High gravity fermentation of softwood derived sugar streams

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE
in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Forestry)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

June 2014

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Abstract

For a biochemically based biomass-to-ethanol process, one of the advantages of using softwoods as the substrate is the predominance of hexose sugars, which means that most of the sugars should be readily fermented by *Saccharomyces cerevisiae*. However, one of the biggest challenges with fermenting softwood derived sugars is the presence of both process derived and naturally occurring inhibitory compounds that are detrimental to both the growth and metabolism of yeasts. The presence of inhibitory compounds together with “low” initial sugar concentrations typically result in poor ethanol yields and titres which limit the economic viability of the process. In the work reported here, we tried to improve the fermentation of Douglas-fir derived sugar streams by enhancing the sugar concentration of the upstream processes (steam pretreatment and enzymatic hydrolysis) while using a combination of strategies to efficiently ferment the resulting liquor. These included the use of industrially relevant *Saccharomyces cerevisiae* strains, high yeast cell density, nutrient supplementation and liquor detoxification. To obtain as high a sugar concentration as possible, a high consistency steam pretreatment and subsequent enzymatic hydrolysis of the combined cellulose and hemicellulose fractions was carried out. Although this “softwood derived liquor” had a final sugar concentration of 18% wt/wt, it also had a very high concentration of inhibitory compounds including phenolics, furan derivatives and organic acids. When the fermentation profile obtained after growth on this liquor was compared to those obtained after growth on glucose and an enzymatically hydrolysed dissolving pulp, it was apparent that these inhibitory compounds severely restricted the growth and fermentation of all of the *S. cerevisiae* strains. Although the Tembec T2 strain that had previously been adapted to growth on spent sulfate liquor demonstrated the best fermentation performance, a detoxification stage was still required before reasonable (77.2%) ethanol yields could be obtained. Even with a prior detoxification stage, a high initial cell density of OD=13 was required before effective fermentation could be achieved. A combination of sulfate detoxification and high cell density fermentation resulted in a final ethanol concentration of about 5.0% (wt/vol) and volumetric productivity 4.9g/l/h.
Preface

This thesis is original, unpublished, independent work by the author, Seena Linoj Kumar. Seena was the lead researcher for all of the work reported in the thesis where she was responsible for all major areas of concept formation, experiments, data collection and analysis, as well as the thesis composition. Dr. Linoj Kumar and Dr. Maya Piddocke were involved in the early stages of concept formation. Dr. Jack Saddler, my supervisor was involved throughout the project in concept formation and thesis edits.
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List of units and abbreviations

ADH  Alcohol dehydrogenase
ALDH  Aldehyde dehydrogenase
α  alpha
β  beta
AD  absorbance detector
°C  degrees Celsius
Ca(OH)₂  Calcium hydroxide
CO₂  carbon dioxide
cm  centimeter
FAN  Free amino nitrogen
FID  Flame ionisation detector
FPB  Forest Products Biotechnology
FPU  filter paper units
ED  electrochemical detector
g  grams
g  acceleration due to gravity (i.e., 2000 x g)
GC  gas chromatography
GLK  glucokinase
h  hour
HCD  High cell density
HCl  hydrochloric acid
HOG  high osmolality glycerol pathway
H₂O₂  hydrogen peroxide
HG  high gravity
HXK  hexokinase
H₂SO₄  sulphuric acid
HMF  5-hydroxymethylfurfural
HPLC  high performance liquid chromatography
IU  international units
L  litre
LYCC 7442  Lallemand industrial yeast strain used in corn ethanol fermentations
M  molar concentration (moles per L)
MAPK  mitogen activated protein kinase pathway
NADH  Nicotinamide adenine dinucleotide (reduced)
MgSO₄.7H₂O  Magnesium sulfate hepta hydrate
min  minutes
mg  milligram
mL  millilitre
mm  millimetre
mM  millimolar concentration
NaOH  sodium hydroxide
Na₂CO₃  Sodium carbonate
Na₂SO₃  sodium sulfite
(NH₄)₂HPO₄  diammonium hydrogen phosphate
nm  nanometer
NREL  National Renewable Energy Laboratory
OD₆₀₀  optical density at 600 nm
p  para
PAD  pulsed amperometric detector
PDH  Pyruvate dehydrogenase
PDU  process development unit
PFK  6-phospho-fructo-2-kinase
rpm  revolutions per minute
Ro  Steam pretreatment severity factor
s  second
SHF  separate hydrolysis and fermentation
SO₂  sulphur dioxide
SSF  simultaneous saccharification and fermentation
SSL  spent sulphite liquor
t  time
T  temperature
TAPPI  Technical Association of the Pulp & Paper Industry
Tembec T2  spent sulfite liquor adapted industrial yeast strain
µL  microlitre
µm  micrometre
v/v  volume per volume
UV  ultraviolet radiation
VHG  very high gravity
w/v  weight per volume
w/w  Weight per weight
Yₑₒ₉(%)  percent theoretical ethanol yield
Y-1528  haploid yeast strain that preferentially ferments galactose
YP  culture media (1% glucose, 1% peptone, 0.5% yeast extract)
Acknowledgements

I would like to express my deepest appreciation and gratitude to my supervisor, Professor Jack Saddler for providing me with an excellent opportunity to do this work in his research group. Without his supervision and constant help, this dissertation would not have been possible. He has been a tremendous mentor and I have no words to express my thanks for his guidance, care, patience and above all his friendliness to both me and my family.

I would also like to thank my committee members Dr. Paul Bicho and Dr. Heather Trajano for providing their valuable and constructive advice on the thesis work.

Special thanks go to Dr. Maya Piddokke, Post-doctoral fellow, who was a great mentor and helping me with her advice in the lab and outside throughout my thesis work. I would also like to thank Dr. Nuwan Sella Kapu, Jinguang Hu, and Dr. Valdeir Arantes for their suggestions with my experiments in the lab. Finally, I would like to thank each and everyone in the Forest Products Biotechnology/Bioenergy research group for their great friendship and invaluable support throughout this thesis work.

Words cannot express how grateful I am to my husband, Linoj, who has stood by me all along this thesis work as well as my life. Your prayers and support for me was what sustained me thus far. I also thank my daughter, Ishika Kumar and my five months old son, Shivano Kumar for making my life bright and colourful. I thank my parents, sister and my brother for their continuous support and motivation in every step of my life. Without them, any of the accomplishments in my life would have been impossible!
1 Introduction

1.1 Background

Ethanol fermentation is one of the most ancient and well-established microbial processes in the world (Suarez-Lepe and Morata, 2012; Kitagaki et al., 2013). Ethanol has been historically used for drinking applications. However, during the industrial revolution, ethanol evolved into a chemical feedstock. Today, ethanol is increasingly being used as feedstock for fuels and chemicals (Amorim et al., 2010; Chum et al., 2014). Although the majority of today’s bioethanol is produced from the fermentation of starch and sugar-based feedstocks (e.g. corn, sugar cane, sugar beet), further expansion in ethanol production, to the levels needed for it to be used as a global fuel and chemical, means it is likely to be sourced from the world’s abundant lignocellulosic biomass (Cheng and Timilsina, 2011; Walker, 2011). Despite being nature’s largest source of carbohydrates, there are several technical challenges that need to be addressed for the efficient fermentation of lignocellulosic sugars to ethanol as compared to the fermentation of starch or sugar cane based feedstocks (Koppram et al., 2014). The overall goal of the work described in this thesis was to assess the influence of both naturally occurring and process-derived inhibitors in restricting the ethanol yield and titre during the fermentation of the sugar streams derived from softwoods, one of the most abundant lignocellulosic biomass sources in the Pacific North West.

The production of ethanol from softwoods generally consists of the four main steps of pretreatment, fractionation, enzymatic hydrolysis of the cellulose and fermentation. During the first steps, the polysaccharides present in biomass are converted to soluble monosaccharides, which can then be fermented downstream by the appropriate microorganisms to produce ethanol (Saddler et al., 1983). However, in addition to releasing sugars, both pretreatment and enzymatic hydrolysis also generates a range of compounds that are inhibitory to the fermentative microorganisms. The inhibitory compounds can be both process-derived or can be originally present in native softwoods and released during the processing (Azhar et al., 1981; Clark and Mackie., 1984; Palmqvist et al., 1999a). These
inhibitory compounds can suppress the growth and metabolism of microorganisms during fermentation, thus reducing the yield, titre and productivity of ethanol production (Almeida et al., 2007; Jonsson et al., 2013). This is one of the most severe challenges in efficiently fermenting sugars derived from lignocellulosic substrates as compared to starch and sugar cane fermentation. Characterising these materials and trying to derive ways to overcome the inhibitory effects of the compounds was one of the primary goals of the work reported in this thesis.

Among many pretreatment methods developed for biochemical conversion of lignocellulosic biomass, acid catalysed steam pretreatment has been shown to be highly efficient in both facilitating the recovery of hemicellulosic sugars in a monomeric form and in enhancing the enzymatic hydrolysis of the cellulosic component (Mackie et al., 1985; Bura et al., 2002; Ewanick et al., 2007; Galbe and Zacchi, 2012). The dissolution of the hemicellulose sugars in the water soluble fraction and their subsequent removal by washing enhances the accessibility of the cellulose to cellulase enzymes (Ohgren, et al., 2007; Varnai et al., 2010). The net result is good recovery of both the hemicellulose and cellulose derived sugars after steam pretreatment and subsequent enzymatic hydrolysis. However, the total sugar recovery will be dependent on the enzyme loading employed. At 20FPU/g cellulose enzyme loading, total recovery from softwoods was found to be close to 65% of the starting sugars even with an extensive washing of the substrate (Ewanick et al., 2011; Kumar et al., 2011). Sugar recovery can be increased to 80% by doubling the enzyme loadings (Kumar et al., 2012). The high hemicellulosic sugar recovery in the pretreatment liquid is generally achieved at milder pretreatment conditions and requires extensive washing of the water insoluble component (~20:1 water to biomass ratio) (Tengborg et al., 2001; Merino and Cherry, 2007; Galbe and Zacchi, 2012). This, together with the low consistency steam pretreatment employed in the previous work, significantly dilutes the pretreatment liquid leading to a very low (<4% w/v) sugar concentration. The low sugar concentration can lead a faster depletion of the sugars, which in combination with the presence of inhibitory compounds can cause high levels of stress to the fermenting microorganisms, thus reducing the overall ethanol yield and titre (Robinson et al., 2003; Helle et al., 2004). In addition to the stress to microbial growth, low sugar concentration corresponds to a low ethanol titre. For the distillation process and
ethanol recovery to be economically viable, a minimum ethanol concentration of fermentation broth should be 6%, which means >12% starting sugar concentration (Lin and Tanaka, 2006; Zacchi and Axelsson., 1989) indicating that the sugar concentration of the pretreatment liquid must be increased.

There have been several attempts to concentrate the pretreatment liquor. In the work by Robinson et al. (2003), the dilute sugar streams were concentrated by evaporation. But the sugar concentration levels were still quite low (<8%). Evaporating water is also more energy intensive than evaporating ethanol from the final fermentation broth. Subsequently, the authors enhanced sugar concentrations by spiking the original lignocellulosic sugar stream with pure sugars (Robinson et al., 2003, Liu et al, 2010). Although this strategy enhanced fermentation efficiency and ethanol titre, the approach was not realistic and the inhibitor concentration was not proportionately enriched. The authors subsequently enhanced the sugar concentration by spiking the water soluble fraction with the enzymatic hydrolysates of peroxide delignified substrate, which represented an almost pure cellulosic substrate with almost no inhibitors. However, the sugar concentration was still around 7-8%, which was substantially lower than what is typically used in the sugar and starch based ethanol industries (~30%). Although earlier work has tried to enhance the sugar concentration by increasing the concentration of cellulose used for enzymatic hydrolysis, this work has focused on using almost ‘pure’ cellulosic substrates such as kraft or organosolv pulps. Zhang et al. (2009) achieved a sugar concentration of ~20% from the hydrolysis of organosolv pretreated poplar and the fermentation of this liquor resulted in a 6% ethanol titre. However, the hemicellulose fraction was completely ignored in this work, which reduced the overall ethanol yield. In the work described in this thesis, we carried out both the steam pretreatment and enzymatic hydrolysis at high consistencies so as to enhance the final sugar concentration. Subsequently, we investigated how we could efficiently ferment the resulting “sugar rich” as well as “inhibitor containing” hydrolysate prepared from softwood.

Although the low concentration of sugars derived from the cellulose and hemicellulose is likely to be a significant challenge for the yeasts when fermenting a dilute pretreatment liquid, earlier work has shown that the inhibitory compounds can severely restrict
fermentation even at this low substrate concentration (Jonsson et al., 1998; Palmqvist and Hahn-Hagerdal., 2000a; Robinson et al., 2003; Liu et al., 2010). Thus, it can be anticipated that a greater inhibition will be encountered when fermenting more concentrated sugar streams. There have been several attempts to detoxify the pretreatment water soluble fraction to overcome the inhibition and enhance the efficiency of fermentation including physical, chemical and biological detoxification methods (Leonard et al., 1945; Roberto et al., 1991; Jonsson et al., 1998; Lee et al., 1999; Persson et al., 2002; Robinson et al., 2003; Carvalho et al., 2006; Alriksson et al., 2011; Cavka et al., 2011; Jonsson et al., 2013). While the physical techniques have tended to remove toxic compounds from the hydrolysate, the biological and chemical approaches usually convert the toxic compounds to less toxic products (Roberto et al., 1991; Jonsson et al., 1998; Lee et al., 1999; Persson et al., 2002; Robinson et al., 2003; Alriksson et al., 2011; Cavka et al., 2011; Jonsson et al., 2013). Although these methods have resulted in some success, so far, none of these methods has been able to simultaneously remove the wide range of biomass derived inhibitory compounds and attain a fast and complete fermentation of the biomass derived sugars. In addition, the efficiency of detoxification was found to be highly dependent on the pretreatment conditions, the type of substrate used, the robustness of microbial strain and the mode of fermentation (Palmqvist and Hahn-Hagerdal., 2000a; Jonsson et al., 2013).

Some groups have reported that the inhibitory compounds primarily restrict fermentation by limiting microbial growth rather than metabolic ethanol yield (Palmqvist and Hahn-Hagerdal., 2000a; Jonsson et al., 2013). Therefore, an alternative approach to detoxification could involve fermentation strategies for maintaining the cell viability and cell count to enhance the fermentation performance. Fermentation using a high cell density is widely practiced in starch and sugar cane industries to maintain active cells and obtain a fast fermentation of high gravity liquor with enhanced titre and productivity (Thomas and Ingledew., 1992; Godoy et al., 2008). Therefore, it is possible that a high cell density approach may work well in efficiently fermenting softwood derived sugar streams. It should be noted that in most of the previous work, the influence of detoxification has been investigated using low cell densities. Similarly, nutrient supplementation has been shown to be a promising
strategy in the starch and sugar cane industries for high gravity fermentation to enhance the productivity and maintain cell viability (Casey et al., 1984; Nguyen and Viet Man, 2009).

In addition to being inhibitor tolerant, the microorganisms used to ferment softwood derived sugar streams should also be able to simultaneously or at least consecutively ferment multiple sugars. Unlike starch and sugar cane derived glucose, lignocellulosic biomass contains five sugar sub-units, which act as the building blocks for hemicellulose. These sugars are both C6 sugars and C5 sugars including glucose, galactose and mannose (C6 sugars) and arabinose and xylose (C5 sugars) (Sjostrom, 1993). Therefore, efficient utilisation of the feedstock requires all of these multiple sugars to be effectively fermented to ethanol. Although the yeast, *Saccharomyces cerevisiae* is usually the preferred microorganism for industrial ethanol fermentations (due to its high robustness, low susceptibility to bacterial contamination and exceptional ethanol tolerance), the wild type yeast cannot ferment pentose (C5) sugars (Zaldivar et al., 2001; Hahn-Hagerdal., 2004). Compared to agricultural residues and hardwood, softwoods have the significant advantage of having a hemicellulosic component rich in C6 sugars (mannose, galactose and glucose) (Schwald et al., 1989). However, the uptake of galactose is usually delayed in *Saccharomyces cerevisiae* due to carbon catabolite repression (Zaldivar et al., 2001; Sanchez et al., 2010).

It is likely that applying detoxification alone would not be sufficient to result in efficient fermentation of “inhibitor-rich”, high gravity sugar streams derived from softwoods. Appropriate fermentation strategies using suitable microbial strains at high cell density may be equally important for obtaining high ethanol yield and titre. In the work described in the thesis, in addition to assessing various detoxification strategies, the possibility of using high cell densities and nutrient supplementation were also investigated using industrially robust strains of *S. cerevisiae*. Inhibitor tolerant *Saccharomyces cerevisiae* strains from Tembec Ltd. have been adapted to grow on the hexose sugars in spent sulfite liquor and they were compared to ethanol tolerant strains from Lallemand Inc., and a galactose fermenting strain received from the USDA Agricultural Service. As will be described in the main body of the thesis, a high gravity approach was used to grow the strains on sugars derived from Douglas-
fir wood chips which had been subjected to steam pretreatment and enzymatic hydrolysis to give a high sugar concentration (Keating et al., 2004a & b; 2006, Helle et al., 2003; 2004).

1.2 Softwoods as a bioethanol feedstock

Softwood species (conifers) represent one of the most abundant sources of cellulose on the planet. They are more evolutionarily primitive compared to hardwoods (angiosperms). Compared to hardwoods feedstocks, softwoods therefore have a more uniform cellular structure with more than 95% of the volume consisting of longitudinal tracheids (Sjostrom, 1993). Conifers currently constitute ~40% of the total growing stock in the world’s forest (FAO, 2010) and are particularly abundant in the Nordic countries, Canada and Russia where the taiga/boreal forest predominates. Softwoods are already a significant commercial commodity being the source of about 80% of the world’s timber, with traditional centers of production being the Baltic region (including Scandinavia and Russia) and North America (Ekstrom, 2012).

Softwoods have been shown to be one possible feedstock for an enzyme-based biomass-to-ethanol process (Mabbe et al., 2006). In addition to their abundance, it is worth noting that, compared to agricultural and hardwood-derived biomass sources, softwoods such as Lodgepole pine (*Pinus contorta*), Norway spruce (*Picea abies*), Western hemlock (*Tsuga heterophylla*), and Douglas-fir (*Pseudotsuga menziesii*), have the advantage of having a hemicellulose component that is primarily composed of hexose sugars. About 60 – 65% of a typical softwood is composed of C6 sugars including glucose, mannose and galactose whereas agricultural residues or hardwoods comprise less than 50% hexose sugars by weight and the rest of the carbohydrates are pentose sugars, primarily xylose and arabinose. It has been well established that the hexose sugars can be much more readily fermented to ethanol by conventional yeasts such as *Saccharomyces cerevisiae* (Matsushika et al., 2009; Olsson and HahnHagerdal, 1996) and they do not generally ferment C5 sugars (Hahn-Hagerdal and Pamment, 2004). Therefore, when using conventional microorganisms such as *Saccharomyces cerevisiae*, the theoretical ethanol yield for softwoods becomes significantly higher compared to that of hardwoods or agricultural residues (Olsson and HahnHagerdal, 1996; Matsushika et al., 2009). However, as was mentioned earlier, there are numerous challenges associated with
recovering softwood derived sugars in high concentration and subsequently fermenting those sugars to ethanol.

### 1.3 Overview of bioconversion and the need for steam pretreatment

The first step in the conversion of lignocellulose to ethanol is pretreatment. The pretreatment step disrupts the complex structure of lignocellulose to enable cellulase enzyme access to the cellulosic component. While doing so, the hemicellulose fraction is solubilised in liquid component. A subsequent enzymatic hydrolysis converts the insoluble cellulosic component to monomeric sugars followed by fermentation of both the hemicellulose and cellulose-derived sugars to ethanol (Olsen et al., 2012).

Although, no best option currently exists, SO$_2$ catalysed steam pretreatment and dilute acid pretreatment are two of the pretreatment processes closest to commercialisation (Sims et al., 2010; Galbe and Zacchi, 2012). Compared to other pretreatment methods, steam pretreatment is effective at recovering most of the hemicellulose sugars. Steam pretreatment provides good recovery of the hemicellulose derived sugars in the water soluble liquid stream, which can be relatively easily fermented by using traditional yeast strains. Use of an acid catalyst such as SO$_2$ during steam pretreatment facilitates the recovery of hemicellulose mostly in monomeric form. Other pretreatments such as organosolv/kraft pulping or ionic liquid pretreatments results in significant dissolution of lignin together with the hemicellulose fraction making the recovery and fermentation of hemicellulosic sugars extremely difficult (Pan et al., 2008). In addition to the recovery of hemicellulose, steam pretreatment also opens up the cellulose so that more effective enzymatic hydrolysis can occur as well as helping ensure sterile conditions required to minimise bacterial contamination.

In addition to being more complementary to the fermentation step, steam pretreatment has additional advantages of requiring limited capital, energy, and chemical inputs while being applicable to a wide range of biomass feedstocks including softwoods (Brownell and Saddler, 1984; 1987; Brownell et al., 1986; Holtzapple et al., 1989; Schwald et al., 1989a; Ramos et al., 1992a; 1992b; Bura et al., 2002; 2003; 2009; Hendriks and Zeeman, 2009; Kumar and Murthy, 2011). With woody feedstocks, steam pretreatment offers a unique
benefit of processing wood chips ‘as is’ without requiring a size reduction step before or after the process. The use of an acid catalyst also helps in this disintegration process. Moreover, direct steam injection on a dry substrate makes it possible to generate high consistency (high solids) slurries. Flexibility of controlling steam to biomass ration provides an opportunity to increase the sugar concentration, which is a pre-requisite for an enhanced ethanol titre in a downstream fermentation. Because of these various techno-economic benefits, almost all of the emerging pilot scale and demonstration bioconversion plants are using variations of steam pretreatment (IEA Task 39, 2011; Galbe and Zacchi, 2012). Despite many of these benefits, there are still numerous challenges associated with the conversion of steam pretreated substrates to ethanol (Wingren et al., 2003).

Early work on steam pretreatment was largely focussed on optimising the pretreatment conditions mostly for enhanced cellulose hydrolysis with little regard to the recovery of hemicellulosic sugars (Saddler et al., 1982; Saddler et al., 1983; Brownell and Saddler, 1984). This earlier work developed a parameter called the severity factor:

\[ Ro = \int_0^t e^{(T-100)/14.75} \, dt, \]  

This effectively combines the effects of time \( t \) and temperature \( T \) into a single parameter \( R_o \). These workers observed that, increasing the pretreatment severity generally enhanced the amenability of the cellulosic component to subsequent enzymatic hydrolysis. Later, the importance of recovering the hemicellulose and the need for the subsequent fermentation of these sugars was recognised as being essential to enhance the overall ethanol yields. Researchers subsequently realised that, although increasing the pretreatment severity had the beneficial effect of enhancing the enzymatic hydrolysis of the water insoluble, cellulosic rich fraction, the use of these high treatment severities also resulted in the degradation of the solubilized hemicellulosic sugars thereby diminishing the overall sugar recovery and the fermentability of the water soluble fraction (Schwald et al., 1989; Ramos et al., 1992; Wu et al., 1999; Boussaid et al., 1999). Thus, subsequent work recommended using a moderate severity as a compromise between optimizing hemicellulose recovery while ensuring reasonable cellulolytic digestibility of the water insoluble, cellulose rich component to obtain a
high overall sugar yield (Clark and Mackie, 1987; Boussaid et al., 2000; Ewanick et al., 2007). Despite obtaining a good overall recovery of both cellulose and hemicellulose derived sugars, some degree of hemicellulose degradation will always occur, even at moderate conditions. Although these studies focussed on the influence of treatment severity on sugar recovery, it was difficult to establish a quantitative relationship between the generation of inhibitors and severity factor. This is mainly because inhibitors are numerous and their formation involves a series of multiple reactions including volatilisation of some of the inhibitor components (Clark and Mackie, 1987; Olsen et al., 2012).

1.4 Challenges with fermenting steam pretreated softwood liquor

1.4.1 Low sugar concentration

A high ethanol titre is one of the pre-requisites for enhancing the economic viability of lignocellulosic ethanol. It has been shown that, doubling the ethanol concentration from 2.5 to 5% can result in a one third reduction in the energy required to distill a fermentation broth to high-purity ethanol (>93.5%) using conventional distillation techniques (Zacchi and Axelsson, 1989). For the distillation process to be economically viable, a minimum ethanol concentration of 6% is required, which means greater than 12% starting sugar concentration. The sugar concentration generally used in the sugar-cane and starch-based industries is 20 - 30% (w/v) (Devantier et al., 2005; Lin and Tanaka, 2006; Amorim et al., 2011). This can potentially generate a high ethanol titre of around 10-15% wt/vol ethanol (Lin and Tanaka, 2006; Amorim et al., 2011). In contrast, the lignocellulosic process streams are very dilute (<4% sugars) (Ewanick et al., 2007; Modenbach and Nokes, 2012) resulting a low ethanol titre (1-2%).

The primary reason for the low sugar concentration obtained in the lignocellulosic process streams are, a) the low solids consistency previously employed during pretreatment and enzymatic hydrolysis processes and, b) a solid-liquid separation and water washing generally employed between pretreatment and enzymatic hydrolysis steps. Both approaches were likely used in much of the previous work since the focus of these investigations was mostly to increase the sugar yield rather than the sugar concentration (Schwald et al., 1989;
Ewanick et al., 2007; Galbe and Zacchi, 2002). However, the sugar concentration of the steam pretreatment liquor needs to be substantially enhanced to obtain an overall high ethanol titre. In this work, we tried to increase the solids loading and to avoid a solid liquid separation and water washing step after the pretreatment step.

1.4.2 How to obtain a high sugar concentration in the softwood derived liquors after steam pretreatment?

Previous efforts to increase the sugar concentration of pretreatment/hydrolysis process streams had limited success (Robinson et al., 2003; Zhang et al., 2009; Liu, 2010; Modenbach and Nikes, 2011). In the study of Robinson et al.(2003), the pretreatment liquors derived from steam exploded Douglas-fir were concentrated by two physical concentration methods of rotary evaporation and freeze drying. The authors could reach up to 7% sugar hexose sugar concentration, still lower than what is generally employed in starch fermentation. In addition, evaporating water is more energy intensive than ethanol evaporation. Although the approach will not contribute much to improving the economic viability of the process, the authors have used this approach to investigate whether providing a minimum required sugar concentration can help maintaining the cell viability (Robinson et al., 2003). However, they noticed that by enhancing the sugar concentration, the fermentation rate decreased due to the proportionate accumulation of fermentation inhibitors (Robinson et al., 2003). These earlier workers subsequently supplemented the steam pretreatment liquor with glucose derived from the enzymatic hydrolysates of peroxide delignified substrate, which is an ‘ideal’ substrate with almost no inhibitors. Although this improved the fermentability, they only achieved a titre of about 2% ethanol as both the steam pretreatment liquor and enzymatic hydrolysates were very dilute (Robinson et al., 2003). Other work has looked at spiking the original lignocellulosic sugar stream with pure sugars (Robinson et al., 2003, Liu et al, 2010). Although this strategy enhanced ethanol production, the approach is not realistic as the inhibitor concentration was not proportionately enhanced.

There have been several studies looking at increasing the sugar concentration of enzymatic hydrolysates by enhancing the consistency of the slurry. However, such studies have mostly focused on using ‘pure’ cellulosic substrates such as kraft pulps or organosolv and
consequently, did not make efficient use of the hemicellulose component. Zhang et al. (2009) achieved a high sugar concentration of 16% from the hydrolysis of organosolv pretreated poplar. The fermentations of this liquor (enzymatic hydrolysate) resulted in a 6% ethanol titre, but the hemicellulosic fraction was ignored even though it represents almost 25% of the weight of the starting feedstock. This means that, although the authors were able to obtain a high ethanol concentration, this was achieved at the expense of a reduced ethanol yield. Maximum utilization of the entire substrate will be required for improved process economics and therefore, bioconversion strategies should focus on recovering both hemicellulose and cellulose derived sugars at high concentration and effectively fermenting them to obtain a high yield and concentration.

It would be desirable if we can concentrate the water soluble hemicellulosic sugars by substantially minimising the amount of condensate formed from the steam pretreatment step. However, all of the previous studies on the optimisation of steam pretreatment have been conducted at low biomass loadings leading to a high liquid to solid ratio of the resulting slurry of about 10-to-1 (Ewanick et al., 2007; Sipos et al., 2010). Even conducting the enzymatic hydrolysis of the combined fractions does not result in high enough sugar levels due to the extremely low sugar concentration of water soluble hemicelluloses in the pretreatment liquid. Therefore, a high pretreatment consistency, which applies a low amount of steam per weight of biomass, should be a focus of future work. Although such an approach would lead to high sugar concentration, the inhibitors are also expected to be proportionately enhanced. Therefore, it might be challenging to achieve efficient fermentation of this “realistic lignocellulosic substrate” containing high concentration of both sugars and inhibitors.

1.4.3 Presence of inhibitory compounds – Naturally occurring and process derived

During pretreatment and subsequent enzymatic hydrolysis of lignocellulosic biomass, certain compounds are released that are detrimental to the microbial growth during subsequent fermentation (Azhar et al., 1981; Delgenes et al., 1996). These inhibitory compounds can be naturally occurring and process derived (Table 1).
Wood naturally contains compounds that resist microbial attack and these are mostly present in the form of components that can be extracted with a combination of polar and non-polar organic solvents (Kurth and Becker, 1953; Haygreen and Bowyer, 1996). Naturally occurring extractives can be generally classified as a) terpenoids and steroids b) fats and waxes c) phenolic constituents. There are numerous and diverse types of inhibitory compounds in each of these categories. For example, there are ~7500 types of terpenoids and steroids identified in wood (Sjostrom, 1993). Depending on the frequency of isoprene subunits, they can be further classified as mono, sesqui, di, tri and poly terpenoids. Similarly, more than 30 different types of fats and waxes (both saturated and unsaturated) have been identified in softwoods with oleic, linoleic and pinolenic being the most common components. Phenolic extractives are also diverse and mostly present in the heart wood component. The most common phenolics are stilbenes, lignans, hydrolysable tannins, flavonoids and condensed tannins (Sjostrom, 1993).

In addition to the diverse and numerous naturally occurring inhibitory compounds, compounds produced during upstream processing (pretreatment and enzymatic hydrolysis) can also be detrimental to fermentation (Leonard et al., 1945; Sears et al., 1971; Wilson et al., 1989; Larsson et al., 1999a; Lee et al., 1999; Palmqvist and Hahn-Hagerdal, 2000; Luo et al., 2002; Sassner et al., 2008). They are generally classified as furan derivatives, organic acids and phenolics (Larsson et al., 1999a; Almeida et al., 2007). These are mostly the degradation products of carbohydrates and lignin components (Sears et al., 1971; Sassner et al., 2008). During steam pretreatment, dissolved sugars can be further degraded to furan derivatives (Cantarella et al., 2004) although the pretreatment process is typically optimised to minimise the formation of sugar degradation products (Wu et al., 1999).

While the hexose sugars are primarily degraded to hydroxy methyl furfural, pentose sugars are degraded to furfural (Dunlop, 1948; Popoff et al., 1976; Suortti, 1983; Parajo et al., 1998; Palmqvist and Hahn-Hagerdal, 2000; ) (Figure 1). The furan derivatives can also be further degraded to a range of organic acids including levulinic acid, acetic acid and formic acid (Weingarten et al., 2012; Megawati et al., 2011; Qi et al., 2008). Short to long chain organic acids can also be released as a consequence of the hydrolysis of fats and waxes (Kim,
Acetic acid can also be produced by the deacetylation of hemicellulose (Palmqvist and Hahn-Hagerdal, 2000). Although the depolymerisation of lignin during steam pretreatment largely occurs in the solid phase, these primary reactions are generally accompanied by the generation of diverse spectrum of soluble phenolic compounds (Adler, 1977), which are reported to be very detrimental to fermentation (Clark and Mackie, 1984; Bardet and Robert, 1985; Ando et al., 1986; Palmqvist and Hahn-Hagerdal, 2000a; 2000b; Jonsson et al., 2013) (Figure 1).

The variety and the concentration of inhibitory compounds vary greatly with the type and conditions of pretreatment and also the raw material used. For example, the abundance of furfural may be higher in agricultural residues and hardwoods compared to softwoods due to the higher concentration of pentoses in their hemicellulose fraction. Similarly, hardwood hemicellulose is generally more acetylated, which can lead to a higher organic acid concentration. Lignin structure also varies significantly between softwood, hardwoods and agricultural residues and therefore the type and concentration of soluble phenolics can also vary (Palmqvist and Hahn-Hagerdal, 2000a).
Table 1 Some of the common constituents of “naturally occurring” and “process derived” inhibitors present in the lignocellulosic hydrolysates

| Type of inhibitors | 
|--------------------|---|
| Terpenoids and steroids | a) Monoterpenoids: borneol, bornylacetate, 3-carene, camphene, limonene, β-myrcene, β-phellandrene, α-pinene, β-pinene, β-thujaplicin.  
b) Sesqui terpenoids: δ-cadinene, α-cadinol, α-cedrene, chanootin, juniperol, longifolene, α-murolene, nootkatin  
c) Diterpenoids: geranyl-linalool, β-Epimanool, cis-abienol, manoyloxdse, pimaral, pimarol, cembrene.  
d) Resin acids: pimaric acid, sandaracopimaricacid, isopimaricacid, abietic acid, levopimaric acid, palustric acid, neoabietic acid, dehydroabietic acid, lambertianic acid, communic acid, mercusiacid, secodehydroabietic acid.  
d) Steroids and triterpenoids: sitosterol, campesterol, sitostanol, citrostadienol, cycloartenol, betulinol, serratenediol.  
| Fats and waxes | a) Esters of; arachidic, behenic, eicosatrienoic acids, lignoceric, linoleic, linolenic, oleic, palmitic, pinolenic, stearic  
b) Esters of; arachinol, behenol, lignocerol  
| Phenolics | a) Stilbenes: pinosylvln  
b) Lignans: conidendrin, hydroxymatairesinol pinoresinol, plicatic acid, c) Hydrolyzable tannins: ellagic acid, gallic acid  
d) Flavonoids: chrysin, catechin, genistein, taxifolin  
| Furan derivatives | Furfural, 2-furoic acid, HMF  
| Weak acids | Acetic acid, formic acid, levulinic acid  
| Phenolic compounds | Coumaric acid, 4-hydroxy benzoic acid, Syringaldehyde, syringic acid, vanillin, |
Figure 1  A few of the typical degradation reactions of biomass components to a range of process derived inhibitory compounds (drawn based on reactions described in Almeida et al., 2007; Palmqvist and Hahn-Hagerdal, 2000a)
1.4.4 Mechanism of inhibition on yeasts

Although the degree of inhibition varies among different strains of yeasts and bacteria (Delgenes et al., 1996; Durre, 1998; Converti et al., 1999; Palmqvist and Hahn-Hagerdal, 2000b; Dien et al., 2003; Klinke et al., 2003; Brandberg et al., 2004; Klinke et al., 2004; Jeffries et al., 2007; Cann and Liao, 2008; Cho et al., 2009; Canilha et al., 2010) there is no microbial strain reported to date, which is entirely resistant to all types of inhibitory compounds present in lignocellulosic hydrolysates (Helle et al., 2003). The exact mechanism by which these compounds inhibit fermentation is not known, however, there are three proposed mechanisms: 1) chemical interference with the cell maintenance 2) direct inhibition of ethanol production pathway 3) osmotic pressure effect on the yeast cells (Palmqvist and Hahn-Hagerdal, 2000b; Liu et al., 2008; Liu et al., 2011).

The mechanisms by which different compounds inhibit fermentation vary with the structure of the chemical compounds (Figure 2). Some chemical functional groups were reported to be more toxic to yeasts. More specifically, much of the previous works have reported that aldehyde functional groups are more inhibitory relative to other functional groups such as carboxylic acids (Palmqvist and Hahn-Hagerdal, 2000b; Liu et al., 2005; 2008; 2009). Therefore, researchers have also attempted to categorise the inhibitory compounds in terms of their functional groups (Table 2) such as aldehydes, ketones, phenolics and carboxylic acids. Within this classification, low molecular weight compounds were proposed to be more detrimental to microbes than the high molecular weight compounds (Table 2) due to the higher rate of passive diffusion of smaller molecules across the cell membrane (Clark and Mackie, 1984).

Aldehyde derivatives were shown to inhibit microbial growth more than metabolic yield (yield of ethanol for every g of consumed sugar molecules) (Navarro, 1994). Previous work has shown that furan derivatives particularly furfural inhibit the dehydrogenase enzymes including alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH) both in vivo and in vitro measurements (Banerjee et al., 1981; Ulbricht et al., 1984; Sanchez et al., 1988; Modig et al., 2002) (Figure 2). Other work has also shown that furfural and HMF can be metabolised by S. cerevisiae to the corresponding alcohols and
this reduction process leads to a depletion of NADH (Liu et al., 2005; Petersson et al., 2006; Liu et al., 2008; 2009; 2011;). The depletion of NADH can in turn result in the accumulation of acetaldehyde. Redirection of yeast’s energy to fix the damage of reduced intracellular ATP and NADH levels are the inhibitory effects of furan derivatives. At higher concentrations (<15mM), the furan derivatives are also reported to damage plasmid DNA (Figure 2) (Khan and Hadi., 1993).

Organic acids inhibit the yeasts primarily in the undissociated form and therefore largely depend on the pKa of the corresponding acid (Jonsson et al., 2013). However, despite a lower pKa, formic acid was reported to be more detrimental compared to acetic or levulinic acid (Larsson et al., 1998). The higher toxicity of formic acid has been largely attributed to its low molecular size, which enables a faster molecular diffusion through the plasma membrane. This indicated that molecular weight also plays an important role together with the pKa of the acid. Undissociated organic acids are liposoluble and can enter the cytoplasm via diffusion through the cell membrane. Dissociation of the acids in the cytosolic pH can reduce the intracellular pH, which is maintained by pumping out protons through the plasma membrane via ATP hydrolysis (Stouthamer, 1979) (Figure 2). Continuous inflow of undissociated acids may either lead to eventual acidification of the cytosol or intracellular anion accumulation leading to cell death (Russel, 1992; Palmqvist and Hahn-Hagerdal, 2000b).

As the phenolic compounds are diverse and heterogeneous, it has been difficult to understand the exact mechanism of inhibition. Many of the lignin derived phenolics carry multiple functional groups and it has been difficult to attribute the inhibitory effects to a particular functional group. However, it was generally agreed that many of the phenolic compounds could potentially rupture the cell membrane and therefore influence the fraction of active cells and the cell growth (Verduyn et al., 1992). Weakly acidic phenols can also influence the electrochemical gradient across the cell membrane similar to that of other organic acids. In addition, the molecular weight, hydrophobicity, type and diversity of functional groups, including methoxylation are critical factors determining the extent of inhibition (Palmqvist and Hahn-Hagerdal, 2000b; Klinke et al., 2004; Almeida et al., 2007;
Jonsson et al., 2013;). Generally phenolic aldehydes were found to be more toxic compared to the corresponding acids.

It is apparent that although inhibitory compounds can restrict both the cell growth, viability and fermentation, growth and viability seems to be more sensitive than ethanol production (Palmqvist et al., 1999; Almeida et al., 2007; Stoutenburg et al., 2011). In addition, the degree of inhibition does not seem to be solely dependent on a particular class of inhibitory compound, but rather it is a cumulative and synergistic effect (Almeida et al., 2009; Canilha et al., 2010; Liu et al., 2011). It has been shown previously that even at a low concentration a mixture of inhibitors can be detrimental to yeast cells (Liu et al., 2004). However, almost all of these studies on inhibitors were done at low sugar and inhibitors concentrations (Boussaid et al., 1999; Robinson et al., 2003). Although, there were a few studies focused on higher inhibitor concentrations (10-20 mM syringaldehyde or vanillin and 15-30 mM furan derivatives etc.), the authors typically used pure glucose with added ‘synthetic’ inhibitors (Zaldivar et al., 1999., Persson et al., 2002; Liu, 2010).

The yeast, *Saccharomyces cerevisiae* is the preferred microorganism for industrial ethanol fermentation due to its robustness, low susceptibility to bacterial contamination and exceptional ethanol tolerance of <18% w/v with more than 90% of theoretical yield (Amorim et al., 2011; Lin and Tanaka, 2011). In the case of lignocellulosic ethanol fermentations, *S.cerevisiae’s* ability to tolerate inhibitory compounds depends on the strain and type and abundance of inhibitory compounds. Industrial polyploid strains, in general, are more tolerant to inhibitory compounds than laboratory strains. Although its growth can be suppressed, *S.cerevisiae* was reported to be generally tolerant to furans due to their innate ability to convert the furan aldehyde to corresponding alcohols or acids under both aerobic and anaerobic conditions (Taharzadeh et al., 1999; Liu et al., 2004; Nilsson et al., 2005; Peterssson et al., 2006; Liu et al., 2011). However, *S.cerevisiae’s* ability to assimilate furan derivatives is clearly strain dependent. Also, the concentration of inhibitors (10 – 120 Mm HMF will influence the different strains (Almeida et al., 2007). Concentrations from 15-50 mM for HMF have been reported in softwood hydrolysates depending on the pretreatment conditions used (Almeida et al., 2007). Under anaerobic conditions, furfural is first converted to furan...
methanol and further reduced to furoic acid whereas furan is oxidised to formic acid under aerobic conditions (Horvath et al., 2004; Taharzadeh et al., 1999). HMF is converted to 2, 5-bis-hydromethylfuran during fermentation (Taharzadeh et al., 1999; Liu et al., 2004; 2011). The presence of phenyl acrylic acid decarboxylase enables the yeasts to assimilate some of the phenolic inhibitors such as cinnamic, p-coumaric and ferulic acids (Goodey et al., 1982; Clausen et al., 1994). While several of the inhibitors can be metabolised by the yeasts in vivo, these metabolic actions can interfere with growth and productivity. More importantly, accumulation of non-metabolised inhibitors can destroy the functioning of the cells.

Despite several efforts using metabolic engineering to make the yeasts more resistant to lignocellulose derived inhibitors, none of the efforts have so far reached commercial success (Zaldivar et al., 2001; Helle et al., 2003; 2004; Almeida et al., 2007; Almeida and Hahn-Hagerdal, 2009; Dellomonaco et al., 2010; Sanda et al., 2011; Chandel et al., 2013; ). The inherent complexity involved with the wide variety of inhibitors makes it difficult to simulate the real situation using synthetic inhibitory compounds. It should be noted that the success of metabolic engineering depends on the knowledge of targets that should be altered or introduced in order to achieve a desired improvement. Inducing a genetic make up to tolerate a wide range of inhibitory compounds of varying properties while enabling the efficient uptake of multiple sugars present in the lignocellulose hydrolysate is incredibly complex (Zaldivar et al., 2001). Many of the genetic engineered strains that are tolerant to some of the inhibitors were shown to be far less efficient in fermenting multiple sugars and vice versa (Zaldivar et al., 2001; Helle et al., 2003; Wahlbom et al., 2003; Almeida et al., 2007; Almeida and Hahn-Hagerdal, 2009; Sanchez et al., 2010; Sanda et al., 2011; ). One of the possible alternatives to circumvent inhibitor problems is to improve the fermenting microorganism by long term adaptation to the inhibitors present in the lignocellulosic hydrolysates. Under laboratory conditions, evolutionary engineering is usually a phenomenon of long term adaptation of cells under selective pressure, where variants of cell populations with a selective advantage take over the population (Sauer, 2001). The advantage of using evolutionary engineering is that a detailed understanding of the action of inhibitors and their complex nature of interaction with biochemical networks is not needed. Rather than develop suitable yeast strains, researchers have also attempted to employ several detoxification strategies prior to fermentation (
Larsson et al., 1999; Palmqvist and Hahn-Hagerdal, 2000a; Almeida et al., 2007 Stoutenburg et al., 2011; Jonsson et al., 2013).
Table 2  Classification of compounds inhibitory to ethanol fermentation based on the functional groups, key examples, their structures and molecular weights (adapted from Liu and Blaschek, 2010; Almeida et al., 2007).

<table>
<thead>
<tr>
<th>Type inhibitors</th>
<th>Chemical structures</th>
<th>Common name</th>
<th>Molecular weight</th>
<th>Concentration range reported in softwood hydrolysates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldehydes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Furfural" /></td>
<td>Furfural</td>
<td>96.09</td>
<td>5 – 10 mM</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="HMF" /></td>
<td>HMF</td>
<td>126.11</td>
<td>15 – 50 mM</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Vanillin" /></td>
<td>Vanillin</td>
<td>152.15</td>
<td>0.8 mM</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Syringaldehyde" /></td>
<td>Syringaldehyde</td>
<td>182.17</td>
<td>0.6 mM</td>
</tr>
<tr>
<td>Ketones</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="4-hydroxyacetophenone" /></td>
<td>4-hydroxyacetophenone</td>
<td>136.15</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Acetovanillin" /></td>
<td>Acetovanillin</td>
<td>166.17</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Acetosyringone" /></td>
<td>Acetosyringone</td>
<td>196.2</td>
<td>---</td>
</tr>
<tr>
<td>Phenolics</td>
<td><img src="image" alt="Phenol" /></td>
<td>Phenol</td>
<td>94.11</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Catechol" /></td>
<td>Catechol</td>
<td>110.11</td>
<td>0.02 – 0.08 mM</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Guaiacol" /></td>
<td>Guaiacol</td>
<td>124.14</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Coniferyl alcohol" /></td>
<td>Coniferyl alcohol</td>
<td>180.2</td>
<td>0.2 – 0.3 mM</td>
</tr>
<tr>
<td>Organic acids</td>
<td><img src="image" alt="Acetic acid" /></td>
<td>Acetic acid (pKa – 4.75)</td>
<td>60.05</td>
<td>40 – 60 mM</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Formic acid" /></td>
<td>Formic acid (pKa – 3.75)</td>
<td>46.03</td>
<td>34 – 60 mM</td>
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<td></td>
<td><img src="image" alt="Levulinic acid" /></td>
<td>Levulinic acid (pKa – 4.6)</td>
<td>116.12</td>
<td>8 – 26 mM</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Gallic acid" /></td>
<td>Gallic acid (pKa – 4.41)</td>
<td>170.12</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td><img src="image" alt="Syringic acid" /></td>
<td>Syringic acid (pKa – 9.6)</td>
<td>198.17</td>
<td>---</td>
</tr>
</tbody>
</table>
Figure 2  A diagrammatic representation of the possible interference of inhibitory compounds on yeast cell growth and metabolism (modified from Liu et al., 2008; Pretorius, 2000).
1.4.5 Detoxification: An approach to overcome inhibition effects

Detoxification is the process of either removing the inhibitory compounds or modifying them to compounds that are less toxic to fermentation microorganisms (Jonsson et al., 2013). There have been several studies which looked at detoxifying the lignocellulosic process streams to overcome inhibition (Larsson et al., 1999; Carvalho et al., 2006; Cho et al., 2009; Jonsson et al., 2013; Lee et al., 1999; Nilvebrant et al., 2001; Stoutenburg et al., 2011). The degree of their success was highly dependent on the diversity and abundance of inhibitory compounds, mode of detoxification, robustness of the yeast strain and the fermentation conditions used (Nilvebrant et al., 2001; Rodrigues et al., 2001; Klinke et al., 2003; Cantarella et al., 2004; Klinke et al., 2004; Talebnia et al., 2005; Carvalho et al., 2006; Chandel et al., 2007; Caniha et al., 2012; Jonsson et al., 2013). Based on the mode of action, detoxification methods can be classified as physical, chemical and biological/biochemical detoxification (Palmqvist and Hahn-Hagerdal, 2000b; Jonsson et al., 2013).

Evaporation, membrane separation, adsorption using activated carbon/diatomaceous earth, ion exchange, solvent extractions are some of the common physical methods previously investigated (Lee et al., 1999; Robinson et al., 2003). A review of the literature indicates that there has been a mixed response to the effectiveness of these physical methods. Although evaporation was found to be efficient for the removal of volatile inhibitors including furan derivatives and some organic acids, this method can result in the accumulation of non-volatile inhibitors such as lignin derivatives (Robinson et al., 2003). Depending on the original concentration of non-volatile inhibitors, the evaporation methods can lead to either positive or negative results. Adsorption using activated carbon on the other hand was more efficient in removing inhibitory compounds particularly acetic acid and phenolics (Roberto et al., 1991; Rodrigues et al., 2001). However, a fraction of the sugars were also removed by this method (Roberto et al., 1991). The efficiency of solvent extraction is restricted to removing particular types of inhibitory compounds and largely depends on the type of solvent used (Parajo et al., 1997; Persson et al., 2002). The efficiency of ion exchange is also highly dependent on the type of resins used. It is unlikely that one type of resin can remove all of the inhibitory compounds due to their wide range of properties (Nilvebrant et al., 2001; Horvath et al., 2004). Anion
exchange generally removes the majority of the aliphatic acids, phenolics and Hibbert’s ketones (Horvath et al., 2004) whereas cation exchange is more efficient in removing furans and phenolics (Nilvebrant et al., 2001). Although a combination of resins can remove a majority of the inhibitory compounds, this method is costly due to the expensive resins and need for their regeneration (Palmqvist and Hahn-Hagerdal, 2000b; Nilvebrant et al., 2001; Jonsson et al., 2013).

Chemical detoxification involves the use of chemical reagents that either precipitate the inhibitory compounds or modify them in the soluble phase itself. Overliming is the most commonly used detoxification method by which the pH of the hydrolysate is raised above 10 (generally using Ca (OH)₂) followed by re-acidification to the fermentation pH (Roberto et al., 1991; Martinez et al., 2001; Persson et al., 2002; Horvath et al., 2005). Overliming was found to be highly effective in removing both furan and phenolic derivatives. It has been suggested that under highly alkaline conditions, aldehyde forms enolate ion, which is a reactive nucleophile and can readily undergo “aldol-like” reactions (Persson et al., 2002). Although these nucleophilic addition reactions can potentially reduce the concentration of aldehydes, the influence of resulting reaction products on yeast toxicity is not completely understood (Persson et al., 2002). Similarly, phenolic compounds readily form phenolate ion under alkaline conditions, which can results in a series of reactions including that of the side chain reactions of phenyl propane units. Despite the removal and modification of phenolics and aldehydes, the amount of organic acids mostly remains unchanged after overliming (Persson et al., 2002; Alriksson et al., 2006). Although the method is reported to be relatively inexpensive, the formation of significant amount of salts (gypsum in the case of sulfuric acid/SO₂ catalysed pretreatments) can pose issues related to their disposal (Mohagheghi et al., 2006). In addition, a decrease in the sugar concentration was also reported after over liming (Alriksson et al., 2006; Mohagheghi et al., 2006).

In situ detoxification using reducing agents is a recent advancement in this field and appears to have several promising traits (Alriksson et al., 2011; Cavka et al., 2011). The method was reported to overcome many challenges encountered in over liming such as sugar degradation, formation of precipitate, re-adjustment of the pH and extra processing steps.
The most commonly used and the most effective reducing agents are the sulfur oxyanions: sodium sulfite and sodium dithionite (Cavka et al., 2011). Although the complete mechanism of the high efficiency of reducing agents is not yet elucidated, sulfonation of phenolics and their subsequent reduction in hydrophobicity are proposed to be the major contributing factors towards the improvement effect (Jonsson et al., 2013).

Biological detoxification can be carried out by either directly employing the microbes (particularly fungi) to modify or assimilate the inhibitory compounds or by using enzymes to change the structure and composition of inhibitory compounds (Palmqvist et al., 1997; Jonsson et al., 1998; Martin et al., 2002; Lopez et al., 2004; Okuda et al., 2008). *Trichoderma reesei* and *Trametes versicolor* have been extensively studied for their ability to modify phenolics, furan derives and also organic acids (Palmqvist et al., 1997; Jonsson et al., 1998). Although a significant reduction in the inhibitory compounds can be achieved (~45 – 70%), a large amount of sugars (up to 35%) were also consumed by the fungi. The removal/modification of inhibitory compounds can be made more selective by using pure enzymes for detoxication. The enzymes used for detoxification were mostly ligninases; laccase and peroxidase produced by white rot basidiomycetes (Palmqvist et al., 1997; Jonsson et al., 1998; Palmqvist and Hahn-Hagerdal, 2000b; Jonsson et al., 2013). Oxidative polymerisation of phenolics to higher molecular weight compounds and reduction in the monomeric phenols were the major effects of enzyme treatment (Palmqvist and Hahn-Hagerdal, 2000b; Jonsson et al., 2013). Although environmentally friendly, the need for expensive enzymes and stabiliser chemicals makes it less attractive on an industrial scale (Jonsson et al., 1998; Johansson et al., 2011; Jonsson et al., 2013). In addition, despite long incubation time, furan derivates and organic acids are largely unaffected by this treatment (Palmqvist and Hahn-Hagerdal, 2000b).

It is also worth noting that almost all of the previous work on detoxification assessed the efficiency of detoxication on “very dilute” pretreatment hydrolysates. The challenges with inhibitors are expected to be considerably greater when using highly concentrated lignocellulosic process streams (Palmqvist et al., 2000b; Jonsson et al., 2013). Some of the *S. cerevisiae* strains developed by Tembec were shown to be able to ferment spent sulfite liquor (Helle et al., 2003; 2004). The spent sulfite liquor contains the similar spectrum of inhibitory
compounds as released from a typical pretreatment process for bioconversion and the spent sulfite liquor adapted strains are likely to be highly tolerant to inhibitors (Helle et al., 2003; 2004; Ewanick et al., 2007). However, their tolerance to higher amounts of inhibitors has not been well studied. We have used the Tembec T2 strain in this thesis work and assessed its ability to tolerate high concentration of inhibitory compounds in the high gravity softwood liquor.

Since inhibitory compounds present in lignocellulosic process streams are numerous and diverse, detoxification alone may not be a feasible approach in the commercial bioconversion process scheme (Ando et al., 1986; Stoutenburg et al., 2011). As will be described in more detail, one of the possible strategies to overcome inhibition is to employ high cell density (Chung et al., 1985; Cantarella et al., 2004; Jonsson et al., 2013). Since cell growth was found to be more sensitive to inhibitors than ethanol production, a high cell density approach appears to be a highly promising way to partially overcome some of the inhibitory effects while maintaining reasonable cell viability. It should be noted that the high cell density approach is widely practiced in the fermentation industry to enhance the ethanol titre within a short time (Amorim et al., 2011).

1.4.6 Presence of multiple sugars, their metabolism in yeasts and carbon catabolite repression

Although *Saccharomyces cerevisiae* was shown to thrive on a variety of carbon sources, it is apparent that glucose is its preferred carbon source (Larsson et al., 2001; Reijenga et al., 2001; Zaldivar et al., 2001; Robinson et al., 2003; Sanchez et al., 2010). In the case of softwood, the yeast has the potential to consume all three hexose sugars present in softwood hydrolysates (glucose, mannose and galactose). However, the presence of glucose represses the expression of genes required to catabolise mannose and particularly galactose, thereby, ensuring the immediate and full allocation of resources to the acquisition of glucose. The uptake of mannoses and galactose in particular is delayed as the result of this carbon catabolite repression (Zaldivar et al., 2001; Sanchez et al., 2010). The efficiency of simultaneous uptake of all of the sugars and conversion to ethanol varies considerably between strains.
Glucose uptake is characterized by rapid plasma membrane transport through a variety of transporter proteins with subsequent phosphorylation in the glycolytic pathway (Horak, 2013). Glucose and mannose have the same constitutive transport and phosphorylation systems (Ozcan and Johnston, 1999). However, hexose transport and phosphorylation is considerably distinct in galactose metabolism of yeasts compared to that of glucose or mannose (Frey, 1996; Holden et al., 2003; Sellick et al., 2008). Galactose is a C4-epimer of glucose meaning that they have opposite hydroxyl group orientations at the C4 carbon whereas mannose is a C2-epimer of glucose (Frey, 1996). Galactose requires the additional induction of permease for transport and also the enzymes of Leloir pathway (Douglas and Condie, 1954; Hawkins and Smalke, 2006; Sellick et al., 2008; ). Subsequent to transport, galactose enters the Leloir pathway, where galactose undergoes phosphorylation, group transfer, and epimerization prior to glycolysis. The key enzymes involved in this conversion are galactose mutarotase, galactokinase, galactose-1-phosphate uridyl transferase, UDP-galactose 4-epimerase, phosphoglucomutase. In the presence of glucose, genes encoding Leloir pathway enzymes (GAL genes) are completely repressed. However, fortunately, when glucose is absent and galactose is present, GAL genes are activated almost a thousand fold. The Leloir pathway is one of the few metabolic pathways in yeast that is regulated in a nearly 'all-or-nothing' mode depending on the presence of galactose and other sugars especially glucose. While inhibition of glucose and mannose on galactose utilisation is most common, in certain cases and in some strains of S. cerevisiae, galactose can also slow down glucose and mannose utilization. This occurs particularly with a prior acclimatisation to galactose. When galactose is the primary carbon source, this inhibition occurs to prevent the negative influence of glucose and mannose on galactose utilisation (Nevado et al., 1994).

While very few studies have been conducted on the catabolite repression of mannose, there is no consensus on whether or not catabolite repression exists with mannose (Larsson et al., 2001; Reijenga et al., 2001; Robinson et al., 2003). When S. cerevisiae is grown in a medium containing glucose, mannose and fructose, glucose gets metabolised first. The phosphorylation step has been attributed as the main factor that triggers catabolite repression in the presence of mannose, hence directly associated with the activities of hexokinase PI and PII, which phosphorylate glucose and mannose upon their entry to the cells.
(Nevado et al., 1994). Many authors reported that mannose utilisation is far more efficient in *S. cerevisiae* compared to galactose (Boles and Hollenberg, 1997; Reijenga et al., 2001). Some authors reported that glucose and mannose can be fermented with simultaneous and complete consumption of both sugars. Mannose consumption was found to be only slightly delayed, which could be attributed to the competition between common hexose transporters rather than catabolite repression (Boles and Hollenberg, 1997; Larsson et al., 2001; Reijenga et al., 2001; Okuda et al., 2008). Significantly higher consumption rates for glucose and mannose is indeed beneficial for fermenting the softwood derived sugar streams as mannose is present at three times higher concentration than galactose in the softwood hemicellulose (Sjostrom, 1993).

Due to the presence of multiple hexose sugars in softwoods, the development of an efficient microbial strain which can ferment all of the sugars is important for enhancing the ethanol titer in a lignocellulose-based fermentation industry. Previous work from our group has shown that a natural isolate of *S. cerevisiae* strain Y-1528 (Keating et al., 2004a; b) preferably utilised galactose over mannose and glucose and the strain was able to metabolise all three hexose sugars - glucose, galactose and mannose, while tolerating high sugar concentrations (Keating et al., 2004a; b). This particular strain appears to hold great promise for fermenting all of the C6 sugars released from certain lignocellulosic substrates, particularly softwoods (Keating et al., 2004a; b). However, the efficiency of the strain was only tested on pure sugar solutions and the performance of this strain on the ‘real’ lignocellulosic process stream is not known. It is possible that the strain may perform differently in a real pretreatment liquor/enzymatic hydrolysate due to the interference of the inhibitory compounds on their growth and metabolism. Since this strain had the promising feature of multiple sugar utilisation, we have also used this strain to assess the fermentability of high gravity softwood liquor.

### 1.4.7 Ethanol tolerance

In addition to the diverse spectrum of naturally occurring and process derived inhibitors, which are unique to lignocellulosic substrates, ethanol inhibition is a common problem encountered in the ethanol industry regardless of the choice of feedstock (Jones,
1989). This issue is more prominent in high gravity fermentations as the process leads to a higher ethanol concentration in the fermentation broth (Alper et al., 2006). In the case of lignocellulosic feedstocks, ethanol inhibition together with the previously discussed inhibitors would generate even higher levels of stress to the yeasts. The ethanol tolerance of the yeast is largely dependent on the yeast strain and the fermentation environment. Therefore, it is important to use yeast strains that are tolerant to high levels of ethanol concentration and facilitate an appropriate fermentation environment to overcome this inhibition.

It has been suggested that the cell membrane is the primary target of ethanol toxicity as ethanol has been shown to fluidize the cell membrane (Jones et al., 1987; Mishra et al., 1988; Lloyd et al., 1993). Increased membrane fluidity also results in uncontrolled transport of solutes that can decrease the proton flux across the membrane (Leao et al., 1984; Jimenez et al., 1988; Lentini et al., 2003) and cause leakage of amino acids and enzymes. Some work has also reported damage to mitochondrial DNA in yeast cells and inactivation of enzymes, such as hexokinase and dehydrogenases (Chi et al., 1999; Gibson et al., 2009).

*Saccharomyces cerevisiae* generally exhibit intrinsically high ethanol tolerance compared to other strains because of niche acclimation. The progressive increase in ethanol concentration in the fermentation broth provides an opportunity to yeasts for physiological adaptation to some extent (Watanabe et al., 2009). It is well established that the yeast cells which adapted to high levels of ethanol fermentation often show a higher degree of ethanol tolerance compared to the ones that has not been exposed. It has been reported that yeasts increase their ethanol tolerance by changing the fatty acid composition of the cell membrane (Heipieper et al., 1994; Chi and Arneborg, 1999). Yeasts that have membranes with increased proportions of unsaturated fatty acids and more complex lipid components can potentially minimize their membrane fluidity and thus are more tolerant to higher ethanol concentrations (Heipieper et al., 1994; Chi and Arneborg, 1999).

Since ethanol tolerance was expected to be an additional challenge in this particular work due to the high gravity lignocellulosic liquor used, we have attempted to use some of the robust industrial yeast strains that have been pre-exposed to high level of ethanol concentrations and thus have greater tolerance limits. However, cumulative or synergistic
inhibitory effect of lignocellulose derived inhibitors and high sugar/ethanol concentration is largely unknown. Therefore, although these industrial strains are generally robust in starch or sugar cane ethanol fermentations, it may be difficult for these strains to efficiently handle numerous stress factors simultaneously during the fermentation of high gravity lignocellulosic liquors.

1.5 Fermentation of lignocellulose derived sugars: Lessons from the sugar cane/starch ethanol industry

1.5.1 High gravity fermentation

High gravity fermentation is the fermentation of mash having high dissolved solids and thus has a very high starting sugar concentration (16-18°P) (Amorim et al., 2011). The concept was proposed in 1980s and widely employed in the brewing industry to drastically enhance the fermentation rate and overall ethanol titer in the final fermentation broth without major additional investment/infrastructure (Amorim et al., 2011; Koppram et al., 2014). The approach is particularly beneficial to making fuel ethanol as we need to obtain absolute alcohol (>93.5%) to be an effective substitute for gasoline and the distillation cost can be substantially minimised by increasing the ethanol concentration in the final fermentation broth (Amorim et al., 2011). In addition to reducing the distillation cost, the approach can prevent bacterial contamination and significantly decrease process water requirements and hence reduce the effluent treatment costs. Given these benefits, the ethanol industry is attempting even higher gravity (VHG, very high gravity fermentation) fermentation approaches (20 – 25°P) and the starch based fuel ethanol industry ferments a starting mash containing more than 27% dissolved solids in pilot scale demonstration plants (Thomas and Ingledew., 1992; Koppram et al., 2014). If it is possible to obtain a high sugar concentration in the lignocellulosic process stream, a high gravity fermentation approach holds great promise to obtain high ethanol yields and titers with minimum energy consumption and waste water generation while significantly reducing capital cost (Koppram et al., 2014).

Despite many advantages, there are several challenges with high gravity environment in the fermentation medium, which imparts high levels of stress to the fermenting microorganisms (Amorim et al., 2011; Koppram et al., 2014). Low water activity and higher
sugar concentrations potentially cause high osmotic pressure particularly at concentrations above yeasts’ tolerance limit (>30% wt/vol). Increased external osmolarities can lead to cell dehydration and collapse of the plasma membrane ion-gradients and a subsequent loss in cell viability. One of the major responses of the yeast to enhanced external solute concentration is the higher accumulation of glycerol through the activation of the high osmolarity glycerol pathway (HOG) (stimulation of 6-phosphofructo-2-kinase - PFK2), which essentially helps to prevent the cell collapse thus, facilitating the survival and proliferation of the cells (Petrovska et al., 1999). It has been reported that glycerol accumulation can be three times higher under conditions of the high osmotic stress (Petrovska et al., 1999). High osmotic pressure can also lead to an intracellular accumulation of ethanol, which inhibits the intracellular enzymes involved in ethanol production. Even with certain osmotolerant yeasts such as *Saccharomyces rouxii*, which thrives reasonably well on high sugar concentration, ethanol production was found to be significantly lowered. Additional stressful conditions that can be created by high osmolarity include nutrients limitation, especially free amino nitrogen (FAN) and lack of dissolved oxygen (Casey et al., 1984; Nguyen and Viet Man, 2009; Amorim et al., 2011). Therefore, high osmotic pressure can compromise both the cell viability as well as the metabolic ethanol yield. As was mentioned earlier, during the course of fermentation, enhanced ethanol concentration can also contribute to the inhibition of growth and decreased fermentation performance.

When lignocellulosic substrates are used as the feedstocks for high gravity fermentations the influence of all multiple stress factors can be anticipated such as the presence of high concentration of inhibitory compounds, the presence of multiple sugars and a lack of any in-situ nutrient availability (Figure 3). However, some of the process strategies typically used in the starch or sugarcane ethanol industry can be adapted to at least partially overcome some of these stress factors. For example, there have been several research efforts to better understand the yeast mechanisms for adapting to extreme conditions of osmolarity (Koppram et al., 2014). Various methods have subsequently been suggested to overcome these drawbacks including nutritional supplementation, the use of mutant yeast strains, more efficient aeration than conventional brewing, higher pitching rate/cell density and the use of
immobilized yeast (Casey et al., 1984; Barber et al., 2002 a; b; Erten et al., 2007; Nguyen and Viet Man, 2009).

Figure 3 Key stress factors anticipated during the fermentation of high gravity softwood hydrolysates (adapted from Puligundla et al., 2011).

1.5.2 Use of high cell density during high gravity fermentation

Increasing the initial cell density (increasing the pitching rate) is one of the most attractive methods currently practiced by the ethanol industry to overcome the stress associated with high gravity fermentation. Ethanol fermentation processes in Brazil use very high cell densities, in the range of 10-15% w/v on a fresh weight basis (Amorim et al., 2011). More than 90% of the yeast cells are reused from one fermentation cycle to another. This
approach enhanced the ethanol titre to 8-11% w/v within a period of six to 10 hours (Amorim et al., 2011).

Since the fermentation rate is directly linked to the active yeast count (Erten et al., 2007), high cell density (HCD) fermentation can potentially increase the number of viable cells in the culture leading to a higher sugar uptake and enhanced ethanol production rates (Suihko et al., 1993; Erten et al., 2006; (Nguyen and Viet Man, 2009). In addition, the use of high cell density can lead to a reduction in the lag phase and limited growth, thus more carbon will be utilized for ethanol production (Palmqvist et al., 1998; Kapu et al., 2013). In the brewing industry, it is generally agreed that, the higher the original gravity, the more yeast that is needed. A higher initial cell density would therefore imply a fast and complete fermentation. Some work has reported that increasing the cell density is more efficient than supplementing wort with osmoprotectants or nutrients (Tween 80/yeast extract) in terms of sugar utilization and ethanol production rates (Nguyen and Viet Man, 2009). Some other workers have reported that a combination of both high cell density and nutrient supplementation can significantly enhance the cell viability and fermentation performance (Suihko et al., 1993; Erten et al., 2007; Amorim et al., 2011). In the case of lignocellulosic fermentation in particular, a high cell density of yeast inoculum is expected to help partially overcome some inhibitory effects. Since cell growth was found to be typically more sensitive to inhibitors than ethanol production (Palmqvist and Hahn-Hagerdal, 2000), the use of high cell density could result in fast and efficient fermentation of high gravity lignocellulosic liquors. In some earlier work using traditional alcoholic fermentations, there were some limitations reported when employing high cell density such as higher yields of glycerol and other long chain alcohols such as 2-methyl-1-propanol and 2- and 3-methyl-1-butanol (Radler and Schütz, 1982). However, this observation has been primarily attributed to the type of strain employed rather than a function of inoculum size (Radler and Schütz, 1982).

1.5.3 Nutrient supplementation

Nutrient supplementation has been extensively reported to help maintain cell viability in high gravity fermentations (Casey et al., 1984; Barber et al., 2002 a; b; Dragone et al., 2003; Pereira et al., 2010). Nutrients can be osmoprotectants (such as ergo sterol, long chain fatty
acids/Tween, triglycerides/ vegetable oils, skimmed milk powder, chitin, calcium and magnesium salts, assimilatory nitrogen sources (such as urea, yeast extract, proline, glycine, betaine, glycine, tryptone, adenine-uracil-cytosine, ammonium salts, and soya flour), protein–lipid complexes or other additives (Barber et al., 2002 a&b). These nutrients either alone or in combination were shown to promote cell growth and metabolism in both laboratory and pilot scale trials (Barber et al., 2002 a&b; Dragone et al., 2003). Casey et al. (1984) reported that, with combined supplementation of ergosterol and oleic acid, ethanol concentrations of >16\% (w/v) could be achieved during very high gravity (VHG) fermentation. Ergosterol and unsaturated lipid content helps preventing interdigitation and maintain an optimal membrane thickness (Casey et al., 1984; Dragone et al., 2003). Use of osmoprotectants such as Tween 80 was also reported to overcome the additional side effects of high gravity fermentation such as the limitation of dissolved oxygen due to their surfactant effect. Supplementation of osmoprotectants was also found to significantly reduce the formation of byproduct glycerol in high gravity fermentations, apparently due to the significant reduction in hyperosmotic stress (Reddy et al., 2005).

Assimilatory nitrogen sources were also found to be highly effective in enhancing the cell viability and growth, thereby increasing the rate of ethanol production (Casey et al., 1984; Bafrncová et al., 1999; Devantier et al., 2005; Breisha, 2010; Pereira et al., 2010). Supplementation of the free amino nitrogen (FAN) was reported to result in enhanced substrate utilisation rates and an up to 20\% improvement in ethanol concentrations and an up to 50\% improvement in ethanol productivity during VHG fermentation while reducing the required fermentation time (Bafrncová et al., 1999; Pereira et al., 2010). It has been also reported that the extent of improvement due to nutrient supplementation is largely dependent on the initial cell density employed (Casey et al., 1984; Devantier et al., 2005). Yeast extract, urea and corn steep liquor are the mostly commonly studied nitrogen supplements. It has been shown that the addition of yeast extract together with thiamine enabled the complete consumption of 30\% sucrose generating 14\% w/v ethanol in the fermentation broth (Breisha, 2010). Other extensively used nitrogen sources are urea and corn steep liquor, which are relatively cheap and therefore widely used in the commercial corn ethanol industry (Pereira et al., 2010).
Although lignocellulosic feedstocks will contain some nutrients, the nitrogen may not be in a form which cannot be readily assimilated by yeasts. In addition, the availability of nutrients in lignocellulosic feedstocks can vary considerably depending on the substrate used. Agricultural residues will likely have a higher amount of nitrogen sources as well as calcium and magnesium salts. However, with woody feedstocks, the availability of these nutrients will most likely be limited and the external supplementation of nutrients may be required for effective fermentation. Jorgensen et al (2009) showed that supplementation of yeast extract, peptone, corn steep liquor and magnesium sulfate to wheat straw hydrolysates resulted in higher fermentation rates, higher ethanol yields and lower glycerol formation. However, high levels of nutrient supplementation will increase the processing cost and enhance the risk of contamination and therefore unlikely to be used in an industrial scale. In addition, these authors used a low inoculum size and a non-adapted Baker’s yeast compared to other studies and they also reported that supplementation of urea did not improve the fermentation rate (Jorgensen, 2009).

1.6 Research objectives and approach

The work described in this thesis covers the preparation of, as well as the efficient fermentation of, concentrated sugar streams from Douglas-fir wood chips. The goal was to achieve a high ethanol titre, yield and productivity. The thesis had three specific objectives. The first was to assess the general role of inhibitors in determining the fermentability of high gravity softwood liquor using selected strains of *Saccharomyces cerevisiae*. Second, we evaluated the role of increasing cell density of the best strain (selected from the previous comparison) and nutrient supplementation to overcome stress factors and enhance the overall ethanol yield and titre. The third objective was to assess whether a detoxification step is still required prior to the high gravity fermentation of softwood liquor when using high cell density to obtain efficient fermentation performance.

To assess the role of inhibitors in the high gravity fermentation of softwood sugar streams, we prepared two high gravity softwood liquors. One was a steam pretreatment liquor (containing hemicellulose and cellulose derived sugars) prepared from Douglas-fir wood
chips. This liquor was expected to contain high concentration of inhibitors, both naturally occurring and process derived that were released during steam pretreatment and subsequent enzymatic hydrolysis. The second liquor was derived from the enzymatic hydrolysis of softwood derived dissolving pulp (received from Tembec). The dissolving pulp was expected to be an almost pure cellulosic substrate and the liquor derived from this substrate was expected to contain a minimal amount of inhibitors. While the high gravity liquor prepared via steam pretreatment was used to represent a more realistic substrate, the dissolving pulp liquor was used as a model cellulosic substrate to compare against the inhibitor rich, steam pretreatment liquor.

In order to prepare high gravity liquor via steam pretreatment of Douglas-fir wood chips, we first assessed whether we could do a high consistency steam pretreatment. As was mentioned earlier, all previous work on steam pretreatment was conducted at very low biomass loadings. Therefore, we conducted some initial trials by progressively increasing the biomass to steam ratio and determining the overall sugar recovery and water soluble sugar concentration at each of the biomass loadings. The best substrate loading, which resulted in good overall hemicellulose recovery and concentration was selected and subsequently subjected to a direct enzymatic hydrolysis of the cellulosic component (at high enzyme loading) without any intermediate steps of solid-liquid separation and water washing. After enzymatic hydrolysis, the resulting slurry was filtered and the supernatant was used for fermentation. The dissolving pulp hydrolysate was prepared by conducting a high solids enzymatic hydrolysis of the substrate at high enzyme loadings. Since the sugars in both the liquors are expected to be concentrated, the inhibitory compounds, particularly in the steam pretreatment liquor was also anticipated to be proportionately enhanced.

For the high gravity fermentation of softwood liquors, we used three different yeast strains to assess the fermentability of steam pretreatment liquor and dissolving pulp hydrolysates. Although some bacteria such as *E.coli* have wider substrate preferences and higher substrate utilisation rates (Ingram et al., 1987; Ingram, 1990), we expected that the yeasts strains would still be far more relevant organisms even in the lignocellulosic fermentations in the industrial scale. Yeasts have many promising features such as their shear
stress tolerance, reasonably stable genetic composition, short generation times, intrinsically high sugar and ethanol tolerances, minimal generation of undesired metabolic by-products (due to well-controlled glycolysis) and a unique extracellular growth-permitting pH range (Amorim et al., 2011; Kitagaki et al., 2013). All of these factors make their selection for alcoholic fermentation favorable. In addition, some yeasts such as *Saccharomyces* are able to flocculate and therefore can be easily filtered out of culture broth, a useful feature for cell recycle fermentations and product recovery.

The three different strains of *Saccharomyces* used in this work are all known to be efficient in fermentation, but specialised in different aspects of importance to fermenting lignocellulosic substrates. One strain, the Tembec T2 is known to be inhibitor tolerant. However, it has not been tested for its ability to ferment liquors with high levels of toxicity (Robinson et al., 2003; Ewanick et al., 2007). The second one (Y-1528) has been shown to consume multiple sugars, however the strain’s ability to tolerate inhibitors is not known (Keating et al., 2004). The third strain (LYCC-7442) has been shown to have a high level of ethanol tolerance based on its use in the starch/sugar cane industry. All of these strains will be compared for their ability to perform efficiently in highly concentrated softwood derived liquors.

Subsequent to the comparison of yeast strains, we investigated the possible benefits of increased cell density in being able to successfully ferment softwood liquors. A high cell density approach has been widely employed in the fermentation industry to enhance ethanol titres. In addition, since inhibitors tend to predominantly affect cell growth, a high-cell density approach was expected to partially overcome the inhibition problems and enhance fermentation performance. Since the various stress factors, particularly the presence of inhibitory compounds, can potentially influence cell viability and vitality during high gravity fermentation, we also investigated whether nutrient supplementation could help maintain cell vitality and enhance fermentation performance.

Finally, we assessed whether a detoxification step is still needed for effective fermentation of softwood liquors. Although several previous reports employed a range of detoxification strategies, the effectiveness of these methods were typically tested using dilute
softwood liquors. In addition, fermentation of the detoxified liquor was conducted using very low cell densities. Therefore, the work reported in this thesis investigated the effectiveness of various detoxification approaches when treating a highly concentrated sugar stream. We primarily employed two different detoxification strategies including in-situ detoxification using sodium sulfite, and laccase treatment.
2 Materials and methods

2.1 Steam pretreatment

Douglas-fir (*Pseudotsuga menziesii*) wood chips were used as a substrate for steam pretreatment and subsequent preparation of high gravity liquor. The wood chips were made from 27-year-old Douglas-fir trees which had been grown in coastal regions of British Columbia (Kumar et al., 2011). All of the logs were debarked, split, chipped, and screened to approximately 2 cm x 2 cm x 0.5 cm. The moisture content of the wood chip samples was 10.2±0.9% and the chemical composition of the wood chips on a dry weight is given in the Table 3.

<table>
<thead>
<tr>
<th></th>
<th>Douglas-fir wood chips</th>
<th>Dissolving pulp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>47.3</td>
<td>91.1</td>
</tr>
<tr>
<td>Mannan</td>
<td>11.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Xylan</td>
<td>4.4</td>
<td>2.1</td>
</tr>
<tr>
<td>Galactan</td>
<td>2.2</td>
<td>---</td>
</tr>
<tr>
<td>Arabinan</td>
<td>1.2</td>
<td>---</td>
</tr>
<tr>
<td>Lignin</td>
<td>30.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Extractives</td>
<td>1.1</td>
<td>---</td>
</tr>
<tr>
<td>Total</td>
<td>98.2</td>
<td>96.4</td>
</tr>
</tbody>
</table>

Pretreatment of the wood chips with saturated steam was conducted in a 2.6L StakeTech steam gun at 200°C for 5 minutes and 4% SO₂ impregnation levels (Ewanick et al., 2007; Kumar et al., 2010). The steam gun consisted of a vertically mounted and insulated 316L stainless steel pipe closed at both top and bottom by ball valves (7.6 cm and 3.8 cm diameter opening respectively). The reactor was supplied with steam from a high pressure boiler and the steam was introduced through a narrow orifice (1.3 cm controlled by a steam supply valve) located almost in the centre of the reactor. Immediately before use, the gun was preheated to steam temperature by allowing it to stand filled with steam at the boiler pressure. It was then emptied and the substrates were immediately loaded by opening the upper ball valve directly into the preheated empty gun. Biomass loading ranged from...
50–300 g to achieve different levels of solids concentration in the pretreated substrate. After the reaction, the substrates were rapidly discharged into the receiving cyclone (~150 litres capacity) by the automated fast opening of the bottom ball valve. After collecting the majority of the slurry, the receiving cyclone was rinsed off with a minimal amount of water to collect the residual slurry separately, which was also processed in a similar fashion, to obtain a better overall mass closure (Bura et al., 2002).

After the pretreatment, the total weight of the slurry was determined. The solid and liquid fractions were separated by vacuum filtration using a Buchner funnel. The liquid fraction was analyzed for the carbohydrate concentration (both monomeric and oligomeric sugars) as described in section 2.5.1. A small, representative fraction of the solid sample was washed extensively with water and vacuum filtered to a final moisture content >60%. This water insoluble component, after drying and grinding was subjected to a Klason chemical compositional analysis as described in the section 2.5.1.

2.2 Preparation of the substrates for fermentation

2.2.1 High gravity liquor derived from steam pretreated Douglas-fir

The steam pretreated substrate with high dry matter content was directly subjected to enzymatic hydrolysis without any solid-liquid separation or water washing. Prior to conducting enzymatic hydrolysis, a small fraction of the steam pretreated substrate was diluted to make 2% slurry and the diluted slurry was subsequently titrated against 1M NaOH with the intermittent measurement of the pH and the amount of NaOH required for the increasing the pH value to 5 for a given amount of dry matter equivalent was calculated. For enzymatic hydrolysis, the wet weight corresponding to a 25% dry matter was directly weighed into a 2L Erlenmeyer flask followed by the addition of appropriate amount of water and the base (NaOH) until a consistency of 25% was obtained (~300 mL of additional water for a one litre reaction volume). After a thorough mixing in a vortex mixer, the appropriate amount of buffer was added to a final dry matter consistency of 25%. The whole mixture was subsequently stirred at 150 rpm in the controlled environment incubator shaker (New Brunswick Scientific Co., Edison, NJ, USA) at 50°C for 2 hours prior to the addition of the enzymes. The hydrolysis
was initiated by adding the enzymes (Ctec.2, Novozymes Inc.) at a loading of 60 filter paper
units (FPU) per g dry matter and the reaction was conducted for a period of 72 hours.

2.2.2 High gravity liquor derived from dissolving pulp liquor

Dissolving pulp was used as a control cellulosic substrate as it contained mostly
 cellulose. The initial dry matter content of the dissolving pulp was 41% which was diluted with
buffer and water to a final dry matter consistency of 25% prior to enzymatic hydrolysis. The
hydrolysis was conducted at an enzyme loading of 60 FPU/g cellulose in 50 mM acetate buffer
and the hydrolysis conditions were similar as was described for the steam pretreated
substrates.

After the enzymatic hydrolysis of steam pretreated substrate and the dissolving pulp,
the solid and liquid fractions of the slurry were separated by vacuum filtration using a Buchner
funnel. The resulting supernatants were used for fermentation experiments. The chemical
compositional analysis of each liquor was carried out as described in 2.5.

2.3 Microbial strains, pre-culture conditions and fermentation

Three industrial strains of *Saccharomyces cerevisiae*, Tembec strain T2, LYCC 7442 and
Y-1528 were compared. The strain T2 was provided by Tembec Limited (Temiscaming,
Quebec, Canada). The strain had been previously adapted to growth on spent sulfite liquor
and had been traditionally used by the sulfite mills to ferment the softwood derived,
hemicellulose hydrolysate (Helle et al., 2003). The Y-1528 was obtained from the US
Agricultural Research Center strain collection, (USDA, USA) and was previously shown to be
efficient in the effective fermentation of galactose (Keating et al., 2004). The strain LYCC 7442
was a traditional industrial yeast strain provided by Lallemand, Inc., (Québec, Canada) and was
shown to be effective in handling the high gravity liquors found in the corn ethanol industry.
All of the strains were able to ferment hexose sugars, but not the pentose sugars (Helle et al.,
2003; Keating et al., 2004).

All three yeast strains were maintained at 4°C on YPD agar plates containing 20 g/L
glucose (Sigma), 20 g/L peptone (Fisher), 10 g/L yeast extract (Fisher) and 18 g/L agar (Fisher).
The inoculum cultures were prepared by propagating the yeast at 30°C for two days. The cells were grown by transferring a single yeast colony to 5 mL of YPD media containing 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract in a sterile 50 mL Falcon tube and incubated overnight in a rotary shaker with constant shaking (at 150 rpm) at 30°C. About 1 mL of the pre-culture was subsequently transferred to a shake flask containing 800 mL of YPD media and incubated until an optical density (OD$_{600}$) of ~0.8 was reached (Bura et al., 2001; Ewanick et al., 2007).

The cells were harvested by centrifuging the media at 5000 rpm (3000g) at 4°C for 10 minutes. The collected pellets were washed three times with sterile deionized water to remove the residual media components. Subsequently, the cell pellet was diluted in distilled water to appropriate cell concentration required for fermentation experiments. 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). For fermentation, the pH of the sample was first adjusted to 5.5 using 50% NaOH and the flasks containing 20mL sample and the appropriate amount of yeasts were incubated at 30°C and 150 rpm in 100mL septa bottles. Initial yeast cell densities corresponding to an OD$_{600}$ of 2, 8, 13 and 20 were used in the fermentation experiments. The fermentation profiles of T2 strain in dissolving pulp and steam pretreatment liquor at all these cell densities were compared to that of the synthetic glucose medium. 200 µL samples were taken periodically over 48 h, centrifuged at 10,000 rpm (10621 g force) for 5 minutes, and the supernatant was immediately stored at -20°C until further use. The aliquots were analysed for ethanol and sugar concentration profiles and selected samples were also analysed 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, mannose, and galactose) 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:
where \( P_{\text{EtOH}} \) is the total amount of ethanol (g) formed in the fermentation and \( S_0 \) is the initial amount of C6 sugars (g) 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 first eight hours of fermentation:

\[
Q_{\text{EtOH}} = \frac{C(t_2) - C(t_1)}{t_2 - t_1}
\]

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

2.4 Detoxication methods

2.4.1 Laccase treatment

Laccase enzymes from the fungus, Trametes versicolor (Sigma) with a specific activity of 10 U/mg were used for the laccase detoxification experiments (Jonsson et al., 1998). An enzyme loading of 3 U/mL was employed in all experiments. 1 unit of enzyme activity is defined as the amount of enzyme which converts 1 mol catechol per minute at pH 4.5 and 25°C. After adjusting the pH to 5.5 using 50% NaOH, the samples were incubated with the equivalent amount of enzymes at 30°C for 12 hours in a rotary shaker at 90 rpm (Jonsson et al., 1998). Laccase treated samples were subsequently centrifuged and the supernatants were analyzed to quantify the concentration of inhibitory compounds.

2.4.2 Sodium sulfite treatment

Sulfite detoxification was carried out according to Alriksson et al. (2011). Sodium sulfite (\( \text{Na}_2\text{SO}_3 \)) (Sigma) was added to the steam pretreatment liquor to a final concentration of 15mM. 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 analysed to assess the concentration of inhibitory compounds.
Both the detoxified and non-detoxified samples were fermented in parallel using three different cell densities of OD, 2, 8 and 13 respectively.

2.5 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 0.5g/L \((\text{NH}_4\text{)}_2\text{HPO}_4\), 1g/L yeast extract, and 0.025g/L MgSO\(_4\).7H\(_2\)O (Ewanick et al., 2007). 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 100g/L \((\text{NH}_4\text{)}_2\text{HPO}_4\), 200g/L yeast extract, and 5g/L MgSO\(_4\).7H\(_2\)O (Jorgensen et al., 2009). The nutrients were added prior to 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 Analytical methods

2.5.1 Determination of carbohydrates

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\(\mu\)m HV filters (Millipore, MA, U.S.) and a volume of 20\(\mu\)L 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.

The water-insoluble components of steam pretreated Douglas-fir substrates were subjected to a Klason compositional analysis via a two stage acid hydrolysis (TAPPI, 2002;). Approximately 0.2-0.3 g oven dry weight of the ground sample (40-mesh) was added to 3 mL of 72% sulfuric acid. In this first stage acid hydrolysis, the sample was stirred every 10 minutes for 2h before being diluted to 4% acid concentration by the addition of 112ml of water. The diluted samples were subsequently transferred to an autoclave set at 121˚C for 1 hour for a second stage of acid hydrolysis. A batch of sugar standards was also autoclaved in parallel with the samples to correct for the hydrolysis loss factor. After cooling, the acid hydrolyzed samples were vacuum filtered through oven dried (105°C) and pre-weighed sintered glass crucibles (medium-coarseness). The filtrate was analyzed for sugars using HPLC as described above. The acid insoluble lignin recovered in the crucibles was rinsed with deionized water, subsequently dried in the hot air oven and amount of lignin was gravimetrically determined.

2.5.2 Determination of 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°C respectively. The oven was heated to 45°C for 2.5 minutes and the temperature was raised to 110°C at a rate of 20°C/min and later held at 110°C for 2
minutes. Standards were prepared using ethanol (Sigma) and butanol (0.5 g/L) (Fisher) was used as an internal standard.

2.5.3 Determination of inhibitory compounds

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 0.45μm syringe filter (Chromatographic Specialties, Brockville, Canada). 5 mM H₂SO₄ was used as an eluent at a flow rate of 0.6 ml/min.

The concentration of total phenolics in the high gravity liquors were quantified using Folin–Ciocalteu reagent (Sigma), as proposed by Singleton and Rossi (1965). A 100µL 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) Na₂CO₃ and the total volume was brought up to 5mL 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.
3 Results and discussion

For a biomass-to-ethanol conversion process to be economically viable, it is essential that maximum recovery of both the hemicellulose and cellulose derived sugars can be achieved and that these sugars can be effectively fermented (Humbird et al., 2011; Kazi et al., 2010). Compared to other lignocellulosic substrates, softwoods have a considerable advantage as they have a hemicellulosic component predominantly composed of C6 sugars which can be readily fermented by conventional *S. cerevisiae* strains (Olsson and Hahn-Hagerdal, 1996; Tengborg et al., 2001; Matsushika et al., 2009). Typically, more than 90% of the total carbohydrates present in softwoods are C6 sugars (Stenberg et al., 1998; Soderstrom et al., 2002; Ewanick et al., 2007; Monavari et al., 2009; 2011).

In order to recover as much of this fermentable sugars as possible, some form of pretreatment and fractionation is required. Although earlier work has shown that pretreatment processes such as organosolv can result in softwood substrates that are readily susceptible to subsequent enzymatic hydrolysis, the hemicellulosic sugars were difficult to recover from the solvent stream, which is rich in soluble lignin and extractives (Pan et al., 2008). In contrast, SO2 catalysed steam pretreatment resulted in the selective dissolution of the hemicellulose in the water soluble component leaving a water insoluble cellulosic component that can be readily hydrolysed to monomeric glucose in a subsequent enzymatic hydrolysis step (Brownell and Saddler, 1987; Clark and Mackie., 1987; Stenberg et al., 1998; Boussaid et al., 2000; Ewanick et al., 2007). Due to the higher susceptibility of hemicellulose derived sugars to thermal degradation, milder temperatures and shorter reaction times (lower severity) generally favour maximum dissolution and recovery of the hemicellulose derived sugars in the water soluble fraction with minimum sugar degradation. In contrast, harsher temperature and longer reaction time are usually required to achieve greater enzymatic digestibility of the cellulosic component (Boussaid et al., 1999; Ewanick et al., 2007; Kumar et al., 2011). Therefore, the optimum steam pretreatment conditions needed to achieve high overall sugar recovery are a compromise between the recovery of hemicellulose and the cellulose derived sugars. For softwoods, 200°C, 5 minutes and 4% SO2 were found to be the best set of compromised steam pretreatment conditions to achieve a high overall sugar yield.
(Ewanick et al., 2007; Kumar et al., 2010, 2011). However, despite this high overall sugar recovery, partly due to extensive washing of the steam pretreated material to recover the hemicellulose derived sugars, the final sugar concentration was always low, typically less than 5 %w/v (Ewanick et al., 2007; Kumar et al., 2010, 2011). One of the benefits of working with these low sugar concentrations was that both “naturally occurring” and “process derived” inhibitory compounds obtained after pretreatment were also low. However, for so-called “second generation” biomass-to-ethanol processes to ever compete with “first generation” processes, it is essential that the sugar and ethanol concentrations and titres are raised and that appropriate fermentation strategies are developed to enhance the fermentability of the inhibitor-rich sugar stream generated from softwood.

3.1 Preparation of high gravity softwood liquors and their chemical composition

One of the benefits of using steam as a pretreatment method is that there is a possibility to increase the consistency of pretreated substrate by controlling the ratio of biomass to steam injected. This is in contrast to other pretreatments where the water/solvent ratio is generally defined prior to the addition to the wood chips (for example, kraft pulping, organosolv, dilute acid etc.). The main reason why low sugar concentrations are typically obtained after steam pretreatment is that low consistency solids (low total solids) are usually employed in both the pretreatment and subsequent enzymatic hydrolysis processes steps (Modelbach and Nokes, 2011). As mentioned earlier, the steam condensate formed can significantly dilute the water soluble hemicellulosic sugars (<4%w/v) and this dilution can carry over to the subsequent enzymatic cellulose hydrolysis step.

The low consistency approach was likely used in much of the previous work since the focus of these investigations was mostly to increase the sugar yield rather than the sugar concentration (Schwald et al., 1989; Ewanick et al., 2007; Galbe and Zacchi, 2012). Other methods that were employed to enhance the sugar yield also compromised the sugar concentration in the pretreatment liquid and the enzymatic hydrolysates. For example, a solid liquid separation and water washing of the pretreated solid (water insoluble cellulosic component) was shown to enhance the sugar yields during enzymatic hydrolysis while
reducing the required enzyme loadings (Palmqvist et al., 1998; Tengborg et al., 2001; Merino and Cherry, 2007; Wiman et al., 2011). However, washing substantially dilutes the pretreatment liquid stream. Similar to the pretreatment step, majority of previous work has conducted enzymatic hydrolysis also at lower substrate concentrations as the primary goal was to enhance the sugar yields using reduced enzyme loadings (Schwald et al., 1989; Boussaid et al., 1999; Tengborg et al., 2001; Ewanick et al., 2007; Monavari et al., 2011; Galbe and Zacchi, 2012). Although some researchers have reported using high cellulosic substrate concentrations and subsequently obtained high sugar concentrations, this previous work typically ignored the water soluble hemicellulosic sugars (Pristavka et al., 1998; Roche et al., 2009; Zhang et al., 2009; Humbird et al., 2010; Lu et al., 2010; Di Risio et al., 2011; Olsen et al., 2011; Wiman et al., 2011; Ran et al., 2012; Wang et al., 2012). In the work reported within the thesis, we have increased the solids loading for both the steam pretreatment and subsequent enzymatic hydrolysis steps without any intermediate steps of solid liquid separation or water washing. Although this approach was expected to enhance both the sugar yield and concentration, we also anticipated that, by increasing the sugar concentration we would also, consequently, increase the concentration of inhibitors.

Unlike our previous steam pretreatment work which typically used less than 15% of the reactors volume capacity when loading in the biomass substrate (Schwald et al., 1989; Robinson et al., 2003; Ewanick et al., 2007), we tried to increase the biomass loading up to 80% of the reactor volume. The goal was to increase the ratio of biomass to the condensate formed and thus obtain high consistency slurry and a water soluble fraction containing a higher sugar concentration. We hoped that the production of a high consistency steam pretreated substrate would then enable us to conduct a direct enzymatic hydrolysis of the whole slurry without any significant dilution. However, it was likely that the increased the solids loading would reduce the amount of steam accessing the chips, thus influencing the mass transfer limitations of the process and consequently the overall sugar yield or recovery (fraction of the sugars recovered relative to the initial loading). Therefore, in the first part of the work, we carried out a preliminary investigation to determine the “near optimal” biomass loading which would give us the highest concentration of pretreated substrate without significantly compromising sugar recovery. We used 50, 200 and 300 g of biomass loading in
the steam reactor and each of the samples had 4% SO₂ added to it prior to pretreatment at 200°C for 5 minutes.

**Figure 4** Influence of increasing the substrate loading during steam pretreatment on the water soluble sugar concentration (% wt/wt), consistency (% wt/wt) of the resulting slurry and the recovery of hemicellulose (Sum of mannose, galactose, xylose and arabinose) from the Douglas-fir wood chips. Consistency of the slurry refer to the total amount of dry matter (both soluble and insoluble dry matter) contained in 100g of the pretreated slurry.
It was apparent that increasing the biomass loading resulted in an increased sugar concentration obtained in the water soluble fraction (Figure 4). Compared to the typically less than 4% sugar concentration obtained after low consistency steam pretreatment, high consistency steam pretreatment provided a 14% w/v sugar concentration in the water soluble fraction. However, despite this increase in sugar concentration, the higher biomass loading had a detrimental influence on the overall sugar recovery. Compared to the low biomass loading, which typically resulted in 82% recovery of hemicellulose derived sugars (fraction of hemicellulosic sugars recovered relative to the initial loading), the high biomass loading decreased the recovery of hemicellulose derived sugars to about 60% (Figure 4). This lower recovery of sugar suggested a greater extent of sugar degradation, indicating that a greater degree of severity had been encountered when using a high biomass loading approach with the reactor. It may also be possible that lowering the degree of condensation favoured a
higher extent of pyrolysis and other sugar degradation reactions and lesser extent of hydrolysis reactions thus diminishing the overall sugar recovery (Brownell et al., 1986). This was also evident by the degree of fractionation of the hemicellulose derived sugars between the water soluble and insoluble fractions after steam pretreatment (Figure 5). While some of the hemicellulose sugars were still present in the water insoluble component (~6%) after the low consistency pretreatment (Figure 5), almost all of the hemicellulosic sugars were solubilised after a high consistency pretreatment and they were detected as mostly monomeric sugars (>97%) (Figure 5).

Since medium biomass loading (200g wood chips) was found to be the best compromise between overall sugar recovery and consistency of the pretreated substrate, this concentration was chosen for subsequent enzymatic hydrolysis. After “medium consistency” steam pretreatment, the water soluble sugar concentration was 12% while at the same time we could recover 68% of the original hemicellulose derived sugars (Figure 4). We found that it was difficult to separate the hemicellulose rich water soluble fraction from the “semi-solid” using filtration as most of the water was tightly associated with the substrate. To try to resolve this issue we subsequently neutralised the substrate with alkali (1M NaOH) and added the required amount of sodium acetate buffer to bring it to a final dilution of 25% consistency and 50 mM final buffer concentration. We subsequently hydrolysed the substrate using a high enzyme loading of 60 FPU/g substrate (CTec. 2). This enabled faster liquefaction of the substrate, which resulted in a relatively high sugar concentration at the end of 72 hours of enzymatic hydrolysis (Table 4).

The high sugar concentration liquor derived from the dissolving pulp was prepared by carrying out enzymatic hydrolysis at a consistency of 25% using an enzyme loading of 60 FPU/g cellulose. The chemical composition of both the high sugar concentration liquors prepared from both the steam pretreated Douglas-fir and dissolving pulp are summarized in Table 4.

The results of sugar analysis showed that we were able to generate a softwood liquor with a very high sugar concentrations (Table 4). The total C6 sugar concentration was 17.7% while the dissolving pulp hydrolysate contained 16.3% total sugar concentration. As was mentioned earlier, previous authors have used significantly lower sugar concentrations when
fermenting steam pretreatment/dilute acid hydrolysates. Keating and coworkers (2004) used a water soluble fraction from Douglas-fir which contained 3.2% total sugar concentration including glucose, mannose and galactose. Similarly, the spent sulfite liquor, which these and other authors (Helle et al., 2003) used, also had a low sugar concentration. The softwood derived spent sulfite liquor contained primarily mannose and the total sugar concentration was 2%, which is far more dilute compared to the softwood liquors used in the work reported in this thesis (Helle et al., 2003).

As was expected, with an increase in the sugar concentration, the amount of probable inhibitors, which included acetic acid, furfural, hydroxymethylfurfural and phenolics, also increased significantly (Table 4). The total phenolic concentration detected was 64 mM, almost 4-6 times higher than that of the liquors used in previous work (Larsson et al., 2001; Robinson et al., 2003). The acetic acid concentration was also substantially higher than that reported by other workers with the softwood hydrolysates that they used (50 – 100 mM) (Larsson et al., 1999; Horvath et al., 2005).

Although the concentration of furan derivatives was higher (~25%) than the values reported by previous workers, their concentration did not increase as much the values for the phenolics or acetic acid. The low concentration of furfural could be attributed to the lower amount of C5 sugars present in softwoods, which is the main precursor molecule that would be dehydrated to furfural (Girisuta et al., 2006; Marzialetti et al., 2008). The HMF concentration was approximately 16mM (Table 4), which is 25% higher than the 12.7 mM reported previously by Robinson and coworkers (2003). These authors used steam pretreatment hydrolysate prepared by using a low biomass loading in the steam gun. However, given the higher biomass loading used in the current work, the HMF concentration was anticipated to be much higher. Relatively lower concentration of HMF and “very high” concentration of acetic acid (Table 4) indicated that the furan derivatives might have been further degraded to organic acids. It may also be possible that due to the lower amount of condensate formed during steam pretreatment, a major fraction of the HMF might have been volatilised during steam pretreatment (Qi et al., 2008; Megawati et al., 2011; Weingarten et al., 2012). As was expected, we did not observe any significant amount of inhibitors in
dissolving pulp hydrolysate except some acetic acid. The acetic acid concentration was likely not high enough to result in any detrimental impact on the fermentation microorganisms (Jonsson et al., 2013).

**Table 4** Chemical composition of high gravity softwood liquor prepared for fermentation

<table>
<thead>
<tr>
<th></th>
<th>Sugars(% wt/v)*</th>
<th>Inhibitors(mM)</th>
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<tbody>
<tr>
<td></td>
<td>Arabinose</td>
<td>Galactose</td>
</tr>
<tr>
<td>Steam pretreatment</td>
<td>0.2 (0.0)**</td>
<td>0.6 (0.0)</td>
</tr>
<tr>
<td>and enzymatic</td>
<td>14.5 (0.8)</td>
<td>0.8 (0.1)</td>
</tr>
<tr>
<td>hydrolysate*</td>
<td>2.6 (0.1)</td>
<td>216.5 (0.2)</td>
</tr>
<tr>
<td></td>
<td>15.9 (0.0)</td>
<td>1.04 (0.0)</td>
</tr>
<tr>
<td>Dissolving pulp</td>
<td>BDL*****</td>
<td>BDL</td>
</tr>
<tr>
<td>enzymatic</td>
<td>15.9 (0.3)</td>
<td>BDL</td>
</tr>
<tr>
<td>hydrolysate**</td>
<td>0.3 (0.0)</td>
<td>BDL</td>
</tr>
<tr>
<td>**</td>
<td>0.4 (0.1)</td>
<td>BDL</td>
</tr>
<tr>
<td>**</td>
<td>32.4 (1.1)</td>
<td>BDL</td>
</tr>
<tr>
<td>**</td>
<td>BDL</td>
<td>BDL</td>
</tr>
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</table>

*The hydrolysate was prepared by the direct enzymatic hydrolysis of steam pretreated Douglas-fir. The steam pretreatment was conducted at high consistency (~40%) and the resulting slurry had no free water. Enzymatic hydrolysis was conducted on this substrate without any solid-liquid separation or water washing.

**Values in the bracket represent standard deviations from the mean (n=3).

***The hydrolysate was prepared by the enzymatic hydrolysis of dissolving pulp at 25% consistency.

****Since “phenolics” is the mixture of different types of phenols, molecular weight of vanillin (152.15) is used for calculating mM concentration (Vanillin was used as a standard)

*****Below detectable level

3.2 Comparison of different microbial strains for their effectiveness in fermenting lignocellulosic process streams

We used three different yeast strains in this work including Tembec T2, LYCC 7442 and Y-1528. In order to assess the role of inhibitors, we compared the fermentation profile of an inhibitor rich liquor derived from steam pretreated softwood to that of a dissolving pulp enzymatic hydrolysate with almost no inhibitors.

These results showed that the inhibitory compounds had indeed a detrimental role in restricting the fermentability of the softwood derived sugar streams. This was apparent by the observation that all of the three yeast strains were able to ferment the dissolving pulp hydrolysate substantially better than the liquor derived from steam pretreated softwoods.
The liquor derived from the steam pretreated softwood had been shown to contain a much higher diversity and abundance of inhibitory compounds (Table 4) which likely restricted the fermentability of the liquor derived from steam pretreated softwoods compared to dissolving pulp hydrolysate, which had substantially less inhibitors present.

The fermentability of the softwood liquors was highly dependent on the yeast strain that was used as strains LYCC 7442 and Y-1528 produced less than 1% ethanol when grown on the steam pretreatment liquor (Figure 6b & 6c), while the Tembec T2 strain was able to ferment the liquor resulting in a final 4.2 % (w/v) ethanol titre (Figure 6a). Although the LYCC 7442 and Y-1528 strains had several promising features, it should be noted that these strains had not previously been adapted to growth in the presence of lignocellulose derived inhibitors. In contrast, Tembec T2 strain had been isolated after many months growth on spent sulfite liquor, which would have contained a similar spectrum of inhibitory compounds such as the acetic acid, extractives, lignin degradation products and sulfur compounds that have been detected in steam pretreatment liquors (Bura et al., 2001; Ewanick et al., 2007). Thus it was likely that the ability of the Tembec T2 strain to tolerate these types of inhibitory compounds enabled this strain to ferment the inhibitor rich sugar streams derived from steam pretreated softwood.

During the fermentation of dissolving pulp hydrolysate, the LYCC 7442 resulted in the highest ethanol titre (6.8%) within 48 hours followed by the Tembec T2 (6.5%) and Y-1528 (5.2%). Despite the poor performance of LYCC 7442 in steam pretreatment hydrolysate, the superior performance in dissolving pulp hydrolysate indicated the robustness of this strain to handle high sugar and ethanol concentrations, probably because this strain was previously employed in conventional corn fermentation systems (Figure 6a & 7a). The Tembec T2 was also able to effectively ferment the dissolving pulp hydrolysate due to the faster fermentation of glucose and mannose (Figure 7a & 7b). Although the final ethanol titre was slightly lower than that obtained with the LYCC 7442 strain, the T2 strain consumed glucose and mannose at a faster rate during the initial period of fermentation.
Surprisingly, although strain Y-1528 had been previously shown to have the ability to consume multiple sugars, the strain did not perform well, either in the dissolving pulp hydrolysate or the steam pretreatment liquor used in this particular work. As was expected, the galactose consumption was highest for strain Y-1528 and approximately 85% of the original galactose was consumed (Figure 7b). However, since the concentration of galactose was significantly lower compared to glucose and mannose in both softwood liquors, galactose consumption did not apparently result in any real improvement in the overall ethanol yield or titre. It appears that substantially lower uptake of glucose and mannose by Y-1528 compared to the other two strains resulted in a poor ethanol titre. Keating et al (2004) reported that, when fermenting Douglas-fir hydrolysate and spent sulfite liquor, despite a faster consumption of galactose, the uptake of glucose and mannose proceeded at a much lower rate compared to Tembec T1 strain. The authors recommended using a mixed culture fermentation to enhance the multiple sugar consumption and overall ethanol yield (Keating et al., 2004). Compared to the results obtained by Keating and coworkers (2004), the consumption of mannose and glucose was substantially lower in this work as well as when the Y-1528 was used alone. In Keating’s previous work, the performance of Y-1528 had been assessed on pure sugar medium or Douglas-fir hydrolysates and both of them contained a lower sugar concentration (<9 %). The much higher sugar concentration used in the work reported in this thesis might have resulted in feedback inhibition, influencing Y-1528, which together with the abundance of inhibitors led to a poor uptake of mannose and glucose compared to the other two yeast strains.

It is also interesting to note that although the Tembec T2 strain had been isolated from spent Sulphite Liquor, which contained many lignocellulose derived inhibitory compounds, the concentration of the known inhibitory compounds in the steam pretreatment liquor used in this work was considerably higher than that of the spent sulfite liquor (~8 times higher) (Nigam et al., 2001; Helle et al., 2008; Lai 2010). Despite this high concentration, the Tembec T2 strain was able to exhibit a good fermentation performance (Figure 6a). It should be noted that we have also used a relatively high cell density (OD=13) which might have also helped the T2 strain to overcome the challenges with inhibitors. Therefore, we next carried out a detailed
investigation of the influence of cell density on the fermentation of the highly gravity liquor derived from steam pretreated softwoods.
Figure 6  Possible influence of inhibitory compounds on the fermentation performance of a) Tembec T2 strain, b) LYCC 7442, c) Y-1528. The initial cell density was OD$_{600}$=13. Error bars represent deviations from the average of the two replicate runs.
Figure 7  Sugar consumption profiles of the three yeast strains during the fermentation of sugars derived from, a) dissolving pulp, b) steam pretreatment liquor. The initial cell density used was OD$_{600}=13$. Error bars represent deviations from the average of two replicate runs.
3.3 Influence of cell density on the fermentation of high gravity softwood liquor by T2 Tembec strain

To investigate the influence of increasing cell density on high gravity fermentation, we used the T2 Tembec strain at four different optical densities of 2, 8, 13 and 20 corresponding to approximately 1, 5, 9 and 13 g/l of yeast concentration respectively. Most of the previous work that has looked at the fermentation of lignocellulosic hydrolysates has mostly employed cell concentrations over a range of 1-5 g/l corresponding to an OD$_{600}$ of 3-6 (Bura et al., 2001; 2002; Ewanick et al., 2007; Larsson et al., 2001; Cantarella et al., 2004; Shen et al., 2012). However, as mentioned earlier, high cell density fermentation is widely practiced in starch and sugar cane industries to enable the fast and efficient fermentation of high gravity liquors (Wheals et al., 1999; Ueno et al., 2002; Lin and Tanaka, 2005; Amorim et al., 2010; 2011).

As was expected, by increasing the cell density this resulted in a significant improvement in the fermentability of all of the substrates (Figure 8 a-f). When using synthetic glucose medium, low and medium cell density fermentation resulted in a yield of 3 and 5% ethanol respectively after 48 hours. However, increasing the cell densities (OD$_{600}$) to 13 and 20 enabled a fast and complete fermentation within 48 hours, resulting in an ethanol concentration higher than 6% (Figure 8a). The fermentation performance of T2 when using the dissolving pulp hydrolysate was slightly lower than that of the synthetic sugar medium at low and medium cell densities. At low cell density, the total ethanol titre obtained for dissolving pulp hydrolysate was 3.9% compared to 4.4% obtained for synthetic sugar medium. This was probably due to the presence of multiple sugars and the presence of low levels of inhibitors (Figure 8c and 8d). However, the use of high cell density clearly enabled the yeast to overcome these challenges and the fermentation profile was close to that obtained after growth on the synthetic sugar medium (Figure 8c and 8d).

Increasing the cell density was most influential when trying to ferment the inhibitor-rich liquor derived from the steam pretreated Douglas-fir (Figure 8e and 8f). While the low cell density resulted in an ethanol titre of 4%, (after the fermentation of synthetic glucose medium or dissolving pulp hydrolysate, Figure 8a and 8c), the same cell concentration resulted in
<0.5% ethanol titre when using the steam pretreatment derived liquor. An inoculum size greater than OD$_{600}$ = 8 was needed to result in a reasonable fermentation performance when the steam pretreated biomass derived liquor was used (Figure 8e and 8f). As was discussed earlier, it is likely that the inhibitory compounds derived from the pretreated substrates limit cell growth, the number of viable cells and cell vitality (Olsson and Hahn-Hagerdal., 1993; Heipieper et al., 1994; Palmqvist et al., 1999a; Palmqvist et al., 2000; Larsson et al., 2001). Therefore, a high cell density approach should be used to maintain sufficient number of active cells to result in reasonable fermentation of softwood derived sugars. It has been documented in the literature that, to deal with stressful conditions that result in a faster yeast cell death, a higher inoculum size will help sustain a greater number of active cells, at least in the initial hours of fermentation (Palmqvist et al., 2000; Ueno et al., 2002; Jorgensen, 2009; Basso et al., 2011). Palmqvist and coworkers (1998) have shown that organic acid, furan derivatives and phenolics synergistically decreased the apparent specific growth rate, ethanol yield and biomass yield of *S. cerevisiae* during the fermentation of pure glucose medium. It has been reported that some of the biomass derived compounds, such as phenolics, can cause increased membrane fluidity resulting in rupturing of the microbial cells thus reducing cell growth and causing cell death (Heipieper et al., 1994). Larsson and coworkers (2001) observed little cell growth when they tried to cultivate yeast, *S. cerevisiae INVSC1* on lignocellulosic hydrolysates.

It appears that, when the microorganisms were already stressed due to the presence of inhibitors, the introduction of additional harmful factors resulted in a faster cell death. In addition to the inhibitory compounds, high sugar and ethanol concentration are recognised additional stress factors when using high gravity fermentation of lignocellulosic hydrolysates. Mohagheghi et al. (1992) reported that viability of yeast cells dropped to zero during the high gravity fermentation of pretreated wheat straw due to the combined effect of inhibitors and accumulating ethanol. In a similar work, Jorgensen et al. (2009) reported a loss of cell viability in the last 48 hours of simultaneous saccharification and fermentation at 35% dry matter. In their work on high gravity fermentation of pure sugars, Ueno and co-workers (2002) reported a significant reduction in the viable cell concentration. After 12 hours of fermentation, the initial viable cell count of 35 ($10^8$ cells/mL) decreased to 24 ($10^8$ cells/mL) and the number of...
viable cells almost completely disappeared after 24 hours, mostly due to the stress from the high ethanol concentration.

When the ethanol production rate, ethanol yield and the metabolic yield were summarised as a function of inoculum size and substrate (Table 5), as was expected, the rate of ethanol production was clearly stimulated in all of the substrates by increasing the cell density. This could again be attributed to the higher number of living cells at enhanced cell densities, which resulted in good amounts of the ethanol being produced in the early stages of fermentation. When high cell density is employed, there is also less of a lag phase and less of a carbon drain for growth and, as a consequence, more carbon can be used for ethanol production (Kapu et al., 2013; Melzoch et al., 1991 and Palmqvist et al., 1998). Even with the use of high cell density, the ethanol productivity and yield (both theoretical and metabolic yield) were still lower for steam pretreatment liquor compared to the synthetic sugar medium or dissolving pulp hydrolysates (Table 5). The ethanol productivity was 3.65 g/l/h when the steam pretreatment liquor was used as compared 4.98 g/l/h for the reference fermentation, indicating that fermentation proceeded at a much slower rate on the steam pretreatment liquor. Similarly, the ethanol yield from steam pretreatment liquor was only 67%, even after employing the highest cell density, compared to 88% for the reference fermentation of the synthetic glucose medium. All of these results suggested that, in addition to influencing the cell viability, the inhibitory compounds might have negatively influenced the extent of sugar uptake during fermentation. The lower metabolic yield compared to the fermentation of synthetic sugar medium also indicated that the inhibitory compounds might have interfered with the metabolic pathway of sugars to ethanol and resulted in a greater formation of byproducts such as glycerol (Abbott and Ingledew, 2004; Ingledew, 2012). For example, it has been shown that acetic acid can cause protons to accumulate inside the cell resulting in the conversion of dihydroxyacetone phosphate to glycerol (Ingledew et al., 2008).

The interference of inhibitory compounds in restricting the cell growth and metabolism was also evident by their high residual concentration in the fermentation broth even after 48 hours of fermentation using high cell density (Figure 9). Acetic acid and phenolics were still
detected in the fermentation broth at high concentration, which could still be the major limiting factors of fermentation (Figure 9).

These results suggested that, although the use of high cell density helped enhance the ethanol titre, this approach alone may not be sufficient to obtain a high enough ethanol yield and productivity. Since the nutrient supplementation was previously shown to be necessary in order to maintain cell viability and enhance the rate of fermentation in high gravity corn wort (Casey et al., 1984), we next investigated whether nutrient supplementation could also enhance the productivity and yield of high gravity fermentation of softwood liquors.
Figure 8  The influence of increasing cell density on the fermentation profile of synthetic sugar medium (a & b), dissolving pulp hydrolysate (c & d), and softwood liquor (e & f) using the yeast strain Tembec T2. Low, Medium, High, and Very high refer to the initial cell densities of $OD_{600} = 2, 8, 13$ and 20, respectively. Error bars represent deviations from the average of two replicate runs.
Table 5  The influence of cell density on ethanol yield and productivity after the fermentation of synthetic glucose medium, dissolving pulp hydrolysates and steam pretreatment liquors.

<table>
<thead>
<tr>
<th></th>
<th>Initial cell density</th>
<th>Ethanol yield (% theoretical)</th>
<th>Metabolic ethanol yield (g/g)</th>
<th>Volumetric ethanol productivity (g/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synthetic glucose medium</strong></td>
<td>Low</td>
<td>58.2</td>
<td>0.39</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>76.2</td>
<td>0.41</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>87.8</td>
<td>0.45</td>
<td>5.06</td>
</tr>
<tr>
<td></td>
<td>Very high</td>
<td>87.1</td>
<td>0.44</td>
<td>4.98</td>
</tr>
<tr>
<td><strong>Dissolving pulp hydrolysate</strong></td>
<td>Low</td>
<td>49.2</td>
<td>0.33</td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>68.4</td>
<td>0.37</td>
<td>3.59</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>82.6</td>
<td>0.43</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td>Very high</td>
<td>85.6</td>
<td>0.45</td>
<td>4.94</td>
</tr>
<tr>
<td><strong>Steam pretreatment liquor</strong></td>
<td>Low</td>
<td>10.9</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>40.0</td>
<td>0.31</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>67.2</td>
<td>0.37</td>
<td>3.01</td>
</tr>
<tr>
<td></td>
<td>Very high</td>
<td>67.0</td>
<td>0.37</td>
<td>3.65</td>
</tr>
</tbody>
</table>

Figure 9  The influence of high cell density fermentation (48hrs) on the concentration of inhibitory compounds present in the liquor derived from steam pretreated softwood. Error bars represent deviations from the average of two replicate runs.
3.4 Effect of nutrient supplementation on the fermentation of softwood liquors

When the high gravity liquor derived from the steam pretreatment of softwood was fermented with nutrient supplementation (100g/L (NH₄)₂HPO₄, 200g/L yeast extract, and 5g/L MgSO₄·7H₂O) there did not appear to be a substantial improvement in fermentation performance (Figure 10a and 10b) although the small degree of improvement seemed to be dependent on the initial cell density employed (Figure 10a and 7b). Interestingly, it seems that the nutrients had the highest influence at medium cell density compared to low and high cell density fermentation. The ethanol titre obtained at low cell density was 0.8 and 1% with and without nutrients respectively. Earlier work had reported that nutrients can help improve fermentation, mostly in the medium containing low cell density (Casey et al., 1984; Devantier et al., 2005). However, when using a low cell density with this particular substrate, the numbers of viable cells may be too low for nutrient supplementation to have any beneficial effect. It was also likely that nutrient uptake was severely restricted by the presence of inhibitors. It has been reported that inhibitors, particularly at higher acetic acid concentrations, can reduce ATP levels which can severely restrict nutrient transport (Ding et al., 2013). However, at medium cell density the nutrients seem to have helped maintain cell viability and helped enhance cell growth. The influence of nutrient supplementation was again marginal at high cell density, possibly implying that a large inoculum size can potentially limit the need for nutrient addition (Figure 10a and 10b). Previous work has also reported that at high cell density the numbers of viable cells may be high enough that the influence of nutrients becomes low particularly when adapted yeast strains are used (Casey et al., 1984; Jorgensen, 2009).

In contrast to the results obtained with the steam pretreated derived liquor, nutrient supplementation did influence the fermentation of the synthetic glucose medium (Figure 11a and 8b). As was expected, the effect was greatest when a lower cell density was used compared to the high cell density fermentation. When the synthetic glucose medium was used, there should be no inhibitors present, thus the nutrient supplementation helped maintain cell viability and facilitated cell growth when low cell density was used. At high cell densities, the extent of fermentation was almost the same with and without nutrient
supplementation indicating that a sufficient number of active cells were present. This seemed to minimise any beneficial influence that nutrient supplementation might have had on growth (Figure 11a and 11b; Figure 12a and 12b).

The ability of the nutrients to enhance ethanol yield or productivity was also negligible during the fermentation of the steam pretreatment liquor (Table 6). The ethanol yield at the highest cell density employed only increased by 2% after nutrient supplementation. While the metabolic ethanol yield remains unchanged, the ethanol productivity was only slightly improved from 3.7 to 4 g/l/h with the addition of nutrients (Table 6). In contrast, addition of nutrients enhanced the productivity of fermentation of pure glucose solution at both low and high cell densities (Table 7). At low cell density, nutrient supplementation resulted in a 90% improvement in ethanol productivity (4 g/l/h compared to 2.1 g/l/h) while at high cell density, the productivity was enhanced by 20%. This indicated a clear difference in the degree of influence that nutrients could make when either trying to ferment pure glucose or the steam pretreatment softwood liquor. In order to obtain adequate levels of ethanol production it is likely that detoxification of the steam pretreatment softwood liquor will be required prior to fermentation so as to enable effective uptake of the nutrients and enhance the viability and vitality of the cells.
Figure 10  Influence of different initial cell densities on the fermentation of softwood liquor with and without nutrient supplementation (Fermentation was conducted using T2 Tembec strain) a) ethanol production profile b) sugar consumption profile. Error bars represent deviations from the average of two replicate runs.
Figure 11 Influence of nutrient supplementation on the fermentation of synthetic glucose medium at low and very high cell densities of T2 Tembec strain a) ethanol production profile b) glucose consumption profile. Error bars represent deviations from the average of two replicate runs.

Figure 12 Influence of nutrient supplementation on the fermentation of synthetic medium containing glucose and mannose using T2 Tembec strain a) ethanol production profile b) glucose consumption profile. High cell density was used for fermentation. Error bars represent deviations from the average of two replicate runs.
Table 6  Influence of increasing cell density on the ethanol yield and productivity during the fermentation of softwood liquor with and without nutrient supplementation

<table>
<thead>
<tr>
<th>Cell density/nutrient supplementation</th>
<th>Ethanol yield (% theoretical)</th>
<th>Metabolic ethanol yield (g/g)</th>
<th>Volumetric ethanol productivity (g/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>10.8</td>
<td>0.27</td>
<td>0.3</td>
</tr>
<tr>
<td>Low + Nutrients</td>
<td>13.8</td>
<td>0.31</td>
<td>0.4</td>
</tr>
<tr>
<td>Medium</td>
<td>39.8</td>
<td>0.30</td>
<td>0.8</td>
</tr>
<tr>
<td>Medium + Nutrients</td>
<td>45.6</td>
<td>0.31</td>
<td>2.2</td>
</tr>
<tr>
<td>High</td>
<td>62.4</td>
<td>0.34</td>
<td>3.0</td>
</tr>
<tr>
<td>High + Nutrients</td>
<td>64.1</td>
<td>0.34</td>
<td>2.8</td>
</tr>
<tr>
<td>Very high</td>
<td>66.3</td>
<td>0.37</td>
<td>3.7</td>
</tr>
<tr>
<td>Very high + Nutrients</td>
<td>68.2</td>
<td>0.37</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Table 7 Influence of nutrient supplementation on the ethanol yield and productivity during the fermentation of synthetic glucose medium at low and high cell densities

<table>
<thead>
<tr>
<th>Cell density/nutrient supplementation</th>
<th>Ethanol yield (% theoretical)</th>
<th>Metabolic ethanol yield (g/g)</th>
<th>Volumetric ethanol productivity (g/l/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>56.4</td>
<td>0.38</td>
<td>2.1</td>
</tr>
<tr>
<td>Low + Nutrients</td>
<td>75.6</td>
<td>0.42</td>
<td>4.0</td>
</tr>
<tr>
<td>Very high</td>
<td>86.6</td>
<td>0.44</td>
<td>5.4</td>
</tr>
<tr>
<td>Very high + Nutrients</td>
<td>90.2</td>
<td>0.46</td>
<td>6.5</td>
</tr>
</tbody>
</table>
3.5 Is detoxification necessary for the effective fermentation of steam pretreatment softwood liquor?

As was mentioned earlier, some of the previous work in this area has attempted several detoxification strategies to overcome the inhibition effects of lignocellulosic hydrolysates (Carvalho et al., 2006; Palmqvist and Hahn-Hagedal, 2000). However, all of this work has been done using “dilute” liquors that contained sugars as well as inhibitors at comparatively low concentrations (Lee et al., 1999; Palmqvist and Hahn-Hagedal, 2000; Nilvebrant et al., 2001; Rodrigues et al., 2001; Luo et al., 2002; Robinson et al., 2003; Chandel et al., 2007; Ewanick et al., 2007). Therefore, we investigated whether the previously optimised detoxification methods would work effectively on the highly concentrated softwood produced in this thesis. Previous work had also assessed the influence of detoxification using relatively low cell densities (Jonsson et al., 1998; Larson et al., 1999; Okuda et al., 2008; Alriksson et al., 2011; Cavka et al., 2011). As we had already observed that increasing the cell density had a pronounced beneficial effect on the fermentability of high gravity softwood liquor, we wanted to assess whether detoxification would be necessary when fermenting the softwood liquor using a high cell density.

Two different detoxification strategies, a laccase and a sulfonation treatment, were compared on the high gravity liquor derived from steam pretreated softwood. Although these two methods had previously been reported to be effective in enhancing the fermentability of softwood hydrolysates, the biomass derived sugar liquor was at a comparatively low concentration (Jonsson et al., 1998; Larson et al., 1999; Alriksson et al., 2011; Cavka et al., 2011). We also assessed the fermentability of the detoxified liquor at three different cell densities, low, medium and high corresponding to an OD\textsubscript{600} of 2, 8 and 13, respectively (equivalent to a cell concentration of 1, 5, 9 g/l).

The results showed that, even with a prior detoxification step, the use of high cell density was necessary for the effective fermentation of the steam pretreatment liquor (Figure 13, a-f). Among the two detoxifications investigated, sulfite detoxication was found to be better than the laccase treatment (Figure 13, a-f). Laccase treatment was only effective at high cell density fermentation whereas sulfite treatment enhanced the fermentation performance.
at all of the cell densities employed (Figure 13, a-f). Although the sulfite treatment substantially improved the fermentation at low and medium cell densities, the final ethanol titre reached only 3.1 and 3.9% respectively. However, the high cell density fermentation, which did not employ any prior detoxification, resulted in a 4.5% ethanol titre. Applying a sulfite or laccase treatment prior to high cell density fermentation resulted in a 10% improvement in the final ethanol titre (Figure 13e).

Despite a fairly similar ethanol titre, the sulfite treatment resulted in a substantial increase in ethanol productivity during high cell density fermentation (Table 8). The productivity was enhanced by almost 60%, reaching 4.9 g/l/h compared to the 3.1 g/l/h obtained for the non-detoxified liquor (Table 8). It is likely that the sulfite treatment enhanced the maintenance of a high population of viable cells in the early hours of high cell density fermentation thus increasing the fermentation rate. In addition, the ethanol yield after the high cell density fermentation of detoxified liquor reached 77% of the maximum theoretical yield compared to 67% obtained for the non-detoxified liquor (Table 8). These results suggested that a combination of a detoxification step with a high cell density fermentation was an effective approach to ferment high gravity softwood liquor, resulting in a high ethanol titre, productivity and a reasonably high ethanol yield.

Although the ethanol yield was substantially enhanced by the detoxification followed by high cell density fermentation, the yield was still low, about ~11% lower than what we obtained after the fermentation of the reference glucose medium with a similar sugar concentration (Table 5, Table 8). Despite the complete consumption of the hexose sugars (Figure 13f), the lower theoretical ethanol yield together with the lower metabolic yield compared to the reference glucose medium, indicated that some of the sugars were converted to products other than ethanol. It is possible that the sulfur compounds present in the steam pretreatment hydrolysates, resulted in the greater formation of glycerol which may have been needed to maintain the cytosolic redox balance encountered under anaerobic conditions (Freeman and Donald, 1957 a;b; Wang et al., 2001). The formation of byproducts could also be one consequence of the stress response to the residual inhibitory compounds remaining even after detoxification (Olsson and Hahn-Hagerdal., 1993; Tao et al., 2012).
Therefore, future work should try to refine both the pretreatment and the detoxication methods to further minimise the effect of inhibitory compounds of the liquor and enhance the overall ethanol yield.

To try to assess the contributions that the detoxification and high cell density fermentation approach made to overcome the influence of inhibition, we measured the concentration of inhibitors after both the detoxification step and fermentation at high cell density (Figure 14a and 14b). As was reported previously, laccase treatment resulted in a significant reduction in the phenolic and furan derivatives present in the softwood derived liquors. While the concentration of the phenolic compounds decreased by about 70%, a 25% reduction in the HMF concentration was also observed (Figure 14a). Although the sulfite treatment did not reduce the concentration of phenolic compounds (Figure 14b), it has been shown that sodium sulfite can sulfonate inhibitory aromatic compounds and furan aldehydes at low temperature and detoxify them (Alriksson et al., 2011; Cavka et al., 2011). Although both the laccase and sulfite treatments decreased the detrimental effects of inhibitory compounds, complete removal of all of the inhibitory compounds was not achieved with either detoxification treatment. Therefore, a substantial amount of inhibitors are still likely to be present in the liquor, even after a detoxification, thus requiring the use of a high cell density approach during subsequent fermentation. It was apparent that the use of high cell density for the subsequent fermentation further helped assimilate a significant fraction of the remaining inhibitory compounds (Figure 14a and b). The ability of the yeasts to assimilate furan derivatives and phenolics in the sulfite detoxified liquor was indicated by a reduction in their concentration after the fermentation of detoxified liquor (Figure 14b). These results again confirmed that a combination of both an effective detoxification and a high cell density fermentation approach could potentially overcome the major inhibitory effects resulting in the relatively fast and efficient fermentation of steam pretreatment softwood derived liquor. Acetic acid was still present in high concentration even after a detoxification and fermentation steps. The significant improvement in the fermentation rate and yield obtained after the detoxification of steam pretreatment liquor albeit a high acetic acid concentration indicates their relatively lower impact compared to other types of inhibitors. Previous work on steam/dilute acid pretreated softwood has reported that compared to phenolics and furan
derivatives, acetic acid has a much lower inhibitory effect (Jönsson et al., 1998; Larsson et al., 1999a; Palmqvist and Hahn-Hagerdal, 2000b). The inhibitory effect of acetic acid has been primarily attributed to the uncoupling and intracellular anion accumulation particularly at pH values lower than their pKa of 4.76. We have carried out the fermentation at a pH of 5.5, higher than 4.76, therefore, the effective concentration of undissociated acetic acid, which interferes with fermentation must be lower (Larsson et al., 1999a). However, given the high concentration, it is apparent that any modifications in pretreatment/detoxication strategies, which will reduce the acetic acid concentration, might further improve the efficiency of fermentation.
Figure 13  The influence of detoxification on the fermentation of softwood derived liquor by the strain Tembec T2 at low initial cell density (a & b), medium cell density (c & d) and high cell density (e & f). Error bars represent deviations from the average of two replicate runs.
Table 8  The influence of increasing cell density on the ethanol yield and productivity during the fermentation of softwood derived liquor with and without detoxification.

<table>
<thead>
<tr>
<th>Cell density/method of detoxification</th>
<th>Ethanol yield (% theoretical)</th>
<th>Metabolic ethanol yield g/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-Low OD</td>
<td>11.1</td>
<td>0.25</td>
</tr>
<tr>
<td>Control-Medium OD</td>
<td>40.1</td>
<td>0.31</td>
</tr>
<tr>
<td>Control-High OD</td>
<td>67.3</td>
<td>0.37</td>
</tr>
<tr>
<td>Laccase-Low OD</td>
<td>6.1</td>
<td>0.20</td>
</tr>
<tr>
<td>Laccase-Medium OD</td>
<td>22.7</td>
<td>0.30</td>
</tr>
<tr>
<td>Laccase-High OD</td>
<td>73.6</td>
<td>0.38</td>
</tr>
<tr>
<td>Sulfite-Low OD</td>
<td>42.9</td>
<td>0.22</td>
</tr>
<tr>
<td>Sulfite-Medium OD</td>
<td>60.0</td>
<td>0.37</td>
</tr>
<tr>
<td>Sulfite-High OD</td>
<td>77.2</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Figure 14  The influence of detoxification and a subsequent high cell density fermentation on the concentration of inhibitory compounds present in the high gravity liquor derived from steam pretreated softwood a) laccase detoxification b) sulfite detoxification. Error bars represent deviations from the average of two replicate runs.
4 Conclusions

A highly efficient fermentation implies that good ethanol yield, productivity and concentration can be achieved. In the starch or sugar cane ethanol industries, the use of high initial sugar concentrations and high cell densities result in good ethanol titres and yields within a short period of time. Generally, ethanol titres of 8-11% w/v can be achieved within six to ten hours, resulting in a more than a 90% yield (Amorim et al., 2011). Although lignocellulosic feedstocks have been proposed as future feedstocks for sustainable bioethanol production, the efficient fermentation of lignocellulosic sugar streams to ethanol is considered to be quite complex due to several challenges such as low sugar concentration, the presence of multiple sugars and numerous inhibitory compounds, both naturally occurring and also derived from the upstream processes such as pretreatment. The work described in this thesis focussed on preparing a highly concentrated sugar streams from softwood as well as efficiently fermenting the resulting high gravity liquor to obtain a high ethanol titres, yields and productivities.

Most of the previous work on the fermentation of lignocellulosic substrates has been carried out using very dilute pretreatment or enzymatic hydrolysates. While the starch ethanol industry can generate high gravity sugar solutions (18-25%), previous work on lignocellulosics substrates used a maximum sugar concentration of 5-7%. This low initial sugar concentration generally resulted in the fast depletion of sugars in the initial stages of fermentation, which together with the presence of inhibitory compounds results in poor ethanol (<3% w/v) titres, productivities and yields.

Since a high sugar concentration is a pre-requisite to obtain a high ethanol titre, the first part of the thesis work looked at preparing a high gravity sugar stream from softwood by conducting a high consistency steam pretreatment followed by a high consistency enzymatic hydrolysis. The objective of the work was to obtain a highly concentrated water soluble hemicellulose during steam pretreatment without significantly compromising the high overall sugar yield. By increasing the biomass loading in the steam reactor we were able to minimise the formation of condensate for a given amount of substrate and therefore increase the consistency of the resulting slurry and concentrate the water soluble hemicellulosic sugars.
(14% w/v). However, this increased sugar concentration was obtained at the expense of decreased sugar recovery, likely due to the pyrolysis and degradation of hemicellulosic sugars due to the low amount of condensate formed during steam pretreatment. It was noted that the higher the substrate loading, the lower the hemicellulose recovery although an increase in the water soluble sugar concentration could be achieved. Medium substrate loading (200g wood chips in the steam reactor used in this work) was found to be the best compromise for obtaining a high concentration of water soluble hemicellulosic sugars while obtaining a reasonable hemicellulosic sugar recovery (~68%).

When the concentrated substrate obtained from the steam pretreatment was subsequently subjected to a direct enzymatic hydrolysis at high enzyme loading, a high gravity sugar solution (containing both hemicellulose and cellulose derived sugars) could be effectively prepared. We did not employ any intermediate processing steps of solid-liquid separation or water washing between the steam pretreatment and enzymatic hydrolysis, which enabled the carryover of all of the water soluble hemicellulosic sugars to enzymatic hydrolysis step. A high enzyme loading employed during the hydrolysis process helped to effectively liquefy and convert the insoluble cellulose to glucose, which was released to the already concentrated water soluble hemicellulose fraction, thereby making a highly concentrated sugar solution with a total of ~18% hexose sugars. This sugar concentration is very close to the high gravity solutions used in the starch/sugar cane ethanol concentrations. However, as mentioned earlier, a major problem anticipated with such high gravity lignocellulosic liquor was the presence of inhibitory compounds. A subsequent analysis of the inhibitory compounds indicated that compounds including furan derivatives, organic acids and phenolics were also concentrated during high consistency upstream processing. This increased concentration of inhibitors together with the high concentration of multiple sugars was expected to be problematic during the subsequent fermentation step to produce ethanol. In order to clearly assess the role of inhibitors, we prepared a high gravity liquor from dissolving pulp (derived from softwood) which should not contain as many inhibitors.

When we subsequently compared the fermentation profile of steam pretreatment liquor to that of dissolving pulp hydrolysate the role of inhibitors in restricting fermentation
was clearly evident in the fermentation performance of all of the strains. The yeasts (LYCC-7442, Tembec T2, and Y-1528) could ferment the dissolving pulp hydrolysate substantially better than steam pretreatment liquor, which had a much higher concentration of inhibitors. Since the concentration of galactose is significantly lower in softwood liquor compared to mannose and glucose, galactose consumption by Y-1528 did not result in any real improvement in ethanol yield or titre compared to the other strains. Only the T2 Tembec strain fermented the steam pretreatment liquor reasonably well reaching 4.2% ethanol titre. The better performance of the T2 Tembec strain could be attributed to the fact that the strain was previously exposed to the inhibitory compounds present in spent sulfite liquor for months. Although in a much lower concentration, the spent sulfite liquor contained similar spectrum of inhibitory compounds as in steam pretreatment hydrolysates. Interestingly, the T2 strain was also efficient in the simultaneous uptake of both glucose and mannose compared to other strains.

Since the steam pretreatment liquor was fermented fairly well by the T2 Tembec strain, we next investigated the influence of increasing the initial cell density of this strain on the fermentation performance. Since the previous work had shown that inhibitory compounds primarily limit the cell growth rather than the ethanol production (Palmqvist and Hahn-Hagerdal., 2000a), we expected that an increase in cell density would help improve the fermentability of steam pretreatment liquor. The results showed that increasing the cell density had substantial influence on enhancing the fermentability of inhibitor liquor and that a high initial cell density seemed to have helped maintain a greater number of active cells, thus enhancing both the rate and extent of fermentation. Increasing the cell density from low (OD=2) to a high (OD=20) increased the ethanol yields from 11% to 67% with an approximately 5-fold improvement in volumetric ethanol productivity.

Although high cell density helped improve the efficiency of fermentation the ethanol yield and productivity was still poor as compared to that of dissolving pulp hydrolysates, indicating that inhibitory compounds still play a critical role in limiting fermentation. Interestingly, the yield and productivity obtained with high cell density fermentation of dissolving pulp liquor was very close to that of synthetic glucose medium. It was likely that, in
addition to the detrimental influence on cell growth, the lower metabolic ethanol yield indicated that high sugar/ethanol concentration together with that of high concentration of inhibitory compounds resulted in the formation of byproducts such as glycerol during the fermentation of steam pretreatment liquor.

As it has been previously shown that nutrient supplementation helped to overcome osmotic stress and facilitate the cell proliferation and growth, we also investigated the influence of nutrient supplementation. The results indicated that extent of improvement by nutrient supplementation was highly dependent on the nature of the fermentation liquor and cell density employed. At low cell densities, nutrient supplementation did not help improve the fermentation of the liquor. It appears that at low cell density (OD$_{600}$=2), even if the nutrient supplementation can help to maintain the cell viability, the initial cell densities may too low to result in any significant growth or fermentation performance. The improvement effect of nutrients on steam pretreatment liquor was highest at the medium cell density employed (OD$_{600}$=8) resulting in a 6% improvement in the ethanol yield. Interestingly, at very high cell densities, the improvement effects were minimal on both the steam pretreatment liquor and synthetic glucose medium, probably because the number of viable cells was sufficient enough to mask any effect of nutrient supplementation.

Previous work had looked at several detoxification strategies with some degree of success. However, all of these studies have been carried out in dilute lignocellulosic hydrolysates, having low concentration of inhibitors as well as sugars. Therefore, we investigated the effectiveness of sulfite and laccase detoxification treatments in improving the fermentability of the steam treatment liquor. Among the two detoxification methods compared, detoxification with sulfite was more promising than laccase treatment.

A comparison of the fermentability of detoxified liquor and non-detoxified steam pretreatment liquor at various cell densities indicated that the use of high cell density is a prerequisite to obtain efficient fermentations regardless of a prior detoxification employed. At low and medium cell densities, sulfite detoxification could only achieve 40 and 60% ethanol yields within 48 hours corresponding to an ethanol titre of 3.1 and 3.9% respectively whereas high cell density alone enabled us to achieve a 67% ethanol yield with another 10%
improvement when applying a prior detoxification step. A combination of both a prior detoxification and use high cell density makes the fermentation of high gravity softwood liquor fast and efficient.

The work reported here showed that a combination of a prior detoxification and high cell density fermentation could provide a high ethanol titre (5%) when the combined hemicellulose and cellulose fractions obtained from pretreated softwoods were used as substrates. Future work should continue to refine the upstream processing steps to enhance the overall sugar recovery and minimise the formation of inhibitors. For example, it seemed that by increasing the biomass loading enhanced the severity of steam pretreatment, either due to the higher concentration of acid catalyst or the more direct interaction of the steam with the biomass prior to condensation. Therefore, when using a higher biomass loading, there may be an opportunity to use milder pretreatment conditions and lower catalyst loadings to enhance the overall sugar recovery. If enhanced sugar recovery could be obtained, this should result in less sugar degradation which would minimise the formation of inhibitory compounds. In addition, since the steam pretreatment is conducted at temperatures much higher than the boiling points of furan derivatives and furfural (boiling points of furfural and HMF are 162°C and 116°C, respectively), this could potentially lead to the “in-situ” volatilisation of most of these components. For enzymatic hydrolysis step, the current work used very high enzyme loadings and there is an opportunity to minimise this enzyme loading by optimising the suitable combination of enzyme cocktails, which are tolerant to both high sugar and inhibitor concentration. In addition, applying a lignin modifying/removing post treatment could also significantly enhance the cellulose accessibility of the enzymes while lowering the required enzyme loadings.
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