Strain improvement of *Scheffersomyces stipitis* for the bioconversion of lignocellulosic biomass into ethanol.

by

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ABSTRACT

Strain improvement of *Scheffersomyces stipitis* using for the bioconversion of lignocellulosic biomass into ethanol.

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Dr. Hung Lee and Dr. Jack Trevors

Pretreatment of recalcitrant lignocellulosic biomass to release sugars for bioconversion into ethanol produces fermentation inhibitors. Increasing yeast inhibitor tolerance should reduce production time and cost. UV mutagenesis followed by genome shuffling using cross mating was performed on *Scheffersomyces stipitis* strain GS301, a genome shuffled strain with increased tolerance to spent sulphite liquor (SSL). The main fermentation inhibitors in SSL are acetic acid, hydroxymethylfurfural (HMF), and various phenolics. UV mutagenesis resulted in acetic acid tolerant mutants, but they were phenotypically unstable. However, two rounds of UV mutagenesis followed by five rounds of genome shuffling resulted in strains EVB105, EVB205 and EVB505 with increased SSL tolerance and improved acetic acid and HMF tolerance. When fermenting undiluted SSL at pH 5.5, the three strains utilized sugars faster producing higher maximum ethanol than GS301. This study demonstrates that UV mutagenesis with genome shuffling can significantly improve inhibitor tolerance and fermentation performance of yeast.
Acknowledgements

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Finally, thank you to my partner and best friend, Eric, for all of his love, encouragement and understanding over the years.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>6-4PPs</td>
<td>pyrimidine (6-4) pyrimidone adducts</td>
</tr>
<tr>
<td>CPDs</td>
<td>Cyclobutane pyrimidine dimers</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethyl methane sulfonate</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HMF</td>
<td>Hydroxymethylfurfural</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HWSSL</td>
<td>Hardwood spent sulphite liquor</td>
</tr>
<tr>
<td>MNNG</td>
<td>N-methyl-N-nitro-nitrosoguanidine</td>
</tr>
<tr>
<td>SSL</td>
<td>Spent sulphite liquor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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1.0 Introduction

Worldwide increases in energy consumption, energy demands, declining fossil fuel reserves and concerns over the effect of greenhouse gas (GHG) emissions have led to efforts to develop clean and/or renewable fuel sources. Ethanol is an alternative energy source that burns more cleanly than gasoline (Environment Canada 2009), making it both a substitute and additive for petroleum-derived fuels. Adding ethanol to gasoline oxygenates the fuel, allowing for a cleaner combustion and a reduced emission of pollutants. In Canada, gasoline blends containing up to 10% (v/v) ethanol have been used (Environment Canada 2009).

In Canada, the storage carbohydrates in agricultural crops such as corn and wheat are used as substrates for bioethanol production (Chamanrokh et al. 2008; Champagne 2008). These crops are traditionally grown for food and animal feed, and may not be sustainable for large-scale ethanol production (Chu and Lee 2007; Hahn-Hagerdal et al. 2006). In contrast, vast quantities of sugars occur as structural polysaccharides in the form of cellulose and hemicellulose in lignocellulosic biomass residues. Lignocellulosic residue can be residues from agricultural crop such as straw, stalks, leaves, branches etc., forestry byproducts, such as logging residues and processing liquors, or from municipal wastes, such as scrap wood. Lignocellulosic residues are more abundant than food crops, have fewer competing uses, and can be harvested with considerably less interference to the food economy and less impact on environmental resources. The bioconversion of lignocellulosic sugars to ethanol is complex and expensive relative to starch conversion. Thus, there is interest to refine existing technology or
develop new technologies to produce the second generation lignocellulosic ethanol to provide the large volume needed to satisfy the demand for the transportation sector.

Lignocellulosic substrates envisioned for bioconversion include crop residues, municipal solid wastes, forestry residues and discarded material from paper mills. These low-value waste materials can be converted to produce ethanol. Lignocellulosic ethanol can aid in the reduction of GHG emissions, provide a new market for agricultural industries and reduce concern over lack of energy supply (Demain 2009).

Producing ethanol from lignocellulosic biomass consists of four main steps. First, the biomass undergoes a pretreatment process to break down the structure of the lignocellulosic matrix. Second, enzymatic hydrolysis is used to depolymerize the cellulose and hemicellulose to create sugar monomers. Third, microorganisms ferment sugars in the biomass to ethanol. Finally, the ethanol is distilled and purified to meet fuel specifications.

1.1 Structure of lignocellulose:

Obtaining fermentable sugars from lignocellulose is a major bottle-neck for industrial production of ethanol and other value added products from lignocellulose. Lignocellulosic biomass is highly recalcitrant due to its physical and chemical structure, which confers rigidity to the plant cell wall. Lignocellulosic biomass is comprised of lignin (10-25%) as well as the structural carbohydrates hemicellulose (20-35%) and cellulose (35-50%) (Saha 2003; Webb and Lee 1990). The composition of various lignocellulosic materials is summarized in Table 1.1. The lignin, cellulose and hemicellulose are combined together in a heterogeneous matrix (Agbor et al. 2011). Cellulose in the plant cell wall provides the main support for the plant. Cellulose is a
linear polymer of glucose molecules linked by β-1, 4 glycosidic bonds, making it highly crystalline, compact and resistant to biological attack (Gray et al. 2006). The cellulose chains are arranged together, forming microfibrils, which are bundled together to form cellulose fibres (Agbor et al. 2011).
Table 1.1: Composition of selected lignocellulosic biomass.

<table>
<thead>
<tr>
<th>Lignocellulosic Material</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Lignin (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hardwood</td>
<td>41-50</td>
<td>11-33</td>
<td>19-30</td>
<td>(Klinke et al. 2004)</td>
</tr>
<tr>
<td>Birch</td>
<td>40</td>
<td>39</td>
<td>21</td>
<td>(Olsson and HahnHagerdal 1996)</td>
</tr>
<tr>
<td>Aspen</td>
<td>51</td>
<td>29</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Spruce</td>
<td>43</td>
<td>26</td>
<td>29</td>
<td>(Olsson and HahnHagerdal 1996)</td>
</tr>
<tr>
<td>Herbaceous plants</td>
<td>24-50</td>
<td>12-38</td>
<td>6-29</td>
<td>(Klinke et al. 2004)</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>45</td>
<td>30</td>
<td>12</td>
<td>(Saha 2003)</td>
</tr>
<tr>
<td>Agricultural residues</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn stover</td>
<td>40</td>
<td>25</td>
<td>17</td>
<td>(Saha 2003)</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>30</td>
<td>50</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>
Hemicellulose is class of branched heteropolymers including xylan (xylose), glucuronoxylan (glucoronic acid, xylose), arabinoyxylan (arabinose and xylose), glucomannan (glucose and mannose) and xyloglucan (glucose, xylose, galactose) (Webb and Lee 1990). Hemicellulose connects cellulose fibres and lignin, providing rigidity to the lignocellulose matrix (Laureano-Perez et al. 2005).

Xylose is the predominant sugar in xylan and may comprise up to 25% of the dry weight in some biomass (Bicho et al. 1988; Webb and Lee 1990). Hemicellulose composition can vary between different biomasses. The hemicellulose in agricultural residues is mainly composed of xylan. Glucomannan is the primary hemicellulose in softwood (Agbor et al. 2011). Lignin is a cross linked heteropolymer of phenolic monomers (p-coumaryl, coniferyl and sinapyl alcohol) (Kumar et al. 2009). Located in the primary cell wall, lignin provides the plant with structural support, impermeability, microbial resistance and oxidative stress resistance. Lignin is closely associated with cellulose microfibrils and is a hindrance to enzymatic and microbial hydrolysis of biomass (Avgerinos and Wang 1983). Other disadvantages associated with lignin include: adsorption of hydrolytic enzymes, binding to cellulolytic enzymes, and toxicity of lignin derived fermentation inhibitors created during pretreatment of biomass (Agbor et al. 2011).
1.2 Pretreatment of biomass:

To yield fermentable sugars, lignocellulosic substrates are subjected to a pretreatment process which breaks apart the lignin, hemicellulose and cellulose matrix. The objective of pretreatment processes is to remove lignin and hemicellulose, reduce crystallinity of cellulose and to increase the porosity of the lignocellulose to allow hydrolytic enzymes access.

Several criteria should be met for an effective pretreatment process: The pretreatment method should be effective on a wide variety of biomass feedstocks. For instance, acid based pretreatment methods are effective on a wide variety of feedstocks (Mosier et al. 2005), whereas alkaline based methods effectively remove lignin, but are less effective at overcoming the recalcitrance of softwoods (Chandra et al. 2007). The method should yield cellulose that is easily digested by hydrolytic enzymes, with minimal sugar degradation. It is beneficial to minimize the formation of byproducts that are toxic to the enzymes and microbes of the downstream processes, hydrolysis and fermentation. The method should also be cost effective. Cost effectiveness is maximized by minimizing milling or grinding of the biomass, maintaining a high yield of sugars, minimizing the production of waste material necessitating disposal, minimizing heat and power usage, and maximizing the recovery of lignin and other constituents for value added products (Alvira et al. 2010).

Pretreatment processes can be physical (milling and grinding, steam treatment/explosion), chemical (alkali, dilute acid, organic solvents, oxidizing agents), or biological (fungal treatment) in nature. Or they can be a combination of these, usually physical and chemical (steam pretreatment with autohydrolysis, hydrothermolysis and wet oxidation) (Kumar et al. 2009).
The milling of biomass serves to reduce crystallinity and particle size, and shear the biomass, which leads to an increase of specific surface availability and a reduction in polymerization (Palmowski and Muller 1999). Depending on the type of biomass, and the type and extent of the milling used, an increase in the specific surface availability, the degree of depolymerization, and shearing can increase the hydrolysis yield 5-25%, while also reducing the digestion time by 23-59% (Delgenés et al. 2002; Hartmann et al. 1999).

Steam pretreatment involves placing the biomass into a large vessel where the biomass is subjected to steam, high temperatures (up to 240 °C) and pressure for a few minutes, after which the steam is released and the temperature quickly lowered (Alvira et al. 2010). Steam explosion involves similar conditions, but the release of pressure and cooling is much faster and leads to the water in the biomass exploding (Hendriks and Zeeman 2009). The aim of steam pretreatment is to solubilise the hemicellulose to allow hydrolytic enzymes access to the cellulose. In this treatment some of the hemicellulose hydrolyzes to form acids, which can then solubilise the hemicellulose further.

Acid pretreatment of biomass is used to solubilise the hemicellulose, resulting in increasing the accessibility of cellulose to hydrolytic enzymes. Dilute or concentrated acids can be used, but concentrated acids are generally avoided for ethanol production as there are more inhibitors generated with concentrated acids and also more corrosion of the equipment used. Dilute acid treatment can be executed at high temperatures (180 °C) for shorter time periods, or at lower temperatures (120 °C) for longer time periods (30-90 minutes) (Alvira et al. 2010). Dilute acid treatment converts solubilised hemicellulose to fermentable sugars, but some sugar
degradation products, namely furfural and HMF (Fengel and Wegener 1984; Ramos 2003) and lignin degradation products are produced as well (Amartey and Jeffries 1996; Liu and Wyman 2003; Shevchenko et al. 1999).

Brown, white and soft-rot fungi, which degrade lignin, hemicellulose, and a small amount of cellulose, are used for the biological pretreatment of biomass. White-rot fungi degrade lignin using the lignin-degrading enzymes, peroxidase and laccase (Kumar et al. 2009). Biomass pretreatment with fungi is usually low cost, of low energy expenditure, and does not require chemicals. However, this method is too slow for efficient ethanol production on an industrial scale, as it has a very low hydrolysis rate (Sun and Cheng 2002).

A physicochemical pretreatment that is commonly used is SO$_2$-steam treatment. This method involves the addition of an external acid to catalyze the solubilisation of hemicellulose, lower treatment temperatures and results in better enzymatic hydrolysis of the substrate (Brownell et al. 1986; Gregg and Saddler 1996). The biomass is impregnated with SO$_2$, which is converted to H$_2$SO$_4$ during the first 20 seconds of the treatment, after which the catalytic hydrolysis of the hemicellulose begins (Hendriks and Zeeman 2009). During SO$_2$-steam treatment some of the lignin and hemicelluloses are degraded to produce furan derivatives, weak acids and phenolic compounds.

1.3 Formation of inhibitors during pretreatment:

A number of inhibitory compounds are formed in lignocellulosic hydrolysates from the harsh pretreatment processes. The main inhibitors include furan derivatives, organic acids and phenolics. These compounds adversely affect the viability, growth, and fermentative ability of
yeasts (Klinke et al. 2004; Lohmeier-Vogel et al. 1998), thereby limiting the efficiency and economic feasibility of the bioconversion process. Generally, native pentose-fermenting yeasts are more sensitive to inhibitors than *S. cerevisiae* (Olsson and HahnHagerdal 1996). The reasons for this are unknown.

Acetic acid is a weak acid released from acetylated xylan (du Preez 1994; Lee et al. 1987). A concentration of 5 g/L can be inhibitory to *S. stipitis* and *P. tannophilus* (Lee and McCaskey 1983) and hydrolysates may contain higher concentrations. For example, hydrolysates of sugarcane bagasse and corn stover may contain 10.4 g/L (Watson et al. 1984) and 13 g/L (Lu et al. 2009) of acetic acid, respectively. During dilute acid pretreatment, furfural and hydroxymethylfurfural (HMF) are formed from the dehydration of pentose and hexose sugars, respectively. Furfural can be further degraded to formic acid, while HMF can be broken down to levulinic and formic acids (Taherzadeh et al. 1997). Various phenolic compounds such as vanillin, syringaldehyde and 4-hydroxybenzaldehyde are formed from lignin degradation (Palmqvist and Hahn-Hägerdal 2000). The phenolics are typically found at low concentrations in hydrolysates (Clark and Mackie 1984; McMillan 1994). Nevertheless, when the inhibitors are present together, they may act synergistically, so even low concentrations of the inhibitors may contribute to the overall toxicity.

1.4 Hydrolysis:

Enzymatic hydrolysis is used to depolymerize the polysaccharides that remain after pretreatment (Margeot et al. 2009). Cellulose is the main polysaccharide remaining after pretreatment. Three main classes of enzymes are used to synergistically hydrolyze cellulose, the
endo-β-1,4-glucanases, the cellobiohydrolases, and the β-glucosidases (Margeot et al. 2009). The endo-β-1,4-glucanases cleave the interior bonds of the cellulose polymer, whereas the cellobiohydrolases cleave the ends of the polymer, releasing cellobiose, which is then cleaved in two glucose units by β-glucosidases (Lynd et al. 2002). Additional accessory enzymes, such as hemicellulases (Berlin et al. 2005) and ligninases (Palonen and Viikari 2004) can be used to clear access to the cellulose for the primary enzymes. The high cost and excessive enzyme loading required for enzymatic hydrolysis is a limiting factor for cost effective ethanol production.

There are several limiting factors in enzymatic hydrolysis, including the crystallinity of cellulose, the degree of polymerization, moisture content, available surface area and lignin content (Chang et al. 2001; Koullas et al. 1992; Puri 1984). Lignin affects hydrolysis by acting as a physical barrier, preventing the enzymes access to the cellulose (Mooney et al. 1999) and by non-productively binding the enzymes (Eriksson et al. 2002; Yang and Wyman 2006). Hemicellulose also acts as a barrier between the cellulose and the hydrolytic enzymes (Varga et al. 2004). Increasing the solubility of hemicellulose during steam pretreatment has resulted in better enzymatic hydrolysis of cellulose (Fernández-Bolaños et al. 2001). However, a balance of increasing hemicellulose solubility and avoiding degradation of hemicellulose into fermentation inhibitors must be maintained. Some inhibitors released during the pretreatment have been found to adversely affect some of the hydrolytic enzymes. Cantarella et al. (2004) studied the effects of inhibitors released from steam pretreated poplar wood on the enzyme activities for endo-glucanase, exo-glucanase, and β-glucosidase. The enzymes did not appear to be affected by acetic acid (2 g/L), furfural HMF, syringaldehyde, 4-hydroxy benzaldehyde and vanillin (0.5g/L). Formic acid (11.5 g/L) inactivated the enzymes. Levulinic acid (29 g/L) partially affected the
enzymes resulting in a 21% reduction in the formation of glucose during hydrolysis (Cantarella et al. 2004).

**1.5 Spent Sulphite Liquor**

For bioconversion, byproducts or industrial wastes, which contain fermentable sugars, are an appealing alternative to recalcitrant lignocellulosic biomass, which requires pretreatment and hydrolysis to release fermentable sugars. Sugar-rich byproducts obviate pretreatment and enzymatic hydrolysis steps. Also, they are more readily available on an industrial scale than lignocellulosic hydrolysates. Spent sulphite liquor (SSL) is a byproduct of the pulping industry. It contains monomeric fermentable sugars, ranging from 3 to 4% (w/v) depending upon the source of wood being pulped. SSL from hardwoods have a higher pentose sugar concentration (52-70% of total sugar) than softwoods (26% of total sugar) (Helle et al. 2004). The acid sulphite chemical pulping takes place in batch digesters using $\text{SO}_2/\text{MHSO}_3/\text{MSO}_3$ under acidic conditions (pH 1-2) and at 135-145 °C for 8 to 12 h (Pereira et al. 2013). During pulping the lignin is sulphonated and removed from the wood as lignosulphonates. The integrity of cellulose and hemicellulose is maintained, providing cellulose fibres with the desired composition and yield (Pereira et al. 2013). Acid sulphite pulping partially hydrolyzes the hemicellulose to sugar monomers and oligosaccharides, which can be fermented to ethanol. After pulping the pulp is washed and the SSL containing lignosulphanates and sugars are concentrated by evaporation (Pereira et al. 2013). The lignosulphonates are removed from SSL and can be used as binders in animal feed, limestone, fertilizer, cement, concrete, wax and asphalt emulsions among other products (Magdzinski 2006). Similar to lignocellulosic hydrolysates, SSL also has inhibitors due the high temperature and acid condition.
of the pulping process. Common inhibitors include acetic acid, furfural, HMF and various phenolic compound, as well as heavy metals released from the corrosive pulping process (Fernandez et al. 2011).

**Functional Effects of Inhibitors**

1.6 Acetic acid:

Acetic acid is one of the most abundant weak acids found in lignocellulosic hydrolysates. Common weak acids in lignocellulosic hydrolysates include acetic acid, formic acid, and levulinic acid among others (Table 1.2). Weak acids dissociate only partially in a solution and have pKa values over 4. In solutions with pH values below their pKa the acid will be mostly in their undissociated form and above their pKA the weak acids will be mostly in the dissociated form. The undissociated form of the acid is liposoluble and can traverse the cell membrane via simple diffusion, thus the toxicity of acetic acid is influenced to a great extent by pH of the medium. Inside the cell the pH is neutral and the weak acid dissociates leading to intracellular acidification and anion accumulation resulting in growth inhibition of the yeast cell (Pampulha and Loureiro-Dias 1989; Pampulha and Loureiro-Dias 2000). The dissociation of the weak acid inside the cell acidifies the cytosol and contributes to the deterioration of the electrochemical gradient across the membrane. To restore intracellular pH to physiological values the H⁺-ATPase is stimulated to pump H⁺, which couples ATP hydrolysis to proton extrusion. This process results in increased ATP consumption and has been attributed to the decrease in growth when yeasts are exposed to weak acids (Maiorella et al. 1983). Above a critical external concentration of a
weak acid the diffusion rate may exceed the transport capacity of the cell and acidification of the cytosol occurs (Lohmeier-Vogel et al. 1998; Verduyn et al. 1990).
Table 1.2: Common organic acids found in selected lignocellulosic hydrolysates.

<table>
<thead>
<tr>
<th>Organic acids</th>
<th>pKa</th>
<th>Chemical structure</th>
<th>Concentration in hydrolysates (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spruce (Larsson et al. 1999)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olive Tree Cuttings (Diaz et al. 2009)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yellow Poplar (Cho et al. 2010)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspen Wood (Almeida et al. 2007; Wilson et al. 1989)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic</td>
<td>4.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formic</td>
<td>3.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Levulinic</td>
<td>4.66</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND: No data available
Low acetic acid concentrations in lignocellulosic hydrolysates have been associated with increased ethanol yields (Pampulha and Loureirodias 1989). Additional ATP is required for the H⁺-ATPase to pump out protons from the cell to maintain intracellular pH, and this is accomplished by increasing ethanol production under anaerobic conditions at the expense of biomass (Maiorella et al. 1983). The ethanol yield for an anaerobic batch fermentation of 50 g/L glucose increased 20% in the presence of 3.3 g/L undissociated acetic acid compared to fermentations without acetic acid (Taherzadeh et al. 1997). In the presence of 3.3 g/L undissociated acetic acid, the biomass yield decreased 45% and the glycerol yield decreased 33%. Similarly, Larsson (1999) (Larsson et al. 1999) found that low weak acid concentrations (<100 mmol/L) increased ethanol yield at pH 5.5, but the ethanol yield decreased at higher concentrations. At higher acid concentrations, the energy reserves are depleted and the cytosol becomes acidic, leading to cell death (Imai and Ohno 1995). Casey et al. (2010) also found lower acid concentrations increased ethanol yields from the co-fermentation of glucose and xylose by a S. cerevisiae strain engineered to ferment xylose. At pH 5 the ethanol yield increased by 20% with 7.5 g/L acetic acid, and 30% with 15 g/L acetic acid.

In a fermentation medium of chardonnay grape juice (glucose 88 g/L, fructose 90 g/L, pH 2.97), 1 g/L acetic acid has been shown to adversely affect S. cerevisiae growth by increasing the lag phase, reducing the specific growth rate from 0.074 to 0.061 h⁻¹ and by reducing the maximum biomass production (Vasserot et al. 2010). A decreased biomass yield (64.7 mg/g to 50.8 mg/g) was also observed.

The effect of acetic acid on the transport of glucose and glycolytic enzymes of S. cerevisiae has been investigated (Pampulha and Loureirodias 1989). At 0.26 M acetic acid and
pH 3.5, acetic acid did not affect glucose transport. Hexokinase was inhibited the least by acetic acid, but enolase was the most affected. At pH 6.6 it required greater than 1000 mM of acetic acid to reduce the enzyme activity of hexokinase by 50%, whereas enolase only required 120 mM of acetic acid before its enzyme activity was reduced.

*S. cerevisiae* has been demonstrated to commit to programmed cell death in response to higher concentrations of acetic acid at low pH (Ludovico et al. 2001). In this experiment exponentially growing cells were placed in YEPD pH 3 containing 0, 20, 40, 80, 120, 160 and 200 mM acetic acid for 200 minutes at 26°C. There was chromatic condensation along the nuclear envelope in cells treated with 20 to 80 mM acetic acid. The control cells had nuclei that were homogeneous in shape and density. The exposure of phosphatidylserine at the outer surface of the membrane occurs during the early stages of apoptosis (Martin et al. 1995). Phosphatidylserine exposure on the outer membrane occurred in cells exposed to 40 to 80 mM acetic acid. DNA breakage was apparent for cells exposed to 20, 40 and 80 mM of acetic acid. This study suggests that *S. cerevisiae* is induced to undergo programmable cell death by acetic concentrations below 80 mM at pH 3. Acetic concentrations above 80 mM seem to elicit a non-active, necrotic cell death mechanism in *S. cerevisiae*.

**1.7 Furfural:**

High heat and acidic conditions during pretreatment cause a dehydration reaction of xylose to form furfural. Furfural has a heteroaromatic furan ring and an aldehyde functional group (Table 1.3). Yeasts reduce furfural to the less toxic furfuryl alcohol using NAD(P)H dependant reactions (Almeida et al. 2007). This conversion occurs during a growth lag phase, during which ethanol production and many enzymes are inhibited (Modig et al. 2002;
Taherzadeh et al. 2000b). After furfural conversion is complete growth resumes. Furfural extends the lag phase of yeast growth (Heer et al. 2009). In particular, furfural reduces the specific growth rate (Banerjee et al. 1981), cell mass yield on ATP (Palmqvist et al. 1999a), the volumetric (Navarro 1994) and specific ethanol productivities (Palmqvist et al. 1999a). Furfural is metabolized by *S. cerevisiae* under aerobic, anaerobic and oxygen limited conditions (Taherzadeh et al. 2000b).

One study examined the effects of furfural on *S. cerevisiae* being grown on ethanol, a non-fermentable carbon source (Taherzadeh et al. 2000b). This allowed the study of the effects of furfural outside of glycolysis. Aerobic batch cultivations with 15 g/L of ethanol as the carbon source were carried out. Pulse additions of 4 g/L furfural to the medium during exponential growth resulted in severe inhibition of cell growth. While furfural was present, no biomass was produced. Within 25 minutes after the addition of the furfural, the carbon dioxide evolution rate (CER) decreased 73%. After 25 minutes the CER started to increase. Cell growth resumed after furfural was converted to furfuryl alcohol and furoic acid. After furfural was added to the culture with ethanol as the sole carbon source, there was initially a rapid conversion of furfural to mainly furfuryl alcohol (85%) and furoic alcohol (15%). For the first two minutes the specific conversion rate was 9 ± 3 g/g⁻¹/h⁻¹, but then it dropped to 0.15 ± 0.02 g/g⁻¹/h⁻¹ for the remaining furfural. The author suggested the drop in the specific conversion rate could be due to the exhaustion of the supply of the cofactor NADH. Following conversion of furfural to furfuryl alcohol, the furfuryl alcohol was slowly converted to furoic acid. After the pulse addition of furfural the consumption of ethanol did not stop, but the specific uptake rate slowed from 0.23 to 0.1 g/g⁻¹/h⁻¹ while furfural was undergoing conversion.
Table 1.3: Furan derivatives found in selected lignocellulosic hydrolysates.

<table>
<thead>
<tr>
<th>Furan Derivatives</th>
<th>Chemical structure</th>
<th>Concentration in hydrolysates (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfural</td>
<td><img src="image" alt="Furfural structure" /></td>
<td>0.4</td>
</tr>
<tr>
<td>HMF</td>
<td><img src="image" alt="HMF structure" /></td>
<td>1.4</td>
</tr>
</tbody>
</table>
Within one minute after the pulse addition of furfural the formation of acetaldehyde occurred (Taherzadeh et al. 2000b). Ethanol metabolism in *S. cerevisiae* involves the initial steps of oxidation of ethanol to acetaldehyde, which is then oxidized to acetic acid. The first oxidation step is catalyzed by alcohol dehydrogenase II, and results in the formation of NADH, and the second oxidation step catalyzed by alcohol dehydrogenase, yields either NADH or NADPH (Wang et al. 1998). The concentration of acetaldehyde increased from 0.3-0.5 g/L within the first minute, reaching a final concentration of 0.6 g/L when about half of the furfural had been converted. Taherzadeh et al. (2000b) suggested NADH is necessary for the conversion of furfural and it is generated through the oxidation of ethanol to acetaldehyde. The reduction of furfural to furfuryl alcohol seems necessary for mitigating the toxicity of furfural. However, this places an extra demand on the cell for NADH. This extra demand for NADH may leave insufficient NADH for respiration, resulting in inadequate ATP formation for growth (Taherzadeh et al. 2000b). The proposed mechanisms for the conversion of furfural and HMF have been suggested to be NADH-dependent reduction and NAD⁺-dependent oxidation by oxidoreductases (Horvath et al. 2003; Liu et al. 2008). Furfural and HMF likely replace acetaldehyde as substrate for alcohol dehydrogenase and aldehyde dehydrogenase (Modig et al. 2002). (Figure 1.1)
Figure 1.1: Proposed mechanism for conversion of furfural and HMF. (A) Conversion of furfural to furfuryl alcohol via alcohol dehydrogenase. (B) Conversion of furfural to furoic acid via aldehyde dehydrogenase. (C) Conversion of HMF to 2,5-bis-hydroxymethylfuran.
The effect of the furfural conversion products furfuryl alcohol and furoic acid on *S. cerevisiae* growing on ethanol were also tested (Taherzadeh et al. 2000b). Furfuryl alcohol (4 g/L) was added to the batch of *S. cerevisiae* cells in exponential growth phase. This resulted in a decrease in the specific growth rate from 0.13 to $0.8 \pm 0.01 \text{ h}^{-1}$, and a decrease in the specific rate of ethanol uptake from 0.23 to $0.19 \text{ g/g} \cdot \text{h}^{-1}$. The CER decreased 26% within 15 minutes of the addition of furfuryl alcohol. It was also found there was no increase in acetaldehyde after the addition of furfuryl alcohol. The furfuryl alcohol was converted to furoic acid with a specific rate of $0.03 \pm 0.01 \text{ g/g} \cdot \text{h}^{-1}$. The addition of 4.5 g/L of furoic acid (pH adjusted to 4.5) to exponentially growing *S. cerevisiae* cells resulted in the specific growth rate decreasing from 0.13 to $0.10 \text{ h}^{-1}$. The addition of furoic acid resulted in a 22% decrease in the CER, but this value returned to the initial value within 30 minutes after the addition of the furoic acid. The formation of acetaldehyde following furoic acid addition was not observed.

One research group studied the effect of furfural on the key enzymes of carbon metabolism of *Candida blankii* 35 and *Candida psuedotropicalis* 11 (Hristozova et al. 2006). The enzymes studied were: hexokinase, glucosephosphate isomerise, gylceraldehyde-3P-deyrogenase of glycolysis, and glucose-6P-dehydrogenase from the pentose phosphate cycle, and β-galactosidase. Fermentations were carried out in a chemostat under carbon limitation and at two dilution rates, 0.1 and 0.25 h$^{-1}$. The media contained 10 g/dm$^3$ lactose, with 0.04% (v/v) furfural added to the medium.

The addition of 0.04% furfural to *C. blankii* 35 in a chemostat at a dilution rate of 0.1 h$^{-1}$ resulted in a drastic decrease in lactose assimilation within the first few hours after adding
Correspondingly, the activity of β-galactosidase, the enzyme responsible for lactose assimilation, decreased. The activity of β-galactosidase remained three times lower compared to the control. The activity of glucose phosphate isomerase decreased 3.7 times, whereas the activity of glyceraldehyde-3P-dehydrogenase increased nearly three times. Hexokinase activity was not affected. Initially glucose-6P-dehydrogenase activity decreased to 3.8 times lower at 4 hours after exposure to furfural, but the activity reached almost control levels in the new steady state, indicating the culture had overcome the toxic effect of furfural. However, at a dilution rate of 0.25 h⁻¹, the addition of furfural did not influence the activity of glucose-6P-dehydrogenase.

After adding 0.04% furfural to *C. pseudotropicalis* 11 at 0.1 h⁻¹ dilution rate, there was an initial decrease in activity for β-galactosidase, hexokinase, glucose phosphate isomerase, glyceraldehyde-3P-dehydrogenase, and glucose-6P-dehydrogenase for about 3 hours, followed by an increase in enzyme activity. After the new steady state was reached the activity of β-galactosidase was 3.8 times higher than the control. An increase of activity was observed for the glycolytic enzymes following exposure to furfural. Activities of hexokinase, glucose phosphate isomerase, and glyceraldehyde-3P-dehydrogenase increased 4.6, 2.3, and 1.4 times, respectively. The activity of glucose-6P-dehydrogenase was variable for about 6 hours following furfural exposure, but once culture growth was stabilized, the activity was comparable to the control without furfural added.

After adding 0.04% furfural to *C. pseudotropicalis* 11 at the dilution rate of 0.25 h⁻¹, β-galactosidase activity was 1.7 times higher than in the control. However, there was a decrease
in glycolytic enzyme activities even after reaching steady state. Activities of hexokinase, glucose phosphate isomerase, and glyceraldehyde-3P-dehydrogenase decreased 4.4, 3.8, and 2.9 times, respectively. Activity of the pentose phosphate pathway enzyme glucose-6P-dehydrogenase also decreased about two times.

The results from Hristozova et al. (2006) indicate the importance of growth rate with respect to the inhibiting effect of furfural. At a lower growth rate, 0.1 h⁻¹, *C. psuedotropicalis* 11 overcame the influence of furfural and the activities of β-galactosidase, hexokinase, glucose phosphate isomerase and glucose-6P-dehydrogenase increased, indicating 0.04% furfural did not inhibit the yeast. However, at a higher growth rate, 0.25 h⁻¹, the glycolytic and pentose phosphate pathway enzymes were inhibited by 0.04% furfural. This indicates the effect of furfural was related to the physiological state of the culture, with higher growth rates being more susceptible to furfural.

Activities of β-galactosidase and glucosephosphate isomerase in *C. blankii* 35 cells were inhibited at both dilutions. However, in contrast to *C. psuedotropicalis* 11, hexokinase activity was not affected by furfural; nor was glucose-6P-dehydrogenase activity at the dilution rate of 0.25 h⁻¹; indicating the inhibitory effect of 0.04% furfural is dependent upon the inherent properties of specific yeast enzymes.

A subsequent study investigated the effect of furfural on the following nitrogen assimilating enzymes of *C. blankii* 35 and *C. psuedotropicalis* 11 (Hristozova et al. 2008): NADPH-dependant glutamate dehydrogenase (NADPH-GDH), NADH-dependant glutamate dehydrogenase (NADH-GDH), glutaminesynthetase (GS), glutamate synthase (GOGAT) and
alanine dehydrogenase (ADH). The growth experiments were carried out in a chemostat under carbon limitation and at two dilution rates, 0.1 and 0.25 h\(^{-1}\). The media contained 10 g/dm\(^3\) lactose supplemented with 0.04% (v/v) furfural. Furfural (0.04%), added to \textit{C. blankii} 35 at dilution rate of 0.1 h\(^{-1}\), resulted in an inhibition of glutamate dehydrogenase activity and the alanine metabolite routes of ammonium ion assimilation. The assimilation of nitrogen was reduced and at a dilution rate of 0.1 h\(^{-1}\), the glutamine synthetase/glutamate synthase system was activated. The addition of furfural to \textit{C. pseudotropicalis} 11 did not inhibit the activities of NADPH-GDH and NADH-GDH, GS, GOGAT or ADH; in fact they increased 6.3, 4.9, 5.6 and 4.1 times, respectively. At the higher dilution rate, 0.25 h\(^{-1}\), GOGAT activity was reduced 1.9 times compared to the control, and stayed reduced for the remainder of the fermentation. GS activity was 13.8 times lower than the control. At the higher dilution rate, activities of NADPH-GDH, NADH-GDH and ADH of \textit{C. pseudotropicalis} 11 were unaffected by furfural.

An \textit{in vitro} study found that furfural adversely affected the activity of the enzymes alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and pyruvate dehydrogenase (PDH) (Modig et al. 2002). ADH and ALDH were purified from baker’s yeast and the PDH was isolated from porcine heart. At 1.3 mM furfural, the activities of ALDH and PDH were reduced 80%. At 10.4 mM furfural, ALDH and PDH activities were reduced more than 90%. However, ADH activity only decreased 20% in 1.3 mM furfural. Modig (2002) suggested that the inhibition of these enzymes \textit{in vitro} could account for \textit{in vivo} furfural inhibition of \textit{S. cerevisiae}. However, it is not known how easily furfural is transported into the yeast cell.

Furfural has been reported to induce the accumulation of reactive oxygen species (ROS) and causes cellular damage to mitochondria, vacuoles, actin and nuclear chromatin in
exponentially growing *S. cerevisiae* cells (Allen et al. 2010). In this study, exponentially growing yeast cells were transferred to either 25 or 50 mM furfural, 5 mM hydrogen peroxide, or a control with no inhibitors. The cells went into an immediate lag phase upon transfer to the furfural medium. Cell staining for a positive ROS signal was used to determine the percentage of cells containing ROS. After eight hours of growth in control with no inhibitors 10% of the cells had a positive ROS signal. In contrast, in media supplemented with 20 mM furfural, 31% of the cells stained positive for ROS. In media supplemented with 50 mM furfural, 36% of the cells stained positive for ROS. Transmission electron microscopy was used to evaluate the cellular damage. The cells from the 25 mM medium had mitochondria that appeared very aggregated, swollen with less structured cristae and were gathered in the cell interior. The vacuoles remained the same size as the control cells, but their edges were not smooth. The nuclear membrane of the furfural treated cells appeared to be unaltered. Cells containing a mitochondrial membrane targeting green fluorescent protein were used to further assess damage. At six hours, 80% of the mitochondria in control cells were tubular. The mitochondria in cells exposed to 25 mM furfural were either fragmented (41%) or aggregated to one side of the cell (9%), whereas those exposed to 50 mM furfural were 45% fragmented or 45% aggregated to one side. The 25 mM furfural exposed cells had vacuoles that were divided into two to four medium sized vacuoles, whereas the 50 mM furfural cells had more than four vacuoles, which were more fragmented and aggregated. The control cells had single large vacuoles. DNA specific dye, diaminophenylindole was used to visualize damage to chromatin. At 6 hours, 18.5% of the cells exposed to 25 mM furfural had disorganized and diffuse nuclear chromatin; and 21.5% of cells exposed to 50 mM furfural had damaged chromatin. Alexa Fluor®
568 phalloidin was used to determine the actin cytoskeleton damage. Seventy percent of the actin structure was abnormal in cells exposed to 25 or 50 mM furfural, whereas the control cells had 33% abnormal actin.

Hydroxymethyl furfural (HMF) is comprised of a heteroaromatic furan ring and an aldehyde functional group. HMF is formed during pretreatment from hexose sugars at high temperatures in an acid catalyzed dehydration reaction. Similar to furfural, HMF can cause cells to reduce their fermentation rate or stop growing and enter an extended lag phase (Almeida et al. 2007). Some yeast cells can reduce the aldehyde group on the furan ring to an alcohol, resulting in 2,5-bis-hydroxymethylfuran (Taherzadeh et al. 1999). HMF and furfural have similar effects on yeast metabolism; however, furfural severely inhibits the fermentation rate and yeast growth as compared to HMF (Almeida et al. 2008b).

The effect of HMF on S. cerevisiae CBS 8066 was studied (Taherzadeh et al. 2000a). Pulse additions of HMF were added to batch cultivations of 50 g/L glucose. After 4 g/L HMF was added there was a 32% decrease in the CER. HMF was taken up at a specific uptake rate of 0.14 ± 0.03 g/g/h⁻¹ under both aerobic and anaerobic conditions. When both furfural (2 g/L) and HMF (2 g/L) were added to the medium, transformation of HMF did not occur until the complete conversion of furfural. The CER decreased by 62%. Also, the conversion rates for furfural and HMF were slower than when they were individually tested. Growth was completely inhibited until conversion of both furfural and HMF was complete. The specific furfural uptake rate was 0.24 g/g/h⁻¹, which was decreased from 0.6 g/g/h⁻¹ the rate when furfural alone was added. The specific uptake rate for HMF was 0.03 g/g/h⁻¹, but it increased to 0.08 g/g/h⁻¹ after all the furfural was converted.
1.8 Phenolic compounds:

Phenolics are derived from lignin during pretreatment processes. Various phenolic compounds such as vanillin, syringaldehyde and 4-hydroxybenzaldehyde are formed from lignin degradation (Palmqvist and Hahn-Hägerdal 2000) (Table 1.4). Vanillin is formed from the degradation of the guaiacylpropane units of lignin and may be present in spruce, poplar, willow and pine hydrolysates (Klinke et al. 2004). The phenolics are typically found at low concentrations in hydrolysates (Clark and Mackie 1984; McMillan 1994). Phenolics have toxic effects on cell membranes of yeast during fermentations of lignocellulosic hydrolysates. They partition into membranes, affecting their integrity to act as a selective barrier (Heipieper et al. 1994). Low molecular weight phenolics are the most toxic (Larsson et al. 2001).

The enzyme laccase was used to remove phenolics from a willow hemicellulose hydrolysate (Jönsson et al. 1998). The main phenolics in the hydrolysate were hydroxybenzoic acid, vanillin and catechol. After removal of the phenolics from a steam and S0₂ pretreated willow hydrolysate, the inhibition of fermentation by S. cerevisiae decreased. The willow hydrolysate had sugar added to it so the starting glucose concentration was 50 g/L. Yeast extract (2.5 g/L) was also added to the willow hydrolysate. The ethanol productivity improved from 0.8 g to 2.7 g ethanol/L/h. The rate of glucose consumption went from 1.5 to 4.8 g glucose/L/h. However, the ethanol yield was similar: the control had 0.51 g/g and the laccase treated had 0.49g/g.
Table 1.4: Some common phenolic compounds found in lignocellulosic hydrolysates.

<table>
<thead>
<tr>
<th>Phenolic Compounds</th>
<th>Chemical Structure</th>
<th>Concentration in hydrolysates (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillin</td>
<td><img src="image" alt="Vanillin Structure" /></td>
<td>0.23</td>
</tr>
<tr>
<td>Syringaldehyde</td>
<td><img src="image" alt="Syringaldehyde Structure" /></td>
<td>ND</td>
</tr>
<tr>
<td>4-Hydroxy-benzaldehyde</td>
<td><img src="image" alt="4-Hydroxy-benzaldehyde Structure" /></td>
<td>0.82</td>
</tr>
</tbody>
</table>

ND: No data available
1.9 Synergistic Effects:

Many inhibitors act in synergy to adversely affect yeast cells, meaning the biological effect on a microorganism can be enhanced in the presence of other inhibitors beyond the effects of the single inhibitors (Palmqvist et al. 1999b). For instance, acetic acid and furfural interact to reduce the specific growth rate to a greater extent than the decreases seen with individual inhibitors (Palmqvist et al. 1999b).

Another study examined the interactive effects of acetic acid, furfural and phenol on the growth and metabolism of *S. cerevisiae* in defined fermentation media containing 20 g/L glucose (Ding et al. 2011). One key finding was that of the three inhibitors, acetic acid played a central role in the combined effects on some amino acids and central carbon metabolic intermediates. For instance, when only acetic acid was in the fermentation media there was a reduction in pyruvate, 2-oxoglutarate and succinate to similar levels seen when all three inhibitors were present. This indicates acetic acid likely play a key role in the combined effects of the inhibitors.

1.10 Strategies to Overcome Inhibition:

Physical, chemical and biological methods have been used to overcome inhibitors present in lignocellulosic hydrolysates (Table 1.5). Many of these methods focus on removing or converting inhibitors through a detoxification step prior to fermentation. Recently, biological methods have focused on increasing yeast tolerance to inhibitors, thereby, obviating the need for a detoxification step. It would be advantageous to not have a detoxification step as it represents a significant portion of production cost. For example, the addition of calcium
hydroxide and sodium sulphite to a willow hydrolysate has been reported to account for 22% of the production costs for ethanol (Sivers et al. 1994). The focus of this review is recent advances in yeast strain improvement, rather than detoxification. See Table 1.5 for a list of detoxification methods.
Table 1.5: Common detoxification methods to increase the fermentability of lignocellulosic hydrolysates

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical Methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacuum evaporation</td>
<td>Evaporation of volatile inhibitors, such as furfural, acetic and formic acids.</td>
<td>(Larsson et al. 1999; Palmqvist, et al.1996; Wilson et al. 1989)</td>
</tr>
<tr>
<td>Solvent extraction</td>
<td>Removal of phenolic compounds.</td>
<td>(Cantarella et al. 2004; Cruz et al. 1999; Wilson et al. 1989)</td>
</tr>
<tr>
<td>Ion exchange resins</td>
<td>Adsorb aliphatic acids, furans and phenolic compounds. Ion exchange resins are expensive.</td>
<td>(Chandel et al. 2007; Horvath et al. 2004; Larsson et al.1999; Nilvebrant et al. 2001)</td>
</tr>
<tr>
<td>Activated charcoal</td>
<td>Adsorb furans, phenolic compounds, and acetic acid.</td>
<td>(Berson et al. 2005; Chandel et al. 2007;Priddy et al. 2003)</td>
</tr>
<tr>
<td>Lignin residue</td>
<td>Adsorb furans and phenolic compounds.</td>
<td>(Bjorklund et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chemical Method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkali treatment</td>
<td>Addition of an alkali to adjust pH to 9-10, followed by readjustment of pH to 5.5. Inhibitor removal is though chemical conversion. Loss of sugars has been reported.</td>
<td>(Alriksson et al. 2005; Grohmann et al. 1985; Horvath et al. 2005; Leonard et al. 1945; Martinez et al. 2000; Martinez et al. 2001; Nilvebrant et al. 2003; Perego et al. 1990; Persson et al. 2002b; Sjolander et al. 1938; Taherzadeh 1997)</td>
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<td>Table 1.5 (con’t)</td>
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<tr>
<td><strong>Biological Methods</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method</td>
<td>Description</td>
<td>References</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bioabatement</td>
<td>Treat hydrolysate with microorganisms that utilize or convert inhibitors to less toxic compounds.</td>
<td>(Lopez et al. 2004; Nichols et al. 2005; Nichols et al. 2008; Okuda et al. 2008; Schneider et al. 1996)</td>
</tr>
<tr>
<td>Fed-batch fermentation</td>
<td>Allows <em>in-situ</em> detoxification of inhibitors by fermenting yeast by keeping inhibitor concentration low through control of substrate feed rate.</td>
<td>(Rudolf et al. 2004; Taherzadeh et al. 2000a; Taherzadeh et al. 2000b; Taherzadeh et al. 2000c; Taherzadeh et al. 1999)</td>
</tr>
<tr>
<td>Continuous fermentation with cell retention system</td>
<td><em>In-situ</em> detoxification coupled with a cell retention system to maintain high cell density. Faster fermentation times than with fed-batch.</td>
<td>(Brandberg et al. 2005; De Bari et al. 2004; Horvath et al. 2001; Lee et al. 1996; Palmqvist et al. 1998; Purwadi et al. 2008; Purwadi et al. 2007; Talebnia et al. 2006)</td>
</tr>
<tr>
<td>Yeast adaptation</td>
<td>Utilizes yeasts adaptation ability to increase inhibitor resistance.</td>
<td>(Alkasrawi et al. 2006; Amartey et al. 1996; Keller et al. 1998; Martin et al. 2007; Parekh et al. 1986)</td>
</tr>
<tr>
<td>Recombinant yeast</td>
<td>Molecular techniques are used to create recombinant yeast with increased resistance to inhibitors</td>
<td>(Petersson et al. 2006; Larroy et al. 2003; Larroy et al. 2002; Dickinson et al. 2003; Gorsich et al. 2006; Almeida et al. 2008b; Chu et al. 2007; Larsson et al. 2001; Cassland et al. 1999)</td>
</tr>
<tr>
<td>Evolutionary engineering of yeast</td>
<td>Development of inhibitor tolerant yeast through iterative genetic diversification and functional selection.</td>
<td>(Bajwa et al. 2009; Bajwa et al. 2010)</td>
</tr>
</tbody>
</table>
1.11 Yeast Strain Improvement for Increased Inhibitor Tolerance:

Yeast strains have been improved using three main techniques: adaptation, recombinant molecular techniques, and evolutionary engineering. The main focus of strain improvement for the fermentation of lignocellulosic hydrolysates is to improve the yeast’s tolerance to the inhibitors present in the hydrolysates. There are several advantages of yeast strain improvement over detoxification. First, it eliminates a costly processing step. Second, detoxification can be difficult to standardize especially with variable hydrolysate feedstocks. Third, most of the detoxification methods remove only selected classes of inhibitors. One distinct advantage of strain improvement is that inhibitor tolerant yeasts are likely able to ferment a variety of hydrolysates with varying concentrations of related inhibitors (Bajwa et al. 2011).

1.12 Yeast Adaptation for Improved Inhibitor Tolerance:

Adaptation of fermenting yeasts to toxic compounds in hydrolysates is a low-cost technique to overcome inhibition from toxic compounds in lignocellulosic hydrolysates. This approach reduces reliance on more expensive detoxifying steps, thereby, reducing the cost and time required to produce ethanol from lignocellulosic hydrolysates. Adaptation can decrease fermentation time and increase yeast tolerance to inhibitors present in hydrolysates. Adaptation of yeast cells to inhibitors can be done by growing the strain in a dilution of hydrolysate, cultivating the yeast in increasing concentrations of hydrolysate, or cultivating the yeast on the hydrolysate before fermentation (Alkasrawi et al. 2006; Amartey and Jeffries 1996; Martin et al. 2007).
Recombinant xylose-fermenting S. cerevisiae TMB 3001 was adapted to inhibitors present in sugarcane bagasse hydrolysate by growing the yeast in a medium containing hydrolysates with increasing concentrations of toxic inhibitors (Martin et al. 2007). This approach led to an adapted yeast strain that had better ethanol yields and productivity compared to the original unadapted yeast strain. After a 24 hour fermentation of 50% sugarcane bagasse hydrolysate containing 25.8 g/L glucose and 5.9 g/L xylose, the adapted yeast strain gave an ethanol yield of 0.38 g/g, while the unadapted strain gave an ethanol yield of 0.18 g/g.

The adapted yeast converted inhibitors in the hydrolysate faster than the non-adapted yeast. With the 50% bagasse hydrolysate, both yeast strains converted furfural to furfural alcohol within 24 hours, but the adapted yeast had a maximal conversion rate of 0.150 g/L/h, while the unadapted yeast achieved a rate of 0.075 g/L/h, only half that of the adapted yeast (Martin et al. 2007). Likewise, in 75% bagasse hydrolysate with a total furan concentration of 3.8 g/L, the adapted strain converted 74% of the initial furfural and 40% of the initial HMF over 48 hours whereas the unadapted strain converted 22% of the initial furfural and 20% of the initial HMF (Martin et al. 2007).

How yeast cells become acclimatized to the inhibitors present in hydrolysates is not known. It is hypothesized that growth in the presence of inhibitors modifies gene expression, leaving the yeast “primed” for future exposure to inhibitors. Alkasrawri et al. (2006) suggest yeast cells grown on hydrolysate are likely to have a different set of genes being expressed compared to yeast cells grown on glucose (Alkasrawi et al. 2006). The generation of mutants
occurring during adaptation is possible, but limited due to small number of generations occurring during adaptation. This again places emphasis on the potential of different gene expression profiles for adapted yeast compared to unadapted yeasts. The adaptation of fermenting microbes that already have substantial tolerance to inhibitors could be especially useful to develop microorganisms with even higher inhibitor tolerance for ethanol production from lignocellulosic hydrolysates.

1.13 Recombinant Yeasts with Improved Inhibitor Tolerance:

The development of genetically engineered yeasts with greater inhibitor tolerance is a promising option to the traditional costly detoxification steps. One way to improve yeast tolerance to inhibitors is to create recombinant yeasts expressing enzymes that confer resistance to specific inhibitors.

1.14 Improved Furfural and HMF Tolerance:

Some researchers have created yeast strains that are more efficient at reducing furfural and HMF to their corresponding alcohols, 2-furanmethanol (Alkasrawi et al. 2006) and 2,5-bis-hydroxymethylfuran, which are significantly less toxic (Alkasrawi et al. 2006). *S. cerevisiae* TMB3000 is naturally resistant to inhibitors present in spruce hydrolysates (Brandberg et al. 2004). TMB3000 has increased resistance to furfural and is more efficient at metabolizing furfural compared to *S. cerevisiae* CBS8066, a laboratory strain (Modig et al. 2008; Nilsson et al. 2005). Correspondingly, furfural and HMF reduction activity was higher in TMB3000 than in CBS8066. Petersson et al. (2006) performed a genome wide transcription analysis of known reductase and dehydrogenase genes in TMB3000 and CBS8066 cultured with 0.5 g/L HMF.
TMB3000 showed higher expression levels for almost all the reductase and dehydrogenase genes examined. Three of the eighteen genes studied were selected to further examine their role in HMF reduction. These genes, \textit{ADH2}, \textit{ADH6} and \textit{SFA1}, were cloned from both TMB3000 and CBS8066 and over expressed in CBS8066 (Petersson et al. 2006).

The gene product of \textit{ADH6} reduces HMF using NADPH as a cofactor. \textit{ADH6} encodes an alcohol dehydrogenase that only uses NADPH as a cofactor; and it has high affinity for long chain aliphatic substrates (Larroy et al. 2003). The \textit{ADH6} gene product can also reduce furfural, cinnamaldehyde and veratraldehyde (Larroy et al. 2002). Sequence analysis confirms that the increased HMF reduction capacity of TMB3000 is not due to mutations in the coding region of its \textit{ADH6}. Petersson et al. (2006) suggested that high expression of \textit{ADH6} in TMB3000 could be due to mutations in regulator sequences.

\textit{ADH2} encodes an ethanol dehydrogenase and has high expression in TMB3000, but when overexpressed in CBS8066 there was no evidence of HMF reducing capacity (Petersson et al. 2006). \textit{SFA1} encodes an enzyme that converts aldehydes to ketones, by catalyzing amino acid catabolism (Dickinson et al. 2003). Overexpression of \textit{SFA1} from CB8066 and TMB3000 in CB8066 resulted in improved HMF reduction activity.

Over expression of \textit{ADH6} in yeast when cultured in glucose media containing HMF resulted in increased glycerol yield under anaerobic and aerobic conditions, and decreased biomass yield under aerobic conditions (Petersson et al. 2006). Decreased biomass yield is likely a result of a redirection of NADPH from biosynthetic pathways to HMF reduction, but increased glycerol yield is not understood (Petersson et al. 2006). Since HMF reduction is solely
dependent on NADPH as a cofactor it is unexpected that an increase in NADPH production would result in increased glycerol production, which is used to regenerate NAD\(^+\) for anabolic reactions. Other researchers reported that over expression of \(ADH6\) (isolated from \(S. cerevisiae\) TMB3286 (Petersson et al. 2006) in \(S. cerevisiae\) CBS8066 results in an increase in glycerol production, a decrease in biomass, and an increase in acetate production (Almeida et al. 2008b). HMF reduction by the NADPH-dependent enzyme encoded by \(ADH6\) places a demand for NADPH, which may be responsible for increased acetate, as production of acetate has a role in redox balancing and creation of acetate from acetaldehyde regenerates NADP\(^+\) (Almeida et al. 2008b; van Dijken and Scheffers 1986).

In \(S. cerevisiae\) the pentose phosphate pathway (PPP) has been linked to furfural tolerance (Gorsich et al. 2006). The PPP is a central pathway in carbohydrate metabolism and is a primary source of NADPH (Flores et al. 2000). To identify genes involved in furfural tolerance, a \(S. cerevisiae\) gene disruption library was screened for mutants exhibiting decreased growth in the presence of furfural (Gorsich et al. 2006). It was observed that a number of mutants that were inefficient at reducing furfural were defective in the PPP. PPP disruption mutants of \(S. cerevisiae\) with deletions of PPP genes, \(zwf1\), \(gnd1\), \(rpel\) and \(tkl1\) displayed decreased growth in comparison to the control strain when grown with furfural. Over expression of \(zwf1\), encoding glucose-6 phosphate dehydrogenase, resulted in growth at concentrations of furfural up to 50 mM, a concentration toxic to \(S. cerevisiae\) strains not over expressing \(zwf1\) (Gorsich et al. 2006). Increased levels of glucose-6 phosphate dehydrogenase, the first enzyme in the PPP, may commit its substrate, glucose-6 phosphate, to the PPP at the expense of other pathways (eg. glycolysis), resulting in high levels of NADPH (Gorsich et al. 2006).
tolerance has not yet been elucidated, it is likely due to increased NADPH, the cofactor of ADH6p and many other enzymes involved in stress response (Carmel-Harel and Storz 2000).

Recombinant xylose-fermenting *S. cerevisiae* strains have been created by insertion and over expression of heterologous xylose isomerase, or xylose reductase and xylitol dehydrogenase (Chu and Lee 2007). The fermentative ability of recombinant xylose-fermenting *S. cerevisiae* strains has been well characterized and is summarized by Chu and Lee (2007). One research group found enhanced tolerance to furfural and HMF by the xylose-fermenting *S. cerevisiae* TMP3400 strain, which expresses the *S. stipitis* xylose reductase (Ps-XR) (Almeida et al. 2008a). Strain TMB3400 is a mutant of a recombinant strain of *S. cerevisiae* USM21, which was created by chromosomal integration of *S. stipitis* genes encoding xylose reductase (XR) and xylitol dehydrogenase (XDH), and a *S. cerevisiae* gene encoding xylulokinase (XK) (Wahlbom et al. 2003). TMB 3400 has higher tolerance to the inhibitors in spruce hydrolysate during anaerobic batch fermentation in comparison to the control strain USM21, and has three times higher in vitro HMF and furfural reduction activity (Almeida et al. 2008a). Ps-XR is capable of both in vitro and in vivo HMF reduction. Creation of *S. cerevisiae* TMB3290, a new strain over expressing Ps-XR, resulted in a 20% increase in the in vivo HMF reduction rate, and improved HMF tolerance (Almeida et al. 2008a).

1.15 Improved Phenol Tolerance:

Exploiting *S. cerevisiae’s* innate ability to metabolize phenolic compounds to create strains that have increased tolerance has been a means to increase inhibitor tolerance (Larsson et al. 2001b). *S. cerevisiae’s* phenylacrylic acid decarboxylase is encoded by the *PAD1* gene, and
its substrates include cinnamic, p-coumaric, and ferulic acids (Clausen et al. 1994; Goodey and Tubb 1982). Under aerobic conditions ferulic acid is converted to vinyl guaiacol; and under anaerobic conditions ferulic acid is converted to dihydroferulic acid (Larsson et al. 2001a; Larsson et al. 2001b). Cinnamic acid is converted to styrene and dihydrocinnamic acid (Larsson et al. 2001b). The *in vivo* performance of genetically engineered *S. cerevisiae* strains overexpressing *PAD1* in the presence of ferulic and cinnamic acid containing media, and in spruce hydrolysate was examined (Larsson et al. 2001b). Overexpression of *PAD1* resulted in enhanced growth rate and ethanol productivity in the presence of cinnamic and ferulic acids, as well as in spruce hydrolysate. *S. cerevisiae* overexpressing *PAD1* converted ferulic acid 1.5 times, and cinnamic acid four times faster than the control yeast. *PAD1* overexpression led to yeast consuming glucose and mannose at rates 22-25% and 40-45%, faster than yeast not overexpressing *PAD1*, respectively. Additionally, ethanol production rate was 24-29% faster in yeast overexpressing *PAD1*.

Strain engineering represents an effective tool to improve tolerance of yeasts to inhibitors with the end result of improved ethanol production from lignocellulosic substrates. Recombinant yeast strains created to date exhibit increased tolerance to specific inhibitors. As more inhibitor-metabolizing enzymes are indentified, a better understanding of the stress response of yeast is gained, and even greater tolerant recombinant yeasts can be engineered for optimal fermentation of lignocellulosic hydrolysates.
1.16 Evolutionary engineering via genome shuffling for yeast strain improvement:

Evolutionary engineering is a method mimicking natural selection through directed evolution to obtain a desired phenotype. The goal of directed evolution is improvement of cellular properties through iterative genetic diversification (mutagen induced or not) and functional selection (Koffas and Cardayré 2005). Unlike molecular techniques used to create recombinant yeasts, evolutionary engineering does not require prior knowledge of the genes involved in a desired phenotype, or the modifications needed to generate the desired phenotype. Whole genome shuffling is a technique using iterative cycles of multi-parental crossing with recombination of entire genomes to generate single cells with useful alleles expressing an improved phenotype. Genome shuffling is an appropriate technique to use to improve complex multi-genic phenotypic traits not easily modified by traditional molecular techniques. Genome shuffling may prove to be a very useful tool in the creation of yeast strains that are highly tolerant to a variety of inhibitors as individual mutants expressing alleles conveying resistance to a particular inhibitor can be combined in one cell to create a multi-inhibitor tolerant strain. The use of genome shuffling should ideally result in an accumulation of beneficial mutations and elimination of deleterious mutations. Furthermore, genome shuffling is a convenient and low-cost technique that is easy to operate and does not require expensive equipment (Gong et al. 2009).

Evolutionary engineering, via UV mutagenesis and selection has been used to increase yeast tolerance to inhibitors in lignocellulosic hydrolysates (Bajwa et al. 2009; Bajwa et al. 2010). Although yeast tolerance to inhibitors can be increased through continuous adaptation
by growing the yeast in the hydrolysate (Nigam 2001), it is time-consuming. Random
mutagenesis can quickly generate mutant yeast strains that show improved inhibitor tolerance
(Bajwa et al. 2009). UV mutagenesis has been used to generate mutants of *S. stipitis* NRRL Y-
7124 that exhibited improved tolerance to inhibitors in hardwood spent sulfite liquor (HW SSL)
(Bajwa et al. 2009). A UV dose causing 50% cell survival and then selection upon gradient plates
of HW SSL and diluted liquid HW SSL were used to generate and select mutants tolerant of the
mixed inhibitors in HW SSL. The *S. stipitis* mutants were able to grow in 75% (v/v) HW SSL broth,
whereas the wild type (WT) was only able to grow in 65% (v/v) HW SSL. Two of the six, 3rd
generation mutants, PS301 and PS302, survived in 80% (v/v) HW SSL, but no growth was
observed. Compared to the WT, the growth rate was slightly increased for the mutants, but the
mutants had lower growth yields on xylose and glucose. The mutants displayed improved
fermentation efficiency on 4% (w/v) xylose, and the same fermentation efficiency on 4% (w/v)
glucose compared to the WT. The mutants had a higher ethanol yield than the WT when a
mixture of 4% (w/v) each of glucose and xylose was fermented. After four days of fermentation,
the mutants utilized 100% of the xylose in the medium, whereas the WT only utilized 26.2% of
the xylose. The reason for improved xylose utilization of the mutants is not known (Bajwa et al.
2009). Glucose repression of xylose utilization, where glucose is utilized before xylose, is a
common problem with mixed sugar fermentations (Lee 1992). The mutants started to utilize
xylose when 1% glucose remained in the fermentation medium (Bajwa et al. 2009).
Additionally, in 60% (v/v) HW SSL, the mutants were able to utilize and ferment glucose,
mannose, galactose and xylose, while the WT was unable to ferment any of the sugars.
Subsequent research focused on the use of genome shuffling of the *S. stipitis* mutants to further improve HW SSL tolerance (Bajwa et al. 2010). (Figure 1.2) Bajwa et al. (2010) created genome shuffled mutants of *S. stipitis* by starting with a pool of UV-induced mutants displaying improved HW SSL tolerance. The mutants were recombined using sexual mating followed by sporulation to regenerate the haploid cells. After each round of genome shuffling, growth on HW SSL gradient plates was used to select improved mutant strains to serve as the starting pool for the next round of genome shuffling. A total of four rounds of genome shuffling were completed. Improved growth in liquid HW SSL was observed for four of the mutants. GS301 and GS302, from the third round of genome shuffling, and GS401 and GS402 from the fourth round, were able to grow in 80% (v/v) HW SSL (Bajwa et al. 2010). GS301 and GS301 displayed the best HW SSL tolerance as they were able to grow in 85% (v/v) HW SSL, with GS301 showing viability in 90% (v/v), but no growth was observed. Fermentations of a mixture of 4% (w/v) each of glucose and xylose using GS301 and GS301 were carried out. GS301 and GS302 utilized glucose and xylose more efficiently and produced more ethanol than the WT. Both strains utilized all the glucose within 20 hours, while the WT took 36 hours to consume all the glucose. Due to glucose repression, xylose was poorly utilized by the WT, taking 150 hours to consume only 1% (w/v). The genome shuffled strains consumed all the xylose in approximately 108 hours. The maximum ethanol concentration accumulated by GS301 and GS302 ranged from 2.8 to 2.9% (w/v), while it was only 1.8% (w/v) for the WT. The genome shuffled strains produced low levels of ethanol, 0.15 to 0.18% (w/v), from the sugars in undiluted HW SSL, but the WT was unable to utilize or ferment any of the sugars. This research demonstrated genome shuffling of *S. stipitis* can be used for the improvement of HW SSL tolerance.
Figure 1.2 Schematic of genome shuffling in yeast
Lignocellulosic hydrolysates contain a large assortment of inhibitors, which have synergistic effects on yeast cells. Overcoming the synergistic effects of multiple inhibitors will likely require the creation of a genome shuffled *S. stipitis* strain that combines mutations that confer tolerance to key inhibitors in hydrolysates (Bajwa et al. 2010). Since HW SSL has many of the same inhibitors found in many of the lignocellulosic hydrolysates, it was hypothesized that the genome shuffled strains from Bajwa et al. (2010) would be tolerant of inhibitors in other lignocellulosic hydrolysates.

A subsequent study tested the growth and fermentation of GS301 and GS302, two genome shuffled strains of *S. stipitis* on three wood hydrolysates (Bajwa et al 2011). Both GS301 and GS302 displayed improved fermentation and inhibitor tolerance for all three of the hydrolysates. The hydrolysates were: steam-pretreated enzymatically hydrolyzed poplar hydrolysate from Mascoma Canada, a steam pretreated poplar hydrolysate from the University of British Columbia Forest Products Biotechnology Laboratory, and mixed hardwoods pre-hydrolysate from FPInnovations. Both GS301 and GS302 completely utilized all the sugars in all three of the hydrolysates. The WT did not utilize or inadequately utilized the sugars in the hydrolysates and did not produce any or just minimal ethanol. The initial sugar concentrations of the poplar hydrolysate from Mascoma were 3.3% w/v glucose and 3.0% xylose. GS301 and GS302 completely utilized the sugars within 96-120 hours, while the WT consumed 75% after 11 days. GS301 produced 1.4% (w/v) ethanol and GS302 produced 1.2% (w/v), whereas the WT only produced minimal ethanol. In the mixed hardwoods pre-hydrolysate from FPInnovations, GS301 and GS302 utilized all of the 0.2% (w/v) glucose within 42 to 66 hours, whereas the WT only consumed the first 35% of the 0.2% glucose after 18 hours, but the remaining glucose was
not consumed by 186 hours. GS301 and GS302 had used most of the initial 3.5% (w/v) xylose by 138 and 120 hours, respectively. GS301 produced 0.80% (w/v) ethanol, and GS301 produced 0.96%. The WT had not utilized any of the xylose by 186 hours, and ethanol was not produced. In the steam pretreated poplar hydrolysate from UBC, GS301 and GS302 completely consumed the initial 0.33% (w/v) glucose within 72 hours, the WT took 192 hours. Almost all of the initial 1.8% (w/v) xylose was utilized by GS301 and GS302 by 120 hours, whereas WT had only consumed 11% of the initial xylose. GS301 and GS302 produced 0.42 and 0.39% (w/v) of ethanol in 120 and 96 hours, respectively, while the WT produced minimal ethanol. These fermentations of three wood hydrolysates displayed that the improved tolerance of the genome shuffled strains to HW SSL extends to other hydrolysates.

Another research group used genome shuffling to create a high ethanol producing strain, *S. cerevisiae* GS3-10 (Jingping et al. 2012). *S. cerevisiae* is the preferred strain for ethanol production because it has high tolerance to inhibitors, and high ethanol production and tolerance. However *S. cerevisiae* cannot ferment xylose, one the main sugars in lignocellulosic hydrolysates. Three recombinant strains of *S. cerevisiae* W5 were created in previous experiments, YX-3, LX-4, and CXS-5 (Ge et al. 2010). Each strain contained one gene encoding an enzyme necessary for xylose metabolism. The YX-3 strain contained the xylose reductase (XR) gene, the LX-4 strain contained the xylitol dehydrogenase (XDH) gene, and the CXS-5 strain contained the xylulokinase (XK) gene. Their genome shuffling technique involved recursive rounds of protoplast fusion of the three starting recombinant strains. The screening of genome shuffled strains was accomplished by using the triphenyl tetrazolium chloride colour development reagent, which will react with yeast metabolites to produce a red colour. The
darker the red colour of the colony, the greater enzyme activity and, therefore, a greater level of ethanol production. The starting recombinant strains could not ferment xylose on their own, so colonies that stained red had all three genes for xylose metabolism and they were selected for the next round of genome shuffling. A secondary screening of a co-fermentation of xylose and glucose was done to ensure the genome shuffled strains were fermenting both glucose and xylose. Three rounds of genome shuffling yielded *S. cerevisiae* GS3-10. GS3-10 will metabolize both xylose and glucose and produce high levels of ethanol. After 84 hours of fermentation of a defined medium containing 5% (w/v) glucose and 2.5% (w/v) xylose, GS3-10 utilized 100% of the glucose, and 69.48% of the xylose, compared to 100% of glucose, and 14.83% of xylose for *S. cerevisiae* W5, the WT strain. GS3-10 produced 26.65 g/L ethanol, which is 47.08% times more than W5, at 18.12 g/L. The ethanol yield was 17.65% higher for GS3-10 at 0.40 g/g compared to W5 at 0.34 g/g.

Zhang et al. (2012) used a modified genome shuffling method to create a new xylose-fermenting yeast strain from *S. cerevisiae* and *S. stipitis*, which also exhibited better tolerance to xylose and ethanol, and improved rates of xylose consumption and ethanol production compared to the parental strains (Zhang and Geng 2012). This research group used a method based on the recombination of whole genomes in vivo from two different yeast strains, *S. cerevisiae* ATCC 24860 and *S. stipitis* CBS 6054. Two rounds of genome shuffling and screening were used. Whole genomic DNA from *S. stipitis* was extracted and transferred to *S. cerevisiae* via electroporation. After seven to ten days of incubation at 30°C eight hybrid yeast strains were selected on YNBX plates containing 50 g/L xylose, 6.7 g/L yeast nitrogen base without amino acids and 20 g/L agar. Next the eight recombinants were tested for their ethanol
production capability in YNB broth containing 6.7 g/L YNB, 150 g/L xylose and 50 mM phosphate buffer at pH 7.0 and 30°C for 72 hours. The hybrid yeast strain with the greatest ethanol production was F1-8, with an ethanol yield of 0.31 g/g and an ethanol productivity of 0.38 g/L/h. The parental strain S. stipitis had an ethanol yield of 0.27 g/g and an ethanol productivity of 0.33 g/L/H. For the second round of genome shuffling the whole genome of S. cerevisiae was transferred into F1-8 by electroporation. The screening was carried out on YNBXE plates containing 6.7 g/L yeast nitrogen base, 50 g/L xylose, 50 g/L ethanol and 20 g/L agar. F1-8 would not grow on these plates. Three hybrid yeast strains were obtained from this selection and hybrid yeast strain, ScF2 showed the greatest aptitude for ethanol production. In a fermentation medium containing 120 g/L xylose, ScF2 had improved ethanol production rate and ethanol titre compared to its parental strains, S. stipitis and F1-8. ScF2 produced about 50 g/L ethanol, F1-8 produced about 0.45 g/L ethanol and S. stipitis produced about 0.42 g/L ethanol (Zhang and Geng 2012). The xylose fermentation ability of ScF2 was tested in media containing high concentrations of xylose (100-205 g/L). ScF2 consumed xylose faster and produced ethanol faster than S. stipitis. With a xylose concentration of 200 g/L ScF2 produced 49 g/L ethanol and consumed all the xylose by day five; whereas S. stipitis produced 43 g/L ethanol and took until day eight to consume all of the xylose. ScF2 was also more tolerant of higher xylose concentrations. At 250 g/L xylose the xylose consumption and ethanol productivity of S. stipitis was inhibited by the high xylose concentration and only produced 20 g/L ethanol by day seven. ScF2’s xylose consumption and ethanol productivity was only slightly affected when xylose concentrations were 250 g/L. By day six, ScF2 had produced 47 g/L in media containing 250 g/L xylose. However, higher initial xylose concentrations resulted in a
small decrease in the maximal ethanol amount and an increase in fermentation time. ScF2 produced the most ethanol, 0.51 g/L, after 5 days in media containing an initial xylose concentration of 150 g/L. Fermentation in defined media containing glucose, xylose and a mixture of the two by ScF2, *S. stipitis* and *S. cerevisiae* was evaluated. *S. cerevisiae* completely consumed the glucose in a media containing an initial glucose concentration of 100 g/L within 24 hours. *S. stipitis* consumed all the glucose within 48 hours, and ScF2 within 56 hours. Although ScF2 took longer to consume all the glucose it produced more ethanol (47 g/L) than *S. stipitis* (45 g/L) (Zhang and Geng 2012). In a mixed sugar fermentation initially containing 50 g/L xylose and 50 g/L glucose, ScF2 fermented both sugars, but consumed glucose before xylose. ScF2 also showed a decreased xylose consumption rate with the mixed sugar fermentation compared to the fermentation of only xylose. Similar trends were observed with *S. stipitis*. In the mixed sugar fermentation ScF2 did show higher xylose consumption and ethanol production than *S. stipitis*. At 144 hours ScF2 had a maximal ethanol concentration of 40 g/L and for *S. stipitis* the maximal ethanol concentration was 31 g/L at 96 hours.

In order to improve the ethanol productivity and acetic acid tolerance in *S. cerevisiae* 308, drug resistant marker aided genome shuffling was used (Zheng et al. 2011a). UV mutants of 308 were selected for their acetic acid tolerance by being plated on YNB plates (pH 4.5) containing 0.3% (v/v) acetic acid. Two hundred of the fastest growing acetic acid tolerant UV mutants were used as the starting strains for the first round of genome shuffling. The starting strains were divided into two groups: one group was transformed with a plasmid conferring Zeocin resistance (pSH65) and the other group was transformed with a plasmid conferring G418 resistance (pYK). The two groups of transformed UV mutants then underwent sporulation and
were crossed together. Cell suspensions from the crossing medium were plated on drug selection plates containing both Zeocin and G418. After the shuffled yeast cells were selected from the drug selection plates they were selected for their acetic acid tolerance by being spread on YNB plates containing 0.35% (v/v) acetic acid (pH 4.5). From the 0.35% (v/v) acetic acid, the fastest growing colonies were selected for fermentation analysis with and without acetic acid stress in order to determine their ethanol yield and fermentation rate. To progress to the second round of genome shuffling the first generation shuffled strain had to meet two criteria: 1) higher ethanol yield than the original 308 strain while under acetic acid stress; 2) the fermentation rate, ethanol yield and glucose to ethanol conversion could not be inferior to 308 while not under acetic acid stress (Zheng et al. 2011a). Five first generation shuffled strains were used as the starting strains for the second round of genome shuffling. For the selection of the second generation shuffled strains the acetic acid concentration was increased to 0.4% (v/v) in the acetic acid selection plates. Of the second generation shuffled strain, YZ2 showed the best fermentation tests. When grown in YNB liquid medium (pH 4.5) containing 0.4% (v/v) acetic under anaerobic conditions YZ2 had a lag phase that was 20 hours shorter than that of the parental strain, 308. In the fermentation medium of corn mash, which contained about 210 g/L glucose the fermentation performance of YZ2 was similar to 308 and the glucose was nearly depleted by 45 hours, and both produced about 90 g/L of ethanol. However, when 0.5% (v/v) acetic acid was added to the medium the fermentation rate and ethanol yield were lower for 308. YZ2 had a shorter lag phase and improved ethanol production of 21.6% compared to 308. All the glucose was consumed by YZ2 by 60 hours and about 90 g/L of ethanol was produced,
whereas with 308 about 30 g/L of glucose remained at 60 hours and less than 75 g/L of ethanol was produced at that point.

Zheng et al. (2011b) used genome shuffling to create *S. cerevisiae* strains with improved tolerance to osmotic, heat and acid stresses and better ethanol production than their parent strains. Initially fifteen *S. cerevisiae* strains were screened for their tolerance to multiple stresses and their fermentation ability. The high stress conditions were: high sugar content stress (30% (w/v) glucose), heat stress (incubation at 42°C), oxidative stress (4 mM H$_2$O$_2$), ethanol stress (7% (v/v) ethanol), low pH stress (pH adjusted to 2.5 using HCl), salt stress (0.8 M NaCl), acetic acid stress (4 g/L acetic acid), and complex stress conditions (20% glucose (w/v), 5% ethanol (v/v)) and incubated at 38°C. Of the fifteen *S. cerevisiae* strains tested, Z15 was the most-stress tolerant strain showing highest tolerance in high sugar content, salt stress, heat stress, acetic acid stress and complex stress conditions. Strain Z8 had the highest ethanol yield while under high heat and high gravity conditions, followed by Z15. Strain Z15 and Z8 served as the starting strains for the first round of genome shuffling. The strains were sporulated and crossed. The first generation shuffled strains were spread onto YPD selection plates containing 20% glucose and 5% ethanol and incubated at 38°C. One hundred first generation shuffled colonies were selected from the plates and analyzed in shake-flask fermentation under high gravity (300 g/L glucose) and high heat (42°C) conditions. Four first generation shuffled strains that produced more ethanol and had higher glucose/ethanol conversion rates than the parental strains, Z15 and Z8, were selected for the second round of genome shuffling. The following two rounds of genome shuffling were carried the same way as the first round, except the YPD selection plates in the second round were incubated at 39°C and for the third round the plates
were incubated at 40°C. YZ1 had the best fermentation performance after three rounds of genome shuffling and selection and also grew faster on the YPD selection plates. YZ1, along with its parental strains, Z8 and Z15, were tested in an industrial fermentation medium of a mash under three different conditions: regular (224.5 g/L glucose at 34°C), high heat (224.5 g/L glucose at 42°C) and high gravity (299.5 g/L glucose at 34°C) (Zheng et al. 2011b). The source of the mash and composition is not given (Zheng et al. 2011b). YZ1 performed slightly better than Z8 and Z15 under regular fermentation conditions, with a faster fermentation rate during the middle and late period and it produced 3.11% more ethanol. Under increased heat conditions YZ1 had decreased fermentation performance, but it was more tolerant than Z8 and Z15, and it had an ethanol yield of 11% (v/v) which was 10.31% higher than that of Z8. Under high gravity conditions YZ1 had decreased fermentation performance, but it still had improved ethanol yields over Z15 by 10.55%. Zheng et al. (2011b) note that the genome shuffled strain YZ1 has produced over 1 million tons of ethanol for Henan Tianguan Fuel Co. Ltd., China.

1.17 Pentose fermenting yeast:

In the bioethanol industry, fermentation is typically conducted with *S. cerevisiae*. This yeast utilizes hexose sugars in lignocellulosic substrates, but is unable to ferment pentose sugars, despite the presence of a xylose transporter and all the enzymes needed for a full xylose-metabolic pathway (Batt et al. 1986). Both pentose and hexose sugars in potential lignocellulosic hydrolysates must be efficiently converted to ethanol to maximize the economic feasibility of a commercial bioconversion process.
Native pentose-fermenting yeasts such as *Candida shehatae*, *Candida tropicalis*, *Pachysolen tannophilus* and *S. stipitis* can utilize the dominant pentose and hexose sugars and convert them to ethanol. However, when presented with lignocellulosic hydrolysates, this conversion becomes inefficient for several reasons. First, native pentose-fermenting yeasts suffer from catabolite repression and inactivation (Bicho et al. 1988; Lee 1992). When glucose and xylose are present, preferential utilization of hexose sugars occurs and pentose metabolism is inhibited (Bicho et al. 1988; Zhao et al. 2008). Second, they convert little substrate to ethanol in the presence of inhibitors arising from the pretreatment of lignocellulosic substrates, thereby limiting their fermentation performance (Lohmeier-Vogel et al. 1998). Third, they suffer from low ethanol tolerance which limits the amounts of ethanol that can be accumulated in the medium (Barbosa et al. 1990). To illustrate the problem, in a study of SSL fermentation (Lindén and Hahn-Hägerdal 1989), *S. cerevisiae* was found to give a greater ethanol yield (0.38 g/g total sugar) than *P. tannophilus* (0.12 g/g total sugar), *S. stipitis* (0.17 g/g total sugar) and *Candida tropicalis* (0.21 g/g total sugar), even though the latter three ferment both pentose and hexose sugars. At low concentrations ethanol can negatively affect yeast growth by inhibiting cell division (Birch and Walker 2000), while at high concentrations ethanol can decrease cell vitality and increase rates of cell death (Marza et al. 2002). The use of genetic engineering to improve ethanol tolerance is limited by the complexity of ethanol-stress tolerance pathways. Thus, there is considerable scope for improving of the ability of the native pentose-fermenting yeasts to ferment potential lignocellulosic substrates.
**1.18 Scheffersomyces stipitis:**

*S. stipitis* is a homothallic hemiascomycetous yeast and is normally found in its haploid state (Melake et al., 1996), which is advantageous for mutagenesis as only one copy of the genome is available for expression. *S. stipitis* is also amenable to mating and mates as a diploid, allowing recombination, followed by sporulation to form stable haploid cells (Melake et al. 1996).

Glucose is the most abundant sugar in biomass, but xylose can account for up to 25% dry weight of forestry and agriculture residues (Woodward 1984). It is estimated that the cost of biomass represents up to one third of production costs for lignocellulosic ethanol (Wingren et al. 2003). *S. stipitis* can ferment glucose, xylose, galactose, mannose, cellobiose and xylan into ethanol (du Preez 1994; Lee et al. 1986). It can also utilize arabinose and rhamnose (Koivistoiten et al. 2008). Xylose fermentation in *S. stipitis* is slow or blocked in the presence of glucose due to catabolite repression (Jeffries 1984), resulting in lower productivity and yield (Panchal et al. 1988). Glucose represses the activities of xylose reductase, an enzyme involved in xylose metabolism (Bicho et al. 1988).

Inhibition of *S. stipitis* growth can be seen at ethanol concentrations exceeding 30 g/L (Meyrial et al. 1995), whereas *S. cerevisiae* growth is not inhibited until ethanol concentrations reach 70 g/L or greater (Casey and Ingledew 1986). Low ethanol tolerance in *S. stipitis* is likely due to ethanol causing an uncoupling of the ATP hydrolysis activity and the proton pumping activities of the H⁺-ATPase, resulting in a reduction in the proton extrusion rate from the cell, and ultimately collapse of the proton gradient (Meyrial et al. 1997).
*S. stipitis* has low ethanol and inhibitor tolerance and it suffers from catabolite repression. Despite these short-comings, *S. stipitis* is a candidate for further improvement to increase its robustness for bioethanol production for several reasons. *S. stipitis* is widely studied and one of the better native xylose-fermenting yeast able to convert xylose quantitatively to ethanol (du Preez et al. 1989; van Dijken et al. 1986). Genes for xylose metabolism have been isolated from *S. stipitis* and engineered into *S. cerevisiae* for xylose metabolism. However, the rate of xylose consumption is low (Chu and Lee 2007) and *S. cerevisiae*’s regulatory responses have not been optimal (Jeffries et al. 2009). Other pentose fermenting yeast may have greater inhibitor tolerance, but *S. stipitis* produces primarily ethanol with minimal xylitol, unlike *P. tannophilus* which also produces xylitol in addition to ethanol (du Preez et al. 1984; Sanchez et al. 2002). Also, the mating protocol for *S. stipitis* has been described and is easily done in the laboratory, whereas the mating protocol for *C. shehatae* has not been described yet and cannot be done in the laboratory (Jeffries et al. 1994).

1.19 Thesis Objectives and Hypothesis:

This project focused on further improving GS301, a genome shuffled strain of *S. stipitis*, for the production of ethanol from lignocellulosic biomass. GS301 was produced from recursive cross mating of UV-induced SSL-tolerant mutant strains of *S. stipitis* (Bajwa et al. 2010). GS301 has increased tolerance to SSL, a byproduct of the sulphite pulping industry. SSL is a mixed sugar lignocellulosic hydrolysate that is rich in xylose and contains fermentation inhibitors (Bajwa et al. 2009). Improving the tolerance of *S. stipitis* to the inhibitors in SSL led to improved fermentation of the sugars in the SSL and higher ethanol yields by the genome shuffled strains, GS301 in particular (Bajwa et al. 2010). The improved tolerance of GS301 to the inhibitors in SSL
also conferred cross tolerance to the inhibitors in other lignocellulosic hydrolysates, leading to improved ethanol yields compared to the WT (Bajwa et al. 2011).

While GS301 is greatly improved compared to the WT the overall objective of this study was to further improve GS301 with a focus on further increasing its tolerance to the inhibitors in SSL, acetic acid and ethanol, while also reducing its glucose repression. Further improvement of the SSL tolerance of GS301 should lead to improved fermentation of the sugars in SSL. If the yeast tolerance to the inhibitors in the SSL is improved the yeast is better able to utilize the sugars in the SSL, leading to increased ethanol yields. Acetic acid is the main inhibitor in SSL (Pereira et al. 2013). Improving the tolerance of GS301 to acetic acid specifically may provide to increased tolerance to SSL. GS301, like most pentose fermenting yeast, suffer from low ethanol tolerance (Meyrial et al. 1997). Improving the ethanol tolerance of GS301 may lead to improved ethanol yields in fermentations of chemically defined media or lignocellulosic hydrolysates.

While GS301 does show improved utilization of the biomass sugars in SSL compared to the WT it still suffers from glucose repression and will utilize glucose preferentially over xylose (Bajwa et al. 2010). In order for economically viable ethanol production from biomass, all the sugars must be utilized quickly. Improving the glucose repression of GS301, by creating glucose derepressed mutants may lead to faster and more efficient fermentations of lignocellulosic hydrolysates.
Objectives

1) Use mutagenesis to produce four lines of improved mutant pools: SSL-tolerant line, acetic acid tolerant line, ethanol tolerant line, and glucose derepressed line.

2) Use these four mutant lines as the starting strains for genome shuffling to produce a genome shuffled strain displaying all four improved characteristics: improved tolerance to SSL, acetic acid, ethanol and reduced glucose repression.

Hypothesis

Mutagenesis and genome shuffling can be used to further improve GS301 by producing a new genome shuffled strain with improved SSL, acetic acid and ethanol tolerance and reduced glucose repression.
2.0 Materials and Methods

2.1 Yeast Strains and Chemicals:

*Scheffersomyces stipitis* NRRL Y-7124 (NRC 2548) wild type (WT) was obtained from the National Research Council Canada Culture Collection, Ottawa, Canada. GS301 is a genome shuffled mutant of WT that has increased tolerance to the inhibitors in hardwood spent sulfite liqour (Bajwa et al. 2010). In this study GS301 was subject to further improvement through UV mutagenesis and genome shuffling.

All the chemicals used were purchased from Sigma-Aldrich or Fisher Scientific. SSL was provided by Tembec (Temiscaming, Quebec, Canada). The batches of SSL were generated from a mixture of hardwoods and softwoods. We received different batches of SSL with variable toxicity. We adjusted the pH or added acetic acid to adjust the toxicity of the SSL. BP Biofuels supplied a sugar cane bagasse hydrolysate designated BP hydrolysate.

2.2 Culture Maintenance and Inoculum Preparation:

*S. stipitis* WT, GS301, and putative SSL–tolerant mutants were maintained on Yeast Peptone Dextrose (YPD) plates at 4°C and subcultured at one month intervals. The genome shuffled SSL-tolerant mutants were maintained on YPD plates containing 50% (v/v) SSL. The strains were lyophilized for long term storage. For inoculum preparation a loopful of cells from a YPD plate was aseptically transferred to a 20 mL broth containing 0.67% (w/v) yeast nitrogen base (YNB) without amino acids and 2% (w/v) xylose in a 125-mL Erlenmeyer flask and incubated in a shaker incubator set at 28°C and 180 rpm for 48 hours.
2.3 Improvement Strategy: Mutant Lines

The objective was to select for four different lines of mutants, an SSL-tolerant line, an acetic acid tolerant line, an ethanol tolerant line, and a glucose derepressed line. These mutant lines were to be mated together via genome shuffling to yield a yeast cell with all four of the traits: SSL, acetic acid, and ethanol tolerance and glucose derepression. (Figure 2.1)

To select for SSL-tolerant mutants, plates with a concentration gradient of SSL were used as described in (Bajwa et al. 2009; Bajwa et al. 2010). To select for ethanol-tolerant mutants, plates with a concentration gradient of benzyl alcohol were used. Preliminary testing determined WT and GS301 do not metabolize benzyl alcohol. A non-metabolizable alcohol was used so as to not select for mutants that consume ethanol in response to greater ethanol concentrations. Acetic acid tolerant mutants were selected using solid media containing fixed concentrations of acetic acid. All mutants were screened for their ability to continue utilizing xylose as the sole carbon source. The mutants were grown on plates of Yeast Nitrogen Base (YNB) without amino acids, supplemented with 2% (w/v) xylose. The mutants were assessed for their ability to ferment xylose as the sole sugar using initial low cell density inocula. Mutants that retained the ability to utilize xylose were used for further mutagenesis and genome shuffling.
Figure 2.1: Strategy for strain improvement. Mutagenesis and screening on selective media was used to create four mutant lines with improved tolerance to SSL, acetic acid, ethanol and glucose derepression. The mutant lines were used as the starting strains for genome shuffling. Several rounds of genome shuffling and selection were used to obtain one yeast strain combining the beneficial mutations of the mutant lines.
2.4 UV Mutagenesis:

UV mutagenesis was performed as described by Bajwa et al (2009). One mL of GS301 inoculum was transferred to an empty Petri dish, which was placed 40 cm from the UV light and exposed for 20 seconds to achieve a 50% survival rate, which is a low dose of mutagen. Higher doses of mutagenic agents can result in multiple mutations which may mask the effect of single or few beneficial mutations. A 50% survival rate reduces the occurrence of multiple mutations allowing for gradual accumulation mutations (Bajwa et al. 2009). The Petri dish was held stationary during the UV exposure which took place in a windowless room with the lights off. The 1 mL sample of UV irradiated GS301 was transferred to a sterile 15 mL centrifuge tube and placed approximately 60° from the horizontal in a shaker incubator at 28°C and 180 rpm for 24 hours in the dark to limit light mediated DNA repair. A 24 hour incubation period following mutagenesis allowed the mutants to multiply, thereby increasing the chance of selection a mutant with beneficial mutations. The control was a 1 mL sample of GS301 inoculum that had not undergone UV exposure but was otherwise treated in an identical manner. After 24 hours, 100 µl of the UV irradiated GS301 inoculum and the control inoculum were spread in a line, up the concentration gradient of either SSL or benzyl alcohol gradient plates (Bajwa et al. 2009; Syzbalski and Bryson 1952). The plates were incubated at 28°C for 5 to 10 days. Colonies growing at higher concentrations on the gradient plates were presumed to be more tolerant than the original strain and were isolated and transferred on to YPD plates. Putative mutant colonies were maintained on YPD plates. The mutants were grown on plates of YNB without amino acids, supplemented with either 2% (w/v) xylose to confirm they continued to utilize xylose as the sole carbon source. These putative mutant colonies were designated first
generation mutants. In the second round of mutagenesis the first generation mutants were pooled together and mutagenized followed by screening on SSL gradient plates to select second generation mutants. The procedure for the following rounds of mutagenesis and mutant selection were the same as for the first round.

2.5 Genome Shuffling of Improved Strains:

Genome shuffling was performed as described in Bajwa et al. (2010). Each mutant strain was grown separately in 5 mL YPD broth in a 15 mL centrifuge tube and incubated overnight at 28 °C at 180 rpm. For mating 2 x 10⁸ cells of each yeast strain were combined and a 100 µl aliquot was dropped on the center of a 3% malt extract and 1.5% agar plate. The plates were incubated at 28 °C for 10 days to allow for mating and sporulation. After 10 days all the cells were scraped off the plates and added to 10 mL of YPD broth in a 15 mL centrifuge tube and incubated overnight at 28 °C at 180 rpm. A 100 µl aliquot of the cells was spread up a gradient plate. Colonies growing at higher concentrations than the starting strains were picked to create a mutant pool to serve as the starting strains for the next round of genome shuffling.

2.6 Concentration Gradient Plates for Initial Screening and Selection of Putative Mutants with Improved SSL and Ethanol Tolerance:

Concentration gradient plates were prepared according to Syzbalski and Bryson (1952). Square plastic plates measuring 120 mm x 120 mm x 17mm were propped on a glass rod creating a slant, approximately 30 ° from the horizontal. A bottom layer of 2% agar with either SSL or benzyl alcohol was poured into the plates and allowed to solidify on a slant, creating a concentration gradient. Then a top layer of 2% agar was poured over top of this solid layer,
while the plate was level. After UV mutagenesis and incubation, 100 µl of the UV-exposed culture was streaked up the gradient plates, and incubated for 5-10 days at 28°C. Colonies growing at higher concentrations were selected and maintained on YPD plates for further characterization. Putative mutants were checked for growth on YNB plates without amino acids and supplemented with 2% (w/v) xylose to ensure their continued utilization of xylose.

The SSL gradient plates had 0.7% (w/v) acetic acid added to 100% SSL to increase the toxicity. After the addition of the acetic acid the pH of the SSL was adjusted to 4.5 using 10 M NaOH. The benzyl alcohol gradient plates used for ethanol tolerance selection consisted of 0.1-1% (w/v) benzyl alcohol, 2% xylose and 0.67% YNB without amino acids.

2.7 Fixed Concentration Acetic Acid Plates:

Petri plates containing a fixed concentration of 0.25% (w/v) acetic acid, 2% (w/v) xylose and 0.67% (w/v) YNB without amino acids, were used to select for acetic acid tolerant mutants. The pH of the plates was not adjusted and a concentration at which GS301 would not grow was used to select for acetic acid tolerant mutants. An aliquot (50 µl) of GS301 was spread on one side of the plate and an aliquot (50 µl) of UV exposed culture was spread on the other side. The plates were incubated at 28 °C for 10 days. The plates were inspected daily for fast growing mutants, which were picked and maintained on YPD plates for further characterization.

2.8 Mutagenesis and Selection for Glucose Derepressed Mutants using 2-Deoxyglucose (2DG):

2DG is a glucose analog that can be used to obtain mutants derepressed for pentose metabolism (Sreenath and Jeffries 1999). Solid media containing fixed concentrations of 4.5%
(w/v) 2DG, 0.67% YNB without amino acids, and 2% (w/v) xylose were used to generate spontaneous mutants with reduced catabolite repression due to glucose. GS301 inoculum was prepared as described and after 48 hours of growth a 50 µl aliquot was spread on a 4.5% (w/v) 2DG plate. The 2DG plates were incubated at 28°C and inspected every 24 hours for the appearance of fast-growing colonies, which were selected and spread on YPD plates. The putative mutants were grown on plates of YNB without amino acids and 2% (w/v) xylose. These putative mutants were evaluated for growth on 5% and 6% (w/v) 2DG plates. GS301 did not grow at 5% and 6% (w/v) 2DG. Subsequently the putative mutants were evaluated for their fermentation performance in mixed sugar fermentations of glucose and xylose, as outlined below.

**Fermentation in Defined Media**

**2.9 Low cell density fermentation:**

First, mutants and genome shuffled strains were assessed for their ability to ferment single sugars, xylose or glucose using low cell density inocula. The fermentation media consisted of 0.67% (w/v) YNB without amino acids and either 4% (w/v) glucose or 4% (w/v) xylose. Inocula were grown as previously described. One hundred mL of the fermentation was placed into a 250 mL flask and 4 mL of the inoculum was added. WT typically produced twice the amount of biomass as GS301 based on the OD$_{600}$. To compensate for this the inoculum of GS301 and the mutant or genome shuffled strains were added at 4% instead of 2% (Bajwa et al. 2009). Four mL of the inocula were collected and centrifuged at 13,000 g for five minutes. Each pellet was washed twice with sterile distilled water and suspended in the fermentation media.
The fermentation flasks were placed in a shaker incubator at 28°C at 180 rpm. At periodic intervals, 2 mL samples were withdrawn and centrifuged at 13,000 g for 5 minutes. The supernatant was removed from the cell pellet and stored at -20°C for subsequent ethanol and sugar analysis.

2.10 High cell density fermentation:

The mutant and genome shuffled strains that performed adequately (ethanol yields and sugar utilization similar to GS301) in low cell density fermentation were assessed in high cell density fermentations of single sugars, glucose or xylose and a mixture of the two [4% (w/v) xylose and 4% (w/v) glucose]. The high cell density inocula were grown as described above, except 100 mL of the medium was used in a 250 mL flask instead. After 48 hours of incubation the cells were centrifuged at 10,000 g for 10 minutes at 4°C. Each pellet was washed twice with sterile distilled water and suspended to an OD$_{600}$ of about 5.0-6.0 in 100 mL of the fermentation medium. Incubation conditions and sample treatment were as described above.

2.11 Growth Assessment in SSL:

Mutant and genome shuffled strains showing improved tolerance to SSL on gradient plates were assessed for growth in liquid SSL. The concentration of the liquid SSL ranged from 85% to 100% SSL. Sterile distilled water was used to dilute the SSL. Due to the variability in SSL composition with each batch we received, the toxicity and degree of inhibition to the yeast was variable from batch to batch. Each batch was assessed to determine the inhibitory concentration to GS301. The SSL was boiled to eliminate any potential contaminating microorganisms, and cooled to room temperature before adjusting the pH to either 4.5 or 5.5
using 10 M NaOH. Sterile distilled water was added to dilute the SSL accordingly. 100 mL were transferred to a sterile 250 mL Erlenmeyer flask and 4% inoculum was added. Inocula were prepared as described above and after 48 hours of growth 4 mL of the inoculum was centrifuged, washed with sterile distilled water and the pellet suspended in the SSL medium. The flasks were placed in a shaker incubator at 28°C at 180 rpm. Growth was monitored periodically by withdrawing 100 µl aliquots and serially diluting with sterile distilled water. Serial dilutions were spread on YPD plates and incubated at 28°C for 48 hours before enumeration.

2.12 Fermentation in SSL:

Improved genome shuffled strains were evaluated for their ability to ferment the sugars in undiluted SSL at both pH 4.5 and 5.5. High cell density inocula were prepared as described above. The SSL was boiled and cooled to room temperature. The pH was adjusted using 10 M NaOH. 100 mL of SSL were transferred to a 250 mL Erlenmeyer flask and the inoculum added. The flasks were placed in a shaker incubator at 28°C at 180 rpm. At periodic time points, two mL samples were taken and centrifuged at 13,000 g for 5 minutes. The supernatant was removed and stored at -20°C for future ethanol and sugar analysis.

2.13 Growth Assessment of Mutants and Genome Shuffled Mutants in the Presence of Acetic Acid and HMF:

The genome shuffled strains were evaluated for growth in liquid media containing 0.67% (w/v) YNB, 2% (w/v) xylose and either 0.4 and 0.5% (w/v) acetic acid. The pH of the media was adjusted to 4.5 using 10 M NaOH. Fifty mL of the media was transferred to a sterile 125 mL Erlenmeyer flask. The inocula were prepared as described as above. After 48 hours of
growth 2 mL of the inoculum was centrifuged, rinsed with distilled water and then the cell pellet was suspended in the medium. The flasks were placed in a shaker incubator at 28°C at 180 rpm. At daily intervals 1 mL samples were taken and the growth monitored by measuring the optical densities at 600 nm (OD$_{600}$) of the cultures against the medium blank using an Ultraspec 3100 Pro spectrophotometer.

The acetic acid tolerant mutants were evaluated for growth in liquid media containing 0.67% (w/v) YNB, 2% (w/v) xylose and 0.3% (w/v) acetic acid. The pH of the media for the acetic acid tolerant mutants was not adjusted because this phase of the study was done earlier than the acetic acid growth assessment of the SSL tolerant mutants, when it was decided to adjust the pH of the acetic acid media to 4.5, close to the pKa of acetic acid. The cultures and inocula were prepared as described above as well as the measurement of the growth of the mutants.

The SSL genome shuffled strains were evaluated for their growth in liquid media containing 0.67% (w/v) YNB, 2% (w/v) xylose and 0.4 or 0.5% (w/v) HMF. Fifty mL of the medium, along with 4% inoculum, was placed in a 125 mL Erlenmeyer flask and incubated at 28°C at 180 rpm. Inocula preparation and sample treatment and measurement were as described above for the acetic acid liquid media.

2.14 Growth Rate and Growth Yield of Genome Shuffled Mutants on Xylose:

An aliquot (0.5 mL) of inoculum culture of GS301 and each of the genome shuffled mutants was inoculated into 25 mL of media contacting 0.67% (w/v) YNB and 4% (w/v) xylose in a 125 mL Erlenmeyer flask. The flasks were incubated at 28 °C and 180 rpm. Growth was monitored by measuring the OD$_{600}$. Growth rate was determined during the early exponential
phase. The growth yield (dry cell weight) was measured gravimetrically after 48 hours. A 15 mL sample of the growth media was centrifuged, the cell pellet washed twice with distilled water and dried at 90 °C to a constant weight.

**Analytical Methods**

**2.15 Ethanol and sugar analysis during fermentations:**

High performance liquid chromatography (HPLC) was used to analyze the sugar and ethanol concentrations in chemically defined fermentation media (Bajwa et al. 2009; Bajwa et al. 2010; Bajwa et al. 2011). A Bio-Rad HPX-87H column was used in an Agilent model 1200 HPLC equipped with a refractive index detector. The eluent was filtered and degassed 5 mM H₂SO₄ at a flow of 0.6 mL/min. The column temperature was 40°C. Ten percent (w/v) glycerol was used as the internal standard for sugar analysis. 10% (w/v) isopropanol was used as the internal standard for ethanol analysis. A 400µl aliquot of the supernatant from a fermentation sample, 50 µl of 10% (w/v) glycerol and 50µl of 10% (w/v) isopropanol were placed in a glass vial for HPLC analysis. The injection volume was 5 µl and the run time was 26 minutes.

The sugar and ethanol concentrations in the SSL fermentations were analyzed using capillary gas chromatography (GC). Alditol acetate derivitisation was used for the sugar analysis (Theander 1991). Glycerol, (10% w/v), was used as an internal standard. Fifty µl of glycerol were added to a 200 µl aliquot of the supernatant from SSL fermentation broth. The SSL aliquots were made alkaline by adding 40 µl of concentrated NH₄OH. Next the aldoses were reduced by adding 100 µl of 2.8M KBH₄ (dissolved in 3M NH₄OH) and incubating in a 40°C water bath for 90 minutes. To stop the reduction reaction, 100 µl of glacial acetic acid was added to the sample
mixture. For the acetylation of the sugar alcohols, 500 µl of 1-methylimidazole and 2 mL of acetic anhydride were added to the sample and vortexed. The sample mixtures were allowed to react for 10 minutes before sterile distilled water (5 mL) was added to stop the reaction. Dichloromethane (2mL) was added to the samples and vortexed. After the phases had separated, about 1 mL of the dichloromethane phase (bottom) was transferred to a GS vial via a Pasteur pipette.

A Shimadzu Gas Chromatograph (GC-2014) with a BP10 capillary column (30 m long, 0.25 mm inner diameter and a film thickness of 0.25 µm) equipped with a flame ionization detector was used for the analysis of the alditol acetate derived samples. Nitrogen was the carrier gas at 30 cm/s and split injection at 1:25. The temperature of the column was 220°C and the injector and detector were 240 °C. The injection volume was 2µl and the total run time was 30 minutes.

For the analysis of the ethanol in the SSL fermentation samples a BP1 column (30 m long, 0.25 mm inner diameter and a film thickness of 0.25 µm) was used in the Shimadzu GC (Bajwa et al. 2009; Bajwa et al. 2010). Nitrogen was the carrier gas at 28.3 cm/s and split injection at 1:20. The temperature of the column was held at 40 °C for 3 minutes and ramped to 150°C at a rate of 15°C/min. The injector was 250°C and detector was 275 °C. The injection volume was 1 µl and the total run time was 10.33 minutes. The samples were prepared for GC analysis by adding 50 µl of 10% (w/v) isopropanol to a 400 µl aliquot SSL in a GC vial.
2.16 Inhibitor concentrations in SSL and BP hydrolysate:

The concentration of acetic acid, furfural, HMF and formic acid in SSL and BP hydrolysate were measured via HPLC analysis using a Bio-Rad HPX-87H column (Ruiz and Ehrman 1996). The standards used were: formic acid at 39.23 mg/mL, acetic acid at 40.23 mg/mL, furfural at 2.67 mg/mL, and HMF at 2.55 mg/mL. One stock solution containing all the inhibitors was used and the stock was diluted by ½ for standard A, 1/10 for standard B, 1/20 for standard C, and 1/100 for standard D. The eluent used was filtered and degassed 5 mM H₂SO₄ at a flow of 0.6 mL/min. The temperature of the column was 65°C. The injection volume was 20 µl and the run time was 50 minutes. Aliquots (500 µl) of hydrolysate were filtered through a 0.2 µm filter syringe into a vial and capped.

2.17 Experimental Replication and Data Analysis:

All liquid culture experiments were conducted three times with independently grown inocula. Figures show the trend of one representative experiment. A student’s t-test with a confidence interval of 95% was used to evaluate the significance in difference between the growth rate and grow yield of GS301 and the genome shuffled mutants.
3.0 Results

The objective was to select for four different lines of mutants, an SSL-tolerant line, an acetic acid tolerant line, an ethanol tolerant line, and a glucose derepressed line. These mutant lines were to be mated together via genome shuffling to yield a yeast cell with all four of the traits: SSL, acetic acid, and ethanol tolerance and glucose derepression. Of the four lines of mutants, only the SSL-tolerant line yielded workable mutants. Some acetic acid-tolerant mutants were obtained, but they proved to be unstable and reverted after five to six subcultures. Using 2DG to select spontaneous mutants did not yield glucose derepressed mutants. Ethanol tolerant mutants were unable to be obtained due to the difficulties associated with the screening and selection of ethanol tolerant mutants. Work was discontinued with the acetic acid, ethanol and glucose derepressed mutant lines. Work continued only with the SSL-tolerant mutant.

SSL Tolerant Line

3.1 Concentration of Inhibitors in SSL and BP Hydrolysate:

HPLC was used to quantify the concentration of the common inhibitors furfural, HMF, acetic acid and formic acid in SSL* and BP hydrolysate (Table 3.1).
Table 3.1: Concentration of furfural, HMF, acetic acid and formic acid in SSL and BP hydrolysate.
* This batch of SSL was used for all the characterization of the genome shuffled SSL-tolerant mutants.

<table>
<thead>
<tr>
<th>Hydrolysate</th>
<th>Furfural (mg/mL)</th>
<th>HMF (mg/mL)</th>
<th>Acetic Acid (mg/mL)</th>
<th>Formic Acid (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSL</td>
<td>0.039</td>
<td>0.11</td>
<td>3.37</td>
<td>0.29</td>
</tr>
<tr>
<td>BP</td>
<td>4.30</td>
<td>1.0</td>
<td>11</td>
<td>2.84</td>
</tr>
</tbody>
</table>
3.2 SSL-Tolerant UV Mutants of GS301:

GS301 was subjected to two rounds of UV mutagenesis. After UV mutagenesis the cells were allowed to recover 24 hours before being spread on SSL gradient plates. After the first round of mutagenesis the putative mutants growing at higher concentrations on the SSL gradient plates were retested on SSL gradient plates. Mutants still growing at a higher concentration on the gradient plate than GS301 were pooled and used in the second round of UV mutagenesis. The second round of UV mutagenesis yielded six SSL-tolerant UV mutants that grew at a higher concentration on the SSL gradient plates relative to GS301 (Figure 3.1). These SSL-tolerant mutants (designated M1, M2, M3, M4, M5, M6) were grown three times on SSL gradient plates to confirm their SSL tolerance and stability. The ability of SSL-tolerant mutants to utilize xylose as the sole carbon source was verified by spreading them on plates of YNB without amino acids and 2% (w/v) xylose.
Figure 3.1: Growth of second generation SSL-tolerant UV mutants on a SSL gradient plate, pH 5.5. The plate was incubated at 28 °C for 10 days.
3.3 Growth of second generation SSL-tolerant UV mutants in SSL:

After confirming the SSL-tolerance of the mutants on SSL gradient plates, the mutants were evaluated for their tolerance to the inhibitors in liquid cultures of SSL. The six mutants were tested for their ability to grow in liquid SSL. As previously mentioned the degree of inhibition was variable with each batch of SSL that was received from Tembec. As such each batch of SSL had to be characterized to determine an inhibitory concentration for GS301 before testing the mutants. At 90% (v/v) SSL (pH 5.5) the GS301 cell number decreased by about three log units on day two. By day four GS301 started to grow slowly, achieving about 1.8 log units of growth by day eight (Figure 3.2). At 90% (v/v) SSL (pH 5.5), the cell numbers of mutant 2, 5 and 6 decreased by about one log unit by day two. By day four, their cell numbers increased by one log unit. By day ten, the cell number of mutant 2, 5 and 6 had increased by another 1.5 log units. The cell number of mutant 1 remained fairly stable at about log 7.8 CFU/ml for the first four days of growth, after which it started to grow, increasing one log unit between day four and day six, and then growing a further 1.5 log units to reach the highest cell number of the mutants, log 9.94 CFU/mL. The cell number of mutant 3 and 4 decreased by half by day two of the growth experiment in 90% (v/v) SSL. After day four, mutant 3 and 4 started to grow slowly, increasing by two log units by day nine. All of the mutants grew to a cell number 2 to 4 log units higher than GS301. At 95% (v/v) SSL (pH 5.5), GS301 survived, but did not grow. The number of GS301 cells decreased by 4.5 log units by day two and did not increase over the ten day incubation period. The mutants grew at 95% (v/v) SSL, but at a slower rate than 90% (v/v) SSL. The cell numbers of mutant 1 and 2 did not decrease; their cell number increased by two log units from day zero to day ten. The cell number of mutant 5 and 6 decreased by about one log
unit by day two. The cell numbers of mutant 5 and 6 started to increase after day two and increased by about 1.5 log units by day ten. Mutants 3 and 4 experienced a decrease in cell number of about two log units by day two. After day two the cell number of mutants 3 and 4 increased by about one log unit by day ten.
Figure 3.2: Growth of second generation SSL-tolerant UV mutants in (A) 90% (B) 95% (v/v) SSL, pH 5.5. * One replicate graph representing a triplicate experiment.
3.4 Fermentation of second generation SSL-tolerant mutants in chemically defined media:

Glucose and xylose are abundant in lignocellulosic hydrolysates (Limayem and Ricke 2012). It was important that the mutants retained the ability to ferment glucose and xylose into ethanol. If the mutants did not ferment glucose and xylose into ethanol at a comparable rate to GS301, the mutant would have been culled from the mutant pool. The UV mutants were tested for their ability to ferment glucose (Figure 3.3) and xylose (Figure 3.4) individually in chemically defined media using low initial cell density inocula. In the fermentation of 4% (w/v) glucose, GS301 and all the mutants had consumed all of the glucose within 48 hours. GS301 produced a maximum ethanol concentration of 1.4% (w/v), while the mutants produced maximum ethanol concentrations ranging from 1.42 to 1.64% (w/v). In the fermentation of 4% (w/v) xylose, GS301 did not completely consume the xylose and about 0.13% (w/v) xylose remain in the medium at 120h. The maximum ethanol concentration produced by GS301 was 1.05% (w/v). Mutant 4 consumed all the xylose within 96 hours and produced a maximum ethanol concentration of 0.89% (w/v). Mutants 3 and 4 consumed all the xylose within 120 hours and produced maximum ethanol concentrations of 0.95 and 0.89% (w/v), respectively. Xylose was not completely consumed by mutants 1, 2, 5 and 6, and about 0.3 to 0.97% (w/v) xylose remained after 120 hours of incubation. These mutants achieved maximum ethanol concentrations ranging from 0.73 to 0.77% (w/v).
Figure 3.3: Fermentation of glucose by the second generation SSL-tolerant mutants

* One replicate graph representing a triplicate experiment.
Figure 3.4: Fermentation of xylose by the second generation SSL-tolerant UV mutants

* One replicate graph representing a triplicate experiment.
3.5 Genome Shuffled Strains:

After confirming the SSL tolerance of the SSL-tolerant mutants and checking their ability to ferment xylose and glucose, genome shuffling was conducted. The objective with genome shuffling was to combine the beneficial mutations of the six UV mutants into one yeast strain with the aim of generating even greater SSL-tolerance than the starting UV mutant strains. The six second generation SSL-tolerant UV mutants served as the starting strains for five rounds of genome shuffling. After each round of mating, approximately 30 colonies showing improved tolerance compared to the second generation SSL-tolerant UV mutants on SSL gradient plates were selected. These mutants were retested on SSL gradient plates (Figure 3.5) and those still showing improved tolerance were used as the starting strains in the next round of genome shuffling. Three mutants showing the greatest tolerance on the gradient plates were selected for further testing in liquid SSL. The genome shuffled mutants were grown on plates of YNB supplemented with 2% (w/v) xylose to confirm they continued to utilize xylose. These genome shuffled mutants were designated EVB105, EVB205 and EVB505. EVB105 was obtained from the first round of genome shuffling, EVB205 from the second round, and EVB505 from the fifth round.
Figure 3.5: Growth of GS301 and genome shuffled SSL-tolerant mutants on an SSL gradient plate. The SSL was supplemented with 0.7% (w/v) acetic acid, and the pH adjusted to 4.5 to increase the toxicity of this particular batch of SSL.
3.6 Growth of SSL-tolerant Genome Shuffled Strains in Liquid SSL:

After testing the SSL tolerance of the genome shuffled mutants, EVB105, EVB205 and EVB505, on SSL gradient plates their tolerance to inhibitors were evaluated in liquid SSL. The same batch of SSL was used for all of the characterization of the mutants obtained from genome shuffling. In this batch of SSL, GS301 would grow in undiluted SSL at pH 5.5. In order to select for improved tolerance in liquid SSL the adjusted pH was lowered to 4.5 to increase the toxicity. The lower pH of the SSL increased the amount of protonated acetic acid in the SSL, and this enhanced its toxicity. With this pH adjustment, GS301 grew in 85% (v/v) SSL (Figure 3.6). However, during the first two days of the growth experiment the cell number of GS301 dropped 6 log units to below detectable limits. After the second day, GS301 started to grow and its number increased by 2.7 log units by day three. The mutants grew in 85% SSL. Similar to GS301, the mutants also experienced an initial drop in cell number (about 1 log unit) for the first day of growth, but the mutants recovered and their numbers increased by about 1 log units by day two. The cell numbers of the mutants increased by another log unit by day five. While 90% (v/v) SSL was found to be completely inhibitory to GS301 with no viable cells being recovered, the mutants grew at this concentration. Initially the mutants experienced a 1.5 log unit decrease, but increased by about 2 log units by day three. EVB105, EVB205, and EVB505 grew similarly to one another in SSL with no strain growing better or worse than the others. The mutants were more tolerant than GS301 to the inhibitors in SSL and grew in 90% SSL, a concentration of SSL that was completely inhibitory to GS301.
Figure 3.6: Growth of SSL-tolerant genome shuffled mutants in diluted SSL. (A) 85% (B) 90% SSL
pH 4.5

* One replicate graph representing a triplicate experiment.
3.7 Growth of SSL-tolerant Genome Shuffled Mutants in the Presence of Acetic Acid and HMF:

SSL contains an assortment of inhibitors (Bajwa et al. 2009; Bajwa et al. 2010). Acetic acid and HMF are two main inhibitors in SSL (Table 3.1). To assess if SSL-tolerant mutants may also exhibit tolerance to the main inhibitors in SSL, the genome shuffled mutants were evaluated for their ability to grow in chemically defined media containing either acetic acid or HMF. GS301 grew in the presence of 0.4% (w/v) acetic acid (pH 4.5), but only after a 24 hour lag period (Figure 3.7A). The OD$_{600}$ of GS301 increased by about 1.8 log units by day five. The mutants did not experience a lag phase in the presence of 0.4% (w/v) acetic acid and grew faster than GS301. For the three mutants the OD$_{600}$ increased by about 2 log units by day four; followed by a further increase of 0.5 log units by day five. GS301 survived but did not grow in the presence of 0.5% (w/v) acetic acid (pH 4.5). The mutants grew in the presence of 0.5% (w/v) acetic acid but at a decreased rate compared to their growth in the presence of 0.4% (w/v) acetic acid (Fig. 3.7B). In 0.5% (w/v) acetic acid, the OD$_{600}$ of the mutants increased about 0.8 log units by day two. By day four the OD$_{600}$ increased another log unit for the mutants. The mutants show increased tolerance to acetic acid compared to GS301, since the mutants would grow faster in 0.4% (w/v) acetic acid and grew in 0.5% (w/v) acetic acid, whereas GS301 did not grow in this concentration.
Figure 3.7: Growth of SSL-tolerant genome shuffled mutants in (A) 0.4% (B) 0.5% (w/v) acetic acid, pH 4.5

* One replicate graph representing a triplicate experiment.
GS301 grew in the presence of 0.4% (w/v) HMF after a 24 hour lag phase (Figure 3.8). The OD$_{600}$ of GS301 increased by 1.5 log units by the fourth day. The mutants grew in the presence of 0.4% (w/v) HMF, and at a faster rate than GS301. The mutants’ OD$_{600}$ increased by about 1.5 log units by the second day. GS301 survived in the presence of 0.5% (w/v) HMF, but did not grow. The mutants grew in the presence of 0.5% (w/v) HMF and their OD$_{600}$ increased by about 1.5 log units by the third day.

The mutants, EVB105, EVB205, and EVB505 grew at very similar rates to each other in the presence of acetic acid and HMF. They all showed increased tolerance to acetic acid and HMF compared to GS301.
Figure 3.8: Growth of SSL-tolerant genome shuffled mutants in 0.4% and 0.5% (w/v) HMF

* One replicate graph representing a triplicate experiment.
3.8 Growth of SSL-tolerant Genome Shuffled Strains in BP Hydrolysate:

Lignocellulosic hydrolysates can differ widely in inhibitor composition and concentration. To determine if the SSL-tolerant mutants were tolerant to inhibitors in other hydrolysates the mutants were grown on gradient plates of BP hydrolysate (Figure 3.9), and in liquid BP hydrolysate (Figure 3.10). On the BP hydrolysate gradient plate, the streaks of EVB105, EVB205 and EVB505 grew higher up the plate and thus at a higher concentration of BP hydrolysate than GS301. The BP hydrolysate was more toxic than the SSL. GS301 did not survive in 40% (v/v) BP (pH 5.5). The mutants survived and grew slightly in 40% (w/v) BP; their cell numbers increased by one log unit by day three.
Figure 3.9: Growth of genome shuffled SSL-tolerant strains on gradient plates of BP hydrolysate, pH adjusted to 5.5.
Figure 3.10: Growth of SSL-tolerant genome shuffled mutants in 40% BP hydrolysate, pH 5.5.

* One replicate graph representing a triplicate experiment.
3.9 Growth Rate and Growth Yield of Genome Shuffled Mutants on Xylose:

EVB105, EVB205 and EVB505 were evaluated for their growth rate (h\(^{-1}\)) and growth yield (g/L dry cell weight) in 4% (w/v) xylose, and the results are shown in Table 3.2. GS301 had a growth rate of 0.51 ± 0.05 h\(^{-1}\) and a growth yield of 1.56 ± 0.08 g/L. EVB105 had a growth rate of 0.58 ± 0.02 h\(^{-1}\) and a growth yield of 1.51 ± 0.16 g/L. EVB205 had a growth rate of 0.51 ± 0.03 h\(^{-1}\) and a growth yield of 1.73 ± 0.14 g/L. EVB505 had a growth rate of 0.61 ± 0.08 h\(^{-1}\) and a growth yield of 1.48 ± 0.16 g/L. A two tailed t-test was done to determine if the difference in growth rate and growth yield of the mutants and GS301 were statistically different. There is not a significant difference (p > 0.05) between the growth rate and growth yield of GS301 and the mutants.
Table 3.2: Growth rate (h\(^{-1}\)) and growth yield (dry weight in g/L) of GS301 and genome shuffled mutants on 4% (w/v) xylose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth Rate (h(^{-1}))</th>
<th>Growth Yield (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS301</td>
<td>0.51 ± 0.05</td>
<td>1.56 ± 0.08</td>
</tr>
<tr>
<td>EVB105</td>
<td>0.58 ± 0.02</td>
<td>1.51 ± 0.16</td>
</tr>
<tr>
<td>EVB205</td>
<td>0.51 ± 0.03</td>
<td>1.73 ± 0.14</td>
</tr>
<tr>
<td>EVB505</td>
<td>0.61 ± 0.08</td>
<td>1.48 ± 0.16</td>
</tr>
</tbody>
</table>

N = 3; ± SD
3.10 Fermentation Assessment of SSL-tolerant Genome Shuffled Mutants in Chemically Defined Media:

EVB105, EVB205, and EVB505 were initially screened for their ability to ferment xylose and glucose as single sugars using low initial cell density inocula. Mutants that performed adequately (ethanol yields and sugar utilization rates similar to GS301) were selected for further characterization. Fermentations of single and mixed sugars (xylose and glucose) using high initial cell density inocula were done. In 4% (w/v) glucose using high initial cell density inocula, EVB105, EVB205 and EVB505 performed similarly to GS301 (Figure 3.11). GS301 and the mutants had consumed all of the glucose within 18 hours of fermentation. GS301 produced a maximum ethanol concentration of 1.4% (w/v). The mutants produced maximum ethanol concentrations ranging from 1.38 to 1.49% (w/v). In the fermentation of 4% (w/v) xylose using high initial cell density, GS301 consumed all the xylose within 48 hours of fermentation and produced a maximum ethanol concentration of 1.2% (w/v) (Figure 3.12). The mutants exhibited slower rates of xylose consumption than GS301, consuming all of the xylose within 60 hours of fermentation. Their maximum ethanol concentration ranged from 1.42 to 1.48% (w/v). In summary, the mutants utilized glucose at a very similar rate to GS301 and produced similar ethanol yields, while GS301 utilized xylose faster than the mutants, but the mutants produced higher ethanol yields than GS301.
Figure 3.11: Fermentation of 4% (w/v) glucose by genome shuffled SSL-tolerant mutants

* One replicate graph representing a triplicate experiment.
Figure 3.12: Fermentation of 4% (w/v) xylose by genome shuffled mutants.

- GS301
- EVB105
- EVB205
- EVB505

* One replicate graph representing a triplicate experiment.
In the fermentation of mixed sugars (4% w/v xylose and 4% w/v glucose) using high initial cell density inocula the glucose was consumed before xylose by both GS301 and the mutants (Figure 3.13). Glucose was consumed by 18 hours by both GS301 and the mutants. GS301 consumed the xylose at a faster rate than the mutants and completely consumed the xylose by 48 hours. The mutants had consumed all the xylose within 96 hours of fermentation. The maximum ethanol concentration produced by GS301 was 1.52% (w/v), while the mutants ranged from 1.43 to 1.50% (w/v). No difference in sugar consumption and ethanol production was observed between the mutants. In the mixed sugar fermentation GS301 and the mutants utilized glucose at a similar rate, but GS301 utilized xylose at a slightly faster rate than the mutants. The ethanol yields were similar for GS301 and the mutants.
Figure 3.13: Mixed sugar fermentation of 4% (w/v) glucose and 4% (w/v) xylose by genome shuffled mutants.

* One replicate graph representing a triplicate experiment.
3.11 Fermentation Performance of SSL-tolerant Genome Shuffled Strains in Undiluted SSL:

In the preceding studies, the genome shuffled mutants were evaluated for their growth in liquid SSL, and in the presence of acetic acid and HMF. The mutants were also evaluated for their ability to ferment the sugars in SSL to ethanol. However, it is important that the mutants could not only grow in the presence of the inhibitors in SSL, but also ferment the sugars to ethanol in the presence of the inhibitors. The mutants were further tested for their ability to ferment the sugars in undiluted SSL at pH 5.5 and 4.5. GS301 utilized the glucose, xylose, mannose and galactose, but not the arabinose in the SSL. Like GS301, the mutants utilized glucose, xylose, mannose and galactose, but not arabinose. In the SSL fermentation at pH 5.5, GS301 consumed all the glucose within 36 hours of fermentation (Figure 3.14). Within 72 hours of fermentation GS301 had not consumed all of the xylose; 1.0% (w/v) xylose remained. GS301 had not utilized all the galactose and mannose by 72 hours of fermentation; 0.34% (w/v) and 1.3% (w/v), respectively remained. The mutants consumed the glucose faster than GS301. EVB105 and EVB205 consumed all the glucose within 8 hours of fermentation, and EVB505 within 12 hours of fermentation. The mutants EVB205 and EVB505 also had xylose remaining at 72 hours of fermentation, but they consumed more xylose than GS301 did. EVB105 had consumed all of the xylose within 72 hours. EVB105 reached maximum ethanol production at 60 hours, reaching 0.96% (w/v) ethanol. GS301 produced maximum ethanol of 0.54% (w/v) ethanol at 60 hours. EVB205 produced maximum ethanol (0.88% w/v) at 48 hours. EVB505 produced maximum ethanol (0.65% w/v) at 72 hours. GS301 and the genome shuffled mutants produced a small amount of xylitol during the fermentation of undiluted SSL at pH 5.5 (Figure 3.15). GS301 produced 0.072% (w/v) xylitol. The genome shuffled mutants produced slightly
more xylitol than GS301. EVB105 produced 0.098% (w/v) xylitol, EVB205 produced 0.09% (w/v) xylitol and EVB505 produced 0.089% (w/v).
Figure 3.14: Sugar utilization and ethanol production of SSL-tolerant genome shuffled strains in undiluted SSL at pH 5.5. * One replicate graph representing a triplicate experiment.
Figure 3.15: Xylitol production during the fermentation of undiluted SSL at pH 5.5.

* One replicate graph representing a triplicate experiment.
GS301 performed poorly in the fermentation of undiluted SSL at pH 4.5 (Figure 3.16). It did not utilize all of the glucose within 72 hours of fermentation; only 0.25% (w/v) of the xylose was consumed. GS301 utilized a small amount of xylose (0.3% w/v) and mannose (0.4% w/v). Galactose was not utilized by GS301. The maximum ethanol yield of GS301 was 0.1% (w/v). The mutants did utilize the sugars in the SSL at pH 4.5 however, it was at a slower rate than at pH 5.5. EVB105 had consumed all the glucose within 48 hours of fermentation. EVB205 and EVB505 had consumed all the glucose within 60 hours of fermentation. The mutants utilized xylose, mannose and galactose, but not arabinose. The mutants produced similar maximum ethanol concentrations at 72 hours. EVB105 had a maximum ethanol concentration of 0.57% (w/v), EVB205 had 0.52% (w/v) and EVB505 had 0.5% (w/v). GS301 and the mutants produced a very small amount of xylitol during the fermentation of SSL at pH 4.5 (Figure 3.17). GS301 produced 0.07% (w/v) xylitol. The genome shuffled mutants produced slightly higher amounts. EVB105 produced 0.096% (w/v) xylitol, EVB205 produced 0.1% (w/v) and EVB505 produced 0.097% (w/v).
Figure 3.16: Sugar utilization and ethanol production of SSL-tolerant genome shuffled strains in undiluted SSL at pH 4.5.

* One replicate graph representing a triplicate experiment.
Figure 3.17: Xylitol production during fermentation of undiluted SSL at pH 4.5.

* One replicate graph representing a triplicate experiment.
3.12 Stability of EVB105, EVB205, EVB505:

The mutants were subcultured monthly onto YPD plates. After five subcultures, EVB205 and EVB505 showed some phenotypic instability. EVB205, and EVB505 seemed to have decreased tolerance to SSL compared to when they were initially characterized (appendix, Figure A 3.1). EVB105 did not have any phenotypic instability. After this the mutants were taken from the glycerol stock and spread on YPD plates containing 50% (v/v) SSL. Characterization of the mutants continued using the cells from the YPD plates containing 50% (v/v) SSL.

3.13 Acetic Acid-Tolerant UV Mutants of GS301:

GS301 was subjected to UV mutagenesis and selection on pH unadjusted fixed concentration acetic acid plates to obtain acetic acid-tolerant mutants. Four first generation putative mutants showing increased tolerance to acetic acid were obtained. The mutants were tested for their growth on pH unadjusted, fixed concentration (0.25 and 0.3% w/v) acetic acid plates and the results are shown in Figure A 3.2 and Figure A 3.3, respectively. The mutants were able to grow on the fixed concentration plates, whereas GS301 was not able to grow. The mutants were tested for their ability to grow in chemically defined liquid media containing 0.3% (w/v) acetic acid, pH unadjusted (Figure A 3.4). GS301 survived in 0.3% (w/v) acetic acid, but no increase in cell number was observed. The acetic acid-tolerant mutants had a long lag phase of five days before any increase in cell number was observed. From day five today six the mutants increased in cell number by approximately one log unit.

3.14 Acetic-Acid Tolerant Mutants- Summary:

Three main results with the acetic acid mutants were identified. First, further mutagenesis did not yield mutants with increases in acetic acid tolerance. In fact, further
mutagenesis resulted in mutants that were less tolerant to acetic acid than GS301. The putative second generation mutants would not grow at acetic acid concentrations in which GS301 would grow. Second, first generation mutants being used as the starting strains for the first round of genome shuffling did not yield any viable cells. The mating procedure was carried out as outlined in the materials and methods section. After ten days of incubation the cells were scraped from the malt extract plates and incubated over night in YPD and then spread on acetic acid gradient plates. However, no cells appeared on the gradient plate from the mated first generation acetic acid-tolerant mutants. Cells from the control GS301 which had undergone the same mating protocol as the mutants did have viable cells on the gradient plate. Third, after four to five subcultures on to YPD plates the acetic acid-tolerant mutants reverted to their original state losing any increased acetic acid tolerance they had. The combination of these three results led to the cessation of further work with these mutants.

3.15 Glucose Derepressed Mutants of GS301:

GS301 was spread on solid media containing 4.5% (w/v) 2DG and 2% (w/v) xylose. The plates were inspected daily and spontaneous putative mutants were picked. The putative glucose derepressed mutants were tested for their ability to grow on solid media containing 5 and 6% (w/v) 2DG. GS301 did not grow above 4.5% (w/v) 2DG. Three putative glucose derepressed mutants could grow at 5 and 6% (w/v) 2DG. The mutants grew faster on 2DG solid media than GS301 (Figure A 3.5). The mutants were evaluated for their fermentation performance using initial low cell density inocula in single sugar fermentations of either xylose or glucose and in mixed sugar fermentations containing both xylose and glucose. In the single sugar fermentation of 4% (w/v) xylose mutant 1 consumed xylose at a faster rate than GS301
(Figure A 3.6). Mutant 2 consumed xylose at a similar rate to GS301, and mutant 3 consumed xylose at a slower rate. By 72 hours of fermentation GS301, mutant 1 and 2 consumed all of the xylose. Mutant 3 had consumed all of the xylose by 120 hours of fermentation. GS301 had the greatest maximum ethanol concentration at 0.67% (w/v). Mutant 1 had a maximum ethanol concentration of 0.52% (w/v). In the fermentation of 4% (w/v) glucose GS301, mutant 1 and mutant 2 had a similar rate of glucose consumption with all the glucose being consumed by 48 hours of fermentation (Figure A 3.7). Mutant 3 had consumed all the glucose by 72 hours of fermentation. GS301 had the greatest maximum ethanol concentration of 1.2% (w/v) ethanol. Mutant 1 and mutant 2 each had a maximum ethanol concentration of 1% (w/v). Mutant 3 had a maximum ethanol concentration of 0.74% (w/v). In the mixed sugar fermentation of 4% (w/v) xylose and 4% (w/v) glucose GS301 had consumed all the glucose by 48 hours of fermentation (Figure A 3.8). Mutants 1 and 2 had also consumed all of the glucose by 48 hours and displayed a faster rate of consumption. Mutant 3 consumed glucose at a slower rate and had consumed all of the glucose by 72 hours of fermentation. GS301 and the mutants did not consume all the xylose in 120 hours of fermentation. Mutant 1 and mutant 2 consumed xylose slightly faster than GS301. Mutant 3 had the slowest rate of xylose consumption. GS301 had the highest maximum ethanol concentration at 1.65% (w/v). Mutant 1 had a maximum ethanol concentration of 1.51% (w/v), mutant 2 had 1.48% (w/v) and mutant 3 had 0.97% (w/v). None of the mutants appeared to have less glucose repression as glucose was utilized preferentially over xylose in the mixed sugar fermentation. The mutants also reverted after they were subcultured 5 to 6 times on YPD plates. Work with the mutants was discontinued due to their tendency to revert and lack of glucose derepression.
3.16 Difficulties with Selection for Ethanol Tolerant Mutants:

Ethanol tolerant mutants were unable to be selected due to the difficulties associated with the screening and selection of these mutants. The benzyl alcohol used for selection seemed to evaporate out of the gradient plates, based on the observation of liquid water droplets that appeared on the top of the plastic lids and the smell of the alcohol. Various benzyl alcohol concentrations were tried (0.1 to 1%, w/v), but it was uncertain if and for how long the alcohol would remain in the plates after preparation. After the 24 hour rest period following UV mutagenesis selection in liquid media containing (0.05 to .5%, w/v) benzyl alcohol was tried, but no viable cells were collected from the media. After a few unsuccessful attempts, work with obtaining ethanol tolerant mutants was discontinued due to the difficulty with the selection following mutagenesis.
4.0 Discussion

Ethanol from lignocellulosic biomass is a promising alternative renewable energy source to conventional non-renewable fossil fuel-based energy sources. The recalcitrance of biomass necessitates the use of harsh pretreatment methods to yield fermentable sugars. Harsh pretreatment generates fermentation inhibitors, which are toxic to fermenting yeasts. Increasing the tolerance of the fermenting yeasts to inhibitors is a good alternative to costly and time consuming detoxification methods. Lignocellulosic hydrolysates have a mixture of glucose and pentose sugars. For cost effective ethanol production all the sugars in the hydrolysate should be efficiently utilized and fermented. Traditional S. cerevisiae strains cannot utilize pentose sugars. Native pentose-fermenting yeast, such as S. stipitis can ferment both glucose and xylose into ethanol. However, pentose-fermenting yeast preferentially utilize glucose before xylose. They also suffer from low inhibitor and ethanol tolerance. The objective of this study was to use mutagenesis and genome shuffling to further improve GS301, an SSL-tolerant genome shuffled mutant of S. stipitis. The aim was to isolate a new genome shuffled mutant with improved tolerance to SSL, acetic acid and ethanol, and better co-utilization of glucose and xylose. While this study had difficulty improving the ethanol tolerance and co-utilization of glucose and xylose, it did yield genome shuffled strains with further increased tolerance to the inhibitors in SSL. UV mutagenesis and genome shuffling is an inexpensive method that can be used for yeast strain improvement.
4.1 Mutagenesis and Genome Shuffling for Yeast Strain Improvement:

Mutagenesis is an effective method of strain improvement. Using mutagenesis in combination with genome shuffling can lead to further strain improvement. Protoplast fusion is commonly used for genome shuffling (Jingping et al. 2012; Lu et al. 2012; Shi et al. 2009). Protoplast fusion can help to overcome the problems associated with poor mating ability, poor sporulation and poor spore viability (Wenge and Laustsen 1937), but it does not allow for genetic recombination. Mating allows for genetic recombination, which theoretically combines multiple beneficial mutations into one yeast strain. Iterative cycles of protoplast fusion results in an increase in DNA content in the yeast cells. For instance, the DNA content of *S. cerevisiae* increased when using protoplast fusion for genome shuffling (Shi et al. 2009). The first generation UV mutant of *S. cerevisiae* was 5.089 mg/g, the first generation genome shuffled strain’s DNA content was 5.144 mg/g, the 2nd generations was 6.289 mg/g and the third generations was 7.447 mg/g. The increased DNA content may affect the cell’s stability. Mating-based genome shuffling has been used to improve the tolerance of *S. cerevisiae* to the inhibitors in SSL (Pinel et al. 2011; Wang and Hou 2010). UV mutagenesis and EMS exposure followed by mating was used to generate mutants of *S. cerevisiae* which exhibit shorter fermentation time, improved stress tolerance and improved flavour compound production (Wang and Hou 2010). Similarly, mutagenesis followed by genome shuffling via mating was successful with improving the tolerance of *S. stipitis* to the inhibitors present in SSL (Bajwa et al. 2009; Bajwa et al. 2010). UV mutagenesis was used to create *S. stipitis* mutants with increased SSL tolerance (Bajwa et al. 2009). These SSL-tolerant mutants served as the starting strains for recursive genome shuffling to yield genome shuffled mutants with increased SSL-tolerance.
compared to the starting mutant strains (Bajwa et al. 2010). GS301 was the genome shuffled strain showing the greatest SSL-tolerance.

Among several well-known native pentose-fermenting yeasts, *S. stipitis* exhibits the poorest tolerance to inhibitors in HW SSL (unpublished, our lab). Thus, despite the significant improvement in tolerance seen in GS301, as described in Bajwa et al. (2009, 2010), there was still considerable scope for further improvement. One of the key benefits of using mating-based approaches for strain improvement is that the improved strains obtained are genetically stable, unlike some of those obtained by protoplast fusion. In this study, UV mutagenesis and genome shuffling were used to further improve GS301. The original aim of the study was to select for mutant lines showing tolerance to SSL, acetic acid, ethanol and reduced glucose repression, with the objective of combining these beneficial mutations into one yeast strain. However, only the SSL line proved successful in yielding useful or stable mutants, which were used for genome shuffling to yield four strains with increased SSL-tolerance compared to GS301. The acetic acid mutants exhibited greater acetic acid tolerance than GS301, but the mutants were unstable after several subcultures. The selection of ethanol tolerant mutants was unsuccessful, and the glucose derepressed mutants did not exhibit reduced glucose repression. Thus, the focus of the strain improvement shifted exclusively to the SSL-tolerant mutant line.

Two rounds of UV mutagenesis yielded mutants that were more SSL-tolerant than GS301. A total of five rounds of genome shuffling were done. Three genome shuffled mutants with even greater SSL tolerance than GS301 were obtained. EVB105 is from the first round, EVB205 is from the second round, and EVB505 is from the fifth round of genome shuffling.
EVB105, EVB205 and EVB505 grew in 90% (v/v) SSL, a concentration GS301 would not grow in. The mutants fermented the sugars in undiluted SSL (pH 5.5) to ethanol, with EVB105 producing a 46% greater ethanol yield than GS301. EVB105, EVB205 and EVB505 produced slightly more xylitol during the fermentation of undiluted SSL than GS301 (0.09% vs 0.07%). The reason for increased xylitol production is not known. EVB105, EVB205 and EVB505 also exhibited increased tolerance to HMF and acetic acid compared to GS301.

Undetoxified lignocellulosic hydrolysates typically contain a complex mixture of inhibitors which may act synergistically to exert toxic effects on fermenting yeasts. The major inhibitors, furfural, HMF, acetic acid and phenolic compounds are found in all lignocellulosic hydrolysates, albeit in differing concentrations based upon the type of lignocellulosic biomass and the pretreatment methods and conditions used. As such, mutants tolerant to the inhibitors in one hydrolysate are likely to be tolerant of similar inhibitors in another hydrolysate. It is costly to remove the inhibitors from the hydrolysates through physicochemical methods, and biological methods require too much time. Hence it is cost-effective to select for inhibitor tolerant mutants. EVB105, EVB205 and EVB505 were tested for their tolerance to the inhibitors present in BP hydrolysate. The BP hydrolysate was more toxic than the SSL, and contained 11 g/L acetic acid and 4.3 g/L furfural, whereas the SSL used in this study contained 3.37 g/L acetic acid and 0.039% g/L furfural. While EVB105, EVB205 and EVB505 did not grow to the same extent in BP hydrolysate as they did in SSL they still grew in it, whereas GS301 did not. Thus, the mutants also exhibited cross tolerance to the inhibitors in another hydrolysate, as expected. A previous study evaluated the cross tolerance of GS301 and GS302, SSL-tolerant genome shuffled strains of *S. stipitis*, to the inhibitors in other lignocellulosic hydrolysates (Bajwa et al.)
GS301 and GS302 were evaluated for their growth and fermentation in a mixed hardwood pre-hydrolysate and two different poplar hydrolysates. GS301 and GS302 completely utilized the glucose and xylose in each hydrolysate and produced 0.39 to 1.4% (w/v) ethanol, whereas the WT did not or poorly utilized the sugars and produced no to minimal ethanol.

4.2 Similarity of EVB105, EVB205 and EVB505:

The three genome shuffled mutants all displayed similar growth on SSL gradient plates (Figure 3.5) and in liquid SSL (Figure 3.6). Their growth in media containing acetic acid (Figure 3.7) or HMF (Figure 3.8) was comparable. Fermentation performance in SSL at pH 4.5 and 5.5 (Figure 3.14 and 3.15) was similar for the genome shuffled mutants as well. It is possible that EVB105, EVB205 and EVB505 are the same mutant that was selected through each round of genome shuffling and selection. However, EVB105 was the only mutant that did not show any signs of instability during its testing for growth in liquid SSL. Comparison of the genome sequences of EVB105, EVB205 and EVB505 could be used to determine if they are the same mutant.

4.3 Instability of the Mutants:

Instability and reversion of the mutants was observed in this study. The acetic acid tolerance of the acetic acid mutants was tested over five months. Within that time each mutant was subcultured five times; by the fifth subculture the mutants had lost their increased level of acetic acid tolerance, and were no more tolerant than GS301. Within five subcultures EVB205 and EVB505 had shown some decreased tolerance to liquid SSL. In contrast, EVB105 did not show any decreased tolerance. To continue with the characterization of the genome shuffled
mutants the glycerol stocks of the mutants were spread on YPD plates containing SSL and they were subcultured monthly onto these plates. Kumari and Pramanik (2012) also reported reversion of several multi-stress tolerant mutants of a hybrid yeast strain of *S. cerevisiae* and *P. tannophilus*. The mutants were created using sequential chemical and UV mutagenesis. Three of the eight mutants reverted by the third subculture, and another two mutants had reduced thermotolerance after four months (Kumari and Pramanik 2012). Their best mutant, RPRT90 did not revert and remained stable.

It’s not known why the UV mutants in this study were unstable and reverted after several subcultures. UV radiation induces the formation of covalent linkages between nearby pyridimidines (Ravanat et al. 2001) with cyclobutane pyrimidine dimers (CPDs) being the most common (Taylor et al. 2012) and pyrimidine (6-4) pyrimidone adducts (6-4PPs) being less common (Abdulovic et al. 2006b). The nucleotide excision repair (NER) pathway is the principal pathway for repairing UV-induced pyrimidine dimers (Abdulovic and Jinks-Robertson 2006a). Yeasts have the ability to correct CPDs using photoreactivation (PR), but not 6-4PPs. In PR, DNA photolysase binds to a CPD and uses energy from 365 to 385 nm light to cleave the cyclobutane ring to restore the two neighbouring pyrimidine bases (Sancar 2006). UV mutagenesis of yeast was conducted in the dark and followed by an incubation period in the dark to prevent PR repair of the mutations

**4.4 Glucose Derepression:**

The glucose analog, 2DG, was used to select spontaneous mutants that are glucose derepressed. Three mutants were selected from the 2DG plates and tested for their co-
fermentation of xylose and glucose. No improvement in glucose derepression was observed as the mutants utilized glucose preferentially before xylose during a mixed sugar fermentation. Sreenath and Jeffries (1999) used chemical (EMS) mutagenesis and selection on solid media containing 2DG and xylose to isolate glucose derepressed mutants of *S. stipitis*. The glucose derepressed mutants could grow on solid media containing up to two percent (w/v) 2DG and two percent xylose (w/v) (Sreenath and Jeffries 1999). The mutants isolated from 2DG in this study would grow on solid media containing up to six percent (w/v) 2DG and two percent xylose (w/v). Sreenath and Jeffries (1999) note that one of their mutants, FLP-DX26 completely consumed xylose in the presence of glucose and produced 33 g/L ethanol in about 45 hours from 40 g/L of xylose and 40 g/L of glucose in a chemically defined medium. However, the simultaneous consumption of glucose and xylose was not clearly demonstrated in their graph showing the fermentation of 80 g/L of xylose and glucose. The samples for this fermentation experiment were taken every 20 hours from time 0 to 120 hours of fermentation. From 0 to 20 hours of fermentation the glucose concentration dropped from 40 g/L to 10 g/L, but no xylose was consumed during this time. By the next time point at 40 hours all the glucose was consumed as well as all of the xylose. It is not clear whether FLP-DX26 consumed the rest of the glucose and then the rest of the xylose after. More frequent sampling time points would have given better evidence of simultaneous consumption of glucose and xylose. It may be possible FLP-DX26 can utilize xylose quickly rather than showing true glucose derepression.
4.5 Acetic Acid Mutant Line:

When the acetic acid mutant line was being characterized the pH was not standardized to a consistent pH. Thus, it was not known how much of the acetic acid was in the more toxic protonated form. Using a standardized pH would have ensured a more uniform concentration of the protonated form of acetic acid during mutant assessment and allowed for more consistency throughout the thesis as the pH was adjusted to 4.5 when the genome shuffled mutants were characterized for their acetic acid tolerance. In the future using a standardized pH may help with selecting stable acetic acid mutants as there will be a consistent selective media.

4.6 Stress Tolerance in Yeast:

Stress tolerance in yeast is regulated by complex gene interactions. Very little research has focused on improving the stress tolerance of native pentose-fermenting yeast. Recent research has focused on improving *S. cerevisiae*’s multi-stress tolerance to a variety of stressors for the production of ethanol from lignocellulosic biomass (Kumari and Pramanik 2012; Zheng et al. 2011b). Improving the tolerance of *S. cerevisiae* to one type of stress has been shown to confer tolerance to another type of stress (Causten et al. 2001). The multiple stress tolerance of *S. cerevisiae* was improved by creating a hybrid strain by crossing a heterothallic strain with high temperature tolerance with a homothallic strain with high ethanol productivity (Bengaphokee et al. 2012). The resultant hybrid strain, TJ14 had improved ethanol productivity under high temperature conditions. The acid tolerance of TJ14 was also improved compared to the parental strains. In a fermentation of a chemically defined medium containing 10% (w/v)
glucose at high temperature (41 °C) and low pH (3.5), TJ14 produced 46 g/L ethanol after 24 hours. The parental strains TISTR5606 and 3ADip produced about 7 g/L and 13 g/L ethanol, respectively.

Similarly, the thermotolerance and tolerance to acetic acid, and furfural of *S. cerevisiae* was improved using chemical mutagenesis and genome shuffling (Lu et al. 2012). UV mutagenesis followed by three rounds of genome shuffling and selection yielded a genome shuffled strain of *S. cerevisiae* designated R32. R32 showed much improved thermotolerance and acetic acid tolerance compared to the WT strain CE25. In a chemically defined medium containing 200 g/L glucose and 0.5% (v/v) acetic acid with a temperature of 40°C, R32 utilized 194 g/L of glucose to produce 84.2 g/L of ethanol, whereas CE25 produced 10.8 g/L of ethanol. Zheng et al. (2011b) used genome shuffling to obtain *S. cerevisiae* strains with improved tolerance to osmotic, heat and acid stresses and better ethanol production than their parent strains. YZ1 was their best genome shuffled strain. YZ1 along with its parental strains, Z8 and Z15, were tested in an industrial mash fermentation medium under high heat (224.5 g/L glucose at 42°C) or high gravity (299.5 g/L glucose at 34°C) (Zheng et al. 2011b). The source of the mash and composition was not given (Zheng et al. 2011b). At higher temperatures, YZ1 was more heat tolerant than Z8 and Z15, and it had an ethanol yield of 11% (v/v), which was 10.31% higher than that of Z8. Under high gravity conditions, YZ1 had improved ethanol yields over Z15 by 10.55%. Zheng et al. (2011b) noted that the genome shuffled strain YZ1 has been used to produce over 1 million tons of ethanol for Henan Tianguan Fuel Co. Ltd., China.
4.7 Future Perspectives:

This study successfully used mutagenesis and genome shuffling to further improve the SSL tolerance of *S. stipitis*. Although there are several studies describing the improvement of *S. cerevisiae* strains using genome shuffling, very little research has focused on improving native pentose-fermenting yeasts. This study has shown that *S. stipitis* is amenable to further improvement using genome shuffling. While the inhibitor tolerance of *S. stipitis* has been improved it is not yet an industrially viable strain, as further improvement in ethanol tolerance and glucose derepression is needed. Future work could be directed at improving the multi-stress tolerance of EVB105, EVB205 or EVB505. Some yeasts exposed to a mild stress have been shown to confer cross tolerance to another stress. For example, *S. cerevisiae* cells exposed to mild heat shock were reported to be resistant to subsequent stresses from a lethal dose of H₂O₂, ethanol, NaCl and heat (Berry et al. 2008). There is evidence that heat stress and ethanol stress induce the same stress response because both stresses induce changes in the cell membranes (Piper et al. 1993). One study revealed there is some overlap between genes required for ethanol tolerance and those required for thermotolerance (Auesukaree et al. 2009). Improving the tolerance of yeasts to stresses such as high heat, osmotic pressure or acid for example, may confer multi-stress tolerance to the inhibitors in lignocellulosic hydrolysates. Recent research focusing on improving multi-stress tolerance in *S. cerevisiae*, as outlined above, has led to mutants showing improved ethanol production in biomass hydrolysates (Zheng et al. 2011).

Whole genome sequencing of WT, GS301 and EVB105 could be done to compare the genetic sequences to determine where in the genome the mutations have occurred.
Microarray analysis can be used to detect changes in gene expression when the yeasts are exposed to different inhibitors. The information from the whole genome sequencing and changes in gene expression when exposed to different inhibitors could be used to identify which mutations collectively confer increased tolerance to inhibitors.

Once it is known where in the genome the mutations are occurring, gene disruption or deletion can be used to determine which mutations are contributing to the increased inhibitor tolerance in the genome shuffled strains. For example, Auesukaree et al. (2009) used genome screening of deletion mutants of *S. cerevisiae* to identify the genes involved in the tolerance to alcohol, heat, oxidative stress, and osmotic stress. Once the mutations that play a role in increased inhibitor tolerance have been identified, further gene manipulation can be done by random or site-directed mutagenesis and the functional effect of the mutations assessed in the yeasts in vivo after gene replacement.


Casey, E., Sedlak, M., Ho, N.W.Y., Mosier, N.S. 2010. Effect of acetic acid and pH on the cofermentation of glucose and xylose to ethanol by a genetically engineered strain of Saccharomyces cerevisiae. FEMS Yeast Res. 10 (4): 385-393.


Figure A 3.1 Growth of EVB105, EVB205, EVB505 and GS301 in 90% (v/v) SSL, pH 4.5.

* One replicate graph representing a triplicate experiment.
Figure A 3.2: Growth of GS301 and an acetic acid-tolerant mutant on pH unadjusted solid media containing 0.25% (w/v) acetic acid and 2% (w/v) xylose. Picture taken after a 24 hour incubation period. * One replicate graph representing a triplicate experiment.
**Figure A 3.3:** Growth of GS301 and an acetic acid-tolerant mutant on pH unadjusted solid media containing 0.3% (w/v) acetic acid and 2% (w/v) xylose. Picture taken after a 24 hour incubation period. * One replicate graph representing a triplicate experiment.
Figure A 3.4: Growth of acetic acid-tolerant mutants in pH unadjusted chemically defined media containing 0.3% (w/v) acetic acid.

* One replicate graph representing a triplicate experiment.
**Figure A 3.5:** Two days of growth of GS301 and a mutant on solid media containing 3% (w/v) 2DG and 2% (w/v) xylose. * One replicate graph representing a triplicate experiment.
Figure A 3.6: Fermentation of 4% (w/v) xylose by GS301, Mutant 1, 2 and 3.

One replicate graph representing a triplicate experiment.
Figure A 3.7: Fermentation of 4% (w/v) glucose by GS301 and Mutant 1, 2 and 3.

* One replicate graph representing a triplicate experiment.
Figure A 3.8: Sugar utilization and ethanol production of GS301 and three mutants in defined media containing 4% (w/v) xylose and 4% (w/v) glucose. Panels: (A) glucose utilization (B) xylose utilization (C) ethanol production. Symbols: * One replicate graph representing a triplicate experiment.