FERMENTATION OF HEMICELLULOSE RICH LIQUID FRACTION DERIVED FROM STEAM PRETREATED SOFTWOODS

by

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ABSTRACT

One of the main challenges in the bioconversion of lignocellulosics into bioethanol is to maximize the recovery of hemicellulosic sugars while increasing ethanol production through fermentation of these sugars. Steam pretreatment of Douglas-fir (DF) and Lodgepole pine (LPP) at a severity factor of $\log R_o = 3.64$ resulted in water soluble fractions (WSFs) containing monomeric hexose sugars up to 86 g/L. The crude WSFs were not fermentable by four yeast strains: T_1 and T_2 (spent sulfite liquor adapted strains), Y1528 (haploid strain that preferentially ferment galactose first) and BY4742 (haploid laboratory strain). Dilution of fermentation inhibitors in crude WSFs led to appreciable improvements in their fermentability, especially by the SSL-adapted yeast strain T_2.

The four yeast strains were tested against several model furan and phenolic compounds to examine their tolerance to these fermentation inhibitors. All four yeast strains produced comparable ethanol productivity when 3 g/L of HMF or 0.8 g/L of furfural were added to medium containing 2% glucose. However, T_1 and T_2 exhibited higher ethanol productivity compared to Y1528 and BY4742 when 5 g/L of 4-hydroxybenzoic acid and 5 g/L of vanillic acid were added to media as supplements. This provides evidence that the robustness of SSL-adapted T_1 and T_2 yeast strains probably originates from their tolerance to certain phenolic compounds.

Overliming improved ethanol production from Douglas-fir 1 (DF1) WSF by T_2 from 1.7 g/L to 13 g/L. When DF1 WSF was spiked with glucose up to 100 g/L, it produced ethanol yields similar to that of the glucose reference fermentation media. Since it is not

practical to spike WSF with glucose in an industrial process, we investigated the applicability of separate hydrolysis and fermentation (SHF) and hybrid hydrolysis and fermentation (HHF) with the whole slurry to achieve higher initial fermentable sugar concentration. SHF of combined WSF and hydrolysates recovered after enzymatic hydrolysis of water insoluble fraction (WIF) by T_2 produced up to 90% ethanol yield. HHF produced ethanol concentrations comparable to those of SHF with or without overliming. This result indicated that SHF and HHF of the whole slurry can help improve the fermentability of WSFs.

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%	percent
µL	microlitre
BY4742	haploid laboratory yeast strain derivative of S288C strain
$Ca(OH)_2$	calcium hydroxide
CBU	cellobiase unit
CO_2	carbon dioxide
Csc	consistency
DF	Douglas-Fir
FPU	filter paper unit
g	gram(s)
GC	gas chromatography
h	hour
H_2SO_4	sulphuric acid
HHF	hybrid hydrolysis and fermentation
HMF	5-hydroxymethyl furfural
HPLC	high performance liquid chromatography
l (L)	litre
R_o	severity factor
LPP	Lodgepole pine
M	molar
min	minute
mL	millilitre

mM	millimolar
MTBE	Methyl tertiary butyl ether
NaOH	sodium hydroxide
ºC	degrees Celsius
OD	optical density
rpm	revolutions per minute
s	second
SHF	separate hydrolysis and fermentation
SO_2	sulphur dioxide
SSF	simultaneous saccharification and fermentation
SSL	spent sulphite liquor
T	temperature
t	time
T_1	spent sulphite liquor adapted industrial yeast strain 1
T_2	spent sulphite liquor adapted industrial yeast strain 2
TAPPI	Technical Association of the Pulp and Paper Industry
UBC	The University of British Columbia
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
WIF	water-insoluble fraction
WSF	water soluble fraction, liquid fraction
Y1528	haploid yeast strain that ferments galactose preferentially

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1. Introduction

1.1 Background

Bioconversion of lignocellulosic feedstocks to ethanol is an attractive option to reduce greenhouse gas emissions from the transportation sector that currently relies heavily on fossil fuel. Lignocellulosic biomass is recognized as a viable feedstock for bioconversion because of its abundance and lower cost than agricultural crop-based sources. Its widespread abundance worldwide also relieves geopolitical concerns over fossil fuels (Asif and Muneer, 2007).

Bioethanol as a transportation fuel is cleaner burning than non-renewable gasoline because it is more oxygenated (Wheals et al., 1999). A 10% bioethanol blend can effectively replace methyl tertiary butyl ether (MTBE) as an oxygenation additive to gasoline and potentially prevent MTBE-associated health risks (Hartley et al., 1999). When bioethanol replaces aromatic and sulfur-containing compounds used in gasoline, it may also reduce nitrogen oxide (NOx) emissions to improve air quality which can reduce urban smog (McCloy and O'Connor, 1999). The high oxygen content in bioethanol could reduce the generation of known hazardous volatile organic compounds (VOCs) and carbon monoxide in vehicle exhaust (Wyman, 1996; Yoon et al., 2009).

Bioethanol such as that derived from starch is often produced by the fermentation of hexoses by micro-organisms. Hexoses, such as glucose, are often stored as starch in crops such as corn or wheat. Starch can be readily hydrolyzed by commercially available amylases and later fermented by *Saccharomyces cerevisiae*. Utilization of starch from grain is currently the main source of bioethanol in Canada and the US while sugars from

sugar cane are the main source for bioethanol production in Brazil (Wyman, 1996; Balat and Balat, 2009). Although producing ethanol from starch and sugar cane is a relatively cheap and simple process, it only utilizes a small fraction of the total carbohydrate components in the plant. Plants also contain cellulose, the most abundant biopolymer on earth. Furthermore, life cycle analysis has raised concerns, both economic and environmental, with regard to large scale bioconversion of corn grain (Wang and Huo, 2007; Kim and Dale, 2005). If large quantities of corn grain are utilized for fuel rather than food, this raises a 'food vs fuel' concern (Cadenas and Cabezudo, 1998). Furthermore, the process generates steam through natural gas, a form of fossil fuel that contributes to net CO_2 green house gas emissions (Wang and Huo, 2007).

Lignocellulosic feedstocks, which contain cellulose, such as softwood, hardwood and agricultural residues, would not pose the same environmental and societal concerns as the use of starch from grain feedstocks (Fu et al., 2003; Kim and Dale, 2005). The main chemical components of lignocellulosics are cellulose, hemicellulose and lignin. Cellulose, the most abundant component in lignocellulosics is a linear biopolymer comprised of D-glucose sugars linked by β-1, 4 bonds. Hemicelluloses are polymers of various pentoses and hexoses. While softwood has galactoglucomannan as the predominant hemicellulose component, hardwood contains more arabinoglucuronoxylan (Sjostrom, 1993). Softwoods and hardwoods contain a greater amount of lignin in general compared to agricultural residues. Softwoods produce more guaiacyl phenylpropane units derived from coniferyl alcohol, while hardwood produces more syringyl phenylpropane units derived from sinapyl alcohol (Sjostrom, 1993). Agricultural residues tend to contain a greater number of *p*-hydroxyphenyl lignin

subunits. In addition, softwood and hardwood species produce a range of extractives for various natural functions. The diversity of the chemical composition of lignocellulosic feedstocks can significantly affect the choice of pretreatment/hydrolysis/fermentation technologies employed in a bioconversion scheme.

Bioconversion technologies conventionally are composed of feedstock size reduction, pretreatment, hydrolysis, fermentation and distillation steps. Size reduction of the feedstock effectively increases the accessible surface area during pretreatment. The purpose of pretreatment technologies is to reduce the recalcitrance of the lignocellulosic structure to enable efficient enzymatic hydrolysis and fermentation. In addition pretreatment can fractionate the cellulose, hemicellulose and lignin components. The main advantage of steam pretreatment is its ability to break down the lignocellulosic structure and produce an aqueous slurry that includes a solid fraction (water-insoluble fraction, WIF) composed of mainly cellulose and lignin and a liquid fraction (water soluble fraction, WSF) composed of mainly partially hydrolyzed hemicellulosic sugars (Ramos et al., 1992). Ideally, steam pretreatment of lignocellulosic feedstocks under an optimized severity should produce a readily hydrolysable WIF and a readily fermentable WSF in addition to a lignin fraction which can potentially be used in other applications. However, this is often not the case due to the heterogeneity of the feedstock and the ongoing challenges associated with the process include requirement of high enzyme loadings and the formation of fermentation inhibitors during pretreatment. For example, the presence of lignin in the WIF often restricts access of cellulolytic enzymes to cellulose, while the presence of process-derived and natural inhibitors often prevent microorganisms from fermenting the sugars into ethanol (Berlin et al., 2006; Palmqvist et

al., 1996). Baker's yeast, *Saccharomyces cerevisiae*, is one of the best characterized and most robust micro-organisms available to date due to its high ethanol productivity and relatively high tolerance to fermentation inhibitors compared to other known micro-organisms (Clark and Mackie, 1984; Larsson et al., 2001; Martin et al., 2003). Previous studies have indicated this yeast's ability to tolerate the presence of known process derived and natural fermentation inhibitors below a certain "threshold" concentration (Palmqvist et al., 2001; Hahn-Hagerdal et al., 2006). However, fermentation inhibitors at concentrations above the threshold level tolerable by *Saccharomyces cerevisiae*, this results in a lowering of the ethanol yield below theoretically attainable levels (Robinson, 2003). Moreover, a low concentration of ethanol requires significant energy and capital investment for downstream product recovery thereby leading to negative impacts on the financial viability of a commercial bioconversion process (Quershi and Manderson, 1995; Wingern et al., 2003).

1.2 Feedstocks for bioconversion

The feedstocks generally available for bioconversion are sugar-containing sugar cane, starch-containing maize grain and lignocellulosic-containing forestry products or agricultural residues (McKendry, 2003). Lignocellulosic feedstocks have distinctive advantages with lower raw material costs and higher per annum mass production per hectare of land compared to starch and sugar-based feedstocks (Wheals et al., 1999). Lignocellulosic feedstocks including softwood, hardwood and agricultural residues account for half of the biomass on Earth with an estimated annual production of 50×10^{12} kg available for bioconversion. Moreover lignocellulosics are not part of the food supply

and hence does not result in "food versus fuel" economic issues (Claassen et al., 1999; Wheals et al., 1999).

Lignocellulosic feedstocks to be utilized in bioconversion should be selected based on several factors including consistent availability and ease of processing. Softwoods such as Douglas-fir (*Pseudotsuga menziesii*) and Lodgepole pine (*Pinus contorta*) rank among the most abundant lignocellulosic feedstocks available in the Northern hemisphere especially in the province of British Columbia (BC). Douglas-fir is a common softwood species in the Pacific Northwest of North America, found both in the coastal and interior regions (Hermann and Lavender, 1990). This species accounts for approximately 6% of British Columbian coastal forest standing volume and 17.6% lumber production due to its density and strength properties (COFI, 2000). Therefore a significant amount of sawdust, shavings and rejected lumber can be available for bioconversion (Robinson, 2003). Lodgepole pine is another abundant softwood species in western Canada with a coverage area of approximately 20 million hectares that spans over BC, Alberta and the Yukon. Over the past few years, the Lodgepole pine forests in British Columbia have been decimated by the mountain pine beetle epidemic prompting research efforts directed at finding new applications for the dead trees. For this reason bioconversion of beetle-killed trees has been investigated extensively (Ewanick et al., 2007; Pan et al., 2007). In general, softwoods such as Douglas-fir and Lodgepole pine possess hemicellulose mainly composed of yeast fermentable hexose sugars such as galactoglucomannan (Sjostrom, 1993).

1.3 Chemical composition of lignocellulosic feedstocks

The cell wall of softwood, hardwood and agricultural feedstock is composed of a complex lignocellulosic matrix. Lignocellulose is mainly composed of cellulose, hemicellulose and lignin. Cellulose is a linear biopolymer constituting units of D-glucose sugar linked by β-1, 4 glycosidic bonds. Hemicellulose, unlike cellulose, has an amorphous structure and is composed of heterogeneous polysaccharides. The chemical composition and the proportion of various hemicelluloses are highly divergent among different types of lignocellulosics. Lignin is a biopolymer that is composed of heterogeneous units of phenylpropane. These phenylpropane units are linked to polysaccharides via various covalent bonds (Iversen, 1986).

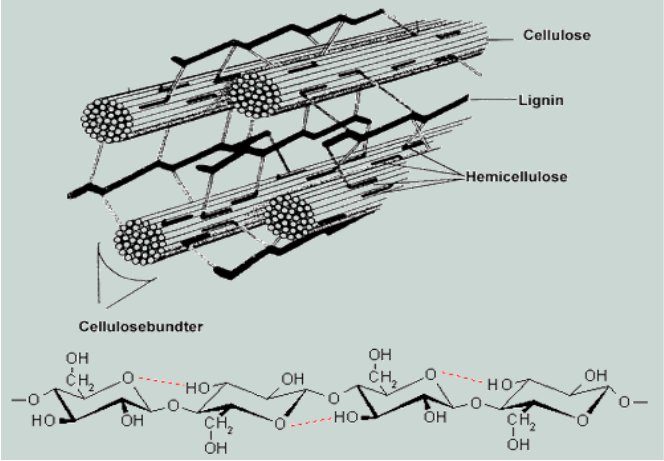

Figure 1-1. Structure of lignocellulosic matrix (Fengel and Wegner, 1984).

1.3.1 Cellulose

Cellulose is the predominant constituent found in the cell wall of green plants and is the most abundant biopolymer on the Earth. Cellulose is a homogeneous polysaccharide

composed of β-D-glucopyranose units linked by β-1, 4 glycosidic bonds. This highly linear structure combined with the tendency to form intra- and intermolecular hydrogen bonds provides high tensile strength and solvent insoluble properties that are vital for plant cells (Sjostrom, 1993). Cellulose fibers are composed of macrofibrils which are formed by the aggregation of microfibrils containing glucopyranose units. Microfibrils have alternating crystalline regions and amorphous regions and vary in the degree of polymerization (DP). The DP is measured on the basis of the number of repeating units of glucans in the microfibrils which is typically about 10000 for softwood (Fengel and Wagner, 1984). The DP of the cellulose component of pretreated substrates has been shown to influence its hydrolyzability using cellulases (Pan et al., 2007). The cellulose fibers are intermingled with hemicellulose and lignin to form the lignocellulose matrix.

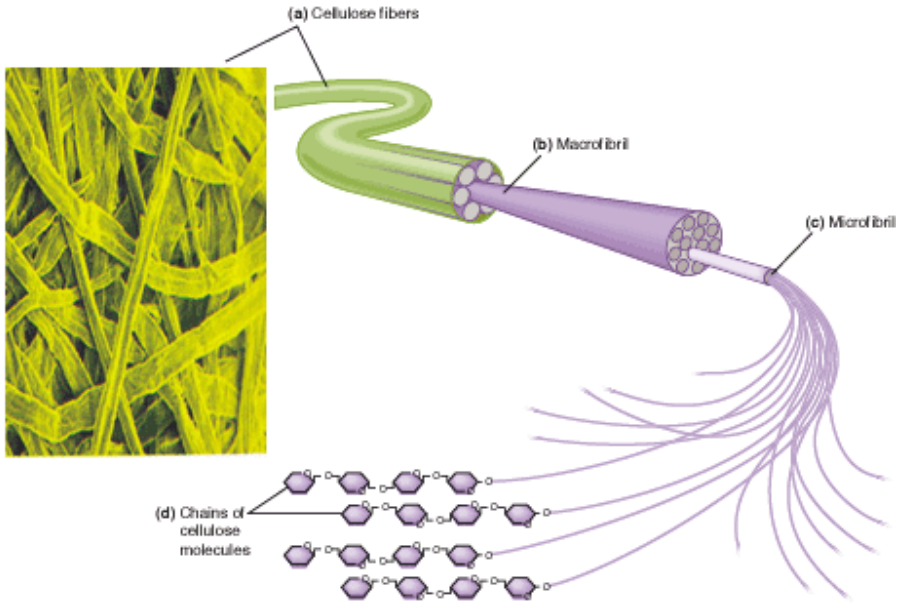

Figure 1-2. Cellulose fiber, macrofibril and microfibril.

Source: http://nutrition.jbpub.com/resources/images/images/fiber.gif

1.3.2 Hemicellulose

Hemicelluloses are amorphous polymers of heterogeneous polysaccharides with a degree of polymerization of approximately 200 (Fengel and Wegner, 1984). Hemicelluloses, unlike cellulose, have a random combination of monosaccharides mainly including D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose (Fengel and Wegner, 1984; Sjostrom, 1993). These monosaccharides are linked by an assorted combination of ether bonds and weak hydrogen bonds which can be readily hydrolyzed in acid or alkaline conditions. The β-linkage forms the 'backbone' of the polymer, while the α-linkages often generate the 'branches' (Fengel and Wegner, 1984). The composition of hemicellulose varies between softwood and hardwood. Softwoods have a higher proportion of D-mannose derived hemicelluloses such as galactoglucomannans, while hardwoods have a higher proportion of D-xylose derived hemicellulose such as arabinoglucuronoxylan (Sjostrom, 1993). The xylose and mannose residues of hemicellulose often form ester linkage with the α-carbon of lignin to provide a strong shield against water permeation and enzymatic degradation by parasites (Fengel and Wegner, 1984).

1.3.3 Lignin

Lignin is an amorphous network biopolymer known as the second most abundant biopolymer found on earth (Sjostrom, 1993). Lignin is composed of three common phenylpropane structures including p-hydroxyphenyl, syringyl, and guaiacyl units. These phenylpropane units have seven types of common alkyl or ether linkages, which includes β-O-4, α-O-4, β-5, 5-5, 4-O-5, β-1, β-β and dibenzodioxocin (Baucher et al., 1998).

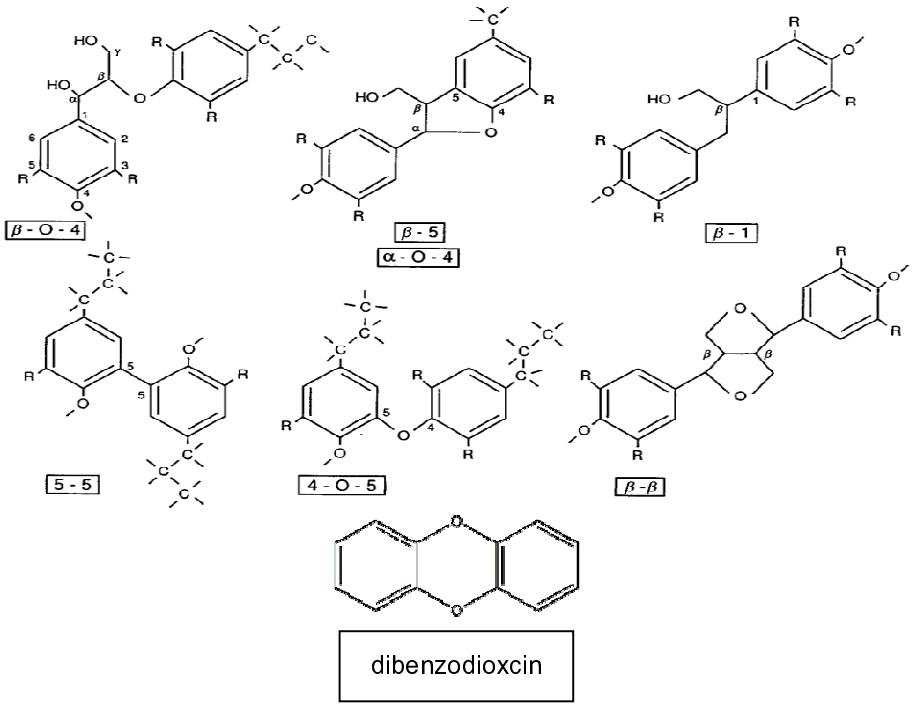

Figure 1-3. Common intermolecular linkages in lignin (Baucher et al., 1998).

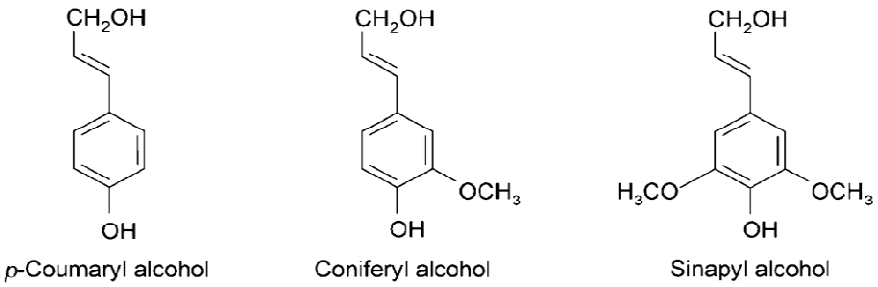

Figure 1-4. Chemical structure of the hydroxycinnamyl alcohol precursors (Fengel and Wegner, 1984).

Diverse heterogeneous polymers of lignin are formed due to the different possible combination of phenylpropane structures and their common linkages. The type of phenylpropane units present in the plant cell wall determines the type of linkages and

alters the overall recalcitrance due to the linkage options presented by the three main subunits and their methoxyl groups. The lignin co-polymers are found in different proportions among various lignocellulosic feedstocks. The lignin in softwood has a higher portion of guaiacyl phenylpropane units derived from coniferyl alcohol, while in hardwood, a higher portion of syringyl phenylpropane units derived from sinapyl alcohol is present. The guaiacyl phenylpropane does not have a C5-methoxy group whereas it is present in the syringyl phenylpropane, on the other hand, β-5, 5-5, 4-O-5 linkages are present in guaiacyl phenylpropane but not in syringyl phenylpropane (Sarkanen, 1990; McDonough, 1993). The extra cross-linkage at C5 among guaiacyl phenylpropane units as compared to syringyl phenylpropane provides a higher thermodynamic stability in softwoods and therefore it is more recalcitrant during pulping as compared to hardwood.

1.3.4 Additional minor chemical components

The chemical components in addition to cellulose, hemicellulose and lignin are mainly composed of extractives and inorganic compounds. Extractives are compounds that are soluble in water (hydrophilic) or neutral organic solvents (lipophilic) such as acetone, ethyl ether, ethyl acetates and others and are mainly composed of neutral compounds such as glycerides, resin acids, free fatty acids, higher alcohols, steryl esters, waxes, hydrocarbons, various polyphenols and oxidized compounds (Fengel and Wegener, 1984). Essentially, extractives are classified based on the solvent by which they can be extracted as a change in solvent results in variations in the type of compounds extracted. Extractives are mostly concentrated in resin canals and ray parenchyma cells. Upon attack by insects or fungi, trees naturally secrete phenolic compounds through secondary metabolism as a defense mechanism. Phenolic compounds such as vanillic acid,

coniferyl aldehyde, 4-hydroxybenzoic acid and cinnamic acid have been found in the WSF of steam pretreated woody residues and are suspected to possess anti-fungal activities that prevent fermentation by yeast (Larsson et al., 2000; Luo et al., 2002; Klinke et al., 2004).

1.4 Pretreatment

1.4.1 Overview

Pretreatment is a critical process for bioconversion that can improve accessibility of cellulase to cellulose by partially hydrolyzing hemicelluloses and by removing or modifying lignin in the lignocellulosic matrix. An ideal pretreatment process would also have characteristics of preserving the hemicellulose fraction, limiting the formation of degradation products that could inhibit fermentation, while minimizing both energy and chemical requirements (Mosier et al., 2005). Furthermore, ideally, pretreatment would fractionate the lignin, cellulose, hemicellulose and extractive components in a usable form to maximize the value obtained from the feedstock.

A wide range of pretreatment methods have been developed for the bioconversion process (Wyman, 1996; Sun and Cheng, 2002). Pretreatment methods can be categorized into physical, chemical, biological and physico-chemical (Chandra, 2007; Sun and Cheng, 2002). The main physical pretreatment methods include mechanical comminution (Millet et al., 1976) and pyrolysis (Shafizadeh and Stevenson, 1982). The main chemical pretreatment methods include ozonolysis (Neely, 1984), acid hydrolysis (Sivers and Zacchi, 1995), alkaline hydrolysis (Bjerre et al., 1996) and organosolv based processes (Kleinert, 1974; Aziz and Sarkanen, 1989). The main biological pretreatment methods involve utilizing different fungal species to degrade lignin and hemicelluloses in

lignocellulosic biomass (Boominathan and Reddy, 1992; Sun and Cheng, 2002; Taniguchi et al., 2005). The main physico-chemical pretreatment methods include ammonia fiber explosion (AFEX) (Mes-Hartree et al., 1988; Holtzapple et al., 1992) and steam pretreatment (Morjanoff and Gray, 1987; Saddler et al., 1993).

1.4.2 Acid-catalyzed steam pretreatment

Steam pretreatment is one of the most attractive and most comprehensively studied pretreatment methods because it has a lower energy cost than physical pretreatments, lower chemical cost than chemical pretreatment and a shorter reaction time requirement than biological pretreatment (Boussaid et al., 2000; Robinson et al., 2002; Ewanick et al., 2006). Steam pretreatment involves the exposure of lignocellulosic feedstock to high pressure steam in a vessel at a temperature from 200°C to 250°C for 20 seconds to several minutes. This is followed by a decompression stage in which the steam pressure is released facilitating the disintegration of the feedstock (Brownell and Saddler, 1987). Due to the explosive decompression stage, steam pretreatment is also referred to as steam explosion (Morjanoff and Gray, 1987).

Although it has been demonstrated that steam pretreatment can be an effective pretreatment method without the addition of a catalyst with hardwood and non-woody feedstocks such as Poplar and corn stover (Bura et al., 2002; Bura et al., 2009), due to the recalcitrant nature of softwood feedstocks such as Douglas-fir and Lodgepole pine, the addition of an acid catalyst is required in order to improve the pretreatment efficiency (Boussaid et al., 2000; Shevchenko et al., 2001; Mais et al., 2002; Robinson et al., 2002; Ewanick et al., 2006; Mabee et al., 2006). For example steam pretreatment of Poplar has been shown to produce a readily hydrolysable substrate for enzymatic hydrolysis with 80-

90% glucose conversion with 10-15 FPU/g of substrate in 72 hours (Grous et al., 1986; Brownell and Saddler, 1987; Ramos et al., 1992; Robinson, 2003; Wyman et al., 2009; Bura et al., 2009) while softwoods substrates such as Douglas-fir have been demonstrated to be difficult to hydrolyze unless high enzyme loadings (20-40 FPU/g of cellulose) are utilized (Pan et al., 2004). The addition of acid to catalyze the steam pretreatment of softwood is favorable to overcome the lower amount of acetylated groups in softwood hemicellulose compared to hardwood and non-wood hemicelluloses which facilitate auto-hydrolysis during the steam pretreatment. The addition of an acid catalyst can also reduce the required reaction temperature and therefore improve overall sugar recovery (Galbe and Zacchi, 2002). Sulfuric acid (H_2SO_4) was initially determined to be an ideal acid catalyst. However, impregnation with H_2SO_4 requires a longer incubation time and involves limited permeability issues depending on the feedstock. Therefore gaseous SO_2 is chosen for shorter incubation times. The use of SO_2 has also been shown to result in reduced steam requirement for pretreatment thus leading to potential savings in energy (Schwald et al., 1989; Saddler et al., 1993). Sulfur dioxide acts as a catalyst by undergoing a combination of oxidation and disproportionation reactions that yield sulfuric acid within the wood chips (Shevchenko et al., 1999). The resulting acid catalyst improves the partial hydrolysis and solubilization of the hemicellulose and hydrolytic reactions of lignin (Clark et al., 1989), thereby allowing treatment at lower severities, improving carbohydrate recovery and the ease of enzymatic hydrolysis of the solid fraction.

1.4.3 Severity of steam-pretreatment

The extent of solubilization of hemicellulose and hydrolysis of lignin during acid-catalyzed steam pretreatment is dictated by residence time, temperature and concentration of SO_2 selected. The optimal pretreatment conditions may vary for different feedstocks. These conditions were quantified by Overend and Chornet (1987), who proposed a severity factor R_o (Eq. 1) with two variables where t is the residence time (min), T is temperature in degrees Celsius.

$$R_o = te^{(T-100)/14.57} \quad \text{(Eq. 1)}$$

The severity factor R_o provides a reference to compare different steam pretreatment conditions with varying residence times and temperatures. However, the severity factor has its limitations since it does not account for the applied acid catalyst concentration. Therefore the severity factor R_o is only valid when comparing pretreatments with identical concentrations of acid catalyst. The combined severity (CS) (Eq. 2) takes into account the acid concentration and is more suitable for comparing pretreatments with different concentrations or type of acid catalyst.

$$CS = \log R_o - pH \quad \text{(Eq. 2)}$$

The limitation of CS is that the initial pH of the feedstock reaction mixture is difficult to measure accurately because for example, the pH is difficult to measure if the substrate is in solid state as in the case of SO_2-impregnated woodchips. In addition the pH of the reaction mixture is known to change during pretreatment as the acetylated residues are released to form acetic acid especially in the case of hardwoods and non-woody biomass.

At a mild severity, limited physical and chemical changes will be imparted on the feedstock in the reaction mixture. However, as the severity increases, the hemicellulose will be the first to be hydrolyzed since it has a low degree of polymerization and an amorphous structure (Sjostrom, 1993). When the severity is increased further, cellulose can begin to break down and lignin can condense. Although it is ideal to have a higher severity to hydrolyze the cellulose during pretreatment and to aid in subsequent enzymatic hydrolysis, it is not practical because at such severity cellulose and hemicellulosic sugars degrade to furans such as furfural and 5-hydroxymethyl furfuraldehyde (HMF) which compromise sugar recovery and inhibit fermentation. Therefore, pretreatment severities are ideally selected as a "medium severity" based on overall sugar recovery from the initial feedstock, and the production of a substrate which can be readily hydrolyzed by enzymes and fermented by yeast.

In addition, as the pretreatment severity increases, lignin undergoes a more severe oxidation reaction that turns hydroxyl and ether groups at the α-carbon position to carbonyls or benzylic cations where they can undergo condensation by forming carbon-carbon bonds with electron rich centers of lignin (Shevchenko et al., 1999). The resulting condensed lignin may be less reactive and thus may potentially render the lignin component less usable for co-product applications. Oxidation of lignin can also form diverse species of low molecular weight phenolic compounds which can dissolve in the water soluble fraction with the hemicellulosic sugars during pretreatment (Ohgren et al., 2007). The low molecular weight phenolic compounds can be composed of one to several phenol propanoid subunits that act as potent fermentation inhibitors (Almeida et al., 2007).

1.5 Enzymatic hydrolysis and fermentation

As mentioned previously, the hydrolysis of both cellulose and hemicellulose can be accomplished during the pretreatment stage with the application of a sufficiently high pretreatment severity. However, the overall sugar recovery and fermentation is compromised when the pretreatment severity is high enough to degrade hemicellulosic sugars to furans. Therefore, while the hydrolysis of hemicellulose is partially accomplished depending on the type and condition of pretreatment, hydrolysis of cellulose is performed with an additional enzymatic hydrolysis step.

1.5.1 Enzymatic hydrolysis

Cellulolytic enzymes are produced extracellularly by different fungi such as *Trichoderma*, *Penicillium* and *Aspergillus* to hydrolyze cellulose to glucose to be used both as a nutrient and energy source (Eriksson et al., 1990). These organisms produce different glycolytic enzymes primarily composed of three different activities. The endo-1,4-β-D-glucanases can cleave β-1-4 glycosidic linkages, while exo-1,4-β-D-glucanases can cleave off glucose or cellobiose from both the reducing and non-reducing ends (Lee et al., 1983; Vinzant et al., 2001). Subsequently, the cellobiose produced is further hydrolyzed by β-glucosidase to two glucose monosaccharides (Eriksson et al., 1990). Cellobiose exhibits end-product inhibition to both endo- and exo-1,4-β-glucanases, therefore hydrolysis reactions are supplemented with 1,4-β-glucosidase (Holtzapple et al., 1990). Furthermore, ethanol and glucose are also inhibitors of cellulases above a certain concentration (Holtzapple et al., 1990; Wu and Lee, 1997). This presents a particular challenge for the bioconversion processes since the glucose produced by enzymatic hydrolysis and ethanol produced through fermentation by yeast all inhibits enzymatic hydrolysis of cellulose.

Each of cellulose, hemicellulose and lignin presents obstacles to efficient enzymatic hydrolysis. The partially hydrolyzed hemicellulose releases monomeric glucose that can also be implicated in end-product inhibition. Lignin blocks potential binding sites and has been shown to irreversibly bind with cellulases (McMillan 1994; Sun and Cheng, 2002; Berlin et al., 2004). Crystallinity of cellulose affects the binding of exoglucanases to reducing ends (Carrard et al., 2000). Porosity of the substrate and the degree of polymerization of substrate cellulose affect the accessible surface area for the cellulases to bind (McMillan 1994; Sun and Cheng, 2002; Berlin et al., 2006). Each of these obstacles to enzymatic hydrolysis affects the amount of sugar available for downstream fermentation.

1.5.2 Choice of microorganism for fermentation

Numerous species of bacteria, filamentous fungi and yeast have been studied for their fermentation of hydrolysates derived from pretreatment and enzymatic hydrolysis (Jeffries, 1983; Toivola et al., 1984; Skoog and Hahn-Hagerdal, 1990; Robinson, 2003; Keating et al., 2006). Each species tested has its advantages and disadvantages. *Zymomonas mobilis* is a species of bacteria that is known to produce good ethanol yields during fermentation and high specific ethanol productivity (Swings and De Ley, 1977; Lee et al., 1980; Hahn-Hagerdal et al., 2007). However haploid laboratory *Z. mobilis* is restricted in its fermentation substrates, since it can only ferment glucose. It is unable to ferment any other common hexoses or pentoses found in lignocellulosic substrates which limits it ability to ferment the hemicellulosic sugars in the water soluble fractions from the pretreatment/hydrolysis of lignocellulose. In addition, similar to other bacteria such as *Escherichia coli*, *Z. mobilis* is only minimally tolerant to common fermentation

inhibitors such as weak acids, furans and low molecular weight phenolic compounds (Hahn-Hagerdal et al., 2007). Unlike the bacterial species, filamentous fungi such as *Fusarium oxysporum* and *Monilia sp.* are capable of fermenting different hexoses and pentoses while exhibiting a high level of tolerance to different fermentation inhibitors present in the water soluble fraction (Skoog and Hahn-Hagerdal., 1988; Hahn-Hagerdal et al., 1994). However, wild type filamentous fungi exhibit a low rate of sugar uptake and ethanol production (Skoog and Hahn-Hagerdal., 1988). Wild type yeasts such as *S. cerevisiae* offer some of the beneficial attributes of both the bacteria and filamentous fungi as yeasts are capable of producing high levels of ethanol while possessing both the robustness to tolerate certain fermentation inhibitors up to a threshold concentration and the ability to ferment different hexose sugars (Hahn-Hagerdal et al., 2007; Keating et al., 2004a; Keating et al., 2004b). However, a major disadvantage of *S. cerevisiae* is its inability to ferment pentoses derived from the hydrolysis of hemicellulose such as xylan in hardwood and non-wood feedstocks. Since softwood hemicellulose is composed mostly of hexoses derived from galactoglucomannan, *S. cerevisiae* is still the microorganism of choice for fermentation of steam pretreated softwood derived water soluble fractions (Sjostrom, 1993).

1.5.3 Fermentation inhibitors

The amount of ethanol produced from hemicellulosic sugars in the water soluble fraction (WSF) by *S. cerevisiae* depends on the chemical composition of the WSF including the types of sugars present (hexoses versus pentoses and monomers versus oligomers) and the concentration of various fermentation inhibitors. These inhibitors can be categorized

into different groups including weak acids, furans and phenolics. These compounds can be grouped further to naturally-occurring inhibitors and process derived inhibitors.

1.5.3.1 Naturally-occurring inhibitors

The naturally-occurring inhibitors which primarily consist of extractive compounds are derived from the tree's self-defense against fungal decay (Haygreen and Bowyer, 1996). Since *S. cerevisiae* is a fungal species, the extracted compounds can be expected to reduce fermentation efficiency. The composition of extractives is complex and varies significantly among tree species, between trees of the same species and even within parts of the tree (Dellus et al., 1997). For example polyphenolic extractives are more abundant in Douglas-fir than Lodgepole pine, and the bark contains more extractives than some other parts of the tree (Gao et al., 1995; Dellus et al., 1997; Robinson, 2003). The extractives are mainly composed of glycerides, resin acids, free fatty acids, higher alcohols, steryl esters, waxes, hydrocarbons and oxidized compounds (Fengel and Wegener, 1984). Aromatic extractives derived from lignin such as stilbenes, lignans, flavonids and tannins possess anti-fungal properties and have been shown to be abundant in Douglas-fir (Manter et al., 2007; Gao et al., 2008).

Previous studies have indicated that the presence of various extractives in the WSF derived from southern pine resulted in a significant reduction in fermentability by *S. cerevisiae* (Tran and Chambers, 1986). However, studies by Robinson (2002) have shown that ethanol yields were not significantly influenced by extractive-rich bark during fermentation of WSF derived from steam pretreatment of Douglas-fir (Robinson et al., 2003). The results were thought be caused by the hydrophobic nature of the Douglas-fir

derived extractives which limited their solubility in the WSF thereby decreasing their inhibitory effect on fermentation (Lomax et al., 1994).

1.5.3.2 Process-derived inhibitors

Since it has been shown that the naturally-occurring inhibitors in the extractive-rich bark did not have a significant effect on the fermentability of WSF derived from steam-pretreated Douglas-fir by *S. cerevisiae*; it is likely that the process-derived inhibitors contributed to the poor fermentability of the WSF (Robinson, 2003). Process derived inhibitors are those which are produced during pretreatment and hydrolysis processes which precede fermentation in a typical lignocellulosic bioconversion scheme. Process-derived inhibitors are mainly composed of sugar and lignin degradation products (Almeida et al., 2007). These process-derived inhibitors are categorized into three main groups; furans, weak acids and phenolics (Almeida et al., 2007).

1.5.3.2.1 Furans

The low degree of polymerization and amorphous properties of hemicelluloses increase their susceptibility to hydrolysis during pretreatment which results in the release of hexoses (glucose, galactose and mannose) and pentoses (arabinose and xylose) into the water soluble fraction (WSF). When the severity of pretreatment is too high, the hexoses undergo a dehydration reaction to form 5-hydroxymethyl furfuraldehyde (HMF) while the pentoses also undergo a similar dehydration reaction to form furfural (Palmqvist et al., 2000a). These furans are known to inhibit fermentation by various mechanisms. Modig et al. (2002) performed an *in vitro* experiment to show furfural and HMF directly inhibited alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde

dehydrogenase (ALDH). These enzymes are essential to the metabolism of the organism. Banerjee et al. (1981) used crude-cell extracts to show that furans decreased the activity of glycolytic enzymes such as hexokinase and glyceraldehydes-3-phosphate dehydrogenase. Palmqvist et al. (1999) showed that the reduction of furans by yeast which detoxified the aldehyde group can result in the depletion of NAD(P)H and ATP, which are vital energy storing compounds. Gorsich et al. (2006) indicated that furfural causes reactive oxygen species to accumulate causing damage to cell components such as vacuoles, mitochondrial membranes, chromatin and actin.

Although the furans have a range of mechanisms for fermentation inhibition, the yeast *S. cerevisiae* possesses innate detoxification mechanisms to overcome their inhibitory effects. Palmqvist et al. (1999) found that furfuraldehyde can be reduced to furfural alcohol by alcohol dehydrogenase (ADH) that only slightly inhibits the anaerobic growth of *S. cerevisiae*. Larsson et al (1999) noticed HMF is converted to 5-hydroxymethyl furfuryl alcohol by ADH but at a lower rate compared to furfuraldehyde. This lower rate of conversion is the reason that HMF is thought to be more inhibitory to yeast than furfuraldehyde (Larsson et al., 1999). Palmqvist et al. (1999) showed that the reduction of furan aldehyde to alcohol by yeast as a mechanism to detoxify this compound can result in the depletion of NAD(P)H and ATP. This suggests that the inhibitory effect depends on the proportion of furans versus the available NAD(P)H and ATP. It is apparent that the generation of NAD(P)H and ATP depends on available hexoses, therefore, available hexoses may also potentially have an indirect effect on the inhibitory effect of furans.

1.5.3.2.2 Weak acids

Weak acids such as acetic acid are formed through the de-acetylation of hemicellulose during acid-catalyzed steam pretreatment (Dunlop, 1948). Other species of weak acids such as formic acid and levulinic acid are formed through the reduction of furfural under acidic conditions at elevated temperatures during acid-catalyzed steam-pretreatment (Ulbricht et al., 1984). Verduyn et al. (1992) identified that undissociated forms of weak acids can diffuse across the plasma membrane and dissociate resulting in higher intracellular pH, thus reducing the cytosolic pH to inhibit metabolic activities such as fermentation. Despite the observation that weak acids can change intracellular pH, the yeast possesses the innate ability to maintain internal physiological pH by pumping out excess protons through the plasma membrane ATPase (Verduyn et al., 1992). However, during the same study, it was found that the yeast can deplete its ATP reservoir while pumping out the excess protons to maintain physiological pH. Similar to furans, available hexose uptake dictates the ATP reservoir which presents an interesting indirect relationship between available hexoses and the detoxification of weak acids by *S. cerevisiae*. While ATP is being depleted the yeasts will increase their rate of ATP production to maintain their physiological pH. Pampulha and Loureiro-Dias (2000) noticed increased rates of both hexose metabolism and ethanol productionupon exposure to low concentrations of weak acids. Therefore, the toxicity of weak acids on yeasts can vary based on their species and concentrations.

1.5.3.2.3 Phenolic compounds

Process derived phenolic compounds are lignin degradation products which can be released during pretreatment (Bauchert et al., 1998; Klinke et al., 2004). The toxicity of a given phenolic compound is thought to be proportional to its molecular weight, where the lower the molecular weight, the higher the toxicity (Clark and Mackie, 1984). The type of lignin degradation product and associated functional groups depend on the para-hydroxyphenyl (H) residue, guaiacyl (G) residue and syringyl (S) residue ratio (H/G/S ratio of lignin) and the type of pretreatment (Klinke et al., 2004).

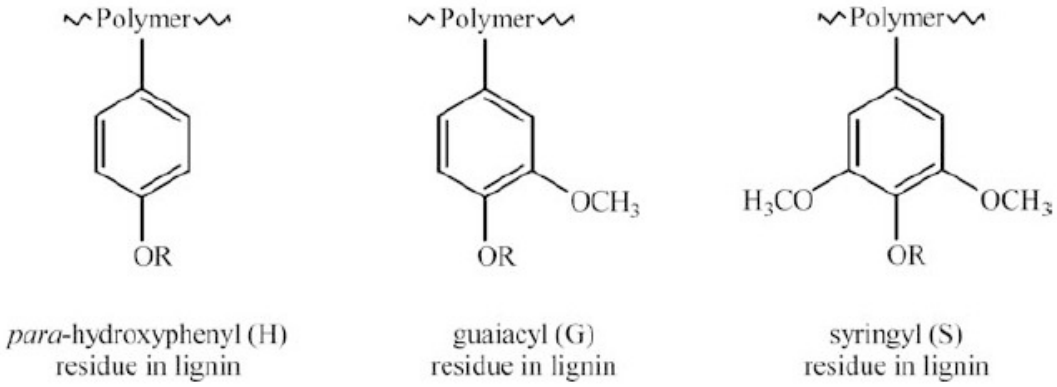

Figure 1-5. Chemical structure of para-hydroxyphenyl, guaiacyl and syringyl residues in lignin (Fengel and Wegner, 1984).

A previous study described that the specific removal of phenolic monomers and phenolic acids from a willow hemicellulose hydrolysate by laccase treatment, which likely oxidized, the phenolic acids resulted in an improved fermentation yield (Jonsson et al., 1998). Terada (1990) found that weakly acidic phenolic compounds can alter the electro-chemical gradient by transporting protons back across the mitochondrial membrane, suggesting a mechanism for the synergistic effect between phenolic compounds and weak

acids. The hydrophobic nature of different phenolic compounds was suspected to disrupt the amphiphilic biological membranes in yeast causing loss of membrane integrity and their function as a selective barrier (Heipieper et al., 1994; Klinke et al., 2003).

Previous attempts to survey phenolic compounds have suggested that variations in hydrophobic properties and functional groups at different positions of the aromatic ring exhibit different degrees of toxicity (Ando et al., 1986). The inhibitory nature of various functionalities of the phenolic compounds has been ranked as double bonds (CH=CH)> aldehyde (CHO)> para-OH phenols> carboxylic acid (COOH) > meta-OH phenol (Ando et al., 1986). In contrast to other functionalities, Meta-OH phenols have been shown to exhibit no inhibitory effect on fermentation while methoxy (OCH3) groups are thought to promote ethanol production (Ando et al., 1986). The mechanism of detoxification by microorganisms such as S. cerevisiae often converts more toxic functional groups to less toxic functional groups. However, the exact nature of fermentation inhibition exhibited by different phenolic compounds is yet to be completely elucidated largely due to the heterogeneity and diversity of compounds in this group which varies with each lignocellulosic feedstock in addition to a lack of quick and accurate qualitative and quantitative chemical analytical methods to identify these compounds.

1.5.3.3 Current detection methods of fermentation inhibitors

Phenolic compounds isolated from both naturally-occurring extractives and process-derived lignin degradation products are commonly detected by a two stage process. The first stage involves extraction using an organic solvent such as ethyl acetate followed by mass spectrometry coupled gas chromatography (GC-MS) analysis (Luo et al., 2002; Gao

et al., 2008). The detection of both furans and weak acids can be accomplished through high-performance liquid chromatography (HPLC), but with different combinations of detector wavelength. A reversed phase hydrophobic column can be used to separate compounds while using a spectrophotometer-based UV detector at 280 nm for furans (Ewanick et al., 2007) while a wavelength of 205 nm can be used for weak organic acids (Robinson, 2003).

1.5.4 Detoxification methods

Detoxification methods are in general divided into three categories: Biological, physical and chemical (Palmqvist et al., 2000a). In the case of biological detoxification, as mentioned previously, compared to bacteria, fungi have a higher tolerance for fermentation inhibitors, in particular the phenolic compounds. For example, a fungus such as *Trametes versicolor* can produce oxidases and laccase that can detoxify phenolic monomers (Jonsson et al., 1998). Detoxification is achieved by oxidative polymerization of low molecular weight phenolic compounds. Palmqvist et al. (1997) has shown that *Trichoderma reesei* helped improve ethanol productivity during fermentation of a hemicellulose hydrolysate derived from steam-pretreated willow. They hypothesized that the mechanism of detoxification involved the removal of weak organic acids (acetic acid), furan (furfural) and phenolic compounds (benzoic acid) based on a 30% decrease in absorbance at 280 nm.

Physical detoxification often involves an extraction process with roto-evaporation or a combination of chemical solvents and roto-evaporation (Clark and Mackie, 1984; Palmqvist et al., 1996). Roto-evaporation of an acidic hydrolysate of aspen by *Pichia stipitis* has reportedly improved ethanol yield by up to 13% (Wilson et al., 1989).

However, roto-evaporation of the water soluble fraction recovered from steam-pretreated Douglas-fir revealed the opposite result, where the ethanol yield upon fermentation was significantly reduced (Robinson, 2003). Thus it is apparent that the volatility of the various inhibitors governs the efficacy of the roto-evaporation method. The decrease in the fermentation yield from Douglas-fir hydrolysates is likely due to concentration of the non-volatile inhibitors during evaporation.

In the case of chemical detoxification, alkali treatment such as overliming is often employed (Leonard and Hajny, 1945; van Zyl et al., 1998). The detoxification mechanism of overliming involves precipitation of the inhibitory compounds and increased instability of some inhibitory compounds at high pH (Palmqvist et al., 2000a). Horvath et al. (2005) has obtained a 120% increase in ethanol production after overliming up to pH 11 at 30°C. Minimal amounts of weak organic (formic acid, acetic acid) acids were removed; however, up to 65% of the furans and 22% of the phenolic compounds contained in the water soluble fraction were removed. The results suggest that in this particular case, the removal of furans and phenolic compounds was the main contributing factor to the increased fermentability of the dilute-acid hydrolysate obtained from Norway spruce, *Picea abies*.

1.5.5 Separate hydrolysis and fermentation (SHF) and hybrid hydrolysis and fermentation (HHF)

In a bioconversion scheme to produce ethanol from lignocellulose, the enzymatic hydrolysis and fermentation processes can either be performed separately in separate hydrolysis and fermentation (SHF) or simultaneously in simultaneous hydrolysis and fermentation (SSF) or a combination of SHF and SSF with a process referred to as hybrid

hydrolysis and fermentation (HHF). Optimum temperature is the main factor when considering SHF vs SSF and HHF. SHF allows each process to run at its own optimal temperature and avoids the inhibition of the cellulolytic enzymes by ethanol (Wu et al., 1997; Ohgren et al., 2007). However, the tradeoff of SHF is that end product inhibition by glucose and cellobiose can reduce the rate and extent of enzymatic hydrolysis. SSF was developed to overcome end product inhibition since the fermentative microorganism will ferment the hydrolyzed sugars as they are liberated from the substrate by the action of cellulases and β-glucosidase. The typical optimal temperature for enzymatic hydrolysis is approximately 45-50°C while the optimal temperature for fermentation with *S. cerevisiae* is below 40°C (Eklund et al., 1990; Ewanick et al., 2006). Ghosh et al (1982) found that cellulose hydrolysis rates can be increased up to 30% when using SSF rather than SHF. An additional advantage of SSF is the requirement of only a single vessel compared to SHF which could potentially decrease process costs (Hinman et al., 1992). Since the optimal temperature of enzymatic hydrolysis is 50°C, a pre-hydrolysis step for SSF is ideal to obtain a high initial hydrolysis rate and after an initial period of incubation, the incubation temperature of the hydrolysate can be reduced to 37°C for fermentation by *S. cerevisiae* (Sassner et al., 2006). When SHF and SSF are performed for the whole slurry, combined WIF and WSF, there is an additional challenge with end product inhibition by the hemicellulose-derived monomeric sugars in WSF. The process that combines a pre-hydrolysis step as described above, with subsequent SSF is referred to as hybrid hydrolysis and fermentation (HHF) and has been shown to be particularly advantageous (Varga et al., 2004) when dealing with the hydrolysis/fermentation of lignocellulosic substrates.

1.6 Hydrolysis and fermentation

Steam pretreatment of softwoods such as Douglas-fir and Lodgepole pine has been shown to be less effective than the treatment of hardwoods such as Poplar (*Populus*) and agricultural residues such corn stover (Scheald et al., 1989; Excoffier et al., 1991; Boussaid et al., 1999; Bura et al., 2002; Mabee et al., 2006). Previous studies with steam-pretreatment of hardwoods and agricultural residues have obtained up to 90% glucose conversion yields with enzyme loadings of 10-15 FPU/g of substrate cellulose upon subsequent enzymatic hydrolysis (Brownell and Saddler, 1987; Schwald et al., 1989; Eklund et al., 1990; Excoffier et al., 1991; Ramos et al., 1992; Saddler et al., 1993; Ohgren et al., 2007). These previous studies have suggested a combination of high fiber size, low porosity, and high residual lignin content in the softwood derived WIF in addition to a high proportion of guaiacyl lignin in the softwood as possible contributing factors to the greater recalcitrance during steam pretreatment compared to hardwood and agricultural residues.

Variability in recalcitrance was observed between steam-pretreated softwood species for enzymatic hydrolysis and optimal pretreatment severity value $\log R_o$ (Boussaid et al., 2001; Robinson, 2003; Pan et al., 2004; Pan et al., 2005; Ewanick et al., 2007). Boussaid et al. (2000) have performed steam pretreatment with Douglas-fir (DF) at three different severities: $\log R_o$ = 3.08 (low), 3.45 (medium) and 3.73 (high). However the SO_2 was 4.5% for low and medium severity while an SO_2 loading of 2.38% was used for the high severity. Since $\log R_o$ does not take into account the effect of variation in SO_2, the actual level of severity may not be a fair comparison when considering only the $\log R_o$ value. The authors managed to attain sugar recoveries of 90-97%, 82-88% and a 74-77% at

$logR_o$ = 3.08, $logR_o$ = 3.45 and $logR_o$ = 3.73, respectively. In the same study, up to a 100% glucose conversion was obtained with 80 FPU / g of cellulose for $logR_o$ = 3.73. However, at $logR_o$ = 3.45 the glucose conversion was reduced to about 60% and at $logR_o$ = 3.08 glucose conversion was further reduced to about 20% during enzymatic hydrolysis (Boussaid et al., 2000). Ethanol production through fermentation of the water soluble fraction (WSF) at medium severity ($logR_o$ = 3.45) produced ethanol at a concentration of 14.9 g/L, comparable to a glucose control, while low severity ($logR_o$ = 3.08) produced 12.6 g/L and high severity ($logR_o$ = 3.73) did not produce any ethanol (Robinson, 2003) thus indicating the inhibitory nature of the water soluble fraction from the Douglas-fir treated at high severity. Therefore, it is evident that in the case of softwoods such as Douglas-fir a severe treatment is necessary to obtain reasonable enzymatic hydrolysis yields, which compromises both the sugar recovery during pretreatment and increases the amount of process derived fermentation inhibitors.

Ewanick et al. (2007) have investigated steam pretreatment of Lodgepole pine (LP) at three different pretreatment severities including $logR_o$ = 3.67 (low), 3.64 (medium), 4.09 (high). However the SO_2 loading was 4% for low and medium severity while 4.5% was used for high severity. Another limitation of the severity factor ($logR_o$) is seen here; despite having a higher $logR_o$ value for low severity, the actual pretreatment severity was lower than medium severity at $logR_o$. The authors recovered 98%, 96% and 86% of the available hexoses when LP was steam pretreated at low, medium and high severity respectively. Enzymatic hydrolysis at a 2% (w/v) consistency with 20 FPU/g cellulose cellulase dosage and a 10 CBU/g cellulose beta-glucosidase loading, resulted in a 94%, 75%, and 63% glucose conversion for high, medium and low severity pretreatments,

respectively (Ewanick et al., 2007). Ethanol production through fermentation of the WSF at low severity ($logR_o = 3.67$) produced ethanol at 8.5 g/L, while medium severity ($logR_o = 3.64$) produced 7.5 g/L and high severity ($logR_o = 3.73$) produced only 1.3 g/L ethanol after 48 hours of fermentation (Ewanick, 2006) thus indicating once again the detrimental effect of fermentation inhibitors at the high severity pretreatment level even though good hydrolysis yields were obtained.

The optimization of steam pretreatment for softwoods presents an interesting dilemma of balancing increasing the pretreatment severity to improve the susceptibility of cellulose to cellulolytic hydrolysis in the solid fraction (WIF) while preserving fermentable sugars in the water soluble stream (WSF) (Boussaid et al., 2000; Robinson, 2003; Ewanick, 2006). Therefore a medium severity for a particular feedstock such as softwoods should be further studied to obtain a WIF amenable to subsequent hydrolysis and a WSF with maximal amounts of available sugars and minimal levels of fermentation inhibitors. The fermentability of WSF recovered in medium severity is far lower than that of low severity in some studies (Stenberg et al., 1998; Boussaid et al., 1999), while in other studies it's only marginally lower (Robinson, 2003; Ewanick, 2006). A proposed alternative to overcome this dilemma is to select pretreatment conditions that are optimized to maximize sugar recovery with a subsequent post-treatment to increase the ease of hydrolysis of the cellulose component of the WIF. Post treatments have been shown to improve the enzymatic hydrolysis of WIF derived from Douglas-fir (Yang, 2002). However, ideally, a post treatment step would not be necessary for the hydrolysis/fermentation of softwood biomass, therefore, another approach could involve a detoxification of the water soluble fraction to increase the fermentability of the WSF.

1.7 Research approach and objectives

The main aim of this project was to improve the fermentability of pretreatment water soluble fraction obtained from the steam pretreatment of softwoods so as to allow effective fermentation even in the presence of significant inhibitory material which may be generated during pretreatment. The increase in fermentability was investigated both from the point of view of the water soluble fractions obtained from steam pretreatment and the various yeasts employed for the fermentation. The MSc project had the following key objectives:

- To obtain a baseline for fermentability of water soluble fractions (WSF) derived from several steam-pretreated DF and LPP samples. Steam pretreatment was performed at a severity factor $\log R_o = 3.64$, a condition considered as medium severity during the pretreatment of Lodgepole pine. Medium severity conditions for steam pretreatment are those which provided adequate fermentable sugar recovery and enzymatic hydrolysis of cellulose in the WIF fraction.

- To compare the ability of non-genetically modified yeast strains T_1, T_2, Y1528 and BY4742 to ferment the WSF derived from steam pretreated softwood. In addition, examine the robustness of different yeast strains against various model fermentation inhibitor compounds.

- To utilize overliming as a detoxification method to increase ethanol production from the fermentation of the WSF derived from the steam pretreated softwoods.

- To evaluate SHF and HHF of the combined WIF and WSF, which increases the initial fermentable sugars and subsequently increase ethanol yields.

1.7.1 Assess the fermentability of WSF derived from several steam-pretreated DF and LPP samples

Previous studies by Ewanick et al. (2007) have identified an optimal steam pretreatment severity factor ($logR_o$ = 3.64; 200°C, 5 mins, 4% SO_2) that resulted in a 96% hexose recovery from original substrate and comparable ethanol production to a WSF recovered from a Lodgepole pine pretreated at a lower severity, while also reaching a 75% glucose conversion during enzymatic hydrolysis. The previous work established the basis for the current pretreatment severity to explore its robustness with other softwood feedstocks such as Douglas-fir which has also been studied intensely by our group over the past years (Boussaid et al., 2000; Robinson, 2003; Pan et al., 2004; Ewanick, 2006). Steam pretreatment at $logR_o$ = 3.64 was applied to several Douglas-fir samples since previous studies on DF by our group were conducted on a single tree. Therefore, it is possible that minor chemical and physical differences amongst trees of the same species treated at an identical pretreatment condition may result in varying levels of recalcitrance of the solid and water soluble fractions to enzymatic hydrolysis and fermentation respectively.

Effective recovery of the partially hydrolyzed hemicellulosic sugars present in the WSF is a critical step for maximizing the overall sugar recovery from the original feedstock which is why we chose a medium severity factor ($logR_o$ = 3.64). Through fermentation with available yeast strains and analysis of known fermentation inhibitors, we hypothesized that the results would indicate whether the WSFs obtained at such a pretreatment condition are similar among the different samples of softwoods.

1.7.2 Assess the robustness of *S. cerevisiae* strains T_1, T_2, Y1528 and BY4742

Previous studies have indicated that industrial strains such as T_1 which have been adapted to survive and ferment spent sulfite liquor from the sulfite pulping process are more robust than haploid laboratory yeast strain such as BY4742 (Keating et al., 2004a). However, the robustness of another yeast strain isolated from spent sulfite liquor, T_2 relative to other strains is unknown. Furthermore, the yeast strain Y1528 possesses the unique ability to completely ferment galactose before fermenting glucose, unlike the haploid laboratory strain (Keating et al., 2006). This could be quite useful in increasing ethanol production from water soluble fractions derived from galactoglucomannan rich softwood species (Boussaid et al., 2001; Robinson et al., 2003; Keating et al., 2006). Other studies have indicated industrial strains which are polyploid similar to T_1 and T_2 exhibit a higher tolerance to fermentation inhibitors than haploid laboratory strains (Larsson et al., 2001; Martin et al., 2003). Therefore, it was proposed that these available yeast strains should be compared for their robustness, which includes their ethanol production capabilities and ethanol yield in the presence of various concentrations and types of fermentation inhibitors such as those prevalent in WSF derived from steam pretreated softwoods and model fermentation inhibitor compounds previously studied. It was envisaged that these studies could possibly elucidate the ability of yeast strains to tolerate these fermentation inhibitors as the main contribution to the robustness of these yeasts.

1.7.3 Assess overliming as a detoxification method for softwood derived WSF

Since a previous study conducted by Horvath et al. (2005) observed a 140% ethanol yield increase from the reference glucose medium by employing overliming to pH 11 at 30°C, it was of interest to assess the ability of overliming to improve the ethanol production from the WSF derived from steam pretreated softwoods. Previous studies on the fermentation of WSFs have observed low ethanol production in WSF recovered with high pretreatment severities for both Douglas-fir ($logR_o = 3.73$) and Lodgepole pine ($logR_o = 4.09$). It is likely that the high severity caused extensive sugar and lignin degradation, thereby resulting in the generation of significant inhibitory material for the yeast strains to contend with during subsequent fermentation (Robinson, 2003; Ewanick, 2006). The selected steam pretreatment severity of $logR_o = 3.64$, is close to the high pretreatment severity ($logR_o = 3.73$) previously used for Douglas-fir by Robinson (2003). Therefore it is possible that at the severity applied in the current study, may have generated high concentration of process-derived inhibitors that may result in poor ethanol production during fermentation of the recovered WSFs using the available yeast strains. Therefore, a detoxification treatment of the WSF derived from steam pretreated softwoods may result in an improvement in its fermentability. In addition, we were also interested in investigating the applicability of overliming in combination with HHF/SSF of the whole slurry (combined WIF and WSF) to assess whether this scheme would improve ethanol yields during fermentation.

1.7.4 Assess applicability of separate hydrolysis and fermentation (SHF) and hybrid hydrolysis and fermentation (HHF) for the whole slurry

A previous study by our group concluded that increasing the initial sugar concentration for fermentation through roto-evaporation was not practical because this process also increased the concentration of non-volatile fermentation inhibitors that were strongly inhibitory to yeast strains during fermentation (Robinson, 2003). An alternative to the roto-evaporation approach is to combine the sugar rich enzymatic hydrolysate from the hydrolysis of the WIF with the hemicellulose rich WSF as a whole slurry to achieve a higher initial sugar concentration. Previous studies revealed that end product inhibition of the existing soluble hemicellulosic sugars and cellobiose released by cellulose resulted in a decreased enzymatic hydrolysis rate in separate hydrolysis and fermentation (SHF) (Robinson, 2003). The same study also indicated that the initial enzymatic hydrolysis is also poor due to end product inhibition during simultaneous saccharification and fermentation (SSF) which was originally designed to overcome such inhibition. It is possible that the end product inhibition is caused by slow sugar uptake by the yeast due to fermentation inhibitors present in the whole slurry. Therefore, the use of a "pre-hydrolysis" of the WIF to increase sugar concentration in an HHF scheme was evaluated to indicate the extent of the reduction in enzymatic hydrolysis caused by end product inhibition during the subsequent SSF process. The use of overliming prior to the addition of yeast after pre-hydrolysis would provide evidence whether removal of fermentation inhibitors would increase the rate of fermentation by yeast thus reducing the potential for end production inhibition of cellulase. If this set-up leads to an increase in the final ethanol concentration after fermentation, it would provide significant insight into

potential cost savings for the bioconversion process by reducing the cost of the distillation process due to increased ethanol concentrations.

2 Materials and methods

2.1 Experimental conditions

The solid particles from size reduction did not have uniform shape or size, therefore the accessible surface area per volume of such feedstock during pretreatment is variable thus producing results that may not be reproducible despite identical pretreatment conditions.

The theoretical ethanol yield for *Saccharomyces cerevisiae* would be 0.51 g ethanol per 1 g of hexose (glucose, galactose and mannose), which is calculated based on:

1 mole of hexose (180 g/mol) → 2 moles of ethanol (total 92 g/mol) + 2 moles of CO_2 (total 88 g/mol)

92 g ethanol / mole ÷ 180 g hexose / mole = 0.51 g ethanol / g hexose

2.2 Softwood samples

Representative samples of Douglas-fir (*Pseudotsuga menziesii*) were collected from both the coastal and the interior regions in BC. Three samples of Douglas-fir at three different age ranges (60, 90 and 120 years old) were collected from both regions for a total of six samples named interior young Douglas-fir (DF1, (60 years old Douglas-fir from interior B.C.), DF2 (90 years old Douglas-fir from interior B.C.), DF3 (120 years old Douglas-fir from interior B.C.), DF4 (60 years old Douglas-fir from coastal B.C.), DF5 (90 years old Douglas-fir from coastal B.C.) and DF6 (120 years old Douglas-fir from coastal B.C).

The Douglas-fir samples collected were subsequently debarked, split, chipped and screened to 20 x 20 x 5mm. These wood chips were stored in sealed plastic bags until used for pretreatment.

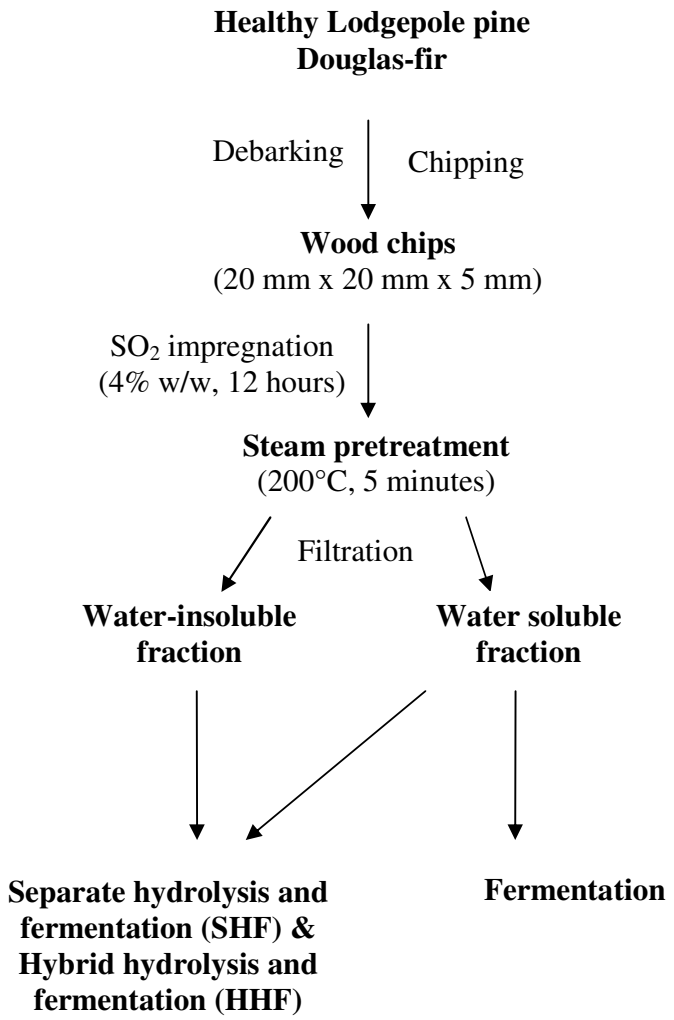

Figure 2-1. Schematic diagram of the steam pretreatment based bioconversion process.

2.3 Composition of wood chips

The moisture content of the wood chips was determined based on weight differential before and after incubation at 105^{o}C for 24 hours. For acetone extraction analysis, the wood chips were oven dried at 50^{o}C for 24 hours and then ground using a Wiley mill and fractions of specific particle sizes were recovered through 1cm mesh, 2 mm mesh, 20-mesh and finally 40-mesh. The ground wood chips (4 g) were added to the thimble and extracted with acetone overnight. The wood chips along with the thimble were removed, oven dried at 50^{o}C overnight and weighed. The acetone solvents collected in the round bottom flask was subjected to roto-evaporation for the recovery of extractives. The round bottom flask along with the extractives was oven dried at 105^{o}C overnight. The percentage of extractives from wood chips was calculated based on ODW (oven dry weight) of extractive / ODW of total wood sample.

2.4 Stream pretreatment of wood chips

The Douglas-fir wood chips were steam pretreated using the conditions shown in Table 2-1. These conditions were selected based on high sugar recovery (96%) and adequate ease of enzymatic hydrolysis (~70%) from previous study using beetle-killed Lodgepole pine (Ewanick et al., 2007).

Table 2-1. Steam-pretreatment conditions and corresponding severities used for pretreatment of Douglas-fir samples.

Time (minutes)	Temperature (^{o}C)	SO_2 (% w/w)	logRo
5	200	4	3.64

Before steam pretreatment, wood chips were impregnated with sulphur dioxide (SO_2) at 4% w/w the proportion illustrated on Table 2-1. The gaseous SO_2 (Praxair Canada) was introduced to the wood chips (300 g dry weight) in sealed pre-weighed plastic bags. The samples were weighed and placed in the fume hood at room temperature overnight. After 12 hours, the unabsorbed gas was released in the fume hood. The bags containing the impregnated samples were then reweighed to measure the weight of SO_2 absorbed.

The impregnated chips were added to a 2-L StakeTech II steam gun (Stake Technology, Norval, Ontario) in 50 g dry weight portions. The steam pretreatment process was performed using conditions as specified in Table 2-1. After pretreatment, the slurry of mixed water soluble fraction (WSF) and water-insoluble fraction (WIF) was recovered. A small portion of slurry that remained deposited on the walls of the vessel was rinsed with tap water and the liquid (steam gun wash liquid) was separately collected and analyzed for sugars in order to provide a more accurate mass balance. The slurry recovered was subsequently vacuum filtered to separate the water soluble and water-insoluble fractions. The chemical composition of the water soluble fraction, water-insoluble fraction and steam gun wash liquid were analyzed to determine the total sugars recovered after pretreatment.

2.5 Analysis of chemical composition of wood chips, water-insoluble fraction, water soluble fraction and steam gun wash liquid

The ground wood chips and the water-insoluble fraction were oven dried at 105 ^{o}C overnight and utilized for Klason lignin analysis. Approximately 0.2 g ODW of ground sample was added to a Klason cup with 3 mL of 72% sulfuric acid (Fisher). The mixture was stirred every 10 minutes during the 2 hours of acid hydrolysis at room temperature.

Upon completion of acid hydrolysis, the mixture was diluted to make a total volume of 115 mL with the final concentration of sulfuric acid at 4 % w/w. The solutions were autoclaved at 121^{o}C for an hour in sealed septa serum bottles after which the samples were cooled to room temperature. The acid hydrolyzed samples were vacuum filtered through pre-weighed, oven dried (105^{o}C) sintered glass crucibles (medium-coarseness). The filtrate was analyzed for sugars using HPLC and acid soluble lignin was analyzed at 205 nm (Dence, 1992). The acid insoluble lignins isolated in the crucibles were rinsed with deionized water and oven dried at 105^{o}C overnight. The percentage of acid insoluble lignin was calculated based on ODW of acid insoluble lignin / ODW of ground sample.

The water soluble fraction and steam gun wash liquid were subjected to oligomer-monomer sugar analysis. The pH of the liquids (Table 2-2) was measured and applied to calculate the amount of 72% sulfuric acid required for hydrolysis. A set of WSF without adding sulfuric acid and another set of WSF with the addition of sulfuric acid were autoclaved at 121^{o}C for an hour in sealed septa serum bottles along with sugar standards. The sugar composition of these liquids was subsequently analyzed by HPLC.

Table 2-2. pH of steam-pretreated Douglas-fir water soluble fraction and volume of sulfuric acid added for oligomer-monomer analysis.

Softwoods	pH	Volume of sulfuric acid added, mL
DF1	1.38	0.662
DF2	1.08	0.628
DF3	1.13	0.635
DF4	1.38	0.662
DF5	0.86	0.582
DF6	1.31	0.656
LPP	1.44	0.667

2.6 Sugar analysis using HPLC

The monosaccharide analysis of the solid substrate and oligomer-monomer analysis of the water soluble fraction were performed on a Dionex (Sunnyvale, CA) HPLC (ICS-2500) fitted with AS50 autosampler, ED50 electrochemical detector, GP50 gradient pump, and anion exchange column (Dionex, CarboPac PA1). Degassed and deionized water at 1 ml/min was used as an eluent and post-column addition of degassed 0.2 M NaOH maintained baseline stability and detector sensitivity. A solution of 1M NaOH was used to recondition the column. The samples injected into the HPLC were pre-filtered through a 0.45µm syringe filter (chromatographic specialties, Brockville, Canada). Standards were prepared using arabinose, galactose, glucose, xylose and mannose (Sigma). The concentration of sugars in the samples was calculated based on the regression line obtained from the known standard sugar composition and the response surface area from HPLC. Fucose (0.2 g/L) (Sigma) was used as internal standard and was added to all samples and standards.

2.7 Analytical determination of the fermentation inhibitors

Sugar degradation products such as furfural and 5-hydroxymethyl furfural (HMF) were analyzed on an HPLC (Alliance 2695) with a Lichrospher RP18 reverse phase column (Varian Instruments, Walnut Creek, CA) and an AD20 (Dionex) detector at a wavelength of 280 nm. HMF (sigma) standards ranged from 0.1 g/L to 4.0 g/L, while the concentration of furfural standards ranged from 0.1 g/L to 2.0 g/L. All standards and samples were filtered through a 0.45µm syringe filter (chromatographic specialties, Brockville, Canada). Three different eluents were used in the HPLC, which included eluent (A) 7.4 mM phosphoric acid (Fisher), eluent (B) acetonitrile (Fisher) and eluent (C) containing 7.4 mM phosphoric acid, methanol (Fisher) and acetonitrile at a ratio of 4:3:3. The elution program consisted of 20 minutes of elution using eluent A (95 %) and C (5 %) reaching to a gradient containing eluent A (50%) and eluent C (50%). This was followed by elution at 100 % of eluent C for 4 minutes and later 1 minute hold. Next step involved the elution with 100 % eluent B for 1 minute followed by 1 minute hold. In the last step of the program, there was 1 minute transition to 100% eluent B and back to 95% eluent A and 5% eluent C, then 10 minutes of re-equilibration to end run.

Total phenolic content of the water soluble fraction (WSF) was determined by the Prussian blue method (Graham, 1992). A suitably diluted sample (600 µL) or catechin (for the standard curve), and 200 µL of $K_3Fe(CN)_6$ (0.016 M) were added to the test tubes. This was immediately followed by the addition of 200 µL of $FeCl_3$ (0.02 M) prepared in HCl (0.1 N). The samples were mixed well and left for 15 minutes at 25 ºC to allow formation of blue precipitate. After incubation was completed, 1000 µL of stabilizer solution (composed of distilled water, 85% phosphoric acid, 1% gum acacia in volume

proportion of 3:1:1) was added. Finally the precipitate color density was measured at 700 nm. All the required blanks were prepared. The test for the samples and the standards was performed in triplicate and were calculate based on a standard curve obtained from catechin.

2.8 Preparation of yeast

The spent sulfite liquor-adapted Tembec T1 and T2 strains of *S. cerevisiae* (provided by Tembec Limited, Temiscaming, Quebec, Canada), galactose fermenting Y1528 (provided by Agricultural Research Service, US Department of Agriculture, Peoria, IL through Dr. Jeff Keating) (Keating et al., 2002) and haploid laboratory strain BY4742 (provided by Dr. Jeff Keating) were maintained on plates of solid media containing 10 g/L yeast extract (Fisher), 20 g/L peptone (Fisher), 20 g/L glucose (sigma) and 18 g/L agar (Fisher) at 4°C. These yeast strains are not genetically modified and only ferment hexoses.

The cells were grown by transferring a colony from a plate to 400 mL liquid media that contained 10 g/L yeast extract (Fisher), 20 g/L peptone (Fisher) and 20 g/L glucose (Sigma) in an orbital shaker at 30°C and 150 rpm. The yeasts were grown in two steps, the starter culture and the inoculation culture. The starter culture was grown by inoculation of a loop-full of single yeast colony on solid media into liquid YPG media. The culture was grown overnight at 30°C and 150 rpm to late log phase. The inoculation culture was obtained by further growing of the washed starter culture to the required cell density and cell mass in YPG. The cell cultures were then centrifuged at 4000 rpm for 10 minutes. The cell pellets at the bottom was washed then centrifuged three more times with sterile distilled water. Afterwards, the minimal volume of sterile distilled water was used to dissolve the cell pellet. The concentration of yeast cells was determined on the

basis of a standard curve of dry yeast cell weight. The concentration of the yeast cells was calculated based on its relationship to the optical density at 600 nm with the slope of the standard curve).

2.9 Fermentation of water soluble fractions

Prior to the fermentation of water soluble fractions, the pH was adjusted to pH 6 using 50% NaOH (Fisher). The yeast was added at a final concentration of 5 g/L dry weight. The fermentation experiments were carried out in 30 mL serum bottles for 48 hours at 30°C and 150 rpm. Samples were collected at 0, 3, 6, 12, 24, 48 hours. Samples obtained were centrifuged at 10,000 rpm for 5 minutes and the supernatant was stored at -80°C.

2.10 Detoxification using overliming

Overliming of water soluble fractions was also performed. The pH of the water soluble fractions was adjusted to 11 with solid $Ca(OH)_2$ (Sigma) with continuous stirring. The samples were then placed in an orbital shaker for 15 minutes at 150 rpm to promote precipitation. The samples were centrifuged at 4000 rpm for 10 minutes. Supernatants were recovered and the pH was adjusted to 5.5 using 72% sulfuric acid (H_2SO_4).

2.11 Separate hydrolysis and fermentation (SHF)

Water-insoluble fractions (WIF) were diluted to 5% (w/v) consistency using the water soluble fractions (WSF) and the slurry was adjusted to pH 4.8. An enzyme dosage containing cellulase at 40 FPU/g glucan (Spezyme) and ß-glucosidase at 40 CBU/g glucan (Novozymes 188) was added to each flask. The hydrolysis experiments were carried out at 50°C, 150 rpm for 72 hours. The time point samples (500 µL) were taken

at 72 hours and boiled at 100°C to deactivate the enzyme then centrifuged at 10,000 rpm for 10 minutes and finally the supernatants were stored at -20°C.

The supernatants obtained after hydrolysis were used for fermentation and their pH was adjusted to 6. Yeast at a concentration of 5 g/L was added to the supernatants to carry out the fermentations. The fermentation experiments were performed in an orbital shaker at 30°C and 150 rpm for 48 hours. Time point samples were collected periodically (0h, 1h, 3h, 6h, 12h, 24h, 48h) and centrifuged at 10,000 rpm for 5 minutes. The supernatants were stored at -80°C.

2.12 Hybrid hydrolysis and fermentation (HHF)

Water-insoluble fractions (WIF) were diluted to 5% (w/v) consistency using the water soluble fraction (WSF) and the pH was adjusted to 5.5. No other nutrients were added to the slurry. The enzyme dosage containing cellulase at 40 FPU/g glucan (Spezyme) and ß-glucosidase at 40 CBU/g glucan (Novozymes 188) was added to each serum bottle containing slurry and incubated in an orbital shaker for prehydrolysis at 50°C and 150 rpm for 12 hours. After prehydrolysis, 5 g/L yeast was added to each bottle and fermentation was performed at 37 °C. Time point samples were taken periodically (1h, 3h, 6h, 12h, 24h, 48h and 72h) , boiled at 100°C for 5 minutes, centrifuged at 10,000 rpm for 5 minutes and then the supernatants were stored at -20°C.

2.13 Fermentation in YPG medium in the presence of fermentation inhibitors

Fermentation in the presence of fermentation inhibitors, furfural (0.8 g/L) (Sigma), HMF (3 g/L) (Sigma), 4-Hydroxybenzoic acid (5 g/L) (Sigma) and vanillic acid (5 g/L) (Sigma) were performed in YPG medium containing (10 g/L yeast extract (Fisher) and 20 g/L

peptone (Fisher), 20 g/L glucose (sigma). Yeast at a final concentration of 5 g/L was used. Fermentation was performed in 30 mL serum bottles at pH 6.0 at 30ºC. Time points were taken at 0, 3, 6 and 12 hours, then 500 µL samples was taken periodically during the 12 hour experiment. The samples obtained were centrifuged 4000 rpm for 5 minutes and the supernatants stored at -80°C.

2.14 Analysis of ethanol production

Ethanol was analyzed using gas chromatography with a Hewlett Packard 5890 GC equipped with a HP-Innowax column (15m x 0.53mm) with helium as the carrier gas (20 mL/min). The temperatures of the injection unit and flame ionization detector (FID) were set at 175°C 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). Butanol (0.5 g/L) (Fisher) was used as an internal standard.

3 Results and discussion

3.1 Background

The initial work in the thesis examined the ability of the selected steam pretreatment condition which has been applied previously for the effective pretreatment of Lodgepole pine (200°C, 5 min 4% SO_2), to recover sugars either in the water insoluble or water soluble fractions after the steam pretreatment of a set of softwood samples. Subsequently, the various water soluble fractions (WSF) derived from the softwood substrates were fermented by non-genetically modified yeast to determine the fermentability. The WSFs were then diluted in an effort to improve fermentability. In the next phase of the work, the effect of various model fermentation inhibitors on the fermentative capability of four different yeast strains was assessed to gain further insight into the origin of the inhibitory material within the WSF derived from steam pretreated softwoods. The next phase of the work focused on evaluating the ability of various non-genetically modified yeasts to ferment the same softwood water soluble fractions (WSF) in both SHF and HHF schemes.

The bioconversion of cellulosic substrates to ethanol involves four main process steps including pretreatment, hydrolysis, fermentation and distillation/ethanol purification. It is widely accepted that pretreatment is required to effectively disintegrate the lignocellulosic structure into a substrate that is readily accessible to enzymes and micro-organisms to carry out hydrolysis and fermentation respectively (Gregg and Saddler, 1996). Steam pretreatment is a potential pretreatment method for bioconversion, because

of its technical advantages over other pretreatment methods including an effective improvement in the ease of hydrolysis of non-wood/woody substrates with relatively low energy and chemical input (Ramos et al 1992; Saddler et al, 1993; Robinson et al., 2002; Ramos, 2003; Ewanick et al., 2006). Steam-pretreatment involves the exposure of the lignocellulosic biomass to medium temperature steam (170-240°C) for a period of time that ranges from a few seconds to a few minutes (Chandra et al., 2007). Generally the effectiveness of the pretreatment depends on two parameters; time and temperature. Based on these two parameters a severity factor was developed, $\log R_o = \log (te^{(T - 100/14.75)})$, where t is duration of pretreatment in minutes and T is temperature in °C (Overend and Chornet, 1987).

Steam pretreatment has a long history of application in the literature for bioconversion studies involving hardwoods and agricultural residues and has resulted in substrates which reach yields between 80-90% upon subsequent enzymatic hydrolysis (Brownell and Saddler, 1984; Schwald et al., 1988; Eklund et al., 1990; Ramos et al., 1992; Ohgren et al., 2007). However, steam-pretreatment of softwood biomass has been shown to be relatively less effective in producing substrates readily susceptible to enzymatic hydrolysis and fermentation. Douglas-fir is a notable example in this regard the feedstock (Robinson et al., 2002; Mabee et al., 2006). A previous study has indicated that a substrate derived from steam-pretreated beetle-killed Lodgepole pine at $\log R_o = 3.64$ reached a considerably higher enzymatic hydrolysis yield compared to previous studies on Douglas-fir (Ewanick et al., 2006). It should be noted that previous softwood studies using steam-pretreatment involved Douglas-fir samples from a single tree, which is highly unlikely to be representative of the species let alone softwoods in general.

Furthermore, previous work with Douglas-fir employed a slightly lower pretreatment severity than the one applied here. Different trees would exhibit varying physical and chemical properties such as fiber size, porosity, extractives, heartwood/sapwood and lignin content that could all potentially affect the recalcitrance of a given softwood sample. Therefore, to gain a better understanding of the recalcitrance of softwoods to bioconversion, in particular Douglas-fir, six samples of Douglas-fir with ages ranging from 60 to120 years from both coastal and interior regions of British Columbia were obtained for the present study. In addition, a sample of Lodgepole pine was included for comparison between different softwood species that were previously studied (Ewanick et al., 2006).

The ideal steam-pretreatment condition for a given feedstock should yield water insoluble fraction (WIF), which is highly susceptible to enzymatic hydrolysis, composed of mainly of cellulose and lignin, while also yielding a highly fermentable water soluble fraction (WSF) composed of mainly partially hydrolyzed hemicellulose and other soluble process-derived aromatic compounds (Boussaid et al., 1999; Robinson et al., 2003). In a previous study, Douglas-fir wood was pretreated with three different severities, which produced WIF and WSF that exhibited a range of susceptibility to enzymatic hydrolysis and fermentability corresponding to the applied pretreatment severity. High pretreatment severity produced a readily hydrolysable WIF with poor WSF fermentation whereas a low pretreatment severity produced the opposite result (Boussaid et al., 1999). Therefore, the severity of the pretreatment selected will always represent a compromise between the ease of hydrolysis of a given substrate and the fermentability of the WSF. In addition, the monomer sugar recovery is also critical to determine the effectiveness of the steam-

pretreatment. It is known that during pretreatment C5 and C6 sugars are degraded to furan compounds which not only reduce overall sugar recovery, but also leads to an increase in process-derived fermentation inhibitors (Mes-Hartree and Saddler, 1983). Therefore a medium severity factor log R_o = 3.64 was selected based on previous criteria which produced a substrate from Lodgepole pine biomass that maximized sugar recovery, with a WIF that was receptive to enzymatic hydrolysis and a WSF that was fermentable (Ewanick et al., 2006).

3.2 Feedstock composition

Douglas-fir wood chips were prepared from wood samples that were debarked and chipped to a size equal to or less than 20 x 20 x 5 mm. This was done to reduce the heterogeneity of chip size, since chip size has been shown to affect the efficacy of pretreatment at a given pretreatment severity (Cullis et al., 2004).

The wood chips were composed of a mixture of heartwood and sapwood, which are known to differ in their chemical composition most likely due to the fact that death of cells in the older heartwood region results in the deposition of various chemical compounds in the wood (Fengel and Wagner, 1984). A previous study reported that Douglas-fir heartwood and sapwood differ in their susceptibility to pretreatment and subsequent enzymatic hydrolysis (Boussaid et al., 2000). An effective and economical pretreatment process is initially judged by sugar recovery. A chemical analysis of the wood chips, the water-insoluble cellulose and lignin-rich fraction and the water soluble hemicellulosic sugar-rich fraction was performed to evaluate the recovery of sugars after steam-pretreatment (Table 3-1, Table 3-2, Table 3-3, Table 3-4).

Table 3-1. Chemical composition of Douglas-fir wood chips (g/100g of substrate) before steam-pretreatment as determined by Klason analysis.

Softwood	Galactan	Arabinan	Glucan	Xylan	Mannan	Acid insoluble lignin
DF1	2.7	3.9	38.2	6.1	14.3	29.0
DF2	3.0	2.9	37.7	4.0	14.3	30.9
DF3	4.2	2.7	38.5	3.2	13.7	28.2
DF4	2.4	2.7	40.3	5.9	10.7	29.1
DF5	2.4	2.0	40.6	3.9	11.7	29.8
DF6	3.0	1.5	38.7	2.4	12.1	31.5
LPP	3.7	1.6	45.6	6.8	10.6	28.5

Due to cellulose being the predominant component in the wood which is a polymer of glucose, the softwood wood chips are expected to contain high proportion of glucan. The abundance of mannan corresponds to the high concentration of galacto-glucomannan hemicellulosic polymers found in softwoods (Sjostrom, 1986). These hexose-rich hemicellulosic polymers are advantageous because they offer the opportunity to produce a water soluble fraction that has an adequate concentration of fermentable monomeric hexose sugars (86 g/L in DF6, Table 3-2) as compared to hardwood and non-woody feedstocks which possess a significant amount of xylan (C5) as part of their overall hemicelluloses component. An effective pretreatment process would ideally hydrolyze most of hemicellulosic sugars to their monomeric forms, since the yeasts generally only metabolize monomeric hexose sugars and disaccharide such as sucrose. However, if the severity of pretreatment is low, there will be partially hydrolyzed short chains of

hemicellulosic sugars that are not fermentable by the yeast. A previous study (Shevchenko et al., 1998) suggested that a mild acid treatment would be sufficient to fully hydrolyze these oligomers, however, since our WSF contained only a small amount of hemicellulosic oligomers (Table 3-3) the mild acid hydrolysis was not performed. Not surprisingly, since cellulose is composed of glucose and softwood hemicellulose is mainly composed of galactoglucomannan, most of the oligomers contain glucan suggesting these may be oligomeric cellulose fragments that went into the WSF.

Table 3-2. Monomeric sugar concentration (g/L) in the water soluble fractions derived from Douglas-fir and Lodgepole pine wood chips at identical pretreatment severity (logRo=3.64).

Softwoods	[Galactose]	[Arabinose]	[Glucose]	[Xylose]	[Mannose]	[Hexose]
DF1	5.2	9.8	23.3	16.6	31.8	60.3
DF2	4.6	9.9	34.6	16.1	31.7	70.9
DF3	4.6	11.7	38.0	13.8	36.7	79.3
DF4	3.9	10.5	39.7	13.8	36.0	79.6
DF5	4.5	15.4	39.5	12.1	38.6	82.6
DF6	3.7	13.9	44.8	10.8	37.8	86.3
LPP	6.2	18.2	30.8	20.8	31.4	68.4

Table 3-3. Oligomeric sugar concentration (g/L) in the water soluble fractions derived from Douglas-fir and Lodgepole pine wood chips at identical pretreatment severity (logRo=3.64).

Softwoods	[Arabinose]	[Galactose]	[Glucose]	[Xylose]	[Mannose]
DF1	n.d.a.	n.d.a.	n.d.a.	n.d.a.	n.d.a.
DF2	n.d.a.	n.d.a.	0.5	n.d.a.	n.d.a.
DF3	n.d.a.	n.d.a.	1.2	n.d.a.	n.d.a.
DF4	n.d.a.	n.d.a.	1.3	n.d.a.	n.d.a.
DF5	n.d.a.	n.d.a.	1.3	n.d.a.	n.d.a.
DF6	n.d.a.	n.d.a.	2.2	n.d.a.	n.d.a.
LPP	n.d.a.	0.7	1.5	n.d.a.	n.d.a.

In addition to the high monomeric hexose sugar concentration in the WSF, an evaluation of the overall sugar recovery from the original wood chips is also very important in determining the effectiveness of the pretreatment since one of the main goals of bioconversion is to maximize the recovery of the chemical components of biomass. The hemicellulosic sugars include arabinose, galactose, xylose, mannose and glucose. However, the glucose recovered from the hemicellulose component is difficult to distinguish from that derived from the cellulose component and was ignored for the calculation. The overall recovery is the sum of the sugars detected in WSF and WIF compared with the sugars detected in the original wood chips before pretreatment.

Table 3-4. Total sugar recovery (gram per 100 g of initial sugars) in water soluble and insoluble fractions after the steam pre-treatment of different softwoods (200°C, 5 minutes and 4% SO_2. Log Ro=3.64).). Douglas-fir from the Interior British Columbia and from the Coastal British Columbia (the numbers 1, 2, 3, 4, 5 and 6 refers to the wood samples from six different Douglas-fir trees), LPP-Lodgepole pine.

Softwoods	Arabinose	Galactose	Glucose	Xylose	Mannose
DF1	38.1	99.7	100.0	85.4	62.3
DF2	32.4	88.2	99.6	74.9	50.0
DF3	38.1	99.7	98.9	84.9	60.1
DF4	41.4	85.1	99.2	73.4	69.1
DF5	38.2	67.3	99.3	75.5	57.9
DF6	31.3	67.2	98.4	56.6	53.9
LPP	48.3	98.4	95.2	62.0	62.4

The total sugar recovery indicated that hemicellulosic sugars, including arabinose, galactose, xylose and mannose, are lost to a greater extent than glucose (Table 3-4). However, over half of all hexoses that originated from both the cellulose and hemicellulose were recovered. When sugars such as hexoses and pentoses are exposed to a given temperature in an acidic environment, they undergo a dehydration reaction that degrades pentoses to furfuraldhydes and hexoses to 5-hydroxymethyl furfuraldehyde (HMF).

Furfural and HMF collectively belong to a chemical group called furans. Furans can act as fermentation inhibitors through various mechanisms. In a previous *in vitro* study, furans were identified to directly inhibit alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALDH) (Modig et al., 2002).

Furans have been shown to decrease the activity in glycolytic enzymes such as hexokinase and glyceraldehyde-3-phosphate dehydrogenase from crude-cell extractions (Banerjee et al., 1981). In addition to their negative effects on metabolic enzymes, furans also deplete NAD(P)H and ATP through reduction reactions (Palmqvist et al., 1999). There is also evidence that furfuraldehyde causes reactive oxygen species to accumulate and thus causes damage to cellular components such as the vacuoles, the mitochondrial membrane, chromatin and actin (Gorsich et al., 2006). Generally the pretreatment conditions are selected to minimize the formation of furans through a reduction in temperature and/or time.

In the WSF recovered with severity factors $\log R_o = 3.64$, the concentration of furfural ranged from 0.3 g/L to 0.50 g/L, while the concentration of HMF ranged from 1 g/L to 2 g/L (Figure 3-1). The concentration of furan detected is reflective of the apparent severity of the pretreatment and the recalcitrance of the substrate.

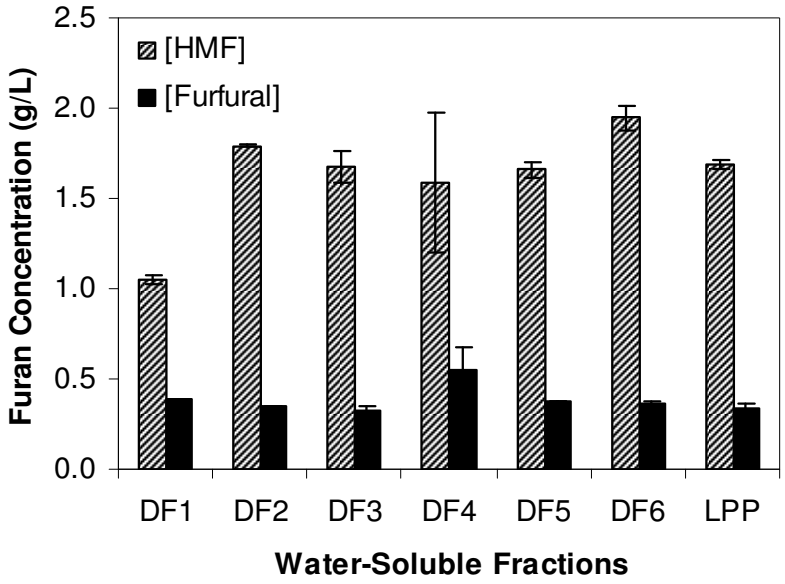

Figure 3-1. Concentration of furfural and 5-hydroxymethyl furfural (HMF) in water soluble fractions after steam-pretreatment of Douglas-firs at identical severity (logRo=3.64). Error bars denote standard deviations.

The degradation of sugars and thus the formation of process-derived inhibitors, such as furan, are proportional to ratio of hexose versus pentose from the recovered sugars in WSFs. Taking into consideration both hexoses and pentoses, DF1 exhibited one of the highest sugar recoveries while DF6 had one of the lowest (Table 3-4). This suggests that the apparent severity experienced by DF1 samples was lower than that experienced by DF6. In addition, the lower concentration of furans in DF1 may also be due to the higher volume of DF1 that may have diluted the WSF.

In addition to furans, phenolic compounds that may be detrimental to fermentation were also examined. These phenolic compounds come from both natural and process-derived sources. Natural phenolic inhibitors originate from a group of chemicals called the

extractives (Tran and Chamber, 1986). Process derived phenolic inhibitors come from the degradation of lignin (Palmqvist et al., 2000). Resin acids are also synthesized by trees for self-defense against fungi and can also be released during the pretreatment.

In this study, the Prussian blue method (Graham, 1992) was selected to detect the total concentration of phenolic compounds including single or short chain phenolic compounds. This method is a two step chemical reaction with an initial redox reaction between the phenolic compound and ferricyanide ions followed by a reaction of the reduced ferricyanide ions with ferric ions to form ferric ferrocyanide (a blue compound called Prussian blue) that is detected using a spectrophotometer. The concentration of phenolic compounds is therefore proportional to the amount of Prussian blue (ferric ferrocyanide) (Figure 3-2). While being a quick method, the Prussian blue technique does not differentiate between different phenolic species or indicate their specific concentrations. The results indicate that all the samples released a similar amount of phenolics which originated either via initial solubilization from the wood or during the steam pretreatment. The released phenolic components may play a key role in determining the fermentability of the WSF samples. It is also interesting to note that the LPP sample released a similar amount of phenolics as the Douglas-fir wood upon steam pretreatment which was unexpected as Douglas-fir, especially from the coast, is known to contain a high amount of phenolic extractives (Graham and Kurth, 1949).

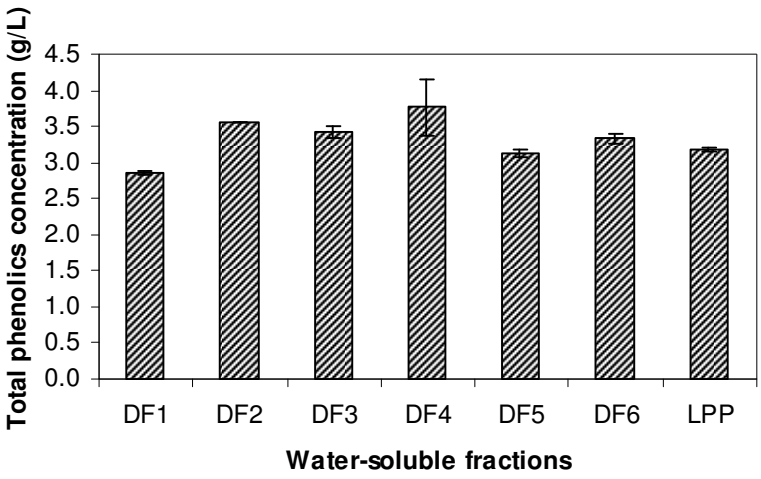

Figure 3-2. Concentration of phenolic compounds in the water soluble fractions derived from steam-pretreatment of Douglas-fir and Lodgepole Pine wood samples. All samples were steam-pretreated at an identical severity (logRo=3.64). Phenolics were quantified by the Prussian blue method. Error bars denote standard deviations.

3.3 Fermentation of the crude water soluble fractions from pretreated softwoods

Each of the WSF from the pretreatment of the seven softwood samples was fermented using a set of yeast strains as described in the sections 2.8 and 2.9. In a previous study on the fermentation of steam-pretreated Douglas-fir water soluble fraction obtained from pretreatment at three different severities (logRo = 3.08, 3.45, 3.73), the authors obtained approximately an 86% ethanol yield at low severity (logRo = 3.08) and medium severity (logRo = 3.45) pretreatments (Robinson, 2003). However, the WSF obtained at a high severity pretreatment (logRo = 3.73), was not fermentable by the yeast strains used (Robinson, 2003). Four yeast strains were selected for this study. T_1 and T_2 are spent sulfite liquor adapted industrial polyploid strains. Y1528 is a haploid strain that preferentially ferment galactose first and BY4742 is the haploid laboratory strain.

Very low ethanol yields were observed during fermentation of the crude WSFs derived from steam pretreatment of softwood performed at a severity of logRo = 3.64 (Table 3-5, Table 3-6). These WSFs appears to be too toxic for the four available yeast strains. The results here indicate that the level of fermentation inhibitors observed was beyond the threshold of tolerance of the yeast. These fermentations were performed with crude water soluble fractions from the steam pretreatment of DF and LPP samples without the addition of minor nutrients including nitrogen or phosphate.

Table 3-5. Ethanol production (g/L) during fermentation of crude steam pretreated softwood derived WSF.

Softwoods	**T1**	**T2**	**BY4742**	**Y1528**
DF1	1.2	1.7	1.7	1.3
DF2	1.2	1.3	1.4	0.8
DF3	1.8	2.3	3.3	1.0
DF4	0.9	0.8	0.8	0.7
DF5	1.7	2.3	0.9	0.9
DF6	0.6	0.7	0.9	1.7
LPP	1.7	2.2	1.4	0.8

Table 3-6. Ethanol yield (%) during fermentation of crude steam pretreated softwood derived WSF based on the theoretical maximum of 0.51 g ethanol per g of hexose.

Softwoods	T1	T2	BY4742	Y1528
DF1	4.0	5.2	3.4	2.9
DF2	1.9	3.2	2.9	2.7
DF3	2.1	6.9	4.8	3.8
DF4	1.7	2.1	2.1	2.3
DF5	2.1	2.0	5.1	3.9
DF6	0	1.8	1.4	1.3
LPP	4.0	5.2	2.9	2.7

3.4 Dilution of fermentation inhibitors improves the fermentability of the WSFs

From the experiments performed thus far, it was evident that the fermentability of the WSFs was significantly impacted by the fermentation inhibitors present. Previous studies (Robinson, 2003) have demonstrated that upon increasing the concentration of fermentation inhibitors in the water soluble fraction (WSF) derived from steam-pretreated Douglas-fir (DF) using roto-evaporation, the fermentability of the WSFs were reduced. Interestingly, in Robinson et al.'s work, unlike the work reported here, the original WSF derived from steam-pretreated DF was fermentable by the selected yeast strains. However, upon concentrating the sugar and fermentation inhibitors, the WSF could no longer be fermented. It should be noted that the previous work utilized a single sample of wood and a slightly lower severity. Therefore, we suspected that in the current study, the high concentration of fermentation inhibitors generated during pretreatment caused poor ethanol production by all the yeast strains (Table 3-5, Table 3-6). These fermentation inhibitors are likely a heterogeneous mix of natural and process derived compounds. Therefore it is difficult to characterize individual chemical species (Larsson et al., 2000; Palmqvist et al., 2000). Thus, in this study, the Prussian blue technique was used to determine the overall phenolic content of the WSF.

Based on the evidence from previous work that the yeast strains T_1 and BY4742 were capable of fermenting the WSFs derived from steam-pretreated DF, it was suspected that the yeast strains have a certain level of tolerance to fermentation inhibitors. Based on this rationale, in order to study the inhibitory effect of the fermentation inhibitors present in the WSF in greater detail, the WSFs with the lowest phenolic content (DF1), the highest phenolic content (DF4) and an intermediate phenolic content (LPP) were diluted to 50%

and 25% of original sugar and fermentation inhibitor concentrations. While fermentation inhibitors remained at the diluted concentration, hexose concentration was restored to the original concentration by glucose supplementation.

It was evident that the different yeast strains varied in their resistance to different concentrations of fermentation inhibitors found in the diluted WSF derived from steam-pretreated DF and LPP (Figure 3-3, Figure 3-4, Figure 3-5, Figure 3-6, Figure 3-7, Figure 3-8). The polypoid, spent sulphite liquor (SSL)-adapted strains (T_1 and T_2) in particular demonstrated a higher ethanol production compared to the haploid laboratory strains (BY4742 and Y1528) when fermentation inhibitors were diluted by 50% (Figure 3-4, Figure 3-5). Thus it is evident that the ability of these SSL-adapted industrial yeasts (T1 and T2) to tolerate fermentation inhibitors is superior to that of the lab-derived strains (BY4742 and Y1528). This is most likely due to the fact that spent sulfite liquor, from which they were isolated from, contains a low concentration of hexose and a high proportion of other potentially toxic compounds. In the case of the WSF derived from DF1, all four yeast strains showed comparable ethanol production after 48 hours. Therefore, it was suspected that the fermentability of the two-fold diluted DF1 was due to the lower furan and a slightly lower phenolic content of DF1 compared to the other WSFs (Figure 3-2). Despite all four strains having similar ethanol production after 48 hours, the rate of ethanol production in the first 24 hours was significantly different between the polyploid, SSL-adapted industrial strains and the haploid laboratory strains for all the diluted WSFs tested including the LPP WSF. It is interesting to note that in the case of the WSF derived from DF1 and LPP the ethanol production from the laboratory strains eventually begins to reach that of the T1 and T2 yeasts, however, this was not the case for

the DF4 derived liquor which contained a greater concentration of phenolic compounds (Figure 3-2). Similarly, further illustrating the inhibitory nature of the WSF, when the concentrations of fermentation inhibitors were reduced to a quarter of the original concentration, the ethanol productivity of the laboratory strains becomes comparable to those SSL-adapted industrial strains (Figure 3-6, Figure 3-7, Figure 3-8). Furthermore, when the water soluble fraction from the DF1, DF4 and LPP were diluted four-fold, the ethanol yields approximately doubled.

These results suggest that for future industrial applications, the fermentation inhibitors present in the WSFs derived from softwood must be reduced to a level that can be tolerated by the yeast strains. However, a potential complication is that the tolerable concentration of fermentation inhibitors varies among different yeast strains and the dilution of the WSF may not be feasible within a bioconversion scheme because dilution places a greater load on downstream ethanol distillation processes where cost of energy increases logarithmically with less than 5% ethanol. A yeast strain such as T_2 which has greater tolerance for fermentation inhibitors has a distinct advantage since it requires less dilution of the WSF inhibitors and therefore may potentially decrease the costs of an additional detoxification process compared to the other yeast strains examined. To gain further insight into the types of inhibitors which were diluted in these experiments, a set of fermentation tests were carried out using various fermentation inhibitors which have been described previously in the literature.

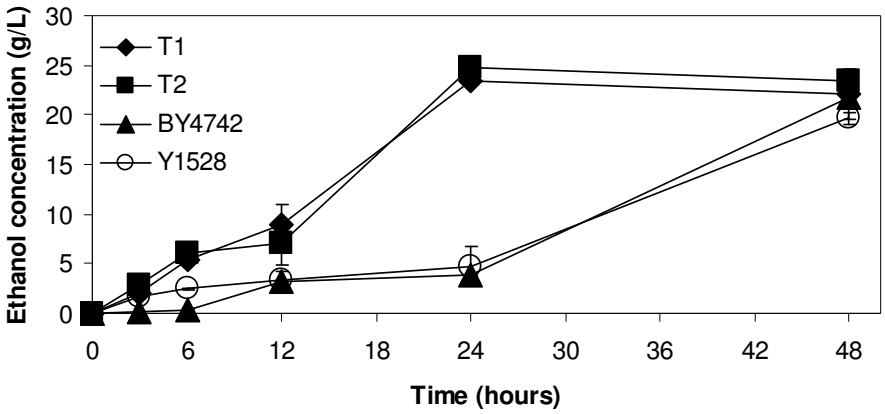

Figure 3-3. Ethanol production (g/L) from DF1 WSF diluted by 50% using four yeast strains. The original hexose concentration was restored by spiking with glucose. Error bars denote standard deviations.

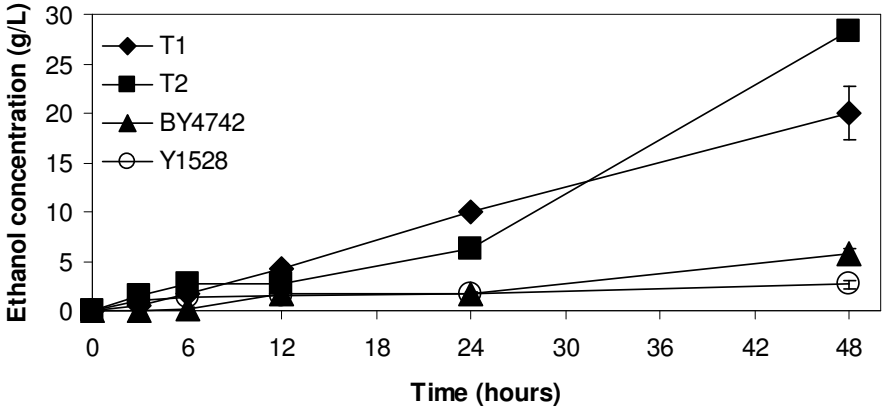

Figure 3-4. Ethanol production (g/L) from DF4 WSF diluted by 50% using four yeast strains. The original hexose concentration was restored by spiking with glucose. Error bars denote standard deviations.

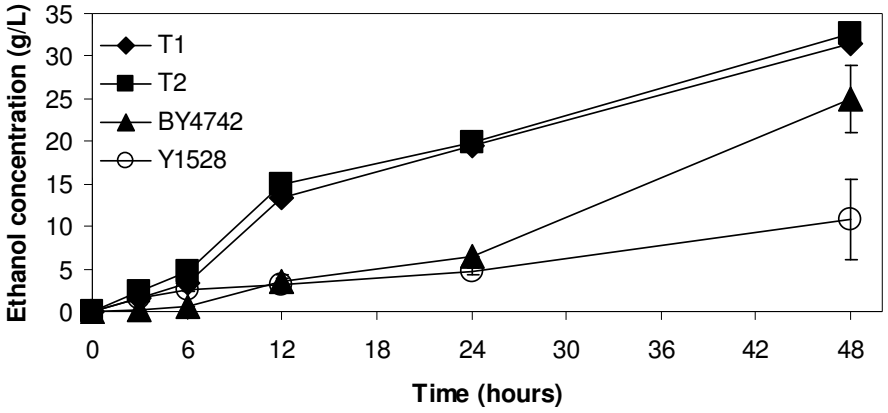

Figure 3-5. Ethanol production (g/L) from LPP WSF diluted by 50% using four yeast strains. The original hexose concentration was restored by spiking with glucose. Error bars denote standard deviations.

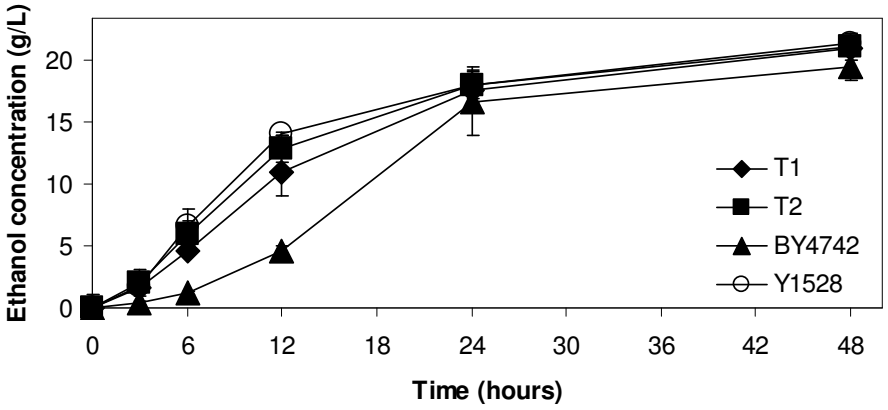

Figure 3-6. Ethanol production (g/L) from DF1 WSF diluted by 75% using four yeast strains. The original hexose concentration was restored by spiking with glucose. Error bars denote standard deviations.

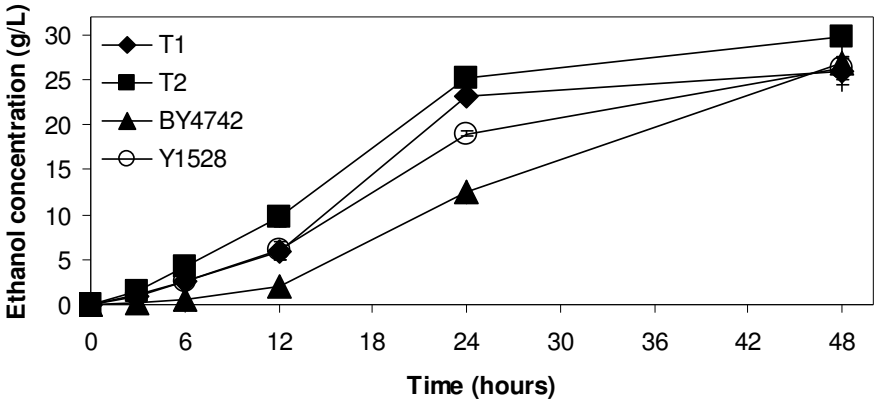

Figure 3-7. Ethanol production (g/L) from DF4 WSF diluted by 75% using four yeast strains. The original hexose concentration was restored by spiking with glucose. Error bars denote standard deviations.

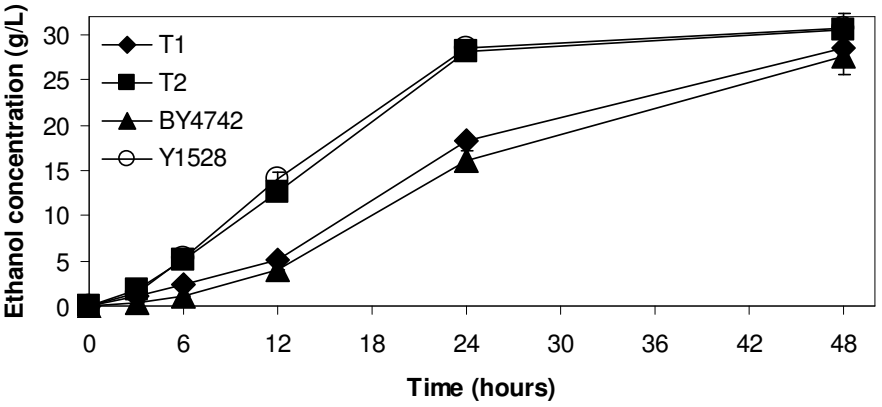

Figure 3-8. Ethanol production (g/L) from LPP WSF diluted by 75% using four yeast strains. The original hexose concentration was restored by spiking with glucose. Error bars denote standard deviations.

3.5 The selected yeast strains exhibit differences in tolerance to fermentation inhibitors identified in previous literature

The fermentation inhibitors associated with lignocellulosics have been classified as furans, phenolic compounds and weak acids. Several process-derived fermentation inhibitors which have been described previously were investigated to elucidate the tolerance of the four yeast strains (Larsson et al., 2001). Since fermentations are conducted at a pH of 6.0, which is above the pKa of many wood derived organic weak acids such as acetic acid, levulinic acid and formic acid these chemicals were not tested since the concentration of their undissociated forms would be relatively low and thus pose a minimal effect on yeast fermentability.

The furans such as HMF and furfural are formed through dehydration of pentose and hexose respectively. Furfural has been shown to exhibit an inhibitory effect on ethanol yields during fermentation at concentrations of 10 mM (1 g/L) and HMF has been shown to reduce ethanol yield at concentrations as low as 24 mM (3 g/L) (Delgenes et al., 1996). Our data (Figure 3-1) indicated that the DF water soluble fraction had lower concentrations of furans than those specified above. The four yeast strains were used to ferment hexose in the presence of 3.0 g/L (23 mM) of HMF and 0.8 g/L (8.3 mM) of furfural. These concentrations are higher than those detected in DF and LPP WSFs. The ethanol productions were compared against a reference fermentation media composed of YPG (2% glucose) (Larsson et al., 2001).

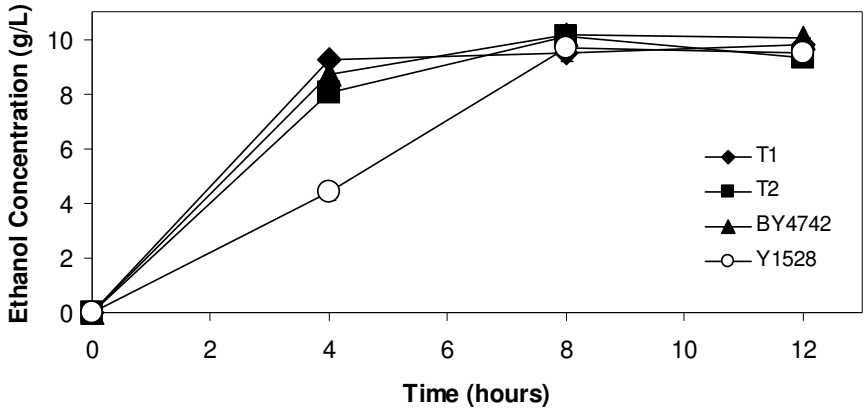

Figure 3-9. Ethanol production (g/L) during fermentation of YPG (2% glucose) media by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

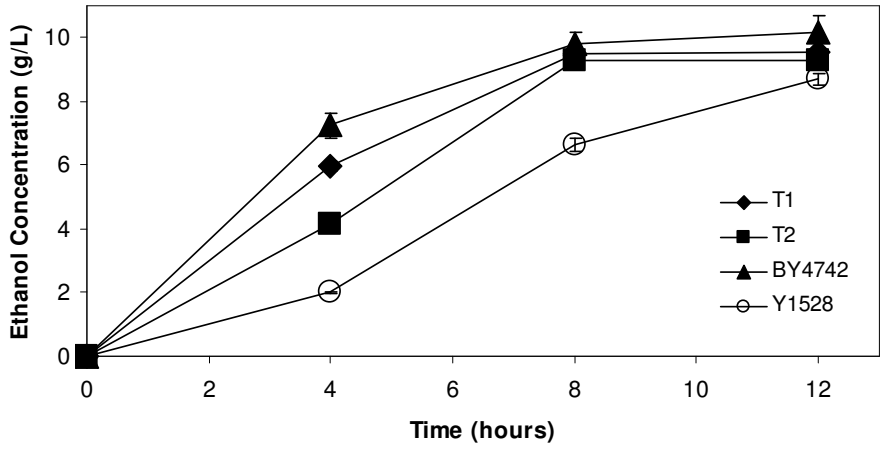

Figure 3-10. Ethanol production (g/L) during fermentation of YPG (2% glucose) media supplemented with 5-hydroxymethyl furfural (HMF) (3 g/L = 23 mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

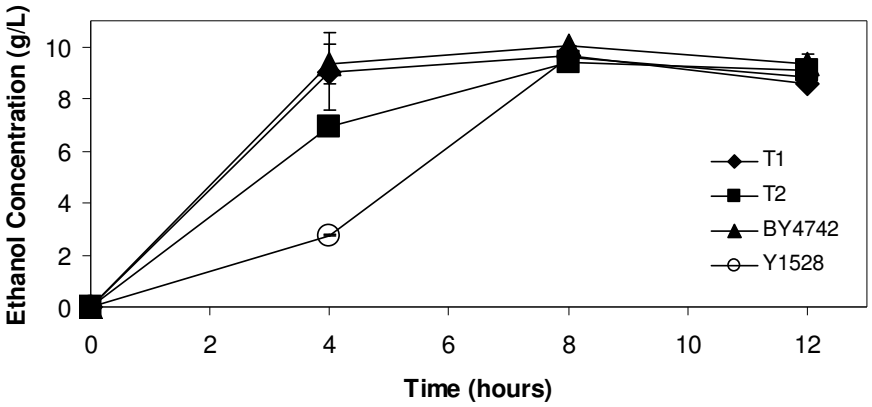

Figure 3-11. Ethanol production (g/L) during fermentation of YPG (2% glucose) media supplemented with furfural (0.8 g/L = 8.3 mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

The four yeast strains showed comparable ethanol production to the reference fermentation after 12 hours in the presence of HMF and furfural at concentrations of 3 g/l and 0.8 g/L respectively (Figure 3-10, Figure 3-11). The concentration of HMF and furfural are higher than the concentrations detected in WSFs evaluated in this study (Figure 3-1). The results suggest that HMF and furfural individually are not the main cause of poor ethanol production observed during fermentation of the crude water soluble fractions (Table 3-5, Table 3-6). However, the rate of ethanol production for Y1528 was distinctively lower than the other three strains in the presence of furan (Figure 3-9, Figure 3-10, Figure 3-11). This suggests that Y1528 may have a longer lag phase before the response to fermentation inhibitors and glucose uptake for Y1528 may be slower than the other strains. Surprisingly, the haploid laboratory strain (BY4742) had the greatest rate of ethanol production during the first four hours of fermentation in the presence of HMF

(3 g/L). It was apparent that the haploid laboratory strain exhibited adaptability in the presence of this tolerable concentration of furans compared to the SSL-adapted strains (T_1 and T_2) and the preferentially galactose fermenting strain (Y1528). This result agrees with a previous study by Keating et al (2006) that T_1 shows tolerance to furans while fermenting in YPG medium. Overall, all four yeasts had a higher ethanol productivity after 12 hours of fermentation than *S. cerevisiae* strain CBS 1200 utilized in a previous study where only a 20% ethanol yield was obtained in the presence of 10 mM of furfural and a 17% ethanol yield in presence of 24 mM of HMF (Delgenes et al., 1996).

Since it was evident that individual furans were not the main inhibitors responsible for the poor fermentation seen with the crude WSF from Douglas-fir, several phenolic compounds which have been shown to be formed during steam pretreatment were investigated (Clark and Mackie, 1984; Lee et al., 1999; Palmqvist et al., 1999; Larsson et al., 2000). These monomeric phenolic compounds were selected based on several criteria. First of all, the H/G/S ratio of softwood lignin which dictates the species of monomeric phenolic compounds present in the WSF. Softwood lignin has predominantly guaiacyl (G) units (82-98%) with a very low concentration of para-hydroxyphenyl(H) and syringyl (S) units (less than 18%) (Sjostrom, 1993). Secondly, the functional groups attached to the phenylpropane monomers are expected to be aldehyde or carboxylic acid groups due to oxidative acidic conditions (Klinke et al., 2002; Larsson et al., 2001; Jonsson et al., 1998; Thomsen et al., 2009). Based on these criteria 4-hydroxybenzoic acid (Ando et al., 1986; Jonsson et al., 1998; Klinke et al., 2002), vanillic acid (Tran and Chambers, 1985; Ando et al., 1986; Klinke et al., 2002), cinnamic acid (Ando et al., 1986; Fenske et al., 1999; Klinke et al., 2002) and coniferyl aldehyde (Burchert et al., 1990) were selected as

model compounds to examine the inhibitory effect of phenolic compounds with different H/G/S unit and functional groups. The 4-hydroxybenzoic acid resembles 4-hydroxybenzyl units of lignin while vanillic acid and coniferyl aldehyde resemble guaiacyl unit structure (Klinke et al., 2004). The acidic groups on some of these compounds also aided their solubility which facilitated the experiments. Finally the concentrations of these phenolic compounds were selected based on previous studies reviewed by Klinke (2004).

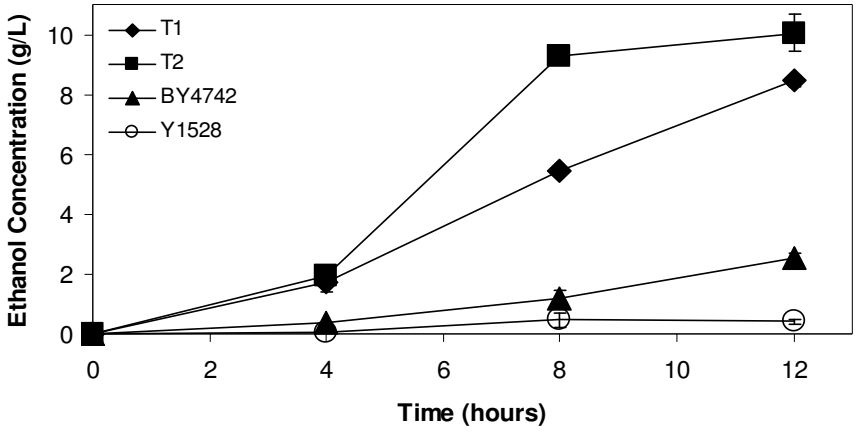

Figure 3-12. Ethanol production (g/L) during fermentation of YPG (2% glucose) media supplemented with 4-hydroxybenzoic acid (5 g/L = 36 mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

Although all four yeast strains had comparable ethanol production after 12 hours of fermentation in the presence of furans (Figure 3-10, Figure 3-11), the SSL-adapted T_1 and T_2 strains had significantly higher ethanol production in the presence of 4-hydroxybenzoic acid compared to both the haploid laboratory strain (BY4742) and Y1528 (Figure 3-12). This is one of the first reports to indicate that the major

contribution to the differences of robustness among the SSL-adapted strains versus haploid strains is their ability to tolerate phenolic compounds such as 4-hydroxybenzoic acid.

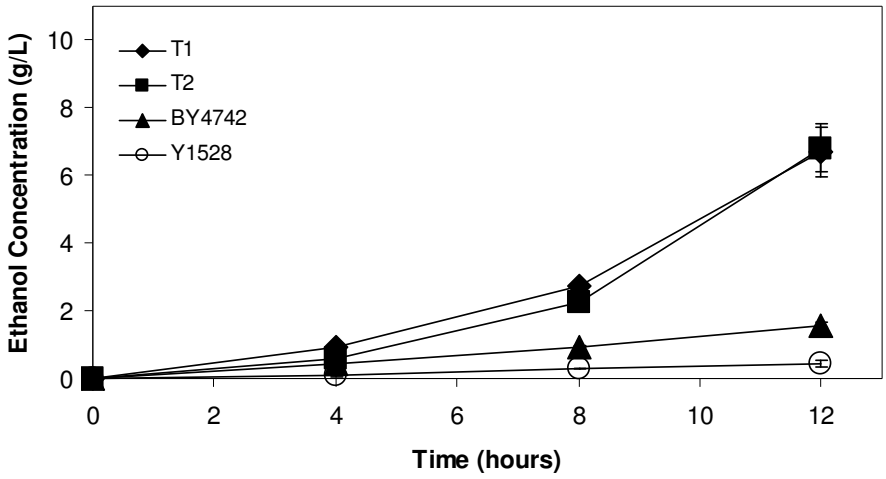

Figure 3-13. Ethanol production during (g/L) fermentation of YPG (2% glucose) media supplemented with vanillic acid (5 g/L = 30mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

Similar to the results observed for ethanol production in the presence of 4-hydroxybenzoic acid (5 g/L), the SSL-adapted strains T_1 and T_2 had higher ethanol production after 12 hours of fermentation in the presence of vanillic acid (5 g/L) compared to the haploid laboratory strain (BY4742) and Y1528. It was evident from the results that the phenolic compounds played a significant role in the toxicity to the yeast, but with both 4-hydroxybenzoic acid and vanillic acid T_1 and T_2 quickly adapted to overcome the toxicity of these phenolic compounds compared to the other yeast strains.

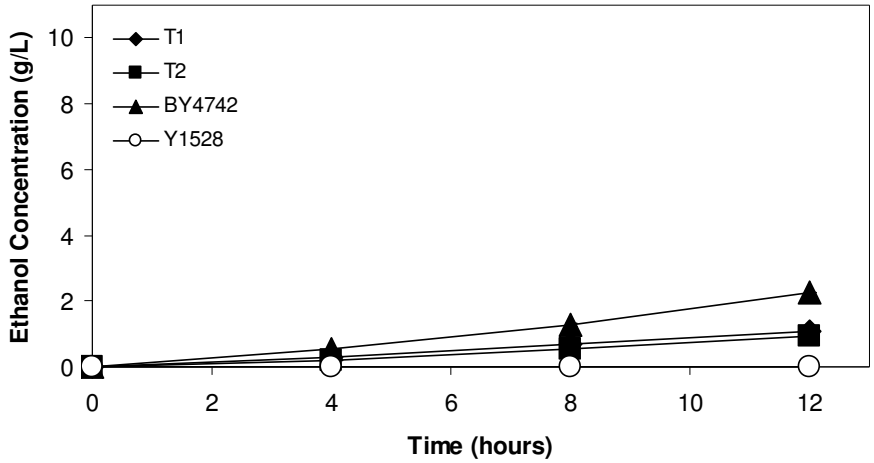

Figure 3-14. Ethanol production during (g/L) fermentation of YPG (2% glucose) media supplemented with cinnamic acid (5 g/L = 33 mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

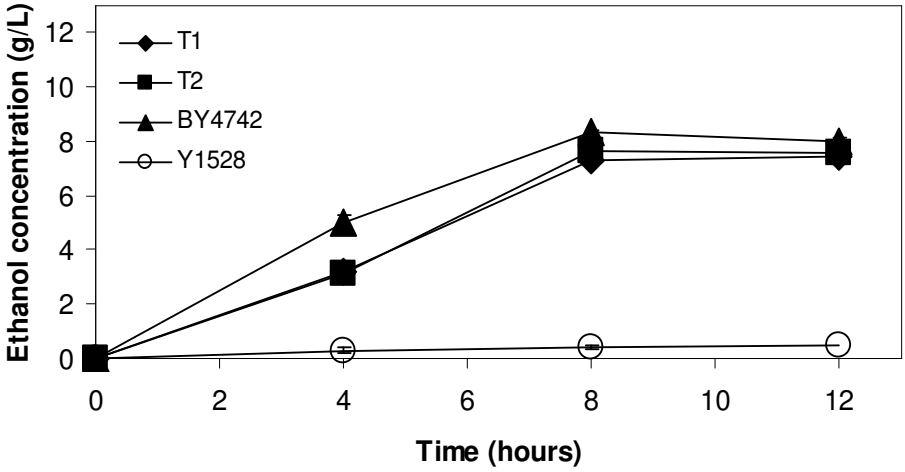

Figure 3-15. Ethanol production during (g/L) fermentation of YPG (2% glucose) media supplemented with cinnamic acid (1 g/L = 7 mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

All four yeast strains had very low ethanol production after 12 hours fermentation in the presence of cinnamic acid (5 g/L) (Figure 3-14). SSL-adapted yeast strains T_1 and T_2 had noticeablely poor ethanol production compared to their performance in the presence of 4-hydroxybenzoic acid and vanillic acid. The results suggest that at comparable concentrations, the toxicity of cinnamic acid is higher than that of 4-hydroxybenzoic acid and vanillic acid, which resembles chemical structures similar 4-hydroxybenzyl unit and a guiacyl unit. These results are supported by previous findings that different functional groups attached on a similar aromatic compound would exhibit varying levels of toxicity (Ando et al., 1986; Palmqvist et al., 2000; Klinke et al, 2004). When the concentration of cinnamic acid is reduced to 1 g/L (7 mM), both SSL-adapted strains and the haploid laboratory strain had significantly higher ethanol production compared to Y1528. The results indicate that Y1528 does not have the same level of tolerance to phenolic compounds such as cinnamic acid as the other three yeast strains.

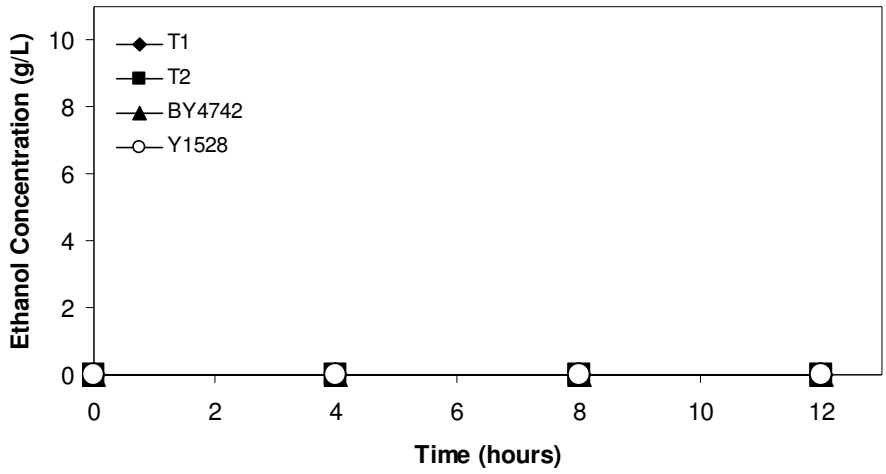

Figure 3-16. Ethanol production during (g/L) fermentation of YPG (2% glucose) media supplemented with coniferyl aldehyde (5 g/L = 28 mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

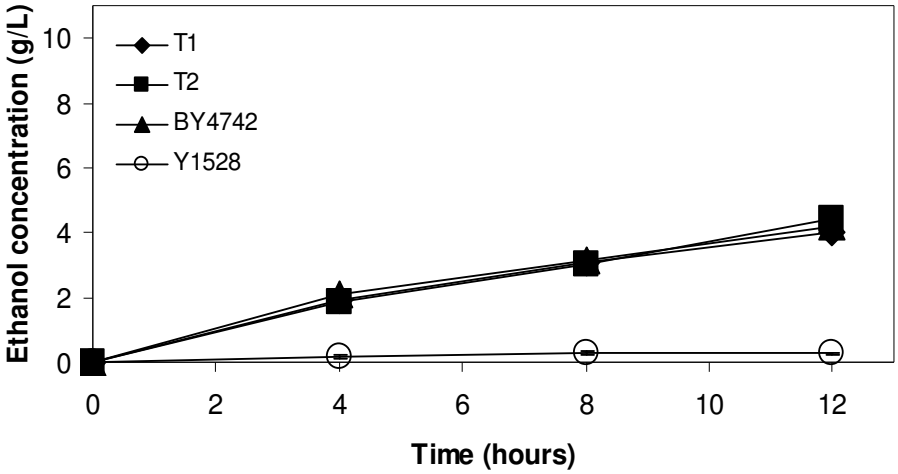

Figure 3-17. Ethanol production during (g/L) fermentation of YPG (2% glucose) media supplemented with coniferyl aldehyde (1 g/L = 6 mM) by yeast strains T1, T2, BY4742 and Y1528. Error bars denote standard deviations.

Coniferyl aldehyde (5 g/L) prevented ethanol production by all four yeast strains (Figure 3-16). However, when the concentration of coniferyl aldehyde was reduced to 1 g/L (6 mM), a similar pattern of ethanol production was found among the SSL-adapted strains and the haploid laboratory strain while Y1528 again produced significantly lower concentrations of ethanol.

Through this series of fermentation experiments with model phenolic compounds, it is evident that the SSL-adapted strains T_1 and T_2 demonstrated a higher ethanol production than the haploid laboratory strain and Y1528. These results are in agreement with previous studies (Robinson, 2003; Keating et al., 2006). Furthermore it was shown that the yeast strain T_2 had a higher tolerance to the phenolic compound 4-hydroxybenzoic acid than T_1. However, T_1 and T_2 were similar in ethanol production when tested in the

presence of other phenolic compounds. Although the haploid laboratory strain had lower a ethanol production in 4-hydroxynbenzoic acid (Figure 3-12), its ethanol production was comparable to the SSL-adapted strains in the presence of vanillic acid, cinnamic acid and coniferyl aldehyde (Figure 3-13, Figure 3-14, Figure 3-15, Figure 3-16, Figure 3-17). The yeast strain Y1528 had a significantly lower tolerance of phenolic compounds compared to the other three strains unlike its tolerance to the furans. Since the Y1528 yeast was not genetically modified, the lower tolerance of phenolic compounds is likely due to a slower or ineffective physiological response. Y1528 has an unusual metabolic pathway for utilization of hexoses, since it preferentially utilizes galactose as the primary carbon source, which may have compromised its ability to immediately metabolize glucose to generate energy through fermentation when it had a significantly lower initial rate of ethanol production (Figure 3-9). A possible explanation is that the lower rate of glucose metabolism through the Embden-Meyerhof pathway may have decreased the rate of energy production to a level below what is required to launch a rapid physiological response against phenolic inhibitors, thereby reducing the tolerance of Y1528 to fermentation inhibitors compared to the SSL-adapted strain and the haploid laboratory strain.

It is evident that the tolerance of different yeast strains to phenolic compounds is based on the specific chemical species and concentration. It is possible that this variation in tolerance contributed to the differential fermentation response to the different crude WSF derived from steam pretreatment of DF and LPP (Table 3-5, Table 3-6). The concentration of fermentation inhibitors appears to be beyond what is tolerable for all four yeast strains and it is not practical to dilute the fermentation inhibitors contained in

the WSFs to a tolerable concentration because that would dilute the initial sugar concentration and thus reduce final ethanol concentration. Therefore, alternative methods to reduce fermentation inhibitor concentration while having minimal reduction of initial sugar concentration would be required.

3.6 Overliming improved the fermentability of the water soluble fractions (WSF) derived from steam pretreated softwood

A reduction in fermentation inhibitors, in particular phenolic compounds may be required to increase ethanol production as described in section 3.5. Although the dilution of WSF effectively reduces the concentration of fermentation inhibitors, dilution also reduces the initial hexose concentration which subsequently will lower ethanol concentrations. When concentration of ethanol is below 5%, it can leads to higher product recovery costs (Galbe and Zacchi, 2002).

Overliming was described as an effective detoxification process in a previous study with WSF derived from steam pretreated softwood (Robinson, 2003). The exact mechanism of detoxification by overliming is not clear, however, it is suspected that at higher pH, the proton on the hydroxyl group on aromatic compounds is removed to form a ketone (quinone) group where the oxygen carries a negative charge. The negatively charged oxygen will form an ionic bond with Ca^{2+} to form a precipitate containing aromatic fermentation inhibitors. The reduction of fermentation inhibitors through precipitation would enhance the fermentability of the overlimed hydrolysate as the yeast would ferment the remaining soluble sugars.

Overliming has the advantage of being a relative by simple detoxification technique, and due to low volume of calcium hydroxide ($CaOH_2$) required, it does not reduce the

concentration of initial sugars significantly. The optimal conditions for overliming were further studied by Horvath et al (2005) who determined that the addition of calcium hydroxide ($CaOH_2$) to dilute an acid-pretreated spruce hydrolysate up to pH 11 at 30°C effectively reduced HMF by 65%, furfural by 53% and total phenolics by 22%. Overliming drastically improved ethanol productivity for the untreated hydrolysate from 6% to 140% of the reference fermentation (Horvath et al., 2005). Interestingly, a 22% reduction of total phenolics in the study by Horvath et al. (2005) was enough to significantly improve ethanol productivity, which supports the notion that the poor ethanol productivity of the crude WSF may be caused by specific fermentation inhibitors.

In the overliming experiments of the WSF from the steam pretreated softwoods, T_2 was selected as the yeast strain to be assessed due to its higher robustness noted in section 3.5. The WSFs of steam pretreated DF1, DF4 and LPP were selected as the hydrolysates to be studied based on their overall phenolic compound concentration (Figure 3-2).

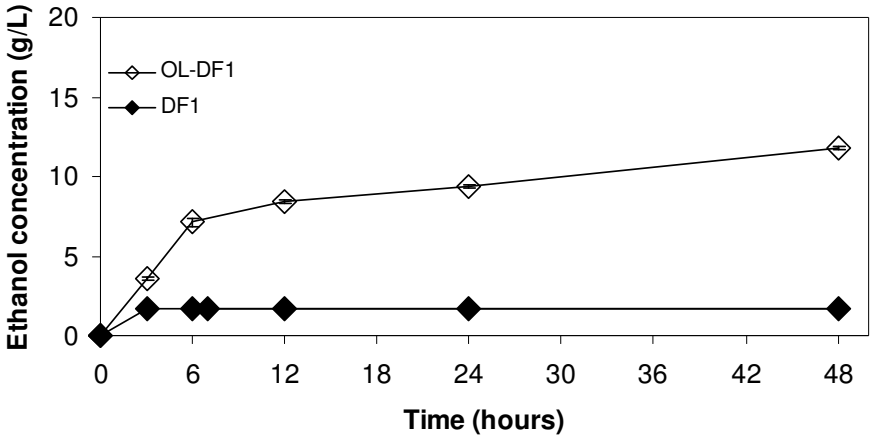

Figure 3-18. Ethanol production (g/L) by yeast strain T2 from fermentation of steam-pretreated DF1 water soluble fraction with and without overliming treatment. Error bars denote standard deviations.

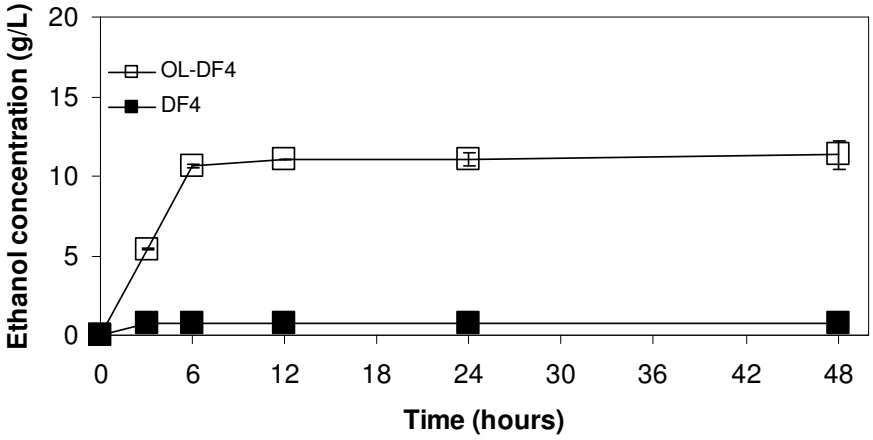

Figure 3-19. Ethanol production (g/L) by yeast strain T2 from fermentation of steam-pretreated DF4 water soluble fraction with and without overliming treatment. Error bars denote standard deviations.

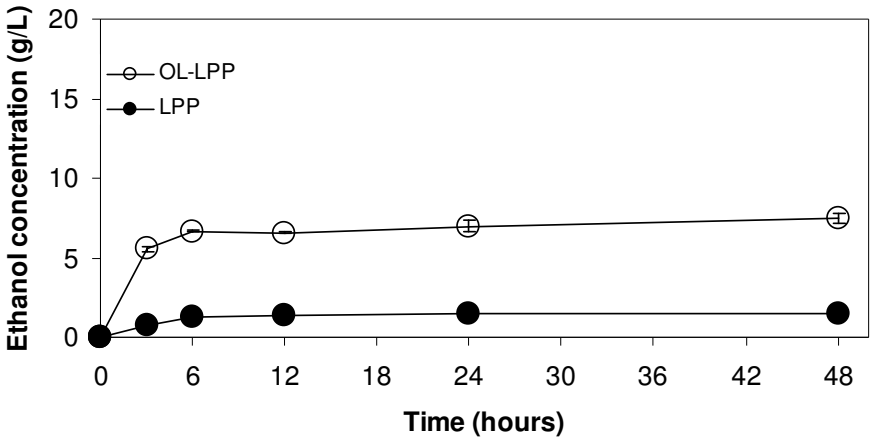

Figure 3-20. Ethanol production (g/L) by yeast strain T2 from fermentation of steam-pretreated LPP water soluble fraction with and without overliming treatment. Error bars denote standard deviations.

Prior to overliming, minimal levels of ethanol production were observed for the WSFs derived from DF1, DF4 and LPP (Figure 3-18, Figure 3-19, Figure 3-20). The ethanol production was significantly improved for all the WSF hydrolysates after overliming treatment (Figure 3-18, Figure 3-19, Figure 3-20). Overliming of the WSF derived from DF1 increased ethanol production from about 1.7 g/L to 11.8 g/L (Figure 3-18). Similar results were noted with DF4 as well. However, overliming of WSF derived from LPP increased ethanol production to a lesser extent from about 1.5 g/L to 7.5 g/L. Overliming of WSF derived from LPP has lower net increase of ethanol production compared to those from DF1 and DF4. This was perhaps caused by different species of fermentation inhibitors present in the different WSFs, since different phenolic compounds exhibits different levels of toxicity on the yeast strains' ethanol productivity seen in section 3.5. Furthermore, Douglas-fir trees, especially those from coastal regions may contain various phenolic compounds with antimicrobial activity (Graham and Kurth, 1949).

3.7 The steam pretreated softwood WSFs exhibit differences in fermentability when supplemented with glucose

The initial monomeric hexose concentration will determine the maximum ethanol produced in addition to the concentration and types of fermentation inhibitors present in the WSF derived from steam pretreated softwood. Each WSF hydrolysate was spiked with glucose up to 100 g/L of total hexose that are fermentable by yeasts. Theoretically 100 g/L glucose should produce 51 g/L of ethanol. The fermentation of WSF hydrolysates was performed with all four yeast strains and was compared against a reference fermentation of 100 g/L glucose, 10 g/L yeast extract and 20 g/L peptone (YPG) media.

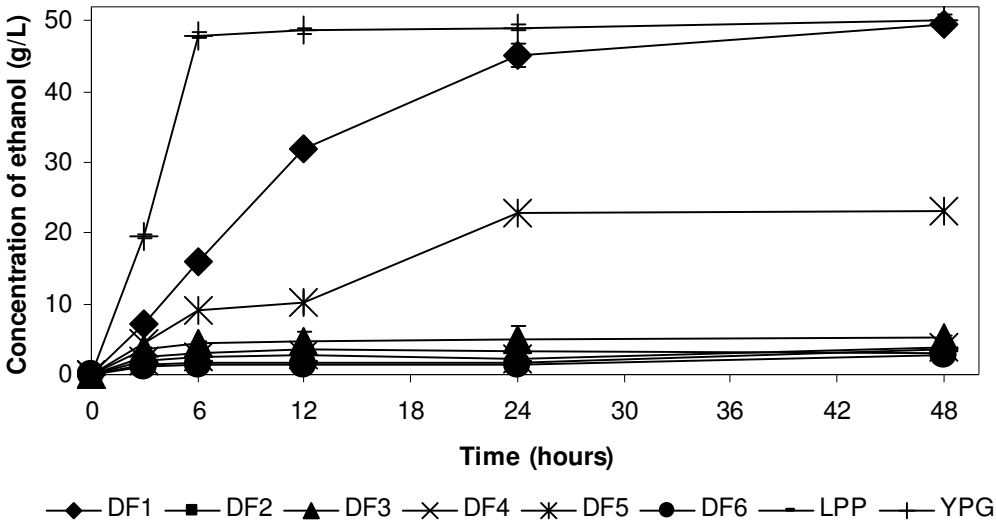

Figure 3-21. Ethanol production (g/L) by yeast strain T1 after spiking steam-pretreated softwood water soluble fractions (DF1, DF2, DF3, DF4, DF5, DF6 and LPP) up to 100 g/L hexose with glucose. YPG indicates a control reaction containing 100 g/L glucose. Error bars denote standard deviations.

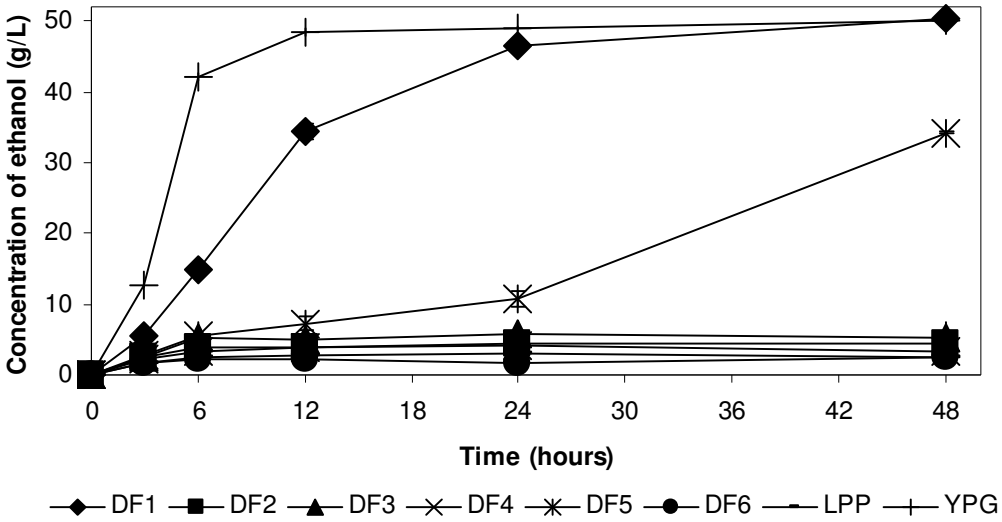

Figure 3-22. Ethanol production (g/L) by yeast strain T2 after spiking steam-pretreated softwood water soluble fractions (DF1, DF2, DF3, DF4, DF5, DF6 and LPP) up to 100 g/L hexose with glucose. YPG indicates a control reaction containing 100 g/L glucose. Error bars denote standard deviations.

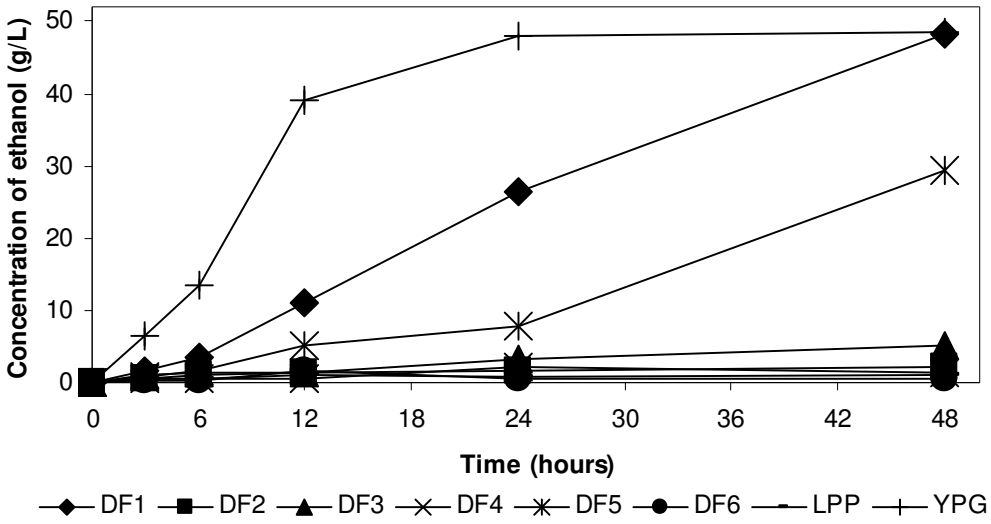

Figure 3-23. Ethanol production (g/L) by yeast strain Y1528 after spiking steam-pretreated softwood water soluble fractions (DF1, DF2, DF3, DF4, DF5, DF6 and LPP) up to 100 g/L hexose with glucose. YPG indicates a control reaction containing 100 g/L glucose. Error bars denote standard deviations.

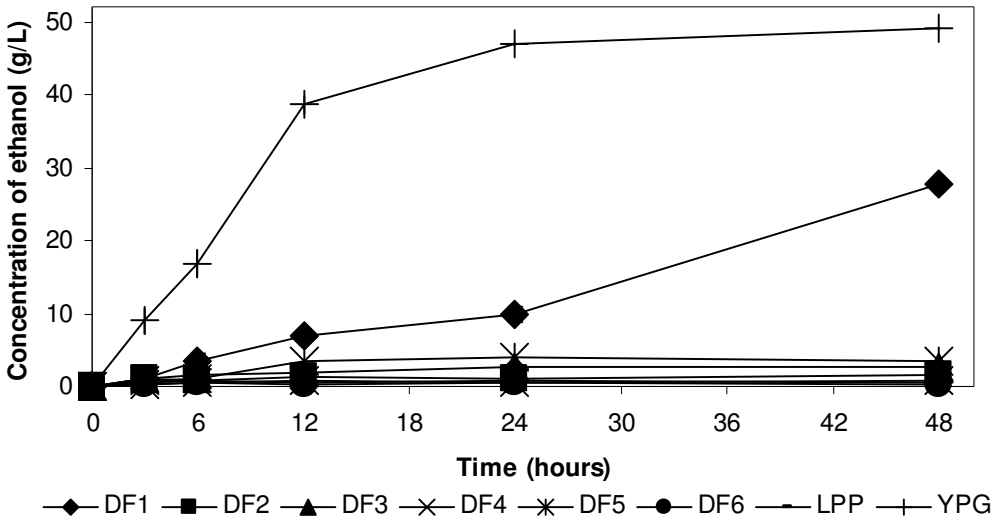

Figure 3-24. Ethanol production (g/L) by yeast strain BY4742 after spiking steam-pretreated softwood water soluble fractions (DF1, DF2, DF3, DF4, DF5, DF6 and LPP) up to 100 g/L hexose with glucose. YPG indicates a control reaction containing 100 g/L glucose. Error bars denote standard deviations.

Both SSL-adapted yeast strains T_1 and T_2 achieved over 95% theoretical ethanol yield while the haploid laboratory strain and Y1528 produced lower ethanol yields (Figure 3-21, Figure 3-22, Figure 3-23, Figure 3-24). Among the different WSFs examined, only the WSF derived from DF1 produced an ethanol concentration comparable to that of the reference fermentation. This is likely due to the presence of phenolic fermentation inhibitors in DF2, DF3, DF4, DF5, DF6 and LPP. Interestingly, the ethanol production from the WSF from DF1was significantly improved when the hexose concentration was spiked to 100 g/L (Table 3-6, Figure 3-21, Figure 3-22, Figure 3-23, Figure 3-24). This is perhaps due to the abundance of fermentable sugar in the supplemented samples which allows the yeast to produce the required energy to overcome some of the inhibitory effect of fermentation inhibitors. DF1 WSF had the lowest furan concentration (Figure 3-1) and

lowest phenolic compound concentration (Figure 3-2) among the WSFs, which may have allowed the yeast strains to overcome the inhibitions. Although the fermentation inhibitors in the WSFs did not completely inhibit ethanol production; the initial rate of ethanol production for DF1 was significantly lower than that of the reference fermentation.

The WSF derived from DF5 was fermented by both SSL-adapted strains and the haploid laboratory strain, while only Y1528 was not able to ferment this liquor stream. The WSF derived from DF5 had the second lowest total amount of phenolic compounds (Figure 3-2). Perhaps phenolic compounds at a concentration between that of DF1 and DF5 may be the threshold tolerance concentration for the yeast. However, this is difficult to determine, due to the chemical heterogeneity of phenolic compounds and their specific inhibitory concentrations. It is also likely that a higher initial concentration of fermentable sugars improved the fermentability of WSF derived from DF5.

3.8 Separate hydrolysis and fermentation (SHF) and hybrid hydrolysis and fermentation (HHF) using whole slurries improved ethanol production

A higher initial fermentable sugar concentration should maximize the final ethanol concentration. It can be envisioned that it would not be practical to supplement the WSF with fermentable sugars such as glucose in an industrial bioconversion process. Therefore, the utilization of fermentable sugars recovered through both the cellulose-rich WIF solid fraction and the hemicellulosic sugar-rich WSF (whole slurry) will be required to achieve a maximal sugar recovery for ethanol production. There are two processes which accommodate the combining of sugars obtained from enzymatic hydrolysis of

cellulose in WIF and hemicellulosic sugars in WSF: separate hydrolysis and fermentation (SHF) and hybrid hydrolysis and fermentation (HHF).

Separate hydrolysis and fermentation has the advantage of allowing enzymatic hydrolysis and fermentation to be performed separately at their individually optimized conditions. The optimal condition for enzymatic hydrolysis is usually pH 4.8 and 50°C while the optimal conditions for fermentation with yeast is usually pH 6.0 and 30°C for 48 hours. To increase the initial sugar concentration after enzymatic hydrolysis, a 5% consistency (w/v) was selected since previous studies have indicated that increasing the consistency could increase the likelihood of end product inhibition for the cellulolytic enzymes (Holzapple et al., 1990). In addition, the presence of hemicellulosic sugars in the WSF also increases the sugar concentration of the whole slurry.

Based on the results of overliming experiments (Figure 3-18, Figure 3-19, Figure 3-20), we explored the possibility of combining SHF with overliming to maximize ethanol production. Since overliming involves a significant fluctuation in pH that can potentially deactivate cellulase enzymes, the WSF was overlimed prior to the addition of WIF for the whole slurry hydrolysis.

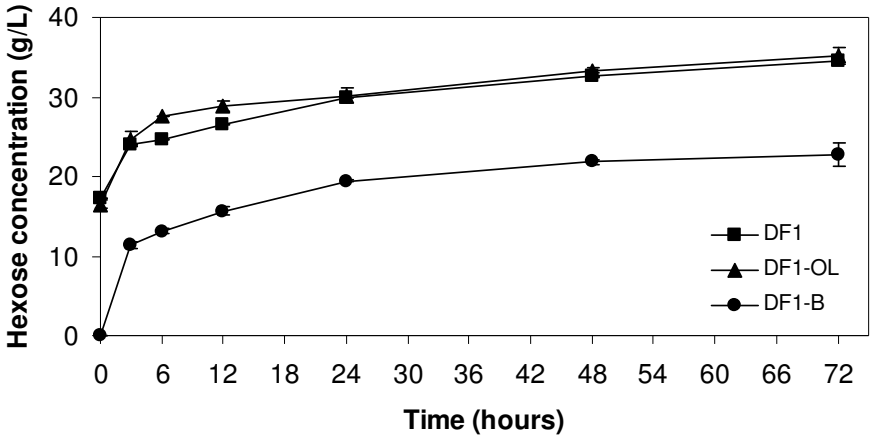

Figure 3-25. Enzymatic hydrolysis of DF1 water-insoluble fraction in water soluble fraction, overlimed water soluble fraction and pH 4.8 acetate buffer at 5% consistency (w/v) with 40 FPU/g glucan (Spezyme) and ß-glucosidase at 40 CBU/g glucan (Novozymes 188). (DF1: DF1 water soluble fraction; DF1-OL: overlimed DF1 water soluble fraction; DF1-B: pH 4.8 acetate buffer) Error bars denote standard deviations.

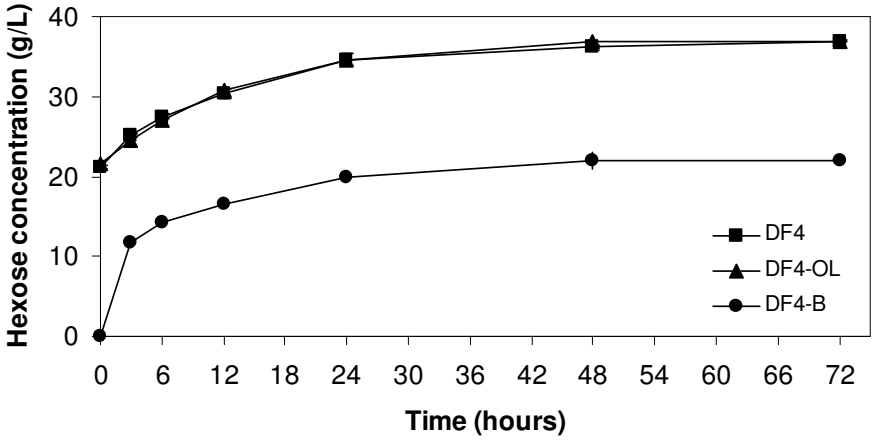

Figure 3-26. Enzymatic hydrolysis of DF4 water-insoluble fraction in water soluble fraction, overlimed water soluble fraction and pH 4.8 acetate buffer at 5% consistency (w/v) with 40 FPU/g glucan (Spezyme) and ß-glucosidase at 40 CBU/g glucan (Novozymes 188). (DF4: DF4 water soluble fraction; DF4-OL: overlimed DF4 water soluble fraction; DF4-B: pH 4.8 acetate buffer) Error bars denote standard deviations.

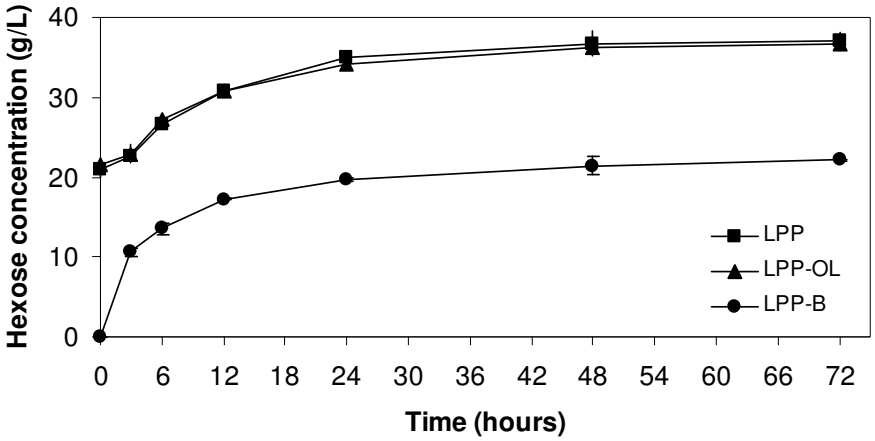

Figure 3-27. Enzymatic hydrolysis of LPP water-insoluble fraction in water soluble fraction, overlimed water soluble fraction and pH 4.8 acetate buffer at 5% consistency (w/v) with 40 FPU/g glucan (Spezyme) and ß-glucosidase at 40 CBU/g glucan (Novozymes 188). (LPP: LPP water soluble fraction; LPP-OL: overlimed LPP water soluble fraction; LPP-B: pH 4.8 acetate buffer) Error bars denote standard deviations.

The glucose yield from the enzymatic hydrolysis of cellulose in the WIF in the presence of WSF has been reduced compared to enzymatic hydrolysis in acetate buffer. This was true for all three softwood samples (Figure 3-25, Figure 3-26, Figure 3-27, Table 3-7). There was no appreciable difference in cellulose conversion between the overlimed and non-overlimed whole slurry hydrolysates (Figure 3-25, Figure 3-26, Figure 3-27) which indicates that the reduction of fermentation inhibitors did not affect enzymatic hydrolysis. The whole slurry approach effectively increased the initial fermentable sugars of the WSFs. The hexose concentration in the whole slurry derived from DF1 increased by 17.3 g/L from 17.3 g/L, DF4 increased by 15.8 g/L from 21.2 g/L and LPP increased by 16.0 g/L from 21.0 g/L (Figure 3-25, Figure 3-26, Figure 3-27).

Table 3-7. Glucan conversion (%) during 72 hours of enzymatic hydrolysis of steam-pretreated softwood WIF in the presence of WSF, overlimed WSF and acetate buffer. Softwood samples pretreated at severity $logR_o$ of 3.64 were used.

Softwood	Water soluble fraction	Overlimed water soluble fraction	pH 4.8 acetate buffer
DF1	60.7	65.9	79.9
DF4	56.9	54.6	79.0
LPP	61.2	57.2	84.5

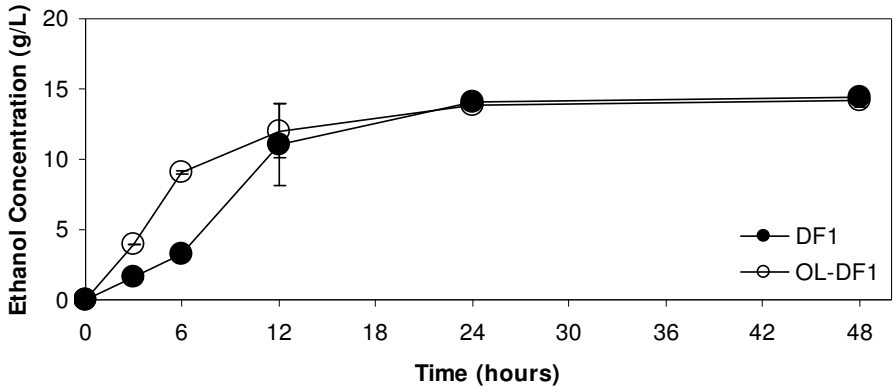

Figure 3-28. Ethanol production (g/L) from separate hydrolysis and fermentation (SHF) of steam-pretreated DF1 whole slurry with and without overliming treatment by yeast strain T2. Error bars denote standard deviations.

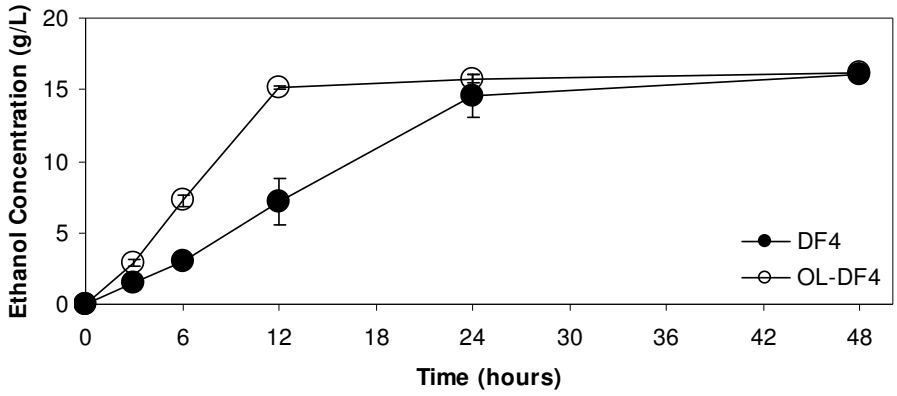

Figure 3-29. Ethanol production (g/L) from separate hydrolysis and fermentation (SHF) of steam-pretreated DF4 whole slurry with and without overliming treatment by yeast strain T2. Error bars denote standard deviations.

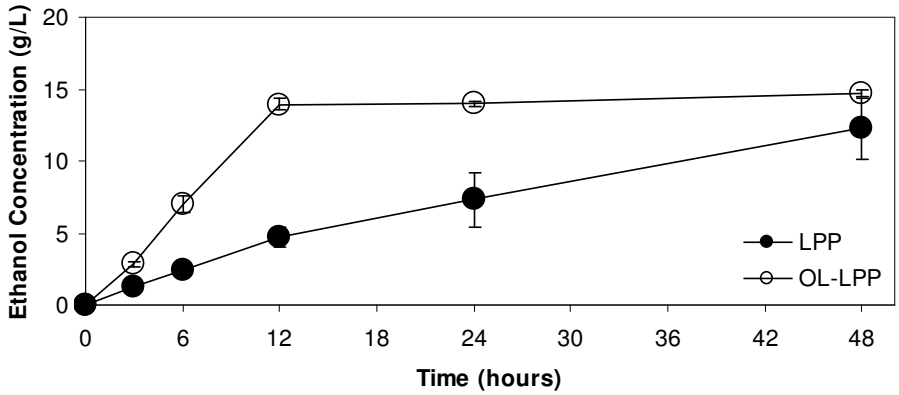

Figure 3-30. Ethanol production (g/L) from separate hydrolysis and fermentation (SHF) of steam-pretreated LPP whole slurry with and without overliming treatment by yeast strain T2. Error bars denote standard deviations.

Table 3-8. Initial hexose concentration (g/L), ethanol production (g/L) and ethanol yield (%) during SHF of whole slurry from steam-pretreated DF1, DF4 and LPP with overlimed and non-overlimed WSF.

	WSF			Overlimed WSF		
Softwood	Hexose (g/L)	Ethanol (g/L)	Ethanol yield (%)	Hexose (g/L)	Ethanol (g/L)	Ethanol yield (%)
DF1	34.6	14.6	82.7	35.3	17.4	96.7
DF4	37.0	17.0	90.1	36.8	16.3	86.8
LPP	37.0	7.5	39.7	36.7	11.5	61.4

* Theoretical ethanol yield was calculated based on 0.51g ethanol produced from every gram of hexose

In addition to examining the effect of detoxification before hydrolysis, overliming after hydrolysis was also examined. We were uncertain whether the whole slurry would contain species of fermentation inhibitors at a concentration higher than that tolerable by yeast. Therefore, we performed overliming after enzymatic hydrolysis to ensure that the whole slurry hydrolysate would be fermentable by yeast.

In contrast to the respective crude WSFs, the whole slurry hydrolysates derived from DF1, DF4 and LPP produced significantly higher ethanol yields (Table 3-5, Table 3-6). Unexpectedly, overlimed and non-overlimed whole slurry hydrolysates derived from DF4 had comparable ethanol production (Table 3-8, Figure 3-29). The results are perhaps due to the addition of the WIF to the WSF which dilutes the concentration of fermentation inhibitors. Another benefit of combining the WIF and WSF is to increase the concentration of initial sugars after 72 hours of hydrolysis for the whole slurry

hydrolysate which gives the yeast strain T_2 access to abundant fermentable sugars which should improve ethanol production. Although overliming drastically increased ethanol productions from crude WSFs (Figure 3-18, Figure 3-19, Figure 3-20) this approach did not produce significant improvements in the overall fermentability during SHF experiments (Table 3-8). The overall ethanol yields from the SHF experiments indicate that there were significant differences in the ability of the T2 yeast to ferment the sugars in the slurries. It was evident that the inhibitory nature of the DF1 sample was overcome to the greatest extent by combining the WIF and WSF fractions, while the LPP sample did not perform well during the SHF indicating that overliming was not able to completely reverse the inhibitory effects on fermentation.

The HHF experiments were performed next to determine whether ethanol yields could be increased by decreasing the end-product inhibition during hydrolysis by adding yeast to the system. Hybrid hydrolysis and fermentation (HHF) was based on the method of simultaneous saccharification and fermentation (SSF) with an additional pre-hydrolysis step (Figure 2-1). Although SHF produced higher overall ethanol than the crude WSF, previous studies with HHF have shown better ethanol yields after fermentation (Soderstrom et al., 2005; Ewanick, 2006). In addition, HHF has the advantage of significantly reducing the duration of the hydrolysis and fermentation steps from 120 hours to 86 hours. However, one disadvantage of HHF is that the latter combined hydrolysis and fermentation are not performed at their optimal conditions but at a medium complementary condition of pH 5.5 and 37°C. Effect of overliming in combination with HHF was also examined.

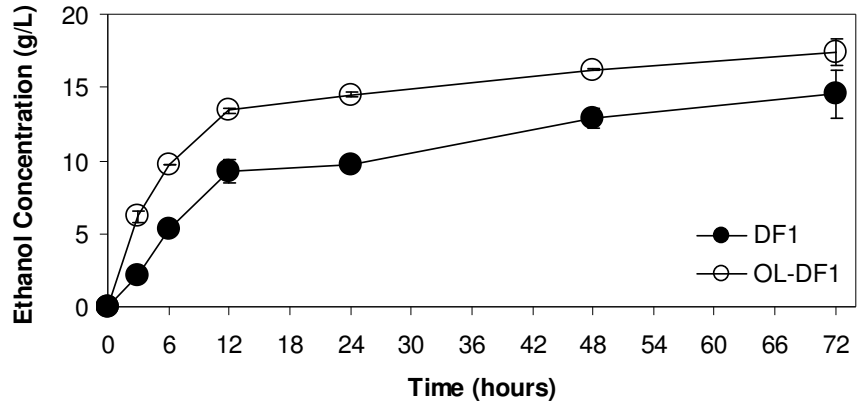

Figure 3-31. Ethanol production (g/L) from hybrid hydrolysis and fermentation (HHF) of DF1 whole slurry by yeast strain T2. Untreated (DF1) or overlimed (OL-DF1) water soluble fractions were used in these experiments.. Error bars denote standard deviations.

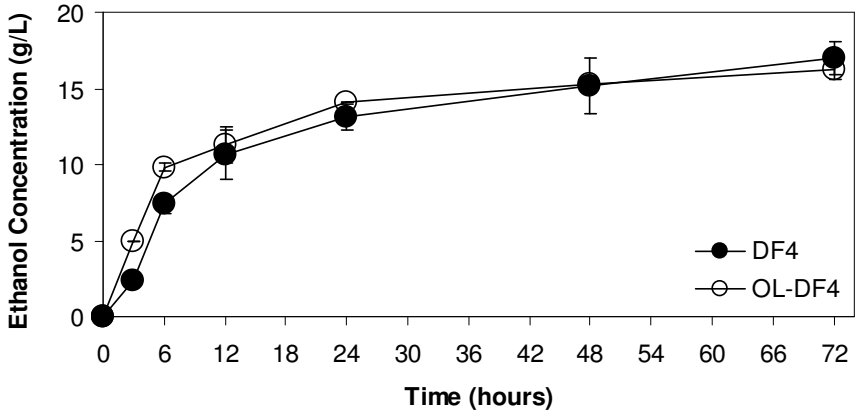

Figure 3-32. Ethanol production (g/L) from hybrid hydrolysis and fermentation (HHF) of DF4 whole slurry by yeast strain T2. Untreated (DF4) or overlimed (OL-DF4) water soluble fractions were used in these experiments. Error bars denote standard deviations.

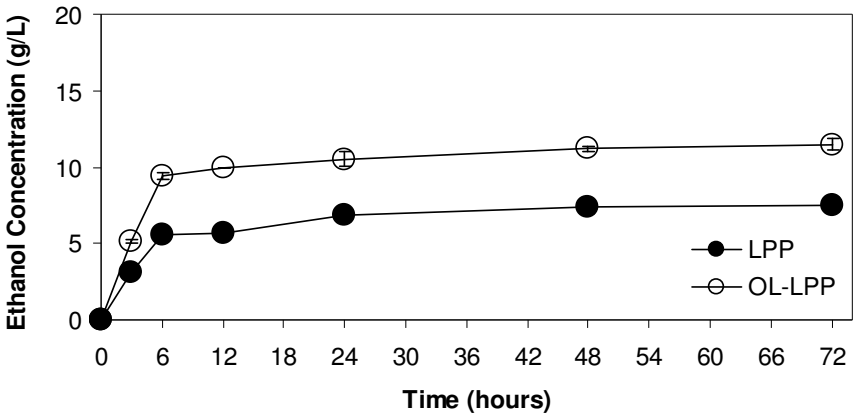

Figure 3-33. Ethanol production (g/L) from hybrid hydrolysis and fermentation (HHF) of LPP whole slurry by yeast strain T2. Untreated (LPP) or overlimed (OL-LPP) water soluble fractions were used in these experiments.. Error bars denote standard deviations.

Similar to SHF, the cellulose conversion during HHF showed limited differences between the non-overlimed and overlimed whole slurries. In both cases, after 12 hours of pre-hydrolysis, approximately 23 to 25 g/L of hexose were detected for DF1, DF4 and LPP (Figure 3-25, Figure 3-26, Figure 3-27). Similar to the SHF experiments, the rate of initial ethanol production and final ethanol concentration for the HHF of the whole slurry derived from DF1 and DF4 were similar between non-overlimed and overlimed hydrolysates. In addition, final ethanol concentration from the HHF was similar to that of SHF at approximately 15 g/L. This suggests that the HHF did not significantly enhance the final ethanol production as seen in previous reports (Ewanick, 2006). For the whole slurry derived from LPP, the overlimed hydrolysate had a higher ethanol production rate than the non-overlimed whole slurry; however, the final ethanol production after 48 hours was similar to that obtained in SHF of the whole slurry. During HHF, the fermentation of the overlimed whole slurry derived from LPP had higher ethanol production compared to

the non-overlimed whole slurry (Figure 3-33). These effects are minor that led to minimal differences between non-overlimed and overlimed hydrolysate. The WSFs from DF4 and LPP could not be fermented in their original state. Even spiking with glucose up to 100 g/L failed to produce improved results (Figure 3-21, Figure 3-22, Figure 3-23, Figure 3-24). Interestingly, SHF and HHF strategies using whole slurries of WSFs and WIFs produced improved ethanol yields from the DF4 and LPP WSFs. The exact mechanism is not clear. However, it is possible either that the solid material in the whole slurry diluted the inhibitors, or that some component of the solid fraction (WIF) was enabling the T_2 yeast to ferment the WSF from DF4 and LPP. Alternatively, WIF may contain minor nutrients (N and P derived compounds) required for the fermentation. Some of the inhibitory material in the WSF may have been adsorbed to the solid substrate when the slurry was formed especially if the inhibitory material was lignin derived because steam pretreated solid added to the slurry contains between 28-31% lignin (Allen et al., 2005).

4 Conclusions

The goal of the current research was to improve the ethanol production from the water soluble fraction from steam pretreated softwoods such as Douglas-fir and Lodgepole pine despite the presence of fermentation inhibitors. Methods that were explored to improve the fermentability of the water soluble fractions included testing for robust yeast strains, examining overliming as a potential detoxification method, increasing the initial sugar concentration and the utilization of SHF and HHF hydrolysis/fermentation schemes. Steam pretreatment of DF and LPP at a severity factor of $\log R_o = 3.64$ produced a WSF that contained mostly monomeric sugars with a range of sugar recoveries observed among the different DF and LPP feedstocks indicating that the different wood samples experienced varying levels of pretreatment severity. Although up to 86 g/L of fermentable hexose sugars were recovered in the WSF, this sugar source was not fermentable using the tested yeasts without the application of either dilution or overliming treatment. This suggested that the level of fermentation inhibitors was above the tolerable threshold for the yeast. Based on the ethanol productivity response of the four yeast strains in model furans and phenolic compounds, it is suspected that low molecular weight phenolic compounds were the most potent inhibitors compared to furans. Based on these observations it is possible that phenolic compounds in the pretreatment derived WSFs may have contributed to the toxicity of these fractions towards the yeast strains tested.

It was apparent that the SSL-adapted yeast strain T_2 was the most robust yeast strain compared to the haploid laboratory strain, Y1528 and the other SSL-adapted strain T_1 due to its higher tolerance of model phenolic compounds. Moreover T_2 had the highest

ethanol production when WSF derived from DF4 which contained abundant fermentation inhibitors was diluted in half. However, dilution is not commercially viable because the initial sugar concentration will be proportionately reduced thereby leading to lower ethanol concentrations and higher product recovery costs. Overliming up to pH 11 at 30°C significantly improved ethanol production from the WSF. Initial sugar concentration is another factor that dictates ethanol production from WSFs. Increasing the initial fermentable sugars up to 100 g/L resulted in improved ethanol production when fermenting the WSF derived from DF1. However this approach was ineffective with the other softwood derived WSFs. However, it can be expected that supplementation with glucose would not be practical in an industrial setting. Therefore, the alternative approach of the fermentation of combined WIF and WSF as a whole slurry was investigated. SHF and HHF of the whole slurry produced similar concentrations of ethanol for the hydrolysates derived from DF1 and DF6 with yeast strain T_2. Overliming did not improve ethanol production compared to the non-overlimed whole slurry hydrolysates which may be a result of a dilution of the WSF stream through the addition of the solid stream. Both SHF and HHF of whole slurries brought the benefit of improved ethanol production because of increased initial sugars from hydrolysates of WIF, while diluting the fermentation inhibitors to overcome the negative effects of fermentation inhibitors without the requirements for a detoxification process or added minor nutrients for yeast fermentation.

5 Future work

The current study attempted to address issues regarding the increase in the fermentability of the hemicellulose rich WSF derived from steam pretreated softwoods, however, numerous issues remain. The following list of experiments describes approaches that would build on current work that could lead to further advances to maximize final ethanol production for an economically viable bioconversion process.

5.1 Fermentation of WSF derived from steam pretreated hardwood and non-wood residues

Although softwood is abundant in the Pacific northwest of North America, different geographical regions in the world would have different types of feedstock available. Therefore it is important to first assess the effectiveness of steam pretreatment on different feedstocks. It is also important to utilize the hemicellulosic sugars recovered in the WSFs from other feedstocks. Since our study has shown that SHF and HHF can effectively increase the initial sugar concentration and dilute the fermentation inhibitors, it will be interesting to investigate the fermentability of those WSF, because they will be composed of different types and concentrations of sugars and fermentation inhibitors.

5.2 Apply other methods of detoxification such as ethyl acetate extraction

Since our study has suggested that low molecular weight phenolic compounds are the most likely group of fermentation inhibitors that have the greatest effect on yeast ethanol productivity, the removal of these compounds should be studied in greater detail. Previous studies by Robinson, 2006 have utilized ethyl acetate extraction as a method of detoxification. It would be interesting to perform ethyl acetate extraction with the WSF. This study might remove the phenolic compounds to a greater extent than overliming and

should give insight into whether these phenolic compounds would affect the enzymatic hydrolysis during SHF and HHF.

5.3 Improve HHF processing of whole slurries

One of the first studies that can be conducted to improve HHF of the whole slurry (WSF+WIF) is to further increase the initial sugar concentration which can be achieved through further increasing the 5% starting solids content. The minimal enzyme loadings for maximal cellulose conversion must also be examined. It would also be of interest to elucidate the underlying mechanism for the improved fermentation of the WSF upon addition of the WIF since it is possible that the added solid substrate adsorbed some of the inhibitory material.

6 References

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