lignin was not removed or modified, excessive mechanical pulping might enhance substrate accessibility to enzymes, despite the origin of the biomass substrate.

During the mechanical pulping process, when fractures occur between the middle lamella and S1 layers of fibres, a high amount of fines will be found in the resulting pulp (Luukko and Maloney, 1999). The fines component has been shown to contain relatively high lignin content, likely due to the presence of fragments from cell wall corners that contain middle lamella lignin (Kangas and Kleen, 2004; Luukko and Maloney, 1999). It was anticipated that removing the fines component could increase cellulose hydrolysis of mechanical pulps by decreasing its overall lignin content. Therefore, another objective of this work was to isolate the fines-free fraction of the mechanical pulps and assess its susceptibility to enzymatic hydrolysis, in comparison to the original pulps.

### 3.2 Results and discussion

As mentioned earlier, the initial thesis work involved the production of a mechanical pulp derived from corn stover, Aspen and Douglas fir using a combination of commercial twin-gear juicer and PFI mill. Previous work had suggested that PFI milling for up to 4000 revolutions was sufficient to fibrillate pretreated biomass substrates (Chen et al., 2013). However, it was soon apparent this level of refining intensity likely did not result in enough fibrillation of fibres that allowed for ready hydrolysis of cellulose. As mentioned previously, the presence of lignin likely significantly hindered the fibrillation process (Hoeger et al., 2013). In the work reported here, we employed PFI milling for 5000, 15000 and 45000 revolutions on the primary refined mechanical pulps made from corn stover, Aspen and Douglas fir. The delignified PHK pulp was also subject to PFI milling at the same refining intensities to represent a biomass substrate from which the lignin had been largely removed.

The morphology of fibres in the pulp substrates were analyzed using a Fibre Quality Analyzer (FQA). Although some studies have considered mechanical pulping as a size-reduction method, it should be noted that it is actually a fibre development process, with the target of retaining long and fibrillated fibres that contribute to the high strength of the resulting pulp. Therefore, fibre-cutting is avoided during the typical mechanical pulping process. This was confirmed in the

study, as FQA analysis indicated the fibre length and fines content of all substrates remained almost the same after PFI milling (Table 1). It was apparent that PFI milling led to the straightening of the fibres as indicated by decreased curl and kink indices (Table 1). Although the fibrillation of fibres could not be clearly captured by FQA analysis, the mean width of the fibres from all of the pulp substrates decreased after PFI milling (Table 1). This was likely because the physical attrition peeled of the surface component of fibre, leading to external fibrillation.

**Table 1.** Fibre morphology of mechanical pulps derived from agriculture residue (Corn stover), hardwood (Aspen) and softwood (Douglas fir) and delignified pre-hydrolyzed kraft (PHK) pulp after PFI milling for 0 - 45000 revolutions.

| Sample          | PFI refining<br>intensity<br>(revolutions) | LW mean<br>Fibre length<br>(mm) | Fines<br>content<br>(%) | Curl<br>index | Kink<br>index | Mean width<br>(um) |
|-----------------|--|---------------------------------|-------------------------|---------------|---------------|--------------------|
|                 | 0  | 0.44                            | 70                      | 0.2           | 2             | 27.2               |
| Com stover      | 5000                                       | 0.5                             | 67.9                    | 0.2           | 1.6           | 25.6               |
| mechanical pulp | 15000                                      | 0.51                            | 67.9                    | 0.2           | 1.6           | 25.8               |
|                 | 45000                                      | 0.5                             | 68.6                    | 0.1           | 1.3           | 24.5               |
|                 | 0  | 0.43                            | 73.9                    | 0.2           | 1.7           | 31.9               |
| Aspen           | 5000                                       | 0.47                            | 79                      | 0.1           | 1.5           | 29.4               |
| mechanical pulp | 15000                                      | 0.48                            | 70.3                    | 0.1           | 1.4           | 30                 |
|                 | 45000                                      | 0.48                            | 70.9                    | 0.1           | 1.2           | 29.5               |
|                 | 0  | 0.51                            | 70.3                    | 0.2           | 1.5           | 35.4               |
| Douglos fin     | 5000                                       | 0.54                            | 66.1                    | 0.1           | 1.1           | 33.4               |
| mechanical pulp | 15000                                      | 0.53                            | 67.2                    | 0.1           | 0.9           | 32.9               |
|                 | 45000                                      | 0.59                            | 66.8                    | 0.1           | 1.1           | 31.5               |
|                 | 0  | 0.65                            | 23.5                    | 0.1           | 1.8           | 16.3               |
| Delignified PHK | 5000                                       | 0.62                            | 25.2                    | 0.1           | 2.4           | 15.9               |
| Pulp            | 15000                                      | 0.64                            | 27.2                    | 0.1           | 2             | 15.8               |
|                 | 45000                                      | 0.58                            | 35.8                    | 0.2           | 2.3           | 15.7               |

The Water Retention Value (WRV) of the pulp substrates were measured with the goal of estimating the extent of fibrillation. The WRV was a method developed by the pulp and paper industry and has been widely used to measure the internal delamination of fibres and estimate the swelling ability of pulps. It was apparent that the WRV of all substrates increased with PFI milling, with PHK pulps showing the greatest improvement. This was likely a result of the lignin hindering the delamination of fibres (Table 2). To better demonstrate the external fibrillation of fibres after PFI milling. Scanning Electron Microscopy (SEM) was conducted on the delignified-PHK pulp, agricultural residue and softwood biomass, before and after PFI milling process. It was apparent that the surface of PHK pulp was much more fibrillated after mechanical pulping (Figure 6a, 6d), as compared to the mechanical pulp substrates made from biomass (Figure 6b, 6c, 6e, 6f). After primary refining and PFI milling for 45000 revolutions, the majority of the fibre surface from agricultural residue and softwood biomass was still poorly fibrillated (Figure 6e, 6f).

| Sample               | PFI refining<br>intensity<br>(revolutions) | WRV          | Direct orange<br>(DO) dye<br>adsorption (mg/g<br>dry substrate) |
|----------------------|--|--------------|---|
|                      | 0  | $2.0\pm0.02$ | 71.8  |
| Corn stover          | 5000                                       | $2.2\pm0.02$ | 73.2  |
| mechanical pulp      | 15000                                      | $2.2\pm0.05$ | 74.0  |
|                      | 45000                                      | $2.3\pm0.13$ | 73.8  |
|                      | 0  | $1.6\pm0.06$ | 72.6  |
| Aspen                | 5000                                       | $1.6\pm0.04$ | 70.0  |
| mechanical pulp      | 15000                                      | $1.6\pm0.03$ | 71.5  |
|                      | 45000                                      | $1.8\pm0.01$ | 73.9  |
|                      | 0  | $1.2\pm0.05$ | 70.0  |
| Douglas fir          | 5000                                       | $1.4\pm0.08$ | 70.1  |
| mechanical pulp      | 15000                                      | $1.4\pm0.05$ | 68.1  |
|                      | 45000                                      | $1.7\pm0.04$ | 74.6  |
|                      | 0  | $2.1\pm0.05$ | 102.8   |
| Delignified DIW auto | 5000                                       | $2.2\pm0.02$ | 109.4   |
| Denginned PHK pulp   | 15000                                      | $2.4\pm0.06$ | 97.7  |
|                      | 45000                                      | $2.7\pm0.03$ | 101.2   |

*Table 1.* Accessibility to water (WRV) and direct orange dye (Simons' staining) of mechanical pulps and delignified PHK pulp and after PFI milling for 0 - 45000 revolutions.



**Figure 6**. Scanning electron microscope (SEM) images of delignified PHK pulp (a, d), corn stover (b, e) and Douglas fir (c, f) before (a, b, c) and after (d, e, f) PFI milling for 45000 revolutions. The scale bar in indicated  $4\mu m$ .

The pulp substrates were subsequently assessed for their susceptibility to enzymatic hydrolysis. The accessibility of enzymes to the cellulose component was measured by the modified Simons' staining technique. This approach used the high-molecular-weight fraction of direct orange (DO) dye as the probe. The dye has been shown to have a size similar to cellulase enzymes and it has a high affinity for cellulose (Chandra and Saddler, 2012). In this study, Simons' staining indicated that the accessibility of mechanical pulps remained unaffected after various stages of PFI milling, despite the observed increases in their WRV. It was likely that the PFI milling generated cellulose that was more accessible to water molecules. However, this accessibility was not enough to allow the penetration of the DO dye or cellulase enzymes whose sizes are much bigger than water molecules. Interestingly, the adsorption of PHK pulp to DO dye also remained relatively unchanged after PFI milling post-treatments, although its initial adsorption was much higher than that of the mechanical pulps (Table 2). As it was likely that the cellulose present in the PHK pulp was already highly accessible, the Simons' staining method was likely not able to capture the increase in cellulose accessibility when it was further increased.

When the mechanical pulp substrates were hydrolyzed using high loadings of the cellulase enzyme mixture (60mg Celluclast and 30mg Novozyme 188/g cellulose), it was apparent that the cellulose component remained highly recalcitrant, as only 10-30% of cellulose was hydrolyzed (Figure 7). Among the mechanical pulp substrates, the softwood-derived pulp was the most recalcitrant, whereas the pulp made from agricultural biomass was better hydrolyzed (Figure 7). It is likely that the recalcitrance of the mechanical pulps was due to their lignin content, as shown in previous work. In contrast, the cellulose component from the PHK pulp was much more susceptible to enzymatic hydrolysis, as up to 80% of the cellulose could be hydrolyzed using low enzyme loadings (5mg of Celluclast and 2.5mg of Novozyme 188/g cellulose). The hydrolysis yield could be improved to greater than 90% and plateaued, after PFI milling for 5000 revolutions (Figure 8). The hydrolysis yields of pulp substrates correlated well with the cellulose accessibility of pulps estimated by the Simons' staining technique (Figure 7, Figure 8, Table 2). This suggested that, although secondary refining was able to enhance the internal delamination of fibres present in mechanical pulps, it was not able to enhance cellulose accessibility to the enzymes. With the absence of lignin, the PHK pulp became more susceptible to enzymatic hydrolysis after PFI milling. However, this was not a significant change as the pulp could be readily hydrolyzed even prior to mechanical post-treatment.

As described earlier, lignin has been shown to impede cellulose hydrolysis through physical blocking effect and adsorbing enzymes that forms non-productive binding. As the mechanical refining process did not involve any chemical or high-temperature treatment, the lignin present in mechanical pulps likely retained its native structure and had a relatively lower tendency to adsorb enzymes compared to more condensed lignin from typical pretreated biomass (e.g., steam pretreated biomass). Therefore, we hypothesized that the lignin present in mechanical pulps hinders the enzymatic hydrolysis of cellulose mostly through physical blocking, restricting the accessibility of enzymes to cellulose. This was confirmed by the enzymatic hydrolysis of mechanical pulps after incubation with Bovine Serum Albumin (BSA). Previous studies have shown that incubation of pretreated substrates with BSA prior to enzymatic hydrolysis could minimize the non-productive binding between lignin and enzymes with BSA blocking the binding sites on lignin via hydrophobic interaction(Lin, 2016). However, in this work, the incubation of mechanical pulps with BSA only resulted in small increases in cellulose hydrolysis. This suggested that, even in the absence of non-productive binding, the enzymes still could not effectively hydrolyze the cellulose, likely due to a lack of accessibility (Figure 8).



**Figure 7.** Enzymatic hydrolysis yields of mechanical pulps derived from agriculture residue, hardwood and softwood. Hydrolysis was conducted at 2% (w/v) solids and protein loading of 60mg Celluclast and 30mg Novozyme 188 ( $\beta$ -glucosidase) /g cellulose with and without the pre-impregnation of bovine serum albumin (BSA) at 50 °C for 72 hours.



*Figure 8.* Enzymatic hydrolysis yields of delignified PHK pulps after PFI milling with various intensities. Hydrolysis was conducted at 2% (w/v) solids and enzyme loading of 5mg Celluclast and 2.5mg Novozyme 188 /g cellulose at 50 °C for 6, 24, 48 and 72 hours.

As it was likely that the lignin present in mechanical pulps hindered cellulose hydrolysis, we next attempted to reduce the overall lignin content of mechanical by removing the fines component. Past studies had indicated that the fines contained a higher lignin content compared to the rest of the fibres. Using a Bauer-Mcnett fibre classifier equipped with a 100-mesh screen, we fractionated the fines-free content of primary and secondary refined mechanical pulps. FQA analysis of the resulting pulps indicated that the majority of the fines were removed after the fractionation, leaving the long fibres that contained lower lignin content (Table 3, Table 4).

*Table 3.* Fibre morphology of primary (using twin-gear juicer) and secondary refined (using PFI mill) mechanical pulps after fines removal.

| Sample      | Revolutions<br>Sample of PFI<br>milling |      | Fines<br>content<br>(%) | Curl<br>index | Kink<br>index | Mean<br>width<br>(µm) |
|-------------|---|------|-------------------------|---------------|---------------|-----------------------|
| Corn stover | 0                                       | 0.70 | 5.8                     | 0.2           | 2.0           | 28.8                  |
|             | 45000                                   | 0.82 | 6.7                     | 0.2           | 1.7           | 26.4                  |
| Aspen       | 0                                       | 0.75 | 2.1                     | 0.2           | 1.9           | 33.1                  |
|             | 45000                                   | 0.77 | 2.2                     | 0.1           | 1.4           | 31.7                  |
| Douglas fir | 0                                       | 0.83 | 4.2                     | 0.2           | 1.3           | 36.6                  |
|             | 45000                                   | 0.88 | 4.3                     | 0.1           | 0.9           | 31.4                  |

Table 4. Chemical composition of mechanical pulps before and after fines removal.

|  |             | Be               | efore fines remov    | al            | After fines removal |                      |               |  |
|--|-------------|------------------|----------------------|---------------|---------------------|----------------------|---------------|--|
|  | Sample      | Cellulose<br>(%) | Hemicellulose<br>(%) | Lignin<br>(%) | Cellulose<br>(%)    | Hemicellulose<br>(%) | Lignin<br>(%) |  |
|  | Corn stover | 42.3             | 28.2                 | 24.6          | 45.9                | 28.8                 | 17.9          |  |
|  | Aspen       | 50.9             | 19.8                 | 22.8          | 51.3                | 20.0                 | 19.8          |  |
|  | Douglas fir | 48.0             | 20.2                 | 29.0          | 50.3                | 20.7                 | 23.9          |  |

Surprisingly, the cellulose present in fractionated mechanical pulps were less accessible to enzymes and more recalcitrant to enzymatic hydrolysis compared to the original pulps (Figure 9), despite the fact that they had lower lignin content (Table 4). It is likely that the smaller particle sizes of fines contributed to their higher cellulose accessibility, despite the high lignin content. Earlier study suggested that the fines present in mechanical pulp could be divided into fibrillar fines and flake-like fines (Kangas and Kleen, 2004; Luukko and Maloney, 1999). Flake-like fines are mainly composed of lignin-rich particles derived from the cell wall corner. In contrast, fibrillar fines normally contain higher cellulose content as they are generated by the peeling of fiber surface (Kangas and Kleen, 2004). It is likely that the highly accessible cellulose present in the fibrillar fines contributed to the higher hydrolysis yields of the fines that were removed in this study. Similar findings were reported in a recent study (Corbett et al., 2020), where fines fractions isolated from mechanical pulps with different refining intensities were all shown to be more susceptible to enzymatic hydrolysis compared to long fibres (Corbett et al., 2020).



**Figure 9.** Cellulose hydrolysis yields and adsorption to direct orange (DO) dye (Simons' staining) of primary and secondary refined mechanical pulps before and after the removal of fines. Hydrolysis was conducted at 2% (w/v) solid and enzyme loading of 60mg Celluclast and 30mg Novozyme 188 /g cellulose at 50 °C for 72 hours.

## 3.3 Conclusions

As a result of the presence of lignin, mechanical pulping only resulted in a limited impact on the susceptibility of the cellulose in TMPs to enzymatic hydrolysis. Although mechanical pulping facilitated internal delamination of fibres, it was not able to significantly increase the accessibility of cellulose to enzymes. The external fibrillation of fibres was also limited with lignin covering the surface. Removing the fines faction decreased the overall lignin content. However, it was also likely removing the more accessible cellulose present in the small cell wall particles, leaving longer fibres that are more recalcitrant to enzymatic hydrolysis.

## 4. Research chapter 2: enhancing enzyme-mediated cellulose hydrolysis by incorporating acid groups onto the lignin during biomass pretreatment

## 4.1 Introduction

Lignin has proven to be a significant obstacle when trying to carry out effective enzyme-mediated hydrolysis of lignocellulosic substrates. Past work has shown that this lignin inhibits hydrolysis by the two major mechanisms of limiting substrate swelling, consequently restricting cellulose accessibility, and binding to the cellulolytic enzymes and limiting their activities (del Rio et al., 2011; Kumar et al., 2012a; Nakagame et al., 2010; Rahikainen et al., 2013). Although lignin removal has been shown to be an effective way to enhance enzyme-mediated cellulose hydrolysis (Mooney et al., 1998), to date, no cost-effective delignification-based methods have been commercialized, primarily due to the high cost associated with the use of chemicals (Takada et al., 2020). As a result, a considerable amount of work has focused on trying to mitigate lignin's inhibitory effect by modifying it. These include using relatively mild reaction conditions that incorporate acid groups onto the lignin macromolecule, consequently enhancing cellulose hydrolysis without the need for complete delignification (Berlin et al., 2006; del Rio et al., 2011; Eriksson et al., 2002; Nakagame et al., 2011b).

Oxidation and sulfonation are the most common methods used as this modifies the lignin structure and charge by the incorporation of acid groups. Both approaches are widely used by the pulp and paper sector. Alkaline oxidation is predominantly used to remove residual lignin from unbleached Kraft pulp (Fengel and Wegener, 1989; Kalliola et al., 2011) and sulfonation predominantly used to soften the lignin prior to mechanically pulping wood chips (Börås and Gatenholm, 1999). More recently, oxidation and sulfonation treatments, which result in the incorporation of carboxylic and sulfonic acid groups respectively, have been incorporated into pretreatment methods to enhance accessibility to the cellulose while retaining the hemicellulose and lignin components (Chandra et al., 2016; Chu et al., 2017a). For example, incorporating acid groups onto the lignin has been shown to both enhance fibre swelling, consequently increasing enzyme accessibility to the cellulose (del Rio et al., 2011), as well as reducing lignin-enzyme interaction resulting from hydrophobic (Eriksson et al., 2002), ionic(Berlin et al., 2006) and hydrogen bond interactions (Pan, 2008; Sewalt et al., 1997). As a result of these treatments the lignin also becomes more hydrophilic, reducing the tendency for it to hydrophobically bind to enzymes (Nakagame et al., 2011b, 2011a). The deprotonation of the acid groups generates a negative charge, facilitating the electrostatic repulsion between the negatively charged lignin and cellulase enzymes (del Rio et al., 2011; Nakagame et al., 2011a, 2011b).

Despite these insights the benefits of acid group integration on biomass deconstruction have yet to be fully resolved. For example, how the structure and characteristic of the sulfonic (strong acid) and carboxylic (weak acid) acids influences cellulose hydrolysis (Ben et al., 1993; Fengel and Wegener, 1989; Yang et al., 2003). At the same time, raising the pH level might have the potential to further ionize the acid groups attached to the lignin. As this has been shown to increase the hydrophilicity and negative charge, it might also facilitate the repulsion between lignin and the negatively charged cellulases (Lan et al., 2013; Lou et al., 2013).

In the work reported in this chapter, similar amounts of carboxylic and sulfonic acid groups were incorporated onto the lignin present in pretreated mechanical pulps. This research helped better elucidate the beneficial effect of sulfonic and carboxylic acid group addition to lignin in terms of enzyme accessibility/deconstruction to/off cellulose. Both the oxidized and sulfonated lignin samples were shown to be less inhibitory to cellulose hydrolysis and also adsorbed less enzymes. The sulfonated and oxidized lignin contained more acid groups and aliphatic hydroxyl groups and less phenolic hydroxyl groups. This enhanced lignin hydrophilicity and increased the negative charge, decreasing the non-productive binding of cellulases to lignin.

## 4.2 Results and discussion

As we anticipated that the presence of hemicellulose and the inherent acid groups (e.g., cinnamic acids and acetyl groups) associated with the biomass might also influence substrate swelling (Ju et al., 2013b; Sjöström et al., 1965) and cellulose hydrolysis, a pretreatment step was first carried out at 170 °C, to primarily solubilize the hemicellulose while minimizing lignin condensation (Garrote et al., 2001; Li et al., 2010). The pretreated Aspen slurry was subsequently pulped using a twin-gear juicer to simulate mechanical pulping. As initial conductometric titration had indicated that the pretreated mechanical pulp (MP) still contained some weak acids (data not shown), the pulp was subsequently acid washed to remove any residual uronic acid groups that were potentially located on the residual hemicellulose (Meng et al., 2015). After this series of treatments, the pulps contained less than 10% hemicellulose and any associated acid groups were below detectable levels (Table 5). The subsequent alkaline oxidation and neutral sulfonation reactions of acid group-free pulp was carried out using sodium percarbonate (Na<sub>2</sub>CO<sub>3</sub>·1.5 H<sub>2</sub>O<sub>2</sub>) and sodium sulfite (Na<sub>2</sub>SO<sub>3</sub>) respectively. Alkaline oxidation and neutral sulfonation had

previously been shown to enhance the enzymatic hydrolysis of pretreated pulps (Kumar et al., 2011; Yang et al., 2002b). As we hoped to modify the lignin rather than remove it, the reaction was carried out at 70 °C to minimize the solubilizing of the more hydrophilic oxidized/sulfonated lignin. As summarized in Table 5, a chemical loading of 16% sodium sulfite and 32% sodium percarbonate resulted in the incorporation of similar amounts of sulfonic and carboxylic acid groups (60 mmol/kg) while recovering more than 90% of the original water-insoluble component (Table 5). As described earlier (Sjöström et al., 1965), sulfonation also increased the exposure of carboxylic acid groups, likely due to the cleavage of ester linkages between lignin and hemicellulose.

|               |                           | Chemical composition |              | Substrate characteristics |                                      |  |                                   |   |
|---------------|---------------------------|----------------------|--------------|---------------------------|--------------------------------------|--|-----------------------------------|---|
| Sample        | Pretreatment<br>yield (%) | Glucan<br>(%)        | Xylan<br>(%) | Lignin<br>(%)             | Sulfonic<br>acid groups<br>(mmol/kg) | Carboxylic<br>acid groups<br>(mmol/kg) | Total acid<br>groups<br>(mmol/kg) | DO dye<br>adsorption<br>(mg/g dry<br>substrate) |
| Unmodified MP | 100.0                     | $63 \pm 1$           | $7\pm0$      | $30\pm0$                  | $0\pm 0$                             | $0\pm 0$                               | $0\pm 0$                          | 84  |
| Sulfonated MP | 93.0                      | $67 \pm 2$           | $4 \pm 1$    | $29\pm0$                  | $44\pm 6$                            | $20\pm5$                               | $64 \pm 3$                        | 93  |
| Oxidized MP   | 91.6                      | $67\pm0$             | $5\pm0$      | $29\pm0$                  | $0\pm 0$                             | $59 \pm 1$                             | $59\pm1$                          | 95  |

*Table 5.* Chemical composition and characteristics of unmodified, sulfonated and oxidized mechanical pulps (MP) derived from pretreated Aspen chips.

Earlier work had shown that acid groups addition to the lignin enhanced substrate swelling, resulting in both increased enzyme accessibility and hydrolysis of the cellulose(del Rio et al., 2011). The Simons' staining method has been successfully used (Stone et al., 1969; Yu and Atalla, 1998) to predict the cellulose accessibility to cellulase enzymes (Chandra et al., 2009) by measuring substrate's adsorption to DO dye. When this method was used it was apparent that sulfonation and oxidation both increased the accessibility of all three pretreated mechanical pulps (Table 5). This was likely due to enhanced substrate swelling resulting from lignin modification. It was also apparent that the sulfonation and oxidation treatments enhanced enzyme-mediated cellulose hydrolysis (Figure 10) with the oxidized substrate being more susceptible, despite the fact that both pulps had similar amounts of lignin and acid groups (Table 5). However, as has been suggested previously (Sjöström et al., 1965), it is likely that the majority of the carboxylic acid groups present in the sulfonated substrate were located on the hemicellulose component, while the carboxylic acid groups associated with the oxidized substrate were associated more with the lignin.



*Figure 10.* Extent of enzyme-mediated hydrolysis of the cellulose present in unmodified, sulfonated and oxidized mechanical pulps (MP). Hydrolysis was conducted at 2% (w/v) solids and enzyme loading of 20mg/g cellulose at 50 °C for 6, 24 and 48 hours.



*Figure 11.* Enzymatic hydrolysis of a cellulose-rich, delignified Kraft pulp at pH 4.8 and 6, and an enzyme loading of 2 and 5mg/g cellulose. Enzymatic hydrolysis was performed at 2% (w/v) solids at 50 °C for 48 hours.

Previous work had suggested that hydrolysis could be enhanced at an elevated pH by further deprotonating the acid groups and consequently increasing the lignin's hydrophilicity and negative charge (Lan et al., 2013; Lou et al., 2013). However, the hydrolysis of both the control and modified substrates was only slightly enhanced when it was carried out at pH 6 (Figure 10). To ensure that activity of the cellulases was not compromised by the use of an elevated pH, a cellulose-rich delignified Kraft pulp was hydrolyzed at both pH 4.8 and pH 6 (Figure 11). As observed previously, it was apparent that slightly better hydrolysis was achieved at pH 4.8 (Ayyachamy et al., 2013; Reese and Mandels, 1980).

As earlier work had shown that lignin inhibits enzyme-mediated hydrolysis of cellulose by both limiting the swelling of the substrate and binding with the enzymes, we next tried to differentiate how sulfonation and oxidation might influence these two mechanisms. As it is difficult to extract lignin from biomass substrates without modifying it, Protease Treated Lignin (PTL) has often been used to represent the lignin present in pretreated biomass (Nakagame et al., 2011a; Yuan et al., 2018). This method used a cellulase cocktail to hydrolyse the carbohydrate components to produce a lignin-rich residue which is subsequently treated with protease to remove the enzymes. Despite the removal of much of the carbohydrate by the enzyme treatment, it should be noted that some residual material was still associated with the lignin (Table 6). When the modified PTLs were add to the hydrolysis of a cellulose-rich Kraft pulp (at 1:1 ratio), at both pH 4.8 and 6 (Figure 12), it was apparent that the modified PTLs were both less inhibitory with better cellulose hydrolysis achieved at pH 6, as indicated by around 10% increases in hydrolysis yields at elevated pH and enzyme loading of 5mg/ g cellulose. When the adsorption isotherms of the cellulases enzyme in the presence of the PTLs were measured at pH 4.8 and pH 6, it was apparent that sulfonation and oxidation both decreased the extent of adsorption between the enzymes and the modified lignin (Table 6). Although somewhat unexpected, it is likely that the observed increase in hydrolysis observed at the higher pH was due to the decreased non-productive binding of the enzymes to the lignin.


*Figure 12.* Enzymatic hydrolysis of cellulose-rich delignified Kraft pulp with added protease treated lignin (PTL) isolated from unmodified, sulfonated and oxidized mechanical pulps (MP). Enzymatic hydrolysis was performed at 2% (w/v) solids and enzyme loading of 2 and 5mg/g cellulose at 50 °C for 48 hours.

**Table 6.** Chemical composition, potential adsorption of cellulases, hydrophobicity (assessed by Rose Bengal adsorption) and negative charge (assessed by Zeta Potential) of lignins isolated from unmodified, sulfonated and oxidized mechanical pulps (MP).

| Comple           | Chemical composition |               | Binding strength | Binding strength         | Hydrophobicity | Zeta      | Zeta     |  |
|------------------|----------------------|---------------|------------------|--------------------------|----------------|-----------|----------|--|
| Sample           | Glucan<br>(%)        | Lignin<br>(%) | (mL/g lignin)    | at pH o<br>(mL/g lignin) | (Ĺ/g)          | at pH 4.8 | at pH 6  |  |
| Unmodified<br>MP | $30\pm 2$            | $70 \pm 1$    | 95.2             | 87.0                     | 1.14           | $6\pm0$   | $0\pm 1$ |  |
| Sulfonated<br>MP | $28\pm1$             | $74\pm2$      | 80.6             | 54.9                     | 0.80           | -6 ± 1    | -8 ± 1   |  |
| Oxidized<br>MP   | $29\pm1$             | $72\pm2$      | 74.1             | 47.6                     | 0.46           | $0\pm 1$  | -6 ± 3   |  |

As acid groups addition is known to influence both the hydrophilicity and negative charge of the lignin, consequently decreasing enzyme binding (Nakagame et al., 2011a), we next wanted to confirm these earlier observations using the Rose Bengal method and Zeta potential measurements (Huang et al., 2017; Nakagame et al., 2011a; Y. Song et al., 2019). It was apparent that sulfonic and carboxylic acid groups addition to the lignin significantly enhanced its hydrophilicity and negative charge (Table 6) with the decrease in the Zeta potential of the oxidized PTL at pH 6 indicating more carboxylic acid group disassociation.

As it was possible that the residual cellulose associated with each of the PTL samples might influence lignin analysis and its structure, we also used the Enzymatic Mild Acidolysis Lignin (EMAL) method to isolate lignin at relatively high yields without changing its structure and properties(Guerra et al., 2006). Subsequent Gel Permeation Chromatography (GPC) analysis of the EMAL samples indicated that both sulfonation and oxidation resulted in a slight increase in the molecular weight of the lignin components, suggesting that the lignin underwent mild repolymerization reaction under alkali conditions (Das et al., 2019) (Table 7). Previous work has used <sup>31</sup>P Nuclear Magnetic Resonance (NMR) analysis to successfully quantify the amount and type of hydroxyl groups (-OH) located on the aromatic and aliphatic structures of lignin(Pu et al., 2011a). As previous studies had indicated that, during the pretreatment of hardwood biomass, syringyl lignin is much easier to remove than Guaiacyl lignin, (Mi Li et al., 2016; Yu et al., 2014), it was likely that a small amount of syringyl subunit lignin was removed during oxidation and sulfonation (Table 7). Interestingly, both sulfonation and oxidation resulted in a slight increase in the aliphatic hydroxyl group content of the lignin. This was probably due to the cleavage of the ester groups connecting the lignin aliphatic chain and carbohydrates during the sulfonation and oxidation of lignin (Crestini and Argyropoulos, 1997). The observed increase in the carboxylic acid groups attached to the EMAL lignin further confirmed that lignin oxidation had occurred (Table 7). When the observed changes in the hydroxyl group content of the lignin was compared to the extent of cellulose hydrolysis, it was apparent that those substrates whose lignin contained more aliphatic hydroxyl groups and less phenolic hydroxyl groups were more easily hydrolyzed (Figure 10, Figure 12, and Table 7). This was consistent with previous studies which had suggested that the aliphatic hydroxyl groups in the lignin made it more hydrophilic, thereby reducing enzyme binding. In contrast, a greater amount of phenolic hydroxyl groups appeared to enhance enzyme binding (Mi Li et al., 2016; Yang and Pan, 2016).

|                  | Molecular Weight |             |      |                  | Hydroxyl groups as determined by <sup>31</sup> P NMR (mmol/g) |                             |                                     |                           |                             |  |  |  |
|------------------|------------------|-------------|------|------------------|---|-----------------------------|-------------------------------------|---------------------------|-----------------------------|--|--|--|
| Sample           | Mn<br>(kDa)      | Mw<br>(kDa) | PDI  | Aliphatic<br>-OH | Syringyl<br>phenolic<br>-OH                                   | Guaiacyl<br>phenolic<br>-OH | p-Hydroxy<br>phenyl<br>Phenolic -OH | Total<br>phenolic -<br>OH | Carboxylic<br>acid<br>-COOH |  |  |  |
| Unmodified<br>MP | 2.3              | 47.5        | 20.9 | 4.19             | 1.11  | 0.61                        | 0.40                                | 2.12                      | 0.10                        |  |  |  |
| Sulfonated<br>MP | 3.1              | 60.6        | 19.4 | 4.35             | 0.94  | 0.50                        | 0.39                                | 1.82                      | 0.05                        |  |  |  |
| Oxidized<br>MP   | 1.4              | 53.5        | 38.0 | 4.53             | 0.84  | 0.59                        | 0.37                                | 1.80                      | 0.19                        |  |  |  |

**Table 7.** Molecular weight and hydroxyl group content of the lignin extracted from unmodified,sulfonated and oxidized pre-hydrolyzed mechanical pulps (MP).

## 4.3 Conclusions

Lignin is known to inhibit effective enzyme-mediated hydrolysis of biomass by both restricting substrate swelling and by binding with cellulase enzymes. Both enzyme accessibility and cellulose hydrolysis could be enhanced by the addition of sulfonic and carboxylic acid groups onto the lignin present in biomass substrates. It was apparent that both oxidation and sulfonation decreased the extent of enzyme binding with the reduction in the number of phenolic hydroxyl groups as well as the incorporation of acid groups enhancing lignin hydrophilicity, consequently enhancing cellulose hydrolysis

# 5. Research chapter 3: enhancing enzymatic hydrolysis of agricultural biomass-derived mechanical pulps by deacetylation and delignification

### 5.1 Introduction

One of the main goals of the pretreatment step in a biochemical-based biorefinery process is to recover as much of the cellulose and hemicellulose as possible while increasing the accessibility of these carbohydrates to enzymes (Chandra et al., 2007). Although pretreatments using dilute acid and steam have been shown to solubilize the hemicellulose component, consequently increasing accessibility to the cellulose component (Linde et al., 2008; Saha et al., 2005), these methods typically produce inhibitors such as acetic acid, phenols, furfural and hydroxymethylfurfural (HMF) that result from the degradation of hemicellulose and lignin (Palmqvist et al., 1996). Acidic pretreatments have also been shown to condense the lignin, decreasing substrate swelling and enzyme accessibility to the cellulose component and contributing to the non-productive binding of cellulases to the lignin (Chandra et al., 2012a). The condensation of lignin also decreased its potential utility as a value-added co-product.

We and other groups have assessed alkaline pretreatments such as sodium hydroxide, alkalineoxygen, sulfite and Ammonia Fibre/Freeze Expansion (AFEX) as one way of retaining all of the biomass components in a "single-pot", while modifying the lignin and/or hemicellulose to enhance enzyme accessibility to the cellulose (Chu et al., 2017a). As described earlier, mechanical pulping is a commercial process that results in high yield pulps that are primarily used to produce newsprint (Unece & Fao, 2006). However, as the newsprint market has declined, primarily due to the growth of digital media (Unece & Fao, 2006), mechanical pulping has been more recently assessed as a potential front-end/pretreatment step for enzyme mediated bioconversion processes (Chen et al., 2014; J. Y. Zhu et al., 2009). The earlier work and the results from Chapter 1 showed that, although mechanical refining did increase the external surface area of the fibres, both the lignin and hemicellulose retention properties of mechanical pulping appeared to limit enzyme accessibility to the cellulose (Kumar et al., 2012a; Selig et al., 2009). Other recent work has shown that mild-alkali treatment (4.8% NaOH) resulted in the deacetylation of mechanically refined corn stover hemicellulose(Chen et al., 2012a), enhancing enzyme accessibility to the cellulose and xylan while decreasing potential inhibitors due to the prior removal of most of the liberated acetic acid (Mitchell et al., 1990; Selig et al., 2009).

As well as deacetylating the hemicellulose (Kong et al., 1992), NaOH addition has also been shown to readily ionize phenols and dissolve low molecular weight lignins (Chen et al., 2012a; de Groot et al., 2009; Karp et al., 2014). As the lignin in agricultural residues is also rich in hydrophilic coumaric and ferulic acid subunits, they are likely to be more susceptible to dissolution after NaOH treatment at mild conditions (Grabber et al., 2008; Min et al., 2014). Earlier work has shown that close to 20% of the lignin could be removed after treatment at these conditions (Chen et al., 2012a).

In the work reported here both lignin removal and modification as well as hemicellulose deacetylation influenced the effectiveness of enzyme mediated hydrolysis of cellulose. As previous work had shown that sulfite addition under alkaline conditions improved hydrolysis by enhancing fibre swelling and reducing non-productive lignin binding (Chandra et al., 2016; Kumar et al., 2012a), its addition was shown to increase lignin removal and hemicellulose deacetylation, enhancing both xylan and cellulose hydrolysis.

#### 5.2 Results and discussion

Previous work has shown that deacetylation enhances enzyme-mediated hydrolysis of biomass (Chen et al., 2012a; Garrote et al., 2001; Kong et al., 1992) with the alkali concentration, residence time and temperature all influencing the extent of deacetylation. At room temperature, longer residence times (24 hours) and higher alkaline concentrations (0.1 - 0.2 M) have been used to deacetylate agricultural and hardwood substrates (Kong et al., 1992) while at higher temperatures (70-100  $^{\circ}$ C), lower alkali charges(< 0.01 M) and shorter residence times have been successfully used (Zanuttini and Marzocchi, 1997). As the temperature is increased, up to 20% of the lignin can be removed (Chen et al., 2012a), likely due to both the hydrolysis of LCC ester linkages (Giummarella et al., 2019) and the ionization of the phenolic and carboxylic functionalities in lower molecular weight lignin fragments. This process has some similarity to the alkaline extraction step employed during pulp bleaching (Biermann, 1996b; Fengel and Wegener, 1989; Sun and Tomkinson, 2002). As mentioned earlier, lignin has been shown to impede cellulose hydrolysis by restricting substrate swelling and non-productively binding cellulase enzymes (dos Santos et al., 2019; Li et al., 2018; Rahikainen et al., 2013). As previous work had shown that the removal of even a small amount lignin from the pretreated biomass could significantly enhance enzymatic hydrolysis as well as decrease the non-productive binding of cellulases (Pan et al., 2005; Tu et al., 2007), the primary goal of this chapter was to better

elucidate the relative contribution of deacetylation and delignification to enhancing the enzymatic hydrolysis of corn stover.

As NaOH-based deacetylation had been successfully used by Chen and others (Chen et al., 2016, 2012a, 2012b), we first wanted to compare this method to a milder, KOH treatment at room temperature, that had previously been shown to selectively remove acetyl groups (Jiang and Xu, 2016; Kong et al., 1992). Controls, in the absence of added alkali, were carried out at both 80 °C and at room temperature. Likely due to the labile nature of the ester linkages within the hemicelluloses the control at 80 °C resulted in the removal of about 30% of the acetyl groups, the solubilization of around 25% of the carbohydrates and about 10% of the lignin (Table 8). In contrast, the room temperature control retained the majority of the carbohydrates and lignin within the water-insoluble fraction (Table 8). It is probable that the hot water (80 °C) control was acting as a milder version of "auto-hydrolysis", where the initial removal of acetyl groups was facilitated, consequently releasing protons(Li et al., 2010; Tunc and Van Heiningen, 2008) which resulted in mild acidolysis and the dissolution of a small amount of lignin (Fengel and Wegener, 1989).

It was apparent that the addition of either the NaOH or KOH enhanced deacetylation, as 80% of the acetyl groups were removed from the corn stover regardless of whether the reaction was performed at 80 °C or at room temperature (Table 8). However, the NaOH treatment at 80 °C for 3 hours resulted in the removal of more lignin (>25%) as compared to the KOH 24-h treatment at room temperature (Table 8). As the two alkaline treatments resulted in similar amounts of deacetylation, but differed in the extent of lignin removal, these treatments were next compared to try to better elucidate the relative influence of deacetylation and delignification on the enzymatic hydrolysis of pretreated corn stover.

| Treatments                         | Cellulose<br>content<br>(%) | Lignin<br>content<br>(%) | Xylan<br>content<br>(%) | Acetyl<br>content<br>(%) | Pretreatment<br>yield<br>(%) | Cellulose<br>recovery<br>(%) | Xylan<br>recovery<br>(%) | Lignin<br>removal<br>(%) | Acetyl<br>removal<br>(%) |
|------------------------------------|-----------------------------|--------------------------|-------------------------|--------------------------|------------------------------|------------------------------|--------------------------|--------------------------|--------------------------|
| Starting corn stover               | $43\pm3$                    | $19\pm0$                 | $22 \pm 1$              | $3.1\pm0$                | N/A                          | N/A                          | N/A                      | N/A                      | N/A                      |
| 25 °C 24 h<br>water control        | $44\pm0.4$                  | $20 \pm 1$               | $20 \pm 1$              | $2.1\pm0$                | 95                           | 99                           | 86                       | 1.2                      | 32                       |
| 25 °C 24 h<br>KOH<br>Deacetylation | $49 \pm 1$                  | $20\pm1$                 | $23 \pm 1$              | $0.6\pm0$                | 93                           | 107                          | 97                       | 5.9                      | 81                       |
| 80 °C 3 h<br>water control         | $42\pm3$                    | $22\pm0$                 | $21\pm2$                | $2.2\pm0$                | 78                           | 76                           | 73                       | 13                       | 29                       |
| NaOH<br>deacetylation              | $53\pm4$                    | $18 \pm 1$               | $25 \pm 1$              | $0.6\pm0$                | 81                           | 100                          | 92                       | 26                       | 80                       |

 Table 8. Chemical composition, sugar recovery and lignin/acetyl removal of the various

mechanically pulped corn stover samples.

As previous work has shown that the addition of accessory enzymes such as xylanases enhanced the hydrolysis of the cellulose component of pretreated corn stover (Hu et al., 2011), it was anticipated that these xylan rich substrates could provide a good indication of the influence of deacetylation on the hydrolysis of both of the cellulose and the xylan components (Zhai et al., 2018b). As indicated in Figure 13, deacetylation significantly enhanced the hydrolysis of both cellulose and hemicellulose. However, it was likely that the observed 25% delignification resulting from NaOH treatment at 80 °C also helped increase cellulose and xylan hydrolysis, suggesting that both deacetylation and delignification contributed to the observed increase in hydrolysis.



*Figure 13. Enzymatic hydrolysis of various mechanically pulped corn stover samples. Hydrolysis was conducted at 2% (w/v) solids and enzyme loading of 20 mg/g cellulose at 50 °C for 48 hours.* 

Although both the mild KOH and NaOH treatments resulted in about the same amount of deacetylation (Table 8), it was apparent that the xylan contained in the substrate resulting from the KOH treatment at room temperature was less susceptible to enzymatic hydrolysis than the xylan component of the NaOH-treated substrate (Figure 13). This result suggested that in addition to deacetylation, partial delignification had also enhanced xylan accessibility, we next added xylanases (Novozymes HTec) to each of the pretreated substrates to see if enzyme accessibility had in fact increased. Medium-to-relatively-high-loadings of xylanase (25 and 50 mg xylanase /g of cellulose) were used, to ensure that the use of low enzyme concentrations did not influence the results. The xylan in the deacetylated corn stover substrates appeared to be more accessible to the xylanases, particularly within the first 3 hours (Figure 14), although the xylan in all of the substrates was partially hydrolyzed after 48 hours (Figure 13). The slightly higher hydrolysis yields of the xylan present in the NaOH treated substrates after hydrolysis with the CTec 3 cellulase mixture was likely due to the synergistic action of the cellulases hydrolyzing the cellulose which consequently exposed the xylan previously 'buried' within the fibre structure to the xylanases (Hu et al., 2011). Although the 3-hour water treatment at 80 °C increased the CTec 3 induced hydrolysis of both the cellulose and xylan (Figure 13), much lower hydrolysis yields were obtained when xylanase alone (HTec) was added (Figure 14). It is likely that the hot water

treatment resulted in the removal of the more easily accessible xylan, consequently exposing more of the cellulose that was more readily hydrolyzed by the CTec 3 mixture (Figure 14).



*Figure 14. Xylan hydrolysis of deacetylated and control samples of mechanically pulped corn stover at high enzyme loadings and short incubation times. Hydrolysis was conducted at 2\% (w/v) solids at 50 °C for 3 and 24 hours.* 

Although the NaOH and KOH deacetylation treatments appeared to enhance xylan accessibility to similar extents, it was not clear if the major benefit of the treatments was due to deacetylation rather than delignification. As well as enhancing xylan hydrolysis, it was apparent that the NaOH treatment resulted in the removal of 25% of the lignin which also likely contributed to the observed increase in cellulose hydrolysis. Previous work has shown that, as well as the overall total lignin content, the surface lignin in particular plays a significant role in influencing enzymatic hydrolysis (Ju et al., 2013a). The relative surface lignin of the deacetylated and untreated corn stover biomass was measured using X-ray Photoelectron Spectroscopy (XPS). As detailed previously, the XPS method measures the oxygen to carbon ratio (Johansson et al., 1999; Laine et al., 1994) of the substrate surface, at a depth of 5-10 nm, to estimate the relative amount of lignin that is present, with an increase in surface lignin indicated by an increase in measured surface carbon and a reduction in oxygen (Tshabalala, 2005). It was apparent that, compared to the negligible effect of KOH treatment at room temperature, the NaOH treatment at 80 °C was able to remove more than 12% of the surface lignin (Table 9). This suggested that the removal of the lignin, particularly at the substrate surface, resulted in the increased overall enhanced enzyme

accessibility and hydrolysis of the NaOH treated substrates.

In past work, the water retention value (WRV) has been used to assess a pulp or pretreated substrate's accessibility to water and this value is typically used to provide an estimate of fibre swelling (Bendzalova et al., 1996; Ogiwara and Arai, 1968). In contrast to the WRV, the Simons' staining technique utilizes a direct orange (DO) dye that has a similar size to the predominant cellulase enzyme, cellobiohydrolase, and has been successfully used to estimate the enzyme accessibility of a substrate (Chandra and Saddler, 2012; Grethlein, 1985; Stone et al., 1969; Yu and Atalla, 1998). When the five substrates were compared (Table 9), the NaOH treatment resulted in the largest increase in cellulose accessibility when compared to the mechanical pulp control (65% increase in WRV and 43% increase in DO dye adsorption). This enhanced accessibility was likely due to the removal of much of the surface lignin as well as the embedded lignin which facilitated substrate swelling (Table 9). Although the KOH and water treatments at 80 °C also enhanced substrate swelling and cellulose accessibility, it was to a considerably lesser extent (Table 9). Although the removal of around 30% of the xylan as well as 13% of the lignin after hot water (80 °C) treatment likely resulted in enhanced swelling and cellulose accessibility, KOH treatment did not cause a significant change in the chemical composition of the substrate other than deacetylating the xylan (Table 9). It should be noted that the KOH treatment of corn stover gave results that were similar to those previously observed after Ammonia Fibre/Freeze Expansion treatment (AFEX). This pretreatment has been shown to selectively deacetylate the xylan present in agricultural biomass such as corn stover, consequently increasing cellulose accessibility while resulting in only limited changes to the chemical composition of the substrates (Chundawat et al., 2011b; R. Kumar et al., 2009). Thus, as suggested previously, it is likely that selective deacetylation, through the cleavage of ester bonds between the xylan backbone and the pendant acetyl groups, increased enzyme accessibility to the xylan and the cellulose (Chen et al., 2012a; Mitchell et al., 1990; Selig et al., 2009). As there are also a significant number of ester bonds that link uronic acids and cinnamic acids to corn stover xylan (Gupta et al., 2014; Mao et al., 2010; Mueller-Harvey et al., 1986), it is also likely that the cleavage of ester bonds during the alkaline treatments exposed more of the weaker acid groups (Sjöström et al., 1965). Thus, it is probable that this contributed to the observed increase in substrate swelling, as described previously (Lindström and Carlsson, 1982). In addition, it was also possible that the cleavage of ester bonds contributed to enhancing the accessibility of the xylan backbone to xylanases. When the strong and weak bulk acid groups in the various substrate were assessed using conductometric titration (Table 9), the alkaline treatments were shown to result in a 50% increase in overall total

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acid groups. This suggested that both the alkaline and hot water treatments enhanced swelling and cellulose accessibility through a combination of lignin/hemicellulose removal as well as through the enrichment of acid groups within the substrate.

*Table 9.* Substrate swelling, accessibility and enzymatic hydrolysis of mechanically pulped corn stover.

Surface

| Treatment                    | lignin<br>(%) | Retention<br>Water | adsorption<br>(mg/g) | groups<br>(mmol/kg) |
|------------------------------|---------------|--------------------|----------------------|---------------------|
| Mechanical refining          | 82.6          | $2.0\pm0$          | 58.3                 | $113\pm14$          |
| 25 °C 24 h water control     | -             | $2.3\pm0$          | 54.3                 | $100\pm7$           |
| 25 °C 24 h KOH deacetylation | 85.1          | $2.6\pm0$          | 76.3                 | $157 \pm 10$        |
| 80 °C 3h water control       | -             | $2.8\pm0$          | 75.0                 | $106 \pm 17$        |
| 80 °C 3h NaOH deacetylation  | 71.9          | $3.3 \pm 0$        | 83.3                 | $166 \pm 10$        |

Water

**DO** dve

**Total acid** 

Due to the mild nature of the NaOH treatment at 80 °C it was anticipated that the solubilized lignin would be of a low molecular weight. When alkaline pulping is used to produce paper making pulps, it is typically far more aggressive than the deacetylation treatments used here and results in the cleavage of ether bonds and the formation on new phenolic end groups (Chakar and Ragauskas, 2004; Gierer, 1980). Therefore, the milder alkaline treatment that had been used for deacetylation were expected to solubilize lignin that retained its native ether and carbon-carbon bond structure to a greater extent than would occur after either pulping or acid pretreatments (Jensen et al., 2017; Liu et al., 2018). When the molecular weight and chemical characteristics of the lignin present in the deacetylation liquor were compared to that of enzyme mild acidolysis lignin (EMAL) isolated from the original corn stover biomass (Guerra et al., 2006; Y. Song et al., 2019), the average molecular weight of the deacetylation liquor lignin was much lower than that of the EMAL lignin (Table 10). As anticipated, the lower temperature alkaline treatment had removed the smaller fragments of lignin that were more readily ionized under alkaline conditions. When Heteronuclear Single-Quantum Correlation NMR (HSQC-NMR) was used to analyze the low-molecular-weight lignin, it was apparent that this solubilized lignin retained up to 40% of its native  $\beta$ -O-4 bonds (Table 10). This was comparable to the amount  $\beta$ -O-4 linkages detected in corn stover milled wood lignin, which has been shown to have a similar structure to native lignin, indicating that the solubilized lignin retained much of its native structure (Min et al., 2014). This, in combination with other features from lignin such as < 1% of  $\beta$ - $\beta$  bonds and an syringyl/guaiacyl (S/G) ratio that was close to the original corn stover lignin (Table 10) (Li et al., 2012), strongly suggested that this lignin fraction underwent limited changes during

solubilization. This was consistent with recent work which showed that lignins isolated at lower temperature by methods such as DES and p-TsOH hydrotrope were structurally similar to the original biomass lignin (Y. Song et al., 2019). The HSQC analysis also indicated the presence of p-coumarate and ferulic acids in corn stover lignin, as reported previously(Min et al., 2014; Takada et al., 2018), supporting the notion that carboxylic groups, which are readily ionized at pH >5, facilitated the dissolution of this low molecular lignin fraction. The attractive properties of this lignin fraction, such as its low molecular weight, high percentage of native linkages and semi-hydrophilic properties, make it a possible source of aromatics for the production of renewable chemicals via both chemical (Ma et al., 2014; Xiang and Lee, 2000) and biological pathways (Abdelaziz et al., 2016; Dupont et al., 2007).

*Table 10.* The molecular weight and structure of isolated lignin from the deacetylation liquor and enzymatic mild acidolysis lignin (EMAL) derived from corn stover, analyzed by Gel Permeation Chromatography (GPC) and HSQC NMR.

| Weight average<br>molecular<br>weight<br>(kDa) |              |                |         | Lign                | in stru  | cture <sup>(a</sup> | l)     |             |          |       |
|--|--------------|----------------|---------|---------------------|----------|---------------------|--------|-------------|----------|-------|
|  | Inte         | er-linkages (  |         | Lignin subunits (%) |          |                     |        |             |          |       |
|  | β-Ο-4        | α-Ο-4/<br>β -5 | β-β     | Cinnamyl<br>alcohol | S        | G                   | Н      | р-СА<br>(b) | Ferulate | ratio |
| 8.1  | 40.1         | 5.2            | 0.9     | 2.3                 | 43.3     | 44.4                | 12.3   | 24.7        | 9.5      | 0.98  |
| (a): calculated base                           | ed on the to | otal S+G+H a   | romatic | ring. Results e     | expresse | ed per 1            | 00 Ar. |             |          |       |

(b): p-Coumarate (p-CA) and Ferulate levels are expressed as a fraction of S + G + H

As it was likely that the removal of low molecular weight lignin, especially from the surface of corn stover during the mild NaOH treatment at 80 °C, played a significant role in enhancing cellulose accessibility to enzymes, we wanted to further investigate how much deacetylation had contributed to this enhancement. Previous work had shown that an alkaline environment was beneficial to the incorporation of sulfonic acids to lignin by deprotonating phenolic lignin moieties that were susceptible to nucleophilic attack by sulfite anions (Chandra et al., 2016; Fengel and Wegener, 1989). In related work, sulfonated lignin has been shown to be less restrictive to substrate swelling and also has a lower tendency to bind enzymes due to its increased hydrophilicity (del Rio et al., 2011; Nakagame et al., 2011a). Although several studies have looked at sulfonation at temperatures above 100 °C, recent work by Zhong et al. (Zhong et al., 2019) showed that 106 mmol/kg of sulfonic acid groups could be incorporated into softwood

lignin at a temperature of 70 °C using 2% alkali. In the work reported here, as the NaOH had been added at a loading of 4.8% during the deacetylation reactions, it is possible that the NaOH treatment at 80 °C was able to simultaneous deacetylate the hemicellulose and sulfonate the corn stover lignin, further enhancing subsequent hydrolysis.

It was apparent that the addition of 3% Na<sub>2</sub>SO<sub>3</sub> during the deacetylation reaction further enhanced the removal of lignin and acetyl groups from corn stover to 34% and 100% respectively, while retaining most of the carbohydrate (Table 11). Although lignin removal was enhanced by the addition of sulfite during the deacetylation reaction, enhanced sulfonation of the residual lignin was not detected by conductometric titration (Table 11). Similarly, elemental analysis of the NaOH/Na<sub>2</sub>SO<sub>3</sub> treated sample also did not show any sulfur incorporation onto the corn stover during treatment (Table 12). However, it is likely that the addition of sulfonic acid groups facilitated the dissolution of a greater amount of the low molecular weight lignin as the lignin became more hydrophilic and ionizable. It was also likely that the increased dissolution of lignin exposed a greater amount of xylan, which could be further deacetylated by the added hydroxide ions. Consequently, the addition of Na<sub>2</sub>SO<sub>3</sub> to the NaOH during the deacetylation process also resulted in a >20% enhancement in the hydrolysis of the xylan (Figure 13, Table 11). In contrast, the addition of Na<sub>2</sub>SO<sub>3</sub> to the corn stover in the absence of alkali did not result in any further increase in the solubilization of acetyl groups, lignin or carbohydrates (Table 11), suggesting the synergistic action of alkali with the sulfite had enhanced both lignin removal and xylan deacetylation.

| Samples   | Total Acid<br>groups<br>(mmol/kg) | WRV          | DO dye<br>adsorption<br>(mg/g) | Cellulose<br>recovery<br>(%) | Xylan<br>recovery<br>(%) | lignin<br>removal<br>(%) | Acetyl<br>Removal<br>(%) | Cellulose<br>hydrolysis<br>(%) <sup>(a)</sup> | Xylan<br>hydrolysis<br>(%) <sup>(a)</sup> |
|---|-----------------------------------|--------------|--------------------------------|------------------------------|--------------------------|--------------------------|--------------------------|---|---|
| 80 °C 3hr<br>NaOH/<br>Na <sub>2</sub> SO <sub>3</sub><br>deacetylation<br>80 °C 3hr | $163 \pm 10$                      | $2.8\pm0.1$  | 83                             | 102.1                        | 92.9                     | 34.3                     | 100.0                    | 71 ± 1  | $78\pm2$                                  |
| 3% Na <sub>2</sub> SO <sub>3</sub><br>control                                       | $147\pm13$                        | $2.3\pm0.1$  | 62                             | 98.0                         | 93.1                     | 4.5                      | 30.0                     | $41 \pm 1$                                    | $40 \pm 1$                                |
| <sup>(a)</sup> : Enzymatic  | hydrolysis was                    | conducted at | 2 % solid and p                | orotein loadir               | ng of 20mg /             | g cellulose              | at 50 °C for             | 48 h  |   |

*Table 11.* The recovery of biomass chemical components and substrate characteristics after the treatments of NaOH/Na<sub>2</sub>SO<sub>3</sub> and Na<sub>2</sub>SO<sub>3</sub> combined with mechanical refining to corn stover.

 Table 12. Elemental analysis of mechanically pulped corn stover.

| Sample                         | Nitrogen<br>(%)     | Carbon<br>(%) | Hydrogen<br>(%) | Sulfur<br>(%) |  |  |  |  |  |
|--------------------------------|---------------------|---------------|-----------------|---------------|--|--|--|--|--|
| NaOH deacetylation             | <LOD <sup>(a)</sup> | 44            | 6.0             | < LOD         |  |  |  |  |  |
| KOH deacetylation              | < LOD               | 44            | 6.0             | < LOD         |  |  |  |  |  |
| NaOH/ $Na_2SO_3$ deacetylation | < LOD               | 43            | 6.1             | < LOD         |  |  |  |  |  |
| (a): Limit of detection        |                     |               |                 |               |  |  |  |  |  |

## 5.3 Conclusions

Previous work had suggested that, during alkaline pretreatment of agricultural biomass, deacetylation of the hemicellulose was the predominant mechanism which enhanced enzymemediated cellulose hydrolysis. However, alkaline mediated deacetylation also resulted in partial delignification, particularly at the substrate surface. It was apparent that lignin removal also enhanced enzyme accessibility to the cellulose, resulting in increased cellulose hydrolysis. The delignification and deacetylation mechanisms were further enhanced by supplementing the alkaline solution with sodium sulfite. It was likely that the sulfonation reaction facilitated lignin removal, which in turn exposed more of the xylan to xylanases.

# 6. Research chapter 4: enhancing enzymatic hydrolysis and carbohydrate recovery of hardwood by alkaline oxygen and thermomechanical pulping pretreatment

## **6.1** Introduction

As described in detail earlier, the pretreatment step is one of the main components that has been investigated for the potential development of a biomass-based biorefinery process (Chandra et al., 2007; Mosier et al., 2005). At an industrial scale, one of the most effective ways to pretreat and fractionate biomass is the pre-hydrolysis Kraft pulping process, where the majority of the hemicellulose is separated from the woody biomass followed by a subsequent Kraft pulping process that removes most of the lignin (Li et al., 2010). However, the high cost of fractionating lignin from biomass precludes its use for biochemical conversion. Unlike Kraft pulping, mechanical pulping typically converts wood chips into fibres at yields close to 95% of the original biomass. As mentioned earlier, the demand for mechanical pulp-derived newsprint has dwindled significantly (Unece & Fao, 2006), leading to considerable interest in the possible "repurposing" of existing mechanical refining/pulping infrastructure as the "front-end" of an enzyme/microbial based biorefinery process (Chen et al., 2016, 2012a; Koo et al., 2011; Wang et al., 2009; J. Y. Zhu et al., 2009). As described in Chapter 1 and other studies (Boussaid and Saddler, 1999; Chandra et al., 2016; Mooney et al., 1998), a key challenge with utilizing a mechanical pulping approach to pretreating woody substrates is that the substrates continue to be quite recalcitrant to enzymatic hydrolysis. This is mainly because the lignin remains associated with the mechanically refined substrates significantly limits the accessibility of the enzymes to the cellulose and binds the enzymes through hydrophobic interactions (Berlin et al., 2006, 2005; Boussaid and Saddler, 1999; Kumar et al., 2011; Mooney et al., 1998; Tu et al., 2007).

Considering the high cost associated with complete delignification, more recent research has looked at the use of milder chemical treatments to modify lignin rather than targeting complete lignin removal (Chandra et al., 2016; Wang et al., 2009; J. Y. Zhu et al., 2009). It was shown that the incorporation of acid groups into the lignin macromolecule using oxidation or sulfonation increased the hydrophilicity of lignin (Lou et al., 2013; Palonen et al., 2004b), consequently enhancing cellulose accessibility by facilitating fibre swelling (del Rio et al., 2011) and by reducing non-productive binding of enzymes to lignin (Chandra et al., 2016; Nakagame et al., 2010). The negative charges on lignin have also been implicated in decreasing non-productive enzyme binding to lignin by electrostatic repulsion since the majority of enzyme components exhibit a net negative charge at the pH (4-5) where hydrolysis is being performed (Chandra et al., 2016; Lou et al., 2013; Nakagame et al., 2010).

Pretreatments that employ sulfonation and mild oxidation should also be easily implemented since they are already utilized at a commercial scale by the pulp and paper industry (Smook, G, 1989). Previous work has shown that sulfonation of refiner mechanical pulps and steam pretreated softwood and hardwood biomass improved hydrolysis yields without removing lignin (Chandra et al., 2016; Kumar et al., 2012a; Mooney et al., 1998). However, the improvements in enzymatic hydrolysis were still impeded by the physical presence of the lignin in the substrate (Chandra et al., 2016; Kumar et al., 2012a; Mooney et al., 1998). In subsequent work the application of either oxygen to wood chips prior to steam pretreatment was shown to improve enzymatic hydrolysis yields and also resulted in a high retention of carbohydrates (Chu et al., 2017a).

One of the goals of the work reported in this chapter was to determine whether a similar approach could also be used to modify the lignin in Aspen (hardwood) biomass prior to the application of mechanical pulping, as a demonstration of a modified chemi-thermomechaical pulping (CTMP) process. As described below, alkali and oxygen were integrated into a pre-steaming step (110-130°C) that is normally employed during the production of thermo-mechanical pulps with the hope that it would increase the acid groups on the resulting mechanical pulp substrate. In this way we could retain most of the carbohydrate components in the pulp associated "water-insoluble fraction" while improving the cellulose accessibility and ease of enzymatic hydrolysis. It was apparent that the use of a mild (130°C) oxygen/steaming-based CTMP process was effective at both improving enzymatic hydrolysis while retaining/recovering most of the hemicellulose in the water insoluble substrate as part of a "one-pot" approach.

### 6.2 Results and discussion

As described earlier, one of the goals of this study was to assess the potential of integrating an

alkaline oxygen step into the initial steaming of wood chips prior to mechanical pulping/refining. Previous work had shown that the addition of an alkaline oxygen treatment at the milder temperatures of 110 and 135°C prior to steam pretreatment/explosion at 210°C resulted in enhanced hemicellulose recovery while improving the ease of hydrolysis of the cellulose component (Chu et al., 2017a). Therefore, we first wanted to assess if a similar alkaline oxygen pretreatment could be successfully applied to Aspen chips during steaming, prior to mechanical pulping.

As discussed in earlier studies (Chandra et al., 2016; Mooney et al., 1998), the residual lignin remaining in the substrate after refining was likely restricting more effective hydrolysis. Thus, a range of pretreatment conditions were next assessed to see if we could modify or solubilize the lignin while retaining as much of the hemicellulose content with the insoluble cellulosic fraction as possible (Table 13). We first looked at utilizing alkaline oxygen treatment to modify lignin in the biomass to improve the ease of hydrolysis of the carbohydrates contained in the mechanically refined substrates. Initially, the alkaline-oxygen-steam treatment was applied at 190°C to original Aspen chips prior to the mechanical refining. Previous work which had integrated alkaline oxygen into the steam pretreatment process showed that a temperature of 190°C was effective for enhancing enzymatic hydrolysis while retaining much of the hemicellulose (Chandra et al., 2016; Chu et al., 2017a) in the water insoluble fraction.

It was apparent (Table 13) that an alkaline pH was beneficial to retaining and recovering the hemicellulose component in the solid pulp. In contrast, treatments performed in the absence of alkali, using either hot water or oxygen, resulted in lower hemicellulose recoveries in the water insoluble fraction (Table 13). The "190°C Water" treatment likely solubilized the hemicellulose via "auto-hydrolysis" where the inherent acetyl groups on the hemicellulose component as an acid catalyst to hydrolyze and solubilize the hemicellulose. The use of oxygen in the absence of alkali has also been shown (Biswas et al., 2015) to result in the oxidation of a portion of the hemicellulose component to carboxylic acids. This, consequently acidifies the pretreatment resulting in the solubilization of hemicellulose and likely occurred with the "190°C Oxygen" treatment. Although the addition of alkali to the pretreatment increased the recovery of hemicellulose in the solid fraction, it was evident that pre-steaming at 190°C may have been excessively severe as the hemicellulose retention in the solid pulp fraction was just over 50% (Table 13). In earlier work, increased solubilization of hemicellulose at higher temperature oxidative treatments has been shown to result from radical initiated reactions that damage and

solubilize hemicellulose (Yang et al., 2003).

| Sample                     | Cellulose<br>(%) | Hemicellulose<br>(%) | Lignin<br>(%) | Cellulose<br>recovery<br>(%) | Hemicellulose<br>recovery<br>(%) | Lignin<br>removal<br>(%) |
|----------------------------|------------------|----------------------|---------------|------------------------------|----------------------------------|--------------------------|
| Aspen chips                | 46               | 19                   | 28            | N/A                          | N/A                              | N/A                      |
| Mechanical pulping<br>Only | 46               | 19                   | 28            | 100                          | 100                              | 0                        |
| 190 °C AO                  | 66               | 15                   | 23            | 98                           | 56                               | 42                       |
| 190 °C Alkali              | 57               | 13                   | 24            | 90                           | 53                               | 35                       |
| 190 °C Oxygen              | 56               | 9.4                  | 26            | 91                           | 38                               | 28                       |
| 190 °C Water               | 62               | 9.0                  | 29            | 96                           | 35                               | 24                       |

*Table 13.* Chemical composition, carbohydrate recovery and lignin removal of the Aspen Chemithermomechanical pulp (CTMP) samples after various pretreatment conditions.

Explanation of treatments:

190 °C AO: alkali-oxygen impregnation prior to pre-steaming at 190 °C for 15 min and mechanical refining;

190 °C Alkali: alkali impregnation prior to pre-steaming at 190 °C for 15 min and mechanical refining;

190 °C Oxygen: oxygen impregnation prior to pre-steaming at 190 °C for 15 min and mechanical refining;

190 °C Water: water impregnation prior to pre-steaming at 190 °C for 15 min and mechanical refining;

As well as retaining approximately 50% of the hemicellulose in the water insoluble substrate, the 190°C AO and 190°C Alkali pretreatments were also quite effective in solubilizing the lignin (Table 13). This was consistent with previous studies (Chandra and Saddler, 2013; Siqueira et al., 2017) which showed a higher selectivity for lignin removal when alkaline treatments were compared to acidic treatments. It has been shown that, under alkaline conditions, the de-

protonation of phenolic groups by hydroxide ions leads to the formation of quinone methide structures, which facilitates the cleavage of  $\alpha$ -aryl ether bonds (Fengel and Wegener, 1989). The  $\beta$ -aryl ether bonds are also cleaved by hydroxide ions and the lignin fragments are solubilized by the alkaline solution. In the work reported here, alkali treatment without the addition of oxygen removed up to 35% of the lignin from the Aspen chips. The addition of oxygen to the alkaline treatment increased lignin removal to more than 40%, likely due to the synergistic action of alkali and oxygen oxidizing the lignin macromolecule (Asgari and Argyropoulos, 1998). The higher lignin removal after 190°C AO treatment was likely due to the increased solubility of the oxygenmodified lignin in an alkali solution (Asgari and Argyropoulos, 1998). The addition of oxygen to the alkaline treatments (190°C AO) also increased the recovery of the cellulose (Table 13), which is also indicative of the higher selectivity of the oxygen treatment.

It has been shown that the main effect of alkaline oxygen (AO) treatment on substrate lignin is a stepwise oxidation, typically commencing on the phenolic groups (Asgari and Argyropoulos, 1998) and that alkaline oxygen treatment prior to steaming partially removed lignin, while increasing the amount of acid groups on the substrate lignin. It was apparent that, in addition to solubilizing 40% of the lignin, the 190°C AO treatment also left the residual substrate lignin in a highly oxidized state as it contained 78 mmol of carboxylic acid group per kg of substrate (Table 14). Interestingly, the high level of sodium carbonate used for the alkali treatment in the absence of oxygen also resulted in the generation of a 67 mmol carboxylic acid groups/kg of substrate (Table 14). It has been suggested that alkaline treatment results in the formation of acid groups within biomass due to the alkaline induced cleavage of ester bonds between lignin and hemicellulose. This has been observed to occur during the sodium carbonate (Alkaline) treatment (Kokta and Ahmed, 1998; Sjöström et al., 1965).

| Sample        | Carboxylic acid<br>groups (mmol/kg) | Water Retention<br>Value | DO dye<br>adsorption (mg/g<br>dry fibre) | LW fibre<br>length<br>(mm) | Viscosity<br>(mPa*s) |
|---------------|-------------------------------------|--------------------------|--|----------------------------|----------------------|
| 190 °C AO     | 78                                  | $3.1\pm0.1$              | 95                                       | 0.80                       | $5.2\pm0$            |
| 190 °C Alkali | 67                                  | $2.8\pm0.1$              | 90                                       | 0.81                       | $8.8\pm0$            |
| 190 °C Oxygen | 23                                  | $2.6\pm0.0$              | 88                                       | 0.66                       | $6.7\pm0$            |
| 190 °C Water  | 22                                  | $2.8\pm0.0$              | 77                                       | 0.60                       | $8.6 \pm 0$          |

*Table 14.* Substrate characteristics of the Aspen CTMP samples after various pretreatment conditions.

When substrate swelling was assessed by water retention value (WRV) measurement (Bendzalova et al., 1996; Ogiwara and Arai, 1968) the 190°C AO treated substrate also exhibited the highest WRV (Table 14). Both the WRV and the adsorption of the DO dye that constitutes the Simons' staining have been successfully used to predict the susceptibility of pretreated biomass to enzymatic hydrolysis (Chandra and Saddler, 2012; Luo et al., 2011). However, unlike the WRV which uses water to probe for substrate swelling, the Simons' staining utilizes the DO dye that has a high specificity for cellulose and a similar size to a typical cellulase enzyme (Grethlein, 1985; Stone et al., 1969; Yu and Atalla, 1998). As well as having the highest accessibility, as indicated by the Simons' staining, it was also apparent that the 190°C AO treatment affected the average viscosity/molecular weight of the cellulose. When oxygen was combined with alkali (190°C AO), the viscosity decreased substantially compared to the alkaline treatment in the absence of oxygen (190°C Alkali). As mentioned above, the alkaline oxygen treatment at 190°C may have been excessively severe, resulting in the solubilization of hemicellulose and the nonspecific radical induced molecular weight reduction of cellulose (Table 13) (Dence, 1996). The non-specific nature of alkaline oxygen is one of the recognized limitations of oxygen delignification when applied at a commercial scale during pulp bleaching (Biermann, 1996a). However, although the 190°C AO and 190°C alkaline treatments decreased the molecular weight of the cellulose and/or compromised hemicellulose yield, these treatments resulted in substrates with the longest fibres. These results suggested that alkaline treatments primarily targeted the lignin in the middle lamella to provide cleaner fibre separation (Table 14). Overall, it appeared that both the 190°C alkali and 190°C AO treatments were the most effective as these treatments resulted in substrates that had the highest accessibility as measured by Simons' staining and WRV while retaining more than 50% of the hemicellulose in the solid fraction.

As mentioned earlier, a major goal of this work was to recover as much of the hemicellulose as possible by retaining hemicellulose in the solid pulp substrate component. However, as shown previously, the retention of hemicellulose in lignocellulosic substrates can compromise the ease of cellulose hydrolysis (Mussatto et al., 2008; Öhgren et al., 2007) unless enzyme cocktails are supplemented with hemicellulase activities (Hu et al., 2015, 2011). Therefore, considering the high xylan content (ranging from 11 to 14%) of the substrates pretreated under alkaline conditions, the enzyme cocktails were supplemented with xylanases to assess the potential benefits of this enzyme addition.

In order to achieve high hydrolysis yields at the solids loading of 10% (w/v), the total protein loading was used at 20mg/g cellulose (15 mg cellulase and 5 mg xylanase). It was apparent that the hydrolysis yields reflected the incorporation of acid groups in the substrates (Figure 15, Table 14) as previous work had shown that an increase in acid groups improved the swelling and hydrophilicity of the substrate as well as decreasing the non-productive binding of the enzyme to the lignin (del Rio et al., 2011; Nakagame et al., 2010). It was also evident that the xylan component of the substrates treated under alkaline conditions (190°C AO, 190°C alkali) were more prone to hydrolysis (Figure 15). Although the enzyme preparations were loaded to the substrate based on cellulose content, the hydrolysis of the alkaline pretreated substrates that contained less cellulose and a higher xylan content resulted in almost complete hydrolysis of the xylan, despite the use of a lower enzyme/protein loading (Table 13, Figure 15). This was likely due to the deacetylation of the xylan as mild alkaline treatments have been shown to facilitate the removal of acetyl groups on the hemicellulose from hardwood (Castro et al., 2017; Cho et al., 2010) and agriculture biomass (Chaudhary et al., 2012; Chen et al., 2012a). The removal of acetyl groups has also been shown to result in an increase in subsequent xylan and cellulose hydrolysis yields by liberating the reaction sites on xylan from xylanases (Mitchell et al., 1990).



*Figure 15.* Cellulose and xylan hydrolysis the Aspen CTMP samples after various pretreatment conditions. Hydrolysis was conducted at 10% (w/v) solids and enzyme loading of 15mg cellulase and 5mg xylanase/ g cellulose at 50 °C for 72 hours.

Despite its high yield of xylan hydrolysis, the 190°C AO treatment recovered just over 50% of the hemicellulose in the solid substrate. To determine if the use of less severe conditions would help we next assessed alkaline oxygen treatment with a steaming temperature of 130°C, which is closer to the pre-steaming conditions used in a commercialized mechanical pulping process (Vena, 2005). At the lower 130°C temperature, although hemicellulose recovery increased from 55 to 73% (130°C AO, Figure 16a) the lignin removal decreased from 42 to 25%. However, the carboxylic acid groups on the 130°C AO substrate increased from 100 to 107 mmol/kg, indicating that the alkaline oxygen reaction with lignin had shifted from lignin removal towards lignin modification (Figure 16a). The 130°C AO substrate also showed a slightly higher accessibility to the DO dye as well as a decrease in the viscosity of cellulose (Figure 16b, Figure 16c). This suggested that the cellulose underwent some oxidative damage even at the lower 130°C steaming temperature. Despite the higher amount of carboxylic acid groups on the 130°C treated substrate, they were less susceptible to enzymatic hydrolysis (Figure 16a, Figure 16d), probably due to the lower amount of lignin removed at 130°C.



**Figure 16.** The recovery of biomass chemical components(*a*), substrate characteristics (*a*, *b*, *c*) and enzymatic hydrolysis (*d*) after the application of alkaline and alkaline-oxygen (AO)- based CTMP pretreatments at 130°C to Aspen biomass. Enzymatic hydrolysis was conducted at 10% (*w*/*v*) solids at enzyme loading of 15mg cellulase and 5mg xylanase/ g cellulose at 50 °C for 72 hours.

When the 130°C Alkali and the 130°C AO treated substrates were compared, the addition of oxygen only resulted in a slight improvement (~7%) in cellulose hydrolysis (Figure 16d). As this result was unexpected, we reduced the enzyme loading to ensure that the higher 20 mg/g protein loading had not "masked" any differences between the two substrates. As well as potential "masking" of substrate differences at the higher enzyme loading, non-productive binding between

enzymes and lignin through hydrophobic interactions has been shown to have negative impacts on enzymatic hydrolysis (Palonen et al., 2004b; Rahikainen et al., 2013), especially at low enzyme loadings (Kumar et al., 2012a). The addition of bovine serum albumin (BSA) to enzymatic hydrolysis has long been known to bind to lignin through hydrophobic interactions, thereby acting as a lignin "blocking agent" to increase enzymatic hydrolysis yields (Yang and Wyman, 2006). Recent studies have also shown that the increase in enzymatic hydrolysis of a given substrate that has been pre-incubated with BSA is an indication of the hydrophobic nature of lignin and its potential to non-productively bind cellulase enzymes through hydrophobic interactions (Rollin et al., 2011; Siqueira et al., 2017). Therefore, the enzymatic hydrolysis of the substrates treated under alkaline conditions at 190 and 130°C was performed at enzyme loadings of 5 and 10 mg/g cellulose on substrates both with and without pre-incubation with BSA. When comparing the 190°C AO and 190°C Alkali substrates, it was apparent that reducing the enzyme loading to 10 mg/g cellulose exposed differences between these substrates as the hydrolysis yield of the 190°C AO was 26% higher than the 190°C alkali (Figure 17). However, when hydrolyzing the substrates pre-incubated with BSA, the substrates reached similar hydrolysis yields, indicating the hydrophobic nature of the lignin component of the 190°C alkali substrate (Figure 17). Therefore, despite containing a high amount of acid groups, likely through the cleavage of lignincarbohydrate complexes, the lignin in the 190°C alkaline treatment also appeared to be quite hydrophobic. As mentioned earlier, under alkaline conditions the phenolic groups in lignin are deprotonated to form quinone methides. In the absence of a strong nucleophile, the quinone methides undergo alkaline condensation reactions that increase lignin hydrophobicity (Fengel and Wegener, 1989). This likely occurred with the 190°C alkaline substrate. Typically, when oxygen is added to the alkaline reaction, it removes an electron from the phenolate anion and subsequently reacts with the lignin to incorporate acid groups, consequently increasing the hydrophilicity of lignin. The increased lignin hydrophilicity in the alkaline oxygen reaction likely explains the negligible effect of hydrolysis after adding BSA to the 190°C AO substrate.

The trends observed with the substrates treated at 130°C were similar but less pronounced. This was probably a result of the decreased amount of lignin condensation occurring as the temperature was decreased from 190 to 130°C. However, reducing the temperature of the AO pretreatment from 190 to 130°C increased the hemicellulose recovery in the water insoluble component (Table 13, Figure 16a) while the cellulose in this fraction was readily hydrolyzed, resulting in a yield of 70% at enzyme loading of 20mg/g cellulose (Figure 17).



**Figure 17.** Enzymatic hydrolysis of aspen substrates after alkaline and alkaline-oxygen (AO)based CTMP pretreatments at 130°C, with and without the addition of BSA. Hydrolysis was conducted at 2% (w/v) solids using enzyme loading of 8mg cellulose/2mg xylanase and 4mg cellulase/1mg xylanase/ g cellulose at 50 °C for 48 hours.

# 6.3 Conclusions

Alkaline-oxygen (AO)-based CTMP pretreatment of Aspen chips, using a pre-steaming temperature of 130°C resulted in lignin modification while recovering more than 70% of hemicellulose in association with the cellulose fraction in the water insoluble fraction. When a commercial cellulase mixture was supplemented with xylanases, this substrate could be readily hydrolyzed, recovering most of the hemicellulose and cellulose as monomeric sugars. Following this "one-pot" approach it was possible to enhance enzyme-mediated hydrolysis of the cellulose and hemicellulose components of mechanically refined biomass substrates without the need for extensive lignin removal.

# 7. Research chapter 5: enhancing enzymatic hydrolysis of softwood chips and pellets by alkaline sulfonation and thermomechanical pulping pretreatment

### 7.1 Introduction

Enzyme-mediated deconstruction of lignocellulosic materials typically requires a pretreatment step that increases enzyme accessibility to the cellulose while recovering the majority of the hemicellulose and lignin components in a usable form (Chandra et al., 2007). It is well recognized that the lignin component of the biomass limits the effectiveness of cellulose hydrolysis, mostly due to lignin physically blocking access to the cellulose and its non-productive binding of enzymes (del Rio et al., 2011; Kumar et al., 2012a). Softwoods have been shown to be particularly recalcitrant due to their higher lignin content and the high proportion of guaiacyl lignin subunits that are prone to condensation during pretreatment.

Although earlier studies have shown that good cellulose hydrolysis can generally be achieved if most of the lignin is removed (Mooney et al., 1998; Öhgren et al., 2007), the cost of lignin removal is likely to remain too high to provide an acceptable route to renewable fuels and chemicals. Alternatively, steam pretreatment has been shown to migrate lignin by employing temperatures above the glass transition point of biomass lignin, consequently enhancing enzyme accessibility to the cellulose (Donaldson et al., 1988). Subsequent work has shown that cellulose accessibility can be further enhanced by solubilizing the hemicellulose and disrupting the cellulose structure during steam pretreatment (Mosier et al., 2005). However, more recent work showed that, due to softwood's more complex structure, it is much more difficult for the steam to access the softwood lignin (Takada et al., 2019). Thus, more severe pretreatment conditions are typically required, invariably enhancing lignin condensation and the degradation of the carbohydrate components, resulting in the formation of furfural and hydroxymethylfurfural (HMF) that inhibit downstream fermentation processes (Jönsson and Martín, 2016; Ragauskas and Huang, 2013).

Past work has assessed the potential of mild steam pretreatment followed by mechanical size reduction (Lloyd et al., 2017) in an analogous process to thermomechanical pulping (TMP) which is used to make newspaper pulps. The work described in Chapter 4 has shown how the integration of an alkaline-oxygen step during chemi-thermomechanical pulping (CTMP) resulted in partial delignification and the oxidation of the residual lignin while retaining the majority of cellulose and hemicellulose in the water-insoluble fraction. The cellulose present in this pretreated

substrate could be readily hydrolyzed using relatively low enzyme loadings. Although alkaline conditions have been shown to aid in the retention of the hemicellulose component, related work has shown that oxidation at high temperatures can lead to the removal/degradation of these carbohydrates (Fengel and Wegener, 1989). This was observed in Chapter 4 when pre-steaming at 190°C resulted in the loss or solubilization of more than 50% of the hemicellulose from pretreated Aspen chips. As softwoods are known to be more recalcitrant when compared to hardwoods, lignin oxidation might not be an ideal approach when using relatively severe pretreatment. Thus, neutral/alkaline sulfonation, which has been proven to be an effective method to selectively modify the lignin when combined with mechanical pulping (Chandra et al., 2016; Zhong et al., 2019), might better enhance the enzymatic hydrolysis of softwoods.

As well as being a major producer of pulps, British Columbia is a large producer and exporter of softwood pellets, thanks to its significant sawmill industry providing the sawdust that is predominantly used to make pellets (Pa et al., 2012). As the biochemical-based biorefinery industry develops, it is likely that a more densified feedstock will be desired, to achieve economies of scale with the smaller particles that predominate in wood pellets possibly facilitating enzyme-mediated deconstruction. However, recent work has indicated that the temperatures used during pelletisation, which are typically greater than 100°C (Takada et al., 2020; Whittaker and Shield, 2017) lead to lignin condensation. Thus, one of the objectives of the study was to compare the ease of conversion of softwood pellets and chips to assess the possibility that smaller particle size of the pellet constituents might compensate for the likely negative impact of lignin condensation occurring during pelletisation.

### 7.2 Results and discussion

Earlier work has shown that neutral sulfonation enhances the cellulose hydrolysis of steampretreated softwoods by mitigating the inhibitory effect of lignin though the incorporation of sulfonic acid groups (Kumar et al., 2012a, 2011). As subsequent research showed that the lignin underwent condensation during acidic steam pretreatment, restricting the sulfonation reaction (Rahikainen et al., 2013), more recent work focused on lignin modification prior to steam pretreatment (Chandra et al., 2016; Chu et al., 2017a). This work showed that sulfonation resulted in both greater hemicellulose recovery and increased cellulose hydrolysis by the incorporation of acid groups onto the lignin when applied prior to steam pretreatment (Chandra et al., 2016; Chu et al., 2018). This earlier work also showed that alkali addition enhanced lignin sulfonation (Chandra et al., 2016), with the addition of 2% sodium carbonate during sulfonation significantly increasing the amount of sulfonic acid groups on pretreated hardwoods (polar) from 73 to 83 mmol/kg, when using a lower sulfite loading of 8% compared to initial loading of 16%. In the work reported here we assessed if a similar approach might be successful with softwood chips steam-pretreated at 190°C prior to mechanical pulping (CTMP-based pretreatment) as well as the potential impact of increasing alkalinity (0, 2, 4, 6 and 8% of sodium carbonate) on sulfonation and enzyme-mediated cellulose hydrolysis.

As reported earlier, alkaline pretreatment resulted in a higher retention of the hemicellulose in the cellulose-rich, water-insoluble fraction (Table 15), when compared to the water control and previous acid based pretreatments (Siqueira et al., 2017). Although the alkaline treatments resulted in the retention of more than 80% of the original material in the solid fraction (Table 15), the increasing alkalinity of sulfonation removed more of the mannan, leaving the xylan component relatively unchanged. It was likely that the side chains associated with softwood xylan backbone, which was mainly composed of mainly 4-O-methylglucuronic acid, protected the xylan from alkali attack, whereas the galactoglucomannan was more prone to solubilization under alkaline conditions (Fengel and Wegener, 1989). As shown earlier, lignin is more difficult to solubilize than the hemicellulose (Kumar et al., 2012a). Thus, as expected, compared to previous work on poplar chips (Chandra et al., 2016), the alkaline treatment resulted in much lower lignin removal from softwood chips. However, the addition of sodium sulfite enhanced lignin removal (up to 30% lignin removal as compared to 13% removal with the alkali control), likely due to the sulfonation reducing the hydrophobicity of the softwood lignin. This was confirmed by the conductometric titration of acid groups in each of the pretreated substrates (Table 15) which also indicated that increasing alkalinity had a very limited impact on lignin sulfonation. It was also apparent (Table 15) that sulfonation combined with different levels of alkali addition resulted in similar levels of lignin removal (20-30%) and sulfonic acid group's addition (80mmol/kg, measured as strong acid during the conductometric titration process). In addition, each of the substrates contained about 40 mmol/kg of carboxylic acid, likely due to the cleavage of uronic acid esters in the hemicellulose (Ben et al., 1993).

*Table 15.* Chemical composition, water insoluble yields, lignin removal, carbohydrate recovery and acid groups of Chemi-thermomechanical pulp (CTMP) made from softwood chips after alkaline sulfonation.

| Sample <sup>a</sup> | Ch            | emical con   | nposition (% | <b>(</b> 0) | pretreatment<br>vield | cai        | Lignin<br>bohydrate | Acid groups<br>(mmol/kg) |              |          |           |
|---------------------|---------------|--------------|--------------|-------------|-----------------------|------------|---------------------|--------------------------|--------------|----------|-----------|
|                     | Glucan        | Xylan        | Mannan       | Lignin      | ·(%)                  | Lignin     | Glucose             | Xylose                   | Mannose      | Weak     | Strong    |
| 8%/0%               | $52\pm 2$     | $3.9\pm 0$   | $11 \pm 1$   | $25\pm1$    | 80                    | 30         | 89                  | 54                       | 72           | 40       | 80        |
| 8%/2%               | $51\pm0$      | $4.8.\pm0$   | $11\pm0$     | $26\pm1$    | 82                    | 27         | 90                  | 67                       | 68           | 40       | 86        |
| 8%/4%               | $50\pm3$      | $5.0\pm0$    | $10\pm1$     | $26\pm3$    | 81                    | 26         | 86                  | 70                       | 61           | 66       | 81        |
| 8%/6%               | $56\pm4$      | $5.1\pm0$    | $8.1\pm0$    | $27\pm0$    | 82                    | 20         | 98                  | 73                       | 51           | 54       | 80        |
| 8%/8%               | $52\pm 4$     | $5.1\pm0$    | $6.9\pm0$    | $27\pm0$    | 79                    | 25         | 88                  | 69                       | 44           | 67       | 80        |
| Water control       | $57\pm5$      | $3.4\pm0$    | $4.5\pm0$    | $34\pm2$    | 69                    | 18         | 94                  | 41                       | 24           | 40       | 0         |
| Alkali control      | $50\pm1$      | $5.0\pm0$    | $7.6\pm0$    | $29\pm1$    | 87                    | 13         | 91                  | 74                       | 50           | 40       | 0         |
| a: The CTMP sub     | ostrates were | e pre-sulfon | ated via soa | lium sulfit | e/sodium carbon       | ate loadin | g (e.g., "8%        | %/8%" ind                | dicates 8% s | odium su | lfite and |

": The CTMP substrates were pre-sulfonated via sodium sulfite/sodium carbonate loading (e.g., "8%/8%" indicates 8% sodium sulfite and 8% sodium carbonate).

As shown earlier, acid group addition enhances substrate swelling (del Rio et al., 2011) while mechanical pulping induces fibre separation and fibrillation(De Assis et al., 2018; Hoeger et al., 2013). As the Water Retention Value (WRV) has been successfully used by the pulp and paper industry to estimate fibre swelling (Ogiwara and Arai, 1968) we next used it to assess if neutral/alkaline sulfonated softwood CTMP substrate showed a higher WRV as compared to an alkali control (Figure 18) and if this increase in the WRV corresponded to an increase in cellulose hydrolysis. It was apparent that all of the sulfonated substrates were more easily hydrolyzed than either the water or alkali controls, with the addition of 4% alkali resulting in both the greatest hydrolysis and swelling, as determined by WRV (Figure 18).





To try to better clarify the possible influence of alkaline sulfonation on cellulose accessibility Transmission Electron Microscopy (TEM) was used to assess possible lignin re-distribution within the fibres. When the softwood substrates were sulfonated at 0, 4 and 8% alkali loadings and subsequently stained with 1% KMnO4, the darker areas indicated the relocation and higher concentrations of lignin (Figure 19). It was apparent that alkali addition to the sulfonation reaction resulted in increased cellulose accessibility as less lignin was observed in the secondary walls of the 4% and 8% alkali treated samples. It was likely that some of the lignin located in the cell wall was more readily attacked by the alkali enhanced sulfite ions as, at an 8% sodium sulfite loading, the addition of 4% sodium carbonate resulted in enhanced cellulose accessibility and hydrolysis with little effect on hemicellulose retention in the water-insoluble component.



**Figure 19.** Transmission Electron Microscopy (TEM) micrographs of cross-sections of softwood CTMP after sulfonation at different alkalinities. The CTMP substrates were pre-sulfonated via sodium sulfite/sodium carbonate loading (e.g., "8%/8%" indicates 8% sodium sulfite and 8% sodium carbonate).

As discussed earlier, it is likely that, to achieve needed economies of scale, densified biomass such as softwood pellets might become the predominant feedstock for any future biorefinery process. Although it was possible that the smaller particle size of wood pellet components may contribute to enhanced penetration of chemicals (Lin, 2016), a major apprehension was that the condensation/repolymerization of lignin during the pelletisation process might further increase softwood recalcitrance (Nanou et al., 2018; Takada et al., 2020; Whittaker and Shield, 2017).

It was apparent that alkaline sulfonation was significantly less effective on softwood pellets, resulting in the removal of only 12% of the original lignin and 32% of the lignin present in the CTMP treated substrate (Figure 20a, Figure 20b). Subsequent conductometric titration of the acid groups also indicated that the sulfonated CTMP substrate derived from pellets contained significantly less sulfonic acid groups (38 mmol/kg compared to 81 mmol/kg for the sulfonated chips) (Figure 20b). This indicated that the lignin in the softwood pellets was considerably more resistant to sulfonic acid group incorporation, making it less hydrophilic thus lowering its solubility in alkaline solutions. As indicated by the WRV (Figure 20c) the pellet derived



sulfonated CTMP substrate was also less swollen and more poorly hydrolyzed, likely due to the more hydrophobic lignin and residual mannan restricting enzyme access to the cellulose.

*Figure 20.* Chemical composition (a), lignin removal (b), cellulose hydrolysis and WRV (c) of alkaline sulfonated CTMP derived from softwood chips and pellets, using 8% sodium sulfite and 4% sodium carbonate. Hydrolysis was performed at 2% (w/v) solids and enzyme loading of 40 mg and 60 Ctec3 /g cellulose, at 50 °C for 72 hours.

To try to better elucidate the differential recalcitrance of softwood chips and pellets to alkaline sulfonation, we used the enzymatic mild acidolysis lignin (EMAL) extraction method (Guerra et al., 2006) to try to obtain lignin with relatively high yield while retaining its native structure. Although <sup>31</sup>P Nuclear Magnetic Resonance (<sup>31</sup>P NMR) has been successfully used to quantify the hydroxyl groups on lignin's aromatic and aliphatic structures (Pu et al., 2011b), no significant differences were observed between the lignin isolated from softwood chips or pellets (Table 16).

However, when Two-Dimensional Heteronuclear Single-Quantum Correlation Nuclear Magnetic Resonance (2D HSQC NMR) was used to assay the linkages between the subunits of the two EMAL samples it was apparent that the softwood pellet lignin contained significantly lower amount of native  $\beta$ -O-4 linkages (Table 16). This was likely due to the breakage of the ether linkages between the lignin subunits during the pelletisation process. Parallel Gel Permeation Chromatography (GPC) analysis indicated that the pellet derived lignin had a higher molecular weight (Table 16), probably due to the re-polymerization of lignin after the breakage of  $\beta$ -O-4 linkages at high temperatures(Funaoka et al., 1990). It is likely that the pellet derived lignin underwent condensation/repolymerization during softwood pelletisation, resulting in enhanced recalcitrance to subsequent pretreatment and enzymatic hydrolysis.

*Table 16.* The molecular weight, hydroxyl groups and interlinkages of EMAL isolated from softwood chips and pellets, analyzed by GPC, <sup>31</sup>P NMR and 2D HSQC NMR.

|                          |                  | Hydroxyl groups (mmol /g) |                                      |                        |                    |       |     |     | Molecular weight |             |     |
|--------------------------|------------------|---------------------------|--------------------------------------|------------------------|--------------------|-------|-----|-----|------------------|-------------|-----|
| Samples                  | Aliphatic<br>-OH | Guaiacyl<br>-OH           | C5<br>substituted<br>Phenolic<br>-OH | p–hydroxyphenyl<br>-OH | Carboxylic<br>acid | β-Ο-4 | β-5 | β-β | Mn<br>(kDa)      | Mw<br>(kDa) | PDI |
| Softwood chips<br>EMAL   | 5.7              | 1.2                       | 0.08                                 | 0.09                   | 0.14               | 39.5  | 3.2 | 0.0 | 4.9              | 33.6        | 6.8 |
| Softwood pellets<br>EMAL | 5.5              | 1.2                       | 0.13                                 | 0.12                   | 0.14               | 34.0  | 2.8 | 0.9 | 6.1              | 42.7        | 7.0 |

### 7.3 Conclusions

Neutral/alkaline sulfonation-based chemic-thermomechanical pulping (CTMP) treatment of softwood chips enhanced substrate swelling, due to the combined effects of acid groups incorporation, partial delignification and mechanical pulping, consequently enhancing cellulose hydrolysis. Increasing the alkalinity of sulfonation increased cellulose accessibility, resulting in an increase cellulose hydrolysis. Softwood pellets proved to be more recalcitrant to alkaline sulfonation than chips, resulting in poorer cellulose hydrolysis. Subsequent 2D NMR and GPC analysis indicated that the starting softwood pellets contained lignin with less  $\beta$ -O-4 linkages and a higher molecular weight, likely due to the high temperature condensation of lignin during the pelletisation process.

# 8. Overall conclusions

### 8.1 Thesis conclusions

Initial work confirmed that, although a traditional mechanical pulping approach enhanced the fibrillation of various lignocellulosic feedstocks, it resulted in only a limited increase in enzymemediated cellulose hydrolysis. For agricultural feedstocks, which are largely recognized as being more susceptible to hydrolysis, extended mechanical pulping only resulted in about 30% cellulose hydrolysis, even when using high enzyme loadings. Initial work indicated that it was likely that the lignin component which limited the effectiveness of mechanical pulping, significantly restricting cellulose accessibility and decreasing the biomass substrates susceptibility to enzymatic hydrolysis.

As past work had shown that complete delignification was too expensive to be justified as a pretreatment method, we next focused on lignin modification as one way of reducing the inhibitory effects of lignin on cellulose hydrolysis. It was apparent that both sulfonation and oxidation, which incorporated sulfonic and carboxylic acid groups respectively onto the lignin, reduced lignin's inhibitory effect on cellulose hydrolysis. Both methods facilitated substrate swelling consequently enhancing enzyme accessibility to the cellulose. When this modified lignin was isolated from sulfonated and oxidized substrates it was also shown to be more hydrophilic and had a lesser tendency to adsorb enzymes.

In the subsequent work we pretreated various biomass feedstocks under mild alkaline conditions. In this way we could take advantage of mechanical pulping's ability to retain high solid yields, while mitigating lignin's inhibition of cellulose hydrolysis by sulfonation and oxidation. Compared to acid pretreatments, alkaline approaches were more selective at retaining the labile hemicellulose in the water-insoluble fraction. They also partially removed the surface lignin and induced the deacetylation of the hemicellulose component of agricultural feedstocks, thereby increasing enzymatic hydrolysis.

As the hardwood hemicellulose also contained acetyl groups, the alkali treatment of hardwoods was anticipated to enhance its accessibility via deacetylation. However, hardwoods also contain

more hydrophobic and higher-molecular-weight lignin, which might be more resistant to dissolution via the alkali solution. Thus, when we pretreated hardwood chips, pre-impregnation of alkali and oxygen was employed to facilitate lignin oxidation. This was done prior to a thermomechanical pretreatment conducted at a higher temperature of 190°C, as a demonstration of the CTMP-based pretreatment process. The resulting substrate was rich in carboxylic acid groups, indicating the oxidation of lignin. Although the addition of alkali improved hemicellulose retention in the water-insoluble component, pre-steaming at 190°C still resulted in around 50 % removal of the hemicellulose from the hardwood substrate. However, by lowering the pre-steaming temperature to 130 °C, which was close to the temperature of a typical thermomechanical pulping approach, we managed to increase the hemicellulose retention to 70%. Although this reduced delignification from 40% to 25%, the enzymatic hydrolysis yield of the resulting substrate was not significantly reduced as the residual lignin contained a much greater number of carboxylic acid groups.

Alkali pretreatment had a limited impact on lignin removal of softwoods, even at a pre-steaming temperature of 190 °C. However, the addition of sulfite facilitated lignin removal by sulfonic acid group incorporation. Alkali addition also benefited the sulfonation reaction that predominantly occurred within the secondary-cell-wall lignin. However, excessive alkali addition compromised the recovery of hemicellulose, especially the mannan. Although softwood pellets have the advantage of smaller particle size their lignin contained less native  $\beta$ -O-4 linkages and had higher molecular weight in comparison to softwood chips. This condensed lignin structure was likely due to the temperatures and conditions used during the pelletisation resulting in the higher recalcitrance of softwood pellets to pretreatment and subsequent enzymatic hydrolysis.

Under optimum sulfite and alkali loadings, only half of the sulfonic acid groups were incorporated onto the pellets compared to the chips. This resulted in limited lignin removal and substrate swelling. Unlike the CTMP-pretreated softwood chips, where up to 80% of the cellulose component could be hydrolyzed, less than half of the cellulose within the pellets was hydrolyzed.

### 8.2 Possible future work

# 8.2.1 Can the hydrolysis of the pretreated biomass be enhanced by optimizing the pretreatment conditions and enzyme cocktails?

Softwoods, especially softwood pellets, proved to be more recalcitrant to CTMP-based pretreatment

compared to hardwood and agricultural biomass. We could try to improve the effectiveness of CTMPbased pretreatment of softwood pellets by adjusting the impregnation approaches, with the goal of enhancing chemical penetration and lignin sulfonation.

As well as the pretreatment process, the hydrolysis of softwood substrates could likely be enhanced by optimizing the enzyme cocktails. Unlike agricultural residues and hardwoods, softwood hemicellulose contains mannan which was retained in the water-insoluble component after CTMP-based pretreatment. However, the current enzyme cocktail (Cellic CTec 3) lacks mannanase activities. Supplementing the enzyme cocktail with mannanases could facilitate mannan hydrolysis, enhancing cellulose accessibility by exposing the cellulose previously "buried" within the fibre structure.

As lignin is recognized as the main hurdle in readily hydrolyzing softwoods, future work could try to improve hydrolysis by supplementing the cocktail with "lignin-degrading" enzymes such as lignin peroxidases and laccases. This might degrade the lignin during enzymatic hydrolysis, mitigating lignin's negative impact on cellulose accessibility.

#### 8.2.2. Better quantification of cellulose accessibility

Simons' staining proved to be a good method to estimate cellulose accessibility of pretreated biomass. This method utilizes the high-molecular-weight fraction of direct orange dye, which has a high affinity for cellulose over hemicellulose and lignin. However, the major limitation of Simons' staining is the heterogeneous nature of the direct orange dye. The current method isolates the high-molecular-weight fraction of direct orange dye through ultrafiltration. However, it only collects the dye within a specific molecular weight range (>100 kDa). This is likely one reason why the Simons' stain showed low batch to batch reproductivity. Future work should look at further improving the isolation process. Perhaps using a more precise filtration procedure.

In addition to Simons' staining, recent studies have shown that cellulose binding modules (CBMs) isolated from actual cellulase enzymes can better quantify cellulose accessibility to enzymes. However, most of the current work has focused on the binding CBMs to substrates with a low lignin content (CBMs also bind to lignin through hydrophobic interactions). Recent work has indicated that blocking the lignin with bovine serum albumin (BSA) in advance might overcome this issue. Therefore, future work could use a similar approach to better quantify the cellulose accessibility of CTMP-pretreated biomass substrates using specific CBMs combined with prior "lignin blocking".
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