Steam pretreatment as an effective front end for a softwood based biorefinery

by

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Abstract

For any bioconversion/biorefinery process, the nature of the pretreatment process used has a significant influence on all of the subsequent process steps. Although steam pretreatment has proven effective on agricultural residues and hardwoods, softwoods are considerably more recalcitrant, usually requiring an acid catalyst to ensure effective pretreatment. One of the initial objectives of the work was to assess how effective acid catalysed steam pretreatment would be on a range of softwood substrates as past work had utilized wood chips that were obtained from one tree. It was apparent that similar pretreatment conditions could be used for a range of softwood substrates, resulting in comparable hemicellulose recovery while providing a cellulosic component which could be readily hydrolysed, but at the expense of using high enzyme loadings. To try to enhance cellulose hydrolysis we assessed the role of the various substrate components that are thought to limit hydrolysis. Lignin was shown to restrict hydrolysis at low enzyme loadings (5 – 10 FPU/g glucan), primarily by limiting the accessible cellulose surface area, but also by unproductive binding of the enzymes. To achieve effective hydrolysis at low enzyme loadings, a post-treatment step that removed/modified lignin to enhance the cellulose accessibility was assessed. Steam pretreatment and post-treatment were further optimised to result in a >85% cellulose hydrolysis at an enzyme loading of 10 FPU/g glucan. To try to increase the concentration of final sugars obtained we next evaluated the use of high substrate concentrations. Increased biomass loading during steam pretreatment not only minimised steam and SO_2 consumption, it also resulted in good recovery of the sugars at high concentration. However this was done at the expense of high enzyme loadings. Past work has primarily utilised pulp chips as the feedstock. However, they are unlikely to be used as a commercial bioconversion feedstock. A more likely feedstock, wood pellets were presoaked and steam pretreated. Surprisingly, little hemicellulose loss occurred while the cellulosic rich, water insoluble fraction was readily enzymatically hydrolysed. It was also possible to apply a single steam pretreatment to facilitate both pelletisation and subsequent enzymatic hydrolysis without the need for subsequent steam pretreatment.

Preface

List of publications

Paper 1 Kumar L, Chandra R, Chung PA, Saddler J. 2010. Can the same steam pretreatment conditions be used for most softwood to achieve good, enzymatic hydrolysis and sugar yields? Bioresour. Technol. 101:7827-7833.

Paper 2 Kumar L, Arantes V, Chandra R, Saddler J. 2012. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. Bioresour. Technol. 103:201-208.

Paper 3 Kumar L, Chandra R, Saddler J. 2011. Influence of steam pretreatment severity on post-treatments used to enhance the enzymatic hydrolysis of pretreated softwoods at low enzyme loadings. Biotechnol. Bioeng. 108:2300-2311.

Paper 4 Kumar L, Tooyserkani Z, Sokhansanj S, Saddler JN. 2012. Does densification influence the steam pretreatment and enzymatic hydrolysis of softwoods to sugars? Bioresour. Technol. 121:190-198.

Paper 5 Kumar L, Tooyserkani Z, Sokhansanj S, Saddler JN. 2012. Influence of increased substrate concentrations in steam pretreatment to enhance the sugar concentration in the subsequent process streams. *In preparation.*

Linoj Kumar (LK) helped develop the research and carried out the majority of the experimental work leading to Papers 1, 2 and 3 and drafted the manuscript with the help of Jack Saddler (JNS). For Paper 4, LK planned the initial phase of the work with the other authors and carried out the experimental work on steam pretreatments, characterisation of the process streams and enzymatic hydrolysis. Ms. Zahra Tooyserkani made the wood pellet samples required for the study and measured their physical properties. LK drafted the manuscript together with JNS. LK with JNS planned the work leading to Paper 5. LK carried out the experimental work and interpreted the results with the help of JNS. Other authors provided the wood pellet samples required for the study and helped with the planning of the study and interpretation of the results.

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List of units and abbreviations

A°	Angstrom
AHP	Alkaline hydrogen peroxide treatment
ALK	Alkaline treatment
°C	Degrees Celsius
AD	Absorbance detector
AFEX	Ammonia fibre explosion treatment
CD	Catalytic domain
CBM	Carbohydrate-binding modules
CHL	Chlorite treatment
CHP	Combined heat and power
CTMP	Chemithermomechanical pulp
DF	Douglas-fir
DP	Degree of polymerisation
CS	Combined severity
DNS	Dinitrosalicylic acid
FPU	Filter paper units
ED	Electrochemical detector
EDTA	Ethylenediaminetetraacetic acid
g	Grams
x g	Acceleration due to gravity (i.e., 2000 x g)
GC	Gas chromatography
h	Hour
HSP	High severity steam pretreatment
HMF	5-hydroxymethylfurfural
HPLC	High pressure liquid chromatography
IEA	International energy agency
IU	International units
L	Litre
kDa	kilodalton
Log Ro	Steam pretreatment severity factor
LPP	Lodgepole pine
LSP	Low severity steam pretreatment
Lw	Weighted average fibre length
M	Molar concentration (moles per L)
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar concentration
MSP	Medium severity steam pretreated
nm	Nanometer

NMR	Nuclear magnetic resonance
PAPTAC	Pulp and paper technical association of Canada
PDU	Process development unit
rpm	Revolutions per minute
SPF	Spruce , Pine and Fir
SPDF	Steam pretreated Douglas-fir
SPLPP	Steam pretreated Lodgepole pine
SPORL	Sulfite pretreatment to overcome the recalcitrance of lignocellulose
SULF	Sulfonation treatment
t	Time
T	Temperature
TAPPI	Technical association of the pulp and paper industry
μL	Microlitre
μm	Micrometre
wt/vol.	Weight per volume
wt/wt.	Weight per weight

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all my loving teachers, mentors and supervisors
for the affectionate guidance, knowledge and training,
you have given to me all along…

1 Introduction

1.1 Background

During the last few years there has been increasing interest in the biorefinery concept. Unlike pulping where the primary role is to obtain a bright, strong fibre that constitutes the feedstock for various paper products, the biorefinery approach considers the biomass feedstock as the source of a variety of chemicals and polymers, in an analogous fashion to where an oil refinery gives us the thousands of chemicals we use routinely in our daily life. The first step in a biorefinery or biomass-to-ethanol process is the pretreatment component. This is a critical component as it influences the efficiency of all subsequent downstream processing steps and the overall process. There has been a considerable amount of work done on pretreatment and the nature of the substrate has been shown to heavily influence the effectiveness of the various pretreatment processes. Agricultural residues and hardwoods are generally considered to be more easily treated with softwoods shown to be typically more recalcitrant. However, it would be desirable if any pretreatment could be feedstock agnostic, proving to be effective on a wide range of biomass feedstocks. As will be described in more detail, steam pretreatment, with or without acid catalysts, has been shown to be effective on a wide range of substrates, providing generally good recovery of the lignin and hemicellulose fractions and a cellulosic fraction that can be readily enzymatically hydrolysed.

Over the past few decades a range of pretreatment processes have been developed, covering mechanical, chemical and a combination of physicochemical approaches. Some of the most common methods include steam pretreatment, dilute acid pretreatment, ammonia fiber expansion, cellulose dissolution processes (such as phosphoric acid, ionic liquids etc.) and variations on pulping processes (for example, Sulfite Pretreatment to Overcome the Recalcitrance of Lignocellulose (SPORL), green liquor pretreatment, organosolv etc.) (Hendriks and Zeeman, 2009; Kumar and Murthy, 2011; Mosier et al., 2005b). Although most of these pretreatments are effective in processing agricultural

feedstocks, with more recalcitrant substrates, such as softwoods, the list of effective pretreatment options is more limited. Although many of the pulping related pretreatments are effective on softwoods the conditions used to enhance the accessibility of the enzymes to the cellulosic component often result in the dissolution of hemicelluloses in the lignin-rich liquid fraction thereby making their recovery difficult. In contrast steam and dilute acid pretreatments have been shown to be effective on both woody and non-woody feedstocks resulting in the good hemicellulose derived sugar recovery while generating a cellulosic component which is readily hydrolysed (Elander et al., 2009; Galbe and Zacchi, 2012; Gregg and Saddler, 1998).

It has been suggested that the steam pretreatment of biomass for bioconversion had its origins in both the Masonite process (for making particle board) (Boehm, 1930; Mason, 1926) and in the early pulp and paper industry. Aqueous phase prehydrolysis is used in Kraft pulping to eliminate a large fraction of the hemicelluloses before manufacturing the high-purity alpha-cellulose (Overend and Chornet, 1987). Steaming is also a critical component of mechanical refining processes to generate thermomechanical pulps (Overend and Chornet, 1987; Biermann, 1996). Thus, many of the process engineering concepts for steam pretreatment were adapted from pulp and paper processes. For example, the H factor, used in Kraft pulping to predict the degree of delignification, combines the effect of time and temperature into a one single factor. This concept was later modified and used for steam pretreatment as the severity factor (Ro). In subsequent work the influence of an acid catalyst was also incorporated into this model which referred to the combined severity factor (Chum et al., 1990) as the key calculation in determining optimum pretreatment conditions.

Earlier work, which did not recognize the need to recover all of the available sugars while using low enzyme loadings, mainly used the severity factor to predict the enzymatic digestibility of the pretreated solid fraction with little regard to the recovery of hemicellulosic sugars (Brownell and Saddler, 1985; Schwald et al., 1989). However, the importance of also recovering the water soluble hemicelluloses was recognised as being

essential to enhancing the overall sugar yield and to ensure efficient utilisation of all of the sugars present in the starting feedstock. It was also soon recognised that although increasing the pretreatment severity had the beneficial effect of enhancing the enzymatic hydrolysis of the water insoluble cellulosic rich fraction, these high severities also contributed to hemicellulosic sugar degradation, thereby diminishing the overall sugar recovery (Brownell and Saddler, 1984; 1987; Brownell et al., 1986). This previous work recommended using a medium severity, which is a compromise between optimizing hemicellulose recovery while ensuring the reasonable cellulolytic digestibility of the water insoluble, cellulose rich component (Brownell et al., 1986; Schwald et al., 1989).

Steam pretreatment at these compromise conditions was found to be highly effective in pretreating agricultural residues and hardwoods, resulting in good recovery of both hemicellulosic and cellulosic derived sugars (Mackie et al., 1985; Ramos et al., 1992; Bura et al., 2002; Ohgren et al., 2007a). However, with softwoods, identifying the compromise conditions that result in good hemicellulose recovery while providing a readily digestible cellulose rich water insoluble fraction has proven to be more challenging. Past work (Schwald et al., 1989; Clark and Mackie, 1987; Clark et al., 1989) showed that an acid catalyst could be used to reduce the pretreatment temperature and reaction time, leading to the recovery of most of the hemicelluloses in a monomeric form. However, at the pretreatment conditions that resulted in reasonable hemicellulose recovery, the water insoluble, cellulose rich fraction has proven to be difficult to hydrolyze, typically requiring relatively high enzyme loadings (>20 FPU/g glucan) to achieve hydrolysis yields of greater than 60%. Thus, the challenge is to recover as much of the hemicellulose-derived sugars as possible, while providing a cellulosic component that can be readily hydrolyzed with low enzyme loadings. One of the general goals of the work described in this thesis was to optimise steam pretreatment so that it could be used more effectively on softwoods, recovering both hemicellulose- and cellulose-derived sugars in high yield and concentration with reduced enzyme and chemical loadings.

One of the initial objectives of the work was to assess how effective acid catalysed steam pretreatment would be on a range of softwood substrates. A review of the literature had indicated that much of the past work in this area had utilized wood chips that had been obtained from one biomass source (Boussaid et al., 1999; 2000; 2001; Wu et al., 1998; Cullis et al., 2004; Cullis and Mansfield, 2010; Pan et al., 2004; 2005b; Yang et al., 2002). Recent studies by the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) group compared leading pretreatment options across different feedstocks and they reported some variations between different batches of switch grass, corn stover and poplar feedstocks (Wyman et al., 2009; 2011; Elander, et al. 2009). This particular comparison did not include softwoods as a feedstock as some of the pretreatment processes that were compared would have difficulty in processing softwoods.

One key element of the work that will be described in the thesis was the use of both low and high enzyme loadings for assessing the susceptibility of the substrates to enzymatic hydrolysis. Although the high enzyme loadings that have been generally used in previous work resulted in decent hydrolysis yields, this was done at the expense of using uneconomically high protein loadings. As the cellulosic component of steam pretreated softwoods has generally been found to be significantly recalcitrant to enzymatic hydrolysis even at high enzyme loadings (>30 FPU/g glucan) (Boussaid, et al. 2000), attaining high sugar yield at low enzyme loadings was anticipated to be challenging. Thus, one of the main objectives of the work reported in this thesis was to increase our understanding of the substrate and enzyme factors which restrict the effective hydrolysis of pretreated softwoods.

As will be discussed in detail within the thesis, various substrate characteristics have been shown to influence enzymatic hydrolysis (Saddler, 1986; Gregg and Saddler, 1996; Mansfield and Saddler, 1999; Arantes and Saddler, 2010; Hidayat et al., 2012; Henrissat, 1994; Lynd et al., 2002; Zhang and Lynd, 2004; Zhao et al., 2012). In addition to characteristics within the cellulose itself (i.e. crystallinity, degree of polymerization, etc.), the lignin and hemicellulose are known to both restrict the accessibility to the cellulose and,

in the case of lignin in particular, to bind to the enzymes (Ohgren et al., 2007; Berlin et al., 2006; Nakagame et al., 2010). Although the hemicellulose can be almost completely removed by steam pretreatment, the residual hemicellulose and the lignin have been shown to significantly influence the enzymatic hydrolysis of cellulosic component. In addition to being a barrier, lignin can also unproductively bind to the cellulase enzymes reducing the effective amount of enzymes available to cellulose hydrolysis (Nakagame et al., 2010). The relative contributions of these two mechanisms of lignin inhibition and how both these relate to enzyme loading employed on a typical steam pretreated softwood was further investigated in this work. By better elucidating the influence of the various mechanisms at various enzyme loadings we hoped to identify more effective pre/post-treatment strategies for enhancing the ease of hydrolysis at reduced enzyme loadings.

Past work has recognised the potential of a delignification post-treatment step to achieve a fast and complete hydrolysis of steam pretreated softwoods (Pan et al., 2005b; Yang et al., 2002). Post treatment is typically applied after pretreatment primarily to remove or modify lignin to enhance the accessibility of the cellulosic fraction to the cellulase enzymes. This strategy was also adapted from the pulp and paper industry, the biggest industries processing lignocellulosic substrates for over a century. Pulp and paper processes generally employ a range of different methods in sequence for delignification/lignin modification to obtain the desired cellulose based products (Gierer, 1985). A typical pulping/bleaching regime is a multi-step process due to the unique chemistry of lignin and the complexity of its selective removal/modification without destroying cellulose. Therefore, it is highly likely that the pretreatment and fractionation of wood-to-ethanol will also require a multi-step process if we are to use low enzyme loadings and achieve a fast and complete hydrolysis.

Although there has been some work (Yang et al., 2002; Pan et al., 2005b; Cullis and Mansfield, 2010) on evaluating post-treatment as one way of enhancing the enzymatic hydrolysis of softwoods, several issues still need to be resolved. The optimum combination of pre and post-treatments is poorly understood since most of the previous research which

has evaluated the effectiveness of post-treatments has tended to be done after a single set of steam pretreatment conditions have been used (Yang et al., 2002; Pan et al., 2005b; Cullis and Mansfield, 2010). Just as important is that the chemical loading used to post-treat the substrate has generally been very high and, to make a post-treatment economically viable, it is imperative to reduce the amount of chemicals used in the process. Thirdly and most importantly, in most of the previous studies looking at post-treatment, high enzyme loadings were typically used to assess the hydrolysability of cellulose (Yang et al., 2002; Pan et al., 2005b; Cullis and Mansfield, 2010). Therefore, we felt it was important to assess the ability of the post-treatment to generate substrates from steam pretreated softwoods which would be susceptible to enzymatic hydrolysis at lower enzyme loadings. We addressed these aspects by optimising a suitable pre/post-treatment combination with the objective of enhancing the overall sugar yield with minimum consumption of enzymes and chemicals.

As well as increasing the sugar yield by employing appropriate pre-/post-treatment strategies, equally important is obtaining these sugars at high concentration. Previous studies used low biomass to steam ratios during steam pretreatment and consequently the large amount of condensate formed after steam pretreatment diluted the water-soluble hemicellulose fraction (Sipos et al., 2010; Ewanick, et al., 2007). Even combining the two streams and using a direct enzymatic hydrolysis of the whole slurry did not result in desired sugar concentration levels primarily due to the low consistency of the pretreatment slurry (Robinson et al., 2003; Tengborg et al., 2001b; Pettersson et al., 2002). Unlike other pretreatments, where water/solvent has to be impregnated with wood chips using special equipments prior to processing (for example, kraft pulping, organosolv, dilute acid etc.), steam pretreatment has the flexibility of controlling the ratio of biomass to which the steam injected. The biomass to steam ratio has been rarely studied although it could potentially help minimise steam usage and lead to the recovery of sugars at high concentration.

Most past work in this area has utilised pulp chips as the starting material (Galbe and Zacchi, 2012; Mabee et al., 2006). However, pulp chips are unlikely to be used as

commercial bioconversion feedstock for large scale facilities since their high moisture content and low bulk density limit their transport over long distances. Therefore, in order to benefit from economies of scale it is highly likely that densified feedstocks, such as wood pellets, will be used to meet the large scale biomass demand similar to what is occurring in the rapid growth of large scale combustion and cogeneration plants (Richard, 2010; Stephen et al., 2010). Despite extensive work evaluating the use of wood pellets for thermochemical applications and combined heat and power (CHP), the suitability of wood pellets as a feedstock for biochemical conversion has not yet been studied.

1.2 Why develop a lignocellulosic biorefinery?

Global demand for energy and commodity chemicals is increasing at an alarming pace in parallel with industrialization and economic development (IEA, 2011), while access to traditional fossil reserves are declining. The fast depletion of petroleum deposits is one of the many reasons why alternative carbon and energy sources and energy independence have become policy priorities around the world. The non-uniform distribution of fossil resources in the globe, an ever-increasing crude oil price in the international market, and the impact of fossil fuel use on the global climate and the consequent effect on biodiversity - all reflect the inevitability of a transition to a clean, renewable and sustainable bio based economy (Bauen et al., 2009). Plant biomass, formed by the biochemical fixation of inorganic carbon, is the only alternate and renewable carbon source produced by nature. It is more uniformly distributed across the planet than oil and has the potential to produce the equivalent of any product derived from fossil fuels (Smeets and Faaij, 2007). Compared to other renewable energy options such as wind and solar, only biomass has the potential to provide energy-dense liquid transportation fuels and chemicals currently based on petroleum (Smeets and Faaij, 2007).

Global development of sugar/starch/plant oils derived biofuels has already been given a kick-start for the use of biomass-to-biofuels as a partial replacement of petroleum. A number of major policy initiatives have been introduced such as the United States' "Energy Dependence and Security Act" of 2007, which set a goal to produce 36 billion gallons of

bioethanol to substitute ~15% gasoline by 2022 paralleling an earlier initiative known as the "European Union Directive" of 2003 to replace 5.75% of all gasoline and diesel transport fuels with biofuels by 2010. Partly in response to these directives global bioethanol and biodiesel production increased three fold in the last decade reaching 22 and 5 billion gallons respectively in 2010. However, current production capacity of liquid biofuels is only 2% of petroleum derived fuels (IEA, 2011). In the US it has been shown that gasoline can be substituted up to 15% without any changes in the engine and supply chain infrastructure. However, the so called 'blend wall' has limited the US market for ethanol. In addition, it is increasingly being realised that in order to make a complete transition to a true biorefinery based bioeconomy, the whole barrel of oil need to be replaced by the equivalent products derived from biomass (Holladay et al., 2007; Ragauskas et al., 2006). These realities demonstrate a need to expand and diversify the dedicated biofuel production into a biorefinery in order to significantly reduce our reliance on petroleum.

Development of biofuels in the past has relied exclusively on energy intensive food crops such as sugar cane, corn, wheat etc. (conventional biofuels) (Solomon et al., 2007). Even the most efficient crop-based production is insufficient to meet the current gasoline/diesel usage levels without a dramatic increase in cultivation. Meanwhile, the use of food crops for biofuel production is constrained by food security, economic and environmental implications (Solomon and Johnson, 2009; Solomon et al., 2007). Owing to both physical and political limitations on the expansion of biofuel production on arable land, new production capacity will be mostly likely based on abundantly available cellulosic biomass (Solomon and Johnson, 2009; Solomon et al., 2007). Being the largest resource of carbohydrate, 5% of the cellulosic biomass on the planet has the potential to fulfill the current levels of petroleum consumption (Smeets and Faaij, 2007). Therefore, cellulosic biomass is expected to produce the vast majority of the liquid biofuels and chemicals in future, predominantly from wood and agricultural residues (referred to as advanced biofuels). Despite high demand and significant research advancements, lignocellulosic biorefineries have yet to be successfully commercialised because of the lack of economic competitiveness and unfavorable market conditions in comparison to conventional starch

or sugarcane based production. Therefore, process efficiency and economics of cellulose based advanced biofuels and biorefinery need to be substantially improved.

1.3 Overview of bioconversion process and key ingredients for commercial success

A biochemical conversion process is one of the most prominent routes of producing different liquid fuels and chemicals, employing pretreatment and enzymatic hydrolysis steps to convert lignocellulosic biomass into platform sugars, which in turn can be fermented by appropriate microorganisms to produce ethanol, butanol and long chain alcohols (Foust et al., 2009; Sims et al., 2010) (Figure 1). Although fermentation conditions and microorganisms may vary in the production of different types of products, all these processes have to deal with similar upstream processes. Simple sugars are present as complex polymers in cellulosic biomass (namely cellulose and hemicellulose) which form higher order complexes with lignin, a phenol based polymer, that are less amenable to biodegradation. Once released, the simple sugars can be metabolised in the downstream fermentation processes by a variety of microorganisms to produce the desired fuels or chemicals (Peralta-Yahya et al., 2012; Yu et al., 1985b).

In the overall bioconversion process, feedstock cost is the single largest expense (Kazi et al., 2010). Therefore, it is important that we extract the maximum yield of sugar from biomass to ensure the efficient utilisation of the feedstock. As the enzyme costs are the second largest contributor (Humbird et al., 2011; Kazi et al., 2010), the high sugar yield should be achieved with the minimum use of enzymes. Finally, the simple monomeric sugars should be obtained at high concentration to increase the product titre during the fermentation process. This will enable the minimum use of energy during subsequent distillation/concentration steps (Sassner et al., 2008). In much of the published work, high sugar yields were typically obtained at the expense of using high enzyme dosages, particularly from softwoods, and final sugar concentrations were relatively low (Galbe and Zacchi, 2002; Mabee et al., 2006; Tengborg et al., 2001a).

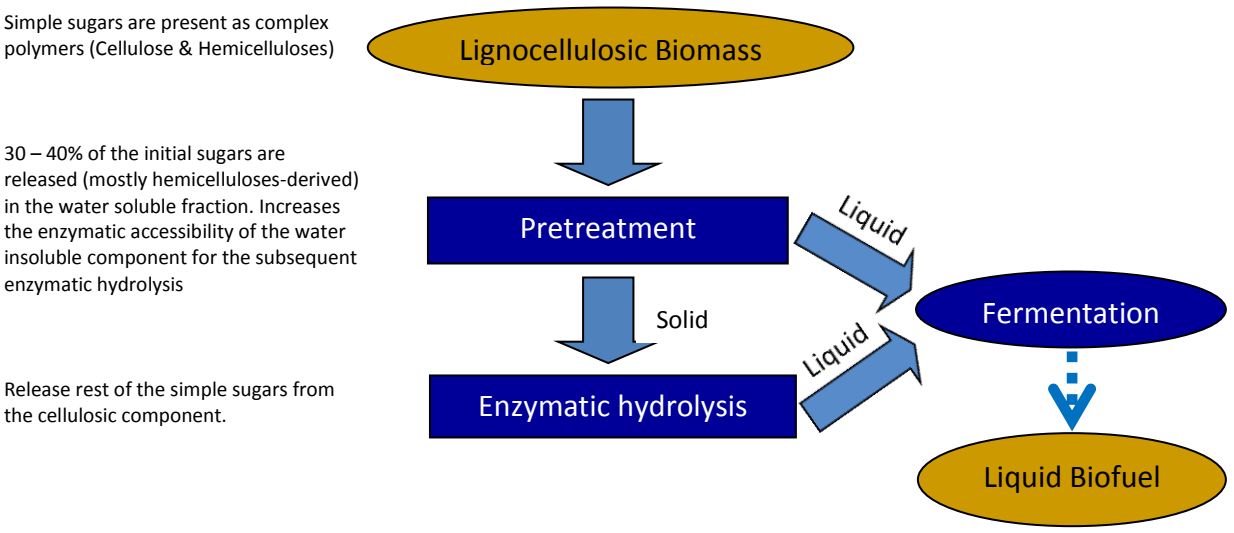


Figure 1 Bioconversion process scheme

1.4 Pretreatment as a prerequisite for the bioconversion of lignocellulose to sugars

For an industrial process to be economically viable, the enzymatic breakdown of lignocellulose to fermentable sugars must occur rapidly, preferably in hours or minimally in days. The wood structure, including how the hemicellulose and lignin are associated with the cellulose microfibrils, reduces the accessibility to the cellulose in the cell wall in such a way that only a very small fraction of cellulose in the untreated wood is accessible to the cellulase enzyme molecules (Cowling, 1975). Therefore, a pretreatment step is employed to disrupt the complex lignocellulosic structure and enhance the accessibility of the enzymes. Releasing simple sugars from the lignocellulosic materials generally consists of two main steps. These are pretreatment to fractionate and recover the hemicellulose and/or lignin while increasing the enzyme accessibility of the water insoluble cellulosic component and, subsequent enzymatic hydrolysis of the cellulosic fraction to release the remaining simple sugars (Mabee and Saddler, 2010).

To make lignocellulose more amenable to breakdown by enzymes, a wide range of biological, mechanical, physical, and chemical pretreatments have been developed (Eggeman and Elander, 2005). Some of the most common approaches includes steam

pretreatment, hot water/dilute acid pretreatment, ammonia fiber expansion, biomass dissolution processes, and variations on pulping processes with different chemicals and reaction conditions (Hendriks and Zeeman, 2009; Kumar and Murthy, 2011; Mosier et al., 2005b) (Table 1). Many of these pretreatments are effective on agricultural residues and are comparable to each other in terms of overall sugar yield (Elander et al., 2009; Wyman et al., 2009) despite many pros and cons in other technical aspects (Table 1).

To be effective, processes such as AFEX or biomass dissolution requires finer particle sized feedstocks (For example, saw dust instead of wood chips), demanding the need for a size reduction step prior to pretreatment. In contrast, other pretreatments such as steam pretreatment can handle feedstocks with a wide range of particle size and moisture contents. The requirement for chemicals such as in the AFEX, and other cellulose dissolution and pulping processes can be quite high (Mora-Pale et al., 2011; Sathitsuksanoh et al., 2012; Bals et al., 2011). For example, ionic liquid or concentrated phosphoric acid pretreatments need an almost 10:1 solvent to biomass ratio and an even higher amount of anti-solvents for an effective dissolution of biomass and subsequent precipitation of cellulose (Mora-Pale et al., 2011; Sathitsuksanoh et al., 2012). AFEX also requires a loading of 1:1 ratio of ammonia to biomass in this process, although it is claimed that most of this ammonia can be potentially recycled after the process (Bals et al., 2011). It should be noted that steam and dilute acid pretreatments use a chemical to biomass ratio of <0.05:1 (Less than 5% of the biomass weight), which is an order of magnitude lower than the chemical loading, required for other pretreatments (Galbe and Zacchi, 2012). Another key difference between these pretreatments is the ability of the pretreatments to produce a high consistency slurry. AFEX is very effective in producing a substrate with very high consistency since it is essentially a dry pretreatment. As direct steam injection is possible on a dry biomass feedstock, steam pretreatment also has advantages of producing high consistency slurries. However, with other pretreatments such as pulping processes or dilute acid, the biomass has to be impregnated with the reagent liquor using special equipment to raise the consistency of the slurry, and therefore, has limitations to reach the same level of solids as AFEX or steam pretreatment (Modenbach and Nokes, 2012).

When using woody biomass as a feedstock, there are not as many effective pretreatments. AFEX and biomass dissolution pretreatments are largely ineffective on woody biomass, particularly softwoods (Balan et al., 2009; Mora-Pale et al., 2011). Although pulping processes are effective in processing softwoods, at conditions which results in enhanced cellulose accessibility, hemicelluloses are dissolved in the lignin-rich liquid fraction making their recovery difficult. Steam and dilute acid pretreatments can process both woody and non-woody substrates and can recover the hemicellulosic sugars in the water soluble fraction resulting in water insoluble cellulosic component amenable to enzymatic hydrolysis.

1.5 The case for steam pretreatment

Although, no best option exists, steam pretreatment and dilute acid pretreatment appear to be the two processes closest to commercialisation, though R&D (Research and Development) continues to improve cost and performance goals of other technologies (Sims et al., 2010) (Table 2). As mentioned above, steam pretreatment has the advantages of requiring limited capital, energy, and chemical inputs while being applicable to a wide range of biomass feedstocks (Table 2) (Brownell and Saddler, 1984; 1987; Brownell et al., 1986; Bura et al., 2003; 2009; Hendriks and Zeeman, 2009; Holtzapple et al., 1989; Kumar and Murthy, 2011; Ramos et al., 1992a; 1992b; Schwald et al., 1989a). Particularly with woody feedstocks, steam pretreatment offers a unique benefit of processing wood chips 'as is' without requiring a size reduction step before or after the process. Moreover, direct steam injection on a dry substrate makes it possible to generate high consistency (high solids) slurries. In addition, steam ensures sterile conditions required for subsequent bioconversion steps. Because of these various techno-economic benefits, almost all of the emerging pilot scale and demonstration bioconversion plants are using variations of steam pretreatment (Table 2) (IEA Task 39, 2011). However, it is also worth noting that most of these companies are using agricultural residues as their starting feedstocks. As will be described later in more detail, despite many advantages, there are several technical issues that still need to be

resolved when using steam pretreatment as a front end for processing woody feedstocks particularly softwoods.

Table 1 Comparison of different pretreatments for the bioconversion of lignocellulose

	Capital investment	Chemical consumption	Recovery of hemicellulose in a fermentable form****	Potential to form high consistency slurry	Water use	Robustness to handle a range of biomass substrates	Requirement for size reduction before/after pretreatment	Range of enzyme loadings for reasonable hydrolysis (>60%) of softwoods *****	Value addition for lignin
AFEX*	+++	+++	+	++++	-	-	++++	NA	NA
Hot water	++	-	+	+	+++	+	+++	++++	+
Dilute acid	+++	+	+++	+	+++	+++	++	+++	+
Dilute alkali	++	+	-	+	+++	++	++	+++	++
Steam pretreatment	++	+	+++	+++	++	+++	-	+++	+
SPORL**	++	++	++	+	+++	++	++	++	++
Organosolv	++	+++	-	+	+++	++	+	+	+++
Wet oxidation	+++	+	+	+	+++	+	++	+++	+
Green liquor pretreatment	++	++	-	+	+++	++	++	++	++
Ionic Liquid pretreatment	NA***	++++	-	+	++++	+	++++	++	+++
Phosphoric acid dissolution	NA	++++	-	+	++++	+	++++	++	+++
- negligible ; + Low; ++ Medium ; +++ High , ++++ very high *Ammonia fibre expansion; **Sulfite pretreatment to overcome the recalcitrance of lignocellulose; ***Information not available for comparison ****without an extensive processing; ***** Without a post-treatment;									
References on which this comparison was based: (Brownell and Saddler, 1987 ; Bura and Saddler, 2004 ; Eggeman and Elander, 2005 ; Ewanick et al., 2007 ; Galbe and Zacchi, 2002 ; Galbe et al., 2007 ; Gregg and Saddler, 1995 ; Gregg et al., 1998 ; Hendriks and Zeeman, 2009 ; Kumar and Murthy, 2011 ; Mabee et al., 2006 ; Mosier et al., 2005a ; Pan et al., 2005a ; Saddler et al., 1982; Sassner et al., 2008 ; Schwald et al., 1989a ; Shevchenko et al., 2001a ;2001b; Shuai et al., 2010 ; Tengborg et al., 2001b ; Tengborg et al., 2001b ; Wyman et al., 2009)									

Table 2 Commercial/demonstration facilities that are using variations of steam pretreatment as part of their biomass-to-biofuels process

Company and location	Feedstock	Capacity (MG*)	Pretreatment technology
Poet-DSM, US	Corncobs, leaves, husk, and some stalk	20 – 25	Two stage steam explosion (Andritz)
Abengoa Bioenergy, US	Wheat straw/corn stover/sorghum grain	23	Acid catalysed steam explosion
Mascoma Inc. US	Hardwood waste	20	Steam explosion (SunOpta Bioprocess)
Inbicon owned by Dong Energy, Denmark	Wheat straw	1	Hydrothermal pretreatment
Maabjerg Energy Concept owned by Dong Energy, Denmark	Wheat straw	13 - 18	Hydrothermal pretreatment
Beta Renewable, Crescentino, Italy	Arundo donax and wheat straw	20	Steam pretreatment
BP Biofuels, England	Corn stover/cobs, wheat straw	110	Steam pretreatment
Iogen Corporation, Canada	Agricultural residues	>1	Steam explosion
*Million gallons			

1.6 A brief history of steam pretreatment

The concept of steam explosion was first introduced in a patent in 1926 by William H. Mason (Boehm, 1930; Mason, 1926). This patent demonstrated the way by which steam explosion of wood chips could be performed on an industrial scale for the production of fibre boards (Boehm, 1930; Koran et al., 1978; Mason, 1926). In this process called Masonite process, the wood chips are steam heated at a very high temperature, ~285°C, at a pressure of 3.5 MPa for about 2 minutes followed by a sudden increase in pressure to 7 MPa for about 5 seconds and subsequently the chips are exploded to atmospheric pressure through restricted orifices to form a pulp. Subsequent, two stage low consistency refining results in a dark pulp of about 75% yield suitable for the manufacture of high-density fiberboard (Mason, 1926). Despite the coarse structure and brown colour, the process was very effective for achieving fiber separation with low energy consumption (Koran et al., 1978). In very early work, Babcock used steam explosion as an effective method to extract fermentable sugars from pine to produce ethanol (Babcock, 1932). Iotech Corporation (1978) later extended the process as a way to enhance the cellulolytic digestibility of aspen in order to make animal feed for ruminants (Kokta and Ahmed, 1999). Further research by the company Forintek revealed more of the fundamental chemistry of steam pretreatment and assessed the robustness of the process on a range of cellulosic biomass in the context of producing different types of fuels and chemicals including ethanol, butanol, and butanediol (Brownell and Saddler, 1984; 1985; 1987; Brownell et al., 1986 ; Saddler et al., 1982 ; Saddler et al., 1983 ; Yu et al., 1985a; 1985b).

The Masonite process was also later extended to produce ultra-high-yield pulps suitable for paper making (Vit and Kokta, 1986; Kokta and Vit, 1987). The process scheme consisted of chemical impregnation of wood chips, short-duration steaming followed by sudden pressure release, refining and bleaching (Vit and Kokta, 1986) and this process was later optimized and advanced into today's chemi-thermo-mechanical pulping (Law and Bi, 1989; Heitner et al., 1991). Steaming is also employed in chemical pulping to produce

dissolving pulp where the objective of steaming is to remove the hemicelluloses prior to sulfite or Kraft pulping.

1.6.1 Pretreatment severity factor

In early work Vroom (1957) had shown that the time and temperature of kraft pulping was inter-dependent for a given pulp yield and it was possible to combine this effect into a single parameter. The resulting H factor is used in the pulp and paper industry to predict the degree of delignification and pulp yield. This H factor was later modified to trade the effects of time and temperature of steaming to obtain equivalent final effects, which is called the severity factor (Heitz et al., 1991; Overend and Chornet, 1987). The severity factor can be expressed by the equation, $\mathrm{Ro} = \int_0^{\mathrm{t}} \mathrm{e}^{(\mathrm{T}-100/14.75)}\,\mathrm{dt}$ where *T* is the temperature in °C and *t* is the time in minutes (Heitz et al. 1991; Overend and Chornet, 1987).

The severity factor has also proven to be useful in the field of bioconversion for comparing different pretreatment conditions and feedstocks (Heitz et al., 1991; Overend and Chornet, 1987). However, in this earlier work it was largely used to predict the effect on enzymatic hydrolysis, with little regard to the recovery of hemicellulosic sugars (Saddler et al., 1982; Saddler et al., 1983; Brownell and Saddler, 1984). The researchers found that increasing the pretreatment severity was beneficial to enhance the digestibility of biomass. In subsequent work an acid catalyst was added prior to steam pretreatment as this step reduced the required temperature and reaction time. The influence of the acid catalyst was also integrated into the model by incorporating pH as an additional variable. The resulting equation was $\mathrm{Ro} = \left(\int_0^{\mathrm{t}} \mathrm{e}^{(\mathrm{T}-100/14.75)}\,\mathrm{dt}\right) - \mathrm{pH}$ (Chum et al., 1990).

More recently, the importance of recovering the hemicellulosic sugars was recognized to be crucial to ensure a high overall sugar recovery. Although it has been shown that increasing the pretreatment severity has the beneficial effect of enhancing the enzymatic hydrolysis of the water insoluble, cellulosic rich fraction, the use of these high severities also results in the degradation of solubilized hemicellulosic sugars thereby

diminishing the overall sugar recovery and the fermentability of the water soluble fraction (Schwald et al., 1988; Ramos et al., 1992; Wu et al., 1999; Boussaid et al., 1999). Thus, subsequent work recommended using a medium severity, which is a compromise between optimizing hemicellulose recovery while ensuring the reasonable cellulolytic digestibility of the water insoluble, cellulose rich component for obtaining a high overall sugar yield (Boussaid et al., 2000).

Steam pretreatment has been shown to be largely effective in processing both hardwoods and agricultural residues when processed at their optimum conditions (Bura et al., 2002; 2009; Ohgren et al., 2007; Ramos et al., 1992a). Therefore, most of the commercial and demonstration facilities currently employ steam pretreatment to process primarily agricultural residues or hardwood feedstocks (Table 2). However, when softwoods are used, providing good hemicellulose recovery in the liquid fraction and a cellulose rich water insoluble fraction, which was amenable to hydrolysis at relatively low enzyme loadings, has proven to be more challenging (Ewanick et al., 2007; Monavari et al., 2009; Pan et al., 2005b; Wu et al., 1999). For example, at pretreatment conditions that provide reasonable hemicellulose recovery, the resulting softwood-derived solid fraction has been shown to be difficult to hydrolyze, typically requiring relatively high enzyme loadings (>20 FPU/g glucan) to achieve somewhat reasonable (>60%) hydrolysis yields (Boussaid et al., 2000). Although recent work has shown that other pretreatments such as organosolv pretreatment results in softwood substrates that have been readily susceptible to subsequent cellulolytic hydrolysis (Pan et al., 2005a; 2008), the hemicellulose containing liquid resulting from such delignification pretreatments is difficult to ferment (Pan et al., 2005). The recovery of hemicellulose is extremely important for softwoods as it contains mostly hexose sugars, which are easier to ferment, compared to pentose sugars (Robinson et al., 2003; Matsushika et al., 2009). Steam pretreatment provides good recovery of the hemicellulose derived sugars in the water soluble liquid stream, which can then be fermented by using traditional yeast strains. Therefore, one of the key challenges is to recover as much hemicellulose as possible while enhancing the ease of enzymatic hydrolysis of water insoluble cellulosic component at low enzyme loadings.

1.6.2 Effect of mechanical shearing and rapid decompression

Early research into steam pretreatment emphasized the importance of mechanical effects of shear and explosive decompression for the production of highly accessible substrates for enzymatic hydrolysis (DeLong, 1982). However, later work showed that explosion contributes essentially nothing to the development of cellulose accessibility (Brownell et al., 1986). In a study on eucalyptus wood chips, partial bleed-down of the pressure (slow release) resulted in comparable sugar and subsequent ethanol yields as compared to rapid explosive decompression. The former process resulted in a product that was almost entirely in a chip form in contrast to a highly defibrillated pulp after rapid decompression (explosion) (Brownell et al., 1986). This observation was an important finding since the slow release of the pressure can remove the undesirable volatile inhibitors, which would otherwise condense during an explosive decompression. These findings provide a technical advantage, but the most critical elements of the steam pretreatment process are the dissolution of hemicellulose, the extent of lignin depolymerisation/subsequent redistribution and their effect on the sugar recovery and subsequent enzymatic hydrolysis.

1.7 Why softwoods?

Softwood species (conifers) represent one of the most abundant sources of cellulose on the planet. As of 2010, conifers constitute ~40% of the total growing stock in the world's forest (FAO, 2010) and are particularly abundant in the Nordic countries, Canada and Russia where the taiga/boreal forest predominates. Softwoods are already a significant commercial commodity being the source of about 80% of the world's timber, with traditional centers of production being the Baltic region (including Scandinavia and Russia) and North America (Ekstrom, 2012).

In British Columbia, the ongoing outbreak of the mountain pine beetle (*Dendroctonus ponderosae*) has created an abundance of beetle-killed Lodgepole pine at advanced stages of infection with limited value for traditional structural applications, and

can be potentially repurposed for biofuel production (Kim et al., 2005; Pan et al., 2008). In addition, as softwoods are already an established commercial entity, utilisation of industrial residues for energy applications has been shown to result in immediate financial gains (Mabee et al., 2006; White et al., 2010). Pelletisation is the process by which softwood residues are densified to wood pellets. Wood pellets are one of the most globally traded bioenergy commodities with ~2 million tonnes of softwood pellets exported from British Columbia to Europe in 2011 (Bradley et al., 2011).

Softwoods have been shown to be one possible feedstock for an enzyme-based biomass-to-ethanol process. In addition to their abundance, it is worth noting that, compared to agricultural and hardwood-derived biomass sources, softwoods including species of Lodgepole pine (*Pinus contorta*), Norway spruce (*Picea abies*), Western hemlock (*Tsuga heterophylla*), and Douglas-fir (*Pseudotsuga menziesii*), have the advantage of having a hemicellulose component that is primarily composed of hexose sugars (>90%) that can be much more readily fermented to ethanol by conventional yeasts such as *Saccharomyces cerevisiae* (Matsushika et al., 2009; Olsson and HahnHagerdal, 1996). About 60 – 65% of a typical softwood is composed of C6 sugars including glucose, mannose and galactose (Table 3). However, softwoods generally have a higher lignin content and a significantly different fibre structure and chemical composition, which make them more recalcitrant to break apart by the enzymes and microbes compared to hardwoods and non-wood residues (Schwald et al., 1989; Sjostrom, 1993). Therefore, if a process can be made effective on softwoods, the strategies can be most likely adapted universally to all lignocellulosic feedstocks to process them more efficiently. The unique physical characteristics and chemical composition of softwoods create particular challenges and potential opportunities in a biorefinery perspective to produce fuels and chemicals.

Table 3 Typical chemical composition of softwoods reported in previous studies (% dry weight)

Species	Carbohydrates			Lignin	Extractives ***	References
	Pentose sugars*	Hexose sugars**	Total			
Norway spruce	6 – 8	58 – 63	64 – 71	28 – 32	3 – 4	Stenberg et al., 1998; Monavari et al., 2009; 2011; Soderstrom et al., 2002; 2003; Tengborg et al., 2001
Lodgepole pine	7 – 8	56 – 60	63 – 68	27 – 29	3 – 4	Ewanick et al., 2007
Douglas-fir	4 – 6	63 - 68	67 – 74	27 - 31	2 – 3	Cullis and Mansfield, 2010 Robinson et al., 2002 Boussaid et al. 2000
*refer to arabinose and xylose **refer to galactose, glucose and mannose ***extracted by organic solvents (acetone in some studies and ethanol in some other)						

1.7.1 Acid catalyst is essential for the effective steam pretreatment of softwoods

Earlier work has shown that the use of an acid catalyst, such as H_2SO_4 or SO_2, is particularly beneficial during steam pretreatment of more recalcitrant substrates such as softwoods (Brownell et al., 1986; Clark and Mackie, 1987; Schwald et al., 1989a). This allows the use of less severe pretreatment conditions such that more of the hemicellulose component is solubilized and recovered in a readily fermented, monomeric form while providing a cellulosic rich water insoluble fraction amenable to subsequent enzymatic hydrolysis. Clark et al (1987) found that addition of even small concentration of SO_2 (0.3%) significantly increased the digestibility of the solid fraction when pretreating Radiata pine wood chips.

When previous studies compared the influence of SO_2 and H_2SO_4 (Eklund et al., 1995; Mackie et al., 1985; Tengborg et al., 1998) the overall influence was found to be similar for both catalysts. It was hypothesized that sulfuric acid is the actual catalyst during

the steam pretreatment of SO_2 impregnated wood chips as the SO_2 which is incorporated into the wood chips interacts with water to form sulfurous acid, which in turn is oxidized to sulfuric acid under the high temperature during steam pretreatment (Brownell et al., 1986 ; Gregg and Saddler, 1996). Despite this similar mechanism, SO_2 has several benefits. Being a gaseous catalyst, SO_2 ensures a uniform penetration in the wood chips and the residual unabsorbed catalyst can be easily recycled back prior to steam pretreatment (Brownell et al., 1986; Gregg and Saddler, 1996). The addition of dilute sulfuric acid increases the moisture content of the wood chips during the impregnation step, consequently demanding higher amounts of steam during the subsequent steam pretreatment to heat up the additional moisture to obtain equivalent effect of a gaseous catalyst (Brownell et al., 1986; Gregg and Saddler, 1996). In previous studies, where the two catalysts were compared, there were some differences noted in the fermentability of the liquid fractions (Tengborg et al., 1998). The difference in fermentability suggested that H_2SO_4 impregnation lead to higher concentrations of fermentation inhibitory compounds resulting in lower ethanol yields (Tengborg et al., 1998).

At a constant temperature and duration of steam pretreatment, increasing the impregnation levels of SO_2 was found to increase the hemicellulose recovery and digestibility of the solid fraction (Clark and Mackie, 1987; De Bari et al., 2007; Schwald et al., 1989b; Wong et al., 1988). There appears to be an optimal loading, which results in maximum sugar yield, but this optimal loading varies, perhaps due to the difference in the feedstock characteristics and pretreatment conditions used (Clark and Mackie, 1987; De Bari et al., 2007; Wong et al., 1988). However, 3 – 5% SO_2 concentration was reported to be sufficient to generate enough sulfuric acid required for an efficient pretreatment. Clark and Mackie (1987) reported that optimum SO_2 concentration for the impregnation of softwood chips was 2 – 3 % for pretreatment at 213°C for 3 minutes. This optimization was based on the overall sugar yield. In a related work, enzymatic hydrolysis of steam pretreated Radiata pine (195°C, 9min) almost doubled when SO_2 impregnation levels were increased from 1 to 6.5% (Wong et al., 1988). However, when the SO_2 concentration was further increased, only a marginal improvement was observed in the hydrolysis yield (Wong et al., 1988).

1.8 Compromised pretreatment conditions for softwoods

As mentioned earlier, the best steam pretreatment conditions for obtaining the maximum recovery of hemicellulosic sugars and the most susceptible water insoluble cellulosic component are not the same. Efficient enzymatic conversion of cellulose requires higher severities and conversely, the best hemicellulose recovery with lowest concentration of inhibitors results from milder pretreatment conditions. Lower steam pretreatment severities (Log Ro = 3 - 3.5) results in almost complete recovery of hemicellulosic sugars (Boussaid et al., 1999; Ewanick et al., 2007). Higher pretreatment severities (Log Ro >4.0) results in a water insoluble component that can be readily hydrolysed (albeit at high enzyme loadings), but at the expense of considerable destruction of hemicellulosic sugars (Boussaid et al., 2000). Therefore, many previous studies recommended using a medium severity, which is a compromise between optimizing hemicellulose dissolution and recovery in a monomeric form while ensuring a reasonable cellulolytic digestibility of the water insoluble component so that a high overall sugar yield can be obtained (Boussaid et al., 2000; Ewanick et al., 2007; Wu et al., 1999). Depending on the nature of the feedstock, such conditions for softwoods generally result in 65 - 85% recovery of hemicellulosic sugars and 60 – 80% sugar yields in the enzymatic hydrolysis of the water insoluble component while using the enzyme dose in the range of 20 – 40 FPU/g glucan. An overview of the compromised conditions, used for different softwood feedstocks and relative yields can be found in Table 4.

Table 4 Optimum pretreatment conditions (single stage) and the corresponding sugar yield reported for different softwood species with varying physical properties (Sugar yield is the % original sugars released during pretreatment and subsequent enzymatic hydrolysis)

Pretreatment severity (Log Ro)	SO_2/ H_2SO_4 loading	Maximum yield reported (% of initial sugars)*	Nature of the feedstock			References
			Species	Moisture content (%)	Particle size (mm)	
3.9	2.6% SO_2	69	Radiata pine	43	30×30×5	Clark and Mackie, 1987
3.6	2.5% SO_2	71	Black spruce	46	20×20×5	Schwald et al., 1989
4.0	3.5% SO_2	66	Norway spruce and Scots pine mixed	47	2 – 10	Stenberg et al., 1998
3.6	0.5% H_2SO_4	67	Norway spruce	57	30	Tengborg et al., 1998
3.6	0.7% H_2SO_4	75	Mixed softwood forest thinnings**	50	13	Nguyen et al., 2000
3.5	4.5% SO_2	59	Douglas-fir	30	50×50	Cullis et al., 2004
3.5	4.5% SO_2	57	Douglas-fir	12	0.4	Cullis et al., 2004
3.6	4% SO_2	71	Lodgepole pine	8	20×20×5	Ewanick et al., 2007
3.6	2.5% SO_2	73	Norway spruce	47 – 50	1 -2	Monavari et al., 2009a
3.6	2.5% SO_2	69	Norway spruce	47 – 50	5 – 6	Monavari et al., 2009a
3.6	5-6% SO_2 + Ferrous sulfate	75	Norway spruce	52 - 55	2 – 10	Monavari et al., 2011

*This is the total soluble sugars released during pretreatment and subsequent enzymatic hydrolysis at enzyme loadings in the range of 20 – 40 FPU/g glucan
**70% White fir & 30% Ponderosa pine

With a single stage steam pretreatment and high enzyme loadings (20 - 40 FPU/g glucan), the highest overall sugar yield (both hemicellulose and cellulose derived sugars) reported to date was 75% (Nguyen et al., 2000). This recovery was accomplished with a mixture of forest thinnings White fir (*Abies concolor*) and Ponderosa pine (*Pinus ponderosa*) using the following pretreatment parameters; Impregnation with 0.65% H_2SO_4 followed by pretreatment at 215°C for 100 seconds (log Ro – 3.6). For Norway spruce, the highest overall yields have been 66 and 67% of the theoretical value using impregnation with SO_2 and H_2SO_4, respectively (Stenberg et al., 1998b; Tengborg et al., 1998). Despite a wide range of feedstock characteristics used in the different studies, it is interesting to note that optimum pretreatment conditions for a reasonable hemicellulosic sugar recovery (>60%) and reasonable enzymatic hydrolysis of the cellulosic component (>60% of the cellulose present in the water insoluble component) lies within the range of $3.5 \leq \log Ro \leq 4.0$ (Table 4). The slight variation in the optimal conditions might be due to the difference in the acid concentration used in different studies and also on the nature of the feedstocks. The combined total monomeric sugar recovery from these optimal conditions (followed by enzymatic hydrolysis) appears to be in the range of 60-75% of the original sugars present in the raw material (Table 5)

As noted in Table 4, several different softwood species including Radiata pine, Douglas-fir, Lodgepole pine, Black spruce (*Picea mariana*), Norway spruce were assessed to try to determine the pretreatment severity that results in maximum sugar recovery. A comparison of these studies does give an indication that the optimum pretreatment severity for maximum sugar yield for a range of softwoods is fairly close. However, most of these studies were carried out using a single wood source (i.e. chips from one log), which may not be a true representative of the behavior of the respective wood species. In other words, the ability of the optimum conditions to handle the apparent variations between the trees or species has not yet been properly validated. This is particularly relevant in the context of the recent studies by Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) which compared leading pretreatment options across different varieties of agricultural and hardwoods feedstocks. This work reported some variations between

different batches of switch grass, corn stover and poplar feedstocks (Wyman et al., 2009; 2011; Elander et al., 2009) in terms of their overall sugar yield after a pretreatment and enzymatic hydrolysis. However, softwood feedstocks were not included in these studies since several of the assessed pretreatment processes were likely not effective on softwoods.

Although variation is likely to be less substantial as in hardwoods or agricultural residues, variability in fibre type and topochemistry does exist between different softwood species and at different life stages. This variability means an assessment of how different trees/species respond to the same set of steam pretreatment conditions may be necessary. Previous studies had indicated that Douglas-fir was relatively more recalcitrant to bioconversion when compared to Lodgepole pine, Norway spruce, Black spruce or Radiata pine (Schwald et al., 1989; Boussaid et al., 2000; Wu et al., 1999; Ewanick et al., 2007). Achieving reasonable cellulolytic hydrolysis has been difficult, even at optimal steam pretreatment conditions and high enzyme loadings of >20 FPU/g glucan. Therefore, post-treatments such as alkali, alkaline peroxide, and oxygen delignification have been explored to achieve reasonable hydrolysis yields from Douglas-fir (Boussaid et al., 2000; Cullis et al., 2004 ; Ewanick et al., 2007 ; Mabee et al., 2006 ; Pan et al., 2004 ; 2005b ; Stenberg et al., 2000 ; Wu et al., 1999 ; Yang et al., 2002). Again, most of this past work was based on a single log bolt derived from a ~150 year old tree. It therefore, may not be representative of the behavior of a range of Douglas-fir samples as subsequent work also indicated that the Douglas-fir sapwood was more amenable to steam pretreatment and enzymatic hydrolysis compared to the heartwood fraction. This observation indicated that differently aged Douglas-fir may respond differently to different processing conditions. In order to provide a comprehensive assessment of how sample heterogeneity influences the robustness of steam pretreatment, it is important to process them at standard set of conditions. To date, this approach has not been performed.

Table 5 A typical material balance of carbohydrates in softwood during steam pretreatment at near optimal conditions

Initial sugars in softwood per 100 g	Monomeric sugars (g)		Total recovery (g)	References
	Pretreatment	Enzymatic hydrolysis (20 – 40 FPU/g glucan)		
56 – 63	15 – 18	22 – 25	37 – 43	Stenberg et al., 1998; Monavari et al., 2009; 2011; Soderstrom et al., 2002; 2003; Tengborg et al., 2001; Ewanick et al., 2007; Yang et al., 2002; Boussaid et al., 2003

1.9 Influence of initial feedstock properties (particle size and moisture content) on the robustness of steam pretreatment

In addition to the type of biomass, the initial physical characteristics of biomass can also influence the requisite severity of steam pretreatment and thus the overall sugar recovery. Total sugar recovery varies with the original sugar composition and characteristics of the starting biomass even when they are pretreated at their near optimal pretreatment conditions. The two major physical properties that can vary from batch to batch of feedstock and that have been shown to influence the relative severity of steam pretreatment are the particle size and moisture content of the wood chips.

When controlling for moisture content, a smaller particle size facilitates better mass transfer and thus slightly increases the relative severity of pretreatment (Cullis et al., 2004). Substantially larger particle size was shown to lead to an uneven cooking resulting in increased degradation of the sugars, especially on the outside of the wood chips (Brownell et al., 1985; Cullis et al., 2004). To assess the effect of moisture content, steam pretreatment has been carried out with wood chips ranging from air-dried to green conditions. Small chips, whether green or air-dried have been shown to result in similar sugar recoveries upon pretreatment and subsequent enzymatic hydrolysis (Brownell and Saddler, 1985; Gregg and Saddler, 1996; Cullis et al., 2004). However, especially when larger

chips were used, the air-dried material appeared to reach the temperature of the injected steam within a shorter time, resulting in higher apparent severities (Brownell et al., 1986). Higher initial moisture content (~50%) such as that found in green chips was shown to require additional energy to heat up, thus decreasing the apparent severity of the steam pretreatment. Lower relative severity due to increased moisture content was also observed during the steam pretreatment of corn stover (Tucker et al., 2003) where the required duration of steaming was almost doubled for higher moisture containing corn stover to obtain equivalent release of hemicellulosic sugars. This could also be the reason, when sulfuric acid was directly used as a catalyst during the steam treatment of willow, that the digestibility was found to be significantly less compared to using SO_2 (Eklund et al., 1995 ; Gregg and Saddler, 1996). However, a minimum moisture content appears to be essential to maintain a sufficient retention of SO_2 within the wood chips and facilitate the formation of sulfuric acid (Gregg and Saddler, 1996; Ramos et al., 1992a). Similarly, drying the wood chips prior to pretreatment can also lead to irreversible aspiration of the bordered pits resulting in a lower permeability of the SO_2 and water during pretreatment and can also influence the accessibility of the enzymes during enzymatic hydrolysis. In short, moisture content seems to have a non-linear influence on the requisite severity of steam pretreatment with a minimum critical moisture necessary for effective steam pretreatment and excessive moisture increasing the energy requirements (Olsen et al., 2012).

When the feedstock consists of relatively dry and smaller particles, the overall sugar recovery at their near optimal conditions is generally on the lower range (~60 %) compared to ~70% overall sugar recovery reported for feedstocks with sufficient moisture and moderate particle size (Cullis et al., 2004; Monavari et al., 2009). The variability in sugar recovery due to the changes in feedstock characteristics can be minimised by either fine tuning the steam pretreatment conditions or conditioning the feedstock for a desired moisture or particle size. Comparison of the data from previous studies indicated that, regardless of the variations in the particle size or moisture content, steam pretreatment generally results in a total sugar yield in the range of 60 – 70% when pretreated at their

near optimal conditions and subsequently hydrolysed at 20 – 40 FPU/g glucan (Cullis et al., 2004; Monavari et al., 2009).

1.10 Mechanism of steam pretreatment: structural changes in lignocellulosic biomass

Steam pretreatment causes the physical rupture of woody structure by adiabatic expansion of absorbed water and hydrolysis reactions of the cell wall components (Kallavus and Gravitis, 1995). Changes in the wood structures during steam pretreatment can be generally classified into macro-, micro- and molecular-level changes (Table 6).

1.10.1 Macro and micro level changes in softwood structure during steam pretreatment

As a result of steam pretreatment, wood chips (~30×30×5 mm) are ruptured into smaller particles approximately <2mm in length. The degree of reduction in fibre size is significantly higher with the addition of an acid catalyst (Boussaid et al., 2000). Despite a reduction in size, separation of fibres along the longitudinal direction seems to be relatively rare in softwoods as the fibres appear to rupture more in the transverse direction. This phenomenon is the opposite to what is generally observed in pulping (Tanahashi et al., 1983). In contrast, hardwood species demonstrated a higher extent of fibre separation upon steam pretreatment (Fujii et al., 1985). This difference is likely due to the lesser extent of middle lamella disruption in softwoods due to the stable lignin structure of this region of the cell wall (Donaldson et al., 1988; Michalowicz et al., 1991). It should be noted that transverse rupturing results in the longitudinal separation of fibers after steam pretreatment of softwoods especially when an acid catalyst is used.

A combination of light and electron (both scanning and transmission) microscopy has been used to study the cell wall structural changes during steam pretreatment (Brownell and Saddler, 1984; Donaldson et al., 1988; Kallavus and Gravitis, 1995; Michalowicz et al., 1991; Tanahashi et al., 1983; Zhang and Cai, 2006). Fractures were observed in the cell wall structure of both hardwoods and softwoods even after mild

steaming especially at pit membranes of bordered/half bordered pit pairs between different cells. This observation indicated the initial sites of steam penetration and heat transfer (Brownell and Saddler, 1984; Kallavus and Gravitis, 1995; Zhang and Cai, 2006). Different cell types also respond differently during steam pretreatment. In hardwoods, it was reported that vessel elements and ray cells behaved in opposite ways. While vessel elements collapsed readily during steam pretreatment and accelerated the degree of disruption of the wood (Fujii et al., 1985; Tanahashi et al., 1983), ray cells were relatively unaffected. In contrast to vessel elements, which are abundant in hardwoods, longitudinal tracheids in softwoods (being more lignified), are generally less affected (Tanahashi et al., 1983). This anatomical variation could also be the reason why softwoods require higher pretreatment severities compared to hardwoods in order to obtain the same level of structural disruption.

Lignin redistribution during steam or dilute acid pretreatment was another common observation reported in these microscopy studies (Donaldson et al., 1988; Donohoe et al., 2008; Excoffier et al., 1991; Kallavus and Gravitis, 1995; Michalowicz et al., 1991). As the temperature of steam pretreatment is substantially higher than the glass transition temperature of lignin, lignin softens and transforms to a higher elastic state, which together with the surface tension effects of water and hydrophobicity of lignin makes them agglomerate to form spherical droplets (Angles et al., 2001; 2003; Vignon et al., 1995). The redistributed lignin appeared as spherical droplets in size ranging from 100 – 10000 nm size, both on the inner and outer surface of the cell wall (Donaldson et al., 1988; Donohoe et al., 2008; Toussaint et al., 1991).

1.10.2 Molecular level changes

The main consequences at the molecular level are reactions within the polysaccharides (hemicellulose and cellulose) and lignin. Steam pretreatment largely hydrolyses and solubilises the hemicellulosic fraction, which is recovered in the water soluble component, leaving a water-insoluble component mainly composed of partially depolymerised cellulose and lignin (Boussaid et al., 2000; Wu et al., 1999).

1.10.2.1 Changes in hemicellulose

Relatively high pretreatment severities (Log Ro≥3.3) and the use of an acid catalyst lead to complete dissolution of hemicelluloses from softwood (Boussaid et al., 2000; Ewanick et al., 2007). The lower degree of acetylation in softwood hemicelluloses makes them easier to remove at lower severities compared to the heavily acetylated hemicelluloses in hardwood and agricultural residues (Sun et al., 1999; Sixta, 2006). The addition of an acid catalyst facilitates recovery of hemicelluloses in a monomeric form (Angles et al., 2001; Boussaid et al., 2000) in contrast to the oligomeric fractions of galactoglucomannans obtained in the water soluble component when no acid catalyst is used (Song et al., 2008).

Although the goal is to obtain complete hemicellulose dissolution and their recovery as monomeric sugars, at severities where complete dissolution of hemicellulose is achieved, a small fraction of the solubilized sugars also degrades to furan derivatives or secondary degradation products such as a levulinic/acetic/formic acid (Brownell and Saddler, 1984; Mussatto and Roberto, 2004). During pretreatment, sugar degradation can be attributed to the three distinct processes: pyrolysis, oxidation and dehydration (Brownell and Saddler, 1984). Pyrolysis occurs in the absence of sufficient oxygen and results in the thermal decomposition of organic matter (Ramos et al., 2003). Among the three polymers, hemicellulose, cellulose and lignin, hemicellulose is most sensitive to thermal decomposition (Ramiah, 1970). Oxidation promotes degradation of organic matter to carbon dioxide and water and also contributes to a partial conversion of pentoses to carboxylic acids and other by-products. Dehydration reactions of pentose and hexose sugars produce furfural and hydroxymethylfurfural respectively (Mussatto and Roberto, 2004). There were several studies indicating that these products, when produced at high concentration are strong inhibitors of microbial growth during fermentation and prior detoxification strategies are required to ensure good fermentability (Boussaid et al., 2001; Cavka et al., 2011; Mussatto and Roberto, 2004; Robinson et al., 2003; Sundstrom et al., 2010). Therefore, while maximising the recovery of hemicellulosic sugars in a monomeric

form, pretreatment also needs to be optimised to minimise the degradation of simple sugars to inhibitory compounds.

Different sugar moieties in hemicellulose differ in their sensitivity to steam pretreatment. Arabinose and galactose sugars are released in the initial stages of steam explosion (Korte et al., 1991), while fractions of xylose and mannose require higher temperatures for dissolution since they are located in the most interior of the cell wall and are tightly bound to cellulose microfibrils (Sun and Tomkinson, 1999). Despite an earlier release, galactose has been shown to exhibit higher rate of survival during steam pretreatment.

1.10.2.2 Changes in cellulose

Relative to hemicellulose, cellulose undergoes lesser, though still substantial, chemical changes during steam pretreatment. At near optimal conditions, most of the cellulose (>90%) is recovered in the water insoluble component. However, the degree of polymerisation (DP) decreases from >10000 to <400 (Ramos et al., 1993; Tanahashi et al., 1983). The degree of crystallinity is also affected during steam pretreatment with some studies reporting an increase in crystallinity. However, this observation is likely to be largely due to the removal of amorphous components present in the biomass. At higher severities a good fraction of the amorphous cellulose could be dissolved during steam pretreatment along with hemicellulose, leading to a relative increase in crystallinity of the remaining cellulose (Ramos et al., 1993; Tanahashi et al., 1983). However, related work with a more definitive measure of crystallinity found a near complete transformation of cellulose III to cellulose I during steam pretreatment at very high severities (log Ro = 4.5). This indicated that the rearrangement of less crystalline regions to crystalline allomorphs is possible thus, increasing the intrinsic crystallinity of the cellulose itself (Yamashiki et al., 1990).

1.10.2.3 Changes in lignin

Softwood lignin does not become solubilised during steam pretreatment and therefore the vast majority of the initial lignin (>95%) is recovered in the water insoluble component (Clark et al., 1989; Hemmingson, 1987). However, lignin undergoes dramatic changes in its structural features during steam pretreatment and these changes critically influence the amenability of the steam pretreated substrate to enzymatic hydrolysis as well as lignin's suitability for co-product applications.

Prominent chemical reactions of lignin during steam pretreatment are depolymerisation and repolymerisation (condensation) reactions (Hemmingson, 1987; Marchessault et al., 1982; Robert et al., 1988). Depolymerisation reactions involve extensive cleavage of α-O-4 & β-O-4 linkages, which constitute the most abundant linkages (40-60%) connecting the phenyl propane units in lignin (Adler, 1977; Li et al., 2007; Li et al., 2009). The reactions are initiated by the benzyl cations (carbonium ions) formed by the proton-induced elimination of water (Scheme 1). Subsequent acidolysis or homolytic cleavage reactions of β-aryl ether bonds results in the formation of structures of 'Hibbert's ketones type' compounds (Scheme 2) (Sarkanen and Ludwig, 1971; Li et al., 2009). As a consequence of these reactions, a decrease in β-O-4 linkages was observed with an apparent reduction in molecular weight and a concomitant increase in free phenolic hydroxyl groups (Li et al., 2007; 2009). While the molecular weight of dioxane extracted milled wood lignin is ~11 kDa, the molecular weight of the lignin isolated from steam pretreated softwood was reported to be <2.5 kDa (Hemmingson, 1987; Robert et al., 1988; Sudo et al., 1985). Depolymerisation and low molecular weight of steam pretreated lignin accounts for their higher solubility during the subsequent delignification processes compared to the native lignin in softwood.

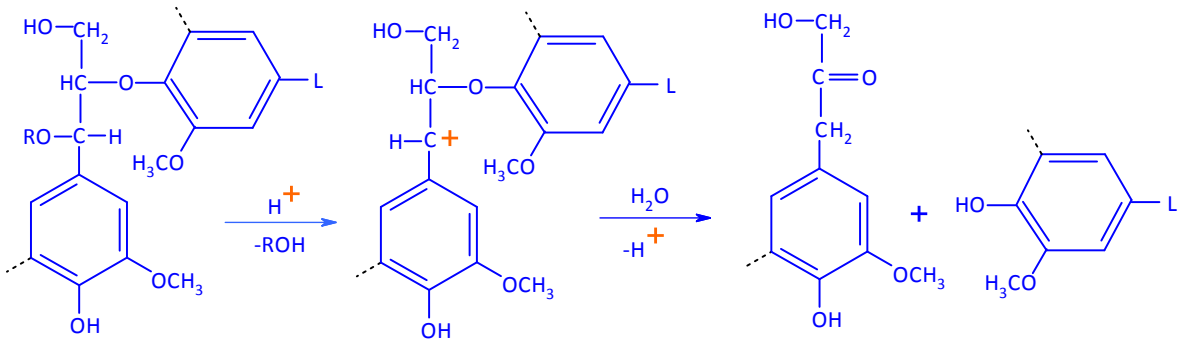
However, as the severity of steam pretreatment goes up, repolymerisation reactions occur as a result of acid catalyzed condensation between the benzyl carbonium ion and an electron rich centre (C6, C2 or C5) of the aromatic ring (Scheme 3) (Li et al., 2007; Robert et

al., 1988; Shevchenko et al., 1999). These reactions can, to a certain extent, counterbalance the degradation of the lignin caused by the cleavage β-aryl ether linkages (β-O-4), resulting in condensed lignin with partly blocked α-C reaction centers (Shevchenko et al., 1999). An increase in the content of C-C condensed structures (as characterised by Nuclear Magnetic Resonance (NMR) spectra) has been suggested as direct evidence for the occurrence of such reactions (Li and Gellerstedt, 2008; Li et al., 2007). The yield of acidolysis products from steam pretreated lignin was also shown to decrease with an increase in pretreatment severity (Robert et al., 1988). At harsher pretreatment severities, where the condensation reaction prevails, the cleavage of β-O-4 links is accompanied by a slight increase in the molecular weight of the lignin (Angles et al., 2003). In subsequent studies on the steam explosion of milled wood lignin (aspen), it was found that, at a treatment severity of log Ro=3.2 – 4.5, the repolymerisation reaction seems to dominate (Li and Gellerstedt, 2008; Li et al., 2007).

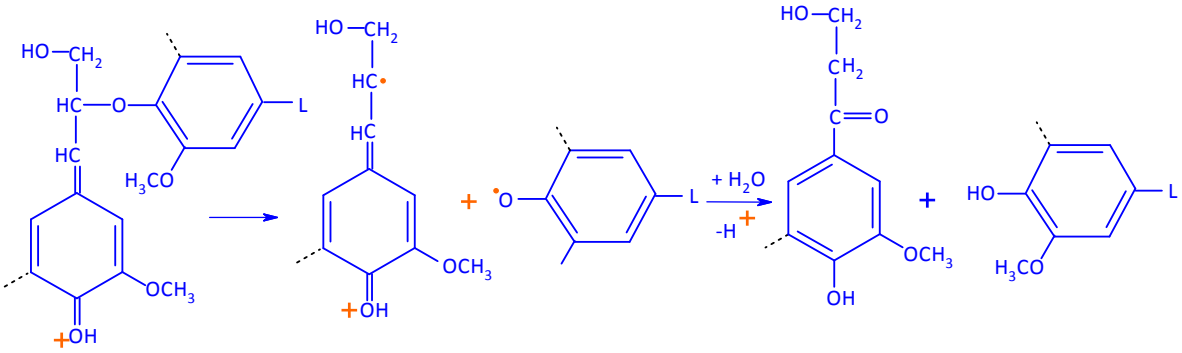
Competition between depolymerisation and repolymerisation reactions in lignin during steam pretreatment is significant as the extent of these reactions can determine the properties of the resultant lignin and its potential for value added co-products and the extent of interference with the bioconversion process. Condensed lignin tends to be more hydrophobic and will increase the extent of unproductive binding with the cellulase enzymes during the enzymatic hydrolysis step (Nakagame et al., 2010; Ooshima et al., 1983; 1990). In addition, unlike depolymerisation reactions, repolymerisation reactions decrease the reactivity of lignin thereby making it more resistant in the subsequent lignin extraction stages. Wayman and Lora, 1979 noticed maximum extractability of the steam exploded lignin only for a limited period of auto hydrolysis. Partial loss of lignin reactivity and low solubility in alkali/organic solvent was reported to occur at harsh conditions of steam pretreatment (Robert et al., 1988). Recent studies have shown that extractability of steam pretreated lignin could be improved by adding carbonium ion scavengers (Li et al., 2007; 2009) such as 2- naphthol during steam pretreatment, which limits the condensation reactions between lignin moieties. The relative occurrence of depolymerisation and repolymerisation reactions is mainly dependent on the pretreatment severity such that

higher temperature, longer reaction time, and stronger acidic medium all favor condensation reactions of lignin.

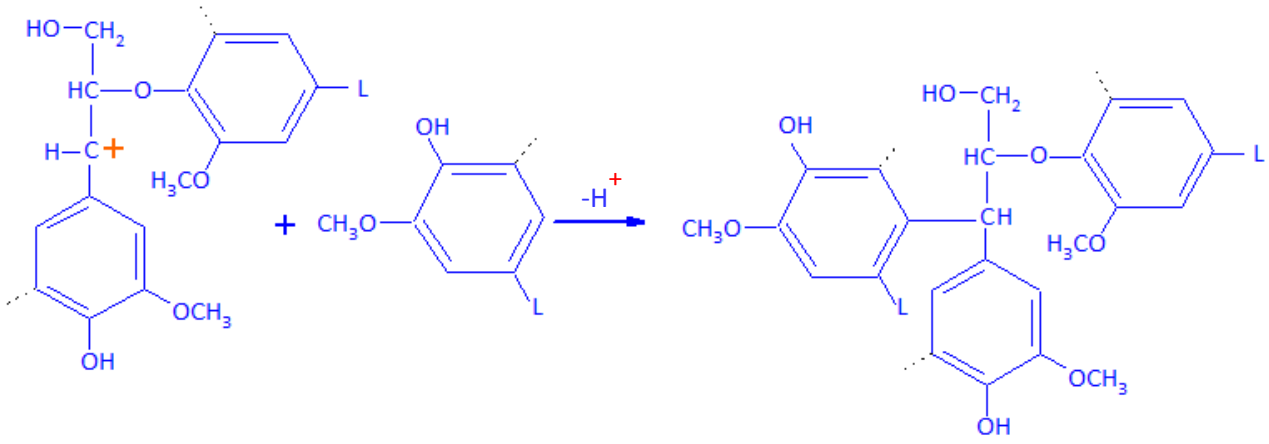
Additional minor changes associated with steam pretreatment involve a small degree of demethylation of aromatic methoxyl groups of lignin (Hemmingson, 1983; 1985; 1986; Robert et al., 1988). Angles et al., 2003 noticed lower methoxyl content in lignin after steam pretreatment. However, as the oxygen content was not significantly reduced and the abundance of p-hydroxy phenyl units increased, it was hypothesized that demethylation rather than demethoxylation occurs during steam pretreatment (Hemmingson, 1983; 1986).



Scheme 1 Formation of benzyl carbonium ions in the acidic medium and consequent formation of Hibbert's ketones. L refers to rest of the lignin polymer.



Scheme 2 Homolytic cleavage reactions of β-aryl ether linkages in lignin. L refers to rest of the lignin polymer.



Scheme 3 Condensation reactions between benzyl carbonium ions and electron rich C6 carbon atom of another aromatic compound. L refers to rest of the lignin polymer.

Table 6 Major structural changes in wood after the acid catalysed steam pretreatment

Macro- and micro-level changes	Molecular level changes		
	Cellulose	Hemicellulose	Lignin
Wood chips are fractionated into water soluble and insoluble components	Mostly recovered in water insoluble component	Almost entirely recovered in water soluble component	Almost entirely recovered in the water insoluble component
Disruption of wood into smaller particle size and fibre length. Transverse rupture predominates the longitudinal fibre separation especially when acid catalyst is used	Substantial reduction in molecular weight	Mostly solubilised as monomeric sugars	Overall decrease in molecular weight
Lignin redistribution as spherical droplets on the fibre surface	Substantial decrease in degree of polymerisation and increase in crystallinity	A fraction of the monomeric sugars are degraded	Significant reduction in α-O-4 & β-O-4 linkages. Higher degree of condensation reactions at harsher pretreatment conditions

1.11 Factors influencing cellulose accessibility and the enzymatic hydrolysis of steam pretreated substrates

Various substrate characteristics have been shown to influence enzymatic hydrolysis (Santos et al., 2012; Nashad et al., 1995; Saddler, 1986; Mansfield and Saddler, 1999). Although it is generally agreed that hydrolysis efficiency cannot be correlated to any single substrate characteristic, it is likely that the enzymes's abilities to access the accessible surface area of the cellulose is the main limitation (Saddler, 1986). However, some substrate factors play more than one role in limiting cellulose accessibility and many of these factors are inter-dependent. In addition to characteristics within the cellulose itself (i.e. crystallinity, degree of polymerization, etc.), the residual lignin and hemicellulose present in the pretreated substrates are known to restrict the cellulose accessibility (Ohgren et al., 2007; Berlin et al., 2006; Nakagame et al., 2010). Accordingly, the relative dominance of the various factors would in turn depend on the initial biomass characteristics and their

respective changes during steam pretreatment. The various substrate properties and their suggested mechanisms of restricting cellulose hydrolysis are briefly discussed below.

1.11.1 Gross fibre characteristics

The average particle size of a substrate has been shown to play a role in the hydrolysis of pretreated lignocellulosic substrates. Since adsorption is a prerequisite for hydrolysis to occur (Zhang and Lynd, 2004; Tanaka et al., 1986; Wang et al., 2011), it seems intuitive that particle size can in turn influence the specific surface area available for adsorption. Fibre length and coarseness have been reported to play important roles in determining the accessible surface area (Mooney et al., 1999). In a study by Mooney and coworkers (1999) on the enzymatic hydrolysis of Douglas-fir kraft pulp, smaller fibre fractions (<2 mm) were found to have a significantly higher digestibility compared to the larger fractions (2 – 5mm) despite a similar chemical composition. They hypothesized that the specific surface area of the small fibres and fines increased the enzyme adsorption, which is critical for the hydrolysis of the substrates.

The relative dominance of other factors may also be critical in determining the influence of particle size on the enzymatic accessibility. For a given steam pretreatment condition and initial particle size, the average particle size of steam pretreated softwoods is generally higher than that of steam pretreated hardwoods. This has been attributed as one of the reasons why softwoods hydrolyse poorly compared to hardwoods (Tanahashi, 1989). However, this inverse correlation between particle size and enzymatic hydrolysability does not carry through when comparing the hydrolysability across pretreatments. As was discussed previously, steam pretreatment generally results in much lower particles size and should ideally favor enzymatic hydrolysis compared to the long fibres produced by kraft pulping or the organosolv process. However, this is not usually the case, which means other substrate factors are playing a critical role in determining the cellulose accessibility and ease of cellulose hydrolysis. The role of particle size in enzymatic hydrolysis is even more questionable when it comes to purer cellulosic substrates. For substrates such as cotton linters and microcrystalline cellulose, several studies have shown no correlation between

average particle size and hydrolysis rates (Rivers and Emert, 1988; Shewale and Sadana, 1979; Sinitsyn et al., 1991).

1.11.2 Residual hemicellulose

Resistant layering of the cellulose elementary fibrils by the hemicellulosic component in native lignocellulose and the precipitation of hemicellulosic fractions on the fibre surface during a pretreatment can both influence the ability of the enzymes to access and hydrolyse the cellulose. In addition to restricting cellulose accessibility, dissolved oligomeric fractions of xylan were also shown to negatively influence cellulose hydrolysis (Qing et al., 2010). Positive correlations between the hemicellulose removal and enzymatic digestibility of the pretreated substrates have been observed in many previous studies (Grohmann, 1984; Bura et al., 2009; Ohgren et al., 2007a; Joeh et al., 2007). The selective removal of xylan by purified xylanase has been shown to increase the digestibility of the holocellulose (Donaldson, 1988; Boutelje and Hollmark, 1971; 1972; Sinner et al., 1973, Hu et al., 2011). However, with steam pretreated softwood, the hemicellulosic component is almost completely removed and therefore the restriction of hemicellulose on the hydrolysis of water insoluble component appears to be minimal.

1.11.3 Residual lignin

Similar to hemicellulose, lignin restricts cellulose swelling and invariably limits accessibility (Grethlein et al., 1984; Stone et al., 1969). Lignin is thought to influence enzyme accessibility to cellulose in more ways than by just acting as a barrier to prevent the enzymes from effectively binding to the cellulose (Ucar and Fenger, 1988). For example, it has been shown that lignin removal increases the porosity of both kraft and sulfite pulps and that consequently increases the median pore width, corresponding to the average molecular weight of the lignin molecules removed (Ahlgren et al., 1971; Stone and Scallan, 1969; Tarkow and Feist, 1969). It has also been shown that the increase in pore volume observed after lignin removal corresponds to the substrate's increased susceptibility to hydrolysis (Grethlein, 1985; Wong et al., 1988). Precipitation of lignin spherical droplets on the fibre surface as a result of pretreatment also limits the access of the enzymes to the

cellulose (Donaldson et al., 1988; Donohoe et al., 2008; Wong et al., 1988). An issue which further complicates the picture is the differences observed in the response of hardwood and softwood substrates to chemical treatments designed to redistribute and/or remove lignin. It has been shown by several authors that softwood substrates are inherently more resistant to lignin removal and redistribution and, consequently, to enzymatic hydrolysis (Grethlein et al., 1984; Ramos et al., 1992; Stone et al., 1969; Maekawa, 1996).

Lignin is also thought to negatively influence the hydrolysis reaction by irreversibly adsorbing the cellulase enzymes, thus preventing their action on cellulose. This interference has been observed with steam-exploded substrates (Clesceri et al., 1985; Converse et al., 1990; Lee et al., 1994; Nakagame et al., 2010; Ooshima et al., 1983; 1990; 1991; Berlin et al., 2005; 2006; Tu et al., 2007). Previous work showed that the extent to which lignin adsorbs enzymes depends very much on the nature of the lignin itself (Sutcliffe and Saddler, 1986). Softwood derived lignin was found to have a significantly higher affinity compared to the lignin derived from hardwood or agricultural residues. Earlier work showed that enzyme adsorption to lignin decreases as the severity of the pretreatment of hardwood increases (Ooshima et al., 1983; 1990; 1991) whereas with softwoods a different trend was observed (Nakagame et al., 2010). Thus, it is not yet clear whether the predominant effect of lignin is in restricting cellulose accessibility, or unproductively binding the cellulase enzymes and how these effects influence the enzyme loadings employed. Gaining a clearer resolution of lignin's effects was one of the goals of the thesis.

1.11.4 Drying and fibre collapse and their influence on cellulose accessibility

Drying the pretreated substrate was shown to have a detrimental effect on enzymatic hydrolysis by limiting the cellulose accessibility. It is generally agreed that drying the substrate prior to enzymatic hydrolysis can lead to fibre collapse and a consequent reduction in cellulose accessibility. Cell wall collapse due to hornification has also been extensively studied in the paper recycling literature (Fernandez et al., 2004; Minor, 1994). Related work also reported that addition of filling agents such as sucrose or glycerol prior to drying prevented the extent of hornification. (Laivins and Scallan, 1993; Higgins and

McKenzie, 1963). Esteghalian et al (2001) reported that the degree of reduction in enzymatic hydrolysis largely depended on the mode and extent of drying. While a 20 – 40% reduction in enzymatic hydrolysis was observed following oven drying and air drying of oxygen delignified kraft pulp, freeze drying had only a minor effect. In more recent work, it was shown that the degree of reduction in enzymatic hydrolysis by hornification largely depends on the type of pretreatment employed and consequent variations in the substrate properties (Wang et al., 2011a).

1.11.5 Cellulose properties (DP and crystallinity)

There have been some contradictory observations when the influences of DP and crystallinity on enzymatic hydrolysis have been examined. There is a general tendency towards decreased hydrolysis rates over the course of hydrolysis, with the residual cellulosic material proving to be quite recalcitrant. However, a number of researchers have shown that the molecular weight or degree of polymerization (DP) of the residual material following hydrolysis is very similar, or only marginally reduced, when compared to that of the original sample (Puls et al., 1991; Reese et al., 1957; Walseth, 1952). In contrast, other workers have shown that the DP of wood-derived cellulose fragments decreased with increasing reaction times (Puri et al., 1984; Ramos et al., 1993). Complementary work to both these contradictory findings showed that the influence of cellulose DP and the extent of its reduction during enzymatic hydrolysis can be a matter of the cellulose ultrastructure and the nature of the cellulase enzyme complex used (both endoglucanases and cellobiohydrolases in particular) (Puls et al., 1991; Kleman-Leyer et al., 1992; 1994a; 1994b; 1996; Stalbrand et al., 1998; Srisodsuk et al., 1998). In addition, although the leveling off of the cellulose DP and the increased recalcitrance of the residual cellulose during the course of hydrolysis can be attributed to the influence of DP (Battista, 1950), it remains unclear as to whether cellulose DP is a contributing limiting factor or whether this structural characteristic is associated with other factors such as crystallinity, accessibility or available surface area, which ultimately result in slower rates and limited degrees of hydrolysis.

The same uncertainty exists with the influence of cellulose crystallinity (Fan et al., 1980; 1981). Some studies have attributed the decreased hydrolysis rate over the course of hydrolysis to the preferential removal of the amorphous cellulose component. (Fan et al., 1980; 1981; Reese et al., 1957; Sasaki et al., 1979; Sinitsyn et al., 1991; Ramos et al., 1993; Tanahashi, 1990; Tanahashi et al., 1989). Several other workers have shown that, when all other substrate factors are similar, the degree of crystallinity of the substrate has no effect on hydrolysis (Puri, 1984; Cowling and Brown, 1969; Ramos et al., 1993, Thompson et al., 1992; Converse et al., 1990; 1993). In studies where crystallinity was suggested to be important (for example, ball milling or phosphoric acid dissolution), the treatments that caused a decrease in crystallinity also resulted in an increase in specific surface area or decreased particle size (Cowling and Brown, 1969; Puri, 1984; Ramos et al., 1993, Thompson et al., 1992; Converse et al., 1990; 1993). Therefore, the observed alterations in the rate and extent of hydrolysis are likely influenced by the associated factors rather than just the crystallinity of the substrate.

It has been shown previously that steam pretreatment of lignocellulosic substrates increases the crystallinity index of the substrate at the same time as it enhances the ease of hydrolysis of the substrate. These observations contradict the theory that crystalline cellulose is hydrolyzed more slowly than amorphous cellulose. However, it is also known that the hydrolysis of crystalline cellulose seems to have more stringent prerequisites for the enzymes (such as carbohydrate binding domains, cellobiohydrolase etc.) than does the hydrolysis of amorphous cellulose and thus synergism seems to be particularly important for crystalline cellulose hydrolysis (Lee et al., 1988).

1.11.6 Why is cellulose specific surface area so important?

In order for cellulases to hydrolyze cellulosic substrates, it seems the prerequisite is to be able to access the cellulose elementary fibrils, which are encased along with hemicellulose and lignin into microfibrils (Stone and Scallan, 1968; Chang and Holtzapple, 2000; Grethlein and Converse, 1991; Arantes and Saddler, 2010). The size of enzyme molecules in the fungal cellulase system is in the range of 24 – 77A° in diameter with an

average of 59A° (Cowling, 1975). When these elementary microfibrils are associated into fibrils and further multifaceted into the fiber walls, the extent to which enzymes can access the cellulose chains is dramatically reduced and factors such as content and location of hemicellulose/lignin, which restricts cellulose accessibility must be much more influential than molecular characteristics such as crystallinity or DP. Therefore, for real lignocellulosic substrates, the cellulose accessibility is generally represented by cellulose specific available surface area. This factor may be more influential than individual substrate properties in determining the rate and extent of hydrolysis. This is the reason when several different substrates' enzymatic hydrolysis was correlated to substrate factors by several workers, the hydrolysis yield did not correlate to a single substrate factor, but rather with the cellulose-specific accessible surface area (Bansal et al., 2012; Wang et al., 2011; Zhang and Lynd, 2004).

1.11.7 Possible substrate factors behind the higher recalcitrance of steam pretreated softwoods

For given pretreatment conditions, it is clear that steam pretreated softwood is more recalcitrant to enzymatic hydrolysis compared to steam pretreated hardwood or agricultural residues. Although steam pretreatment generally results in particle size reduction for both hardwood and softwoods, the extent of particle size reduction is generally higher for hardwoods. On the other hand, at the near optimal conditions, steam pretreatment of softwoods generally results in a near complete dissolution of hemicellulose and therefore, influence of residual hemicellulose is almost negligible. In contrast, steam pretreated agricultural residues and hardwoods have significant amounts of residual xylan, which have been shown to restrict the cellulose accessibility. However, steam pretreated softwood has substantially higher amount of lignin compared to steam pretreated hardwood and non-wood feedstocks, which can potentially limit cellulose accessibility. In addition, lignin present in steam pretreated softwood was found to have a higher affinity to proteins and therefore can contribute more towards the effect of unproductive binding. In summary, under a given steam pretreatment conditions, it appeared that larger particle

size, and more detrimental lignin and overall lower accessibility to cellulose explain the relatively higher recalcitrance of steam pretreated softwood compared to steam pretreated hardwood and agricultural residues. However, it is important to understand the relative contributions of each of these substrate factors in restricting the effective hydrolysis of steam pretreated softwoods and their dynamics with different enzyme loadings.

1.12 Strategies for improving sugar yield

1.12.1 Two stage steam pretreatment

Due to the high recalcitrance of steam pretreated softwoods to enzymatic hydrolysis, two stage steam pretreatment has been applied to improve the overall sugar yield (Table 7). Separate conditions were optimised at each stage to recover the maximum amount hemicellulose and cellulose derived sugars respectively (Stenberg et al., 1998; Tengborg et al., 1998). Nguyen et al., (2000) used a two-stage dilute acid process to hydrolyse a mixture of different softwoods by optimising a first set of conditions for optimum hemicellulose recovery and second set of conditions for optimum ease of cellulose hydrolysis. The overall yield of monomeric sugars, after pretreatment and enzymatic hydrolysis, was 82% of the theoretical value (Table 7), an improvement of 7% over the single stage pretreatment (Nguyen et al., 2000). A related work on Norway spruce reported 77 - 90% theoretical sugar yield with impregnation of either SO_2 or H_2SO_4 compared to 66% obtained for the best, single stage pretreatment (Soderstrom et al., 2002; Stenberg et al., 1998). Despite the improvements in yield, all of these studies still used high enzyme loadings (25 – 60 FPU/g glucan) (Monavari et al., 2009b; Soderstrom et al., 2002; Stenberg et al., 2000). Another major drawback of the two-stage steam pretreatment is the high residual lignin content, which hinders the subsequent enzymatic hydrolysis and possibility of recycling the enzymes (Stenberg et al., 1998; Tengborg et al., 1998).

Despite a slight improvement afforded by the two stage system, it still does not reduce the need for high enzyme loadings (Table 7) (Tengborg et al., 2001a). Although more

sugar was obtained after the two stages the sugar was obtained at lower concentration compared to a single stage due to the additional condensate generated and the use of intermediate solid-liquid separation/washing steps (Galbe and Zacchi, 2002). Therefore, continuing evaluation is required on a variety of technical fronts to determine whether the improvements in sugar yield offset the extra costs involved.

Table 7 A summary of the previous conditions of two stage steam pretreatment of softwoods, enzyme loading employed and the best overall sugar yield reported

Type of feedstock	Conditions (Log Ro)	Acid catalyst	Maximum sugar yield reported (% original sugars)	Enzyme loading (FPU/g glucan)	Highlights	References
Norway spruce (2-10 mm) with 50% moisture	3.0 & 3.9**	3 & 3 % SO_2**	93	33	Hydraulic press for the solid-liquid separation and no washing in between the stages	Monavari et al., 2009
Norway spruce (2-10 mm) with 50% moisture	3.0 & 3.9	3 & 3% SO_2	78	33	Washing in between the two stages	Monavari et al., 2009
Spruce (2-10 mm) with 45% moisture	3.4 & 3.2	0.5 &2% H_2SO_4	77	25	Washing in between the two stages	Soderstrom et al., 2003
Mixed softwood forest thinning* (13 mm) with 47% moisture	3.5 & 3.4	0.4 & 0.6% H_2SO_4	82	60	Washing in between the two stages	Nguyen, 1998
**70% White fir & 30% Ponderosa pine * first stage and second stage respectively						

1.12.2 Lignin modification /extraction post-treatment processes

As mentioned earlier, hemicellulose dissolution during steam pretreatment results in the substantial increase in porosity of the cell wall layers and consequently an increase in the enzyme accessibility compared to the untreated wood. However, even the most successful set of conditions still requires high enzyme loadings to obtain a reasonable level

of cellulose hydrolysis. Many studies have demonstrated the higher recalcitrance of steam pretreated softwood compared to steam pretreated hardwood or agricultural residues (Gregg et al., 1998; Ramos et al., 1992b). As also mentioned earlier, lignin is likely the greatest cause for the recalcitrance of steam treated softwoods to enzymatic hydrolysis, since the remaining water insoluble component after steam pretreatment consists almost exclusively of cellulose and lignin (Gregg et al., 1998; Schwald et al., 1989b; Ewanick et al., 2007).

Some of the previous studies have looked at the partial removal of lignin as a means of enhancing enzymatic hydrolysis of steam pretreated softwoods. Alkaline peroxide treatment was reported to be highly effective in removing lignin and enhancing the enzymatic hydrolysis of a variety of steam pretreated softwoods (Maekawa, 1992 ; 1996 ; Schwald et al., 1989a ; Yang et al., 2002). The process was originally developed and optimised for the pretreatment of agricultural residues (Gould, 1984). It was found that a pH≥11.5 is a prerequisite for obtaining sufficient delignification and the optimum conditions were; 6 hour treatment using 1% H_2O_2 in a 2% biomass slurry (the peroxide loading was 50% by weight of the biomass used) (Gould, 1984; 1985).

In a subsequent work, Schwald et al., 1989 reported ~70% delignification of steam pretreated spruce and a near complete cellulose hydrolysis (at an enzyme loading of ~30 FPU/g glucan) when using the same set of conditions previously optimised for peroxide delignification of wheat straw. In a later study on steam pretreated Douglas-fir, Yang et al. (2002) successfully reduced the required reaction time to 45 minutes by increasing the temperature of the treatment from room temperature to 80°C. However it still required higher peroxide loading to obtain complete cellulose conversion during enzymatic hydrolysis (Yang et al., 2002). Recently, a further 50% reduction in peroxide loading was achieved by increasing the consistency of the peroxide delignification process while also employing stabilising agents such as EDTA or DTMPA to prevent the decomposition of peroxide (Cullis and Mansfield, 2010).

The pulp and paper industry has a long history in the industrial application of peroxide in the bleaching sequence for both chemical and mechanical pulp. In these applications, there are minimal requirements of chemicals and conditions are relatively mild (Dang et al., 2007; Mustajoki et al., 2010; Ni and He, 2010 ; Ramos et al., 2008; Savoye et al., 2011; Zeronian and Inglesby, 1995). However, it should be noted that the primary goal in these lignin-retaining bleaching processes is to increase the brightness of the pulp by selectively oxidising the lignin side chain structures without compromising the pulp yield and fibre strength (Ramos et al., 2008; Zeronian and Inglesby, 1995). Further, peroxide bleaching is generally carried out on a relatively low lignin-containing pulps such as kraft pulp. Although applying such a strategy to high lignin-containing substrate (≥40%) such as steam pretreated softwood seems to be technically challenging, if effective delignification is to be achieved, the selective oxidation of lignin side chain to carboxylic acid structures may help to enhance the swelling of the substrate. It may also reduce the unproductive hydrophobic interactions of lignin with enzymes, further enhancing the hydrolysis yield.

Pan et al (2005) subsequently evaluated several partial delignification strategies with the objective of enhancing the enzymatic hydrolysis of steam pretreated Douglas-fir. Oxygen delignification was reported to result in significant levels of delignification and a substantial increase in the ease of enzymatic hydrolysis. However, the major challenge with oxygen delignification was the requirement of additional chemicals such as magnesium sulfate to minimise cellulose degradation. In addition, at higher temperatures of oxygen delignification, the lignin becomes condensed as a result of radical coupling reactions leading to lower reactivity and reduced delignification. In subsequent work, Cullis and Mansfield (2010) compared the efficacy of wet oxidation and oxygen delignification to alkaline peroxide treatments for enhancing the enzymatic hydrolysis of steam pretreated Douglas-fir. The authors found that alkaline peroxide treatment was better in achieving high levels of delignification (<10% residual lignin content) and high enzymatic hydrolysis yield while wet oxidation and oxygen delignification resulted in substrates with a significantly higher residual lignin content (>20%).

Extraction with alkali alone was also found to result in partial delignification of steam pretreated softwoods (Schwald et al., 1989b). However, despite partial delignification, the alkali treatment generally decreased the enzymatic hydrolysis of steam pretreated softwoods (Schell et al., 1998; Schwald et al., 1989b). Schell et al (1998) showed that post-treatment with alkali decreased the hydrolysis yields of steam pretreated Douglas-fir regardless of the type of alkali used (NaOH, $Ca(OH)_2$, NH_4OH). In an earlier work it was found that when the pretreatment time was increased from 20 to 180 seconds at 210°C, the subsequent alkali extraction decreased the hydrolysis yield from 40% to 30% and 98% to 20%, respectively (Schwald et al., 1989a) . It has been suggested that lignin that has been distributed as spherical droplets during steam pretreatment, becomes redistributed over the surface of cellulose microfibrils during an alkaline extraction thereby limiting the enzyme accessibility to the cellulose (Michalowicz et al., 1991; Schwald et al., 1989b) Other work, which supports this proposed mechanism, showed that the pore volume of steam exploded Radiata pine decreased significantly when the substrate was subsequently alkali extracted (Wong et al., 1988) with a commensurate decrease in enzymatic hydrolysis yield from 84% to 24%. In contrary to this finding, Pan et al (2004) reported that extraction of lignin from steam pretreated Douglas-fir with 1% NaOH at room temperature doubled enzymatic hydrolysis yields compared to the corresponding steam pretreated samples at an enzyme loading of 20 FPU/g glucan. It was postulated that alkali extraction selectively removes the fraction of lignin which has a higher affinity to the enzymes thereby reducing the unproductive binding of the enzymes with lignin.

There are other methods for improving enzymatic hydrolysis without substantial removal of lignin. Previous studies demonstrated the beneficial effect of oxidative enzyme treatment to improve the enzymatic hydrolysis of steam pretreated softwood. Palonen and Viikari (2004) observed a 20% improvement in enzymatic hydrolysis of steam pretreated spruce after treatment with laccase and a mediator chemical, N-hydroxy-N-phenylacetamide (NHA). These authors showed that laccase treatment made the surface of the steam pretreated substrate more hydrophilic due to the oxidation of lignin and consequent enrichment of the carboxylic acid groups. Reducing the hydrophobicity of lignin

by such oxidative enzyme treatments can decrease the non-productive interaction of cellulases with lignin leading to an improvement in enzymatic hydrolysis. Although this work helped elucidate the mechanisms influencing enzymatic hydrolysis, this approach would be economically challenging. The high protein requirement due to the application of both laccase and cellulases and high cost of stabiliser chemicals make the process less attractive compared to a purely chemical post-treatment.

It is apparent that the removal of all lignin present in the steam pretreated substrate may not be necessary for an effective enzymatic hydrolysis. The type and location of the lignin seems to have a significant influence on enzymatic hydrolysis. For example, in addition to lignin removal, certain lignin modification was also reported to enhance the enzymatic hydrolysis of pretreated lignocellulose. Neutral sulfonation has been shown to be efficient in enhancing the enzymatic hydrolysis of mechanical pulp (Mooney et al., 1998). It was hypothesised that during neutral sulfonation, instead of lignin removal, the sulfonic acid groups are incorporated into the lignin, making it more hydrophilic, leading to fibre swelling and an overall increase in cellulose accessibility. Sulfonation of lignin is also expected to decrease the hydrophobic interaction of lignin with cellulase enzymes. However, the suitability of sulfonation as a post-treatment of steam pretreated softwood has not yet been investigated. It is expected that sulfonation will have a higher ability to sulfonate steam pretreated substrate in comparison to a mechanical pulp. Substantial cleavage of β-O-4 linkages of lignin after a steam pretreatment results in a higher abundance of free phenolic groups, which can facilitate a greater degree of sulfonation at a neutral pH. It should be noted that in chemi-thermo-mechanical pulping (CTMP), steaming facilitates sulfonation and increases swelling and the water retention of the pulp (Ahmed, 1994; Area et al., 1995; Beatson et al., 1984).

It is highly likely that steam treated softwood will require some form of post-treatment to partially remove/modify lignin to enhance enzymatic hydrolysis. Currently the best combination of treatments is poorly understood since all of the previous research evaluating the efficiency of post-treatments has been done with a single set of steam

pretreatment conditions. As mentioned earlier, lignin depolymerisation and condensation varies considerably with pretreatment severities and the extent of these reactions will have a substantial influence on the ability of the post-treatments to remove/modify lignin. Therefore, in order for the post-treatment to work effectively, the steam pretreatment conditions need to be optimised in conjunction. Ideally, the steam pretreatment condition should result in good depolymerisation of lignin while minimising the extent of condensation reactions and achieving maximum recovery of water soluble hemicellulosic sugars.

A major challenge with post-treatment is the cost involved with additional steps. The chemical loadings used for post-treatments in research-to-date have been very high. It is important to reduce the amount of chemicals used in the process while retaining the high sugar yield with minimal amount of enzymes. If the chemical loadings can be partially minimised, the additional cost of the post-treatment is expected to be offset by the reduced enzyme requirement for the post-treated substrates. However, in all of the previous studies on post-treatment, high enzyme loadings were used to assess the hydrolysability of cellulose present in the post-treated substrates. Therefore, it is important to assess the ability of the post-treatment to generate substrates from steam pretreated softwoods, which would be susceptible to enzymatic hydrolysis at lower enzyme loadings.

The biggest industries processing lignocellulosic substrates for over a century has been the pulp and paper industries, which employ a range of different methods for delignification/lignin modification to obtain the desired cellulose-based products. A typical pulping/bleaching regime is always a multi-step process due to the unique chemistry of lignin and the complexity of selectively removing/modifying lignin without destroying cellulose. Therefore, it is highly likely that the pretreatment and fractionation of wood-to-ethanol will also require a multi-step process if we are to achieve low enzyme loadings. Further, the modification/removal of lignin during post-treatment can open up new avenues for using lignin in value added co-product applications with the possibility of improving the economics of the overall process.

1.13 Pretreatment effects on sugar concentration

In addition to the recalcitrance to enzymatic hydrolysis, another major challenge with the steam pretreatment of softwoods is the low sugar concentration obtained both in the pretreatment liquid fraction and the enzymatic hydrolysate of the cellulosic component. The starch based ethanol industry uses 20 – 30% (wt/vol.) sugar concentration to obtain an ethanol titre of 10 – 14% in their process (Lin and Tanaka, 2006). To obtain the equivalent titre for cellulosic ethanol, it will be necessary to conduct both the pretreatment and subsequent enzymatic hydrolysis processes at high solids levels (substrate consistency).

1.13.1 Consistency of pretreatment

The primary reason for the low sugar concentration in the steam pretreatment liquid is the low biomass to steam ratio typically employed. Reducing the amount of water right from the pretreatment step will enable us to maintain high sugar concentrations in the downstream processes. However, all of the previous studies on steam pretreatment optimisation have been conducted at low biomass loadings leading to a liquid to solid ratio of the resulting slurry of >10 (Ewanick et al., 2007; Sipos et al., 2010). The large volume of condensate obtained after steam pretreatment results in its separation into two streams. However, even when combining the two streams prior to enzymatic hydrolysis and conducting the hydrolysis at high solids does not result in high enough sugar levels due to the extremely low sugar concentration of water soluble hemicelluloses in the pretreatment liquid. Therefore, a high pretreatment consistency which applies a low amount of steam per weight of biomass should be a focus of future work.

One major advantage of steam pretreatment is the flexibility of controlling the ratio of biomass to steam injected, unlike other pretreatments where the water/solvent ratio has to be developed prior to addition to the wood chips using special equipment prior to processing (for example, kraft pulping, organosolv, dilute acid etc.). However, by adjusting the feedstock filling ratio in the pretreatment reactor we can easily control the biomass to steam ratio during steam pretreatment. Mass transfer limitations are expected to be

greater at high solids content. However, it should be noted that because of the high temperatures and high desired-solids levels, direct steam injection to a relatively dry feedstock is the most practical approach for increasing the sugar concentration.

1.13.2 Influence of solid-liquid separation and water washing after steam pretreatment

Solid-liquid separation and water washing are commonly carried out following steam pretreatment and have been used in many previous studies (Palmqvist et al., 2011; Tengborg et al., 2001b; Wiman et al., 2011). Solid-liquid separation involves separating the steam pretreatment slurry into solid and liquid fractions by vacuum filtration, pressing or centrifuging. Subsequent washing of the residue removes the remaining dissolved solids to leave the water insoluble component. The primary reason for employing these two steps has been to improve the enzymatic hydrolysis. Degradation products resulting from steam pretreatment including phenolics are inhibitory to enzymes, yet can be washed away achieving the same hydrolysis yields with reduced enzyme loading (Merino and Cherry, 2007; Palmqvist et al., 2011; Tengborg et al., 2001b; Wiman et al., 2011). However, a washing step would dilute the sugar streams requiring higher amount of energy for subsequent concentration to obtain the desired levels of biofuel. It may not be economically viable to ignore the water washed fractions as they contain significant amount of soluble sugars. At higher consistency pretreatments in particular, even after an efficient solid liquid separation, there will be a good amount of residual soluble sugars left in the solid fraction, which a washing step can subsequently remove.

When conducting enzymatic hydrolysis of the steam pretreated and subsequently water washed substrate, sugar concentration is generally enhanced by increasing the solids level during enzymatic hydrolysis. But even after increasing the consistencies above 20%, obtaining high sugar concentration was challenging since half of the substrate contained lignin (Palmqvist et al., 2011; Wiman et al., 2011). The whole slurry, however, is expected to have lower relative lignin content in the overall dry matter composition due to the presence of dissolved sugars together with cellulose. If effective mixing and high enzyme loadings are

employed, the direct enzymatic hydrolysis of the whole slurry can result in higher sugar concentration due to the readily available dissolved sugars along with those released during enzymatic hydrolysis (Wiman et al., 2011). Tengborg et al (2001) reported that when all of the material following pretreatment was used in the hydrolysis step, higher enzyme loadings (120 FPU/g glucan) were required to achieve reasonable hydrolysis yield at high concentration.

Further evaluation is required to determine whether the improvements in enzymatic hydrolysis due to a solid liquid separation or water washing outweigh the extra cost involved in concentrating the diluted sugar stream. It is necessary to test the feasibility of high consistency steam pretreatment with the lowest generation of condensate followed by enzymatic hydrolysis without applying any solid-liquid separation or water washing.

1.14 Pellets as a global feedstock for a biorefinery

All of the past work on the pretreatment and subsequent bioconversion of biomass has been done on wood chips, saw dust or loose agricultural residues. These feedstocks are less likely to be used as a feedstock commodity for large scale biofuel production because of their low bulk density and high moisture content. This makes these raw materials transport and storage challenging (Richard, 2010). Wood chips/saw dust when densified to wood pellets, offer benefits in terms of higher density and low moisture content resulting in much more attractive transportation, handling and storage costs. In addition, grinding prior to densification makes the substrates much more uniform and therefore can provide consistent outcome in the processing. Densification, through pelletisation, is one of the reasons that the global wood pellet market has developed so rapidly, with wood pellets from British Columbia being a major bioenergy source for Europe. Global wood pellet production has increased from almost zero in the beginning of the last decade to 5 million tonnes per year in 2012 (WPAC, 2012). Softwood is the main source of wood pellet production in Canada and the vast majority of production is exported to Europe over 16000 kilometers away. In addition to the growing demand in Europe, the market is expanding to

Asian countries and also to US. All of these wood pellets are currently used in combined heat and power, combustion and other thermal applications (Bradley, et al. 2011).

It is therefore likely that wood pellets will become a globally traded commodity that can be used as a feedstock for second generation/advanced of bioethanol. Although pellets have been extensively studied and applied in thermochemical conversion applications such as combined heat and power (CHP)(Alevanau et al., 2011; Erlich et al., 2006), the suitability of pellets for biochemical conversion has not been investigated to any great extent. However, if the US is to meet its suggested target of 16 billion gallons of cellulosic ethanol by 2020, this means that 1–3 billion cubic meters of biomass (assuming completely dry material) will need to be available and readily transported (Richard, 2010) to potential biomass-to-ethanol plants. Thus, it is highly probable that the global production and trade in biomass pellets will grow and, as well as continuing to be a feedstock for thermochemical type process such as combustion, CHP, gasification and pyrolysis, it will be used as a feedstock for second generation/advanced bioethanol production to both benefit from economies of scale and to source an increasing variety of biomass feedstocks (Stephen et al., 2010). Although an increase in density, reduction in the moisture content and the related convenience in handling have made the biomass pellets an attractive commodity for large-scale combustion, combined-heat-and-power (CHP), etc.(Lehtikangas, 2001; Sikkema et al., 2010 ; 2011a; 2011b), it is expected that the low moisture content and increased density of wood pellets would prove problematic in both the pretreatment and downstream processing required for enzyme-based biorefinery processes. Therefore, it is important to study the influence of pelletisation on the suitability of softwoods for steam pretreatment and subsequent enzymatic hydrolysis.

1.15 Are previously optimized steam pretreatment conditions ideal?

Previous optimisations of steam pretreatment for softwoods have mainly focussed on finding the compromised conditions for a reasonable hemicellulosic sugar recovery while achieving a reasonable enzymatic hydrolysis of the cellulosic component. It was apparent

from the review of literature that a range of other technical challenges still need to be resolved in order to make the steam pretreatment more effective on softwoods so that they can be used as a biorefinery commodity. Ideally, steam pretreatment conditions should be able to meet the criteria listed below, which formed the basis of this work for evaluating appropriate pre/post-treatment strategies for the bioconversion of softwoods to platform sugars.

— Robustness for processing a range of softwood feedstocks with different physical characteristics
— Maximum recovery of hemicellulosic sugars in the water soluble component after steam pretreatment
— The water insoluble cellulosic component should be readily hydrolysed at low enzyme loadings
— Preferably, no post-treatment is required or the post-treatment is able to produce readily hydrolysable cellulosic substrate with the use of minimum chemical and enzyme input
— High recovery of hemicellulose and cellulose derived sugars at high concentrations
— No or low levels of fermentation inhibitory compound produced
— Recovery of the lignin in a reactive form with the potential for generating high value co-products.

1.16 Objectives and overview of the thesis

Based on the above criteria, the thesis focussed on how steam pretreatment can be made more effective on a range of softwood feedstocks by looking at the appropriate pretreatment and post-treatment conditions which result in significant increases in enzymatic hydrolysis and overall sugar recovery (both hemicellulose and cellulose derived) at high concentrations by employing low enzyme and chemical inputs. The specific objectives of the thesis are summarised below.

- Assess whether different softwood species or different trees from the same species could be steam pretreated at similar conditions.
- Evaluate the relative contributions of different substrate factors that limit the enzymatic hydrolysis of steam pretreated softwood across a range of enzyme loadings.
- Assess whether a post-treatment is necessary to obtain efficient enzymatic hydrolysis at low enzyme loadings (5-10 FPU/g glucan).
- Optimize the best combination of pre- and post-treatment conditions to enable maximum recovery of hemicellulose and enhanced hydrolysis of cellulose at low enzyme loadings.
- Investigate the influence of increased substrate loading during steam pretreatment and subsequent enzymatic hydrolysis to increase the final sugar concentration.
- Assess the potential of wood pellets as a global feedstock for an enzyme based biorefinery process and evaluate the potential of applying a steam pretreatment prior to pelletisation.

In the first part of the results section (Chapter 3.1), the robustness of the previously optimised steam pretreatment conditions were assessed by evaluating whether different softwoods respond similarly when processed under the same set of conditions. A range of softwood substrates (belonging to different trees and species) was initially pretreated at severities which had previously been found to give reasonable hemicellulose recovery and adequate cellulose hydrolysis. Steam pretreated softwood substrates were then subsequently hydrolyzed over a range of enzyme loadings to determine whether steam pretreated softwood substrates would be susceptible to enzymatic hydrolysis at low enzyme loadings (Chapter 3.1).

To develop appropriate pre/post-treatment strategies, more information on different recalcitrant factors that limit enzymatic hydrolysis and how their influence varies at different enzyme loadings is required. Therefore, various substrate characteristics which influence the enzymatic hydrolysis were evaluated (Chapter 3.1 & Chapter 3.2). The effect

of lignin in either binding the enzyme or limiting the cellulose accessibility is well recognised, yet previous studies had not tried to quantify the relative importance of the proposed mechanisms of action. By being able to assess the influence of one possible mechanism of lignin inhibition when compared to the other, we hoped to determine the influence of enzyme binding of lignin on the extent of enzyme loading required (Chapter 3.2). We also hoped to determine if hydrolysis yields are dependent on the overall accessibly of the cellulose to the enzymes.

Although post-treatments were shown to enhance enzymatic hydrolysis, their efficiency was evaluated on substrates which had been steam pretreated at a single set of conditions. Therefore, in Chapter 3.3 we investigated the influence that steam pretreatment severity might have on the efficiency of a range of different lignin removing/modifying post-treatments. The objective was to identify conditions of steam pretreatment that allow both enhanced recovery of water soluble hemicellulose and facilitate a post-treatment to enhance the enzymatic hydrolysis and thus increase the overall sugar yield.

As sulfonation has been shown to be a promising post-treatment, we carried out an optimization study (Chapter 3.4) with the expectation that we could reduce the chemical and energy requirement of the process compared to what is generally used in the pulping processes. The pulp and paper industry employs higher chemical loadings, temperatures and reaction times in its sulfonation/sulfite pulping processes as they process raw wood chips with the goal of achieving substantial delignification of the substrate by cleaving the majority of the β-O-4 linkages and subsequently sulfonating and solubilising lignin (Biermann, 1996). However, steam pretreatment has already been shown to result in significant cleavage of β-O-4 linkages, which could result in low molecular weight lignin fragments with free phenolic groups and oxidised side chain structures with the potential to be readily sulfonated at milder conditions (Li et al., 2007; 2008; 2009; Robert et al., 1988; Marchessault et al., 1982). Studies on the lignin model compounds indicated that the compounds with free phenolic groups can be readily sulfonated at different pH ranges and at milder temperatures (Lundquist et al., 2007; Shorygina, 1968; Roberts et al., 1988).

Unlike previous studies, which used high enzyme loadings, post-treatment conditions were evaluated using low enzyme loadings (10 FPU/g glucan).

The past work on pre/post-treatments was largely based on wood chips as the feedstock. However, in addition to wood chips, densified feedstocks, such as wood pellets, are also potential bioconversion feedstocks, particularly for large scale plants, since they are an easily transportable commodity over long distance. Therefore, we next assessed the influence of densification on the robustness of optimised conditions of steam pretreatment, post-treatment and enzymatic hydrolysis (Chapter 3.5). It was expected that the low moisture content and increased density of wood derived pellets would prove problematic in both the pretreatment and downstream processing step of a typical enzyme based biomass-to-sugars process. Subsequently, we assessed whether steam pretreatment could be used as an effective preprocessing step both to make durable wood pellets and to then use these wood pellets as a substrate for enzymatic hydrolysis. Although we expected that the conditions for making durable pellets would make it even harder for subsequent bioconversion.

Despite increasing the sugar yield by employing appropriate pre/post-treatment strategies, the sugars were obtained at a low concentration primarily due to the low substrate loading used both in pretreatment and enzymatic hydrolysis. Although increasing the biomass loading in the steam gun has the potential to enhance the consistency of the slurry, the influence of applying such a strategy on the overall sugar yield at high concentration was not yet investigated. Therefore, we examined whether it is possible to increase the biomass loading in the steam gun to ensure there was no free water available after the pretreatment while still obtaining the high recovery of water soluble hemicellulosic sugars at high concentration (Chapter 3.6). We have used both wood chips and wood pellets for this study. Higher density and lower moisture content of the wood pellets is expected to help increase the substrate loading during pretreatment and enhance the consistency of the slurry. As increased biomass loading in the reactor can also lead to a corresponding increase in the partial pressure of SO_2, which would likely influence the sugar

recovery, pretreatment conditions were subsequently fine-tuned to minimise the SO_2 consumption to further enhance the recovery of water soluble hemicellulose sugars at high concentration. Minimum enzyme requirement for efficient enzymatic hydrolysis of the whole slurry was determined to obtain both hemicellulose and cellulose derived sugars at high yield and concentration.

The goal of the thesis was to ascertain whether steam pretreatment would be effective on several different softwoods providing good hemicellulose and lignin recovery and effective hydrolysis of cellulose using low enzyme loadings. As will be explained in the subsequent chapters, the work looked into many of the technical aspects critical for the success of a softwood-based biorefinery process such as lowering the enzyme input and reducing chemical and energy consumption while achieving an increased conversion of softwood to sugars at high concentration.

2 Methods

2.1 Softwood samples used in the study

To assess the robustness of steam pretreatment in processing a range of different softwoods, six different Douglas-fir (*Pseudotsuga menziesii*) wood logs, four different Lodgepole pine (*Pinus contorta*) wood logs (belonging to different trees) were used. The Douglas-fir logs were collected from six different trees ranging from 22-107 years old. Three were obtained from the interior of British Columbia (DF1, DF2, and DF3) and the rest were obtained from coastal regions of British Columbia (DF4, DF5, and DF6). The wood chips from different bolts belonging to four different Lodgepole pine trees in south-central BC, were mixed and used for comparison. This Lodgepole pine is the same sample that had been used previously by Ewanick et al (2007) (101±20 years). All of the wood logs were debarked, split, chipped and screened to an approximate size of 2×2×0.5 cm^3(Figure 2). The moisture contents of the wood chip samples were in the range of 7 – 11% on a wet weight basis.

To assess the influence of pelletisation on the steam pretreatment and subsequent bioconversion, commercial softwood pellets, laboratory prepared pellets (from steam pretreated Douglas-fir) and wood chips were compared. Commercial softwood pellets were received from the Pinnacle Renewable Energy Group, Prince George, BC. This company is one of the major pellet manufacturers in British Columbia. Pellets were mainly sourced from the saw mill residues of spruce, pine and fir, and had a moisture content of 5±1% (Figure 3). The wood chips to which wood pellets were compared were Douglas-fir wood chips (DF4) air dried to a moisture content of 8±1%. The same wood chips were used to prepare the steam conditioned pellets. The steam conditioned pellets were made from the dried steam pretreated substrate using a piston-cylinder unit (Figure 4). The assembly consisted of three parts: (1) a piston with 6.30 mm diameter and 90 mm length, (2) a cylinder with 6.35 mm inside diameter and 70 mm length (3) a heating tape wrapped around the outer body of the cylinder. An MTI (Measurement Technology Inc., Roswell, GA) model 50K universal

mechanical testing machine was used to force the piston into the cylinder. The open bottom of the cylinder was closed during compression by placing a removable block under the cylinder. During pelletisation, the body of the cylinder was heated and maintained at about 70^{o}C. The cavity in the cylindrical die was filled with approximately 0.9 g of ground biomass using a spatula. The MTI was preset to a downward force of 4000 N. The downward measured displacement speed was set at 6.7 mm/min. The bulk biomass in the cylinder was compressed to the preset maximum force and held for 30s to arrest the spring back effect. To eject pellets from the die, the removable lower block was removed from the underneath of the pellet; the cross head was reactivated to move downward at a speed of 10 mm/min. The piston pushed the pellet from the bottom of the cylinder. The produced pellets were cooled to room temperature and stored inside a sealed glass bottle for further experiments. The moisture content of the steam stabilised pellets was 8±0.4% (Figure 5) (Tooyserkani et al., 2013).

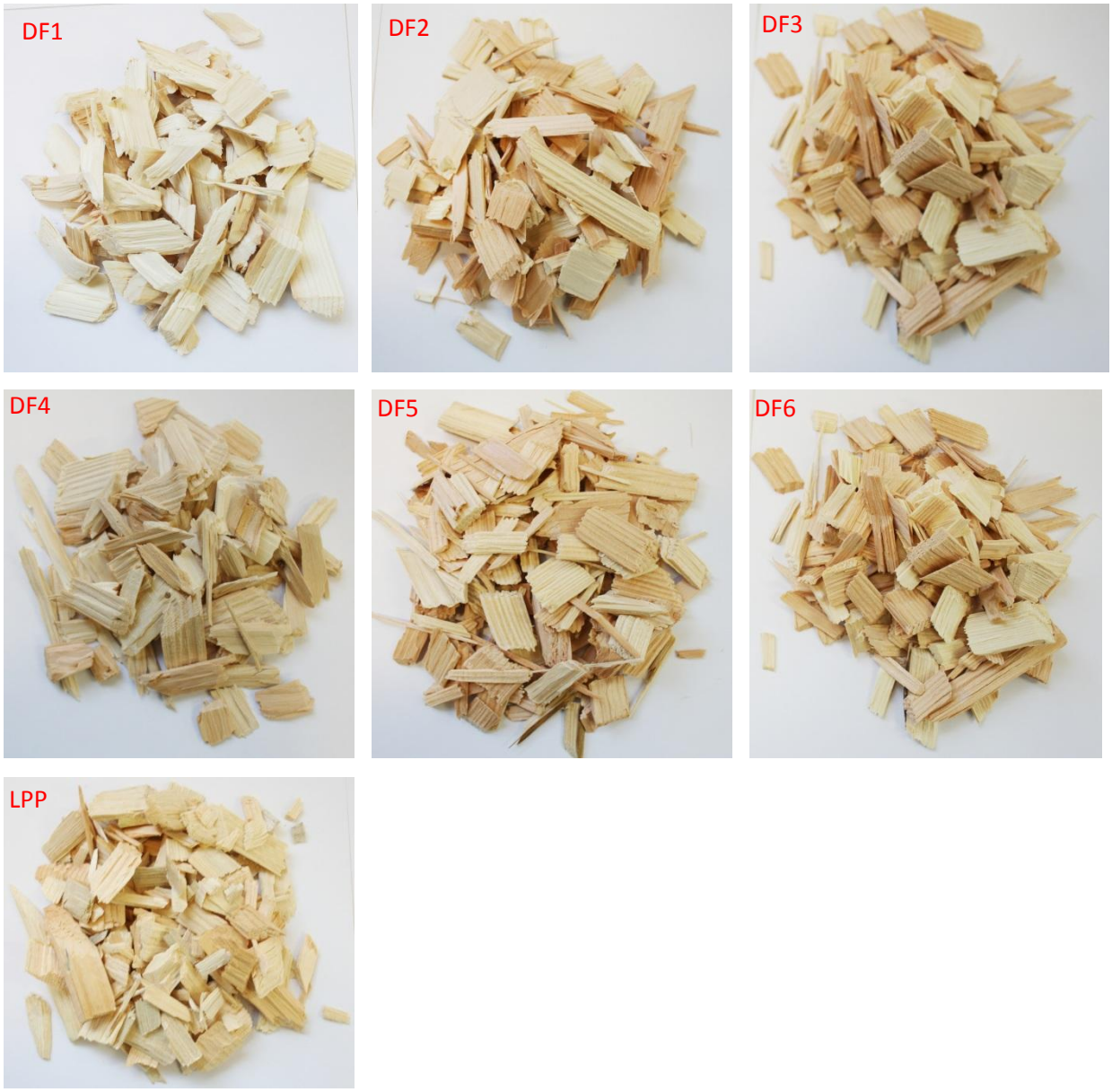


Figure 2 Samples of wood chips used in the study. The numbers DF 1 – 6 refers to the wood chips from six different Douglas-fir trees. LPP refers to wood chips from four different Lodgepole pine trees.

Figure 3 Commercial softwood pellets used in the study

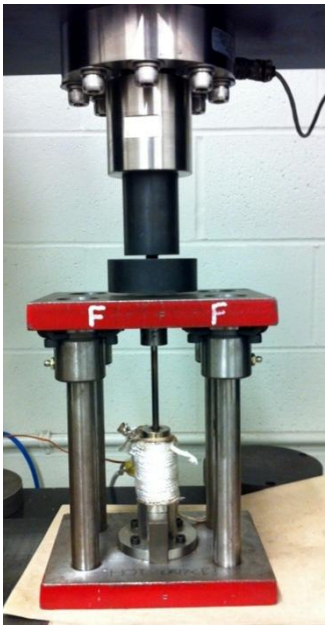


Figure 4 Pelletization set-up consists of a cylinder piston arrangement. The cylinder is wrapped with electric heating tape. The posts guide the movement and alignment of the piston with respect to the die. The entire assembly is placed under MTI for making a single pellet at a time.

Figure 5 Pellets prepared from a) Douglas-fir saw dust and b) steam pretreated Douglas-fir wood chips

2.2 Pretreatment

Prior to steam pretreatment, the wood chips were impregnated by adding a desired amount of SO_2 to sealable plastic bags containing the required amount of substrate (Ewanick et al., 2007). Once the desired amount of SO_2 was added to the bags, the bags were immediately sealed and weighed and left in the fumehood at room temperature for approximately 12 hours. The bags were opened after 12 hours to let the residual SO_2 escape in the fumehood for approximately 30 minutes. SO_2 retention by the biomass samples was determined by weighing the plastic bags again before steam pretreatment was carried out.

Pretreatment with saturated steam was conducted in a 2.6L StakeTech steam gun at the required temperature and reaction time. The steam gun consisted of a vertically mounted and insulated 316L stainless steel pipe closed at both top and bottom by ball valves (7.6 cm and 3.8 cm diameter opening respectively). The reactor was supplied with steam from a high pressure boiler of higher capacity and the steam was introduced through a narrow orifice (1.3 cm controlled by a steam supply valve) located almost in the centre of the reactor. Immediately before use, the gun was preheated to steam temperature by allowing it to stand filled with steam at the boiler pressure. It was then emptied and the substrates were immediately loaded by opening the upper ball valve directly into the preheated empty gun. Biomass loading ranged from 50 g – 400g to achieve different levels of pretreatment consistency depending on the type of biomass pretreated. After the

reaction, the substrates were rapidly discharged into the receiving cyclone (~150 litres capacity) by the fast opening of the bottom ball valve.

After the pretreatment, the total weight of the slurry was determined. The solid and liquid fractions were separated by vacuum filtration using a Buchner funnel. After collecting the large majority of the slurry, the receiving cyclone was rinsed off with minimal amount of water to collect the residual slurry, which was also processed in a similar fashion to obtain a better overall mass closure. The solid fraction of the slurry was washed extensively with water and vacuum filtered to a final moisture content >60%. A small, representative fraction of this sample was subjected to chemical compositional analysis including determination of lignin and carbohydrate contents. The liquid fraction was analyzed for the carbohydrate content (both monomeric and oligomeric) generated during steam pretreatment.

For the experiments using steam pretreatment to make stabilised wood pellets, again a high consistency steam pretreatment was used to keep a high dry matter content for the resulting material. This was done by increasing the amount of biomass aliquots in each batch run to 220g dry weight of the woodchips. Six sets of 220 g wood chips were pretreated and approximately 2/3rd of the resulting wet substrate with ~40% dry matter content was directly dried (a portion was freeze dried and another portion was oven dried) without any water washing and was subsequently used for pelletisation in the lab scale as described in section 2.1. Remaining material was kept for the control experiments to compare the material balance and sugar recovery before and after pelletisation. After pelletizing the steam pretreated substrate, a portion of the pelletised and non-pelletised substrates were disintegrated in water and water soluble and insoluble fractions were separated. After analysing the chemical composition of each of these fractions, the carbohydrate recovery was compared between the pelletised and non-pelletised substrates.

2.3 Post-treatments

2.3.1 Complete delignification and partial delignification of the substrates by sodium chlorite

The complete delignification of the steam pretreated samples was conducted at room temperature according to the procedure of PAPTAC Useful Methods G10, which was the standardized delignification technique for subsequent degree of polymerisation (DP) determination of isolated celluloses (Pulp and Paper Technical Association of Canada (PAPTAC), 1998). Sodium chlorite (0.8 g) and 0.1 mL glacial acetic acid were added per g of steam pretreated substrate with a liquor to wood ratio of 15:1 in a 3 L scale reaction. After an initial gentle mixing, the reaction mixture was kept in the fume hood for 3 hours at room temperature. The slurry was then filtered using a Buchner funnel and the water insoluble fraction was washed extensively with water. This reaction and the washing were carried out multiple times until the substrate was completely delignified. The resulting purely white substrate (with no detectable lignin as characterised by Klason method) was used as a never-dried substrate unless specified.

Substrates with various lignin contents were prepared by using the same conditions of complete delignification, but changing the dosages of the sodium chlorite with respect to the weight of the pulp. Briefly, 100 g of the steam pretreated substrate (on a dry weight basis) was treated in a 5 L Erlenmeyer's flask in a reaction volume of 1.5 L. Sodium chlorite (0.15 – 0.80 g) and 0.1 mL glacial acetic acid were added per g of steam pretreated Douglas-fir on a dry weight basis to obtain different degrees of delignification. The liquor to wood ratio was kept as 15:1. After an initial gentle mixing, the reaction mixture was kept in the fume hood for 3 hours at room temperature. After the reaction, the slurry was filtered using a Buchner funnel and the water insoluble fraction was washed extensively with water (using approximately 5 times the volume of water compared to the original reaction volume).

2.3.2 Alkaline peroxide treatment

The alkaline hydrogen peroxide treatments (AHP) were performed according to Yang et al. (2002). Hydrogen peroxide (Sigma Aldrich 30%) in H_2O was used as a reagent in the reaction. The final hydrogen peroxide loading for the reaction was 1% (wt/wt) of the total reaction mixture and the final pH before starting the treatment was adjusted to 11.5 using 1M NaOH. The reaction was conducted at a final substrate consistency (% wt of dry matter in the total reaction mixture) of 2%. The temperature and reaction time were 80°C and 45 minutes respectively. After the treatment, the resulting slurry was separated into liquid and solid fractions by filtration through a Buchner funnel and the solid fraction was washed extensively with water until the pH of the filtrate dropped to ~7.0.

2.3.3 Cold alkali treatment

The alkali treatments (ALK) were performed according to Pan et al. (2005). The sodium hydroxide was added at a final concentration of 1% (wt/wt) of the total reaction mixture and the process consistency was 2%. The reaction was conducted at room temperature for 72 hours. After the alkali treatment, the resulting slurry was separated into liquid and solid fractions by filtration through a Buchner funnel without the addition of any water during the first stage of filtration. After most of the liquid was removed, the wet solid fraction (~ 70% moisture content) was resuspended in 1 litre of 0.01 N NaOH solution followed by filtration. This step was repeated three times and the resulting solid fraction was finally washed extensively with water until the pH of the filtrate dropped to ~7.0.

2.3.4 Sulfonation

Neutral sulfonation treatment (SULF) was carried out with minor modification of the conditions used earlier (Mooney et al., 1998). Briefly, the treatments were conducted in a Parr high pressure batch reactor (1 litre capacity, T 316 stainless steel, Parr Instrument Company, Illinois) at 10% consistency with the addition of the desired loading (1 – 48%) of Na_2SO_3 based on the dry weight of the substrate. After the addition of the chemicals and the substrate, the final pH was adjusted to pH 7 using 4M sulfuric acid. The reaction was

conducted at desired temperature (120-180°C) for the required amount of time with 20 – 40 minutes allowed for the fibres to come to temperature. The treated slurry was separated into solid and liquid fractions and the resulting wet solid fraction (~70% moisture content) was extensively washed with water.

After quantifying the wet solids yield for the pretreated, delignified and post-treated substrates, the moisture contents of the samples were determined for calculating the yield of dry solids. The moisture content was determined in triplicate by taking a small sample size of the never-dried substrates and oven drying it at 101°C overnight. The moisture content of the pre/post-treated substrates was always >60%. The extent of delignification and carbohydrate dissolution during the post-treatments were calculated based on the yield of water insoluble solids and the composition of the water insoluble component after the process. The carbohydrate content of the post-treatment liquid fraction was not determined as the dissolved carbohydrates could be degraded to a range of different compounds in the post-treatment liquor, especially when using neutral to alkaline medium (Chiang and Sarkanen, 1985).

2.4 Enzymatic hydrolysis

The enzymatic hydrolysis was conducted in duplicates. The solid fractions from the pre/post-treatments were enzymatically hydrolysed in acetate buffer (50 mM, pH 4.8) at 50°C and 150 rpm. The reaction was conducted in a 125 mL capacity screw top Erlenmeyer's flask in 60 mL reaction volume. The enzymes were added based on the filter paper units (FPU) per g glucan present in the substrate. Two antibiotics; tetracycline and cycloheximide (both from Sigma Chemical Co., St. Louis, MO, U.S) was added at concentrations of 40 and 30 µg/mL respectively to prevent the microbial contamination. The reaction mixture containing the substrate, buffer and antibiotics were incubated at 50°C for 30 minutes and the required amount of enzymes was immediately added to the buffered reaction mixture to initiate the hydrolysis reaction. At a given enzyme loading, the enzymatic hydrolysis of most substrates to be compared was conducted simultaneously. Sampling of 500 µL supernatant from the reaction mixture was done at 12, 24, 48 and 72 hours. The enzyme

activity was stopped during sampling by incubating the aliquots of the reaction mixture on a hot plate at 100°C for 10 minutes and the samples were subsequently stored at -20°C until sugar analysis was performed. The great majority of the enzymatic hydrolysis experiments were conducted at a 2% substrate consistency. However, for the experiments designed to increase the sugar concentration, a dry matter consistency of 25% was used for the whole slurry from the high solids steam pretreatment. Prior to conducting enzymatic hydrolysis, a small fraction of the substrate was diluted to make 2% slurry to determine the amount of alkali required to neutralise a given amount of dry matter. The diluted slurry was subsequently titrated against 1M NaOH with the intermittent measurement of the pH. Subsequently, the wet weight corresponding to a 25% dry matter was directly weighed into the 125 mL capacity screw top Erlenmeyer's flask followed by the addition of appropriate amount of water followed by alkali. After a thorough mixing in a vortex mixer, the appropriate amount of buffer was added. The whole mixture was subsequently stirred in the incubator for 2 hours prior to the addition of the enzymes. After the enzymes were added, the mixture was hydrolysed for 72 hours. Sampling was done only at the end of hydrolysis reaction and the sugar concentration was determined by HPLC as described in section 2.7.

Two commercial enzyme preparations were used for enzymatic hydrolysis. A cellulase preparation (Celluclast 1.5L) obtained from the fungus, *Trichoderma reesei*, supplied commercially by Novozymes North America Incorporated (Franklinton, NC, U.S.) was used which was supplemented with additional β-glucosidase enzyme (Novozym-188®) originating from *Aspergillus niger* to achieve an IU (international units, in this case cellobiose units - CBU) to FPU ratio of 2:1. The filter paper activity of the cellulase preparation was 60.4 FPU/mL and the cellobiase activity of the cellulase was 0.2 CBU/mL while the cellobiase activity of the β-glucosidase was 360 CBU/mL.

The measurement of total cellulase activity of the Celluclast preparation was determined by the filter paper assay as reported by Ghose (1987). The detection of glycosidic bond cleavage by this method involves the parallel and identical treatment of

assay mixtures, blanks, and controls and glucose standards. The substrate was a 0.05 g Whatman No.1 filter paper strip (1.0 × 6.0 cm). Initially 0.5 mL of enzyme diluted in an acetate buffer (0.05 M, pH 4.8) (at least two dilutions) was added to the test tubes containing strips of filter paper in 1 mL of sodium acetate buffer and incubated for 1 hour at 50°C with continuous shaking at 150 rpm. After 1 hour, 3 mL of dinitrosalicylic acid reagent (DNS) was added to stop the reaction and the test tubes were incubated in a boiling water bath for 5 min. Formation of reducing sugars were determined spectrophotometrically at 540 nm, and corrected for enzyme and reagent blanks. The final activity was expressed in filter paper units (FPU), where 1 FPU is equivalent to the enzyme concentration required to form one μmol of glucose from the filter paper per minute.

The activity of the betaglucosidase preparation was determined colourimetrically by using 15 mM cellobiose as described by Ghose (1987). 1 mL aliquot of diluted enzyme (at least two dilutions) was added to 1 mL of 15 mM cellobiose (Sigma), prepared in sodium acetate buffer (50 mM, pH 4.8). The mixture was vortexed vigorously and incubated at 50°C for 30 min. The reaction was stopped by immersing the tubes in the boiling water bath for 5 minutes. The enzyme activity was monitored using glucose oxidase assay and 1 unit activity is equivalent to the enzyme concentration required to convert 1 μmol of substrate per minute.

The hydrolysis yield was calculated as the percentage of the glucose content in the feedstock at the start of hydrolysis that was recovered as monomeric glucose during enzymatic hydrolysis. The glucan content was determined by carrying out a chemical compositional analysis as described above. A cellulose to glucose conversion factor of 1.1 was applied to accommodate the water molecules incorporated for every cellobiose unit hydrolysed (Allen et al., 2001).

2.5 Measurement of protein adsorption

For the protein adsorption experiments for the selected steam pretreated samples in Chapter 3.1, the enzyme loading used was 20 FPU cellulase and 40 CBU betaglucosidase/g

glucan. To determine the amount of protein adsorbed to the residual lignin present in the post-treated samples in chapter 3.3, the enzyme loading was increased to 40 FPU cellulase/g and 80 CBU betaglucosidase/g glucan to ensure a near complete enzymatic hydrolysis of the cellulose. Subsequently, the protein content of the enzyme stock preparations and the enzyme present in the supernatant after a near complete cellulose hydrolysis was measured by using a modified ninhydrin assay (Starcher, 2001). Bovine Serum Albumin (Sigma-Aldrich Corporation, St. Louis, MO, USA) was used as a protein standard by preparing a series of concentrations in water ranging from 0-750 µg protein/mL. One hundred µL of 1N HCl was added to 40 µL of each sample and standards and they were incubated in 1 mL Eppendorf tubes at 105°C for 16 hours. The tubes were cooled for 10 minutes and 200 µL of 2% ninhydrin reagent was added. After mixing in a vortex mixer, the solution was heated at 100°C for 20 minutes with subsequent cooling for 10 minutes at room temperature. One millilitre of 50% ethanol was added to each tube, mixed and absorbance values were read at 570 nm using a micro-titre plate.

2.6 Isolation of lignin

Enzymatic residual lignin of steam pretreated softwood was isolated according to methods reported previously (Berlin et al., 2006). Cellulase (Celluclast 1.5L) and beta-glucosidase (Novozymes 188) were added at a loading of 200 FPU/g glucan and 200 CBU/g glucan to the pretreated Douglas-fir substrates in order to achieve near complete enzymatic hydrolysis of cellulose and obtain lignin in as pure a form as possible. The enzymatic hydrolysis was conducted at the conditions described in section 2.5, at a 2% (wt/wt) consistency. After hydrolysis, the lignin residue was recovered by centrifugation at 8000×g for 30 min and re-suspended in distilled water to a final consistency of 2% (w/w). The suspension was sonicated at 40 kHz for 60 min in a TP 680DH ultrasonic water bath (Elma Hans Schmidbauer GmbH & Co., Singen, Germany). The washing was done three times and the lignin residue was incubated overnight at 37 °C in 50 mM phosphate buffer, pH 7, containing 1 U/mL Pronase (Sigma Chemical Company, USA). After protease treatment the residual lignin was heated at 100°C for 15 min to deactivate the protease, followed by

washing four times with acetate buffer, repeating the washing steps with 1 M sodium chloride solution and thereafter with distilled water. The lignin preparations were freeze dried, ground manually, screened through a 180 mesh screen (Figure 6) and stored in sealed vials at room temperature in a desiccator. The lignin was analyzed for lignin and carbohydrate content as described in section 2.7. The nitrogen content of the residual lignin was determined using a LECO CN-2000 elemental analyzer (Leco Inc., MI, USA). The Klason lignin, carbohydrate, and nitrogen contents of the isolated lignin sample were 93.3 ± 0.6, 2.7 ± 0.8, and 0.6 ± 0.1, respectively.

Figure 6 Isolated lignin used in the work

2.7 Analytical methods

All compositional analyses were done in triplicate. Acetone soluble extractives were estimated using Tappi T204 om-88 with the following modifications. Briefly, 10 g of air-dried sample was extracted for 8 h with acetone with 6 cycles/h. The acetone in the round-bottomed flask was then evaporated in the fume hood and then dried in the oven at 101°C overnight and weighed to determine the weight of extractives present in the sample flasks.

The raw biomass, pretreated and post-treated substrates were subjected to a detailed chemical compositional analysis including acid insoluble lignin and carbohydrates

using the Tappi- T-22 om-88 as previously described (TAPPI, 1994). Briefly, the samples were first oven dried at 101°C and milled using a Wiley mill to <40 mesh particle size and again dried in the oven prior to analysis. 0.2 – 0.3 g of the sample was weighed (with actual weight recorded) in Klason cups and 3 mL of 72% sulfuric acid was slowly added and the resulting paste was stirred every 10 minutes to reduce the viscosity and ensure the dissolution of the entire carbohydrates. After 2 hours, the reaction mixture was diluted with nonopure water to a final acid concentration of 3% in a septa bottle, which was sealed and autoclaved at 121°C for 1 hour. After the autoclave, the samples were cooled to room temperature and filtered through a pre-weighed sintered glass filter to separate the residual solid (lignin) and the liquid fraction containing the carbohydrate components. Monomeric sugars present in the hydrolysate were measured on a Dionex (Sunnyvale, CA) HPLC as described below. The hydrolysate from this analysis was also analyzed for soluble lignin by reading the absorbance at 205 nm (Dence, 1992). The residual lignin in the glass filter was washed extensively to remove any remaining acids by passing ~250 mL of nonopure water under vacuum. The weight of the lignin was determined gravimetrically by drying the washed lignin at 101°C in a hot air oven overnight.

The concentrations of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) were determined by HPLC analysis. The HPLC system (Dionex DX-500, Dionex Corp., Sunnyvale, CA, U.S.) was equipped with an ion exchange Carbopac PA-1 column (4 × 250 mm) equilibrated with 0.25 M NaOH and eluted with nanopure water at a flow rate of 1 mL/min (Dionex Corp.), an ED40 electrochemical detector (gold electrode), an AD20 absorbance detector and an autosampler (Dionex Corp., Sunnyvale, CA, U.S.). Sodium hydroxide (0.2 M) was added post-column (for detection) at a flow rate of 0.6 mL/min. Prior to injection, samples were filtered through 0.45 µm HV filters (Millipore, MA, U.S.) and a volume of 20 µL was loaded. Analytical-grade standards: L-arabinose, D-galactose, D-glucose, D-xylose and D-mannose (Sigma) were used to quantify the concentration of sugars in the sample. In addition, L-fucose (Sigma) was used as an internal standard for the normalisation of the HPLC response.

In order to quantify the fraction of oligomeric sugars present in the pretreatment liquid, post-hydrolysis were performed according to Shevchenko et al. (2000). Duplicate samples containing 27 mL of the water soluble fraction were post-hydrolysed after adding concentrated sulphuric acid to achieve a final concentration of 3% acid. The post-hydrolysis was performed by heating the solution at 121°C for 1 hour in an autoclave. A batch of sugar standards was also autoclaved under the same conditions to correct for the hydrolysis loss factor. The monomeric sugars were quantified by HPLC as described above and the fraction of oligomeric sugars was calculated by subtracting the amount of monomeric sugars present in the pretreatment liquid from the total amount of monomeric sugars present after the post hydrolysis of the same sample.

2.8 Substrate characterization methods

2.8.1 Simon`s staining

Simon's staining was performed according to the modified procedure by Chandra et al. (2008). Briefly, direct orange (DO-Pontamine Fast Orange 6RN) and direct blue (DB-Pontamine Fast Sky Blue 6BX) were obtained from Pylam Products Co. Inc. (Garden City, NY, U.S.). For each type of substrate, a wet weight equivalent to approximately 100 mg oven dry weight of the samples were weighed into each of six 15 mL polypropylene centrifuge tubes (Corning Inc. NY, U.S.). 1.0 mL of phosphate-buffered saline (PBS) solution at pH 6 was added followed by the addition of direct orange and direct blue solution (10 mg/mL) in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 mL) to the six tubes resulting in each set of tubes containing a 1:1 mixture of DO and DB dyes at increasing concentrations. Nanopure water was added to make up the final volume to 10.0 mL. The tubes were then incubated overnight at 70°C in an orbital shaker at 200 rpm. After the incubation, the tubes were centrifuged at 5,000 rpm for 5 min, and a sample of the supernatant was placed in a cuvette and the absorbance read on a Cary 50 UV-Vis spectrophotometer at 624 and 455 nm. The amount of dye adsorbed onto the fiber was determined using the difference in the concentration of the initially added dye and the concentration of the dye left in the supernatant after the incubation. The extinction

coefficients were calculated by preparing standard curves of blue and orange dye and measuring the slope of their absorbances at 455 and 624 nm respectively. For determining the cellulose accessibility of the partially and fully delignified substrates mentioned in Chapter 3.2 and Chapter 3.3, a more refined method, which was developed afterward, was employed, which uses the adsorption of purified high molecular weight orange dye fractions (Mw>100KDa) (Chandra et al., 2012).

2.8.2 Fibre quality analysis

Fiber quality analysis (FQA), used to measure the length of the fibers of the pretreated substrates, was performed on an Optest Hi-Resolution bench top fiber quality analyzer (LDA02, OpTest Equipment, Inc., Hawkesbury, ON, Canada) according to the procedure by Robertson et al (1999). The settings on the FQA were adjusted to measure particles down to 0.05 mm. Briefly, a dilute suspension of fibers was transported with a fiber frequency of 25–40 events per second through a sheath flow cell where the fibers are oriented and positioned. The images of the fibers were detected by a built-in CCD camera, and the lengths of the fibers were measured by circular polarized light. The weighted average fibre lengths (Lw) were determined by using the equation $L_w = \frac{\Sigma_i n_i {l_i}^2}{\Sigma_i n_i l_i}$ where $n_i$is the number of fibers in the length class l_i .

2.8.3 Water retention value

The water retention value (WRV) was measured following the Scandinavian test method SCAN-C 62:00 in five replicates. The pulp pad making unit consisted of a 25 mm inner diameter plastic tube with a height of 60 mm with a nylon mesh (pore size ~ 100 µM) at the bottom of the tube. Approximately 0.5 g of the substrate was disintegrated in 50 mL of water and soaked overnight. The resulting suspension was carefully filtered through the unit using suction so as to result in approximately 10% dry matter in the pulp. Subsequently, the whole test pad holding unit was placed in a 50 mL Falcon tube (30 mm internal diameter and 100 mm height) with an open ended plastic tube supporting the unit at the bottom, thus leaving space for water accumulation at the bottom of the unit. The whole unit was

subsequently centrifuged at 3000 × *g* for 15 min in a laboratory centrifuge (Thermo Fisher Scientific, Sorvall ST 16R). The wet centrifuged sample pad was weighed and oven dried at 105 °C overnight and weighed again. The WRV is the percentage of retained water (weight change of the substrate before and after drying) of the dried substrate, i.e. $\mathrm{WRV}\ (\%)=\frac{\mathrm{W_{wet}-W_{dried}}}{\mathrm{W_{dried}}}\times 100$ where w_{wet} and w_{dried} are the wet and dry weights of the substrate, respectively.

2.8.4 Acid groups

Bulk acid groups in the pretreated and post-treated substrates were determined by conductometric titration according to standard methods reported elsewhere (Katz et al., 1984). In brief, wet substrates containing 1 g dry matter were added to 300 mL of 0.10 N HCl and stirred for 1 hour. The pulp was then filtered and washed with 2000 mL of deionised water. The washed pulp was then treated with 0.001 M NaCl (250 mL) and 0.10 N HCl solutions (1.50 mL), stirred and conductometrically titrated with 0.05 N NaOH. The total acid groups were extrapolated from plots of the titration data (volume of NaOH vs. conductivity). Initially, conductometric titration curves indicate a rapid decrease in conductivity which represents the neutralization of strong acid groups. The first equivalence point (intersection of the graph) represents weaker carboxylic acids beginning to dissociate, and the second equivalence point (intersection) represents increases in conductivity due to excess NaOH.

2.8.5 Cellulose accessibility of pure cellulosic substrates

Accessibility of the cellulose for each substrate was measured with the Family 2a cellulose-specific carbohydrate binding module (CBM2a) of xylanase 10A from *Cellulomonas fimi*. Adsorption of CBM2a to the sample was used as a quantitative indicator of the total amount of available cellulose surface area (McLean et al., 2000). Samples containing 450 µg CBM2a and 10 mg substrate (on a dry weight basis) were made up to a final volume of 1.5 mL with 50 mM Tris-HCl at pH 8.0. Samples were incubated at room temperature for 30 min followed by centrifugation at 18,000 ×g for 10 min using Sorvall micro-centrifuge (Sorvall, Asheville, NC). The concentration of CBM2a in the supernatant was determined by

measuring the absorbance of the solution at 280 nm (by using a Cary 50 UV-Vis spectrophotometer – Varian Inc. Mississauga, ON) using an extinction coefficient of 27625 $M^{-1} \cdot cm^{-1}$ (McLean et al., 2002).

3 Results and discussion

3.1 Influence of feedstock variations on the robustness of steam pretreatment to achieve good enzymatic hydrolysis and sugar yields

3.1.1 Background

As discussed earlier, previous work had identified optimum steam pretreatment conditions for several softwood species including Radiata pine, Douglas-fir, Lodgepole pine, Norway spruce and Loblolly pine (*Pinus taeda*) (Boussaid et al., 2000; Ewanick et al., 2007; Huang and Ragauskas, 2012; Monavari et al., 2009; Stenberg et al., 1998). However, most of the work had used wood chips derived from one or two log bolts from a single tree, which was repeatedly used as a substrate for several of the subsequent studies (Boussaid et al., 2000; Cullis and Mansfield, 2010; Pan et al., 2005b; Wu et al., 1999; Yang et al., 2002). Thus it was possible that the samples used in the previous work may not be truly representative of that particular species. Moreover, accurate assessments about the variation across the feedstocks using published data should be used with caution since the studies were conducted in different labs with different types of equipment/process conditions and feedstocks with different physical properties. One effective means of assessing the robustness of previously optimised steam pretreatment conditions is to process several different softwood samples under a standard set of conditions and determine the resulting sugar recovery.

A review of the previous work indicated that there seems to be a mixed impression on how effective steam pretreatment is for processing a range of different softwoods (Boussaid et al., 2000; Clark and Mackie, 1987; Cullis et al., 2004; Ewanick, 2006; Ewanick et al., 2007; Mabee et al., 2006; Maekawa, 1992; Monavari et al., 2009; Pan et al., 2004; 2005; Schwald et al., 1989; Stenberg et al., 2000; Wu et al., 1999). Early studies evaluated steam pretreatment conditions for softwoods merely based on the amenability of the pretreated solid fractions to enzymatic hydrolysis and ignored the amount of sugars recovered in the

water soluble component (Babcock, 1932; Saddler et al., 1982; 1983). Realising the importance of recovering both hemicellulose and cellulose derived sugars; subsequent work had evaluated the pretreatment conditions based on the overall sugar yield (total monomeric sugars released during pretreatment and enzymatic hydrolysis). Related work on Norway spruce (*Picea abies*) and Radiata pine (*Pinus Radiata*) had indicated that it was possible to obtain >70% overall sugar recovery from both these substrates when pretreated at the steam pretreatment conditions log Ro=3.6-3.9 and subsequent enzymatic hydrolysis at ~40 FPU/g glucan (Clark and Mackie, 1987). Despite assessing the overall sugar yield released from steam pretreatment and enzymatic hydrolysis, the fermentability of the pretreatment liquid was not assessed. Later work at the University of Lund (Stenberg et al., 1998) had shown that a mixture of fresh Norway spruce and Scots pine (*Picea abies* and *Pinus sylvestris*) could be pretreated at a level of severity (210°C, 5.5 minutes and 3.5% SO_2 ; Log Ro=4.0) which resulted in 72% sugar yield with a high fermentability. As these authors used fresh pieces of wood (green) the moisture content (~50%) of the feedstock seems to have shifted the optimum pretreatment conditions towards a higher severity, resulting in a high overall sugar/ethanol yield.

In other work Douglas-fir, (*Pseudotsuga menziesii*) a dominant softwood species in the Pacific Northwest, was studied extensively for its potential as a biomass source for bioconversion (Boussaid et al., 2000; Cullis et al., 2004; Pan et al., 2004; 2005; Wu et al., 1999; Yang et al., 2002). This particular work had indicated that Douglas-fir was relatively more recalcitrant as the overall sugar yield was lower despite high enzyme loadings employed. The compromised optimum conditions were 195°C, 4.5 minutes and 4.5% SO_2 (Log Ro=3.5), which resulted in 57 – 59% overall sugar yield while using an enzyme loading of 20 FPU/g glucan. Higher enzyme loadings and/or subsequent delignification steps were applied to achieve more than 80% sugar yield from Douglas-fir substrates indicating that Douglas-fir is relatively more recalcitrant to enzymatic hydrolysis compared to other softwood species.

Subsequent work compared the recalcitrance of a dominant North West America softwood (Lodgepole pine) with a dominant Scandinavian softwood (Norway spruce). This work identified pretreatment conditions, 200^{o}C, 4% SO_2, 5 min, Log Ro = 3.6, as the best compromise that could result in reasonable hydrolysis yields (60 – 70%) for both species, albeit at relatively higher enzyme loadings (≥20 FPU/g of glucan), without the need for a subsequent delignification step (Ewanick, 2006; Ewanick et al., 2007; Monavari et al., 2009). The conditions were slightly more severe than those previously used for Douglas-fir. However this approach resulted in a good recovery of hemicellulose and the resulting pretreatment liquid could be readily fermented. The work also showed that both healthy Lodgepole pine and mountain pine beetle killed Lodgepole pine could be pretreated under a similar set of conditions to result in comparable sugar yields. As these pretreatment conditions were different than the conditions that had been used previously for the earlier Douglas-fir work, we first wanted to determine if the previously observed recalcitrance of the Douglas-fir substrate might have been due to some unique features of that particular wood sample or differences in the conditions applied. It should also be noted that the Douglas-fir wood chips utilized in the previous studies (Cullis et al., 2004) were obtained from a log bolt derived from a single tree, which was more than 150 years old, and the log bolt had been stored in an air-dried condition for many, several years. Thus, it was possible that recalcitrance observed with this particular sample may not have been representative of different aged or stored Douglas-fir wood chip samples and that fresh samples may in fact show optimized steam pretreatment conditions that were similar to those observed with other softwood species. Moreover, the variations in the yield could have been due to the different pretreatment conditions/equipment and physical characteristics of samples used in each study.

The variability among the typical softwoods such as spruce, pine or fir is relatively less apparent compared to that of hardwoods. Anatomically, while hardwoods have more differentiation of cells with specialised structure and function, softwoods have a relatively uniform cell structure and mostly dominated (>90% by weight) by a single cell type called longitudinal tracheids (Sjostrom, 1993). Since these similarities lead to comparable

properties for most softwoods, both pulp and paper, and lumber industries generally group most softwoods together (For example, SPF, Hem-fir) while grading the quality of their wood products. It should be noted that the prevalent softwoods such as spruce, pine, fir are generally subjected to the same set of pulping conditions to obtain pulps with fairly similar properties (Lanouette et al., 1998; Dalpke et al., 2007). However, hardwoods such as aspen may respond substantially different to a chemical/mechanical pulping compared to some other hardwoods such as beech or birch (Santos et al., 2011; Collins et al., 1990; Pinto et al., 2005; Bose et al., 2009). Therefore, the similarities among typical softwoods are hoped to enable us to use the same set of optimised steam pretreatment conditions for most softwoods to result in almost similar sugar yield.

The work described here compared Douglas-fir wood chip samples from six different trees with a representative Lodgepole pine sample, to assess their responsiveness to a single pretreatment condition (200°C, 5 minutes, 4% SO_2**)** which had previously been shown to be effective for Lodgepole pine and Norway spruce in terms of both sugar recovery (hemicellulose and cellulose) and subsequent ease of cellulose hydrolysis at both high and low enzyme loadings. As mentioned previously, the majority of the studies on steam pretreated softwood have evaluated the hydrolysis of the substrate at a relatively high enzyme loading (20 – 80 FPU/g of glucan) (Boussaid et al., 2000; Ewanick et al., 2007; Pan et al., 2004; 2005; Wu et al., 1999) which will likely not be economically feasible in a commercial bioconversion scheme (Gregg and Saddler, 1996; Merino and Cherry, 2007; Donghai and Junshe, 2007; Sun and Cheng, 2002). Consequently, we assessed the substrate characteristics, which would lead to the high enzyme requirements and investigated whether the substrates could be generated from steam pretreated softwoods that are effectively hydrolysed at reduced enzyme loadings.

3.1.2 Results and discussion

3.1.2.1 Chemical composition of the raw softwood chips

It was apparent that the carbohydrate and lignin contents were quite similar for the softwood samples (Table 8). It should be noted that, primarily because of the relatively high hexose content of the softwood hemicellulose, 60-65% weight of the original softwood was comprised of hexose sugars (Table 8) which is significantly higher when compared to typical hardwood and agricultural residues. Typically, the hexose sugar content of these latter substrates is in the range of 34 – 41 (Guo et al., 2009; Hamelinck et al., 2005) and 48 – 52% respectively (Bura et al., 2009; Hamelinck et al., 2005). This higher hexose content of softwood hemicelluloses is beneficial since the hexose sugars liberated during pretreatment and hydrolysis may be easier to utilize directly for fermentation (Matsushika et al., 2009) compared to the pentose sugars that are typically found in the hemicelluloses of hardwoods and agricultural derived biomass.

Table 8 Composition of softwood chips before steam pretreatment (% dry weight)

Softwood	Arabinan	Galactan	Glucan	Xylan	Mannan	Acid Insoluble Lignin	Acid Soluble Lignin	Extractives
DF1*	1.6 (0.1)**	2.8 (0.1)	44.7 (0.3)	4.9 (0.2)	12.9 (0.0)	28.0 (0.2)	0.4 (0.1)	2.2 (0.2)
DF2	1.3 (0.1)	3.1 (0.0)	46.2 (0.3)	3.4 (0.1)	13.6 (0.2)	28.5 (0.4)	0.4 (0.1)	1.3 (0.4)
DF3	1.1 (0.0)	3.9 (0.0)	46.6 (0.6)	3.2 (0.1)	13.7 (0.2)	28.7 (0.7)	0.4 (0.0)	1.5 (0.6)
DF4	1.2 (0.0)	2.2 (0.0)	47.3 (0.4)	4.4 (0.1)	11.7 (0.1)	29.8 (0.8)	0.5 (0.1)	1.1 (0.4)
DF5	0.9 (0.1)	2.3 (0.0)	48.9 (0.3)	3.6 (0.0)	12.6 (0.1)	29.4 (0.2)	0.4 (0.2)	1.2 (0.1)
DF6	0.9 (0.0)	3.3 (0.0)	46.9 (0.4)	2.8 (0.0)	12.9 (0.2)	30.2 (0.2)	0.5 (0.1)	2.4 (0.5)
LPP	1.7 (0.2)	3.2 (0.2)	45.4 (0.1)	6.2 (0.2)	11.5 (0.3)	28.5 (0.6)	0.5 (0.1)	3.4 (0.5)

*The number 1 – 6 refers to the biomass samples from six different Douglas-fir trees, LPP-Lodgepole pine.
** Numbers in the brackets represents standard deviations from the mean (n=3)

3.1.2.2 Sugar recovery after steam pretreatment

After steam pretreatment at 200°C, 5 minutes and 4% SO_2, it was apparent that all of the softwood samples responded in a fairly similar fashion, resulting in overall combined sugar recoveries of 54 – 61 g per 100g of the original softwood (Table 9). Recovery of hexose and pentose sugars correspond to 83 – 92% and 62 – 82% of the original hexose and pentose sugars respectively present in the starting feedstock (Table 9). The sugar recovery data is comparable to the results reported previously with Lodgepole pine and Norway spruce when pretreated at the same conditions (Ewanick et al., 2007; Monavari et al., 2009). Although the pentose sugar recovery is slightly lower at these treatment conditions, pentoses represent less than 10% of the total sugars present in the initial substrates (Table 8). The majority of the hemicellulose present in each of the softwood substrates were recovered (Table 9). This hemicellulose removal and recovery in the water soluble fraction was likely beneficial to the subsequent enzymatic hydrolysis of the cellulose rich, water insoluble fraction, as the removal of hemicellulose has been shown to influence the ease of hydrolysis of pretreated solid substrates (Bura et al., 2009; Grethlein et al., 1984; Wong et al., 1988). Complete hemicellulose dissolution was also reported in previous studies on the

steam pretreatment of Douglas-fir at a severity of log Ro = 3.76 (Wu et al., 1999). However, the severity applied in this study was slightly lower (log Ro = 3.64).

In all of the substrates, greater than 95% of the initial glucan was recovered out of which 12 – 25% of the original glucan was recovered in the water soluble fraction (Table 9). This was similar to the work reported by (Clark et al., 1989) on steam pretreated Radiata pine (*Pinus radiata*) where 26% of the total glucan was recovered in the liquid fraction when pretreated at a severity of log Ro = 3.9. More recent studies (Monavari et al., 2009) indicated that steam pretreatment of spruce lead to a glucan dissolution of nearly 15% under the same pretreatment conditions as used in the present study. The dissolution of glucan into the liquid stream during pretreatment varied between the substrates. Consequently, there were slight differences in the lignin and glucan content in the resulting solid fraction, which varied from 40 – 47% and from 51-57% respectively (Table 10). Considering the general similarities among the pretreated samples in their chemical composition, we next wanted to determine their susceptibilities to enzymatic hydrolysis at both relatively low and high enzyme loadings.

Table 9 Recovery of sugars after the steam pretreatment of different softwoods expressed as g/100g of starting softwood (Steam pretreatment condition: 200°C, 4% SO_2 for 5 minutes; log Ro=3.64).

Softwood	Hemicellulose recovered*			Glucose recovered			Total hexose sugars	Total pentose sugars
	Liquid fraction**	Solid fraction	Total	Liquid fraction****	Solid fraction	Total		
DF1	14.5 (0.3)***	1.6 (0.2)	16.1 (0.3)	5.3 (0.1)	39.7 (0.7)	45.0 (0.7)	55.8 (0.2)	5.3 (0.2)
DF2	10.3 (0.1)	1.0 (0.1)	11.3 (0.2)	10.7 (0.0)	33.8 (0.6)	44.5 (0.6)	52.6 (0.1)	3.2 (0.1)
DF3	13.2 (0.4)	1.1 (0.1)	14.3 (0.4)	10.9 (0.4)	34.1 (0.3)	45.0 (0.5)	56.2 (0.2)	3.2 (0.2)
DF4	12.0 (0.3)	1.2 (0.1)	13.2 (0.3)	10.1 (0.1)	36.2 (0.8)	46.3 (0.8)	55.2 (0.2)	4.2 (0.2)
DF5	9.4 (0.2)	1.0 (0.2)	10.4 (0.3)	8.4 (0.1)	38.4 (1.3)	46.8 (1.3)	54.0 (0.1)	3.2 (0.1)
DF6	8.8 (0.1)	0.9 (0.1)	9.7 (0.2)	11.8 (0.0)	32.9 (0.4)	44.7 (0.4)	52.1 (0.1)	2.3 (0.1)
LPP	15.1 (0.4)	1.1 (0.1)	16.2 (0.4)	7.8 (0.2)	36.6 (0.4)	44.4 (0.5)	54.5 (0.2)	6.1 (0.2)

*Hemicellulose refer to the sum of arabinan, galactan, xylan and mannan
**The proportion of monomeric sugars were 94-103%
*** Numbers in the brackets represents standard deviations from the mean (n=2 for the analysis of water soluble fractions and n=3 for the compositional analysis (Klason) of insoluble fractions)
****The proportion of monomeric sugars were 89 - 96%

Table 10 Chemical composition of the water insoluble component of steam pretreated softwood (% dry weight) (Steam pretreated at 200°C, 4% SO_2 for 5 minutes; log Ro=3.64).

Softwood	Solids yield (%)	Glucan	Xylan	Mannan	Acid insoluble Lignin
DF1	69.7 (1.1)	57.0 (0.3)*	0.9 (0.1)	1.4 (0.2)	40.1 (0.3)
DF2	63.1 (0.9)	53.5 (0.6)	0.5 (0.0)	1.1 (0.1)	42.9 (0.9)
DF3	66.6 (0.3)	51.2 (0.4)	0.5 (0.2)	1.2 (0.1)	44.7 (0.5)
DF4	67.7 (0.7)	53.4 (1.1)	0.6 (0.1)	1.1 (0.1)	44.2 (0.4)
DF5	69.0 (1.0)	55.6 (1.7)	0.4 (0.1)	1.1 (0.3)	43.0 (1.1)
DF6	65.1 (0.7)	50.6 (0.1)	0.4 (0.2)	1.0 (0.1)	47.0 (0.3)
LPP	69.9 (0.6)	52.4 (0.4)	0.6 (0.1)	1.0 (0.1)	45.9 (1.1)
*Numbers in the bracket represent standard deviations from the mean (n=3).					

3.1.2.3 Enzymatic hydrolysis of the steam pretreated substrates at high enzyme loadings

At relatively high enzyme loadings (20 FPU/g of glucan), the cellulose to glucose conversion for all the pretreated softwoods was in the range of 60 - 72% after 72 hours (Figure 7). The hydrolysis yield of 67% for Lodgepole pine is close to the previously reported results for the same original wood samples (Ewanick et al., 2007). Within the narrow range of hydrolysis yields, the enzymatic hydrolysis of DF6 and DF1 substrates resulted in the highest and lowest yields of the pretreated samples respectively. It should be noted that the percent hexose and pentose recovery data during pretreatment (Table 9) show that DF6 and DF1 exhibited the lowest and highest total sugar recovery of the pretreated samples respectively (Table 9). This observation was similar with the trend of glucan dissolution during pretreatment (Table 9). Therefore, it is possible that the different Douglas-fir samples (DF 1-6) may have varied slightly in the level of severity they experienced, resulting in some samples undergoing somewhat increased amounts of sugar degradation and a small increase in enzymatic hydrolysis yields. In previous work on the steam pretreatment of Lodgepole pine, samples with greater amounts of lignin tended to have been treated more severely (resulting in solubilisation of some of the cellulose component) and consequently, increased hydrolysis yields of the cellulosic rich water insoluble fraction

(Chandra et al., 2009). This was similar to what was observed with sample DF6 in this study which contained the highest amount of lignin (Table 10) and the highest enzymatic hydrolysis yield.

When the sugars released from steam pretreatment and enzymatic hydrolysis were added, 64 – 70% of the original carbohydrates (42 – 47 g per 100 g of the starting substrate) present in the starting samples were found to be released as soluble sugars (Figure 8). This was in the range of previously reported values for different softwoods at their optimum conditions (Cullis et al., 2004; Ewanick et al., 2007; Monavari et al., 2009; Stenberg et al., 1998). Therefore, the results indicate that Douglas-fir, Lodgepole pine and Norway spruce could be processed at the same set of conditions to result in almost similar sugar yield.

Although the wood samples in this study behaved in a similar fashion during the pretreatment, it has been suggested (Boussaid et al., 2000; Brownell et al., 1986; Cullis et al., 2004; Ramos et al., 1992a; 1992b; Ramos, 2003) that slight differences in wood samples might, potentially, lead to variations in response to steam pretreatment and subsequent enzymatic hydrolysis. For example, the amount of heartwood (which is higher in older trees) present in the wood chips, has been shown to play a role in the effectiveness of pretreatment of Douglas-fir wood on subsequent enzymatic hydrolysis (Boussaid et al., 2000). Other factors may include latewood to early wood ratio, the presence of reaction wood, the ratio of juvenile to mature wood etc. (Boussaid et al., 2000; Sjostrom, 1993; DeMartini and Wyman, 2010). The variations in the ease of hydrolysis obtained with different fractions within the same wood sample have been reported previously (Boussaid et al., 1999). It was shown that the susceptibility to enzymatic hydrolysis of the sapwood fractions of Douglas-fir was nearly 30% higher than that of corresponding heartwood fractions when the substrates were pretreated at a severity of Log Ro=3.45.

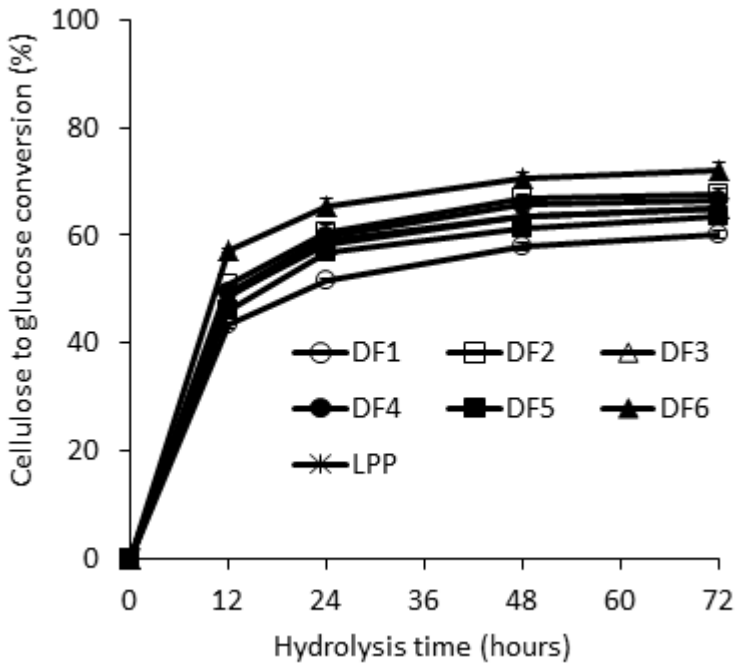


Figure 7 Enzymatic hydrolysis of the water insoluble cellulosic component of different softwood samples steam pretreated at the same set of conditions (Log Ro=3.64). Enzyme loading: 20 FPU cellulase and 40 CBU beta glucosidase per g of glucan. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis with subsequent HPLC analysis of the sugars in two replicates for each sample).

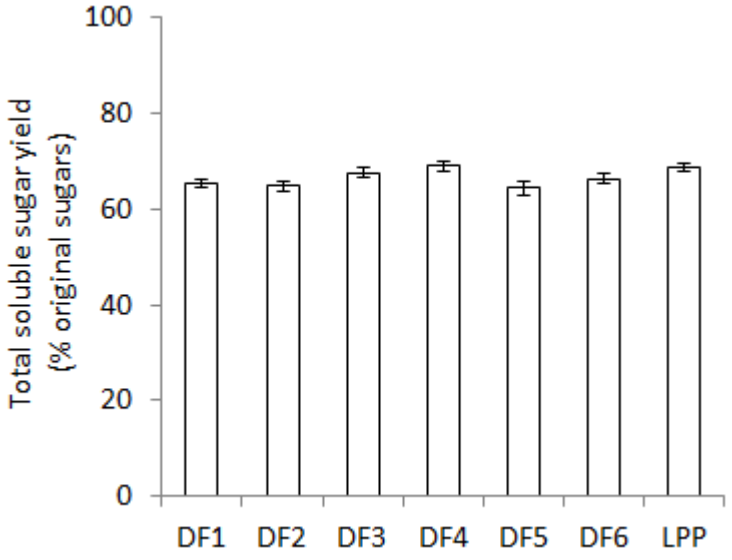


Figure 8 Total soluble sugar yield after steam pretreatment and subsequent enzymatic hydrolysis of a range of different softwood samples. Enzyme loading: 20 FPU cellulase and 40 CBU beta-glucosidase per g of glucan. Error bars represent standard

deviations from the mean (n=2 for the analysis of water soluble fractions and n=3 for the compositional analysis of water insoluble fractions).

3.1.2.4 Enzymatic hydrolysis of the steam pretreated substrates at low enzyme loadings

Previous work on the enzymatic hydrolysis of pretreated LPP and Douglas-fir samples usually employed enzyme loadings in the range of 20-80 FPU/g glucan (Cullis et al., 2004; Ewanick, 2006; Ewanick et al., 2007; Pan et al., 2005; Wu et al., 1999). However, it is now recognized that such high enzyme loadings would be cost prohibitive in a commercial bioconversion process (Humbird et al., 2011; Kazi et al., 2010; Merino and Cherry, 2007). Since DF 1-6 and LPP wood chips all resulted in hydrolysis yields in the 60-72% range, similar to previous work on LPP and Norway spruce using the 20FPU/g glucan loading (Ewanick, 2006; Ewanick et al., 2007), it was also of interest to determine whether lowering the enzyme loading would adversely affect the yields.

When an enzyme loading of 5 FPU/g glucan was used, this severely lowered the hydrolysis yields of all the steam pretreated softwoods (Figure 9) after 72 hours of hydrolysis. Allowing the hydrolysis reaction to continue until 120 hours did not significantly enhance the hydrolysis yield further. It is worth noting that the same hydrolysis yield profile for the pretreated samples was obtained at these lower enzyme loadings, with the DF6 sample again showing the highest cellulose conversion yield.

The results presented here confirmed the previous findings that steam pretreatment was relatively less effective in processing softwoods compared to typical agricultural residues or hardwood biomass. More severe pretreatment conditions and/or a subsequent delignification step were required before good sugar yields could be obtained with softwood substrates. (Brownell and Saddler, 1984; Bura et al., 2002; 2009; DeBari et al., 2007; Grous et al., 1986; Ohgren et al., 2007a; 2007b; Ramos et al., 1992a; Schwald et al., 1988). To try to elucidate some of the key substrate characteristics, which may influence hydrolysis yields, we next performed a preliminary investigation of their fiber length analysis, Simon's stain accessibility and protein adsorption during enzymatic hydrolysis.

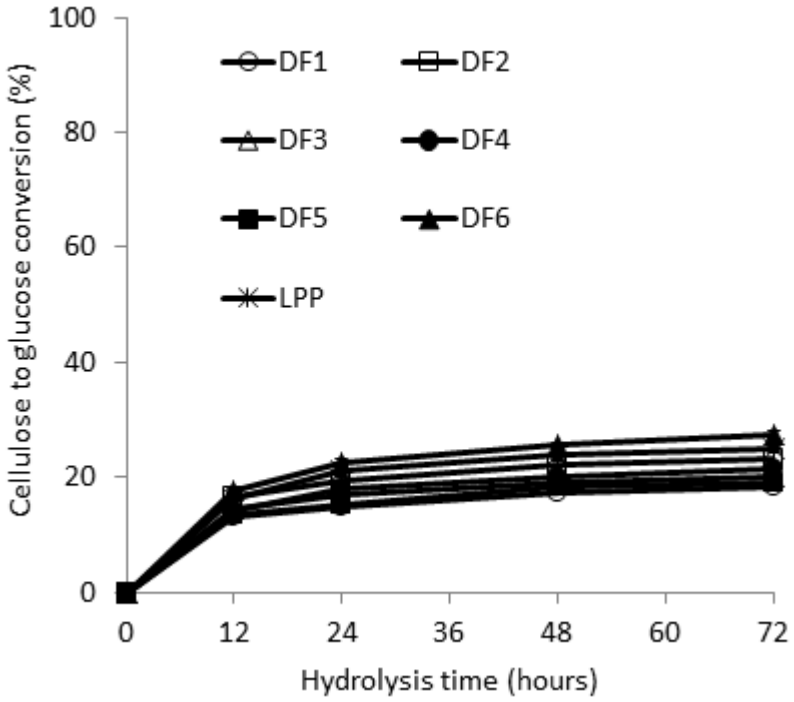


Figure 9 Enzymatic hydrolysis of the water insoluble cellulosic component of different softwood samples steam pretreated at the same set of conditions (Log Ro=3.64). Enzyme loading: 5 FPU cellulase and 10 CBU beta glucosidase per g of glucan. Error bars represent deviations from the mean (n=2 for enzymatic hydrolysis with subsequent HPLC analysis of the sugars in two replicates for each sample).

3.1.2.5 Characterization of the steam pretreated substrates

Accessibility to cellulose has been shown to be essential for an effective cellulose hydrolysis (Arantes and Saddler, 2010). As the steam pretreatment resulted in a near complete dissolution of hemicellulose and a substrate containing exclusively cellulose and lignin, it is highly likely that lignin limits the cellulose accessibility of steam pretreated softwoods thus demanding high enzyme loadings. Although lignin has been shown to restrict the cellulose accessibility, the results presented above indicated that enzymatic hydrolysis does not necessarily correlate with the lignin content of the substrates. Therefore, it appears that accessibility to cellulose depends not only on the lignin content, but also many other factors such as the extent of lignin redistribution, the type and location of lignin and also on the properties within the cellulose itself. Therefore, we next used two

different methods; Simon's staining and fibre length analysis, to indirectly assess the degree of cellulose accessibility in the above substrates and its impact on enzymatic hydrolysis.

Previously it was suggested (Chandra et al., 2009; 2008; Esteghlalian et al., 2001) that the Simons' Stain (SS) technique, which utilitizes the competitive adsorption of smaller and larger sized dye molecules to cellulose, could provide an effective indication of the enzymatic accessibility to the cellulose present in the substrate. The more recent work (Chandra et al., 2009) has shown that, when comparing substrates pretreated by same method, the ratio of the larger orange dye molecules to the smaller blue dye molecules adsorbed by the substrate was an effective indicator of the susceptibility of the substrate to cellulolytic hydrolysis. In the work reported here, the ratios of the orange to blue dye were in the range of 1.9:1 – 2.7:1 indicating the close similarity among the substrates in terms of enzyme accessibility to cellulose (Figure 10). Although the SS values were within this narrow range, the values indicated that DF6 was the most accessible to the orange dye and consequently was the most amenable to subsequent enzymatic hydrolysis.

Similarly, fibre length was measured since it has been shown that the particle size range of pretreated substrates plays a role in influencing a given substrate's accessibility to cellulose and consequent ease of enzymatic hydrolysis (Mooney et al., 1999). Similar to the results obtained with the SS measurements, the fiber length (L_w) of the steam pretreated substrates fell within a narrow range of 0.4 – 0.6 mm (Figure 10). When comparing the average fiber lengths, once again DF6 exhibited the lowest fiber length average, which could possibly indicate that DF6 experienced a slightly greater severity during steam pretreatment, as was suggested earlier. Previous studies have shown that increasing the steam pretreatment severity of LPP wood and Douglas-fir also resulted in smaller particles (Boussaid et al., 2000; Chandra et al., 2009). As was shown in previous studies (Mooney et al., 1999), smaller fibres have an increased surface area and consequently can result in increased cellulose accessibility as was reflected in the Simon`s stain adsorption study (Figure 10). Despite an only 12% variation in hydrolysis yields between the pretreated

substrates, it is interesting to note that both the Simon`s stain adsorption and fiber length values seem to indicate the ease of hydrolysis of the steam pretreated softwood samples.

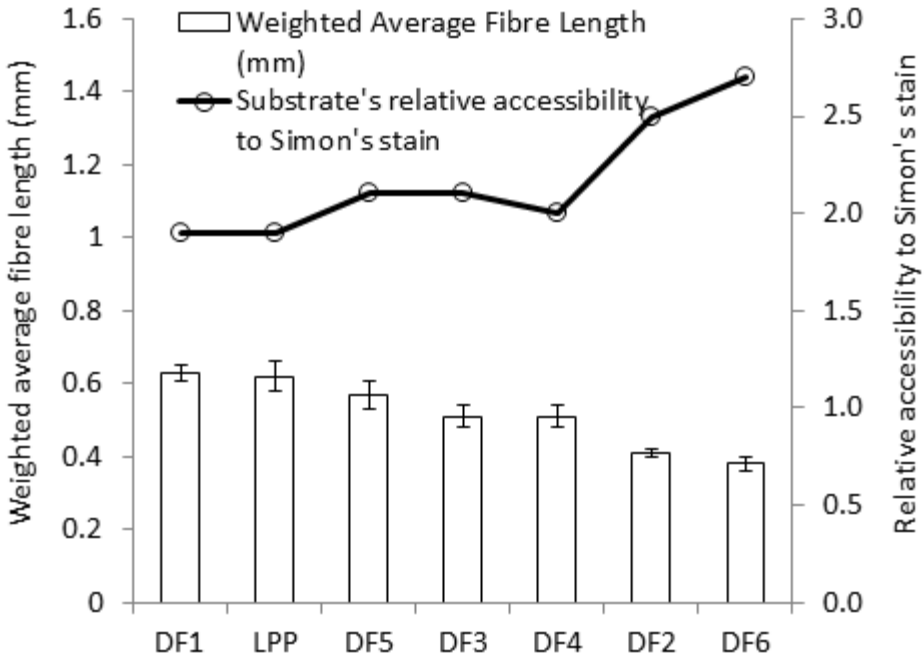


Figure 10 Average Fibre length (L_w) (n=2) and Simon's stain's accessibility of different steam pretreated softwoods processed at the same set of conditions. A_{maxOD} is the maximum adsorption of direct orange dye and A_{maxBD} is the maximum adsorption of direct blue dye during Simon's staining test. The ratio indicates the extent to which the larger orange dye molecules replace the smaller blue dye molecules in the pretreated substrate.

Adsorption of cellulase enzymes to the substrate is an essential step to achieve the enzymatic hydrolysis of the cellulosic component and is generally directly related to the available surface area of cellulose. The incomplete enzymatic hydrolysis of steam pretreated Douglas-fir, even after employing high enzyme loadings and prolonged periods of enzymatic hydrolysis, motivated us to investigate the protein adsorption profiles during hydrolysis (Figure 11). Three steam pretreated samples were selected for the study, which gave the lowest, highest and intermediate hydrolysis yields (DF1, DF6 and LPP). All of these substrates gave a similar trend in protein adsorption profiles. Surprisingly, at the end of 72 hours, 53 – 58% of the original protein was still adsorbed to the water insoluble component

despite the lack of any further improvement in hydrolysis. Despite a substantial decrease in the hydrolysis rate after the preliminary hours of hydrolysis, the fraction of the total protein adsorbed to the solid fraction increased by ~15% in the final hours compared to the beginning of the hydrolysis. This increase in protein adsorption despite the lack of any further improvement in hydrolysis yield indicated that protein might not have been adsorbed to the cellulose. It appears that once the readily accessible cellulose is hydrolysed by the cellulase, the enzymes are adsorbed mostly to the exposed lignin component without getting access to the remaining cellulose present in the substrate. Thus, to further enhance hydrolysis the accessibility to the cellulose need to be enhanced.

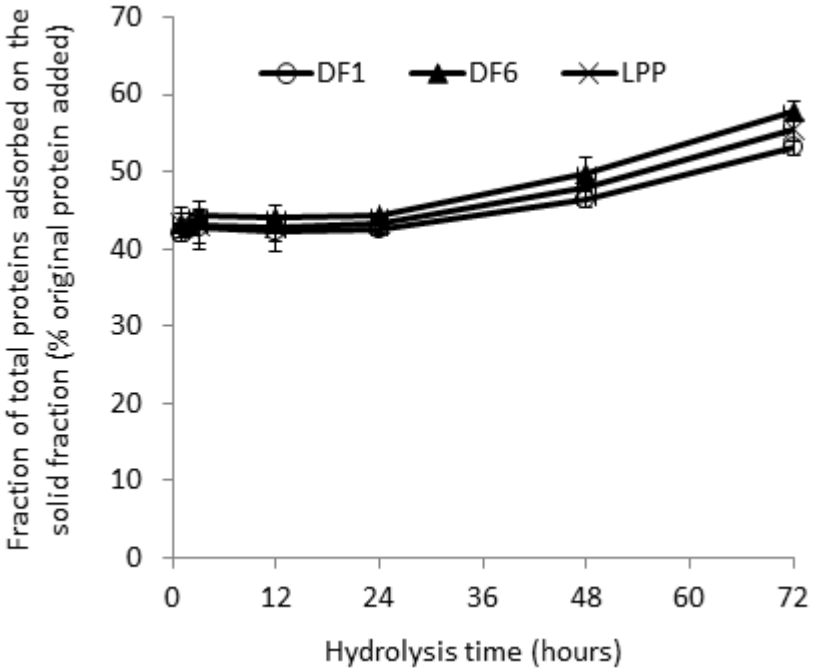


Figure 11 Enzyme adsorption profile during the enzymatic hydrolysis of steam pretreated softwoods at an enzyme loading of 20 FPU cellulase (Celluclast) and 40 CBU beta glucosidase (Novozymes 188) per g of glucan. Error bars represent deviations from the mean (n=2 for enzymatic hydrolysis with subsequent Ninhydrin analysis in two replicates for each sample).

3.1.2.6 Effect of lignin removal on enzymatic hydrolysis of steam pretreated softwoods

Although it was apparent that each of the softwoods samples' responses to the applied steam pretreatment condition was similar, it was clear that, regardless of whether the enzyme loading was at 20 FPU/g or 5 FPU/g of glucan, the complete hydrolysis of the cellulose component could not be achieved. Continued increase in the adsorption of the enzymes without any significant improvement in hydrolysis indicated that enzymes might be stuck to the substrate lignin without getting access to the remaining cellulose present in the substrate. The two potential factors which might limit cellulose accessibility are the residual lignin and the properties of cellulose itself such as degree of polymerization and crystallinity (Mooney et al., 1998, 1999; Ramos et al., 1993; Puri, 1984; Fan et al., 1981). Therefore, we next investigated the selective influence of lignin by trying to remove it from the substrate without altering the cellulose properties. We then monitored the hydrolysis yield of the resulting pure cellulose.

Although previous work with steam pretreated Douglas-fir had applied various delignification post-treatments in order to achieve adequate hydrolysis yields (Cullis et al., 2004; Pan et al., 2004, 2005; Yang et al., 2002) the efficiencies of these post-treatments were typically assessed at relatively high enzyme loadings. Therefore, to assess the potential effect of lignin on the hydrolysis yields, especially at reduced enzyme loadings, selected Douglas-fir samples were selectively delignified and subsequently hydrolyzed and compared at enzyme loadings of 5 FPU/g and 20 FPU/g glucan. This procedure for delignification was the standardized room temperature chlorite method typically used for the determination of the degree of polymerization of isolated celluloses (PAPTAC, 1998). Although other workers had successfully used hot chlorite treatment on substrates with a lignin content as high as 30% (Hubbell and Ragauskas, 2010; Varnai et al., 2010), a hot temperature treatment was avoided to minimize any possible changes/detrimental effects on the cellulose.

As our work had indicated that sample DF4 resulted in both an intermediate level of hydrolysis and sugar recovery (Table 9 and Figure 12), this sample was used to assess the effect that delignification treatment might have on subsequent enzymatic hydrolysis. The room temperature sodium chlorite method was used as a mild delignification method since it is used conventionally for the preparation of holocellulose for subsequent measurements of cellulose viscosity (PAPTAC, 1998) and had also been used previously to remove lignin to assess its effect on enzymatic hydrolysis (Mooney et al., 1998). After chlorite treatment, each pretreated sample contained no detectable acid insoluble lignin. It was evident that the delignification treatment resulted in a significant increase in the hydrolysis yield at 5 FPU/g glucan loading. Near complete hydrolysis was obtained after 72h which was a 4.5 fold increase compared to that obtained with the sample without the delignification treatment (Figure 12). At a loading of 20 FPU/g glucan, complete hydrolysis was obtained after 12 hours or less (Figure 12). These results seem to support previous studies which indicated that the lignin present in steam pretreated softwood such as Douglas-fir was detrimental to hydrolysis by either restricting swelling of the substrate acting as a barrier to enzyme accessibility or by unproductively binding to cellulase enzymes thereby reducing the amount of enzymes available for further hydrolysis of the substrate (Berlin et al., 2006). Similar to the results shown here previous work showed that by removing approximately 90% of the substrate lignin from a steam pretreated Douglas-fir by alkaline hydrogen peroxide this resulted in the rapid and complete hydrolysis of the substrate cellulose at an enzyme loading of 20 FPU/g of glucan (Yang et al., 2002). This current work showed that the hydrolysis yields could be significantly improved by delignification, especially when relatively low enzyme loadings of 5 FPU/g glucan were used.

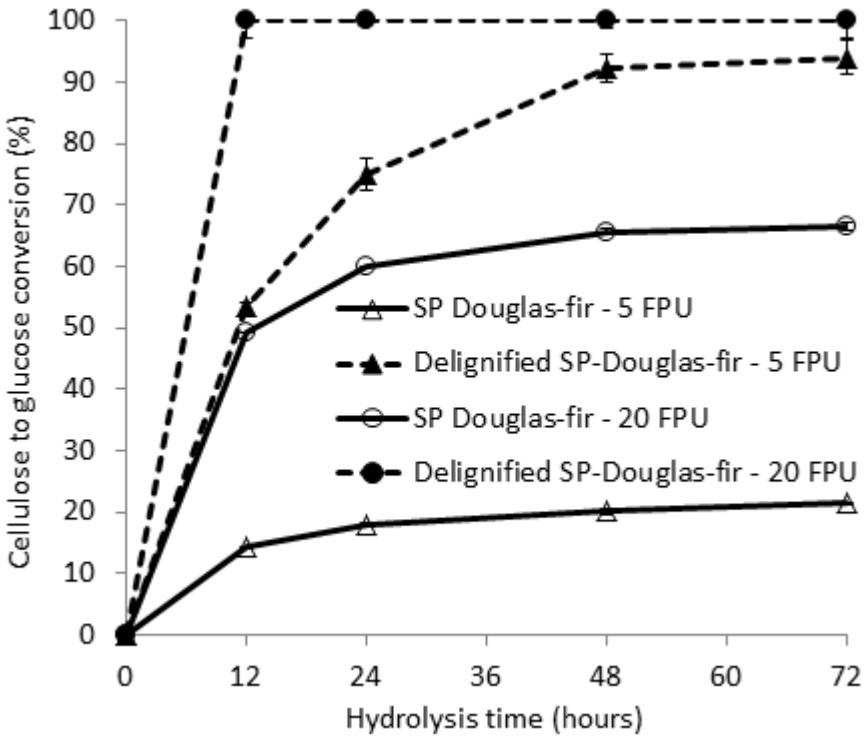


Figure 12 Enzymatic hydrolysis of steam pretreated Douglas-fir and the corresponding delignified substrates at both high and low enzyme loadings. Steam pretreatment conditions were 200°C, 4% SO_2 for 5 minutes. (Log Ro=3.64). The complete delignification was done at room temperature using acidified sodium chlorite solution. Error bars represent deviations from the mean. (n=2 for enzymatic hydrolysis with subsequent HPLC analysis of the sugars in two replicates for each sample).

3.1.3 Conclusions

It was apparent that the same steam pretreatment conditions could be used for a range of the Douglas-fir and a representative Lodgepole pine wood chip samples with similar material balance/chemical composition, sugar recoveries and hydrolysis yields obtained over a narrow range. The enzyme hydrolysis yields of the water insoluble, cellulose rich fractions obtained from the pretreated Douglas-fir substrates were comparable to those obtained in previous studies with Lodgepole pine and Norway spruce, at the higher enzyme loading (20 FPU/g glucan). These results suggest that the previously observed recalcitrance of Douglas-fir could be influenced by factors such as age, storage etc. of the

starting material. Preliminary substrate characterization measurements indicate that techniques such as Simons stain and fiber quality analysis gave good indication with regard to the potential ease of hydrolysis within a narrow range of hydrolysis yields and substrate chemical composition. It was apparent that hydrolysis yields were reduced significantly as the enzyme loading was lowered to 5 FPU/g glucan. A significant fraction of the protein still adsorbed on the substrate without any significant improvement in hydrolysis after 24 hours which indicated that the enzymes are unproductively bound to lignin without getting access to the majority of the cellulose. Subsequently, significant improvements in hydrolysis yields at both low and high enzyme loadings could be achieved upon delignification of the substrate. This finding indicated that, although steam pretreatment can provide good recovery of most of the hemicellulose and cellulose derived sugars, with a concomitant increase in the ease of enzymatic hydrolysis of the water insoluble, cellulosic fraction, a delignification post-treatment of this fraction will be required to achieve high hydrolysis yields when lower enzyme loadings are used.

3.2 Relative contributions of different inhibitory roles of lignin during enzymatic hydrolysis of steam pretreated softwood

3.2.1 Background

Although reasonable hydrolysis yields could be obtained from steam pretreated softwood at high enzyme loadings, it is clear that the economic feasibility of any lignocellulose based ethanol process will require the use of low protein loadings (Gregg and Saddler, 1996; Merino and Cherry, 2007; Donghai and Junshe, 2007). When low enzyme loadings (5 FPU/g glucan) were used to hydrolyze a range of softwoods, poor hydrolysis yields were obtained. To further enhance enzymatic hydrolysis, it is important to increase our understanding of the prominent factors behind the reduced hydrolysis yields at low enzyme loadings. Steam pretreatment of softwoods at severities of Log $R_0 \geq 3.3$ resulted in a complete hemicellulose dissolution resulting in a water insoluble component almost exclusively consisting of cellulose and lignin (Varnai et al., 2010; Ewanick et al., 2007; Boussaid et al., 2000). The work described earlier in Chapter 3.1 showed that either selective or complete delignification could reduce the enzyme requirement by 4-5 folds to attain reasonable hydrolysis yields. This finding implied that the lignin plays a significant role in restricting enzymatic hydrolysis at 5 FPU/g glucan. In addition to delignification, lignin modification processes, which can increase the hydrophilicity of lignin and promoted fibre swelling by incorporating acid groups onto the lignin, can also lead to improvements in hydrolysis yields (Mooney et al., 1998). Lignin modification treatments can also result in a lignin with less unproductive binding sites (Nakagame et al., 2010).

It is known that there are various ways by which lignin limits the accessibility of cellulose in a steam pretreated softwood substrate. Lignin restricts the swelling of cellulose and therefore limits the accessible surface area of the cellulose available to the enzymes (Eriksson et al., 1991; Jeoh et al., 2007; Mooney et al., 1997, 1998; Nakatani et al., 2008; Wong et al., 1988; Zhang et al., 2006). Among the various substrate characteristics, which influence enzymatic hydrolysis, limited cellulose accessibility was suggested to be the major factor that restricts the effectiveness of cellulose hydrolysis (Arantes and Saddler, 2010;

Jeoh et al., 2007; Mooney et al., 1997; Wong et al., 1988; Zhang et al., 2006). Swelling of cellulose, resulting in an increase in the accessible surface area, is essential to achieving efficient cellulose hydrolysis (Arantes and Saddler, 2010). A decrease in molecular weight of lignin during pretreatment, together with its hydrophobicity and the surface tension effects of water, result in the deposition of small spherical lignin droplets on the fiber surface, likely limiting access of the enzymes to the cellulose (Donaldson et al., 1988; Donohoe et al., 2008).

In addition, lignin also reduces the effectiveness of enzymatic hydrolysis by unproductively adsorbing cellulases, thereby reducing the availability of the enzymes (Berlin et al., 2005, 2006; Nakagame et al., 2010; Tu et al., 2007). In the last chapter, we observed that almost 60% of the enzyme protein is still adsorbed to the solid fraction after 72 hours of enzymatic hydrolysis without any further improvement in hydrolysis yield. This observation means the enzymes are not productively adsorbed to the cellulose and likely bound to lignin without getting access to the majority of the remaining cellulose. Although, it is recognized that lignin restricts enzymatic hydrolysis by both limiting cellulose accessibility and unproductively binding cellulases, the relative contribution of these two main mechanisms of lignin inhibition on a typical steam pretreated softwood substrate has not yet been quantified.

Although the role of lignin in restricting the cellulose hydrolysis in steam pretreated softwood is well recognised, there have been ambiguities regarding the primary mechanism of lignin inhibition. For example, it has been shown that enhancing the steam pretreatment severity increased the hydrophobicity of lignin and enhanced affinity to enzymes leading to a higher extent of unproductive binding (Nakagame et al., 2011). However, increase in pretreatment severity, in fact increases the enzymatic hydrolysis despite an increase in the lignin content and the greater extent of unproductive binding to the enzymes (Ewanick et al., 2007; Nakagame et al., 2011). Also in Chapter 3.1, we observed greater hydrolysis yields with the substrates with higher lignin contents. Therefore, it is apparent that the relative contribution of the different roles of lignin and the mechanism by which lignin primarily

restrict enzymatic hydrolysis is not yet fully understood. Thus, further clarification of the role that lignin plays in pre- and post-treated substrates should help optimize pre/post-treatment strategies to enhance the effectiveness of enzymatic hydrolysis so as to obtain high sugar yields at low protein loadings.

To evaluate the relative impact that lignin has on limiting cellulose accessibility vs. unproductive binding of the enzymes, steam pretreated Douglas-fir was selectively delignified and the minimum enzyme dosage required to achieve a near complete conversion of the resulting pure cellulose (SPDF-CD) was determined. While this complete delignification is assumed to eliminate the entire influence of lignin, to separately assess how much the unproductive binding effects of lignin contribute to the generally poor hydrolysis yields obtained with high lignin containing steam pretreated softwoods two strategies were used. One was to use Bovine Serum Albumin (BSA) as a surrogate for cellulases to block the unproductive binding sites prior to the addition of cellulase and assess the improvement in hydrolysis (Kawamoto et al., 1992; Palonen et al., 2004; Pan et al., 2005b; Yang and Wyman, 2006). Secondly, lignin isolated from steam pretreated softwood (Enzymatic residual lignin) was separately added to the completely delignified substrate to assess the reduction in hydrolysis due to lignin when the cellulose is highly accessible.

3.2.2 Results and discussion

3.2.2.1 Influence of enzyme loading on the overall inhibitory effect of lignin

Earlier study (Chapter 3.1) indicated that the hydrolysis yields of steam pretreated softwoods are dependent on the enzyme loading employed. Therefore, it appears that an increased amount of enzymes can partially overcome the inhibitory effects of lignin. However, the detrimental effect of lignin still existed even at reasonably high enzyme loadings such as 20 FPU/g glucan, as incomplete cellulose hydrolysis was obtained. As it is known that lignin can restrict enzymatic hydrolysis by both binding cellulases and restricting access to the cellulose, very high protein loading and prolonged hydrolysis times were

initially employed (realizing this would not be economically viable) to see if it is possible to completely hydrolyze a recalcitrant substrate such as steam pretreated Douglas-fir. Previous work has tended to look at the influence of a lower range of enzyme loadings (20 – 120 FPU/g glucan) (Pan et al., 2004; Tengborg et al., 2001) as the primary goal was to assess the efficiency of enzymatic hydrolysis using a reasonable enzyme loadings. However, in the work described here, it was important to ensure that complete hydrolysis of the cellulose could be achieved, with enough protein and time available. It was apparent that, at very high enzyme loadings (200 FPU/g glucan) and given enough time (72 h), steam pretreated Douglas-fir (SPDF) could be completely hydrolyzed (Figure 13). However, there was a dramatic difference at low (less than 40 FPU/g glucan) enzyme loadings, with slow and incomplete hydrolysis obtained even after 3 days of incubation. Only 16% and 33% of the cellulose present in the SPDF was hydrolyzed after 72 h at enzyme loadings of 5 FPU and 10 FPU/g glucan, respectively (Figure 13).

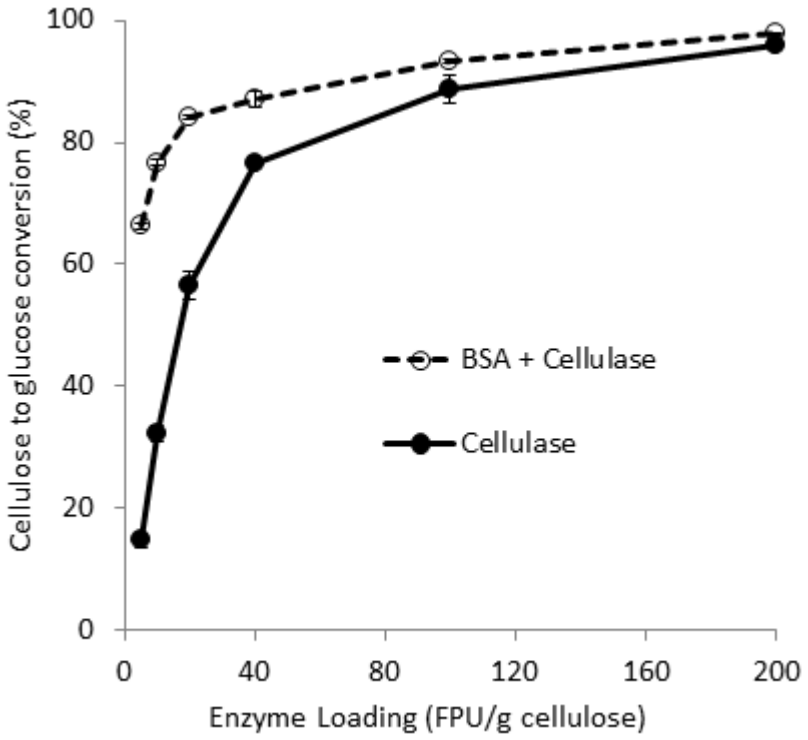


Figure 13 Effect of increasing cellulase loading and BSA pre-incubation on the extent of enzymatic hydrolysis of steam pretreated Douglas-fir (SPDF) after 72 h. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis).

3.2.2.2 Relative contributions of lignin towards enzyme binding and limited cellulose accessibility

To try to differentiate the role that lignin might play in either enzyme binding or restricting enzyme access to the cellulose, Bovine Serum Albumen (BSA) was added to the substrate prior to enzymatic hydrolysis, with the hope that cellulase binding to lignin would be surrogated by prior saturation of the lignin by the binding of the BSA. Initially, the amount of BSA required to saturate the lignin binding sites was optimized by monitoring the improvement in enzymatic hydrolysis of steam pretreated Douglas-fir at different loadings of BSA. The prior addition of 2.5 – 5.0 mg/mL (123 – 245 mg/g of the substrate) of BSA was found to be the optimum range of BSA concentration that could achieve the highest improvement in enzymatic hydrolysis (Figure 14). It was clear that at higher enzyme loadings prior addition of the saturated amount of BSA had only a marginal beneficial effect after 72 h hydrolysis (Figure 13). However, at low enzyme loadings (5 FPU/g glucan), prior addition of the BSA to the non-delignified steam pretreated Douglas-fir (SPDF) increased both the rate and extent of hydrolysis substantially, with about 66% of the original cellulose hydrolyzed after 72 h as compared to only 16% without prior BSA addition (Figure 13 and Figure 15). When the BSA was added to the steam pretreated Douglas-fir which had been delignified (SPDF-CD), no direct beneficial effect was observed (Figure 15). This result indicated that pre-incubation of the higher lignin containing substrate with BSA essentially enhanced the availability of cellulases, which would have otherwise been unproductively adsorbed to lignin. Although lignin binding of the enzymes played an important role in restricting enzymatic hydrolysis, it was also clear that lignin played an important role in limiting the accessibility of the enzymes to the cellulose, as indicated by incomplete hydrolysis of the BSA-treated SPDF substrate at 5 FPU/g glucan enzyme loading compared to the near complete hydrolysis of the completely delignified SPDF-CD substrate (Figure 15). These results indicated that the two main roles of lignin, in both directly binding with the enzymes and restricting the swelling of the substrate, were influential.

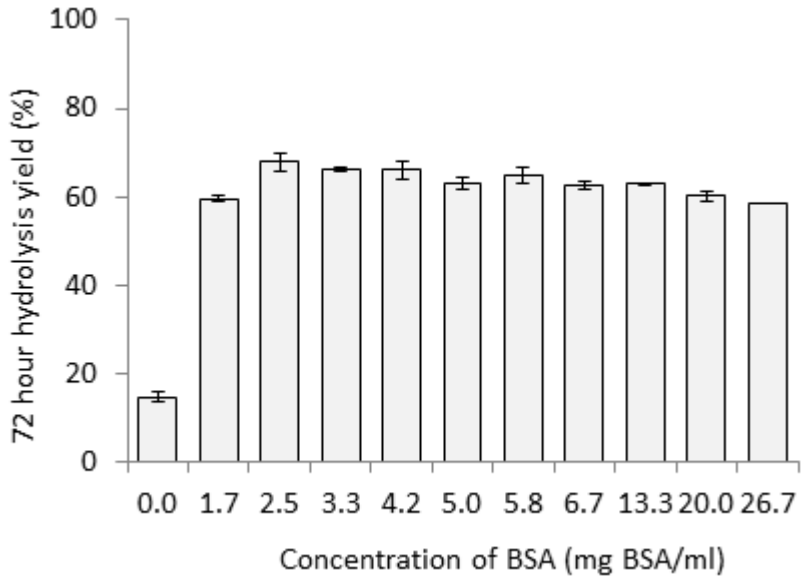


Figure 14 Influence of BSA pre-incubation at various concentrations on the enzymatic hydrolysis of steam pretreated Douglas-fir (Enzyme loading: 5 FPU cellulase and 10 CBU beta glucosidase per g of glucan). Error bars represent standard deviation from the mean (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

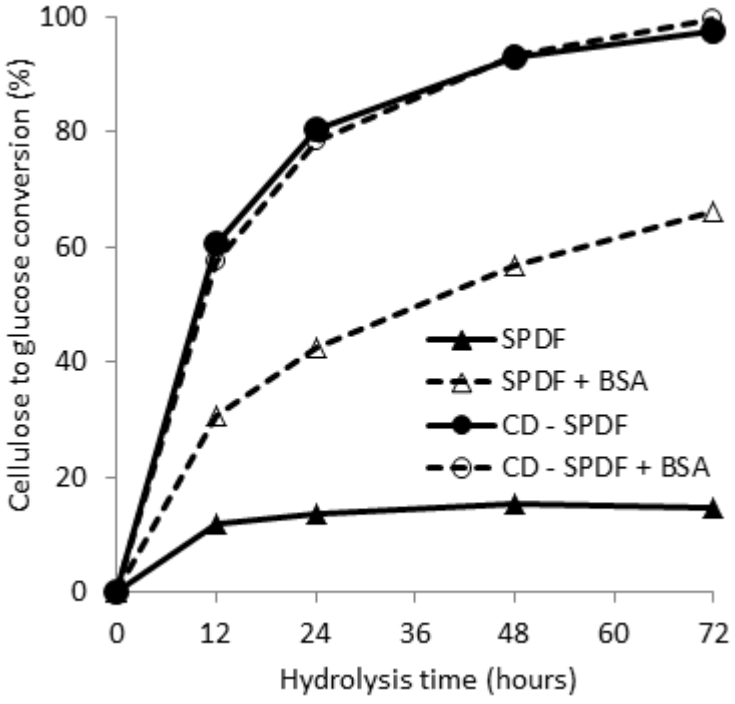


Figure 15 Influence of delignification (SPDF-CD) on the rate and extent of enzymatic hydrolysis of SPDF when low (5 FPU/g glucan) enzyme loadings are used with and without pre-incubation of BSA. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis).

3.2.2.3 Influence of lignin removal/ modification on cellulose accessibility and unproductive binding

To try to further elucidate how the contribution of lignin's unproductive binding of the enzymes to the lignin might be affected by changing the cellulose accessibility, the steam pretreated Douglas-fir substrate was sequentially delignified, using different dosages of sodium chlorite treatment at room temperature, to ensure minimal changes to the cellulose. The partially delignified substrates had a lignin content of 38, 30, 22 and 11% (corresponding to 18, 42, 62 and 84% removal of lignin) and were referred as SPDF-D38, SPDF-D30, SPDF-D22 and SPDF-D11, respectively (Table 11). As was expected, with decreasing lignin content there was an almost equivalent increase in the amount of cellulose present in the substrate, confirming the selectiveness of sodium chlorite delignification. The steam pretreated Douglas-fir sample was also sulfonated at neutral pH to produce a substrate (SPDF-S41), which differed significantly in terms of cellulose accessibility (as characterised by Simon's stain absorption) but was still quite similar in its lignin content (Table 11).

As previous work had shown that the water retention value (WRV) could provide a relatively good indication of cellulose swelling (Luo and Zhu, 2010; Ogiwara and Arai, 1968, 1972), the difference in the cellulose accessibility between SPDF and the corresponding delignified/lignin modified substrates was assessed using this procedure. A more refined Simon's stain method using adsorption of the high molecular weight fractions of the orange dye (>100 kDa) was also employed to assess cellulose accessibility. It was apparent that the accessibility of the cellulose, as indicated by the increase in the WRV and adsorption maxima for the orange dye, increased with increasing lignin removal (Table 11). These observations confirm earlier work that showed that chlorite delignification solubilized the high molecular weight lignin and consequently increased fiber swelling (Ahlgren and Goring, 1971; Ahlgren et al., 1971). The sulfonated steam pretreated substrate (S-41) also showed increased water retention and accessibility to Simon's stain. This was likely due to incorporation of sulfonic acid groups during neutral sulfonation at the α or γ position of the lignin side chain making the lignin more hydrophilic (Mooney et al., 1998 ; Sarkanen and

Ludwig, 1971), suggesting that the sulfonated substrate may also be more accessible to the cellulase enzymes.

To see if this increase in accessibility translated into better hydrolysis at low enzyme loading, 5 FPU/g glucan of enzyme was next added to the partially delignified substrates and the extent of hydrolysis after 72 h, with and without prior addition of BSA (Figure 16) was determined. For the substrates without prior BSA addition, an almost linear increase in cellulose hydrolysis was observed with increasing lignin removal. However, with the high lignin containing substrates, the prior addition of BSA had a significant effect, resulting in substantially increased levels of hydrolysis. As expected, for the low lignin containing substrates, prior addition of BSA resulted in little improvement (Figure 16). However, it was apparent that the lignin-enzyme interaction that led to unproductive binding did not decrease proportionately with a decrease in the lignin content, indicating that unproductive binding was also dependent on other factors. Removal of only 40% of the substrate's lignin substantially decreased (by more than threefold) the beneficial effects of prior BSA addition while still 30% lignin was present in the substrate (Figure 16). It is likely that, the extent of unproductive binding is also dependent on how accessible the cellulose was, not entirely on the proportionate lignin content. As cellulose accessibility increases, lignin plays less and less of a significant role as an unproductive adsorbent, even at high lignin concentrations, as there was enough cellulose available for good hydrolysis.

Table 11 Chemical composition and cellulose accessibility of partially delignified/lignin modified steam pretreated Douglas-fir (SPDF).

Substrate	Lignin (%)	Glucan (%)	Water retention value (%)	A_{max} for orange dye (Mw>100K)
SPDF – Control	42.8 (0.3)***	56.8 (0.6)	121.3 (10.3)	29.8 (4.7)
SPDF-D38*	38.2 (0.1)	61.4 (0.3)	150.9 (12.2)	37.2 (1.3)
SPDF-D30	29.7 (0.3)	68.3 (0.2)	177.2 (3.1)	45.5 (0.9)
SPDF-D22	22.4 (0.2)	75.8 (0.4)	195.4 (3.7)	54.2 (2.5)
SPDF-D11	10.9 (0.6)	87.5 (0.3)	219.7 (8.1)	64.4 (1.2)
SPDF-CD**	BDL****	99.3 (0.8)	250.2 (7.3)	81.2 (1.8)
SPDF-S41	41.1 (0.1)	58.3 (0.4)	170.6 (13.0)	42.8 (2.9)
*Numbers indicate the lignin content; **Steam pretreated Douglas-fir which was completely delignified; ***Numbers in the bracket represent standard deviations from the mean (n=3 for compositional analysis, n=5 for WRV and n=2 for Simon's stain) ****BDL refers to below detectable level				

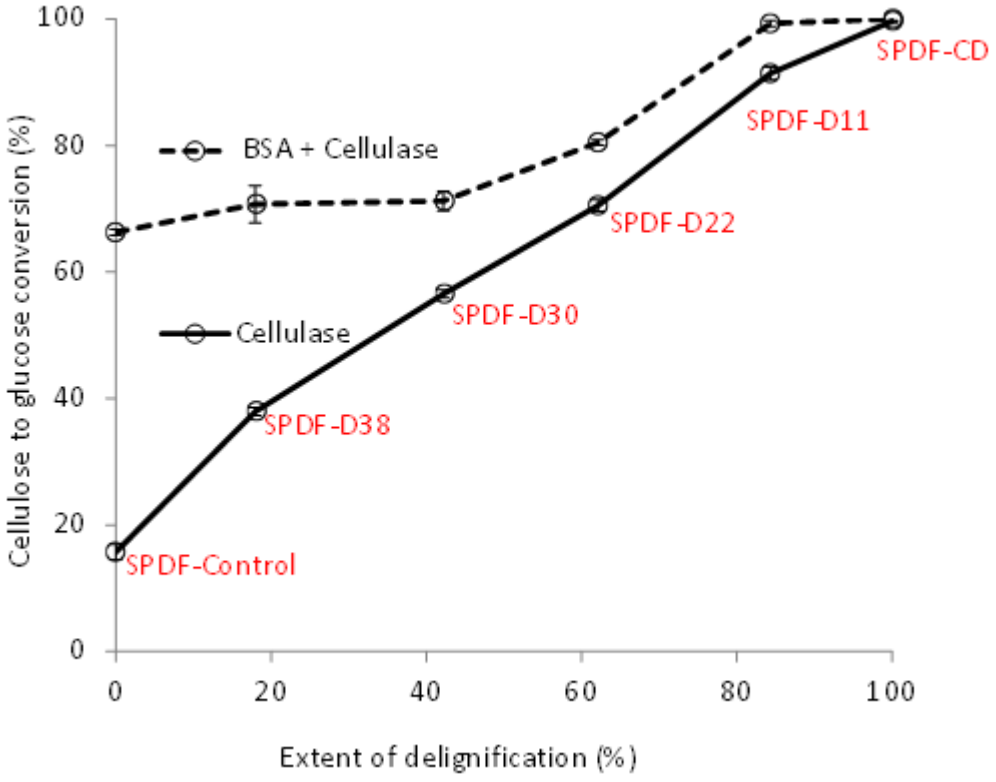


Figure 16 Influence of substrate delignification on 72 h enzymatic hydrolysis of steam pretreated Douglas-fir (SPDF) when low enzyme loadings (5 FPU/g glucan) are used with and without pre-incubation of BSA. Error bars represent deviations from the mean (n=2 for

enzymatic hydrolysis with subsequent HPLC analysis of the sugars in two replicates for each sample).

To try to further elucidate the possible mechanisms involved, the hydrolysis profiles of the steam pretreated Douglas-fir that had been sulfonated (SPDF-S-41), were determined again using low enzyme loadings (5 FPU/g glucan), with or without prior addition of BSA. Although neutral sulfonation resulted in a significant increase in both the rate and extent of hydrolysis, prior incubation with BSA had a much more dramatic effect on the unsulfonated substrate (Figure 17). This result again suggested that when cellulose accessibility is increased (as a result of sulfonation) even though lignin is present in relatively high concentrations, it plays a significantly lesser role in hindering the effectiveness of enzymatic hydrolysis. The small, but significant beneficial effect noted by prior BSA incubation with the sulfonated substrate was also likely due to the decrease in the lignin's hydrophobicity decreasing the unproductive adsorption of the cellulase enzymes.

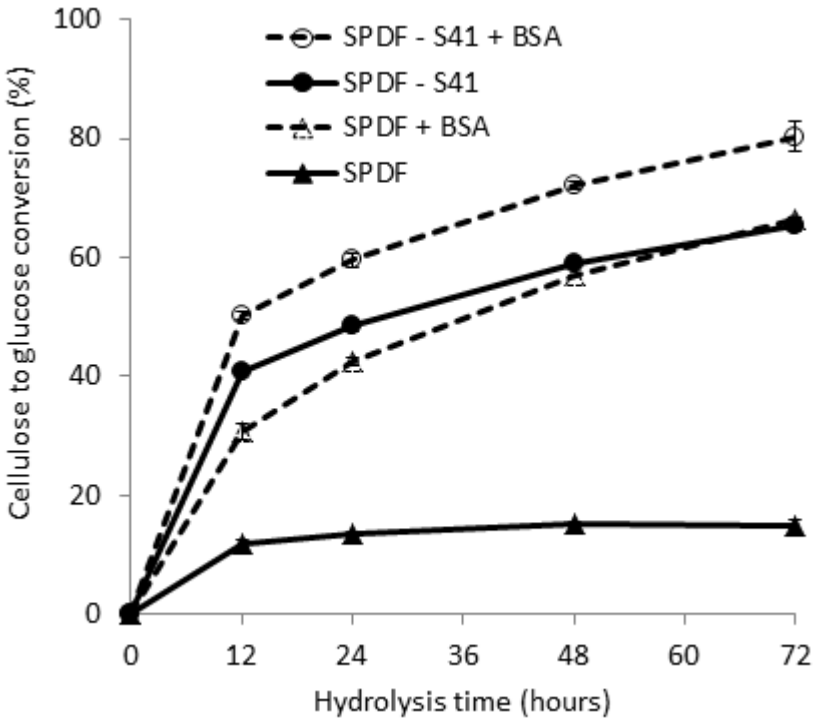


Figure 17 Effect of neutral sulfonation (S-41) on the enzymatic hydrolysis of steam pretreated Douglas-fir (SPDF) when using low enzyme loading (5 FPU/g glucan) with and without BSA pre-incubation. Error bars represent standard deviations from the mean (n=2).

3.2.2.4 Influence of lignin's unproductive binding with changes in cellulose accessibility

In order to further investigate the interaction between lignin's unproductive binding and cellulose accessibility during enzymatic hydrolysis, the influence of isolated lignin was separately assessed on a range of different pure cellulosic substrates having varying degrees of cellulose accessibility. While the water retention values (WRV) and Simon's stain had provided a good indication of the overall accessibility of the whole substrate, a more representative probe that would both assess the specific cellulose surface accessibility and simulate the types of lignin-enzyme interactions that were likely to occur was used in this experiment. Previous work (McLean et al., 2000, 2002; Rollin et al., 2011) had shown that measuring the adsorption profile of the cellulose-specific carbohydrate binding module (CBM2a) could provide a quantitative indicator of the total amount of available cellulose surface area. To see if the CBM probe would give an indication of the anticipated cellulose accessibility, a range of substrates were prepared, including the never-dried, completely delignified steam pretreated Douglas-fir, the same substrate that had been oven dried as well as two cellulose controls, Avicel and Sigmacell. As mentioned earlier in the section 3.2.2.2, the never-dried SPDF-CD was accessible enough such that near complete cellulose hydrolysis was obtained at an enzyme loading of 5 FPU/g glucan, while the other cellulosic substrates were expected to have much lower cellulose accessibilities and, consequently, proved to be more recalcitrant.

As expected, the substrate with the highest cellulose accessibility was the never-dried SPDF-CD while the same substrate when it was oven dried was much less accessible to the CBM probe (Figure 18), likely due to the hornification of the cellulose fibres upon drying and the resulting collapse of the cellulose fibers (Welf et al., 2005). The cellulose accessibility of Avicel and Sigmacell was similar and greater than that of the oven dried SPDF-CD (Figure 18). Avicel and Sigmacell are dried during their manufacture (moisture content ~ 5%) which also probably resulted in the collapse of the pores (Luukkonen et al., 2001). When all of these substrates were hydrolyzed over 72 h at an enzyme loading of 5 FPU/g glucan, the SPDF-CD substrate was completely hydrolyzed while 59%, 57%, and 53%

of the original cellulose present in the respective Avicel, Sigmacell and oven-dried SPDF-CD was hydrolysed. The fact that the cellulose content of the never dried and oven dried substrates (SPDF-CD) is the same indicated that the difference in the enzymatic hydrolysis must be due to differences in the cellulose accessibility.

Subsequently, lignin was isolated from the steam pretreated Douglas-fir using previous reported methods (Berlin et al., 2006) and this lignin was added to each of the four cellulosic substrates described above (Avicel, Sigmacell, never-dried SPDF-CD and oven-dried SPDF-CD) prior to carrying out the same hydrolysis procedure as described previously (5 FPU/g glucan, 72 h). The lignin was added in the same proportions as it was originally found in the steam pretreated Douglas-fir substrate prior to delignification. There was a minimal decrease (less than 10%) in the hydrolysis of the never-dried SPDF-CD substrate while the oven-dried substrate's hydrolysis yield was reduced by about 48% and the Avicel and Sigmacell were reduced by about 45% (Figure 18).

It seems that the degree by which isolated lignin decreased the hydrolysis yields was critically dependent on how accessible the cellulose was. When cellulose was highly accessible, such as in never-dried SPDF-CD, lignin had a minor role in decreasing hydrolysis yields. Alternatively, when the cellulose is not readily accessible, such as occurred with the oven dried SPDF-CD or Avicel and Sigmacell, the decrease in hydrolysis was significant.

These results confirmed that lignin's major role in restricting enzymatic hydrolysis is primarily through limiting cellulose accessibility to the enzymes. At low enzyme loadings (5 FPU/g glucan), only 16% of the cellulose present in steam pretreated Douglas-fir was hydrolysed compared to near complete hydrolysis of the corresponding, completely delignified substrate. To confirm that the beneficial effect of lignin removal observed was due to increase of the specific cellulose surface area, we next compared the hydrolysis profile of the steam pretreated Douglas-fir (SPDF) substrate, this same substrate which had been completely delignified (SPDF-CD) and this later substrate to which had been added back with the same amount of lignin found in the original SPDF substrate (Figure 20). At an

enzyme loading of 5 FPU/g glucan (Figure 20a), the prior addition of the lignin back to the delignified substrate (SPDF-CD) only slightly decreased both the rate and degree of completeness of hydrolysis. However, when an enzyme loading of 10 FPU/g glucan was used, the prior addition of lignin to the delignified substrate had little effect, implying that enough protein had been added to ensure that inhibition caused by the proteins binding to the lignin had been overcome (Figure 20b).

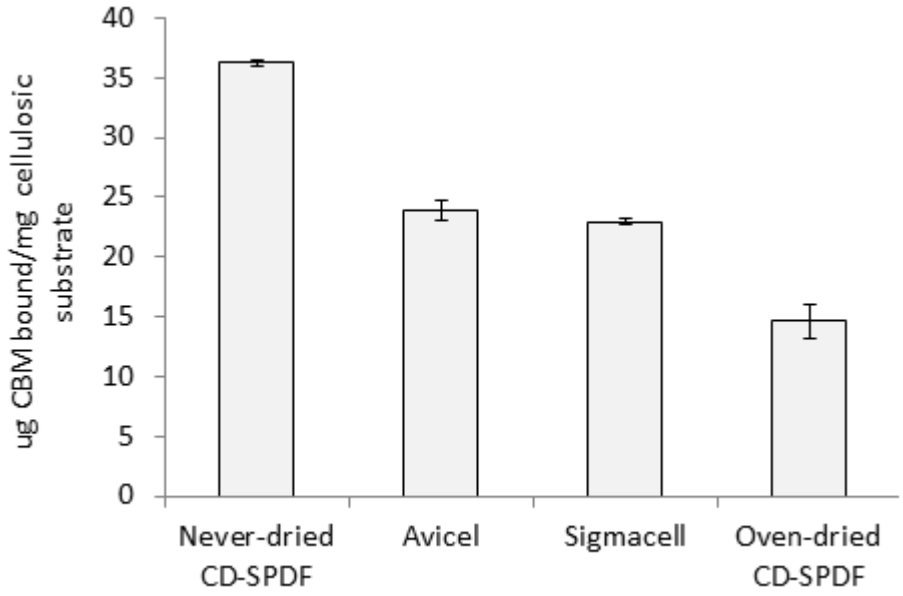


Figure 18 A comparison of the extent of Cellulose Binding Module (CBM) binding, used to assess the likely cellulose accessibility of pure cellulosic substrates. SPDF-CD refers to the completely delignified steam pretreated Douglas-fir. Error bars represent standard deviations from the mean (n=3).

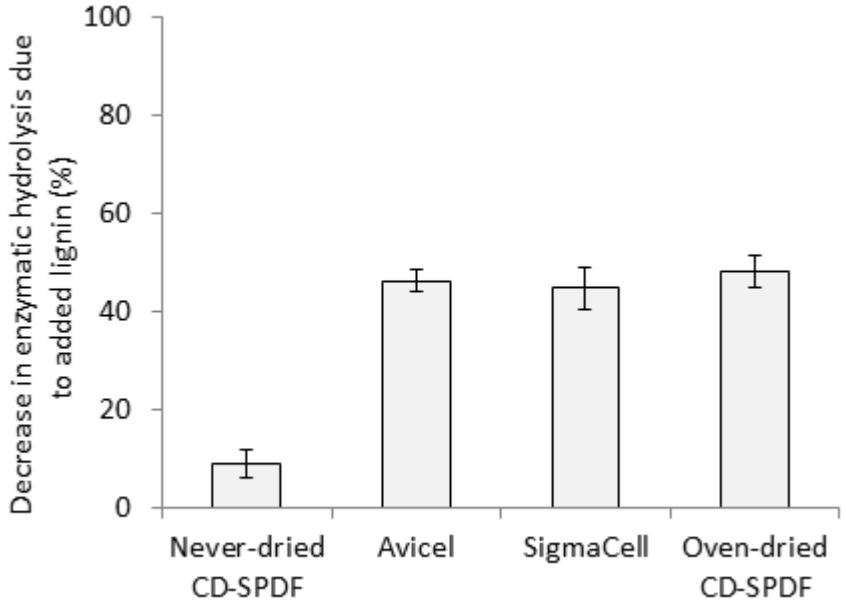


Figure 19 Influence of added lignin on the extent of decrease in cellulose hydrolysis of various pure cellulosic substrates having varying levels of cellulose accessibility. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

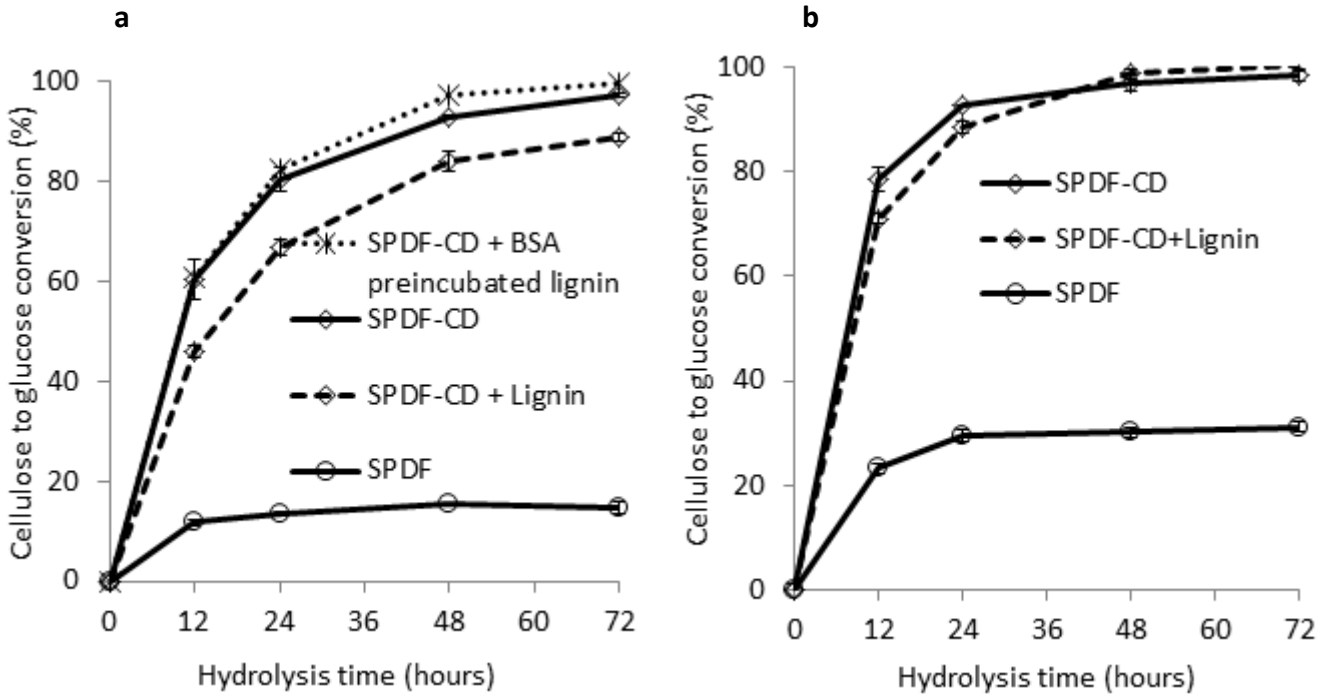


Figure 20 Influence of added lignin, isolated from steam pretreated Douglas-fir (SPDF) on the rate and extent of enzymatic hydrolysis at, a) 5 FPU/g glucan and, b) 10 FPU/g glucan, of never-dried, completely delignified, steam pretreated Douglas-fir (SPDF-CD). Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

The work described here indicated the inability of the enzymes to access the majority of the cellulose present in the steam pretreated softwood, which seems to be the key challenge for the poor hydrolysis yield at low enzyme loadings. Although unproductive binding to lignin is of significance, the influence of lignin binding gets diminished when the cellulose is more accessible to the enzymes. In short, when the cellulose-specific surface area is limited and lignin is present in the immediate vicinity, cellulase enzymes get stuck to lignin without getting access to cellulose. However, when the surface area of cellulose is substantially enhanced, the cellulose-specific cellulase enzymes can effectively hydrolyse cellulose even when lignin is present at high concentrations. This concept is schematically illustrated in the Figure 21.

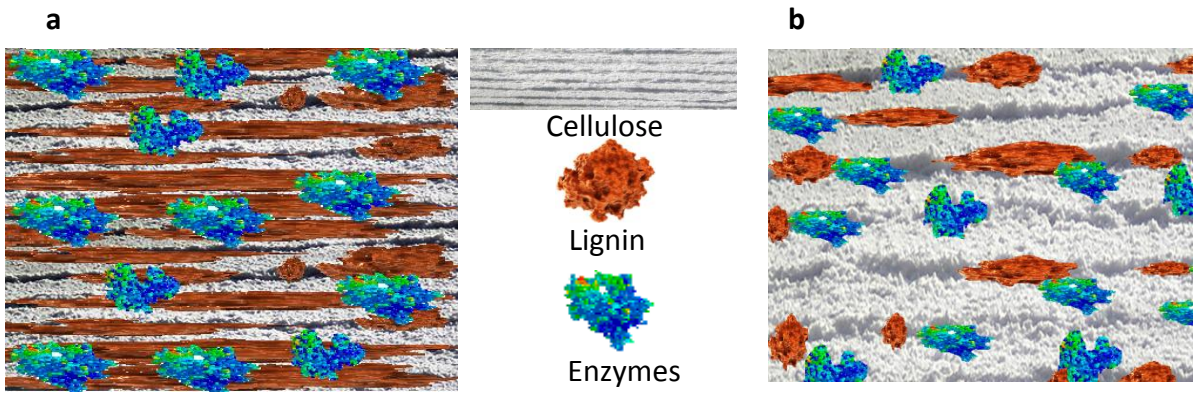


Figure 21 Schematic representation of the primary role of lignin during enzymatic hydrolysis of cellulose a) When the specific cellulose surface area is limited and lignin is present in the immediate vicinity, cellulase binds to lignin without getting access to cellulose. b) When the accessibility of cellulose is enhanced even when the lignin is present, cellulose gets hydrolysed effectively.

3.2.3 Conclusions

This work compared the relative contributions that lignin plays in restricting cellulose hydrolysis through either binding of the cellulases or limiting swelling of the cellulose and therefore limiting accessibility. The inhibitory effect of lignin is dependent on the enzyme loading employed as the use of excessive cellulase loadings resulted in near complete cellulose hydrolysis of steam pretreated Douglas-fir. However, at low cellulase loadings (<10 FPU/g glucan) the presence of lignin resulted in poor hydrolysis yields mainly due to the unproductive binding of the enzymes and limited cellulose accessibility. However, when the lignin does not restrict cellulose swelling and the cellulose is readily accessible to the enzymes, the impact of unproductive binding on the hydrolysis yield is lower. When access to the cellulose was enhanced, even at high lignin concentrations, good hydrolysis yields were obtained, thus minimizing lignin's role as both an unproductive adsorbent and a limitation to cellulose accessibility.

3.3 Influence of steam pretreatment severity on subsequent post-treatments used to enhance the enzymatic hydrolysis of pretreated softwoods

3.3.1 Background

Although it has been shown that increasing the pretreatment severity has the beneficial effect of enhancing the enzymatic hydrolysis of the water insoluble cellulosic fraction, the use of these high severities also contributes to sugar degradation, thereby diminishing the overall sugar recovery and particularly the fermentability of the water soluble, hemicellulose-derived sugars (Boussaid et al., 1999; Ewanick et al., 2007). Thus, previous work recommended using a medium severity, which is a compromise between optimizing hemicellulose recovery while ensuring the reasonable cellulolytic digestibility albeit at high enzyme loadings (Boussaid et al., 2000; Ewanick et al., 2007). The work described in this chapter revisited the previous work on the influence of pretreatment severity both in the context of using lower enzyme loadings for the hydrolysis of cellulosic component and also applying a subsequent post-treatment.

The work described in Chapter 3.2 has shown that lignin played a key role in limiting the cellulose accessibility of steam pretreated softwoods and that the use of a post-treatment step to remove or modify lignin is necessary to obtain good hydrolysis yields, particularly when low enzyme loadings are used. Previous studies had assessed several post-treatments with the objective of enhancing the hydrolysability of the steam pretreated Douglas-fir. These post-treatments included the use of alkali, alkaline peroxide, ozone, and oxygen delignification processes (Pan et al., 2004, 2005b; Yang et al., 2002). A related work has investigated the enzymatic hydrolysability of sulfonated softwood derived mechanical pulps, with the objective of increasing the substrate's accessibility and, consequently, improving hydrolysis yields (Mooney et al., 1998). In the last Chapter, we have shown that sulfonation treatment also enhances the enzymatic hydrolysis of steam pretreated softwood.

Although the potential of some post-treatments to increase enzymatic hydrolysis had been assessed previously, all of these post-treatments were applied to substrates that had been steam pretreated at a single set of conditions and also using relatively high enzyme loadings (Cullis et al., 2004; Pan et al., 2005b; Yang et al., 2002). This past work had ignored the potential effect that the severity of pretreatment conditions might have on the efficacy of these post-treatments. It is likely that, due to substrate changes during pretreatment, characteristics such as particle size distribution, chemical composition, degree of lignin condensation, etc., (Ramos et al., 1993, 1999; Shevchenko et al., 1999) will influence the efficacy of subsequent post-treatments. It is important that the severity of pretreatment is not so high that most of the hemicellulose sugars are lost through degradation while the pretreatment is severe enough to allow the effective hydrolysis of the water insoluble cellulosic fraction. As it was apparent that some form of post-treatment would normally be required for softwoods, it may be possible to use less severe conditions, thus enhancing hemicellulose recovery, while providing a cellulosic component that might be more amenable to post-treatment at these less severe conditions.

To try to elucidate the effect that the pretreatment conditions might have on subsequent post-treatment, Douglas-fir wood chips (DF4) were steam pretreated at three different severities (Log R_0 = 3.34, 3.64, 3.93) and four different post-treatments (alkaline hydrogen peroxide treatment-AHP, alkali treatment-ALK, neutral sulfonation-SULF, and chlorite delignification –CHL) were assessed, to try to enhance subsequent enzymatic hydrolysis. The recovery of hemicelluloses was measured at each of the severities and we compared the effectiveness of the various post-treatments in removing or modifying lignin while enhancing cellulose hydrolysis at low enzyme loadings.

3.3.2 Results and discussion

3.3.2.1 Effect of pretreatment severity on sugar recovery and ease of enzymatic hydrolysis (before applying any post-treatment)

Prior to studying the effectiveness of the various post-treatments, we first revisited the effect of increasing pretreatment severity on hemicellulose recovery and the enzymatic

hydrolysis of the cellulosic component. Although previous work has shown the beneficial effect of increasing the pretreatment severity on enhancing the enzymatic hydrolysis of the water insoluble cellulosic rich fraction, this improvement was achieved at the expense of high enzyme loading. Therefore, we wanted to determine the extent of hydrolysis improvement at low enzyme loadings when the pretreatment severity is enhanced.

Each of the pretreatment severities applied in the study was able to effectively solubilise nearly all of the hemicelluloses (Figure 22). This finding was similar to what was reported in previous studies (Clark and Mackie, 1987; Ewanick et al., 2007; Monavari et al., 2009a). As expected, after low severity pretreatment, the majority of the hemicellulose derived sugars were recovered in the liquid stream. However, sugar recovery decreased as pretreatment severity increased (Figure 22), primarily due to the increased sugar degradation at higher severities resulting in decreased overall carbohydrate yields (Boussaid et al., 2000). The overall hemicellulose recovery decreased from 85 to 74% and further to 54% when the severity was raised from low, to medium, to high respectively (Figure 22). Earlier work with Douglas-fir showed a similar trend of sugar loss with increasing steam pretreatment severity (Wu et al., 1999).

Subsequently, the enzymatic hydrolysis of the water insoluble cellulosic components was assessed at a range of enzyme loadings (5 – 30 FPU/g glucan). At all enzyme loadings, increasing severity resulted in increasing ease of enzymatic hydrolysis of the cellulosic component (Figure 23). It was interesting to note that the cellulose hydrolysis of even the high severity pretreated substrate was substantially lower at low enzyme loadings. At an enzyme loading of 5 FPU/g glucan, less than 21% of the cellulose was hydrolysed, even after the most severe pretreatment (Figure 23). When the severity was increased from medium to high, the hydrolysis yield at 5 FPU increased by 5% although this was achieved at the expense of about a 20% greater hemicellulose derived sugar degradation loss at the higher severity (Figure 23). Thus, increasing the pretreatment severity does not appear to be a solution for increasing the overall monomeric sugar recovery after pretreatment and enzymatic hydrolysis, especially when enzyme loadings as low as 5 FPU/g glucan are used.

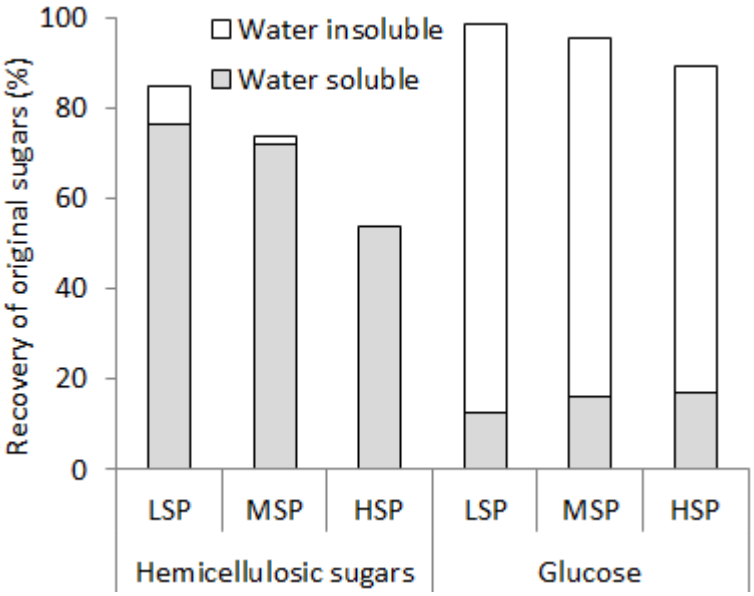


Figure 22 Influence of steam pretreatment severity on the recovery of sugars in the water soluble and insoluble fractions (% of original sugars present in the raw material). LSP, MSP and HSP refer to low, medium and high severity steam pretreatment respectively. Hemicellulose refers to the sum of arabinan, galactan, xylan and mannan.

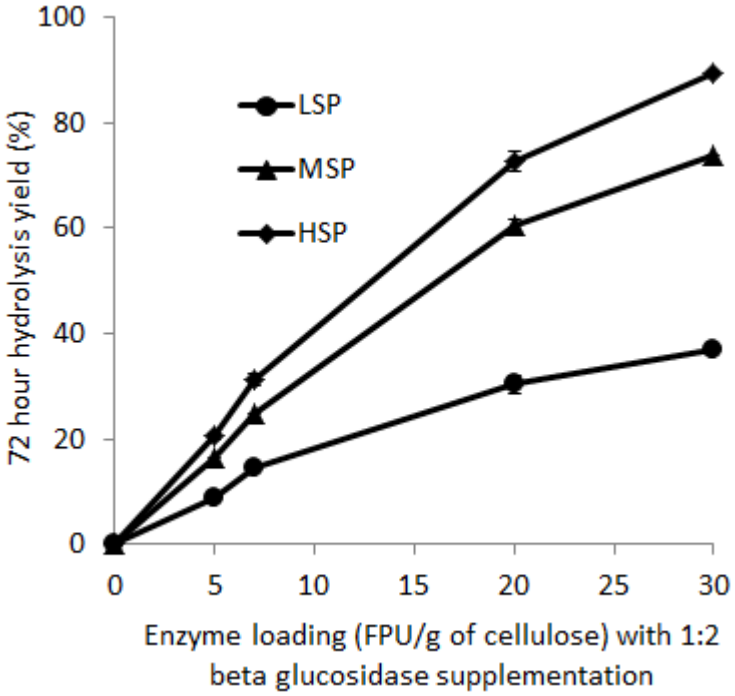


Figure 23 Influence of enzyme loadings on the 72 hour hydrolysis yields of the water insoluble cellulosic component of Douglas-fir after steam pretreatment at different severities. LSP, MSP and HSP refer to low, medium and high severity steam pretreatment respectively. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

In Chapters 3.1 and 3.2, we have shown that steam pretreated and subsequently delignified substrate could be almost completely hydrolysed at an enzyme loading of 5 FPU cellulase/g of glucan compared to an enzymatic hydrolysis yield <20% obtained with the non-delignified substrate. This work showed how influential is lignin in restricting enzymatic hydrolysis of steam pretreated softwoods. In order to further confirm that the main role is played by lignin in restricting enzymatic hydrolysis even at high severities, the substrates pretreated at all three severities were completely delignified and subsequently assessed for their ease of enzymatic hydrolysis at a range of enzyme loadings (Figure 24). When the substrates, which had been pretreated at each of the three severities were completely delignified it was clear that, regardless of the pretreatment severity used, 5 FPU/g glucan was sufficient to achieve near complete hydrolysis (Figure 24). It was interesting to note that although the properties within cellulose such as DP or crystallinity might vary with pretreatment severity, they seem to have not played a role in the range of enzyme loading employed although it is possible that cellulose properties may start playing a role at a lower enzyme loading. It was evident that both the amount and the nature of the lignin played a pivotal role in determining the effectiveness of hydrolysis in pretreated softwoods, particularly when low enzyme loadings are used. Therefore, we next assessed four potential post-treatments for their ability to partially remove or modify lignin and to consequently enhance cellulose hydrolysis and the role that the severity of pretreatment might have on various methods we had assessed previously to remove or modify lignin

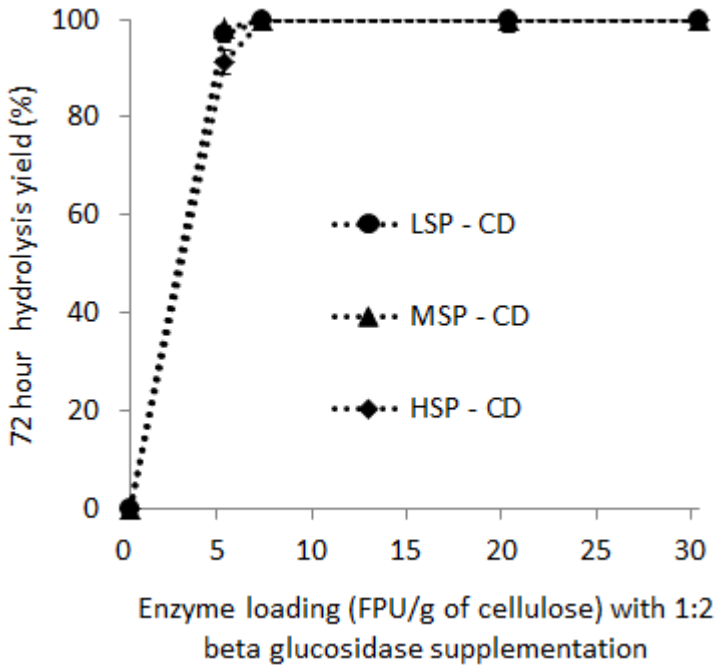


Figure 24 Influence of enzyme loadings on the 72 hour hydrolysis yields of Douglas-fir substrates steam pretreated at different severities and was subsequently completely delignified. LSP, MSP and HSP refer to low, medium and high severity steam pretreatment respectively. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

3.3.2.2 Effect of steam pretreatment severity on the susceptibility of substrates to delignification during post-treatments

We first looked at how changes in the severity of steam pretreatment might influence the ability of subsequent post-treatments to remove lignin, within the range of pretreatment severities studied ($3.34 \leq \text{Log } R_0 \leq 3.93$). It was apparent that, as has been reported previously (Cara et al., 2006), the extent of lignin removed increased as the pretreatment severity increased (Figure 25). Past work has suggested that during the initial stages of the steam pretreatment, depolymerisation of the lignin occurs via cleavage of β-O-4 linkages, resulting in a decrease in molecular weight with a concomitant increase in free phenolic hydroxyl groups (Karina et al., 1990, 1992, 1994; Li and Gellerstedt, 2008; Robert et al., 1988; Tanahashi et al., 1989, Tanahashi, 1990). It is known that an increase in the phenolic hydroxyl group increases the amphiphilic character of the steam exploded lignin (Robert et al., 1988). This effect, in combination with the lignin's lower molecular weight,

may play a role in increasing its solubility and reactivity in subsequent post-treatments, particularly when an alkaline medium is used. Thus, it was possible that the increased lignin extractability observed with increasing pretreatment severity was due to the greater degree of lignin depolymerisation which occurred at the higher severities. However, it is also recognised that when pretreatment severity is increased beyond a certain point, the susceptibility of the substrates to a delignifying post-treatment will likely decrease due to the higher degree of lignin condensation reactions which, at high severities, counterbalance the depolymerisation reactions (Li and Gellerstedt, 2008). Previous work has shown that when the pretreatment severity is raised to a point where the condensation reactions prevail this reduces the reactivity of lignin, consequently making it more difficult to extract (Li and Gellerstedt, 2008; Robert et al., 1988; Wayman and Lora, 1979).

It was clear that the influence of pretreatment severity on the ability of different post-treatments to remove lignin varied depending on the type of post-treatment employed (Figure 25). For example, with acidified hot chlorite treatment the delignification was only slightly increased with increasing pretreatment severity whereas after alkaline hydrogen peroxide treatment, an increase in pretreatment severity resulted in significantly more lignin being removed (Figure 25). The substrates obtained after alkaline hydrogen peroxide delignification (2% consistency, 80°C, 45 minutes, 1% peroxide loading at pH 11.5) had a residual lignin content which ranged from 14% to 25%, which is 1.5 – 3 times lower than the lignin content of the substrates resulting from neutral sulfonation (10% consistency, 16% chemical loading, 160°C, 60 minutes) (Table 12). The higher lignin content of the sulfonated substrates was expected as, at neutral pH, sulfonation does not result in significant delignification but rather results in the incorporation of sulfonic acid groups to the lignin (Mooney et al., 1998). As the post-treated substrates varied considerably in their lignin content, which likely effected cellulose accessibility, we next assessed the effect of these post-treatments on enzymatic hydrolysis.

Table 12 The effect of steam pretreatment severity on the lignin content of the post-treated substrates.

Steam pretreatment Severity	Before post-treatment	ALK**	AHP***	SULF****	CHL*****
Low (LSP)	38.4 (0.1)*	35.9 (0.3)	25.8 (0.7)	38.3 (0.9)	29.2 (0.2)
Medium (MSP)	41.6 (0.4)	35.4 (0.4)	19.7 (0.1)	41.1 (0.1)	30.8 (0.5)
High (HSP)	45.0 (0.5)	35.7 (0.3)	14.0 (0.6)	44.1 (0.1)	34.5 (0.3)
*The numbers in the bracket represent standard deviations from the mean (n=3). **Alkaline pretreatment; ***Alkaline hydrogen peroxide treatment ****Sulfonation; *****Hot Chlorite treatment					

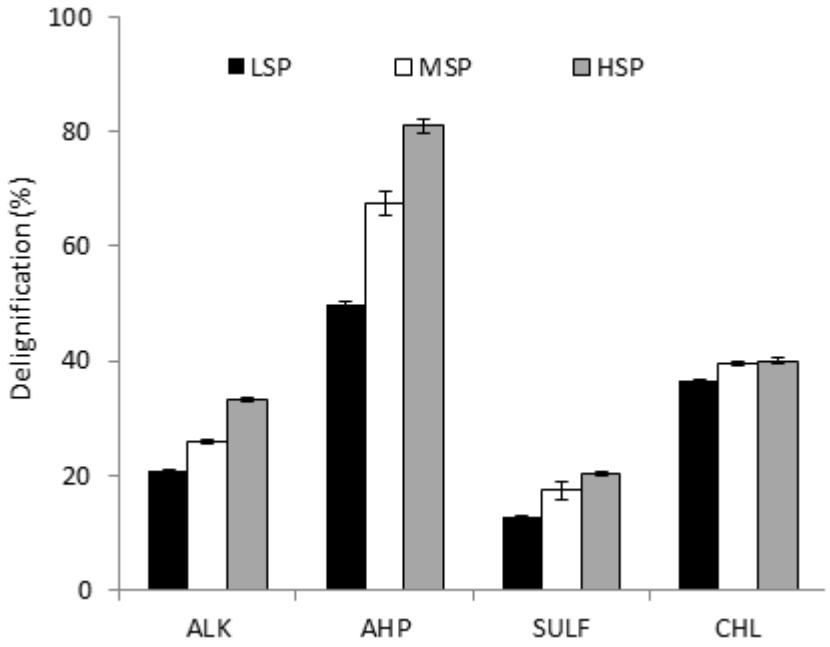


Figure 25 The effect of pretreatment severity on the efficiency of post-treatments to delignify the steam pretreated softwood (Douglas-fir). LSP, MSP and HSP refer to low, medium and high severity steam pretreatment respectively. ALK, AHP, SULF and CHL refer to alkali, alkaline peroxide, sulfonation, and hot chlorite treatment respectively. Error bars represent standard deviations from the mean (n=3 for the compositional analysis).

3.3.2.3 Enzymatic hydrolysis of post-treated substrates at low enzyme loadings

It was clear that post-treatment by alkaline hydrogen peroxide treatment (AHP) resulted in a significant increase in the enzymatic hydrolysis of the substrates treated at all three severities (Figure 26a). After AHP post-treatment, 72 hour hydrolysis yields (at low enzyme loadings) of 42%, 61%, and 51% were obtained after low, medium and high severity steam treatment respectively, which were 2.5 – 4.7 times higher when compared to the samples which had not been post-treated (Figure 26a). It is interesting to note that higher hydrolysis yields were obtained with the medium severity, AHP post-treated substrate than was achieved with the high severity post-treated substrate, despite the slightly lower lignin content of the latter sample. This finding was likely due to variations in the physico-chemical nature of the lignins as related work has shown that hydrophobic interactions are a major mechanism behind the unproductive adsorption of cellulases to lignin with groups such as the carboxylic acid functionality influencing the hydrophobicity of the lignin (Palonen et al., 2004c). When a conductometric titration of these substrates was carried out to quantify the acid groups on the substrates, it was found that the total acid groups in the MSP-AHP substrate was ~5 times greater than that of the HSP-AHP substrate (Figure 27), indicating that the hydrophobicity of the residual lignin in the HSP-AHP was likely higher than that of the MSP-AHP. To try to determine the possible role that non-productive binding of the enzyme to lignin might play, we next completely hydrolysed the MSP-AHP and HSP-AHP substrates, using an enzyme loading of 40 FPU/g glucan, and measured the amount of protein present in the liquid fraction after the complete hydrolysis of the substrates. It was apparent that, even after complete hydrolysis of the cellulose, 47 and 42% of the added protein was still associated with the respective residual lignin fractions in the HSP-AHP and MSP-AHP substrates (Figure 28).

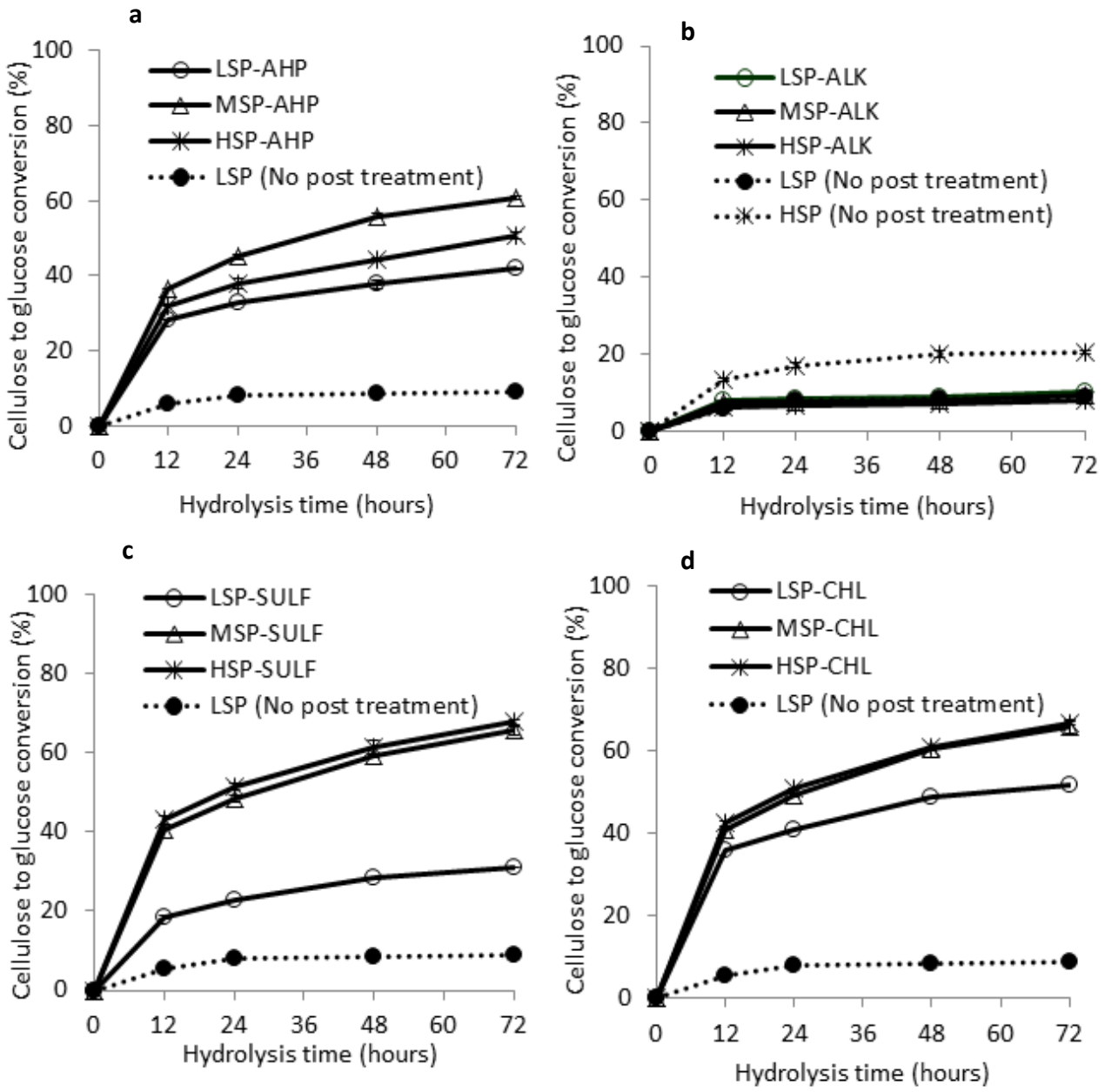


Figure 26 The effect of pretreatment severity on the ease of hydrolysis of post-treated substrates at low enzyme loadings a) Alkaline hydrogen peroxide post-treatment – AHP b) Alkali post-treatment – ALK c) Neutral sulfonation post-treatment – SULF d) acid chlorite post-treatment – CHL. (Enzyme loading: 5 FPU cellulase (Celluclast) and 10 CBU beta glucosidase (Novozymes 188) per g of glucan). Error bars represent deviations from the mean. (n=3 for enzymatic hydrolysis followed by a single HPLC analysis for each sample)

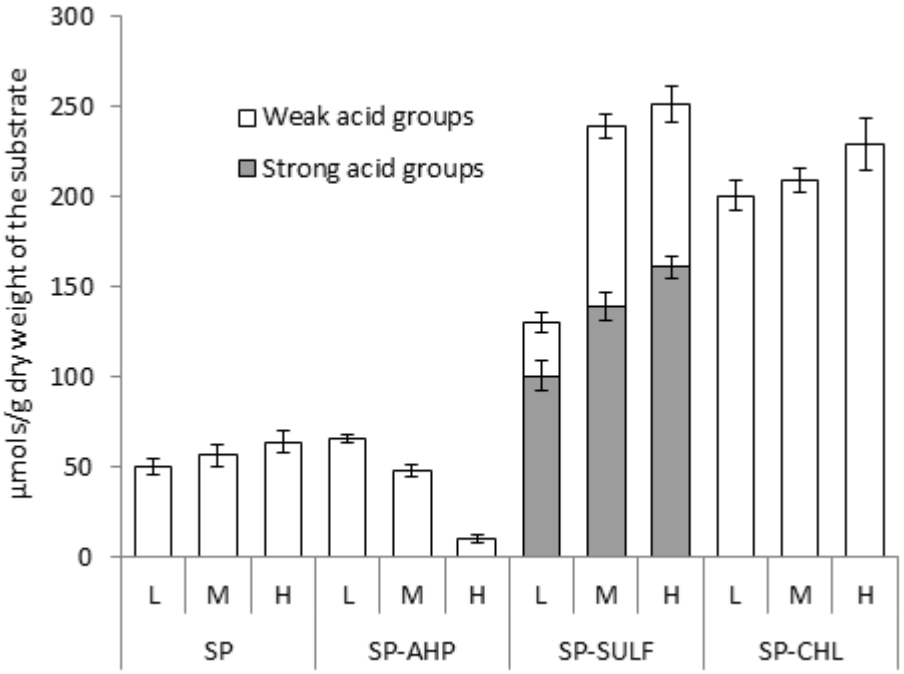


Figure 27 The bulk acid group content of the steam pretreated and post-treated softwood substrates. L, M and H refer to low, medium and high severity steam pretreatment respectively. ALK, AHP, SULF and CHL refer to alkali, alkaline peroxide, sulfonation, and hot chlorite treatment respectively. Error bars represent standard deviations from the mean (n=3).

Although a considerable amount of lignin could be removed by alkali treatment (without the use of peroxide), the enzymatic hydrolysis of the substrates treated at all severities was decreased to less than 10% (Figure 25 & Figure 26b). The same trend was observed with the alkali control (with no peroxide), which was used during alkaline hydrogen peroxide treatment at 80°C. The reduction in hydrolysis yield subsequent to alkali post-treatment of steam pretreated softwood has also been reported previously (Schell et al., 1998) where it was shown that alkali post-treatment decreased the hydrolysis yields of steam pretreated Douglas-fir regardless of the type of alkali used (NaOH, $Ca(OH)_2$, NH_4OH). In earlier work it was found that, when the pretreatment time was increased from 20 seconds to 180 seconds at 210°C, the subsequent alkali extraction decreased the hydrolysis yield from 40% to 30% and 98% to 20% respectively (Schwald et al., 1989b). It has been suggested that lignin softens during steam pretreatment, which combined with the

hydrophobicity of lignin and surface tension effects of water influences the lignin to form spherical droplets on the fibre surface (Kristensen et al., 2008; Selig et al., 2007) which during subsequent alkali treatment is more evenly redistributed thereby limiting the enzyme accessibility to the cellulose (Michalowicz et al., 1991). Other work which supports this mechanism showed that the pore volume of steam exploded *Pinus radiata* decreased significantly when the substrate was subsequently alkali extracted (Wong et al., 1988). These workers also reported that, after alkali extraction, enzymatic hydrolysis yield decreased from 84 to 24%. Other workers (Pan et al., 2005b) observed an improvement in hydrolysis yield of steam pretreated softwood after alkali treatment. However, the reasons behind these apparent contradictions are still not clear. In the present study, it is interesting to note that the highest decrease in hydrolysis yield was observed with the substrate pretreated at highest severity, which is also in accordance with earlier work (Schwald et al., 1989b). It is possible that the greater degree of lignin depolymerisation that occurs at higher pretreatment severities results in a greater redistribution of the lignin over a larger surface after subsequent alkali post-treatment, thus limiting the accessibility of the enzymes to the cellulose (Donohoe et al., 2008). It was apparent that lignin removal does not necessarily correlate with the ease of hydrolysis and that the location and nature of lignin have a considerable influence on cellulose accessibility and the effectiveness of enzymatic hydrolysis.

Similarly to AHP treatment, neutral sulfonation was equally effective in enhancing the hydrolysis of the substrates pretreated at all three severities (Figure 26a and Figure 26c). Although 65 and 68% of the cellulose present in the medium and high severity sulfonated substrates was hydrolysed respectively, only 31% of the cellulose present in the sulfonated, low severity substrate was hydrolysed. Although the AHP treatment resulted in a three times higher delignification when compared to neutral sulfonation for the medium severity pretreated substrates (Figure 25), similar hydrolysis yields were obtained for both substrates (Figure 26c). This finding suggested that, in addition to lignin removal, lignin modification also has the potential to enhance the ease of enzymatic hydrolysis of steam pretreated softwoods. It was clear that the total acid groups present in the sulfonated

substrates were significantly higher than that of AHP treated substrates (Figure 27). Previously it has been reported that, during neutral sulfonation, phenolic lignin structures are readily converted into a quinone methide intermediate and sulfonic acid groups are incorporated to the α or γ carbon on the side chain structures (Sarkanen and Ludwig, 1971). The acid group measurements also confirmed the presence of sulfonic acid groups in the sulfonated substrates (Figure 27). As was shown in Chapter 3.2, the incorporation of sulfonic acid groups on the side chain structures of lignin likely reduced the hydrophobicity of lignin thus potentially decreasing the non-productive binding of enzymes with the substrate lignin in addition to enhancing the cellulose accessibility. Protein adsorption data also indicated that, after the complete enzymatic hydrolysis of cellulose present in the sulfonated and AHP treated substrates, the residual lignin present in the peroxide treated substrates still adsorbed a higher amount of protein when compared to the lignin present in the sulfonated substrates (Figure 28). In addition, incorporation of strong acid groups during neutral sulfonation facilitates the electrostatic repulsion of the fibres and overall swelling, which would likely result in increased enzyme accessibility.

Previous work has shown that enhanced fibre swelling can occur with an increase in the concentration of acid groups in pulp (Dang et al., 2006; Grignon and Scallan, 1980; Katz and Scallan, 1983; Scallan and Grignon, 1979) . The amount of acid groups present in both the medium and high severity sulfonated substrates was about the same and both substrates hydrolysed almost similarly during enzymatic hydrolysis (Figure 27). Through the oxidation reactions which occur during alkaline hydrogen peroxide treatment, carboxylic acid groups are typically incorporated into the lignin and, as a result, a significant portion of the carboxylated fraction will likely be dissolved in the liquid fraction due to the formation of muconic acid type structures. This reaction is particularly relevant to the conditions used in this study, which did not employ stabilising agents for the peroxide (Sun et al., 2001). It has been shown that in the absence of peroxide stabilising agents hydrogen peroxide decomposes to hydroxyl radicals, superoxide ions and oxygen, leading to the decomposition of the phenolic structures and an enhanced degree of lignin removal (Adler, 1977; Agnemo and Gellerstedt, 1979; Kadla and Chang, 2001). This partial depolymerisation of lignin and

the consequential decrease in its molecular weight may increase the susceptibility of the resulting lignin to dissolution during AHP treatment. As a result the residual lignin remaining in the substrate is less hydrophilic.

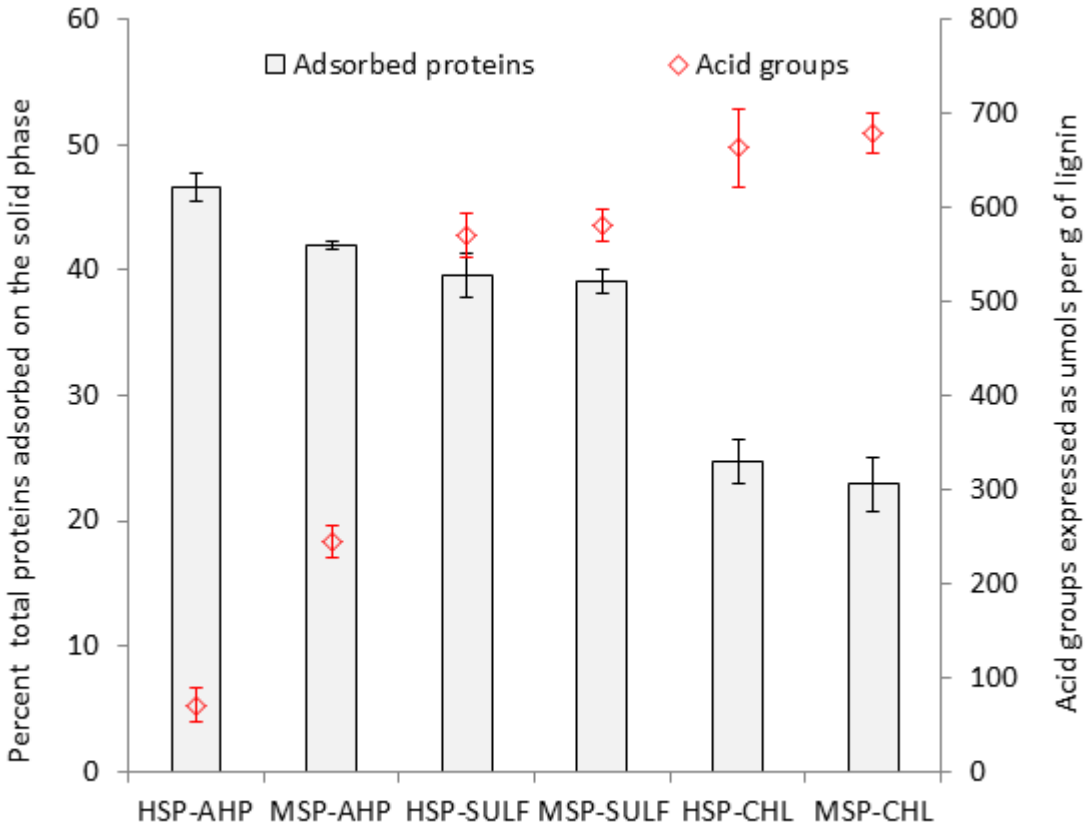


Figure 28 Effect of acid groups on the protein adsorption to the residual lignin fractions resulting from the complete enzymatic hydrolysis of selected post-treated substrates. The enzyme loading was 40 FPU/g glucan and 80 CBU/g of beta-glucosidase and the hydrolysis time was 96 hours. The secondary axis represents the total acid groups expressed in terms of umol/g of lignin present in the sample. Error bars represent standard deviations from the mean (n=3).

Previous work has shown that sulfonation enhanced subsequent enzymatic hydrolysability of mechanical pulp (Mooney et al., 1998). However, this improvement was not as high as was observed with the steam pretreated substrate used in this present study. It was likely that the higher phenolic hydroxyl content of the steam pretreated softwood

arising from the cleavage of β-O-4 linkages during pretreatment resulted in the higher degree of sulfonation of the steam pretreated softwood when compared to the mechanical pulp (Li and Gellerstedt, 2008; Mooney et al., 1998). It was also apparent that an increase in pretreatment severity also influenced the degree of sulfonation and the subsequent enzymatic hydrolysability of the sulfonated substrates (Figure 26c and Figure 27). This was also likely due to the greater degree of β-O-4 cleavage in the lignin that will have occurred at the higher severities, consequently resulting in an increase in phenolic hydroxyl groups which would have facilitated sulfonation at neutral pH. For the low severity steam pretreated substrates, a lower amount of sulfonic acid groups were incorporated, resulting in much poorer hydrolysis yields (Figure 26c).

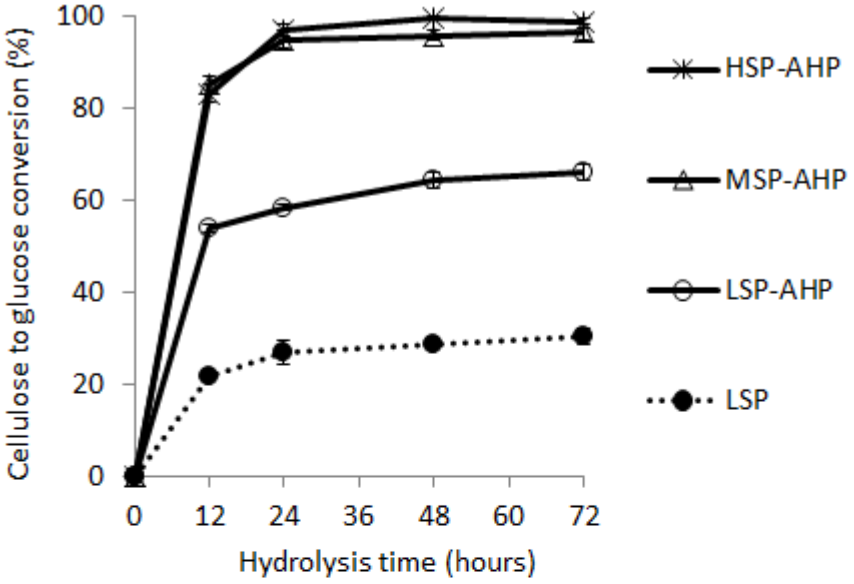
In addition to delignifying the substrate, acid chlorite delignification significantly increased the amount of acid groups in the substrates due to the oxidation of lignin (Figure 27). Therefore, both lignin removal and incorporation of acid groups in the residual lignin could have synergistically enhanced the ease of hydrolysis of the chlorite treated substrates. After chlorite treatment, both the medium and high severity steam pretreated substrates reached similar hydrolysis yields of 66 and 67% after 72 hours hydrolysis (Figure 26d). Substrates pretreated at the lower severity were not as readily hydrolysed as were the medium and high severity treated substrates (Figure 26d). However, when compared to the other post-treatments, the chlorite treatment was the most effective in enhancing the hydrolysis of the low severity treated substrates. After chlorite delignification, 52% of LSP treated substrate was hydrolysed, which was nearly 10% higher than was achieved after alkaline hydrogen peroxide and 20% higher than obtained after neutral sulfonation (Figure 26a, c and d). Previous work has shown that chlorite delignification of thermo-mechanical softwood pulp resulted in increased carboxylation of the pulp and enhanced fibre swelling (Carlsson et al., 1983). It appears that similar mechanisms occurred with the chlorite treated steam pretreated softwood. As was discussed earlier, the increased amount of acid groups present in the lignin will likely reduce the hydrophobic interactions between the lignin and cellulases. The residual lignin from the chlorite treated substrates was shown to have the least amount of protein associated with it after complete enzymatic hydrolysis (Figure 28).

This work indicted that as well as lignin removal, another alternative way to increase hydrolysis yield at low enzyme input is to modify the lignin structure. Generating a more hydrophilic form of lignin would both minimise the non-productive binding of the lignin to the cellulase enzymes and also increase the electrostatic repulsion of the cellulose fibres. These phenomena would consequentially result in enhanced overall enzymatic accessibility due to swelling of the cellulose. While neutral sulfonation treatment will reduce the hydrophobicity of the lignin and increased fibre swelling by the incorporation of acid groups such as sulfonic or carboxylic acid groups, oxidative treatments, such as chlorite treatment, will result in both lignin removal and the generation of oxidised acid group structures in the residual lignin, thereby increasing the overall enzymatic accessibility of the substrate. It was clear that the lignin content or degree of delignification do not necessarily correlate with the ease of enzymatic hydrolysis at low enzyme loading. It is also the type and location of lignin which ultimately impact cellulose accessibility is and are critical in determining the hydrolysis yield of the substrates.

3.3.2.4 Enzymatic hydrolysis of post-treated substrates at high enzyme loadings

When all of the peroxide and sulfonated substrates were hydrolysed at a higher enzyme loading (20 FPU/g glucan) the hydrolysis yields reached completion for both medium and high severity treated substrates (Figure 29). As was observed with low enzyme loading, the low severity treated substrates reached poor hydrolysis yields even after the application of post-treatment and higher enzyme loadings. As well as at lower enzyme loadings, both the sulfonated and peroxide treated substrate were hydrolysed to almost the same extent at higher enzyme loadings. However, the difference in the hydrolysability of medium and high severity AHP treated substrates disappeared at high enzyme loading supporting the earlier hypothesis of the higher extent of unproductive binding for the high severity treated substrates which had been peroxide treated. When enough enzymes were added, it appears that effects of unproductive binding were overcome. This masking effect of excess enzymes resulted in the same extent of hydrolysis as that of the sulfonated substrates.

a



b

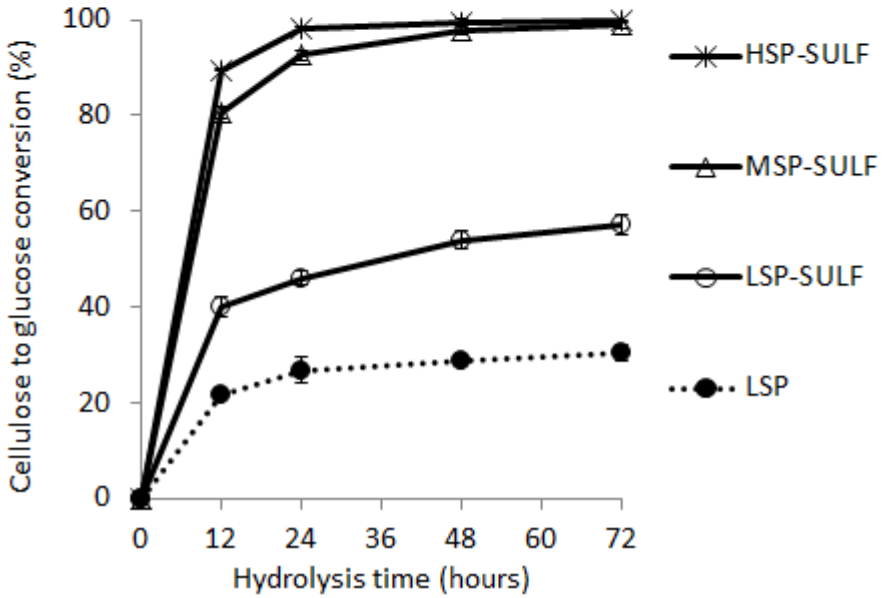


Figure 29 The effect of pretreatment severity on the ease of hydrolysis of post-treated substrates a) Alkaline hydrogen peroxide post-treatment – AHP b) Neutral sulfonation post-treatment – SULF. LSP, MSP and HSP refer to low, medium and high severity steam pretreatment respectively. Error bars represent deviations from the mean (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

3.3.2.5 Effect of post-treatment on overall sugar yield from softwoods

Apart from increasing the ease of enzymatic hydrolysis, another prerequisite of an effective post-treatment is its ability to retain as much carbohydrate as possible during the

process of lignin removal/modification. However, due to factors such as the use of high temperature, oxidising chemicals and the generation of radical species during some of the reactions, it is likely that some carbohydrate degradation occurs during a post-treatment (Chiang and Sarkanen, 1985; Dang et al., 2007; Gratzl, 1987). Thus, we next wanted to determine the overall recovery of sugars in a usable form (or the yield based on the original sugar content of the starting material) after a steam pretreatment followed by a post-treatment and subsequent enzymatic hydrolysis. The total fermentable sugars are the sum of both the sugars present in the steam pretreated, hemicellulose rich water soluble fraction and the sugars released during enzymatic hydrolysis of the cellulose rich, water insoluble fraction. A medium severity steam pretreatment was found to provide the best compromise as it allowed the recovery of much of the hemicellulose sugars in the water soluble fraction while post-treatment of the cellulose rich water insoluble fraction appeared to be quite effective in enhancing enzymatic hydrolysis. As only about 10% and 34% of the original sugars were recovered after hydrolysis at 5 and 20 FPU/g of the cellulosic component of the medium severity pretreated substrate (Figure 30), we compared the total amount of sugars (the combination of hemicellulose and cellulose derived sugars) that could be recovered after neutral sulfonation and alkaline hydrogen peroxide post-treatments.

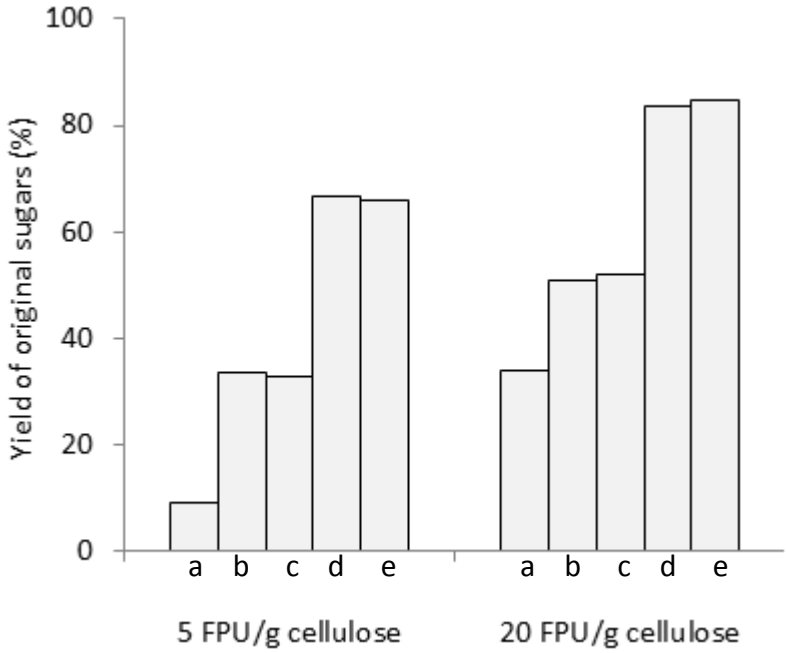


Figure 30 Overall sugar yields of the original substrate after, a) hydrolysis of the medium severity pretreated water insoluble cellulosic fraction, b) this substrate hydrolysed after sulfonation post-treatment, c) the original substrate hydrolysed after peroxide post-treatment, d) total sugars released (hemicellulose and cellulose derived) after sulfonation post-treatment, e) total sugars released (hemicellulose and cellulose derived) after peroxide post-treatment.

Neutral sulfonation and alkaline hydrogen peroxide treatment were found to be equally effective; resulting in similar cellulose recoveries, with about 3 – 7% of the glucose that was present in the water insoluble fraction solubilised by the post-treatment. This slight sugar loss was more than offset by the benefit obtained with significant increase in hydrolysis yield after post-treatment especially when low enzyme loading of 5 FPU/g glucan were used (Figure 30). When the enzyme loading was increased to 20 FPU/g glucan, near complete cellulose hydrolysis could be achieved after both types of post-treatment, compared with just over 60% hydrolysis when there was no post-treatment. When the overall sugar recovery of the original substrate was compared for the various pre-and post-treatments (Figure 30), the compromise medium severity conditions provided a relatively

good recovery of the hemicellulose derived sugars and virtually no sugar loss from the cellulosic component while subsequent post-treatments greatly enhanced the sugar yield from enzymatic hydrolysis. When an enzyme loading of 20 FPU/g glucan was used, close to 85% of the original sugars present in the hemicelluloses and cellulose could be recovered (Figure 30), with virtually all of these sugars being the more readily fermented hexoses.

3.3.3 Conclusions

Although high steam pretreatment conditions typically enhance the enzymatic hydrolysis of the water insoluble cellulosic fraction, this is usually achieved at the expense of poor recovery of the hemicellulose derived sugars due to their degradation during pretreatment. However, softwoods are known to be quite recalcitrant and even the use of high severity conditions did not significantly enhance enzymatic hydrolysis while using low enzyme loadings. It was clear that low or medium severity conditions had to be used, if good hemicellulose recovery was to be achieved. It was also clear that some form of post-treatment would be required if good hydrolysis of the cellulosic fraction was to be obtained when using low enzyme loadings. The medium severity conditions were subsequently shown to be the best compromise, allowing recovery of most of hemicellulose sugars after pretreatment and enhancing subsequent post-treatment and enzymatic hydrolysis of the water insoluble cellulosic fraction. It was apparent that the lignin content or the degree of delignification of the post-treated substrate did not necessarily correlate with the subsequent ease of enzymatic hydrolysis. Despite the three times higher delignification achieved by alkaline hydrogen peroxide treatment compared to neutral sulfonation, both of the substrates were hydrolysed to the same extent. It is likely that, in addition to delignification, post-treatments result in some form of lignin modification such as increase in carboxylic/sulfonic acid groups, which result in an overall increase in cellulose accessibility such that cellulose hydrolysis at low enzyme loadings is greatly enhanced.

3.4 Optimization of the sulfonation post-treatment to enhance the enzymatic hydrolysis at low enzyme input

3.4.1 Background

Neutral sulfonation has several advantages compared to other post-treatments. As the process does not lead to a substantial delignification, it can potentially avoid solid-liquid separation and water washing prior to enzymatic hydrolysis. In addition, sulfonation can be conducted at pH values closer to that of hydrolysis, and therefore, there may not be a need for pH adjustment prior to enzymatic hydrolysis. Most importantly, one key benefit of sulfonation is the possibility of recovering the lignin as a value added co-product (sulfonated lignin).

As mentioned earlier, the pulp and paper industries employ sulfonation reactions to produce both chemical pulps (sulfite and dissolving pulps) and chemi-thermo-mechanical pulp (CTMP). Sulfite pulping is carried out between pH 1.5 and 5, depending on the counter ion and the ratio of base to sulfurous acid. The salts used in the sulfite pulping process are either sulfites (SO_3^{2-}), or bisulfites (HSO_3^-), depending on the pH. The counter ion can be sodium (Na^+), calcium (Ca^{2+}), potassium (K^+), magnesium (Mg^{2+}) or ammonium (NH_4^+). The wood chips are generally in contact with the pulping chemicals for 4 to 14 hours and at temperatures ranging from 130 to 160°C again depending on the chemicals used (Biermann, 1996). The major objective in sulfite pulping is to achieve selective delignification without destroying the fibres. It should be noted that despite substantial delignification, the sulfite process does not degrade lignin to the same extent that the Kraft process does and the lignosulfonates from the sulfite process are useful by-products (Biermann, 1996).

Sulfonation of the wood chips prior to refining is also a well-established commercial practice widely used in production of softwood and hardwood derived chemi-thermo-mechanical pulps (CTMP). The difference in this process is that it is conducted generally in the range of neutral to alkaline pH. The objective is to sulfonate lignin without substantial delignification so that the pulp yield can be maintained. Different sulfonation methods (chip

impregnation, in-refiner sulfonation, reject sulfonation, etc.) have been used to produce mechanical pulps with a wide range of desirable properties (Atack et al., 1982; Barbe et al., 1989, 1993; Heitner et al., 1982; Richardson et al., 1998). Various researchers have extensively studied the effect of sulfonation on the physical properties of mechanical pulp. It was shown that chip sulfonation prior to refining promotes the separation of long, intact fibres and, therefore, improves strength properties of mechanical pulps. Previous studies from our group have also tried to investigate the effect of neutral sulfonation on the enzymatic hydrolysis of mechanical pulp, however, they employed high sulfite loading in their processes to enhance the sugar yield (Mooney et al., 1998).

The pulp and paper industries generally employs higher chemical loading, temperature and longer reaction times for sulfonation as they use raw wood chips or mechanical pulp (Johansson et al., 1997; Lai and Wei, 1992). These severe conditions are required to achieve their goal of obtaining sufficient delignification/lignin modification via the cleavage of β-O-4 linkages and resulting sulfonation of the free phenolic structures. However, steam pretreatment already resulted in substantial cleavage of α-O-4 and β-O-4 linkages meaning that part of the requirement for sulfonation has already been met (Yasuda et al., 1997). Our expectation was that the low molecular weight lignin fragments resulting from steam pretreatment would have a higher abundance of free phenolic groups and carbonyl side chain structures, which might be readily sulfonated at milder conditions compared to the native lignin or lignin present in mechanical pulp (Li et al., 2007). Previous studies on lignin model compounds indicated that compounds with free phenolic groups could get easily sulfonated at different pH ranges and at mild temperatures (Lundquist et al., 2007; Shorygina and Elkin, 1970). This relative ease of sulfonation was confirmed by the work described in the earlier chapter, where it was shown to be possible to reduce the high chemical loading previously used, to a chemical loading of 16% while achieving >60% hydrolysis yield at an enzyme loading of 5 FPU/g glucan compared to <20% hydrolysis yield for the corresponding steam pretreated substrates. We hoped we could further reduce the chemical loading and energy requirement for the sulfonation of steam pretreated substrate by optimizing different process parameters over a wide range of conditions.

3.4.2 Results and discussion

As 5 FPU/g glucan was previously shown to be the minimum dose required for the near complete conversion of a completely delignified substrate, complete enzymatic hydrolysis of a real lignocellulosic substrate appears to be difficult with such a low enzyme loadings even with the application of a most effective post-treatment. Therefore, a slightly higher enzyme loading (10 FPU/g glucan), which is still substantially lower than the previously used loading for steam pretreated substrates, was used for the preliminary screening of the best treatment conditions.

The experiments were divided into two sections. First a mild sulfonation was carried out. This set of experiments was conducted at temperatures closer to enzymatic hydrolysis so that a post-treatment could be directly combined with enzymatic hydrolysis without any washing or solid-liquid separation. A second set of experiments was conducted at harsher conditions (≥120°C) and a solid-liquid separation and water washing were subsequently employed prior to enzymatic hydrolysis.

3.4.2.1 Mild sulfonation of steam pretreated softwood

Surprisingly, sulfonation even at very mild conditions was found to have a substantial influence on enzymatic hydrolysis of steam pretreated softwood. The simple addition of sodium sulfite (2-32%) prior to enzymatic hydrolysis at 50°C, was found to substantially enhance the sugar yield (Figure 31). The enhancement in sugar yield was also dependent on the enzyme loading employed with the greater influence observed in the range of 5 – 10 FPU/g glucan. The addition of 16% sodium sulfite enhanced the hydrolysis yield from 29 to 47% at an enzyme loading of 10 FPU/g glucan (Figure 31). It was apparent that some of the phenolic moieties in lignin were readily sulfonated with the addition of sodium sulfite, thus minimising the impact of lignin's unproductive binding and/or restriction of cellulose accessibility.

Subsequent experiments with varying the reaction time showed that 6 hours of sulfonation enhanced the hydrolysis yield to 55%, an 8% improvement compared to what

was obtained with the instant addition of sodium sulfite (Figure 32). A further increase in reaction time did not substantially increase the hydrolysability even after the reaction was extended to 24 hours (Figure 32). It was also interesting to note that substrates sulfonated at pH 7 and pH 5 responded in a fairly similar fashion in terms of their hydrolysis profiles, which indicated that the same mechanism might have occurred at both pH levels. It appears that the sulfonation mostly likely proceeds through the protonation of oxygen present in carbonyl or ether groups of the lignin side chain. When the reaction was conducted with different chemical loadings, 8% chemical loading was found to be the optimum since further enhancement had a negligible improvement on enzymatic hydrolysis (Figure 33)

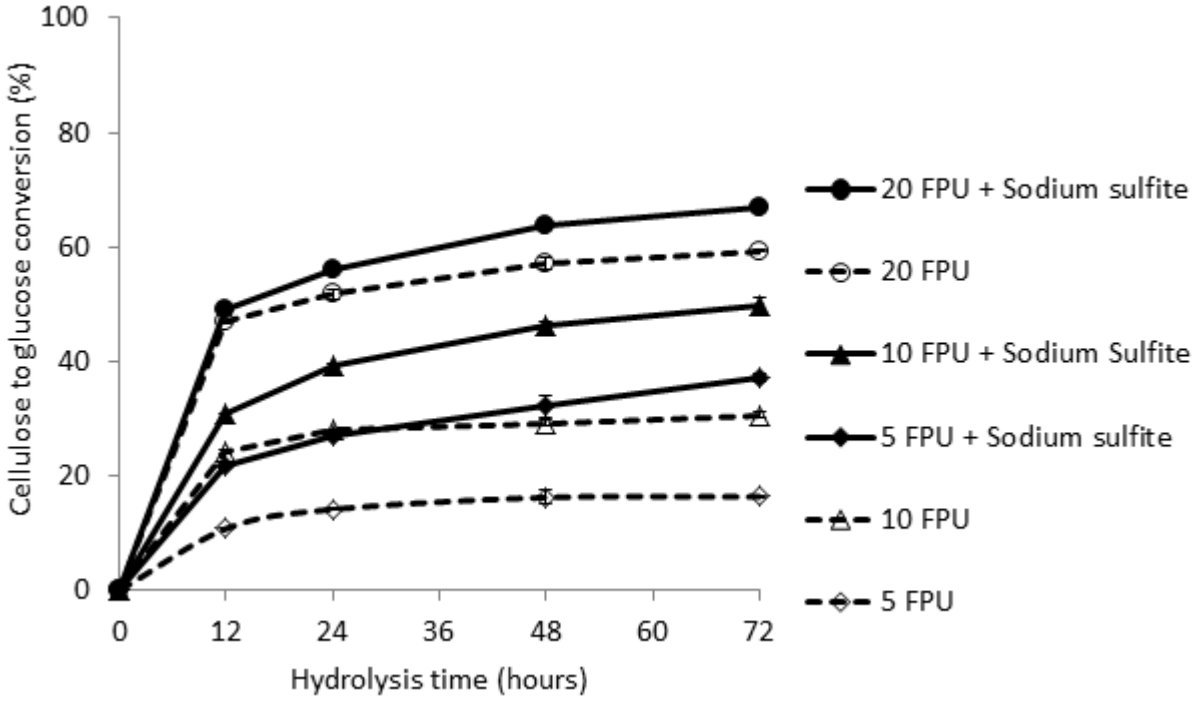


Figure 31 Influence of rapid addition and incubation of sodium sulfite (16% wt/wt of the substrate, 50°C, 2% consistency) prior to enzymatic hydrolysis on the rate and extent of sugar yield at various enzyme loadings (per g of cellulose). Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis with subsequent HPLC analysis of the sugars in two replicates for each sample).

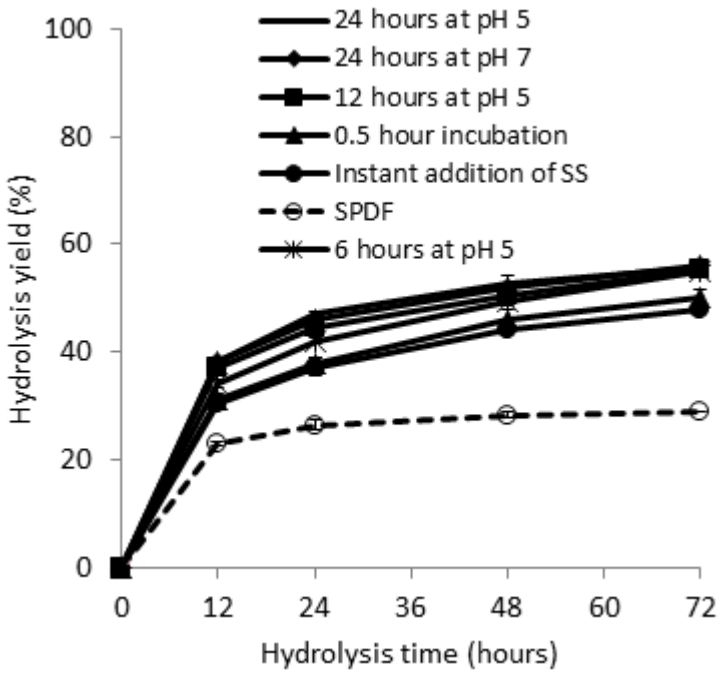


Figure 32 Influence of duration and pH on the efficiency of sulfonation (50°C, 16% loading) to enhance the enzymatic hydrolysis of steam pretreated softwoods. Enzyme loading: 10 FPU/g glucan. Error bars represent deviations from the mean (n=2).

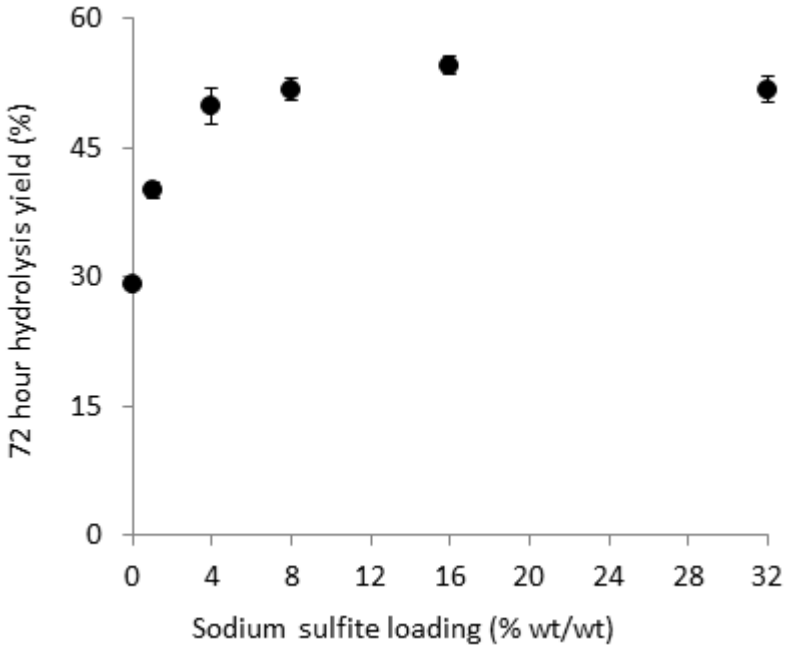


Figure 33 Influence of sodium sulfite loading on the efficiency of milder sulfonation conditions (50°C for 24 hours) to enhance the enzymatic hydrolysis of steam pretreated softwoods. Enzyme loading: 10 FPU/g glucan. Sulfite loading was based on % wt/wt of the substrate. Error bars represent deviations from the mean (n=2).

There are two possible ways by which a mild sulfonation might help improve the hydrolysis yield. It has been previously reported that enzymatic hydrolysis of pretreated lignocellulose can potentially release some phenolic compounds from lignin, thus inhibiting the enzymes (Ximenes et al., 2010; 2011). If this mode of inhibition occurs in steam pretreated softwood, addition of sodium sulfite can sulfonate these phenolics and minimise the inhibitory effects on the enzymes (Soudham et al., 2011). Alternatively, addition of sodium sulfite could readily sulfonate some of the lignin moieties in the solid substrate increasing the cellulose accessibility and minimising the unproductive binding. In order to verify whether the beneficial effect of mild sulfonation occurs in the liquid or solid phase, the substrate after sulfonation were extensively washed prior to enzymatic hydrolysis and hydrolysis yield of this washed substrate was compared to that of the unwashed substrate. If the improvement in hydrolysis was due to the sulfonation of soluble phenolics released during enzymatic hydrolysis, the washed substrate, which contained no free sulfite, was not expected to enhance the enzymatic hydrolysis. However, the hydrolysis profiles of both the substrates were fairly similar indicating that sulfonation almost exclusively occurred to the lignin present in the solid phase (Figure 34). In addition, when the sulfonated substrate was subsequently washed and again sulfonated, the second step of sulfonation did not seem to influence the hydrolysis yield indicating that the first sulfonation had already irreversibly reacted with the available lignin sites (Figure 34).

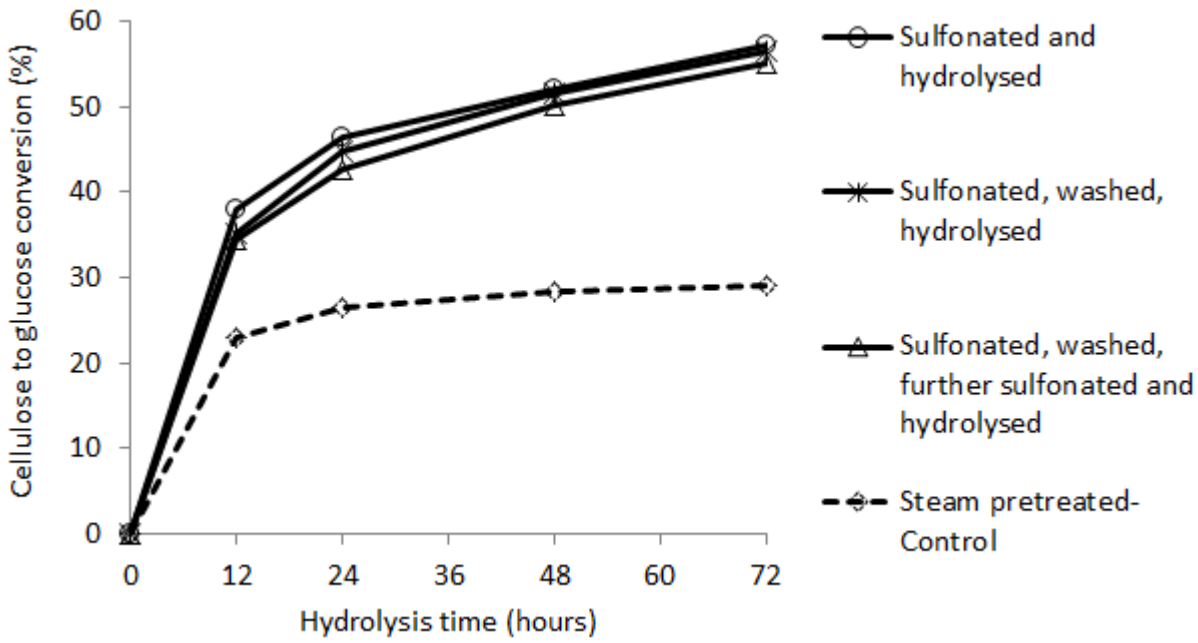
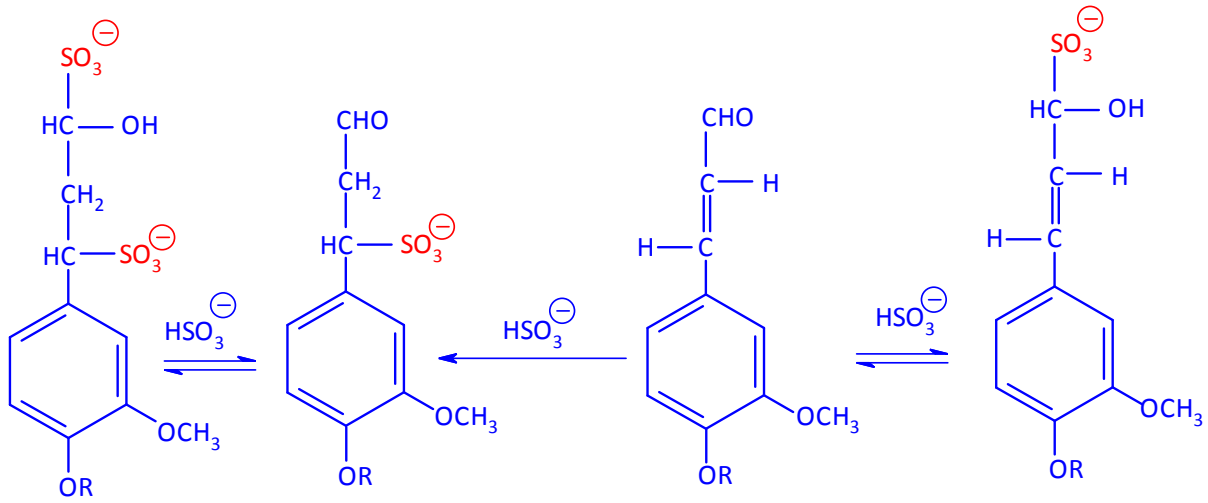


Figure 34 Influence of washing subsequent to sulfonation at milder conditions on the enzymatic hydrolysis steam pretreated softwood. Error bars represent standard deviations from the mean. Error bars represent deviations from the mean (n=2 for enzymatic hydrolysis).

As was discussed earlier, the effectiveness of sulfonation to swell the substrate and enhance cellulose accessibility does not depend much on its ability to delignify the wood, but rather on the manner by which it modifies the lignin present in the substrate. Depending on the conditions, the lignin can be sulfonated to different extents, resulting in the formation of sulfonated lignin and also in some lignin fragmentation (Beatson et al., 1984). It appears that some of the lignin structures present in steam pretreated softwood are readily sulfonated. Steam pretreatment of lignin can result in increased carbonyl groups on the side chain (Li et al., 2007) (for example, Hibbert's ketones type compounds formed during steam pretreatment). The lignin side chain structures will also have a higher aliphatic hydroxyl group substitution as a result of the cleavage of α-O-4 linkages (Hemmingson, 1983). The demethylation of methoxylated phenyl propane units can also lead to the formation of multiple hydroxyl groups in the same benzene ring (Hemmingson, 1983), which can readily form quinonoid structures susceptible to a fast sulfonation. It has been shown

that some phenolic guaiacyl derivatives such as guaiacyl methyl carbinol, guaiacyl glycerol, vanillyl alcohol and p-hydroxy phenyl derivatives can become sulfonated relatively easily and quantitatively at all pHs, even when the reaction mixture is not even stirred (Erdtman et al., 1950a). Therefore, simple quinonoid structures and carbonyl structures of the lignin side chain of the steam pretreated substrate seem to have sulfonated rapidly as illustrated in Scheme 4. It should be noted that quinone methide structures were reported to result in the unproductive binding of proteins/amino acids (Sewalt et al., 1996) and sulfonation of these structures can likely minimise the impact of unproductive binding on enzymatic hydrolysis (Shorygina and Elkin, 1970; Sarkanen and Ludwig, 1971; Lundquist et al., 2010; Erdtman et al., 1950b).

a



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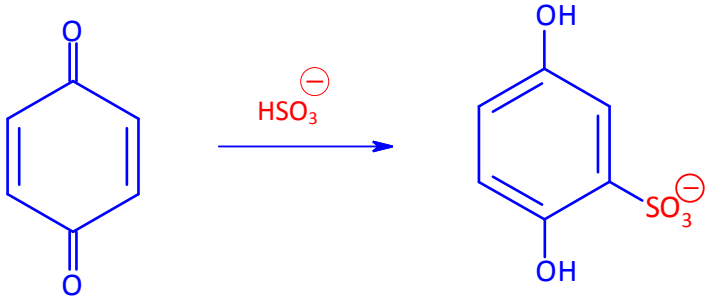
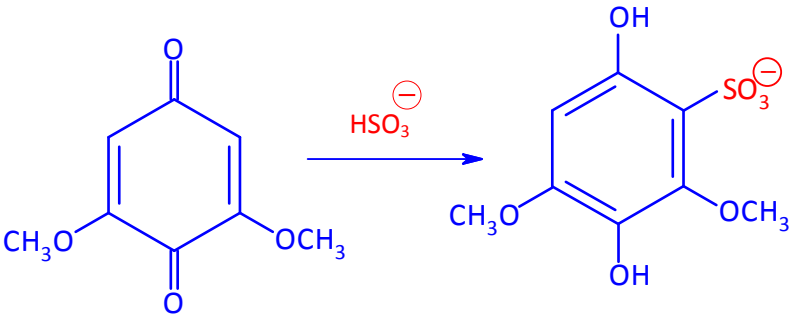
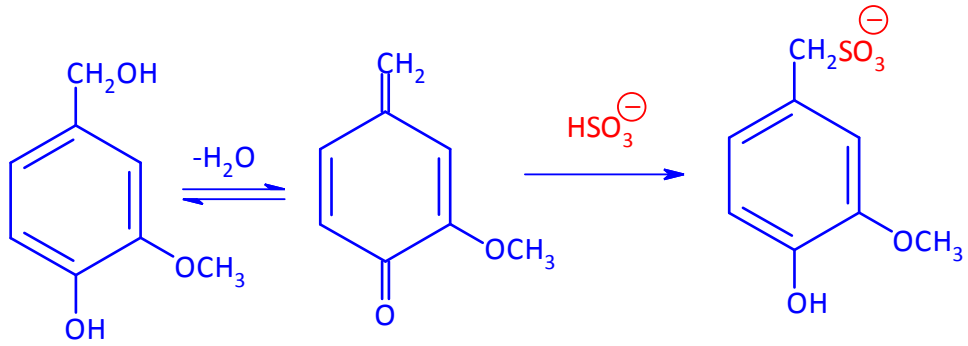




c



Scheme 4 Possible sulfonation reactions of different lignin moieties under milder conditions a) sulfonation of the conjugated side chain and carbonyl structures b) quinonoid structures c) phenolic units with α-hydroxyl groups.

3.4.2.2 High temperature sulfonation of steam pretreated softwoods

It appears that at mild conditions, some of the lignin moieties, particularly those present on the fibre surface are sulfonated, increasing the hydrolysis yield. However, hydrolysis is still incomplete and enhancing the sulfite loading beyond 8% did not enhance the enzymatic hydrolysis perhaps due to the lack of available reactive sites on the lignin for additional sulfonation. However, at high temperatures, the degree of sulfonation is expected to be higher since the conditions can potentially provide the activation energy required both to sulfonate some of the lignin structures and also create more reactive sites. It was expected that the degree of sulfonation could be enhanced to a point where the lignin's restrictions on cellulose accessibility can be largely eliminated. Several past studies

have shown that changing the process variables can change the degree of sulfonation of a refined mechanical pulp (Chagaev et al., 2005; Konn et al., 2002; Stevanic and Salmen, 2008; Ahmed, 1994). In the previous chapter, it was shown that at a 16% sodium sulfite loading, 160°C and 1 hour, these conditions resulted in more than a 60% sugar yield at 5 FPU/g glucan. Therefore, different processing conditions around this range were next evaluated to try to find the optimum conditions for sufficient sulfonation of the lignin structures. Temperatures ranging from 120 – 180°C with reaction times from 30 - 120 minutes at various sulfite concentrations from 4 – 48% were used.

When the chemical composition of the sulfonated substrates was assessed, a similar lignin or glucan content was detected despite the different conditions used (Table 13). However, it was apparent there were significant differences in the yields especially when the severity of the process was enhanced. Since applying more than 1 hour of sulfonation at 180°C lead to a considerable destruction of carbohydrates, a severity beyond this point is considered to be less suitable as a post-treatment (Table 13).

Table 13 The solids yield and chemical composition of some of the selected sulfonated substrates (% dry weight).

Sulfonation conditions			Solids yield (%)	Glucan (%)	Lignin (%)
Chemical loading (%)	Temperature (°C)	Time (Minutes)			
8	180	30	90.3 (0.4)*	55.3 (0.7)	41.5 (0.4)
16	180	120	81.6 (0.7)	54.9 (0.3)	42.8 (0.1)
8	160	60	91.0 (0.3)	54.7 (0.5)	42.8 (0.5)
48	160	60	90.5 (0.9)	54.1 (0.8)	40.1 (0.5)
8	120	30	96.9 (0.7)	54.8 (0.2)	42.9 (0.1)
48	120	60	97.4 (0.5)	55.0 (0.2)	41.8 (0.8)
*The numbers in the bracket represent standard deviations from the mean (n=2 for compositional analysis).					

The more severely sulfonated substrates were more readily hydrolysed at an enzyme loading of 10 FPU/g glucan when compared to the substrates, which had been treated at milder conditions (Figure 35). It was clear that temperatures ≥160°C were required to increase the hydrolysis yield to greater than 90% (Figure 35). Treatments at both 120 and 140°C did not result in complete hydrolysis, despite a continued increase in the reaction time and chemical loading (Figure 35 and Figure 36). Therefore, it appeared that the extent of improvement due to increase in chemical loading or reaction time was critically dependent on the temperature (Figure 35). At 120°C, the maximum hydrolysis yield of 71% was obtained with a chemical loading as low as 8% (Figure 35). Further enhancement in chemical loading had a negligible impact on the sugar yield. Similarly, at 140°C, increasing the chemical loading from 8 to 48% had a marginal influence on enzymatic hydrolysis. At 160°C, a more significant improvement was observed with an increase in chemical loading from 8 to 32% (Figure 35). At 180°C, a chemical loading of 8% was adequate to obtain near complete enzymatic hydrolysis.

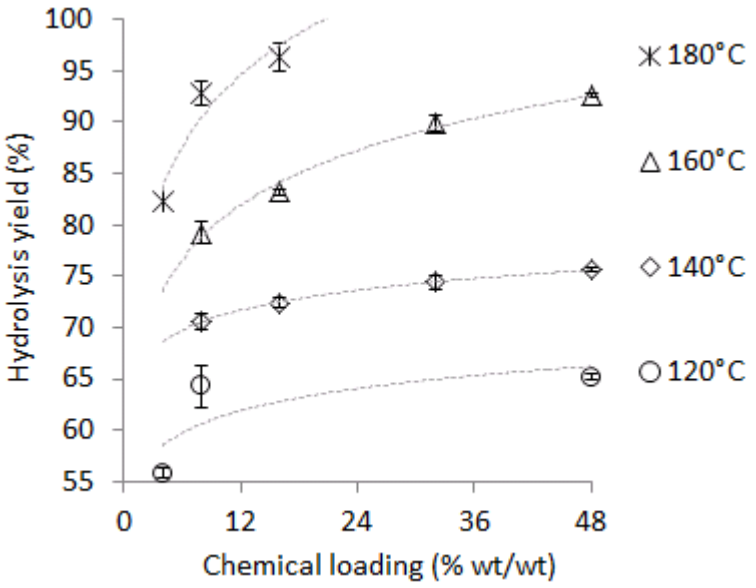


Figure 35 Influence of temperature and chemical loading on the enzymatic hydrolysis of sulfonated substrates (reaction duration: 60 minutes). Error bars represent standard deviations from the mean. Each sulfonation condition was a single run and n=2 for enzymatic hydrolysis.

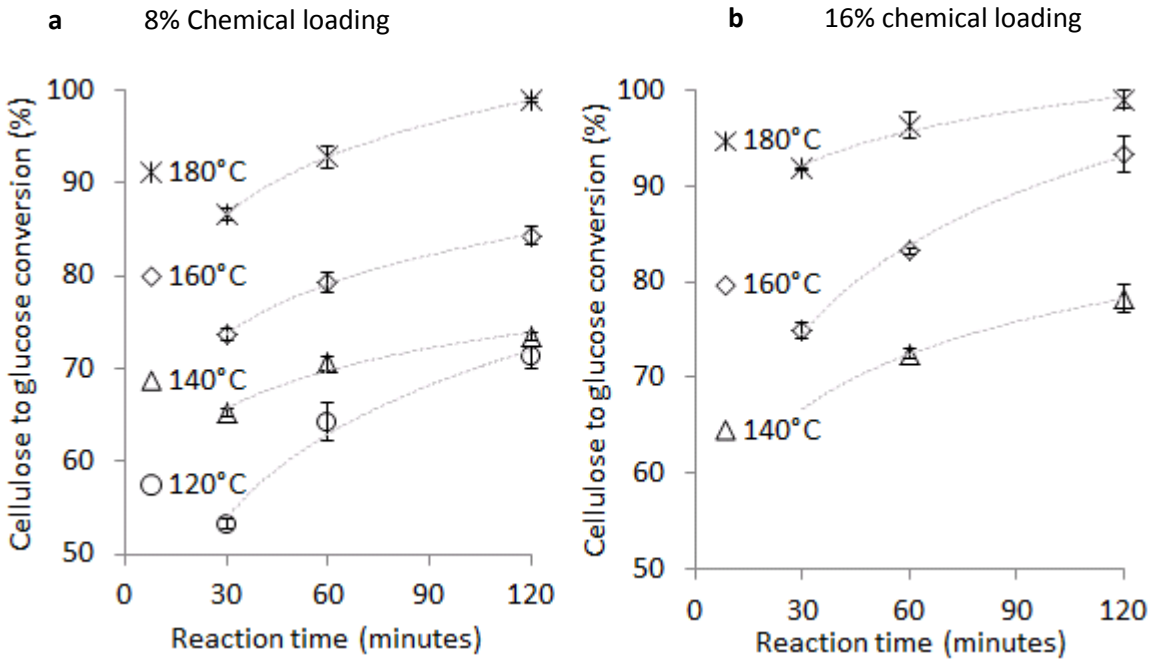
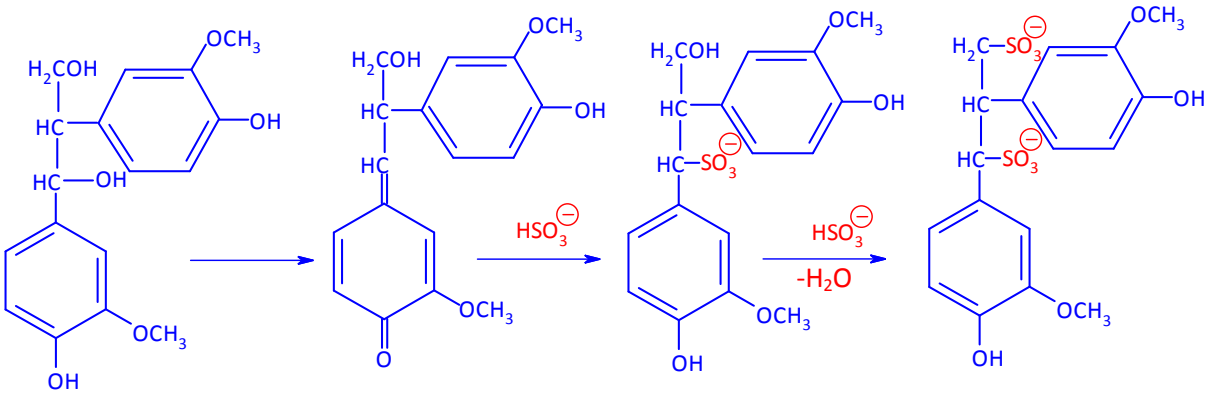
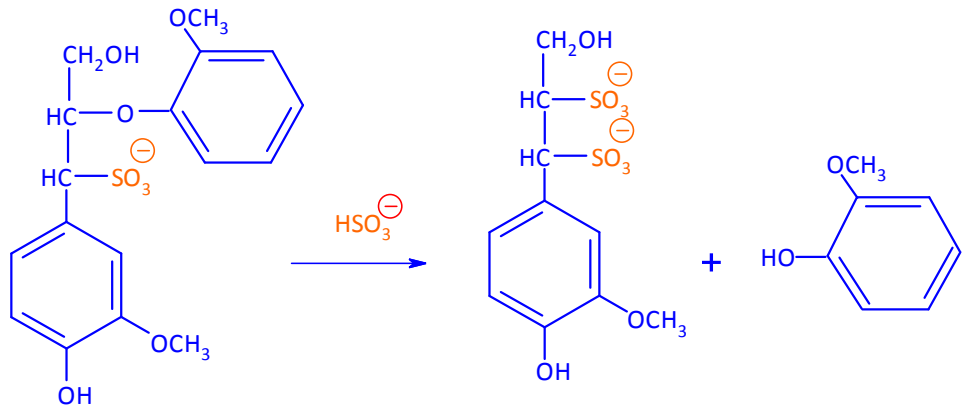


Figure 36 Influence of temperature and reaction time on the enzymatic hydrolysis of sulfonated substrates at, a) 8% sodium sulfite loading b) 16% sodium sulfite loading. Error bars represent standard deviations from the mean. Each sulfonation condition was done in singlet and n=2 for enzymatic hydrolysis.

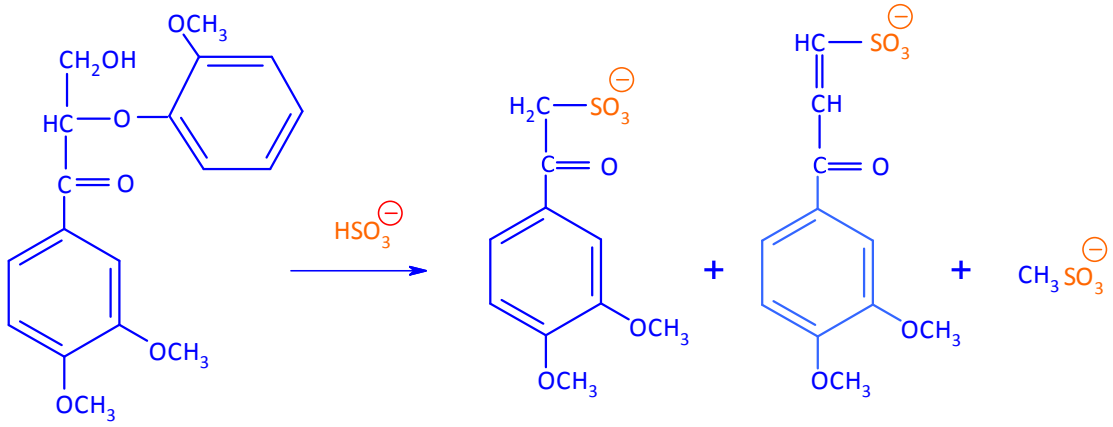
Therefore, at lower temperatures (120 – 140°C), the tendency of the hydrolysis yield to level off and be incomplete, despite a continued increase in reaction time or chemical loading, was likely be due to threshold levels of sulfonation and the degree of cellulose accessibility achieved. There appears to be a limited number of available sites for sulfonation on the steam pretreated lignin. In previous work Heitner et al (1982) reported that there was a maximum attainable sulfonate content when treating mechanical pulp. They suggested that it was a function of sufficient sulfite to sulfonate the available sites (and that the temperature used also had an influence). They reported that a maximum sulfonate content of around 1.5% and 2% respectively for 80°C and 140°C indicating an initial rapid sulfonation followed by an extremely slow rate of sulfonation, which apparently required higher activation energy (Beatson et al., 1984; Erdtman et al., 1950a; Lindgren, 1951). It seems that similar mechanisms also occurred with the steam pretreated substrates. An initial rapid improvement in hydrolysis yield was observed even at mild conditions of sulfonation with subsequent improvement requiring much higher temperatures. The formation of reactive sites at higher temperature was likely partially due to the better diffusion of the sodium sulfite to sulfonate the lignin. It was also likely that some of the lignin structures require higher activation energy for sulfonation to occur (Beatson et al., 1984; Kokta and Ahmed, 1999). It should also be noted that higher severities can lead to the cleavage of more lignin units creating more reactive sites. The initial formation of α-sulfonic acids (Scheme 5) can be followed by β-aryl ether cleavage (Beatson et al., 1984; 1985). This reaction will result in the formation of α-β-disulfonic acids and new phenolic structures, which can be further sulfonated (Scheme 6). Subsequent elimination of α-sulfonic acid group can form styrene-β-sulfonic acid structures. In the non-phenolic unit, the α-carbonyl can facilitate sulfitolytic cleavage at the β-carbon in the same manner as the α-sulfonic acid group in the phenolic units (Scheme 7).



Scheme 5 Sulfonation reactions on α and γ carbon atoms of the lignin side chain



Scheme 6 Sulfitolyic cleavage of β-aryl ether linkages in the α-sulfonic acid structures



Scheme 7 Sulfitolytic cleavage of non-phenolic units in lignin with α-carbonyl groups

The two best reaction conditions, which resulted in >90% hydrolysis yields while using the least chemical input were 160°C, 120 minutes, 16% chemical loading, and 180°C, 30 minutes, 8% chemical loading. These conditions resulted in 94% and 90% (at 10 FPU/g glucan) hydrolysis yields respectively. These two substrates were subsequently hydrolysed at different enzyme loadings and their hydrolysis yields were compared to those of the corresponding steam pretreated substrates (Figure 37). Both the substrates were completely hydrolysed at an enzyme loading >10 FPU/g glucan, while less than half of the cellulose present in the steam pretreated substrates were hydrolysed despite the higher enzyme loading (15 FPU/g glucan) employed.

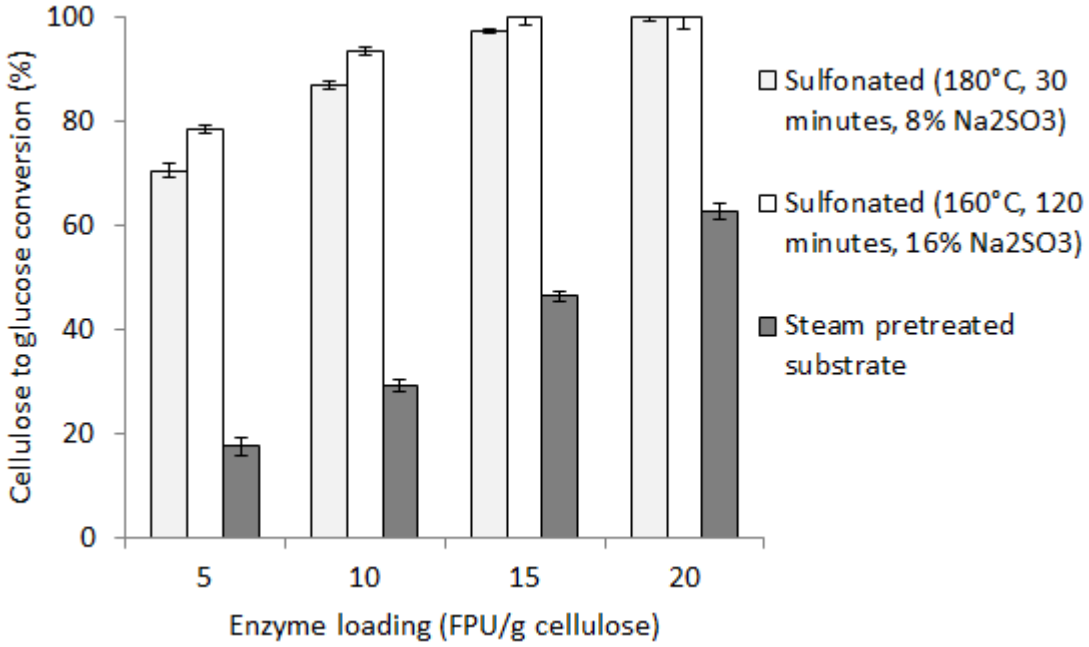


Figure 37 Influence of high temperature sulfonation on the enzymatic hydrolysis of steam pretreated softwood at various enzyme loadings. Error bars represent standard deviations from the mean. Sulfonation was done in singlet and n=2 for enzymatic hydrolysis.

There were two important variables which were not directly addressed in this study but which might potentially help increase the extent of sulfonation while enabling us to reduce the sulfite loading or the energy requirements. First is the change in pH and second is ensuring a better diffusion of the chemicals (Beatson et al., 1985; Heazel and Mcdonough, 1988; 1991). When the liquor pH is more towards the alkaline range, the nature of the

sulfonation is expected to be different from neutral sulfonation conditions since the reaction medium has two principal nucleophiles, hydroxyl and sulfite ions (Gierer, 1985). The extent of sulfitolytic β-aryl ether cleavage and of the subsequent conversion into styrene-β-sulfonic acid structures increases with increasing pH. At slightly alkaline pH, elimination of water and formaldehyde from the side chain lead to the formation of conjugated structures, which can be readily sulfonated. This results in acceleration of α-ether cleavage if any are left after steam pretreatment. The elimination of water from the resulting α-β-disulfonic acid structures and subsequent sulfonation at the γ carbon can lead to the formation of trisulfonic acid. In addition to sulfonation, ionization of lignin phenolates at alkaline pH can also reduce the number of hydrogen bonds and loosen the lignin structure (Heazel and Mcdonough, 1991). All of these reactions can substantially influence cellulose accessibility.

3.4.3 Conclusions

It appears that there are primarily two phases in the sulfonation of steam pretreated softwoods. The initial phase can readily occur under milder conditions where the carbonyl structures of the lignin side chain are sulfonated at the easily accessible reactive sites. This initial sulfonation almost doubled the enzymatic hydrolysis at low enzyme loadings. However, a significant amount of the cellulose still seemed to be inaccessible to the enzymes. The second phase of sulfonation requires substantially higher activation energy (160 – 180°C). This high temperature sulfonation seemed to enhance the enzymatic hydrolysis to completion even at relatively low enzyme loadings while enabling us to reduce the sulfite loading to 8%.

3.5 To determine the potential of pellets as a feedstock for the biomass-to-sugars process

3.5.1 Introduction

Depending on the scale and availability of biomass, wood/pulp chips and wood pellets are likely to be the two most tradable feedstocks used for a forest based biorefinery process. Wood or pulp chips are typically used as the feedstock for most of the world's pulp and paper industry with older mills handling about 1,000 tonnes /day of chips and the biggest, newer mills in places such as those in South America able to handle about 5,000 tonnes/day (Stephen et al., 2010). Larger scale processing of saw dust or wood/pulp chips is constrained by several factors. However, one of the biggest challenges is that the lower bulk density (125 – 200 kg/m^3) and higher moisture content (~50%) of pulp chips limits the economics of moving this type of feedstock over significant distance. Most mills locally access their wood/pulp chip feedstock. In contrast, biomass pellets are traded globally with the densification of wood derived sawdust being one of the main reasons that the economics of shipping this material halfway-around-the-world can be justified (Richard, 2010; Stephen et al., 2010).

Twenty years ago, it would have been difficult to imagine the export of wood pellets from Canada to Europe or Asia. However, a combination of higher oil prices, a desire for relief from oil import dependency and carbon reduction strategies of several nations in Europe have made the co-firing of biomass for electricity production more economically and socially desirable. In the last 20 years, global wood pellets production has increased from zero to 16 million tonnes (Bradley et al., 2011). Canada, together with the USA, are the world's largest pellet exporters exceeding 5 million tonnes in 2011 with British Columbia being the major exporter of pellets, most of which is exported to Europe over a distance of 16,000 kilometers (Bradley et al., 2011). Wood pellets mainly find applications in heat and electricity generation. Although densified biomass holds potential as a feedstock for large scale biorefinery to benefit the economies of scale (Stephen et al., 2010), their suitability for biochemical conversion has not yet been investigated.

It is possible that the pressure, temperature and moisture reduction required to make a stable, transportable pellet would increase its recalcitrance to biochemical conversion processes. For example, pellets are much drier than wood/pulp chips and the heat and pressure generated during drying and pelletisation might result in irreversible aspiration of the pit pores and reduction in the permeability of the wood fibres (Usta and Hale, 2006). This phenomenon would create a mass transfer limitation during steam pretreatment as well as influencing the enzymes' accessibility to cellulose in the subsequent enzymatic hydrolysis of the cellulosic component. In addition, the temperature during drying and pelletisation is generally close to the glass transition temperature of lignin. It has been reported (Biswas et al., 2011; Irvine, 1984; Kaliyan and Morey, 2010) that lignin partially softens and flows during pelletisation and acts as a good binder between the particles resulting in an increased hydrophobicity of the pellets. Thus, it is likely that the melting and surface distribution of lignin will also influence the efficiency of pretreatment and subsequent enzymatic hydrolysis. Therefore, it is important to study the influence of pelletisation on the suitability of softwoods for steam pretreatment and subsequent enzymatic hydrolysis.

In the work reported in Chapter 3.1 we were able to show that various softwoods species can be readily steam pretreated and enzymatically hydrolysed to sugars (Ewanick et al., 2007). We found that the softwood species (Lodgepole pine, Douglas-fir and Norway spruce) behaved similarly during steam pretreatment and subsequent enzymatic hydrolysis. However, a post-treatment to either modify or remove lignin was necessary to obtain fast and efficient cellulose conversion when low enzyme loadings were used. As most of the pellets made in North America are sourced from softwoods (Spelter and Toth, 2009), we wanted to determine whether softwood pellets would prove to be a more difficult to process feedstock when compared to wood/pulp chips. Both commercial softwood pellets and wood chips were steam pretreated at three different steam pretreatment severities which has been previously shown to result in effective overall sugar recovery and good cellulose hydrolysis. We subsequently assessed whether a post-treatment step was also

necessary to obtain a fast and complete enzymatic hydrolysis of the cellulosic component of pretreated pellets.

3.5.2 Results and discussion

3.5.2.1 Influence of pelletisation on the chemical composition of softwood chips

Typically, pelletisation involves the three major steps of; drying the biomass particles to a moisture content of less than 10%; grinding the wood chips to an appropriate size (2-3 mm); and compacting these particles to form the densified pellet or briquette. To make pellets after drying, the biomass is compressed in the pelletiser by small rotating rolls through open-ended cylindrical holes (dies) within the periphery of a ring. The friction between the fed-in particles and the die results in particles that are compressed against each other to form pellets (Kaliyan and Morey, 2010). This process generates heat (normally in the range of 80 – 120°C), due to the friction between the feed particles and the wall of the die (Kaliyan and Morey, 2010). This temperature is much lower than the temperature and pressure normally encountered during a typical steam pretreatment (180 – 220°C) used for bioconversion, where the degradation of some of the carbohydrates especially hemicellulosic sugars is inevitable (Clark et al., 1989; Ewanick et al., 2007). Nevertheless, some degree of carbohydrate degradation was also anticipated during the pelletisation process.

High temperatures and pressures, which are encountered during the pelletisation process, also result in the partial softening of the surface lignin and facilitate the binding of biomass particles, which consequently form a solid bridge upon cooling (Kaliyan and Morey, 2010). The low thermosetting properties and low melting point (70 - 120°C) of lignin means that they play an active role in these binding phenomena (Irvine, 1984; Kaliyan and Morey, 2010). In some cases, the die is preheated to the desired temperature levels to ensure good binding. During this softening and redistribution process it was also anticipated that some of the wood components could form pseudo lignin due to the reaction between lignin and the extractives or the sugar degradation products during pelletisation, resulting in an apparent increase in detectible lignin (Sannigrahi et al., 2011).

To determine whether grinding, drying and pelletisation had any impact on the chemical composition of the pelletised material, its material balance was compared to that of typical softwood pulp/wood chips (Table 14). Surprisingly, it appeared that pelletisation did not have a significant impact on the chemical composition of the feedstock (Table 14). Despite some minor variations in the individual sugar components, the overall carbohydrate content of the wood chips and pellets was quite similar i.e., ~67% while the lignin and extractive content of the pellets was slightly higher when compared to the chips. As the overall pelletisation process was quite rigorous and might in itself prove to be a form of pretreatment, pellets and chips were first milled to a 40 mesh size and subjected to enzymatic hydrolysis at an enzyme loading of 20 FPU/g glucan (Table 14). After 72 hrs only 8 and 13% of the cellulose present in respective chips and pellets was hydrolysed indicating that the pelletisation process alone did not cause enough disruption of the lignocellulosic matrix to result in reasonable hydrolysis yields.

Table 14 Chemical composition of the softwood chips (Douglas-fir) and softwood pellets before steam pretreatment and their ease of enzymatic hydrolysis before pretreatment.

	Wood chips	Pellets
Lignin	29.8 (0.8)**	30.5 (0.3)
Arabinan	1.2 (0.0)	1.3 (0.1)
Galactan	2.2 (0.0)	2.8 (0.1)
Glucan	47.3 (0.4)	51.9 (2.6)
Xylan	4.4 (0.1)	3.7 (0.2)
Mannan	11.7 (0.1)	7.3 (0.3)
Extractives	1.1 (0.4)	3.5 (0.3)
Enzymatic hydrolysis yield after milling* (%)	8.3 (1.2)	12.7 (0.9)
*Enzyme loading: 20FPU/g glucan, Duration: 72 hours **Numbers in the bracket represent standard deviations from the mean (n=3)		

3.5.2.2 Steam pretreatment of softwood chips and pellets

As it appeared that pellets would also require some sort of pretreatment, we next compared their response to three sets of steam pretreatment conditions (low, medium and high, with the severities in the range Log Ro = 3.34 – 3.94). Depending on the physical properties of the feedstock (particle size, moisture content etc.), severities in this range

have been previously shown to result in good hemicellulosic sugar recoveries in the water soluble fraction while obtaining a water insoluble cellulosic rich fraction that was more amenable to enzymatic hydrolysis (Cullis et al., 2004; Ewanick et al., 2007). Surprisingly, the steam pretreatment of softwood-derived chips and pellets resulted in similar product yields (Figure 38 & Table 15) and relatively good hydrolysis of the cellulosic fraction (Figure 39).

Depending on the severity applied, the recovery of original hemicellulosic sugars in the water soluble component was 53-77% for chips and 47-72% for pellets (Figure 38). As was expected, the amount of the hemicellulose sugars that could be recovered decreased with the increasing severity of pretreatment (Figure 38). The sugar recovery in the water soluble fraction was found to be slightly lower for the pellets compared to wood chips and this trend was consistent for all the three severities studied (Figure 38). It is possible that the lower moisture content of the pellets (~5%) compared to wood chips (8%) resulted in undesirable side reactions, such as pyrolysis, leading to slightly lower hemicellulose derived sugar recovery in the water soluble fraction. Previous work has shown that, when dry wood chips are heated up rapidly (compared to the green chips), pyrolysis was the likely reaction that resulted in the destruction of the hemicellulose derived sugars (Brownell and Saddler, 1987; Tomasik et al., 1989)). It has also been shown that at a lower moisture content, the chips did not adsorb the SO_2 as effectively, limiting its further conversion to sulfurous and then sulfuric acid (Brownell and Saddler, 1987; Ewanick and Bura, 2011; Schwald et al., 1989). Only 45±3% of the added SO_2 was retained by the pellets when compared to 58±1% SO_2 absorption by the chips. It should also be noted that, after they had been disintegrated, the pellets had a significantly lower particle size (2- 3 mm) when compared to wood chips (20×40 mm). Previous work (Cullis et al., 2004) has shown that a smaller particle size and lower moisture content will increase the relative severity when Douglas-fir wood chips were pretreated (Cullis et al., 2004). As a result the lower moisture content and smaller particle size of the pellets probably lead to their slightly higher apparent pretreatment severity, when compared to wood chips, leading to a lower overall recovery of hemicelluloses derived sugars.

Enzymatic hydrolysis of the water insoluble cellulosic components at an enzyme loading of 20 FPU/g glucan resulted in reasonable glucose yields (>60%) for both medium and high severity treated wood chips and pellets (Figure 39). As was shown previously for wood chips, despite good hemicellulosic sugar recovery in the water soluble fraction, those substrates which were pretreated at low severities generally resulted in poor hydrolysis of the cellulose, even at relatively high enzyme loadings. The slightly higher apparent pretreatment severity that the pellets were exposed to is also reflected in the greater ease of enzymatic hydrolysis of the water insoluble cellulosic component (Figure 39). Despite the differences in hydrolysis yields, the total sugar released during pretreatment and enzymatic hydrolysis was very similar for both pellets and wood chips. Of the three severities that were compared, the medium severity resulted in the highest overall sugar recovery with 69% and 71% of total sugar released respectively from the chips and pellets after pretreatment and enzymatic hydrolysis.

Table 15 Chemical composition of the water insoluble cellulosic component after the steam pretreatment of softwood chips and softwood pellets pretreated at three different severities (% dry weight)

		Lignin	Glucan	Xylan	Mannan
L*	Wood chips	37.9 (0.8)**	60.2 (1.4)	1.1 (0.2)	1.7 (0.0)
	Pellets	41.3 (1.1)	56.1 (0.8)	BDL	0.4 (0.2)
M	Wood chips	43.3 (0.6)	53.4 (0.7)	0.7 (0.2)	1.3 (0.1)
	Pellets	44.1 (1.2)	52.7 (1.3)	BDL***	BDL
H	Wood chips	44.7 (0.6)	55.0 (2.1)	BDL	BDL
	Pellets	44.2 (1.5)	52.3 (0.4)	BDL	BDL

* L, M and H refer to low, medium and high severity steam pretreatment respectively
** Numbers in the bracket represent standard deviations from the mean (n=3)
*** below detectable level

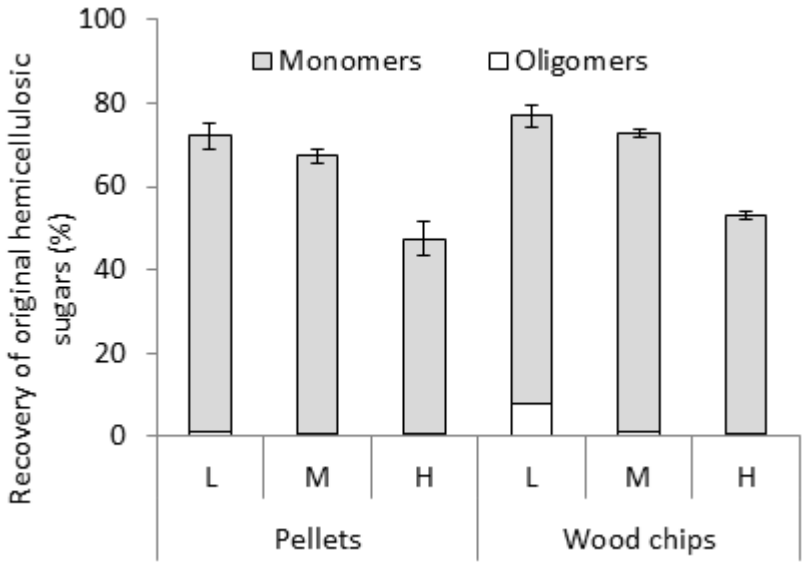


Figure 38 Recovery of original hemicellulosic sugars (arabinose, galactose, xylose and mannose) in the water soluble fraction after the steam pretreatment of softwood chips and pellets pretreated at three different severities. L, M H represents three different pretreatment severities (Log Ro), 3.3, 3.6 and 3.9 respectively. Error bars represent standard deviations from the mean (n=2 for the analysis of water soluble fractions).

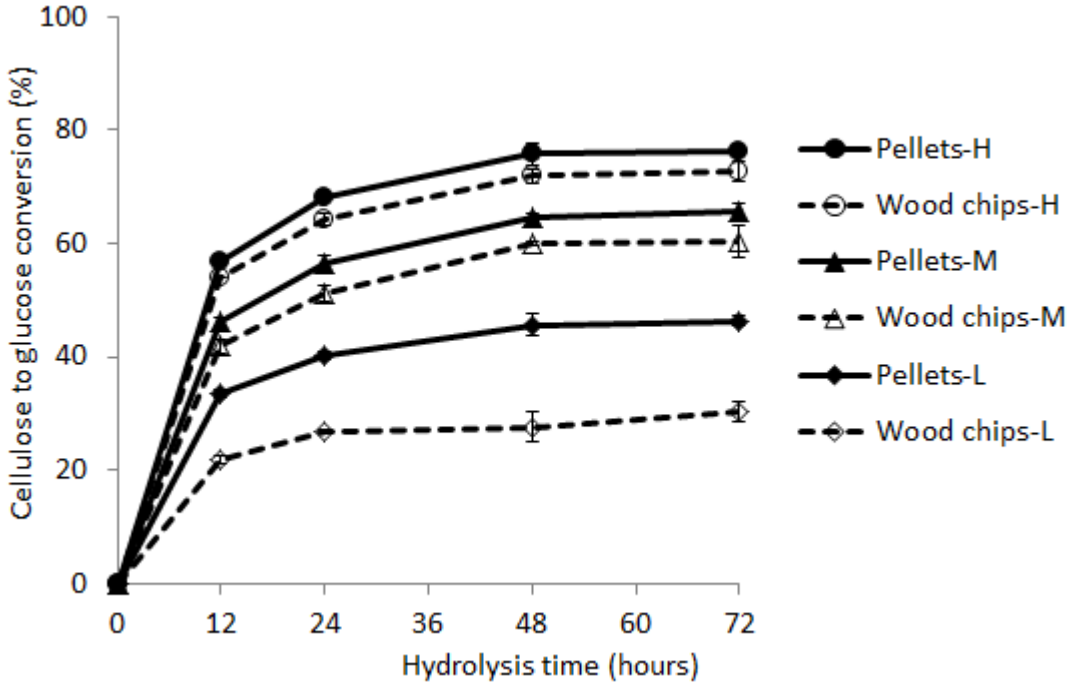


Figure 39 Enzymatic hydrolysis of the water insoluble cellulosic component of steam pretreated softwood chips and pellets pretreated at three different severities. Enzyme loading: 20 FPU cellulase and 40 CBU betaglucosidase/g glucan. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis).

3.5.2.3 Influence of the initial moisture content on the sugar recovery from pretreated pellets

As mentioned earlier, a biomass substrate which has a moderate moisture content can readily take up added SO_2, resulting in minimal sugar decomposition and a greater overall sugar recovery in both the water soluble and water insoluble fraction after pretreatment (Ewanick and Bura, 2011). To see if we could further improve sugar recovery from pellets we presoaked them in water, adjusted the pellets to two different moisture contents and then impregnated them with SO_2 prior to steam pretreatment. It was hoped that the increased moisture content would minimise hemicellulose degradation as well as open up the cellulosic substrate, thus enhancing accessibility to the cellulase enzymes.

To ensure sufficient water penetration, the pellets were immersed in water overnight and subsequently vacuum filtered to remove any excess water. The resulting disintegrated pellets had a moisture content of approximately 46% (wt/wt on a wet weight basis). Subsequent air drying of this material for 24 hours reduced the moisture content to ~18% (wt/wt on a wet weight basis). It was apparent that, by increasing the moisture content of the pellets, the SO_2 uptake increased dramatically with SO_2 absorption increased from 45±3% for the regular pellets to 81±3% for the soaked pellets which had been subsequently dried back to 18% (wt/wt) moisture content. Increasing the moisture from 5% to 18% resulted in significant increase in the recovery of hemicellulosic sugars in the water soluble fraction to 86%. Despite, the possible reduction in the severity of pretreatment due to the additional moisture, the water insoluble cellulosic fraction appeared to be as readily hydrolysed as were the original pellets (Figure 40b). Previously it had been shown that, when moisture content was increased, more of the steam's energy is used to heat up the water within the biomass substrate (Brownell and Saddler, 1987; Cullis et al., 2004; Tucker et al., 2003). Despite this apparent reduction in severity, the increased enzymatic hydrolysis was likely due to the opening up of the cellulose structure at the fibre level due to increased water penetration (Brownell and Saddler, 1987). The increased SO_2 uptake will also have facilitated enhanced cellulose accessibility and consequently its improved hydrolysability (Clark et al., 1989). Although increasing the moisture from 18 to 46% did not significantly

increase SO_2 retention, it did seem to have reduced the severity of the pretreatment resulting in lower enzymatic hydrolysis and thus the overall sugar yield (Figure 40a and b).

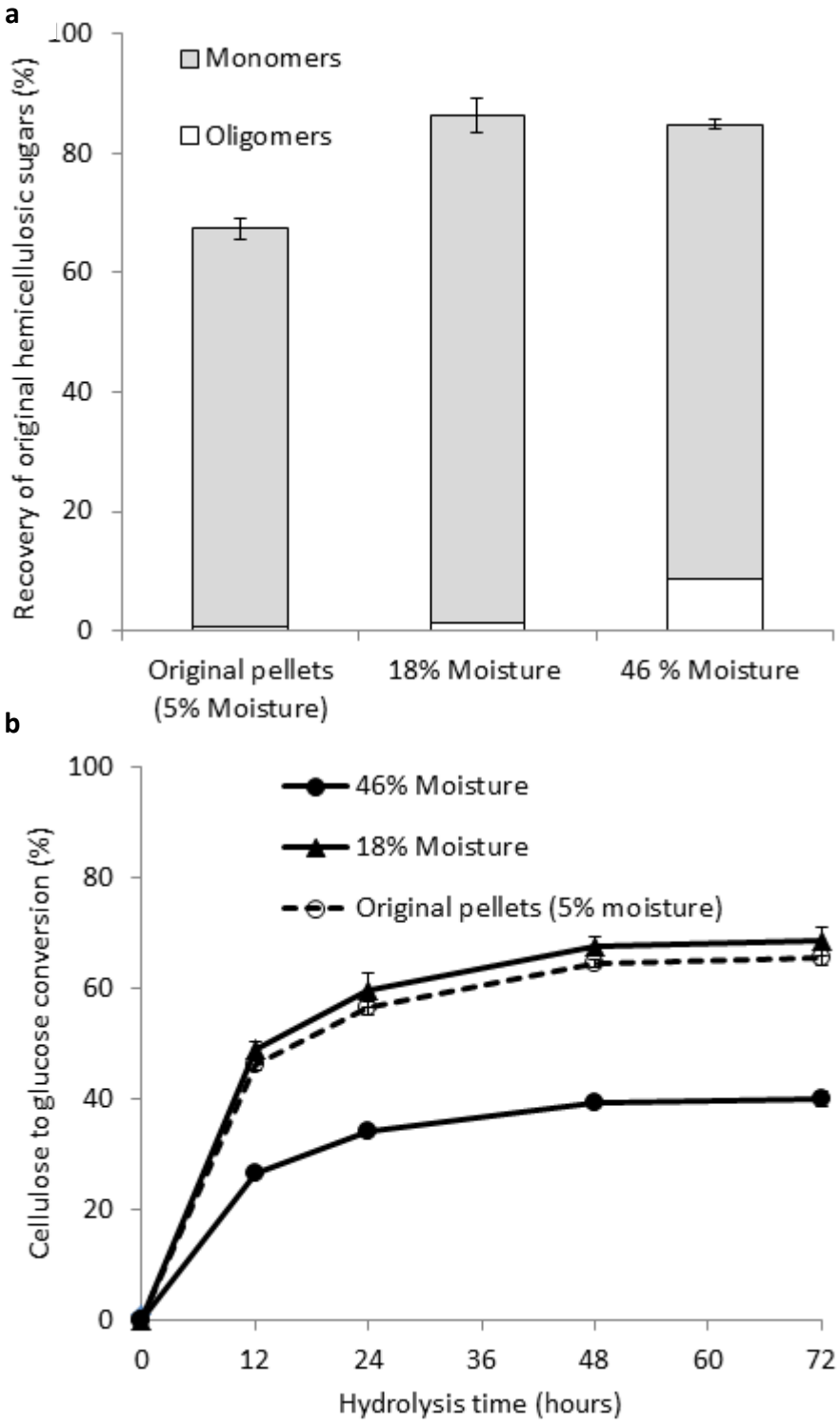


Figure 40 Influence of increasing the initial moisture content of softwood pellets on steam pretreatment's (200°C, 5 minutes 4% SO_2) ability to, a) recover the hemicellulose derived sugars in the water soluble fraction b) enhance the enzymatic hydrolysis of water insoluble cellulosic component at an enzyme loading of 20 FPU cellulase and 40 CBU beta-

glucosidase /g glucan. Error bars represent standard deviations from the mean. Hemicellulose refers to the sum of arabinan, galactan, xylan and mannan.

3.5.2.4 Can steam pretreatment prior to pelletisation facilitate improved pellet properties and subsequent bioconversion?

Despite a significant increase in density achieved by pelletisation, one of the major challenges, which the current pellet sector faces, is the lower durability of the pellets (Vinterback, 2004) and lower volumetric energy density compared to other energy commodities such as coal or crude oil (Kaliyan and Morey, 2010). Low durability results in breakage of pellets and consequent dust formation during handling. Broken pellets and dust aggravate problems associated with the loss of the material, health impacts and dust explosions (Vinterback, 2004). Applying steam pretreatment prior to pelletisation of saw dust has been reported to increase the durability and strength for the resulting pellets (Biswas et al., 2011; Lam et al., 2011). Therefore, in addition to comparing regular wood chips and pellets for their amenability to steam pretreatment and subsequent enzymatic hydrolysis, we also investigated whether a single steam pretreatment could be applied to facilitate both the improved stability of the pellets and its subsequent pretreatment and hydrolysis.

Generally, steam pretreatment is carried out at a low biomass to steam ratio, which typically results in very dilute slurry with liquid to solid ratio of greater than 7:1 (Ewanick et al., 2007; Sipos et al., 2010). This biomass to steam ratio, if used in a steam pretreatment prior to pelletisation, will require higher amounts of energy for drying the material prior to pelletisation. Therefore, we first adjusted the biomass to steam ratio to ensure that there was no free water available such that the resulting slurry could be directly dried and pelletised. To minimize water use, 220 g dry weight of wood chips were used instead of 50 g. Steam pretreatment (200°C, 5 minutes, 4% SO_2) at this higher biomass concentration resulted in a pretreated substrate with an initial moisture content of 60% (1:1.5 solid to liquid ratio). This material was subsequently dried and pelletised. It should be noted that a 60% moisture content prior to drying was close to the typical moisture content of green wood chips (~ 50% moisture).

The SO_2 catalysed steam pretreatment reduced the particle size of the wood chips from ~20×40 mm to <2 mm (Figure 41), allowing the pelletisation process to proceed without the need for a prior grinding step. This observation is worth noting as the energy inputs for steam pretreatment could likely be off-set by the savings from not having to grind the wood chips prior to pelletisation. Steam pretreatment of wood chips at the higher biomass to steam ratio resulted in a 68% recovery of hemicellulose derived sugars (Table 16). This recovery is slightly lower than what was obtained after pretreatment at the lower biomass to steam ratio, possibly due to the lower amount of steam applied. Surprisingly, subsequent drying followed by pelletisation had a minimal impact on sugar degradation and almost all of the sugars that were recovered after steam pretreatment survived subsequent pelletisation (Table 16). Freeze drying and oven drying followed by pelletisation, showed similar levels (65 - 68% of the original hemicellulose) of sugars were still detected in the water soluble component (Table 16) with most of the sugars (>98%) present in a monomeric form.

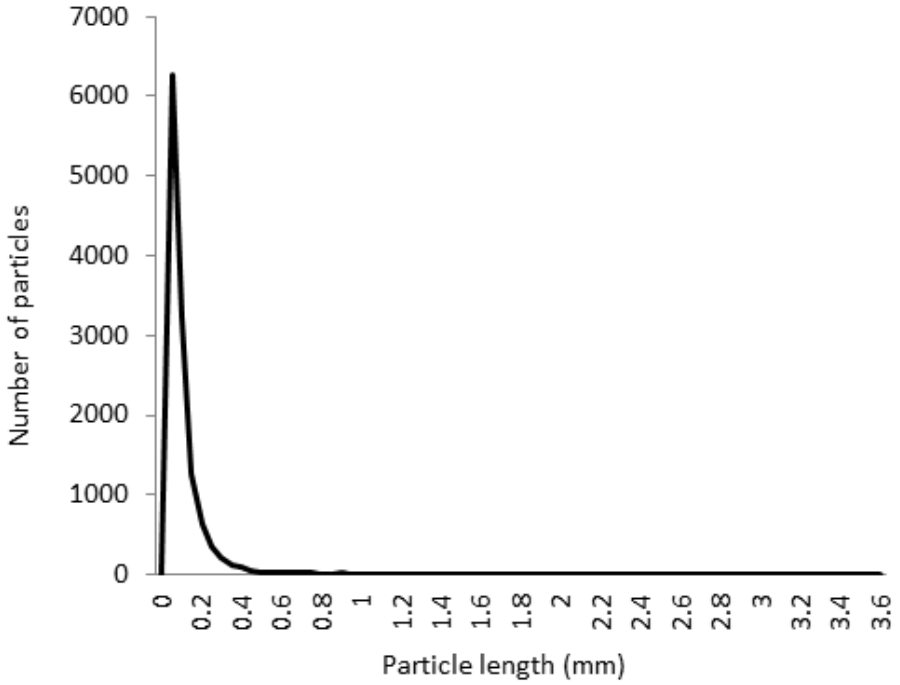


Figure 41 Particle size distribution of steam pretreated Douglas-fir (Pretreatment condition: 200°C, 5 minutes and 4% SO_2) as determined by Fibre Quality Analyser.

When the water insoluble, cellulosic rich fraction, before and after pelletisation were compared little hemicellulose was detected while the cellulose and lignin content was similar for both the pelletised and non-pelletised material (Table 17). Subsequent enzymatic hydrolysis of these water insoluble cellulosic components indicated that 64% of the cellulose of the never-dried steam pretreated substrate was hydrolysed while only 52-53% of the cellulose present in the dried and subsequently pelletised material was hydrolysed (Figure 42). The lack of difference in the hydrolysability between the oven and freeze dried material (Figure 42) was surprising. Previous work had shown that the mode of drying can have a significant impact on hydrolysis yield, likely due to fibre collapse and a reduction in enzyme accessibility (Esteghlalian et al., 2001; Luo and Zhu, 2010). However, in this earlier work, the pretreated material was first water washed and subsequently dried (Esteghlalian et al., 2001; Luo and Zhu, 2010). In the work reported here the pretreated material was dried without any prior water washing, meaning that greater than 20% of the dried material was still soluble sugars. It was possible that the soluble sugars present in the steam pretreated substrate minimised the collapse of the cellulose microfibrils and thus did not significantly reduce enzymatic accessibility. Therefore, we water washed the steam pretreated substrate, dried it to a ~10% moisture content and subjected it to enzymatic hydrolysis. As anticipated, the washed and dried material resulted in poor hydrolysis yield (26% after 72 hours) (Figure 43). This agrees with previous observations that the presence of soluble hemicellulose during drying can result in the preservation a considerable amount of the specific fibre surface area and restrict collapse of the swollen fibers (Köhnke et al., 2010).

Table 16 Influence of drying and pelletisation on the recovery of original carbohydrates after the steam pretreatment of softwood chips (expressed as g per 100 g starting material).

	Arabinan	Galactan	Glucan	Xylan	Mannan	Lignin
Untreated wood chips	1.2 (0.0)*	2.2 (0.0)	47.3 (0.4)	4.4 (1.0)	11.7 (0.1)	29.8 (0.8)
Steam pretreated substrate	0.6 (0.0)	1.8 (0.0)	44.1 (0.7)	2.0 (0.1)	8.9 (0.4)	32.1 (0.2)
Steam pretreated and subsequently freeze dried and then pelletised	0.6 (0.1)	1.9 (0.1)	43.7 (2.2)	1.9 (0.2)	8.3 (0.6)	31.4 (0.3)
Steam pretreated and subsequently oven dried and then pelletised	0.6 (0.1)	1.9 (0.0)	45.2 (0.8)	2.0 (0.1)	8.8 (0.3)	30.4 (0.9)
*Numbers in the bracket represent standard deviations from the mean (n=3).						

Table 17 Influence of drying and pelletisation on the chemical composition of water insoluble cellulosic component of steam pretreated wood chips (% wt/wt)

	Glucan	Xylan	Mannan	Lignin
Steam pretreated and water washed	53.9 (0.9)	0.1 (0.0)	0.4 (0.1)	46.8 (0.5)
Steam pretreated, freeze dried, pelletised and subsequently water washed	54.1 (0.6)	0.3 (0.0)	0.7 (0.1)	45.8 (0.8)
Steam pretreated, oven dried, pelletised and subsequently water washed	53.6 (1.0)	0.4 (0.0)	0.9 (0.2)	46.2 (0.3)
*Numbers in the bracket represent standard deviations from the mean (n=3).				

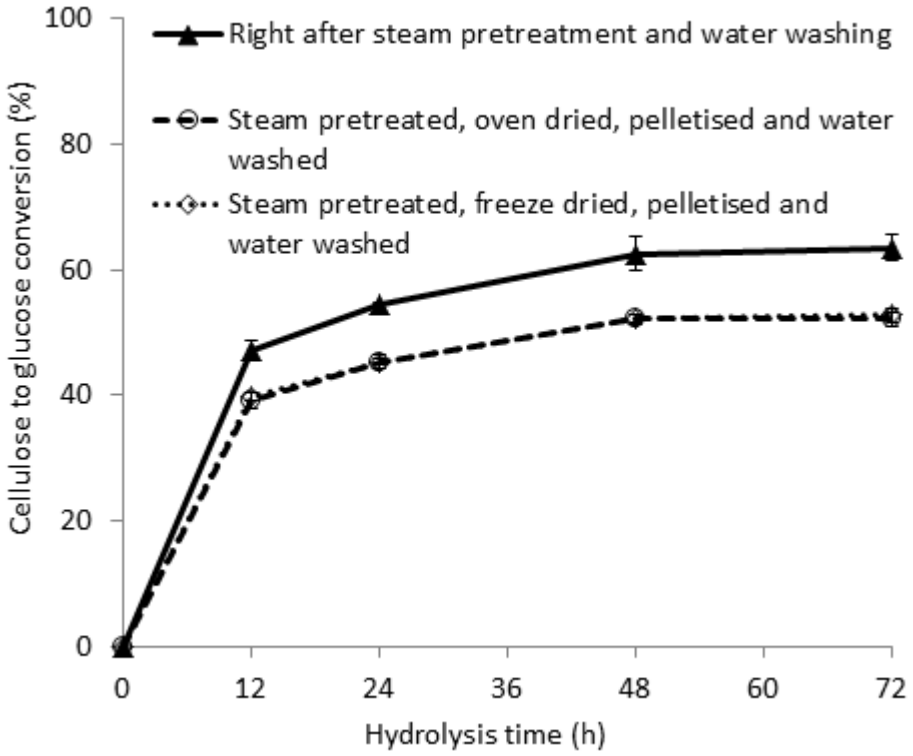


Figure 42 Enzymatic hydrolysis of water insoluble component right after steam pretreatment and after steam pretreatment, drying and pelletisation. Enzyme loading: 20 FPU Cellulase and 40 CBU beta-glucosidase/g glucan. Error bars represent standard deviations from the mean (n=3 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

It was apparent that steam pretreatment had the multiple benefits of reducing the requirement of a grinding step, producing a pellet that was likely more robust, retaining high levels of the original hemicellulose in a monomeric form while providing a substrate that could be as readily hydrolysed as steam pretreated wood chips, without the need for a subsequent steam pretreatment of the pellets. However, the total sugars recovered after steam pretreatment and enzymatic hydrolysis was 60-63% for the pelletised and 67% for never-dried and non-pelletised material respectively, even when high enzyme loadings and prolonged incubation times are used. To achieve relatively fast and complete hydrolysis of softwoods at low enzyme loadings it is likely that a post-treatment step, that modifies or

removes lignin will be required. This has been reported previously for pretreated wood chips (Yang et al., 2002).

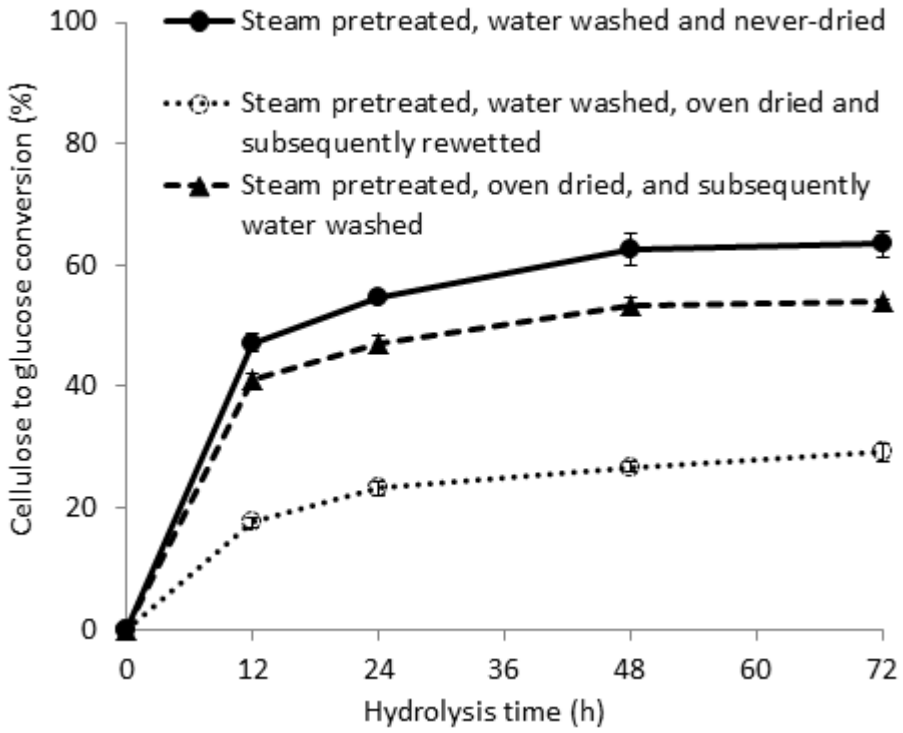


Figure 43 Influence of drying on enzymatic hydrolysis of washed and unwashed steam pretreated softwood (Douglas-fir). Error bars represent standard deviations from the mean (n=3 for enzymatic hydrolysis followed by a single HPLC analysis for each sample)

3.5.2.5 Post-treatment of steam pretreated wood chips and pellets for a fast and effective cellulose hydrolysis

Earlier work described in the thesis had shown that the lignin present in the water insoluble component of steam pretreated softwood played a key role in limiting enzyme accessibility to the cellulose and that a neutral sulfonation post-treatment could significantly enhance the ease of enzymatic hydrolysis of steam pretreated softwoods. We next assessed the effectiveness of a similar post-treatment to try to obtain higher sugar yield from pellets and steam stabilised pellets when similar enzymes loadings were used.

As expected, although sulfonation did not result in significant delignification of the substrates, both the rate and extent of enzymatic hydrolysis increased considerably for all

of the substrates compared to the control steam pretreated substrate (Figure 44). For those substrates which were steam pretreated and subsequently pelletised, the sulfonation was found to be less effective. Approximately 85% of the cellulose present in this substrate was hydrolysed after 72 hours, implying that drying and pelletisation decreased the effectiveness of a post-treatment step when compared to the never-dried and non-pelletised material. Although the rate of hydrolysis was slightly higher for pellets as compared to wood chips (Figure 44) this slight difference was likely due to the slight variations in the severity experienced by these substrates during steam pretreatment and its subsequent influence on sulfonation. In Chapter 3.3, it was shown that the severity of pretreatment could result in structural changes to the lignin, which in turn influences the post-treatments ability to remove or modify the lignin.

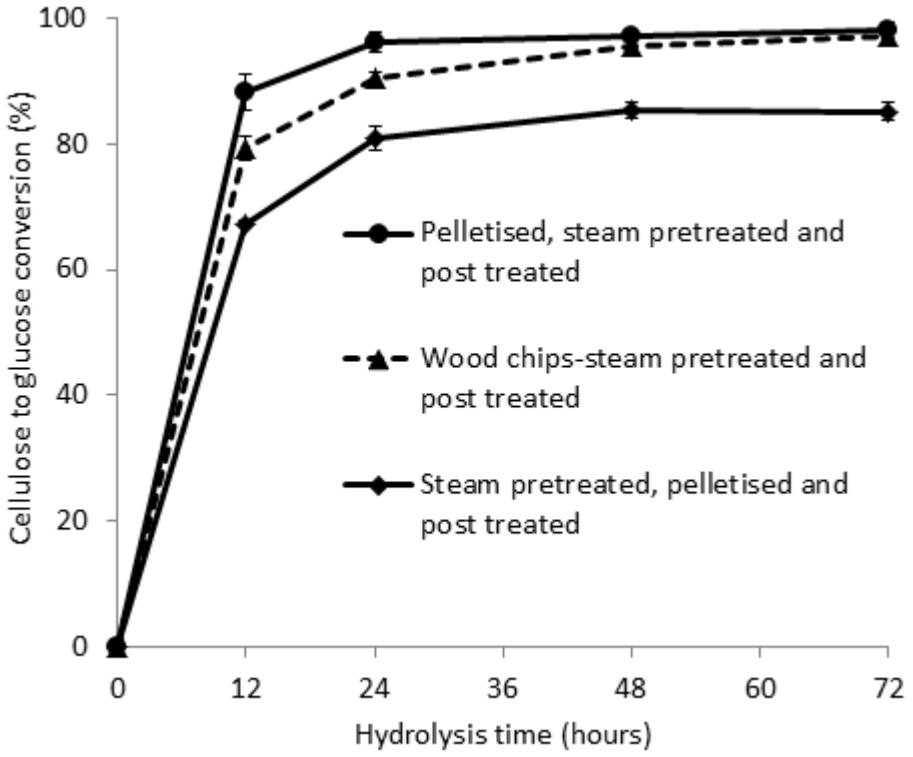


Figure 44 Influence of a post-treatment (neutral sulfonation) step on the enzymatic hydrolysis of the cellulose present in pretreated pellets, wood chips and steam stabilised pellets. Error bars represent standard deviations from the mean. (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

3.5.3 Conclusions

In contrast to what was anticipated, pelletisation did not result in significant hemicellulose loss and steam pretreated pellets were found to be as readily hydrolysed as were steam pretreated chips. When chips were steam treated prior to pelletisation the material did not require further grinding prior to pelletisation and good enzymatic hydrolysis of the cellulose could be obtained without the need for a subsequent steam pretreatment step after pelletisation. It appears that steam pretreatment could enhance the pelletisation of chips, providing good hemicellulose recovery and a cellulosic component that could be readily hydrolysed. Post-treatment further enhanced the hydrolysis of both the chips and pellets. We anticipated that the reduced moisture content and pressure exerted on the wood biomass during the pelletisation process would result in some carbohydrate loss as well as making the biomass more recalcitrant to pretreatment and subsequent hydrolysis. However, when softwood chips and pellets were steam pretreated at medium severity, little hemicellulose loss occurred while more than two-thirds of the cellulose present in the cellulose rich water insoluble fractions were hydrolysed (at 20 FPU cellulase/g glucan). Prior steaming substantially reduced the particle size of the wood chips enabling direct pelletisation without the need for grinding. It was possible to apply a single steam pretreatment to facilitate both pelletisation and subsequent enzymatic hydrolysis without the need for a further pretreatment step.

3.6 The use of high substrate concentrations during pretreatment and enzymatic hydrolysis to enhance the overall sugar yield and concentration

3.6.1 Background

Earlier work had shown that we could obtain good sugar yields with low enzyme loading by using appropriate steam pretreatment and post-treatment strategies. However, the sugar concentrations in the pretreatment liquid stream and enzymatic hydrolysate were generally very low (<4%). An ethanol titre of 10 – 15% is desirable for downstream purification/distillation, and in order to achieve this titre, the corn ethanol industry (starch based) uses a 20 – 30% sugar concentration (Lin and Tanaka, 2006). Therefore, it is very likely that a higher sugar concentration will be needed if a cellulose-based biofuel process is to be successfully established. To obtain an equivalent sugar concentration as a grain-based ethanol process, it will be necessary to conduct both the pretreatment and subsequent enzymatic hydrolysis at high substrate concentrations (Modelbach and Nokes, 2011). Although data for high-solids pretreatment and hydrolysis is limited, it has been suggested that the combination of a high-solids pretreatment followed by high-solids hydrolysis has great potential to improve the process economics by increasing sugar and ethanol yields while decreasing capital costs (Hodge et al., 2008; Roche et al., 2009a, 2009b).

For cellulose-based ethanol production, reducing the amount of liquid generated in the pretreatment step is necessary to minimise the dilution of sugars in the subsequent process streams. Although, direct steam injection to a relatively dry biomass would be the most practical way of generating a high consistency slurry from cellulosic biomass, almost all of the previous studies have used very low biomass loading for a given amount of steam (Schwald et al., 1989; Galbe and Zacchi, 2002; Ewanick et al., 2007). Consequently, a high amount of condensate was typically formed resulting in the separation of the slurry into liquid and solid fractions for further processing. Even combining the two streams and conducting the enzymatic hydrolysis at a high solids concentration did not result in the desired sugar concentration levels, primarily due to the extremely low sugar concentration

of the pretreatment liquid (Robinson et al., 2003). Although increasing the biomass loading in the steam gun has the potential to enhance the consistency, the influence of such a strategy on the overall sugar yield at high concentration has not yet been investigated.

All of the previous studies on increasing the sugar concentration primarily focussed on increasing the consistency of enzymatic hydrolysis (Di Risio et al., 2011; Humbird et al., 2010; Lu et al., 2010; Olsen et al., 2011; Pristavka et al., 1998; Ran et al., 2012; Roche et al., 2009; Wang et al., 2012; Wiman et al., 2011; Zhang et al., 2009). When Zhang et al. (2009) studied the high consistency enzymatic hydrolysis of unbleached hardwood Kraft pulp and organosolv pretreated poplar they obtained a 21% glucose concentration in the enzymatic hydrolysate. Although this approach will also bring economic savings to the bioconversion process, this work ignored the hemicellulosic fraction as it was difficult to recover these sugars at high concentration in a typical pulping pretreatment such as Kraft pulping or organosolv treatment (Pan et al., 2005a, 2008). As was shown in the previous chapters, steam pretreatment can potentially result in good recovery of the hemicellulosic sugars and these sugars, if obtained at high concentrations, can be relatively easily fermented thus increasing the overall biofuel titre and yield per kg of feedstock used.

A high biomass to steam ratio during steam pretreatment is important for both enhancing the sugar concentration of the pretreatment liquid and subsequent enzymatic hydrolysate and it also helps reduce the energy consumption and minimises the generation of waste water in the following processing steps. Therefore, capital and production cost can be greatly reduced since smaller reactors and equipment can be utilised for equivalent sugar and product yield. Furthermore, energy usage for heating, cooling, mixing and product distillation is reduced, which makes the overall conversion process more efficient on an energy basis.

Therefore the influence of increasing the biomass loading in the steam gun was next investigated. We expected that a high biomass to steam ratio would decrease the steam consumption and generate a high consistency biomass slurry. However, this may also lead

to mass transfer challenges leading to the decreased efficiency of steam pretreatment and subsequent enzymatic hydrolysis. An initial goal was to verify whether it was possible to increase the biomass to steam ratio to ensure there was no free water available while still obtaining hemicellulose dissolution and its recovery in the water soluble fraction of the wet pretreated cake. Obtaining such a substrate with no separate liquid stream was expected to enable us to proceed with the enzymatic hydrolysis at high consistency for the whole substrate without any water washing or solid-liquid separation.

Two different feedstocks, wood chips and wood pellets were compared. Wood pellets have an advantage of higher bulk density and thus more substrate could be loaded in the steam gun compared to wood chips. In addition, wood pellets are drier, which was expected to increase the consistency of the resulting slurry after pretreatment. The recovery and concentration of water soluble sugars and the ease of enzymatic hydrolysis of the water insoluble cellulosic component were determined. Finally, the whole slurry resulting from the best pretreatment conditions was subjected to enzymatic hydrolysis at a 25% dry matter consistency without any solid-liquid separation or water washing. The minimum enzyme requirement to obtain sufficient liquefaction and high concentrated sugar stream was subsequently determined.

3.6.2 Results and discussion

3.6.2.1 Influence of biomass loading on the consistency, sugar recovery and sugar concentration

In the previous work 50g of wood chips were added to the 2.6 L capacity steam gun indicating that approximately 15% of the reactor volume was filled with biomass (Boussaid et al., 2000, 2001; Bura et al., 2003, 2009; Cullis et al., 2004; Ewanick et al., 2007; Wu et al., 1999). In the work described here, we added 50 g, 200 g and 300 g of wood chips corresponding to approximately 15%, 60% and 85% of the volume of the reactor respectively. We also compared the response of the wood pellets to steam pretreatment at increased biomass loadings to investigate whether pellets would respond differently to wood chips compared to what was observed previously at low solids loadings. As greater

weights of pellets could be loaded in the steam reactor, a maximum loading of 400g of wood pellets was used in this work whereas with wood chips the maximum loading was 300g. The steam pretreatments were conducted at conditions of 200°C, 5 minutes and 4% SO_2 impregnation, which had previously been shown to be effective at lower solids loadings.

It was apparent that steam pretreatment at increased biomass loading (≥200g) resulted in high consistency substrates with no free water. All of the condensate was absorbed by the pretreated substrate forming a wet cake. Compared to the <12% dry matter consistency when using a low biomass loading, a higher, 33 – 49% dry matter consistency was obtained at high biomass loading depending on the extent of loading and the type of feedstock pretreated (Figure 45). This high consistency could be attributed to the lower steam consumption and consequently lower levels of condensate formed. Reduced formation of condensate should be advantageous as it could potentially enable us to minimize the dilution of the sugar streams and maintain high consistencies in the subsequent processing steps. A slightly higher consistency was obtained with wood pellets compared to wood chips (Figure 45), possibly due to the lower moisture content of the starting feedstock. It has previously been shown that for a given biomass loading, the moisture content of the biomass is the biggest contributor to the formation of condensate during steam pretreatment (Brownell and Saddler, 1987).

As expected, an increase in substrate consistency lead to an increase in the concentration of soluble sugars that could be obtained. An increase in the loading of wood chips from 50g to 300 g increased the concentration of water soluble sugars from 4 to 14%. A similar trend was also obtained with wood pellets, although a slightly lower concentration was observed compared to wood chips (Figure 45).

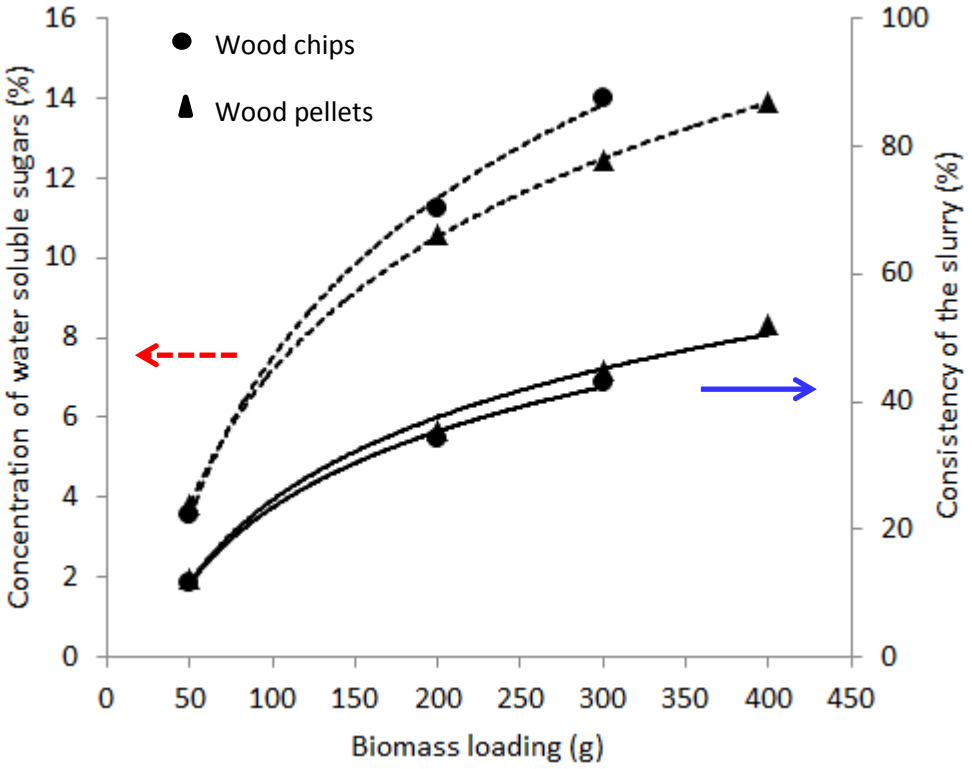


Figure 45 Influence of increasing the biomass loading on the consistency of the resulting slurry and also on the concentration of sugars in the water soluble fraction.

Despite achieving higher concentration of water soluble sugars, a decrease in the overall sugar recovery was observed with increasing in biomass loading. When the loading of wood chips was increased from 50 to 300g, the recovery of hemicellulose decreased from 73 to 59% respectively (Figure 46). As was observed previously with low solids loading, sugar recovery at high solids loading was also lower for wood pellets compared to wood chips (Figure 46 and Figure 47). However, the difference between wood chips and wood pellets was more apparent at high substrate loadings (Figure 46 and Figure 47). The recovery of hemicellulose from wood pellets was less than 40% at the highest biomass loading (Figure 47). Similar to hemicellulosic sugars, the amount of glucan recovered (combined water soluble and insoluble components) was also significantly decreased with increasing biomass loading, indicative of the higher extent of sugar degradation at higher solids loading.

When the chemical composition of the water insoluble component of the pretreated substrates was analyzed, almost complete hemicellulose dissolution was observed at all biomass loadings (Table 18). The glucan content was fairly similar between the substrates. However, the lignin content of the substrates increased with an increase in the biomass loading. This increase was likely due to the higher dissolution of carbohydrates or to the formation of pseudo lignin (Table 18). Contrary to our expectations that increased biomass loading would lead to mass transfer challenges during steam pretreatment, the results indicated that increased biomass loading in the reactor resulted in an apparent increase in relative severity as indicated by a higher dissolution of carbohydrates and higher extent of their degradation. In order to investigate whether this higher severity translated to an improved enzymatic hydrolysis of the cellulosic component, we next assessed the enzymatic hydrolysability of the substrates at an enzyme loading of 20 FPU/g glucan.

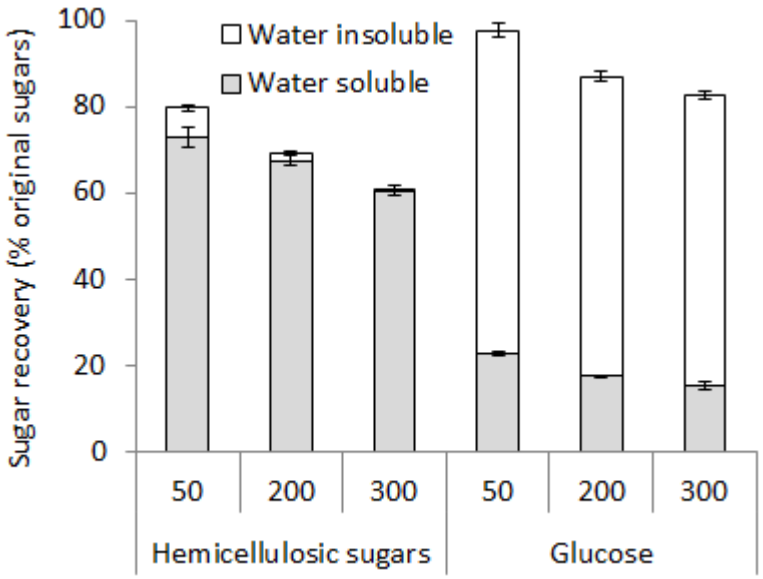


Figure 46 Influence of increasing the substrate loading on the recovery of original carbohydrates from wood chips. Error bars represent standard deviations from the mean (n=2 for the analysis of water soluble fractions and n=3 for the compositional analysis (Klason) of water insoluble fractions). Hemicellulose refers to the sum of arabinan, galactan, xylan and mannan.

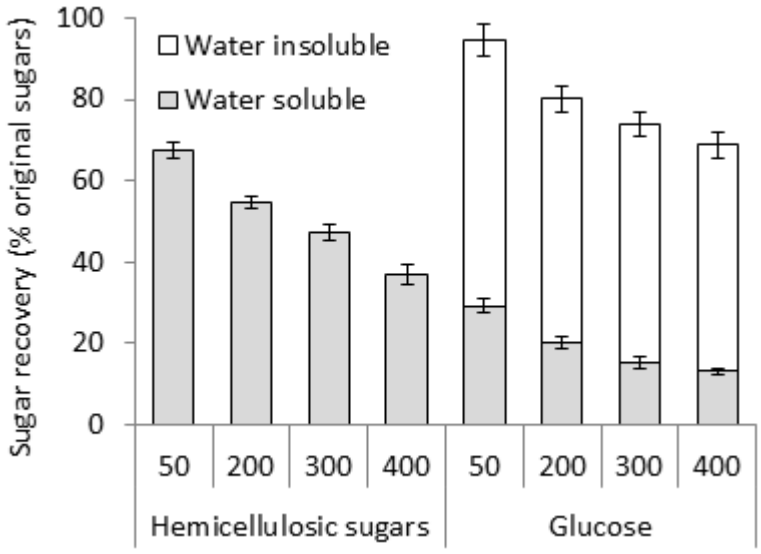


Figure 47 Influence of increasing the substrate loading on the recovery of original carbohydrates from wood pellets. Error bars represent standard deviations from the mean (n=2 for the analysis of water soluble fractions and n=3 for the compositional analysis (Klason) of water insoluble fractions). Hemicellulose refers to the sum of arabinan, galactan, xylan and mannan.

Table 18 Chemical composition (% dry weight) and enzymatic digestibility of the water insoluble cellulosic component of steam pretreated wood chips and wood pellets pretreated at various substrate loadings.

Feedstock filling (g)	Glucan	Xylan	Mannan	Lignin	72 hour hydrolysis yield (%)
Wood chips					
50	53.4 (0.7)*	0.7 (0.2)	1.3 (0.1)	43.3 (0.6)	60.5 (0.7)
200	53.9 (0.7)	0.1 (0.0)	0.5 (0.1)	46.8 (0.5)	69.2 (0.1)
300	52.3 (0.5)	BDL	BDL	47.5 (1.0)	72.9 (1.4)
Wood pellets					
50	52.7 (1.3)	BDL**	BDL	44.1 (1.2)	65.0 (1.4)
200	53.0 (0.1)	BDL	BDL	45.4 (0.7)	70.5 (0.2)
300	50.2 (0.9)	BDL	BDL	48.1 (1.0)	72.9 (0.8)
400	49.4 (0.1)	BDL	BDL	49.0 (1.1)	70.2 (0.5)
*Numbers in the bracket represent standard deviations from the mean (n=3 for compositional analysis and n=2 for enzymatic hydrolysis). **Below detectable level					

Although hydrolysis yield did show an increase with increased biomass loading during pretreatment the improvement was not substantial. When the loading of wood chips was increased from 50 to 200g, the corresponding hydrolysis yields were 61 and 69% respectively (Table 18). However, when the biomass loading was increased to 300g, the enzymatic hydrolysis was only improved by about 4%. Similarly, with wood pellets, an increase in substrate loading from 200 to 400g, resulted in little difference in the hydrolysis yield (Table 18). This result indicated that increasing the biomass loading, although it did influence the degradation of the hemicellulosic sugars, did not significantly change the amenability of the cellulosic component to enzymatic hydrolysis.

The steam gun used in the current work has steam supplied through a narrow orifice located near the center of the reactor. When the biomass loading is lower, it is possible that the initial amount of steam entering the reactor is rapidly condensed at the bottom of the reactor, with the condensate consequently covering up almost all of the wood chips located at the bottom of the reactor. As the biomass immersed in the condensate would not have much contact with the rest of the steam the lack of interaction of the steam with the biomass would likely lead to a dilute acid hydrolysis type of situation. However, at higher solids loading, the substrate occupies most of the reactor space and the steam is directly condensed on the biomass, where it readily reacts with SO_2 present in biomass. This direct interaction with steam would result in an efficient mass transfer in the vapor phase and an increase in the apparent severity of steam pretreatment.

In addition to a more effective interaction of the steam with biomass, one of the key reasons for lower recovery of carbohydrates at high solids loading could also be the increased SO_2 concentration in the system. Since the loading of SO_2 for a given amount of biomass remains the same for all biomass loadings, the SO_2 concentration in the reactor is proportionately enhanced with increasing biomass loading. For example, while ~1g of SO_2 enters the steam gun with a loading of 50g of the wood chips, a six times higher SO_2 concentration would be present in the reactor when 300g of wood chips is loaded and subsequently pretreated. This higher amount of SO_2 will increase the partial pressure of the gas in the reactor and would consequently result in the formation of sulfuric acid (Goldberg and Parker, 1985; De Bari et al., 2007 ; Lua and Yang, 2009a, 2009b; Papanicolaou et al., 2009). An increased concentration of sulfuric acid would potentially lead to more dehydration reactions of the soluble sugars to furan derivatives and then to organic acids (Girisuta et al., 2006; Marzialetti et al., 2008). Therefore, we next tried to decrease the SO_2 loading at high substrate loading to assess whether a reduced SO_2 consumption would help minimise the destruction of hemicellulosic sugars.

3.6.2.2 Influence of SO_2 loading on the high consistency steam pretreatment

Wood chips were used as the substrate for this work and all experiments were conducted at 300g biomass loading in the reactor, which is approximately 85% of the reactor volume. Three different SO_2 impregnation levels were compared, 1.5%, 3% and 4%. The SO_2 adsorption at these three impregnation levels were 72, 65 and 60% resulting in 1.1, 1.9 and 2.4 g of SO_2 adsorbed by 100g wood chips respectively (Table 19). When the wood chips were transferred to the steam gun, roughly 3, 6 and 7g of SO_2 would likely to be present in the 2.6L volume leading to equivalent partial pressures 18, 37 and 42 mM/m^3 of SO_2 respectively before the beginning of the reaction (Table 19).

Table 19 Influence of SO_2 loading on the potential SO_2 partial pressure, consistency of the pretreated substrate and concentration of water soluble sugars

SO_2 loading (g per 100g wood chips)	SO_2 retention (g per 100g wood chips)	Theoretical SO_2 concentration in the reactor (mM)	Consistency of the resulting slurry (% wt/wt)	Concentration of water soluble sugars (% wt/wt)
1.5	1.1	18	39.5	15.9
3	1.9	37	42.3	14.6
4	2.4	42	43.1	14.2

It was apparent that varying the SO_2 loading did not result in a significant variation in the consistency of the resulting slurry, probably due to the amount of condensate produced being the same for the same substrate loading (Table 19). However, the SO_2 concentration in the reactor does seem to play a role in the recovery of hemicellulosic sugars (Figure 48). When the SO_2 loading was reduced from 4 to 1.5%, the recovery of hemicellulosic sugars increased from 59 to 81% (Figure 48). In addition, 94% of the glucan was also preserved in the combined solid and liquid fractions when 1.5% SO_2 loading was used compared to 82% glucan recovered at 4% SO_2 loading (Figure 48). Despite this substantial difference in the recovery of soluble sugars, the chemical composition of the water insoluble components was fairly similar for all SO_2 loadings (Table 20). In addition, when the water washed cellulosic component was subsequently hydrolysed, the variation in SO_2 impregnation did

not appear to influence the sugar yields (Figure 49). These results seemed to indicate that it was the degradation kinetics of dissolved sugars that was mainly influenced by the SO_2 concentration at high solids loadings.

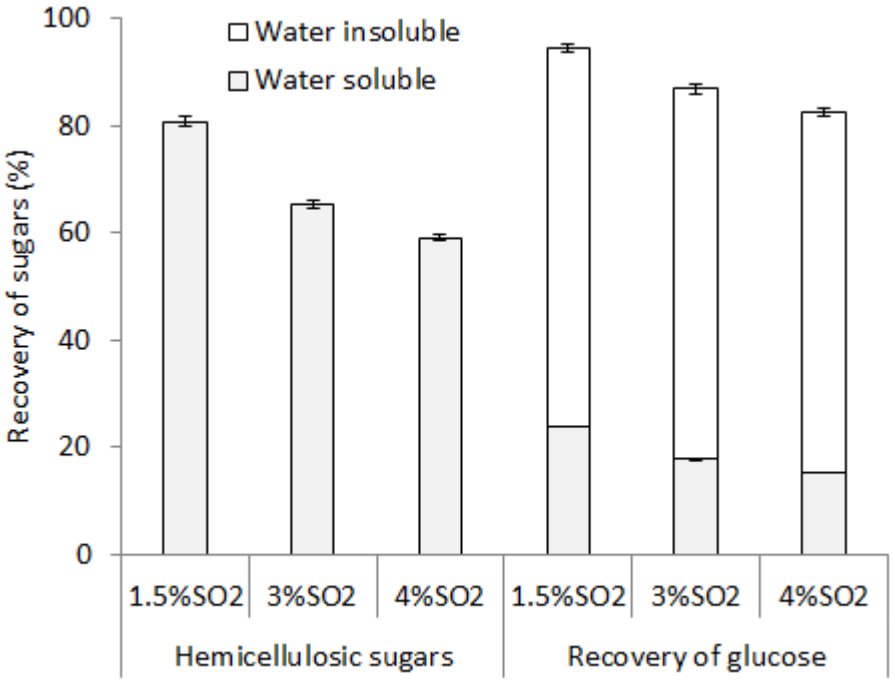


Figure 48 Influence of varying the SO_2 loading on the recovery of original sugars after high consistency steam pretreatment. Error bars represent standard deviations from the mean (n=2 for the analysis of water soluble fractions and n=3 for the compositional analysis (Klason) of water insoluble fractions).

Table 20 Influence of SO_2 loading on the chemical composition of the water insoluble cellulosic component (% dry weight) resulting from the steam pretreatment of Douglas-fir wood chips at high substrate loading*

SO_2 loading (% dry wt of the wood chips)	Glucan	Lignin
1.5	53.1 (0.36)**	45.1 (0.54)
3	53.5 (0.61)	45.4 (0.88)
4	52.3 (0.47)	47.5 (1.04)
*Hemicellulosic sugars were below detectable level **Numbers in the bracket represent deviations from the mean (n=3)		

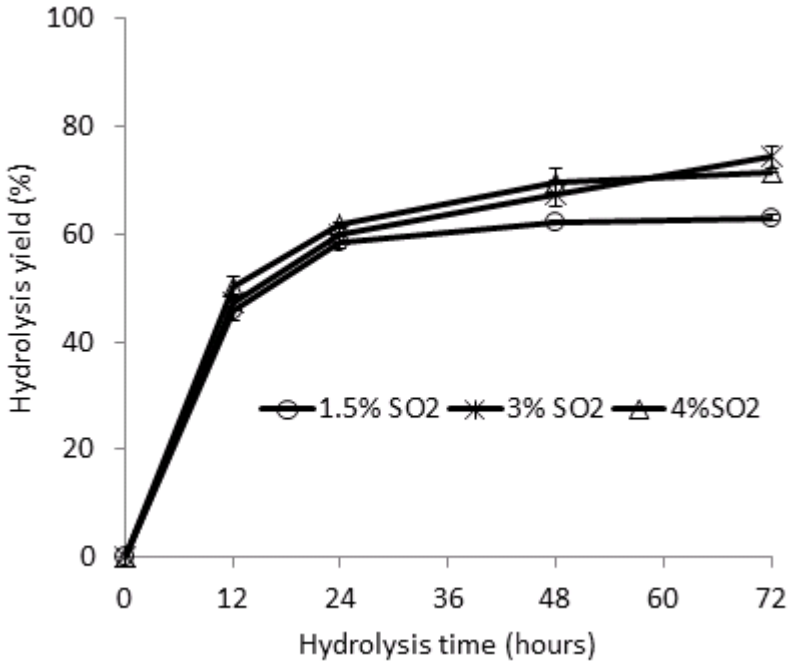


Figure 49 Influence of varying the SO_2 loading on the enzymatic hydrolysis of water insoluble cellulosic component. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis followed by a single HPLC analysis for each sample).

Previously it was reported that, when aspen wood chips were impregnated with SO_2 and subjected to steam at 200°C, much of the SO_2 in the chips was almost immediately converted to sulfuric acid (Gregg and Saddler, 1996). It was suggested that, in the presence of air, sulfurous acid is oxidised to sulfuric acid (Equation 1) (Brownell and Saddler, 1986). Sulfuric acid can also be formed in the absence of air apparently by disproportionation reactions (Equation 2 & Equation 3) (Holland, 1965; Symonds et al., 2001). The SO_2 can react with steam to produce sulfuric acid and hydrogen gas, particularly in the gaseous phase. Alternatively, SO_2 can also react with water leading to the formation of native sulfur and sulfuric acid.

$SO_2 + H_2O + ½ O_2 \Leftrightarrow H_2SO_4$ Equation 1 (Brownell and Saddler, 1986)

$SO_2 + 2H_2O \Leftrightarrow H_2 + H_2SO_4$ Equation 2 (Holland, 1965)

$3SO_2 + 2H_2O \quad \Leftrightarrow \quad S + 2H_2SO_4$ Equation 3 (Symonds et al., 2001)

All these chemical reactions illustrate that the increased partial pressure of SO_2 can lead to the increased formation of sulfuric acid. The maximum solubility of SO_2 in water at atmospheric pressure decreases from 10.4 g per 100 g at 20°C to 3.8 g per 100 g at 50°C (Hasenberg, 2010) . Therefore, it is possible that the SO_2 absorbed in the wood chips during room temperature impregnation would be largely desorbed back to the steam reactor at 200°C and occupy the reactor space. This free SO_2 would likely react with steam forming equivalent amounts of sulfuric acid and this sulfuric acid vapor can readily react with the wood chips. As the loading of SO_2 is based on the amount of biomass present, a higher biomass loading results in a greater partial pressure of SO_2 in the reactor. Consequently, the greater formation of sulfuric acid for almost the same amount of condensate leads to a much higher acid concentration compared to low substrate loadings. When the concentration of sulfuric acid is increased, the extent of sugar degradation increases, leading to the formation of furan derivatives, furfural and secondary degradation products such as organic acids. This loss of sugars could explain the lower recovery of water soluble sugars at the higher partial pressure of SO_2. Other workers have previously suggested that the degradation of both the pentose and hexose sugars to furan derivatives, and their further conversion to organic acids, is mainly dependent on the temperature of the reaction and the sulfuric acid concentration (Megawati et al., 2011; Weingarten et al., 2012; Qi et al., 2008).

It was interesting to note that decreasing the SO_2 impregnation from 4 to 3% did not make a substantial difference in the recovery of sugars (Figure 48). However, reducing the partial pressure of SO_2 down to 1.5% impregnation substantially preserved both the hemicellulose and cellulose derived sugars. It appears it may be possible to further reduce the required SO_2 loading while maintaining the process efficiency. In addition to the increased recovery of hemicellulosic sugars, other possible advantages of lowering the SO_2 loading were the lower amount of alkali required to bring up the pH for subsequent processes as well as the lower levels of degradation products.

3.6.2.3 Direct enzymatic hydrolysis of the steam pretreated substrate without any solid-liquid separation/water washing

Earlier work had shown that it was possible to enhance the substrate loading in the steam gun while obtaining an even better overall sugar yield. This high consistency pretreatment not only decreased the energy and water requirements, it also allowed us to reduce the required amount of catalysts for the pretreatment. An additional advantage of the higher biomass to steam ratio is the enriched hemicellulosic sugar recovery in the water soluble component which can be combined with the water insoluble fraction. However, the challenge is to recover these water soluble sugars without dilution. Dewatering the substrate is difficult as there is no free water and all of the condensate is absorbed by the pretreated solids.

One possible way to maintain/further increase this sugar concentration is to directly conduct an enzymatic hydrolysis of the whole slurry at high consistency without any water washing or solid liquid separation. Therefore, in the next step, we carried out a direct enzymatic hydrolysis of steam pretreated substrates over a range of enzyme loadings with the objective of obtaining a highly concentrated sugar stream. Douglas-fir wood chips steam pretreated at previously determined optimum conditions (feedstock filling of 300g wood chips, 1.5% SO_2 loading, 200°C for 5 minutes) were used as the substrate for direct enzymatic hydrolysis. Enzymatic hydrolysis was conducted at a final consistency of 25%. After adding the required amount of alkali and buffer followed by a thorough mixing, the enzymes were subsequently added and substrate was hydrolyzed at 50°C for 72 hours. After the enzymatic hydrolysis, the solid and liquid fractions were separated by vacuum filtration and the liquid was analyzed for its sugar composition.

As was expected, high sugar concentrations (140 – 180 g/L) were obtained after the direct enzymatic hydrolysis of steam pretreated substrates without any washing or solid liquid separation (Figure 50). This is one of the highest sugar concentration reported from the steam pretreatment process streams (Figure 50). It should also be noted that it is almost impossible to obtain this high concentration (180g/L) from the hydrolysis of the water

insoluble component alone as the lignin occupies almost half of the substrate and, consequently, the hydrolysis has to be conducted at consistencies above 35%.

It was apparent that despite a substantial increase in sugar concentration, high enzyme loadings (40FPU/g glucan) were needed to obtain >140 g/L sugar concentration (Figure 50). Although nearly 120g/l of sugars were obtained at reasonable enzyme loadings such as, 20 FPU/g glucan, a substantial fraction of the cellulose was not hydrolysed even at this high enzyme loading. When the extent of cellulose hydrolysis was determined, less than 40% of the cellulose present in the water insoluble component was hydrolysed at an enzyme loading of 20 FPU/g glucan. This relatively high enzyme requirement could be due to a) the toxicity of the inhibitors which are concentrated in the high consistency pretreated substrate, b) end product inhibition, or c) the lack of sufficient mixing in the Erlenmeyer flasks.

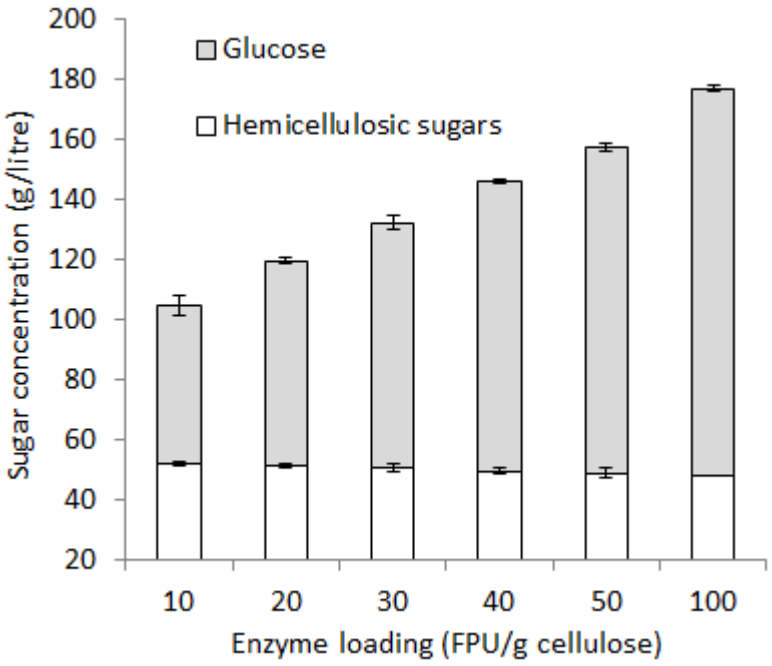


Figure 50 Influence of enzyme loading in increasing the sugar concentration by the direct enzymatic hydrolysis (without any solid-liquid separation/water washing) of steam pretreated substrate pretreated at high substrate loading. Error bars represent standard deviations from the mean (n=2 for enzymatic hydrolysis followed by the HPLC analysis of each sample in two replicates).

3.6.3 Conclusions

Biomass loading during steam pretreatment was successfully enhanced to result in a high consistency slurry. The chemical composition and enzymatic hydrolysis of the cellulosic component were somewhat similar to those obtained at low biomass loading. However, high biomass loading resulted in the significant destruction of the soluble sugars with wood pellets exhibiting higher levels of sugar degradation compared to wood chips. Lowering the SO_2 loading minimised the extent of sugar degradation while generating a cellulosic component amenable to enzymatic hydrolysis. Therefore, the higher substrate loading in the steam reactor not only helped recover the majority of the water soluble hemicellulose at high concentration, enhancing the consistency of the slurry, it also enabled us to reduce water, energy and the amount of catalyst required. Enzymatic hydrolysis of the whole substrate without any water washing or solid liquid separation resulted in very high sugar concentrations (140 – 180g/l). However, this concentration was achieved at the expense of needing high enzyme loadings (≥40FPU/glucan) for effective hydrolysis.

4 Conclusions and future recommendations

4.1 Conclusions

As the earlier work had used Douglas-fir wood chips derived from a log of an older tree that had been stored for an indeterminate time, we first wanted to assess how effective steam pretreatment would be on a range of Douglas-fir sources and how they are compared to other softwoods such as pine and spruce. It was apparent from both this work and a comparison with the results reported in the literature that most softwoods could be processed at the same set of compromise conditions. This resulted in a comparable material balance, overall sugar recovery and subsequent ease of enzymatic hydrolysis of the water insoluble cellulosic fraction. The work described in this thesis suggests that, in an analogous fashion to the pulp and paper sector, a biorefinery process can process varying types of softwoods in the steam pretreatment unit without substantial variations in the process conditions used. Despite the so-called "robustness" of the process including good recovery of hemicellulose sugars when compromise conditions are used, one of major challenges was the need to use relatively high enzyme loadings so that reasonable cellulose hydrolysis could be achieved. It was clear that, when lower, more realistic enzyme loadings were employed (5-10 FPU/g glucan), poor sugar yields were obtained from steam pretreated substrates. Even after enhancing the pretreatment severity at the expense of a lower hemicellulose recovery, the cellulosic fraction was still poorly hydrolysed.

Although, it is recognised that lignin restricts the enzymatic hydrolysis of steam pretreated softwoods, complete removal of lignin from steam pretreated softwood at an industrial scale is economically unattractive. Our subsequent investigations on the relative contributions of the two major mechanisms of lignin inhibition (limiting cellulose accessibility vs. unproductively binding of cellulase enzymes) showed that the inhibitory effect of lignin was critically dependent on the enzyme loading employed. The use of high cellulase loadings resulted in near complete cellulose hydrolysis of steam pretreated Douglas-fir. However, at low cellulase loadings (<10 FPU/g glucan), both the unproductive

binding of the enzymes to the lignin and the limited accessibility to the cellulose were at play. Interestingly, when the lignin does not restrict cellulose accessibility, the impact of unproductive binding on the hydrolysis yield is low, even at low enzyme loadings. It was apparent that a post-treatment to eliminate lignin`s restrictions on cellulose accessibility will be a prerequisite if lower enzyme loadings and high hydrolysis yields are to be attained with softwood substrates.

As some form of post-treatment will be required if lower enzyme loadings are used, we next tried to determine the optimum combination of pre- and post-treatments. Although there has been some work on evaluating post-treatments as one way of enhancing the enzymatic hydrolysis, their effectiveness had been generally assessed on substrates which had been steam pretreated under a single set of conditions. However, to try to minimise the cost and increase the effectiveness of post-treatment, the preceding pretreatment step first had to be optimised. It was thought that the pretreatment conditions used would influence the resulting substrate characteristics such as particle size distribution, chemical composition, degree of lignin condensation, etc., subsequently influencing the post-treatments ability to remove/modify lignin and enhance enzymatic hydrolysis. Subsequent work showed that the pretreatment conditions used had a significant influence on the recovery of hemicellulosic sugars as well as on the ability of post-treatment to enhance the enzymatic hydrolysis of the cellulosic component. The medium severity conditions were shown to provide the best compromise, allowing recovery of most of hemicellulose derived sugars while enhancing subsequent post-treatment and enzymatic hydrolysis of the cellulosic fraction. It was found that the lignin content or the degree of delignification of the post-treated substrate did not necessarily correlate with the subsequent ease of enzymatic hydrolysis. For example, despite three times higher delignification achieved by alkaline peroxide process compared to neutral sulfonation, both of these substrates were hydrolyzed to the same extent. This observation indicated that, in addition to delignification, lignin modification post-treatments such as neutral sulfonation could also increase cellulose accessibility and enzymatic hydrolysis at low enzyme loadings.

It was expected that higher abundances of free phenolic and carbonyl groups resulting from the cleavage of α-O-4 and β-O-4 linkages during steam pretreatment would readily sulfonate lignin at milder conditions compared to the lignin present in native wood. Surprisingly, sulfonation at very mild conditions (50°C, 6 hours, 16% sulfite loading) almost doubled the hydrolysis yield from 29 to 55% at an enzyme loading of 10 FPU/g glucan although to achieve a further increase in the hydrolysis yield required more severe conditions. The post-treatment conditions that provided the best hydrolysis yields were at an 8% Na_2SO_3 loading, 180°C, 0.5 hour or 16% Na_2SO_3 or 160°C 2 hours, resulting in >90% hydrolysis yield at 10 FPU/g glucan. Close to 85% of the original sugars (both hemicellulose and cellulose derived) could be recovered after a steam pretreatment, post-treatment and enzymatic hydrolysis at an enzyme loading of 10 FPU/g glucan.

In addition to increasing the sugar yield by employing appropriate pre/post-treatment strategies, equally important is obtaining these sugars at high concentration. Previous work has tended to use low biomass loading for a given amount of steam and, consequently, a large amount of condensate was formed after steam pretreatment, diluting the water soluble hemicellulose. Our work on increasing the substrate loading in the steam gun showed that it was possible to obtain high consistency slurry (35-50%) with water soluble hemicellulosic sugars recovered at high concentration (~140g/L). This approach not only decreased the energy and water consumption, but also reduced the required amount of SO_2 to 1.5%. When the whole slurry was subjected to enzymatic hydrolysis at a 25% dry matter consistency without any solid-liquid separation or water washing, high sugar concentrations were achieved (140 – 180 g/L). However this achievement was at the expense of needing high enzyme loadings (40 – 100 FPU/g glucan).

Most past work which looked at pretreatment of wood substrates utilised pulp chips as the starting material (Galbe and Zacchi, 2012; Mabee et al., 2007). However, pulp chips are unlikely to be used as commercial bioconversion feedstock for large scale facilities since their high moisture content and low bulk density limits their transport over long distances. Therefore, in order to benefit from economies of scale it is highly likely that densified

feedstocks, such as wood pellets, will be used to meet the large scale biomass demand similar to what is occurring in the rapid growth of large scale combustion and cogeneration plants (Richard, 2010; Stephen et al., 2011). Despite extensive work evaluating the use of wood pellets for thermochemical applications and combined heat and power (CHP), the suitability of wood pellets as a feedstock for biochemical conversion had not previously been studied. Therefore, the influence of pelletisation on steam pretreatment, post-treatment and enzymatic hydrolysis was investigated. Although it was anticipated that the conditions used to make strong and durable wood pellets would prove to be problematic it was shown that pelletisation did not result in significant loss of hemicellulosic sugars and that the steam pretreated pellets were readily hydrolysed. In addition, when wood chips were steam treated prior to pelletisation, the material did not require further grinding prior to pelletisation and the resulting pellets were more stable and durable. Good enzymatic hydrolysis of the cellulose could be obtained without the need for a subsequent steam pretreatment step after pelletisation. It appears that steam pretreatment could enhance the pelletisation of chips, provide good hemicellulose recovery and a cellulosic component that could be readily hydrolysed. However, as noted earlier, a further post-treatment will likely be required to reduce the enzyme loadings to a more acceptable level.

4.2 Possible future research directions

Although the work described in the thesis was focused on softwoods, many of the research findings can also be potentially adapted for hardwoods or non-wood residues to enhance their efficiency of fractionation and subsequent bioconversion. The influence of densification on the steam pretreatment and enzymatic hydrolysis of hardwoods and non-wood residues should be assessed. In addition, the strategies of using high substrate loading in steam pretreatment can be employed for other feedstocks to enhance the final sugar concentration.

The challenge of needing high enzyme loading when using high consistency substrate slurry was not resolved. One potential way to partially reduce the high enzyme loading is to ensure better, more homogenous enzyme-substrate interactions during the

process. In addition, applying a milder post-treatment (such as neutral sulfonation) on the whole slurry might also reduce the enzyme loading while enhancing the overall sugar yield at high concentration.

Enzyme loadings in the range of 10 FPU/g glucan were still needed to recover the majority of the sugars present in the original feedstock. This requirement indicated that improved efficiency of the enzymes is crucial for a further reduction in the enzyme requirement. The improvements in hydrolysis reported in the current work should also be tested with the recently improved enzyme formulations. There have been recent claims by the enzyme companies regarding the improvements in hydrolysis yield by their newly released enzyme products. For example, the recent Cellic CTec 3 cellulase mixture released by Novozymes Inc. is expected to have higher tolerance towards inhibitors generated during pretreatment. This efficiency might possibly reduce the enzyme requirements for hydrolyzing the whole slurry while obtaining the same levels of yield and sugar concentration.

The work on steam pretreatment could also be extended to assess the possibility of recovering lignin in a reactive form and its potential for value added applications. From both a fundamental and applied view point, it would be valuable to understand what lignin structures were prone to sulfonation, how to obtain them in usable fractions and their potential uses. This research could be initiated by elucidating the structure of both the dissolved and residual lignin fractions from the best steam pretreatment and post-treatment conditions obtained in this work.

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