# Investigation of a two-stage steam/organosolv pretreatment approach for the fractionation of softwood biomass

by

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#### Abstract

In order to improve the economic viability of a bioconversion process it would be extremely beneficial to maximize the recovery of all of the lignocellulosic components while enhancing enzyme accessibility to the cellulosic component. Wood residue derived pellets are already a prominent Canadian commodity and the existing supply-chain produces a high density, low moisture feedstock suitable for mass collection and transport. However, pellets are almost exclusively used for combustion and not as a possible biorefinery feedstock. As a result, there is limited information on the influence of the pelletization process (e.g. grinding, drying, compressing) on the susceptibility of pellets, as opposed to chips, to the various pretreatment, fractionation and cellulose hydrolysis steps that are components of a typical bioconversion process.

The work described in the thesis assessed the potential of a two-stage steam/organosolv pretreatment process to fractionate and isolate the hemicellulose and lignin components from softwood pellets, yielding a more accessible, cellulose-rich substrate. Various steam pretreatment conditions were compared for their ability to enhance hemicellulose solubilisation while minimizing lignin condensation (first-stage), to improve subsequent organosolv delignification (second-stage). Carbocation scavengers were compared for their ability to minimize lignin condensation during either stage. When softwood chips and pellets were compared, the effectiveness of the pretreatment was determined by hemicellulose solubilisation, delignification capability and the ease of enzymatic hydrolysis of the cellulosic component.

It was apparent that pellets were more responsive than chips to pretreatment due to their smaller particle size, which facilitated both hemicellulose solubilisation and delignification. At conditions that solubilized and recovered hemicellulose, acid-catalyzed steam pretreatment induced lignin condensation. This impeded subsequent organosolv delignification and enzymatic hydrolysis. The addition of lignosulfonates as a potential carbocation scavenger during acid-catalyzed steam pretreatment resulted in increased hemicellulose solubilisation and carbohydrate recovery while improving delignification during subsequent organosolv

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treatment. Adding lignosulfonates during acid-catalyzed steam pretreatment also enhanced enzymatic hydrolysis. It was likely that the added lignosulfonates increased lignin hydrophilicity which facilitated lignin dissolution and decreased non-productive enzyme inhibition. It was apparent that the addition of lignosulfonates prior to pretreatment reduced the detrimental effects of lignin condensation which benefited subsequent fractionation of the pretreated biomass.

## Preface

All the work presented henceforth was conducted by Mandy Lin in the Forest Products Biotechnology/Bioenergy Laboratories at the University of British Columbia, Point Grey campus.

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## List of units and abbreviations

4HBA	4-hydroxybenzoic acid
Å	Angstrom
AIL	Acid insoluble lignin
ALCELL	Alcohol-cellulose
ASAM	Alkaline sulfite anthraquinone methanol
BC	British Columbia
BSA	Bovine serum albumin
С	Celsius
cal	Calorie
cm	Centimeter
δ	Hildebrand solubility parameter (cal/cm <sup>3</sup> ) <sup>1/2</sup>
Da	Dalton
DP	Degree of polymerization
EAS	Electrophilic aromatic substitution
EOL	Ethanol organosolv lignin
FPU	Filter paper unit
g	Gram
GPC	Gel permeation chromatography
h	Hour
$H_2SO_4$	Sulfuric acid
ha	Hectare
HCI	Hydrochloric acid
HPLC	High performance liquid chromatography
kg	Kilogram
km	Kilometer
λ	Wavelength
L	Liter
LCC	Lignin-carbohydrate complexes

LS	Lignosulfonates
μm	Micrometer
m	Meter
М	Mole per liter
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimole per liter
mmol	Millimole
M <sub>n</sub>	Number average molecular weight
Mt	Million metric tonnes
Mw	Weight average molecular weight
$Na_2SO_3$	Sodium sulfite
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
o.d.	Oven dry
РНК	Prehydrolysis kraft
Ro	Severity factor
rpm	Revolutions per minute
SO <sub>2</sub>	Sulfur dioxide
SPORL	Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose
Т	Temperature
t	Time
ΤΑΡΡΙ	Technical Association of the Pulp and Paper Industry
THF	Tetrahydrofuran
ТМР	Thermo-mechanical pulp
v/v	Volume per volume
w/	with

w/o Witho
-----------

- w/w Weight per weight
- XPS X-Ray photoelectron spectroscopy

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#### 1 Introduction

#### 1.1 Background

Factors such as the global economic downturn, increase in paperless communications as well as a push for more sustainable practices have been the impetus for the shift of the forest products sector from traditional paper products towards a "biorefinery" model. Lignocellulosic biomass is a major natural resource in Canada, with more than 2 million km<sup>2</sup> of managed forests and an estimated 30 to 66 m<sup>3</sup> ha<sup>-1</sup> of forest biomass residues being made available annually in Canada (Paré et al., 2011). In addition, the mountain pine beetle epidemic which has ravaged the lodgepole pine supply on the Canadian west coast in the last decade has also provided a large abundance of softwood biomass that will have limited application as lumber. Softwoods represent a prime source for the production of cellulose, hemicellulose and lignin, but are the most recalcitrant biomass compared to hardwoods and agricultural biomass. In addition, with woody biomass in general, their low density and high moisture content makes transportation of this material inefficient. Therefore, pelletized lignocellulosic biomass presents itself as an alternative feedstock choice that would be viable for use in a biorefinery process. The pelletization process decreases the moisture and increases the density, benefitting efficient transportation. In addition, pelletization decreases the particle size of the lignocellulosic biomass, which has been shown to increase the susceptibility of lignocellulosic biomass to further processing by methods such as pretreatment and pulping (Cullis et al., 2004). With these underutilized resources available, they represent the prime feedstock for future biorefinery operations.

In a hypothetical biorefinery, lignocellulosic biomass will be treated more as a chemical feedstock to produce a wide array of products, analogous to that of a petroleum refinery. Unlike fossil fuels, lignocellulosic biomass has the marked advantage of being sustainable, renewable and with the added benefit of acting as a carbon sink to sequester carbon-dioxide emissions. In the same vein, innovations in the forest sector have expanded from traditional forest products into products such as textile fibres, bio-plastics, bio-fuels, neutral-pharmaceuticals, dyes and chemicals, transforming lignocellulosic biomass from a bulk material

into a higher value chemical feedstock (Cherubini, 2010). Lignocellulosic biomass has been shown to have the potential for producing analogous products to those made in a petroleum refinery, establishing lignocellulosic biomass as a candidate feedstock to replace fossil fuels in the future (Cherubini, 2010; Hu *et al.*, 2011). However, the success of a biorefinery initiative is contingent on its ability to separate the biomass into the cellulose, lignin and hemicellulose components to maximize the potential value obtainable from the biomass. Unfortunately, woody biomass was designed by nature to remain intact, which makes it ideal for use as a building material but presents significant obstacles toward strategies that aim to cleanly separate and recover the individual components for downstream applications. A prime example illustrating the challenges associated with fractionating woody biomass is the many processing steps required to make pulp, including debarking, chipping, pulping with high chemical loadings, washing, screening and bleaching. Therefore, it is crucial to determine an effective, simplified pretreatment method for fractionating lignocellulosic biomass into clean and usable components.

Bioconversion has been one of the main technologies that have been investigated as the "backbone" of a biorefinery. The bioconversion process typically consists of three major steps: pretreatment to improve biomass accessibility, enzymatic hydrolysis of the cellulose to glucose and fermentation of the hemicellulose and cellulose derived sugars. The pretreatment step increases the biomass accessibility as part of a bioconversion process and is paramount in facilitating the fractionation of the cellulose, hemicellulose and lignin components in an overall biorefinery scheme. When considering current pretreatment methods that have been shown to be effective for processing recalcitrant softwood biomass, each has roots in the pulp and paper industry, including steam, organosolv and SPORL (Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose) pretreatments which originated from the Masonite, Alcell and chemithermomechanical pulping processes respectively. However, current research in biomass pretreatment has mainly focused on the challenges of maximizing cellulose hydrolysis, often compromising the recovery or "usability" of the hemicellulose and lignin components. This is mainly because increasing the ease of cellulose hydrolysis has been a sufficiently challenging objective in its own right. However, there have been breakthroughs in the development of

cellulolytic enzymes that have enabled lower enzyme loadings to be employed for enzymatic hydrolysis (Lane, 2012). Therefore, maximizing the clean separation and recovery of each of the constituents of biomass to maximize the value obtainable is now becoming a bigger focus. It is likely that developing effective fractionation methods that facilitate the purification of the cellulose component through its clean separation from the hemicellulose and lignin components would also increase the ease of hydrolysis of the resulting substrates by cellulases.

Lignin potentially has high value as a natural source of phenolics and as a sustainable replacement for many fossil fuel derived compounds (Hu *et al.*, 2011; Wang *et al.*, 2014). However, lignin is also one of the most complex components of lignocellulosic biomass to fractionate, due to its heterogeneous physical and chemical structure. In addition, the large, lignin macromolecules within the biomass structure are prone to both depolymerization and repolymerization during pretreatment, resulting in a need to design experiments to favour the depolymerization pathway in order to promote efficient extraction. Softwood lignin, in particular, is known to be particularly recalcitrant towards pretreatment when compared to lignin in other feedstock types such as hardwood and agricultural residues. Its recalcitrance is partially due to its higher guaiacyl lignin content that is more sensitive to forming stable condensation products during pretreatment. This can potentially impede hydrolysis, extraction and downstream applications. Thus, by better understanding the mechanisms which influence the behaviour of lignin under different conditions, it should be possible to gain some insights into designing pretreatment processes suitable for lignocellulosic biomass fractionation resulting in more value for a biorefinery approach.

Overcoming the recalcitrance of softwood biomass will be needed if we are to fully maximise a proposed biorefinery process. One of the major objectives of the work described in this thesis was to assess the effectiveness of a two-stage pretreatment process to cleanly fractionate softwood biomass while determining how much guaiacyl lignin contributed to the recalcitrance of softwoods. In addition, the application of pellets in a biorefinery can potentially benefit the logistics of the process and help realize economies of scale during industrialization. This thesis studied the ability of a two-stage steam/organosolv pretreatment process to cleanly

fractionate softwood biomass and assessed the pretreatment susceptibility of pellets to the process as compared to wood chips.

#### 1.2 Pellets

One of the major limitations to realising a lignocellulose based biorefinery is reliable access to large quantities of biomass (Kurian et al., 2013). Previous work has suggested that biorefineries will be limited to a processing capacity of less than 5000 tonnes of biomass per day due to the challenges in and cost of transporting and storing low density lignocellulosic biomass (Kim and Dale, 2015). In comparison, most kraft pulp mills can operate at an average production volume of 1000-2000 tons of pulp per day at a product yield of 45%, with larger pulp mills like Eldorado Papel e Celulose Ltda of Eldorado, Brazil being capable of producing more than 5000 tons of pulp per day (Pulp Paper News, 2014; Sjöström, 1993; Smith 1997). The low density and high moisture qualities of lignocellulosic biomass makes collection and transportation of these residues to a centralized processing facility a costly endeavor. It will be influenced by factors such as geographic location, collection method and biomass type. Estimates have placed delivery costs at 35-50% of the total cost of biofuel production (Kumar et al., 2006). A potential solution to improve transportation efficiency is biomass densification methods such as pelletization. Through pelletization, biomass density more than doubles while the moisture content decreases to less than 10%, thus improving the logistics of transporting biomass to and from remote locations (Gilbert et al., 2009; Kurian et al., 2013; Stephen et al., 2010; Sultana and Kumar, 2011; Wu et al., 2011). Although the pelletization process incurs additional costs and results in a more expensive feedstock compared to wood chips (approximately \$280 and \$200 CAD/tonne respectively as of December 2016 according to FOEX), pellets tend to be shipped over long distances compared to pulp chips. The enhanced mobility of pellets is demonstrated by their use in biomass fueled power plants such as the Drax Power Station in the UK that will have an estimated consumption of seven to eight million tonnes of pellets per year by 2017, with most of the pellets derived from North America (FOEX, 2016; Wood Pellet Association of Canada, 2016). Currently, pellets are primarily produced and used as a solid biofuel for residential and industrial heating and power generation, with global production estimated at 14.3 Mt in 2010 (Goh et al., 2013). With increasingly ambitious

government policies to promote the switch to renewable energy consumption (20% renewable by 2020), the use and production of wood pellets are projected to continue to rise in the coming years (Sikkema *et al.*, 2011). In Canada, almost 85% of the country's pellet production, amounting to 1.3 million tonnes, are exported to Europe. This demonstrates the feasibility and extent of the market for pellets as a future feedstock choice for a biorefinery operation (Natural Resources Canada, 2016). In addition, the mountain pine beetle epidemic in BC also presents an abundance of beetle killed wood that has limited application as lumber and could be used as a feedstock for pellet production. Pellets also present a more "normalized" substrate with a relatively consistent particle size and moisture content that can eliminate many feedstock variables and further streamline biomass handling and experimental design (Cullis *et al.*, 2004; Kurian *et al.*, 2013). However, while pellets are appealing from a logistics standpoint, there is limited information as to how the pelletization process may affect the biomass characteristics and whether it will affect how they respond to subsequent treatments.

During the pelletization process, the starting wood chips are mechanically ground to a smaller particle size and dried to reduce their moisture content before being compressed into the final pellet. Depending on the pelletization method used, the biomass may be repeatedly exposed to high temperatures, which can potentially encourage "lignin flow" during the densification step of the pellets (Stelte *et al.*, 2011b; Stelte *et al.*, 2012). Lignin flow occurs when the biomass is exposed to temperatures greater than the lignin's glass transition temperature, which is the temperature at which lignin changes from a glassy to a plastic state. The glass transition temperature for lignin has been shown to range between 50°C to greater than 100°C and is dependent on various factors such as moisture content and lignin type (Stelte et al., 2011b). As a result, lignin can flow and be redistributed onto the surface of the biomass during pelletization which, although improving pellet durability, potentially reduces the accessibility of the cellulose component to deconstructive enzymes (Kumar et al. 2010; Stelte et al., 2011b). Drying of the substrate can also result in irreversible closing of the pits in the cell wall, limiting liquor penetration between the cell walls during treatment (Petty and Puritch, 1970). In addition, the densification step may also physically collapse the internal pore structure, which can further reduce pretreatment and enzymatic susceptibility (Mani et al.,

2004, Grethelein, 1985). In contrast, the size reduction steps could also be advantageous in increasing the overall surface area and improve substrate susceptibility to treatment when compared to wood chips (Cullis *et al.*, 2004). However, previous work on hybrid poplar has also contradicted the effect of particle size on treatment response to acid-catalyzed steam pretreatment (Morales-Vera *et al.*, 2016). Therefore, there is still much to learn with regards to the influence that the pelletization process might have on the subsequent processability of the biomass feedstock.

While the pelletization process conditions have an effect on the resulting pellet characteristics, the starting biomass used also has an influence on the quality of the pellets. Woody biomass has already been industrially established as the primary lignocellulosic feedstock choice for pellet production. Wood pellets are generally produced from sawdust and wood shavings (sawmill by-products) but can also be produced from tree tops and branches (Andersson et al., 2006). Densification of agricultural residues in the form of pellets or briquettes has also been investigated to improve the transportation, handing and storage of the material, with varying success (Gilbert et al., 2009; Kaliyan and Morey, 2010; Stelte et al., 2011a). As mentioned earlier, the high temperature experienced by the biomass during pelletization helps to soften and "plasticize" the lignin, transforming it into a binder to increase particle cohesion (Gilbert et al., 2009; Stelte et al., 2011b; Stelte et al., 2012). Agricultural residues typically have a lower lignin content that results in lower strength properties compared to woody residues. Agricultural residue derived pellets have been shown to benefit from binding additives such as heavy pyrolysis oil to improve pellet properties (Gilbert et al., 2009; Stelte et al., 2011a). The densification of agricultural residues such as wheat straw is also complicated by the presence of surface waxes that weaken the strength of the pellets due to the formation of a weaker boundary layer (Stelte et al., 2011a; Stelte et al., 2012). Therefore, feedstocks that are typically higher in lignin content, such as hardwoods and softwoods, have been shown to respond more favourably to pelletization and produce pellets with better strength properties (Stelte et al., 2011a). In the Canadian Pacific Northwest, softwoods are the most prominent tree type and are consequently the primary feedstock source for pellet production. In addition, the mountain pine beetle epidemic combined with the prominent

forestry industry in the region provides a rich source of underutilized softwoods such as lodgepole pine. While softwood biomass has been successfully demonstrated for producing durable pellets, the recalcitrant nature of softwoods towards bioconversion still poses additional challenges towards successfully utilizing softwoods pellets for a biorefinery approach.

#### 1.3 Softwoods as a feedstock

More than 80% of BC's forests are composed of softwood species, with lodgepole pine dominated forests being the most common type, followed by spruce, true firs, hemlock and Douglas-fir (*The State of British Columbia's Forests*, 2010). Softwoods contain approximately 43-45% cellulose and 20-23% hemicellulose that, theoretically, can be converted to 455 L of ethanol per metric ton of dry material (Galbe and Zacchi, 2002). In addition, softwoods on average have the highest lignin content, at 25-35% compared to 10-30% and 18-25% for agricultural residues and hardwoods respectively (Sun and Cheng, 2002). The higher lignin content found in softwoods presents an attractive prospect for a natural phenol source that can be transformed into various downstream applications, such as chemical precursors or phenol replacement for applications such as in phenol-formaldehyde resins (Wang *et al.*, 2014). However, the fractionation and utilization of softwoods presents various challenges when compared to other lignocellulosic biomass sources such as hardwoods or agricultural residues.

Cellulose, the most prominent biomass component, is a homogeneous, linear polymer that is chemically and structurally similar across different biomass types. In contrast, hemicellulose is a heterogeneous polymer that is composed of a range of different sugars, including arabinose, galactose, glucose, xylose and mannose (Sjöström, 1993). Depending on the biomass type, the relative proportions of these monomeric units will vary. Agricultural residues primarily consist of arabinoxylans, hardwoods of glucuronoxylan and softwoods of galactoglucomannan (Saha, 2003; Sjöström, 1993). In addition to differences in their sugar composition, hemicellulose from different biomass sources also differ by their degree of acetylation, defined as the ratio between the total acetyl groups to the total amount of monomers that can have those groups. Comparing the hemicellulose derived from the two

predominant woody biomasses, glucuronoxylan from hardwoods have been shown to have a degree of acetylation between 0.6 to 0.75 while galactoglucomannan in softwoods are acetylated at around 0.3-0.4 (Pawar *et al.*, 2013; Teleman *et al.*, 2000; Teleman *et al.*, 2003).

Since glucuronoxylan and galactoglucomannan are the predominant hemicellulose types in hardwoods and softwoods respectively, softwoods generally possess a lower overall acetyl content when compared to hardwoods. The lower degree of acetylation contributes to one of the major reasons why softwoods do not respond as favourably to auto-catalyzed conditions when compared to xylan rich biomass types (Dekker, 1987; Galbe and Zacchi, 2002). Autocatalyzed pretreatment involves the hydrolysis and release of the hemicellulose acetyl groups under elevated temperatures, providing the required acid catalyst for hydrolysing the carbohydrate component. The released acid can also result in acidolysis and/or condensation of the lignin component. This will be covered in more detail in the subsequent section. Since softwoods do not possess as many acetyl groups to be hydrolyzed and released, additional acid is often required to produce a substrate amenable to enzymatic hydrolysis, contributing to extra costs (Dekker, 1987; Galbe and Zacchi, 2002). In addition to having the potential to act as an acid catalyst, the acetyl groups on the hemicellulose can also act as a "protective layer" to prevent degradation under alkaline conditions (Helmerius et al., 2010). In contrast, hemicelluloses that are less substituted, such as glucomannan, will be more prone to degradation and, therefore, more difficult to recover and valorize (Helmerius *et al.*, 2010). However, the higher mannan content and lack of acetylation can also be seen as a benefit for softwoods. Mannose, a hexose sugar like glucose, can be easily fermented with baker's yeast while xylose, a pentose sugar, requires the use and development of genetically engineered yeast strains (Galbe and Zacchi, 2002). In addition, the release of acetyl groups (acetic acid) during pretreatment can become inhibitory to downstream processes such as fermentation, which decreases ethanol concentration and yield (Chen et al., 2012; Keating et al., 2006; Kundu et al., 2015). As a result, additional steps such as deacetylation or anionic exchange resins are implemented to remove the generated inhibitors when utilizing substrates with highly substituted hemicelluloses, resulting in additional cost concerns (Cho et al., 2010; Nilvebrant et al., 2001).

Lignin is the third major component in lignocellulosic biomass and differs greatly, both chemically and physically, from the carbohydrate component. It is a heterogeneous, highly branched, amorphous polymer that is composed of three types of phenylpropanoid monomers: p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) (Figure 1) (Lin and Dence, 1992). There is an absence of a regular, repeating or ordered pattern in its polymeric structure, leading to a much more physically and chemically heterogeneous component when compared to the carbohydrate fraction (Lin and Dence, 1992). As with hemicellulose, lignin composition also varies between different biomass types. For example, agricultural residue contains all three lignin types, hardwoods contain syringyl and guaiacyl lignin and softwoods contain mostly guaiacyl lignin (Lin and Dence, 1992).



# Figure 1. Chemical structures of phenylpropanoid monomers (a) p-hydroxyphenyl (pcoumaryl alcohol, H), (b) guaiacyl (coniferyl alcohol, G) and (c) syringyl (synapyl alcohol, S).

The chemical structure of the native lignin is a factor that greatly influences the behaviour of the lignin when the biomass is subjected to different pretreatments. Considering the two main lignin subunit types in woody biomass, guaiacyl and syringyl, the correlation between their relative proportions and ease of delignification has been demonstrated in previous alkaline extraction research (del Río *et al.*, 2005; Santos *et al.*, 2011; Tsutsumi *et al.*, 1995). When Santos *et al.* (2011) compared a range of hardwood species that had differing syringyl/guaiacyl ratios they found that an increasing syringyl/guaiacyl ratio correlated to an increase in delignification during kraft pulping. The difference between the two subunits stem from the fact that the aromatic ring in a guaiacyl unit is only methoxylated at the C-3 position,

while a syringyl unit is methoxylated at both the C-3 and C-5 positions. The open C-5 position on guaiacyl units allows the formation of additional linkages, such as 5-5' carbon-carbon bonds, with other monolignols during treatment (Kisimoto *et al.*, 2010; Lourenço *et al.*, 2012). The formation of these stable carbon-carbon bonds will, in turn, hinder further delignification as they are virtually impossible to break under conventional pretreatment or pulping conditions. As a result, the increased potential to form these additional linkages gives rise to the more recalcitrant nature of softwood lignin to extraction as they contain the highest proportion of guaiacyl lignin (Shimada *et al.*, 1997; Tsutsumi *et al.*, 1995; Wikberg and Maunu, 2004). Therefore, in order to access the potential of softwoods as a lignin source, it is necessary to optimize the pretreatment methods to facilitate a clean separation of lignin.

#### 1.4 Lignin applications

Currently, the majority of lignin is produced and supplied by the pulp and paper industry, with lignin produced from sulfite pulping methods dominating the existing market (Norgren and Edlund, 2014). Sulfite lignin, also termed lignosulfonates, are modified during the pulping process by reaction with bisulfite ions, which introduces sulfonate groups to the lignin and increases its overall hydrophilicity (Lora, 2008). As a result, lignosulfonates are coveted for their water soluble properties and have an application as binders, complexing agents, dispersants and emulsifying agents (Lora, 2008). Although Kraft pulping is the most commonly used pulping process, the produced alkali soluble lignin has a much smaller market in applications such as pesticides and dispersants for dyes (Lora, 2008). Despite the large quantity of lignin being extracted from pulp and paper production, less than 2% of that lignin actually makes it to commercial applications, with the remaining lignin being combusted at the mill for energy production and chemical recovery (Lora, 2008). However, it is estimated that only 60% of the lignin from the processed biomass would be required to provide energy for the process while the remaining lignin can and should be used for the development of value added products (Ragauskas et al., 2014). In addition, it may make more sense to combust lignin for energy in areas where electricity is at a premium while using the lignin for other value added products may be better where electricity is more economical.

The lack of lignin applications to higher value bioproducts is largely due to its complex and heterogeneous nature, both physically and chemically, which poses challenges during its extraction, isolation, characterization and utilization (Norgren and Edlund, 2014). During pulping and pretreatment processes, native lignin will undergo changes that will greatly influence the ease of extraction and final applications of the lignin. Ideally, one would want to produce a lignin of high purity, low polydispersity, high functionality (e.g. phenolic, hydroxyl) and high reactivity (Pan *et al.*, 2005; Sannigrahi *et al.*, 2010). Being able to produce a homogeneous yet versatile lignin would be invaluable for use as a chemical feedstock. Therefore, it is important to better understand how different pretreatment conditions change the chemical and physical structure as well as the distribution of lignin so that pretreatments can potentially be "tuned" to produce lignin with properties desirable for a particular application.

#### **1.5 Delignification**

Economically, removing and recovering the lignin component in a usable form is one of the major obstacles to using woody lignocellulosic biomass in a biorefinery approach. While pulping processes have been able to develop effective delignification methods over the course of the last century, the high capital and process costs precludes the use of the cellulose for fuel. When subjected to acid/alkaline pretreatment/pulping, lignin has the potential to depolymerize (delignification) as well as re-polymerize (condensation). Effective lignin depolymerization assists in the extraction and solubilization of the lignin into the treatment liquor while increased crosslinking during lignin condensation can greatly reduce the extractability of lignin and would require the removal of the carbohydrates in order to utilize the remaining lignin as a by-product (Zhang et al., 2014). In addition, previous work has shown that decreasing the molecular weight of the lignin is beneficial to its subsequent extractability and solubility (Schuerch, 1952; Ziebell et al., 2010). While the heterogeneous, non-repeating structure of lignin presents a challenge in understanding lignin chemistry, there are some common linkage types between the phenylpropanoid monomers that are of more interest. The most common linkage type,  $\beta$ -O-4, comprises over 50% of the total linkages and, alongside  $\alpha$ -O-4, are the most studied when investigating delignification mechanisms (McDonough, 1993; Sjöström, 1993). Therefore, better

understanding of lignin chemistry and the mechanisms that affect the breakdown of the larger lignin macromolecule will be important if an effective pretreatment process is to be developed.

#### 1.5.1 Acidolysis

Alkaline processes are predominantly used in pulping for their ability to effectively delignify woody substrates while retaining hemicellulose and preserving fibre quality. However, the high cost of alkaline processes limits its application in bioconversion and, as a result, most pretreatment processes that have been investigated for bioconversion, such as steam and organosolv, have been conducted under acidic conditions. While acidic conditions can be more favourable towards lignin condensation, delignification via acidolysis of the lignin will also occur at both the  $\alpha$ -O-4 and  $\beta$ -O-4 aryl-ether linkages (McDonough, 1993). The mechanism of cleavage of the  $\alpha$ -O-4 bond involves the protonation of the oxygen atom in the ether bond, providing a good leaving group and thus cleaving the  $\alpha$ -O-4 bond and producing a carbocation (Figure 2a). The formed benzyl carbocation can be resonance stabilized, such as through the formation of a quinone methide intermediate.

Cleavage of the  $\beta$ -O-4 bond is initiated in a similar fashion as the  $\alpha$ -O-4 mechanism, in that the hydroxyl group on the alpha carbon becomes protonated, leaves and produces a carbocation at the alpha carbon (Figure 2b). To stabilize this carbocation, a double bond is formed between the alpha and beta carbon and a proton on the beta carbon is lost. The oxygen atom in the  $\beta$ -O-4 bond then becomes protonated and, once again, produces a good leaving group that produces a carbocation at the  $\beta$ -carbon. Water is then able to add to the  $\beta$ -carbon (losing a proton in the process) and an enol is formed which then tautomerizes to form a ketone at the  $\beta$ -carbon (Li and Gellerstedt, 2008; McDonough, 1993). However, delignification can be inefficient as there are competing acidolysis and condensation pathways once the carbocation species is formed.

The tendency for the lignin to undergo condensation can also be minimized by removing the end products as they are formed. An example is hot water flow-through pretreatment, which has been shown to achieve near complete removal of lignin from poplar biomass with mild modifications to the lignin structure (Zhang *et al.*, 2015). During the pretreatment,

significant cleavage of  $\beta$ -O-4 bonds in the lignin was observed, which assisted in the lignin removal, while the flow-through action helped to remove and dilute the end products and prevent their re-polymerization to the residual lignin in the biomass. The ability to shift the balance between depolymerisation and re-polymerization by varying the pretreatment conditions, using chemical additives (as discussed in more detail below) and/or changing the biomass source can increase the likelihood of developing strategies to fragment lignin to enable its clean separation.



Figure 2. Acidolysis of lignin through the cleavage of (a)  $\alpha$ -O-4 or (b)  $\beta$ -O-4 aryl-ether linkages. Adapted from McDonough (1993) and Del Rio (2012).

## 1.5.2 Solvolysis

Solvolysis is another method of delignification that can be applied under acid or alkaline conditions. Solvolysis is the general description for reactions that involve lignin cleavage via the

solvent that occur during pretreatment methods such as organosolv. Under acidic conditions, solvolysis can occur at both  $\alpha$ -O-4 and  $\beta$ -O-4 aryl-ether linkages (Figure 3-4) (McDonough, 1993; Meshgini and Sarkanen, 1989).



Figure 3. Solvolysis of lignin through the cleavage of  $\alpha$ -O-4 aryl-ether linkages. Adapted from McDonough (1993) and Del Rio (2012).



Figure 4. Solvolysis of lignin through the cleavage of  $\beta$ -O-4 aryl-ether linkages by (a) nucleophilic attack or (b) release of formaldehyde. R' = -OH, -OCH3, etc., L = lignin. Adapted from McDonough (1993) and Del Rio (2012).

Cleavage at the  $\alpha$ -carbon via solvolysis starts similarly to acidolysis and the oxygen in the ether linkage is first protonated, generating an alcohol leaving group. A carbocation intermediate is then subsequently formed, allowing the nucleophilic addition of either water or an alcohol solvent such as ethanol (Figure 3). For cleavage at the  $\beta$ -carbon, two mechanisms have been proposed, both of which involve the formation of a carbocation intermediate at the  $\alpha$ -carbon. In the first, faster mechanism, the carbocation intermediate formed at the  $\alpha$ -carbon is stabilized by the formation of a carbon-carbon double bond and the release of the proton at the  $\beta$ -carbon (Figure 4a). The  $\beta$ -carbon is then vulnerable to nucleophilic attack by water/alcohol solvent, resulting in the rapid cleavage of the  $\beta$ -O-4 bond (Sarkanen, 1990). The second, slower mechanism involves the release of formaldehyde ( $\gamma$ -carbon) to stabilize the carbocation intermediate, after which a double bond is formed between the  $\alpha/\beta$ -carbons and cleavage of the  $\beta$ -O-4 bond occurs (Figure 4b) (McDonough, 1993).

Cleavage at  $\alpha$ -O-4 has been shown to require a lower activation energy and, therefore, occurs at a faster rate under the same reaction conditions (Meshgini and Sarkanen, 1989). However, while cleavage of  $\beta$ -O-4 requires more severe conditions to occur, it is one of the most common linkage types in lignin and accounts for 50-60% of interunit linkages (McDonough, 1993; Sjöström, 1993). Therefore, understanding the mechanisms at both  $\alpha$ -O-4 and  $\beta$ -O-4 linkages are important for designing experiments to promote lignin fragmentation.

#### **1.6 Condensation (electrophilic aromatic substitution)**

As mentioned earlier, lignin has the potential to undergo both depolymerization and repolymerization reactions during treatment. During re-polymerization, lignin will undergo electrophilic aromatic substitution (EAS) which, in a general sense, is when an electrophile will replace an atom (typically hydrogen) on an aromatic system (McDonough, 1993; Sjöström, 1993). In the case of lignin re-polymerization, the electrophile will be another lignin molecule (e.g. carbocation), resulting in increased, intermolecular crosslinking of the lignin within the biomass. On the lignin aromatic ring, substitution can happen at both the C-2 and C-6 position and also the C-5 position in the case of guaiacyl lignin (Sjöström, 1993). The additional open C-5 position on guaiacyl lignin reduces steric hindrance and allows the potential to form more linkages when compared to syringyl lignin. This increases the likelihood of guaiacyl lignin to undergo lignin condensation (Kisimoto *et al.*, 2010; Lourenço *et al.*, 2012). Previous research using model compounds has shown that, while the syringyl aromatic system is more susceptible to substitution, the resulting products were also found to be more unstable under acidic cooking conditions (Shimada *et al.*, 1997). In contrast, while guaiacyl aromatic systems are less susceptible, the condensation products formed are more stable under acidic conditions (Shimada *et al.*, 1997). In addition, guaiacyl lignin was also found to form carbocations more rapidly through the cleavage of the benzyl ether bond than syringyl lignin (Shimada *et al.*, 1997). The combination of these factors further contributes towards the tendency of guaiacyl lignin to form stable condensation products, thus producing lignin that is more recalcitrant towards subsequent delignification strategies.

#### 1.6.1 Carbocation scavengers

Another method that can indirectly facilitate delignification by preventing repolymerization is the addition of carbocation scavengers. Carbocation scavengers such as 2napthol and 4-hydroxybenzoic acid are electron rich, aromatic compounds that have similar properties to mimic the aromatic rings that are present in lignin (Li *et al.*, 2007; Wayman and Lora, 1978). When lignin undergoes depolymerization via the cleavage of aryl-ether linkages during acidic pretreatment, the formation of electrophilic carbocation intermediates presents the opportunity for the lignin to re-polymerize through the formation of carbon-carbon bonds with the aromatic rings of other lignin moieties. With the addition of carbocation scavengers, this introduces a competing, electron rich aromatic source that can "quench" the carbocation intermediates before they are able to re-polymerize and form a more condensed lignin structure (Li *et al.*, 2007; Wayman and Lora, 1978, 1980) (Figure 5). As a result, carbocation scavengers are chosen for their ability to stop further re-polymerization and have been shown to enhance lignin extractability by preventing high molecular weight lignin from forming (Li and Gellerstedt, 2008).



Figure 5. Reaction mechanism of the lignin carbocation with the carbocation scavenger, 2napthol, to prevent intermolecular lignin condensation. Adapted from Li et al. (2007).

As will be described in the results section of the thesis, selecting a compatible carbocation scavenger is an important aspect of the thesis experimental design. Since carbocation scavengers essentially act as a "receptor" for the carbocation intermediates, it is also necessary to ensure that the carbocation scavenger is not able to undergo electrophilic substitution at multiple positions themselves. Compounds such as resorcinol are an example of molecules that allow for substitution at multiple positions on their aromatic ring and further promote lignin condensation by acting as a cross-linking agent between other lignin molecules (Pielhop et al., 2015). The compound 2-napthol has been shown to be one of the more effective carbocation scavengers (Wayman and Lora, 1978). However, its low water solubility, complex impregnation procedures as well as environmental toxicity have limited its applications in larger scale, water-based pretreatments that would also require more costly waste water treatment. In comparison, the less studied 4-hydroxybenzoic acid is a more attractive alternative, being both water soluble and more environmentally benign (Wayman and Lora, 1978). However, the increase in water solubility from the carboxylic functional group may withdraw electron density from the aromatic ring and decrease its effectiveness as a carbocation scavenger. As the research studying carbocation scavengers has mostly been performed at longer residence times (e.g. 1-4 hours) it was of interest to study their effectiveness when applied to shorter pretreatments, such as steam pretreatment (e.g. 0-30 min). Due to its shorter reaction time, steam pretreatment could benefit from a carbocation scavenger with an increased water

solubility which would assist in the diffusion and transfer of the chemical within the biomass. Ultimately, considering how most commercially implemented pretreatment processes for bioconversion are water based, the application of carbocation scavengers with greater water solubility such as 4-hydroxybenzoic acid should be a great asset in helping facilitate integration into existing aqueous pretreatment processes.

#### **1.7 Single-stage pretreatments**

#### **1.7.1 Steam pretreatment**

Steam pretreatment is a water-based, high temperature, high pressure pretreatment method that has been investigated over the past few decades for its potential to process lignocellulosic biomass. The process was originally developed by the pulp and paper industry to deconstruct wood chips into fibre at a high yield to produce the high-density fibreboard, Masonite (Overend and Chornet, 1987; Schultz et al., 1983). During steam pretreatment, the biomass is loaded in a vessel which is sealed and heated under pressure by injecting steam. After the biomass has reached the desired temperature and treatment time, usually between 160-260°C and from few seconds to minutes respectively, it is released from the vessel and rapidly released (exploded) into a collection tank (Taherzadeh and Karimi, 2008). Typically, when being implemented for bioconversion, the major objective of steam pretreatment is to increase the overall cellulose accessibility of the starting biomass to enzymatic treatment. To do so, steam pretreatment is most commonly carried out under acid-catalyzed or auto-catalyzed conditions. Auto-catalysis occurs when the more labile ester groups that attach the acetyl groups to the hemicellulose are hydrolyzed, releasing the acetyl groups as acetic acid which functions as the acid catalyst during steam pretreatment. However, as mentioned earlier, softwood hemicellulose generally contains significantly fewer acetyl groups compared to hardwood and agricultural biomass and requires additional acid to achieve comparable hemicellulose removal and enzymatic conversion (Dekker, 1987; Galbe and Zacchi, 2002). As a result of the pretreatment, the hemicellulose component is hydrolysed and solubilised into the liquor stream and the overall size of the biomass is reduced through mechanical shearing during the explosive decompression. The relatively cheap and simplistic nature of this

pretreatment method makes it ideal for producing low cost biofuel and is thereby one of the few pretreatment methods that are currently being implemented worldwide at an industrial scale by companies such as Chemtex, Inbicon, DSM-POET and Raizen (Olsen *et al.*, 2015). However, the majority of commercialization initiatives have been focused on the processing of agricultural residues where the lignin component typically constitutes less of the biomass and does not play as large of a role in inhibiting enzymatic hydrolysis when compared to more recalcitrant woody biomass.

When processing woody biomass using steam pretreatment, higher severity conditions are typically required to achieve good cellulose hydrolysis yields at low enzyme loadings. However, with increasing reaction severity, the recovery of the more labile hemicellulose component will consequently decrease as a result of conversion into degradation products (Chandra *et al.*, 2007). The "severity factor" is often used to describe and compare the intensity of the steam pretreatment conditions and takes into consideration the time (t, min) and temperature (T, °C) to form the following parameter (Overend and Chornet, 1987):

$$R_o = te^{[(T-100)/14.75]}$$

When Douglas-fir wood chips were pretreated at increasing severity (log  $R_0 = 3.45$  vs. 4.21), cellulose hydrolysis increased from 87% to 98% while hemicellulose recovery in the water soluble stream drastically decreased from 65% to 38% (Wu *et al.*, 1999). In addition, there is minimal removal of the lignin component during steam pretreatment, with the exception of the dissolution of some lower molecular weight lignin into the water soluble stream as a result of acidolysis reactions (Shevchenko *et al.*, 1999). The removal of hemicellulose from the biomass also significantly increases the proportion of lignin in the pretreated residual solids to as high as 50% for softwoods, depending on the treatment severity (Kumar *et al.*, 2012a). The lack of lignin removal and higher tendency towards lignin re-polymerization during acidic steam pretreatment also greatly decreases the subsequent extractability of the lignin (Shevchenko *et al.*, 1999). Despite limited removal, high severity conditions have also been demonstrated to cause lignin redistribution and the formation of lignin agglomerates, resulting in an overall increase in cellulose accessibility (Donaldson *et al.*, 1988; Ewanick *et al.*, 2007). However, the

increase in lignin condensation with increasing pretreatment severity also results in the production of a more hydrophobic lignin that negatively impacts the efficiency of enzymatic hydrolysis (Eriksson *et al.*, 2002; Nakagame *et al.*, 2010; Nakagame *et al.*, 2011a). Previous research isolated lignin fractions from steam pretreated substrates and tested the effect of their addition on the enzymatic hydrolysis of Avicel (Nakagame *et al.*, 2010). The addition of lignin isolated from steam pretreated corn stover resulted in minimal effect on enzymatic hydrolysis while the addition of lignin isolated from steam pretreated poplar and lodgepole pine resulted in a decrease of 9% and 18% to the conversion yield respectively (Nakagame *et al.*, 2010). Furthermore, the addition of lignin isolated from Douglas-fir substrates that were steam pretreated at increasing severity (190-210°C) to Avicel resulted in a greater decrease in the enzymatic hydrolysis yield with increasing steam pretreatment severity (Nakagame *et al.*, 2011a). Analysis of the isolated lignin also showed an increased degree of lignin condensation (more C-C bonds) and increased cellulase absorption and binding affinity at increasing treatment severity, further demonstrating the detrimental interactions of the residual lignin on enzymatic conversion (Nakagame *et al.*, 2011a).

As discussed earlier, the impact of lignin condensation has been shown to be more significant for softwoods due to the higher proportion of more easily condensed guaiacyl lignin. In addition, the solubilisation of the carbohydrate component also effectively enriches the relative lignin content in the substrate, which will further magnify the lignin effects. As a result, softwood biomass has had less success with steam pretreatment and typically requires much higher enzyme loadings to compensate for the low conversion compared to steam pretreated hardwoods or agricultural residues (Chandra *et al.*, 2007; Nakagame *et al.*, 2010). As mentioned earlier, the increase in lignin condensation modifies the chemical structure of lignin and will also impact its subsequent extractability and downstream applications. Analysis of steam pretreated lignin showed that pretreatment caused a decrease in aryl-ether bonds, increase in carbon-carbon bonds, increase in the hydrophobicity of the lignin (Li *et al.*, 2009; Nakagame *et al.*, 2011a; Shimizu *et al.*, 1998). Modifications of the lignin functionality can potentially limit the type of downstream products that the lignin can be applied to. When lignin
isolated from steam pretreated hardwood biomass was tested for resin production, slower reaction and cure rates were observed when compared to commercial phenolic-resins (Gardner and McGinnis, 1988; Shimizu *et al.*, 1998). Lastly, the physical and chemical characteristics of the steam pretreated lignin will also be greatly dependent on the extraction method used to isolate the lignin (Nakagame *et al.*, 2011a). Considering the effects of the pretreatment severity on both cellulose accessibility and hemicellulose and lignin recovery, it is typical to select compromised conditions that allow for enhancements in enzymatic digestibility while minimizing sugar degradation and lignin condensation.

#### 1.7.2 Organosolv

Compared to the aqueous-based steam pretreatment which largely focuses on carbohydrate solubilisation, organosolv pretreatment is a solvent-based process geared for lignin removal from woody biomass (de la Torre et al., 2013; Zhao et al., 2009). Organosolv pretreatment was originally developed by the pulp and paper industry as an environmentally friendly substitute to the Kraft pulping process (Johansson et al., 1987). This interest spawned various commercial endeavors such as the ALCELL (ALcohol-CELLulose), ASAM (Alkaline Sulfite Anthraquinone Methanol) and Organocell processes (Young and Akhtar, 1998). The ALCELL method is most effective in processing hardwood and agricultural residues that have a greater amount of acetyl groups but has difficulty processing softwood that contain hemicellulose with less acetyl groups (Gilarrahz et al., 1998; Rodríguez et al., 2010; Young and Akhtar, 1998). In comparison, Organocell and ASAM employ alkali conditions to improve the delignification of softwoods but consequently require more complex chemical recovery methods (Rodríguez et al., 2010; Young and Akhtar, 1998). While the organosolv processes are capable of producing pulps of competitive quality to Kraft pulps, they encountered challenges such as requiring high energy input, explosion hazards as well as solvent cost and recovery, preventing them from replacing the Kraft pulping method (Alvira et al., 2010; Rodríguez et al., 2010). However, the production of a lignin co-product, relatively lower capital cost (for non-alkali conditions) and environmental benefits make the organosolv pulping method an attractive for further investigation as a pretreatment for a biorefinery process (Pan et al., 2005).

For use in pretreatment for bioconversion, organosolv has been most commonly performed using ethanol as the extraction solvent under acidic conditions but has also been studied using different solvents as well as under alkaline conditions (Arague *et al.*, 2008; Holtzapple and Humphrey, 1984; Thring et al., 1990; Yawalata, 2001). Currently organosolv has not been realized as a commercial pretreatment process and is still being investigated at the pilot scale by companies such as Fibria Innovations (previously Lignol Innovations). When used as the front end of a bioconversion process, downstream bleachability and fiber preservation are not a concern as the main objective of organosolv for pretreatment is to improve enzymatic accessibility to the cellulose component through lignin and hemicellulose removal. Therefore, more severe acidic conditions can be utilized to help achieve increased cellulose accessibility. The process involves cooking the starting biomass under pressure in the selected solvent in a sealed digester at elevated temperatures anywhere between 100-250°C (Zhao et al., 2009). After pretreatment, both the lignin and hemicellulose are solubilised through the cleavage of lignin-carbohydrate complex (LCC) and lignin-lignin bonds, leaving behind a solid rich in cellulose and low in hemicellulose and lignin content (Zhao et al., 2009). However, while this method is effective in solubilizing the lignin component and improving cellulose accessibility, the harsh conditions required to fragment and solubilize lignin also compromises hemicellulose recovery (Pan et al., 2007).

Delignification is one of the major defining features of organosolv, as most acidic, aqueous pretreatment methods do not focus on the removal and recovery of the lignin component. The solvent environment of organosolv helps both the delignification (via solvolysis) as well as solubilisation of the lignin in the organic solvent (McDonough, 1993). The Hildebrand solubility parameter ( $\delta = (cal/cm^3)^{1/2}$ ) is an estimate of the miscibility of two materials, with materials of similar values having a higher probability of miscibility (Hildebrand and Scott, 1950). Lignin, which has a  $\delta$  value ranging from approximately 11-14 (cal/cm<sup>3</sup>)<sup>1/2</sup> is predicted to be more compatible with solvents such as ethanol and methanol ( $\delta = 12.7$ , 14.5 (cal/cm<sup>3</sup>)<sup>1/2</sup>) than water ( $\delta = 23.4$  (cal/cm<sup>3</sup>)<sup>1/2</sup>), explaining the improvement in lignin solubility in a solvent based system (Pan and Sano, 1999; Schuerch, 1952; Wang *et al.*, 2011). With that in mind, the concentration of the solvent in the pretreatment liquor will also play a role in affecting the treatment response of the biomass. Previous work that tested organosolv pretreatment on mountain pine beetle killed lodgepole pine over a range of ethanol concentrations (48-82% v/v) observed that higher ethanol concentrations helped promote increased lignin solubility and lower klason lignin content on the substrate (23% and 10% klason lignin content for 48% and 82% v/v ethanol concentration respectively) (Pan *et al.*, 2007). However, higher ethanol concentrations also result in a decrease in the hydrogen ion concentration needed to help promote cleavage of the aryl-ether linkages in lignin. This results in an inflection point at 75% v/v ethanol concentration where the lowest klason lignin content and highest EOL yield were achieved (Pan *et al.*, 2007). The introduction of solvents to the water system effectively reduces the  $\delta$  value and brings it closer to that of lignin and improves its solubility but will still require a balance of conditions to achieve both high delignification and enzymatic hydrolysis yields of the biomass during organosolv (Pan *et al.*, 2007; Wang *et al.*, 2011).

In addition to the pretreatment conditions, the biomass characteristics are also important in determining the efficiency of the organosoly process. Organosoly pretreatment of softwood biomass has yielded substrates of 10-15% klason lignin content, 60% ethanol organosolv lignin recovery and approximately 85% glucan conversion (Bouxin et al., 2014; Pan et al., 2008). In comparison, organosolv pretreatment on hardwoods have generally resulted in higher ethanol organosolv lignin recovery (74%) and a lower substrate lignin content (6-8%), indicating a higher specificity of the process towards the delignification of hardwoods (Del Rio, 2012; Pan et al., 2006a). When comparing the delignification specificity (defined as the solid yield divided by the percent recovery of the initial lignin in the solid fraction) of hardwoods and softwoods pretreated with the same conditions, the organosolv pretreatment of the hardwood substrate had almost double the selectivity for lignin compared to the pretreatment of softwoods (3.0 vs. 1.6 respectively) (Del Rio, 2012). This difference in delignification selectivity between the two woody substrates has been largely attributed to the difference in lignin type and their relative ease of extraction. During organosolv pretreatment both depolymerization and re-polymerization reactions occur but the higher proportion of guaiacyl lignin in softwoods results in a higher potential to undergo the re-polymerization pathway when compared to

hardwoods (Wikberg and Maunu, 2004). Consequently, NMR analysis of ethanol organosolv extracted lignin and residual lignin from softwood biomass showed an increase in the degree of condensation compared to native lignin (Sannigrahi *et al.*, 2010).

Lignin isolated through the organosolv process provides a potential, value-added byproduct to a biorefinery process. Compared to water based pretreatments, the presence of an organic solvent in organosolv presents a more favourable medium for lignin solubilisation. However, the use of an organic solvent to increase lignin solubilisation also necessitates the implementation of proper washing procedures to prevent the redistribution of the solubilised lignin on the substrate surface (Zhao et al., 2009). To isolate the lignin component, the treatment liquor is diluted with water which causes the lignin to precipitate. In doing so, the lignin component can be isolated in high purity through filtration or centrifugation (Pan et al., 2005). The high purity, low molecular weight, low polydispersity and high phenolic/hydroxyl functionality gives organosolv lignin properties suitable for applications in products such as phenolic, epoxy, and isocyanate resins as well as polyurethane foams, antioxidants or green diesel (Pan et al., 2005; Sannigrahi et al., 2010). In contrast to the sulfite or Kraft process, the organosolv process is able to produce a more "neutral" lignin that is sulfur and ash (sodium) free (de la Torre et al., 2013; Pan et al., 2005). In addition, the organosolv process is also less polluting to both water and air. However, the lack of hemicellulose recovery after organosolv processes sacrifices a major value-added component extractable from the biomass.

#### **1.8 Two-stage pretreatments**

Hemicellulose, being the most labile of the three major components, would benefit the most from a milder extraction stage to improve its overall recovery (Wu *et al.*, 1999). In comparison, lignin and cellulose are polymers of higher stability due to their aromatic and crystalline structure respectively and require more severe pretreatment conditions in order to increase delignification and enzyme digestibility respectively. Pretreatment methods such as those described above were originally developed by the pulp and paper sector but have since been optimized to increase cellulose accessibility for further deconstruction. However, the relatively more severe conditions required to deconstruct biomass and increase cellulose

accessibility often result in the compromise of the other lignocellulosic components. Steam pretreatment, for example, is capable of solubilising hemicellulose but the high temperature, acidic conditions can also cause carbohydrate degradation and lignin condensation. In comparison, organosolv pretreatment solubilizes hemicellulose and lignin but the longer retention times required to increase delignification results in increased degradation of the hemicellulose component. Therefore, multi-stage pretreatment of lignocellulosic biomass have been proposed and investigated as a means of extracting each component individually and achieve high hemicellulose recovery, high delignification and increased enzymatic conversion (Amiri and Karimi, 2016; Ibrahim and Glasser, 1999; Panagiotopoulos *et al.*, 2013; Romaní *et al.*, 2011).

The most common approach to multistage pretreatment that has been investigated and is suitable for a biorefinery process involves a milder, water-based step to extract and recover the hemicellulose fraction followed by a lignin extraction step to yield a substrate containing a more purified cellulose. The removal of both the hemicellulose and lignin component will ultimately be beneficial in partially relieving some of the physical blocking effects and has been shown to greatly improve the enzyme conversion of the cellulose fraction (Bura et al., 2009; Kumar et al., 2010). Lignin removal, in particular, is of great importance and provides significant benefit to more recalcitrant substrates such as softwoods. As mentioned previously, the higher guaiacyl content of softwood lignins means they are more readily condensed during pretreatment, thus hindering enzymatic hydrolysis through non-productive binding (Nakagame et al., 2011a). Previous work has shown how complete delignification of steam pretreated Douglas-fir increased the hydrolysis yield from <27% to 94% at low enzyme loadings (Kumar et al., 2010). This further demonstrates the importance of lignin removal for softwoods. Thus, the implementation of multiple pretreatment stages that can extract and recover the hemicellulose and lignin component in a useful manner while producing a high cellulosic substrate amenable to enzymatic hydrolysis would be invaluable to a biorefinery process.

## 1.8.1 Prehydrolysis kraft (PHK)

A prime example of a two-stage process that is currently being used as an effective method to fractionate woody biomass is the prehydrolysis kraft (PHK) process to produce dissolving pulp. In contrast to traditional acid sulfite methods of producing dissolving pulp that remove both hemicellulose and lignin simultaneously, the PHK process produces a highly pure cellulosic pulp by removing hemicellulose and lignin in separate stages (Hamaguchi *et al.*, 2013). During the PHK process, the biomass is first subjected to a hot water or steam prehydrolysis step to extract hemicellulose, followed by a Kraft/alkaline pulping process for delignification. Compared to acidic delignification methods such as organosolv, Kraft pulping is more specific towards delignification and retains a large proportion of the hemicellulose component in the pulp. In addition, the alkaline pulping conditions can also promote peeling reactions, converting the hemicelluloses to organic acids, and complicating recovery efforts (Sjöström, 1993). Therefore, in order to cleanly remove the hemicellulose fraction, alkaline processes such as Kraft pulping greatly benefit from a prehydrolysis step to produce a highly pure cellulosic pulp.

The previous work that investigated the effects of prehydrolysis on Kraft pulping and the resulting pulp properties showed changes to all three biomass components. During prehydrolysis a large fraction of the hemicellulose is preferentially solubilised from the biomass into the water soluble stream. The degree of extraction has been shown to be dependent on the prehydrolysis conditions. Increasing retention times (10-60 min) showed an increase in xylan solubilisation from 12.3 to 61.4% for eucalyptus wood chips (Colodette *et al.*, 2011). In addition, the remaining hemicellulose is also more readily removed during the subsequent pulping process. Implementation of the prehydrolysis step before Kraft pulping has been shown to reduce the substrate xylan content from 20-22% to 4-6% in sugar maple, which results in the typical hemicellulose content found in dissolving pulps (Duarte *et al.*, 2011; Fišerová and Opálená, 2014). The addition of acid during prehydrolysis results in higher severity conditions that have been shown to degrade the cellulosic component and reduce the degree of polymerization of cellulose, as reflected by a decrease in viscosity (Saukkonen *et al.*, 2012). Prehydrolysis has also been shown to affect the rate of delignification of the hydrolysed biomass. Treatment of both hardwoods and softwoods has been shown to increase the rate of

delignification and decrease the time needed during Kraft pulping to achieve the same lignin content (Duarte et al., 2011; Frederick et al., 2008; Rauhala et al., 2011; Schild et al., 1996; Yoon and van Heiningen, 2008). The improvement in delignification has been proposed to be due to the cleavage of  $\beta$ -O-4 bonds, formation of more phenolic hydroxyls and lignin fragmented during the acidic treatment through partial acidolysis, thus allowing for more effective removal during the subsequent alkaline pulping (Leschinsky et al., 2008; Rauhala et al., 2011). In addition, pulps that have been subjected to prehydrolysis were also easier to bleach and showed increased brightness compared to untreated kraft pulps (Colodette et al., 2011; Saukkonen et al., 2012). However, the severity of the prehydrolysis conditions can also have an impact on delignification during Kraft pulping. Prehydrolysis of slash pine at increasing temperatures resulted in a decrease in delignification of the substrate during pulping (Paulson, 1971). In contrast, changing the prehydrolysis conditions for eucalyptus wood chips still yielded pulps with the same kappa number when using constant Kraft pulping conditions (Mendes et al., 2009). The decrease in delignification observed for softwoods is suggested to be due to an increase in lignin condensation in response to the increasing prehydrolysis severity due to their higher guaiacyl lignin content (Paulson, 1971). Overall, prehydrolysis has been shown to be capable of extracting hemicellulose while benefiting delignification during subsequent kraft pulping for both hardwood and softwood biomass.

While the PHK process has been demonstrated to be effective for hemicellulose removal and delignification, the process is not without its complications when being implemented at the commercial scale. One such complication involves the use of a hot water prehydrolysis stage. When hot water is used in lieu of steam to improve hemicellulose solubilisation, the extraction of acid soluble lignin also increases (Hamaguchi *et al.*, 2013). Previous work that investigated auto-catalyzed hot water prehydrolysis on eucalyptus resulted in approximately 20% lignin removal after treatment (Leschinsky *et al.*, 2009). The extraction of and subsequent condensation of the acid soluble lignin results in the formation of "sticky lignin" precipitates that complicate the recovery and use of the hemicellulose extracted (Leschinsky *et al.*, 2009). In addition, the pH swing from an acidic prehydrolysis step with subsequent neutralization to transition to an alkaline kraft pulping step results in both increased water demand and salt formation which can cause more strain on the chemical recovery process (Grace and Tran, 2009). Lastly, the high operating and capital costs of a Kraft process requires the end product to be equally valuable (e.g. paper pulp, dissolving pulp). This would require the production of additional high value product streams from the hemicellulose and lignin components in order to justify its application in a biorefinery process focused on utilizing the cellulose component for biofuel production. Therefore, despite being effective in fractionating biomass and producing a high cellulose purity substrate, the application of the PHK process as a commercial biorefinery process would prove challenging.

# 1.8.2 Steam/organosolv pretreatment

As mentioned earlier, steam and organosolv pretreatment are two methods that are capable of processing lignocellulosic biomass and have a long history of investigation (Brownell and Saddler, 1987; Paszner and Cho, 1989). When considering them as single-stage pretreatments, both processes have their advantages and disadvantages; steam pretreatment is effective in solubilising hemicellulose but causes lignin condensation while organosolv is effective in solubilising lignin but causes hemicellulose degradation and difficult recovery. As demonstrated by the effectiveness of the PHK process for making dissolving pulp, a similar twostage approach can also be adapted with steam/organosolv pretreatment for a biorefinery process. Similarly, steam pretreatment has also been previously utilized in the prehydrolysis step for the PHK process and it is anticipated that milder steam pretreatment conditions could possibly help improve hemicellulose extraction and recovery while minimizing lignin condensation. An organosolv pretreatment step can then be subsequently implemented to both delignify the hemicellulose-poor substrate and increase cellulose accessibility to enzymatic hydrolysis. In addition, in contrast to the PHK process that utilizes alkaline delignification, the use of an "acid-acid" two-stage process should be more economically feasible due to lower chemical loadings, limited pH swing and lack of a need for an extensive chemical recovery system. Therefore, by implementing both steam and organosolv pretreatments consecutively at milder conditions it is potentially possible to minimize the detrimental effects of each pretreatment while still allowing the advantageous qualities to complement each other, as with the PHK process.

Previous work has assessed a two-stage, steam/ethanol organosolv approach using hardwood as the substrate (Panagiotopoulos et al., 2013). The use of an optimized, mild steam pretreatment first-stage facilitated the recovery of 90% of the xylan from poplar wood chips. Similar to the work described using the PHK process, the first-stage steam pretreatment resulted in an increase in the effectiveness of the second-stage organosolv delignification, allowing for a delignification yield of 66%. In addition, this enhanced delignification was achieved at a shorter residence time of 30 min compared to the 60 minutes residence time typically required for single-stage organosoly pretreatment of hardwoods. When the substrates pretreated in two stages were subjected to enzymatic hydrolysis, 88% of the cellulose was hydrolyzed at an enzyme loading of 5 FPU/g cellulose as compared to 76% for the single stage control substrate (Panagiotopoulos et al., 2013). Similar to the results reported for poplar wood chips, previous work on eucalyptus using an auto-hydrolysis pre-extraction and non-catalyzed organosolv pretreatment also showed similar results, with optimal two-stage conditions yielding 83% hemicellulose solubilisation, 65% delignification and 94% glucan conversion at an enzyme loading of 10 FPU/g substrate (Romaní et al., 2011). Fractionation of red oak has also demonstrated the benefits of high severity steam pretreatment in improving delignification (approx. 70%) during low temperature solvent extraction, albeit at the expense of hemicellulose recovery (Ibrahim and Glasser, 1999). Similar success in two-stage biomass fractionation has also demonstrated increased delignification and enzymatic hydrolysis on less recalcitrant substrates such as rice straw (Amiri and Karimi, 2016). Other organic solvents such as methanol and butanol were also tested on hardwood biomass and displayed similar trends in improved delignification (Rughani and McGinnis, 1989). It has been proposed that the increase in lignin removal was due to the depolymerization of the lignin component during the firststage steam/hot water pretreatment which resulted in more efficient removal during the subsequent organosoly pretreatment stage at milder conditions (e.g. shorter retention time, no additional acid) (Panagiotopoulos et al., 2013; Romaní et al., 2011). However, while the majority of the published literature has demonstrated the benefits of applying a two-stage pretreatment process, contradictory results have been reported as well. Previous work applying a two-stage hot water-organosolv pretreatment process on elm showed a decrease in

organosolv delignification from 59% to 41% when comparing the single-stage control and twostage process respectively (Amiri and Karimi, 2016). The decrease in delignification yield was attributed to the increase in lignin re-polymerization during the acidic conditions of the twostage pretreatment.

As discussed earlier previous work investigating the effectiveness of a two-stage steam/organosolv pretreatment process to fractionate lignocellulosic biomass have focused on less recalcitrant substrates such as hardwoods and agricultural residues. In comparison, little work has been on softwoods and the higher guaiacyl lignin content in softwood biomass can potentially present increased challenges, particularly under acidic condition where lignin condensation is more likely to occur. The limited work that has been done on softwoods found that applying a hot-water extraction to pine resulted in the solubilisation of 60% of the hemicellulose and improve delignification from 43% to 50% after subsequent organosolv pretreatment. However, enzymatic hydrolysis yields after the two stages were still poor, at only 23% cellulose conversion (25 FPU/g substrate, 5% solids, 72 h) despite improved delignification (Amiri and Karimi, 2016). While the authors reported an increase in delignification yield, the single-stage organosolv delignification yield of 43% achieved in their study is low compared to previously optimized single-stage conditions for beetle killed lodgepole pine (79% delignification) (Pan et al., 2007). The decrease in enzymatic hydrolysis yield observed was likely due to non-productive enzyme binding to the residual lignin as a result of increased lignin condensation after the acidic two-stage pretreatment.

The presence of guaiacyl lignin in softwood biomass increases the potential for lignin condensation compared to less recalcitrant feedstock such as hardwood and agricultural residues. Guaiacyl lignin has been shown previously to be more prone to form stable condensation products which can affect subsequent removal and increase hydrophobic interactions with the hydrolytic enzymes (Shimada *et al.*, 1997; Tsutsumi *et al.*, 1995; Wikberg and Maunu, 2004). In contrast to the two-stage steam/organosolv pretreatment processes discussed earlier, several more chemically intensive "post-treatments" or "pseudo two-stage pretreatments" have also been investigated. These have typically been implemented after an initial steam pretreatment first-stage to overcome the recalcitrance of softwood biomass to

enzymatic conversion. In most cases, the post-pretreatment has focused on substrate modifications after the initial pretreatment step to further improve enzyme digestibility of the substrate. For softwoods, the post-treatment typically focuses on the modification or removal of the lignin component to reduce the inhibitory effects of lignin condensation on enzymatic hydrolysis. Previous work which has studied the application of an alkaline hydrogen peroxide or neutral sulfonation post-treatment on acid-catalyzed steam pretreated softwood substrates resulted in a four times increase in enzymatic hydrolysis yield to 62% at an enzyme loading of 5 FPU/g cellulose after post-treatment (Kumar et al., 2011). An alkaline hydrogen peroxide posttreatment increased enzymatic conversion by reducing the residual substrate lignin content from 42% to 20% while neutral sulfonation resulted in insignificant lignin removal but incorporated sulfonic acid groups onto the lignin (>100 mmol/kg) which decreased lignin hydrophobicity (Kumar et al., 2011). The necessity to either remove or modify the chemical characteristics of the residual lignin component to improve enzymatic hydrolysis yields further accentuates the inhibitory effects of the residual softwood lignin after acidic pretreatment. However, as mentioned earlier, post-treatment is a chemically demanding process (e.g. 16% w/w Na<sub>2</sub>SO<sub>3</sub> for neutral sulfonation post-treatment) and would be unattractive to implement in a biorefinery process. In addition, lignin recovery from either process will be challenging since alkaline hydrogen peroxide post-treatment degrades the lignin and neutral sulfonation posttreatment does not result in lignin extraction. Therefore, the need for a milder pretreatment process to minimize the amount of lignin condensation that will occur during acidic pretreatment becomes even more crucial. Thus, a much more detailed understanding of the mechanisms and impacts that result from lignin condensation is needed if we are to overcome the recalcitrance of softwoods. This was assessed by using a two-stage steam/organosolv pretreatment process that has a goal of optimising overall sugar recovery and ease of cellulose hydrolysis using low enzyme loadings.

# 1.9 Rationale and objective of the thesis

A lignocellulose based biorefinery aims to maximize the value extractable from the biomass by producing a wide array of products, analogous to contemporary oil based refineries. In addition, to compensate for the cost of the biomass which includes harvesting, processing

and transport, it would be extremely beneficial if the pretreatment step of a biorefinery process is able to separate and recover as much of the cellulose, hemicellulose and lignin components as possible in a clean and usable form. However, the recalcitrance of the lignin component, particularly for softwoods, usually necessitates the use of pretreatment conditions that sacrifice the recovery of the more labile hemicellulose component in order to extract the lignin. Earlier work has shown that a two-stage, steam/organosolv pretreatment process was effective in fractionating hardwood biomass (Panagiotopoulos et al., 2013; Romaní et al., 2011). The steam pretreatment step selectively solubilized the hemicellulose, while the subsequent organosolv pretreatment solubilized the lignin. In comparison, the increased amount of guaiacyl subunits that comprise softwood lignin resulted in the creation of more crosslinks within the lignin macromolecule, rendering it more resistant to fragmentation and solubilisation. As well as its inherent recalcitrance, the acidic conditions that are effective in solubilising the hemicellulose causes softwood lignin to undergo condensation reactions that further compromise its extractability. However, softwoods represent a prime biomass source for utilization in the Canadian Pacific Northwest and are the main feedstock used for the production of pellets. Pelletization, and the existing pellet industry in the region, presents a viable method to improve the logistics of collecting and transporting the available softwood biomass in order to realize the benefits of economies of scale.

While pelletization is an attractive option for improving biomass logistics, there has been limited research on the effects of the pelletization process (e.g. size reduction, drying, densification) on the subsequent susceptibility of the feedstock to pretreatments which facilitate lignin extraction in a usable form. Earlier work which compared pelletized biomass to air-dried wood chips demonstrated a similar level of hemicellulose recovery in the water soluble stream after steam pretreatment (Kumar *et al.*, 2012b). However, the work mainly focused on biomass and pretreatment effects on the hemicellulose and cellulose components and little emphasis was placed on subsequent extraction of the lignin fraction. In addition, this earlier work lacked controls that compared the two biomass types at equalized particle size. Particle size has been shown previously to play a role in influencing the "apparent severity" experienced by the biomass during pretreatment (Cullis *et al.*, 2004). Thus, the goal of the work outlined within this thesis was to investigate the ability of a two-stage, steam/organosolv pretreatment approach to effectively fractionate hemicellulose and lignin from pelletized softwood biomass.

It was hypothesized that a milder steam pretreatment could be utilized to selectively solubilize the hemicellulose component while limiting condensation of the lignin component, thereby still allowing for the extraction of lignin during a subsequent organosolv treatment. The initial work indicated that steam pretreatment could be used at mild conditions (175-185°C) to enhance the solubilisation and recovery of the hemicellulose. Under mild conditions, pelletization appeared to result in enhanced hemicellulose recovery and solubilisation when compared to wood chips. However, when wood chips and pellets were ground and sieved to equalize their particle size, it appeared that both ground substrates performed similarly after steam pretreatment. This confirmed the role that size reduction during pelletization appeared to be reversible and, when using thermomechanical pulp as a model substrate, lignin extraction yields could be restored provided they were first rehydrated. This work confirmed that pellets were as processable as wood chips when used as a feedstock in a likely enzyme mediated biorefinery process.

Due to the potential of lignin condensation during steam pretreatment the initial thesis work compared the use of acid vs. auto-catalyzed pretreatments for hemicellulose solubilisation and recovery. It was apparent that the lower amount of acetyl groups in softwood hemicellulose necessitated the addition of acid to solubilize >60% of the hemicellulose at the milder conditions utilized. However, acid-catalyzed pretreatment was shown to cause more lignin condensation, which compromised subsequent organosolv delignification. The enzymatic hydrolysis of the two-stage pretreated substrates also resulted in lower yields when compared to the single-stage organosolv controls. The use of bovine serum albumin (BSA) to "block" hydrophobic lignin binding sites significantly improved the hydrolysis of the cellulose component. Therefore, while the two-stage steam/organosolv pretreatment seemed to generate sufficient accessibility for enzymatic hydrolysis, the non-productive binding of

enzymes to the residual softwood lignin that condensed during the two pretreatment stages resulted in lower enzymatic hydrolysis.

Based on the results from the Chapter 3.1, Chapter 3.2 focused on methods to limit lignin condensation during the two-stage pretreatment of pelletized softwood biomass. Carbocation scavengers have previously been investigated as an additive to prevent the formation of high molecular weight lignin during acidic pretreatment (Li et al., 2007; Pielhop et al., 2015; Wayman and Lora, 1978, 1980). During the pretreatment process, these carbocation scavengers add to the lignin during the condensation process, thereby preventing the formation of cross-links within the native biomass lignin during acidic pretreatment. While 2naphthol has been the most frequently utilized scavenger in the literature, its limited water solubility makes it impractical to apply to biomass. Therefore, an alternative carbocation scavenger that has had relative success in application, 4-hydroxybenzoic acid (4HBA), was chosen for its water solubility (Wayman and Lora, 1978). Our previous work had also shown that lignosulfonates, which are water soluble, amphiphilic lignin derivatives from the sulfite pulping process, could also attach to native lignin during acid-catalysed steam pretreatment via a potential condensation mechanism, resulting in an enhancement in enzymatic hydrolysis. Therefore, we tested the ability of 4-hydroxybenzoic acid and lignosulfonates to act as carbocation scavengers which attach to the native biomass lignin.

Initially, we tested the effect of increasing the temperature (175-205°C) of steam pretreatment on lignin condensation and post-treatment extraction using a neutral, dioxane extraction. Surprisingly, increasing the steam pretreatment temperature produced substrates more amenable to subsequent lignin extraction using dioxane. However, the same extraction trend was not observed when using organosolv delignification, as the samples treated using higher steam temperatures were more resistant to subsequent lignin removal using an acidic ethanol/water extraction. Analysis of the substrate that resulted from the addition of 4HBA to the steam pretreatment suggested that 4HBA acted as a secondary acid source rather than as a carbocation scavenger. In contrast to 4HBA, the addition of lignosulfonates during steam pretreatment indicated that the lignosulfonates were incorporated into the substrate. Although the incorporation of lignosulfonates into the native biomass lignin resulted in modest increases

in delignification, the resulting substrate was more susceptible to enzymatic hydrolysis. The successful incorporation of lignosulfonates demonstrates the potential to use condensation as a vehicle for lignin modification while limiting crosslinking within the native lignin. In addition, the difference in lignin extractability in dioxane vs. ethanol/water systems suggests the need to tailor organosolv solvent systems based on the substrate lignin modifications in order to optimize extraction. Overall, the addition of lignosulfonates to steam pretreatment appeared to increase lignin hydrophilicity and benefit both delignification and enzymatic hydrolysis after a two-stage steam/organosolv pretreatment.

Therefore, based on the above rationale, the main objectives of the thesis work can be summarized as follows:

- (1) Investigate the ability of a two-stage, steam/organosolv pretreatment approach to effectively fractionate hemicellulose and lignin from softwood biomass.
- (2) Compare the treatment response of softwood pellets to chips to both single and twostage steam/organosolv pretreatment.
- (3) Elucidate the effect of carbocation scavengers (e.g. 4-hydroxybenzoic acid, lignosulfonates) on minimizing lignin condensation, benefiting subsequent delignification and enzymatic hydrolysis.

## 2 Materials and methods

#### 2.1 Feedstock preparation

The softwood pellets used in this study were commercial pellets manufactured by Okanagan Pellet Company (formerly known as Westwood Fibre Products), produced from white pine and spruce sawdust without bark contamination. Commercial, grey-stage beetle killed lodgepole pine (*Pinus contorta*, LPP) wood chips were obtained in 2012 from Canfor Pulp Ltd. Partnership (Vancouver, BC, Canada). The wood chips were screened and a size fraction between 2.5 x 2.5 cm and 5.0 x 5.0 cm was collected and used as the feedstock for further pretreatments. The wood chips were air-dried to equilibrium moisture and stored at room temperature. The chemical composition of the wood chips were 47% glucan, 12% mannan, 7.2% xylan, 2.8% galactan, 1.6% arabinan and 29% lignin (0.5% acid-soluble lignin). The chemical composition of the pellets were 46% glucan, 11% mannan, 7.8% xylan, 3.5% galactan, 1.7% arabinan and 29% lignin (0.6% acid-soluble lignin).

For the ground substrates used in section 3.1.5, softwood chips and pellets were size reduced using a laboratory, heavy-duty hammer mill (10HMBL, Glen mills Inc., NJ). Biomass was fed into the hammer mill through a vibrating feeder and the mill was fitted with a 0.125" and 0.4" mesh for softwood chips and pellets respectively to screen for particles of visually similar sizes. Due to the different shapes of the resulting ground material (shives vs. flakes for ground wood chips and pellets respectively) visual identification was used to determine the appropriate mesh size to yield particles of approximately similar size (refer to Figure 8 in section 3.1.5). Ground samples were sieved through a metal mesh with a 1.0 mm opening to remove fines. Larger, improperly ground particles (rejects) were manually removed from the sample.

The thermomechanical pulp (TMP) used in section 3.1.4 was a commercial TMP generously supplied by Howe Sound Pulp and Paper Ltd. (Port Mellon, BC, Canada). The pulp was a mixture of western hemlock and Douglas-fir and was received as a wet slurry.

# 2.2 Pretreatments

# 2.2.1 Steam pretreatment

A 2-L Stake Tech II steam gun (Stake Tech II batch reactor, Sun Opta (formerly Stake Technologies) of Norval, ON, Canada) was used for all steam pretreatments. For substrates used in Chapter 3.1, two batches of 200 g wood chips/pellets (o.d. basis) were soaked with water (and 0.7% w/w sulfuric acid on biomass for acidic conditions) at a solid to liquid ratio of 1:1 overnight at room temperature for each treatment condition. Steam pretreatment was performed at 175°C with a retention time of 7.5 minutes, as detailed by Wu et al. (1999). For each treatment condition, the two batches were pretreated consecutively and combined into a single, larger batch for further processing. Steam pretreated ground wood chips and pellets used in section 3.1.5 were prepared in a similar manner but with a single batch of 200 g biomass (o.d. basis). For SO<sub>2</sub> pretreated wood chips and pellets used in section 3.1.1, substrates were prepared and pretreated as described by Ewanick et al. (2007). A summary of the pretreatment conditions and chemical loadings used are shown in Table 1.

For substrates used in Chapter 3.2, 200 g pellets (o.d. basis) were soaked with water, acid and respective chemical loadings of 4HBA or lignosulfonates (LS) at a solid to liquid ration of 1:1 overnight at room temperature. A summary of the pretreatment conditions and chemical loadings use are shown in Table 2.

Sample Code	Substrate	Temperature (Celsius)	Time (min)	Chemical and Loading (% dry matter basis)
NC-0	Chips	175	7.5	0% H <sub>2</sub> SO <sub>4</sub>
AC-0	Chips	175	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>
SC-0	Chips	200	5.0	4% SO <sub>2</sub>
GC-0	Ground Chips	175	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>
NP-0	Pellets	175	7.5	0% H <sub>2</sub> SO <sub>4</sub>
AP-0	Pellets	175	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>
SP-0	Pellets	200	5.0	4% SO <sub>2</sub>
GP-0	Ground Pellets	175	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>

Table 1. Summary of steam pretreatment conditions studied in Chapter 3.1

Sample Code	Substrate	Temperature (Celsius)	Time (min)	Chemical and Loading (% dry matter basis)
175°C	Pellets	175	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>
185°C	Pellets	185	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>
195°C	Pellets	195	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>
205°C	Pellets	205	7.5	0.7% H <sub>2</sub> SO <sub>4</sub>
185°C 4HBA	Pellets	185	7.5	0.7% H <sub>2</sub> SO <sub>4</sub> + 4% 4HBA
185°C LS	Pellets	185	7.5	0.7% H <sub>2</sub> SO <sub>4</sub> + 4% LS

Table 2. Summary of steam pretreatment conditions studied in Chapter 3.2

After pretreatment, the water soluble and water insoluble (solid) fractions of the steam pretreated biomass were separated by vacuum filtration. The water soluble stream was collected and analysed for sugar content and the water insoluble stream was further washed with water to remove excess, solubilized sugars and unreacted chemicals. The washed substrate was collected for chemical composition analysis and used as the feedstock for subsequent organosolv pretreatments.

# 2.2.2 Organosolv pretreatment

A custom, four-vessel (2 L capacity each) rotating digester (Aurora Products, Savona, BC, Canada) was used for all organosolv pretreatments. For single-stage organosolv pretreatments used in section 3.1.3, 200 g wood chips/pellets (o.d. basis) were either soaked in water overnight at a solid to liquid ratio of 1:1 prior to treatment ("wet") or pretreated directly with no prior preparation ("dry"). Remaining liquor was added as necessary shortly before treatment to achieve a final solid to liquid ratio of 1:10 at an ethanol concentration of 65% v/v with a sulfuric acid loading of 1.1% w/w on the biomass. Samples were pretreated at 170°C for 30 or 60 minutes with a heating rate of 3°C/min. For two-stage organosolv pretreatment on the steam substrates used in section 3.1.6, the reaction was scaled down to using 50 g o.d. basis of substrate. At the end of the treatment, the vessels were cooled in a cold water bath to room temperature. A summary of the single and two-stage pretreatments conditions used in Chapter 3.1 are shown in Table 3 and 4 respectively

 Table 3. Summary of single-stage organosolv pretreatment conditions studied in section 3.1.3

Sample Code	Soaking Condition	Substrate	Time (min)
DC-30	Dry	Chips	30
DC-60	Dry	Chips	60
DP-30	Dry	Pellet	30
DP-60	Dry	Pellet	60
WC-30	Wet	Chips	30
WC-60	Wet	Chips	60
WP-30	Wet	Pellet	30
WP-60	Wet	Pellet	60
DGC-30	Dry	Ground Chips	30
DGP-30	Dry	Ground Pellets	30

and 3.1.5

# Table 4. Summary of two-stage steam/organosolv pretreatment conditions studied in section

3.1.6

		Steam Condition <sup>a</sup>	Organosolv Condition <sup>b</sup>
Sample Code	Substrate	H <sub>2</sub> SO <sub>4</sub> level (% dry matter basis)	Time (min)
NC-30	Chips	0	30
NC-60	Chips	0	60
AC-30	Chips	0.7	30
AC-60	Chips	0.7	60
GC-30	Ground Chips	0.7	30
NP-30	Pellets	0	30
NP-60	Pellets	0	60
AP-30	Pellets	0.7	30
AP-60	Pellets	0.7	60
GP-30	Ground Pellets	0.7	30

<sup>a</sup>All steam pretreatments were performed at 175°C for 7.5 min

 $^b\mbox{All}$  organosolv pretreatments were performed at 170°C with a  $H_2SO_4$  level of 1.1% w/w dry biomass

Pretreated slurries (solid and liquor fractions) for the single-stage treatments were homogenised in a standard British disintegrator for 10 minutes and then separated by vacuum filtration. The liquor was collected and analysed for sugar content. To wash the substrate, the substrate was re-suspended in 1000 mL of 65% w/w aqueous ethanol that was heated to 70°C and filtered. The filtrate was reheated to 70°C and filtered through the substrate a second time.

Lastly, a fresh 500 mL portion of 65% w/w aqueous ethanol heated to 70°C was poured over the top of the filter cake and filtered through the substrate. The resulting filtrate and substrate was collected separately for analysis. For two-stage treatments (section 3.1.6), the pretreated slurry was homogenized in a Waring Commercial, 2-speed laboratory blender (model 51BL30) on the low setting for 10 seconds and then separated by vacuum filtration. A similar washing procedure was followed with the washing volumes scaled down accordingly.

For substrates used in section 3.1.4, never-dried TMP was filtered to remove excess moisture (W-TMP), oven dried overnight at 105°C (OD-TMP) and rewetted overnight to the same moisture content as W-TMP (RW-TMP). Fifty grams biomass (o.d. basis) was used and each sample was organosolv pretreated at a 30:1 liquid to wood ratio, 3.3% w/w H<sub>2</sub>SO<sub>4</sub> loading and 65% v/v ethanol at 170°C for one hour. After pretreatment the substrates were filtered to separate out the solid. The solid portion was washed with warm water, filtered and collected for further analysis.

For second-stage organosolv pretreatment of substrates used in Chapter 3.2, the steam pretreated substrates were impregnated with the organosolv pretreatment liquor overnight and respective chemical loadings as shown in Table 5. Samples were then pretreated at 170°C for 30 minutes. The pretreated slurry was separated by vacuum filtration and the solid fraction was washed with hot, aqueous ethanol as described above.

Table 5. Summary of two-stage steam/organosolv pretreatment conditions studie	d in
Chapter 3.2	

Sampla	Chemical Loading (% dry matter basis)		
Sample	Steam <sup>a</sup>	Organosolv <sup>b</sup>	
205°C + organosolv w/ acid	0.7% H <sub>2</sub> SO <sub>4</sub>	1.1% H <sub>2</sub> SO <sub>4</sub>	
205°C + organosolv w/o acid	0.7% H <sub>2</sub> SO <sub>4</sub>	0% H <sub>2</sub> SO <sub>4</sub>	
185°C + organosolv	0.7% H <sub>2</sub> SO <sub>4</sub>	1.1% H <sub>2</sub> SO <sub>4</sub>	
185°C + organosolv w/ 4HBA	0.7% H <sub>2</sub> SO <sub>4</sub>	1.1% H <sub>2</sub> SO <sub>4</sub> + 4% 4HBA	
185°C + organosolv w/ LS	0.7% H <sub>2</sub> SO <sub>4</sub>	1.1% H <sub>2</sub> SO <sub>4</sub> + 4% LS	
185°C 4HBA + organosolv	0.7% H <sub>2</sub> SO <sub>4</sub> + 4% 4HBA	1.1% H <sub>2</sub> SO <sub>4</sub>	
185°C LS + organosolv	0.7% H <sub>2</sub> SO <sub>4</sub> + 4% LS	1.1% H <sub>2</sub> SO <sub>4</sub>	

<sup>a</sup>All steam pretreatments were heated at the indicated temperature (185/205°C) for 7.5 min <sup>b</sup>All organosolv pretreatments were heated at 170°C for 30 min

## 2.3 Compositional analysis of feedstock, pretreated substrates and hydrolysate

Klason lignin and carbohydrate content of the insoluble fractions for the untreated and pretreated biomass was determined according to the Technical Association of the Pulp and Paper Industry (TAPPI) standard method T-222. Samples from the pretreatment soluble fractions were autoclaved with 0.7 mL of 72% H<sub>2</sub>SO<sub>4</sub> (sample volume used varied, remaining volume made up to 20 mL with water) at 121°C for 1 hour to yield monosaccharides for analysis. Hydrolysates from enzymatic hydrolysis experiments were not further acid-hydrolysed prior to monosaccharide analysis. All monosaccharide analysis was performed using an ICS-3000 HPLC (Dionex, Sunnyvale, CA) equipped with an anion exchange column (Dionex CarboPac PA1). Fucose was used as an internal standard and all samples were filtered through a 0.22 μm nylon filter prior to HPLC analysis.

# 2.4 Enzymatic hydrolysis of pretreated substrates

Enzymatic hydrolysis was conducted at a 10% solids consistency unless otherwise noted with 50 mM acetate buffer (pH 4.8) and incubated at 50°C at 150 rpm. Commercial cellulose preparation, Cellic<sup>®</sup> CTec3 from Novozymes (Franklington, NC) was used at an enzyme loading of 25 mg/g cellulose for all hydrolysis experiments unless otherwise noted. Hydrolysis was terminated at 72 h by incubating the samples in a 100°C water bath for 30 minutes. A sample of the hydrolysis was centrifuged to collect the supernatant for further analysis by HPLC.

# 2.5 Enzymatic hydrolysis with BSA blocking of lignin

Enzymatic hydrolysis of select pretreated substrates were performed with and without BSA addition. Samples blocked by BSA were done so by incubating the reaction at a final concentration of 10 mg/mL of BSA for half an hour at room temperature prior to enzyme addition. Reactions were conducted at a 2% solid consistency with an enzyme loading of 12 mg Ctec3/g cellulose and incubated at 50°C for 72 hours. A sample of the hydrolysate was then collected for further analysis by HPLC.

# 2.6 Neutral dioxane extraction

Steam pretreated substrates were extracted using a dioxane water solvent system in section 3.2.5. Substrates were air dried and ground to powder (40 mesh) using a Wiley Mill. Approximately 10 g of substrate (o.d. basis), accurately weighed, was transferred into a cellulose extraction thimble and placed in a soxhlet extractor. A 9:1 v/v dioxane to water solvent system was used that was targeted towards the extraction of lignin. The extraction was heated under reflux for 18 hours. A rotary evaporator was used to concentrate the resulting extraction solution which was then precipitated by drop-wise addition to water to recover the lignin. The lignin precipitate was filtered, washed, collected and dried in a 40°C vacuum oven for further analysis.

# 2.7 Substrate properties

# 2.7.1 Viscosity

Viscosity of selected substrates were measure according to TAPPI Standard Method T230 om-08 using a capillary viscometer (Cannon Ubbelohde Viscometer, Cannon Instrument Co., State College, PA). Prior to viscosity measurements, substrates were delignified with sodium chlorite following the Useful Method G.10U of the Pulp and Paper Technical Association of Canada (PAPTAC).

# 2.7.2 Acid-group titration

To determine the acid groups on the steam pretreated substrates, approximately 0.25 g (o.d. basis) of substrate was soaked in 50 mL of 0.01 M HCl overnight. The substrate was then filtered under gentle suction, washed with deionized water and re-suspended in 100 mL of 0.001 M NaCl solution. A 0.1 mL aliquot of 0.1 M HCl of known concentration was added and the resulting suspension was then titrated with 0.05 M NaOH of known concentration. Conductivity measurements were recorded at 20 µL aliquot additions using the conductivity probe attachment on the Accumet AR50 from Fisher Scientific.

# 2.7.3 X-Ray photoelectron spectroscopy (XPS)

Samples were prepared by thoroughly washing with distilled water, filtered to remove excess moisture, air dried and ground to powder (40 mesh) using a Wiley Mill. The samples were further dried in a vacuum oven at 40°C overnight and then affixed to an adhesive strip for analysis. Measurements were carried out using a Leybold Max-200 XPS with a Mg Kα X-ray source. A sample area of 4x7 mm<sup>2</sup> was analyzed, with the survey scan and narrow scan conducted at 192 and 48 eV respectively. The samples were measured for carbon, oxygen and sulphur and the data was analyzed using the software provided with the spectrometer.

#### 2.8 Lignin characterization

# 2.8.1 Gel permeation chromatography (GPC)

To determine the molecular weights (M<sub>n</sub> and M<sub>w</sub>) and polydispersity of the dioxane lignin and ethanol organosolv lignin, GPC was performed using an Agilent 1200 series liquid chromatograph equipped with a Wyatt Optilab T-rEx differential refractometer ( $\lambda$ =658 nm, 40 °C). Vacuum dried lignin samples were prepared by dissolution in tetrahydrofuran (THF) at a concentration of approximately 2 mg/mL. An injection volume of 100 µL and flow rate of 0.5 mL/min was used and samples were pumped through a series of Phenomenex Phenogel 5 mm narrow bore columns (104 Å (5000-500 000 Da), 500 Å (1000-15 000 Da) and 103 Å (1000-75 000 Da)). Molecular weights of the samples were determined by comparison to polystyrene standards.

# 2.8.2 <sup>31</sup>P nuclear magnetic resonance (NMR)

Approximately 20 mg of lignin was dissolved in 500 μL of anhydrous pyridine/deuterated chloroform (1.6:1) solution. The samples were stirred to ensure complete dissolution. A hundred microliters of the internal standard solution containing both cyclohexanol (10.85 mg/mL; internal standard) and chromium (III) acetylacetonate (5 mg/mL; relaxation agent) dissolved in anhydrous pyridine/deuterated chloroform solution was mixed into the sample tube. Prior to analysis, 100 μL of the phosphitylating reagent, TMDP (2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphosphlane), was introduced into the sample tube, which was shaken for approximately 20 seconds. The sample was then transferred to a 5 mm NMR tube for analysis. The NMR spectrum was acquired using a Bruker AV II 600 MHz with a QNP cryoprobe using an inverse gated decoupling pulse sequence with a 90° pulse angle with a 25 second delay. A minimum of 40 scans were collected. The chemical shifts were calibrated relative to the peak signal for cyclohexanol at 145.1 ppm and the integration regions used for signal assignment are summarized in Table 6.

Functional Group	Integration Range (ppm)
Aliphatic OH	149.9-145.5
Cyclohexanol (internal standard)	145.5-144.9
C-5 substitution	144.5-140.4
Guaiacyl phenolic OH	140.2-139.0
p-hydroxyphenyl OH	138.2-137.6
Carboxylic acids OH	136.0-134.0

Table 6. Integration regions for <sup>31</sup>P NMR used for quantification of hydroxyl groups on lignin

**3** Results and discussion

# 3.1 Application of a two-stage steam/organosolv pretreatment process for the fractionation of softwood chips and pellets

## 3.1.1 Introduction

Recent years have seen a rise in innovation in the forestry sector and expansion from traditional forest products to alternative products such as bio-fuels, bio-plastics, textile fibres and chemicals to name a few (Cherubini, 2010). The shift from utilizing lignocellulosic biomass as a bulk material to that of a feedstock of higher chemical value is reliant on the effectiveness of pretreatments to separate and recover the individual components (e.g. hemicellulose, lignin, cellulose) into a usable form. In addition, efficient use of the biomass is especially imperative when considering the cost of the biomass, including harvesting, processing and transport. However, the resilient nature of woody biomass, which makes it ideal for use for construction, presents a significant obstacle towards strategies that target clean separation and recovery of the individual chemical components. The existing pellet industry in the Canadian Pacific Northwest region presents an attractive method for improving the overall logistics of biomass collection and transportation, to realize the benefits of economies of scale. In addition, softwoods are the primary feedstock utilized by the pellet industry in the region and, thus, represents a prime biomass source for investigation.

In order to extract the most value out of the biomass being used, technologies previously designed for deconstruction in bioconversion are now also being investigated for use as part of a biorefinery approach. The pretreatment step employed in a bioconversion schematic assists in deconstructing and increasing the accessibility of the biomass, which is necessary to facilitate the fractionation of the biomass into its hemicellulose, lignin and cellulose components. However, the focus of most pretreatments have been on overcoming the challenges of maximizing cellulose hydrolysis and often require more severe pretreatment conditions to achieve. As a result, the more severe conditions required to increase cellulose accessibility often compromise the recovery and usability of the hemicellulose and lignin components. While increasing the ease of cellulose hydrolysis is still a challenge in its own right,

recent developments in cellulolytic enzyme cocktails have greatly improved hydrolysis efficiency and tolerance towards more recalcitrant substrates (Lane, 2012). These improvements reduce the requirements for excessively severe pretreatments, thus, milder pretreatment conditions can also be explored to focus on maximizing clean separation and recovery of each of the biomass constituents.

Of the pretreatments available for processing lignocellulosic biomass, steam and organosolv are two such pretreatment methods that have a long history of investigation at UBC and are capable of overcoming the recalcitrance of softwood biomass (Brownell and Saddler, 1987; Paszner and Cho, 1989). The steam pretreatment process solubilizes hemicellulose in an aqueous stream, but decreases the extractability of the lignin component due to the acidic condensation of lignin which is promoted by increases in temperature, time and acidity (Shevchenko et al., 1999). In contrast, the organosolv process is a solvent extraction method that solubilizes lignin, but compromises hemicellulose recovery due to the harsh conditions required to fragment and solubilize the lignin (Pan et al., 2007). In order to realize the benefits of both pretreatments to fractionate both hemicellulose and lignin, a two-stage approach combining milder versions of both pretreatments may be a viable alternative. Previous work on hardwoods and agricultural residues utilizing a similar two-stage steam/organosolv approach was successful in increasing hydrolysis yields and the recovery of hemicellulose and lignin (Amiri and Karimi, 2016; Panagiotopoulos et al., 2013; Romaní et al., 2011). However, compared to biomass such as hardwoods, softwood biomass has a higher lignin content (25-35% compared to 18-25% in hardwoods) as well as a higher proportion of guaiacyl lignin subunits, which results in an increased potential for lignin condensation that may compromise its removal during organosolv treatment (Pan et al., 2007). It has already been shown that softwood biomass is far less amenable to organosolv delignification than hardwoods and the additional likelihood of lignin condensation occurring during a first-stage steam pretreatment could potentially further compromise lignin removal from softwoods.

During the same timeframe where biomass fractionation has been a key area of investigation, facilitating biomass transportation to supply potential biorefineries has also become a major research focus (Carolan *et al.*, 2007; Kumar *et al.*, 2006). Wood pellets greatly

aid in biomass transport and can be regarded as a potential tradeable biomass feedstock (Gilbert *et al.*, 2009; Kurian *et al.*, 2013; Stephen *et al.*, 2010; Sultana and Kumar, 2011; Wu *et al.*, 2011). There has been limited research on the effects of the pelletization process, which includes size reduction, drying and densification, on the susceptibility of the feedstock to pretreatments that aim to solubilize the lignin component in a usable form. Earlier work in our group indicated that pelletized biomass respond similar to air-dried wood chips during steam pretreatment (Kumar *et al.*, 2012b). However, the previous work did not optimize the steam pretreatment for hemicellulose recovery and did not evaluate the effects of steam pretreatment on the ease of subsequent extraction of the lignin component. This earlier work on comparing the steam pretreatment of pellets to chips also lacked controls that equalized the particle size of the feedstocks, which can also play a role in influencing the "apparent severity" undergone by the biomass during steam pretreatment and, thus, the overall hemicellulose recovery (Cullis *et al.*, 2004).

The objective of this chapter was to assess the ability of a two-stage, steam/organosolv pretreatment approach to effectively fractionate hemicellulose and lignin from pelletized softwood biomass. Similar to the work with hardwoods mentioned above, it was hypothesized that a milder steam pretreatment could be utilized to selectively solubilize the hemicellulose component, while limiting lignin condensation, thereby still allowing for lignin extraction during a subsequent organosolv treatment. The application of the two-stage pretreatment to softwood pellets was compared to wood chips to determine whether the pelletization process (e.g. size reduction, drying, densification) affected the response of the biomass to pretreatment. The initial work examined the response of pellets and wood chip to separate steam and organosolv pretreatments prior to the application of the two-stage steam/organosolv pretreatment.

# 3.1.2 Steam pretreatment for hemicellulose recovery

As mentioned above, it was hoped that milder steam pretreatment conditions (low temperature, low chemical loading) could be used as part of a two-stage, steam/organosolv pretreatment to maximize hemicellulose solubilisation and recovery while minimizing lignin

condensation. To test the effectiveness of mild steam pretreatment to improve hemicellulose recovery, two pretreatment conditions were selected for investigation: auto-catalyzed and acid-catalyzed steam pretreatment. During auto-catalyzed steam pretreatment, the ester bonds that connect the acetyl groups to the hemicellulose backbone can be cleaved, releasing acetic acid into the system to hydrolyze and solubilise the hemicellulose into the water soluble stream. It was hypothesized that, compared to acid-catalyzed steam pretreatment, the autocatalysis approach could remove a portion of the hemicellulose while limiting lignin condensation. However, as softwoods contain fewer acetyl groups when compared to other lignocellulosic biomass such as hardwoods, auto-catalyzed steam pretreatment at low temperatures was not anticipated to be as effective for hemicellulose solubilisation compared to acid-catalysis (Dekker, 1987). Therefore, acid-catalyzed steam pretreatment conditions using sulfuric acid as the acid catalyst were also tested as a comparison to increase hemicellulose solubilisation. Sulfuric acid was chosen as the catalyst due to previous work demonstrating its effectiveness at solubilising hemicellulose during steam pretreatment when compared to SO<sub>2</sub>, which was shown to be more beneficial towards sugar release during enzymatic conversion (Tengborg *et al.*, 1998).

As anticipated, acid-catalyzed steam pretreatment conditions (AC-0 and AP-0) solubilized a greater amount of hemicellulose (49-62%) than auto-catalyzed steam pretreatment (NC-0 and NP-0, 28-33%) but only suffered a slight decrease in total hemicellulose recovery (2-7% decrease) (Figure 6). Previous work on single stage steam pretreatment of Douglas-fir wood chips using similar conditions (175°C, 7.5 min, 4.5% SO<sub>2</sub>) was able to achieve a higher hemicellulose recovery of 77% in the liquor stream (Wu *et al.*, 1999). The difference in hemicellulose solubility is likely due to the lower acid loading of 0.7% w/w H<sub>2</sub>SO<sub>4</sub> that was used in this study. In addition, in this work, the biomass was pre-wetted to a 50% moisture content. The higher moisture content within the biomass likely produced a "buffering effect" against the rapid steam heating, effectively decreasing the "apparent severity" of the pretreatment and contributing to a lower hemicellulose solubilisation (Cullis *et al.*, 2004). To elaborate, the "apparent severity" refers to the actual severity experienced by the substrate when considering all pretreatment effects whereas the "severity factor", the parameter typically used to describe

steam pretreatment severity, only considers the effects of temperature and time. Therefore, the lower apparent severity experienced due to the "buffering effect" could likely also contribute to a lower amount of lignin condensation, which was desired with the milder steam pretreatment conditions chosen.





When wood chips and pellets were pretreated using previously optimized single-stage steam pretreatment conditions for softwoods (200°C, 5 min, 4% SO<sub>2</sub>), an increase in hemicellulose solubilisation was observed when compared to the milder steam pretreatment conditions tested (Figure 6). However, as expected, the more severe SO<sub>2</sub>-catalyzed steam pretreatment conditions resulted in a decrease in the overall hemicellulose recovery from 87-

88% to 62-81% when compared to the milder steam pretreatment conditions at 175°C. For all pretreatment conditions investigated, pellets consistently showed both higher hemicellulose recovery in the water soluble stream (5-13% higher) and overall recovery (1-19% higher) compared to wood chips. These results contrast previous work by Kumar *et al.* that found that the pretreatment of pellets and wood chips resulted in similar amounts of hemicellulose solubilisation after steam pretreatment (2012). However, it should be noted that in the work by Kumar *et al.*, dry wood chips were used. The low moisture content of either substrate likely resulted in uneven SO<sub>2</sub> uptake during the chemical impregnation. As a result, they observed a lower uptake of SO<sub>2</sub> by the pellets which may have contributed to an unfair comparison and underestimation of the hemicellulose solubilization. For the conditions tested in this study, the difference in hemicellulose recovery for the pellets in the water soluble stream was likely due to the smaller particles experiencing a higher apparent severity, resulting in increased hemicellulose removal from the solid fraction (Cullis *et al.*, 2004). Overall, acid-catalyzed steam pretreatment at milder conditions solubilized and recovered from pellets in each case.

## 3.1.3 Organosolv pretreatment of pellets and wood chips

Similar to the experiments above using steam pretreatment, the initial wood chips and pellets were subjected to organosolv pretreatment to assess the ability of organosolv to remove lignin from the pelletized and chipped softwood biomass. Previous work has shown that, similar to the hornification effect observed after drying wood pulps, drying biomass can also be an impediment towards the accessibility of the wood structure to chemical reagents during treatment (Ewanick and Bura, 2016; Suchy *et al.*, 2010). Therefore, wood chips are often pre-steamed prior to kraft pulping to increase their susceptibility to the pulping chemicals (Bäckström *et al.*, 2016). Similarly, it was hypothesized that the drying and compression steps undergone by the biomass during pelletization could result in a compromised response of the pellets to the organosolv delignification process. Therefore, the potential to "rehydrate" the substrate prior to organosolv pretreatment to remediate any detrimental drying effects towards delignification was investigated.

When compared to the dry biomass, it was apparent that wetting was beneficial towards increasing delignification when the organosolv pretreatment was performed on wood chips and pellets for a residence time of 60 minutes (Figure 7a). However, when the residence time of the organosolv pretreatment was decreased to 30 minutes, the opposite trend was observed, with wetting becoming detrimental towards delignification. The difference in the effects of pre-wetting at the two residences times was likely due to the shorter pretreatment time of 30 min being insufficient to allow for a complete exchange between the water in the wood chips and the ethanol rich pretreatment liquor. This incomplete exchange between the pretreatment liquor containing ethanol and the water in the wood chips likely diluted the effect of the pretreatment liquor and decreased the overall delignification rate. However, extending the residence time to 60 minutes allowed for the ethanol rich liquor to penetrate and exchange with the water within the wood chips. In addition, with the longer residence time, the pre-wetting could have potentially helped with fibre swelling within the biomass to improve the amount of delignification (Berry and Roderick, 2005).

Previous work on the organosolv pretreatment of beetle killed lodgepole pine at similar conditions to those that were used here obtained 75-79% delignification (Pan *et al.*, 2007; Pan *et al.*, 2008). However, in this study, 60 and 64% delignification was achieved during the organosolv pretreatment on chips and pellets respectively (dry, 60 min). The lower delignification yield obtained in this study may be due to the higher liquor to wood ratio of 10:1 used in this work, compared to Pan *et al.* who used 7:1 (2008). In this study, the higher liquor to wood ratio was necessary to ensure proper mixing of the pelletized biomass in the pulping digester. However, the higher liquor, resulting in a lower effective acid concentration. Previous work has shown that, by decreasing the acid loading in the organosolv cooking liquor, a corresponding decrease in the delignification of softwood chips was also observed (Pan *et al.*, 2008). Overall, for all of the conditions tested, pellets showed a higher degree of delignification during organosolv when compared to chips. The higher amount of delignification observed in pellets was likely due to the difference in particle size between chips and pellets, whereas the

smaller particle size in pellets could promote better liquor penetration and likely benefit lignin extraction.



**Figure 7.** The (a) delignification and (b) enzymatic hydrolysis yields of dry and rewetted softwood chips ("C") and pellets ("P") after single-stage organosolv pretreated. The softwood biomass was organosolv pretreated at 30 or 60 minutes. Enzymatic hydrolysis was conducted at 10% solids at an enzyme loading of 25 mg Ctec3/g cellulose.

While particle size has been observed to affect biomass response during steam pretreatment, the shorter retention time of steam pretreatment may not be an adequate indicator of the effects of particle size on long retention, pulping-type pretreatments such as organosolv. Pretreatment methods such as hot water and ionic liquid pretreatment are likely more comparable and previous work has also demonstrated the effect of decreasing particle size on increasing pretreatment susceptibility and solubilisation of the woody biomass components (Kilpeläinen et al., 2007; Song et al., 2012). On the contrary, previous work investigating organosolv pretreatment of wheat straw found that particle size had little effect on delignification (Wildschut et al., 2013). However, it has been shown that the size of the biomass should be sufficiently large to reach what has been referred to as a "threshold size" before the particle size will actually play a role in affecting the ease of pretreatment. The threshold size has also been found to depend on both the pretreatment and biomass type, which could explain the contradictory results on the effect of particle size on the pretreatments described previously (Vidal et al., 2011). Therefore, the effect of particle size and its effect on organosolv delignification of softwood chips and pellets will be investigated in later sections of this thesis.

While wetting effects on organosolv pretreatment were dependent on the residence time that was used, it was apparent that, regardless of the pretreatment residence time, rewetting the biomass was detrimental for enzymatic hydrolysis when compared to dry wood chips and pellets (Figure 7b). The negative effects of rewetting were more evident at shorter retention times (30 min), especially with wood chips. Wood chips that were pre-wetted and organosolv treated for 30 min resulted in a substrate with a lower cellulose content, a higher hemicellulose content and a higher solid yield when compared to the same organosolv conditions applied to dry wood chips (Appendix A). In contrast, the other pretreated substrates showed similar chemical compositions between their dry and rewetted counterparts. Similar to what was described above, the lower hydrolysis yields due to rewetting were likely due to the additional time needed for the pretreatment liquor to displace the water within the biomass. Without efficient penetration of the pretreatment liquor into the biomass, the response of the water saturated portions of the biomass would be similar to auto-catalyzed pretreatment,

which has been shown to be less effective on increasing the cellulose accessibility of softwood biomass (Dekker, 1987). In the case of wood chips, the larger particle size and internal pore structure would further impede efficient liquid exchange, which could exacerbate the decrease in enzymatic hydrolysis after rewetting when compared to the organosolv treatment of rewetted pellets. As the organosolv pretreatment retention time was increased from 30 to 60 minutes, the difference in hydrolysis from rewetting decreases, further supporting the hypothesis of a time dependent liquid exchange between the biomass and the pretreatment liquor.

The similarity in the response to organosolv pretreatment between wet and dry pellets was likely due to the smaller particle size allowing for more efficient liquor exchange between the water in the pellets and the pretreatment liquor. From these results, it appears that the wetting of pellets resulted in minor differences in pretreatment response while the wetting of wood chips impeded treatment susceptibility at shorter pretreatment times. In addition, pellets were more easily delignified when compared to softwood chips. The results thus far indicate that, compared to wood chips, pellets appear to a better feedstock with regards to both sugar recovery and delignification for steam and organosolv pretreatments respectively.

# **3.1.4** Substrate drying effects on organosolv delignification of softwood thermomechanical pulp (TMP)

A limitation in the work above that investigated drying effects by testing the rehydration of chips and pellets is that both starting substrates were dried to a certain degree prior to the pretreatments (air dried for wood chips and oven dried for pellets). The air dried wood chips had a moisture content of approximately 10% while the pellets had a moisture content of nearly 5%. Although both samples contained some moisture, in the case of bleached paper pulps it has been shown that when the moisture content drops below a threshold of 18%, the drying effects become irreversible due to hornification (Newman, 2004). Similar to the effects of hornification on pulps mentioned above, it was hypothesized that the drying of the biomass may compromise the ability of the organosolv pretreatment to penetrate the biomass. Therefore, to investigate the effects of drying on delignification during organosolv, a set of

single stage organosolv pretreatments were performed on never dried thermomechanical pulp (TMP). Softwood TMP was chosen as the TMP process retains >95% of the biomass components. Therefore, the chemical composition of TMP would be similar to both the softwood chips and pellets. In addition, the use of never dried pulp was anticipated to accentuate the effects of biomass drying on pretreatment susceptibility.

From the results, it can be seen that rewetted oven-dried TMP (RW-TMP) showed similar levels of delignification and solid yield to never dried TMP (W-TMP) at 72-73% and 43-47% respectively (Table 7). In comparison, it was apparent that the oven-dried TMP (OD-TMP) was less amenable to delignification as the organosolv pretreatment resulted in a lower delignification of 61% and a higher solid yield of 62%. Interestingly, all of the substrates had a similar pulp lignin content of 17-18%, despite the differences in delignification and solid yields. However, it is important to note that the difference in response for OD-TMP could largely be attributed to the hornification of the fibres into stiff pulp aggregates during the drying step. Due to the formation of these aggregates, solvent penetration appeared to be compromised and, without proper mixing to help disrupt these aggregates, there were still visually identifiable pulping "rejects" (e.g. insufficiently treated pulp aggregates) present after the organosolv pretreatment of the dried pulp. In contrast to the hornification effect observed in the case of paper pulp, rewetting TMP was effective for remediating the effects of drying and restoring delignification to levels similar to that of the never dried pulp.

Table 7. Effect of drying and rewetting on organosolv delig	gnification of thermomechanical
pulp (TMP)	

TMP (Sample Code)	Pulp Lignin Content (%)	Delignification (%)	Solid Yield (%)
Never Dried (W-TMP)	17±1	72±1	47±0
Oven Dried (OD-TMP)	18±0	61±0	62±0
Rewetted Oven Dried (RW-TMP)	18±0	73±0	43±0

# 3.1.5 Particle size effects on steam and organosolv pretreatment

In the results described thus far, many of the observed differences in hemicellulose recovery, delignification and enzymatic hydrolysis between pellets and wood chips were to be

due to particle size differences. To test this theory, both wood chips and pellets were size reduced and subjected to the same pretreatment conditions as unground biomass. It should be noted that the size reduction of wood chips and pellets resulted in ground materials of different geometry (Figure 8). This difference in geometry after size reduction was likely due to the prior processing that the pellets had undergone during production. As a result, different mesh sizes were used during the hammer milling of the wood chips and pellets to produce particles of similar visual size. The ground material was then passed through a sieve with a 1.0 mm opening to remove fines. The resulting material was used in further pretreatments. The first pretreatment condition tested was acid-catalyzed steam pretreatment. As expected, an increase in hemicellulose recovery from 49% to 70% in the water soluble stream was observed once the particle size of the wood chips was reduced (Figure 9). In addition, both of the ground substrates (wood chips and pellets) yielded similar results and even slightly outperformed unground pellets. The improvement in hemicellulose recovery in the water soluble stream for the ground substrates was likely due to the further reduction of biomass particle size and the removal of the fine particles during sieving. Through sieving, the biomass size was homogenized and previous work had shown that doing so increases the efficiency of steam pretreatment by allowing more uniform pretreatment (Cullis, 2003).



**Figure 8. Ground softwood (a) chips and (b) pellets.** The biomass was size reduced using a hammer mill and sieved with a screen with a 1.0 mm opening to remove fines.


Figure 9. Percent hemicellulose recovery in the water soluble and water insoluble streams of ground and unground softwood chips and pellets after steam pretreatment. The biomass was impregnated with 0.7% w/w  $H_2SO_4$  and steam pretreated for 7.5 minutes at 175°C. Hemicellulose recovery was calculated relative to the untreated biomass.

When subjected to a single-stage organosolv pretreatment for 30 minutes without prior rehydration, ground chips and ground pellets both showed an increase in delignification but a decrease in enzymatic hydrolysis yields when compared to their unground counterparts (Figure 10). The decrease in enzymatic hydrolysis yields, despite similar levels of delignification, was likely due to the ground material experiencing a higher "apparent severity" in the organosolv treatment due to their smaller particle size. The higher apparent severity (increased mass transfer of acidic pulping liquor, temperature and residence time) likely caused the formation of a greater amount of condensed residual lignin. Consequently, the increase in acid-catalyzed lignin condensation was most likely responsible for the reduced hydrolysis yields due to nonproductive enzyme inhibition (Nakagame *et al.*, 2011a). While the pretreatment response between wood chips and pellets were largely normalized after grinding, the organosolv compared to the ground chips. As mentioned earlier, the difference in response could be due to differences in the geometry between the hammer milled pellets and wood chips as pellets have already gone through a size reduction prior to pelletization (Figure 8). Despite the use of a sieving step to isolate particles of similar size, it was apparent that the hammer milled wood chips and pellets differed in their appearance, which could explain the slight increase in delignification observed during the organosolv delignification of the ground pellets compared to the ground wood chips.



Figure 10. Delignification and enzymatic hydrolysis yields of ground and unground softwood chips and pellets pretreated using a 30 min single-stage organosolv pretreatment. The biomass was organosolv pretreated with 1.1% w/w H<sub>2</sub>SO<sub>4</sub> at 170°C. Enzymatic hydrolysis was conducted at 10% solids at an enzyme loading of 25 mg Ctec3/g cellulose.

#### 3.1.6 Two-stage steam/organosolv pretreatment of softwood chips and pellets

#### 3.1.6.1 Delignification

Having determined that both softwood chips and pellets responded positively to singlestage organosolv delignification, we next examined the efficiency of organosolv pretreatment of softwood chips and pellets that had already undergone steam pretreatment as part of a twostage steam/organosolv pretreatment process. Single-stage organosolv pretreatment of rewetted biomass (referred to as "single-stage organosolv" or "control") was chosen as the control to best reflect the high moisture content of the steam pretreated substrates (auto or acid-catalyzed) that were subjected to a second-stage organosolv pretreatment (30 or 60 minute retention time).

Unlike previous work on hardwoods, when the acid-catalyzed steam pretreated softwood substrates were subjected to the organosolv pretreatment second-stage, a decrease in delignification was observed for all of the substrates when compared to the single-stage organosolv pretreatment (Figure 11) (Panagiotopoulos *et al.*, 2013). The pellets and chips that were first steam pretreated without an acid catalyst, or "auto-catalyzed", prior to organosolv pretreatment at 30 minutes were the only samples that maintained a comparable delignification yield to their respective controls. When the auto-catalyzed steam pretreated samples were organosolv pretreated at a longer retention time of 60 minutes, a decrease in delignification was also observed. It was evident that applying the acid-catalyzed steam pretreated prior to organosolv pretreatment at both 30 and 60 minutes resulted in the largest decrease in delignification (18-32% lower) (Figure 11). In comparison, an auto-catalyzed firststage also decreased delignification during organosolv but to a lesser extent (0-22% lower). The compromised delignification observed with the samples that were steam pretreated prior to organosolv pretreatment was likely due to the condensation of lignin during the steam pretreatment step. As a result, the condensation of lignin decreased the ability to fragment and solubilize the lignin during the subsequent organosolv pretreatment (Li et al., 2007). The higher severity of the acid-catalyzed steam pretreatment compared to the auto-catalyzed steam pretreatment promoted the increased formation of condensation products, thus the autocatalyzed steam pretreated samples were more amenable to subsequent organosolv

delignification (Nakagame *et al.*, 2011a). These results highlight how milder steam pretreatment conditions during the first-stage are necessary to reduce lignin condensation and benefit further delignification during subsequent organosolv pretreatment. However, in the case of softwood, milder auto-catalyzed conditions resulted in poor hemicellulose solubilisation which is detrimental when aiming to develop a two-stage clean fractionation process.





When the retention time during the organosolv pretreatment was increased from 30 to 60 minutes, the delignification of the acid-catalysed steam pretreated substrates increased while delignification of the auto-catalyzed steam pretreated substrates decreased (Figure 11). The opposite trend when extending the residence time of the organosolv pretreatment of the acid vs auto-catalyzed steam pretreated substrates was likely due to the state of the lignin after steam pretreatment. The results suggest that the auto-catalyzed substrates underwent lignin condensation during steam pretreatment but not to the same extent as the acid-catalyzed steam pretreated substrates. As a result, organosolv pretreatment of the auto-catalyzed steam pretreated substrates at 30 min was still effective for delignification. However, when the organosolv pretreatment was extended to a residence time of 60 min, the mildly condensed lignin in the auto-catalyzed steam pretreated sample provided a platform for further lignin condensation to occur under the acidic organosolv environment, limiting delignification and increasing the solid yield by 5%. Lignin condensation during acidic organosolv pretreatment has been observed previously by Sannigrahi *et al.* (2010).

In contrast, the acid-steam pretreated substrates showed an increase in delignification when the residence time during organosolv pretreatment was increased from 30 to 60 min. The increase in delignification was likely due to the overall deconstruction of the substrate with prolonged treatment, allowing the lignin to be more easily liberated from the biomass along with the carbohydrate component. These results are supported by an overall decrease in the solid yield (4-14% decrease) for acid-catalyzed steam pretreated substrates when the residence time during organosolv pretreatment was extended to 60 min. It is hypothesized that, due to the higher severity steam pretreatment undergone by the biomass under acid-catalyzed steam pretreatment conditions, the subsequent acidic organosolv pretreatment at an extended residence time would result in an increase in the acid-hydrolysis of the carbohydrate component. To determine if the carbohydrates were indeed being hydrolyzed, viscosity was used as an indirect measurement to assess the degree of polymerization of cellulose (DP) for the single-stage acid-catalyzed steam pretreated pellets (AP-0), acid-catalyzed steam pretreated pellets that had undergone organosolv pretreatment at 30 minutes (AP-30) and the acid-catalyzed steam pretreated pellets that were organosolv pretreated at 60 minutes (AP-60). It was apparent that the viscosity of the substrates decreased when the biomass was subjected to two pretreatment stages and further decrease was observed when the residence time of the organosolv pretreatment was extended to 60 minutes (Table 8). Previous work by Pan et al. also observed a decrease in cellulose DP with increasing organosoly pretreatment severity, which also correlated with increasing enzymatic conversion (2007).

Table 8. The effect of increasing the residence time during the organosolv pretreatment of acid-catalyzed steam pretreated pellets (0 vs. 30 vs. 60 min) on the viscosity of the bleached substrate

Substrate	Viscosity (mPa*s)
AP-0	3.93±0.02
AP-30	2.41±0.01
AP-60	2.17±0.01

Increasing the organosolv pretreatment retention time from 30 to 60 minutes was also seen to slowly diminish the differences in delignification observed between auto- and acidcatalyzed steam pretreatments. With increasing retention time, the difference in delignification between auto- and acid-catalyzed steam pretreated substrates decreased from 15-21% at 30 minutes to 1-10% at 60 minutes. As organosolv pretreatment severity increases with increasing retention time, the acidic organosolv pretreatment environment results in continual condensation of the residual lignin with simultaneous acid hydrolysis of the cellulosic component. Eventually, the overall deconstruction of the biomass at increasing pretreatment severity will limit the effects of the characteristics of the starting biomass on its ease of pretreatment.

As discussed earlier, the consistently higher amount of delignification achieved during the organosolv pretreatment of pellets after both single and two-stage organosolv conditions was likely attributed to the particle size effects. The smaller particle size of the pelletized biomass when compared to wood chips likely allowed for more efficient heating and mass transfer of the pretreatment liquor during pretreatment. To further confirm these particle size effects, acid-catalyzed steam pretreatment was performed on the ground and sieved pellets and wood chips with subsequent organosolv pretreatment at a retention time of 30 minutes. Similar to what was observed with single-stage organosolv pretreatment, the organosolv pretreatment of the acid-catalyzed steam pretreated ground material resulted in a higher amount of delignification when compared to the unground material (Figure 12). The ground pellets also underwent a greater amount of delignification compared to the ground wood chips during the two stage process. The increase in delignification during organosolv pretreatment observed with the ground material supports the significance of particle size effects on affecting the pretreatment response between softwood chips and pellets. Overall implementing an acidcatalyzed steam pretreated first-stage was detrimental to delignification for both biomass types while auto-catalyzed steam pretreatment was less detrimental toward delignification, but compromised hemicellulose solubilisation, thus decreasing the efficacy of the two-stage fractionation approach.





#### **3.1.6.2** Enzymatic hydrolysis

Originally, it was anticipated that a multi-stage pretreatment design would ultimately increase enzymatic hydrolysis due to the increase in cellulose accessibility created by the removal of hemicellulose and lignin during the steam and organosolv pretreatment processes. In the case of hardwood biomass, the ease of hydrolysis, delignification and hemicellulose

recovery all improved when implementing a two-stage steam/organosolv pretreatment (Panagiotopoulos *et al.*, 2013). However, as shown above, rather than enhancing delignification, the use of a steam pretreatment step to enhance hemicellulose recovery when pretreating softwood chips and pellets resulted in compromised delignification during organosolv pretreatment.

Similar to the results observed with organosolv delignification, when the two-stage steam/organosolv pretreated substrates were subjected to enzymatic hydrolysis, a decrease in the hydrolysis yield was observed for all of the substrates, with the exception of auto-catalyzed steam pretreated chips that were organosolv pretreated for 30 min (Figure 13). It was hypothesized that, compared to the control where the moisture within the rewetted chips proved to be inhibitory towards organosolv pretreatment, the structure of the auto-catalyzed steam pretreated chips became more permeable for the exchange between the water contained in the chips and the organosolv pretreatment liquor after steam pretreatment. This effect is similar to increasing the severity of the pre-steaming conditions prior to Kraft pulping, which has been shown to increase the susceptibility of the biomass to Kraft pulping (Bäckström et al., 2016). In addition, organosolv pretreatment of the auto-catalyzed steam pretreated chips at a retention time of 30 minutes could be considered the mildest treatment condition tested as the chips have a larger particle size than the pellets and the auto-catalyzed steam pretreatment conditions were the mildest steam pretreatment conditions tested. Therefore, the degree of lignin condensation and resulting non-productive enzyme binding was also expected to be the least pronounced with this substrate that was pretreated at the mildest conditions. As anticipated, the hydrolysis of the organosolv pretreated auto-catalyzed steam pretreated wood chip sample had an enhanced hydrolysis yield compared to the control sample (Figure 13). However, it should be noted that auto-catalyzed steam pretreatment of the wood chips only solubilized 28% of the hemicellulose in a usable form. Therefore, it was evident that in order to obtain effective hydrolysis of the cellulose component after a two-stage steam/organosolv process, steaming conditions that compromise the clean separation of the hemicellulose component must be applied. It appeared that conditions that facilitated the clean

separation of hemicellulose compromised both the ability to delignify the biomass and the ease of hydrolysis of the resulting cellulosic substrate



Figure 13. Enzymatic hydrolysis yields of organosolv pretreated (30 or 60 min) softwood chips ("C") and pellets ("P") that were pretreated with a single-stage organosolv (rewetted) pretreatment or a two-stage steam/organosolv pretreatment with a prior auto-catalyzed or acid-catalyzed steam pretreatment (SE) first-stage. Enzymatic hydrolysis was conducted at 10% solids at an enzyme loading of 25 mg Ctec3/g cellulose.

Similar to what was observed with organosolv delignification of the steam pretreated substrates, two opposing trends were observed for the organosolv pretreatment of auto- or acid-catalyzed steam pretreated substrates when the retention time was increased from 30 to 60 minutes. As explained previously, mild lignin condensation during auto-catalyzed steam pretreatment likely created a platform for subsequent lignin condensation to occur during the acidic organosolv pretreatment as the pretreatment retention time was extended from 30 to 60 minutes. As a result, increased lignin condensation produces more hydrophobic lignin that is more prone to non-productive binding with the hydrolytic enzymes, effectively decreasing the overall hydrolysis yield. In contrast, the increase in enzymatic hydrolysis after organosolv pretreatment retention time was increased from 30 to 60 min for acid-catalyzed steam pretreated substrates was likely due to the overall deconstruction of the biomass, further opening up the cellulosic structure at extended pretreatment times. As discussed above, the conditions for acid-catalyzed steam pretreatment were more severe compared to autocatalyzed steam pretreatment and resulted in a greater amount of lignin condensation, which was reflected by the decrease in delignification observed after organosolv. However, since the lignin in the acid-catalyzed steam pretreated substrates prior to organosolv pretreatment were already more condensed, lignin condensation effects during organosolv would, consequently, be less significant as well, allowing the beneficial effects of overall deconstruction to overtake that of lignin condensation. Previous work investigating single-stage steam pretreatment at increasing severities also observed the same phenomenon where, despite increasing lignin condensation, the effects of disrupting the cellulosic structure eventually overtook the condensation effects and resulted in an overall increase in enzymatic hydrolysis (Nakagame *et al.*, 2011a).

As mentioned previously, hardwood and agricultural residue substrates such as poplar, eucalyptus and rice straw have shown an increase in enzymatic hydrolysis after a two-stage steam/organosolv pretreatment (Amiri and Karimi, 2016; Panagiotopoulos *et al.*, 2013; Romaní *et al.*, 2011). In contrast, previous work investigating a two-stage steam/organosolv pretreatment process on pine observed a decrease in enzymatic hydrolysis after both treatments, with a low cellulose conversion of only 23% (Amiri and Karimi, 2016). The increased recalcitrance of softwood biomass has largely been attributed to the difference in lignin composition between the biomass types. Compared to hardwoods and agricultural resides, softwoods not only typically have a higher lignin content but also contain a higher proportion of guaiacyl lignin. As mentioned in previous sections, guaiacyl lignin has been found to form more stable condensation products that can negatively impact both delignification and enzymatic hydrolysis (Shimada *et al.*, 1997; Tsutsumi *et al.*, 1995; Wikberg and Maunu, 2004). From the results, it appears that a 2-stage steam/organosolv pretreatment was mostly detrimental towards increasing enzymatic hydrolysis of softwood chips and pellets when compared to a single-stage organosolv pretreatment.

#### 3.1.6.3 BSA blocking

As described previously, enzymatic hydrolysis was substantially decreased for most substrates after a two-stage steam/organosolv pretreatment. The results were somewhat surprising as it was originally anticipated that the cumulative effect of the two-stage steam/organosolv pretreatment would be effective in disrupting the cellulosic structure and increase enzymatic hydrolysis. Instead, the results suggest that lignin condensation during the two-stage steam/organosolv pretreatment interfered with both delignification and enzymatic hydrolysis. Interestingly, the substrate lignin content after organosolv pretreatment was highly dependent on the condition of the first-stage steam pretreatment but independent of biomass type and length of organosolv pretreatment. Substrates that had undergone auto-catalyzed or acid-catalyzed steam pretreatment in the first stage all had a substrate lignin content of 24-25% and 30-32% after organosolv pretreatment respectively, despite differences in delignification and solid yield (Appendix B). In comparison, the substrate lignin content of biomass subjected to a single-stage organosoly pretreatment was lower at 19-23%. However, despite similar substrate lignin contents, the enzymatic conversion of those substrates differed considerably, suggesting that the different pretreatment conditions produced lignin of varying degrees of condensation.

Lignin condensation in softwood biomass has been shown to be particularly detrimental towards enzymatic conversion, where non-productive binding between the hydrophobic lignin and hydrolytic enzymes results in decreased enzyme efficiency (Eriksson *et al.*, 2002; Nakagame *et al.*, 2010; Nakagame *et al.*, 2011a). Previous research has demonstrated the use of "BSA blocking" as a method to increase enzymatic hydrolysis by decreasing the non-productive binding between the enzymes and substrate lignin (Yang and Wyman, 2006). Briefly, the incubation of lignocellulosic substrates with the protein, BSA, prior to enzyme addition results in hydrophobic interactions between BSA and the substrate lignin. The hydrophobic interactions leads to BSA effectively occupying the potential "non-productive binding sites" on the substrate lignin that would otherwise bind the enzymes. Since cellulases and BSA are both proteins that can bind with the substrate lignin through hydrophobic interactions, it was anticipated that BSA binding would also be more substantial for substrates treated at more

severe conditions and that have more condensed lignin, as observed in previous work on enzyme binding to residual lignin after pretreatment (Nakagame *et al.*, 2011a). Therefore, we anticipated that the hydrolysis of the substrates that contained a greater amount of more condensed hydrophobic lignin would similarly benefit from BSA blocking to a greater extent. The increase in enzymatic hydrolysis yields as a result of BSA blocking could then be used as an indirect measurement for estimating the inhibitory effects of condensation of the substrate residual lignin after pretreatment.

As anticipated, the substrates that had undergone acid-catalyzed steam pretreatment prior to organosolv showed the most substantial relative increase in enzymatic hydrolysis after BSA blocking, at 3.3-4.3 compared to a 1.8-2.7 relative increase for the auto-catalyzed steam pretreated substrates that had undergone subsequent organosolv pretreatment (Figure 14). In addition, organosolv pretreatment of auto-catalyzed steam pretreated chips at a 30 minute retention time had the smallest relative increase (1.8), further supporting the prior discussion that this milder pretreatment likely underwent the least amount of lignin condensation. In addition, BSA blocking generally appeared to be more effective for pellets than wood chips, which supports the prior hypothesis that the smaller particle size for pellets experienced a higher apparent severity, resulting in increased lignin condensation. Overall, the BSA blocking treatment showed that cellulose accessibility was in fact being generated during the two-stage, steam/organosolv pretreatment as the hydrolysis yields of these substrates increased after the effects of non-productive enzyme binding to the residual lignin component were minimized. It was apparent that the condensation undergone by the lignin during the acidic steam and organosolv pretreatments was the most likely cause of the decrease in enzymatic conversion due to the non-productive binding of the enzymes to the condensed residual lignin during hydrolysis. Therefore, in order to minimize the impact of lignin condensation on enzymatic conversion after a two-stage steam/organosolv pretreatment of softwood biomass it will likely be necessary to investigate methods to either minimize lignin condensation and/or to modify the lignin to reduce lignin hydrophobicity.



**Figure 14. Relative increase in enzymatic hydrolysis of two-stage steam/organosolv pretreated substrates after BSA blocking treatment.** Prior to enzyme addition, BSA blocked substrates were incubated at room temperature with 10 mg BSA/mL hydrolysate. Enzymatic hydrolysis was conducted at 2% solids at an enzyme loading of 12 mg Ctec3/g cellulose. Relative increase was calculated as cellulose conversion of substrate after BSA blocking divided by the conversion of the unblocked substrates.

### 3.1.7 Conclusions

One of the main objectives of this chapter was to investigate the response of softwood chips and pellets to fractionation during a two-stage steam/organosolv process. The ability of a mild steam pretreatment to solubilize hemicellulose and enhance overall hemicellulose recovery was investigated. However, it was found that the auto-catalyzed steam pretreatment conditions were too mild and resulted in low hemicellulose solubilisation (<35%) despite high overall recovery. In comparison, the more severe acid-catalyzed, steam pretreatment conditions solubilized more than 60% of the hemicellulose in the pellets with minimal compromise to the overall hemicellulose recovery (2% decrease). Single-stage organosolv pretreatment was also found to be effective in achieving >60% delignification and an enzymatic

hydrolysis yield of >75% when the organosolv pretreatment was performed using a 60 minute residence time. However, while both single-stage steam and organosolv pretreatments were found to be effective in solubilising hemicellulose and extracting lignin respectively, applying steam pretreatment prior to organosolv pretreatment as a two-stage process resulted in the decrease of both delignification and enzymatic hydrolysis yields. It was hypothesized that the acidic steam pretreatment conditions resulted in increased lignin condensation which impeded subsequent organosolv delignification and inhibited enzymatic hydrolysis through nonproductive interactions between the hydrophobic lignin and hydrolytic enzymes. In addition, BSA blocking of the two-stage, steam/organosolv pretreated substrates was capable of minimizing the effects of non-productive enzyme inhibition and increased the yields during enzymatic hydrolysis of the substrates. The results of the BSA blocking experiments indicated that cellulose accessibility was being generated after the two-stage pretreatment and further supports the inhibitory effects of lignin condensation that occurs during the acidic two-stage pretreatment. Overall, softwood biomass was less receptive towards a two-stage, steam/organosolv pretreatment process when compared to other biomass types such as hardwood and agricultural residues.

From the previous results it was observed that softwood chips and pellets had responded differently to the pretreatment conditions tested. It was anticipated that the pelletization process (size reduction, drying) likely contributed to the difference in pretreatment response between wood chips and pellets. When wood chips and pellets were ground and sieved to a comparable particle size, ground wood chips showed an increase in hemicellulose solubilisation and lignin extraction and achieved yields comparable to that of pellets. In addition, it was found that rewetting the biomass prior to pretreatment was able to remediate the effects of drying on organosolv pretreatment, thereby eliminating the concerns of drying during pelletization adversely affecting pretreatment susceptibility. Therefore, the difference in pretreatment response observed in this study was largely due to the difference in particle size between wood chips and pellets. Generally, it was apparent that pellets were more favourable towards extraction (e.g. hemicellulose solubilisation, lignin removal) but had lower enzymatic conversion when compared to wood chips. The lower enzymatic hydrolysis yield was

likely attributed to the smaller particle size of the pellets experiencing a higher "apparent severity" during pretreatment, resulting in an increase in lignin condensation. Overall, pellets appeared to be a viable alternative to wood chips for use in a biomass pretreatment fractionation process.

From the pretreatment conditions investigated, it was realized that the lignin component played the largest role in impeding the effective fractionation of softwood biomass in a two-stage, steam/organosolv pretreatment process. In order to achieve adequate hemicellulose solubilisation under mild conditions, acid-catalyzed steam pretreatment was necessary to compensate for the low hemicellulose acetyl content on softwoods. However, the acidic steam pretreatment environment also resulted in an increase in condensation of the more recalcitrant softwood guaiacyl lignin. Overall, the acidic two-stage, steam/organosolv pretreatment resulted in an increase in lignin condensation which correlated with a decrease in delignification and enzymatic hydrolysis yields. Therefore, in order to more effectively fractionate softwood biomass, it was necessary to devise alternative methods to minimize and/or overcome the inhibitory effects of lignin condensation.

# 3.2 Impact of lignin condensation on the pretreatment efficiency of a two-stage steam/organosolv pretreatment process on softwood pellets

#### 3.2.1 Introduction

The previous chapter showed that acid-catalyzed steam pretreatment was necessary to solubilize greater than 60% of the hemicellulose component from softwood pellets, which were general more responsive to pretreatments than softwood chips. However, the implementation of an acid-catalyzed steam pretreatment also resulted in a substantial decrease in the ability to extract lignin during subsequent organosoly pretreatment. The results in the previous chapter illustrated the compromise when attempting to apply a two-stage steam/organosolv approach to cleanly fractionate the cellulose, hemicellulose and lignin from softwood chips and pellets. As mentioned above, it was necessary to add an acid catalyst during steam pretreatment in order to solubilize sufficient hemicellulose to justify the use of a first-stage steam pretreatment. However, the use of the acid catalyst during the first steam pretreatment stage compromised downstream delignification during the second organosolv pretreatment stage, likely through increased lignin condensation which increased the recalcitrance of the residual lignin component. It was also apparent that acid-catalyzed lignin condensation not only occurred during the first steam pretreatment stage, but also continued to occur during the second acidic organosolv stage. As well as hindering delignification, the condensed lignin also acted to inhibit enzymatic hydrolysis through increased non-productive binding despite the generation of cellulose accessibility, as shown by the BSA blocking experiments.

As demonstrated in the previous chapter, the increase in lignin condensation after a two-stage, acidic steam/organosolv pretreatment resulted in a >50% decrease in enzymatic hydrolysis when compared to a single-stage organosolv pretreatment. While complete lignin removal would be ideal to generate a more accessible substrate and to eliminate non-productive enzyme binding, such a process is generally too costly and chemically intensive to be practical for implementation in a biorefinery scheme (Kumar *et al.*, 2010). Alternatively, lignin modification has been explored as a potential method to circumvent the need for complete lignin removal while minimizing the effects of lignin on enzymatic hydrolysis (Kumar *et al.*,

2011). Lignin modification through sulfonation has been shown previously to benefit delignification and enzymatic hydrolysis by increasing lignin solubility and reducing non-productive, hydrophobic interactions between the residual lignin and hydrolytic enzymes (Kumar *et al.*, 2011; Zhang *et al.*, 2014). Similarly, the addition of lignosulfonates during steam pretreatment of poplar chips has also seen an increase in subsequent enzymatic hydrolysis (75 to 92%) and was hypothesized to benefit hydrolysis through a similar mechanism (Chandra *et al.*, 2015).

Due to the lack of acetyl groups that decorate the hemicellulose backbone, the use of an acid catalyst is unavoidable when trying to achieve adequate hemicellulose solubilization during the steam pretreatment of softwood biomass. However, there have been alternative methods to improve hemicellulose solubilization and recovery that have been applied previously to hardwoods and may also be effective when treating softwood biomass. Previous work by Chandra et al. mentioned above studied the effect of lignosulfonate addition during acidcatalysed steam pretreatment of poplar biomass and demonstrated an overall increase in hemicellulose recovery and a concomitant enhancement in the ease of enzymatic hydrolysis of the biomass (2015). This previous work also showed that the sulfonic acid content of the biomass lignin increased upon addition of the lignosulfonates to steam pretreatment, suggesting a coupling between the lignosulfonates and biomass lignin. The aromatic moieties present within lignosulfonates have the potential to serve as carbocation scavengers to minimize the intermolecular lignin re-polymerization (condensation) that occurs during steam pretreatment as a result of electrophilic aromatic substitution reactions that are catalyzed by the acid that is added during steam pretreatment to solubilize hemicellulose. Therefore, it is possible that lignosulfonates acted as a carbocation scavenger and coupled to the biomass lignin through the condensation mechanism.

Steam pretreatment promotes both depolymerization (cleavage of  $\beta$ -O-4 bonds) as well as re-polymerization (increase in lignin molecular weight through condensation) of the lignin fraction. Briefly, carbocation scavengers function as a "quenching agent" and provide an electron rich, aromatic moiety to react with the benzyl carbocations that form in the lignin as a result of the acid added during steam pretreatment, thus preventing intermolecular lignin

reactions. The use and effectiveness of carbocation scavengers, such as 2-naphthol, to improve delignification and minimize lignin re-polymerization have been demonstrated repeatedly on hardwood biomass, but there have been limited studies on the effect of these scavengers during the pretreatment of softwoods (Li et al., 2007; Pielhop et al., 2015; Wayman and Lora, 1978, 1980). In addition, the few studies that have been performed previously to test the effectiveness of carbocation scavengers on the ease of pretreatment of softwoods have employed liquid hot water pretreatment and required long residence times (>60 min), which compromised carbohydrate recovery (Pielhop et al., 2015). The study also demonstrated that shorter residence times (<20 min) limited the effectiveness of the 2-naphthol addition but it has yet to be seen whether other carbocation scavengers would also respond similarly during shorter residence times such as those employed during steam pretreatment (5-10 min) (Pielhop et al., 2015). Although, 2-naphthol has been the most extensively studied carbocation scavenger to date, its minimal water solubility involves complicated and tedious solvent-based impregnation procedures. These characteristics of 2-napthol pose challenges for scale-up as well as for aqueous pretreatments such as steam pretreatment. Alternatively, previous work by Wayman and Lora has shown that 4-hydroxybenzoic acid (4HBA), a compound with moderate water solubility, could also act as a carbocation scavenger to limit lignin condensation during the acid-catalyzed hot water treatment of hardwood biomass (1978).

Based on the findings from the previous chapter and the results of prior studies employing targeted carbocation scavengers and lignosulfonates, the objective of this chapter was to minimize lignin condensation through the introduction of potential carbocation scavengers (e.g. 4HBA and lignosulfonates) during the 2-stage pretreatment of softwood pellets. During pretreatment, the acidic conditions utilized for both the steam and organosolv pretreatment processes can promote lignin condensation. Therefore, the application of 4HBA or lignosulfonates was tested during the steam and organosolv pretreatment stages to determine the stage where lignin condensation had the greatest impact. It was hypothesized that the addition of 4HBA or lignosulfonates as potential carbocation scavengers would be most effective during the first, steam pretreatment step as 4HBA or lignosulfonates would minimize the effects of lignin condensation, thereby increasing subsequent delignification by organosolv pretreatment. The incorporation of either 4HBA or lignosulfonates into the lignin macromolecule would increase acidic groups on the lignin and thus also potentially reduce nonproductive, hydrophobic enzyme interactions (Nakagame *et al.*, 2011b). The initial work reexamined the steam pretreatment conditions to determine if the hemicellulose solubilization could be improved during the initial steam pretreatment stage while the subsequent work involved the use of carbocation scavengers during the steam and organosolv pretreatments.

#### 3.2.2 Influence of steam pretreatment severity on hemicellulose solubilization and recovery

The previous chapter established that pellets were more responsive to the steam pretreatment conditions investigated when compared to wood chips. It should be noted that the steaming conditions employed for the first steam pretreatment stage (175°C at 7.5 min) in the previous chapter were based on the steam pretreatment of wood chips, rather than pellets. The difference in response between wood chips and pellets to steam pretreatment observed in the previous chapter suggests that the optimal steam pretreatment condition to further improve hemicellulose solubilization and recovery for pellets are likely different as well. Therefore, the optimal condition for maximizing hemicellulose solubilization and recovery during the pretreatment of pellets was examined in this chapter.

When softwood pellets were steam pretreated at increasing temperatures (175-205°C), the overall hemicellulose recovery (water soluble + water insoluble streams) decreased and the greatest amount of hemicellulose recovery was obtained at the mildest condition (175°C) (Figure 15). The decrease in hemicellulose recovery with increasing steam temperature was anticipated and has been shown previously to be due to the degradation of the more labile hemicellulose component to furans as pretreatment severity increases (Chandra *et al.*, 2007; Wu *et al.*, 1999). However, while steam pretreatment at 175°C had the highest overall recovery, the optimal condition for hemicellulose recovery in the water soluble stream was observed at 185°C. As the primary purpose of the first-stage steam pretreatment step in the two-stage process was to recover hemicellulose in the water soluble stream, the ideal condition would achieve the best combination of the highest hemicellulose solubilization and a minimal decrease in overall hemicellulose recovery. It was apparent that steam pretreatment at 185°C

achieved this compromise and thus was chosen as the optimal condition used in subsequent steam pretreatment experiments described below.



**Figure 15. Percent hemicellulose recovery in the water soluble and water insoluble streams from softwood pellets after steam pretreatment at increasing temperatures (175-205°C).** The pellets were impregnated with 0.7% w/w H<sub>2</sub>SO<sub>4</sub> and steam pretreated for 7.5 minutes at the indicated temperature. Hemicellulose recovery was calculated relative to the untreated biomass.

## **3.2.3 Effect of lignosulfonate and 4-hydroxybenzoic acid (4HBA) addition during steam** pretreatment

As shown in the previous chapter the addition of acid was necessary to solubilize hemicellulose during the steam pretreatment of softwood biomass. However, the addition of acid condenses lignin which compromises its solubilization during the second-stage organosolv pretreatment. Lignin condensation also decreases the ease of hydrolysis of the resulting pretreated substrate, mostly through non-productive binding of enzymes. One approach to mitigate the detrimental effects of lignin condensation is to add a carbocation scavenger that reacts with the native biomass lignin during the acidic steam pretreatment process, thereby decreasing the tendency for lignin to couple with itself and re-polymerize. As mentioned above, 2-napthol is challenging to solubilize and use for larger scale steam pretreatments. In contrast, 4-hydroxybenzoic acid (4HBA) has higher water solubility than 2-napthol and has been shown to function as a carbocation scavenger. Earlier work by Chandra *et al.* (2015) demonstrated an increase in hemicellulose solubilization and recovery with the addition of lignosulfonates during steam pretreatment. In addition, lignosulfonates possess the necessary aromatic moieties to function as a potential carbocation scavenger such as 4HBA. In particular, it was surprising that lignosulfonates were able to increase the hemicellulose solubilization and recovery during acid-catalyzed steam pretreatment. There has yet to be any studies focusing on the effects of carbocation scavengers on hemicellulose solubilization and recovery. Therefore, the addition of lignosulfonates or 4HBA during acid-catalyzed steam pretreatment of softwood pellets was investigated to determine the effect of both compounds on hemicellulose recovery and lignin condensation.

The addition of 4HBA and lignosulfonates during acid-catalysed steam pretreatment resulted in limited changes to the hemicellulose recovered in the water soluble fraction at 185°C (60-62%) (Figure 16). The 4HBA addition during steam pretreatment also did not appear to impact the total hemicellulose recovery, relative to the control without the addition of 4HBA or lignosulfonates (71% vs. 72% respectively). However, steam pretreatment with lignosulfonates resulted in an increase in overall hemicellulose recovery as a result of the addition of lignosulfonates is not fully understood, it has been theorized to be due to the surfactant properties of the lignosulfonates allowing the hydrophilic portion of lignosulfonates to aid in "protecting" the hemicellulose during the typically aggressive steam pretreatment (Chandra *et al.*, 2015). This is supported by similar improvements to hemicellulose recovery that have also been observed with the use of surfactants such as Tween 80 in the pretreatment of corn stover (Qing *et al.*, 2010).



**Figure 16.** Percent hemicellulose recovery in the water soluble and water insoluble streams of softwood pellets steam pretreated at 185°C with and without the addition of 4hydroxybenzoic acid (4HBA) and lignosulfonates (LS). The pellets were impregnated with 0.7% w/w H<sub>2</sub>SO<sub>4</sub> and 4% w/w of either 4HBA or lignosulfonates. Impregnated pellets were then steam pretreated for 7.5 minutes at 185°C. Hemicellulose recovery was calculated relative to the untreated biomass.

In contrast to the addition of 4HBA, it was apparent that the addition of lignosulfonates increased the overall hemicellulose recovery during steam pretreatment compared to the acidcatalyzed control pretreatment. However, as it was anticipated that the additives would function as potential carbocation scavengers, it was of interest to determine whether 4HBA or lignosulfonates had successfully reacted with the substrate lignin. As a preliminary investigation, the samples steam pretreated at 185°C and at 185°C with lignosulfonates were analyzed using X-ray photoelectron spectroscopy (XPS) to detect for the presence of sulphur on the substrate surface. The presence of sulphur would indicate the addition of lignosulfonates to the pellet biomass during steam pretreatment. The XPS analysis of the sample steam pretreated at 185°C with lignosulfonates to the pellet biomass during steam pretreatment. the control, 185°C, did not result in detectable levels of sulphur. Since XPS only examines the surface of the substrate, bulk acid group titration was also utilized to quantify the total addition of either lignosulfonates or 4HBA during steam pretreatment. The presence of lignosulfonates would increase the amount of strong acid groups (sulfonic acid groups) on the substrate while the presence of 4HBA would increase the amount of weak acid groups (carboxylic acid groups).

Acid group titration of the sample steam pretreated with 4HBA indicated that the addition of 4HBA did not result in an enhancement of carboxylic acid groups, suggesting a lack of 4HBA incorporation onto the substrate (Table 9). The inability of 4HBA to incorporate into the substrate lignin was likely due to the incompatible pretreatment conditions utilized in this study. Previous work that demonstrated the successful addition of carbocation scavengers to hardwood or softwood biomass utilized higher temperatures and longer pretreatment times (e.g. 210°C for up to 4 hours) (Pielhop *et al.*, 2015). Therefore, it is likely that the shorter pretreatment time and milder steam pretreatment conditions chosen for this study were not sufficient to "activate" the 4HBA to act as a carbocation scavenger and react with the substrate lignin.

In contrast to the addition of 4HBA to steam pretreatment, steam pretreatment with lignosulfonates resulted in an increase in strong acid groups compared to the control, supporting the successful reaction of lignosulfonates with the substrate lignin. Previous work on poplar chips measured 50-60 mmol/kg of strong acid groups after the poplar chips were subjected to acid-catalyzed steam pretreatment with a 3-6% w/w loading of lignosulfonates (Chandra *et al.*, 2015). In comparison, acid-catalyzed steam pretreated pellets with a 4% w/w loading of lignosulfonates achieved 23 mmol/kg of strong acid groups in this thesis. The lower amount of acid groups observed in this thesis could possibly be due to the difference in pretreatment conditions employed in the two studies (0.7% w/w H<sub>2</sub>SO<sub>4</sub>, 185°C @ 7.5 min vs. 3% w/w H<sub>2</sub>SO<sub>4</sub>, 160°C @ 15 min for this thesis and work by Chandra *et al.* (2015) respectively). The higher acid loading used in the previous study on poplar would encourage increased acidolysis of the lignin component which could allow for the increased formation of carbocation intermediates necessary for reaction with the added lignosulfonates. In addition, the longer pretreatment time could also provide more time for the reaction with lignosulfonates to occur,

resulting in a higher incidence of attachment of the lignosulfonates to the biomass lignin in the study by Chandra *et al.* (2015). Overall, the successful incorporation of lignosulfonates demonstrates the potential for lignin modification through the condensation pathway while using a pulping by-product at relatively low chemical loadings.

Table 9. Acid group titration of substrates steam pretreated with and without the addition of
4-hydroxybenzoic acid (4HBA) or lignosulfonates (LS)

Substrate	Strong Acid Groups (mmol/kg)	Weak Acid Groups (mmol/kg)
185°C	8	12
185°C 4HBA	12	12
185°C LS	23	14

## **3.2.4** Enzymatic hydrolysis of substrates steam pretreated with the addition of 4hydroxybenzoic acid (4HBA) or lignosulfonates

Previous work observed an increase in enzymatic hydrolysis when lignosulfonates were added during the steam pretreatment of hardwood biomass and when carbocation scavengers were added during the autohydrolysis pretreatment of spruce (Chandra et al., 2015; Pielhop et al., 2015). The addition of 4HBA during steam pretreatment of softwood pellets resulted in a similar enzymatic conversion yield of 25% at an enzyme loading of 12 mg/g cellulose compared to the control which yielded 23% conversion (Figure 17). In comparison, the addition of lignosulfonates during steam pretreatment resulted in a surprising decrease in enzymatic hydrolysis yield to 15%. While these results contradict the previous results observed by Chandra et al., it is important to note that the substrates in their study were subjected to two sequential steam pretreatment stages, which could result in additional substrate modifications that could further improve cellulose accessibility (2005). In addition, lignosulfonates have a much higher molecular weight (typically >1000 Da) when compared to small carbocation scavengers such as 2-naphthol and 4HBA. Incorporation of the high molecular weight lignosulfonates into the substrate during steam pretreatment could potentially result in an increase in the physical blockage of the enzyme accessible sites on cellulose, resulting in a decrease in enzymatic hydrolysis yields.





Increasing the enzyme loading to 42 mg/g cellulose further accentuated the differences in enzymatic hydrolysis response. At the higher enzyme loading substrates with 4HBA addition during steam pretreatment resulted in a slightly higher enzymatic conversion yield of 45% compared to the control (40%). As mentioned earlier, the addition of 4HBA likely functioned as a secondary acid source during steam pretreatment and would result in the substrate experiencing a higher severity. The higher severity experienced by the pellets steam pretreated with 4HBA would promote increased cellulose accessibility and higher enzymatic hydrolysis yields, which is similarly observed when increasing the temperature for steam pretreatment (Appendix C). However, the higher severity will also result in increased lignin condensation which can result in enzyme inhibition through non-productive binding with the more hydrophobic lignin. The application of a higher enzyme loading has been shown previously to be able to overcome the effects of non-productive binding by "sacrificing" some of the enzymes towards saturating the non-productive lignin binding sites (Kumar *et al.*, 2012a). As a result, the increase in the difference between the substrate with 4HBA addition and the control at higher enzyme loadings further supports the function of 4HBA as a secondary acid source during steam pretreatment. In comparison, the substrate with the addition of lignosulfonates during steam pretreatment still resulted in a lower enzymatic hydrolysis yield of 26% compared to the control which further supports the hypothesis of physical blocking by the incorporated lignosulfonates.

When the samples were blocked with BSA, the substrate pretreated with 4HBA during steam pretreatment showed the largest relative increase in hydrolysis yield after blocking (Figure 18). Considering how an increase in weak acid groups was not detected on the substrate steam pretreated with 4HBA, these results further support the hypothesis that 4HBA may have acted as a secondary acid source rather than adding to the substrate as a carbocation scavenger. As mentioned previously, the presence of an additional acid source would be anticipated to result in an increase in pretreatment severity, cellulose accessibility and consequently lignin condensation, which is supported by the higher relative increase in hydrolysis observed after BSA blocking of the substrate steam pretreated with 4HBA. In contrast, BSA blocking of the substrate pretreated with lignosulfonates showed little change in the hydrolysis yield (1.2x increase). As anticipated, the increase in lignin hydrophilicity as a result of the incorporation of lignosulfonates would likely diminish the ability of BSA to bind to the lignin via hydrophobic interactions. Therefore, the use of BSA on the lignin that was modified by lignosulfonates to increase its hydrophilicity would not result in an increase in hydrolysis yields since BSA was utilized to prevent non-productive binding between more hydrophobic lignin and hydrolytic enzymes. The detection of sulphur on the substrate surface (XPS) and the increase in strong acid groups (acid group titration) also supported the results of the BSA blocking and, collectively, they indicate the successful incorporation of the more hydrophilic lignosulfonates with the residual substrate lignin. Interestingly, despite the difference in conversion and response to BSA blocking, all three substrates showed similar klason lignin (40~42%) and glucan (53~54%) contents. The difference in response despite similar chemical composition after steam pretreatment further supports the varying roles that lignosulfonates and 4HBA play in substrate modification when included as an additive during the first-stage steam pretreatment.



Figure 18. Relative increase in enzymatic hydrolysis of substrates steam pretreated with and without the addition of 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS) after BSA blocking treatment. Relative increase was calculated as cellulose conversion of substrate after BSA blocking divided by the conversion of the unblocked substrates. Prior to enzyme addition, BSA blocked substrates were incubated at room temperature with 10 mg BSA/mL hydrolysate. Enzymatic hydrolysis was conducted at 2% solids at an enzyme loading of 12 mg Ctec3/g cellulose.

# 3.2.5 Influence of steam pretreatment severity on hemicellulose solubilization and dioxane lignin extraction of softwood pellets

The previous section determined the steam pretreatment conditions that maximized hemicellulose solubilization and recovery. It was also apparent that the addition of carbocation scavengers such as 4HBA and the addition of lignosulfonates resulted in the simultaneous modification of lignin during the steam pretreatment stage aimed at solubilizing hemicellulose. From the previous chapter investigating two-stage steam/organosolv fractionation of softwood biomass, it was found that lignin condensation during the steam pretreatment stage was particularly detrimental towards lignin extraction during subsequent organosolv pretreatment. Therefore, to assess the effects of the steam pretreatment conditions on the ease of lignin extraction, an indirect screening method using dioxane was employed. Extraction using aqueous neutral dioxane was chosen as an indicator of the ease of delignification of steam pretreated substrates based on previous work by Li and Gellerstedt (2008). It was anticipated that the dioxane/water system would serve as a potential indicator to how effectively the substrates will subsequently respond to delignification under the ethanol/water system during organosolv pretreatment.

Previous work by Li *et al.* (2007) demonstrated an increase in dioxane lignin extraction of hardwoods after pretreatment at increasing steam pretreatment severity, which was correlated with a decrease in  $\beta$ -O-4 linkages. However, the authors also observed an increase in the formation of higher molecular weight lignin, indicating an increasing tendency for both repolymerization and depolymerization to occur simultaneously at increasing pretreatment severities. It was anticipated that a similar trend of increasing re-polymerization with increasing pretreatment severity would also be observed for softwood substrates. However, the higher guaiacyl lignin content in softwoods was predicted to result in an even higher tendency to favour re-polymerization and, contrary to the results observed for hardwoods, result in a decrease in overall dioxane lignin extraction at increasing steam pretreatment severity. Therefore, acid-catalyzed steam pretreated substrates produced at increasing temperatures from 175-205°C were extracted with neutral dioxane to determine the relationship between increasing steam pretreatment severity and the extractability of the lignin from the pretreated pellets using dioxane.

When the steam pretreated softwood pellets were subjected to dioxane lignin extraction, the results were unexpected as it was hypothesized that the higher steam pretreatment temperatures would compromise the extractability of the lignin due to increased lignin condensation. Surprisingly, the trend during the dioxane extraction of softwood pellets pretreated at a range of temperatures were similar to that observed by Li *et al.* for hardwood biomass (2007). As the steam pretreatment temperature was increased from 175°C to 205°C, the dioxane lignin extraction yield of the substrates steam pretreated at increasing severity also increased from 12% to 17%, with untreated pellets only achieving 7% dioxane lignin extraction

(Table 10). As mentioned, it was originally anticipated that the increasing steam pretreatment severity of softwood biomass would result in a decrease in lignin extraction due to the formation of more condensed lignin structures, similar to what was observed in the previous chapter with the two-stage steam/organosolv pretreatment. In contrast, unlike the previous chapter where it was shown that acid-catalyzed steam pretreatment decreased the ability of the organosolv pretreatment to extract lignin, the dioxane extraction was able to extract increasing amounts of lignin as the temperature (severity) was raised during the initial steam pretreatment. However, it is also important to note that under the neutral dioxane extraction conditions employed only a small fraction of the overall residual lignin is being extracted during the procedure. While the extraction yields are comparable to previous work that have also utilized neutral dioxane extraction, the lack of acid will likely limit the extraction ability to lower molecular weight lignin that is more easily solubilized. The extractability of the lignin from the steam pretreated substrates using organosolv pretreatments will be discussed further in section 3.2.6-7.

Table 10. Dioxane extracted lignin yield of substrates steam pretreated at increasing temperatures with and without the addition of 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS)

Sample <sup>a</sup>	Lignin Extraction (g/100g substrate)	Substrate Lignin Content (%)	Lignin Extraction Relative to Substrate Lignin (%)
Untreated pellet	2.1	29±1	7.4
175°C	4.5	39±1	11.7
185°C	5.9	42±1	14.0
195°C	7.0	42±0	16.5
205°C	7.8	46±0	17.1
185°C 4HBA	6.7	42±0	16.0
185°C LS	5.4	40±0	13.4

<sup>a</sup>Samples were steam pretreated with 0.7% w/w H2SO4 and respective chemicals (e.g. 4HBA or LS) at the indicated temperature for 7.5 min.

The addition of 4HBA or lignosulfonates to the substrates during steam pretreatment increased and decreased the yield of dioxane lignin extracted respectively when compared to the acid-catalyzed steam pretreated control at 185°C (Table 9). Based on previous work, it was

anticipated that the incorporation of carbocation scavengers would result in an increase in lignin extraction due to the limited formation of higher molecular weight lignin (Li et al., 2007; Pielhop et al., 2015). While the sample where 4HBA was added during steam pretreatment resulted in an increase in dioxane lignin extraction, the increase can also support the hypothesis that 4HBA acted as an additional acid source (i.e. increasing pretreatment severity). It should be noted that the data from BSA blocking and acid group measurements indicated that 4HBA was not attaching to the substrate during steam pretreatment. Therefore, the higher extraction yield observed with the 4HBA sample was most likely due to 4HBA acting as an additional weak acid source that likely participated in acidolysis reactions that can consequently enhance lignin extraction with the dioxane. In comparison, the addition of lignosulfonates resulted in a slight decrease in dioxane lignin extraction. An interesting observation was made during the dioxane extraction of substrates steam pretreated with lignosulfonates. When biomass pretreated with lignosulfonates was subjected to dioxane extraction, the formation of a dark, sticky precipitate along the edges of the extraction flask just above the liquid level of the solvent was observed. The observed precipitate was only present in the extraction of substrates pretreated with lignosulfonates and raised the question of the compatibility of the dioxane/water solvent system with the more hydrophilic, sulfonated lignin. A lack of compatibility could potentially result in an underestimation of the ease of lignin extractability for substrates steam pretreated with lignosulfonates due to solubility limitations. To test the solubility of lignosulfonates in different solvent systems, a small sample of the lignosulfonates was dissolved in water and then added to the ethanol/water (65:35) and dioxane/water (9:1) systems to achieve the final solvent proportions indicated (Figure 19a-c). From the results it was apparent that lignosulfonates were soluble in both the water and ethanol/water systems. In comparison, the addition of solubilized lignosulfonates to the dioxane/water system resulted in a cloudy solution upon mixing. Over time, the mixture of lignosulfonates and dioxane/water resulted in the formation of a dark, sticky precipitate and the solution became less cloudy as the precipitate was formed (Figure 19d).



Figure 19. Solubility of the commercial lignosulfonates in (a) water, (b) ethanol/water (65:35), (c) dioxane/water (9:1) and (d) dioxane/water (9:1) after two weeks.

As mentioned earlier, previous work on hardwood biomass showed an increase in dioxane lignin extraction and higher molecular weight lignin with increasing steam pretreatment severity (Li et al., 2007). A similar increase in the yield of lignin during dioxane extraction was also observed in this study for softwood pellets steam pretreated at increasing severities and GPC analysis of the extracted lignin also resulted in a general trend of increasing molecular weight (Table 11). The trend towards extracting higher molecular weight lignin at higher steam pretreatment severities may have been due to the simultaneous lignin repolymerization reactions that occur during acidolysis of lignin. Analysis of the dioxane lignin also showed an increase in C-5 substitution and overall phenolic OH content at increasing steam pretreatment severity. The increase in C-5 substitution has been shown to be indicative of lignin condensation while the increase in total phenolic OH is due to the cleavage of  $\beta$ -O-4 bonds, thus indicating the parallel depolymerization/re-polymerization reactions (Sun et al., 2014) (Table 12). As expected, very little p-hydroxyphenyl OH was observed in the pretreated substrates and is consistent with the low p-hydroxyphenyl content initially found in softwood biomass (Pu et al., 2011; Sannigrahi et al., 2008). A decrease in aliphatic OH and carboxylic OH groups was also observed with increasing steam pretreatment severity. While the cause for the decrease is not entirely clear, a similar trend depicting a decrease in aliphatic OH after pretreatment has been observed in previous work on lignin isolated from two-stage acid

pretreated loblolly pine and dilute acid pretreated switchgrass compared to the starting biomass (Samuel *et al.*, 2010; Sannigrahi *et al.*, 2008). A potential explanation for the decrease in both aliphatic and carboxylic OH groups is the increased cleavage of lignin-carbohydrate complex (LCC) bonds at increasing pretreatment severities. The cleavage of LCC bonds could liberate carbohydrate contaminates (e.g. glucuronic acid) that could potentially contribute to the aliphatic and carboxylic OH signals observed during NMR analysis. Overall, the results from the NMR analysis are in agreement with the findings from GPC and dioxane lignin extraction and support the concomitant depolymerization and re-polymerization reactions occurring during steam pretreatment at increasing severities.

Analysis of the lignin obtained from the dioxane extraction of substrates steam pretreated with lignosulfonates showed an increase in aliphatic OH and a slight decrease in C-5 substitution and total phenolic OH compared to the 185°C control without the addition of lignosulfonates. As mentioned earlier, the higher aliphatic OH content despite little change to the other functional groups could also be due to carbohydrate contamination in the sample. Interestingly, steam pretreatment of softwood pellets with the addition of 4HBA resulted in a slight decrease in C-5 substitution while the total phenolic OH content increased. Previous work observed an increase in  $\beta$ -O-4 cleavage with the addition of carbocation scavengers as well (Li *et al.*, 2007, Pielhop *et al.*, 2015). In addition, a decrease in C-6/C-2 and minimal change to C-5/C-2 substitution was observed after the pretreatment of spruce with 2-naphthol (Pielhop *et al.*, 2015). It was originally anticipated that the addition of carbocation scavengers would reduce the amount of C-5 substitution detected by NMR analysis. However, the decrease in C-5 substitution observed was unexpected because prior analysis of the substrate steam pretreated with 4HBA did not support the reaction of 4HBA with the substrate lignin. Table 11. Summary of  $M_n$ ,  $M_w$  and polydispersity data from GPC analysis of dioxane extracted lignin from substrates steam pretreated at increasing temperatures with and without the addition of 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS)

Somploi	Aver	age	Polydispersity	
Sample	Mn	Mw	(M <sub>w</sub> /M <sub>n</sub> )	
175°C	246	1507	6.13	
185°C	236	1475	6.25	
195°C	246	1615	6.57	
205°C	274	1774	6.46	
185°C 4HBA	244	1535	6.29	
185°C LS	256	1547	6.04	

<sup>a</sup>Samples were steam pretreated with 0.7% w/w H2SO4 and respective chemicals (e.g. 4HBA or LS) at the indicated temperature for 7.5 min.

Table 12. Summary of functional groups detected by <sup>31</sup> P NIVIR on dioxane extracted lightin
from substrates steam pretreated at increasing temperatures with and without the addition
of 4-hydroxybenzoic acid (4HBA) and lignosulfonate (LS)

	Functional Groups (mmol/g)					
Sample <sup>a</sup>	Aliphatic OH	C-5 substitution	Guaiacyl phenolic OH	p- hydroxyphenyl OH	Carboxylic acids OH	Total phenolic OH
175°C	2.2	0.4	0.7	0.1	0.7	0.8
185°C	2.0	0.6	1.0	0.1	0.6	1.1
195°C	1.9	0.6	1.1	0.1	0.5	1.2
205°C	1.8	0.9	1.3	0.1	0.4	1.4
185°C 4HBA	2.0	0.3	1.1	0.1	0.6	1.2
185°C LS	2.3	0.5	0.9	0.1	0.6	1.0

<sup>a</sup>Samples were steam pretreated with 0.7% w/w H2SO4 and respective chemicals (e.g. 4HBA or LS) at the indicated temperature for 7.5 min.

### 3.2.6 Organosolv pretreatment of substrates steam pretreated at 205°C

Based on the dioxane lignin extraction results in the previous section, steam pretreatment of pellets at 205°C showed the highest delignification yield. Therefore, it was of interest to assess whether the extractability of the lignin in the steam pretreated pellets at 205°C would also be enhanced during a second-stage organosolv pretreatment. When the pellets steam pretreated at 205°C were subjected to organosolv pretreatment, a delignification yield of only 36% was achieved (Table 13). In comparison, a higher delignification yield of 40% but a similar selectivity of 1.22 was achieved during the acidified organosolv pretreatment of pellets that were steam pretreated at 175°C (data shown previously in section 3.1.6.1). These results from the organosolv pretreatment using acidic aqueous ethanol are opposite to those observed with the dioxane extraction as discussed above. However, since the dioxane extraction was conducted under neutral conditions, it was uncertain whether the acidic environment of the organosolv pretreatment resulted in additional condensation that interfered with efficient extraction (Sannigrahi *et al.*, 2010). Similar to the effects of steam pretreatment, acidic organosolv treatment has also been shown to both polymerize and depolymerize lignin simultaneously. Therefore, pellets steam pretreated at 205°C was also subjected to organosolv pretreatment without the addition of the sulfuric acid catalyst to provide a better comparison to the neutral dioxane extraction conditions.

When the substrates that were subjected to steam pretreatment at 205°C were organosolv pretreated without the addition of the acid, the delignification yield further decreased from 36% to 26% while little change to lignin selectivity was observed. From these results it was evident that additional acid was still necessary to facilitate the depolymerization and extraction of lignin from the steam pretreated substrates during the second-stage organosolv pretreatment. In addition, the lower delignification yield during organosolv pretreatment of steam pretreated substrates at 205°C compared to 175°C indicates that the neutral dioxane/water extraction system was not a good predictor of the delignification response of an ethanol/water organosolv system. As well as the inability of dioxane extraction to indicate organosolv delignification, the formation of a precipitate during the dioxane extraction of substrates steam pretreated with lignosulfonates also illustrates the limits of the dioxane/water system for assessing the substrates in this work. However, despite these limitations, dioxane lignin extraction still serves as a valuable analytical tool to assess the chemical and physical changes the lignin undergoes during steam pretreatment. To better reflect the effect of 4HBA and lignosulfonates on the ease of extracting lignin from the pellets steam pretreated at 185°C, acid-catalyzed ethanol organosolv was utilized in subsequent studies.

Table 13. Delignification, klason lignin content and selectivity after organosolv pretreatment of steam pretreated substrates at 205°C with and without the addition of 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS)

Substrate	Delignification <sup>a</sup> (%)	Klason Lignin Content (%)	Lignin Selectivity <sup>b</sup>
205°C + organosolv w/ acid	36±1	37±0	1.24
205°C + organosolv w/o acid	26±1	38±0	1.20

<sup>a</sup>Delignification yield relative to the treated biomass.

<sup>b</sup>Lignin selectivity is calculated as the solid yield divided by the percent retention of the lignin in the solid substrate relative to the steam pretreated substrate.

### 3.2.7 Organosolv pretreatment of substrates steam pretreated at 185°C

The previous sections determined that the optimal condition for hemicellulose recovery in the water soluble stream was steam pretreatment at 185°C and that the addition of lignosulfonates during steam pretreatment increased hemicellulose recovery and resulted in substrate lignin modifications. Therefore, the samples steam pretreated at 185°C were used as the model substrate in subsequent sections. When the substrates steam pretreated at 185°C with and without the addition of 4HBA or lignosulfonates were subjected to a 30 minute organosolv pretreatment, delignification yields in the range of 46-56% was observed (Table 14). Unlike the results from the dioxane lignin extraction of the steam pretreated substrates observed above, organosolv pretreatment of the substrate steam pretreated with lignosulfonates resulted in the highest amount of delignification while the substrate steam pretreated with 4HBA resulted in the lowest delignification yield. These results further support the importance of choosing the correct solvent system to accommodate the type of lignin modifications that the substrate may have undergone. In addition, despite having similar starting lignin contents (40-42%), organosolv pretreatment of the substrates steam pretreated with and without the addition of 4HBA or lignosulfonates resulted in residual substrate lignin contents ranging from 25-32%. The addition of lignosulfonates during steam pretreatment also resulted in an increase in the lignin selectivity during organosolv pretreatment compared the acid-catalyzed steam pretreatment control (1.55 vs 1.41). It was hypothesized that the addition of lignosulfonates during steam pretreatment resulted in lignin modifications which increased lignin hydrophilicity and increased solubility of the residual substrate lignin to the organosolv

system, which resulted in more effective delignification during the second-stage pretreatment (Iakovlev and Heiningen, 2012). In comparison, the addition of 4HBA during steam pretreatment was detrimental towards subsequent delignification. This further implied that 4HBA may have played a role as a secondary acid source rather than as a carbocation scavenger.

Table 14. Delignification, klason lignin content and selectivity after organosolv pretreatment of steam pretreated substrates at 185°C with and without 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS)

Substrate	Delignification <sup>a</sup> (%)	Klason Lignin Content (%)	Lignin Selectivity <sup>b</sup>
185°C + organosolv	52±1	29±0	1.41
185°C 4HBA + organosolv	46±1	32±0	1.33
185°C LS + organosolv	56±1	25±0	1.55

<sup>a</sup>Delignification yield relative to the treated biomass.

<sup>b</sup>Lignin selectivity is calculated as the solid yield divided by the percent retention of the lignin in the solid substrate relative to the steam pretreated substrate.

Interestingly, while organosolv pretreatment of pellets that were steam pretreated at 205°C resulted in a decrease in organosolv delignification compared to the organosolv pretreatment of pellets steam pretreated at 175°C, substrates steam pretreated at 185°C saw an overall increase in organosolv delignification yield and a higher lignin selectivity. It is possible that steam pretreatment at 185°C achieved a greater amount of acidolysis compared to steam pretreatment at 175°C, while steam pretreatment at 205°C shifted lignin reactions toward condensation. Regardless, in the case of pellets, steam pretreatment at 185°C with an acid catalysis provided an efficient combination of increased hemicellulose recovery in the water soluble stream and improved susceptibility to organosolv delignification, when compared to steam pretreatment at milder temperatures (175°C).

# **3.2.8** Organosolv pretreatment in the presence of 4-hydroxybenzoic acid (4HBA) and lignosulfonates

The previous sections of this chapter have mainly focused on the effects of lignin condensation occurring during the first-stage steam pretreatment where the solubilization and
recovery of hemicellulose was the main goal. However, the acidic conditions employed during organosolv pretreatment can also result in further lignin re-polymerization (Sannigrahi *et al.*, 2010). Ethanol acts as both a solubilization medium for lignin that has been cleaved via acidolysis reactions as well as participating in the delignification reaction itself through solvolysis (McDonough, 1993). However, if the residence time, acidity or temperature is raised, the reactions of the lignin component have been shown to shift from fragmentation to re-polymerization (Sannigrahi *et al.*, 2010). Therefore, the effect of the addition of 4HBA and lignosulfonates during the organosolv pretreatment of pellets that were already steam pretreated under acid-catalyzed conditions at 185°C was investigated. While previous sections demonstrated that 4HBA did not seem to act as a carbocation scavenger during steam pretreatment, it was anticipated that the longer residence time utilized during organosolv pretreatment may provide additional time for the 4HBA to combine with lignin and thus act as an effective carbocation scavenger.

A second-stage organosolv pretreatment with the addition of 4HBA or lignosulfonates resulted in an overall decrease in delignification yield from 52% (control) to 45% and 50% for substrates pretreated with the addition of 4HBA and lignosulfonates respectively (Table 15). The addition of 4HBA during the second-stage organosolv pretreatment resulted in a similar delignification yield and substrate lignin content compared to 4HBA addition during the firststage steam pretreatment. The similar pretreatment response elicited by 4HBA addition during either pretreatment stage suggests that 4HBA also did not act as a carbocation scavenger during organosolv pretreatment. Similar to the previous observations, the results suggest that 4HBA acted as an additional acid source and resulted in further lignin condensation during the second-stage organosolv pretreatment. In the case of lignosulfonates, the addition of lignosulfonates during the second-stage organosolv pretreatment did not result in a similar increase in delignification as when lignosulfonates were added during the first-stage steam pretreatment. However, a decrease in residual substrate lignin and an increase in delignification selectivity was observed, albeit to a lesser degree compared to the addition of lignosulfonates during steam pretreatment. The response observed with the addition of lignosulfonates during the second-stage organosolv pretreatment suggests that lignin modification can still occur

during the second-stage organosolv pretreatment, despite some condensation already having occurred in the prior, steam pretreatment stage. However, an alternative explanation is that the increase in delignification with the addition of lignosulfonates could be attributed to the lignosulfonates in the organosolv pretreatment liquor acting as a surfactant to improve lignin solubility, which has been previously demonstrated to occur with surfactants such as Tween80 (Qing *et al.*, 2010).

Table 15. Delignification, klason lignin content and selectivity during the organosolv stage of softwood pellets two-stage steam/organosolv pretreated with and without the addition of 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS)

Substrate	Delignification <sup>a</sup> (%)	Klason Lignin Content (%)	Lignin Selectivity <sup>b</sup>		
185°C + organosolv	52±1	29±0	1.41		
185°C + organosolv w/ 4HBA	45±1	31±0	1.31		
185°C + organosolv w/ LS	50±1	27±0	1.52		

<sup>a</sup>Delignification yield relative to the residual lignin in the steam pretreated biomass. <sup>b</sup>Lignin selectivity is calculated as the solid yield divided by the percent retention of the lignin in the solid substrate relative to the steam pretreated substrate.

To determine whether lignosulfonates acted as a surfactant to increase lignin solubility or if the addition of lignosulfonates resulted in hydrophilic lignin modifications to produce more soluble lignin, acid group titration of the two-stage steam/organosolv pretreated substrates were performed. As mentioned previously, the detection of strong acid groups (e.g. sulfonic acid groups) would support the addition of lignosulfonates to the substrate while the detection of weak acid groups (e.g. carboxylic acid groups) would indicate the addition of 4HBA. In agreement with previous observations, the addition of 4HBA during the second-stage organosolv pretreatment did not result in an increase in weak acid groups compared to the control. It was apparent that, despite the longer retention time employed during organosolv pretreatment, 4HBA did not act as a carbocation scavenger in either steam or organosolv pretreatment stages for the conditions tested in this study. In contrast, the addition of lignosulfonates during the second-stage organosolv pretreatment resulted in a mild increase in strong acid groups (11 mmol/kg) compared to the control without the addition of lignosulfonates (5 mmol/kg) (Table 16). Interestingly, a second-stage organosolv pretreatment of pellets steam pretreated with lignosulfonates observed a decrease in strong acid groups from 23 to 7 mmol/kg compared to the single-stage steam pretreated substrate prior to organosolv pretreatment. The 70% reduction in strong acid groups after organosolv pretreatment was greater than the delignification yield (56%), which suggests the more selective removal of the hydrophilic lignosulfonate attached lignin. In comparison, the addition of lignosulfonates during organosolv pretreatment yielded a lower quantity of strong acid groups (11 mmol/kg) when compared to substrates steam pretreated with lignosulfonates. The lower quantity of strong acid groups could potentially be due to lignin condensation during the first-stage steam pretreatment limiting the amount of incorporation of lignosulfonates that could occur during the subsequent second-stage organosolv pretreatment. However, a more likely explanation for the low quantity of strong acid groups measured on the residual solid is that the addition of lignosulfonates during organosoly pretreatment resulted in both the incorporation of lignosulfonates and increased lignin removal as a result of the simultaneous lignin extraction occurring during organosolv pretreatment. The acidic environment of the organosolv pretreatment would encourage both re-polymerization and depolymerization reactions, resulting in lignin modification via the incorporation of lignosulfonates followed by subsequent selective removal of the lignin modified with lignosulfonates. As a result, the quantity of strong acid groups on the substrate that was organosolv pretreated with the addition of lignosulfonates would remain relatively low. This is similar to the reduction in acid groups observed during the organosolv pretreatment of substrates that were steam pretreated in the presence of lignosulfonates where the sulfonic acid groups decreased because of enhanced lignin solubilization. Overall, it was observed that the addition of lignosulfonates during the first-stage steam pretreatment was more effective for the incorporation of lignosulfonates than when lignosulfonates were added during the second-stage organosolv pretreatment of the steam pretreated substrates. The incorporation of lignosulfonates into the substrate lignin enhanced the selectivity of the delignification during organosolv pretreatment.

Table 16. Acid group titration of substrates two-stage, steam/organosolv pretreated with and without the addition of 4-hydroxybenzoic acid (4HBA) or lignosulfonates (LS)

Substrate	Strong Acid Groups (mmol/kg)	Weak Acid Groups (mmol/kg)
185°C + organosolv	5	11
185°C + organosolv w/ 4HBA	5	7
185°C + organosolv w/ LS	11	7
185°C 4HBA + organosolv	4	5
185°C LS + organosolv	7	7

# 3.2.9 Enzymatic hydrolysis of two-stage steam/organosolv pretreated softwood pellets with and without the addition of 4-hydroxybenzoic acid (4HBA) or lignosulfonates

Previously in this chapter it was observed that the addition of lignosulfonates during single-stage steam pretreatment of softwood pellets resulted in a decrease in the enzymatic hydrolysis yield. This result differed from those shown in previous work by Chandra *et al.* where steam pretreatment of hardwood biomass in the presence of lignosulfonates improved the overall enzymatic hydrolysis yield (2005). However, in that study, the biomass that was steam pretreated in the presence of lignosulfonates was subjected to a second, more severe steam pretreatment step prior to enzymatic hydrolysis. It was hypothesized that the second, more severe steam pretreatment step resulted in further modifications to the substrate and the lignosulfonate-modified residual substrate lignin which contributed to the overall increase in enzymatic hydrolysis observed. Therefore, it was of interest to determine whether the implementation of a second organosolv pretreatment stage after the addition of 4HBA or lignosulfonates during the steam pretreatment step would induce similar modifications that would result in an overall increase in enzymatic hydrolysis in this study as well.

As expected and in agreement with the data seen so far, the addition of 4HBA during the second-stage organosolv pretreatment did not change the enzymatic hydrolysis yield compared to the control (39-40%) (Figure 20). In comparison, organosolv pretreatment of pellets steam pretreated with 4HBA resulted in a slight decrease in enzymatic conversion (36%). A decrease in the enzymatic hydrolysis yield to 32% was also observed for substrates pretreated with a second-stage organosolv pretreatment in the presence of lignosulfonates. The decrease in hydrolysis after the addition of lignosulfonates during the second-stage organosolv pretreatment was similar to the decrease in enzymatic hydrolysis yield observed in the previous section when hydrolyzing pellets steam pretreated with lignosulfonates. In the previous section it was hypothesized that the decrease in enzymatic hydrolysis yield after steam pretreatment with lignosulfonates was possibly due to the attachment of the high molecular weight lignosulfonates to the residual substrate lignin, resulting in physical blocking of the enzyme accessible cellulose sites. However, the lower strong acid group content in the substrate with the addition of lignosulfonates during the second-stage organosolv pretreatment (11 mmol/kg vs 23 mmol/kg for pellets steam pretreated with lignosulfonates) suggests that physical blockage by high molecular weight lignosulfonates may not be the only contributing factor in impeding enzymatic hydrolysis. An alternative hypothesis for the decrease in enzymatic hydrolysis yield that was observed when lignosulfonates were added during the second-stage organosolv pretreatment could be due to the lack of acid hydrolysis of the cellulose component. During the second-stage organosolv pretreatment in the presence of lignosulfonates, a near complete recovery of the cellulose was observed, relative to the cellulose content of the first-stage steam pretreated substrate. In contrast to these results, the two-stage steam/organosolv pretreated substrate without the addition of 4HBA or lignosulfonates resulted in a glucan recovery of 84%. The higher glucan recovery suggests a lack of acid-hydrolysis of the cellulosic component and the increase in recovery was similar to the increase in overall hemicellulose recovery observed after the first-stage steam pretreatment of softwood pellets in the presence of lignosulfonates. The increase in carbohydrate recovery observed when lignosulfonates were added in either pretreatment stage suggests that lignosulfonates have some sort of a "protecting function" that minimized the degradation of the carbohydrate component. Previous work demonstrated that a decrease in cellulose DP as a result of acid-hydrolysis of the cellulose correlated with an increase in enzymatic hydrolysis yield (Pan et al., 2007). Therefore, the results with the addition of lignosulfonates during the organosolv pretreatment suggests a reduction in the generation of cellulose accessibility due to limited cellulose hydrolysis during the second-stage organosoly pretreatment.





As mentioned previously, the addition of lignosulfonates during steam pretreatment of softwood pellets resulted in a decrease in enzymatic hydrolysis of the steam pretreated substrate. However, when the substrate steam pretreated with lignosulfonates was subjected to a subsequent second-stage organosolv pretreatment an increase in enzymatic hydrolysis yield (58%) was observed compared to the control (39%) (Figure 20). Subsequent organosolv pretreatment of the substrate steam pretreated with lignosulfonates resulted in an increase in delignification and decrease in substrate lignin content compared to the control substrate pretreated without the addition of 4HBA or lignosulfonates. In addition, the retention of a low quantity of strong acid groups (7 mmol/kg) after the second-stage organosolv pretreatment suggests the retention of some hydrophilicity on the residual substrate lignin. From these results it was apparent that the higher enzymatic hydrolysis yield observed after the two-stage

steam/organosolv pretreatment of pellets with the addition of lignosulfonates during the initial steam pretreatment step was possibly due to lignin modifications from the incorporation of lignosulfonates during the steam pretreatment stage. As a result, the modifications from the addition of lignosulfonates likely generated more hydrophilic lignin and enhanced subsequent organosolv delignification. When the substrate was blocked with BSA, the lowest relative increase in hydrolysis of 2.9 was observed (Figure 21). The results suggests that the substrate likely retained hydrophilicity after the second-stage organosolv pretreatment, which likely contributed to the overall higher enzymatic hydrolysis yield and diminished the effect of BSA blocking in preventing non-productive enzyme binding.



Figure 21. Relative increase in enzymatic hydrolysis after BSA blocking of softwood pellets two-stage steam/organosolv pretreated with and without the addition of 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS). Substrates were subjected to a first-stage steam pretreatment at 185°C with the respective chemicals indicated. Relative increase was calculated as cellulose conversion of substrate after BSA blocking divided by the conversion of the unblocked substrates. Prior to enzyme addition, BSA blocked substrates were incubated at room temperature with 10 mg BSA/mL hydrolysate. Enzyme hydrolysis was conducted at 2% solids at an enzyme loading of 12 mg Ctec3/g cellulose.

The substrate pretreated with the addition of lignosulfonates during the second-stage organosolv pretreatment also showed a lower relative increase in enzymatic hydrolysis yield after BSA blocking treatment compared to the control (3.7 vs 4.5) (Figure 21). The reduced effect of the BSA blocking suggests that the addition of lignosulfonates during the second-stage organosolv pretreatment also increased the hydrophilicity of the residual lignin which consequently decreased non-productive enzyme binding. The increase in lignin hydrophilicity is supported by the presence of strong acid groups on the substrate, indicating successful attachment of the lignosulfonates to the residual substrate lignin. However, while the substrate with the addition of lignosulfonates during the second-stage organosolv pretreatment yielded a slightly higher amount of strong acid groups compared to the substrate where the addition of lignosulfonates occurred during the first-stage steam pretreatment (11 vs 7 mmol/kg), the relative increase in enzymatic hydrolysis after BSA blocking was still higher (3.7 vs. 2.9). The difference between the results observed with substrates where the addition of lignosulfonates occurred during steam vs during the organosolv pretreatment stage suggests that the addition of lignosulfonates during the second-stage organosolv was not as effective in producing a more hydrophilic lignin due to the condensation that had already occurred during the initial steam pretreatment stage.

The pretreatment of substrates with 4HBA addition in either steam or organosolv stages showed little difference in the relative increase in hydrolysis after BSA blocking when compared to the control (4.3-4.7) (Figure 21). The results of the BSA blocking further support the function of 4HBA as an additional acid source as opposed to as a carbocation scavenger. However, it should be noted that the two-stage steam/organosolv pretreatment of the control substrate without the addition of 4HBA or lignosulfonates and substrates where 4HBA was added in either the steam or organosolv pretreatment stages achieved complete conversion during enzymatic hydrolysis after the BSA blocking treatment. The large increase in hydrolysis after BSA blocking was also in agreement with the results observed in the previous chapter, indicating that cellulose accessibility was in fact being generated after the two-stage, steam/organosolv pretreatment but the acidic conditions of the pretreatment resulted in condensed lignin that inhibited enzymatic hydrolysis.

## **3.2.10** Characterization of ethanol organosolv lignin (EOL) extracted from two-stage steam/organosolv pretreatment

As noted earlier, the extraction of lignin in a clean and usable form is a major challenge in the fractionation process. Increased lignin condensation during acidic conditions that are typically employed during steam pretreatment have been found to reduce the reactivity of the lignin and, thereby, limit the potential downstream applications of the extracted lignin (Gardner and McGinnis, 1988; Shimizu *et al.*, 1998). However, acidolysis of the lignin and cleavage of aryl ether bonds during pretreatment have also been shown to increase the formation of free phenolic OH groups on the resulting lignin, which have been previously shown to increase the antioxidant properties of the extracted lignin (Pan et al., 2006b; Sannigrahi et al., 2010). As a result, lignin with a narrow polydispersity, low molecular weight and high amounts of functional groups (e.g. phenolic, carboxylic) are generally more desirable for higher value applications (Pan et al., 2006b). In the 2-stage, steam/organosolv pretreatment scheme proposed in this thesis, milder pretreatment conditions and chemical additives (e.g. 4-hydroxybenzoic acid (4HBA), lignosulfonates) were employed to limit lignin condensation and to improve the extraction of useful lignin. Therefore, this section analyzed the resulting properties of the ethanol organosolv lignin (EOL) extracted from the two-stage, steam/organosolv pretreated substrates.

Analysis of the EOL from the two-stage, steam/organosolv pretreated substrates observed a decrease in M<sub>w</sub> (3460 to 1715-1959 g/mol) and polydispersity (6.28 to 4.85-5.64) compared to the single-stage organosolv pretreatment at 175°C investigated in the previous chapter (Table 17). The wider polydispersity and overall higher M<sub>w</sub> observed for the EOL extracted from the single-stage organosolv control was due to the presence of a second, smaller peak of higher molecular weight lignin (M<sub>w</sub>=112800 g/mol) that was observed during GPC analysis. A similar higher molecular weight fraction peak was not observed for the twostage pretreated samples. It was hypothesized that the two-stage, steam/organosolv pretreatment resulted in increased lignin condensation during the first-stage steam pretreatment that inhibited the efficient extraction of the more condensed, higher molecular weight lignin during subsequent organosolv pretreatment. The analysis of the EOL lignin using NMR saw an increase in both C-5 substitution and phenolic OH functional groups compared to the single-stage control (Table 18). The results support the increase in both lignin condensation and depolymerization reactions that are expected to occur with increased pretreatment severity which corresponds with the trends observed in previous work (Sannigrahi *et al.*, 2010). In contrast to the lignin isolated from the two-stage pretreatments, the single-stage organosolv pretreatment was capable of extracting lignin from the initial biomass that had not undergone a prior steam pretreatment step. Therefore, the single-stage organosolv pretreatment of the starting biomass resulted in the extraction of lignin of both higher and lower molecular weight fractions and a wider range could be more effectively solubilized. As a result, the presence of a higher molecular weight lignin fraction in the EOL contributes to the higher polydispersity and lignin M<sub>w</sub> observed for the single-stage organosolv control.

Table 17. Summary of $M_n$ , $M_w$ and polydispersity data from GPC analysis of ethanol
organosolv lignin (EOL) from substrates two-stage steam/organosolv pretreated with and
without the addition of lignosulfonates (LS)

Sampla	Ave	rage	Polydispersity		
Sample	Mn	Mw	(M <sub>w</sub> /M <sub>n</sub> )		
175°C (single-stage)	551	3460	6.28		
185°C + organosolv	354	1715	4.85		
185°C + organosolv w/ LS	363	1919	5.29		
185°C LS + organosolv	347	1959	5.64		

The addition of lignosulfonates during either the first-stage steam or second-stage organosolv pretreatment resulted in a similar increase in M<sub>w</sub> and polydispersity compared to the two-stage steam/organosolv control without the addition of 4HBA or lignosulfonates. It appeared that the increase in molecular weight that resulted from the addition of lignosulfonates occurred regardless of the stage were the lignosulfonates were added. Extraction of EOL in the M<sub>w</sub> range of 1900 g/mol from the substrates pretreated with lignosulfonates suggests that the lignosulfonates that were incorporated into the substrate also experienced further acidolysis and depolymerization since the starting M<sub>w</sub> of the lignosulfonates used was 8000 g/mol (M<sub>n</sub> = 3000 g/mol). The increase in both M<sub>w</sub> and polydispersity suggests that the addition of lignosulfonates during pretreatment either resulted

in an increase in the ease of extraction of larger molecular weight lignin or a decrease in the severity of lignin condensation during pretreatment. Further analysis of the EOL using NMR suggests that the addition of lignosulfonates did not prevent lignin condensation nor did it encourage increased cleavage of aryl-ether bonds compared to the two-stage control. In contrast, addition of lignosulfonates during the second-stage organosolv pretreatment resulted in a slight decrease in total phenolic OH groups (1.8 to 1.7) while the addition of lignosulfonates during the first-stage steam pretreatment resulted in a slight increase in C-5 substitution (1.0 to 1.1). An increase in C-5 substitution was also observed with the addition of 4HBA during either stage. However, an increase in total phenolic OH groups was also observed with 4HBA addition which further supports the action of 4HBA as a secondary acid source that promotes both lignin cleavage and re-polymerization.

Table 18. Summary of functional groups detected by <sup>31</sup>P NMR of ethanol organosolv lignin (EOL) from substrates two-stage steam/organosolv pretreated with and without the addition of 4-hydroxybenzoic acid (4HBA) and lignosulfonates (LS)

	Functional Groups (mmol/g)							
Sample	Aliphatic OH	C-5 substitution	Guaiacyl phenolic OH	p- hydroxyphenyl OH	Carboxylic acids OH	Total phenolic OH		
175°C (single-stage)	2.3	0.7	1.4	0.1	0.2	1.5		
185°C + organosolv	1.7	1.0	1.7	0.1	0.3	1.8		
185°C + organosolv w/ 4HBA	1.9	1.2	1.8	0.1	0.4	1.9		
185°C + organosolv w/ LS	1.8	1.0	1.6	0.1	0.3	1.7		
185°C 4HBA + organosolv	1.7	1.1	1.8	0.1	0.3	1.9		
185°C LS + organosolv	1.8	1.1	1.8	0.1	0.3	1.8		

Precipitation of the organosolv liquor for recovery of the EOL after pretreatment resulted in the observation of some unexpected lignin properties for the substrates that were pretreated with lignosulfonates. Typically, precipitation of EOL results in the formation of lignin particulates that settle at the bottom of the solution (Figure 22a-c). In comparison, precipitation of EOL from samples pretreated with lignosulfonates during either stage resulted in the formation of cloudy suspension of finer particulates that did not settle over time (Figure 22d-e). It was hypothesized that the lignosulfonates reacted with the substrate lignin during pretreatment and produced more hydrophilic lignin that diminished the tendency for the lignin to form agglomerates in water. However, precipitation of the EOL from the two-stage steam/organosolv control substrate without 4HBA or lignosulfonate addition in the presence of lignosulfonates also resulted in the formation of a similar cloudy suspension (Figure 23c). Similarly, the addition of lignosulfonates to precipitated EOL from the two-stage control also reverted the initial characteristics of settling and formed a cloudy suspension after mixing (Figure 23d). These results suggest that the interaction of lignosulfonates with the substrate lignin may be through physical association as opposed to covalent bonding through the condensation mechanism as originally thought. The phenomenon of lignin association is not novel and has been previously observed as well (Norgren and Edlund, 2014). However, the substrate where lignosulfonate addition occurred during the first-stage steam pretreatment had undergone extensive washing after steam pretreatment and was subjected to a secondstage organosoly pretreatment. The survival of the lignosulfonate interaction with the substrate lignin (extracted and/or residual) after a two-stage pretreatment suggests a more stable interaction mechanism which supports the theory of covalent bonding between the lignosulfonates and the substrate lignin. In addition, when the precipitated samples were left undisturbed over a period of 14 days, the two-stage control substrate that was precipitated in the presence of lignosulfonates showed varying degrees of settling while the samples that were pretreated with lignosulfonates did not (Figure 23). The difference in stability of the precipitated solutions suggests that lignosulfonates may be capable of interacting with the substrate lignin through both covalent bonding and physical association while imparting similar characteristics. The method of association may also impact the stability of the modifications that the lignosulfonates impart on the substrate lignin. In addition, the presence of lignosulfonates during pretreatment may also contribute to additional modifications to the lignin component that have yet to be fully elucidated. Therefore, further study on the attachment and effect of the addition of lignosulfonates towards the modification of lignin

properties will be invaluable in understanding their role in improving the effectiveness of a twostage, steam/organosolv pretreatment process in fractionating softwood biomass.



Figure 22. Precipitation of ethanol organosolv lignin (EOL) extracted from two-stage, steam/organosolv pretreated softwood pellets: (a) 185°C + organosolv, (b) 185°C + organosolv w/ 4HBA, (c) 185°C 4HBA + organosolv, (d) 185°C + organosolv w/ LS, (e) 185°C LS + organosolv.



Figure 23. Precipitation and settling of ethanol organosolv lignin (EOL) from (a) 185°C + organosolv w/ LS, (b) 185°C LS + organosolv and 185°C + organosolv, with the addition of lignosulfonate (c) during precipitation or (d) after precipitation. Precipitated samples were allowed to settle undisturbed over a course of 14 days.

#### 3.2.11 Conclusions

The work in this chapter focused on improving the acidic two-stage steam/organosolv pretreatment process investigated for softwood pellets in the previous chapter. The work in this chapter was also aimed at gaining a better understanding of the underlying mechanisms of the changes that occurred during steam pretreatment that were limiting lignin removal during the subsequent organosolv pretreatment. As well as the changes undergone by lignin during steam pretreatment that decreased the subsequent extractability of lignin, the amount of lignin condensation that may have been imparted by the acidic organosoly pretreatment was also studied. Further optimization of the acid-catalyzed, steam pretreatment process for softwood pellets found that steam pretreatment at 185°C yielded the highest hemicellulose recovery in the water soluble stream, unlike previous work that examined wood chips. The addition of lignosulfonates during steam pretreatment further improved the overall hemicellulose recovery by 10%, which was in agreement with previous work on hardwood biomass (Chandra et al, 2015). In addition to improving hemicellulose recovery it was hypothesized that the aromatic moieties found on lignosulfonates could enable them to act as carbocation scavengers such as 2-napthol and 4HBA which have been shown previously to reduce lignin condensation during pretreatment (Pielhop et al., 2015; Wayman and Lora, 1978). The results from the acid group titration and XPS confirmed the successful incorporation of lignosulfonates into the substrate lignin which suggests the potential for lignin modification through the condensation pathway.

It was anticipated that the addition of water soluble 4HBA could be used as a carbocation scavenger to limit the condensation of lignin. The incorporation of 4HBA could also be readily measured through the incorporation of carboxylic acid groups. However, it appeared that 4HBA did not act as a carbocation scavenger under the pretreatment conditions tested. The inability for the 4HBA to react with the substrate lignin was likely due to the shorter pretreatment retention times studied for both steam (7.5 min) and organosolv (30 min) pretreatment stages compared to the conditions typically used in previous studies investigating carbocation scavengers (>1 hour) (Li *et al.*, 2007; Pielhop *et al.*, 2015; Wayman and Lora, 1978, 1980). As a result, 4HBA appeared to act as an additional acid source which seemed to increase

lignin condensation as a result of increased pretreatment severity which consequently impeded subsequent organosolv delignification and decreased enzymatic hydrolysis yields.

In contrast to 4HBA, adding lignosulfonates to the initial steam pretreatment resulted in an increase in delignification and delignification selectivity during the subsequent organosolv pretreatment. Consequently, the increased delignification resulted in an enhancement in enzymatic hydrolysis yields. In addition, experiments applying BSA blocking to the substrate prior to enzymatic hydrolysis suggested that the pretreatment with lignosulfonates resulted in a more hydrophilic residual lignin. In contrast to the addition of lignosulfonates during steam pretreatment, the addition of lignosulfonates during the second-stage organosolv pretreatment was less effective and did not improve delignification or enzymatic hydrolysis yields, even though both steam and organosolv pretreatments were performed under acidic conditions that promote lignin condensation. However, the addition of lignosulfonates to organosolv pretreatment improved the selectivity of the organosolv delignification for lignin. It was apparent that the addition of lignosulfonates to the steam and organosolv pretreatments resulted in an increase in the recovery of hemicellulose and cellulose respectively. The higher glucan recovery after the two-stage steam/organosolv pretreatment and higher hemicellulose recovery observed during steam pretreatment of pellets suggests that lignosulfonates have a "protecting function" towards the carbohydrate component. While the underlying mechanism for the increased carbohydrate recovery upon the addition of lignosulfonates remains to be elucidated, the application of lignosulfonates during a two-stage steam/organosolv pretreatment to improve fractionation and recovery is a promising option.

#### **4** Overall conclusions

One of the main challenges that a biorefinery process faces is to design a pretreatment process that can effectively fractionate and maximize the extractable value from the feedstock. In addition, the cost of collection and transportation of low density biomass such as wood chips to supply a biorefinery constitutes a large percentage of the overall process cost. In BC, softwoods are the predominant wood species and are the primary feedstock for pellet production. With their high density and low moisture, pellets present a viable alternative to wood chips to enable efficient transportation. However, the effects of the pelletization process (e.g. drying, size reduction, compression) on the ease of pretreatment have not yet been extensively studied. In addition, softwoods have been demonstrated to be particularly recalcitrant towards pretreatment due to their higher lignin content and higher proportion of more condensed guaiacyl lignin. Due to this high recalcitrance, single-stage pretreatment processes designed for bioconversion typically require severe conditions to increase cellulose accessibility. However, these severe conditions frequently sacrifice the recovery of a portion of the labile hemicellulose component and/or result in the condensation of the lignin structure that decreases its utility for downstream value-added application. Therefore, a two-stage, mild steam/organosolv pretreatment approach was investigated for its ability to effectively fractionate softwood biomass in a step-wise fashion to improve overall recovery of the individual components. As well as assessing the two-stage pretreatment approach, the work in this thesis also compared the ease of pretreatment of softwood chips vs softwood pellets.

Previous work investigating two-stage steam/organosolv pretreatment approaches have been successful on less recalcitrant hardwood biomass and agricultural residues (Amiri and Karimi, 2016; Ibrahim and Glasser, 1999; Panagiotopoulos *et al.*, 2013; Romaní *et al.*, 2011). Implementation of a steam pretreatment step prior to organosolv pretreatment was demonstrated to improve hemicellulose recovery, delignification and enzymatic hydrolysis yields in those studies. However, steam pretreatment is typically carried out under acidic conditions and softwoods have been shown to be more prone to lignin condensation during acidic pretreatments which could potentially compromise subsequent organosolv delignification (Shevchenko *et al.*, 1999). It was hypothesized in this thesis that a two-stage

steam/organosolv pretreatment process could be an effective option for fractionating softwood biomass if milder pretreatment conditions were used to minimize lignin condensation. It was observed that applying a milder two-stage steam/organosolv pretreatment condition (autocatalyzed steam, 30 min organosolv) to softwood chips and pellets was able to achieve comparable hydrolysis and delignification yields to the single-stage organosolv controls at the same 30 min retention time. However, the milder auto-catalyzed steam pretreatment conditions tested resulted in limited solubilization of the hemicellulose into the water soluble stream (28-33%) due to the lower amount of acetylation of softwood hemicellulose (Pawar et al., 2013; Teleman et al., 2000; Teleman et al., 2003). In contrast to auto-catalyzed steam pretreatment, acid-catalyzed steam pretreatment was more successful in recovering hemicellulose in the water soluble stream (49-62%). However, the acid catalyzed first-stage steam pretreatment compromised delignification and enzymatic hydrolysis yields during subsequent organosoly pretreatment. It was proposed that the more severe conditions during acid-catalyzed steam pretreatment resulted in increased lignin condensation which reduced its extractability during organosolv (Shevchenko et al., 1999). In addition, the increase in lignin condensation had been shown previously to be detrimental towards enzymatic hydrolysis yields due to increased non-productive binding between the enzymes and hydrophobic lignin (Nakagame et al., 2011a). Unlike less recalcitrant biomass such as hardwood and agricultural residues, the higher proportion of guaiacyl lignin that is more prone to lignin condensation in softwoods appeared to result in greater inhibition towards fractionation and subsequent hydrolysis (Nakagame et al., 2010). Therefore, from the results, it was apparent that lignin condensation played a critical role in limiting the ability of a two-stage steam/organosolv pretreatment to achieve a clean fractionation of softwood biomass.

As mentioned earlier, reducing the cost and logistics of biomass collection and transport is crucial for improving the economic viability of a biorefinery process. Wood pellets are a high density, low moisture feedstock that represents a viable alternative to the more traditionally studied wood chips but the effect of the pelletization process (e.g. drying, size reduction and compression) had yet to be extensively studied in the literature using comparable conditions. In this thesis, pellets and wood chips were ground and sieved to a similar particle size and

subjected to both single and two-stage, steam/organosolv pretreatment. After grinding, the hemicellulose recovery in the water soluble stream and the delignification yield of both ground wood chips and ground pellets achieved levels comparable to pellets. In addition, it was initially anticipated that drying would decrease the ease of pretreatment but the effects of drying on the biomass susceptibility to organosolv pretreatment was found to be reversible with prior rewetting, which had also been previously demonstrated with the steam pretreatment of previously dried poplar biomass (Ewanick and Bura, 2016). Therefore, the comparison of pretreatment response between softwood chips and pellets to both single and two-stage, steam/organosolv pretreatment suggested that the difference in pretreatment response observed was likely attributed to the difference in particle size between wood chips and pellets. As a result, pellets were found to be more susceptible to pretreatment and achieved higher hemicellulose recovery in the water soluble stream during steam pretreatment and higher delignification yields during organosolv pretreatment. However, enzymatic hydrolysis yields after two-stage steam/organosolv pretreatment of pellets were found to be consistently lower compared to wood chips. Previous work observed that biomass at a smaller particle sized experienced a higher "apparent severity" during pretreatment (Cullis et al., 2004). Therefore, it was hypothesized that the higher apparent severity experienced by the pellets resulted in increased condensation of the residual lignin during pretreatment which decreased enzymatic hydrolysis yields due to non-productive binding. Overall, pellets were demonstrated to be a viable feedstock alternative to wood chips but further optimization of the pretreatment conditions would likely be required to fully take advantage of their smaller particle size.

It was also demonstrated that the increase in lignin condensation during the acidic twostage steam/organosolv pretreatment resulted in a decrease in delignification and enzymatic hydrolysis yield of softwood biomass. Previous work had demonstrated the effectiveness of carbocation scavengers such as 4-hydroxybenzoic acid (4HBA) and 2-naphthol towards minimizing lignin condensation during the acidic pretreatment of both hardwood and softwood biomass (Pielhop *et al.*, 2015; Wayman and Lora, 1978). In addition, lignosulfonates contain aromatic moieties that have the potential to operate as carbocation scavengers and have been shown previously to improve hemicellulose recovery and enzymatic hydrolysis of steam

pretreated poplar (Chandra et al., 2015). Therefore, it was hypothesized that the addition of 4HBA or lignosulfonates to the two-stage steam/organosolv pretreatment studied in this thesis would similarly minimize lignin condensation and improve the delignification and enzymatic hydrolysis yields. The addition of lignosulfonates during the first-stage steam pretreatment or second-stage organosolv pretreatment of softwood pellets was found to increase overall hemicellulose or cellulose recovery respectively. Analysis of the substrate determined that lignosulfonates were incorporated with the substrate lignin and likely resulted in an increase in hydrophilicity of the lignin. As a result, the addition of lignosulfonates during the first-stage steam pretreatment resulted in an increase in delignification and lignin selectivity during subsequent organosolv pretreatment. In comparison, 4HBA did not appear to act as a carbocation scavenger and did not result in an increase in delignification or enzymatic hydrolysis yields. Instead, 4HBA appeared to act as a secondary acid source under the conditions tested in this thesis. It was apparent that the conditions for the two-stage steam/organosolv pretreatment investigated were not sufficient for the 4HBA to function as a carbocation scavenger while the application of lignosulfonates showed promising results for further investigation.

#### 4.1 Future work

Through the findings in this thesis, it was demonstrated that softwood pellets can be investigated further as a viable feedstock substitute for wood chips in a biorefinery process. The smaller particle size of the pellets appeared to enhance its susceptibility to pretreatments (i.e. higher apparent severity) which increased the extraction of hemicellulose and lignin during steam and organosolv pretreatment respectively compared to wood chips. However, most of the pretreatment conditions studied in this thesis were previously optimized for the pretreatment of wood chips and can potentially be unnecessarily severe for pellets and result in increased lignin condensation. As a result, milder steam and organosolv pretreatment conditions (e.g. shorter retention time, lower acid loading) should be investigated in the future to compensate for the higher apparent severity experienced by the pellets and reduce the formation of lignin condensation. In addition, experimental determination of the solvent exchange rate for rewetted substrates will assist in understanding the role of time and particle

size on solvent exchange and allow for more optimized substrate preparation procedures prior to pretreatment in the future. The synergistic action between the two stages of pretreatment should also be taken into consideration when attempting to optimize the pretreatment conditions. Previous work by Panagiotopoulos et al. on poplar wood chips demonstrated that the implementation of a prior steam pretreatment step reduced the required pretreatment severity for the second-stage organosolv pretreatment when compared to a single-stage organosolv pretreatment (60 to 30 min) (2013). It was apparent from the organosolv pretreatment of steam pretreated substrates at different temperatures that the effects of the first-stage steam pretreatment resulted in modifications to the lignin characteristics (e.g. depolymerization, re-polymerization) that influenced the subsequent response of the substrate during the second-stage organosolv pretreatment. In addition, dioxane lignin extraction of the first-stage steam pretreated substrates was demonstrated to be capable of extracting increasing amounts of lignin despite increasing steam pretreatment severity which contradicted the trend observed with organosolv pretreatment. The difference in trends demonstrates that alternative solvent systems that are better suited for solubilizing more hydrophobic lignin can also be explored to improve delignification yields. Overall, the results from the thesis suggest that milder organosolv conditions should be investigated in the future and that the severity of the second-stage organosolv conditions should be designed to complement the first-stage steam pretreatment conditions used.

As mentioned previously, the results from this thesis support the importance of overcoming lignin condensation to facilitate the fractionation of softwood biomass. Lignosulfonates were investigated for their ability to function as carbocation scavengers during acidic pretreatment and minimize lignin condensation. The successful application of lignosulfonates to increase delignification and enzymatic hydrolysis yields provides insight into the potential for utilizing the lignin condensation mechanism as a pathway for lignin modification in the future. The potential modification of the residual substrate lignin by lignosulfonates was seen to affect the solubility characteristics of the residual lignin as observed during dioxane extraction when compared to the samples steam pretreated without lignosulfonates. As mentioned earlier, modifications to the lignin characteristics suggest that

the solvent system currently used for organosolv may not be suitable for achieving maximum delignification for the modified lignins. Therefore, further optimization using different solvent systems will be beneficial for increasing the effect of applying lignosulfonates during pretreatment. The addition of lignosulfonates during pretreatment also yielded organosolv lignin of unique properties, notably the precipitation of finely dispersed lignin that did not agglomerate and settle like typical organosolv lignin. However, precipitation of organosolv liquor from substrates pretreated without the addition of lignosulfonates also yielded a similar effect when precipitation was performed in the presence of lignosulfonates. Therefore, additional confirmation is still needed to determine whether the addition of lignosulfonates to the residual substrate lignin is through covalent bonds or physical association.

In addition to increasing delignification and enzymatic hydrolysis yields, lignosulfonates were also observed to have a "protecting function" towards carbohydrates and resulted in an increase in both hemicellulose and cellulose recovery during steam and organosolv pretreatment. While this phenomenon was also observed previously, the mechanism of this "protecting function" has yet to be fully understood (Chandra *et al.*, 2015). Investigation of the mechanism behind the "protecting function" of lignosulfonates could potentially uncover alternative pathways to improve carbohydrate recovery by minimizing the degradation that typically occurs under the more severe pretreatment conditions required for delignification and generating cellulose accessibility. The application of lignosulfonates during the two-stage steam/organosolv pretreatment is still a rather novel concept and further study of its mechanism of action will be necessary to better understand and refine the process to overcome the recalcitrance of softwood biomass and achieve clean fractionation for a biorefinery process.

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### Appendices

Cultaturate	Solid Yield	Chemical Composition (% w/w)							
Substrate	(%) <sup>a</sup>	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin <sup>b</sup>	Total	
NC-0	83	0	1	51	5	10	31	98	
AC-0	73	0	1	55	3	6	35	101	
SC-0	60	0	0	58	0	1	41	100	
GC-0	71	0	0	59	2	2	39	102	
NP-0	83	0	1	50	5	9	33	99	
AP-0	71	0	0	55	3	5	39	102	
SP-0	65	0	0	55	1	4	40	100	
GP-0	72	0	0	59	3	2	37	102	
DC-30	59	0	0	74	3	2	21	101	
DC-60	52	0	0	76	2	3	22	102	
DP-30	50	0	0	73	2	3	21	100	
DP-60	51	0	0	78	2	3	20	104	
WC-30	62	0	1	66	4	6	23	99	
WC-60	45	0	0	76	1	2	21	100	
WP-30	52	0	0	72	2	3	22	100	
WP-60	47	0	0	78	1	2	19	100	
DGC-30	54	0	0	76	3	3	19	102	
DGP-30	52	0	0	78	3	4	17	103	
175°C	71	0	0	55	3	5	39	102	
185°C	68	0	0	54	1	2	41	98	
195°C	68	0	0	54	1	2	41	98	
205°C	61	0	0	52	0	1	46	99	
185°C 4HBA	68	0	0	54	1	1	42	99	
185°C LS	73	0	0	53	3	3	40	98	

## Appendix A. Chemical composition and solid recovery of single-stage steam or organosolv pretreated substrates.

<sup>a</sup>Solid yield is calculated relative to the untreated biomass

<sup>b</sup>Lignin = acid soluble + acid insoluble (klason) lignin

Reported values have a standard deviation within the range of  $\pm 2$ 

Appendix B. Chemical composition and solid recovery of two-stage steam/organosolv pretreated substrates.

Substrate		lid eld 6)	Chemical Composition (% w/w)						
	Α	В	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin <sup>a</sup>	Total
NC-30	64	53	0	0	71	2	2	24	100
NC-60	69	57	0	0	68	4	4	24	101
AC-30	80	59	0	0	65	1	1	32	99
AC-60	76	55	0	0	68	1	1	30	100
GC-30	74	53	0	0	68	1	1	31	101
NP-30	60	50	0	0	70	2	2	24	99
NP-60	64	53	0	0	64	4	4	25	98
AP-30	73	52	0	0	66	1	1	32	100
AP-60	59	42	0	0	66	1	1	32	99
GP-30	69	50	0	0	67	1	1	30	99
205°C + organosolv w/ acid	79	48	0	0	61	0	0	37	99
205°C + organosolv w/o acid	89	54	0	0	58	0	0	38	97
185°C + organosolv	68	46	1	0	67	1	0	29	98
185°C + organosolv w/ 4HBA	71	49	1	0	66	1	0	31	99
185°C + organosolv w/ LS	75	52	0	0	71	1	0	27	99
185°C 4HBA + organosolv	72	49	1	0	66	1	0	32	100
185°C LS + organosolv	68	50	0	0	74	1	0	25	101

A = solid yield relative to the first-stage steam pretreated substrate

**B** = solid yield relative to the untreated biomass

<sup>a</sup>Lignin = acid soluble + acid insoluble (klason) lignin

Reported values have a standard deviation within the range of ±2

### Appendix C. Enzymatic hydrolysis of softwood pellets single-stage steam pretreated at increasing temperatures (175-205°C).



Substrates were hydrolyzed at 2% solids with an enzyme loading of 12 mg Ctec3/g cellulose.