SULPHITE TREATMENT TO ENHANCE THE ETHANOL PRODUCTION OF SOFTWOOD WHOLE SLURRIES

by

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Abstract

To try to compete with conventional bioethanol production from sugar/starch feedstocks, “whole-slurry”, high solids loading hydrolysis and fermentation of steam pretreated softwood biomass was employed to obtain high sugar, high ethanol concentrations. However, two major challenges were encountered; substrate recalcitrance limiting effective enzymatic hydrolysis and high concentrations of inhibitors inhibiting effective sugar fermentation. The major focus of the work described in this thesis was to assess the benefits of integrating sulphite treatments prior, during and after steam pretreatment to enhance the effectiveness of the enzymatic hydrolysis of the whole cellulose and hemicellulose derived slurry and the fermentation of the biomass derived sugars.

Initial work focused on improving fermentation at high sugar concentrations (up to 25% w/v) by improved strain selection, nutrient supplementation and the use of high cell density growth Subsequently, sulphite post treatment was assessed to see if it could improve both the hydrolysis of the whole slurry, containing both cellulose and hemicellulose, and the fermentation of the softwood derived sugars. To try to maximize the beneficial influence of sulphite treatment the softwood chips were treated prior to steam pretreatment, to result in sulphonation ahead of lignin condensation, hopefully improving the extent of sulphonation with less sulphite loading. This approach resulted in some improvements. However, it proved difficult to balance some of the factors that influence the effectiveness of enzyme mediated hydrolysis, such as the extent of suphonation, substrates size reduction and lignin condensation, although an improvement in whole slurry fermentation was observed. To try to further improve this strategy a two stage, alkali- followed by acid-sulphite approach was assessed, using sulphite in the first stage to sulphonate the lignin and SO_2 in the second stage to further sulphonated the lignin while

decreasing particle size. This resulted in high degree of sulphonation, enhanced delignification and substantial substrate size reduction. Minimum lignin condensation and fermentation inhibitors were detected. More than 160 g/L fermentable sugars and 80 g/L ethanol could be achieved when using the two stage (alkali and acid) sulphite pretreatment of lodgepole pine approach to generate a hemicellulose and cellulose whole slurry that could be readily hydrolysed and fermented.

Lay Summary

As past US President Bush once said, “the world is addicted to oil”. Bioethanol is one way to find alternatives to oil for transport. In countries such as Brazil and the US, more than 10% of their transportation fuels are bioethanol. However, this bioethanol is made from sugar or starch crops such as corn or wheat. The work in this thesis looks at the possibility of making bioethanol from cheaper and more sustainably sourced wood residues. The complex nature of lignocellulosic substrates compared to starch/sugar makes it difficult for biodegradative enzymes to overcome the biomass recalcitrance structure to make fermentable sugars. A one-step sulfite treatment was assessed to enhance both the enzymatic hydrolysis and the fermentation of wood residues to bioethanol. A novel sulfite steam pretreatment allowed us to achieve more than 160 g/L fermentable sugars and 80 g/L ethanol using high solids loading softwood lodgepole pine whole slurries.

Preface

I. Zhong N, Chandra R, Piddocke M, Hu J, Saddler JN (2017) Assessing the challenges of high residual lignin and fermentation inhibitors in steam pretreated softwood whole slurry. *In preparation (Chapter I)*

II. Zhong N, Chandra R, Saddler JN (2017) One step sulfite post-treatment to enhance both hydrolysis and fermentation in steam pretreated softwood whole slurry. *In preparation (Chapter II)*

III. Zhong N, Chandra R, Saddler JN (2017) Sulfite steam pretreatment to enhance sugar and ethanol production from softwood whole slurry. *In preparation (Chapter III)*

IV. Zhong N, Chandra R, Saddler JN (2017) Two stages of sulfite (alkali and acid) steam pretreatment to achieve high sugar and ethanol production from softwood whole slurry. *In preparation (Chapter IV)*

V. Chandra R, Chu Q, Hu J, Zhong N, Lin M, Lee J, Saddler JN (2015) The influence of lignin on steam pretreatment and mechanical pulping of poplar to achieve high sugar recovery and ease of enzymatic hydrolysis. Bioresource Technology 199: 135-141. *(Chapter III)*

For paper I to IV, Na Zhong, Jack Saddler and Richard Chandra contributed to the planning of the experimental work, interpretation of the results and drafting of the manuscripts. Na Zhong carried out most of the laboratory work. For paper V Na Zhong helped develop the research and performed some of the experimental work.

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List of Abbreviations

AISL	acid insoluble lignin
Ara	arabinose
ASL	acid soluble lignin
BG	β-glucosidase
CBH	cellobiohydrolase
CBM	carbohydrate-binding module
CD	catalytic domain
CrI	crystallinity
DP	degree of polymerization
DsP	dissolving-grade pulp
DW	dry weight
EG	endoglucanase
FE-SEM	field emission scanning electron microscopy
FPU	filter paper unit
FQA	fibre quality analyzer
FTIR	fourier-transform infrared spectroscopy
Gal	galactose

Glu	glucose
HMF	hydroxymethylfurfural
HPLC	high performance liquid chromatography
LCC	lignin-carbohydrate complex
Man	mannose
Mono	monomer
NBSK	northern bleached softwood kraft
NMR	nuclear magnetic resonance
OD	optical density
Olig	oligomer
P*I*	isoelectric point
SP	steam pretreatment
SPLP	steam pretreated lodgepole pine
WIF	water insoluble fraction
WRV	water retention value
WS	whole slurry
WSF	water soluble fraction
w/w	weight/weight
w/v	weight/volume

XRD	X-ray diffraction
Xyl	xylose

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Dedication

This thesis is dedicated to my forever beloved father Shiqin Zhong.

Chapter 1: Introduction

The main drivers for the production of renewable transportation fuel such as bioethanol include safeguarding the environment/reducing greenhouse gas emissions and ensuring energy security (Walker, 2011). The carbohydrate feedstocks that can be used to make bioethanol can come from sugary, starchy or cellulosic biomass sources. Conventional bioethanol production from sugar/starch feedstocks are now mature industries where a high gravity fermentation approach is routinely used to result in ethanol concentrations of 10-15% (v/v), yields of >90%, within a relatively short period of time (6-10 hours) (Walker, 2011). However, biomass-derived ethanol is still largely at the research, development and demonstration stage of deployment, mostly because of the high recalcitrance of the lignocellulosic substrates towards the bioconversion process and the poor fermentability of the biomass derived sugars.

So far, several demonstration/commercial scale plants, such as Poet/DSM, Inbicon, Dupont, Iogen, Butalco, and Chemtex have been running in various locations throughout the world (Janssen, Turhollow, Rutz, & Mergner, 2013). However, most of these plants have employed agriculture residues as a feedstock due to their lower recalcitrant compared to woody substrates (hardwood and softwood). Most of the pretreatment approaches used by these companies have involve some form of steam pretreatment to maximize hemicellulose recovery while opening up the structure of substrate for the following enzymatic deconstruction process (Chandra et al., 2007). Steam pretreatment has been frequently utilized for these initial commercialization efforts most likely due to its simplicity and its ability to liberate hemicellulose sugars into the water soluble stream. The hemicellulose sugars can also be further combined with

the water insoluble cellulose enriched fraction and treated as a “whole slurry” to minimize the processing steps and maximize final sugar concentrations. Despite these advantages, there are still several challenges that need to be overcome if we are to achieve economically feasible softwood derived biofuel production. Much of the biomass lignin is typically condensed during normal acid catalyzed steam pretreatment. Thus, this condensed lignin is commonly combusted to provide energy which does not maximize its potential value in the attractive biorefinery concept (Lin, 2016). Another challenge for hardwood and agricultural feedstocks is that the hemicellulose component contains mostly C_5 sugars (xylose and arabinose) which cannot be directly used by prevalent ethanol producing yeasts such as *Saccharomyces cerevisiae* (Parreiras et al., 2014; Tomás-Pejó, Oliva, Ballesteros, & Olsson, 2008).

Softwoods are the most abundant lignocellulosic feedstock in British Columbia and Lodgepole pine is the predominant softwood species in the interior BC (L. Kumar, Chandra, & Saddler, 2011). Compared with agriculture residues and hardwoods, softwoods have the advantage of containing primarily C_6 fermentable sugars (glucose, galactose and mannose) as part of their hemicellulose component, which can be readily utilized by *Saccharomyces cerevisiae* along with the glucose that results from enzymatic hydrolysis of the cellulose component. Over the last 15 years, much of BC’s lodgepole pine forest has been devastated by the mountain pine beetle infestation. As a result, the timber derived from the infected can loses its suitability for traditional lumber applications and has even been shown to have varying and unpredictable behavior when utilized as a feedstock for making pulp and paper (Kapu, Piddocke, & Saddler, 2013; S. L. Kumar, 2014). Therefore, an alternative process to utilize this softwood biomass could be as a biorefinery feedstock to produce bioenergy, biofuels and biochemicals.

However, when compared to agricultural and hardwood biomass, due to their higher lignin content and more interconnected lignin structure, softwoods tend to be more challenging substrates for bioconversion. When pretreating when utilizing compromised acid catalyzed steam conditions to treat softwoods, although the hemicellulose could be broken down and solubilized into the water soluble stream, at the same time the water insoluble fraction becomes enriched in lignin to levels approaching 50%. This significantly limits the efficacy of the enzymatic hydrolysis process. In addition, the use of an acid catalyst and relatively severe steam pretreatment conditions to treat softwoods results in the production of fermentation inhibitors (e.g. furans, aliphatic acid and phenolic compounds) derived from sugar and lignin degradation (Jönsson, Alriksson, & Nilvebrant, 2013; Cavka, Alriksson, Ahnlund, & Jönsson, 2011; Eva Palmqvist, 2000). This severely limits the ability of yeast to ferment the liberated sugars. As well as end-product inhibition, it is difficult to produce high sugar concentrations for fermentation from steam pretreated softwoods. This is primarily the result of the high lignin content of the substrate physically restricting the accessibility of the cellulose to cellulase enzymes. As a result, high enzyme loadings are required to get anywhere close to acceptable enzymatic hydrolysis yields (Mooney, Mansfield, Touhy, & Saddler, 1998; Xuejun Pan, 2005; Zhou, Lou, Yang, Zhu, & Qiu, 2013). To try to overcome these challenges, various types of post treatment such as alkaline peroxide, oxygen and sulphite treatments, have been explored with a goal of either modifying the lignin or partially removing the lignin as a means of enhancing the accessibility/hydrolyzability of pretreated substrates (Xuejun Pan, 2005). Among all these post treatments, sulphite post treatment has the advantage of being highly specific for lignin removal/modification while possibly enhancing the recovery of lignosulfonates as a value added co-product. Coincidently, sulphite has also been reported by several other groups to effectively

detoxify fermentation inhibitors at milder temperature, primarily because it has a good reducing capacity (L. Kumar et al., 2011).

Even though sulphite has been used to both detoxify fermentation inhibitors and modify lignin as a method of enhancing enzymatic hydrolysis, previously these two kinds of sulphite treatment have only been studied separately and conducted at different conditions in order to maximize the individual effects according to their proposed reaction mechanisms (sulphonation VS. reduction). Therefore, in the work reported here, considering the issues faced when processing steam pretreated softwood whole slurry, a one-step sulphite treatment was developed to simultaneously modify lignin and detoxify fermentation inhibitors, with the goal of achieving high ethanol yields and titers using a reasonable/low enzyme loading.

1.1 Lignocellulosic biomass

1.1.1 Lignocellulosic biomass components

All lignocellulosic biomasses are potentially valuable sources of carbohydrates. In nature, the dominant simple sugars which serve as building blocks for the production of complex carbohydrates include the hexose sugars (C_6) D-glucose, D-mannose, and D-galactose, as well as the pentose sugars (C_5) D-xylose and D-arabinose (Walker, 2011). Among them, glucose and mannose are the fermentable sugars which can be readily utilized by the traditional ethanol producing yeast *Saccharomyces cerevisiae*.

In the plant cell wall, these monomeric sugars are polymerized into complex carbohydrates, namely cellulose and hemicellulose. Cellulose has a highly organized crystalline

structure constituted by linear glucan chains, while hemicelluloses has less organized structure formed by typically branched polysaccharides (Carpita & McCann, 2000). Besides cellulose and hemicellulose, another major component of lignocellulosic biomass is lignin, which is a highly branched, three-dimensional polymer derived from three phenylpropane units (monolignols), namely guaiacyl (G, conniferyl alcohol), syringyl (S, sinapyl alcohol) and p-hydroxyphenyl (H, p-coumaryl alcohol).

1.1.2 Cellulose

In wood, around 45%-50% of the dry biomass is cellulose, which is a homo-polysaccharide, composed of β-D glucopyranose units. All of the β-D glucoses are linked by the β (1-4) glucosidic bonds into a linear structure with the basic repetitive unit of cellobiose (glucan chain), which has a strong tendency to form inter- and intra-molecular hydrogen bonds to keep the chains straight and stacked in a sheet-like structure (Figure 1). Although the chain length (which is also described as the degree of polymerization) of the native cellulose is not very clear, it is widely agreed that wood cellulose is approximately made of 10,000 glucose unites (Sjostrom, 1993). Although no hydrogen bonds are found between the sheets, van der Waals forces appear to hold the sheets together (Payne et al., 2015). Cellulose forms a highly organized, crystalline structure under these strong hydrogen bonds and van der Waals forces, interspersed by some disorganized amorphous or paracrystalline regions (Carpita & McCann, 2000). It is widely acknowledged that the tightly packed nature of the cellulose structure is one of the major reasons for its low hydrolyzability by enzymes.

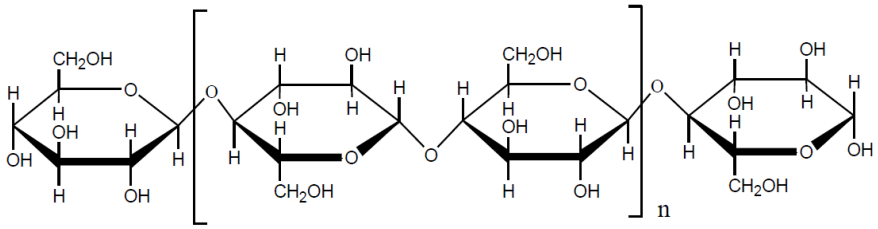


Figure 1 The chemical structure of cellulose. (Adapted from Nakagame 2010)

Although cellulose chains can pack into different forms including cellulose I, II, III, IV, the most abundant cellulose allomorphic form in nature is defined as cellulose I (Payne et al., 2015). Cellulose I has two allomorphic form where the monoclinic cellulose Iβ is dominant in higher plant such as in the softwood cell wall. Triclinic cellulose Iα, which is dominant in bacterial and algal cellulose, has a different hydrogen bond pattern as well as an interlayer chain stacking arrangement as compared to cellulose Iβ (Payne et al., 2015). In general, the cellulose Iβ form is more thermostable than the Iα form (Lynd, Weimer, Zyl, & Isak, 2002). During thermochemical treatment processed, cellulose I can transform into other allomorphic forms such as cellulose II, III, and IV, when NaOH, ammonia, and glycerol are added respectively (Ishikawa et al., 1997). All these different cellulose allomorphs have been shown to greatly influence the efficacy of the enzymatic hydrolysis process (Lynd et al., 2002).

1.1.3 Hemicellulose

Unlike cellulose which is a homopolysaccharide made of pure glucose, hemicelluloses are complex heteropolysaccharides comprised of glucose, xylose, mannose, galactose and arabinose (Sjöström, 1993). Hemicellulose accounts for approximately 25%-30% of the dry biomass in most woody materials although its structure and composition varies widely among

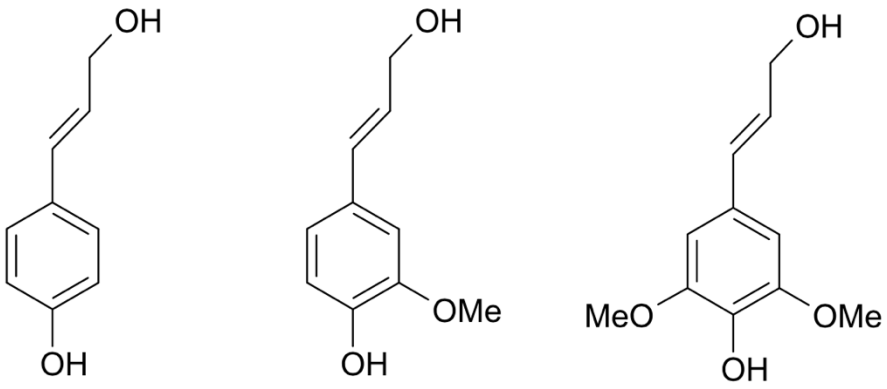
different biomass species (Sjostrom, 1993). Most hemicellulose are extensively branched, so consequently relatively easy to be accessed/hydrolyzed (compared with cellulose) by acids or hemicellulase enzymes (Saha, 2003). The degree of polymerization of most hemicellulose is about 200 which is much smaller compared to the DP of cellulose (Sjostrom, 1993).

Unlike the xylan enriched hemicellulose components in hardwood biomass and annual plants, the major hemicelluloses in softwood is galactoglucomannans (accounting for ~20% of the dry biomass) (Carpita & McCann, 2000). The backbone of galactoglucomannan is built up of (1-4) linked β-D-glucopyranose and β-D-mannopyranose units (mannose/glucose ratio 3:1), which are often branched with galactose at C_6 position and acetylated at C-2 and C-3 positions (on the average one group per 3-4 hexose units) (Carpita & McCann, 2000). As well as the galactoglucomannan, a small portion of arabinoglucuronoxylan (~5% of the biomass) also exists in the softwood hemicellulose. This polymer has a framework containing (1-4)-linked β-D xylopyranose units which also be partially substituted at C-2 with 4-O-methyl-α-D-glucuronic acid groups by, on the average, two residues per ten xylose units (Scheller & Ulvskov, 2010). Thus, compared to hardwood and agricultural residues, the high hexose (mannose, galactose and glucose) content of softwood hemicellulose makes it an attractive candidate for bioethanol production, since these C_6 sugars could be easily fermented to ethanol by industrially relevant yeast strains.

1.1.4 Lignin

Lignin is a Latin derived term from the original word "*lignum*" which means "wood" (Sjostrom, 1993). Lignin is a highly branched, three-dimensional polymer that can be composed

of three phenolpropane units (monolignols), namely guaiacyl (G, conniferyl alcohol), syringyl (S, sinapyl alcohol) and p-hydroxyphenyl (H, p-coumaryl alcohol) (Figure 2). Lignin is synthesised by free-radical coupling of phenoxyl radicals which are formulated through enzyme induced dehydrogenation of the monolignol units (Humphreys & Chapple, 2002). Although the phenoxyl radicals can be linked through various carbon-carbon and carbon-oxygen bonds, the β-O-4 linked aryl ether linkages is dominant in lignin, accounting for more than 50% of the total monolignol joint linkages (Adler, 1977; Huang et al., 2016). The physicochemical properties of lignin are known to be highly dependent on the sources of lignocellulosic biomass. Softwood lignins are primarily composed of G units while the hardwood lignins (18-25%) are dominant in G and S units. Grass lignin's contain all three G, S, and H monolignol units (Kelley et al., 2004; Popper, 2008). Compared to sinapyl (S) units, the guaiacyl (G) units (only the third position of the aromatic ring is methoxylated) are able to form linkages at the position 5 with other monolignols (Cai, Bhuiya, Shanklin, & Liu, 2015). The formation of 5-5` carbon-carbon bonds between two guaiacyl units make lignin more problematic for the biomass bioconversion process(Zhou, Zhu, et al., 2013; Modenbach & Nokes, 2012).



a b c

Figure 2 Chemical structures of the phenylpropanoid lignin precursors a) para-hydroxyphenyl (p-coumaryl alcohol, H) b) guaiacyl (coniferylalcohol, G) c) syringyl (sinapyl alcohol, S) adapted from Adler (1977).

The main role of lignin is to provide mechanical strength and structural support in the plant cell wall. The structural support of lignin is mainly achieved by covalent bonding to the side chains of branched hemicelluloses to formulate the lignin carbohydrate complexes (Sjostrom, 1993). The amount and distribution of lignin is highly dependent on the types of biomass. Generally lignin plays a negative role in the bioconversion process by physically blocking enzyme assess to cellulose, by restricting fiber swelling and by unproductively binding cellulase enzymes through hydrophobic interactions (Zhang, Lei, & Li, 2013; Z. Wang, Lan, & Zhu, 2013). Many pretreatment and/or post-treatment strategies such as SPORL, organosolv pretreatment, wet oxidation and sulphonation have been developed to try to overcome the recalcitrant nature of biomass lignin (Sannigrahi, Miller, & Ragauskas, 2010; Zhu, Pan, Wang, &

Gleisner, 2009; Chu, Chandra, Kim, & Saddler, 2017). These methods can remove and/or modify the lignin structure thereby improving enzyme performance during cellulose hydrolysis (L. Kumar et al., 2011; Leu, Zhu, Gleisner, Sessions, & Marrs, 2013).

For softwood biomass, the higher lignin content and the high proportion of guaiacyl subunits (G) have been shown to increase its recalcitrance towards bioconversion as compared to hardwoods and agricultural residues (L. Kumar, Chandra, & Saddler, 2012). Of all the biomass pretreatment methods (which will be discussed in detail below), steam pretreatment has been shown to be one of the most promising methods for softwoods (R P Chandra et al., 2007). However, under typical acid catalyzed steam pretreatment conditions, the softwood lignin is also more prone to condensation reactions, which are not only more problematic to the bioconversion process but also limit its reactivity for the downstream value added applications (Ewanick, Bura, & Saddler, 2007; Li, Henriksson, & Gellerstedt, 2007; Sannigrahi, Ragauskas, & Miller, 2010). For example, the increased lignin hydrophobicity that results from the condensation of softwood lignin significantly increases the non-productive binding between cellulases and lignin, therefore decreasing hydrolysis yields under acid catalyzed steam pretreatment conditions (Pan, Zhang, Gregg, & Saddler, 2004).

1.2 Softwood biomass for bioethanol production

1.2.1 Softwood cell types

Softwood cell wall are mainly composed of two types of cells namely the tracheids (90%-95%) and the ray cells (5%-10%) (Sjostrom, 1993). These two cells are specialized for different

functions where the longitudinal tracheids (1.5-5.0 mm in length and 20-70 um in diameter) mainly play the role of mechanical support and conduction while the ray cells (contains ray parenchyma and ray tracheids) primarily assist with nutrient transport and storage (Booker & Sell, 1998). Aside from these two cells, softwoods also contain resin canals in both the longitudinal and transverse direction, which serve a protective role in the tree (Booker & Sell, 1998).

1.2.2 Softwoods for bioconversion

As mentioned earlier, softwoods are the most abundant lignocellulosic feedstock in British Columbia and Lodgepole pine is the predominant softwood species in the interior of BC (Nakagame, 2010). As of 2010, the world's conifers constitute around 40% of the total forest stock and they are particularly abundant in the countries where the taiga/boreal forests predominate such as the Nordic countries, Canada and Russia. (Hakan Ekstrom, 2012). Softwoods are already a significant commercial commodity being the source of about 80% of the world's timber. Traditional centers of production are the Baltic region and North America. However, over the last 15 years, a significant component of this Lodgepole pine forest has been devastated by the mountain pine beetle infestation (Forests, 2012). In British Columbia, the ongoing outbreak of the mountain pine beetle has created an abundance of beetle-killed Lodgepole pine at advanced stages of infection. This forest derived material has limited value for traditional structural applications. As a result, there is lower overall lumber recovery from these infected trees, due to splitting and cracking. Chips have been shown to have varying and unpredictable behavior when utilized as a feedstock for pulp and paper manufacture (Les Safranyik, 2006). In addition, since softwoods are an already established commercial entity,

utilization of industrial residues for energy applications such as palletization has been shown to result in immediate financial gains. Therefore, there is a much greater amount of softwood residues available and some of this material is used in BC's growing pellet sector. However, an alternative process to utilize these softwood residues could be for the production of bioenergy, biofuels and bio-chemicals (Luo et al., 2010).

Softwoods have been shown to be one possible feedstock for an enzyme-based biomass-to-ethanol biological conversion process (Safari, Karimi, & Shafiei, 2017; Qin et al., 2016). As mentioned earlier, in addition to their abundance, compared to agricultural and hardwood-derived biomass sources, softwoods including species of Lodgepole pine, Norway spruce, Western hemlock, and Douglas-fir, have the advantage of having a hemicellulose component that is primarily composed of hexose sugars that can be much more readily fermented to ethanol by conventional yeasts such as *Saccharomyces cerevisiae* (Kapu et al., 2013; Larsson et al., 1999). However, as also mentioned earlier, softwoods generally have a higher lignin content and a significantly different fiber structure and chemical composition, which make them more recalcitrant towards deconstruction by biochemical processes compared to hardwoods and agricultural biomass (Mooney et al., 1998; Xuejun Pan, 2005; L. Kumar, Arantes, Chandra et al., 2012). Thus, if a biochemical conversion scheme can be demonstrated to be effective on softwoods, similar strategies could likely be adapted universally to all lignocellulosic feedstocks. These unique physical characteristics and chemical composition of softwoods create particular challenges but also potential opportunities when attempting to utilize softwoods as a biorefinery feedstock. Considering the recalcitrance of softwood biomass, the most important step for applying biochemical conversion to softwood biomass is the pretreatment process, which aims to

reduce biomass recalcitrance and separate/recover all of the biomass chemical components in a useful form (Pienkos & Zhang, 2009; Stelte, 2013).

1.3 Steam pretreatment and post treatment

Some form of pretreatment is the prerequisite in all the bioconversion process. A "good" pretreatment can be defined as an economical and scalable process that allows for an increase in the accessibility of the biomass to hydrolytic enzymes to allow enzymatic hydrolysis at low enzyme loadings while separating cellulose, hemicellulose and lignin and good recovery in a useful form (R P Chandra et al., 2007). Pretreatment strategies have generally been categorized into biological, physical and chemical processes, or a combination of these approaches (R P Chandra et al., 2007). Among these processes, pretreatments that combine both chemical and physical elements are generally referred to as physiochemical processes. This type of pretreatment has received most attention in recent years. Unfortunately, many pretreatment conditions that allow the production of an easily-digestible cellulose enriched substrate (usually at very high pretreatment severity) typically result in significant loss of the hemicellulose component (L. Kumar, 2013; Zhang et al., 2013). The degradation of the hemicelluloses not only reduces the carbohydrate recovery, but also results in the formation of various degradation products which are inhibitory to the downstream enzymatic hydrolysis and subsequent fermentation processes (Jönsson et al., 2013; P. E. R. Persson et al., 2002). Therefore, the work reported in this thesis has focused on adapting pretreatment conditions and assessing their suitability for application on softwood lignocellulosic substrates.

1.3.1 Steam pretreatment

Steam pretreatment has been derived from the Masonite pulping process which was initially developed by William H. Mason in 1926 (R P Chandra et al., 2007). In the original process the wood chips were steamed at a high temperature and pressure (~285°C and 3.5 MPa) for approximately 2 minutes, followed by a rapid increase of the pressure (up to 7 MPa) for about 5 seconds and then exploded to a sample collector at atmospheric pressure. Subsequent, two stage low consistency refining resulted in a dark pulp of about 75% yield suitable for the manufacture of high-density fiberboard (Stelte, 2013). The process showed great potential to achieve efficient fiber separation with relatively low energy input (Koran et al., 1978). The Masonite process was later substantially modified into today's chemi-thermo-mechanical pulping process (Vit and Kokta, 1986; Kokta and Vit, 1987; Law and Bi, 1989; Heitner et al., 1991).

Steam pretreatment has been shown to be an efficient and industrially relevant method for the newly developed biorefinery industry (Stelte, 2013). Generally, steam pretreatment is conducted at relatively high temperatures and pressures (160-230 °C and 3.5 MPa) without or with acid catalysts (e.g. SO_2 and H_2SO_4) for about 5 to 10 min. This results in the dissolution of large amounts of the hemicellulose sugars into a water soluble liquid stream while retaining the cellulose and most of the lignin in the water insoluble solid stream. Steam pretreatment combines both physical and chemical processes, solubilizing the hemicellulose through acid hydrolysis while relocating lignin by causing it to flow above its glass transition temperature, increasing overall cellulose accessibility (Donaldson, Wong, & Mackie, 1988). The extent of steam pretreatment can be measured by the severity factor. In early work, it was reported by Vroom

that the time and temperature of Kraft pulping was inter-dependent for a given pulp yield and that it was possible to combine this effect into a single parameter (Vroom, 1957). The resulting H factor is used in the pulp and paper industry to predict the degree of delignification and pulp yield (Chum, Johnson, Black, & Overend, 1990). A lesson has been learned from the pulp and paper industry as the severity factor which is used to assess the pretreatment effects was modified from the H factor. The severity factor can be expressed by the equation as follows, where "T" is the temperature in °C and "t" is the time in minutes (Overend & Chornet, 1987).

$$\text{Ro} = \int_0^t e^{(T-100/14.75)}\ dt$$

Since the severity factor is used to describe the pretreatment effects, it has proven to be useful in the field of bioconversion for comparing different pretreatment conditions and feedstocks. Initially, it was largely used to predict the effect on enzymatic hydrolysis but not much for the recovery of hemicellulosic sugars (Overend & Chornet, 1987). It was widely acknowledged that the digestibility of biomass could improve with an increase of the pretreatment severity (Ewanick et al., 2007). Subsequently, acid was introduced in the pretreatment process as a catalyst to reduce the required temperature and reaction time. Thereby, the influence of the acid catalyst was also integrated into the model by incorporating pH as an additional variable (Chum et al., 1990). The resulting equation was as follows.

$$\text{Ro} = \int_0^t e^{(T-100/14.75)}\ dt - \text{pH}$$

Most of the commercial and demonstration facilities currently employ steam pretreatment to process primarily agricultural residues or hardwood feedstocks (Janssen et al., 2013).

However, when softwoods are used, providing good hemicellulose recovery in the liquid fraction and a cellulose rich water insoluble fraction that is amenable to hydrolysis at relatively low enzyme loadings, has proven to be more challenging (Hu, Chandra, et al., 2015). The recovery of the hemicellulosic sugars in the liquid fraction has been recognized to be crucial if we want to achieve a high overall sugar recovery (Olsen, Arantes & Saddler, et al, 2012). Even though the ease of hydrolysis of the water insoluble, cellulosic rich fraction could be largely enhanced by increasing the pretreatment severity, the usage of high severity pretreatment also diminishes the overall sugar recovery and fermentability of the water soluble fraction through the degradation of solubilized hemicellulose sugars (Pienkos & Zhang, 2009; Shuai et al., 2010). On the other hand, pretreatment conditions which could provide reasonable hemicellulose recovery, usually do not result in a cellulosic fraction that can be easily hydrolyzed. Thus, higher enzyme loadings are required to achieve somewhat reasonable hydrolysis yields (Richard P. Chandra et al., 2016). It should be noted that the recovery of the hemicellulosic sugars is extremely important for softwoods for bioethanol production, since these are mainly hexose sugars that can be directly used by the conventional *Saccharomyces* yeast. Therefore, one of the key challenges for steam pretreatment is to recover as much hemicellulose as possible while enhancing the ease of enzymatic hydrolysis of water insoluble cellulosic component at low enzyme loadings.

1.3.2 Post-treatment

As discussed above, variations on steam pretreatment have been the predominant methods used in many bioconversion demonstration/commercialization processes (Ewanick et al., 2007; Modenbach & Nokes, 2012). However, the solubilisation of hemicellulose enriches the

lignin content in the resulting water insoluble substrate, consequently increasing substrate recalcitrance. As mentioned earlier, a reasonable enzymatic hydrolysis yield of acid catalysed steam pretreated softwood substrates at compromised condition usually requires unfavourable high enzyme loadings (Pribowo, 2014). Although high severity steam pretreatment can produce more hydrolysable substrate, it also creates large amount of hemicellulose sugar degradation products such as acetic acid, furfural and HMF (hydroxymethylfurfural) which inhibit the activity of enzymes and yeast during subsequent hydrolysis and fermentation processes (Modig, Lidén, & Taherzadeh, 2002; Eva Palmqvist & Almeida, 1999). In addition, high severity pretreatment also cause lignin condensation, which strongly limits the potential of further lignin valorization (Ragauskas et al., 2014). Alternatively, in order to improve the ease of hydrolysis of lignocellulosic substrates after steam pretreatment at a low severity, post treatment has been applied which aims at lignin modification and/or removal thus to decrease substrate recalcitrance. So far, the most common post treatment methods includes peroxide post treatment, sulphite post treatment, and oxygen post treatment (Xuejun Pan, 2005). For example, the alkali peroxide could be used to remove the residual lignin in the steam pretreated softwood biomass which significantly improved the digestibility of the substrates during enzymatic hydrolysis process (Yang, Boussaid, & Saddler, 2002). In addition, the alkali oxygen delignification has been successfully applied to deligninfy the acid steam pretreated softwood biomass (Xuejun Pan, 2005; Pan, Zhang, & Saddler, 2004). Other work has shown that sulphite post treatment can both remove and modify lignin thereby improving the hydrolysability of the steam pretreated softwood substrates. This indicated that not only lignin removal but also lignin modification plays an important role in enhancing substrate digestibility (Mooney et al., 1998;L. Kumar, 2013). However, all of the previous research only focused on the hydrolysis of washed steam

pretreated softwood substrates. Plus, the fermentability of these post treated substrates was also not assessed. Although it has been suggested that chemicals used in post-treatment might have some negative effects on the enzymes and yeasts, since high amount of sulphite and peroxide have been proven to poison yeast (Petrovska, Winkelhausen, & Kuzmanova, 1999), the influence of post-treatment on a whole slurry hydrolysis/fermentation system (no washing involved) need to be further investigated.

1.3.3 Sulphite pretreatment to overcome the recalcitrance of lignocellulosic biomass

Sulphite pretreatment to overcome the recalcitrance of lignocellulosic biomass (SPORL) has been shown to be one of the most effective pretreatments for softwood biomass (G. S. Wang, Service, & Rockwood, 2009; Zhu et al., 2009; Qin et al., 2016). This pretreatment approach combines mild acid sulphite pulping and mechanical refining to achieve effective hemicellulose removal and lignin modification The SPORL process initially involved treating biomass using 2-4% sulfuric acid and 4-10% sodium bisulphite at temperatures from 160-180 ℃ for 30-75 minutes (G. S. Wang et al., 2009; Zhu et al., 2009). Since the pretreatment is performed under acidic conditions in the presence of bisulphite, the hemicellulose and the lignin are partially solubilized in the initial acid sulphite cooking stage prior to a subsequent mechanical refining stage which is employed for size reduction. Although SPORL is performed under acidic conditions that can promote lignin condensation when treating softwood biomass, the combined effects of the addition of sulphite to the reaction and the modulation of the reaction pH between 3.5-5 favors lignin solubilisation. Due to the removal/modification of lignin and hemicellulose, softwood substrates treated by the SPORL process can be readily hydrolyzed at enzyme loadings

of approximately 20 FPU/g cellulose at 18 % total solid loading(Zhu, Gleisner, & Tian et al., 2011). Another potential issue with SPORL pretreatment is the production of potential fermentation inhibitors. It was shown that the pretreatment liquor in SPORL process contained furfural 2.2 g/L, HMF 2.7 g/L, and phenolic compounds 5.3 g/L (Tian, Luo, & Zhu et al., 2010). However, despite these possible drawbacks, it is apparent that sulphite mediated processes such as SPORL are most effective when attempting to pretreat softwoods.

1.3.4 Sulphite treatment during steam explosion (pretreatment) for the production of pulp and pretreated substrates

As well as utilizing acidic and neutral sulphite prior to mechanical refining for the production of pretreated substrates for bionconversion or pulp, previous work has also examined the effects of utilizing sulphite during the steam pretreatment process itself (Richard P. Chandra et al., 2016). Most of the work applying sulphite during steam explosion has been performed under neutral sulphite conditions. One objective has been to produce high-yield pulps while consuming less energy for their production. As will be discussed in more detail below, the use of sulphite under neutral conditions results in limited solubilization of the lignin component while preserving most of the carbohydrates in the pulp furnish. So called “explosion pulps” have been produced by impregnating wood chips with sulphite with subsequent steam explosion where the high temperatures facilitate the sulphonation of the lignin component, while the explosive decompression results in size reduction of the biomass (Kokta & Ahmed, 1998). Much of the work on steam explosion pulping is summarized in a review by Kokta and Ahmed (Carrasco, Kokta, & Garceau et al., 1994; Kokta & Ahmed, 1998). The process involves several steps that

include soaking the chips at 60 °C for 24 h in solutions containing 8% Na_2SO_3 and various concentration of either Na_2CO_3 or $NaHCO_3$, ranging from 0 to 2%. Next the chips are cooked at a temperature of 190-200 °C for a short period of time (1-2 mins). This material is then rapidly released with a pressure drop after the cooking followed by a further step of mechanical refining (Carrasco et al., 1994). As mentioned earlier, this process was derived from the Mason process that was used to produce fibers for the manufacture of fiberboard consisting of similar steps but without chemical impregnation. The drawback of the Mason process was the weak mechanical resistance and dark colour (Kokta & Ahmed, 1998). The new steam explosion pulping processed that was suggested by Kokta was a result of the chemical impregnation step now used by the chemi-mechanical pulping process (Carrasco et al., 1994). The addition of sulphite in Kokta's steam explosion work improved the mechanical properties of the pulps and decreased the refining energy compared with that required in the Mason process. Although this method has mostly been applied to hardwoods, it was shown that sulphite impregnated Black Spruce and Douglas-fir exploded at temperatures of >200 °C cause fibre separation in the middle lamella and limited fibre damage, thus preserving carbohydrate yield (Kokta & Ahmed, 1998).

Recognizing the ability of this process to sulphonate lignin while preserving the carbohydrate components in the water insoluble fraction to potentially increase sugar concentrations and reduce fermentation inhibitors, researchers recently applied the neutral sulphite steam explosion to poplar wood (Richard P. Chandra et al., 2016). The resulting substrate was rich in sulfonate groups and was readily hydrolyzed by cellulases at a solid loading of 10% while recovering >85% of the carbohydrates present in the original biomass. However,

this neutral sulphonation steam explosion approach has yet to be applied to softwood biomass at high solids loadings with subsequent fermentation.

1.4 Mechanism of sulphite treatment of biomass

Sulphite reacts with biomass under acidic, neutral and alkaline conditions. Each of these approaches is derived from various types of sulphite pulping (Gierer, 1985). At all pH values (ranging from pH 2-11), sulphonation readily occurs on lignin macromolecules containing free phenolic hydroxyl groups due to the formation of quinone-methide type intermediates that react with sulphite and bisulphite (Gierer, 1985). Generally, acidic conditions operate through the nucleophilic addition of bisulphite to lignin which results in the solubilisation of larger molecular weight portions of the lignin macromolecule. Under neutral conditions, lignin is sulfonated and is mostly retained in the biomass due to the lack of acidolysis or alkanolytic lignin cleavage at neutral conditions. Alkaline sulphite pulping operates similarly to alkaline Kraft pulping with the breakdown and solubilisation of lignin via sulphonation and alkaline induced lignin scission. The amount of sulfur dioxide, bisulphite, is dependent on the pH of the solution. At acid conditions in the range of pH 3-5, the predominant sulphur species is bisulphite while at alkaline conditions (pH 8-10), sulphite is the predominant species (Figure. 3). At a given temperature, the extent of delignification depends largely on the pH of the cooking liquor.

During acid sulphite pretreatment, the cleavage of less prevalent α-aryl ether bonds by bisulphite is the most significant mechanism of lignin fragmentation. Thus, as mentioned above, lignin is solubilized in larger molecular weight fragments. As well as lignin fragmentation, under acidic conditions, competing condensation reactions can induce new stable carbon-carbon

linkages which inhibit the solubilisation of lignin. As well as lignin solubilisation and condensation, the labile hemicellulose is typically solubilized, de-acetylated and/or de-branched under acidic conditions due to hydrolysis and de-acetylation reactions.

Increasing the pH to neutral or alkaline conditions shifts the reactions towards mostly lignin phenolic groups (Sjostrom, 1993). Initially, quinone methides are formed by the deprotonation of the phenolic group under alkaline conditions, which cleaves the α-hydroxyl or α-ether group. The quinone methide can then be attacked by bisulphite or sulphite with subsequent displacement of the β-substituent an additional sulphite or bisulphite ion. The hemicelluloses on the biomass typically undergo deacetylation under the alkaline conditions. As well as hemicellulose debranching, depending on the level of alkalinity, the carbohydrate components can also undergo peeling reactions and alkaline hydrolysis (chain scission). Due to their amorphous nature, the hemicellulose components are more prone to the peeling reactions which result in the formation of saccharinic acids and carbohydrate losses under alkaline conditions.

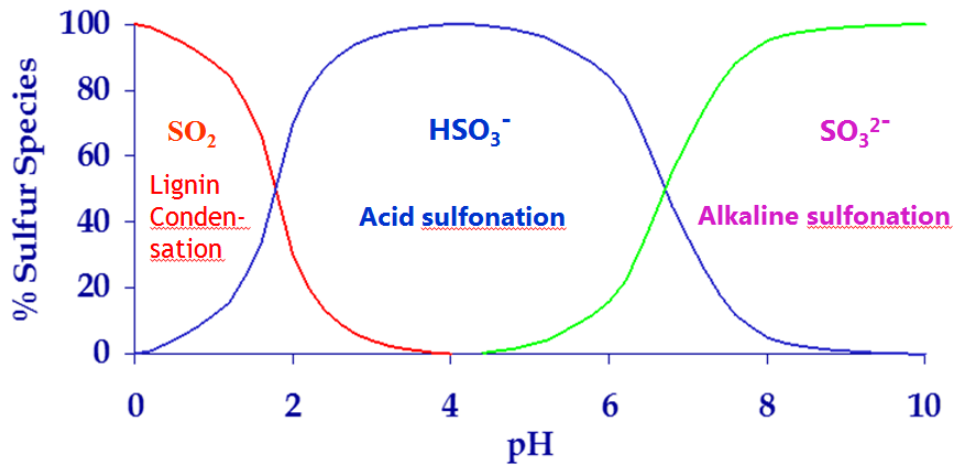


Figure 3 Effect of pH on Sulfur Dioxide Species. Adapted from a pulping and bleaching lecture by University of Washington.

Sulphonation leads to the generation of hydrophilic sulphonic acid groups (Chagaev, Heitner, & Hellstern, 2005). Sulphonation and hydrolysis are the reactions which result in delignification and the increase in the hydrophilicity of lignin. The solubilized lignin, or lignosulfonates that result from sulphonation are currently one of the only marketable lignin derived products. Although lignin has been shown to play an influential role in softwood recalcitrance (L. Kumar et al., 2012), the sulfonated residual lignin in the substrate has been shown to be far less detrimental towards enzymatic hydrolysis compared to the native biomass lignin. This is thought to be due to the sulfonic acid induced swelling of the substrate and a decrease in non-productive binding between cellulolytic enzymes and the hydrophilic sulfonated lignin(Zhu et al., 2009; L. Kumar, 2013).

1.5 Enzymatic hydrolysis

Besides pretreatment, subsequent enzymatic hydrolysis of the pretreated lignocellulosic substrates plays an essential role in the cost effective bioconversion process due to the high price of the enzymes. Previous work by many research groups and enzyme companies has significantly decreased the required enzyme dosage by 20-30 fold, by improving the individual and collective enzyme activities. However, much of the research on enzyme development was aimed at optimizing enzyme cocktail for biomass substrates such as corn stover rather than woody biomass substrates.

1.5.1 Bioconversion enzyme system

Glycoside hydrolases (GH) have been grouped into 132 families as described in the Carbohydrate Active Enzymes Database (CAZY) in which cellulolytic enzymes belong to at least 14 families (Henrissat & Daviest, 1997). An effective cellulase mixture typically consists of three major types of cellulase enzymes namely endoglucanase, exoglucanase and β-glucocidase. As well as these three main types of cellulases, some so called "accessory enzymes" such as hemicellulases, ligninases, swollenins and lytic polysaccharide monooxygenases also contribute significantly to the efficient deconstruction and hydrolysis of various types of lignocellulosic substrates (Hu, Chandra, et al., 2015; Hu, Gourlay, et al., 2015; Gourlay, Hu, Arantes, Penttilä, & Saddler, 2015; Hu, Arantes, & Saddler, 2014). The mechanism of enzymatic hydrolysis of the glycosidic bond takes place via an acid/base catalysis, which requires two critical carboxylate groups that play the role of a proton donor and a nucleophile/base (Payne et al., 2015). The major organism that has been used to express cellulase enzyme cocktail is the filamentous fungi

Trichoderma reesei due to its tremendous ability to secrete significant amounts of cellulolytic enzymes (Aker, Inter, & Immel et al., 2001; I. Persson, Tjerneld, & Hahn-Hagerdal, 1991).

In the fungal cellulase system, the main exoglucanases are Cel7A and Cel6A (Payne et al., 2015). The Cel7A which is also named as CBH I, accounts for 50-70% of the total protein expressed from a *Trichoderma reesii*, and is thus a major component of the cellulase mixture (I. Persson et al., 1991), while the Cel6A named as CBH II accounts for another 10-15% of the total protein in the cellulase system (Payne et al., 2015). Two types of exoglucanases process the hydrolysis from the reducing (by Cel7A) and non-reducing (by Cel6A) ends of the cellulose chain respectively, releasing cellobiose as the major hydrolysis products (Jørgensen, Kristensen, & Felby, 2007). Both Cel7A and Cel6A have a two domain structure, of which a large catalytic domain consists of a tunnel-like structure with 10 separate binding subsites around the active center (Payne et al., 2015). Through a glycosylated linker peptide, the catalytic domain connects with a small family 1 cellulose-specific carbohydrate binding module (Lou et al., 2014). A large number of interactions with a single cellulose chain and a processive style of hydrolytic action probably result from this special structure (Beckham et al., 2010).

As well as exoglucanases, endoglucanases (e.g. Cel7B and Cel5A) are the other major cellulase components in the fungal cellulase system. They normally account for 6-10% of the total protein secreted by *T.reesei* (I. Persson et al., 1991). The function of the endoglucanases during hydrolysis is to dramatically decrease the degree of polymerization (DP) of cellulose chains and create more reactive sites (reducing and non-reducing ends) for the exoglucanases (Van Dyk & Pletschke, 2012). The endoglucanases typically have a similar two domain structure as the exoglucanases, but unlike exoglucanase, their catalytic domain has a more open active site

cleft which is more suitable for grabbing the glucan chains within the amorphous cellulose region (Kleywegt et al., 1997). The major hydrolysis products of endoglucanases are cellotriose, cellobiose and glucose (Payne et al., 2015).

Unlike endo/exo-glucannases, β-glucosidases have only one domain which has a pocket-like structure (Chirico & Brown, 1987; Jäger, Brumbauer & Re et al., 2001). The major role of β-glucosidases is to hydrolyze the soluble cellodextrins created by the exo/endo-glucanases to glucose (Jäger et al., 2001). Although different types of disaccharides and cellodextrins can be effectively hydrolyzed (Langston et al., 2006; Korotkova et al., 2009), glucose and gluconolactone have been shown to be strong competitive inhibitors to its activity (Nishimura and Ishihara, 2009). This inhibition can become a big challenge when hydrolyzing substrates at high solids loadings or in the presence of solubilized sugars during the whole slurry hydrolysis of steam pretreated substrates. This problem is described in more detail later in the thesis.

1.5.2 Factors affecting enzymatic hydrolysis of lignocellulosic substrates

1.5.2.1 Enzyme factors

The ease of hydrolysis of a given substrate can be affected by enzyme factors such as the enzyme inhibition as well as the constituents of the enzyme cocktail (Zhai, Hu, & Saddler, 2016; Hu, Chandra, et al., 2015). It is widely acknowledged that high concentrations of "end products" such as glucose or cellobiose can significantly limit the rate and yields during cellulose hydrolysis (Quintanilla, 2013). For example, exoglucanases and endoglucanases are directly inhibited by cellobiose while glucose strongly inhibits all types of cellulase enzymes (Mandels &

Reese, 1965; Eric Johnson, 1982). In terms of inhibition mechanism, it mainly involves competitive and uncompetitive as well as some others such as interfering enzyme-substrate interactions and exoglucanase processive movements (Payne et al., 2015; Zhai, Hu, & Saddler, 2016).

The hydrolysis of lignocellulosic biomass requires a combination of cellulase components and so called "accessory enzymes" whose synergistic action is more efficient than the sum of the actions of the individual enzymes (Hu, Chandra, et al., 2015; Hu, Gourlay, et al., 2015; Hu et al., 2014). Much of the initial research in this area focused on optimizing cellulase components for the hydrolysis of "model" cellulosic substrates such as cotton fiber, Avicel, and filter paper (Baker et al., 1998; Kim et al., 1998; Boisset et al., 2001). However, it is increasingly realized that the optimized cellulase mixtures based on "model" cellulosic substrates do not reflect their hydrolysis potential on "real-life" pretreated lignocellulosic biomass (Meyer et al., 2009; Gao et al., 2010). More recently, it has been recognised that some so called cellulase "accessory enzymes" and/or disrupting proteins, such as hemicellulases, lytic polysaccharide monooxygenase (LPMOs), loosenin and swollenin, although not directly hydrolyze cellulose, can significantly enhance the hydrolytic performance of cellulase enzymes on various cellulosic substrates (Gourlay et al., 2015; Hu, Chandra, et al., 2015). For example, we and others have found that LPMO AA9, swollenin and xylanases can synergistically cooperate with cellulase and significantly improve the hydrolytic efficiency of cellulases over a range of pretreated biomass substrates (Gourlay et al., 2015; Hu, Chandra, et al., 2015). In addition, small amounts of other "accessory enzymes" such as acetyl esterase, arabinofuranosidase (Arb), glucuronidase (Gl), and mannanase can also contribute to cellulose hydrolysis. However, the observed synergism

between cellulase and accessory enzymes has been shown to be highly depended on the type of pretreated biomass (Hu et al., 2014). In the case of softwood biomass, mannanases are anticipated to be more beneficial than xylanases since the majority of softwood hemicellulose components have mannan in their backbone structure (Section 1.2.2.2). However, the development of mannanases in the cocktail has received far less attention as compared other "accessory enzymes". Although some authors have shown that mannanases addition can contribute to the hydrolysis of pretreated softwoods, the limited improvement (Várnai, Huikko, & Viikari et al., 2011) might be have been due to less mannan being present in their pretreated biomass. Thus, it is likely that using the "right" enzyme cocktail will help achieve fast and efficient hydrolysis of certain types of pretreated biomass.

1.5.2.2 Substrate factors

As well as enzyme related factors, the physico-chemical characteristics of pretreated biomass substrates also play a major role in influencing the ease of hydrolysis of a given substrate. It has been shown that the content, type, location and arrangement of the cellulose, hemicellulose and lignin govern the overall "accessibility" of the lignocellulosic substrate (Richard P Chandra et al., 2009; L. Kumar et al., 2012; C. A. Mooney, Mansfield, & Saddler et al., 1999). Substrate characteristics are both physical and chemical and range from the macroscopic to the nano-scale. Physical characteristics include, particle size, crystallinity and porosity while chemical characteristics refer to properties such as the substrate chemical composition and the chemical linkages between individual cell wall components. Many substrate properties such as lignin/hemicellulose content and location, gross fiber properties, cellulose

crystallinity (CrI)/degree of polymerization (DP)/accessibility have been shown to influence cellulose hydrolysis (Hu & Saddler et al., 2014).

For softwoods, as mentioned earlier, lignin plays a major role in overall substrate recalcitrance (Richard P. Chandra et al., 2016; L. Kumar et al., 2012; Li et al., 2007; C. Mooney et al., 1998). The removal of lignin from steam pretreated softwood biomass has been shown to improve cellulose accessibility by increasing substrate swelling and porosity, as well as decreasing the non-productive binding of enzymes to the lignin (L. Kumar et al., 2012; C. Mooney et al., 1998). As mentioned earlier, softwoods contain higher amounts of lignin than hardwoods and are rich in guaiacyl subunits, which increases the inherent recalcitrance of the softwood lignin due to the increase carbon-carbon bonds on the more accessible C_5 position in the benzyl ring (Cai et al., 2015). In addition, the softwood lignin is also more prone to condensation reactions during thermochemical pretreatment process which increases the hydrophobicity of the lignin and thus its tendency to non-productively bind cellulases during hydrolysis (Lin, 2016).

1.5.3 High solid loading hydrolysis

It has been reported that the final ethanol distillation process plays a significant role in the overall commercial viability of the biomass-to-ethanol process (W.-D. Huang & Percival Zhang, 2011). Normally, the energy consumed in the ethanol distillation process will increase rapidly if the ethanol concentration is less than 4.5%. This will be detrimental to the cost effective bioethanol production process. Therefore, high solid loading hydrolysis and high gravity fermentation could lead to the high sugar/ethanol concentrations, resulting in the biomass

to ethanol process being an efficient one. Besides saving distillation costs, high solid loading hydrolysis would also result into other significant operational savings such as water and vessel input.

Although high consistency hydrolysis of lignocellulosic biomass seems attractive, unlike the hydrolysis of starch-based first generation bioethanol production that can easily be carried out at a 20-30% solid content, there are lots of challenges with a biomass substrate. These include inefficient mass transfer and increased levels of enzyme inhibitors from both sugar and various degradation products derived from thermo-mechanical pretreatment associated with the high solid loading hydrolysis of lignocellulosic biomass (Kristensen, Felby, & Jørgensen, 2009). Several strategies have been attempted to overcome the challenges associated with the high solid hydrolysis. For example, specially designed reactors and fed-batch hydrolysis have been explored to overcome the rheological problems (Jorgensen et al., 2007; Zhang et al. 2009; Varga et al., 2004), while simultaneous saccharification and fermentation (SSF) processes has been performed to reduce cellulase end product (glucose) inhibition (Varga et al., 2004; Rudolf et al., 2005; Jorgensen et al., 2007). Recent work has also indicated that some of the so called cellulase "accessory enzymes" such as lytic polysaccharide monooxygenase (LPMO) and xylanases can significantly facilitated high solid loading hydrolysis by selectively modifying the carbohydrate network within the pretreated biomass (Hu et al., 2016).

Although effective enzymatic hydrolysis at a 30% of solid loading can be achieved by using the specially designed reactors such as horizontally placed drum with a horizontal rotating shaft mounted with paddlers (Jorgensen et al., 2007) and the peg mixer (Zhang et al., 2009), many authors have claimed that hydrolysis at a lower, 20-25% solid loading would be more

practical (Cara et al., 2007; Zhang et al., 2009; Di Risio et al., 2011). A 20-25% solid loading hydrolysis could achieve more than 100g/L of glucose (pretreated biomass substrates typically contains more than 60% of cellulose) without sacrificing too much of the hydrolysis yield, and a pulp consistency between 20-25% is also typically encountered in the "pulp and paper" industry (Zhang et al., 2009). Therefore, the 25% solid loading hydrolysis was selected in the work reported here to try to mimic an industrial scenario.

The high lignin content of the steam pretreated softwood substrate (~50%) was anticipated to be a challenge for conducting high solid loading hydrolysis, since large amount of cellulase enzymes would tend to unproductively bind to these condensed lignin matrixes due to their hydrophobic properties (C. Mooney et al., 1998). In addition, these lignin will significantly block the accessibility of the carbohydrates within the substrate towards their hydrolytic enzymes (L. Kumar et al., 2012). Although lignin sulphonation should help overcome these two challenges, the sulfonated lignin might induce another challenge at high solid loading hydrolysis due to its increased hydrophilicity (increased difficulty for mixing). Therefore, the degree of lignin sulphonation might have to be compromised between carbohydrate accessibility, lignin-enzyme interaction and lignin-water interaction at high solid loading hydrolysis.

However, as mentioned before, even if a reasonable cellulose hydrolysis yield can be obtained at a 25% of solid loading, the water soluble fraction derived from the steam pretreated softwood biomass (mainly contains hemicellulosic sugars) still need to be integrated in order to obtain a sugar concentration that approaches first generation bioethanol production. However, the use of a "whole-slurry" at solids loadings results in another challenge as the various soluble components generated during steam explosion, including various degradation products derived

from the hemicellulose and lignin fractions, are known to be inhibitory to both the enzymatic hydrolysis and fermentation processes. It has been shown that the sugars and the phenolics are the major group of inhibitors (Zhai et al., 2016). Therefore, another main objectives of the thesis work is to overcome these soluble inhibitors present in the "whole-slurry" solids loadings hydrolysis process. It has been shown that sulphite post treatment can also detoxify phenolic inhibitors through a reduction mechanism (Cavka et al., 2011). Thus, in the project, we will try to develop an one step sulphite treatment process to both sulfonate water insoluble lignin and detoxify the soluble phenolics to improve the "whole-slurry" solids loading hydrolysis of steam pretreated softwood substrates. In addition, the simultaneous saccharification and fermentation (SSF) process will also be employed to try to mitigate the sugars induced end products inhibition of cellulase enzymes, with the main goal of bring the second generation bioethanol production (based on lignocellulosic biomass) as close as possible to first generation bioethanol production (based on sugar/starch feedstocks).

1.6 Fermentation

1.6.1 Yeast strain selection

Yeast strain selection is of great practical importance for the ethanol production and the identification of commercial and wild yeasts is of major concern in many industrial processes (Dition, 2003). For example, in first generation ethanol production, wild yeast contamination will lead to a decrease in the final ethanol production and this will likely be even more problematic for second generation lignocellulosic bioethanol production (Walker, 2011). Therefore, in order

to guarantee the usage of proper yeast strains in fermentation, some yeast selection/identification technologies which allow the detection and recognition of the most suitable yeast strains should be carried. The general yeast identification methods which have been developed over the past several decades include culture characterization, morphological characterization, physiological characterization, replica plating and some genetic approaches. However, the use of these traditional techniques might not reflect the real performance of yeast strains during fermentation of lignocellulosic hydrolysates due to system complexity and the multi-stress environment. Therefore, a direct comparison of the fermentation profiles (e.g. ethanol productivity, yield and titer) might be more suitable for selecting the best yeast strains for lignocellulosic bioethanol production.

Among all the yeast species, *Saccharomyces cerevisiae* is the predominant species that has been utilized for the production of ethanol since it has the capability to ferment a range of sugars from different sources to ethanol alcohol (Walker, 2011). Ethanol fermentation is a biological process which converts sugars such as glucose, fructose and sucrose into cellular energy, producing ethanol and carbon dioxide, as shown in (Figure 4). Generally, yeast species such as *S.cerevisiae* first utilize glycolysis which breaks down glucose (six-carbon) to pyruvate and two molecules of ATP to provide energy. The glycolysis process consists of 10 steps that can be generally separated into 2 phases including the preparatory and the pay-off phases. In the preparatory phase (first five steps of glycolysis), the glucose is converted into two three-carbon molecules of glyceraldehyde-3-phosphate, while the remaining phase is referred to as the "pay-off" phase where 2 molecules of pyruvate, NADH and ATP are produced (Nelson & Cox, 2013). Alcoholic fermentation has to be conducted in an anaerobic environment (conducted without oxygen), since pyruvate and NADH will go to respiration (oxidative phosphorylation) in the

presence of oxygen which generates much more ATP than glycolysis alone. Under anaerobic conditions, two molecules of pyruvate are converted to acetaldehyde and carbon dioxide while the reducing power of NADH is used to convert acetaldehyde to ethanol while regenerating NAD+ for the glycolysis process.

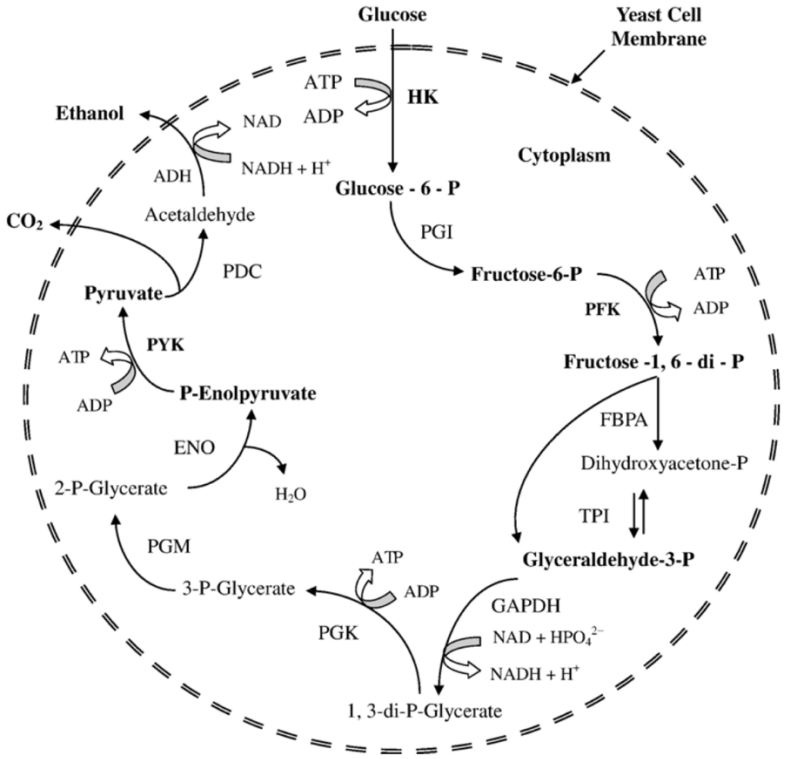


Figure 4 Ethanol fermentation pathway in Saccharomyces cerevisiae (adapted from Bai, Anderson, & Moo-Young, 2008a)

1.6.2 High gravity fermentation

To try to increase the efficiency of first generation ethanol production considerable attention has been paid to improving bioethanol production. Of the various approaches that have been adopted high gravity fermentation technology, which was initially developed in the 1980's,

has been increasingly implemented (Puligundla, Smogrovicova, & Ko et al., 2011). High gravity fermentation can be defined as fermentation of higher concentrations of sugar substrate resulting in an increase in the final ethanol concentration (Koppram, Tomás-Pejó, & Olsson et al., 2014). High gravity fermentation has the benefit of using less water and optimizing the use of available reactor volume, while also reducing energy costs required for cooling during fermentation and the volume of liquid that is distilled following fermentation (Jones & Ingledew, 1994; Kapu et al., 2013).

Before high gravity fermentation was introduced, corn ethanol production rarely occurred at solid corn slurries greater than 20 %, w/w, with final ethanol titers seldom reaching 10 % (v/v), mainly due to yeast inhibition at higher ethanol concentrations (Singh et al., 2010). High gravity fermentation has expanded primarily as a result of improvements in yeast strains (either through classical mutation/directed evolution and selection or through genetic engineering) and optimization of fermentation conditions (Nichols et al., 2006; Caspeta et al., 2014). As a result ethanol yields of 20–23 % (v/v) are now possible.

Despite many advantages there are several challenges with high gravity fermentation such as resulting high levels of stress to the fermenting microorganisms. Low water activity and higher sugar concentrations can also result in high osmotic pressures at concentrations above the yeasts' tolerance limit (>30% wt/vol) (S. L. Kumar, 2014). Increased external osmolarities can lead to cell dehydration, the collapse of the plasma membrane ion-gradients and a subsequent loss in cell viability (Bai, Anderson, & Moo-Young, 2008). One of the responses of the yeast to increased external solute concentration is to accumulate glycerol through the activation of the high osmolarity glycerol pathway (HOG) (stimulation of 6-phosphofructo-2-kinase-PFK2). This

essentially helps prevent cell collapse and facilitates the survival and proliferation of the cells (Petrovska et al., 1999).

When using lignocellulosic hydrolysates as the feedstocks for high gravity fermentations, the influence of multiple stresses can be anticipated. These include the presence of high concentration of inhibitory compounds, the presence of multiple sugars and the lack of any in-situ nutrient availability (Figure 5) (S. L. Kumar, 2014). However, some of the strategies that have been successfully used in the starch or sugarcane ethanol industries should be able to be adapted to at least partially overcome some of these multiple stress factors. For example, genetically modified yeast have been shown to better deal with extreme conditions of osmolality (Caspeta et al., 2014; Lam, Ghaderi, & Stephanopoulos, 2014). Various methods, such as nutritional supplementation, the use of mutant yeast strains, more efficient aeration than conventional brewing, higher pitching rates/cell densities and the use of immobilized yeast have also been successfully used to enhance fermentation (Claesson, 2011; Jørgensen, 2009; Harner et al., 2013; Yu, Zhang, & Tan, 2007)

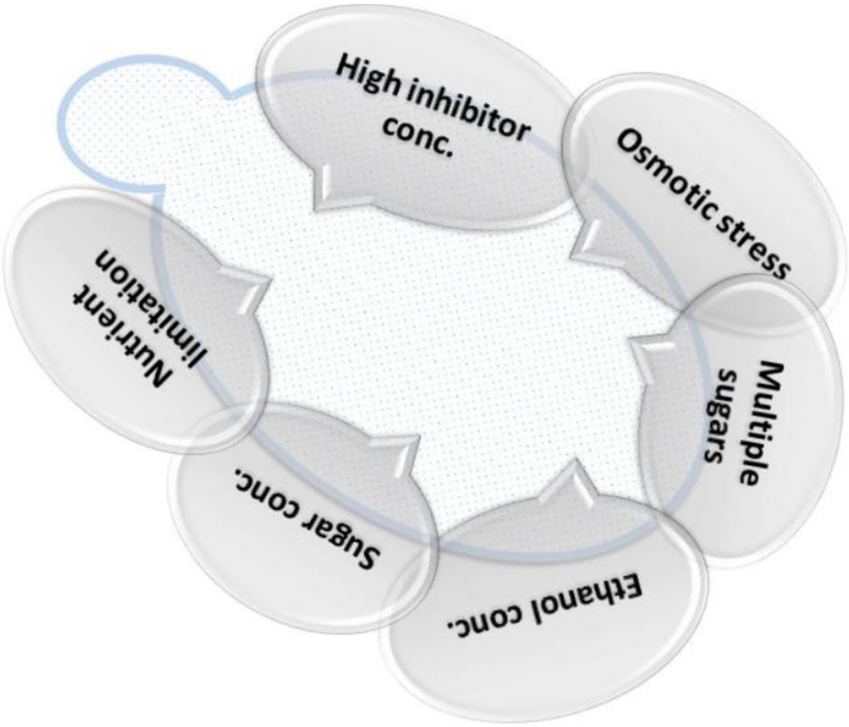


Figure 5 Key stress factors that are anticipated to be encountered during high gravity fermentation of softwood hydrolysates (adapted from Puligundla et al., 2011)

1.6.3 Nutrient supplementation

During high gravity fermentaion, nutrient supplementation is typically required to improve yeast stress tolerance in the multi-stress conditions resulting from high concentrations of inhibitors, sugars and ethanol (Xiros & Olsson, 2014). Nutrient supplementation has been shown to further improve final ethanol titers and overall productivity (Claesson, 2011; Jørgensen, 2009). For example, the utilization of yeast extract, an enriched nitrogen source, has been shown to greatly reduce the fermentation times (improved the productivity) of very high gravity fermentations of dissolved wheat mash (~ 350 g/L) from 7 days to 3 days while resulting in similar final ethanol titers (around 17%) (Thomas & Ingledew, 1990; Bai et al., 2008). Considering the relatively high cost of yeast extract, several industrial relevant nutrients (e.g.

urea, $MgSO_4$, NH_4SO_4) have been used instead of yeast extract (Claesson, 2011). In addition to looking at alternative nitrogen sources such as urea, numerous other types of nutrients such as oxygen, magnesium and phosphorus have also been reported to improve ethanol fermentation, likely by improving the inhibitors tolerance of the yeasts (Brandberg & Carl Johan Franzen, 2005; C.G. Liu, Lin, & Bai, 2011; Jørgensen, 2009). Since lignocellulosic hydrolysates typically lack several key nutrients that are needed for optimal yeast growth and stress tolerance (Kapu, 2013), nutrient supplementation was anticipated to play an essential role in the current study where the whole-slurry high solid loading softwood biomass hydrolysis and fermentation was assessed. Recent studies have shown that adding supplementary nutrients enhanced yeast growth and ethanol production when grown on the sugars present on un-modified/detoxified softwood hydrolysates (Rudolf et al. 2007, Bertilsson et al. 2009, Olofsson et al. 2010).

1.6.4 High cell density fermentation

High cell density fermentation is commonly applied during high gravity fermentation processes. This results in higher volumetric ethanol productivities due to the increased number of "working cells" (Borzani et al., 1993, Navaro 1994, Cardona and Sanchez 2008, Walker 2011; E. Palmqvist, 1998). During high cell density fermentation, large amounts of yeast cells are inoculated into the fermentation tank. This directly initiates ethanol production by reducing the lag phase and down-regulating yeast cell growth (reducing carbon drain for cell growth), resulting in much better ethanol yields (Melzoch et al., 1991; E. Palmqvist, 1998). In addition, since the large yeast population has significantly improved its inhibitor tolerance, due to decreased cell stress, it should have great potential for the production of cellulosic ethanol where much more inhibitory factors are likely (Alriksson, Cavka, & Jönsson, 2011; Kapu et al., 2013).

For example, it has been shown that increasing yeast cell density from 1 g/l to 3 g/l led to a more than three-fold increase in ethanol productivity during the fermentation of the hydrolysate from steam pretreated sweet sorghum bagasse (Shen et al. 2012). Significant improvements in ethanol productivity/yield/titer have also been reported when using molasses and hydrolysates from pretreated sugarcane bagasse (Palmqvist et al. 1998; Canilha et al., 2010; Canihla et al. 2012; Nofemele et al., 2012). Thus, it is anticipated that the use of high cell density fermentation should also facilitate the fermentation of whole-slurry, high solid softwood biomass hydrolysates.

1.7 Inhibition of the fermentation of lignocellulosic hydrolysates and possible detoxification strategies

As mentioned earlier, naturally occurring (i.e. extractives) and process derived (i.e. furans, lignin's) inhibitors in the whole-slurry hydrolysis system limit both the hydrolytic performance of enzymes and significantly decrease ethanol productivity and yield (Jönsson et al., 2013; Larsson, Reimann, Nilvebrant, & Jonsson, 1999). Considerable effort has been expended in trying to identify these fermentation inhibitory components as well as to develop effective detoxification methods (Keating, Panganiban, & Mansfield, 2006; Eva Palmqvist & Hahn-Hägerdal, 2000; P. E. R. Persson et al., 2002; Xiros & Olsson, 2014). It has been suggested that lignocellulosic biomass derived fermentation inhibitors can be generally divided into the three categories of; 1) furan and HFM resulting from C_5 and C_6 sugars degradation; 2) aliphatic acids, liberated from hemicellulose and furan aldehydes decomposition; and 3) phenolic compounds that naturally existed in the biomass (extractives) or derived from lignin degradation (Larsson,

Palmqvist, et al., 1999). The formation of various fermentation inhibitory compounds from lignocellulose during diluted acid pretreatment is illustrated in (Figure 6).

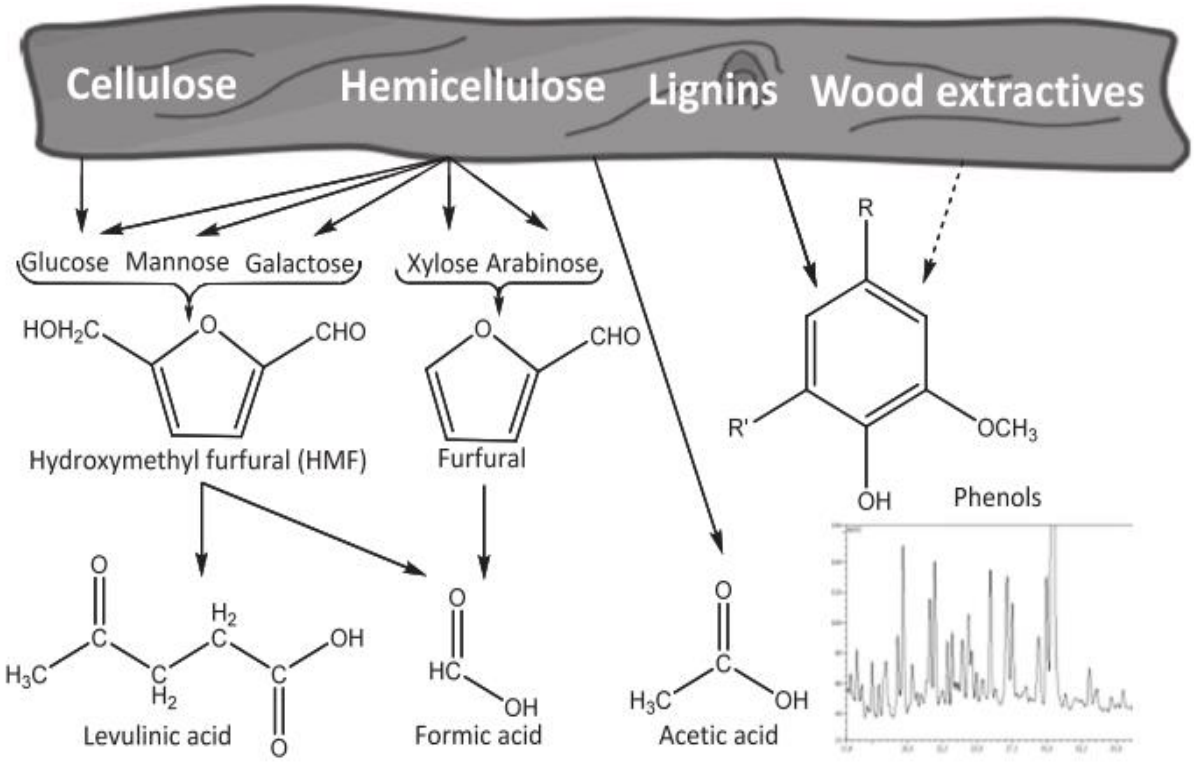


Figure 6 Formation of inhibitors. Schematic indicating the main routes of inhibitors formation. (Note: Furan aldehydes and aliphatic acids are carbohydrate degradation products, while lignin is the main source of phenolic compounds, as indicated by guaiacyl (4-hydroxy-3- methoxyphenyl) and syringyl (4-hydroxy-3,5-dimethoxyphenyl) moieties found in many phenolics. While the furan aldehydes and aliphatic acids content are relatively easy to determine, the quantification and identification of phenolic compounds remains challenging. The insert shows the variety of peaks representing phenolic compounds found in a hydrolysate of Norwegian spruce, as indicated by analysis using liquid chromatography-mass spectrometry (LC-MS)) Adapted from Jonsson et al, 2013.

Furan aldehydes (Furfural and HMF) are the degradation products derived from cellulose and hemicellulose sugars. The C_6 sugars (glucose, mannose, galactose) and the C_5 sugars (arabinose, xylose) undergo dehydration reactions to yield hydroxyethyl furfural (HMF) and furfural, respectively (Jönsson et al., 2013). Although most of the aliphatic acids, such as levulinic acid and formic acid, come from the further decomposition of HMF and furfural, the acetic acid originates from the acetyl groups originally substituted on the hemicellulose backbone. The phenolic compounds are mainly generated from heteropolymeric lignin degradation at higher temperature and acid-catalyzed conditions (Larsson, Reimann, & Jonsson, 1999; Pienkos & Zhang, 2009; Romaní, Ruiz, & Domingues, 2014).

1.7.1 Inhibition mechanism

The inhibition mechanism of these biomass derived inhibitors on the yeast fermentation metabolism has been extensively studied (Chandel, Silvério, & Singh, 2011; Guo, Cavka, Jönsson, & Hong, 2013; Jönsson et al., 2013; Modig et al., 2002; Eva Palmqvist, 2000; Eva Palmqvist & Almeida, 1999; Eva Palmqvist & Hahn-Hägerdal, 2000; Xiros & Olsson, 2014). Although the specific underlying mechanisms responsible for fermentation inhibition remains unclear, it has been shown that these inhibitors can interact with yeasts to decrease their fermentation performance via several mechanisms such as chemical interference with cell membranes, direct inhibition of ethanol production pathways, and/or osmotic pressure effects on the yeast cells (Z. L. Liu, 2011; E Palmqvist, Grage, Meinander, & Hahn-Hägerdal, 1999).

It has been suggested that the furans adversely affect the activity of some enzymes in the glycolysis metabolism pathway, such as alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase, significantly reducing ethanol productivity and yield (Modig et al., 2002; Modig, n.d.; Eva Palmqvist & Almeida, 1999). The inhibitory effects of aliphatic acids have been shown to be highly dependent on their acid dissociation constant pKa (E Palmqvist et al., 1999). Since the pH of fermentation medium is around 5.5 while the pH of the yeast cell cytoplasm is about 7.0, an aliphatic acid with a pKa between 5.5 and 7.0 will dissociated in the cell cytoplasm, consequently decreasing the intracellular pH. Therefore, if the aliphatic acid concentration is high, a greater amount of plasma membrane ATPase needs to be utilised to pump out the accumulated protons to maintain the intracellular pH. In contrast, if the aliphatic acetic concentration is moderate, under anaerobic conditions, ATP can be provided by enhanced ethanol fermentation, thus increasing the ethanol yield (Taherzadeh, Niklasson, & Lidn, 1997). However, if the aliphatic acid concentration is too high, ATP must be generated in order to maintain the intracellular pH. Under anaerobic conditions this is achieved by increased ethanol production at the expense of yeast biomass formation, thereby inhibiting the yeast (Eva Palmqvist & Hahn-Hägerdal, 2000). Although the exact inhibitory action of phenolic compounds is still unclear, some possible mechanisms have been suggested. For example, the double bond in the side chain of some phenolics was believed to inhibit some key enzymes (e.g. alcohol dehydrogenase, aldehyde dehydrogenase) in fermentation metabolism pathway (Adeboye, Bettiga, & Olsson, 2014). In addition, the hydrophobic character of phenolics might interfere with yeast cell membranes, changing the cells lipid : protein ratio (Jurado, Prieto, Martínez-Alcalá, Martínez, & Martínez, 2009).

1.7.2 Detoxification methods

The strategies that have been used to detoxify fermentation inhibiters include chemical (e.g. overliming, sulphite, dithionite etc.), biological (bacteria, fungi, laccase etc.) and physical methods (anion exchange, membrane separation, evaporation etc.) (Jönsson et al., 2013; Parawira & Tekere, 2011; Pienkos & Zhang, 2009). Chemical detoxification approaches have tended to predominate due to their efficiency and lower capital costs (Jönsson et al., 2013). Alkali treatments such as overliming (by using calcium hydroxide) has been studied for a long time (Ranatunga, Jervis, Helm, Mcmillan, & Wooley, 2000). The detoxification mechanism of overliming was originally thought to be a result of the precipitation of inhibitory components (Zyl, Prior, & Preez, 1988). However, subsequent investigations have suggested that, in addition to the removal of inhibitors through precipitation, overliming also results in the chemical conversion of inhibitors. This was result of sodium hydroxide treatments which didn't generate much precipitation but significantly improved ethanol yields and productivity (P. E. R. Persson et al., 2002). Although overliming is an effective detoxification strategy, one key limitation associated with overliming is that sugar degradation also occurred, considerably decreasing final ethanol yields. Overliming also requires the use of relatively high temperature (70 °C) which is also energy intensive.

Aside from overliming, in-situ detoxification using reducing agents such as sulphite and dithionite has received considerable attention in recent years. (Alriksson et al., 2011). Recent work has indicated that in-situ sulphite/dithionite treatment showed similar effects as overliming without the input of extra energy that is needed to increase the temperature during overliming

treatment. It has been suggested that the detoxification mechanism of sulphite and dithionite is the reduction and sulphonation of inhibitiors in the water soluble stream and that dithionite was more efficient than sulphite at similar molar loadings such as 10 mM (Alriksson et al., 2011). Coincidentally, the sulphonation of water insoluble lignin within the steam pretreated substrate has been shown to be one of the most effective approaches to improve enzymatic hydrolysis yields at reduced enzyme loadings (L. Kumar et al., 2011; C. Mooney et al., 1998). Along with lignin/phenolic sulphonation, it has been reported that the reduction of phenolic compounds also happens when sulphite is used to detoxify the water soluble streams resulting from the steam pretreatment of softwoods (Cavka et al., 2011). Recent work has shown that a reduction in the amount of phenolics can significantly improve enzymatic hydrolysis by reducing phenolic toxicity towards cellulase enzymes (Zhai et al., 2016). Thus, one of the main objectives of this thesis was to develop a one step sulphite treatment process to improve both the enzymatic hydrolysis and the fermentation of whole-slurry, high solid loading, steam pretreated softwoods.

1.8 Thesis overview and objectives

As mentioned earlier, although conventional sugar/starch based bioethanol (first generation bioethanol) production has been successfully commercialized by using high gravity fermentation (10-15% v/v ethanol within 10 hours), the commercialization of biomass-derived (second generation) bioethanol is still challenging. This is mainly due to the relatively low concentration of fermentable sugars that are typically derived from enzymatically hydrolyses pretreated biomass and the high fermentation inhibitors derived from both the biomass itself and the physicochemical pretreatment process. Although whole-slurry, high solid loading hydrolysis of softwood biomass could potentially result in high concentrations of fermentable sugars

(glucose and mannose), it faces several challenges. These include a high lignin content, a more condensed lignin structure and high concentrations of fermentation inhibitors. Thus, one of the major goals of the thesis work was to optimize the pretreatment, post-treatment, enzymatic hydrolysis and fermentation conditions of whole-slurry high solid loading softwood biomass such that high sugars concentrations, and consequently high ethanol productivities and titers could be achieved using a relatively low enzyme loading within a short time period.

Since high cell density fermentation with nutrient supplementation has been used at industrial scale, one of the initial objectives of the thesis work was to establish "optimized" fermentation conditions for cellulosic ethanol production. Therefore, model/"pure" cellulosic substrate dissolving pulp and a "realistic", pretreated lignocellulosic substrate, steam pretreated lodgepole pine, were hydrolyzed at high solids loadings (>20% w/w) to obtain a high concentration of fermentable sugars. The fermentability of various potential cellulosic ethanol production strains including strain 6391 from sugar cane ethanol fermentation, strain 7442 from starch/lignocellulose ethanol fermentation, strain 6469 starch/lignocellulose ethanol fermentation, and strain T_2 from spent sulphite liquor fermentation, were assessed at low/medium/high cell densities were compared. It is hoped that these optimized fermentation condition (e.g. yeast strain, cell density, nutrients etc.) would form a good basis for the subsequent fermentation of the whole-slurry, high solid, softwood biomass hydrolysate.

At the established, "compromise" steam pretreatment conditions that were used, most of the softwood hemicellulose components were solubilized. However, the high lignin content of the water insoluble cellulosic fraction still strongly limited the efficiency of enzymatic hydrolysis of the cellulose. Sulphite post-treatment at an elevated temperature has been used to sulphonate lignin, significantly increasing cellulose accessibility and decreasing cellulase adsorption. In

addition, when whole-slurry high solids loading hydrolysis is carried out, high inhibition occurs for both the enzymatic hydrolysis and fermentation processes. However, sulphite treatment at room temperature has been shown to be an effective detoxification method to deal with these inhibitory components. Thus, one of the major goal of the proposed work was to develop a sulphite treatment process to alleviate these challenges during the steam pretreated, softwood whole-slurry, high solids loading hydrolysis and fermentation processes.

To achieve this goal, a sulphite post-treatment was initially assessed to see if it could simultaneously enhance both the enzymatic hydrolysis and the fermentation of the whole slurry. Sulphite post treatment at different temperatures (room temperature and elevated temperatures of 70, 121, 160 ˚C) and times with a range of chemical loading were evaluated. However, as will be described later, this process proved problematic since the sulphite post-treatment conditions which sulfonate lignin (improve cellulose accessibility) are accompanied by sugar degradation and the carryover of too high a sulphite content in the whole slurry hydrolysis and fermentation processes.

To try to overcome these problems, an innovative sulphite steam pretreatment approach was assessed. The goal was to achieve lignin sulphonation ahead of lignin condensation during pretreatment process, thereby improving the extent of sulphonation while decreasing sulphite loading. Various sulphite pretreatment at both alkali and acid conditions were assessed. Since lignin sulphonation prefers alkaline conditions while typical steam pretreatment performs better in acidic conditions, a two stage sulphite pretreatment using sulphite in the initial stage to sulphonate the lignin (at alkaline condition) and SO_2 at second stage to further sulphonate lignin and open-up the substrates (at acid condition) was evaluated. Various substrate physicochemical characteristics such as particle size/morphology, lignin and hemicellulose content/location,

cellulose crystallinity/degree of polymerization/accessibility were also assessed. As will be described in the thesis, this latter strategy was relatively successful.

Chapter 2: Materials and Methods

2.1 Biomass

Dissolving pulp used in this research was kindly supplied by Neucel. Inc. Mountain beetle killed lodgepole pine was kindly provided by Canfor. Inc, with the moisture content of 7%.

2.2 Pretreatment

2.2.1 Acid steam pretreatment

The lodgepole pine wood chips were screened to retain chips between 6 and 38 mm. Prior to pretreatment, wood chips with 200 g dry weight were impregnated with 4% SO_2 (wt/wt of the dry substrate) for around 12 hours at room temperature. The amount of SO_2 was determined by weighing the total substrates weight before and after the addition of the gas. Steam pretreatment was conducted in a 2L StakeTech II batch steam gun (constructed by Stake Tech-Norvall, Ontario, Canada) at 200 °C for 5 min.

2.2.2 Post treatment

Post-treatment was carried out with minor modification of the conditions used earlier. Briefly, the treatments were conducted in different equipments according the different temperature required (160 °C in Parr high pressure batch reactor, 121 °C in the autoclave machine and 70 °C in a water bath) with the addition of the desired loading of Na_2SO_3 or Na_2SO_3

+ Na_2CO_3 based on the dry weight of the substrate for certain amount of time. Post treatment conducted in Parr reaction at 160 °C was based on 10% solid loading. 25% solid loading of substrates sealed by aluminum foil in a glass baker was treated in the autoclave machine at 121 °C. Post-treatment conducted in water bath is based on 25% solid loading with 100 Ml volume reaction bottles at 70 °C.

2.2.3 Sulfite steam pretreatment

Prior to steam pretreatment, 200 g of dry lodgeple pine chips were placed in plastic bags, mixed with water containing the specified chemicals which consisted of either sodium sulphite or sodium suphite + sodium carbonate at a solid : liquid ratio of 2:1 and left at 70 °C in water bath for a certain period of time. For another set of experiment by using acid sulfite prior to steam pretreatment also follow the same experiments protocol as above. In each case, the chips that had been impregnated with the chemical solution were steam pretreated in steam gun, as specified above at 140 °C for certain time ranging from 10mins to 70mins. The chips, and the liquor resulting from the steam pretreatment were then separated by filtration. If required, the chips were then subjected to a second steam pretrement at certain temperature and time based on different experiment design.

2.3 Enzymatic Hydrolysis

The enzymatic hydrolysis was conducted in duplicates. Commercial Ctec-3 preparation were obtained from Novozymes (Franklinton, NC). The enzyme activity of the Ctec-3 is 71.7

FPU/mL using Whatman No. 1 filter paper as a substrate and the protein concentration was 277mg/ml. Both dissolving pulp and pretreated Lodgepole Pine substrates enzymatic hydrolysis were conducted at 50 °C in a rotary shaker or horizontal mixer at 150 rpm. After 2-3 days, hydrolysis was ceased and liquid fractions obtained by centrifugation or the whole slurry were used for downstream fermentation.

2.4 Microorganisms and yeast propagation

Four different strains, 6391, 7442, 6469, (Lallemand, Inc. Quebec, Canada) and T_2 (Tembec, Inc, Quebec, Canada) were used in this study. All strains were maintained in Glycerin tube and stored at – 80 °C. For the preculture, 300ul yeast was inoculum in 20 ml of YPD media in a sterile 50 ml Falcon tube and incubated overnight at 30 °C in a rotary shaker at 150 rpm. Subsequently all the broth was transferred to a shake flask with 800 mL of YPD media and incubated until an OD of ≈ 0.8 was reached. The cells were harvested by centrifugation at 5000 rpm at 4 °C. Pellets were washed three times with 0.9% NaCl solution and re-suspended in 0.9% NaCl solution for inoculation in fermentation trials. The final cell concentration was calculated based on a standard curve correlating the inoculum's dry weight and optical density at 600nm. The final dry weight of the cells was confirmed by overnight drying of the cell culture at 105 °C (Bura et al., 2001; Ewanick et al., 2007).

2.5 Media and fermentation

2.5.1 Media and fermentation protocol

The fermentation trials were carried out in 30 ml septic bottles with butyl-PFTE seals, with a working volume of 10 ml or 20 ml. Before fermentation, the pH of the Lodgepole Pine hydrolysate or dissolving pulp hydrolysate were adjusted to 5.5 with NH_4OH. The reaction bottles loaded with cultures were incubated in an orbital shaker at 30 °C and 150 rpm. During the course of fermentation, 400 µl samples were taken at different sample hours. The samples were centrifuged at 5 000 rpm for 5 min and the supernatant was stored at −80 °C for further analysis. Final yeast cell densities corresponding to an OD_{600} of 6.5, 13, 20 and 25 were used in the fermentation experiments. The fermentation profiles of T_2 strain in dissolving pulp and steam pretreatment liquor at all these cell densities were compared to that of the synthetic glucose medium. 300 µL samples were taken periodically over 48 h, centrifuged at 10,000 rpm (10621 g force) for 10 minutes, and the supernatant was immediately stored at – 20 °C until further use. The aliquots were analyzed for ethanol and sugar concentration profiles and selected samples were also analyzed for the concentration of inhibitory compounds.

Ethanol yield were expressed as a percentage of the maximum theoretical yield obtained. The calculation was based on the total amount original fermentable sugars (including glucose and mannose) present in the liquor. By using a maximum stoichiometric ethanol yield of 0.51 g per gram of sugar, the percentage of ethanol yield was calculated as:

$$Y_{EtOH} = \frac{P_{EtOH} \times 100}{S_0 \times 0.51}$$

where P_{EtOH} is the total amount of ethanol formed in the fermentation and *So* is the initial amount of C_6 sugars present in the original liquor. The metabolic yield was calculated as the average ethanol produced for every gram of consumed sugars. Volumetric ethanol productivities were determined based on the exponential phase of the fermentation and is calculated as:

$$Q_{EtOH} = \frac{C_{(t_2)} - C_{(t_1)}}{t_2 - t_1}$$

where t_2 and t_1 are the initial and final time points of the exponential phase and Ct_2 and Ct_1 are the corresponding ethanol concentrations (% wt) respectively.

2.5.2 Nutrient supplementation of fermentations

In the experiments used to assess the influence of nutrient supplementation, the fermentation medium was supplemented with a nutrient cocktail containing urea or 1 g/L yeast extract. Due to the higher sugar concentration of the liquor and a high cell density employed, we later assessed a higher level of nutrient supplementation using medium containing 10 g/L $(NH_4)_2HPO_4$, 10 g/L yeast extract, and 5 g/L $MgSO_4.7H_2O$ (Jorgensen et al., 2009). The nutrients were added prior to the fermentation, the pH adjusted to 5.5 and the resulting liquor sterilized by filtration. Any improvement in fermentation was evaluated against a fermentation of the control medium with the same conditions and characteristics, but with no nutrient supplementation.

2.5.3 Hybrid hydrolysis and fermentation (HHF) and Separate hydrolysis and fermentation (SHF)

Prior hydrolysis, the pH of the whole slurry or dissolving pulp substrates with 25% consistency were adjusted to 5.5 by NH_4OH. After that the substrates were subjected to Ctec-3 for hydrolysis with desired protein loading. No other nutrients were added to the slurry. All the serum bottles containing substrates are incubated in an orbital shaker for prehydrolysis at 50 °C and 150 rpm for 24 hours. For HHF, after prehydrolysis, yeast was added to each bottle and fermentation was performed at 37 °C. For the SHF, after hydrolysis, liquid and solid were separated by centrifuge at 5 000 rpm for 15 min. Time point samples were taken periodically (2h, 4h, 8h, 18h, 24 h, 48 h and 72h), centrifuged at 10,000 rpm for 10 minutes and then the supernatants were stored at -20 °C.

2.5.4 Sulfite detoxification

Sulfite detoxification was carried out according to Alriksson et al. (2011). Sodium sulfite (Na_2SO_3) (Sigma) was added to the steam pretreatment liquor to a final concentration of 10 mM and 15 mM. After the addition of the reagent, the pH was adjusted to 5.5 using 50% NaOH. The samples were then thoroughly mixed by stirring at 150 rpm for 10 min at room temperature (23 °C). The samples were analyzed to assess the concentration of inhibitory compounds.

2.6 Analytical methods

2.6.1 Sugars

The concentrations of monomeric sugars (arabinose, galactose, glucose, xylose and mannose) were determined by HPLC analysis (Bura et al., 2001; Sluiter et al., 2004). 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 1 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 auto sampler (Chromatographic Specialties, Brockville, Canada). 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 sample 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 HPLC response.

In order to quantify the fraction of oligomeric sugars present in the samples, post-hydrolysis was 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% w/v 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 hydrolysis loss. 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.

2.6.2 Ethanol

Ethanol was determined using gas chromatography and a Hewlett Packard 5890 GC equipped with a HP-Innowax column (15mx0.53mm). Helium was used as the carrier gas at a flow rate of 20 mL/min. The temperatures of the injection unit and flame ionization detector (FID) were set at 175 and 250 ℃ respectively. The oven was heated to 45 ℃ for 2.5 minutes and the temperature was raised to 110 ℃ at a rate of 20 ℃/min and later held at 110 ℃ for 2 minutes. Standards were prepared using ethanol (Sigma) and butanol (0.5 g/L) (Fisher) was used as an internal standard.

2.6.3 Inhibitors

The sugar degradation products, furfural and 5-hydroxymethyl furfural (HMF) as well as acetic acid were analyzed using a HPLC (ICS-500) with Aminex HPX-87H column (Bio-Rad, Hercules, CA). The HPLC has fitted with an AS3500 auto sampler, a UV detector at a wavelength of 280 nm and a GP40 gradient pump (NREL, 2008). Standard concentration of HMF (Sigma) ranged from 0.1 - 4.0 g/L, while the concentration of furfural ranged from 0.1 - 2.0 g/L. All of the standards and samples were filtered through a 0.45 μm syringe filter (Chromatographic Specialties, Brockville, Canada). 5 mM H_2SO_4 was used as an eluent at a flow rate of 0.6 ml/min.

The concentration of total phenolics in the pretreated substrates were quantified using Folin–Ciocalteu reagent (Sigma), as proposed by Singleton and Rossi (1965). A 100 μL aliquot of the diluted sample was first mixed with 250 μL of the Folin–Ciocalteu reagent. After 5 minutes, the reaction was stopped by adding 750 μL of 20% (w/v) Na_2CO_3 and the total volume was brought up to 5 mL using nanopure water. The flasks were incubated for 2 hours at 22 °C with constant stirring on a magnetic stir plate. The absorbance of each reaction was measured spectrophotometrically at 760 nm. Reaction blanks with nanopure water were also run in parallel. Calibration was done using vanillin as the standard. The reactions were performed in duplicate for each sample and the standard and average values were reported.

2.7 Determining pretreated biomass composition

The chemical composition of the pretreated materials was determined according to the Technical Association of the pulp and paper industry (TAPPI) standard method T222 om-88. 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 weighted (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 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 in section 2.6. 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.

2.8 Substrates characterization

2.8.1 Water retention value (WRV)

The fiber swelling property could be measured by the water retention value which was initially developed by the Technical Association of the Pulp and Paper Industry. Briefly, 0.5-1.0 g (based on oven dried weight) of never-dried substrates was suspended in 50 ml nanopure water and shake strongly in order to break the pulp apart. After that, the suspended pulp was soaked overnight at room temperature followed by filtration with a 200 mesh screen in a falcon tube. To avoid the loss of fines, the filtrates were recirculated three times and the resulting pulp pads centrifuged at 900 g for 30 mins. The wet (centrifuged) pulp pads were weighted, dried overnight at 105 degrees and reweighted. The Water Retention Values (WRVs) were calculated as the weight of water retained in the pulp pad after centrifugation divided by the dry weight of the fibers according to the following equation:

$$WRV = \frac{(W_w - W_d)}{W_d}$$

Where Ww is the weight of the wet sample after centrifuging, and W_d is that of the dried sample. To determine the effect of residual lignin on the (fiber) swelling of hybrid poplar and lodgepole pine pretreated at varying severities, the substrates were subjected to sodium chlorite-mediated delignification as described in section 2.8 followed by determination of the WRV. All WRV measurements were performed in triplicate.

2.8.2 Simon's stain

Simons' Stain (SS) was used to evaluate the surface area of cellulosic material, thereby assessing the cellulose accessibility to cellulases. It was initially derived from a staining technique used in the pulp and paper industry to examine changes in the physical structure of pulp fibers. Here, cellulose accessibility to cellulases was estimated by Simons' stain according to the procedure modified by Chandra (R. Chandra, Ewanick, & Saddler et al., 2008). Briefly, direct orange was obtained from Pylam Products Co. Inc. Fractionation of the orange dye was performed according to Esteghlalian (Esteghlalian, Bilodeau, & Saddler et al., 2001). For each substrate, approximately 100 mg of never dried pulp samples were weighed into six 1 mL polypropylene centrifuge tubes, each tube received 1.0 mL of phosphate buffered saline solution at pH 6. The DO solution (10 mg/mL) was added in a series of increasing volumes (0.25, 0.5, 0.75, 1.0, 1.5 and 2.0 mL) to the six tubes containing pulp sample and PBS. Distilled water was added to increase the final volume of the samples to 10.0 mL. The tubes were then incubated overnight at 70 degree with shaking at 200 rpm. After the incubation period, the tubes were

centrifuged at 5000 rpm for 5 min, and a sample to the supernatant was placed in a cuvette and the absorbance read on a carry 50 UV-Vis spectrophotometer at 624 and 455 nm. The amount of dye adsorbed onto the fiber was determined using the difference in the according to the Beer-Lambert law. The extinction coefficients were calculated by preparing standard curves of each dye and measuring the slope of their absorbance at 455 and 624 nm. The values calculated and used in this thesis were εO_{455} = 35.62, εB_{455} = 2.59, εO_{624}= 0.19, εB_{624}=15.62 (L g^{-1} cm^{-1}).

2.8.3 Fiber quality analysis

Fiber length, width and size distribution of the substrates was measured using a Fiber Quality Analyzer. Briefly, a dilute suspension of fibers with a fiber frequency of 25-40 events per second was transported through a sheath flow cell where the fiber are oriented and positioned. The images of the fibers were detected by a built-in CCD camera, and the length of the fibers was measured by circular polarized light. The experiments were conducted according to the procedure described by Robertson et al (Robertson, Olson, & Seth et al.,1999). All samples were run in triplicate.

2.8.4 Degree of polymerization

To determine the degree of polymerization, the pretreated substrates were carefully dignified as described on section 2.3. The viscosity of substrate solutions containing 0.06%,

0.1%, 0.125%, and 0.5% (w/v) delignified substrates in 0.5 M cupriethylenediamine was measured on a capillary viscometer (Cannon Ubbelohde Viscometer, Cannon Instrument Co., State College, PA) according to the guidelines found in TAPPI standard method T230 om-99. The specific viscosity of the substrate solutions was determined according to the following equation:

$$\eta_{sp} = \frac{(\eta_c - \eta_o)}{\eta_o}$$

where η_c is the viscosity of the sample at concentration c and η_o is the viscosity of the solvent. The intrinsic viscosity (η_{int}) of each substrate was calculated by extrapolating a plot of η_{sp}/c as a function of c to c = 0 as described by Lapierre et al, (Lapierre, Bouchard, & Berry, 2009). The viscosity average cellulose degree of polymerization (DP_v) was calculated from the intrinsic viscosity by the following equation (Robertson et al., 1999):

$$DPv = (1.65\eta_{int})^{1.11}$$

where η_{int} is the intrinsic viscosity of the substrate. All viscosity measurements were performed in triplicate.

2.8.5 Acid group

Bulk acid groups in the pretreated and post-treated substrates were determined by conductometric titration according to standard methods reported elsewhere. In brief, wet substrates containing 1 g dry matter were added to 300ml of 0.1 N HCl and stirred for 1hour. The pulp was then filtered and washed with 2000 ml of deionised water. The washed pulp was then treated with 0.001 M NaCl (250ml) and 0.1 N HCl solutions (1.5ml), stirred and conductometrically titration data (colume 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.

2.8.6 SEM imaging

Lyophilized biomass samples were mounted on aluminum SEM stubs using double sided tape and sputter-coated with 10nm Au/Pd (80:20 mix) then imaged on a Hitachi S-2600 VP-SEM (Tokyo, Japan).

2.8.7 FTIR

To investigate and quantify the chemical groups in the pretreated substrates, a FTIR system (Varian 3100, Varian Inc. Palo Alto, CA) with MIRacle Accessory (Pike technologies,

Madison, WI) was used and id-IR spectra were obtained by averaging 128 scans from 4000 to 600 cm^{-1} at a spectral resolution of 4 cm^{-1}.

Chapter 3: Results and Discussion

3.1 Assessing the challenges of having high residual lignin and fermentation inhibitors in steam pretreated softwood whole slurries.

3.1.1 Background

It is well known that increasing the concentration of sugars and thus ethanol during a biomass to ethanol process would result in considerable savings in energy and water consumption (M.R. Ladisch et al., 1983; Huang & Percival Zhang, 2011). "High gravity" fermentation which involves using sugar concentrations of greater than 20% has been used for first generation ethanol to obtain ethanol concentrations of close to 10% (w/v). However, the complex nature of lignocellulosic substrates compared to starch/sugar and the multiple challenges encountered by both the cellulolytic enzymes and the fermentative yeasts limits the sugar/ethanol concentrations achievable. Therefore, the highest sugar concentrations reported for lignocellulosic substrates usually are in the range of 10% with the resulting ethanol concentrations of approximately 4% ethanol after fermentation (Öhgren, Bura, Lesnicki, Saddler, & Zacchi, 2007; Z.-H. Liu, Li, & Yuan, 2014; Lan, Gleisner, Zhu, Dien, & Hector, 2013; Alriksson et al., 2011; Xiros & Olsson, 2014). Therefore, main objective of the proposed work in this chapter will be to assess the challenges associated when attempting to achieve 18-20% fermentable sugars and 8-9% ethanol in a relatively short period of time (2-3 days) when processing steam pretreated softwoods as substrates.

As mentioned above, the relatively high proportion of C_6 sugars in the hemicellulose component of softwoods is one advantage of processing softwoods. However, softwoods are

particularly recalcitrant when trying to achieve high ethanol concentrations due to both enzyme and fermentation related factors, especially when attempting to process whole slurries (WSF+WIF). These challenges include the high lignin content of softwoods, end product (sugar) inhibition resulting from high substrate loading and inhibition of fermentation (Kristensen et al., 2009). These main challenges will be investigated in this chapter.

The initial enzymatic hydrolysis will be performed on the whole slurries (WSF+WIF) with different enzyme loadings to obtain a baseline hydrolysis at a 25% solids loading. To determine the effect of the WSF on the enzymatic hydrolysis process, a model cellulose substrate, dissolving pulp, which is composed of over 95% cellulose will be hydrolyzed when combined with the WSF. To determine the effect of the non-sugar inhibitors (phenols, organic acids, furans) dissolving pulp will be hydrolyzed in buffer containing the same concentration of sugars that are found in the WSF. This will also enable the assessment of the effects of the sugars in the WSF on enzymatic hydrolysis of the dissolving pulp when compared to the hydrolysis of the dissolving pulp in buffer alone.

To improve ethanol yields in the presence of the anticipated high amounts of inhibitors, several yeast strains will be tested, including Saccharomyces cerevisiae strains 6391(Lallemand strain for sugarcane industry), 7442(Lallemand strain for starch bioethanol industry), 6469(Lallemand strain for starch bioethanol industry) and T_2 (Tembec strain for lignocellulosic bioethanol research). The T_2 yeast is of particular interest since it was isolated from the fermentation of spent sulfite liquor which contains significant amounts of degraded sugars and lignosulfonates(Kapu et al., 2013).

High cell density fermentation has been commonly applied in high gravity fermentation because of its boosting effect on ethanol productivity(E. Palmqvist, 1998). Therefore, to test the

effects of cell density, the yeast strains will be applied at densities ranging from OD 3.25 - OD 25, using the WIF derived from steam pretreated lodgepole pine supplemented with model glucose media and a model lignocellulsic DsP hydrolyzate as control. This will be used to determine the optimum cell density needed to ferment the steam pretreated softwood substrates. In addition to high cell density, additional nutrient supplementation was also shown to improve yeast stress tolerance in the presence of high inhibitor concentrations, high sugars, and high ethanol multi-stress conditions(Xiros & Olsson, 2014). Therefore, the effect of supplementing with various nutrients will be assessed using pure glucose media, dissolving pulp hydrolysate and a steam pretreated lodgepole pine hydrolysate. It was anticipated that the evaluation of these main factors that are expected to influence the hydrolysis and fermentation of whole-slurries of recalcitrant steam pretreated lodgepole pine, would allow for the development of strategies to improve the performance in subsequent Chapters of the thesis.

3.1.2 Results and Discussion

There were several substrates utilized in this chapter which are summarized in Table 1.

Table 1 Summary of substrates that were utilized in this chapter.

Substrates	Explanation
Synthetic glucose media	Media composed of glucose and various of nutrients (yeast extract, urea and mineral salts)
Liquid fraction of dissolving pulp hydrolysates	Supernatant generated from the centrifugation of the slurry after the enzymatic hydrolysis of the dissolving pulp substrate (removal of residual solid substrate particles after hydrolysis)
Whole slurry	Combined water soluble fraction (WSF) and water insoluble fraction (WIF) generated from the steam pretreatment of softwood biomass
Whole slurry hydrolysates	The hydrolysis of the combined water soluble fraction (WSF) and water insoluble fraction (WIF) generated from the steam pretreatment of softwood biomass (includes both residual solids and the liquid after hydrolysis)
Liquid fraction of steam pretreated softwood whole slurry hydrolysate	Supernatant generated from the centrifugation of the residual slurry at the conclusion of the enzymatic hydrolysis of the whole slurry (removal of residual solid substrate particles after hydrolysis)

Note: In 1.1 and 1.2 sections of this chapter, the liquid fraction of the centrifuged whole slurry hydrolysis of the steam pretreated lodgepole pine was selected as the substrate with the dissolving pulp hydrolysate and synthetic sugar media as controls. In section, 1.3 the whole slurry is used which includes both the solid water insoluble fraction and water soluble fractions that result from steam pretreatment.

3.1.2.1 Hydrolysis of dissolving pulp and whole slurries from steam pretreated softwood over a range of solids and enzyme loadings.

As described above, whole slurry high solids loading hydrolysis is necessary if we are to obtain high sugar concentrations for fermentation. However, increasing solids loadings typically requires higher enzyme loadings due to inefficient mass transfer, increased end-product inhibition and unproductive enzyme binding to lignin. Therefore, the minimum enzyme loading based on a range of substrate solids loading (2%, 10%, 20%) was initially assessed. Dissolving pulp was selected as the model substrate since it contains >95% cellulose. It was apparent that when hydrolyzing dissolving pulp at a 2% solid loading, a hydrolysis yield of 90% could be achieved using 15 mg enzyme loading per gram cellulose. However, when the substrate loading was increased to 10%, the hydrolysis yield decreased to 80%. As well as a lower hydrolysis yield, the required enzyme loading to achieve this yield also increased to 20 mg enzyme per gram cellulose (Figure. 7). These results indicate, that even for the case of pure cellulose such as dissolving pulp, raising the solids loading reduces the hydrolysis yield and increases the required enzyme protein dosage. When the solids loading was increased to 20%, the highest hydrolysis yield that was obtained was 62%, regardless of the enzyme dosage used. These results illustrate the detrimental effects of increased substrate solids loading on hydrolysis, including inefficient mixing and end-product inhibition. The effects of performing enzymatic hydrolysis at elevated solids loadings are even more pronounced when attempting to hydrolyze lignocellulosic substrates, where non-cellulosic components such as lignin and hemicellulose as well as the overall cellulose accessibility of the substrate play a significant role. End product inhibition has been shown to be a major contributor towards the poor performance of hydrolytic enzymes at

high solids loadings(Kadic & Liden, 2017)(Hsieh, Felby, & Thygesen et al., 2014). It was evident, that increasing the solid loading during hydrolysis resulted in a higher, final glucose concentration which likely inhibited the activity of enzymes through end product inhibition (Figure 8).

Visually, another phenomenon that was observed was that when the solids loading during hydrolysis was increased, the required time for liquefaction was increased. At a 10% solid loading, the time required for liquefaction was close to 12 hours. However, the observed time for liquefaction doubled when the solids loading was increased to 20%. Liquefaction has been shown to be an essential step in high solid loading hydrolysis which is directly related with the final hydrolysis yield(van der Zwan, Hu, & Saddler, 2017).

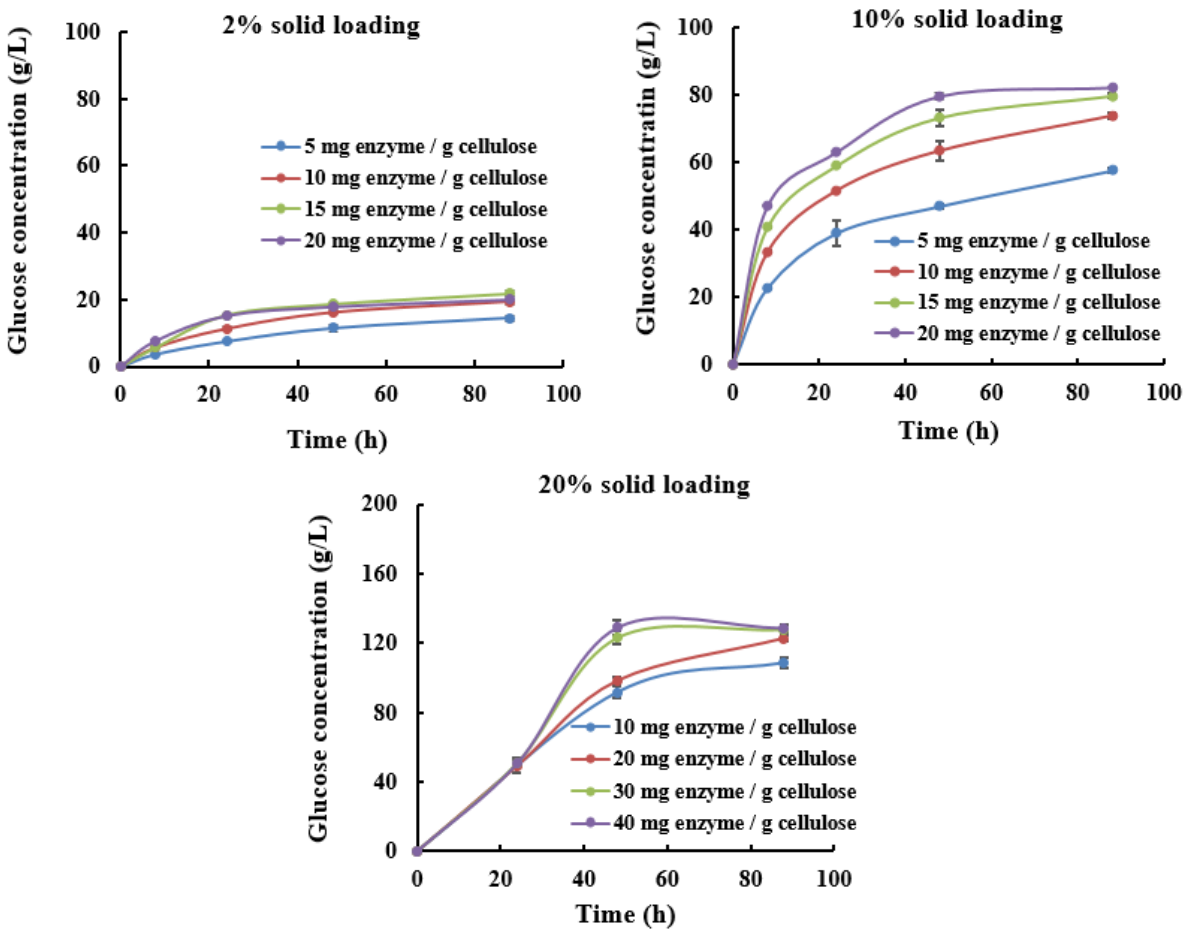


Figure 7 Glucose concentration of the enzymatic hydrolysis of dissolving pulp (DsP) with various substrates loading (2%，10%，and 20% w/w) at different Ctec-3 enzyme loadings (5-40 mg protein per g cellulose).

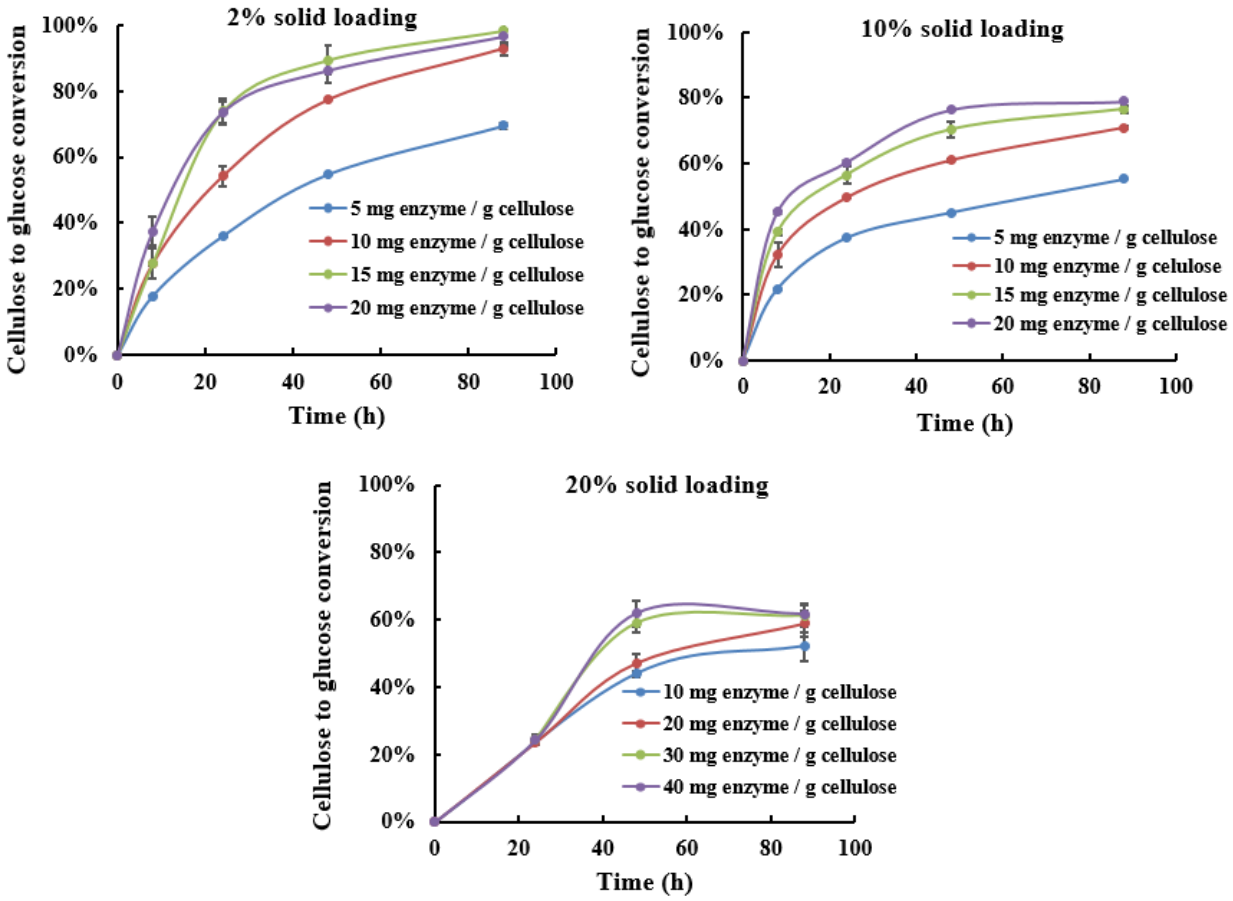


Figure 8 Enzymatic hydrolysis of dissolving pulp (DsP) with various substrates loading (2%，10%，and 20% w/w) at different Ctec-3 enzyme loadings (5-40 mg protein per g cellulose).

After testing the hydrolysis of the dissolving pulp (DsP), the enzyme loading for the steam pretreated softwood whole slurry was assessed using dissolving pulp as a control. A range of different enzyme loadings were selected with the aim of achieving a target of 70% cellulose hydrolysis (Figure. 9). It was apparent that the DsP substrate could not reach a hydrolysis yield of 70% regardless of the amount of enzyme that was added, while the steam pretreated substrate could reach a hydrolysis yield of approximately 80%, but required an enzyme loading of 180 mg /g of glucan (Figure 9A). These results illustrated the effects of end product inhibition in the case

of the DsP substrate which contained >94% cellulose, where end product inhibition likely did not allow for the hydrolysis to surpass a 65% conversion at a 25% solids loading. It should be noted that although the steam pretreated substrate could reach a conversion of 80%, albeit at a protein loading of 90 mg/g glucan, the resulting glucose concentration was only 137 g/L (Figure 9B). The result with the SPLP was quite different than that of the hydrolysis of the DsP where a sugar concentration 167 g/L was obtained at a conversion of only 66% (Figure 9 A and B). However, as will be discussed below, when considering the total hexoses in the "whole slurry" (WSF + WIF) after this enzymatic hydrolysis the total hexose concentration reaches approximately 160 g/L, similar to that of the DsP substrate (Table 2). These results illustrate the necessity to use the whole slurry (WSF + WIF) for the SPLP substrate rather than just the WIF.

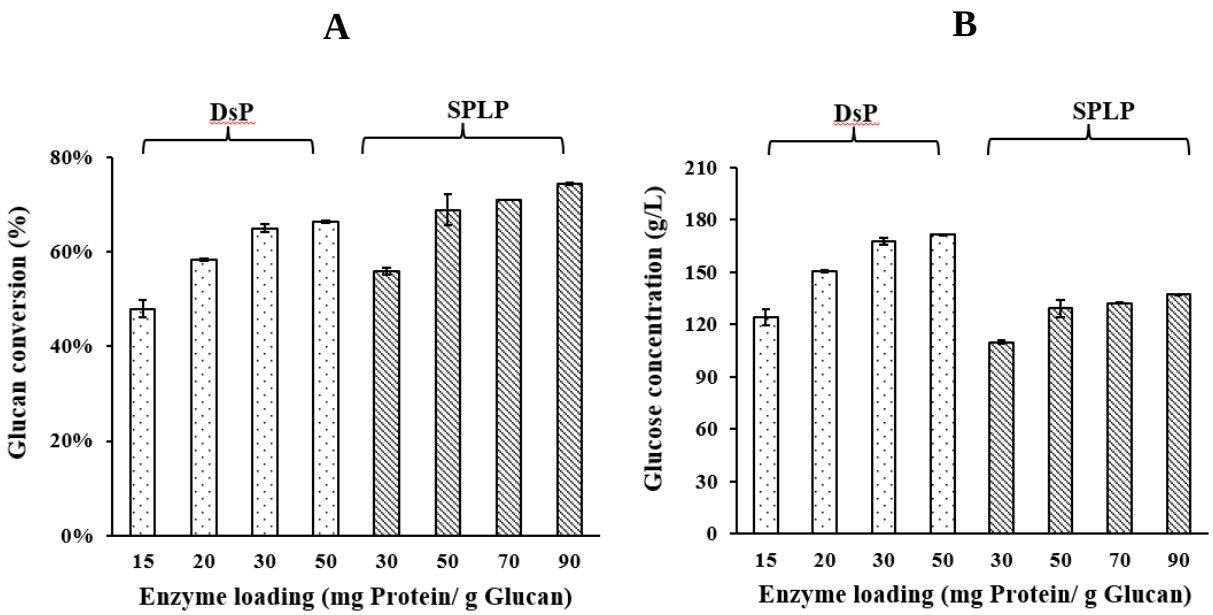


Figure 9 Enzymatic hydrolysis of Dissolving pulp (DsP) and whole-slurry steam pretreated Lodgepole pine (SPLP) with 25% (w/w) solid loading at different Ctec-3 enzyme loadings (15-90 mg protein per g cellulose) for 48 h. (A) Percentage of cellulose to glucose conversion (%) and (B) Glucose concentration (g/L).

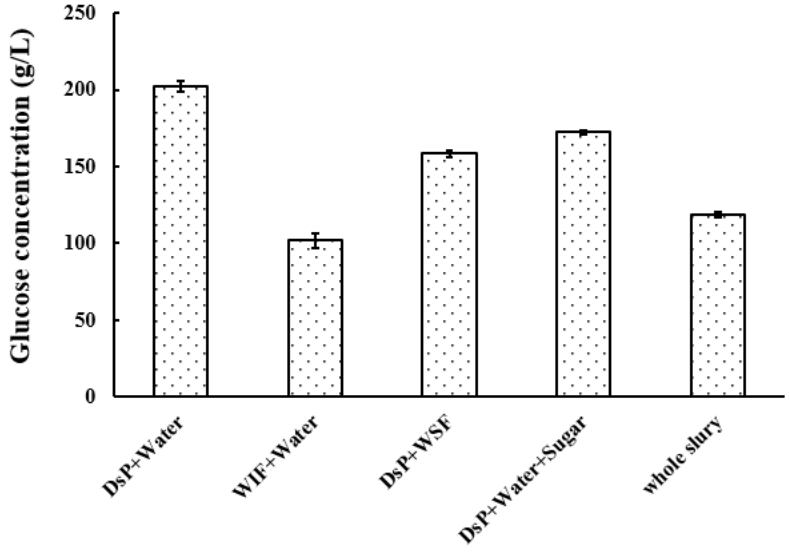


Figure 10 The effects of steam pretreated Lodgepole pine water soluble fraction (WSF) on the enzymatic hydrolysis of its water insoluble fraction (WIF) and dissolving pulp (DsP) substrates with 25% (w/w) solid loading at Ctec-3 loading of 50 mg protein per g cellulose for 72 h.

It was apparent that a higher enzyme loading was needed for the hydrolysis of the whole slurry SPLP compared with DsP to obtain an equivalent sugar concentration, which suggested that the high residual lignin in SPLP would physically and chemically inhibit enzymatic hydrolysis (Figure. 9 A). As mentioned above, another aim in the initial work was to assess the magnitude of the effects of the different factors such as end product inhibition, water soluble compounds in the WSF and high residual lignin to figure out which factor had the most influential role in affecting enzymatic hydrolysis. Different hydrolysis model systems were set up to demonstrate individual inhibitory effects (Figure 10). The enzymatic hydrolysis of dissolving pulp with buffer was used as a control. The influence of high residual lignin on hydrolysis was assessed using the hydrolysis of the water insoluble fraction (WIF) from the SPLP in buffer (Figure 10). Two other sources of potential inhibition of the enzymatic hydrolysis

include the presence of water soluble inhibitors and end product inhibition. These were assessed by performing the hydrolysis of the dissolving pulp with the water soluble fraction from the stream pretreatment and performing the hydrolysis of dissolving pulp in a solution containing the same sugar composition as the water soluble stream after steam pretreatment (Kim, Ximenes, & Ladisch et al., 2011)(Zhai et al., 2016). As expected, the dissolving pulp hydrolysis in buffer resulted in the highest sugar concentration while both the water soluble fraction and the whole slurry enzymatic hydrolysis gave much less sugar production. Among all of the inhibitory effects, it was apparent that the high residual lignin or limited accessibility of the steam pretreated softwood substrate, was the most significant limitation towards obtaining high sugar concentrations. For example, the addition of the steam pretreated WSF to the steam pretreated WIF had a similar result to the just using the WIF alone.

3.1.2.2 Testing the fermentability of the liquid fraction of the steam pretreated softwood whole slurry hydrolysates.

Prior to fermentation, the composition of the hydrolysates (supernatant from centrifuged hydrolysis reaction) from the enzymatic hydrolysis of the whole slurry of the SPLP at an enzyme loading of 100 mg/g and DsP at 35 mg/g were analyzed (Table 2). Since the DsP substrate was rich in cellulose, the DsP hydrolysate contained mostly glucose. In the case of the SPLP hydrolysate, with the exception of glucose monomers and oligomers, there were some mannose monomers and oligomers since mannan comprises a large proportion of softwood hemicellulose. The SPLP hydrolysates also contained significant amounts of inhibitors, surpassing the levels observed in previous studies (Alriksson et al., 2011; Cavka & Jönsson, 2013; Chandel et al., 2011; Xiros & Olsson, 2014). Therefore, as an initial strategy to contend with likely fermentation

inhibitors, the fermentation conditions were assessed by strain selection, nutrient supplementation, and cell density using the SPLP whole slurry hydrolysate fraction from the enzymatic hydrolysis as the substrate.

Table 2 The major soluble components of Dissolving pulp (DsP) and steam pretreated Lodgepole pine (SPLP) whole-slurry hydrolysates after 48 h enzymatic hydrolysis with Ctec-3 at an enzyme loading of 45 mg protein per g cellulose and 100 mg protein per g cellulose.

Components	DsP hydrolysate (g/L)	SPLP hydrolysate (g/L)
Glucose	171	135
Mannose	3	19
Cellooligomers	20	20
Mannoligomers	2.8	9
Acetic acid	-	9.2
Furfural	-	2.8
HMF	-	3.0
Total phenolics	-	4.9

It was anticipated that the pretreatment process will play an influential role in affecting the ease of hydrolysis and fermentation of the softwood substrate. However, the fermentation conditions also require some optimization for processing the toxic streams generated from pretreated softwood substrates in order to relieve likely end product inhibition toward cellulases during SSF and to ultimately maximize fermentation yields. After enzymatic hydrolysis at a high solids loadings (25%), the resulting hydrolysates were fermented using various Saccharomyces

yeasts strains including 6391(Lallemand strain used in the sugarcane industry), 7442 (Lallemand strain used in the starch bioethanol industry), 6469 (Lallemand strain used in the starch bioethanol industry) and T_2 (Tembec strain used for lignocellulosic bioethanol research) to determine which yeast would provide the most robust choice for at least partially overcoming the effects of fermentation inhibitors present in the steam pretreated softwood substrate (Figure 11). Subsequent work assessed the effects of nutrient supplementation, increasing cell density during fermentation and detoxification of the hydrolysate using sodium sulphite to further increase the ability of the yeasts to withstand the inhibitory nature of the steam pretreated softwood slurries toward fermentation. After testing the initial ease of fermentation, we assessed the influence of various pretreatment strategies by incorporating sulphonation. Of the yeast strains tested, T_2 is of particular interest as the fermentation of a spent sulphite liquor is the closest example to the fermentation of an actual lignocellulosic water soluble stream. It was apparent that the performance of these four strains were quite similar in synthetic sugar media as well as the DsP hydrolysate (Figure 11 A and B). However, the differences between the strains became apparent during the fermentation of the SPLP hydrolysate. As was anticipated, the T_2 strain exhibited the highest ethanol productivity of 5.2 g/L h in the SPLP hydrolysate, while the least effective strain was 6391 which had an ethanol productivity of 2.3 g/L h (Table 3). Therefore, in subsequent experiments, strain 6469 was selected to be used for fermenting the synthetic sugar media and the DsP hydrolysate, while T_2 was employed as the model strain for the SPLP hydrolysate when testing the effects of nutrient supplementation on fermentation performance.

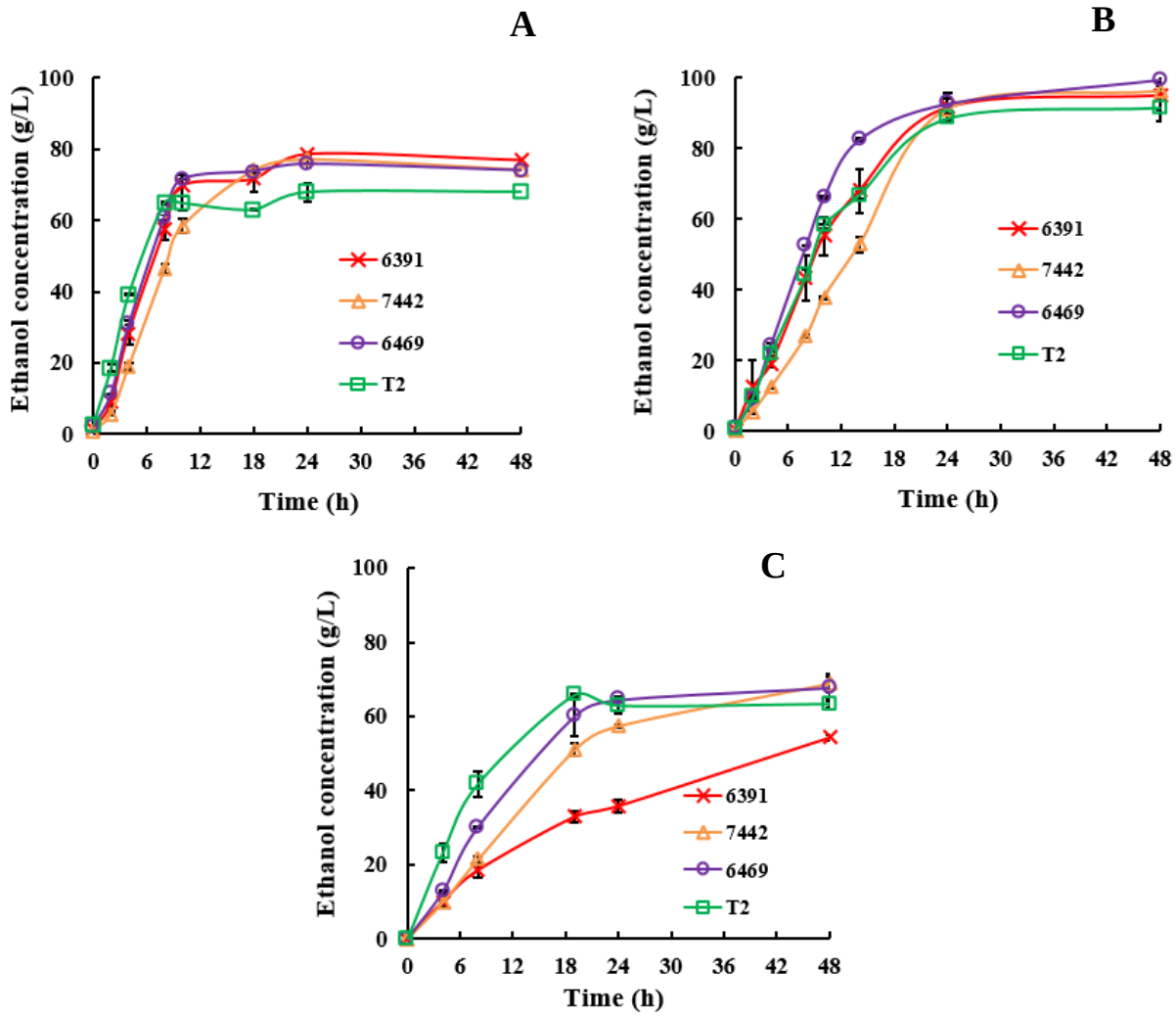


Figure 11 Ethanol production profile after growth of S. cerevisiae strains 6391, 7442, 6369 and T_2 on (A) synthetic sugar media, (B) Dissolving pulp (DsP) hydrolysate, and (C) the hemicellulose rich water soluble fraction of steam pretreated lodgepole pine whole slurry hydrolysate.

Table 3 Ethanol productivity and yield after growth of S. cerevisiae strains 6391, 7442, 6369 and T_2 in synthetic sugar media, dissolving pulp hydrolysate and the hemicellulose rich water soluble fraction of steam pretreated lodgepole pine whole slurry hydrolysate (SPLP).

Strains	Synthetic glucose media		Dissolving pulp		Real lignocellulosic hydrolysate	
	Productivity (g/L h)	Yield (%)	Productivity (g/L h)	Yield (%)	Productivity (g/L h)	Yield (%)
6391	7.16	87.3%	5.4	86%	2.3	62.6%
7442	5.8	86.3%	3.37	86.9%	2.7	78.6%
6469	7.6	86%	6.6	89.4%	3.8	77.3%
T_2	8.1	88%	5.5	93.1%	5.2	90.8%

Nutrient supplementation such as adding yeast extract, magnesium (Mg), phosphorus (P) has been previously shown to aid yeast in overcoming the inhibitory effects when fermenting highly toxic lignocellulosic substrates(Jørgensen, 2009; Claesson, 2011). Based on this concept, nutrient supplementation was assessed based on three types of media (synthetic sugar media, DsP hydrolysate, and SPLP hydrolysate). Yeast extract plus Mg, P was tested because it was reported to be an effective nutrient supplement for lignocellulosic bioethanol production (Claesson, 2011; Jørgensen, 2009). In addition to yeast extract, urea was also tested since it is more industrially relevant compared to yeast extract. The results show that in glucose media, varying the nutrient supplementation influenced the performance of strain 6469 to the greatest extent (Figure 12 A). When comparing yeast extract with Mg, P, urea, and urea plus yeast

nutrient base, yeast extract with Mg, P provided the best performance in synthetic sugar media, followed by urea with yeast nutrient base. Overall, in all cases where the synthetic sugar media was supplemented with nutrients, the fermentation performance surpassed that of the synthetic sugar media without nutrient supplementation. However, unlike the synthetic sugar media, the difference between the fermentation performances with and without nutrients was not as obvious in the dissolving pulp and steam pretreated LPP hydrolysates (Figure 12 B-C). One potential explanation of the results may be that the presence of chemical components in the hydrolysate act as nutrients (wood extractives, impurities in the enzyme cocktail, etc.). It also might be possible that some nutrients such as amino acids and surfactants that are present in the enzyme cocktail may be responsible for the remarkable fermentability of the DsP hydrolysate in the absence of added nutrients. This is discussed later in greater detail. Nutrient sources may also be liberated from the biomass during pretreatment.

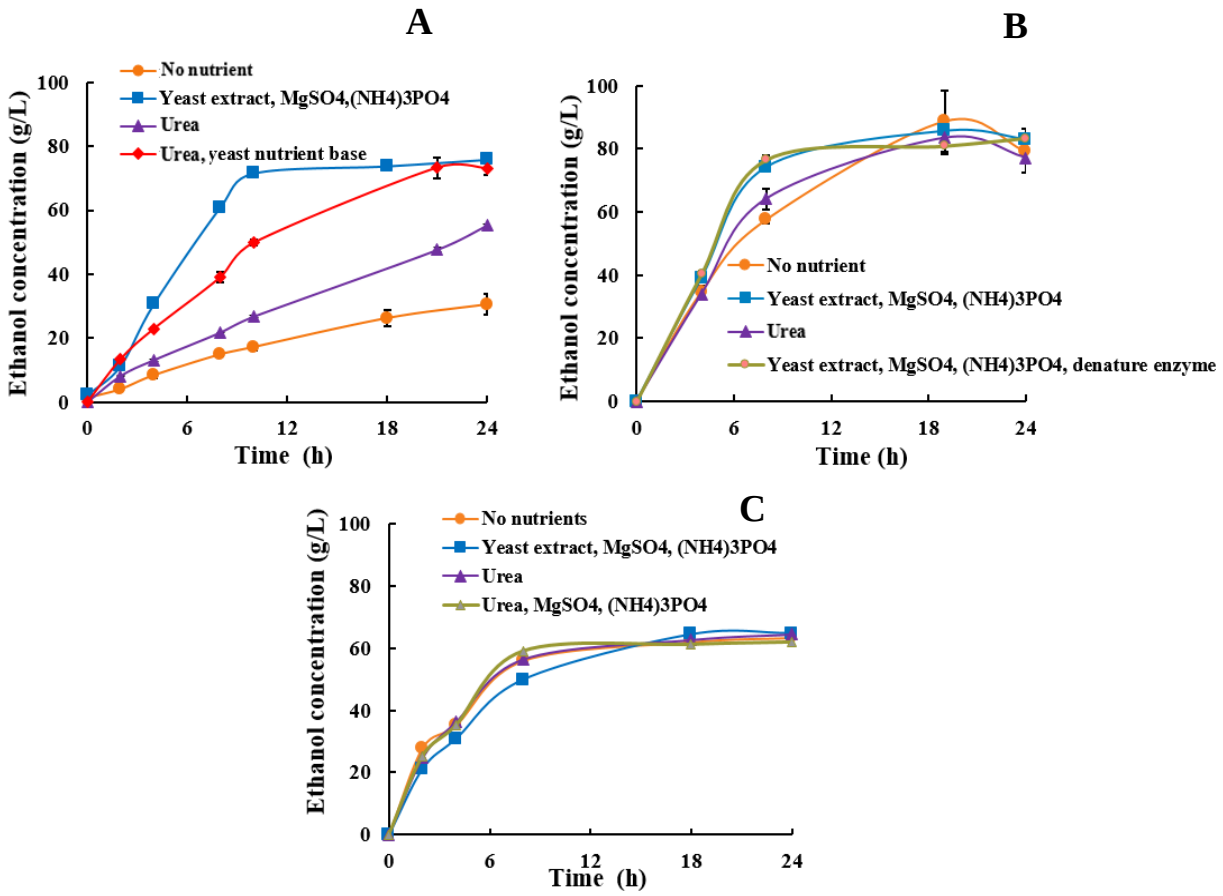


Figure 12 The influence of nutrient supplementation on the ethanol production profile of yeast strain 6469 in (A) synthetic sugar media and (B) Dissolving pulp (DsP) hydrolysate, and strain T_2 (C) on the hemicellulose rich water soluble fraction of steam pretreated lodgepole pine whole slurry hydrolysate.

As discussed above, the dissolving pulp hydrolysate was used as a "model" substrate as it consisted of relatively pure cellulose. However, when compared to the fermentation of synthetic sugar media, the dissolving pulp based hydrolysate still contained additional components from the enzymatic hydrolysis including enzymes, buffer and gluconic acid (by-product of hydrolysis). Therefore, the influence of each of these components on fermentation was tested in synthetic sugar media. Upon testing each potential component present in the dissolving pulp derived hydrolysate, the addition of the enzyme cocktail Ctec-3 was found to boost the ethanol

titer from 65 g/L to 82 g/L (Figure 13 A) even in the presence of nutrients added to the media. To eliminate the possibility of additional sugars being contributed by the enzyme cocktail, the sugar content of Ctec-3 was tested. However, the sugar concentration after dilution of the enzyme was only 0.5 g/L. Thus it was evident that the low level of sugar in the enzyme preparation likely could not induce an extra 17 g/L of ethanol production during fermentation of the synthetic sugar media. A range of different enzyme loadings from 5 mg enzyme / g pulp to 70 mg enzyme / g pulp were tested to determine if the effect of the enzyme addition could be amplified. It was shown that the beneficial effect of enzyme addition was apparent up to an enzyme loading of 30 mg/g cellulose (Figure 13 B). The effects of adding Ctec-3 to the fermentation of pure sugars was compared to another cellulase enzyme cocktail, Celluclast and the fermentation did not seem to improve to the same extent as that which was observed with CTec-3.

It was unclear whether the enhancement of the hydrolysis provided by the CTec-3 cocktail was due to the buffer or the enzyme protein components of the cocktail. Therefore, the enzyme cocktail was separated using an FPLC to yield an enzyme/protein rich component and a buffer/media fraction from the enzyme preparation as provided by the manufacturer (Novozymes). These two fractions were compared for their ability to aid fermentation. It was apparent that the enzyme protein rich fraction from the FPLC had a limited effect on fermentation but the buffer/media fraction provided an enhancement during the fermentation of the synthetic sugar media (Figure 13 C). Based on the results, the inclusion of the enzyme preparation during fermentation which is the case during the simultaneous scarification and fermentation process would be beneficial in both relieving end product inhibition as well as boosting the ethanol production in fermentation.

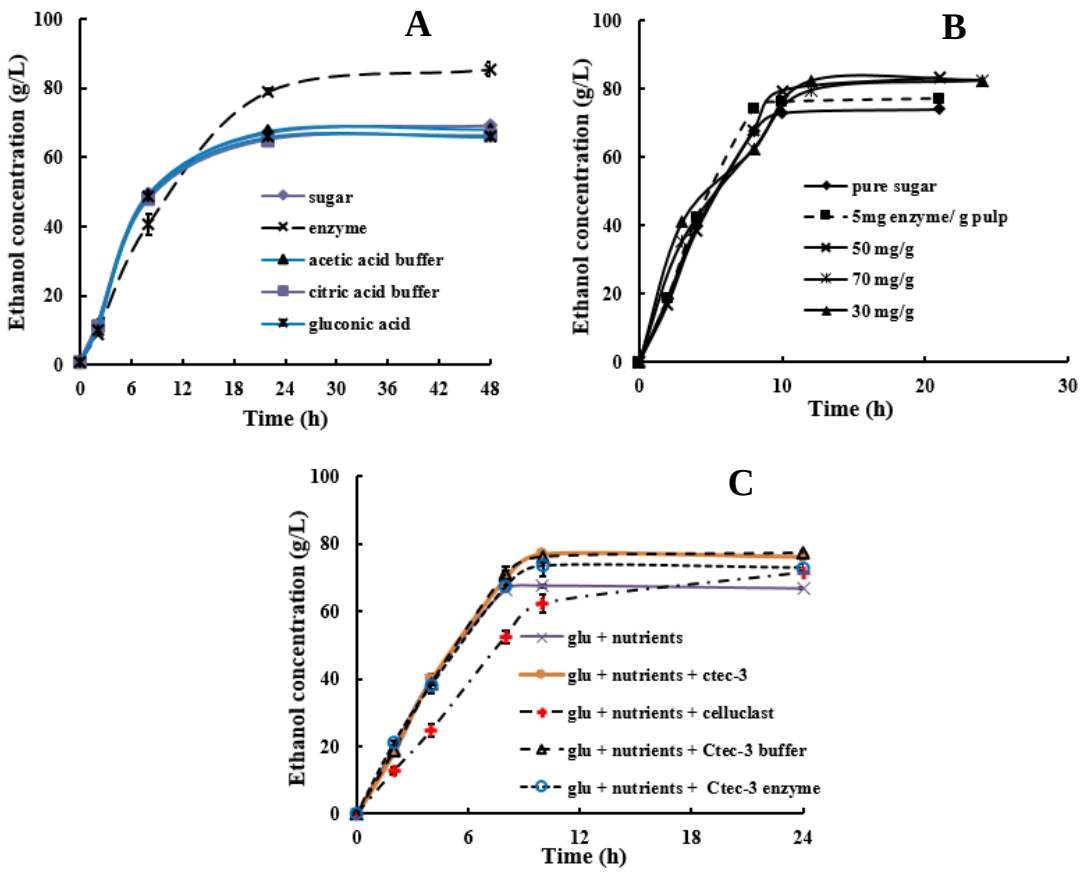


Figure 13 The potential effects of various enzymatic hydrolysis related factors on the ethanol production profile of strain 6469 in synthetic sugar media. (A) The influences of Ctec-3 enzyme preparation, hydrolysis buffer (acetate buffer vs. citrate buffer), and by-product gluconic acid; (B) the influence of various amount of Ctec-3 enzyme addition; and (C) the influence of the protein/non-protein fractions of Ctec-3 and Celluclast enzyme preparations.

As well as the dissolving pulp hydrolysate, the steam pretreated LPP hydrolysate liquid fraction also fermented without the addition of nutrients which could be due to the factors discussed above and/or the relatively higher cell density that was employed during the fermentation. A high cell density may have also helped overcome the inhibitors that were present in the hydrolysate such that the addition of extra nutrients did not enhance the fermentation any further. In previous studies, cell densities of approximately less than OD 5 (Albers & Larsson,

2009; Almeida, Modig, & Petersson, 2007; Bjorn, 2006; Cavka, Alriksson, Ahnlund, & Jönsson, 2011; X. Guo, Cavka, Jönsson, & Hong, 2013; Z. Guo & Olsson, 2014) were used. As these were below the OD of 13 employed in the work presented here, the effects of cell density were investigated in greater detail.

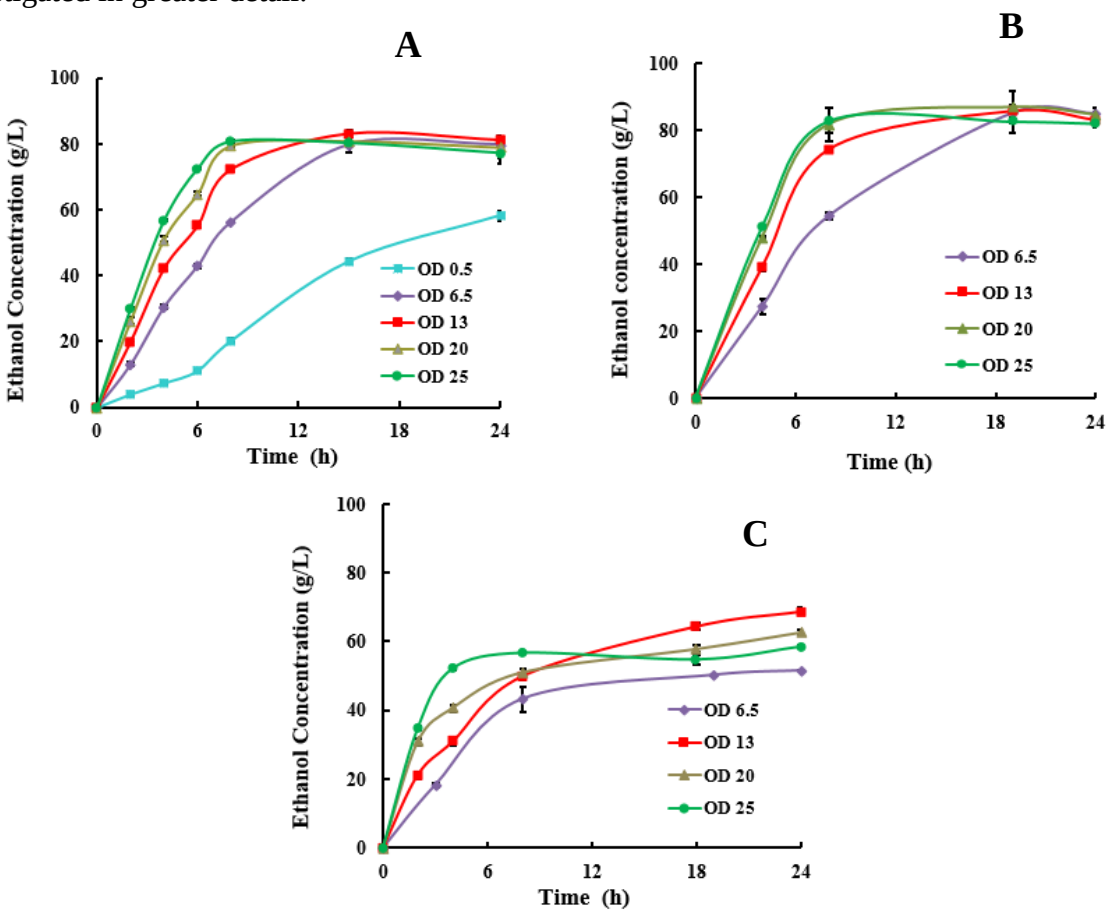


Figure 14 The influence of various yeast concentrations (cell optical density: OD) on the ethanol production profile of strain 6469 in (A) synthetic sugar media and (B) Dissolving pulp (DsP) hydrolysate, and strain T_2 (C) on the hemicellulose rich water soluble fraction of steam pretreated lodgepole pine whole slurry hydrolysate.

As described previously (Kapu et al., 2013), increasing cell density can help improve ethanol productivity as well as aiding the yeast to better overcome inhibitors present in pretreated

lignocellulosic substrates. Therefore a range of cell densities were tested on the same set of media described earlier (synthetic sugar media, DsP hydrolysate and steam pretreated LPP hydrolysate). Ethanol productivity was improved in all of the three media when increasing cell density (Figure 14). However, increasing cell density past OD 20 did not provide any further improvements. It was apparent that at all of the cell densities used (from OD 6.5 to OD 25) the fermentation of the steam pretreated LPP hydrolysate resulted in the worst performance compared to model glucose media or the DsP hydrolysate (Table 4). This was likely due to the presence of high levels of inhibitors (Table 2). However, with increasing cell density, the differences among the productivities based on these three media narrowed (Table 4). Therefore, it was apparent that, with increasing cell density, this can help partially improve the tolerance to high levels of inhibitors in steam pretreated softwood hydrolysates.

Table 4 Ethanol productivity of various yeast concentrations when; strain 6469 was grown in synthetic sugar media and Dissolving pulp (DsP) hydrolysate and; strain T_2 in the liquid fraction of steam pretreated Lodgepole pine whole-slurry hydrolysate (SPLP) with nutrient supplementation.

Different OD	**Synthetic glucose media (g/L. h)**	**DsP hydrolysate (g/L. h)**	**SPLP hydrolysate (g/L. h)**
6.5	7.5	6.3	5.37
13	10.5	9.7	7.7
20	12.8	11.9	10.1
25	14.3	12.8	13

Note: all the samples were taken after 4 hrs of fermentation time except for the productivity of OD 6.5 in steam pretreated hydrolysate. This was calculated based on 8 hrs fermentation.

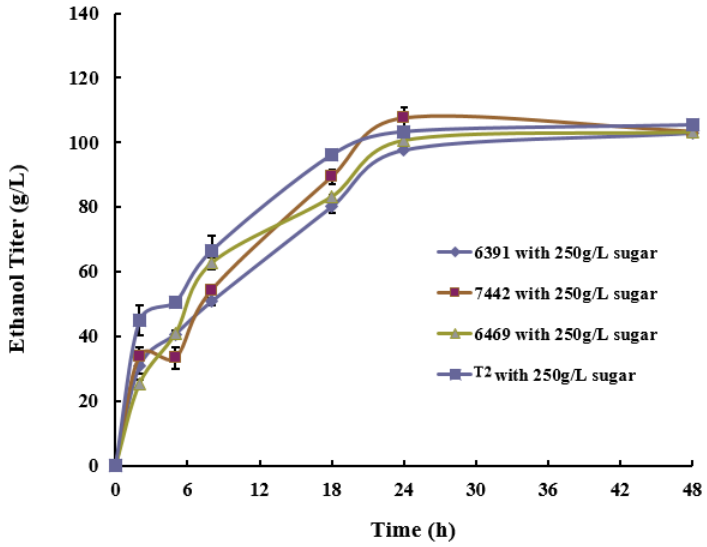


Figure 15 Ethanol production profile after growth of S. cerevisiae strains 6391, 7442, 6369 and T_2 in high concentration synthetic sugar media of 250 g glucose per liter.

One technological approach that has significantly improved industrial ethanol production is the use of very high gravity (VHG) fermentation. Numerous advantages exist through the use of VHG technology including increased ethanol concentration, reduced bacterial contamination and decreased process operating costs. However, it is also recognized that, excessively high sugar concentrations can also stress *Saccharomyces cerevisiae*, limiting ethanol production (Kayikci & Nielsen, 2015). Therefore, the influence of very high gravity fermentation on the different types of strains was assessed using an initial sugar concentration of 250 g/L (Figure 15). In order to deal with the stress induced by high sugar concentrations, very high cell densities (OD 13) were used in the work reported here. It was apparent that the four strains exhibited a similar profile in terms of final ethanol titer using high gravity fermentation and each strain could produce 100-108 g/L ethanol in 24 hours. The T_2 strain was the most effective performer,

yielding 8.5 g/ L. h of ethanol compared to lowest yield of 6.25 g/ for the case of strain 6391 (Figure 15).

3.1.2.3 Comparing fermentation performance in the whole slurry hydrolysate (HHF) to the liquid fraction of whole slurry hydrolysate.

As has been reported, fermentation factors such the type of yeast strain, nutrient supplementation, and different cell densities were assessed using the liquid streams of the whole slurry hydrolysate from steam pretreated lodgepole pine and dissolving pulp. However, the likely scenario for commercial cellulosic ethanol production will be through using the whole slurry that includes the water soluble and residual water insoluble substrate either as part of a simultaneous scarification and fermentation (SSF) or so-called hybrid hydrolysis and fermentation (HHF) configuration. Therefore, a whole slurry hydrolysis and fermentation approach was assessed using an optimized enzyme loading (35 mg/g cellulose, 25% solids) and the fermentation conditions (T_2 yeast, OD 6.5, 13 high cell density) in a HHF process. Interestingly it was found that when the solid substrate particles were included in the hydrolysate (whole slurry hydrolysate) at the 25% solids loading, the sugars in the whole slurry hydrolysate could not be fermented at all. However, the liquid fraction of the whole slurry hydrolysates which contains most of the water soluble inhibitors was shown to be readily fermented (Figure 16). The main difference between these two kinds of media (whole slurry hydrolysate vs. the liquid fraction of whole slurry hydrolysate) was that the whole slurry hydrolysate contained both the water soluble and water insoluble fractions, while the liquid stream of the whole slurry hydrolysate just contained the water soluble fraction. It was evident that the water insoluble fraction contained some inhibitory material that was affecting the fermenting yeast.

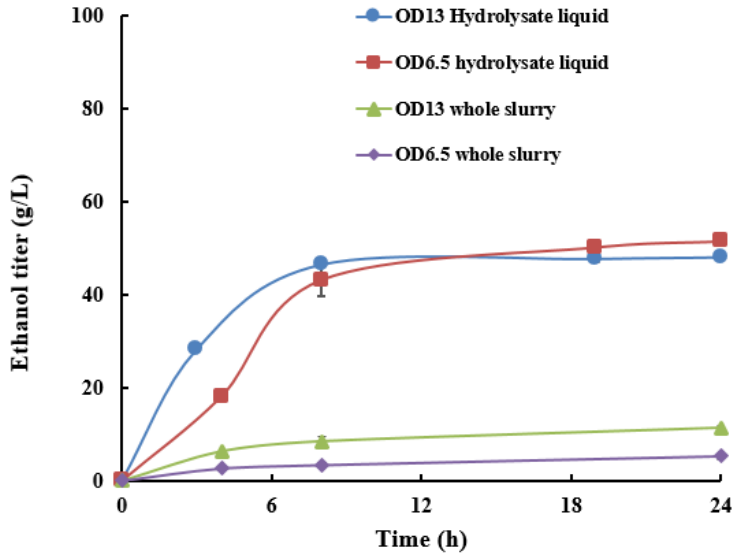


Figure 16 The influence of different yeast concentrations (cell optical density: OD) on the ethanol production profile of strain T_2 grown on steam pretreated lodgepole pine whole slurry hydrolysate with/without the removal of solid hydrolysis residues.

As the water insoluble fraction contains unhydrolyzed polysaccharides and lignin particles which might interact with yeast, the water insoluble fraction was separated from the whole slurry using centrifugation and washed 5 times with water prior to being added to the synthetic sugar media for fermentation. The fermentation of the synthetic sugar media containing the washed water insoluble fraction achieved 67.8% ethanol yield which was much higher compared to that of the whole slurry fermentation process (very weak fermentation was observed). However, it was still not as good as the fermentiablity of the synthetic sugar media which could achieve an 87% ethanol yield. This suggested that the poor performance of the yeast in the whole slurry hydrolysates was due to the synergistic stress from both the water soluble fraction and the water insoluble fraction. This also suggests that, at high solids, the softwood

derived water insoluble fraction of the whole slurry hydrolysate is also inhibitory. Although it has been shown that whole slurries from acidic steam pretreated biomass could be readily fermented, these studies were mostly performed at much lower solids loadings of <15%(Kim et al., 2008; L. Wang, Templer, & Murphy, 2012; Modenbach & Nokes, 2013). Many of these previous studies have also been performed on pretreated agricultural and hardwood biomass rather than the pretreated softwood that was used in this study(Tomás-Pejó, Ballesteros, & Olsson, 2008; Hu, Chandra, et al., 2015; Zhang, Qin, & Saddler, 2009; Yang, Li, & Xing, 2010). The HHF approach reported here was performed using a 25% solids whole slurry of steam pretreated softwood. This presents several obstacles towards fermentation and hydrolysis including higher initial sugar concentrations, high residual recalcitrant solids and an increased amount and concentration of inhibitors. Recent work has shown the SSF processing of corn stover at a solids loading of 15% using a pretreated whole slurry could be successful (Z.-H. Liu et al., 2014). However, the liquid fraction from the steam pretreated corn stover contained only 2.7 g/L acetic acid, 0.7 g/L furfural and 1.0 g/L HMF, while the liquid fraction of the steam pretreated lodgepole used in this research contained 9.2 g/L acetic acid, 2.8 g/L furfural, 3.0 g/L HMF and 4.9 g/L phenolic compounds. Therefore, the higher solids loading, the inhibitory nature of the solid material towards fermentation and enzymatic hydrolysis and the higher amount of known fermentation inhibitors likely compromised the ability to ferment the whole slurry originating from the steam pretreated softwood biomass. Thus, the work that was next tackled was to assess several strategies to improve the hydrolysis and fermentation for the whole slurry material from steam pretreated softwood.

3.1.2.4 Conclusions

To achieve high sugar concentrations for fermentation when processing steam pretreated biomass, both a high solids loading (>25%) and the use of the whole biomass slurry (water insoluble and water soluble fractions) were assessed. As shown in this chapter, even in the case of a lignin-free substrate such as dissolving pulp, the use of high solids loadings presents several challenges for enzymatic hydrolysis including mixing issues and end-product inhibition. These issues are amplified in the case of softwoods. This was due to increased recalcitrance of the substrate as well as high levels of fermentation inhibitors in the water soluble fraction. This necessitated the use of high enzyme loadings and several fermentation strategies to overcome fermentation inhibitors.

When using just the liquid fractions from the whole slurry hydrolysate, strategies such as strain selection, nutrient supplementation and high cell density helped to improve the ethanol productivity and ethanol yield during fermentation. However, when the lignin-rich water insoluble hydrolysis residue was combined with the water soluble hydrolysate to form the "whole slurry hydrolysate", the resulting sugar stream became un-fermentable despite the use of these strategies. Therefore, the work described in the next three chapter investigated the use of detoxification, post-treatments and modified pretreatments to overcome the inhibition presented by the softwood whole slurry.

It is likely that high concentrations of fermentation inhibitors such as acetic acid, furan aldehyde and phenolic compounds that are generated form the steam pretreatment process inhibit the yeast, thereby limiting the ethanol production. It was shown that some cell culture strategies such as strain selection, nutrient supplementation and high cell density could help to improve the ethanol productivity and ethanol yield in the fermentation of whole slurry liquid fraction.

However, even optimizing these factors, the fermentability of whole slurry hydrolysate is still problematic.

3.2 The potential of one step sulphite post treatment to improve both hydrolysis and fermentation of the steam pretreated softwood whole slurry

3.2.1 Background

Building on the work described in the last chapter, the major issues when trying to hydrolyze and ferment whole slurries derived from the steam pretreatment of lodgepole pine include the highly recalcitrant water insoluble fraction that is resistant to enzymatic hydrolysis and the inability to ferment the whole slurry in the presence of the water insoluble fraction. The recalcitrance of steam-pretreated softwoods has been well established in the literature (L. Kumar, 2013; L. Kumar et al., 2012; Ewanick et al., 2007). It was shown in the previous chapter that, during enzymatic hydrolysis at high solids loadings, steam pretreated lodgepole pine required three times more protein to reach a glucose concentration of 120 g/l compared to the model dissolving pulp (DsP) substrate. Due to the solubilisation of hemicellulose, the lignin content of steam pretreated softwood typically approaches 50% of the overall substrate composition (L. Kumar, 2013). Since the cost of producing a "Northern bleached softwood Kraft (NBSK)" pulp is in the range of 800-1000$ per ton, removing lignin to improve enzymatic hydrolysis not economically feasible for a bioconversion process. Therefore, previous work has assessed the use of treatments such as sulphonation (L. Kumar, 2013)(C. Mooney, Mansfield, & Saddler, 1998)(Xuejun Pan, 2005). Sulphonation modifies the lignin to improve its hydrophilicity which

increases substrate swelling while decreasing the tendency of the lignin to non-productively bind with cellulases (C. A. Mooney et al., 1999; Kumar et al., 2011). Under alkaline and neutral conditions, the large amount of phenolic hydroxyl groups on lignin can deprotonate and act as targets for the sulphonation reaction.

The major goal of much of this previous work on sulphonation was to determine if the presence of the lignin was the main issue hindering the hydrolysis of softwood substrates(L. Kumar et al., 2011). Using 160 ℃, a residence time of 1 h and 20 minutes and a sulphite loading of 16% based on dry wood chips, Kumar et al achieved a complete hydrolysis of the cellulosic fraction at an enzyme loading of 15 FPU/g cellulose, despite the high lignin content (44%) of the substrate (L. Kumar et al., 2011). However, it should be noted that the aforementioned post-treatment was applied to a well-washed steam pretreated softwood substrate that was washed again after the post treatment to remove high concentrations of excess sulphite that could potentially inhibit the function of cellulases and yeasts (Bailey & Cole, 1959; Anfinsen & Haber, 1961; Schimz, 1980).

As well as the high recalcitrance of softwood towards enzymatic hydrolysis, the water-soluble fraction originating from the steam pretreatment of softwood is also inhibitory towards fermentation. In the previous chapter, several fermentation strategies such as strain selection, nutrient supplementation, and high cell density were successfully implemented to improve the fermentability of the water-soluble fraction and whole slurry liquid hydrolysate from steam-pretreated softwood. As shown in the previous chapter, the water insoluble fraction was inhibitory towards fermentation as during a hybrid hydrolysis and fermentation configuration the whole slurry resulting from the steam pretreated softwood was virtually “un-fermentable” (thesis section 3.1.2.3).

Co-incidentally, as well as modifying lignin to improve enzymatic hydrolysis of steam pretreated softwoods substrates, sulphite has also been shown to detoxify the fermentation inhibitors in the water soluble fractions originating from the steam pretreatment of softwood. (Cavka et al., 2011; Alriksson et al., 2011; Guo et al., 2013). However, unlike the post-treatments discussed above, these detoxification treatments have typically employed either sulphite or dithionite at very low dosages at room temperature and at a pH similar to that of fermentation (pH 5.5) (Alriksson et al., 2011). Although the underlying mechanisms responsible for the detoxification are not fully understood, it is likely the predominant mechanism is a reduction reaction as dithionite and reducing agents such as sodium borohydride have also been shown to detoxify the water soluble streams from pretreated softwood (Alriksson et al., 2011). However, it should be noted that the amount of potentially inhibitory phenolic compounds and the sugar concentration of the water soluble streams utilized in this previous were relatively low compared to the hydrolysates utilized in this thesis (Alriksson et al., 2011)(Cavka et al., 2011).

Although the use of sulphite improves enzymatic hydrolysis of the water insoluble fraction and fermentability of the water insoluble fractions of steam pretreated softwoods, there has yet to be any research which combines both of these approaches as a single treatment. This type of one-step approach could potentially offer the ability to simultaneously improve hydrolysis and fermentation of the whole slurry (water insoluble and water-soluble fractions) from steam pretreated softwood (Figure 17). However, the conditions that have been employed to apply sulphite to improve fermentation vs enzymatic hydrolysis differ substantially. The detoxification of the water soluble streams likely involves reduction reactions that can be accomplished using low concentrations of sulphite at low temperatures while treatments to improve enzymatic hydrolysis of the water insoluble fractions have typically been applied at

higher temperatures (>140 °C) and sulphite loadings (16 %) (Kumar et al., 2013; L. Kumar et al., 2011). Since the whole-slurry sulphite treatment would not involve washing of the substrate after the sulphite treatment, the presence of excess sulphite that is required to modify the substrate could be inhibitory to subsequent enzymatic hydrolysis and fermentation (Bailey & Cole, 1959)(Anfinsen & Haber, 1961)(Schimz, 1980).

Therefore, the work in this chapter assessed whether it was possible to improve both the hydrolysis and fermentation of a steam pretreated softwood whole slurry using a single step sulphite treatment. The work hoped to find "compromise" conditions where both the hydrolysis of the water insoluble and the fermentation of the water-soluble fraction could be achieved.

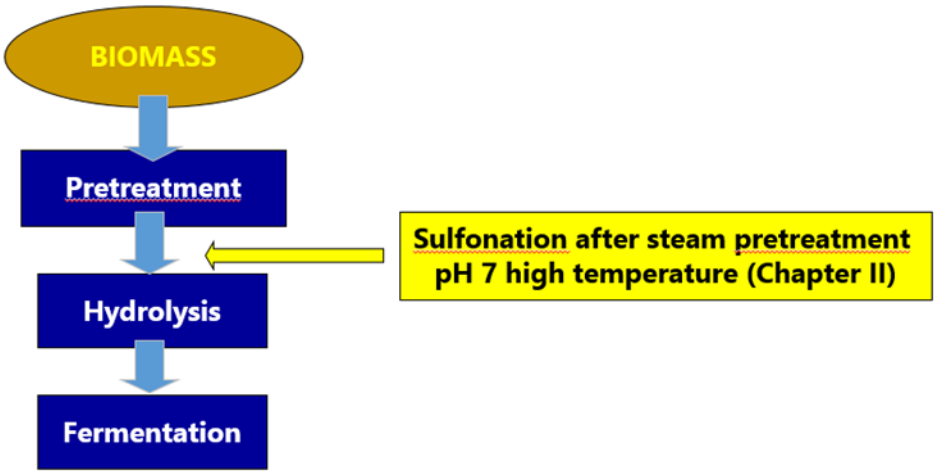


Figure 17 Diagram of post treatment approach followed in whole bioconversion process.

3.2.2 Results and discussion

3.2.2.1 Sulphite detoxification of the water-soluble fraction of steam pretreated softwood

Prior to assessing the effectiveness of sulphite treatment on the whole slurry from the steam pretreatment of lodgepole pine, previous results which described the detoxification of the water-soluble fractions from steam pretreated lignocellulosic substrates (Cavka et al., 2011) were first confirmed using the hydrolysates described in this thesis. This previously reported work was repeated since the steam pretreated LPP hydrolysate used in this thesis contained 9.2 g/L acetic acid, 2.8 g/L furfural, 3.0 g/L HMF and a total phenolic content of 4.9 g/L. This was significantly higher compared to most of the previous literature which has looked at detoxification (Larsson, Nilvebrant, & Jönsson, 2000； Persson et al., 2002; Soudham, Alriksson, & Jönsson, 2011). In order to improve the fermentability of steam pretreated LPP, sulphite detoxification (15 mM sulphite concentration, room temperature) was initially tested on the steam pretreated LPP hydrolysate at cell densities of OD 13 and OD 6.5 using the T_2 yeast strain. It was apparent that the sulphite treatment improved the ethanol productivity at both cell densities (Figure 18).

Since previous work had shown that sulphite treatment aids fermentation of the liquid fraction of the whole slurry hydrolysate, despite its high levels of inhibitors, the ability to aid both the enzymatic hydrolysis and fermentation of the whole slurry at room temperature was next tested.

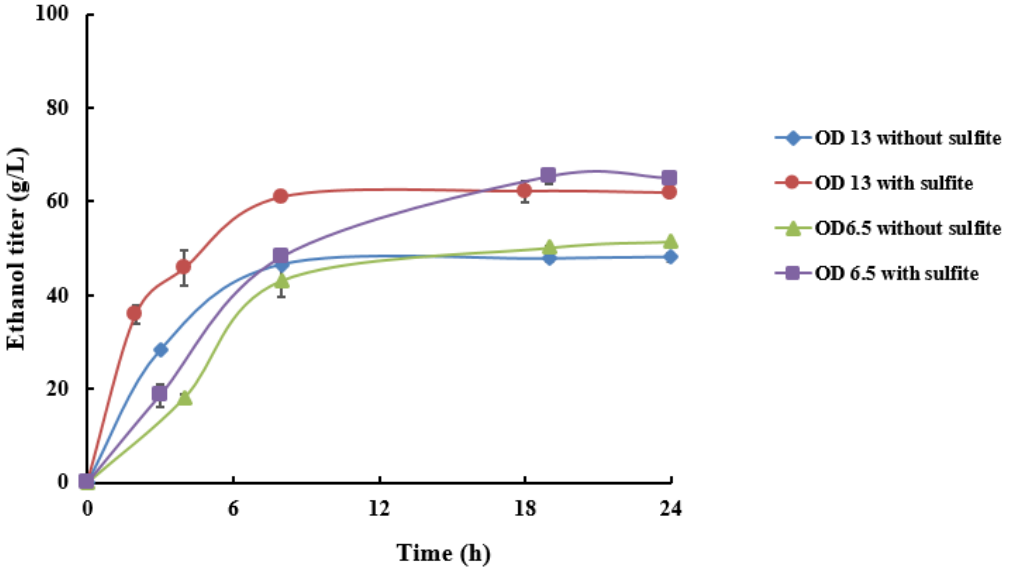


Figure 18 The effect of sulphite detoxification on the ethanol production profile of strain T_2 grown on the water soluble fraction of steam pretreated lodgepole pine whole slurry hydrolysate. The yeast concentrations (cell optical density: OD) were 6.5 and 13 respectively.

3.2.2.2 Sulphite treatment of the whole slurry over a range of sulphite loadings at room temperature

As discussed earlier, neutral sulphonation of steam pretreated softwood substrates was performed previously by applying temperatures of 160 ˚C and sulphite loadings of 16% for 1 hours and 20 minutes to the washed steam pretreated biomass with subsequent washing at the conclusion of the reaction to remove any excess sulphite (L. Kumar et al., 2011; C. Mooney et al., 1998). However, the sulphite treatment of water soluble fractions for detoxification to aid fermentation was performed at room temperature using 10 mM dosages of sulphite. Therefore, the ability to perform the sulphonation reaction using low dosages of sulphite at room temperature without washing would be beneficial and practical in a whole slurry approach. Based on previous pulp and paper literature, it is unlikely that dosages as low as 10 mM would

be capable of providing sufficient sulphonation of the substrate to improve enzymatic hydrolysis while also detoxifying the water soluble inhibitors (Pranovich, Sundberg, & Holmbom, 2003). Therefore, the whole slurry detoxification in this study was initially performed using a range of sulphite loadings from 1% - 6% on the biomass at room temperature.

It was apparent that sulphite treatment at room temperature required a sulphite loading of at least 3% to provide even slight enhancement (7%) while raising the sulphite loading to 6% actually decreased the hydrolysis yield by 15% compared to the control SO_2 steam pretreated softwood substrate (Figure 19). As mentioned above, previous work on sulphonation was performed on washed steam pretreated softwoods with subsequent washing to remove excess sulphite that can potentially inhibit the enzymes during subsequent enzymatic hydrolysis. Sulphite is able to reduce disulfide bonds in proteins, resulting in denaturation of cellulases during enzymatic hydrolysis (L. Zhang & Sun, 2008). It was apparent that these initial levels of sulphonation were insufficient at room temperature as only marginal improvements in hydrolysis yields were obtained and further increases in sulphite loading likely affected the stability of the enzymes since the low temperature did not allow the sulphonation reaction to exhaust the excess sulphite.

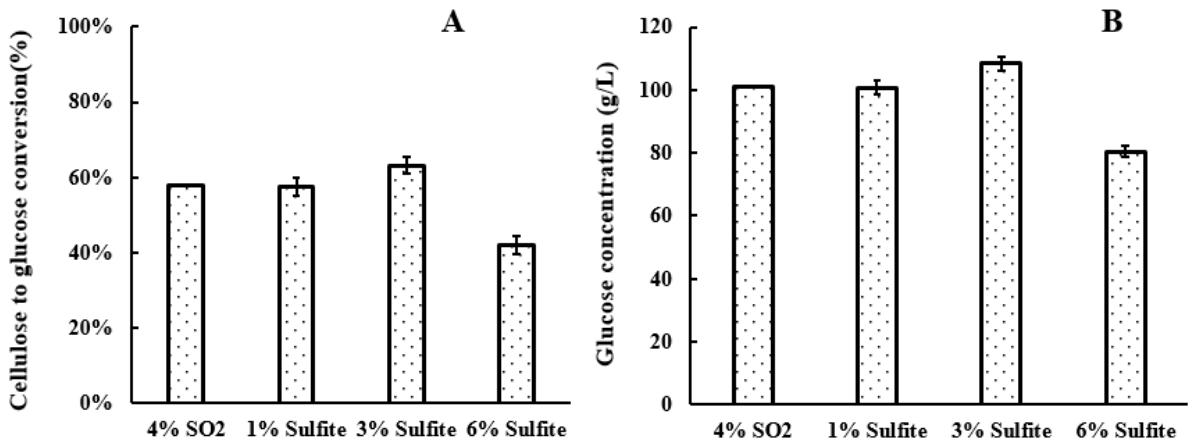


Figure 19 The effect of sulphite post-treatment (1%, 3% and 6% sulphite loading) on the enzymatic hydrolysis of 25% solid loading steam pretreated lodgepole pine whole slurry at a Ctec-3 loading of 40 mg protein per g cellulose for 48 h. (A) Percentage of cellulose to glucose conversion (%) and (B) Glucose concentration (g/L). The sulphite post treatments were conducted at room temperature for 5 mins. 4% SO_2 steam pretreated substrates was selected as control.

The fermentation performance of the sulphite post-treated lodgepole pine whole slurry hydrolysate was also tested. It was shown that the fermentability of the steam pretreated whole slurry could be improved slightly when increasing the sulphite loading from 1%-3% (Figure 20). However, similar to the effects on enzymatic hydrolysis, raising the sulphite loading to 6% was likely toxic to the yeast since fermentation ceased (Figure 20). This is not surprising since sulphite is known to suppress yeast and is used widely in winemaking (and most food industries), because of its antioxidant and antimicrobial properties (Schimz, 1980). It was apparent that sulphite treatment of the whole slurry at room temperature based using whole slurry could not sufficiently aid hydrolysis or fermentation and actually became inhibitory at higher sulphite

loadings. Therefore, the subsequent experiments assessed the ability of higher temperatures to improve the hydrolysis and fermentation of the steam pretreated softwood whole slurries.

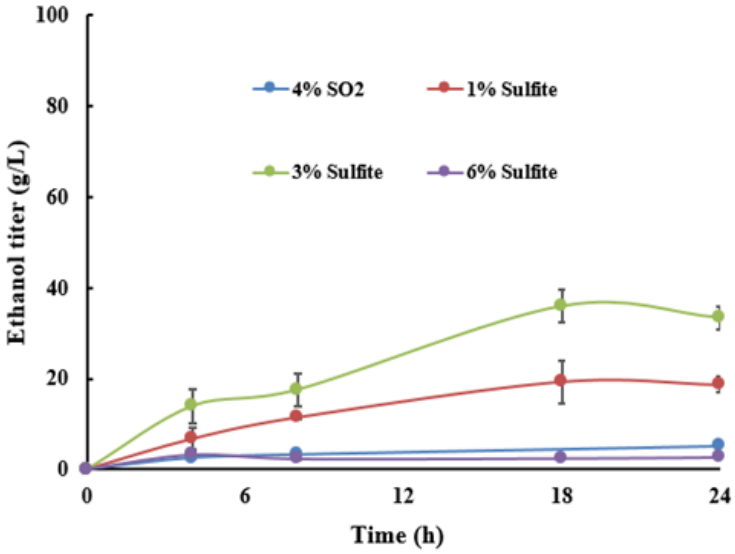


Figure 20 The effect of sulphite post-treatment (1%, 3% and 6% sulphite loading) on the ethanol production profile of strain T_2 grown on the water soluble fraction of steam pretreated lodgepole pine whole slurry hydrolysate. The yeast concentrations (cell optical density: OD) were 6.5. The sulphite post treatments were conducted at room temperature for 5 mins.

3.2.2.3 Sulphite post treatment at high temperature of 160 °C

Several previous studies have reported that higher temperatures of 160 °C can enhance the sulphonation reaction and thus improve the enzymatic hydrolysis of steam and mechanically treated softwood substrates (L. Kumar et al., 2011; C. Mooney et al., 1998). However, when temperatures as high as 160 °C are applied to a steam pretreated whole slurry, where the solubilized hemicellulose derived sugars are present in the water-soluble fraction, they can also undergo degradation during the sulphonation reaction. Initially the sulphonation reaction was applied to the washed steam pretreated substrate to confirm earlier work (C. Mooney et al., 1998;

L. Kumar et al., 2011) using a 10% solid loading, 160 °C for 70 mins with subsequent washing to remove excess sulphite. The results showed that both an 8% and 16% sulphite loading could boost the hydrolysis yield of the washed substrates from 62% (unsulphonated) to 88% (sulphonated) (Figure 21 B). The extent of sulphonation was assessed by the acid group content. The results showed that after sulphonation at 160 °C for 70 mins, the acid groups on the washed substrates were increased from 11 to 92 u mol / g substrates.

Even though the sulphonation of the washed substrates was beneficial towards hydrolysis, applying the sulphite post treatment to the steam pretreated whole slurry did not improve enzymatic hydrolysis compared to the control sample. The likely reason for these results was that, although the higher temperature enhanced the sulphonation of lignin, the residual sulphite in the whole slurry system inhibited the enzyme, which offset the beneficial effects of sulphonation (Figure 21 A).

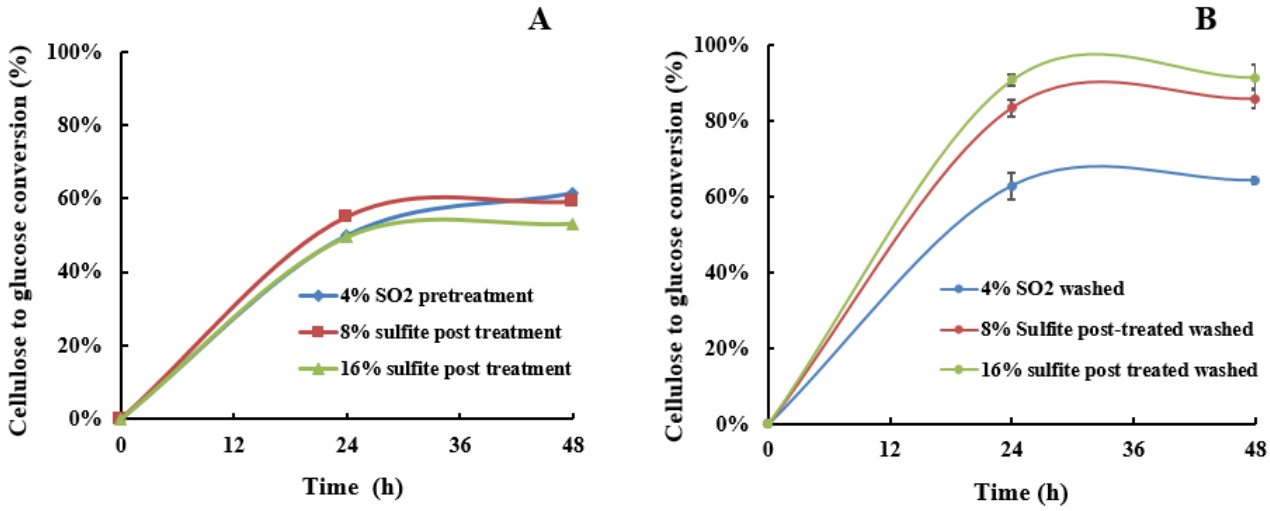


Figure 21 The effect of sulphite post-treatment (8% and 16% sulphite loading) on the enzymatic hydrolysis of 10% solid loading (A) unwashed and (B) washed steam pretreated lodgepole pine whole slurry at Ctec-3 dosage of 15 mg protein per g cellulose for 48 h. The sulphite post treatments were conducted at 160 °C for 70 mins in Parr reactor.

As well as the inability to improve the hydrolysis of the whole slurry system, performing the sulphite treatment at 160 ℃ resulted in the degradation of the hemicellulose-derived sugars solubilized in the water-soluble fraction. The initial concentration of glucose and mannose decreased from 6.95 g/L and 5.5 g/L to an undetectable level after the sulphite post treatment at 160 ℃ (Table 5). Therefore, the influence of sulphite loading and reaction temperature on sugar degradation were next studied to determine if a suitable temperature could be found to perform the sulphonation that would not inhibit enzyme activity and allow for the recovery of the solubilized hemicellulose.

Table 5 Glucose and mannose concentrations and the acid groups content of sulphite post-treated substrates (10% w/v) after treatment with a 8% and 16% sulphite loading at 160 °C for 70 mins in a par reactor.

Substrates	**Glucose (g/L)**		**Mannose (g/L)**		**Acid group (umol /g pulp)**
	Before reaction	After reaction	Before reaction	After reaction	
8% sulphite	6.9	0	5.5	0	86
16% sulphite	6.9	0	5.5	0	92

3.2.2.4 The inhibition of enzymes by sulphite

In order to exclude the influence of substrate characteristics such as lignin content as well as the water-soluble inhibitors on the enzyme, dissolving pulp which contains more than 94%

cellulose was selected as the substrate for this series of experiments to test the inhibition of the enzyme activity by sulphite addition. The hydrolysis was conducted at a 25% solid loading with Ctec-3 enzyme loading of 35 mg protein/ g cellulose. Initially a sulphite loading of 5% sulphite on the pulp was added to the hydrolysis. This was compared to the hydrolysis yield of the dissolving pulp control without sulphite addition. It was found that sulphite completely inhibited the hydrolysis. (Figure 22). As mentioned earlier, the presence of sulphite during enzymatic hydrolysis may result in the denaturation of the enzyme through the reduction of disulfide bonds. Sulphite is also a salt, thus a high concentration of the sodium sulphite can also denature proteins.

To determine if the observed effects of sulphite on the enzyme activity were due to the sodium sulphite salt, sodium chloride was added to the hydrolysis at a 5% loading (Figure 22). The results indicated that the addition of salt did not influence the enzyme activity. Therefore, it was likely that the sulphite was acting as a reducing agent to denature the cellulases. To test this hypothesis, hydrogen peroxide was utilized to oxidize sulphite prior to the enzymatic hydrolysis. In addition, hydrolysis with the addition of sodium sulfate which is the oxidization product of sodium sulphite was also assessed. The results showed that, with the addition of sulfate or peroxide oxidized sulphite, the hydrolysis efficiency is similar with that of the dissolving pulp control. This indicted that the sulphite was acting as a reducing agent which inhibited enzyme activity during hydrolysis (Figure 22).

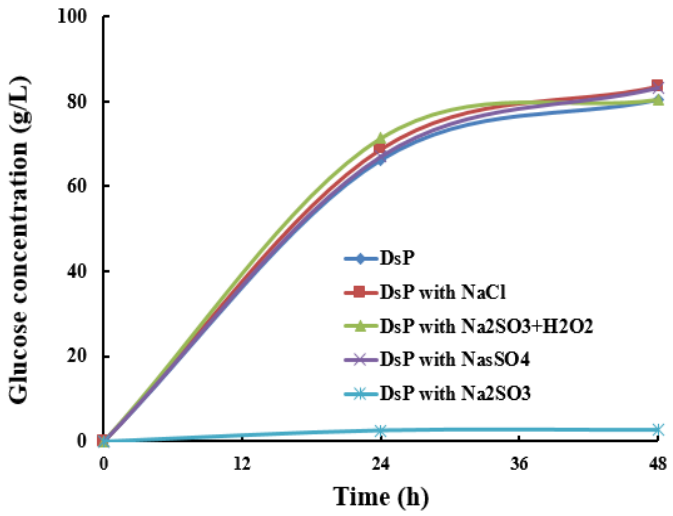


Figure 22 The influence of mineral salts (NaCl, Na_2SO_3 and Na_2SO_4) on the enzymatic hydrolysis of 12% solid loading dissolving pulp (DsP) at Ctec-3 dosage of 30 mg protein per g cellulose for 48 h. The mineral salts loading was 0.13 mol/L.

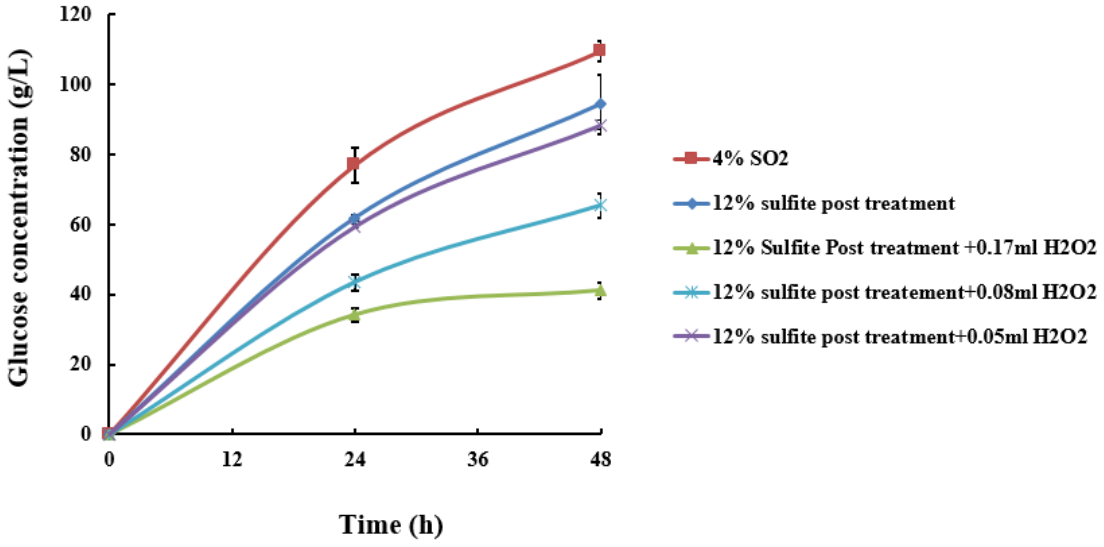


Figure 23 The effect of hydrogen peroxide treatment (0.18 and 0.35 mol/L) on the enzymatic hydrolysis of 25% solid loading sulphite post-treated steam pretreated lodgepole pine whole slurry at a Ctec-3 loading of 40 mg protein per g cellulose for 48 h. The sulphite post treatments were conducted at 121 °C for 40 mins in an autoclave.

Since hydrogen peroxide was shown to aid in eliminating the inhibitory effects of the sulphite on the enzyme during the hydrolysis of dissolving pulp, subsequent experiments used hydrogen peroxide to quench the sulphite in the post treated whole slurry to decrease its detrimental effect on enzymatic hydrolysis. However, the challenge of using hydrogen peroxide is understanding the correct amount of peroxide to add as the amount of residual sulphite after the sulphite reaction was unknown. Therefore, hydrogen peroxide loadings of 0.17 ml, 0.08 ml, 0.05 ml of 30% hydrogen peroxide per 10 ml of the whole slurry (25% solids loading) were added. However, hydrogen peroxide did not show any obvious boosting effect on hydrolysis while, similar to the effects of sulphite, adding too much peroxide actually inhibited the enzyme (Figure 23). In terms of fermentation performance, the inhibition of hydrogen peroxide on yeast was even more obvious. Treating the whole slurry treated with hydrogen peroxide completely inhibited ethanol production (Figure 24). This was likley because hydrogen peroxide acts as a reactive oxygen species which is fatal to yeast cells (Perrone, Tan, & Dawes, 2008; Izawa, Inoue, & Kimura, 1995). These results suggested that the peroxide reacted with the enzymes, yeast as well as the residual sulphite. It should be noted that it was challenging to obtain a homogeneous distribution of peroxide when adding to the high solids whole slurry. This may have compromised the ability of the peroxide to provide a targeted reaction with the sulphite rather than other components such as the enzymes. From previous work, it was apparent that a high temperature for sulphonation was detrimental towards sugar recovery, while excess residual sulphite decreased enzyme activity. Therefore, in order to further improve the post-treatment of the steam pretreated softwood whole slurry, it was necessary to further decrease both the reaction temperature and the sulphite loading.

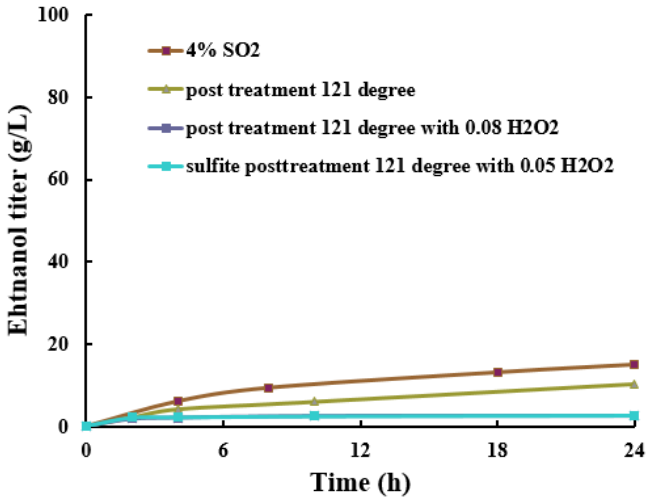


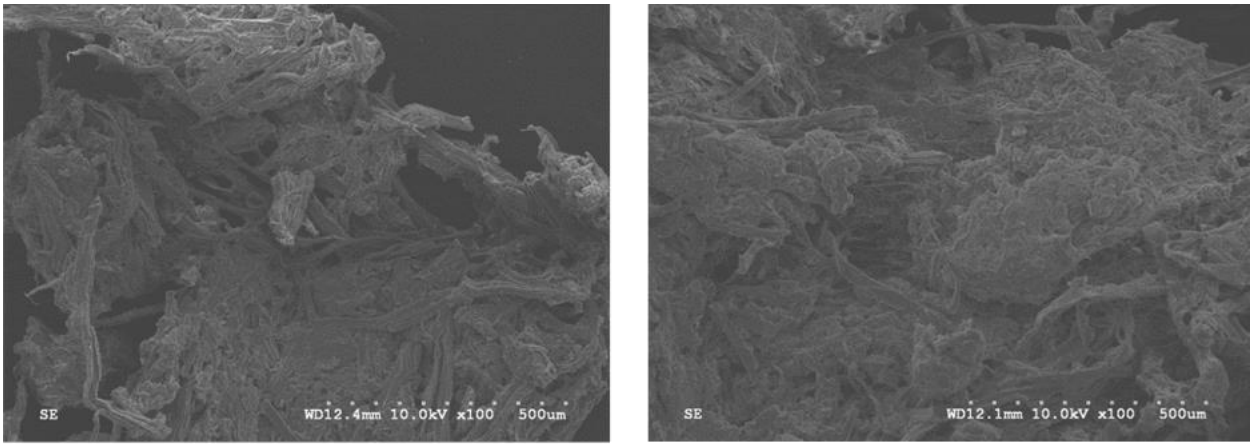
Figure 24 The effect of hydrogen peroxide treatment (0.18 and 0.35 mol/L) on the ethanol production profile of strain T_2 grown on 25% solid loading sulphite post-treated steam pretreated lodgepole pine whole slurry hydrolysate. The sulphite post treatments were conducted at 121 °C for 40 mins in an autoclave. The yeast concentrations (cell optical density: OD) were 6.5.

3.2.2.5 Using an intermediate temperature (121 °C) to simultaneously improve the hydrolysis and fermentation based of the steam pretreated lodgepole pine whole slurry.

To obtain an intermediate temperature, an autoclave was used at 121 °C for the sulphite post treatment. Sulphite loadings of 5% and 12% were applied to the steam pretreated whole slurry at a solids loading of 25%. The results showed that, at the intermediate temperature of 121 °C, the sulphite treatment at 5% did not exhibit sugar degradation. However, the 12% sulphite loading degraded the solubilized hemicellulose in the whole slurry (Table 6). This suggested that, not only the high temperature but also the high amount of sulphite was responsible for the sugar degradation. Scanning electron micrographs also showed that there were no apparent differences in the appearance of the substrates as a result of the post-treatment (Figure 25).

Table 6 The effect of sulphite post-treatment (5% and 12% sulphite loading) on the soluble fermentable sugar concentration and the acid groups content of 25% solid loading steam pretreated lodgepole pine whole slurry. The sulphite post treatments were conducted at 121 °C for 40 mins in an autoclave.

Sulphite loading	Glucose (g/L)		Mannose (g/L)		Acid group (umol /g pulp)	Fiber length (mm)	Fiber width (um)
	Before sulphite treatment	After sulphite treatment	Before sulphite treatment	After sulphite treatment			
4% SO2	-	-	-	-	-	0.41	27.3
5% sulphite	20.8	19.7	12.1	12.5	40	0.46	28.6
12% sulphite	20.8	9.2	12.1	5.1	140	0.42	28.2



4% SO2 acid steam pretreatment

12% sulfite post treatment

Figure 25 The FE-SEM imagines of the 4% SO_2 acid steam pretreated substrates, 12% sulphite post treated substrates. The 12 % sulphite post treatment was conducted at 121 °C for 40 minutes in an autoclave.

The hydrolysis performance of the 25% solid loading autoclave sulphite post treated whole slurry was assessed using 40 mg enzyme/g cellulose with the original SO_2 steam pretreated whole slurry as a control. The results showed that, using a 5% sulphite post treatment in the autoclave, the amount of glucose obtained from the whole slurry hydrolysis was similar to that of the control (4% SO_2 steam pretreated whole slurry). However, the 12% sulphite post treated slurry resulted in a decrease in sugar concentration. The lower yields at the 12% sulphite loading were likely due to the loss of the water soluble sugars at 121 °C as well as the excess residual sulphite at the 12% loading inhibiting the enzymes.

Upon washing to remove the unreacted residual sulphite after the post treatment, the washed 12% sulphite post treated substrate achieved the highest sugar concentration of 123 g/L, followed by the 5% sulphite post treated washed substrate (Figure 26) which both had higher performance than the standard steam pretreated control. These results showed that sulphonation did enhance the hydrolysis yield of the substrates (Figure 26). However, when hydrolyzing the post-treated whole slurry, the excess unreacted sulphite inhibited hydrolysis while the post treatment sacrificed sugar recovery at 121°C (Figure 26).

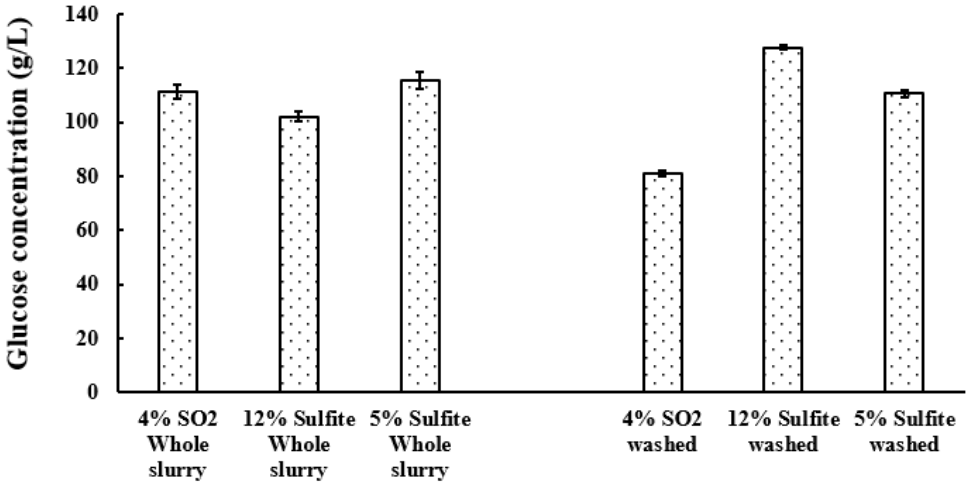


Figure 26 The influence of sulphite post-treatment (5% and 12% sulphite loading) and water washing on the glucose concentration after enzymatic hydrolysis of 25% solid loading steam pretreated lodgepole pine whole slurry (SPLP) at a Ctec-3 loading of 40 mg protein per g cellulose for 48 h. The sulphite post treatments were conducted at 121 degrees for 40 mins in an autoclave.

After assessing the hydrolysis of sulphite post-treated substrates at 121 °C, the fermentability of the 121 °C sulphite post treated whole slurries was next assessed (Figure 27). It was apparent that the 121 °C post treatment with 12% sulphite was detrimental to the yeast since no fermentation was observed. With the addition of 5 % sulphite, the fermentability was improved as an ethanol concentration of 52.9 g/L ethanol was obtained after 24 hours of fermentation (Figure 27). It was apparent that, in order to achieve enhanced hydrolysis, fermentation and sugar recovery, it was necessary to reduce the temperature even further. Therefore, the next set of experiments assessed post treatment at a temperature of 70 °C.

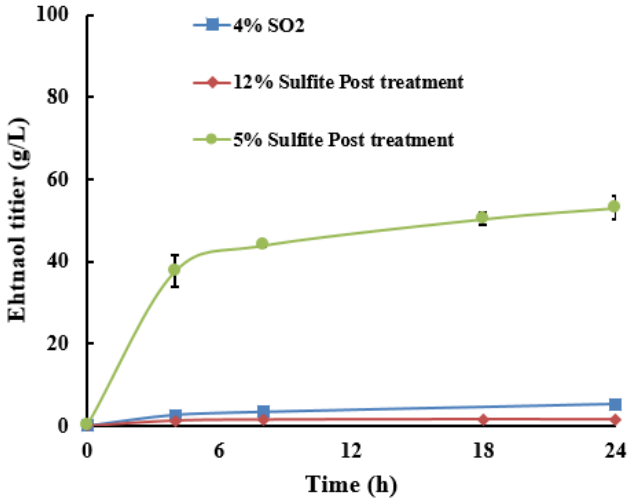


Figure 27 The effect of sulphite post-treatment (5% and 12% sulphite loading) on the ethanol production profile of strain T_2 grown on the steam pretreated lodgepole pine whole slurry hydrolysate (SPLP). The sulphite post treatments were conducted at 121 °C for 40 mins in an autoclave. The enzymatic hydrolysis was performed with 25% solid loading at a Ctec-3 loading of 40 mg protein per g cellulose for 48 h. The yeast concentration (cell optical density: OD) was 6.5.

Table 7 Soluble fermentation inhibitor profiles of two kinds of sulphite (12% and 5% sulphite loading) post-treated of 25% solid loading steam pretreated lodgepole pine whole slurry. The sulphite post treatment was conducted at 121 °C for 40 minutes in an autoclave.

Components	Control (g/L)	12% Sulphite (g/L)	5% Sulphite (g/L)
Acetic acid	7.2	9.9	7.5
Furfural	2.0	0.12	0.09
HMF	2.4	0.08	0.1
Total phenolics	3.8	7.9	5.1

3.2.2.6 Sulphite post treatment at 70 degrees for 121 hrs

It was apparent that a sufficiently high temperature and sulphite loading is necessary to achieve sulphonation to improve hydrolysis of washed substrates (Section 3.2.2.4). However, conditions that improve the hydrolysis of washed substrates are detrimental towards whole slurries as the excess sulphite is toxic towards fermentation and hydrolysis and the high temperatures compromise sugar recovery. In order to avoid sugar degradation, a milder temperature of 70^{o}C was chosen, based on previous research (L. Kumar, 2013). However, in order to process the sulphonation thoroughly at such a mild temperature, a longer residence time was necessary (L. Kumar, 2013). Therefore, we conducted sulphonation using 8% sulphite for 12 hrs based on previous research (L. Kumar, 2013). The results showed that, after the post treatment at 70 °C for 12 hrs using the whole slurry at a solids loading of 25%, the water soluble fraction still contained 20.7 g/L glucose and 12.5 g/L mannose. This was quite similar to the SO_2 steam pretreated control (Table 8). When increasing the sulphite loading to 16% at the 70 °C, the whole slurry also underwent minimal sugar degradation.

Table 8 The influence of sulphite post-treatment on the soluble fermentable sugar concentration of 25% solid loading steam pretreated lodgepole pine whole slurry. Acid group content of the sulphite post treated substrates. Sulphite post treatment was carried out at 70 °C degrees for 12 hours in a water bath.

Substrates	Glucose (g/L)		Mannose (g/L)		Acid groups (umol /g pulp)
	Before reaction	After reaction	Before reaction	After reaction	
8% Sulphite	20.9	20.7	13.5	12.5	85
8% Sulphite+2% Carbonate	20.9	18.2	13.5	10.9	106
16% Sulphite	20.9	18.0	13.5	10.2	129

The hydrolysis of the post treated slurry showed that the substrates treated with 16% sulphite suffered yield losses of 12% (Figure 28) likely due to the excess sulphite inhibiting enzyme activity. The digestibility of the substrates post-treated using 8% sulphite at 70 °C was quite similar to that of the SO_2 steam pretreated control. In order to maximize the sulphonation at this lower temperature, sodium carbonate was added to the post treatment to assist the sulphite react with lignin. This approach was shown previously to enhance the amount of sulphonation at lower sulphite loadings when applied to poplar biomass (Richard P. Chandra et al., 2016). It was shown that the addition of carbonate enhanced the substrates digestibility by 12% (from 55% to 67%). The sulfonic acid groups on the substrate after the post treatment were also shown to increase from 85 u mol/g to 106 u mol / g substrates upon the addition of carbonate during the post treatment (Table 8, Figure 28). Thus, it is likely that the sodium carbonate enhanced sulphonation at the lower temperature, limiting sugar degradation and increasing the hydrolysis

yield of the substrate. The enhancement of sulphonation by sodium carbonate addition also likely decreased the amount of residual excess sulphite, by incorporating more sulphite in the substrate. This likely decreased the sulphite toxicity towards yeast and the enzymes. By testing the final sugar production, it was apparent that, within two days of enzymatic hydrolysis, 108 g/L glucose and 13.3 g/L mannose could be obtained and could be used for downstream fermentation. When the fermentability of the 70 °C post treated substrates with 8% sulphite and 2% carbonate was tested, it was found that this post treatment could also detoxify the whole slurry. The final ethanol titer was 56.4 g/L, which is slightly higher compared with the substrates post treated with 5% sulphite at 121 °C (Figure 29).

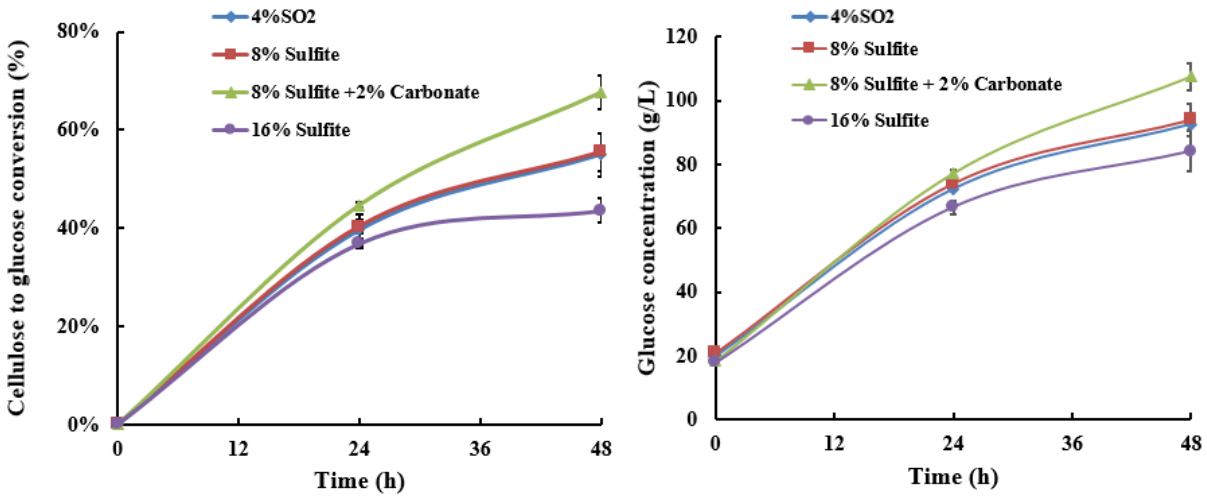


Figure 28 The effect of sulphite post-treatment (8% and 16% sulphite loading) with/without 2% carbonate on the enzymatic hydrolysis of 25% solid loading steam pretreated lodgepole pine whole slurry at a Ctec-3 loading of 40 mg protein per g cellulose for 48 h. (A) Percentage of cellulose to glucose conversion (%) and (B) Glucose concentration (g/L). The sulphite post treatments were conducted at 70 °C for 12 h in a water bath.

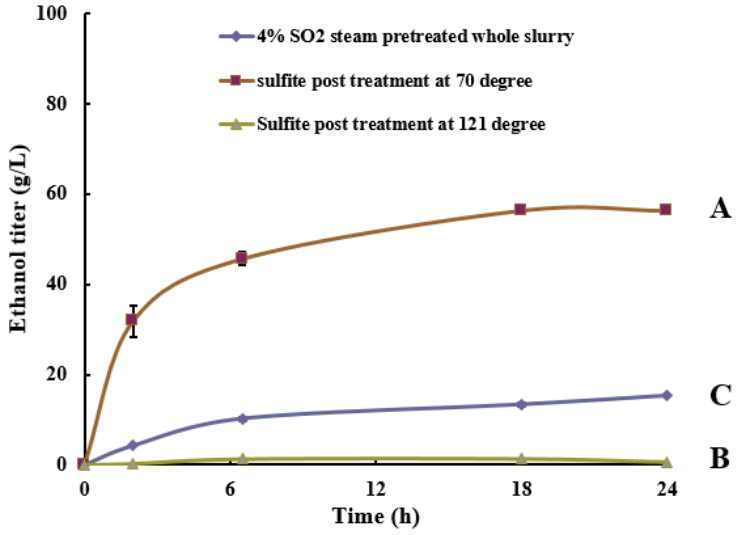


Figure 29 The effect of sulphite post-treatment on the ethanol production profile of strain T_2 grown on the 25% solid loading steam pretreated lodgepole pine whole slurry hydrolysate. The sulphite post treatments were conducted at (A) 70 °C for 12 h with 8% sulphite and 2% carbonate in the water bath and (B) 121 °C for 40 mins in an autoclave with 12% sulphite in an autoclave, respectively. The yeast concentration (cell optical density: OD) was 6.5.

3.2.2.7 Conclusions

A unique approach, using sulphite, was tested to simultaneously detoxify and improve the enzymatic hydrolysis of a steam pretreated softwood lodgepole pine whole slurry. There were several challenges related to the conditions employed during sulphite treatment including sugar degradation in the whole slurry at high post-treatment temperatures and the toxicity of the unreacted sulphite towards enzymes and yeast during subsequent hydrolysis and fermentation of the post treated whole slurry. However, it was shown that the sulphonation reaction at low temperature could be enhanced through the addition of sodium carbonate. The lower temperature limited sugar degradation, while the increased sulphonation improved enzymatic hydrolysis,

presumably by modifying lignin and by decreasing the amount of unreacted sulphite available to interact with enzymes and yeast.

3.3 The potential of using sulphite treatment during steam pretreatment to boost sugar and ethanol production for softwood bioconversion

3.3.1 Background

As described earlier, there were many challenges associated with attempting to use a one stage sulphite post treatment to simultaneously improve the enzymatic hydrolysis (through lignin sulphonation) and the ethanol fermentation (through inhibitor detoxification) of the steam pretreated lodgepole pine whole slurry. These challenges were due to the use of the steam pretreated softwood whole slurry system which results in the degradation of the solubilized hemicellulose during the sulphonation reactions. As well as sugar degradation, the higher amount of sulphite and the increased temperature of the sulphite treatment required also created complications for the single step post treatment approach. However, these challenges were overcome by enhancing the sulphonation reaction at lower temperatures (70 °C) via the addition of carbonate to the single step post treatment of the whole slurry. The sodium carbonate addition allowed for an increase in hydrolysis yields, fermentability and sugar recovery after the post treatment.

Although adding carbonate to the post treatment were promising, one key question was whether the challenges associated with the sulphonation were due to the compromised reactivity of the lignin in the water insoluble fraction of the steam pretreated softwood substrate. During

steam pretreatment of softwoods, guaiacyl rich softwood lignin has been shown to undergo high levels of condensation, which compromises its reactivity during subsequent chemical reactions(Li et al., 2007; Lin, 2016; Nakagame, Chandra, Kadla, & Saddler, 2011). The increase in lignin condensation would also presumably decrease the reactivity of the lignin towards sulphite, especially when attempting to use post treatments at temperatures as low as 70 °C that allow for the preservation of the sugars in the water soluble hemicellulose fraction. Previous work has shown that the neutral sulphonation reaction was more effective when performed on biomass during the steam pretreatment of poplar biomass compared to applying sulphonation post treatment (Richard P. Chandra et al., 2016).

As discussed in the introduction, pretreatments such as SPORL that apply a high temperature acid sulphite treatment to biomass prior to mechanical refining have also been applied effectively to softwood biomass. However, SPORL treatments of softwoods have been shown to still require relatively high enzyme loadings when treating softwood biomass. For example, when treating lodgepole pine, it was shown that the SPORL pretreated whole slurry at an 18% solids loading required 20 FPU/g cellulose and reached an ethanol concentration of 47.1 g/L(Zhou, Zhu, et al., 2013). As shown in the previous chapter, the use of carbonate as a post treatment allowed for a solids loading of 25% to be used in the whole slurry with a similar enzyme loading of approximately 50 mg/g glucan. However, a higher ethanol concentration of approximately 56 g/L was achieved.

One of the most important factors when using sulphite to treat biomass is the reaction pH. When applying “neutral” sulphonation pH (7-10), the reaction proceeds mainly through the deprotonation of the phenols to form quinone methide intermediates. These quinone methides are subsequently sulphonated through nucleophilic addition. Increasing the amount of quinone

methides formed through the addition of alkali can enhance this sulphonation reaction. It also lead to partial delignification by the fragmentation and increases lignin hydrophlicity through the incorporation of sulfonic acid groups (Gierer, 1985). Due to the neutral to alkaline pH, this reaction approach has been shown to be effective in preserving the carbohydrate components of the biomass as a portion of the water insoluble fraction during steam pretreatment (Richard P. Chandra et al., 2016). In particular, the preservation of the hemicellulose as part of the water insoluble fraction may provide opportunities for milder pretreatments where a greater amount of hemicellulose is recovered and subsequently hydrolyzed. A decreased solubilisation of the hemicellulose may also decrease the formation of fermentation inhibitors, such as furans that are derived from the acidic dehydration of carbohydrates during acidic pretreatments such as conventional steam pretreatment. Lignin condensation also occurs under acidic conditions. Thus employing a neutral/slightly alkaline approach should limit acid catalyzed lignin condensation (Li et al., 2007). Alternatively, similar to SPORL, the lignin sulphonation reaction can be performed at acidic pH which tends to fragment lignin through acidolysis while simultaneously sulfonating lignin to result in larger solubilized lignosulfonates. The acidic conditions result in the solubilisation of hemicellulose into a water soluble stream similar to acid catalyzed steam pretreatment. However, the acidic sulphite allows for bisulphite to simultaneously modify lignin while solubilizing hemicellulose.

Therefore, considering the potential increased reactivity of the native lignin in the biomass compared to the lignin in the substrate after steam pretreatment, in this section, sulphite treatment was carried out in the steam gun during the steam pretreatment itself (Figure 30). The neutral approach which tended to retain the hemicellulose in the water insoluble fraction was compared to the acidic sulphonation approach. This latter approach solubilized the hemicellulose

into the water soluble stream. This comparison allowed us to determine which approach provided the greatest enhancement of the hydrolysis/fermentability of the steam pretreated whole slurry.

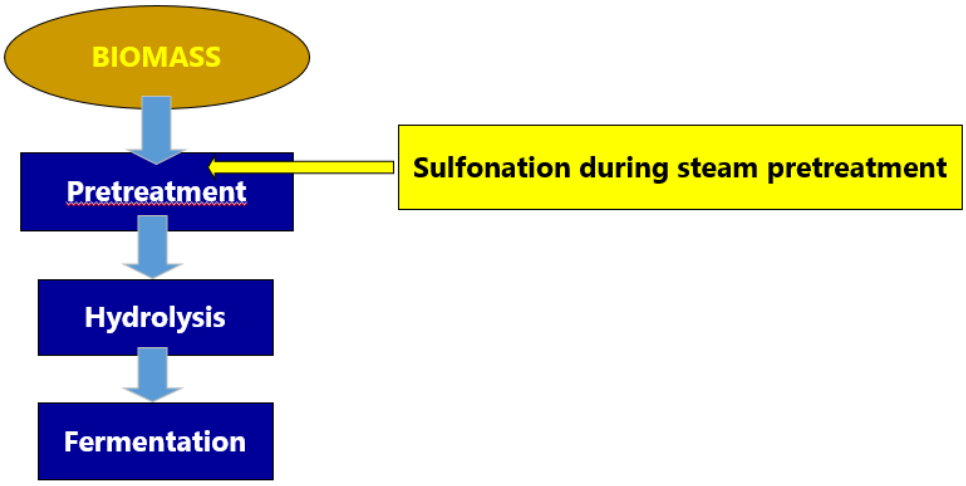


Figure 30 Flow chart of sulphite steam pretreatment in the overall bioconversion process.

3.3.2 Results and discussion

3.3.2.1 Alkali sulphite steam pretreatment with sulphite

Initially, sulphite loadings of 8% and 16% were used while the addition of sodium carbonate was varied from 2-6%. As mentioned above, increasing the pH via the use of alkali has been shown to enhance the sulphonation reaction when sulphite was applied during the steam pretreated of poplar (Richard P. Chandra et al., 2016).

Previous work has shown that an impregnation step prior to performing the sulphonation reaction in the steam gun was beneficial when using poplar biomass (Richard P. Chandra et al., 2016). Therefore, the lodgepole pine chips were soaked overnight in an Na_2SO_3 solution prior to

steam pretreatment over a range of temperatures and residence times using the previous work on poplar as a guide (Table 9) (Richard P. Chandra et al., 2016). Initially it was apparent that the use of neutral/alkaline sulphite in a single steam pretreatment stage was not able to reduce the particle size of the wood chips in a similar manner to steam pretreated softwood (Richard P Chandra et al., 2009). Subjecting the biomass to enzymatic hydrolysis at a solids loading of 5% and an enzyme loading of 15 mg protein / g glucan showed limited sugar release from the substrate after 72 hours (<< 2 g/L glucose).

Table 9 The hydrolysis performance of one step alkali sulphite (16% sulphite loading) steam pretreated lodgepole pine whole slurry at a 25% solid loading. Enzyme loading was 50 mg Ctec-3 /g glucan.

Chemical	Condition
16% Na_2SO_3	160 ℃ 20 mins
16% Na_2SO_3	160 ℃ 40 mins
16% Na_2SO_3	160 ℃ 70 mins
16% Na_2SO_3	180 ℃ 20 mins
16% Na_2SO_3	180 ℃ 40 mins
8% Na_2SO_3	160 ℃ 20 mins
8% Na_2SO_3	160 ℃ 70 mins
8% Na_2SO_3	180 ℃ 10 mins
8% Na_2SO_3	180 ℃ 20 mins
8% Na_2SO_3	200 ℃ 10 mins

Since the neutral sulphite steam pretreatment was not able to improve the enzymatic hydrolysis or effectively separate the softwood fibres in a single pretreatment stage, the next set of neutral sulphonation treatments used steam pretreatment in two stages. In this way we hoped to improve fibre separation and sulphonation. Sulphonation has been shown to improve fibre separation during mechanical pulping processes including chemithermomechnaical pulping processes as well as during neutral/alkali sulphite steam explosion pulping for making paper products(Chagaev et al., 2005)(Konn, Pranovich, Fardim, & Holmbom, 2007). It was hypothesized that, extending the sulphonation reaction at low temperatures (160 ℃) would enable a greater amount of sulphonation, while a second steam pretreatment at higher temperature (210-230 ℃) and thus a higher pressure (300+ psig) would aid in fibre separation/size reduction while extending the sulphonation reaction (Table 10). Since this sulphonation approach during steam pretreatment has not been assessed on softwoods, the incorporation of sulfonic acid groups into the substrate as a result of the sulphite treatment was used to screen for effective sulphonation occurring during steam treatment. Visually, it was apparent that, similar to the substrates from the first pretreatment trials, the softwood underwent limited fibre separation, despite the use of two steaming stages despite and the higher pressure in the second pretreatment step. The highest amount of acid groups were incorporated when using 16% sulphite at 160 ℃ in the first stage and temperatures greater than 200 ℃ in the second stage. This incorporated 96-110 µ mol of acid groups per gram of substrate. Hydrolysis yields of only 35% were obtained during the enzymatic hydrolysis of the resulting substrates, which was far lower than that of the SO_2 steam pretreated “control” substrate (Data not shown).

Table 10 The acid groups content and final pH of the two-stage alkali sulphite steam pretreated substrates with a chemical loading of 8% or 16% sulphite after various pretreatment conditions.

Chemical	Condition	Acid group (μmol/g substrates)	pH
8% Na_2SO_3	1) 160 ℃ 10 mins 2) 210 ℃ 10 mins	91	4.0
8% Na_2SO_3	1) 160 ℃ 20 mins 2) 210 ℃ 10 mins	89	3.7
16% Na_2SO_3	1) 160 ℃ 10 mins 2) 210 ℃ 10 mins	96	4.9
16% Na_2SO_3	1) 160 ℃ 20 mins 2) 210 ℃ 10 mins	110	4.6
16% Na_2SO_3	1) 160 ℃ 20 mins 2) 220 ℃ 10 mins	105	4.1
16% Na_2SO_3	1) 160 ℃ 20 mins 2) 230 ℃ 10 mins	101	3.8

3.3.2.2 Increasing the pH of alkali sulphite pretreatment via the addition of sodium carbonate

As described earlier, increasing alkali conditions can enhance sulphonation (Richard P. Chandra et al., 2016). Therefore, alkali was added to the two stage sulphite steam pretreatment to potentially improve the sulphonation during the two steaming stages (conditions summarized in Table 11). As anticipated, the addition of carbonate to the sulphite provided significant improvements in sulphonation producing substrates that contained up to 140 μ mol / g of sulfonic acid groups. Upon analyzing the chemical composition of the substrate, it was also apparent that the neutral/alkaline sulphonation approach allowed for the retention of the

hemicellulose component in the water insoluble substrate (Table 12). However, the increase in sulphonation and retention of carbohydrates did not result in increased enzymatic hydrolysis yields.

Table 11 The acid group content and final pH of two-stage alkali sulphite pretreated substrates after an initial 16% sulphite loading and various carbonate loadings after different pretreatment conditions.

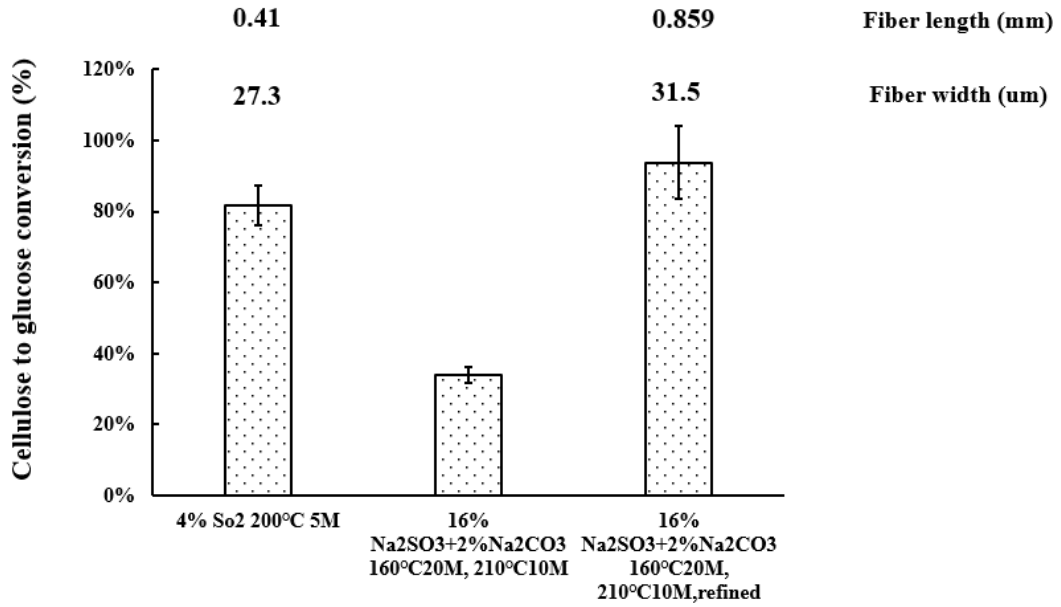
Chemical	Condition	Acid group (μ mol/g substrates)	pH
16% Na_2SO_3 + 2%Na_2CO_3	1) 160 ℃ 20 mins 2) 210 ℃ 10 mins	140	6.4
16% Na_2SO_3 + 2%Na_2CO_3	1) 160 ℃ 20 mins 2) 230 ℃ 10 mins	135	4.4
16% Na_2SO_3 + 2%Na_2CO_3	1) 160 ℃ 20 mins 2) 210 ℃ 10 mins	139	4.9
16% Na_2SO_3 + 4%Na_2CO_3	1) 160 ℃ 20 mins 2) 210 ℃ 10 mins	132	6.8
16% Na_2SO_3 + 4%Na_2CO_3	1) 160 ℃ 20 mins 2) 225 ℃ 10 mins	129	5.2
16% Na_2SO_3 + 6%Na_2CO_3	1) 160 ℃ 20 mins 2) 210 ℃ 10 mins	135	6.6

Table 12 The influence of changes in pH facilitated by carbonate addition on the composition, final pH and acid group content of two-stage alkali sulphite steam pretreated substrates.

Samples	Ara (%)	Gla (%)	Glu (%)	Xyl (%)	Man (%)	ASL (%)	AISL (%)	pH	Acid group (µmol/g pulp)
(1) 4%SO_2 200 °C 5mins	0.1 (0.0)	0 (0.0)	48.1 (0.1)	1.3 (0.1)	3.7 (0.0)	0.8 (0.0)	45.0 (1.3)	1.8	
(2) 16% Na_2SO_3 160°C 20mins, 210 °C 10mins	0.1 (0.0)	0.7 (0.0)	54.3 (0.1)	3.1 (0.0)	9.1 (0.1)	1.0 (0.0)	22.0 (1.1)	4.7	110
(3) 16% Na_2SO_3+2% Na_2CO_3 160°C 20mins, 210 °C 10mins	0.4 (0.0)	1.1 (0.0)	56.1 (0.1)	3.6 (0.0)	9.3 (0.2)	1.0 (0.0)	23.7 (0.5)	6.4	140

It was apparent that the substrates from both the single and two stage alkaline pretreatments underwent limited particle size reduction/fibre separation during steam explosion. This lack of size reduction may have been responsible for the lower enzymatic hydrolysis yields of these substrates. It should be noted, that although the biomass undergoes an intensive chemical treatment, size reduction was also needed to improve the hydrolysis of substrates pretreated using the SPORL process (Wang, Service, & Rockwood, 2009; Zhang, Houtman, & Zhu, 2014; Zhu, Pan, Wang, & Gleisner, 2009). To test this hypothesis, a two stage sulphite pretreated sample, (pretreated with 16% sulphite and 2% carbonate at 160 ℃ for 20 mins followed by 210 ℃ for 10 mins) was size reduced using an industrial strength "juicer" (Chu et al., 2017). The enzymatic hydrolysis of the substrate was assessed using a solids loading of 5%

and an enzyme loading of 10 mg/g using the original substrate (without size reduction) and the 4% SO_2 pretreated substrates as controls. It was apparent that size reduction during the pretreatment was influential in improving the ease of hydrolysis of the substrates as, upon "refining" using the juicer, the sulphonated sample improved the hydrolysis yield from 35 to 91% and also surpassed that of the steam pretreated control (Figure 31). Therefore, it seemed that fibre separation was a key component in increasing cellulose accessibility during alkaline sulphite treatment. It was also apparent that the fibre length (as measured using the FQA) of the alkaline sulphite pretreated steam pretreated softwood was larger than that of the acidic steam pretreated control. This was expected as a similar sulphonation approach is typically utilized to enhance fibre separation during chemithermomechanical pulping (Figure 31). These results suggested that, although the standard acidic steam pretreatment using SO_2 is not aimed at modifying lignin, it is quite effective in providing size reduction during the explosive decompression at the conclusion of the steam pretreatment. Therefore, in the next section, the alkaline and acidic approaches were combined to provide both an increase in sulphonation and better particle size reduction.



Before refining

After refining

Figure 31 The influence of mechanical refining on the enzymatic hydrolysis of washed alkali sulphite steam pretreated lodgepole pine substrate with 5% solid loading at Ctec-3 dosage of 35 mg protein per g cellulose for 48 h. The alkali sulphite steam pretreatment were conducted at 160 °C for 20 mins followed by increasing temperature to 210 °C for another 120 mins. The mechanical refining was performed a lab scale refiner.

3.3.2.3 Sulphite steam pretreatment with Carbonate and SO_2

Typically, during acid sulphite pulping, a base salt is added along with SO_2 in order to achieve sulphonation using bisulphite as the active species and the salt to buffer the system to reduce the amount of lignin condensation when SO_2 is added to the biomass. The chips were initially impregnated with sodium carbonate with subsequent treatment with sulfur dioxide prior to the steam pretreatment. It was hypothesized that maintaining the pH in the acidic 2-4 range, similar to acid sulphite pulping, could help with providing size reduction during the explosive decompression while sulfonating the lignin (Conditions used are shown in Table 13). The size reduction of the substrates from these treatments indicated that the most effective conditions for reducing the particle size of the biomass were the use of a first stage of 200 °C for 10 minutes and a second stage of 225 °C for 5 minutes (Table 13). Analysis of the chemical composition indicated that a higher pretreatment pH resulted in an increase in sulfonic acids on the substrate and a higher retention of hemicellulose. It was also apparent that adding higher amount of sulphonation did not equate to particle size reduction when performing the sulphonation reactions in the steam gun (Table 13, 14). Upon subjecting several of these substrates to enzymatic hydrolyses, the results indicated that even this combination of SO_2 and carbonate was incapable of increasing hydrolysis yield beyond that of the steam pretreated control sample (Figure 32).

Table 13 The influence of chemical loading, pretreatment temperature and pretreatment steps on the effectiveness of substrates size reduction on the sulphite steam pretreatment with the chemicals of Na_2CO_3 and SO_2.

Chemical	Condition	Substrate Size reduction
4% Na_2CO_3 + 4% SO_2	1) 160 ℃ 10 mins 2) 200 ℃ 5 mins	Poor
4% Na_2CO_3 + 4% SO_2	1) 160 ℃ 20 mins 2) 200 ℃ 5 mins	Poor
4% Na_2CO_3 + 4% SO_2	1) 200 ℃ 10 mins 2) 225 ℃ 5 mins	Good
8% Na_2CO_3 + 8% SO_2	1) 210 ℃ 10 mins	Good
8% Na_2CO_3 + 8% SO_2	1) 160 ℃ 20 mins 2) 210 ℃ 10 mins	Medium
8% Na_2CO_3 + 8% SO_2	1) 210 ℃ 10 mins 2) 220 ℃ 10 mins	Good

Note: The morphology of steam pretreated substrates which were referred as Good, Medium and Poor.

Good

Medium

Poor

Table 14 Components of a series of alkali sulphite steam pretreated lodgepole pine substrates including the pH and acid groups of these substrates.

Samples	Ara (%)	Gla (%)	Glu (%)	Xyl (%)	Man (%)	ASL (%)	AISL (%)	pH	Acid group (μmol/g pulp)
(1) 4%SO_2 200 °C 5mins	0.1 (0.0)	0 (0.0)	48.1 (0.1)	1.3 (0.1)	3.7 (0.0)	0.8 (0.0)	45.0 (1.3)	1.8	
(2) 4% Na_2CO_3+4% SO_2 200 °C 10mins, 225 °C 5mins	0.1 (0.0)	0 (0.0)	55.0 (0.1)	1.9 (0.0)	4.0 (0.0)	0.7 (0.0)	35.6 (0.5)	3.1	40
(3) 8% Na_2CO_3+8% SO_2 210 °C 10mins	0.1 (0.0)	0 (0.0)	52.7 (0.6)	2.0 (0.0)	4.8 (0.0)	0.6 (0.0)	34.4 (0.4)	2.7	53
(4) 8% Na_2CO_3+8% SO_2 160 °C 20mins, 210 °C 10mins	0.1 (0.0)	0.3 (0.0)	54.5 (2.4)	2.7 (0.0)	7.4 (0.1)	0.6 (0.0)	23.4 (0.9)	3.8	66
(5) 16% Na_2SO_3 160 °C 20mins, 210 °C 10mins	0.1 (0.0)	0.7 (0.0)	54.3 (0.1)	3.1 (0.0)	9.1 (0.1)	1.0 (0.0)	22.0 (1.1)	4.7	110
(6)16% Na_2SO_3+2% Na_2CO_3, 160°C 20mins, 210 °C 10mins	0.4 (0.0)	1.1 (0.0)	56.1 (0.1)	3.6 (0.0)	9.3 (0.2)	1.0 (0.0)	23.7 (0.5)	6.4	140

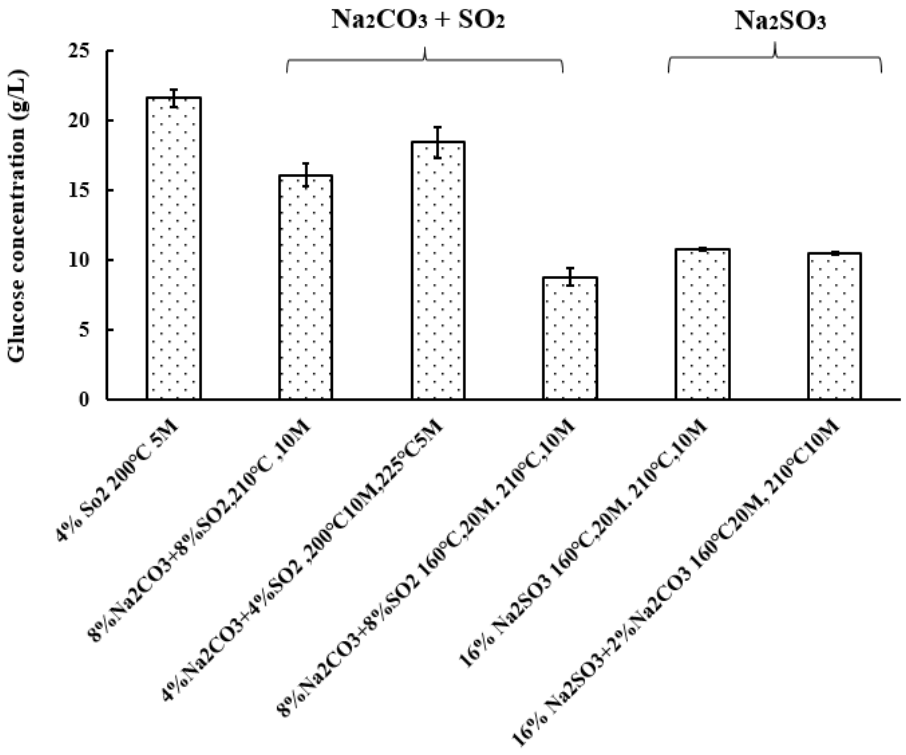


Figure 32 The enzymatic hydrolysis of washed alkali sulphite steam pretreated lodgepole pine with 5% solid loading at a Ctec-3 loading of 35 mg protein per g cellulose for 48 h.

3.3.2.4 Acid sulphite steam pretreatment with bisulphite and sulfuric acid

As mentioned earlier, neutral/alkaline sulphite pretreatment can maximize lignin sulphonation. However, the explosive decompression undergone by the biomass at the conclusion of steam pretreatment was insufficient to separate the fibres in the sulphonated biomass. It was shown that acidic sulphite steam pretreatment was more efficient in providing a combination of particle size reduction and sulphonation in the pH ranges of 3-5. The conditions utilized during the acidic sulphite treatment resembled those utilized during the SPORL process (C. Zhang et al., 2014)(Zhu et al., 2009)(Lan, Gleisner & Hector, 2013). However, rather than combining the SO_2 with carbonate where the amount of SO_2 converted to bisulphite was unclear,

the SPORL process utilized a more controlled approach by directly adding bisulphite and sulfuric acid with subsequent mechanical refining. We therefore adopted this type of approach where bisulphite and sulfuric acid were applied to wood chips, with subsequent steam explosion, to assess whether it would be effective in the steam gun.

The acidic bisulphite pretreatment was applied in two stages. Initially a lower temperature was applied (180 degrees 20 mins or 160 degree 40 mins) which was similar to the first step in SPORL process. After this initial stage, the temperature was elevated to > 200 °C with subsequent explosive decompression. It was hoped that the explosion would help size reduce the biomass similar to the second stage refining step utilized in the SPORL process (Lan et al., 2013; Zhu et al., 2009; Zhou, Zhu, et al., 2013) (Conditions summarized in Figure 33 all using 10% bisulphite and 2% sulfuric acid similar to the SPORL process). It was apparent that utilizing the acidic bisulphite during two stages of steaming was ineffective, as the yields when subjecting the resulting substrates to enzymatic hydrolysis did not surpass 40% (Figure 33). Therefore, the acidic bisulphite treatment was also applied using a single steam pretreatment step (Conditions summarized in Table 15). The results indicated that increasing the steaming temperature to at least 210 °C was necessary to obtain substrates that resulted in significant sugar release during hydrolysis. Visual inspection of the substrates showed that the single stage treatment resulted in a clean fibre separation and relatively long fibres (Table 16, Figure 34).

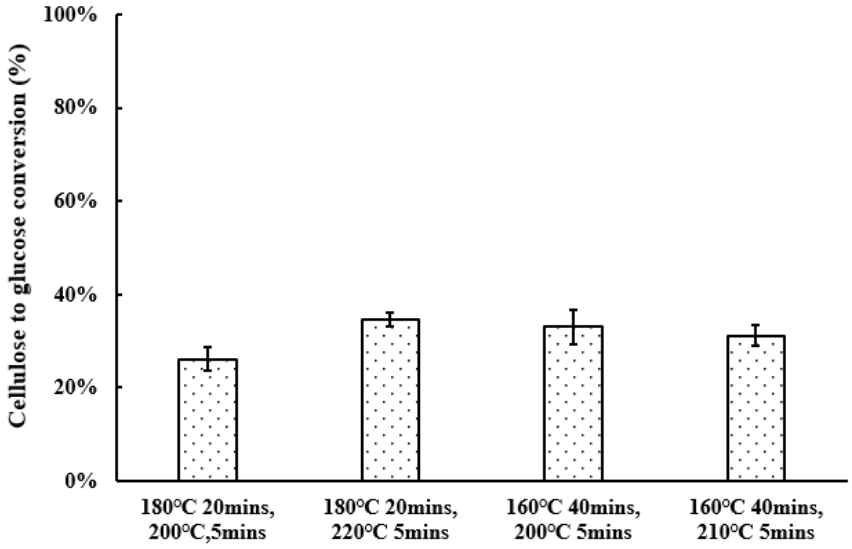


Figure 33 The enzymatic hydrolysis of washed, two stages acid sulphite steam pretreated lodgepole pine with 5% solid loading at Ctec-3 loading of 35 mg protein per g cellulose for 48 h. The two stages of acid sulphite steam pretreatment were conducted with 10% $NaHSO_3$ and 2% H_2SO_4 at various conditions.

Table 15 The hydrolysis performance of a series of single stage acid sulphite steam pretreated lodgepole pine whole slurry at various chemical loading and pretreatment conditions. The hydrolysis was conducted at a 25% solid loading using a Ctec-3 enzyme loading of 40 mg protein/g glucan.

Chemicals	**Pretreatment condition**	**Glucose concentration (g/L) after hydrolysis for 48 hrs**
10% $NaHSO_3$+2% H_2SO_4	165 ℃ 75mins	Not hydrolysable
10% $NaHSO_3$+2% H_2SO_4	180 ℃ 20mins	Not hydrolysable
10% $NaHSO_3$+2% H_2SO_4	180℃ 30mins	Not hydrolysable
10% $NaHSO_3$+2% H_2SO_4	200℃ 10mins	Not hydrolysable
8% $NaHSO_3$+2% H_2SO_4	200℃ 10mins	Not hydrolysable
8% $NaHSO_3$+2% H_2SO_4	210℃ 10mins	103.3g/L
8% $NaHSO_3$+2% H_2SO_4	210℃ 10mins washed	65.4 g/L
4% SO_2	200 ℃ 5 mins	108 g/L

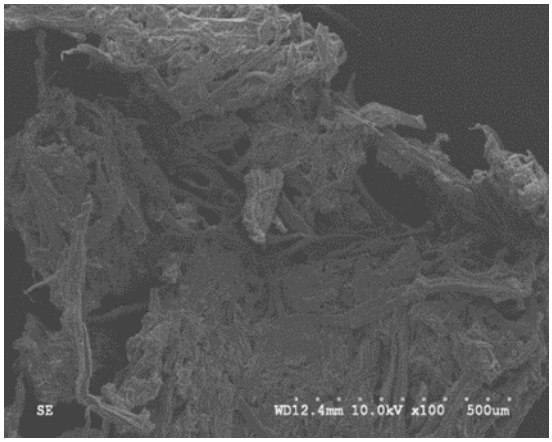
Figure 34 Comparing the gross appearance of the one stage and the two stages acid sulphite steam pretreated lodgepole pine substrates. The one stage acid sulphite steam pretreatment was conducted with 8% $NaHSO_3$ and 2% H_2SO_4 at 210 °C for 10 minutes, while the two stages acid sulphite was conducted with 8% $NaHSO_3$ and 2% H_2SO_4 at 160 °C for 20 mins followed by increasing temperature to 210 °C for another 10 mins.

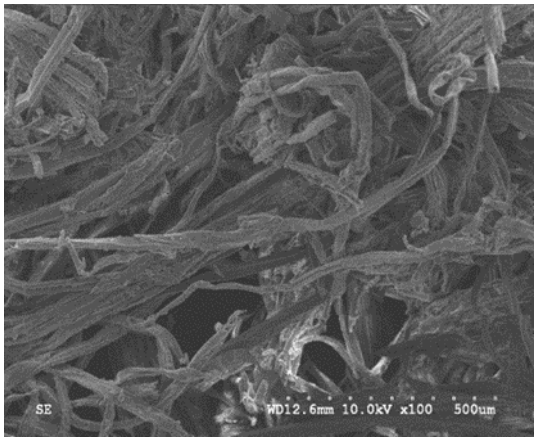
Table 16 Characterizing the acid sulphite steam pretreated substrates and the fermentation inhibitor profiles of 25% solid loading acid sulphite steam pretreated lodgepole whole slurry.

Samples	Acid group (umol/g pulp)	Fiber length (mm)	Fiber width (um)	DP	Acetic acid (g/L)	Furfural (g/L)	HMF (g/L)	Total phenolics (g/L)
4% SO_2, 200 °C 5mins	15	0.41	27.3	197	8.2	3.2	1.6	4.7
8%$NaHSO_3$ +2%H_2SO_4, 210°C 10mins	88	1.3	31.9	1607	0.91	0.27	0.18	7.6

Analysis of the substrate obtained after acidic sulphite treatment indicated that high amounts of acid groups were incorporated when the single step, 8% bisulphite and 2% sulfuric acid approach. This also likely resulted in the longer fibres that were observed. It is probable, that similar to acid sulphite pulping, a greater amount of lignin modification/removal lead to better fibre separation and thus larger fibers. However, these longer fibres may not be as amenable to enzymatic hydrolysis, especially at high solids loadings where longer fibres have been shown to be detrimental to the liquefaction step (van der Zwan et al., 2017). As a result of the higher specificity of the acid bisulphite treatment for lignin, the cellulose degree of polymerization was higher than the control sample which was treated with 4% SO_2 (Table 16). Along with particle size, this change has been shown to play a role in the ease of hydrolysis of pretreated substrates and enzymatic liquefaction at high solids loadings(van der Zwan et al., 2017).

From the SEM images of the substrates it was apparent that, although the acid sulphite steam pretreatment resulted in better fiber separation, the 4% SO_2 steam pretreatment had smaller particles (Figure 35).





4% SO_2 acid steam pretreated substrate

Acid sulphite steam pretreated substrate

Figure 35 FE-SEM images of the one stage acid sulphite steam pretreated and the traditional SO_2 catalyzed steam pretreated lodgepole pine substrates. The one stage sulphite steam pretreatment was conducted using 8% $NaHSO_3$ and 2% H_2SO_4 at 210 °C for 10 minutes.

As well as the variations in substrate characteristics between the acid sulphite steam pretreated softwood and the 4% SO_2 treated control, it was also apparent that the acid bisulphite treatment decreased the amount of fermentation inhibitors in the water soluble fraction (Table 16). In particular, the furans and acetic acid decreased while the phenolics increased likely due to sulphite induced fragmentation of the lignin. With the lower amount of inhibitors, the fermentation of the acid bisulphite steam pretreated lodgepole pine whole slurry resulted in an ethanol concentration of 46.6 g/L after 24 hours. This compared favourably to the SO_2 steam pretreated control whole slurry which was virtually un-fermentable in the presence of the hydrolysis residue as discussed previously in Section 3.1 (Figure 36).

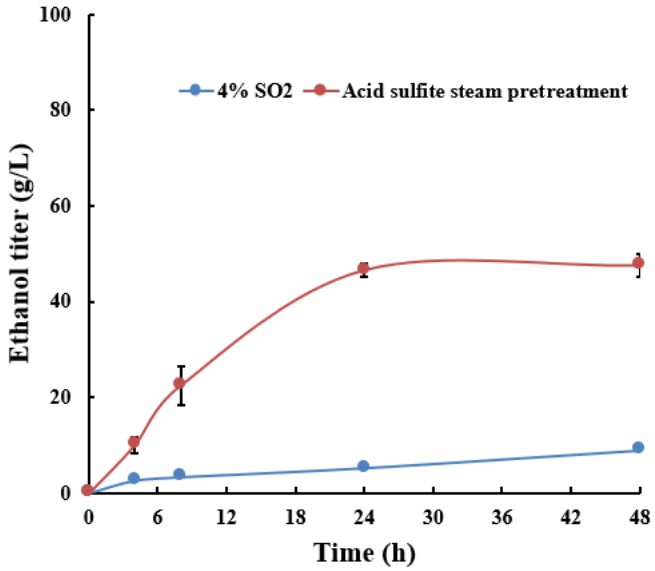


Figure 36 Comparing the ethanol production profile of strain T_2 in the one stage acid sulphite and the traditional SO_2 catalyzed steam pretreated lodgepole pine whole slurry hydrolysate. The one stage sulphite steam pretreatment was conducted using 8% $NaHSO_3$ and 2% H_2SO_4 at 210 °C for 10 minutes. The enzymatic hydrolysis was performed with 25% solid loading at Ctec-3 loading of 40 mg protein per g cellulose for 48 h. The yeast concentrations (cell optical density: OD) were 6.5.

3.3.2.5 Conclusions

Potential approaches to implement sulphite treatment during the steam pretreatment process were assessed, to improve enzymatic hydrolysis of the water insoluble fraction while detoxifying the resulting water soluble fraction. It was hypothesized that the native lignin in the softwood biomass could be readily sulphonated during steam pretreatment as compared to the condensed lignin in the steam pretreated softwood biomass. Initially, based on previous work with poplar biomass and the post treatment of steam pretreated softwood, the sulphonation during steam pretreatment was performed under alkaline conditions. Although the use of alkaline

conditions maximized sulphonation of the biomass, surprisingly, the ease of hydrolysis of the resulting substrates was not improved compared to the conventional SO_2 steam pretreated control. However, the hydrolysis yield of the neutral/alkaline steam pretreated biomass surpassed the SO_2 steam pretreated control after subsequent grinding of the sulphonated biomass. These results indicated that, under neutral/alkaline sulphite treatment, the explosive decompression at the conclusion of steam pretreatment was insufficient to separate fibres as the resulting substrate was rich in "rejects" or "chunks". Therefore, in order to achieve a better fibre separation, size reduction and lignin modification, the subsequent work utilized an acidic sulphite approach. It was apparent that the combination of sulfuric acid and sodium bisulphite, similar to SPORL pretreatment (8% bisulphite and 2% sulfuric acid), was an effective combination in the steam gun when using a single high temperature (>210 °C) pretreatment stage. This acidic sulphite condition was more effective in separating fibres than the neutral/alkaline conditions. It also resulted in lignin sulphonation as well as enabling the hydrolysis and fermentation of the SO_2 steam pretreated softwood whole-slurry (which was shown previously to be virtually un-fermentable in Section 3.1).

3.4 The potential of a two-step (alkali and then acid) sulphite steam pretreatment to boost sugar and ethanol production from sulphite steam pretreated softwood whole slurry

3.4.1 Background

From the work in Section 3.3, it was apparent that neutral/alkaline conditions maximized sulphonation while acidic sulphonation simultaneously sulfonated lignin and aided fibre separation. Although both pretreatment approaches could help fermentation, neither was capable of producing a steam pretreated substrate that could reach hydrolysis yields greater than the washed sulfur dioxide steam pretreated control. As well as maximizing sulphonation, it was evident that the neutral/alkaline approach resulted in the highest amount of hemicellulose retained in the water insoluble substrate fraction. Although the retention of hemicelluloses decreases the amount of furan derived inhibitors, the neutral/alkaline approach was not capable of producing biomass that could undergo effective fibre separation during the explosive decompression step in the steam gun. The use of acidic bisulphite was capable of producing fibres that could be separated, but did not result in as much sulphonation of the lignin as the neutral/alkaline approach. It is also possible that under acidic sulphonation conditions, the lignin component can undergo condensation reactions with decreases its reactivity and is detrimental towards enzymatic hydrolysis.

Based on the beneficial aspects of each pretreatment approach developed in section 3.3, a multi-step approach was followed. In the first step, an alkali sulphite pretreatment was employed to modify/remove lignin. In the second stage, the sulphite pretreatment will be acidified by adding SO_2 to the alkaline sulfonated biomass to decrease the pH, The SO_2 added in the second

pretreatment stage would decrease the pH while providing additional sulfur to the system (Figure 37). It was hypothesized that the increased amount of sulphonation and hemicellulose retention in the first pretreatment stage, combined with the improved fibre separation/size reduction provided by the additional acidic sulphonation and acidification in the second stage would improve the ease of hydrolysis, fibre separation, sugar recovery and fermentability of the resulting substrates. As well as maximizing sulphonation and fibre separation, it was hypothesized that the initial sulphite added in the first stage will likely be washed out prior to the second pretreatment stage, which would limit the toxicity of the residual sulfite towards downstream fermentation after the second acidic sulphonation step was applied.

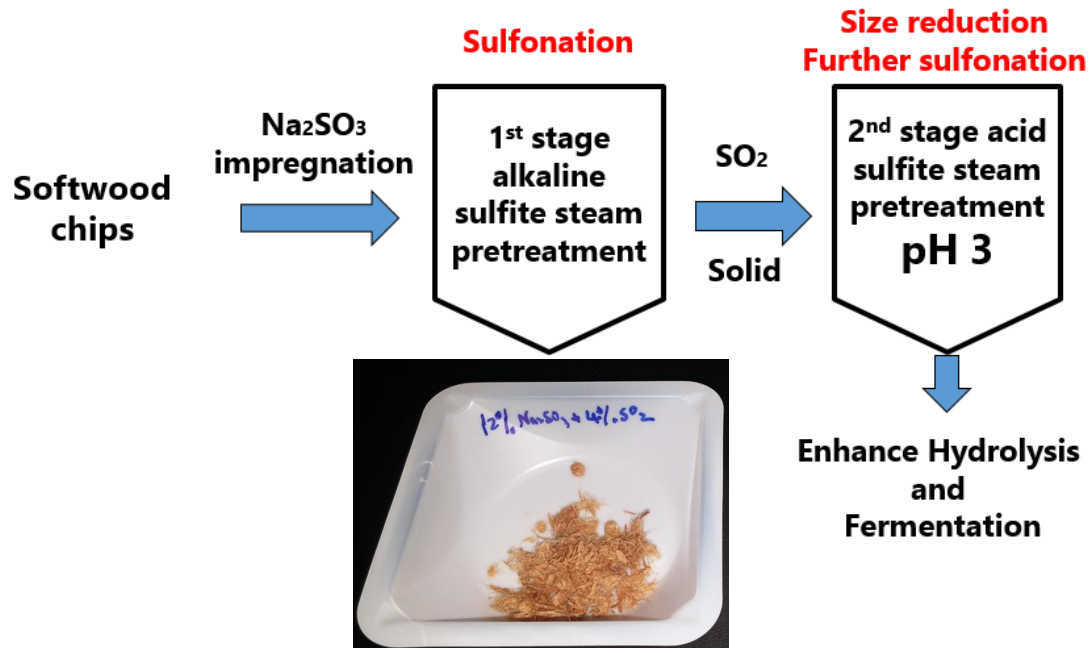


Figure 37 Flow chart of the two stages sulfite steam pretreatment. The first stage is conducted at the alkali condition with Na_2SO_3 pre-impregnation for the purpose of lignin sulfonation, while the second stage is executed at acidic condition (pH=3.0) with SO_2 addition for the purposes of size reduction and further lignin sulfonation.

3.4.2 Results and discussion

3.4.2.1 Applying a two-step (alkaline-acid) sulphite steam pretreatment consisting of an initial sulphite steam pretreatment stage and subsequent SO_2 catalyzed steam pretreatment

In order to implement the two-step (alkaline-acid) steam pretreatment, softwood chips were first impregnated with a solution of 12% sulphite based on the weight of biomass dissolved in 100 ml water. To improve the impregnation of the sulphite, the wood chips were allowed to soak in the sulphite solution overnight in a water bath at 70 °C prior to steam pretreatment. The initial sulphite treatment stage was performed at mild conditions (140 °C, 20 minutes) in order to avoid dissolution or degradation of the biomass carbohydrates. Visually, after the first steam prereatment stage, the wood chip structure remained intact despite the explosive decompression in the steam gun, indicating the mild nature of the pretreatment. The resulting treated wood chips were then impregnated with 4% SO_2 (based on the initial weight of the chips) overnight with subsequent steam pretreatment in the steam gun at a temperature of 200 °C and residence time of 5 minutes. The final pH of the water soluble stream was 3.3 after the second pretreatment stage, indicating the SO_2 impregnation reduced the pH of the treatment. The two stage steam pretreatment seemed to allow for effective homogeneous fibre separation as only a small amount of “rejects” were produced (Figure 37). Analysis of the chemical composition of the water insoluble and soluble streams (Tables 17 and 18) indicated that, compared to the 4% SO_2 steam pretreated substrate, the two stage (alkaline-acid) pretreated substrates retained and recovered far more hemicellulose (Table 17). As well as retaining hemicellulose, the two stage steam pretreatment resulted in the incorporation of acid groups and >60% delignification (Table 17). It was apparent that the two-stage approach had the desired effect as it created unique substrates

that exhibited delignification, homogenous fibre separation and hemicellulose recovery/retention. Likely due to the carbohydrate rich nature of the two stage steam pretreated substrates, the hydrolysis of the two stage treated substrates at an enzyme loading of 40 mg/g cellulose and solids loading of 25%, could generate 163.5 g/L glucose compared to the 121 g/L glucose obtained during the hydrolysis of the 4% SO_2 steam pretreated control after 72 hours (Figure 38).

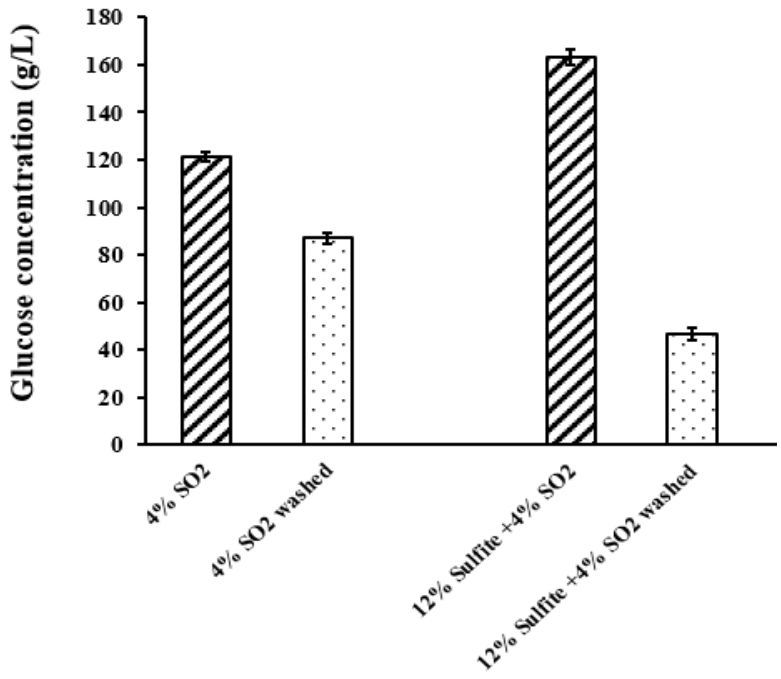


Figure 38 The enzymatic hydrolysis of 25% solid loading washed and unwashed two stages sulfite steam pretreated lodgepole pine whole slurry at Ctec-3 dosage of 40 mg protein per g cellulose for 48 h. The two stages sulfite steam pretreatment was firstly conducted at the alkali condition with sulfite pre-impregnation (6-12% Na_2SO_3 loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of various amount of SO_2 at 200 °C for 5 mins.

Table 17 Components of a series of two steps of (alkali followed by acid) sulfite steam pretreated lodgepole pine substrates and the pH and acid groups of these substrates.

Substrates	Ara (%)	Gla (%)	Glu (%)	Xyl (%)	Man (%)	ASL (%)	AISL (%)	pH	Acid group (μmol/g pulp)
(1) 4%SO_2 200°C	0	0	47.8	0.1	0.3	0.6	49	1.6	
(2) 12% Na_2SO_3 140 °C 20 min, 4% SO_2 200 °C 5mins	0	0.2	67.2	2.6	13.9	0.6	16.4	3.3	113
(3) 8% Na_2SO_3 140 °C 20 min，2% SO_2 200 °C 5mins	0	0	68	3	8.6	0.8	18	3.4	

Table 18 The monomers and oligomers present in the liquid fraction of two steps (alkali followed by acid) sulfite steam pretreated lodgepole pine substrates 25% solid loading whole slurry.

Substrates	Glu Mono (g/L)	Glu Olig (g/L)	Xyl Mono (g/L)	Xyl Olig (g/L)	Man Mono (g/L)	Man Olig (g/L)	Glucose recovery	Mannose recovery	delignification
4% SO_2	28.2	7.1	11.2	2.2	17.7	8.9	98%	75%	0.1%
12% Na_2SO_3+4%SO_2	-0.05	2.3	2.3	2.2	1.9	4.4	104%	105%	61%

The two-stage pretreatment appeared to provide benefits, especially for hemicellulose recovery and the sugar concentration obtainable during enzymatic hydrolysis. However, when analyzing the hydrolysis yields for each of the substrates after a washing step, it was evident that the two-stage sulfonated substrates could not reach the hydrolysis yields of the 4% SO_2 steam pretreated control substrates at a 25% solid loading. This result is similar to the observations made in section 3.1, when comparing the hydrolysis of the SO_2 steam pretreated substrate to the carbohydrate rich dissolving pulp, where a higher sugar concentration was obtained when hydrolyzing the dissolving pulp at a lower overall cellulose conversion yield. The potential end-product inhibition at the high sugar concentrations obtained at a 25% solids loading was thought to be the main factor limiting the enzymatic hydrolysis of the carbohydrate rich dissolving pulp substrate. As well as end-product inhibition, when hydrolyzing substrates at a high solids loading, numerous studies have shown that the ability to rapidly liquefy the substrate slurry is the key factor influencing the hydrolysis yield (Hoyer, Galbe, & Zacchi, 2013; Jung, Kim, & Kim et

al., 2013; Modenbach & Nokes, 2013). It was apparent that the two-stage sulphite treated substrates required 48 hours to liquefy compared to 24 hours in the case of the 4% SO_2 steam pretreated control (Table 19). Previous work has also shown that the length of the fibres can influence the ease of liquefaction of pretreated substrates, as longer fibres have been shown to require a longer time for liquefaction (van der Zwan et al., 2017). Therefore, the better fibre separation imparted by the sulphonation which resulted in longer long fiber length of the newly developed substrates could also be responsible for the longer time that was required for the two-stage pretreated sulphite samples to be liquefied (Table 19).

As well as the cleaner fibre separation which created longer fibres, the higher retention of mannan in the water insoluble substrate also poses challenges for enzymatic hydrolysis. It is well known that the fortification of cellulase cocktails with xylanases can result in significant increases in enzymatic hydrolysis yields (Hu, Chandra, et al., 2015; Hu, Gourlay, et al., 2015). Since the main substrates currently being investigated by the initial commercial cellulosic ethanol companies are agricultural and hardwood biomass, xylanases have been researched extensively and are available as part of the existing cellulase cocktail or can be readily obtained (Mohanram, Amat, & Nain et al., 2013). However, since softwoods have been investigated less frequently, mannanases likely do not currently constitute a significant portion of the conventional commercial cellulase preparations. Previous work has shown that the separation of fibres under acidic conditions such as the use of acidic organosolv vs. alkaline kraft pulping can decrease fibre length and solubilize hemicellulose (Del Rio, Chandra, & Saddler, 2010). Therefore, the next set of pretreatments investigated the effects of increasing the acidification of the second pretreatment step to potentially decrease the fiber length and dissolve the hemicellulose.

Table 19 Liquefaction time of 25% solid loading two stages sulphite steam pretreated substrates with 12% Na_2SO_3 and 4% SO_2. The final pH and the fiber length of the substrates were also shown. The 4% SO_2 steam pretreated substrate was selected as the control.

Substrates	Liquefaction time (day)	Fiber length (mm)	Fiber width (μm)	pH
4% SO_2	1	0.4	27.3	1.6
12% Na_2SO_3 + 4% SO_2	2	1.5	30.4	3.3

Two treatments were applied employing sulphite loadings of 8 and 10% during an initial steaming stage with second steaming stages using loadings of 5.5 and 6% SO_2 respectively. The pH of the pretreatment was decreased to 2.5 in the case of the treatment using 8% sodium sulphite and 5.5% SO_2, which resulted in the dissolution of a greater amount of hemicellulose into the water soluble fraction (Table 20, 21). Consequently, the water insoluble fraction was composed of only 7% mannan which was far lower than the initial experiments where the substrate was composed of up to 14% mannan (Table 18).

Table 20 Components of a series of two steps of (alkali followed by acid) sulfite steam pretreated lodgepole pine substrates and the pH and acid groups of these substrates.

Samples	Ara (%)	Gla (%)	Glu (%)	Xyl (%)	Man (%)	ASL (%)	AISL (%)	pH	Acid group (μmol/g pulp)
(1) 10% Na_2SO_3 140°C 20min, 6% SO_2 200°C 5mins	0	0.3	70.4	3.3	10.0	0.7	14.2	3.2	146
(2) 8% Na_2SO_3 140°C 20min, 5%SO_2 200°C 5mins	0	0.2	71.6	2.8	7.3	1.0	17.2	2.6	99
(3) 6%Na_2CO_3+6%SO_2 140°C 20mins, 4%SO_2 200°C,5mins	0	0.2	69.5	2.7	6.4	0.6	19.4	2.7	115

Table 21 Monomers and oligomers of a series liquid fraction of two steps (alkali followed by acid) sulphite steam pretreated lodgepole pine substrates 25% solid loading whole slurry.

Substrates	Glu Mono	Glu Olig	Xyl Mono	Xyl Olig	Man Mono	Man Olig	Glucose recovery	Mannose recovery	delignification
(1)10%Na_2SO_3+6 %SO_2	0.07	6.0	4.2	5.1	4.1	11.8	100%	108%	66%
(2)8%Na_2SO_3+5.5 %SO_2	0.1	5.0	4.0	5.4	3.3	10.9			
(3)6%Na_2CO_3+6% SO_2+4%SO_2	0. 2	4.0	2.9	4.7	2.4	7.9			

The total measured sugar recovery of these substrates was close to 100% which indicated that lowering the pH of the treatment did not sacrifice the recovery of the hemicelluloses. The treatment using 10% sulphite and 6% SO_2 in two stages also resulted in a delignification of 66% which is similar to the samples treated with 12% sulphite and 4% SO_2 (Table 18). The hydrolysis of the two-stage substrates at a solid loading of 25% resulted in glucose concentrations of > 150 g/L glucose using the whole slurry which was also similar to the substrates in the previous section (Figure 39).

All of the two-stage sulphite steam pretreated substrates from the current and previous section were washed and subjected to enzymatic hydrolysis at a solids loading of 5% and compared to the hydrolysis of the SO_2 steam pretreated control. The results showed that the

hydrolysis yields obtained with all of the two-stage pretreated substrates was lower than that of the SO_2 steam pretreated control sample (Figure 40). It should be noted that, even when reducing the solids loading to as low as 5%, the two-stage sulphite treated samples had only a limited capability to be agitated. The two-stage sulphite steam pretreated softwood absorbed the buffer and was thus difficult to agitate during enzymatic hydrolysis at 5% solids, while the SO_2 steam pretreated substrate began the hydrolysis at 5% solids as a liquefied slurry and thus essentially had a "head-start" for the enzymatic hydrolysis.

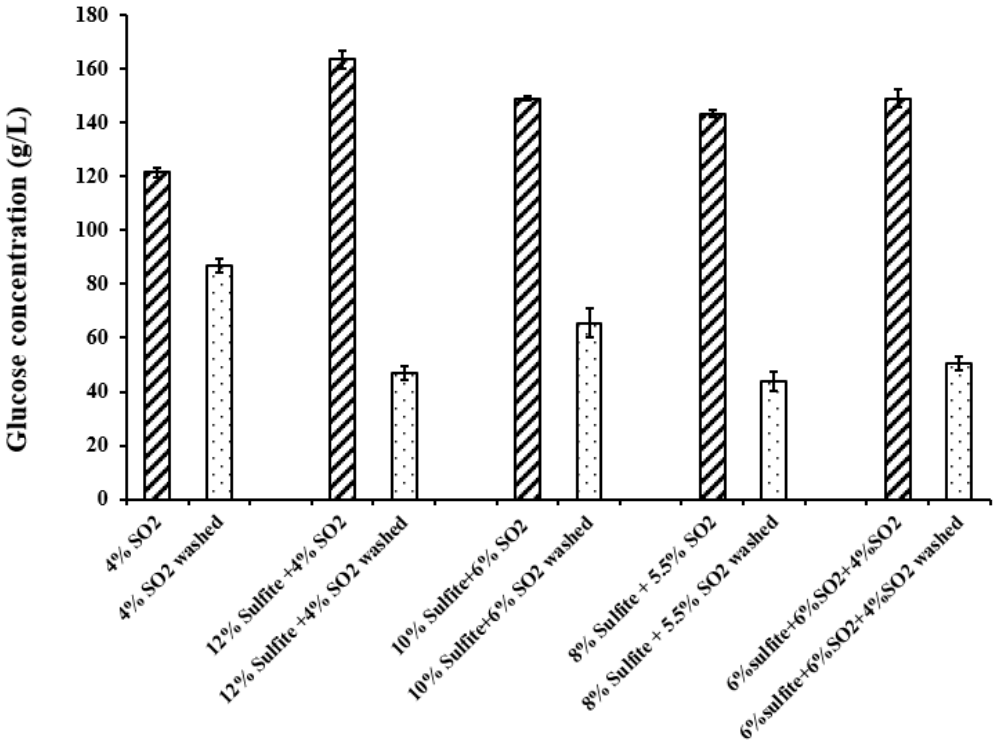


Figure 39 Hydrolysis efficiency of a series of two steps (alkali followed by acid) sulphite steam pretreated whole slurry and washed substrates at 25% solid loading. The enzyme loading in hydrolysis was 40 mg Ctec-3 enzymes/g glucan.

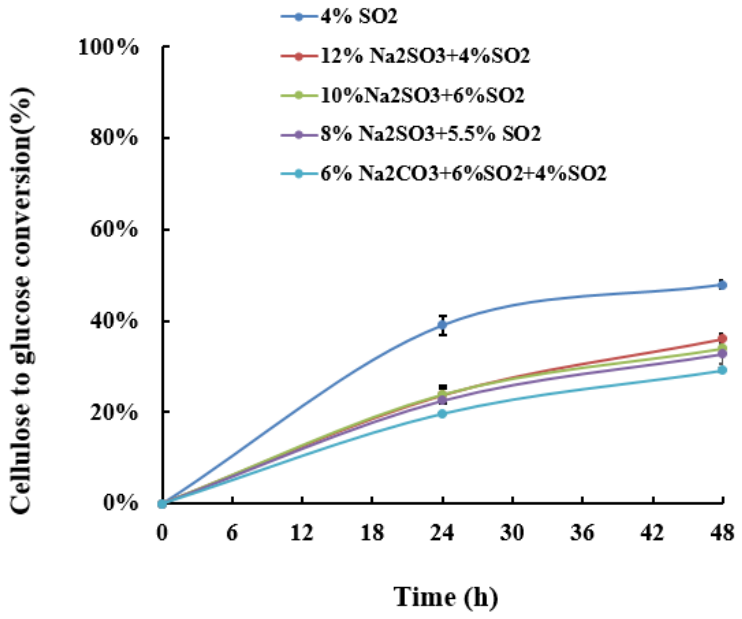


Figure 40 The enzymatic hydrolysis of washed two stages sulfite steam pretreated lodgepole pine with 5% solid loading at Ctec3 dosage of 15 mg protein per g cellulose for 48 h. The two stages sulfite steam pretreatment was first conducted under alkali condition with sulfite pre-impregnation (6-12% Na_2SO_3 loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of various amount of SO_2 at 200 °C for 5 mins.

Although the two-stage sulphite pretreated samples were challenging to agitate, the water soluble fractions resulting from these substrates were virtually devoid of acetic acid furfural or HMF (Table 22). In the case of acetic acid, the initial alkaline sulphite pretreatment stage likely removed the acetyl groups from the softwood hemicellulose, as softwood hemicellulose are less acetylated compared hardwoods. The furfural and HMF are products of the acidic dehydration of the biomass carbohydrates, thus the low concentration of these inhibitors was likely a consequence of the virtual complete recovery of the cellulose and hemicellulose. Therefore, likely due to the low levels of inhibitors compared to the control, the two-stage pretreated substrates treated with either, 12% Na_2SO_3 and 4% SO_2 or 10% Na_2SO_3 and

6% SO_2 were readily fermented to obtain an ethanol concentration of 80 g/L using the whole slurry (Figure 41).

Table 22 Inhibitor profiles of a series of two steps of (alkali followed by acid) sulfite steam pretreated lodgepole pine 25% solid loading whole slurry.

Substrates	**Acetic acid (g/L)**	**Furfural (g/L)**	**HMF (g/L)**	**Total phenolics (g/L)**
4% SO_2	8.2	3.2	1.6	4.7
12% Na_2SO_3+4% SO_2	0.91	0.27	0.19	7.6
10% Na_2SO_3+6% SO_2	1.3	0.34	0.17	10.6
8%Na_2SO_3 + 5.5% SO_2	1.5	0.48	0.23	8.8
8% Na_2SO_3 +2% SO_2	1.2	0.2	0.19	7.5
6%Na_2CO_3+6%SO_2+4%SO_2	1.5	0.42	0.35	7.2

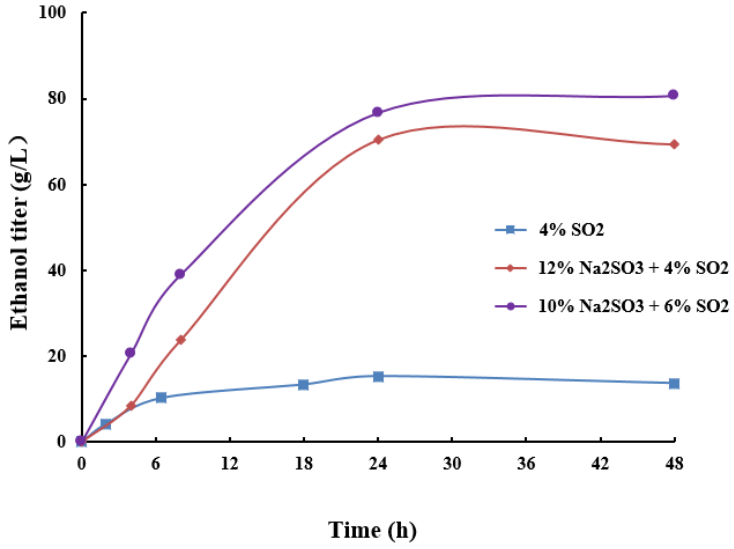


Figure 41 The fermentation performance of 25% solid loadings whole slurry hydrolysates that were treated by two steps sulphite steam pretreated substrates with Na_2SO_3 in the first step and SO_2 in the second step. Initial cell density in fermentation was OD 6.5. Control is 4% SO_2 acid steam pretreated 25% solid loading whole slurry.

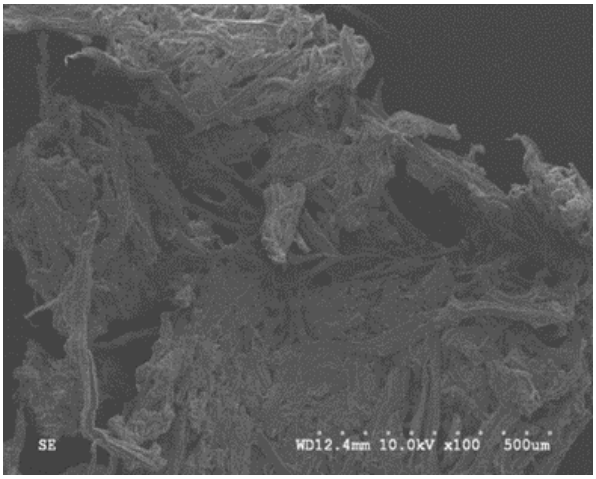
3.4.2.2 Elucidating substrate characteristics to develop strategies to improve enzymatic hydrolysis yields of two-stage sulphite pretreated softwood biomass

The substrate characteristics of the two-stage sulphite pretreated substrates were analyzed in order to obtain a greater understanding of the attributes that would require alteration in order to improve the enzymatic hydrolysis of these substrates. The lignin removal and fibre length data showed that the reactions of sulphite and lignin enabled better fibre separation and thus longer fibres, especially at the conditions that were more alkaline (Table 19, Table 23). The SEM images also showed that the substrate fibres appeared to be quite long compared to the 4% SO_2 steam pretreated substrate (Figure 42). As mentioned earlier, several studies have shown that

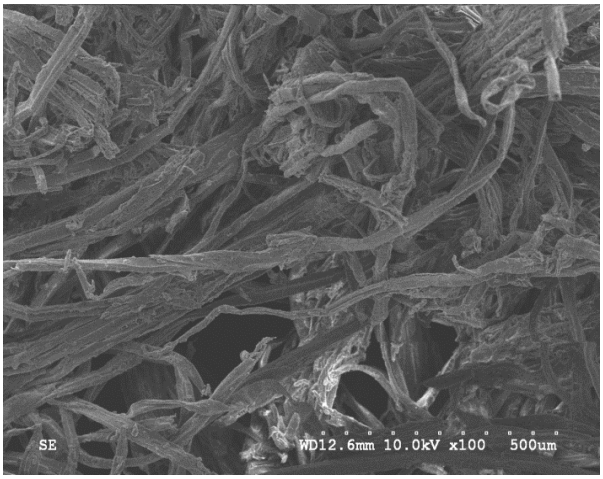
longer fibres are more difficult to liquefy due to their increased likelihood to entangle and limit mixing (Lavenson et al., 2012). As well as fibre length, the degree of polymerization of cellulose (DP) has been shown to play a significant role in influencing the ease of hydrolysis and liquefaction of biomass. The DP values of the substrates were markedly different as the DP of two-stage sulphite pretreated substrates was 9-fold higher than that of the 4% SO_2 steam pretreated substrate (Table 23). The characterization of the substrates also showed the two-stage pretreated substrates had a higher accessibility during the water retention value and Simons staining measurements.

Table 23 Characterizing the two steps (alkali followed by acid) of sulphite steam pretreated

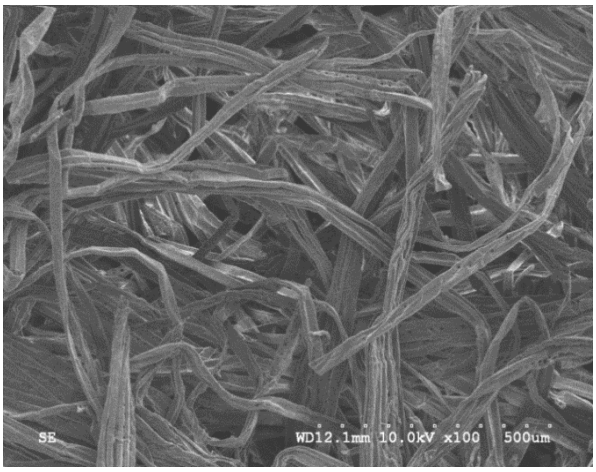
Samples	Fiber length (mm)	Fiber width (um)	Simon stain	DP	Water retention value
4% SO_2	0.41	27.3	80.9	197	150%
12%Na_2SO_3 + 4% SO_2	1.55	30.4	111.2	1757	170.19%
10% Na_2SO_3 + 6% SO_2	1.53	30.8	113.2	1694	173.64%
8% Na_2SO_3 +5.5% SO_2	1.50	31.2	109	1655	184.51%
6%Na_2CO_3 +6%SO_2+4%SO_2	1.60	32.5	109.2	1664	167.93%



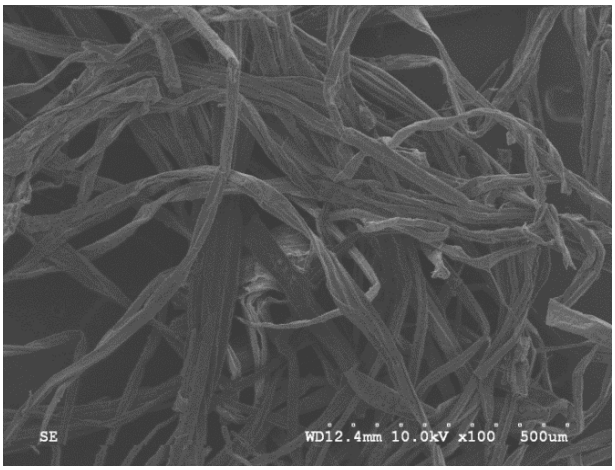
4% SO_2 acid steam pretreatment



Aicd sulphite steam pretreatment



12% Na_2SO_3 + 4% SO_2



6% Na_2CO_3 + 6% SO_2 + 4% SO_2

Figure 42 The FE-SEM imagines of the traditional SO_2 catalyzed and the two stage sulfite steam pretreated lodgepole pine substrates. The two stage sulfite steam pretreatment was firstly conducted at the alkali condition with sulfite pre-impregnation (12% Na_2SO_3 loading, 6% Na_2CO_3 with 6% SO_2 loading, 8% Na_2SO_3 loading, 8% $NaHSO_3$ loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of 4% SO_2 or 3% H2SO4 or 2% H_2SO_4 at 200 °C for 5 mins.

Another limitation of enzymatic hydrolysis at high solids is the inability to mix the substrate slurry since the hygroscopic nature of lignocellulosic biomass consumes water in the slurry and decreases the mobility of the substrate fibres. This phenomenon has been referred to as the "solids effect" in research investigating methods to overcome difficulties encountered during the hydrolysis of lignocellulose at high solids loadings (Kristensen et al., 2009). Jorgensen et al. reported that a horizontal mixer can be used to improve the mix-ability of high solids slurries for enzymatic hydrolysis (Jørgensen, Vibe-Pedersen, & Felby, 2007). Therefore, in an effort to improve the mixing of the two-stage sulphite pretreated biomass, the enzymatic hydrolysis of the whole slurries was performed in a horizontal mixer at a 25% solids loading and an enzyme loading of 40 mg protein/g cellulose. The hydrolysis in the horizontal mixer rotating at 10 rpm was compared to the hydrolysis yields obtained in the lab shaker at 150 rpm. Under these conditions it was apparent that, despite the difference in mixing speed, the horizontal mixer obtained similar hydrolysis yields to the shaking incubator (Figure 43). When applying the two mixers to the washed substrates, the horizontal mixer was unable to improve the hydrolysis of two stage pretreated substrates, thus the yields of the washed two-stage pretreated substrates were still lower than the washed SO_2 steam pretreated control.

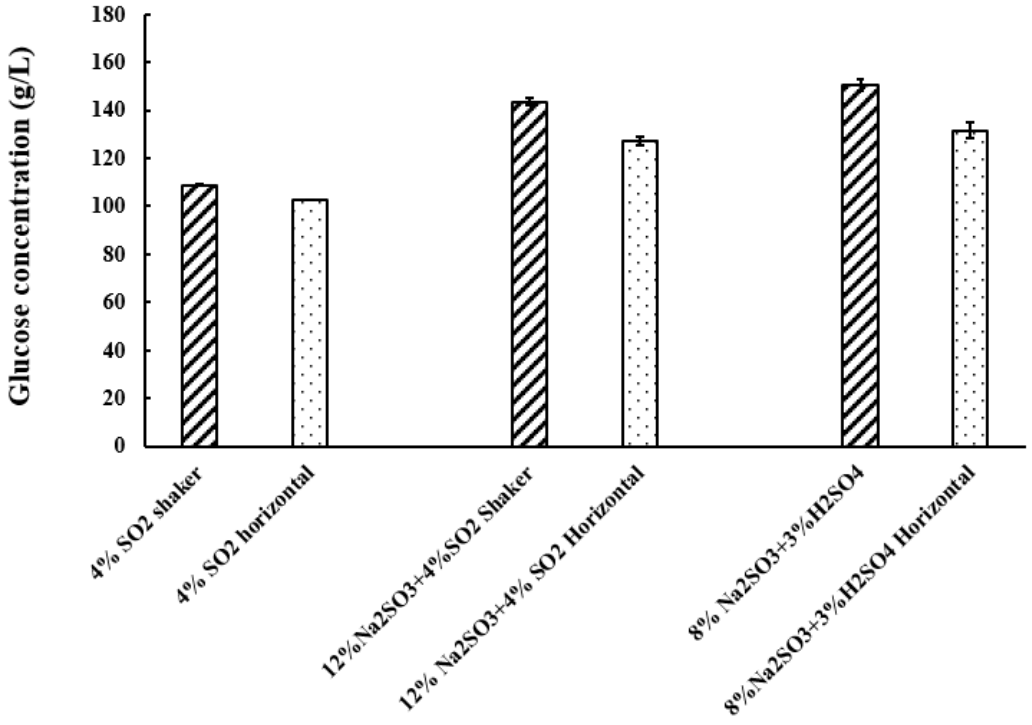


Figure 43 The effect of horizontal mixing on the enzymatic hydrolysis of 25% solid loading washed and unwashed two stages sulfite steam pretreated lodgepole pine whole slurry at Ctec-3 dosage of 40 mg protein per g cellulose for 48 h. The two stages of sulfite steam pretreatment was firstly conducted at the alkali condition with sulfite pre-impregnation (12% Na_2SO_3 loading), followed by a second stage acidic condition treatment with the addition of 4% SO_2.

As discussed above, one of the possible reasons for the poor hydrolysis of the two-stage sulphite pretreated substrates was their high retention of mannan, as current cellulase preparations likely do not contain an abundance of mannanases. It should be noted that the hydrolysis reactions performed thus far using the Ctec-3 enzyme did not result in the hydrolysis of the mannan component of the substrates when an enzyme loading was 40 mg protein/g glucan was applied. Therefore, accessory enzymes were supplemented at a loading of 10 mg protein/g cellulose. The mannanase that was utilized in this work has been shown previously to have

difficulty in hydrolyzing mannan associated with lignin which is likely to be the case for the substrates being tested in this work (Alvarez-Vasco & Zhang, 2017; Du et al., 2014). As anticipated, the addition of the mannanase had a limited effect on the overall sugar concentration in the hydrolyzates (Figure 44).

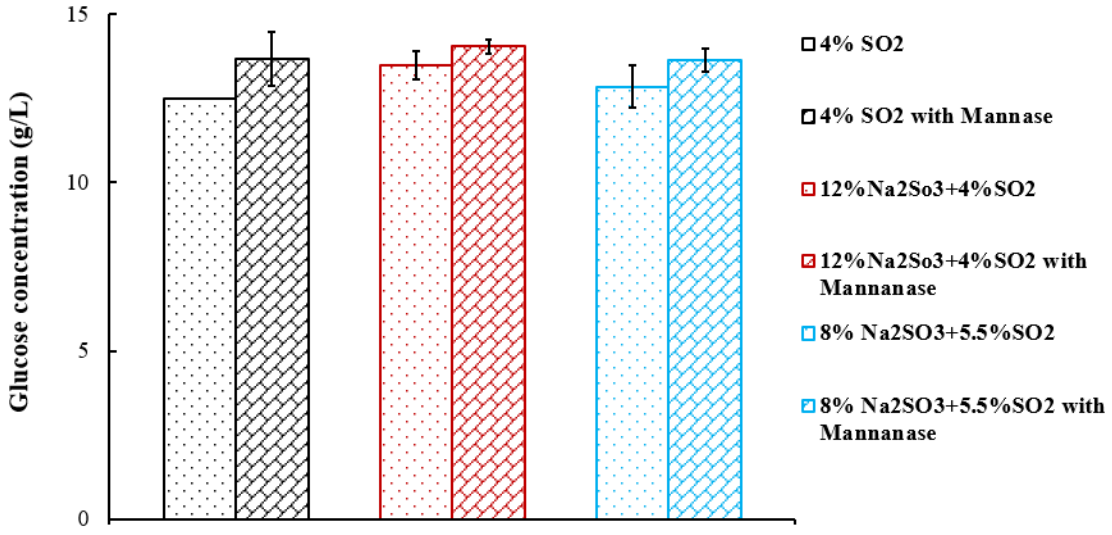


Figure 44 The effect of mannanase supplementation (10 mg/g cellulose) on the enzymatic hydrolysis of washed two stage sulfite steam pretreated lodgepole pine with 5% solid loading at Ctec-3 loading of 15 mg protein per g cellulose for 48 h. The two stages sulfite steam pretreatment was firstly conducted at the alkali condition with sulfite pre-impregnation (8% and 12% Na_2SO_3 loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of 5.5% or 4% SO_2 at 200 °C for 5 mins.

Additional experiments with accessory enzymes were performed on the washed substrates at 5% solids and an enzyme loading of 15 mg CTec-3/g cellulose or 15 mg CTec-3/g cellulose with an additional 10 mg of the specified accessory enzyme/g cellulose. As mentioned above, a high degree of polymerization of cellulose has been shown to inhibit liquefaction (van

der Zwan et al., 2017). Therefore, it is possible that the high DP of the two-stage sulphite steam pretreated substrates may have compromised the ease of hydrolysis of the substrates tested. Endoglucanases are known to cleave cellulose at amorphous regions, resulting in an overall decrease in the cellulose DP. However, similar to the results with the mannanase enzymes, supplementing CTec-3 with additional endoglucanases did not enhance the overall cellulose hydrolysis yields (Figure 45). Previous work has also shown that the addition of xylanases can aid in the hydrolysis of mannan. The supplementation of CTec-3 with two different loadings of xylanases showed that the supplementation of xylanases at a protein loading of 10 mg/g cellulose had a limited effect on the enzymatic hydrolysis yields similar to the other enzymes added (Figure 45). Increasing the protein loading of the HTec xylanase to 30 mg/g resulted in an increase in the cellulose hydrolysis yield of 16% but had no effect on hydrolysis of mannan. Therefore, the only case where the xylanase aided hydrolysis was when the xylanase loading (30 mg/g cellulose) was higher than that of the cellulase (15 mg/g cellulose). Since the substrates contained less than 3.5% xylan, it is possible that the xylanases may have acted as a "blocking agent" by binding to lignin to decrease non-productive binding of cellulases to the lignin in the substrate as shown in previous work adding bovine serum albumin as a "lignin blocker" during the enzymatic hydrolysis of lignocellulosic substrates (B. Yang & Wyman, 2006; Qing & Wyman, 2011).

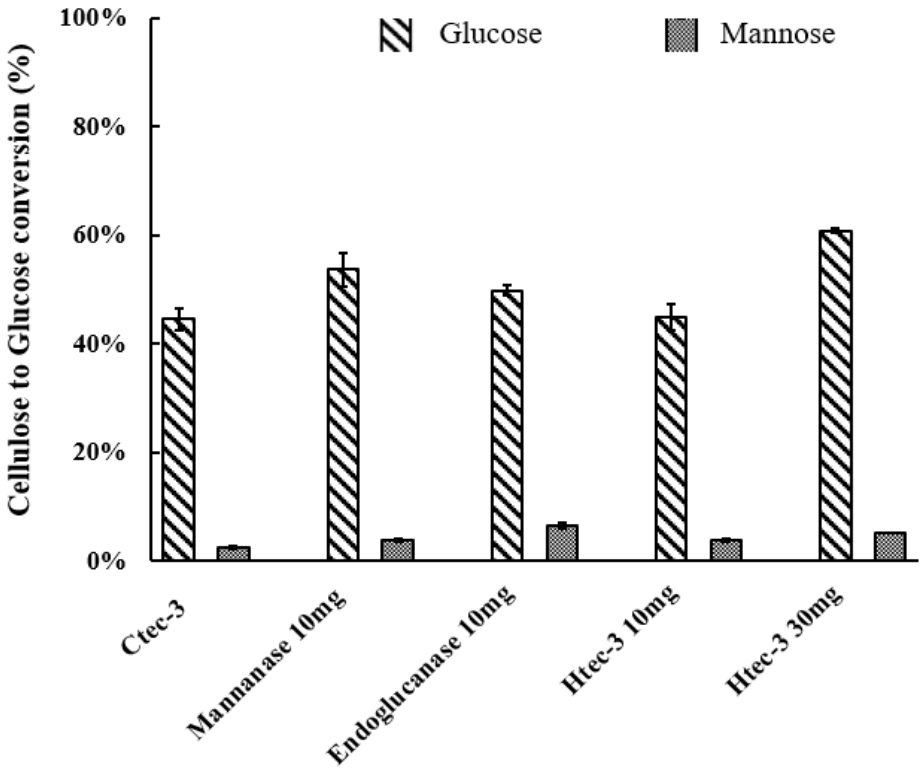


Figure 45 The effect of mannanase, endoglucanase and xylanase (Htec-3) supplementation (10 mg/g or 30 mg/g cellulose) on the enzymatic hydrolysis of washed two stages sulfite steam pretreated lodgepole pine with 5% solid loading at Ctec-3 dosage of 15 mg protein per g cellulose for 48 h. The two stages sulfite steam pretreatment was first conducted at the alkali condition with sulfite pre-impregnation (12% Na_2SO_3 loading), followed by a second stage acidic condition treatment with the addition of 4% SO_2.

3.4.2.3 Using sulfuring acid during the second steam pretreatment stage to reduce the pretreatment to a pH less than 2.5

The newly developed substrates generated from the two-stage sulphite pretreatments discussed thus far have resulted in sugar concentrations of greater than 160 g/L that were readily fermentable to obtain ethanol concentrations of 80 g/L ethanol. However, it was apparent that

the longer fibers, high hemicellulose content and DP of these substrates was quite high which might have played a role in limiting the enzymatic liquefaction of the substrate at high solid loadings. The enzyme cocktails utilized in this work were unable to hydrolyze the hemicellulose in the mannan rich water insoluble substrates which decreases the potential amount of fermentable sugars available from the two-stage sulphite pretreated substrates. Decreasing the pH during pretreatments has been shown to decrease the fiber length and the degree of polymerization of cellulose in the resulting substrate while simultaneously dissolving hemicellulose (Pan, Xie, & Saddler, 2007). Therefore, H_2SO_4 was used as a stronger acid to substitute for the SO_2 that was added during the second steam pretreatment stage with the hope of decreasing the pH to reduce fiber length and DP as well as increasing the solubilization of hemicellulose (Figure 46).

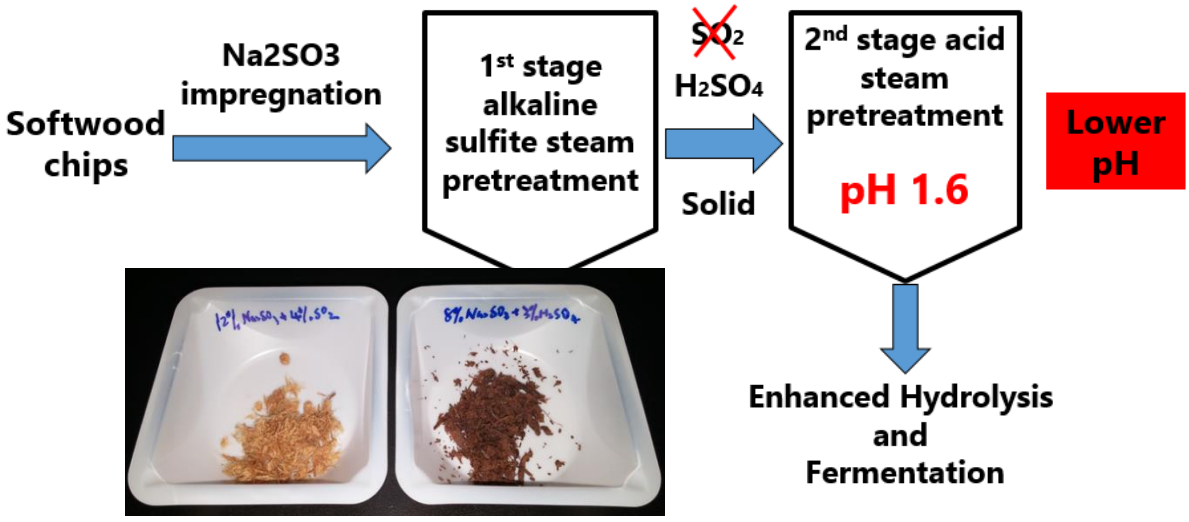


Figure 46 Flow chart of the two stages sulfite steam pretreatment. The first stage is conducted at the alkali condition with Na_2SO_3 pre-impregnation for the purpose of lignin sulfonation, while the second stage is executed at acidic condition (pH=1.6) with H_2SO_4 addition for the purposes of size reduction and further lignin sulfonation.

Similar to the previous sections, the initial sulphite treatment was performed using 8% Na_2SO_3, while 3% H_2SO_4 was applied to the treated chips in the second steam treatment stage which decreased the final pH of the substrates from 3 to 1.6. It should be noted that the use of H_2SO_4 reduced the final pH to the same value observed with the control substrates that used 4% SO_2. Visually, it was apparent that the decrease in pretreatment pH darkened the substrates compared to the light colored substrates described in section 3.4.2.1(two stage sulphite pretreatment). The compositional analysis showed that the lower pH increased the lignin content of the water insoluble substrates from 16 to 26% which corresponded with a decrease in mannan content from approximately 14 to 3% (Table 24). The decrease in mannan content of the water insoluble substrate was likely due to increased hemicellulose solubilization at the lower pretreatment pH, as the water soluble fractions from the substrates exhibited substantial increases in soluble glucose and mannose (Table 25). The lower pH also decreased the overall recovery of mannose to 87%, but the mannose recovery can still be regarded as high as compared to previous work on the SO_2 catalyzed steam pretreatment of lodgepole pine where close to 70% of the hemicellulose was recovered (Ewanick et al., 2007).

As well as utilizing sulphite in the first pretreatment stage, a second trial that was aimed at reducing the pH even further used bisulphite in the initial pretreatment stage. The water soluble streams resulting from the substrates utilizing bisulphite in the first pretreatment stage had a final pH of 1.6 as compared to 1.8 in the case of the substrate that utilized sulphite (Table 24). Consequently, likely due to an increase in hemicellulose solubilization at the lower pH, the lignin content of the water insoluble substrate increased and the hemicellulose recovery decreased further to 80% (Table 25). It was also evident that the substrates underwent less

delignification when H_2SO_4 was added in the second stage, likely because the addition of SO_2 at the neutral/alkaline conditions at the conclusion of the first pretreatment stage allows for additional sulphonation in the second pretreatment stage while H_2SO_4 does not actively delignify, and at a pH <2 can actually condense the lignin (Sjostrom, 1993). The liquid fraction generated bisulphite/sulfuric acid pretreatment contained 8.7 g/L glucose (both monomers and oligomers) and 20.2 g/L mannose (total of monomeric and oligomeric) (Table 25). Depending on the ease of hydrolysis of the water insoluble substrates and the fermentability of the sugars solubilized under the acidic conditions, it was hoped that the potential increase in total sugars would result in a higher overall ethanol concentration.

Table 24 Components of a series of two steps of (alkali followed by acid) sulphite steam pretreated lodgepole pine substrates and the pH and acid groups of these substrates.

Samples	Ara (%)	Gla (%)	Glu (%)	Xyl (%)	Man (%)	ASL (%)	AISL (%)	pH	Acid groups (µm/g substrates)
(1) 4% SO_2 200 °C, 5 mins	0	0	47.8	0.1	0.3	0.6	49	1.6	
(2) 12% Na_2SO_3 140 °C, 20 mins, 4% SO_2 200, 5 mins	0	0.2	67.2	2.6	13.9	2.6	16.4	3.3	113
(3) 8% Na_2SO_3 140 °C, 20 mins, 3% H_2SO_4 200°, 5 mins	0.03	0.03	69	1.87	2.9	1.6	26.0	1.9	53
(4) 8% $NaHSO_3$ 140 °C, 20 mins, 2% H_2SO_4 200 °C, 5 mins	0.02	0.07	65.5	1.3	2.2	1.8	30.5	1.6	33

Table 25 Monomers and oligomers of a series liquid fraction of two steps (alkali followed by acid) sulphite steam pretreated lodgepole pine substrates 25% solid loading whole slurry.

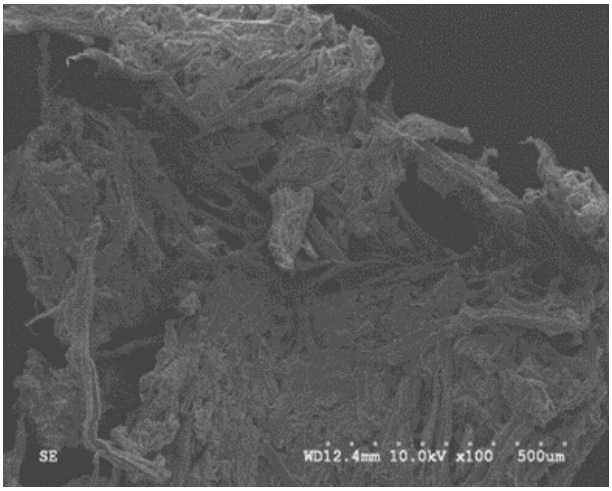
Samples	Glu Mono (g/L)	Glu Olig (g/L)	Xyl Mono (g/L)	Xyl Olig (g/L)	Man Mono (g/L)	Man Olig (g/L)	Glucose recovery	Mannose recovery	Delignif -ication
(1)4% SO_2	28.2	7.1	11.2	2.2	17.7	8.9	98%	79%	0.1%
(2)12%Na_2SO_3 +4%SO_2	-0.05	2.3	2.3	2.2	1.9	4.4	104%	105%	61%
(3)8%Na_2SO_3 +3%H_2SO_4	6.0	6.5	7.5	5.2	11.0	11.0	103%	85.8%	44%
(4)8%$NaHSO_3$ +2%H_2SO_4	3.4	5.3	6.2	4.5	8.6	11.7	99%	80.5%	40%
(5)Acid sulphite steam pretreatment	0.6	3.3	1.5	0.6	2.5	4.8	87%	67%	33%

As mentioned above, the goals of decreasing the pretreatment pH was too decrease the fibre length and cellulose DP of the substrates as decreases in the length and DP have been shown to benefit enzymatic liquefaction at high substrate solids loadings (van der Zwan et al., 2017). It was apparent that the acidic conditions decreased the fibre length from the range of 1.5 mm in the case of the two stage Na_2SO_3-SO_2 to 0.48-0.49 mm which was quite similar with the control 4% SO_2 steam pretreated substrate (Table 23 and 26). The measured degree of polymerization as estimated by the intrinsic viscosity measurement decreased to values between

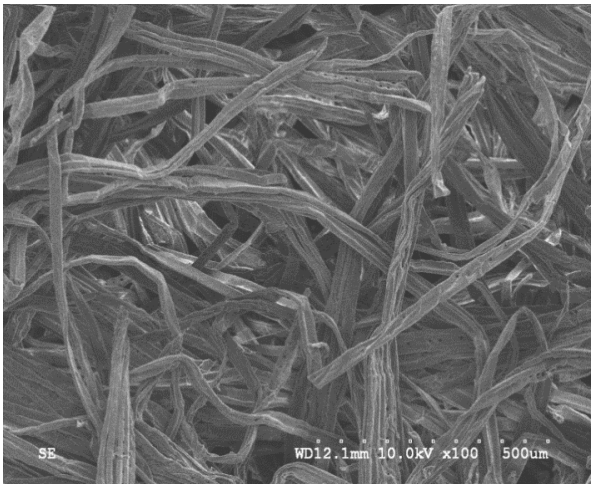
370 and 380 which was a substantial decrease compared to the two stage Na_2SO_3-SO_2 pretreated substrates which had DP values of 1756 (Table 26). In addition to the decrease in DP and fibre length, in particular, the water retention value of the two-stage Na_2SO_3-3% H_2SO_4 pretreated substrate was also increased compared to the other pretreated substrates which indicated a potential increase in the hydrophilicity and porosity of the two-stage Na_2SO_3-3% H_2SO_4 pretreated substrate. Scanning electron microscopy showed that the substrates from the two stage Na_2SO_3-3% H_2SO_4 and the two stage $NaHSO_3$-3% H_2SO_4 also underwent fibre separation similar to the two stage Na_2SO_3-SO_2 pretreated substrates despite their shorter fibre length. The sulfonic acid groups measured on the substrate showed that the two stage Na_2SO_3-3% H_2SO_4 and the two stage $NaHSO_3$-3% H_2SO_4 treatments were both capable of sulfonating lignin but not to same extent as the two-stage Na_2SO_3-SO_2 pretreated substrate (Table 24). Therefore, it is likely that the initial sulphite/bisulphite steam pretreatment stage modified the lignin component to allow fibre separation, while the second sulfuric acid stage solubilized hemicellulose as well as decreasing DP and fibre length similar to conventional SO_2 catalyzed steam pretreatment (Table 24, Figure 47).

Table 26 Characterizing the substrates that were generated from two steps of sulphite steam pretreatment with the sulfuric acid in the second step.

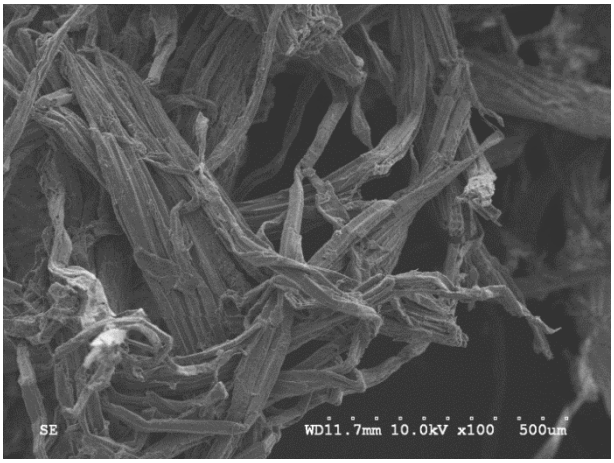
Samples	Fiber length (mm)	Fiber width (um)	Simon stain	DP	Water retention value
4% SO_2	0.41	27.3	80.9	197	150%
12% Na_2SO_3 + 4% SO_2	1.55	30.4	111.2	1757	170.2%
8% Na_2SO_3 +3% H_2SO_4	0.48	28.7	85.9	379	201.7%
8% $NaHSO_3$ +2% H_2SO_4	0.50	29.3	94.3	373	151.6%



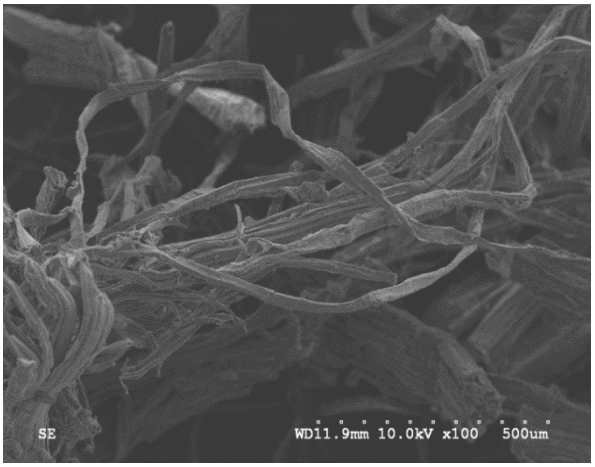
4% SO_2 acid steam pretreatment



12% Na_2SO_3 + 4% SO_2



8% Na_2SO_3 + 2% H_2SO_4



8% $NaHSO_3$ + 2% H_2SO_4

Figure 47 The FE-SEM imagines of the traditional SO_2 catalyzed and the two stages sulfite steam pretreated lodgepole pine substrates. The two stages sulfite steam pretreatment was firstly conducted at the alkali condition with sulfite pre-impregnation (12% Na_2SO_3 loading, 6% Na_2CO_2 with 6% SO_2 loading, 8% Na_2SO_3 loading, 8% $NaHSO_3$ loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of 4% SO_2 or 3% H_2SO_4 or 2% H_2SO_4 at 200 °C for 5 mins.

The hydrolysis of the substrates was initially tested in the whole slurry configuration using a 25% solid loading and an enzyme loading of 35 mg enzyme/ g glucan. The hydrolysis of the two-stage Na_2SO_3-3% H_2SO_4 substrate resulted in a glucose concentration of 150.7 g/L compared to a glucose concentration of 137.6 g/L in the case of the $NaHSO_3$-3%-H_2SO_4 (including sugars solubilized during the pretreatment) (Figure 48). Although both of these values were higher compared to the control SO_2 steam pretreated biomass, these values were lower than substrates generated from the two-stage Na_2SO_3-SO_2 pretreated substrates. It was apparent that reducing the pH of the treatment actually decreased the glucose yield during the whole slurry hydrolysis. It should be noted that the decrease in pH and solubilization of hemicellulose actually increases the overall lignin content of the substrate. As mentioned above, the use of strong sulfuric acid during the second pretreament stage to reduce the pH below 2 minimalizes the chance of additional delignification occurring during the second pretreatment stage compared to the sulfur dioxide used in the previous section that was added to bring the pH to a low of 3 where delignification can still occur. It has been shown during sulphite pulping studies that conducting sulphonation at pH values of 1- 2 also increases the likelihood of lignin condensation rather than delignification (Sjostrom, 1993). To analyze this effect further, the two-stage sulphite pretreated substrates were washed and hydrolyzed at a solids loading of 5% (Figure 49). It was evident that although the hydrolysis of the two-stage sulphite (alkaline-acid) steam pretreated whole slurries at 25% solids resulted in higher sugar concentrations than the SO_2 steam pretreated control, the hydrolysis of the washed substrates was more challenging likely because of the originally reserved higher carbohydrate content of the two-stage sulphite pretreated substrates.

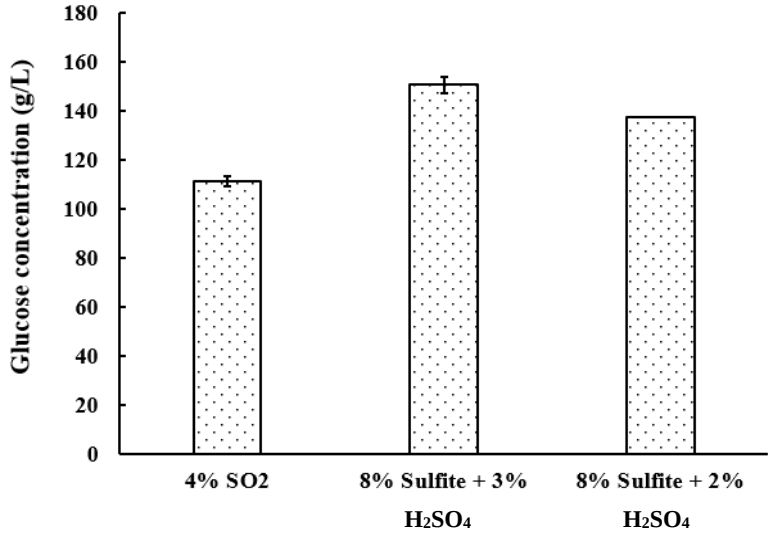


Figure 48 The enzymatic hydrolysis of 25% solid loading two stages sulfite steam pretreated lodgepole pine whole slurry at Ctec-3 loading of 40 mg protein per g cellulose for 48 h. Two kinds of two stages sulfite steam pretreatment were assessed. 1)The substrates was first conducted at the alkali condition with sulfite pre-impregnation (8% Na_2SO_3 loading) at 140 °C for 20 mins followed by a second stage acidic condition treatment with the addition of 3% H_2SO_4 at 200 °C for 5 mins. 2) The substrates was first conducted at the alkali condition with bisulfite pre-impregnation (8% $NaHSO_3$ loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of 2% H_2SO_4 at 200 °C for 5 mins. The 4% SO_2 acid steam pretreated substrate was selected as a control.

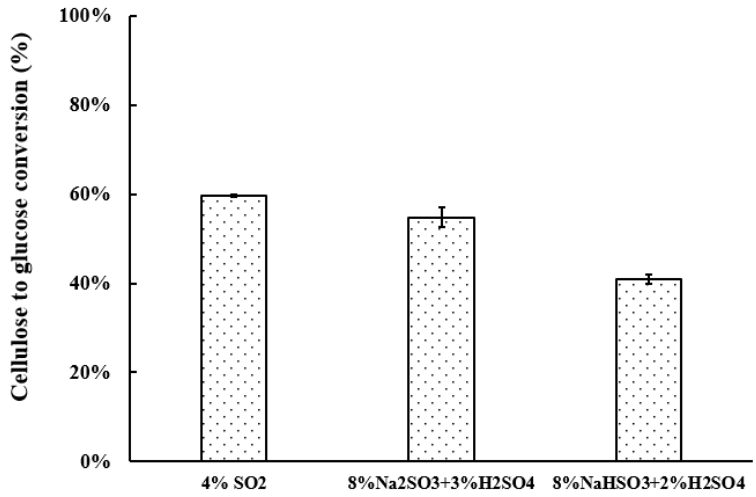


Figure 49 The enzymatic hydrolysis of 5% solid loading two stages sulfite steam pretreated lodgepole pine whole slurry at Ctec-3 loading of 15 mg protein per g cellulose for 48 h. Two kinds of two stages sulfite steam pretreatment were assessed. 1)The substrates was first conducted at the alkali condition with sulfite pre-impregnation (8% Na_2SO_3 loading) at 140 °C for 20 mins followed by a second stage acidic condition treatment with the addition of 3% H_2SO_4 at 200 °C for 5 mins. 2) The substrates was first conducted at the alkali condition with bisulfite pre-impregnation (8% $NaHSO_3$ loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of 2% H_2SO_4 at 200 °C for 5 mins. The 4% SO_2 acid steam pretreated substrate was selected as a control.

The water soluble fractions were characterized for their content of fermentation inhibitors. It was apparent that the two-stage sulphite pretreatments using either SO_2 or H_2SO_4 during the second pretreatment stage decreased the amount of furan inhibitors (furfural and 5-hydroxymethylfurfural) compared to the control steam pretreatment. As mentioned earlier, the likely explanation for the decreased amount of furans was the increased cellulose and hemicellulose recoveries achieved using the two-stage sulphite treatments and the reported ability of sulphite to reduce furan inhibitors (Cavka et al., 2011). Unexpectedly, the total amount

of phenolics in the water soluble fraction was also quite low. As the acidity was increased, this may indicate an increase in the amount of re-condensation of lignin that occurred under the acid conditions utilized in the second pretreatment stage (Table 27).

Table 27 Inhibitor profiles of a series 25% solid loading whole slurry of two steps of sulphite steam pretreated lodgepole pine. The 4% SO_2 acid steam pretreated lodgepole and two steps (alkali followed by acid) sulphite steam pretreated substrates were selected as controls.

Samples	Acetic acid (g/L)	Furfural (g/L)	HMF (g/L)	Total phenolics (g/L)
4% SO_2	8.2	3.2	1.6	4.7
12% Na_2SO_3+4% SO_2	0.91	0.27	0.19	7.6
8%Na_2SO_3+3%H_2SO_4	1.4	0.43	0.21	1.5
8%$NaHSO_3$+2%H_2SO_4	2.6	0.88	0.6	1.2

The fermentability of the two-stage sulphite pretreated substrates (alkali sulphite and acid) using the whole slurry at a solids loading of 25% solid loading was tested by initially hydrolyzing each substrate using an enzyme loading of 40 mg / g glucan for 3 days prior to the addition of yeast to initiate the SSF process. It was apparent that the fermentation of the two-stage sulphite-H_2SO_4 substrates described in this section resulted in ethanol concentrations ranging from 75 to 77 g/L which was slightly lower than those obtained when fermenting the two-stage sulphite-SO_2 steam pretreated substrates but still higher than the control SO_2 steam pretreated softwood whole slurry that was virtually un-fermentable (Figure 50). The hydrolysis

of the washed two-stage acid-alkaline sulphite steam pretreated substrates was comparable or slightly lower than the SO_2 steam pretreated control. However, the hydrolysis challenges appear to be outweighed by the benefits of retaining a greater amount of sugar in the water insoluble substrate. This likely limited the formation fermentation inhibitors which allowed for the production of softwood derived ethanol at concentrations of >80 g/L using the two-stage sulfite steam pretreated substrates.

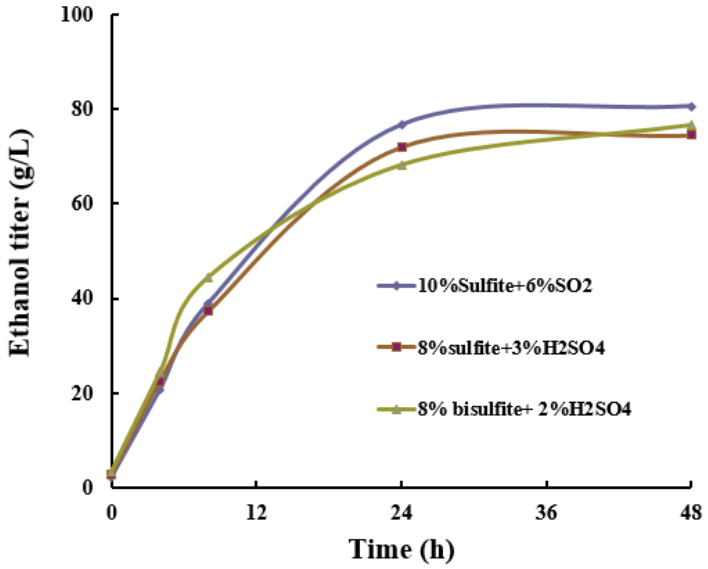


Figure 50 Comparing the ethanol production profile of strain T_2 in the 25% solid loading two stages sulfite steam pretreated lodgepole pine whole slurry hydrolysate with different treatment conditions. The two stage sulfite steam pretreatment was first conducted under alkali conditions with either sulfite (8% and 10% Na_2SO_3 loading) or bisulfite (8% $NaHSO_3$ loading) pre-impregnation at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of 6% SO_2 or 2-3% of H_2SO_4 at 200 °C for 5 mins. The enzymatic hydrolysis was performed at a Ctec-3 loading of 40 mg protein per g cellulose for 48 h. The yeast concentrations (cell optical density: OD) were 6.5.

3.4.2.4 Conclusions

The work in the last two chapters showed that sulphite treatment carried out in an alkaline environment improved the extent of sulphonation (with lower sulphite loading). However, this alkaline-type sulphite treatment was insufficient to allow for; sufficient fibre separation, hemicellulose dissolution and a reduction in particle size and cellulose DP, which should result in enhanced liquefaction at high solids loadings. Therefore, a two stage steam pretreatment was employed utilizing sulphite in an initial alkaline steam pretreatment stage. This allowed the retention of hemicellulose while modifying lignin when either sulfur dioxide or sulfuric acid was added in a second steam pretreatment stage to acidify the reaction. The acidification using SO_2 decreased the pH to 3 and provided enhanced sulphonation. This enhanced fibre separation, but still retained most of the mannan component and had longer fibres and high cellulose DP compared to the conventional sulfur dioxide steam pretreated lodgepole pine. Therefore, sulfur dioxide was substituted with sulfuric acid in the second pretreatment stage. This decreased the fibre size and cellulose degree of polymerization while solubilizing hemicellulose. However, it was apparent that the use of sulfuric acid decreased the total amount of lignin sulphonation as these more acidic substrates had a lower amount of sulfonate groups per gram of lignin and also may have compromised the amount of lignin removal during the second pretreatment stage. Regardless, a final concentration of 160 g/L of fermentable sugars and 80 g/L ethanol could be obtained when a two stage (alkali and acid) sulphonation of pretreated lodgepole pine was carried out a relatively low enzyme loading of 40 mg Ctec-3/g glucan.

The most useful substrates that were produced employed a two stage sulphite-sulfur dioxide steam pretreatment which retained and recovered almost all of the mannan in the

biomass. The ability to enzymatically hydrolyze the mannan in this substrate through the use of an effective mannanase may allow for the liberation of these mannan derived hexose sugars for subsequent fermentation to increase ethanol yields even higher than the 80 g/L achieved in this work.

Chapter 4: Conclusions and Future Work

4.1 Conclusions

Sugar/starch based conventional/first generation bioethanol production routinely uses a high gravity fermentation approach to result in ethanol concentrations of 10-15% (v/v), and yields of >90% within a relatively short period of time (6-10 hours). In contrast, the production of biomass-derived/second generation bioethanol has proven to be more challenging due to several reasons. These include the relatively low sugar concentrations resulting from the enzymatic hydrolyze of the recalcitrant, biomass substrate, and the influence of pretreatment derived inhibitors that limit effective fermentation of the biomass derived sugars. Although softwoods are recognized to be much more recalcitrant than hardwoods or agricultural derived biomass, it was hoped that the predominant hexose composition of softwood hemicellulose would help in the ready fermentation of the combined cellulose and hemicellulose streams during fermentation. The major goal of the thesis work was to integrate sulphite treatments prior, during and after steam pretreatment to enhance the effectiveness of the enzymatic hydrolysis of the whole cellulose and hemicellulose derived slurry and the fermentation of the derived sugars.

Initially, the challenges associated with the high residual lignin and high fermentation inhibitors were assessed using a 25% solid loading steam pretreated whole slurry with a model substrates dissolving pulp as control. It was shown that, compared to the predominantly cellulose containing dissolving pulp, the steam pretreated lodgepole pine whole slurry consumed 3 times more enzyme to achieve similar sugar concentration due to the high content of condensed lignin. Some of the initial fermentation strategies such as strain selection, nutrient supplementation and high cell density also were tested for their potential to deal with fermentation inhibitors. It was

shown that Saccharomyces cerevisiae strain T_2, at a cell density of OD 13, could result in an ethanol productivity of 7.7 g/L.h. However, the fermentablity of whole slurry hydrolysate was still quite problematic even if these fermentation conditions were optimized.

Previous work had shown that sulphite, when used at elevated temperatures, could sulphonate the lignin, likely increasing cellulose accessibility while also decreasing the lignin components inhibitory effect on enzymatic hydrolysis. Although sulphonation aided in enhancing the enzymatic hydrolysis of the cellulose fraction, to achieve higher sugar concentrations the hemicellulose rich water soluble fraction was combined with the cellulose derived sugars. However, "whole-slurries" at a solids loadings of >20% were not readily fermented, likely due to inhibitors associated with the biomass sugar streams.

As other work had shown the potential of sulfite treatments to help detoxify biomass derived sugar streams, post-pretreatment sulphonation was assessed to see if it could simultaneously enhance the hydrolysis of the whole slurry, containing both cellulose and hemicellulose, and the fermentation of the softwood derived sugars. However, little sulphonation was observed when the reaction carried out at room temperature, although fermentation was slightly enhanced. At higher temperatures (70 °C, 121 °C, 160 °C), although sulphonation did occur when high sulfite loadings were used this resulted in a decrease in enzymatic hydrolysis and sugar fermentation due to the poison effects of sulphite to both enzymes and yeast. To try to enhance the beneficial effect of sulphonation and while decreasing chemical loading sulphonation was subsequently carried out in an alkaline environment through the addition of sodium carbonate. It was shown that a sulfite post treatment with 8% sulfite and 2% carbonate at 70 degree for 12 hrs resulted in the production of 107.6 g/L glucose and 13.3 g/L mannose which could be converted to 56.4 g/L ethanol.

To try to maximize the beneficial influence of sulfite treatment on the softwood chips, potential approaches to implement sulphite treatment during steam pretreatment process were assessed. It was thought that, compared with the condensed lignin in the steam pretreated softwood, the native lignin in the softwood biomass could be readily sulfonated during steam pretreatment. Although the use of alkaline conditions maximized sulphonation of the biomass, surprisingly, the ease of hydrolysis of the resulting substrates was not improved compared to the conventional SO_2 steam pretreated control due to the insufficient fiber separation. Therefore, in order to achieve a better fibre separation, size reduction and lignin modification, the subsequent work utilized an acidic sulphite approach. It was apparent that the combination of sulfuric acid and sodium bisulphite was an effective combination in the steam gun when using a single high temperature (>210 °C) pretreatment stage. The new approach was more effective in separating fibres than the neutral/alkaline conditions while providing lignin sulphonation as well as enabling the hydrolysis. In addition, considerably lower amounts of fermentation inhibitors resulted from sulphonation prior to pretreatment. This resulted in an obvious improvement in whole slurry fermentation with 48.5 g/L ethanol detected. This surpassed the fermentation of the SO_2 steam pretreated softwood whole-slurry that was shown to be virtually un-fermentable in Chapter I.

The previous work in this thesis showed that sulphite treatment carried out in an alkaline environment improved the extent of sulphonation (with lower sulphite loading) but the acid type sulphite treatment was more efficient to allow for sufficient fibre separation, hemicellulose dissolution and reductions in particle size and cellulose DP to enhance liquefaction at high solids loadings. Therefore, a two stage steam pretreatment was employed utilizing sulphite in an initial alkaline steam pretreatment stage which retained hemicellulsoe while modifying lignin while

either sulfur dioxide or sulfuric acid was added in a second steam pretreatment stage to acidify the reaction. Acidification, using SO_2, decreased the pH to 3 and provided enhanced sulphonation and enhanced fibre separation while still retaining most of the mannan component. This material also had longer fibres and a higher cellulose DP compared to the conventional sulfur dioxide steam pretreated lodgepole pine. Therefore sulfur dioxide was substituted with sulfuric acid in the second pretreatment stage which further decreased the fibre size and cellulose degree of polymerization while solubilizing hemicellulose. However, it was apparent that the use of sulfuric acid decreased the total amount of lignin sulphonation as these more acidic substrates had a lower amount of sulfonate groups per gram of lignin and also may have compromised the amount of lignin removal during the second pretreatment stage. Regardless, a final concentration of 160 g/L of fermentable sugars and 80 g/L ethanol could be obtained when a two stage (alkali and acid) sulphonation of pretreated lodgepole pine was carried out a relatively low enzyme loading of 40 mg Ctec-3/g glucan.

In summary, we assessed the potential of incorporating sulfite in the steam pretreatment process either prior or after for the purpose of enhancing both the enzymatic hydrolysis and biological fermentation. The work demonstrated that whole slurry sulfite post treatment was not applicable due to the difficulty of finding the optimum sulfite loading. However, a two stage sulfite-SO_2 steam pretreatment could generate a whole slurry substrate that retained and recovered almost all of the glucan and mannan in the solid fraction in an easily hydrolyzed form. This also resulted in smaller particle size, sufficient amount of lignin sulphonation and delignification, as well as lower amount of fermentation inhibitors. Finally more than 80 g/L ethanol could be produced from softwood biomass which is getting into the concentrations currently produced by the first generation bioethanol industry.

4.2 Future work

4.2.1 Does sulphonation of substrates reduce the loading required for effective enzymatic hydrolysis?

It is recognized that sulphonation of lignin improves the overall accessibility of the cellulose component to cellulases by swelling the pretreated substrate and decrease the lignin and enzyme unproductive binding. Previous work has shown that, although enzymatic hydrolysis was improved overall, when treatments such as sulphonation impart negative charges to the lignin component, certain cellulase components become less effective because of the inherent isoelectric point of each enzyme component(Nakagame, 2010). It was shown (Figure 51) that particular enzyme components including EG11and CBH11 do not bind to lignin as effectively when the lignin takes on an overall negative charge. Depending on the properties of amino acids, the enzymes contain both acidic and basic functional groups. In a hydrolysis system at pH 5.0, different enzyme components will be charged differently and/or to a varying extent based on their isoelectric point (pI). Thus, the negatively charged sulphonated lignin will have a distinct influence on each enzyme component during enzyme hydrolysis. In addition, most studies to date have used early generation enzyme preparations such as Celluclast and Spezyme, which may not represent a representative situation when the latest enzyme preparations (ex. Novozymes' CTec system) are applied (Nakagame, 2010; Nordwald, Beckham, & Kaar, 2014; Z. Wang, Lan, & Zhu, 2013). These new enzyme preparations contain genetically modified cellulase monocomponents as well as various accessory enzymes, where the properties of the lignin could play a more significant role when the lignin is sulphonated. Better understanding the influence of lignin sulphonation on the major groups of enzymes present in state-of-the-art

enzyme preparation can provide us invaluable information to further customize the enzyme cocktail for our post-treated lignocellulosic substrates to further reduce enzyme loadings to achieve higher cellulose conversions. Therefore, in future work, the influence of lignin sulphonation on the major group enzyme activities (ex. exo-glucanases/endo-glucanase/β-glucosidase/xylanases/β-xylosidase activities) in the latest enzyme preparation (Ctec-3) could be examined during time course of hydrolysis.

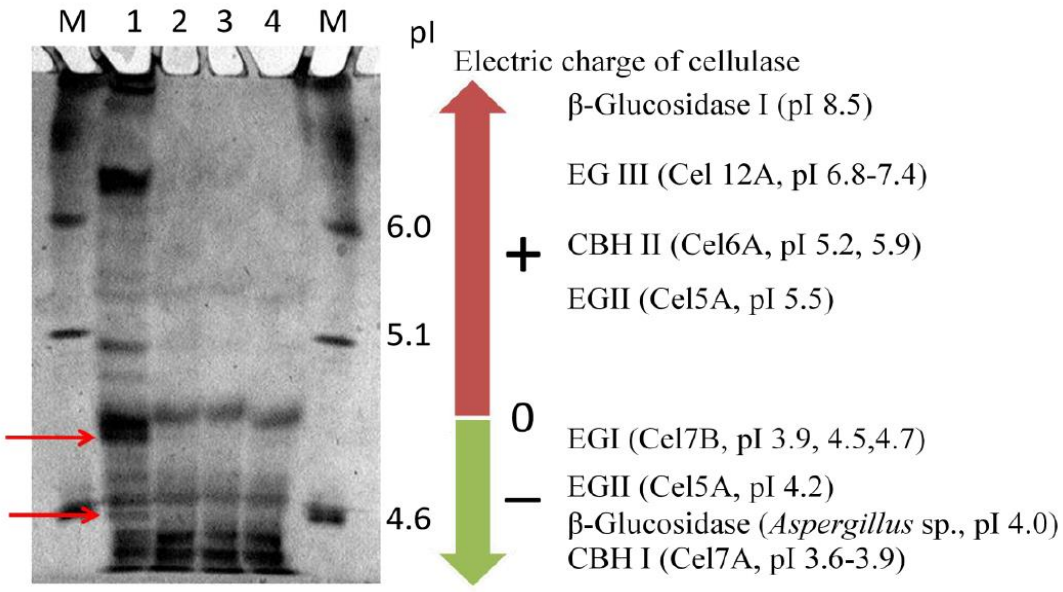


Figure 51 Cellulases adsorption to Protease treated lignin (PTL) isolated from different pretreatment severities. M, IEF markers; lane 1, control (without the PTL); lane 2, PTL 190 °C; lane 3, PTL 200 ºC; lane 4, PTL 210 °C. Cellulases (0.34 mg/ml) and β-glucosidase (0.09 mg/ml) were incubated with 5 mg of PTLs in 500 µl of Na-acetate buffer (pH 4.8, 50 mM) at 50 °C for 3 h. Supernatants after centrifugation were collected, freeze-dried, and analyzed by IEF (pH 5-8). This Figure was adopted from (Nakagame, 2010)

4.2.2 A fed-batch approach to further improve high solids hydrolysis and fermentation using sulphite steam pretreated softwood substrates

Although there have been many accounts showing successful utilization of the fed-batch approach to increase sugar concentrations, most of this previous work did not use solids loadings greater than 20% (Elliston et al., 2013; Gupta et al., 2012; K. Liu et al., 2010; Wanderley, Martín, Rocha, & Gouveia, 2013). In addition, the previous work utilized substrates such as steam pretreated corn stover or fully bleached Kraft pulps which are far less recalcitrant than steam pretreated softwood biomass. In addition, it has been proven that the newly developed substrates are quite difficult to liquefy. Therefore, future work could use novel approaches to boost hydrolysis yield through the use of modified substrates and fermentation strategies combined with fed batch approaches.

As reported before in the example by Liu et al. (Z.-H. Liu et al., 2014), there appears to be a dramatic drop in hydrolysis yield when the solids loading is increased beyond 20%. Therefore, we could conduct the initial hydrolysis at a 20% solid loading in the first batch followed by fermentation and a second batch adding another 10% solid after the fermentation of the first batch. This might result in an improved hydrolysis efficiency compared with that of one batch hydrolysis with total 30% solid loading.

References

Adeboye, P. T., Bettiga, M., & Olsson, L. (2014). The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in Saccharomyces cerevisiae in lignocellulose hydrolysates. *AMB Express*, *4*(1), 46.

Aker, J. O. B., Inter, M. T. K., Herman, N. E. S., Ox, J. W. F., & Immel, M. E. H. (2001). Fingerprinting Trichoderma reesei Hydrolases in a Commercial Cellulase Preparation, *91*, 99–107.

Alriksson, B., Cavka, A., & Jönsson, L. J. (2011). Improving the fermentability of enzymatic hydrolysates of lignocellulose through chemical in-situ detoxification with reducing agents. *Bioresource Technology*, *102*(2), 1254–63.

Alvarez-Vasco, C., & Zhang, X. (2017). Alkaline hydrogen peroxide (AHP) pretreatment of softwood: Enhanced enzymatic hydrolysability at low peroxide loadings. *Biomass and Bioenergy*, *96*, 96–102.

Anfinsen, C. B., & Haber, E. (1961). Studies on the reduction and re-formation of protein disulfide bonds. *The Journal of Biological Chemistry*, *236*, 1361–1363.

Bai, F. W., Anderson, W. a, & Moo-Young, M. (2008). Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances*, *26*(1), 89–105.

Bailey, J. L., & Cole, R. D. (1959). Studies on the reaction of sulfite with proteins. *The Journal of Biological Chemistry*, *234*(7), 1733–1739.

Beckham, G. T., Matthews, J. F., Bomble, Y. J., Bu, L., Adney, W. S., Himmel, M. E., … Crowley, M. F. (2010). Identification of amino acids responsible for processivity in a family 1 carbohydrate-binding module from a fungal cellulase. *Journal of Physical Chemistry B*, *114*(3), 1447–1453.

Booker, R. E., & Sell, J. (1998). The nanostructure of the cell wall of softwoods and its functions in a living tree. *European Journal of Wood and Wood Products*, *56*(1), 1–8.

Brandberg, T., & Carl Johan Franzen, et al. (2005). Continuous Fermentation of Undetoxified Dilute Acid Lignocellulose Hydrolysate by Saccharomyces cerevisiae ATCC 96581 Using Cell Recirculation.

Cai, Y., Bhuiya, M. W., Shanklin, J., & Liu, C. J. (2015). Engineering a monolignol 4-O-methyltransferase with high selectivity for the Condensed Lignin Precursor Coniferyl Alcohol. *Journal of Biological Chemistry*, *290*(44), 26715–26724.

Carpita, N. C., & McCann, M. C. (2000). The Cell Wall. *Biochemistry and Molecular Biology of Plants*, 52–109.

Carrasco, F., Kokta, B. V., Ahmed, A., & Garceau, J. J. (1994). High-yield pulping of aspen wood: Relation between pulp properties and fibre characteristics by multiple linear regression. *Wood Science and Technology*, *28*(6), 409–421.

Caspeta, L., Chen, Y., Ghiaci, P., Feizi, A., Buskov, S., Hallstrom, B. M., … Nielsen, J. (2014). Altered sterol composition renders yeast thermotolerant. *Science*, *346*(6205), 75–78.

Cavka, A., Alriksson, B., Ahnlund, M., & Jönsson, L. J. (2011). Effect of sulfur oxyanions on lignocellulose-derived fermentation inhibitors. *Biotechnology and Bioengineering*, *108*(11), 2592–9.

Cavka, A., & Jönsson, L. J. (2013). Detoxification of lignocellulosic hydrolysates using sodium borohydride. *Bioresource Technology*, *136*, 368–76.

Chagaev, O., Heitner, C., & Hellstern, M. (2005). The effects of sulphonation and high-intensity refining on the ultra-high-yield pulping of spruce. *Pulp and Paper Canada*, *106*, 65–70.

Chandel, A. K., Silvério, S., & Singh, O. V. (2011). Detoxification of Lignocellulosic Hydrolysates for Improved Bioethanol Production.

Chandra, R., Ewanick, S., Hsieh, C., & Saddler, J. N. (2008). <The characterization of pretreated lignocellulosic substrates prior to enzymatic hydrolysis.pdf>, 1178–1185.

Chandra, R. P., Bura, R., Mabee, W. E., Berlin, a, Pan, X., & Saddler, J. N. (2007). Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics? *Advances in Biochemical Engineering/biotechnology*, *108*(May), 67–93.

Chandra, R. P., Chu, Q. L., Hu, J., Zhong, N., Lin, M., Lee, J. S., & Saddler, J. (2016). The influence of lignin on steam pretreatment and mechanical pulping of poplar to achieve high sugar recovery and ease of enzymatic hydrolysis. *Bioresource Technology*, *199*, 135–141.

Chandra, R. P., Ewanick, Æ. S. M., Æ, P. A. C. Æ. K. A., Del, L., Warren, R. Æ., & Jack, M. Æ. (2009). Comparison of methods to assess the enzyme accessibility and hydrolysis of pretreated lignocellulosic substrates, 1217–1222.

Chirico, W. J., & Brown, R. D. (1987). Purification and characterization of a P-glucosidase from Trichoderma reesei, *341*, 333–341.

Chu, Q., Chandra, R. P., Kim, C. S., & Saddler, J. N. (2017). Alkali-Oxygen Impregnation Prior to Steam Pretreating Poplar Wood Chips Enhances Selective Lignin Modification and Removal while Maximizing Carbohydrate Recovery, Cellulose Accessibility, and Enzymatic Hydrolysis. *ACS Sustainable Chemistry and Engineering*, *5*(5), 4011–4017.

Chum, H. L., Johnson, D. K., Black, S. K., & Overend, R. P. (1990). Pretreatment-Catalyst effects and the combined severity parameter. *Applied Biochemistry and Biotechnology*, *24–25*(1), 1–14.

Claesson, K. lisbeth. (2011). Effects of nutrients supplementation on fermentability of lignocellulosic hydrolysates under high gravity conditions . Master of Science Thesis, (2011).

Del Rio, L. F., Chandra, R. P., & Saddler, J. N. (2010). The effect of varying organosolv pretreatment chemicals on the physicochemical properties and cellulolytic hydrolysis of mountain pine beetle-killed lodgepole pine. *Applied Biochemistry and Biotechnology*, *161*(1–8), 1–21.

Dition, D. (2003). *T HE A LCOHOL*.

Donaldson, L. A., Wong, K. K. Y., & Mackie, K. L. (1988). Ultrastructure of steam-exploded wood. *Wood Science and Technology*, *22*(2), 103–114. http://doi.org/10.1007/BF00355846

Du, X., Pérez-Boada, M., Fernández, C., Rencoret, J., del Río, J. C., Jiménez-Barbero, J., … Martínez, A. T. (2014). Analysis of lignin–carbohydrate and lignin–lignin linkages after hydrolase treatment of xylan–lignin, glucomannan–lignin and glucan–lignin complexes from spruce wood. *Planta*, *239*(5), 1079–1090. http://doi.org/10.1007/s00425-014-2037-y

Elliston, A., Collins, S. R. a, Wilson, D. R., Roberts, I. N., & Waldron, K. W. (2013). High concentrations of cellulosic ethanol achieved by fed batch semi simultaneous saccharification and fermentation of waste-paper. *Bioresource Technology*, *134*, 117–26.

Eric Johnson. (1982). inhibition of cellulase by end product.pdf.

Esteghlalian, A. R., Bilodeau, M., Mansfield, S. D., & Saddler, J. N. (2001). Do enzymatic digestibility and Simons' stain reflect the differences in the available surface area of lignocellulosic substrates. *Biotechnol Prog*, *17*, 1049–1054.

Ewanick, S. M., Bura, R., & Saddler, J. N. (2007). Acid-Catalyzed Steam Pretreatment of Lodgepole Pine and Subsequent Enzymatic Hydrolysis and Fermentation to Ethanol, *98*(4), 737–746.

Forests, M. of. (2012). Facts About B . C .' s Mountain Pine Beetle, *i*(May), 1–2.

Gierer, J. (1985). Chemistry of delignification - Part 1: General concept and reactions during pulping. *Wood Science and Technology*, *19*(4), 289–312.

Gourlay, K., Hu, J., Arantes, V., Penttilä, M., & Saddler, J. N. (2015). The use of carbohydrate binding modules (CBMs) to monitor changes in fragmentation and cellulose fiber surface morphology during cellulase- And swollenin-induced deconstruction of lignocellulosic substrates. *Journal of Biological Chemistry*, *290*(5), 2938–2945.

Guo, X., Cavka, A., Jönsson, L., & Hong, F. (2013). Comparison of methods for detoxification of spruce hydrolysate for bacterial cellulose production. *Microbial Cell Factories*, *12*, 93.

Gupta, R., Kumar, S., Gomes, J., & Kuhad, R. C. (2012). Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol. *Biotechnology for Biofuels*, *5*(1), 16.

Hakan Ekstrom. (2012). 4 Wood raw material markets, 2011-2012, 2011–2012.

Harner, N. K., Bajwa, P. K., Habash, M. B., Trevors, J. T., Austin, G. D., & Lee, H. (2013). Mutants of the pentose-fermenting yeast Pachysolen tannophilus tolerant to hardwood spent sulfite liquor and acetic acid. *Antonie van Leeuwenhoek*, 29–43.

Henrissat, B., & Daviest, G. (1997). Structural and sequence-based classification of glycoside hydrolases, 637–644.

Hoyer, K., Galbe, M., & Zacchi, G. (2013). Influence of fiber degradation and concentration of fermentable sugars on simultaneous saccharification and fermentation of high-solids spruce slurry to ethanol. *Biotechnology for Biofuels*, *6*(1), 145.

Hsieh, C. C., Cannella, D., Jørgensen, H., Felby, C., & Thygesen, L. G. (2014). Cellulase Inhibition by High Concentrations of Monosaccharides.

Hu, J., Arantes, V., Pribowo, A., Gourlay, K., & Saddler, J. N. (2014). Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass. *Energy Environ. Sci.*, *7*(7), 2308–2315.

Hu, J., Chandra, R., Arantes, V., Gourlay, K., Susan van Dyk, J., & Saddler, J. N. (2015). The

addition of accessory enzymes enhances the hydrolytic performance of cellulase enzymes at high solid loadings. *Bioresource Technology*, *186*, 149–53.

Hu, J., Gourlay, K., Arantes, V., Van Dyk, J. S., Pribowo, A., & Saddler, J. N. (2015). The accessible cellulose surface influences cellulase synergism during the hydrolysis of lignocellulosic substrates. *ChemSusChem*, *8*(5), 901–907.

Huang, W.-D., & Percival Zhang, Y.-H. (2011). Analysis of biofuels production from sugar based on three criteria: Thermodynamics, bioenergetics, and product separation. *Energy & Environmental Science*, *4*(3), 784.

Huang, Y., Wang, L., Chao, Y., Nawawi, D. S., Akiyama, T., Yokoyama, T., & Matsumoto, Y. (2016). Relationships between Hemicellulose Composition and Lignin Structure in Woods. *Journal of Wood Chemistry and Technology*, *36*(1), 9–15.

Humphreys, J. M., & Chapple, C. (2002). Rewriting the lignin roadmap. *Current Opinion in Plant Biology*, *5*(3), 224–229.

Jäger, S., Brumbauer, A., Feher, E., & Re, K. (2001). Production and characterization of b - glucosidases from di € erent Aspergillus strains. *World Journal of Microbiology and Biotechnology*, *17*, 455–461.

Janssen, R., Turhollow, A. F., Rutz, D., & Mergner, R. (2013). Production facilities for second-generation biofuels in the USA and the EU – current status and future perspectives, 647–665.

Jones, A. M., & Ingledew, W. M. (1994). Fuel alcohol production: Assessment of selected commercial proteases for very high gravity wheat mash fermentation. *Enzyme and Microbial Technology*, *16*(8), 683–687.

Jönsson, L. J., Alriksson, B., & Nilvebrant, N.-O. (2013). Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels*, *6*(1), 16.

Jørgensen, H. (2009). Effect of nutrients on fermentation of pretreated wheat straw at very high dry matter content by Saccharomyces cerevisiae. *Applied Biochemistry and Biotechnology*, *153*(1–3), 44–57.

Jørgensen, H., Kristensen, J. B., & Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars : challenges and opportunities, 119–134. http://doi.org/10.1002/bbb

Jørgensen, H., Vibe-Pedersen, J., Larsen, J., & Felby, C. (2007). Liquefaction of lignocellulose

at high-solids concentrations. *Biotechnology and Bioengineering*, *96*(5), 862–870.

Jung, Y. H., Kim, I. J., Kim, H. K., & Kim, K. H. (2013). Dilute acid pretreatment of lignocellulose for whole slurry ethanol fermentation. *Bioresource Technology*, *132*, 109–114.

Jurado, M., Prieto, A., Martínez-Alcalá, A., Martínez, A. T., & Martínez, M. J. (2009). Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. *Bioresource Technology*, *100*(24), 6378–84.

Kadi??, A., & Lid??n, G. (2017). Does sugar inhibition explain mixing effects in enzymatic hydrolysis of lignocellulose? *Journal of Chemical Technology and Biotechnology*, *92*(4), 868–873.

Kapu, N. S., Piddocke, M., & Saddler, J. J. N. (2013). High gravity and high cell density mitigate some of the fermentation inhibitory effects of softwood hydrolysates. *AMB Express*, *3*(1), 15.

Kayikci, Ö., & Nielsen, J. (2015). Glucose repression in Saccharomyces cerevisiae. *FEMS Yeast Research*, *15*(6), 1–8.

Keating, J. D., Panganiban, C., & Mansfield, S. D. (2006). Tolerance and Adaptation of Ethanologenic Yeasts to Lignocellulosic Inhibitory Compounds. http://doi.org/10.1002/bit

Kim, Y., Hendrickson, R., Mosier, N. S., Ladisch, M. R., Bals, B., Balan, V., & Dale, B. E. (2008). Enzyme hydrolysis and ethanol fermentation of liquid hot water and AFEX pretreated distillers' grains at high-solids loadings. *Bioresource Technology*, *99*(12), 5206–5215.

Kim, Y., Ximenes, E., Mosier, N. S., & Ladisch, M. R. (2011). Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. *Enzyme and Microbial Technology*, *48*(4–5), 408–415.

Kokta, B. V., & Ahmed, A. (1998). Steam Explosion Pulping. *Young*, 191–211.

Konn, J., Pranovich, A., Fardim, P., & Holmbom, B. (2007). Characterisation and effects of new anionic groups formed during chemithermomechanical pulping of spruce, *296*, 1–7.

Koppram, R., Tomás-Pejó, E., Xiros, C., & Olsson, L. (2014). Lignocellulosic ethanol production at high-gravity: Challenges and perspectives. *Trends in Biotechnology*, *32*(1), 46–53.

Kristensen, J. B., Felby, C., & Jørgensen, H. (2009). Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels*, *2*(1), 11.

Kumar, L. (2013). Steam pretreatment as an effective front end for a softwood based biorefinery A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF, (March).

Kumar, L., Arantes, V., Chandra, R., & Saddler, J. (2012). The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. *Bioresource Technology*, *103*(1), 201–8.

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, *108*(10), 2300–2311.

Kumar, S. L. (2014). High gravity fermentation of softwood derived sugar streams A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF, (June).

Lam, F. H., Ghaderi, A., Fink, G. R., & Stephanopoulos, G. (2014). Engineering alcohol tolerance in yeast. *Science*, *346*(6205), 71–75.

Lan, T. Q., Gleisner, R., Zhu, J. Y., Dien, B. S., & Hector, R. E. (2013). High titer ethanol production from SPORL-pretreated lodgepole pine by simultaneous enzymatic saccharification and combined fermentation. *Bioresource Technology*, *127*, 291–7.

Lapierre, L., Bouchard, J., & Berry, R. (2009). The relationship found between fibre length and viscosity of three different commercial kraft pulps. *Holzforschung*, *63*(4), 402–407.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., & Nilvebrant, N.-O. (1999). The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme and Microbial Technology*, *24*(3–4), 151–159.

Larsson, S., Reimann, A., Nilvebrant, N.-O., & Jonsson, L. J. (1999). Comparison of Different Methods for the Detoxification of Lignocellulose Hydrolyzates of Spruce, *77*.

Lavenson, D. M., Tozzi, E. J., Karuna, N., Jeoh, T., Powell, R. L., & McCarthy, M. J. (2012). The effect of mixing on the liquefaction and saccharification of cellulosic fibers. *Bioresource Technology*, *111*, 240–247.

Les Safranyik, B. W. (2006). *The Mountain Pine Beetle： A Synthesis of Biology , Management , and Impacts on Lodgepole Pine*.

Leu, S.-Y., Zhu, J. Y., Gleisner, R., Sessions, J., & Marrs, G. (2013). Robust enzymatic

saccharification of a Douglas-fir forest harvest residue by SPORL. *Biomass and Bioenergy*, *59*, 393–401.

Li, J., Henriksson, G., & Gellerstedt, G. (2007). Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresource Technology*, *98*(16), 3061–3068.

Lin, M. (2016). Investigation of a two-stage steam / organosolv pretreatment approach for the fractionation of softwood biomass by, (December).

Liu, C.-G., Lin, Y.-H., & Bai, F.-W. (2011). Development of redox potential-controlled schemes for very-high-gravity ethanol fermentation. *Journal of Biotechnology*, *153*(1–2), 42–7.

Liu, K., Lin, X., Yue, J., Li, X., Fang, X., Zhu, M., … Xiao, L. (2010). High concentration ethanol production from corncob residues by fed-batch strategy. *Bioresource Technology*, *101*(13), 4952–8.

Liu, Z.-H., Li, B.-Z., & Yuan, Y.-J. (2014). Simultaneous saccharification and fermentation of steam-exploded corn stover at high glucan loading and high temperature. *Biotechnology for Biofuels*, *7*(1), 167.

Liu, Z. L. (2011). Molecular mechanisms of yeast tolerance and in situ detoxification of lignocellulose hydrolysates. *Applied Microbiology and Biotechnology*, *90*(3), 809–25.

Lou, H., Zhou, H., Li, X., Wang, M., Zhu, J. Y., & Qiu, X. (2014). Understanding the effects of lignosulfonate on enzymatic saccharification of pure cellulose. *Cellulose*, *21*(3), 1351–1359.

Luo, X., Gleisner, R., Tian, S., Negron, J., Zhu, W., Horn, E., … Normal, C. (2010). Evaluation of Mountain Beetle-Infested Lodgepole Pine for Cellulosic Ethanol Production by Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose, 8258–8266.

Lynd, L. R., Weimer, P. J., Zyl, W. H. Van, & Isak, S. (2002). Pesquisa Nacional de Saneamento Básico 2008. *Microbiology and Molecular Biology Reviews*, *66*(3), 506–577.

M.R. Ladisch et al. (1983). Process considerations in the enzymatic hydrolysis of biomass, *5*, 82–102.

Mandels, M., & Reese, E. (1965). INHIBITION OF CELLULASES, (January).

Melzoch, K., Rychtera, M., Markvichov, N. S., Pospichalovfi, V., Basalovfi, G., & Manakov, M. N. (1991). Application of a membrane recycle bioreactor for continuous ethanol production,

469–472.

Modenbach, A. a., & Nokes, S. E. (2013). Enzymatic hydrolysis of biomass at high-solids loadings – A review. *Biomass and Bioenergy*, *56*, 526–544.

Modenbach, A. a, & Nokes, S. E. (2012). The use of high-solids loadings in biomass pretreatment--a review. *Biotechnology and Bioengineering*, *109*(6), 1430–42.

Modig, T. (n.d.). Kinetics and inhibition effects of furfural and hydroxymethyl furfural on enzymes in yeast, 0–4.

Modig, T., Lidén, G., & Taherzadeh, M. J. (2002). Inhibition effects of furfural on alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase. *The Biochemical Journal*, *363*(Pt 3), 769–76. Retrieved from

Mohanram, S., Amat, D., Choudhary, J., Arora, A., & Nain, L. (2013). Novel perspectives for evolving enzyme cocktails for lignocellulose hydrolysis in biorefineries. *Sustainable Chemical Processes*, *1*(1), 15.

Mooney, C. A., Mansfield, S. D., Beatson, R. P., & Saddler, J. N. (1999). The effect of fiber characteristics on hydrolysis and cellulase accessibility to softwood substrates, *25*, 644–650.

Mooney, C., Mansfield, S. D., Touhy, M. G., & Saddler, J. N. (1998). The effect of initial pore volume and lignin content on the enzymatic hydrolysis of softwoods, *64*, 113–119.

Nakagame, S. (2010). The roles of lignin in the hydrolysis of pretreated substrates by cellulases, (November).

Nakagame, S., Chandra, R. P., Kadla, J. F., & Saddler, J. N. (2011). The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. *Bioresource Technology*, *102*(6), 4507–4517.

Nelson, D., & Cox, M. (2013). Lehninger principles of biochemistry (4th ed.).

Nishiyama, Y., Langan, P., & Chanzy, H. (2002). Crystal structure and hydrogen-bonding system in cellulose Iβ from synchrotron X-ray and neutron fiber diffraction. *Journal of the American Chemical Society*, *124*(31), 9074–9082.

Nordwald, E. M., Brunecky, R., Himmel, M. E., Beckham, G. T., & Kaar, J. L. (2014). Charge engineering of cellulases improves ionic liquid tolerance and reduces lignin inhibition. *Biotechnology and Bioengineering*, *111*(8), 1541–9.

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J., & Zacchi, G. (2007). A comparison between

simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry*, *42*(5), 834–839.

Olsen, C., Arantes, V., Saddler, J., Columbia, B., & Bc, V. (2012). The use of predictive models to optimize sugar recovery obtained after the steam pre-treatment of softwoods, 534–548.

Overend, R. P., & Chornet, E. (1987). Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments. *Philosophical Transactions of the Royal Society of London*, *321*(1561), 523–536.

Palmqvist, E. (1998). Evaluation of cell recycling in continuous fermentation of enzymatic hydrolysates of spruce with Saccharomyces cerevisiae and on-line monitoring of glucose and ethanol, 545–551.

Palmqvist, E. (2000). Fermentation of lignocellulosic hydrolysates . I : inhibition and detoxi ® cation, *74*.

Palmqvist, E., & Almeida, J. S. (1999). Influence of Furfural on Anaerobic Glycolytic Kinetics of Saccharomyces cerevisiae in Batch Culture.

Palmqvist, E., Grage, H., Meinander, N. Q., & Hahn-Hägerdal, B. (1999). Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. *Biotechnology and Bioengineering*, *63*(1), 46–55. Retrieved from

Palmqvist, E., & Hahn-Hägerdal, B. (2000). Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. *Bioresource Technology*, *74*(1), 25–33.

Pan, X., Xie, D., Yu, R. W., Lam, D., & Saddler, J. N. (2007). Pretreatment of lodgepole pine killed by mountain pine beetle using the ethanol organosolv process: Fractionation and process optimization. *Industrial and Engineering Chemistry Research*, *46*(8), 2609–2617.

Pan, X., Zhang, X., Gregg, D. J., & Saddler, J. N. (2004). Enhanced enzymatic hydrolysis of steam-exploded Douglas fir wood by alkali-oxygen post-treatment. *Applied Biochemistry and Biotechnology*, *113–116*, 1103–14. Retrieved from

Parawira, W., & Tekere, M. (2011). Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. *Critical Reviews in Biotechnology*, *31*(1), 20–31.

Parreiras, L. S., Breuer, R. J., Avanasi Narasimhan, R., Higbee, A. J., La Reau, A., Tremaine, M., … Sato, T. K. (2014). Engineering and Two-Stage Evolution of a Lignocellulosic

Hydrolysate-Tolerant Saccharomyces cerevisiae Strain for Anaerobic Fermentation of Xylose from AFEX Pretreated Corn Stover. *PLoS ONE*, *9*(9), e107499.

Payne, C. M., Knott, B. C., Mayes, H. B., Hansson, H., Himmel, M. E., Sandgren, M., … Beckham, G. T. (2015). Fungal cellulases. *Chemical Reviews*, *115*(3), 1308–1448.

Persson, I., Tjerneld, F., & Hahn-Hagerdal, B. (1991). Fungal cellulolytic enzyme production: A review. *Process Biochemistry*, *26*(2), 65–74.

Persson, P. E. R., Ndersson, J. E. A., Orton, L. O. G., Arsson, S. I. L., Ilvebrant, N. I. L. S. L. O. F. N., & Eif, L. J. J. O. (2002). Effect of Different Forms of Alkali Treatment on Specific Fermentation Inhibitors and on the Fermentability of Lignocellulose Hydrolysates for Production of Fuel Ethanol, 5318–5325.

Petrovska, B., Winkelhausen, E., & Kuzmanova, S. (1999). Glycerol production by yeasts under osmotic and sulfite stress. *Canadian Journal of Microbiology*, *45*, 695–699.

Pienkos, P. T., & Zhang, M. (2009). Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. *Cellulose*, *16*(4), 743–762.

Pranovich, A. V., Sundberg, K. E., & Holmbom, B. R. (2003). Chemical Changes in Thermomechanical Pulp at Alkaline Conditions. *Journal of Wood Chemistry and Technology*, *23*(1), 89–112.

Pribowo, A. (2014). Enzyme-substrate interactions and their influence on enzyme recycling strategies as a way of reusing cellulases, (April).

Puligundla, P., Smogrovicova, D., Obulam, V. S. R., & Ko, S. (2011). Very high gravity (VHG) ethanolic brewing and fermentation: A research update. *Journal of Industrial Microbiology and Biotechnology*, *38*(9), 1133–1144.

Qin, Y., Yu, L., Wu, R., Yang, D., Qiu, X., & Zhu, J. Y. (2016). Biorefinery Lignosulfonates from Sulfite-Pretreated Softwoods as Dispersant for Graphite. *ACS Sustainable Chemistry and Engineering*, *4*(4), 2200–2205.

Qing, Q., & Wyman, C. E. (2011). Supplementation with xylanase and β-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. *Biotechnology for Biofuels*, *4*(1), 18.

Quintanilla, A. L. (2013). A Study of Glucose as an Inhibitor to the Enzymatic Hydrolysis of Cellulose Using a Quartz Crystal Microbalance.

Ragauskas, A. J., Beckham, G. T., Biddy, M. J., Chandra, R., Chen, F., Davis, M. F., … Wyman, C. E. (2014). Lignin valorization: improving lignin processing in the biorefinery. *Science (New York, N.Y.)*, *344*(6185), 1246843.

Ranatunga, T. D., Jervis, J., Helm, R. F., Mcmillan, J. D., & Wooley, R. J. (2000). The effect of overliming on the toxicity of dilute acid pretreated lignocellulosics : the role of inorganics , uronic acids and ether-soluble organics , *27*, 240–247.

Robertson, G., Olson, J., Allen, P., Chan, B. E. N., & Seth, R. (1999). Measurement of fiber length, coarseness, and shape with the fiber quality analyzer. *Tappi Journal*, *82*(10), 93–98.

Romaní, A., Ruiz, H. A., Pereira, F. B., Teixeira, J. A., & Domingues, L. (2014). Integrated approach for effective bioethanol production using whole slurry from autohydrolyzed Eucalyptus globulus wood at high-solid loadings. *Fuel*, *135*, 482–491.

Safari, A., Karimi, K., & Shafiei, M. (2017). Dilute alkali pretreatment of softwood pine: A biorefinery approach. *Bioresource Technology*, *234*, 67–76.

Saha, B. C. (2003). Hemicellulose bioconversion. *Journal of Industrial Microbiology and Biotechnology*, *30*(5), 279–291.

Sannigrahi, P., Miller, S. J., & Ragauskas, A. J. (2010). Effects of organosolv pretreatment and enzymatic hydrolysis on cellulose structure and crystallinity in Loblolly pine. *Carbohydrate Research*, *345*(7), 965–970.

Sannigrahi, P., Ragauskas, A. J., & Miller, S. J. (2010). Lignin structural modifications resulting from ethanol organosolv treatment of Loblolly pine. *Energy and Fuels*, *24*(1), 683–689.

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annual Review of Plant Biology*, *61*(1), 263–289.

Schimz, K. L. (1980). The effect of sulfite on the yeast Saccharomyces cerevisiae. *Archives of Microbiology*, *125*(1–2), 89–95.

Shuai, L., Yang, Q., Zhu, J. Y., Lu, F. C., Weimer, P. J., Ralph, J., & Pan, X. J. (2010). Comparative study of SPORL and dilute-acid pretreatments of spruce for cellulosic ethanol production. *Bioresource Technology*, *101*(9), 3106–14.

SJÖSTRÖM, E., & SJÖSTRÖM, E. (1993). Wood Chemistry. In *Wood Chemistry* (pp. 1–20).

Stelte, W. (2013). Steam explosion for biomass pre-treatment. *Danish Technological Institute*, 1–15.

Taherzadeh, M. J., Niklasson, C., & Lidn, G. (1997). Acetic acid friend or foe in anaerobic batch conversion of glucose to ethanol by Saccharom yces cerevisiae ? *Chemical Engineering Science*, *52*(15), 2653–2659.

Thomas, K. C., & Ingledew, W. M. (1990). Fuel alcohol production: effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes. *Applied and Environmental Microbiology*, *56*(7), 2046–50. Retrieved from

Tian, S., Luo, X. L., Yang, X. S., & Zhu, J. Y. (2010). Robust cellulosic ethanol production from SPORL-pretreated lodgepole pine using an adapted strain Saccharomyces cerevisiae without detoxification. *Bioresource Technology*, *101*(22), 8678–85.

Tomás-Pejó, E., Oliva, J. M., Ballesteros, M., & Olsson, L. (2008). Comparison of SHF and SSF processes from steam-exploded wheat straw for ethanol production by xylose-fermenting and robust glucose-fermenting Saccharomyces cerevisiae strains. *Biotechnology and Bioengineering*, *100*(6), 1122–31.

van der Zwan, T., Hu, J., & Saddler, J. N. (2017). Mechanistic insights into the liquefaction stage of enzyme-mediated biomass deconstruction. *Biotechnology and Bioengineering*, (April), 1–8.

Van Dyk, J. S., & Pletschke, B. I. (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes--factors affecting enzymes, conversion and synergy. *Biotechnology Advances*, *30*(6), 1458–80.

Várnai, A., Huikko, L., Pere, J., Siika-Aho, M., & Viikari, L. (2011). Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. *Bioresource Technology*, *102*(19), 9096–104.

Walker, G. M. (2011). 125th Anniversary Review : Fuel Alcohol : Current Production and Future Challenges, *117*(1), 3–22.

Wanderley, M. C. D. A., Martín, C., Rocha, G. J. D. M., & Gouveia, E. R. (2013). Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. *Bioresource Technology*, *128*, 448–53.

Wang, G. S., Service, U. F., & Rockwood, D. (2009). Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulose (SPORL) for Robust Enzymatic Saccharification of Hardwoods, 0–7.

Wang, L., Templer, R., & Murphy, R. J. (2012). High-solids loading enzymatic hydrolysis of waste papers for biofuel production. *Applied Energy*, *99*, 23–31.

Wang, Z., Lan, T., & Zhu, J. (2013). Lignosulfonate and elevated pH can enhance enzymatic saccharification of lignocelluloses. *Biotechnology for Biofuels*, *6*(1), 9.

Xiros, C., & Olsson, L. (n.d.). Highlights Detoxification is the most efficient method to overcome inhibitory effects The efficiency of detoxification decreases as fermentation temperature increases Fermentation inhibitors is a major challenge for spruce hydrolysates bioconversion Newly.

Xiros, C., & Olsson, L. (2014). Comparison of strategies to overcome the inhibitory effects in high-gravity fermentation of lignocellulosic hydrolysates. *Biomass and Bioenergy*, *65*, 79–90.

Xuejun Pan, J. S. (2005). Strategies to Enhance the Enzymatic Hydrolysis of Pretreated Softwood with High Residual Lignin Content., *121*(3).

Yang, B., Boussaid, A., Mans, S. D., Gregg, D. J., & Saddler, J. N. (2002). Fast and Ef ® cient AIkaline Peroxide Treatment to Enhance the Enzymatic Digestibility of Steam-Exploded Softwood Substrates.

Yang, B., & Wyman, C. E. (2006). BSA treatment to enhance enzymatic hydrolysis of cellulose in lingnin containing substrates. *Biotechnology and Bioengineering*, *94*, 611–617.

Yang, M., Li, W., Liu, B., Li, Q., & Xing, J. (2010). High-concentration sugars production from corn stover based on combined pretreatments and fed-batch process. *Bioresource Technology*, *101*(13), 4884–4888.

Yu, J., Zhang, X., & Tan, T. (2007). An novel immobilization method of Saccharomyces cerevisiae to sorghum bagasse for ethanol production. *Journal of Biotechnology*, *129*(3), 415–20.

Zhai, R., Hu, J., & Saddler, J. N. (2016). What Are the Major Components in Steam Pretreated Lignocellulosic Biomass That Inhibit the Efficacy of Cellulase Enzyme Mixtures? *ACS Sustainable Chemistry and Engineering*, *4*(6), 3429–3436.

Zhang, C., Houtman, C. J., & Zhu, J. Y. (2014). Using low temperature to balance enzymatic saccharification and furan formation during SPORL pretreatment of Douglas-fir. *Process Biochemistry*, *49*(3), 466–473. http://doi.org/10.1016/j.procbio.2013.12.017

Zhang, C., Lei, X., Scott, C. T., Zhu, J. Y., & Li, K. (2013). Comparison of Dilute Acid and Sulfite Pretreatment for Enzymatic Saccharification of Earlywood and Latewood of Douglas fir. *BioEnergy Research*, *7*(1), 362–370.

Zhang, L., & Sun, X. S. (2008). Effect of sodium bisulfite on properties of soybean glycinin. *Journal of Agricultural and Food Chemistry*, *56*(23), 11192–11197.

Zhang, X., Qin, W., Paice, M. G., & Saddler, J. N. (2009). High consistency enzymatic hydrolysis of hardwood substrates. *Bioresource Technology*, *100*(23), 5890–7.

Zhou, H., Lou, H., Yang, D., Zhu, J. Y., & Qiu, X. (2013). Lignosulfonate To Enhance Enzymatic Sacchari fi cation of Lignocelluloses: Role of Molecular Weight and Substrate Lignin.

Zhou, H., Zhu, J. Y., Luo, X., Leu, S. Y., Wu, X., Gleisner, R., … Negron, J. (2013). Bioconversion of beetle-killed lodgepole pine using SPORL: Process scale-up design, lignin coproduct, and high solids fermentation without detoxification. *Industrial and Engineering Chemistry Research*, *52*(45), 16057–16065.

Zhu, J. Y., Gleisner, R., Scott, C. T., Luo, X. L., & Tian, S. (2011). High titer ethanol production from simultaneous enzymatic saccharification and fermentation of aspen at high solids: A comparison between SPORL and dilute acid pretreatments. *Bioresource Technology*, *102*(19), 8921–8929.

Zhu, J. Y., Pan, X. J., Wang, G. S., & Gleisner, R. (2009). Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine. *Bioresource Technology*, *100*(8), 2411–8. http://doi.org/10.1016/j.biortech.2008.10.057

Zyl, C. V. A. N., Prior, B. A., & Preez, J. C. Du. (1988). Production of Ethanol from Sugar Cane Bagasse Hemiceilulose Hydrolyzate by Pichia stipitis Scientific Note.

Appendices

FTIR analysis of substrates to elucidate potential changes to lignin during two-stage sulphite/bisulphite-SO_2/H_2SO_4 steam pretreatments

Fourier transform infra-red (FTIR) analysis was also utilized to characterize the substrates developed in this chapter. When steam pretreated using 4% SO_2 (designated as control steam pretreatment throughout the thesis) the substrate appeared to undergo several changes indicative of lignin structural modifications especially in particular at peaks at 1598, 1511, 1450, 1270, 1140, 1030 cm^{-1} (1598(Aromatic skeletal vibration + C=O stretching), 1511(Aromatic skeletal), 1450(C-H deformation (methyl and methylene)), 1270(C-O of guaiacyle ring), 1140(Guaiacyl C-H and syringyl C-H) cm^{-1}) when compared to the ground lodgepole pine biomass (Figure 52). Increases in the intensity of these peaks has been shown to be due to enrichment in guaiacyl type lignin structures. In contrast to the conventional to the steam pretreated control, analysis of the substrates treated using the two-stage sulphite-SO_2 steam pretreatment showed limited differences in their lignin structure (peaks in the 1200^{-1} and 1600^{-1} region). For the two stages sulphite steam pretreatment, it could decrease the bands near 1270 cm, which represents the C-O stretch in guaiacol rings. And this result might be due to the cleavage of β-O-4 linkages during the sulphite steam pretreatment (Figure 52).

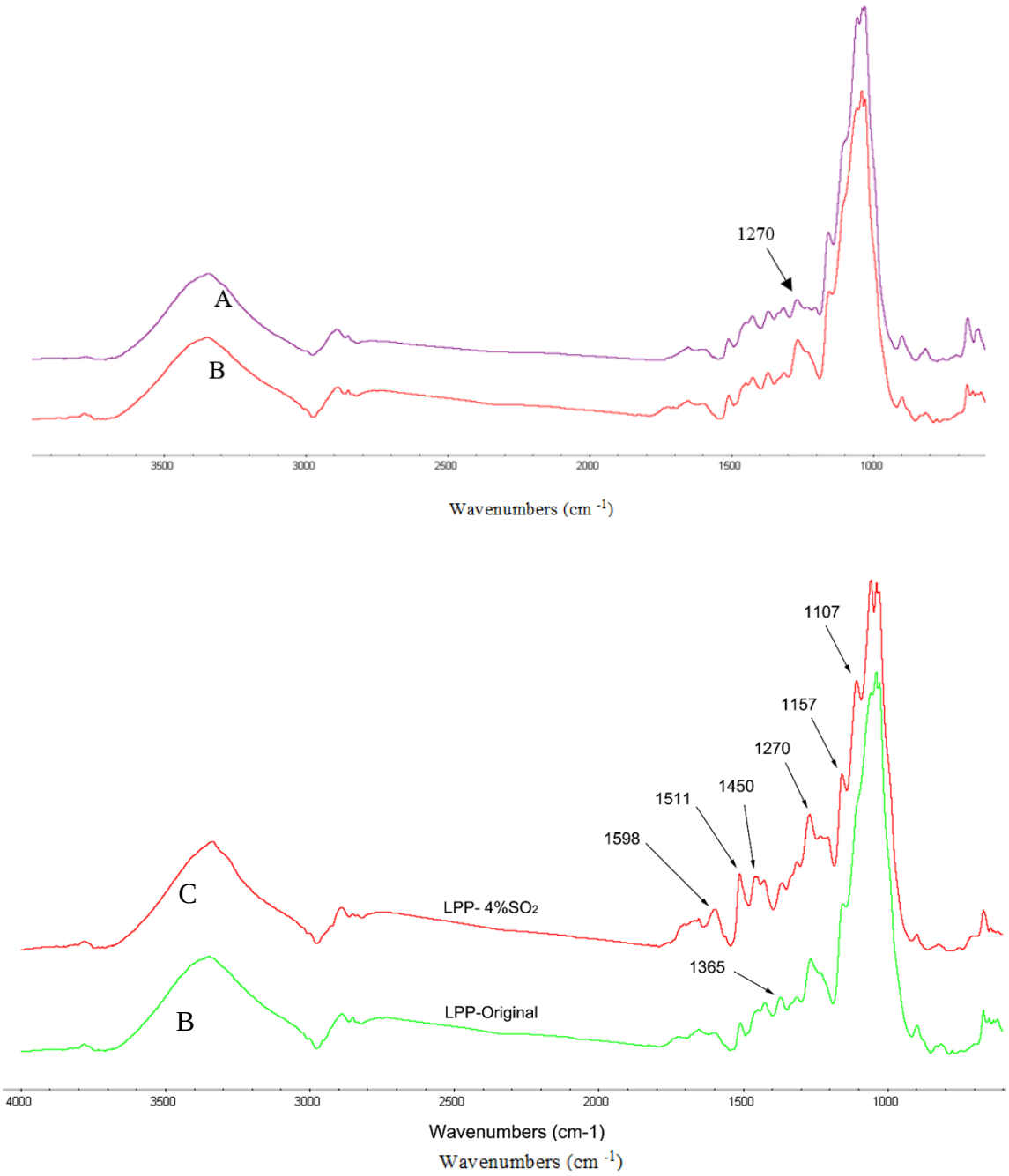


Figure 52 The Fourier transform infrared spectroscopy (FTIR) spectrums of (A) two stages sulfite steam pretreated lodgepole pine, (B) original lodgepole pine (without treatment) and (C) traditional SO_2 catalyzed steam pretreated lodgepole pine. The two stages sulfite steam pretreatment was firstly conducted at the alkali condition with sulfite pre-impregnation (10% Na_2SO_3 loading) at 140 °C for 20 mins, followed by a second stage acidic condition treatment with the addition of 6% SO_2 at 200 °C for 5 mins.

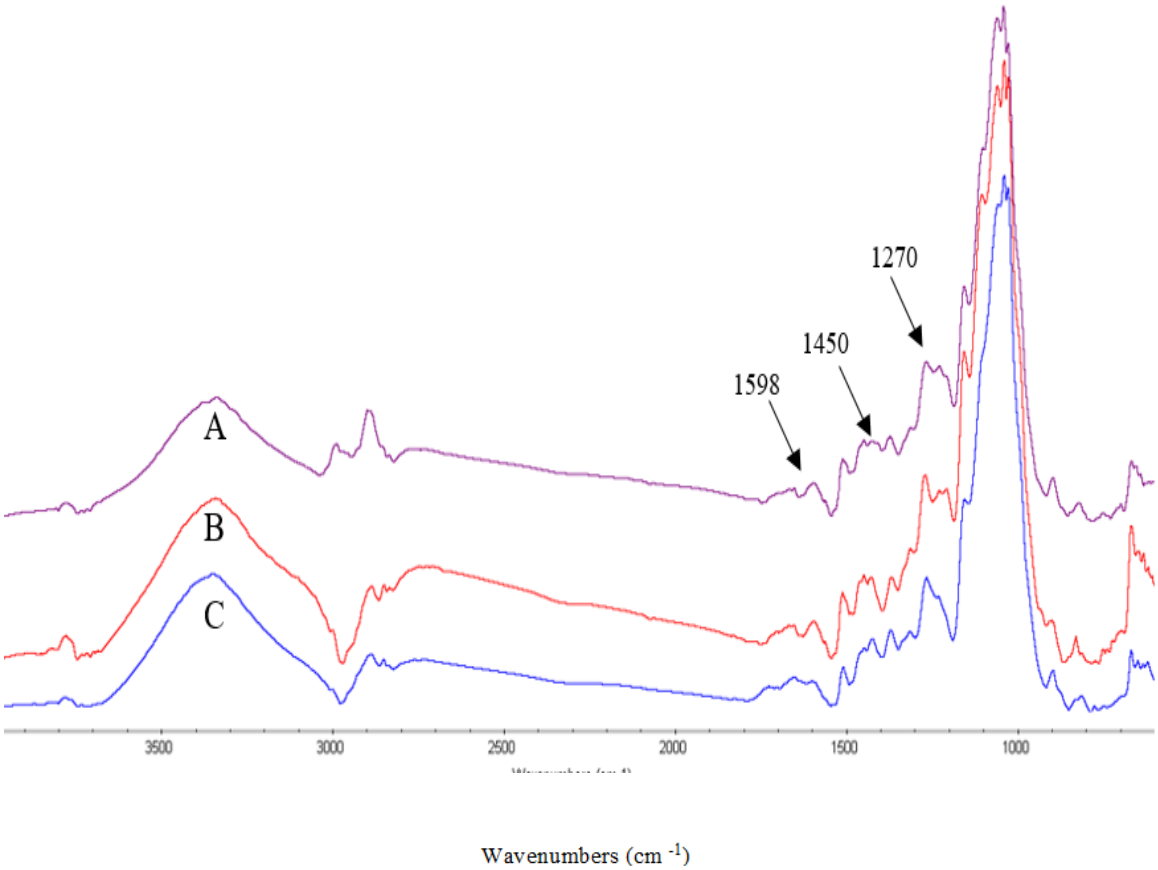


Figure 53 The Fourier transform infrared spectroscopy (FTIR) spectrums of (A) two stages sulfite (8% Na_2SO_3 followed by 3% H_2SO_4), (B) two stages bisulfite (8% $NaHSO_3$ followed by 2% H_2SO_4) and (C) traditional SO_2 catalyzed steam pretreated lodgepole pine.