# DEVELOPMENT AND CHARACTERIZATION OF A ROBUST INDUSTRIAL YEAST FOR HIGH SOLIDS SOFTWOOD FERMENTATION USING ADAPTATION, DIRECTED EVOLUTION, AND TRANSCRIPTOME PROFILING

by

GARY MATTHEW HAWKINS

(Under the Direction of Joy Doran Peterson)

ABSTRACT

Currently almost all the fuel ethanol produced in the United States is generated from processes using corn as the feedstock. To increase production of ethanol, other feedstocks must be developed as viable substrates for fermentation processes. Efficient ethanol fermentation from softwood biomass, which is produced in large quantities globally, at titers suitable for an industrial process is challenging for a variety of reasons. One challenge is the generation of inhibitory compounds from the biomass as it is pretreated prior to enzymatic digestion. These compounds are then present in the fermentation medium where they inhibit the activity of the fermenting organism. To overcome this bottleneck in softwood fermentation, we have developed a *Saccharomyces cerevisiae* yeast strain that is capable of producing ethanol from greater than 17.5% dry weight per volume of pretreated softwood at ethanol yields over 90% of the theoretical maximum. This strain was developed from an industrial corn ethanol yeast by adaptation and directed evolution in increasing concentrations of pretreated softwood. Isolates from the directed evolution experiments were screened for the inhibitor resistant phenotype and for the ability to ferment pretreated softwood at high solids concentrations. Use of a model inhibitor medium for inoculum preparation enhanced fermentation performance with high solids

concentrations of pretreated pine. When the inoculum was prepared for fermentation without the inhibitors present in the medium, divergent phenotypes were observed. One set of isolates retained the ability to ferment high solids of pretreated pine regardless of the method used to prepare the inoculum, whereas another set of isolates displayed reduced performance when the inhibitors were removed during inoculum preparation. Transcriptome profiling was used to characterize the gene expression patterns of a robust strain capable of fermentation of high softwood solids after preparation in either media to a strain showing the divergent phenotype. Many expression differences were observed; including multiple changes not previously known to be related to inhibitor tolerance or high solids fermentation. Potential mechanisms by which the observed expression differences aid in the robust strains' performance in high softwood solids fermentations are presented.

INDEX WORDS: Bioethanol, lignocellulose, *Saccharomyces,* transcriptomics, inhibitors

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# DEDICATION

To my wife Kim. Our relationship will forever be the greatest experiment.

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# CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 OVERVIEW

In recent decades there has been growing interest in the production of liquid transportation fuel from renewable resources, such as lignocellulosic biomass. Lignocellulosic biomass is a broad term encompassing a wide variety of agricultural and forestry products including corn stover, sugarcane bagasse, woody biomass, and grass crops. Softwood biomass is one such forestry product that is both plentiful and able to be cultivated in many different locations world-wide. Use of this biomass for fuel ethanol production presents a number of challenges in the fermentation process. First is the development of a pretreatment method that will successfully enhance the enzymatic digestion of the biomass without demanding large amounts of external energy or expensive catalysts while simultaneously avoiding the creation of too many toxic inhibitory compounds. These compounds are a second challenge in that they inhibit the activity of the biocatalytic organism. This inhibition consequently reduces fermentation yields, especially at high solids loadings. High solids loadings, which lead to high theoretical maximum ethanol yields, are necessary to ensure the economic success of the industry. This is due to the fact that certain minimal ethanol titers are required in order for cost effective distillation. A biocatalyst that can efficiently produce ethanol in the presence of these inhibitory compounds is vital to the success of a softwood fermentation industry. *Saccharomyces cerevisiae* makes an excellent bio-catalyst for softwoods for two reasons. One, it already possesses a relatively high level of resistance to these compounds when compared to other

ethanol producing organisms. Two, these biomass types lack significant amounts of xylose and arabinose, pentose sugars which *S. cerevisiae* cannot use for ethanol production without significant genetic modification. This dissertation begins with a literature review explaining how biofuel can be produced from softwood biomass and why it is desirable to do so. Then three manuscript chapters detail how a strain of *Saccharomyces cerevisiae* was developed to improve this fermentation process as well as phenotypic and genetic details of this unique strain. This is followed by overall conclusions from the dissertation and references.

## 1.2 PURPOSE

One solution to offset oil imports and reduce carbon emissions is to use ethanol generated from biological sources as a liquid transportation fuel or fuel additive. Currently, the most widely used feedstocks are corn in the United States and sugarcane in Brazil [1]. These have the drawback of being difficult to grow in many regions, which prevent these sources from being used for fuel globally. The use of lignocellulosic feedstocks represents a potential alternative to these crops. Compared to corn and sugarcane, lignocellulose has the disadvantage of being more difficult to ferment to ethanol. Prior to fermentation the feedstock must be pretreated to make the biomass amenable to enzymatic digestion, which will release monomeric sugars for conversion to ethanol by the biocatalytic strain. There are many potential organisms that could be employed in this process, but *Saccharomyces cerevisiae* has some advantages over other organisms which will be discussed later in this chapter. Despite their recalcitrance, lignocellulosic sources have advantages over corn and sugarcane: they can be produced cheaply [2], they can be waste streams of current industrial processes for which there is no current economic value [3], a number of them are not edible and their use will have little impact on food prices, some can be

cultivated on marginal land, and they can be produced in a wide variety of locations and climates [4]. There are a number of agricultural and forestry products that are currently being explored for potential use as feedstocks in bioethanol production; one of which is softwood biomass such as loblolly pine. For softwood fermentations to become economically feasible, strains must be developed that are capable of fermenting high concentrations of pretreated biomass. This chapter will provide relevant background information on the fermentation of woody biomass as well as the study and development of *Saccharomyces* strains that display improved performance in the fermentation of this material.

## 1.3 SOFTWOOD BIOMASS

Lignocellulosic biomass represents the most abundant renewable resource on the planet, with an estimated annual production of 10-50 x $10^{12}$ kg worldwide [5]. Softwoods represent a significant fraction of this, and data regarding the annual production in selected countries is summarized in Figure 1.1 [6-9]. Softwoods are not necessarily "softer" than their counterparts the hardwoods but rather describe gymnosperms, which reproduce by seeds that lack coverings. They are a major source of lumber for much of the world and include varieties such as pine, spruce, and fir. Softwoods, like all lignocellulosic biomass, are comprised of three main structural components: cellulose, a polymer of glucose, hemicellulose, a mixed-sugar polymer, and lignin, a complex arrangement of aromatic subunits that cannot be fermented to ethanol [10-12]. Generally, hemicellulose contains a mixture of six-carbon sugars (or hexoses) including mannose, glucose, galactose, and rhamnose and five-carbon sugars (pentoses) such as xylose and arabinose. Neither pentose sugar can be used by genetically unmodified *S. cerevisiae* strain for ethanol production without genetic modification. Softwoods are unique in that their

hemicellulose contains a relatively high amount of mannose, a six carbon sugar which can be fermented to ethanol by *S. cerevisiae*, and little xylose and arabinose. The hemicellulose structure of softwoods alleviates the need for a pentose fermenting biocatalyst, an economic necessity in processes seeking to use other lignocellulosic biomass types [13].

The lignin, cellulose, and hemicellulose that comprise plant material exist as a complex interwoven matrix [14, 15]. The cellulolytic enzymes which release sugar monomers from the biomass for use by *S. cerevisiae* have low activity on untreated plant material; creating the need for pretreatment to open the biomass to enzymatic digestion. The enzymes, generally a mixture of cellulases and cellobiase (or β-glucosidase), break down the cellulose to glucose monomers which can then be fermented into ethanol [12]. A number of processes have been developed for the treatment of softwood with the goal of making the biomass easily enzymatically digested while minimizing the production of inhibitory chemicals which hinder the activity of the biocatalytic strain. These chemicals include both sugar degradation products generated from cellulose and hemicellulose and aromatic compounds derived from lignin [16]. These compounds act in a variety of ways to inhibit the activity of *S. cerevisiae* and reduce ethanol yields, and present a significant challenge to softwood fermentation; the production, mechanisms, and engineering of strains to tolerate these compounds are discussed in later sections.

## 1.4 PRETREATMENTS & FERMENTATION PERFORMANCE

A number of pretreatment methods have been developed to improve the fermentation of lignocellulosic biomass [11, 17-19]. Steam explosion, often with an acid catalyst has been widely used to pretreat softwoods prior to fermentation (Table 1.1), but other pretreatment options have

also been explored (Table 1.2). Acid hydrolysis is a chemical pretreatment that has been used to pretreat woody biomass as early as 1918. This process involves treating the wood with a dilute acid at high temperature, and has been shown to be effective for separation of cellulose from lignin [20]. However, the combination of caustic acid and high temperatures can cause corrosion of the pretreatment apparatus which increases process costs. Numerous inhibitory compounds are also produced during pretreatment which lower the ethanol yields.

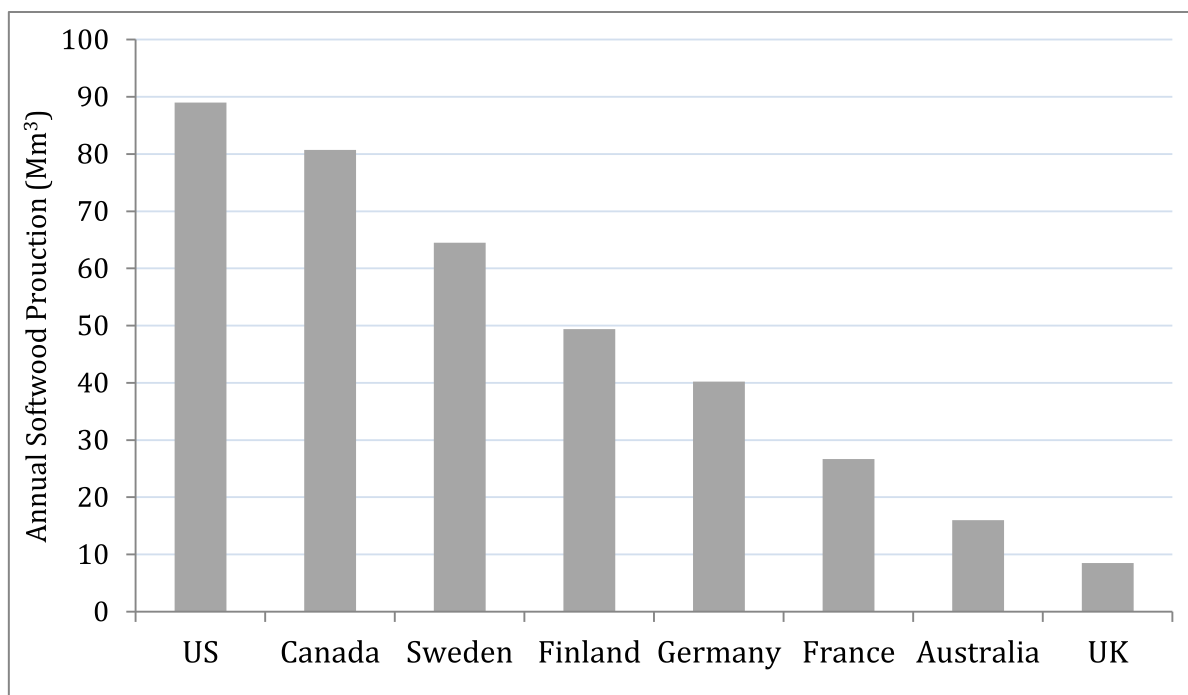



**Figure 1.1.** Approximate annual softwood production (in million cubic meters) in selected countries.

Steam explosion has been explored extensively for the pretreatment of softwoods. This process involves a physical pretreatment by exposing wood to steam at high temperatures. This method is often combined with a chemical pretreatment in the form of an acid catalyst (usually $SO_2$ or $H_2SO_4$). The trees are harvested and chipped prior to pretreatment, and the size of the

wood chips is an important consideration as smaller chips give slightly higher sugar yields, though this must be weighed against the increased energy cost of additional chipping [21]. The chips are then impregnated with the acid catalyst – longer impregnation times have been shown to lead to more complete hemicellulose hydrolysis [21]. The wood is then placed in the pretreatment reactor and treated at the desired temperature and duration. This treatment results in the dissolution of the hemicellulose and removal of lignin, allowing for the cellulose to be partially hydrolyzed. After treatment, the cellulose is open to enzymatic digestion and can be more readily converted to glucose. Both sulfur dioxide and sulfuric acid have been shown to be effective catalysts for steam explosion. Sulfur dioxide has an advantage over sulfuric acid in that it will not corrode the pretreatment apparatus but represents a greater health risk to those performing the pretreatment [11].

The number of steps taken to pretreat the wood is another important consideration in the steam explosion process. In one step pretreatments, the wood is treated once at a single temperature prior to enzymatic digestion and fermentation. Two step pretreatments begin at milder conditions to hydrolyze the hemicellulose. The wood is then washed to remove the solubilized hemicellulose sugars before being treated at harsher conditions for cellulose hydrolysis. The advantage of a two-step pretreatment is in the removal of sugars prior to the second treatment; this reduces the amount of inhibitory sugar degradation products produced from the biomass and results in a more readily fermented product [22]. Two-step pretreatments have the disadvantage of being more costly due to increased usage of process water during the washing of the biomass as well as increased capital and energy costs.

When spruce was used as the feedstock and pretreated with sulfur dioxide in one step, ethanol yields of up to 82% of the theoretical maximum were obtained [23]. One study

compared two-step pretreatments with either $H_2SO_4$ [24] or $SO_2$ [25] as well as a separate hydrolysis and fermentation (SHF) procedure with simultaneous saccharification and fermentation (SSF). In SHF, the wood is incubated with the cellulolytic enzymes for a period of time (generally between 8 and 24 h) prior to the addition of *Saccharomyces*. This allows for the release of some glucose from the biomass prior to yeast addition, and may be performed at a temperature and pH that improves enzyme performance but would be detrimental to the yeast. SSF processes add the enzymes and yeast simultaneously so fermentation occurs as soon as sugar is released from the biomass. It was found that $SO_2$ was a better catalyst for ethanol production from spruce and that SSF was preferable to SHF with regards to ethanol yields [26]. To reduce the energy demand created by using a two-step process, a pretreatment was developed that simulates a two-step dilute acid pretreatment over one single step [27]. This process was found to be more effective with respect to glucose yield than single temperature hydrolysis but not quite as effective as true two step acid hydrolysis.

When Douglas fir was pretreated by $SO_2$ steam explosion under conditions of varying severity, it was found that the wood treated at low severity (7.5 mins, 175°C, 4.5% $SO_2$) had the highest ethanol yields. Despite having increased concentrations of monomeric sugars in the fermentation the higher severity treatments resulted in lower ethanol yields; likely due to the increased formation of inhibitory compounds by the harsh pretreatment conditions [28]. Another study found that that lower temperature pretreatments resulted in more readily fermented hydrolysates for both pine and spruce [29]. Sulfur dioxide steam exploded loblolly pine (*pinus taeda*) was fermented using an industrial *S. cerevisiae* strain, XR122N. Ethanol yields of 98% and 76% of the theoretical maximum were obtained from pine loadings of 10% and 12% dry weight respectively [30].

Typically, bark is removed from the tree before it is chipped and pretreated. This adds to the cost of harvesting and using softwoods as a feedstock. One study found that adding bark back into the pretreatment vessel (simulating incomplete debarking), had negligible effects on the fermentation yields, even at 30% bark concentration [31]. The water soluble fraction, which can be separated from the wood when the pretreated material is washed, of $SO_2$ steam explosion pretreated Douglas fir contains sugars which were found to be readily fermented. Complete hexose consumption occurred in as little as 18 hours [32]. Softwood derived spent sulfite liquor, a byproduct of paper manufacturing using the sulfite pulping process, was fermented even more rapidly, with complete hexose consumption in 10 hours [33]. Lodgepole pine was steam pretreated and fermented to ethanol, with yields of up to 77% [34].

A problem in some forests is the infestation of trees by mountain pine beetles (*Dendroctonus ponderosae*) which can lead to the loss of large regions of forest land. These pests cause damage to the trees which makes the wood unsuitable for many industrial applications. Use of trees affected by beetle infestation in fermentation resulted in increased ethanol yields compared to healthy trees; this could provide a way to use trees in an industrial process that would otherwise be unfit for use in industry and simply be burned for disposal [34]. This increase in yield over healthy trees was attributed to increased $SO_2$ uptake and increased physical degradation of the wood fibers due to beetle activity.

Certain pretreatment methods enhance the enzymatic digestion of cellulose by treating the biomass with solvents and are broadly referred to as organosolv pretreatments. The solvent in these methods is variable; examples include *N*-methylmorpholine-*N*-oxide (NMMO) or a mixture of ethanol or acetone and water at low pH. The goal of pretreatment is the same as in steam explosion: to make the cellulose more accessible to enzymatic degradation by removal of

lignin and hemicellulose. Some of these processes allow for high-quality recovery of the lignin fraction, which can then be used in other industrial processes such as manufacturing automotive brakes, epoxy resins, and biodispersants [35]. It is also possible to recover other chemical products, such as hemicellulose and furfural from the water soluble stream produced during pretreatment. Production of co-products such as these can dramatically increase the overall value of the industrial process.

When a mixture of softwoods was delignified with 1:1 mixture of ethanol and water, the resulting pulp was found to be highly fermentable after washing. Pulp equivalent to 2g of cellulose was fermented at yields of 90% of the theoretical maximum in as little as 8 hours [33]. Treatment of wood with both brown and white rot fungi has been shown to increase the effectiveness of subsequent pretreatment while at the same time lowering the required severity of the pretreatment. Brown rot fungus (*Coniophora puteana*) treatment has been shown to partially depolymerize the cellulose, making the substrate more easily digested by cellulolytic enzymes [36-38]. When Monterey pine was used as the feedstock and treated with brown rot fungus followed by organosolv, ethanol yields of 95% were observed at 5% dry weight. Another study used a 1:1 mixture of acetone and water organosolv pretreatment with Monterey pine as the feedstock. Ethanol yields were observed at greater than 95% for a number of pretreatment conditions when fermentations were performed at 10% dry weight [39]. Another study used 60% ethanol and water organosolv after biopretreatment with white rot fungus (*Coniophora subvermispora)* for Monterrey pine. When the wood was fermented by SSF at 2% weight per volume, a maximum ethanol yield of 65% was obtained [40].

*N*-methylmorpholine-*N*-oxide (NMMO) is a solvent that has been used for softwood pretreatment prior to fermentation. NMMO is currently used in an industrial process known as

Lyocell, which is used to generate cellulose fibers that can be used in manufacturing clothing and other products [41]. Lyocell has the advantages of generating no toxic waste products and allowing for the catalyst to be recovered at 98% efficiency [42]. These two advantages are useful in lowering production costs. This process involves "melting" the biomass in NMMO, which causes the cellulose to swell and dissolve, and then precipitating the cellulose with water [43]. Spruce was pretreated with 85% NMMO at 130°C for 3 hours, and then 5% dry weight of wood was separately enzymatically hydrolyzed and fermented; resulting in ethanol yields of 89% of the theoretical maximum [44].

Ozone has been used as a pretreatment for Japanese cedar [45]. This pretreatment exposes the biomass to 2.9-3.5% $O_3$ with the goal of removing lignin and enhancing enzymatic hydrolysis. Ozone will react specifically with carbon-carbon double bounds, like those found in lignin, to produce a more readily enzymatically digested product [46, 47]. The effects of ozonation are limited to the lignin structure itself; as the hemicellulose is only slightly affected and the cellulose is largely untouched. The large quantities of ozone required could be prohibitively expensive to industrial application of this technique [17]. One study explored the fermentability of ozone pretreated Japanese cedar sawdust. Higher concentrations of ozone produced wood that fermented poorly when compared to wood treated with lower concentrations. The yields in this study were low (<20%); the authors attributed this to the formation of toxic compounds during the pretreatment process, though these compounds were not quantified. It is known that ozone treatment of biomass can yield organic acids and other potentially inhibitory compounds, and the substrate was unwashed, so these compounds would be present in the fermentation medium.

**Table 1.1.** Summary of fermentation data using steam explosion pretreatments

| Year and Reference | Fermenting Strain | Wood Type | Pretreatment | Solids[a] | Max EtOH (g/l) | % Theo. Max |
|---|---|---|---|---|---|---|
| **2000** – Stenberg et al. | Baker's Yeast | Spruce | One step $SO_2$ | 5% dwt/v | 19 | 82 |
| **2002** – Söderström et al. | Compressed Baker's Yeast | Spruce | Two step $SO_2$ | 5% dwt/v | 16.5[c] | 69 |
| **2003** – Söderström et al. | Compressed Baker's Yeast | Spruce | Two step $H_2SO_4$ | 5% dwt/v | Not given | 65 |
| **2003** – Alkasrawi et al. | Baker's Yeast | Spruce | One step $SO_2$ | 5% dwt/v | 23[c] | 92 |
| **2003** – Zacchi et al. | Compressed Baker's Yeast | Spruce | Two step $H_2SO_4$ | 5% dwt/v | 15.2[b] | 65 |
| **2004** – Keating et al. | Y-1528 | Douglas Fir | One step $SO_2$ | 40 ml water soluble fraction | 14.7[b] | 92 |
| **2004** – Zacchi et al. | Compressed Baker's Yeast | Spruce | Two step $SO_2$ | 5% WIS | 30 | 85 |
| **2005** – Söderström et al. | Compressed Baker's Yeast | Spruce | Two Step $SO_2$ | 5% dwt/v | 12.4[b] | 81 |
| **2006** – Zacchi et al. | Baker's Yeast | Spruce | One step $SO_2$ | 8% WIS | 21.65[b] | 92 |
| **2007** – Ewanick et al. | Tembec T1 | Lodgepole Pine | One step $SO_2$ | 5% WIS | 22[c] | 77 |
| **2009** – Hoyer et al. | Baker's Yeast | Spruce | One step $SO_2$ | 10% WIS | 43[cd] | 94.7 |
| **2010** – Zhu et al. | Compressed Baker's Yeast | Spruce | One step $SO_2$ | 8% WIS | 16[b] | 89.4 |

[a]dwt/v=dry weight per volume, WIS=water insoluble solids
[b]calculated using data from the article, an ethanol density of 0.789g/ml, and 0.51 mol of ethanol per mol hexose
[c]inferred from graphical data presented in the article
[d]additional sugar present at 0 h fermentation enabled higher ethanol titers than would have been possible from pretreated wood alone

**Table 1.2.** Summary of fermentation data using other pretreatments

| Reference and Year | Fermenting Strain | Wood Type | Pretreatment | Solids | Inoculum | Max EtOH (g/l) | % Theo. Max |
|---|---|---|---|---|---|---|---|
| **2005** – Pan et. Al | Tembec T1 | Mixed Softwoods | Organosolv (60:40 EtOH:$H_2O$) | Equivalent of 2g cellulose | 5 g/l | 8 | 90 |
| **2006** – Yang et al. | *S. cerevisiae* 2.535 and recombinant *E. coli* | Mixed Softwoods | 2% HCL and 0.5% $FeCl_2$ | Detoxified and supplemented hydrolysate | 3.7 g/l *S. cerevisiae* 1.9 g/l *E. coli* | 17.1 | 88.5 |
| **2008** – Araque et al. | IR2T9 (thermal acclimated) | Pine | Organosolv (50:50 acetone:$H_2O$ w/ $H_2SO_4$ | 10% dwt/v | 2.4 g/l | 36.5 | 99.4 |
| **2009** – Sugimoto et al. | Baker's Yeast | Japanese Cedar | Ozone exposure | 2 kg | 1 g/l | 7.2 | 20 |
| **2010** – Karimi et al. | CCUG 53310 | Spruce | NMMO | 5% w/v | 10 g/l | 12[c] | 89 |
| **2010** – Fissore et al. | IR2-9a | Monterey Pine | Brown Rot Fungus and Organosolv (60:40 EtOH:$H_2O$) | 5% dwt/v | 3 g/l | 19[c] | 95 |

[a]SSF=simultaneous saccharification and fermentation, SHF=separate hydrolysis and fermentation, FPU=filter paper unit, IU=international unit, CBU= cellobiase unit
[b]calculated using data from the article, an ethanol density of 0.789g/ml, and 0.51 mol of ethanol per mol hexose
[c]inferred from graphical data presented in the article

Sulfite Pretreatment to Overcome Recalcitrance of Lignocellulsoe (SPORL) has also been explored as a pretreatment for softwoods. This process involves treating the wood with a mixture of bisulfite and sulfuric acid at high temperature. SPORL is based on sulfite pulping technology which has been used by the paper industry for over 100 years. During this process, the hemicellulose is degraded and removed and the lignin is partially removed and sulfonated, which exposes cellulose fibers allowing for enzymatic digestion [48]. This treatment generates low amounts of furan inhibitors, and can potentially have very low energy inputs [49]. It also has the advantage of being able to use, build on, or adapt existing technologies, which lowers capital costs [48]. A study on the economics and energy production aspects of biomass fermentation found SPORL to be among the most efficient processes [50].

At 8% water insoluble solids, yields of 89% of the theoretical maximum were obtained from SPORL pretreated Lodgepole pine [51]. The authors of this study also performed analysis of the energy yield of the process; which is defined as the net energy gained from the process divided by the energy input in the process. Energy yields of over 175% were obtained, with a maximum of 237%; it should be noted that this only included the energy in the ethanol as the output and the energy consumed for pretreatment as the input. Another study used SPORL pretreatment on trees that had been killed by mountain pine beetles, a problematic pest in some forest areas [52, 53]. Yields were found to reach 65.6% at solids loading of 10%. It was calculated that 200-250 liters of ethanol could be generated per metric ton beetle infested pine wood [54]. Another study examined the fermentation efficiency of beetle killed Lodgepole pine pretreated with SPORL. This study found that 267 liters of ethanol (69% theoretical yield) could be generated from one metric ton of wood that had been infected with beetles for four years, this was 7% more ethanol (250 liters per metric ton) than was obtained from healthy trees [52].

## 1.5 HIGH SOLIDS FERMENTATION

More recent work has focused on using engineered strains of *S. cerevisiae* or modified pretreatment methods to produce ethanol from very high dry weights of pine. Increasing the solids loading in the fermentation process will increase the amount of fermentable sugar, thus leading to higher potential ethanol yields. If these yields are realized they will reduce process costs and make the production of ethanol from softwood biomass economically feasible; primarily by increasing the efficiency of distilling the final ethanol product. When 25% total solids (13.7% water insoluble solids) of stream pretreated spruce was fermented ethanol using an SSF process yields of only 5-6% of the theoretical maximum were observed. Changing from an SSF process to a fermentation process with 48 h presaccharification increased the yields to 72% [55]. At 20% dry solids loading, eastern red cedar was fermented to produce 52 g/L ethanol, 90% of the theoretical maximum, after enzymatic digestion [56]. At 20% solids loading of SPORL treated lodgepole pine, 47 g/L ethanol was produced using a modified SSF fed-batch process [57]. These fermentations show that a variety of softwoods can be pretreated and successfully fermented to high ethanol yields. This is crucial for the success of a bioethanol production process, as concentrations of at least 40 g/L ethanol must be realized in order to have cost effective distillation of the ethanol from the fermentation medium [58].

## 1.6 ENVIRONMENTAL IMPACTS & ECONOMIC POTENTIAL

One of the major reasons for moving from a fossil fuel based economy to one that is based on renewable, biological resources is a net reduction of greenhouse gas emissions. As liquid fuels are combusted, greenhouses gases such as carbon dioxide are released; when fuel derived from biomass such as pine are used these emissions are sequestered by the next

generation of feedstock as it is cultivated. It can be difficult to ascertain the reduction in greenhouse gas emissions when biofuels from lignocellulosic sources are combusted. Calculating this reduction requires life cycle analysis; viewing the entire process from start to finish and determining the net carbon balance. This is a daunting prospect considering the complex and variable emissions associated with crop cultivation, land use change, biomass harvest, transport and pretreatment, ethanol production, and product distribution [59]. The energy cost of pretreating the material prior to fermentation contributes not only to the economics of ethanol production but to the environmental impacts as well, as some pretreatments use more energy (which is often fossil-fuel derived) or may produce environmentally unfriendly byproducts [49].

A 2003 study found the greenhouse gas advantages of bioethanol were more dependent on process energy sources than on biomass cultivation methods; this study analyzed a number of biomass sources, including hay and waste wood [60]. Another study on wheat straw found the environmental impacts of bioethanol production were variable and based on factors including: land use change, production of co-products, and how the ethanol was blended and distributed [61]. Another study on sugar beet generated fuel ethanol found that the fuel production alone had only marginal environmental benefits over fossil fuels, but these benefits could be increased greatly by co-product generation, such as animal feed, electricity, or chemical compounds [62]. A recent Australian study found that there were great environmental benefits when either farmed softwoods or hardwoods were used for ethanol production [63]. To maximize the reduction in greenhouse gas emissions by using lignocellulosic fuels instead of fossil fuels, it is advantageous to use a process with as few energy inputs as possible to reduce the amount of $CO_2$ emitted when producing and distributing the fuel. One study found SPORL was the most efficient process due

to high sugar production from enzymatic hydrolysis, whereas steam explosion had one of the highest energy consumptions [52].

The economics of producing ethanol from softwood must favor production for the industry to flourish. One 2009 study sought to analyze the process of ethanol production from biomass supply to fuel distribution in Europe. The authors found the two most significant factors influencing cost of production were the feedstock cost and the value of the ethanol produced. The feedstock cost is governed primarily by the location of the theoretical ethanol plant as well as the current competing industrial uses for the feedstock; whereas the value of ethanol is determined by the price of oil and governmental policies, which are difficult to predict on long time scales [64]. As previously mentioned, increasing the dry matter fermented is another way to have a positive effect on the process economy, as this results in higher theoretical ethanol yields which if realized would require less energy consumption during distillation [2, 65].

One way to increase the economic gains from ethanol production is to use a low value feedstock or the waste stream from an existing industrial process, both of which could be obtained relatively cheaply. The previously mentioned fermentations using beetle-killed trees represent one way to implement a very low value feedstock into ethanol production processes. There is currently no use for these trees in either the lumber or paper industries, and biomass could potentially be purchased at lower prices than lumber or pulp wood. The paper industry generates paper sludge as a waste product which could be a readily available feedstock with potentially negative costs (meaning they are waste streams from a process that are currently disposed of at a cost to the producer) [66, 67]. This sludge contains some fermentable sugars could be used to produce ethanol. One study found that yields of 51% or 18.6 g/l of ethanol could be obtained from paper sludge using *Pichia stipis* as the fermenting strain [68]. Because

the feedstock is exposed to extensive chemical and mechanical modification during the paper manufacturing process, no further pretreatment of paper sludge is required prior to enzymatic hydrolysis.

In the industrial process of producing ethanol, a number of steps require large amounts of water. Recycling this process water for reuse by the facility could reduce costs by decreasing the freshwater intake as well as by reducing the wastewater produced by the facility [69]. The use of cellulolytic enzymes in the process also adds to the cost, as it can be expensive to purchase the enzyme preparations that are used to hydrolyze the cellulose. One potential way to lower the contribution of enzyme cost is through the use of surfactants, which allow for high ethanol yields while also reducing the needed amount of cellulase in the process [70, 71]. One study on spruce found that the enzyme loading could be reduced by 50% with the addition of tween-20 to the fermentation [72]. The authors of this study do note that for the addition of surfactants to be a feasible alternative, cheaper alternatives to tween-20 must be developed. It is also possible to decrease costs by linking an ethanol production plant with an electricity generating power plant. This plant would burn primarily lignin, which is left over after fuel production, to produce electricity that could be sold to the grid or used to offset the electricity needed to run the ethanol plant [73, 74].

There has been a great deal of progress in the fermentation of softwoods to ethanol in the last few decades, but the process still has challenges that must be met for a softwood bioethanol industry to become viable. Pretreatment processes must consume as little energy and outside materials as possible in order to reduce production costs. The dry matter that can be fermented must be increased; this will involve the continued development of strains that can survive and

ferment in the presence of inhibitory compounds released during pretreatment, because their concentration rises with increasing solids loadings.

### 1.7 INHIBITORS & INHIBITON OF *SACCHAROMYCES CEREVISIAE*

One barrier to the fermentation of high concentrations of biomass is the production of inhibitory compounds during the pretreatment process [75-78]. These compounds are generated from different polymers that comprise the biomass when they are exposed to harsh conditions during pretreatment (Figure 1.2). These compounds are typically categorized by their chemical structure as aromatics, aliphatic acids, or furans [77]. The aliphatic acids and furans are sugar degradation products that are generated from the cellulose and hemicellulose [79, 80]. Acetic acid, which istypically found in greater concentration than the other acid inhibitors, is generated as a sugar degradation product or it can be released from the hemicellulose during pretreatment, as this is acetylated to varying degrees depending on the type of biomass being pretreated [81]. The aromatic inhibitors are released from lignin as it is degraded and separated from the cellulose [82]. Because lignin is a large, complex molecule with many different structural motifs, the variety of aromatics released is much greater than that seen in the other two classes of compounds. Further confounding the fermentation of biomass is the fact that the various inhibitors produced during pretreatment can have synergistic effects; the combination of multiple inhibitors in the media can cause greater inhibition than any single compound [29, 75, 83, 84].

The concentration of these compounds can be reduced after pretreatment using a variety of inhibitor abatement techniques. When applied, these methods improve the performance of biomass fermentations [85, 86]. While this removal of inhibitors does increase ethanol yields, this must be weighed against the cost of adding additional steps into the pretreatment of the

biomass. This may be prohibitively high and hurt the economic performance of the biomass fermentation process [87]. Ideally, the biomass would be fermented without additional treatment. This requires the use of a biocatalytic strain capable of withstanding the inhibitory effects of inhibitors present in the fermentation medium. *Saccharomyces cerevisiae* has greater natural tolerance and resistance to these compounds than many other ethanologens, making it a strong candidate for the fermentation of lignocellulosic biomass [88-91].

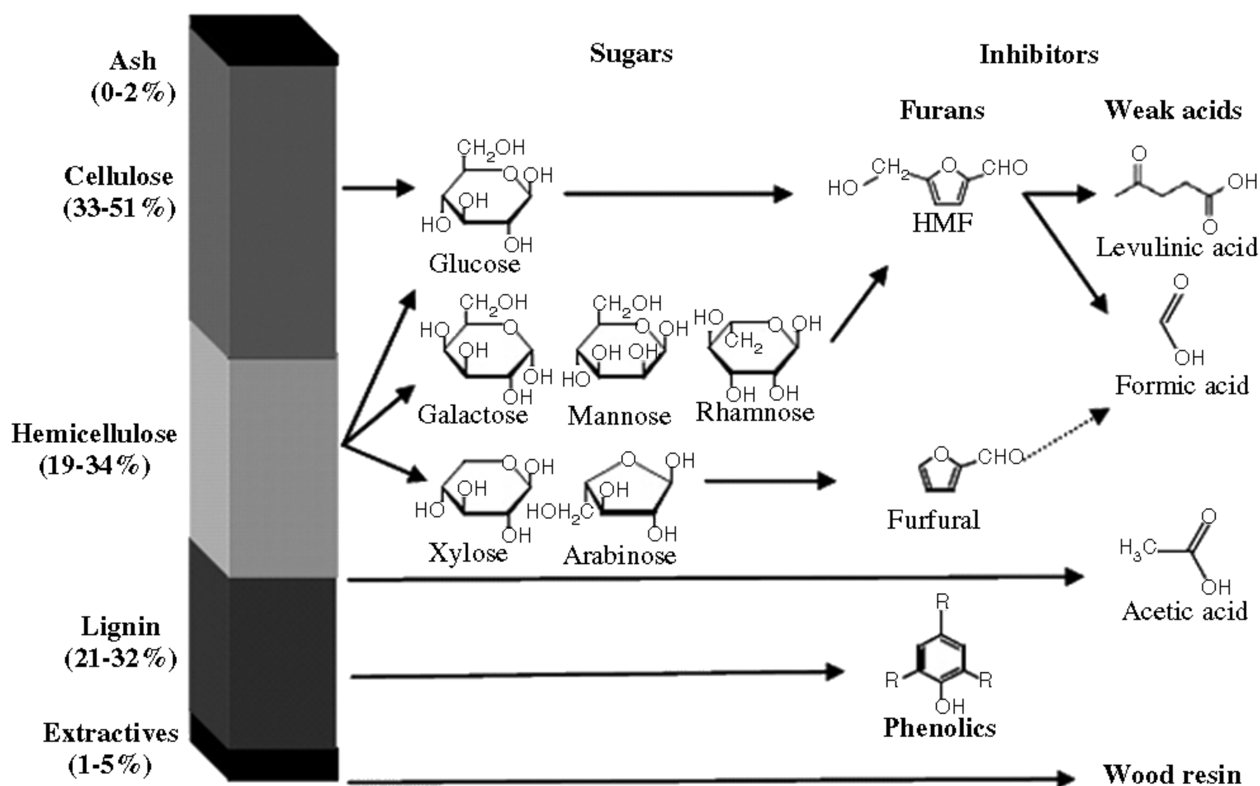



**Figure 1.2.** The production of inhibitory compounds from the various polymers of biomass during pretreatment [78].

The various inhibitory compounds can have a number of negative effects on *Saccharomyces* (Figure 1.3). The furan compounds hydroxymethylfurfural (HMF) and furfural have been most studied and their effects on *Saccharomyces* are best understood of the biomass derived inhibitors. Furfural has been shown to reduce the growth rate of cells, particularly in

aerobic conditions [92-94]. Furfural will also inhibit the activity of cellular enzymes, particularly alcohol, aldehyde, and pyruvate dehydrogenases, which are involved in the metabolism of glucose and the production of ethanol [95, 96]. When furfural and HMF are added into culture medium together, synergistic effects can be observed; meaning that the inhibition of cellular activity observed is greater than in medium containing an equimolar concentration of only one of the inhibitory compounds. No growth or ethanol production was observed in media containing furfural and HMF until these compounds were depleted from the medium [84]. *Saccharomyces* can reduce these furans to their alcohol analogs in an NADPH dependent manner; thus removing the aldehyde inhibitors from the medium and leaving their alcohol forms, which show reduced inhibition of cellular activity. This conversion may cause some of the observed inhibitory effects on cellular growth and metabolism as the cells' supply of NADPH is depleted as the aldehydes are converted to alcohols. Exposure of *Saccharomyces* to these compounds also causes a host of effects including: reactive oxygen damage, mitochondrial disruption, DNA damage, and membrane instability [97].

The aliphatic acids can inhibit cellular activity by acting as uncouplers. In this process the undissociated form of the acid will diffuse into the cell; then the acid dissociates, losing a proton [97]. This dissipates the proton motive force the cell uses to generate ATP and hampers cellular growth and metabolic activity while also reducing the pH of the cytosol. The cell must then correct for this pH imbalance by ATP-dependent pumping of protons out of the cell, a process that depletes the cell's reserves of energy [98]. Acetic acid also has inhibitory effects on certain glycolytic enzymes, particularly enolase [99]. The aliphatic acids have also been shown to inhibit the activity of transport proteins associated with aromatic amino acid import [100].

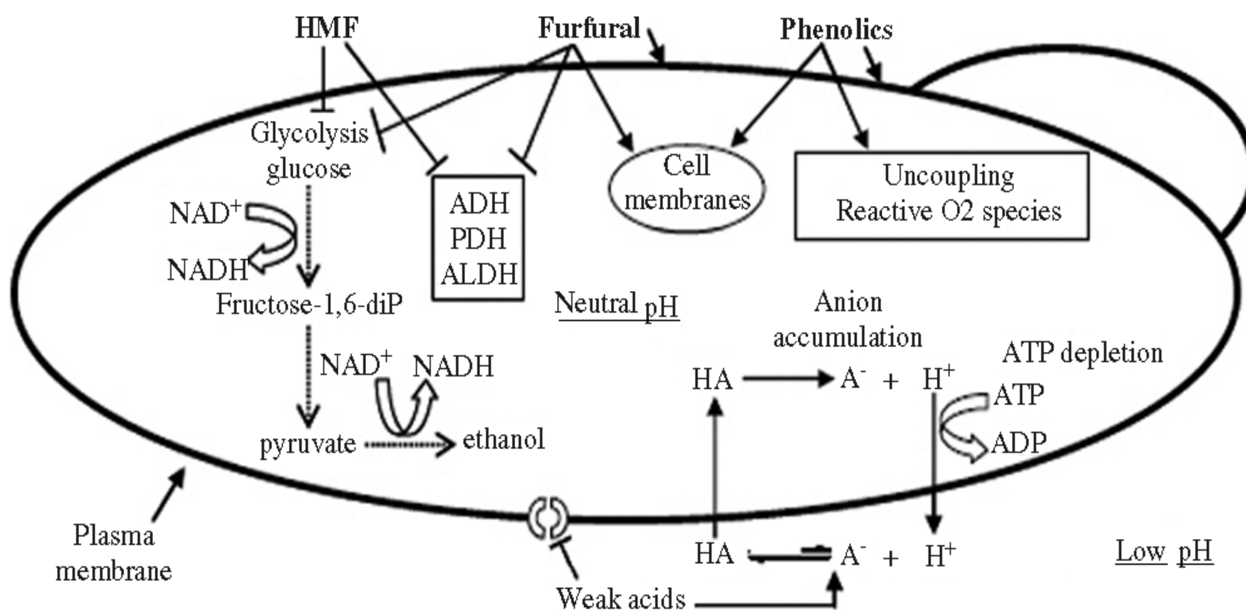



**Figure 1.3.** The effects of certain inhibitory compounds on *Saccharomyces cerevisiae*. Block arrows indicate the inhibition of certain processes or enzymes while arrows indicate damage or inhibition of cellular components without a specific known target [78].

The effects of aromatic inhibitors on *Saccharomyces* are not as well understood as the other two classes of compounds; due at least in part to the much greater variety of compounds present in this class. They are known to destabilize cellular membranes. They have also been shown to act as uncouplers; dissipating the proton gradient the cell uses to generate ATP in a fashion similar to the acid compounds [101]. Certain aromatic compounds target cellular membranes and disrupt their ability to act as selectively permeable barriers [102]. The complex structure of lignin results in the release of a wide variety of aromatic compounds with many different functional groups during pretreatment. In general aldehyde and ketone compounds show greater inhibition of cellular activity than acid compounds with alcohol substituted aromatics showing the least inhibition of *Saccharomyces* [90, 103].

## 1.8 INHIBITOR TOLERANT STRAINS

To improve fermentation performance in the presence of inhibitory compounds, attempts have been made to isolate naturally occurring yeast strains that already possess a high level of inhibitor tolerance. Spent sulfite liquor is a byproduct of paper manufacturing and contains a number of inhibitors found in softwood fermentations, and thus is a likely habitat for yeast strains naturally possessing inhibitor resistance [104]. One *S. cerevisiae* strain, TMB3000, was isolated from spent sulfite liquor and shows higher performance than strain CBS8066 in hydrolysates produced by dilute acid pretreatment [105]. Another study found that strain TMB3000 had the highest specific ethanol production rate when compared to five other *S. cerevisiae* strains in the fermentation of spruce hydrolysates in either fed batch or batch processes [106]. Various strains of *Saccharomyces* naturally show differing levels of tolerance to biomass derived inhibitors as well as performance in biomass hydrolysates [106, 107]. Strains previously used in industrial settings occasionally show increased inhibitor tolerance over lab type strains, but the observed phenotypes differ based on the inhibitor or inhibitor mix used in the medium [108]. Screening of organisms from furfural contaminated soil samples allowed for the isolation of a number of microorganisms with the ability to deplete toxic compounds. However, only one of the strains was capable of high performance in corn stover, another type of lignocellulosic biomass, hydrolysate [109].

The potential for culturing the yeast inoculum on media that is supplemented with hydrolysate, which contains inhibitory sugar degradation products, has been explored as an avenue to increase fermentation performance. It was found that by culturing packaged baker's yeast on media containing hydrolysate, ethanol yields could be greatly increased above what was observed for baker's yeast and strain TMB3000 cultured using standard techniques. The

advantage of the hydrolysate-cultured strain was particularly acute as solids loadings increased to 8 and 10% water insoluble material [110]. One study attempted to increase ethanol yields and limit the negative effects of inhibitory compounds by using two fermenting strains simultaneously to ferment softwood hydrolysate [111]. *S. cerevisiae* was mixed with either *Pachysolen tannophilis* or recombinant *Escherichia coli*; the co-cultures grown in media supplemented with biomass hydrolysate before fermentations were performed. The combination of *S. cerevisiae* and *E. coli* showed higher performance than either strain alone as well as the combination of *S. cerevisiae* and *P. tannophilis*.

Studies have been performed using a variety of techniques to better understand how yeast strains respond to the stress of inhibitory compounds [112]. A library of *Saccharomyces* knockout strains was screened in media containing vanillin, an aromatic compound found in biomass fermentations, to determine which genes increased sensitivity to this inhibitory compound. A number of cellular processes were found to be involved in vanillin tolerance, prominently the biosynthesis of ergosterol, an important component of yeast cell membranes [113]. Microarrays and other methods to profile gene expression changes have been employed to probe the transcriptional shifts that occur when yeast cells are exposed to inhibitor containing environments. Microarray analysis of yeast grown in the presence of inhibitory aromatic compounds suggests that 3 genes are particularly important for resistance to these compounds: *YAP1*, a transcription factor, *ATR1,* and *FLR1,* both of which are multidrug transporter proteins [114]. *YAP1,* and other transcriptional regulators, was also found to be important for HMF tolerance as well [115].

Furfural exposure elicited broad transcriptional changes in *Saccharomyces;* a number of enzymes with alcohol or aldehyde reductase activity show rapid induction upon exposure to this

compound [115-117]. These enzymes convert aldehyde inhibitors to their less toxic alcohol analogs. Transcriptomic analysis of one yeast capable of withstanding a combination of furfural and HMF in media where the parental strain was unable to grow or produce ethanol revealed a number of genetic changes associated with central carbon metabolism as well as the pentose phosphate pathway [118-120]. The PDR gene family has also been shown to be important for biomass derived inhibitor tolerance [115, 121, 122]. These genes are a family of plasma membrane associated proteins that are involved in the transport of a wide variety of substrates and increasing expression of these genes results in higher tolerance to inhibitors [123].

Genetic engineering techniques have been used to generate inhibitor tolerant strains by adding specific genes or altering gene expression in *Saccharomyces* to improve inhibitor tolerance and performance in biomass fermentations. HMF resistance was engineered by increasing the expression of *ADH6,* an alcohol dehydrogenase. Higher expression of this gene allowed for improved growth in the presence of inhibitors relative to the wild-type and fermentation of spruce hydrolysate that could not be fermented by wild-type strains [124]. Overexpression of glucose-6 phosphate dehydrogenase *ZWF1*, the initial enzyme of the pentose phosphate pathway, enhanced the resistance of *Saccharomyces* to furfural [120]. The mechanism for this enhanced furfural tolerance is believed to be linked to more rapid cofactor generation by the increased activity of the pentose phosphate pathway. This would make more NADPH available to the reductases that convert these aldehyde inhibitors to less toxic alcohols [125].

The addition of laccase enzyme to *Saccharomyces* has been shown to improve both resistance to aromatic inhibitors and the fermentation of spruce hydrolysate [121]. This enzyme catalyzes the formation of aromatic radicals, which are highly unstable and will polymerize to form high molecular mass products. This enzyme removes inhibitory low molecular weight

aromatic compounds from the fermentation medium [126]. The laccase expressing strain showed higher ethanol productivity than the control strain in spruce hydrolysate. The overexpression of *PAD1*, a phenylacrylic acid decarboxylase, enhanced strain performance in media containing specific aromatic compounds as well as increased ethanol yields 24-29% over wild-type in spruce hydrolysate [127].

Evolutionary engineering or adaptation has also been used to generate strains capable of more efficient biomass fermentation. This has been accomplished by a variety of methodologies, but in general involves passaging the strain being evolved through media that contains inhibitory compounds and keeping stress on the organism over a large number of generations [128]. When a 12 inhibitor cocktail was used both in repetitive transfer batch culture and long term chemostat adaptation experiments, the resulting isolates were able to better tolerate biomass derived inhibitors and consume sugars than the parental strain [129]. Similar evolutionary engineering approaches have also been successful in increasing acetic acid tolerance [130]. Adaptation of another yeast strain using diluted hydrolysate from wheat straw enabled the fermentation of higher solids loadings of biomass. The original parent strain was unable to produce ethanol in these high wheat straw fermentations [131]. Exposure to furfural in the media for 300 generations enhanced the performance of yeast not only in furfural supplemented media but also in media containing spruce hydrolysate [132]. Pretreated biomass can be used in evolutionary adaptation processes instead of defined media with a known concentration of inhibitors. When pretreated sugarcane bagasse was used, an adapted strain displayed improved performance in biomass fermentations as well as more rapid reduction of furfural and HMF [133].

### 1.9 OBJECTIVES

AJP50, a new strain of *Saccharomyces cerevisiae,* was generated through evolutionary engineering from *S. cerevisiae* XR122N, an industrial strain of yeast used in corn ethanol fermentations. XR122N was chosen as the parent strain because of its robust performance in corn ethanol fermentations and its ability to be readily produced on an industrial scale. AJP50 is able to ferment concentrations of sulfur dioxide steam exploded pinewood biomass of 17.5% dry weight or greater. At these concentrations, the maximum yield of ethanol from properly pretreated samples reaches the required minimum for efficient distillation. This study seeks to evaluate AJP50's performance as well as better understand the genetic changes that have occurred during the evolutionary engineering process which differentiate it from the parent strain XR122N.

Chapter 2 describes the methods used to generate AJP50 as well as compares the performance of the evolved strain to the parent in both pine wood fermentations and inhibitor containing media. Chapter 3 describes culturing methods used to obtain isolated colonies from the AJP50 strain and that allow for the fermentation of pine wood at dry weight concentrations of 22.5%. Chapter 4 is concerned with better understanding selected isolates of AJP50. The performance of isolates is evaluated in pinewood fermentations after growth in rich media lacking inhibitory compounds to determine the retention of the high solids fermentation phenotype. The transcriptomes of two isolates are also sequenced to evaluate the gene expression differences between isolates that are capable of ethanol production from high dry weights of pine wood and an isolate that is incapable of fermenting pine.

## CHAPTER 2

## A STRAIN OF *SACCHAROMYCES CEREVISIAE* EVOLVED FOR FERMENTATION OF LIGNOCELLULOSIC BIOMASS DISPLAYS IMPROVED GROWTH AND FERMENTATIVE ABILITY IN HIGH SOLIDS CONCENTRATIONS AND IN THE PRESENCE OF INHIBITORY COMPOUNDS[1]

[1]Hawkins, G.M., and J. Doran-Peterson. 2011 *Biotechnology for Biofuels* 4.1 (2011): 1-14. Reprinted here with permission of the publisher.

## 2.1 ABSTRACT

**Background.** Softwoods are the dominant source of lignocellulosic biomass in the Northern hemisphere and have been investigated world-wide as a renewable substrate for cellulosic ethanol production. One challenge to using softwoods, particularly acute with pine, is that the pretreatment process produces inhibitory compounds detrimental to growth and metabolic activity of fermenting organisms. To overcome the challenge of bioconversion in the presence of inhibitory compounds, especially at high solids loading, a strain of *Saccharomyces cerevisiae* was subjected to evolutionary engineering and adaptation using pretreated pine wood (*Pinus taeda*).

**Results.** An industrial strain of *Saccharomyces*, XR122N, was evolved using pretreated pine; the resulting daughter strain, AJP50, produced ethanol much more rapidly than its parent in fermentations of pretreated pine. Adaptation by preculturing of the industrial yeast XR122N and the evolved strains in 7% w/v pretreated pine solids prior to inoculation into higher solids concentrations, improved fermentation performance of all strains compared to direct inoculation into high solids. Growth comparisons between XR122N and AJP50 in model hydrolysate media containing inhibitory compounds found in pretreated biomass revealed AJP50 exited lag phase faster under all conditions tested. This ability is due, in part, to AJP50 rapidly converting furfural and hydroxymethylfurfural to their less toxic alcohol derivatives and recovering from reactive oxygen species damage more quickly than XR122N. Under industrially relevant conditions of 17.5% w/v pretreated pine solids loading, additional evolutionary engineering was required to decrease the pronounced lag phase. Using a combination of adaptation by inoculation first into a solids loading of 7% w/v for 24h, followed by a 10% v/v inoculum (approximately equivalent to 1 g/L cell dry wt) into 17.5% the final strain (AJP50) produced ethanol at > 80% of the

maximum theoretical yield after 72h of fermentation and reached > 90% of the maximum theoretical yield after 120h of fermentation.

**Conclusions.** Our results demonstrate that fermentations of pretreated pine containing liquid and solids, including any inhibitory compounds generated during pretreatment, are possible at higher solids loadings than previously reported in the literature. These fermentations demonstrated reduced inoculum sizes and shortened process times, thereby improving the overall economic viability of a woody biomass-to-ethanol conversion process.

## 2.2 BACKGROUND

Cellulosic ethanol could serve as a sustainable biofuel that could replace gasoline use as a transportation fuel [134, 135], and it can be generated from a variety of cellulosic biomass types, such as wood [136]. One challenge that is particularly acute with woody biomass, such as pine, is that the pretreatment process releases a number of compounds that are inhibitory to the growth and/or metabolic activity of the fermenting organism [75]. These chemicals act through a variety of mechanisms to reduce ethanol production efficiency including: inhibition of cell growth, reduction of cell metabolic activity, or inhibition of enzymatic activity. Thus it is important to use a fermenting organism that is able to tolerate these compounds, especially at the high solids loadings required for industrial fermentations to produce ethanol concentrations needed for cost-effective distillation.

Inhibitors found in biomass fermentations are determined by conditions used during pretreatment: temperature, pH, time, and any chemicals used; and act in various ways to inhibit efficient fermentation of sugars to ethanol [77, 84, 97, 101, 137, 138]. Inhibitors can be divided into three general categories; weak aliphatic acids, aromatic compounds, and furan derivatives.

Aromatic compounds, such as vanillin and 4-hydroxybenzaldehyde, are generated when the wood's lignin is degraded [139]. Furan derivatives are generated from sugar portions of the feedstock during pretreatment, furfural from degradation of pentose sugars and 5-hydroxymethylfurfural (HMF) from hexose sugars [140]. HMF can be further degraded during pretreatment to produce the weak acids levulinic acid and formic acid. Acetic acid, another weak acid, is formed by hydrolysis of hemicellulose. HMF and furfural can decrease the ethanol yield and productivity, as well as slow the organism's growth [77]. Furfural and HMF act synergistically to decrease ethanol production [84]. The most concentrated weak acids present in pine wood fermentations are acetic, levulinic, and formic acids, acting to inhibit cellular activity by mechanisms of uncoupling and intracellular anion accumulation [97]. Uncoupling results in a dissipation of the cell's proton gradient; thus hindering its ability to generate ATP [101]. During intracellular anion accumulation, the undissociated form of the acid will diffuse across the plasma membrane, and then dissociate inside the cell, thus decreasing the cytosolic pH [138]. The cell must then correct this pH imbalance. Mechanisms by which aromatics inhibit are not completely elucidated, presumably due to the complex structure of lignin. Proposed mechanisms include a loss of integrity in the cell membrane and destruction of the electrochemical gradient by transporting protons back into the mitochondria similar to the weak acids [101, 102]. Furthermore, it has been shown that furfural and aromatic compounds can lead to reactive oxygen species which cause damage to *S. cerevisiae* [77, 141]. Reactive oxygen species can randomly oxidize proteins, lipids, and other structures and if the damage is too great the cells will not survive.

Inhibitory compounds may be removed prior to fermentation, resulting in increased ethanol production [85, 126, 142]. Although effective, removal of these compounds from

pretreated biomass increases overall production costs. The ethanologenic yeast, *Saccharomyces cerevisiae,* displays relatively robust growth in the presence of inhibitory compounds [143], although the response of individual strains varies widely [106]. Some *Saccharomyces* strains convert HMF to the less toxic 2,5-bis-hydroxymethylfuran [88], and the *ADH6* gene product has been shown to increase the rate at which cells metabolize HMF [124]. *S. cerevisiae* is also able to partially metabolize some of the phenolic compounds, likely via phenylacrylic acid decarboxylase conversion of cinnamic, p-coumaric, and ferulic acids to their less toxic vinyl derivatives [91, 121]. Furan reductase or laccase have been expressed in yeast [121, 144] and increased fermentation rates. Other efforts to reduce detrimental impacts of inhibitors include optimizing process configurations, such as fed batch pulse feeding of hydrolysate instead of immersing the yeast in hydrolysate all at once. *Saccharomyces* strains are able to adapt to some degree by preculturing on hydrolysate or via cell recycle [133, 145, 146], although the exact mechanisms for increased performance are still unknown for many strains.

Previous efforts have described approaches to improve fermentation performance of *Saccharomyces cerevisiae* strains with respect to inhibitor tolerance. When an industrial strain of *Saccharomyces cerevisiae* was cultured in increasing concentrations of furfural, the time spent in lag phase by the adapted strain was significantly reduced as compared to the parental strain [132]. In a later study, this reduction in lag phase was attributed to increased oxireductase activity in the evolved strain [116]. Other efforts have increased xylose utilization in engineered strains using chemostat evolution [147]. In this process, the strain was kept under constant xylose limitation in a chemostat and the resulting pressure selected for strains that are best able to use xylose as a carbon source. Due to a large natural biodiversity in *S. cerevisiae,* other approaches have focused on isolating natural strains from distilleries [148].

In this paper we describe the directed evolution and adaptation of an industrial *Saccharomyces* yeast strain currently used in corn ethanol fermentations for the production of ethanol from pretreated lignocellulose. We selected sulfur-dioxide pretreated pine wood as the substrate due to the high level of inhibitory compounds found in this feedstock. In order to generate a strain with improved tolerance of inhibitory compounds found in pretreated pine, the industrial strain, XR122N, was evolved using $SO_2$ pretreated pine directly without separating the liquid from the solids and without ameliorating the toxic compounds. This approach is in contrast to using a single inhibitory compound such as furfural for directed evolution. The strain was also subjected to additional evolutionary adaptation at high solids loadings in order to increase ethanol concentrations in the fermentation. Growth and ethanol production of the evolved strain in various combinations of 13 inhibitory compounds found in pretreated pine was also investigated. The final evolved strain, AJP50, possesses greater fermentation capability than its parent in both rich liquid media supplemented with various combinations of inhibitory compounds and pretreated pine fermentations at high solids loadings.

## 2.3 RESULTS AND DISCUSSION

**Industrial yeast XR122N pretreated pine fermentations.** Fermentations using $SO_2$ steam explosion pretreated pine (without washing or inhibitor removal) as the substrate at dry weight solids loadings of 5, 10, and 12% w/v were conducted using the industrial yeast *Saccharomyces cerevisiae* XR122N (North American Bioproducts Corporation, Duluth, GA). Compositional analysis of the pine before and after pretreatment is provided in Table 2.1 and a list of thirteen inhibitory compounds and their concentrations in the pretreated pine sample used for fermentations are listed in Table 2.2. XR122N was inoculated in a freeze dried state at an initial

concentration of 4 g/L cell dry weight (cdw) similar to its use in corn ethanol fermentations and enzymes for biomass saccharification were added simultaneously with the cellulase (15 FPU) cellobiase (60 IU cellobiase per gdw of pretreated pine).

**Table 2.1.** Compositional analysis of pine subjected to sulfur dioxide steam explosion[a]

| Sample | Glucan | Xylan | Mannan | ASL[b] | AIL[c] | Sum |
|---|---|---|---|---|---|---|
| Untreated Pine [d] | 42.9 | 6.0 | 12.9 | 0.5 | 33.2 | 99.1[d] |
| 3.3% - $SO_2$ 213°C[e] | 53.0 | 1.2 | 0.4 | 0.4 | 44.0 | 99.0 |

[a] per cent of each component on a dry weight basis
[b] ASL: Acid-soluble lignin
[c] AIL: Acid-insoluble lignin
[d] Pine prior to pretreatment also contained galactan 2.5% and arabinan 1.1% not detected in the pretreated pine sample. Sum includes the galactan and arabinan fractions.
[e]Reaction time of 5 min at $SO_2$ % and temperature indicated

**Table 2.2.** Concentrations (g/L) of inhibitory compound studied, divided into classes

| FURANS | | | AROMATICS | | | ACIDS | | |
|---|---|---|---|---|---|---|---|---|
| | Hyd[a] | Model[b] | | Hyd[a] | Model[b] | | Hyd[a] | Model[b] |
| HMF[c] | 2.153 | 2.000 | 3,4-DHBA[d] | 0.003 | 0.003 | Formic Acid | 0.425 | 0.400 |
| Furfural | 1.180 | 1.000 | 3-HBA[e] | 0.005 | 0.005 | Lactic Acid | 0.100 | 0.100 |
| Furoic Acid | 0.018 | 0.020 | Vanillic Acid | 0.050 | 0.050 | Acetic Acid | 2.153 | 2.000 |
| | | | Vanillin | 0.022 | 0.020 | Succinic Acid | 0.028 | 0.030 |
| | | | Benzoic Acid | 0.015 | 0.015 | Levulinic Acid | 0.410 | 0.400 |

[a] Concentration of compound measured in pretreated pine hydrolysate used in fermentations
[b]Concentration of compound in model inhibitor medium
[c]HMF : hydroxymethylfurfural
[d]DHBA: dihydroxybenzaldehyde
[e]HBA: hydroxybenzaldehyde

Simultaneous saccharification and fermentation (SSF) was desired for fermentations because the added enzymes release monomeric forms of carbohydrates from the solids content of pretreated pine and the fermenting yeast consumes the sugars as soon as they are released, thus minimizing end-product inhibition [149, 150]. Optimal conditions for the fungal enzyme preparations used in these experiments are a pH of 4.5 and a temperature of 45°C, conditions too

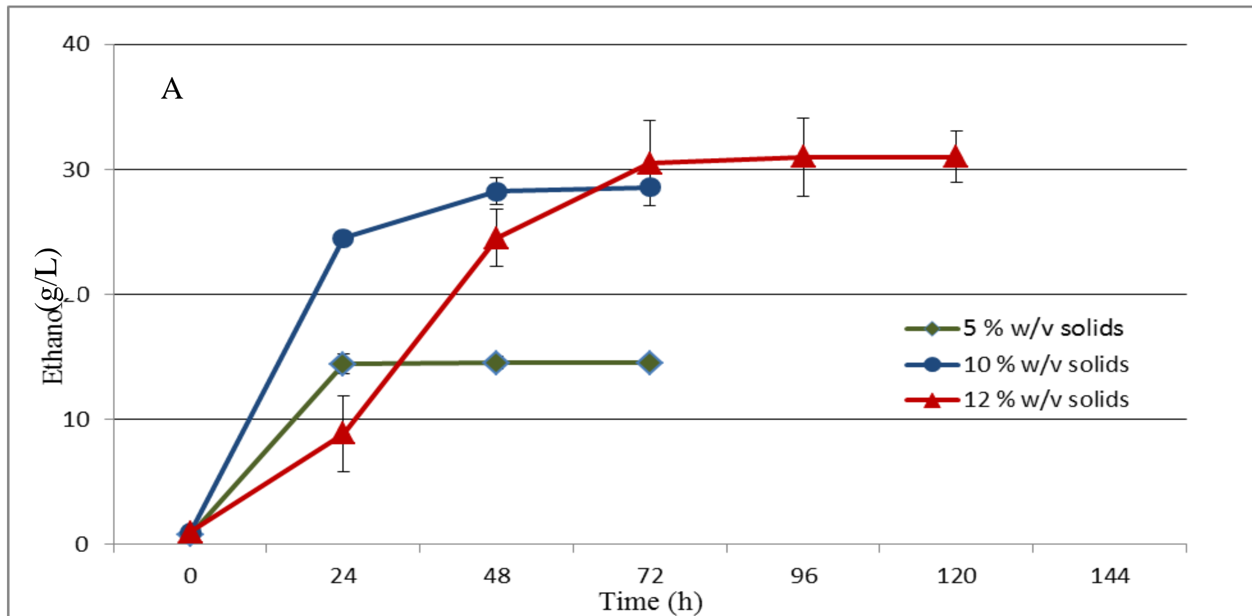
harsh for the fermenting yeast. To optimize enzyme activity during SSF experiments, the pH was held at 5.0, just slightly above the enzyme optimum pH, and the temperature for fermentation decreased from 45°C to 35-37°C. Attempts to increase the fermentation temperature above 37°C dramatically reduced ethanol production (data not shown). Ethanol production from the different biomass concentrations is presented in Figure 2.1A.

The effect of inoculum size on pretreated pine fermentations at a 12 % w/v solids loading is presented in Figure 2.1B. Initial attempts at inoculation of pretreated pine solids at or above 5% w/v using a low inoculum level equal to 0.2 g/L cdw resulted in cell death of XR122N (absence of growth on solid or liquid medium) and no ethanol was detected in these cultures. An inoculum size of 0.5 g/L produced ethanol in pretreated pine fermentations at a 10% w/v solids concentration (data not shown), however, at a 12% w/v solids concentration no ethanol production was detected (Figure 2.1B). Increasing the inoculum level to 1 g/L cdw in 12% w/v solids fermentations resulted in ethanol production, albeit with a pronounced lag phase of 72h. An inoculum of 2 g/L produced almost as much ethanol as 4 g/L and was selected as the inoculum size for further studies.

One of the most promising pretreatments for softwoods, including pine, spruce, and Douglass fir, is $SO_2$ steam explosion [151] and different combinations of $SO_2$ concentration, reactor temperature, and time of reaction have been published. Table 2.3 compares available data on $SO_2$ single-step pretreatments followed by SSF to produce fuel ethanol. Due to the toxic nature of the pretreated softwood, many of the fermentations were conducted with 5-12% w/v solids loadings. The inoculum level for yeasts was routinely 4-5 g/L and enzyme loadings were relatively high at 30-40 FPU cellulase/g d wt of cellulose. Many softwood fermentations were conducted using washed solids [34, 152], diluted solids with filtration [32], or lower solids

loading of 5-8% w/v dry matter [145, 152]. Hoyer and colleagues [65] obtained excellent results (94.7 % of maximum theoretical yield based on glucose and mannose in the pretreated material) during fermentations with 10% w/v solids content. However, when using the same material at 12% w/v dry matter solids loading, the maximum theoretical ethanol yield decreased to only 37%. All of these previous studies highlighted the difficulties involved in fermenting pretreated softwood and we also observed a decrease in the maximum theoretical yield (from 98% to 76%) when increasing the dry matter solids loading from 10% w/v to 12% w/v.

**Evolution of XR122N for fermentation at high solids loading.** In order to reach ethanol concentrations for cost efficient distillation, the solids loading must be increased to 15-20% on a w/v basis [153]. As the biomass content increases in the fermentation, the concentration of inhibitory compounds is also increased. Previous studies with *Saccharomyces* illustrated that some strains are able to adapt to varying degrees by preculturing on hydrolysate or via cell recycle [133, 145, 146]; the exact mechanisms for increased performance are still unknown for many of these strains. Using furfural alone for adaptation experiments resulted in different phenotypes depending upon the method used for selection. Increased furfural reduction rates were observed in selection regimes when furfural was added during logarithmic growth [154]. In contrast, challenging cells at a low inoculum size to relatively high concentrations of furfural did not change furfural reduction rates, but significantly reduced lag phases and allowed growth in glucose minimal medium containing 40% v/v of spruce acid hydrolysate sample that killed the parental strain [132].



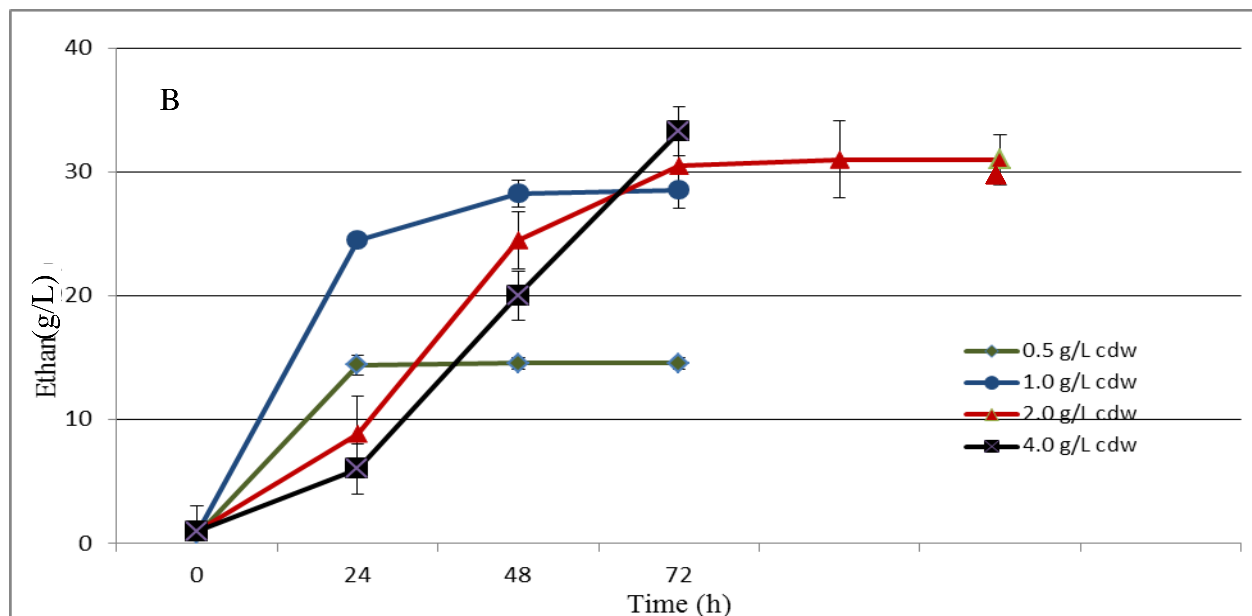




**Figure 2.1.** Effect of solids loading on *Saccharomyces cerevisiae* XR122N. (A) XR122N was inoculated in a freeze dried state at an initial concentration of 4 g/L cell dry weight (cdw) into small scale bioreactors containing pretreated pine at a 5% (green diamonds), 10% (blue circles), and 12% (red triangles) w/v solids loading. Cellulases and cellobiase were added simultaneously with the inoculum (15 FPU cellulase, and 60 IU cellobiase per gdw of pretreated pine). (B) XR122N was inoculated into 12% w/v solids loading of pretreated pine in a freeze dried state at an initial concentration of 4 g/L cdw (black squares), 2.0 g/L cdw (red diamonds), 1.0 g/L cdw (blue circles), or 0.5 g/L cdw (green diamonds). Fermentations were maintained at 35°C, pH 5.0, and performed in triplicate. Error bars represent one standard deviation from the mean.

**Table 2.3.** Comparison of SSF[a] using single step $SO_2$ pretreatment of non-delignified softwoods with *Saccharomyces cerevisiae*

| *Saccharomyces* Yeast Strain | Wood Type | Pretreatment[b] | Solids (% d wt/v) | Cellulase (FPU/g dwt) | Max. ETOH (g/L) | % of Theo Max | Reference |
|---|---|---|---|---|---|---|---|
| Tembec 1 | Lodgepole pine | 4.0-200-5 | 5 % washed solids | 40 FPU 37cellulase/g dw cellulose + 20 IU cellobiase/g cellulose | 17.0 | 68 [c] | Ewanick et al. [34] |
| Tembec 1 | Douglass Fir | 4.5-195-4.5 NOTE output was diluted to 15% w/w | 40 mL wood water-soluble fraction | No enzymes added | 13.8 | 87 [d] | Keating et al. [32] |
| Y-1528 | Douglass Fir | | | | 14.7 | 92 [d] | |
| Baker's Yeast | Spruce | 2.5-215-5 | 8% | 32 FPU cellulase/g dw cellulose + 28 IU cellobiase/ g dw | not presente d | 60[e] | Alkasrawi et al. [145] |
| Baker's Yeast (in hydrolysate) | | | | | | 92 [e] | |
| TMB3000 | | | | | | 89 [e] | |
| Baker's Yeast | Spruce | 3.0-215-5 | 5% | 15 FPU 37cellulase/g dry matter + 23/g cellcellobiase/g dry matter | not presente d | 49[d] | Söderström et al. [152] |
| Baker's Yeast | Spruce | 3.0-210-5 | 12% WIS | | 20.0 (graph) | 37[d] | Hoyer et al. [65] |
| XR122N | Pine | 3.3-215-5 | 10% | 15 FPU 37cellulase/dry g + 60 IU cellobiase/dry g | 28.7 | 98 [c] | This paper |
| XR122N | Pine | 3.3-215-5 | 12% | | 23.6 | 76 [c] | This paper |

[a]Simultaneous saccharification and fermentation
[b]Numbers indicate reaction conditions as follows: First is concentration of $SO_2$, second is reaction temperature, and the third is duration in minutes
[c]Theoretical yield based on cellulose and hemicellulase content derived from gravimetric and analytical analyses of pretreated material
[d]Theoretical yield based on the glucose and mannose content in the pretreated material
[e]Theoretical yield is based on the content of glucose and mannose in the liquid and glucan in the solid material

Inoculation of high solids loading (above 10% w/v solids) using a low inoculum level of XR122N provided multiple stressors (elevated particulate content and increased inhibitory compounds) and selection was targeted at survival and ethanol production. Directed evolution experiments were started at a concentration of 2 g/L cdw inoculum of XR122N added to pretreated pine fermentations at a 17.5% w/v solids loading as described in the methods and illustrated in Figure 2.2. Fermentation was stopped at 168h and aliquots equal to 10% v/v were transferred to fresh 17.5 % w/v fermentations as described. After ethanol was not detected after 96 h and aliquots from the fermenters did not exhibit growth, 2 g/L cdw of XR122N cells were added to the fermentation vessels. Ethanol production was detected after another 24 h of fermentation in one vessel and continued to increase for an additional total of 72h. A 10% v/v inoculum (approximately 1 g/L cdw) was removed from the fermentation vessel where ethanol production was detected and used to inoculate a third fermentation vessel containing 17.5% w/v pretreated pine and enzymes. No additional ethanol was produced after 96h, even though aliquots of the cells grew in liquid media. Again another 2 g/L cdw of XR122N was added to the fermentation. This process of inoculating a 17.5% w/v solids fermentation with a 10% v/v inoculum from a previous fermentation, monitoring ethanol production for 96 hours without observing an increase in ethanol content, and adding 2 g/L cdw of XR122N was repeated for a total of six full cycles. During the seventh cycle ethanol production increased by 24 h, and continued to increase at 48h. At 48 h of fermentation a 10% v/v inoculum was transferred to a fresh 17.5% w/v solids fermentation and ethanol production monitored. Samples from this fermentation (removed after 48h) were frozen in glycerol at -80°C and designated AJP40 (Figure 2.2). A similar set of fermentations using 20% w/v solids failed to produce high concentrations of ethanol, even after the addition of 2 g/L cdw of XR122N (data not shown).

AJP40 was subjected to additional transfers in 17.5% w/v solids loading of pretreated pine. Inoculation of AJP40 into 17.5% w/v solids directly produced little ethanol (Figure 2.3), however, if 10% v/v aliquots from this non-productive fermentation were inoculated into less concentrated solids, ethanol was produced (data not shown). Inoculation of AJP40 glycerol stocks (approximately 0.2 g/L cdw) into a 7% w/v solids fermentation resulted in maximum ethanol production after 24 h of fermentation and a 10% v/v aliquot was used to inoculate a 17.5% w/v solids fermentation. Ethanol production was observed at 48 h and upon transfer of a 10% v/v inoculum into a 17.5% w/v solids fermentation, ethanol was detected after 24 h. Additional transfers into 17.5% w/v solids were made as described in the methods for a total of 50 transfers.

The resulting strain exhibiting the phenotype of increased ethanol production and decreased lag time in high solids fermentations was designated AJP50 and used in additional studies. Inoculation of 17.5% w/v solids fermentations with AJP50 directly from revived freezer stocks (0.2 g/L cdw) did not produce ethanol above 10 g/L. Inoculating AJP50 (0.2 g/L cdw) into 7% w/v solids for a short adaptation period (24h) followed by a 10% v/v inoculum (approximately 1 g/L cdw) for 17.5% w/v solids fermentations improved ethanol production significantly (Figure 2.3). The evolved strain, AJP50, with a short adaptation as described, reduced lag time and produced over 80% of the maximum theoretical yield by 72 h fermentation and over 90% of the maximum theoretical yield of ethanol by 120 h fermentation.

**Growth and ethanol production in the presence of inhibitors.** AJP50 appeared to have acquired the ability to grow and ferment high concentrations of solids and with increasing solids concentrations, there were increased amounts of inhibitory compounds present as well. In order to determine whether or not AJP50 had an advantage over the parental strain in the presence of

inhibitory compounds, growth profiles were compared in different combinations of inhibitory compounds typically found in biomass fermentations (Table 2.2). Growth of AJP50 and XR122N was not inhibited by the aromatic mixture and very weakly inhibited by the acid mixture under concentrations tested (Figure 2.4A, B). Low concentrations of weak acids have stimulated ethanol production in *S. cerevisiae*, but high concentrations were inhibitory to the activity of the organism in previous studies [85, 137].

Because they are present in the largest concentrations in biomass fermentations, both strains were also grown in the presence of a mixture of just HMF, furfural, and acetic acid (Figure 2.4C). Growth of both strains was strongly inhibited by the mixture of the furan compounds (Figure 2.4D). No growth of XR122N was observed over 30 hours; growth of AJP50 showed a longer lag phase than in the other conditions, however the furan inhibited AJP50 cultures did reach the same final optical density as the uninhibited cultures The effects of furfural and HMF on certain strains of *S. cerevisiae* have been described by a number of groups [88, 96, 140]. Furfural completely inhibited the growth of yeast strains at a concentration of 5.76 mg/ml and partially inhibited at a concentration of 2.88 mg/ml over 125 h. HMF completely inhibited one strain and partially inhibited another at 7.6 mg/ml; various degrees of partial inhibition were observed at a concentration of 3.8 mg/ml. These concentrations are higher than previously observed in pine wood biomass fermentations, however, amounts of inhibitory compounds could increase with increased severity of the pretreatment and with increased concentrations of biomass at high solids loadings.

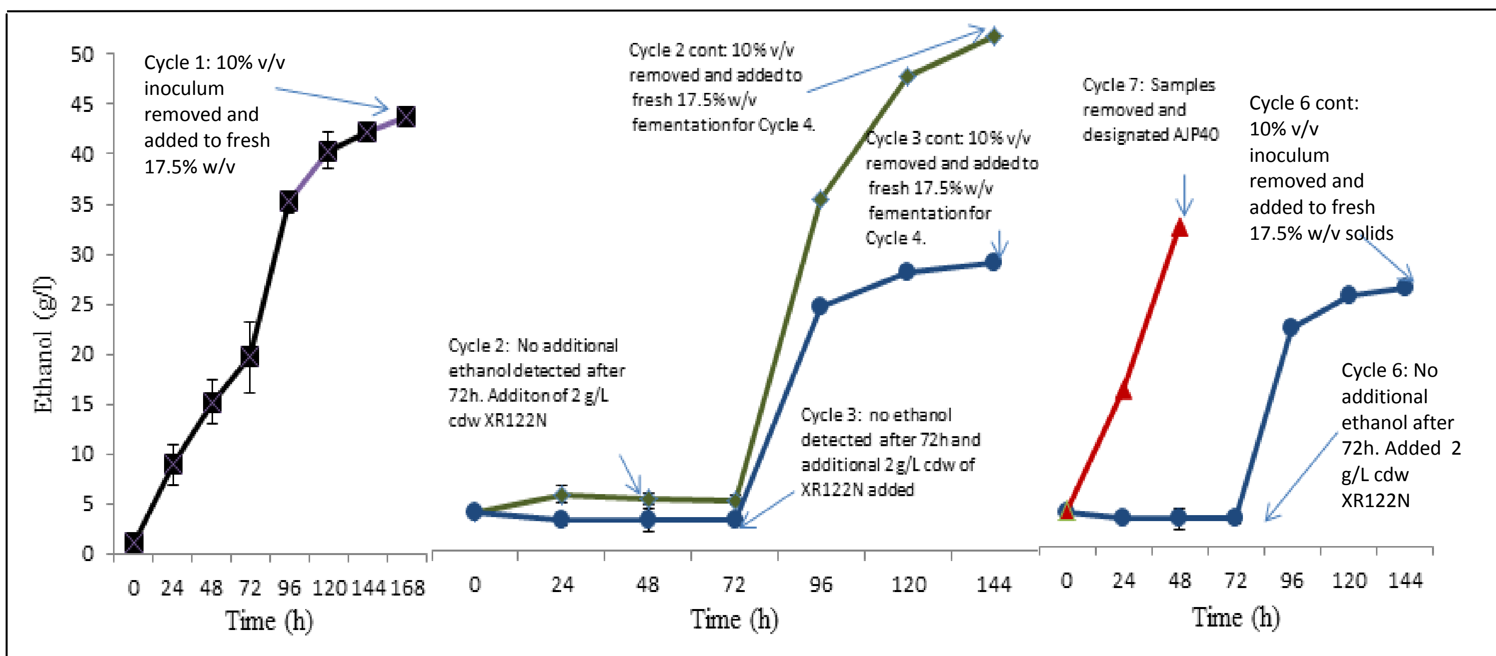



**Figure 2.2.** Evolution and adaptation of XR122N to high solids loading of pretreated pine to produce AJP40. XR122N was inoculated into 17.5% w/v solids and allowed to ferment for 168h. 10% v/v aliquots were removed as indicated and used to inoculate 17.5% w/v solids fermentations. Ethanol was measured every 24hours and additional 2 g/L cdw of XR122N added as indicated. After 6 full cycles the pattern changed and ethanol was produced after 24 and 48h of fermentation without the addition of more XR122N cells.

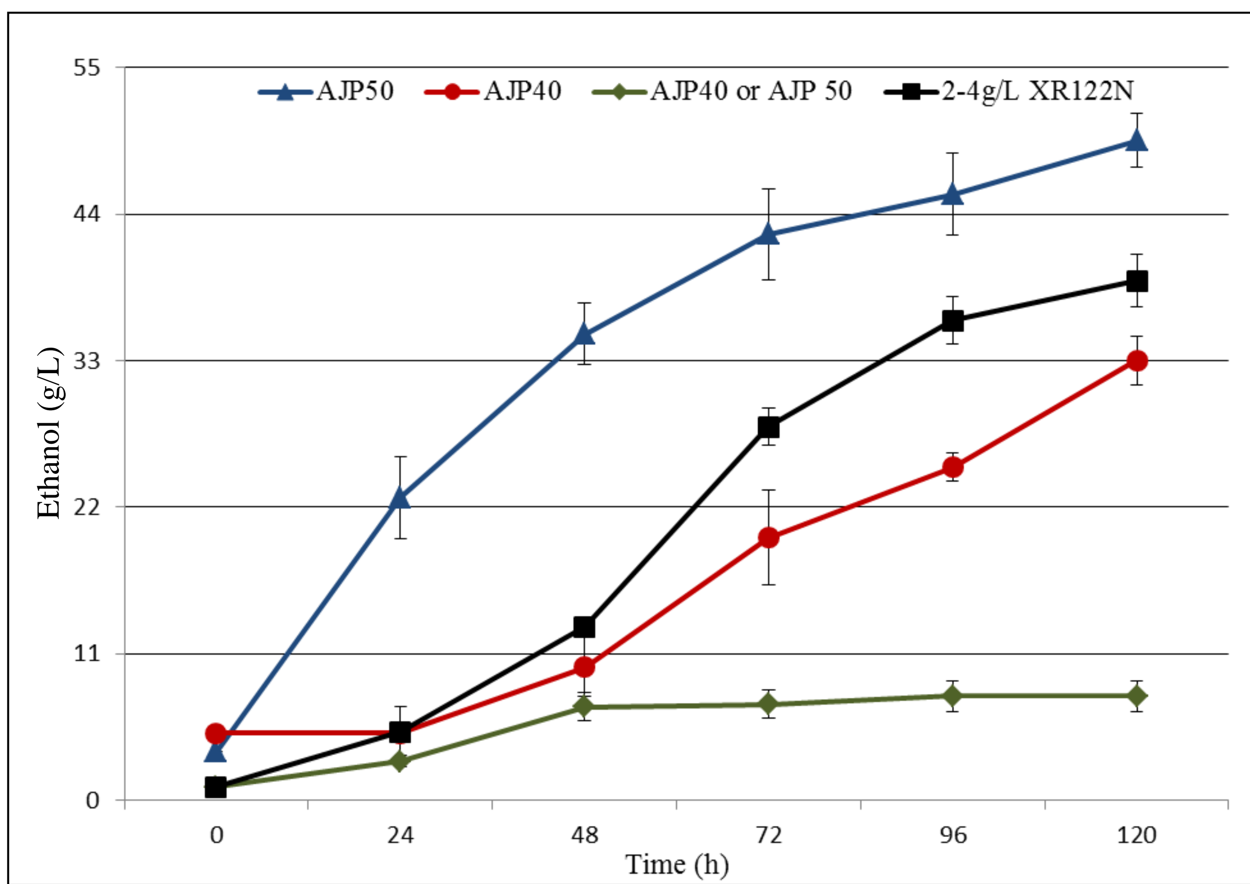



**Figure 2.3.** Comparison of AJP50 and AJP40 in fermentations of 17.5% w/v pretreated pine solids loading. Direct inoculation of AJP40 or AJP50 from freezer stocks (approximately 0.2 g/L cdw) (green diamonds). Short adaptation protocol of inoculation (0.2 g/L cdw) into 7% w/v solids for 24 h, and use of 10% v/v aliquot (approximately 1 g/L cdw) from this fermentation to inoculate 17.5% w/v solids using either AJP40 (red circles) or AJP50 (blue triangles).

To further study inhibition of the strains by these compounds, ethanol production of both strains was compared in the presence of 13 inhibitor compounds (Table 2.2) and in the absence of any inhibitors. Growth data were compared with ethanol data for both strains (Figure 2.5). Even though XR122N failed to grown in the presence of all 13 compounds, it produced a small amount of ethanol after 30h. AJP50 produce the theoretical maximum concentration of ethanol at 18h. Interestingly, in the absence of inhibitory compounds AJP50 was able to produce ethanol after 6h and reached a maximum at 12h; whereas XR122N did not produce ethanol until 12h and reached a maximum at 18h.

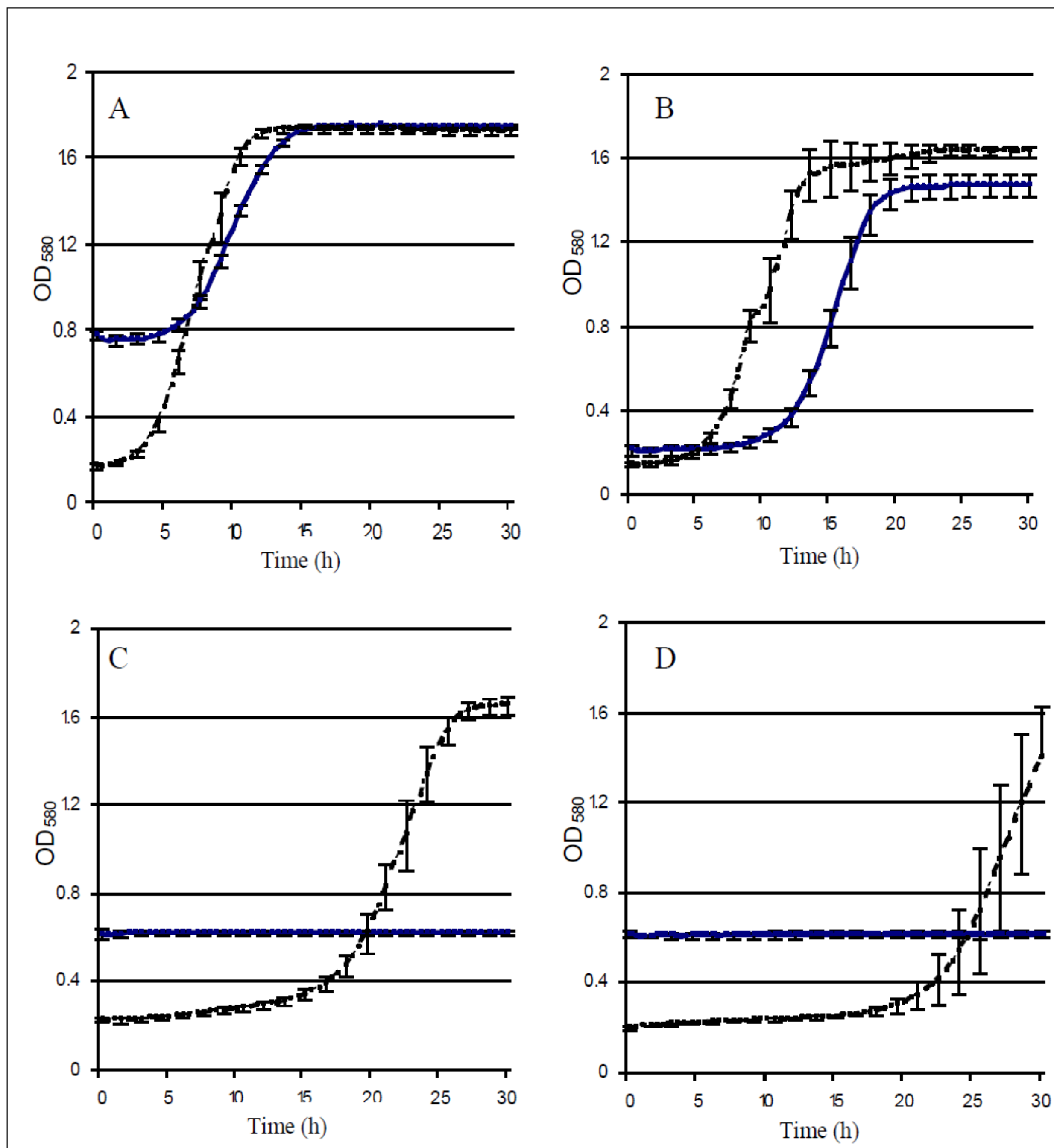



**Figure 2.4.** Growth of XR122N (solid blue) and AJP50 (dashed black) in the presence of various mixtures of inhibitory compounds found in biomass fermentations. Panel A shows growth in the aromatic mixture, Panel B the acid mixture, C a combination of HMF, furfural, and acetic acid, D the furan mixture. All compounds and their concentration in the media can be found in Table 2.2. The inhibitory compounds were dissolved in TSB media with 2% w/v glucose. Error bars represent one standard deviation from the mean. XR122N required a higher volume of revived culture to obtain a cell concentration equivalent 4.0 x $10^5$ cells/ml, this increased the wood particulate matter in the culture and led to the higher initial OD observed in some XR122N cultures.

To further study inhibition of the strains by these compounds, ethanol production of both strains was compared in the presence of 13 inhibitory compounds (Table 2.2) and in the absence of any inhibitors. Growth data were compared with ethanol data for both strains (Figure 2.5). Even though XR122N failed to grow in the presence of all 13 compounds, it produced a small amount of ethanol after 30 h. AJP50 produced the theoretical maximum concentration of ethanol at 18 h. Interestingly, in the absence of inhibitory compounds AJP50 was able to produce ethanol after 6 h and reached a maximum at 12 h; whereas XR122N did not produce ethanol until 12 h and reached a maximum at 18 h.

**Conversion of furfural and hydroxymethylfurfural to alcohol derivatives.** A similar approach to the one used to generate AJP50 was taken to adapt *S. cerevisiae* to the inhibitory compounds in sugar cane bagasse by Martín and colleagues [133]; their study used media with known concentrations of inhibitors added, while the approach taken in this study used pretreated biomass as the media for adaptation and further directed evolution. In the study by Martín et al. the advantage of the evolved strain was attributed to its ability to more rapidly detoxify furfural and HMF [133]. Heer and Sauer were able to evolve another *S. cerevisiae* strain to furfural alone and this evolved strain showed a marked decrease in lag phase, later attributed to increased action of certain oxireductases [116, 132]. Although we used pretreated biomass for the evolutionary adaptation instead of furfural or hydroxymethylfurfural directly, AJP50 is also able to rapidly detoxify furfural and HMF by converting them to their less toxic alcohol derivatives (Figure 2.6).

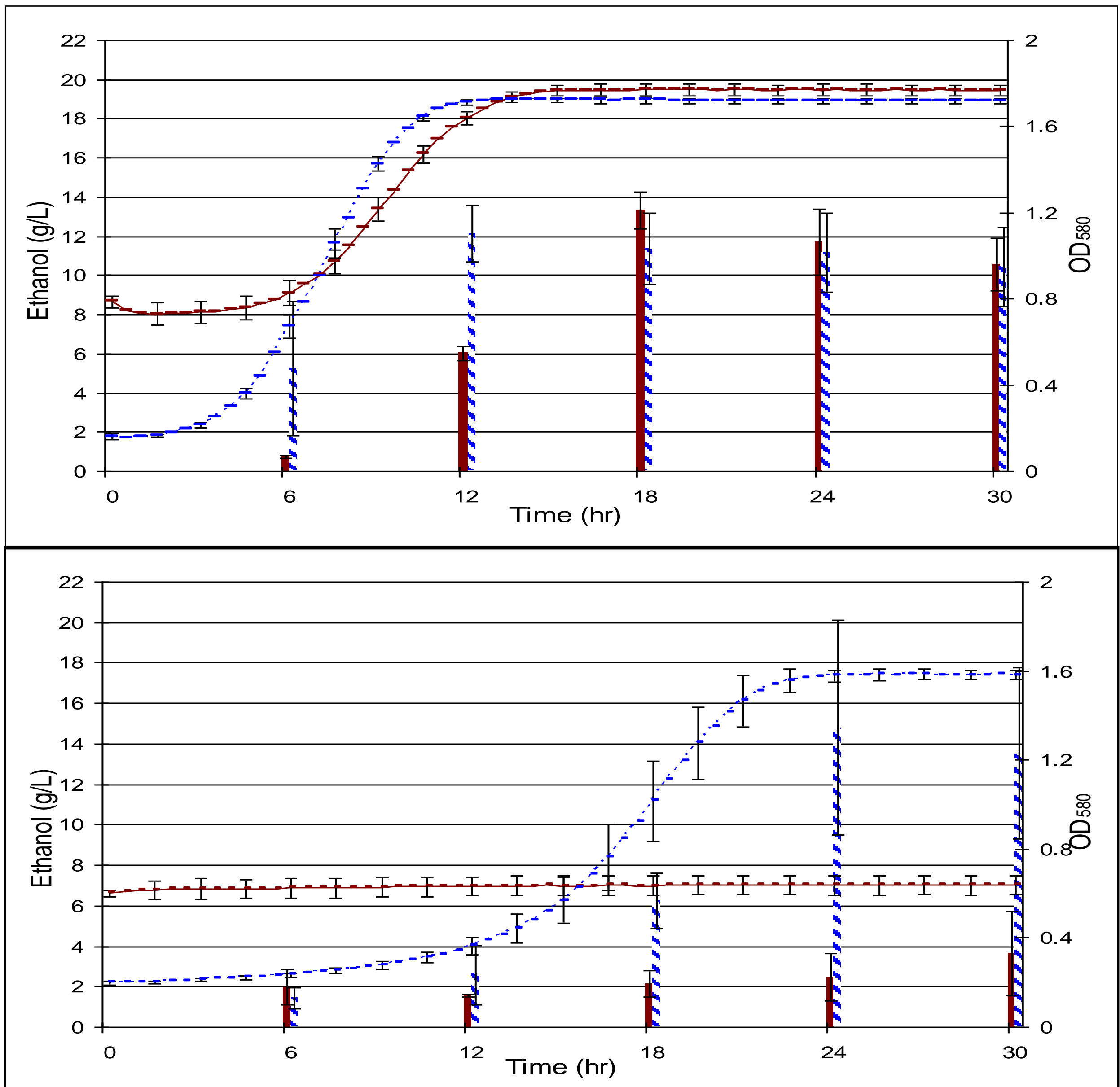



**Figure 2.5.** Growth and ethanol production of both strains in the presence or absence of the selected inhibitory compounds present in pine wood biomass fermentations. Lines represent cell culture density measured at $OD_{580}$. Bars represent ethanol concentration in g/L. The red, solid and blue, dashed lines and bars are data from XR122N and AJP50 at $4.0 \times 10^5$ cells/ml initial cell density. The primary Y axis is ethanol in g/L, the secondary Y axis is $OD_{580}$, and the X axis is time. The upper graph represents growth and ethanol production in the absence of inhibitors; the lower graph represents growth and ethanol production in the presence of all 13 inhibitory compounds.

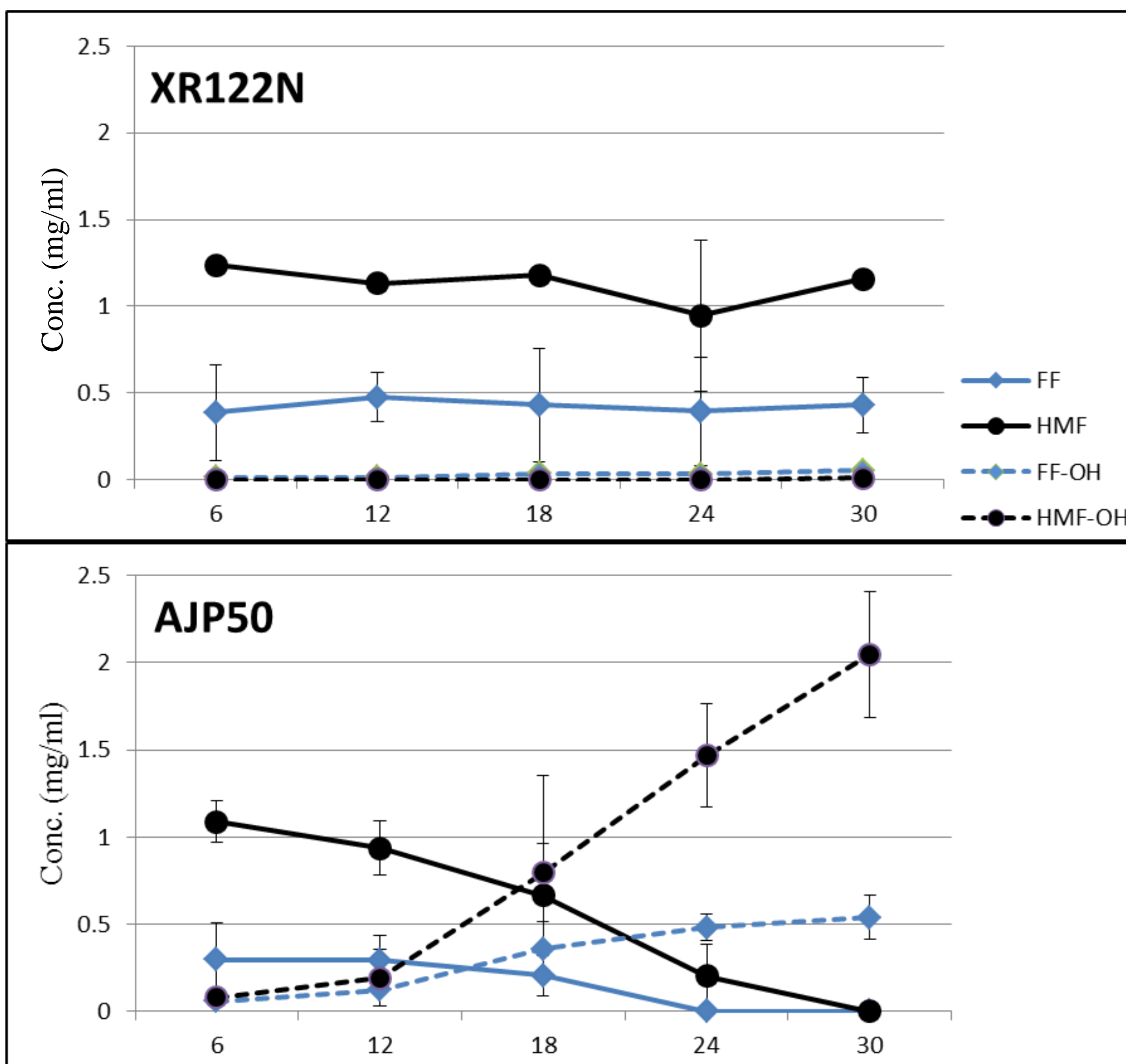



**Figure 2.6.** Conversion of furfural and hydroxymethylfurfural to their less toxic alcohol derivatives. XR122N (A) and AJP50 (B) were compared for their ability to convert furfural (FF) and hydroxymethylfurfural (HMF) to their less toxic alcohol derivatives furfural alcohol (FF-OH) and hydroxymethylfurfural alcohol (HMF-OH). The concentration of each compound is presented for each organism during a 30 h fermentation in TSB with 2% w/v glucose.

**Analysis of isolated clones and verification of inhibitor resistant phenotype.** In order to verify the phenotype of individually isolated clones from the evolved yeast population, samples of AJP50 fermentations from 7% w/v solids were plated onto YPD agar containing all 13 inhibitory compounds in order to obtain isolated colonies. Individual colonies from these plates were subcultured onto a second YPD plate for isolation. Individual colonies from the second

plate were inoculated into YPD broth containing all 13 inhibitory compounds and either plated for isolated colonies again, or used for growth curve experiments. Figure 2.7 depicts the growth curves of individual colonies plated for isolation on a series of two, three, or four plates, prior to inoculation for growth curve measurements. The results were similar for replicates within the same plating series and all individual growth curves for each type of plating regime are plotted as one line with an error bar depicting one standard deviation from the mean. Optical density of ≥1.2 by 24 h in YPD broth with all 13 inhibitory compounds indicates resistance to the inhibitory compounds. XR122N did not grow in YPD broth or agar containing all 13 inhibitory compounds and is omitted from the graph (Figure 2.7).

**Stability of the AJP50 inhibitor resistant phenotype.** To determine if AJP50 would retain its phenotype during routine culturing, the strain was cultured on YPD media without inhibitory compounds. The ability of the resulting culture to grow in inhibitory media was then assessed. After culturing on rich solid and liquid media, AJP50 maintained resistance to the effects of inhibitors found in lignocellulosic biomass fermentations (Table 2.4). After 24 h growth, 14 of 100 cultures had an optical density greater than 1.5 and 40 were between 1.2 and 1.5. Optical densities of this level indicate resistance to the inhibitory compounds. XR122N cultures uniformly have ODs less than 0.3 after 24 hours of growth under these conditions. Only 6 cultures had optical densities below 0.3, indicating only a few cultures displayed no resistance after culturing on YPD media. After 30 hours of growth, the number of cultures with an optical density greater than 1.5 grew to 43; at this time point only 2 cultures possessed optical densities less than 0.3 and 91% of the cultures had optical densities greater than 1.2.

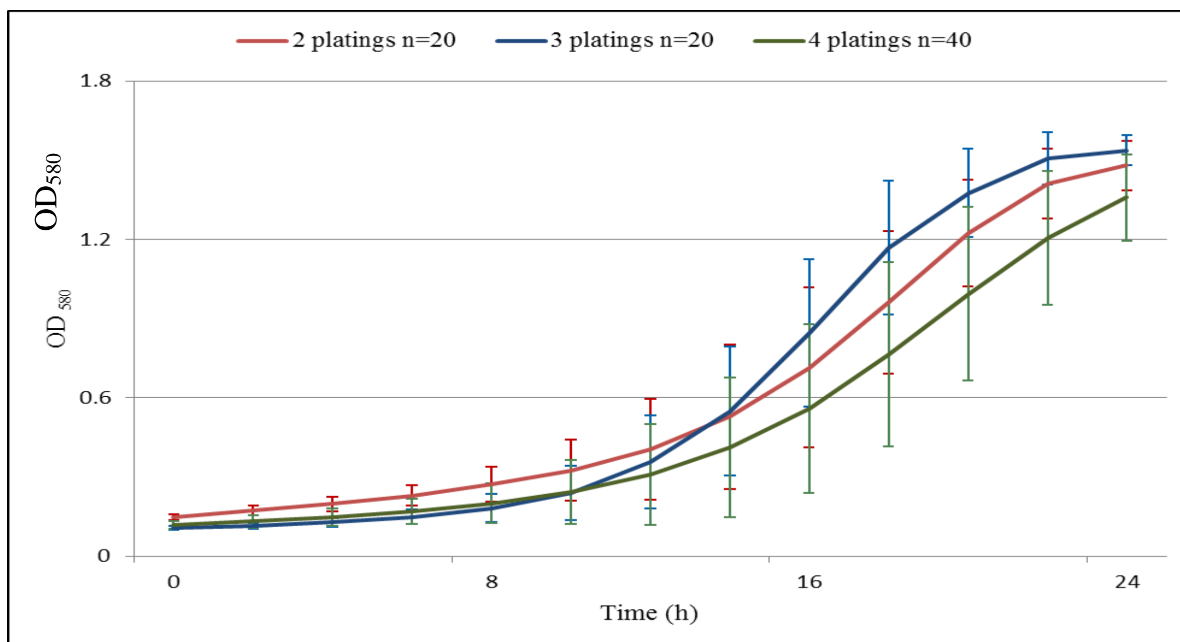



**Figure 2.7.** Verification of phenotype in individually isolated clones from the evolved yeast population. Samples from AJP50 fermentations plated onto YPD agar containing all 13 inhibitory compounds yielded isolated colonies. Individual colonies were then subcultured on additional inhibitor containing YPD agar once more (2 platings), twice (3 platings), or 3 more times (4 platings), as described in the methods. Isolated colonies were used for growth curve measurements in YPD broth containing all 13 inhibitory compounds. Growth curves for each series of platings was plotted as a single line with error bars representing one standard deviation from the mean (2 platings, n=20; 3 platings, n=20; 4 platings, n=40). XR122N did not grow in YPD broth or agar containing the 13 inhibitory compounds and is not represented on the graph.

**Table 2.4.** $OD_{580}$ of AJP50 cultures in inhibitory media after growth on rich media

| $OD_{580}$ | 24hr | 30hr | 48hr |
|---|---|---|---|
| 0.0 - < 0..3 | 6* | 2 | 2 |
| 0.3 - <0.6 | 5 | 1 | 0 |
| 0.6 - < 0.9 | 13 | 2 | 0 |
| 0.9 - < 1.2 | 22 | 4 | 6 |
| 1.2 - < 1.5 | 40 | 48 | 50 |
| ≥1.5 | 14 | 43 | 42 |

*Values are a percentage of the cultures out of 100 replicates

**ROS in AJP50 and XR122N cultures incubated with inhibitory compounds.** Both XR122N and AJP50 suffer considerable damage from reactive oxygen species when revived in media

from glycerol stocks at -80°C. AJP50 is able to recover from this damage more rapidly than XR122N in the presence of inhibitory compounds found in biomass fermentations (Figure 2.8). XR122N is able to reduce its level of ROS in the absence of inhibitory compounds, but is only

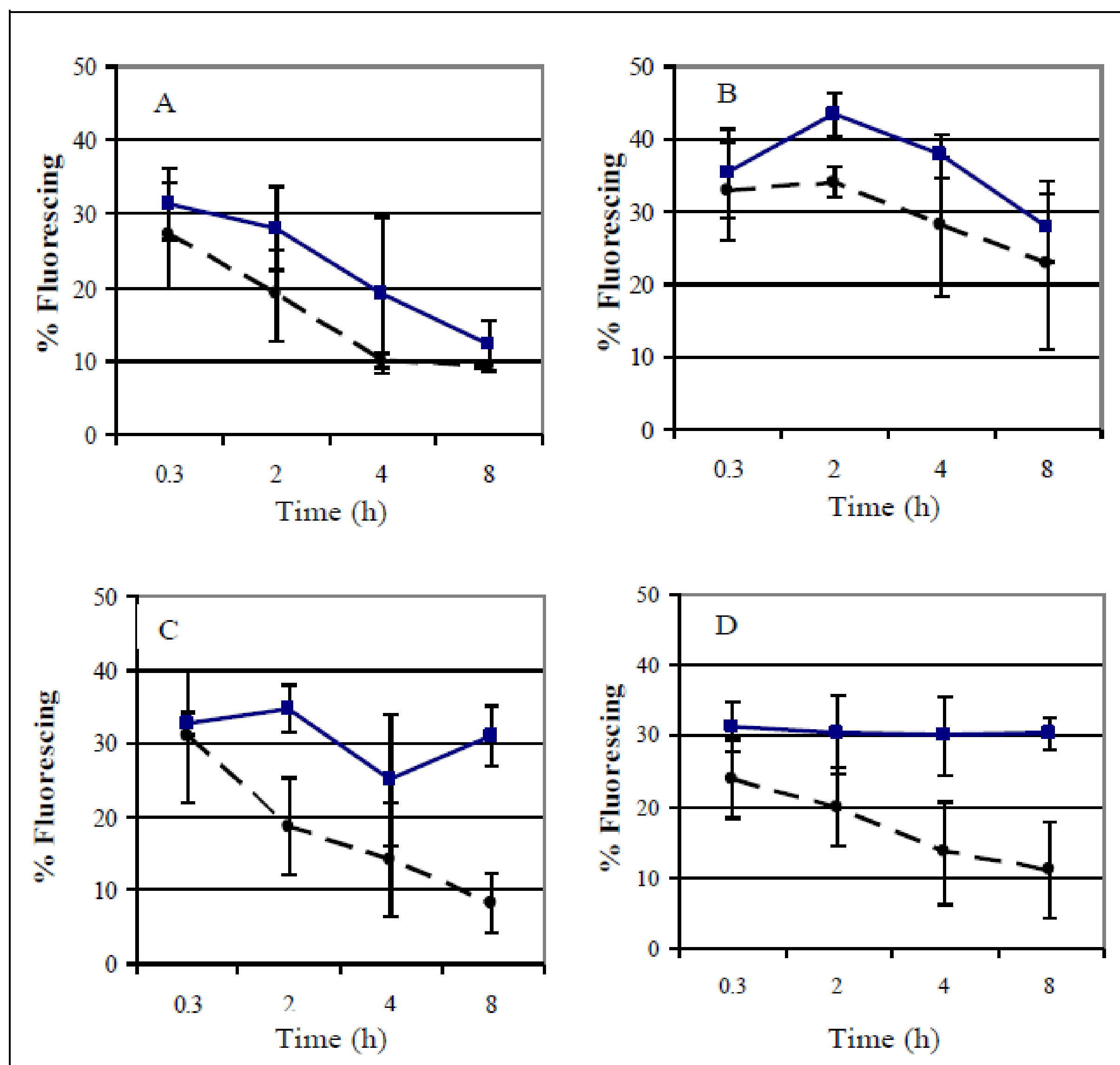



**Figure 2.8.** Levels of reactive oxygen species in XR122N and AJP50 cultures grown in media containing biomass inhibitors. XR122N is represented by solid blue lines and squares, AJP50 is represented by dashed black lines and circles. Panel A represents data from an uninhibited control, B from a culture grown in 5mM $H_2O_2$, C from HMF, furfural, and acetic acid media, D from media containing all 13 inhibitors listed in table 2.1. Error bars represent 1 standard deviation from the mean.

able to slightly alleviate the ROS damage in the presence of inhibitors. In the presence of all 13 inhibitors and the mixture of HMF, furfural, and acetic acid, AJP50 experiences similar levels of ROS to what is seen in the absence of these compounds. XR122N and AJP50 experience similar levels of recovery from reactive oxygen species in the uninhibited and $H_2O_2$ controls, indicating that the faster reduction of ROS by AJP50 in the inhibitory media is related to the presence of the inhibitory compounds.

## 2.4 METHODS

**Pretreatment of pine wood biomass.** Loblolly pine from Georgia, USA was debarked and chipped to a particle size of 10mm or less and the chips were pretreated with gaseous sulfur dioxide [155, 156] and subjected to steam explosion in a Process Development Unit (PDU) located at the Chemical Engineering Department at Lund University, Sweden, or a similar PDU located at the Georgia Institute of Technology under the direction of C2Biofuels (Atlanta, GA). A known weight of chips was pretreated with 3.3% $SO_2$ (w/w moisture content of chips) and held at 215°C for 5 min in the PDU in a single step process. The resulting material consisted of a mixture of liquids and solids and these phases were not separated, pressed, drained, nor washed to remove potentially inhibitory compounds. Materials were stored at 4° C until use.

**Compositional analysis.** Determination of structural carbohydrates, lignin, sugars, byproducts, and degradation products were determined using NREL Biomass Program methods. Moisture content was determined using an IR-35 Moisture Analyzer (Denver Instrument, Denver, Colorado), and all fermentation loadings were determined on a dry matter basis, referred to as percent dry weight per volume (w/v) of pretreated pine. Samples were analyzed by HPLC (Shimadzu, Kyoto, Japan) with refractive index detection, essentially previously as described [157]. Briefly, monomeric sugars were separated using an Aminex HPX-87P column (Bio-Rad,

Hercules, CA), at 80°C, flow rate of 0.5 ml/min, water mobile phase. Samples were filtered prior to analysis. Percent fermentable carbohydrates were defined as the sum of the estimated cellulose and hemicellulose values. Although yeasts used in these studies do not ferment pentose sugars, the low xylose content of the pretreated pine was included in the theoretical yield calculation and included in the "fermentable carbohydrate" total. Percent maximum theoretical yield was calculated from the total fermentable carbohydrate x dry weight of pine x 0.53 (molecular ratio of ethanol/ polymer carbohydrate) x 0.9 for conversion efficiency of 6C sugars.

**Pretreated pine fermentations with XR122N.** Fermentations were performed in small-scale bioreactors with a working volume of 200mL using pretreated pine wood biomass as feedstock essentially as previously described [157]. The percent moisture was determined using an IR-35 Moisture Analyzer and samples containing 5, 10, and 12% w/v dry solids were weighed, added to a 500 ml flask and autoclaved at 121°C for 20 minutes to ensure sterility (although this could be considered an additional pretreatment). Upon cooling, double strength tryptic soy broth (TSB) (Difco, Detroit, MI), containing 15 g pancreatic digest of casein, 5 g papaic digest of soybean meal, and 5 g NaCl per liter, and sterile water were added and the pH adjusted to 5.0 with 2M KOH. *Saccharomyces cerevisiae* XR122N (North American Bioproducts Corporation, Duluth, GA) was inoculated in a freeze dried state at an initial concentration of 4 g/L cell dry weight (cdw) similar to its use in corn ethanol fermentations. Cellulases and cellobiase (Novozymes Inc., Franklinton, NC) were added simultaneously with the inoculum at concentrations of 15 filter paper units (FPU) and 60 cellobiase units (IU) per gram dry weight (gdw) of pretreated pine, respectively. Fermentations were maintained at 35°C and pH 5.0, sampled every 24 h, and ethanol concentration estimated using gas chromatography as previously described [158]. All fermentations were performed in triplicate and error bars

represent one standard deviation from the mean. Inoculation of pretreated pine at 10, and 12% w/v solids loading was performed using 0.2 g/L, 0.5 g/L, 1 g/L, 2 g/L, and 4 g/L cdw.

**Evolutionary Adaptation of XR122N.** A 2 g/L cdw inoculum of XR122N was added to pretreated pine fermentations at a 17.5% w/v solids loading for SFF at 37°C and pH of 5.0. The fermentation was allowed to proceed for 168 h and aliquots equal to 10% v/v were transferred to fresh fermentations containing 17.5% w/v solids, enzymes, and TSB as described previously. Measurements of cell biomass using optical density readings or cell dry weights were not possible due to the particulate matter present from the pretreated biomass, therefore, cultures were monitored for ethanol production every 24h. Cultures were plated during transfer to the fresh 17.5% w/v solids fermentation and were approximately equivalent to 1 g/L cdw. After no ethanol was detected at 96 h, an additional 2 g/L cdw of XR122N cells were added to the fermentation vessels. Ethanol production was measured every 24 hours and ethanol concentrations in one of the fermentation vessels and continued to increase for an additional total of 72 h. A 10% v/v inoculum was removed from the fermentation vessel where ethanol production was detected and used to inoculate a third fermentation vessel containing 17.5% w/v pretreated pine and enzymes. Ethanol production was measured every 24 h and no additional ethanol was produced after 96 h of fermentation. Again another 2 g/L cdw of XR122N was added to the fermentation. This process of inoculating a 17.5% w/v solids fermentation with a 10% v/v inoculum from a previous fermentation, monitoring ethanol production for 96 h without observing an increase in ethanol content, and adding 2 g/L cdw of XR122N was repeated for a total of six full cycles. During the seventh cycle ethanol production increased by 24 h, and continued to increase at 48 h. At 48 h of fermentation a 10% v/v inoculum was transferred to a fresh 17.5% w/v solids fermentation and ethanol production monitored. Samples from this

fermentation were frozen in glycerol at -80°C and designated AJP40. A similar set of fermentations using 20% w/v solids failed to produce high concentrations of ethanol, even after the addition of 2 g/L cdw of XR122N.

Glycerol stocks of AJP40 were subjected to additional transfers in 17.5% w/v solids loading of pretreated pine. Inoculation of AJP40 into 17.5% w/v solids directly produced little ethanol. Inoculation of AJP40 glycerol stocks (approximately 0.2 g/L cdw) into a 7% w/v solids fermentation resulted in maximum ethanol production after 24 h of fermentation and a 10% v/v aliquot was used to inoculate a 17.5% w/v solids fermentation. Ethanol production was observed at 48 h and upon transfer of a 10% v/v inoculum into a 17.5% w/v solids fermentation, ethanol was detected after 24 h. Additional transfers into 17.5% w/v solids were made by removing a 10% v/v inoculum from one 17.5% solids fermentation producing ethanol after 48 h to a new flask containing 17.5% w/v solids and enzymes for saccharification. Transfers were made every 48 h for a total of 50 transfers. Aliquots from the final ($50^{th}$) fermentation were frozen in glycerol stocks and designated AJP50.

**Growth in combinations of inhibitory compounds.** Stock solutions of each inhibitor were prepared fresh on the day they were to be used. Typical compounds found in pretreated pine wood were grouped by inhibitor class and were examined in various mixtures. Weak acids used were: acetic, formic, levulinic, lactic, and succinic. Aromatics examined were: 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde, vanillic acid, vanillin, and benzoic acid. Furans used were: furfural, 5-hydroxymethylfurfural (HMF), and 2-furoic acid. Effects of all 13 compounds were also examined simultaneously, and a mixture of HMF, furfural, and acetic acid was also evaluated. Concentrations of each compound were similar to those observed in pretreated pine wood fermentations (Table 2.2).

Freezer stocks were created from 7% w/v pretreated pine wood fermentations for both AJP50 and XR122N. Freezer stocks were revived briefly (<10 minutes) in 9 ml TSB media and microscopic cell counts performed with a hemocytometer were used to standardize the initial concentration of 4.0 x $10^5$ cells/ml in each well containing 20 g/L glucose and TSB media. The starting OD for XR122N appears higher than that of AJP50 due to the presence of more particulate matter in the original inoculum from the freezer stocks. A larger volume of material was required to obtain an initial cell concentration of 4.0 x $10^5$ cells/ml for XR122N. The initial pH of each well was 5.0 and temperature was maintained at 37°C in a Bioscreen C Growth Curves machine (Oy Growth Curves Ab Ltd. Helsinki, Finland) without shaking. Optical density of the wells was recorded every 30 min at 580nm. Each well was replicated on the plate five times and used to calculate an average and standard deviation.

**Ethanol production in model media containing various combinations of inhibitory compounds and glucose as the carbon source.** Ethanol production was measured by inoculating wells of a plate with the inhibitor stock to be studied and the culture of interest as described above. Ethanol samples were taken in triplicate at each time point. Ethanol was sampled every 6 h by removing the plate and pipetting the full 300 µl volume of the appropriate wells into separate 0.22 µm centrifuge filtration tubes. Tubes were then centrifuged at 14000 rpm for 1 min at room temperature before being frozen at -20°C until further analysis. Ethanol concentration in the samples was determined using gas chromatography as previously described [158].

**Conversion of furfural and hydroxymethylfurfural to alcohol derivatives.** Samples were evaluated for conversion of furfural and hydroxymethylfurfural at 6 h intervals in fermentations

described above. Furfural, hydroxymethyl furfural, furfural alcohol, and hydroxymethylfurfural alcohol concentrations were determined using HPLC essentially as previously described [159].

**Analysis of isolated clones and verification of inhibitor resistant phenotype.** AJP50 glycerol stocks from the directed evolution were used to inoculate 7% w/v pretreated pine solids fermentations and incubated at 37°C for 24 h with shaking. Samples from the 7% w/v solids fermentation were removed and frozen in 40% w/v glycerol stock cultures. Aliquots of these glycerol stocks were revived in YPD containing all 13 inhibitory compounds and incubated for 24 h at 37°C with shaking. Isolated colonies were obtained by plating onto YPD agar containing all 13 inhibitory compounds and incubated at 37°C. Colonies took an average of seven days to appear on the plates containing all 13 inhibitory compounds. Individual colonies from these plates were subcultured onto a second YDP agar plate containing all 13 inhibitory compounds and were incubated at 37°C for approximately seven days. Isolated colonies from the second plate were used to inoculate YPD containing all 13 inhibitory compounds and incubated for 24 h at 37°C with shaking. Aliquots from this broth were used to inoculate individual wells containing 2 mL of TSB with the 13 inhibitory compounds for growth curve measurements. The initial pH of each well was 5.0 and temperature was maintained at 37°C in a Bioscreen C Growth Curves machine without shaking. Optical density of the wells was recorded every 30 min at 580nm. A second round of experiments involved selection of isolated colonies from a second YPD agar plate containing 13 inhibitory compounds and subculture onto a third YPD agar plate containing 13 inhibitory compounds. Isolated colonies from the third plate were then inoculated into YPD broth containing all 13 inhibitory compounds and used for growth curve experiments. A third set of experiments involved selection of isolated colonies from the third YPD agar plate and subculture for isolated colonies onto a fourth YPD agar plate. Isolated colonies were

inoculated into YPD broth containing all 13 inhibitory compounds and screened for growth as described previously. XR122N glycerol stocks from a 7% w/v solids fermentation were used in a similar fashion.

**Examination of the inhibitor resistant phenotype of AJP50.** AJP50 was cultured overnight on YPD agar media at 37°C and a single colony was used to inoculate a 50ml flask of liquid YPD media. The inoculated flask was incubated overnight at 37°C with shaking. The YPD liquid overnight culture was examined in the Bioscreen in the presence of all 13 inhibitors as before. All 100 wells of the plate were identical in media composition and initial inoculum level; the optical density of the wells was determined at 24, 30, and 48 h post inoculation to determine how well AJP50 retained its resistance to the inhibitors after culturing on rich media lacking any inhibitory compounds.

**Comparison of the effect of reactive oxygen species on XR122N & AJP50.** The effect that reactive oxygen species (ROS) had on XR122N and AJP50 was measured using 2' 7'-dichlorofluorescein diacetate (DCF) (Sigma). DCF fluoresces in the presence of ROS, as described by Allen and colleagues [141, 160]. XR122N and AJP50 were inoculated at $4.0 \times 10^5$ cells/ml from freezer stocks into 50 ml YPD media containing either: all 13 inhibitors; HMF, furfural, and acetic acid; 5mM $H_2O_2$; or no inhibitors. Cultures were maintained at 37°C with shaking and samples taken at indicated time points. Samples were examined for fluorescence using an Olympus BX61 reflected fluorescence microscope (Olympus Corp. Tokyo, Japan) with a FITC filter. For each time point, at least 100 cells were examined and the percent fluorescent determined. This percentage of cells exhibiting fluorescence reflects the portion of the cell population experiencing ROS damage.

## 2.5 CONCLUSIONS

A strain of *Saccharomyces cerevisiae* (XR122N) was evolved by continuous exposure to pretreated pine wood biomass to develop the daughter strain AJP50. Adding a preculture or short adaptation phase of 24 h in 7%w/v pretreated pine enhanced performance of the all strains, including AJP50. AJP50 more rapidly fermented pretreated pine wood biomass at a high solids loading than its parent, or other *Saccharomyces* strains reported in the literature. Growth comparisons between XR122N and AJP50 in a model hydrolysate medium containing inhibitory compounds found in pretreated biomass revealed AJP50 exited lag phase faster under all conditions tested. This ability is due, in part, to AJP50 rapidly converting furfural and hydroxymethylfurfural to their less toxic alcohol derivatives and recovering from reactive oxygen species damage more quickly than XR122N. Under industrially relevant conditions of 17.5% w/v pretreated pine solids loading, additional evolutionary engineering was required to decrease the pronounced lag phase. Using a combination of adaptation by inoculation first into a solids loading of 7% w/v for 24 h, followed by a 10% v/v inoculum (approximately equivalent to 1 g/L cell dry wt) into 17.5% w/v solids the final strain (AJP50) produced ethanol at >80% of the maximum theoretical yield after 72 h of fermentation and reached >90% of the maximum theoretical yield after 120 h of fermentation.

Our results demonstrate that fermentations of pretreated pine containing liquid and solids, including any inhibitory compounds generated during pretreatment, are possible at higher solids loadings than previously reported in the literature. These fermentations used reduced inoculum sizes and demonstrated shortened process times, thereby improving the overall economic viability of a pine–to-ethanol conversion process. Results from current studies characterizing the

stability of the strain and demonstrating performance under conditions used with industrial processes (e.g. after lyophilization) will be important for use of AJP50 in industrial applications.

## 2.6 ACKNOWLEDGEMENTS

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# CHAPTER 3

# PRODUCTION OF ETHANOL FROM HIGH DRY MATTER OF PRETREATED LOBLOLLY PINE BY AN EVOLVED STRAIN OF *SACCHAROMYCES CEREVISIAE*[1]

[1]Hawkins, Gary M., D. Ghose, J. Russel, and J. Doran-Peterson. 2013. *Journal of Bioremediation & Biodegradation.*4:6 Reprinted here with permission of the publisher.

## 3.1 ABSTRACT

Obtaining the highest possible yields from a batch fermentation process is desirable for a cellulosic ethanol facility, as this leads to greater profitability by increasing distillation efficiency and reducing costs. One way to increase yields is by increasing the percentage of solids fermented in a batch process which leads to increased fermentable sugars available and thus increased theoretical maximum yields. One factor limiting the solids loading is that during biomass pretreatment a number of inhibitory compounds are released. These confound the fermentation process in a variety of ways, and as the solids concentration increases so do the concentrations of these inhibitors. Therefore a biocatalytic strain that is able to ferment high concentrations of pretreated biomass and withstand the inhibitors present in the fermentation media is desirable. Pine wood has proven to be a particularly difficult biomass type from which to obtain high ethanol titers when fermenting concentrations of solids much greater than 10% dry weight. We previously described a strain of *Saccharomyces cerevisiae,* AJP50, which is able to ferment high concentrations (17.5% dry wt/v) of sulfur dioxide steam exploded pine wood. Present research details the performance of four isolates of AJP50 and their ability to produce ethanol from pretreated pine. We report ethanol yields of over 50 g/l (91% of maximum theoretical yield) from 22.5% dry wt/v of pine fermented using a simultaneous saccharification and fermentation process with strain GHP4 as the biocatalyst.

## 3.2 INTRODUCTION

Cellulosic ethanol represents a potential replacement liquid transportation fuel to offset the use of petroleum derived gasoline [135, 161]. Pine wood represents a potential feedstock that could be used to generate fuel and is widely available, particularly in the northern hemisphere

[50, 162]. Pine wood has been an important forestry resource for both the lumber and pulp and paper industries; the current value of sawtimber is $25/ton while pulpwood is $10/ton [163]. The quality of the wood is considerably less important for biofuel production than for lumber production, thus prices for an ethanol production facility would be closer to and potentially lower than pulpwood prices [51, 54]. The infrastructure and technology for the growth and harvest of this feedstock is already in place allowing for the rapid implementation of pine feedstocks into biofuel pipelines [164]. Pine wood is not without disadvantages to its use as a bioethanol feedstock, one major hurdle to overcome is the release of inhibitory compounds during the pretreatment of the biomass which are then carried over into the fermentation process.

During pretreatment a variety of chemical compounds are released that can inhibit the activity of the biocatalytic organism in many ways [137, 165]. The wide variety of inhibitory compounds released is due to the complex structure of biomass [166]; these can be sorted into three general categories: furans, aliphatic acids, and aromatics. The effects of furans and the aliphatic acids are better understood than the effects of aromatics, possibly due to the greater variety of aromatic compounds produced during pretreatment. This variety is due to the complex and variable structure of lignin. These different compounds have been shown to inhibit cellular metabolism [84, 97], destabilize membranes [102], cause reactive oxygen species damage [141], and acidify the cytoplasm [138]. The presence of these inhibitors is a major factor confounding ethanol production from high concentrations of pretreated biomass; a barrier to an efficient biomass based ethanol fermentation production process [154]. Fermentation of a high concentration of solids could increase maximum ethanol yields and lower process costs by increasing distillation efficiency. However, a high solids loading also brings with it high concentrations of the aforementioned inhibitory compounds, so the development of a strain that

is able to tolerate high concentrations of inhibitory compounds, in addition to the high solids content, is desirable in a cellulosic feedstock based process [75, 165].

To overcome these challenges we developed *Saccharomyces cerevisiae* strain AJP50 [30]. This strain was generated by adaptation and directed evolution from industrial strain XR122N (North American Bioproducts Corportation, Duluth, GA) and is able to catalyze the fermentation of high solids of sulfur dioxide pretreated pine wood [30]. We previously described the ability of AJP50 to withstand the presence of a number of these compounds in defined model fermentation media and its ability to efficiently produce ethanol from 17.5% dry wt/vol of pine. In order to address the question of the maximum solids fermentable by our strain we developed a medium to ensure the retention of the AJP50 phenotype during culturing and isolation; allowing for a large inoculum to be prepared in 24 h. Isolates of AJP50 were then inoculated into various high solids pine fermentations to determine the maximum ethanol titers which could be obtained using a simultaneous saccharification and fermentation process.

## 3.3 METHODS

**Cell Growth and Maintenance.** Methods used to obtain the AJP50 isolates described in this study have been published previously [30]. YPD media (20 g/l peptone, 10 g/l yeast extract, 20 g/l glucose) supplemented with all 13 inhibitors (YPDI media, Table 3.1) was used throughout this study. In brief, AJP50 glycerol freezer stocks were first inoculated into YPDI broth at 4.0 x $10^5$ cells/ml and incubated at 37°C for 24 h before being inoculated onto YPDI agar. This was incubated at 37°C for seven days at which point individual, isolated colonies were inoculated into YPDI broth media. Cell culture samples were frozen at -80°C in 40% w/v glycerol and designated GHP 1, GHP2, GHP 3, and GHP 4. YPDI broth cultures to be used to inoculate pine

fermentations and growth curve experiments were inoculated directly from glycerol freezer stocks at 2.0 x $10^6$ cells/ml and incubated for 24 h at 37°C with 200rpm shaking, reaching cell densities of greater than 5.0 x $10^7$ cells/ml.

**Table 3.1.** Concentrations (g/L) of each inhibitory compound in YPDI

| **FURANS** | | **AROMATICS** | | **ACIDS** | |
|---|---|---|---|---|---|
| HMF[a] | 2.000 | 3,4-DHBA[b] | 0.003 | Formic Acid | 0.400 |
| Furfural | 1.000 | 3-HBA[c] | 0.005 | Lactic Acid | 0.100 |
| Furoic Acid | 0.020 | Vanillic Acid | 0.050 | Acetic Acid | 2.000 |
| | | Vanillin | 0.020 | Succinic Acid | 0.030 |
| | | Benzoic Acid | 0.015 | Levulinic Acid | 0.400 |

[a]HMF : hydroxymethylfurfural
[b]DHBA: dihydroxybenzaldehyde
[c]HBA: hydroxybenzaldehyde

**Pine fermentation experiments.** Pine wood chips were pretreated in a single step $SO_2$ steam explosion reactor [155] prior to fermentation as described previously [30]. The name of the sample indicates pretreatment conditions; for example pine sample 3-210-10 was pretreated with 3% (w/v) $SO_2$ then held at 210°C in the process reactor for 10 min. All pretreated pine wood samples were stored at 4°C without any washing, pressing, or other method of inhibitor abatement. Three samples were chosen for fermentation in this study. Sample A was treatment 3-210-10, sample B was treatment 2.5-213-5, and sample C 5-217-2.

The moisture content of the biomass was determined using an IR-35 Moisture Analyzer (Denver Instrument, Denver, Colorado) and a mass equivalent to the desired dry weight placed into baffled 125ml flasks and autoclaved for 20 min at 121°C (which could be considered an additional pretreatment of the biomass performed for all samples). Prior to cell inoculation, cellulolytic enzymes (Novozymes Inc., Franklinton, NC) at 15 FPU / g dry wt pretreated pine and 60 CBU cellobiase /g dry wt pretreated pine along with tryptic soy broth (TSB) media

without dextrose (Difco, Detroit, MI) were added to the flask and brought to a final volume of 50 ml with sterile water. Cellulolytic enzymes were combined, diluted in TSB media then filter sterilized via 2 micron filters. Hemocytometer readings from YPDI grown cell cultures were used to estimate the number of cells/ml. The appropriate volume of culture was removed to sterile centrifuge bottles and centrifuged at 5,000 rpm for 15 min before inoculation into the fermentation media at an initial concentration of 2 x $10^7$ cells/ml. Fermentations were maintained in baffled flasks at 37°C, pH 5.0, with 200rpm shaking over the course of the experiment.

Fermentations of 22.5% dry wt/v were also performed in 150ml total volume using small scale bioreactors [157]. Mixing was via magnetic stir bars as opposed to the orbital shaker used for the shake flasks. Other than the increased volume and magnetic stirring all conditions for bioreactor fermentations were identical to those in the shake flask fermentations.

Structural analysis of the pretreated pine wood samples has been previously described [30]. Table 3.2 lists the pretreatment conditions, percent cellulose and hemicellulose, and theoretical maximum yields at 17.5, 20, and 22.5% dry weight solids for all pine samples used in this study. Theoretical maximum yields were calculated as follows: total fermentable carbohydrate (cellulose + hemicellulose) x dry weight of pine x 0.53 (molecular ratio of ethanol/ polymer carbohydrate) x 0.9 for conversion efficiency of 6C sugars. Samples were taken for analysis from each fermentation at the indicated time points, and the ethanol concentration was determined using gas chromatography as previously described [157].

**Table 3.2.** Composition and theoretical maximum ethanol titers (g/L) for pine samples

| Pretreatment (%$SO_2$-°C-Minutes) | Cellulose[a] | Hemicellulose[a] | Total Fermentable Carbohydrates[a] | Maximum Ethanol 17.5%[b] | Theoretical Conc. 20%[b] | 22.5%[b] |
|---|---|---|---|---|---|---|
| Sample A (3-210-10) | 29.7 | 4.4 | 34.1 | 28.5 | 32.5 | 36.6 |
| Sample B (2.5-213-5) | 39.2 | 12.3 | 51.5 | 43.0 | 49.1 | 55.3 |
| Sample C (5-217-2) | 31.6 | 10.7 | 42.3 | 35.3 | 40.4 | 45.4 |

[a]as percentage of dry material
[b]dry wt/vol

**Inhibitor growth assays.** Growth of the strains in model fermentation media was performed using a Bioscreen C machine (Oy Growth Curves Ab Ltd. Helsinki, Finland). One hundred well microtiter plates were used in these experiments. Media in each well consisted of TSB media, 13 inhibitors at either the concentration listed in Table 3.1 (1x) or 1.2 times this concentration (1.2x), 2% w/v glucose, with an initial pH 5.0. Cells were counted after 24 h growth in YPDI media using a hemocytometer and 4.0 x $10^5$ cells/ml were inoculated into each well. Twenty replicate wells were prepared from each culture. The plates were incubated at 37°C without shaking or agitation; culture optical density was measured hourly at 580nm.

## 3.4 RESULTS AND DISCUSSION

**12 and 17.5% dry wt/vol fermentations.** We previously described two phenotypes of *Saccharomyces cerevisiae* strain AJP50, the ability to ferment high concentrations of pine solids, and the ability to grow in media that contained biomass-derived fermentation inhibitors. In order to assess the retention of the first phenotype two sets of fermentations were performed with four clonal populations isolated from AJP50 named strain GHP1, GHP2, GHP3, and GHP4. In initial experiments, the isolates were inoculated at a low concentration of cells, 4.0 x $10^5$ cells/ml, into 12% dry wt/vol of sample A (Figure 3.1A). All four isolates produced essentially 100% of the

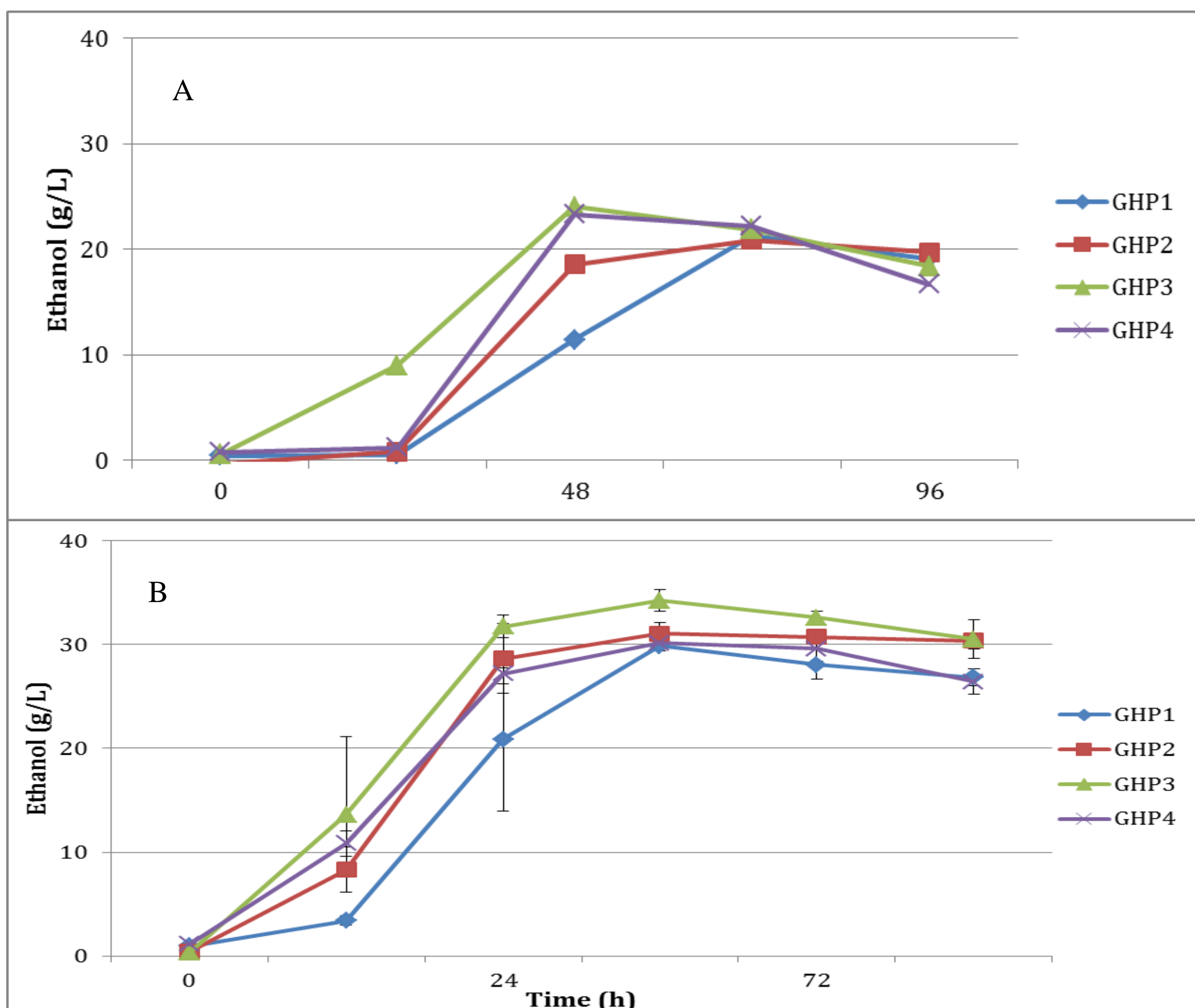


**Figure 3.1.** Ethanol production observed in fermentations of pine sample A by the four GHP isolates. Panel A shows data from two replicate 12% dry wt/vol fermentations which had good agreement. These fermentations were inoculated with 4.0 x $10^5$ cells/ml. Panel B shows data from triplicate 17.5% dry wt/vol fermentations with error bars representing one standard deviation from the mean (no standard deviation was calculated for panel A as only two replicates were performed). These fermentations were inoculated with 2.0 x $10^7$ cells/ml.

theoretical maximum ethanol yield in these experiments. These four isolates were selected for inoculation in higher solids, 17.5% dry wt/vol, fermentations of sample A. These fermentations received a higher inoculum of 2.0 x $10^7$ cells/ml, which is approximately 2 g dry wt/l, an industrially relevant inoculum level. As in the 12% dry wt/vol fermentations, all four isolates performed well reaching essentially 100% of the theoretical maximum (Figure 3.1B).

Based on their performance at 17.5% dry weight solids we concluded that all four isolates retained the ability to ferment high concentrations of pine wood after culturing in YPDI media. In order to obtain higher ethanol titers, pine sample B was selected for fermentation. This sample contains higher concentrations of fermentable carbohydrates than sample A; resulting in higher theoretical maximum ethanol yields (Table 3.2). These fermentations were performed using only isolates GHP1 and GHP4. Both isolates were able to produce over 30 g/L of ethanol, but neither reached the 100% maximum theoretical yield observed with sample A. GHP1 reached 80% of maximum theoretical yield and GHP4 66% of maximum theoretical yield (Figure 3.2). Ethanol titers were not significantly increased over what was observed from 17.5% dry wt/vol sample A; to further increase the amount of fermentable sugars 20% and 22.5% dry wt/vol fermentations were performed. If the percentage of the theoretical maximum amount of ethanol obtained remained constant, the increase in percentage dry weight fermented would allow for higher ethanol titers.

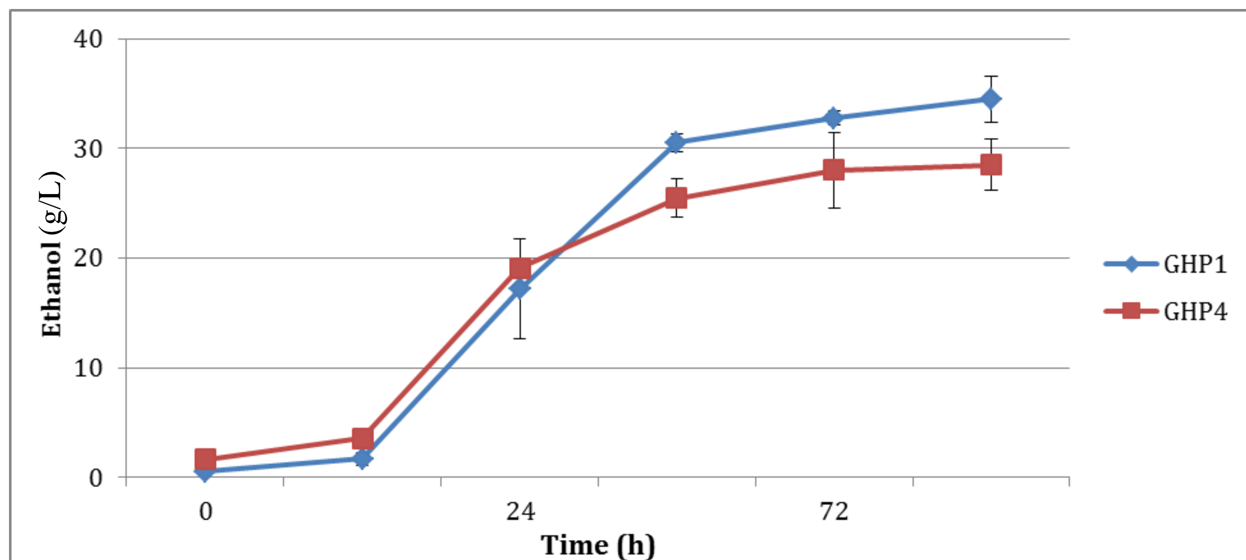


**Figure 3.2.** Ethanol production from 17.5% dry wt/vol pine sample B by GHP1 and GHP4. Both were inoculated at 2.0 x $10^7$ cells/ml, and data represents the average of three replicate fermentations with error bars displaying the standard deviation from the mean.

**20% dry wt/vol fermentations with GHP1 and GHP4.** 20% dry wt/vol fermentations were performed using pine samples B and C. Sample C was selected because it had performed favorably in previous experiments (data not shown) and had 42.3% fermentable sugars. Although ethanol was produced from both pine samples at 20% dry wt/ vol, neither reached greater than 85% of the theoretical maximum ethanol yield (Figure 3.3). For sample B, GHP1 reached 64% of maximum theoretical yield, a slightly lower ethanol titer than was observed in 17.5% dry wt/vol of the same pine sample. Isolate GHP4 was able to produce 69% of maximum theoretical yield, an increase of 3% from the 17.5% fermentations. For sample C higher percentages of the maximum theoretical were realized but ethanol titers remained similar to those observed in 20% dry wt/vol of sample B, reflecting the lowered amounts of fermentable sugar. GHP1 produced 75% of maximum theoretical yield and GHP4 82%. In order to further increase fermentable sugars in the fermentation media 22.5% fermentations were attempted with GHP4.

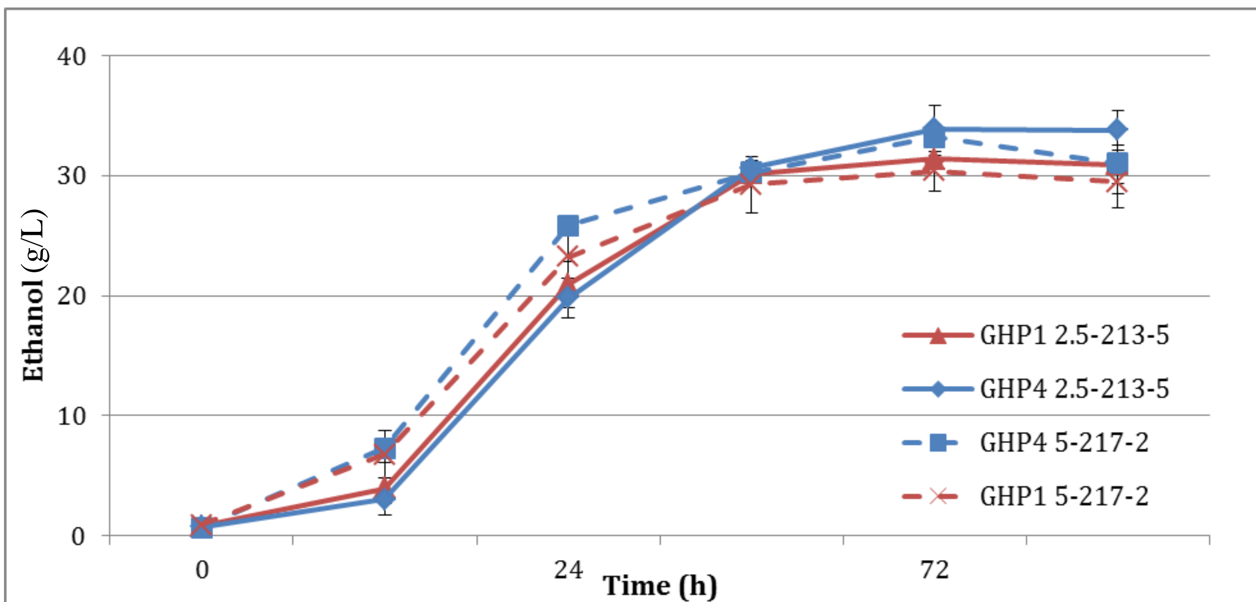


**Figure 3.3.** Ethanol production in 20% dry wt/vol fermentations of either pine sample B (solid lines) or C (dashed lines). Data represents the average of three replicate fermentations with error bars displaying the standard deviation from the mean.

**22.5% dry wt/vol fermentations.** Sample B was selected for fermentations at 22.5% dry wt/vol as it possessed the highest concentrations of fermentable carbohydrate and therefore the greatest theoretical amounts of ethanol could be produced from this sample. As in the 20% dry wt/vol, ethanol production was observed in shake flask fermentations of 22.5% dry wt/vol Sample C (Figure 3.4). The ethanol titer was almost identical to that observed in 20% fermentations, resulting in a decrease in the percentage of the maximum theoretical yield from 69% to 62%. The stalling ethanol titers as the dry weight of pine fermented increased caused concerns that the biomass was being insufficiently mixed with the cellulolytic enzymes; leading to incomplete sugar release from the biomass. In order to promote more efficient mixing of the slurry a bioreactor equipped with a magnetic stir bar was used in 150ml fermentations of 22.5% dry wt/vol. These bioreactor fermentations outperformed the shake flasks reaching just over 50g/l (91% of the maximum theoretical yield) of ethanol (Figure 3.4). These results showed inefficient mixing may be responsible for lower than optimal ethanol yields observed in shake flasks, and strain GHP4 is able to efficiently ferment high loadings pretreated pine with adequate mixing.

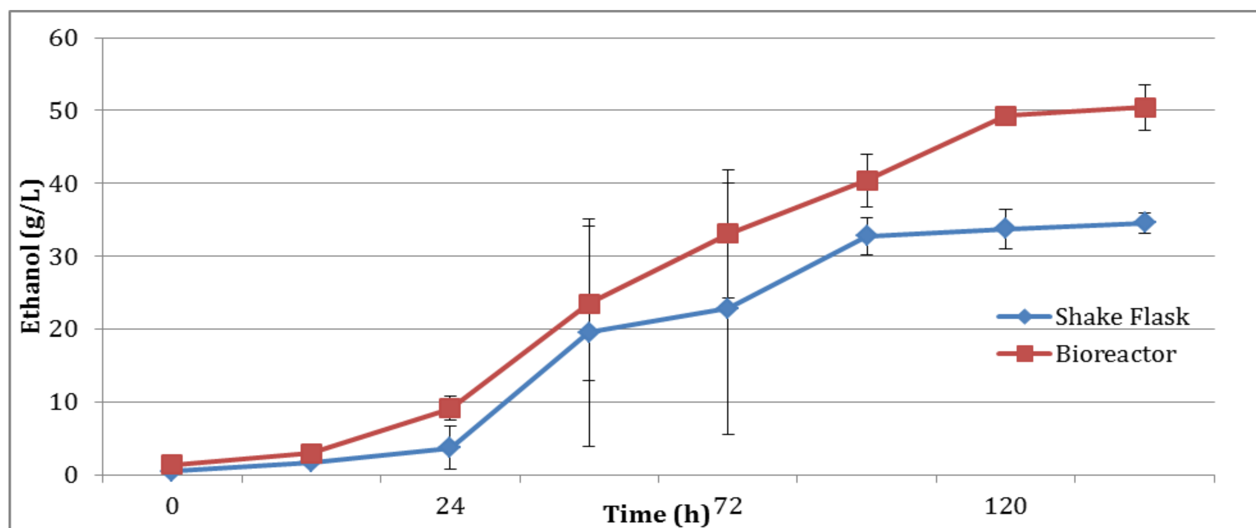


**Figure 3.4.** Fermentation of 22.5% dry wt/vol sample B by isolate GHP4 in either shake flasks with orbital shaking or bioreactors with magnetic stirring.

**Growth in model fermentation media.** To assess the inhibitor tolerances of GHP1 and GHP4 after 24 h of grown in YPDI growth curves were constructed using YPDI grown cultures of these strains. When inhibitors of identical concentration (1x model fermentation media) to those listed in Table 3.1 were added to the media, all three strains showed similar growth (Figure 3.5A). The inhibitor concentrations in 1x model fermentation media were based on 12% dry wt/vol fermentations; in order to better reflect what may be found in higher solids fermentations the concentrations of inhibitors added into the media were increased. In media containing 1.2 times the concentrations in Table 3.1, GHP1 and GHP4 reached maximal optical density at 50h; later than what was observed at 1x concentration (Figure 3.5B). These data suggests that GHP1 and GHP4 retain the inhibitor resistant growth phenotype of AJP50.

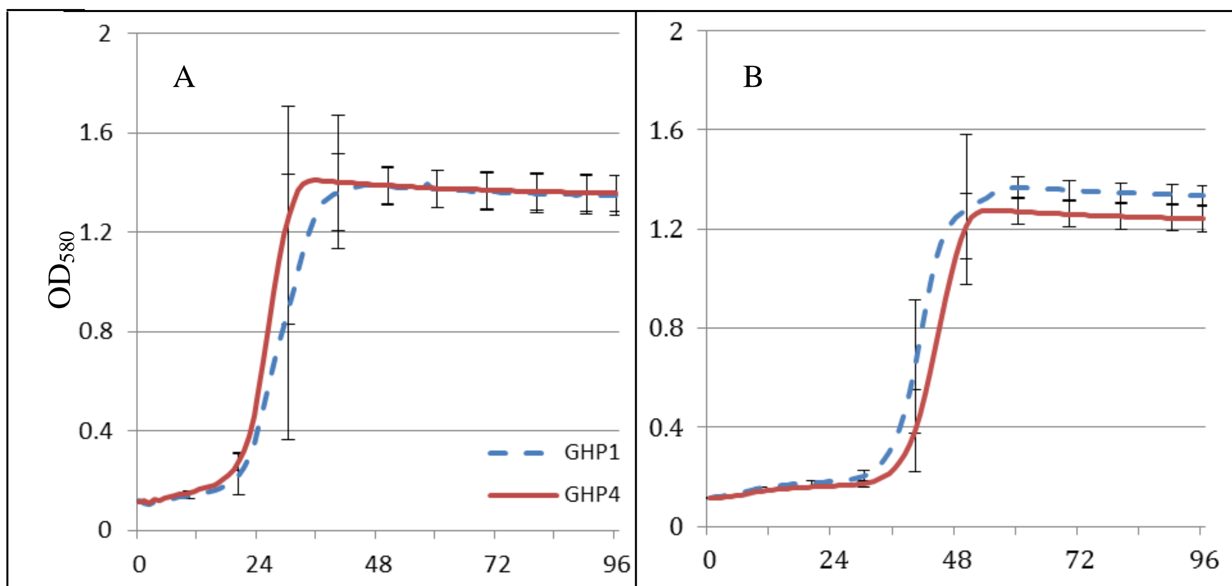


**Figure 3.5.** Performance of YPDI grown GHP1 and GHP4 in either 1x (A) or 1.2x (B) model fermentation media. Data represents the average optical density of twenty replicate cultures with error bars showing standard deviation from the mean.

## 3.5 CONCLUSIONS

Four isolates obtained from AJP50 populations on inhibitor supplemented media retain the desirable characteristics of the original strain when cultured for 24 hours in YPDI media. At least two of these strains also retain the ability to efficiently grow in defined media containing selected compounds typically found in pine wood biomass fermentations. Isolate GHP4 is able to produce ethanol from 22.5% dry wt/vol of sulfur dioxide steam exploded pretreated pine wood at over 90% of the theoretical maximum, provided ample agitation of the pine slurry.

## 3.6 ACKNOWLEDGEMENTS

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# CHAPTER 4

# PHENOTYPIC STABILITY AND TRANSCRIPTOME SEQUENCING OF ISOLATES FROM AN EVOLVED *SACCHAROMYCES CEREVISIAE* STRAIN CAPABLE OF ETHANOL PRODUCTION IN VERY HIGH PINE WOOD BIOMASS FERMENTATIONS[1]

[1]Hawkins, Gary M., and Joy Doran-Peterson. To be submitted to: *Applied Microbiology and Biotechnology*.

## 4.1 ABSTRACT

We previously described a *Saccharomyces cerevisiae* strain capable of producing ethanol from high dry weight loadings of sulfur-dioxide pretreated loblolly pine. To address the stability of this phenotype we cultured isolates from the evolved strain in yeast extract peptone dextrose broth (YPD), a rich medium lacking any biomass or inhibitory compounds that could act as selective pressure on the cells to maintain their phenotype. These cells were then inoculated into high dry weight (17-22% w/v) solids fermentations of pine wood and their performance compared to cells grown with the selective pressure of inhibitory compounds in the media. Some isolates maintained the desired phenotype and were able to perform similarly when grown in rich or inhibitor-containing media; whereas other isolates have reduced performance in pine wood fermentations when propagated in rich media without inhibitors. In order to elucidate the genetic factors involved in the fermentation of high pine solids, the transcriptomes of two isolates grown in two different media were sequenced. One isolate was able to successfully produce ethanol from high pine wood solids after growth in both media types. The other isolate was able to produce ethanol from high pine wood solids only when grown in the presence of inhibitors. By comparing the sequenced transcriptomes of these inocula, we are able to identify a number of genes potentially important for fermentation of high concentrations of pretreated lignocellulosic biomass.

## 4.2 INTRODUCTION

There is growing interest world-wide in moving from a fossil fuel-based economy to one that is based on renewable sources of energy and industrial chemicals. Ethanol is a fuel or fuel-additive that can be generated from renewable lignocellulosic feedstocks via the fermentation of

sugars [1, 135, 167]. Pine wood represents a lignocellulosic feedstock that is abundant worldwide, particularly in the northern hemisphere. As pine has been an important forest product for both the lumber and pulp and paper industries the infrastructure and technology for growing and harvesting this feedstock are already in place; allowing for rapid implementation in biofuel processes [49]. Another advantage of pine is the low concentration of pentose sugars found in the feedstock. *Saccharomyces* strains cannot naturally ferment pentose sugars to ethanol, but they can use the relatively high concentration of mannose in pine as a carbon source. Thus *Saccharomyces* has an advantage in processes using pine as the feedstock.

One difficulty in the conversion of lignocellulosic feedstocks to ethanol is the production of inhibitory compounds during the pretreatment of the biomass prior to fermentation [137]. The biomass must be pretreated in order to make the cellulose fibers amenable to enzymatic digestion, which releases monomeric sugar for fermentation. The various inhibitory compounds are generally grouped based on structure as aliphatic acids, furans, or aromatics [85]. The aliphatic acids and furans are generated from hemicellulose and cellulose, and the aromatics are generated from lignin. During pretreatment a wide variety of chemicals are created that have negative effects on the cells' activity [75]. Different compounds have been shown to inhibit cellular metabolism [84, 97], cause reactive oxygen species damage [141], and acidify the cytoplasm [138].

It is beneficial to have high concentrations of biomass in the fermentation as this will increase the theoretical maximum ethanol yields, which if realized would decrease the cost of distillation and improve the economics of the process. However, as solids loadings increase so do the concentrations of inhibitory compounds. This creates a difficult and challenging environment for the microbial organisms attempting to ferment these biomass derived sugars. To overcome

this challenge it is necessary to have a strain that can ferment sugars efficiently when high concentrations of inhibitory compounds are present.

Directed evolution was used to generate *Saccharomyces* yeast strain AJP50 from an industrial strain, XR122N, by repeated transfer into fresh pine fermentations of increasing solids. AJP50 produced ethanol in pine fermentations where the performance of the parent strain was severely limited [30]. Methods used to culture isolates from AJP50 have been previously described [168]. Fifteen isolates from the AJP50 culture stocks all retained a greater level of inhibitor resistance than what is observed in XR122N cultures. To explore the stability of the described phenotype(s) of AJP50 when all selective pressures, either pine wood biomass or inhibitory chemicals present in fermentations, were removed from the culture media, isolates were screened for performance after growth in rich liquid media.

Transcriptome sequencing and analysis revealed gene expression differences in cultures capable of fermenting high solids loadings of pretreated pine wood, which was the focus of this study. Prior studies to elucidate the genetic response of *Saccharomyces* strains to challenge by lignocellulosic derived inhibitors have described a number of gene expression changes that occur when the yeast cells are exposed to these compounds [114, 115, 169, 170]. We found heightened expression of at least six genes known to provide *Saccharomyces* strains with increased inhibitor tolerance and improved performance in biomass fermentations. In addition to these, we also observed the increased expression of 46 additional genes that may play a role in survival in high solids and in high concentrations of inhibitors not previously shown to be involved in the response to these challenging environments.

## 4.3 METHODS

**Growth of strains.** The methods used to obtain isolates GHP1 and GHP4 have been previously published [168]. To prepare inocula for fermentations, isolates were grown in complex YPD or inhibitor supplemented YPDI broth media. To inoculate flasks of YPD, 2 x $10^6$ cells/ml from a glycerol freezer stock was inoculated directly into the culture flask. Cultures of YPDI were prepared by the addition of 13 inhibitory compounds to the medium at a concentration based on 12% dry w/v pine wood fermentations (Table 4.1). These compounds were chosen because they were present at the highest concentrations in pine wood fermentations. The pH was adjusted to 5.0 and inoculated with 2 x $10^7$ cells/ml. Both YPD and YPDI were incubated at 37°C with 200 rpm shaking for 24h.

**Table 4.1.** Concentrations (g/L) of each inhibitory compound in YPDI media

| FURANS | | AROMATICS | | ACIDS | |
|---|---|---|---|---|---|
| HMF[a] | 2.000 | 3,4-DHBA[b] | 0.003 | Formic Acid | 0.400 |
| Furfural | 1.000 | 3-HBA[c] | 0.005 | Lactic Acid | 0.100 |
| Furoic Acid | 0.020 | Vanillic Acid | 0.050 | Acetic Acid | 2.000 |
| | | Vanillin | 0.020 | Succinic Acid | 0.030 |
| | | Benzoic Acid | 0.015 | Levulinic Acid | 0.400 |

[a]HMF : hydroxymethylfurfural
[b]DHBA: dihydroxybenzaldehyde
[c]HBA: hydroxybenzaldehyde

**Fermentation of pine wood.** Loblolly pine wood chips were treated with $SO_2$ prior to steam explosion as previously described [30]. The name of the sample indicates pretreatment conditions; for example pine sample 3-210-10 was pretreated with 3% w/v $SO_2$ and then held at 210°C in the process reactor for 10 min. All pretreated pine wood samples were stored at 4°C before use without any washing, pressing, or other method of inhibitor abatement.

Fermentations were performed as previously described [30]. The moisture content of the biomass was determined using an IR-35 Moisture Analyzer (Denver Instrument, Denver, Colorado) and a mass equivalent to 17.5% dry w/v weighed and placed into baffled 125ml flasks and autoclaved for 20 min at 121°C (this could be considered an additional pretreatment). Prior to cell inoculation, cellulase (Novozymes Inc., Franklinton, NC) at 15 filter paper units/g dry pine and cellobiase (Novozymes Inc., Franklinton, NC) at 60 cellobiase units/g dry pine were combined and diluted in tryptic soy broth (TSB) medium without dextrose (Difco, Detroit, MI) and then filter sterilized via 2 micron filters. Additional TSB medium was added to a final concentration of 1X and the volume of the fermentation brought to 50 ml with sterile water. Hemocytometer readings were used to estimate the concentration of cells in 24h cultures, and the appropriate volume removed to sterile centrifuge bottles and centrifuged at 5,000 rpm for 10 min and the supernatant removed before inoculation into the fermentation media for an initial concentration of 2 x $10^7$ cells/ml. Fermentations were maintained at 37°C, pH 5.0, with 200 rpm shaking.

**Analysis of fermentation samples.** Samples were taken from fermentations and centrifuged at 14000 rpm to separate out any wood and particulate matter. The supernatant was filtered via 0.2 μm filters and stored at -20°C. Ethanol concentration in all samples was determined by gas chromatography (Shimadzu GC-08A, Columbia, MD) as previously described [158]. Samples from fermentations of pine sample 2.5-213-5 were also examined for 41 different lignocellulosic derived inhibitory compounds using HPLC and HPLC-MS/MS methods [159, 171].

**Growth in model fermentation media.** Experiments to assess the inhibitor tolerance of YPD and YPDI grown cultures were performed using a Bioscreen C machine (Oy Growth Curves Ab Ltd. Helsinki, Finland). Cultures were grown in YPD or YPDI as described, and then 2 x $10^5$

cells/ml were inoculated into microtiter plates. The model fermentation medium in each well was comprised of TSB, 2% w/v glucose, and increased concentrations of inhibitory compounds (Table 4.1). The initial pH of the medium was 5.0 and temperature was maintained at 37°C. The optical density of 20 replicate wells per isolate and growth condition were read hourly at 580nm.

**Transcriptome sequencing.** The transcriptomes of isolates GHP1 and GHP4 were sequenced using Illumina miSeq paired end sequencing and standard methods (Illumina inc. San Diego, CA, USA). GHP1 and GHP4 were grown in either YPD or YPDI medium as described to generate a total of four transcriptome samples. After 24h growth, cell samples were centrifuged at 10000rpm and the supernatant removed. Cells were washed with sterile distilled water and centrifuged again at 10000rpm and the supernatant removed. Total RNA was prepared from each sample using a Zymo YeaStar kit (Zymo Research Corp. Irvine, CA, USA) following the manufacturer's instructions. One library for each isolate and growth condition was prepared, mRNAs isolated, cDNA synthesized, and the libraries finalized according to manufacturer's instructions. All sequencing was performed at the University of Georgia's Georgia Genomics Facility.

**Transcriptome analysis.** All libraries were combined and assembled to create a reference transcriptome using the Trinity pipeline [172]. Individual transcriptomes for each RNA library were assembled to determine the transcriptome of each inoculum. Differential expression and hierarchical clustering analysis was performed to identify which sequences were highly expressed in GHP4 cells grown in YPD or YPDI and GHP1 cells grown in YPDI medium (these three are collectively referred to as the performing inocula) but displayed relatively low levels of expression in GHP1 grown in YPD (referred to as a the nonperforming inoculum) [173]. Sequences highly expressed in the performing inocula but found to have relatively lower

expression in the nonperforming inoculum were annotated using Blastx and the *Saccharomyces* Genome Database (http://www.yeastgenome.org). Each sequence was compared to all yeast genome sequences available in the database to find the sequence with the greatest sequence similarity.

### 4.4 RESULTS

**Pine wood fermentations.** When isolates GHP1 and GHP4 were grown in media that contained the selective pressure of inhibitory compounds, both were consistently able to ferment the sugars present in the pine wood fermentation media to ethanol. However, following growth in YPD media lacking inhibitors, only GHP4 was successfully able to produce ethanol in fermentations of pine samples 3-210-10 and 2.5-213-5. Isolate GHP1 performed at a level similar to that of the original parent strain, XR122N. For pine sample 3-210-10, GHP1 grown in YPDI and GHP4 grown in either YPD or YPDI reached nearly 30 g/l of ethanol, approximately 100% of the theoretical maximum. GHP1 grown in YPD only reached 2 g/l of ethanol; 6.6% of the theoretical maximum and is comparable to the performance of XR122N (Figure 4.1A). In fermentations of 2.5-213-5 both YPDI grown GHP1 and GHP4 displayed similar performance, reaching 28 g/l of ethanol, 68% of the theoretical maximum. Performance of YPD grown GHP4 was reduced compared to YPDI grown cells evidenced by the longer lag in ethanol production. However, by 72h the YPD grown GHP4 produced an ethanol titer equivalent to that of YPDI grown, almost 29 g/l ethanol. The performance of YPD grown GHP1 was again reduced almost to the level of parental strain XR122N; producing only 2 g/l ethanol (Figure 4.1B).

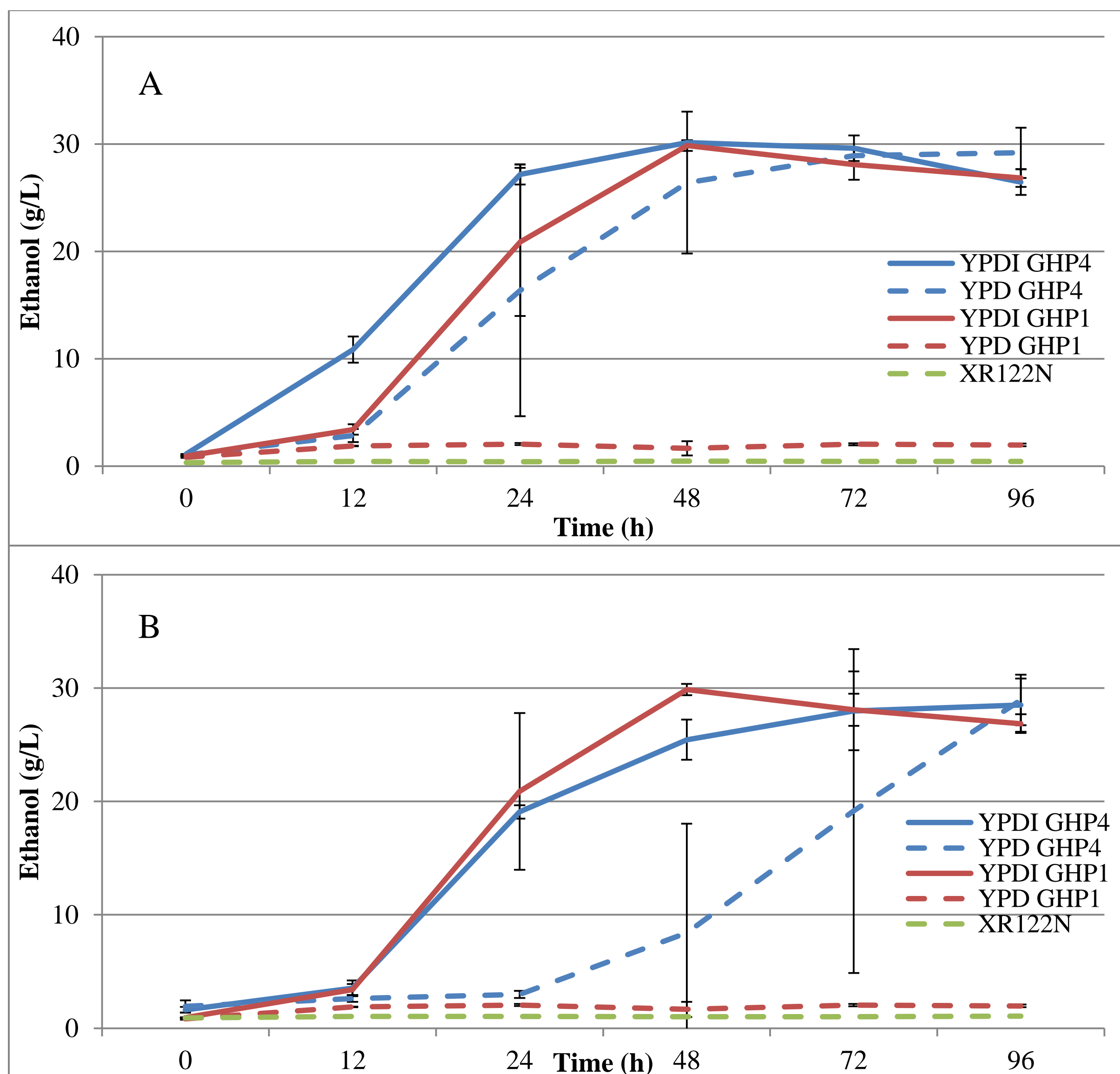


**Figure 4.1.** Ethanol production from two different samples of pretreated pine wood by GHP1 (red), GHP4 (blue), and XR122N (green). Strains were inoculated at 2.0 x $10^7$ cells/ml. Cells were grown in YPD (dashed line) or YPDI (solid line) as indicated in the legend. XR122N was only grown in YPD media, as it grows very poorly in YPDI media. Panel A shows the performance of strains in 17.5% dry weight of pine sample 3-210-10 and panel B shows data for 17.5% of 2.5-213-5. Fermentations were maintained at pH 5.0, 37°C, with 200 rpm shaking and 60 CBU/g cellobiase and 15 FPU/g cellulase. Data shows the average of 3 replicate fermentations and error bars indicate one standard deviation.

To better understand the inhibitory environment of the pine wood fermentations, the concentration of 41 inhibitory compounds were measured during fermentation. Of the 41 compounds only 25 were detected at quantifiable levels, and the initial and final concentration of

these are presented (Table 4.2). Concentrations of 12 of these compounds had only minor fluctuations during the course of fermentation. Eight compounds decreased in concentration: lactic acid, maleic acid, 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde, vanillin, benzoic acid, hydroxymethylfurfural, and furfural. For furfural, hydroxymethylfurfural, vanillin, 4-hydroxybenzaldehyde, and 3,4-dihydroxybenzaldehyde the observed decrease in concentration was much greater in the performing inocula than in the nonperforming inoculum. Five compounds increased in concentration: succinic acid, 2-furoic acid, levulinic acid, 4-hydroxybenzoic acid, and vanillic acid. Of these succinic and vanillic acids had greater increases in performing inocula than in the nonperforming inoculum, while levulinic acid had a much greater increase in the nonperforming inoculum.

**Table 4.2.** Concentrations of inhibitory compounds at the beginning and end of pine sample 2.5-213-5 fermentations

| Compound | Inocula[a] | Initial[b] | Final[b] | Change[b,c] |
|---|---|---|---|---|
| Malonic acid | P | 2.38 | 0.86 | -1.52 |
| | N | 2.29 | 3.70 | +1.41 |
| Lactic acid | P | 327.00 | 176.93 | -150.07 |
| | N | 343.33 | 209.33 | -134.00 |
| Maleic acid | P | 8.13 | 6.48 | -1.66 |
| | N | 8.27 | 1.43 | -6.84 |
| cis-Aconitic acid | P | 2.46 | 5.14 | +2.68 |
| | N | 2.51 | 0.91 | -1.60 |
| Succinic acid | P | 53.73 | 184.33 | +130.60 |
| | N | 59.10 | 131.60 | +72.50 |
| Fumaric acid | P | 3.48 | 1.65 | -1.84 |
| | N | 2.43 | 2.68 | +0.25 |
| trans-Aconitic acid | P | 2.49 | 3.04 | +0.55 |
| | N | 1.40 | 1.03 | -0.37 |
| Glutaric acid | P | 2.75 | 4.10 | +1.35 |
| | N | 2.52 | 3.67 | +1.15 |
| 2-Furoic acid | P | 24.20 | 45.05 | +20.85 |
| | N | 21.70 | 43.83 | +22.13 |
| Itaconic Acid | P | 1.77 | 1.59 | -0.18 |
| | N | 1.67 | 1.38 | -0.29 |
| Levulinic acid | P | 497.00 | 545.00 | +48.00 |
| | N | 408.00 | 596.00 | +188.00 |

| Compound | Inocula[a] | Initial[b] | Final[b] | Change[b,c] |
|---|---|---|---|---|
| 3,4-DiHydroxybenzoic acid | P | 3.51 | 3.55 | +0.04 |
| | N | 2.11 | 3.01 | +0.89 |
| 2,5-Dihydroxybenzoic acid | P | 0.14 | 0.48 | +0.35 |
| | N | 0.07 | 0.18 | +0.11 |
| 3,4-Dihydroxybenzaldehyde | P | 3.01 | 2.09 | -0.93 |
| | N | 3.07 | 3.04 | -0.03 |
| 4-Hydroxybenzoic acid | P | 2.79 | 4.35 | +1.56 |
| | N | 2.37 | 4.33 | +1.96 |
| 4-Hydroxybenzaldehyde | P | 3.72 | 0.27 | -3.46 |
| | N | 4.21 | 2.08 | -2.13 |
| Vanillic acid | P | 30.93 | 57.10 | +26.17 |
| | N | 27.35 | 42.10 | +14.75 |
| Homovanillic acid | P | 4.32 | 4.01 | -0.32 |
| | N | 4.04 | 5.31 | +1.27 |
| Vanillin | P | 21.90 | 1.24 | -20.67 |
| | N | 27.47 | 13.82 | -13.65 |
| Benzoic acid | P | 44.03 | 34.83 | -9.20 |
| | N | 42.80 | 31.33 | -11.47 |
| Syringic acid | P | 3.55 | 3.86 | +0.31 |
| | N | 3.09 | 3.13 | +0.04 |
| 3-Hydroxy-4-methoxycinnamic acid | P | 8.48 | 14.03 | +5.56 |
| | N | 4.10 | 7.38 | +3.28 |
| o-Toulic acid | P | 59.53 | 46.97 | -12.57 |
| | N | 50.15 | 57.45 | +7.30 |
| Hydroxymethylfurfural | P | 1150.00 | 120.00 | -1030.00 |
| | N | 1370.00 | 930.00 | -440.00 |
| Furfural | P | 910.00 | 280.00 | -630.00 |
| | N | 1130.00 | 900.00 | -230.00 |

[a]P performing inocula (Average of YPD GHP4, YPDI GHP4, and YPDI GHP1),
N nonperforming inoculum (YPD GHP1)
[b]concentration in mg/L
[c]final concentration – initial concentration

**Growth in model fermentation media.** Isolates GHP1 and GHP4 grown in YPD or YPDI media were inoculated into model fermentation media that contained 13 different inhibitory compounds found in pine wood fermentations. The concentration of the inhibitors in the model fermentation media was higher than those in YPDI to mimic the higher concentrations of inhibitors the cells are exposed to when inoculated into high dry matter pine fermentations. Isolates grown in YPDI medium were able to reach a maximal optical density more rapidly than

isolates grown in YPD medium (Figure 4.2). Both GHP1 and GHP4 grew similarly to one another in the model fermentation media. GHP1 and GHP4 grown in YPD had significantly shorter lag phases than parent strain XR122N, indicating a much higher level of inhibitor tolerance in both evolved isolates than in the original parent strain.

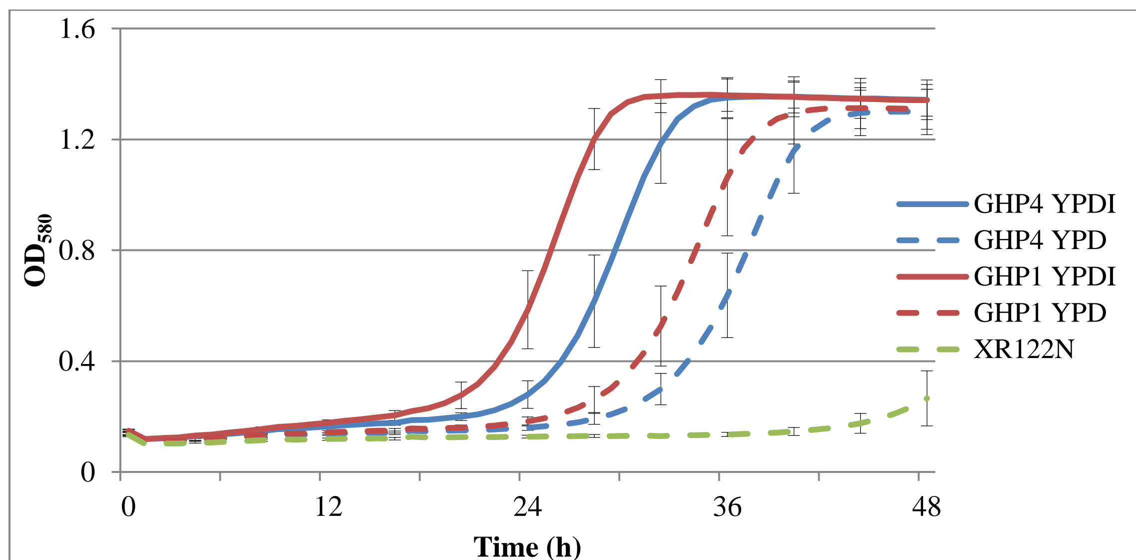


**Figure 4.2.** Growth of strains (GHP4, blue, GHP1, red, XR122N, green) in model fermentation media when inoculated at 4.0 x $10^5$ cells/ml. Inocula were prepared by growth in YPD (dashed line) or YPDI (solid line) and inoculated into media with 1.2X the concentration of inhibitors in table 1, an initial pH of 5.0, at 37°C, without shaking. Data represents the average of 20 replicate culture wells with error bars showing one standard deviation from the mean.

**Transcriptome analysis.** Hierarchical clustering grouped sequences based on the number of times a given sequence was represented in each sample. Six clusters were selected that fit the general pattern of having higher expression in the three performing inocula relative to the nonperforming inoculum (Figure 4.3). The expression level for each sequence was averaged for the three performing inocula (GHP1 YPDI, GHP4 YPD and GHP4 YPDI) and compared to the expression level observed for the nonperforming inoculum (GHP1 YPD). Sequences with expression levels in performing inocula at least two-fold greater than the nonperforming inoculum were queried against all sequences available in the *Saccharomyces* Genome Database

using the Blastx function to determine the most likely protein product encoded by that sequence. A complete list of gene expression changes fitting the described pattern is provided (Table 4.3).

Several genes involved in various cellular metabolic pathways were more highly expressed in the performing inocula. Prominent among these are an alcohol and an aldehyde dehydrogenase, *ADH1* and *ALD2/3,* respectively (both *ALD2* and *ALD3* are listed as we were unable to differentiate the two because of high sequence conservation). *ADH1* was expressed 2.9-fold higher in performing inocula and *ALD2/3* 4.2-fold higher. A transketolase, *TKL1/2*, was also highly expressed in performing inocula, with 3.6-fold higher expression. *FDH1*, which encodes formate dehydrogenase displayed 3.8-fold increased expression. A dihydrofolate reductase, *DFR1*, was also over expressed and is required for aerobic growth [174]. Two genes involved in amino acid metabolism were also upregulated in the performing inocula. These are *ASN1* which is involved in asparagine biosynthesis, and *ARO80* a transcriptional regulator that activates the transcription of aromatic amino acid catabolism genes. Genes related to carbon metabolism such as *TDH3*, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate decarboxylate, *PDC1/6*, were also highly expressed. *TDH3* had the highest increase in expression level among all sequences with a 8.2-fold change.

Four genes related to fatty acid synthesis and degradation were overexpressed in performing inocula. At over 5-fold change, *TES1* a peroxisomal acyl-CoA thioesterase involved in fatty acid oxidation displayed the highest fold change of the four. *PXA2*, a subunit of the peroxisomal ATP-binding cassette transporter was also more highly expressed. This protein is involved in the transport of long chain fatty acid CoA esters into the peroxisome [175]. *OLE1*, a fatty acid desaturase required for the synthesis of monounsaturated fatty acids, and *ETR1*, a

medium chain dehydrogenase/desaturase, displayed 2.6- and 2.2-fold higher expression in performing inocula.

A variety of gene sequences involved in transport, both import/export and intracellular, were found to be significantly overexpressed in performing inocula relative to nonperforming. *SGE1* and *PDR10*, both multidrug exporters, were upregulated in performing inocula. *PCA1* and *CCC2* are two transport proteins involved in cadmium and copper transport respectively; these two transporters play roles in metal homeostasis in the cell [176-178]. A sulfate permease, *SUL1*, had 2.9-fold higher expression and *CHS5* showed 4.4-fold higher expression. *FLC1*, a putative FAD transporter required for FAD transport into the endoplasmic reticulum had 3.4-fold increased expression.

Genes related to cell wall and membrane stability and function include the previously mentioned *FLC1* and *CHS5*. *HES1* is assumed to be involved in the regulation of the biosynthesis of ergosterol, an important component of yeast cell membranes. *HES1* had 4.3-fold increased expression in the performing inocula. Two other genes associated with cell membranes and walls showing increased expression are *PUN1* and *PIR1*.

Many genes highly expressed in our performing inocula are related to the mitochondria. Six of these have no described function but have been described as part of the mitochondrial proteome: *YNL195C, FMP48, YKL187C, YNL208W, MSC1,* and *FMP16* [179, 180]. Among those with known functions were *MIP1*, the mitochondrial DNA polymerase, and *RPO41*, the mitochondrial RNA polymerase. *MSH1* a MutS homolog had 3.4-fold higher expression. *PUT1,* a proline oxidase involved in the breakdown of proline, was also overexpressed with a 2.5-fold change. *DLD3*, a D-lactate dehydrogenase, part of the retrograde regulon displayed higher expression as well.

Certain stress response genes were more highly expressed in performing inocula. *CTT1*, cytosolic catalase T, was expressed at a 3.7-fold increased level in performing inocula, as was *GTT1*, a glutathione transferase at 2.7-fold higher expression. *AHP1*, a peroxiredoxin, was also upregulated at 2.1-fold change in our performing inocula. In addition to the mitochondria specific MutS homologue *MSH1*, four genes were upregulated that have been previously shown to have expression levels increase in response to DNA damage or replication stress. These include: *ENO1*, *IWR1*, *TFS1*, and *TPS2*.

## 4.5 DISCUSSION

GHP1 and GHP4 both displayed excellent performance when inoculated into pine wood fermentations after growth in YPDI media, but had divergent phenotypes when inoculated into fermentations after growth in YPD media. Several isolates from the original AJP50 cultures displayed performance equivalent to either GHP1 or GHP4 (data not shown), indicating the original AJP50 was a mixed population. HPLC and HPLC-MS/MS analysis provided detailed insight into the inhibitor challenge isolates experience during pine wood fermentation. Twenty five different compounds were detected at quantifiable levels in the fermentation media. This does not rule out the presence of other compounds that were not assayed. Different pretreatment methods and the use of different biomass feedstocks would also likely lead to different suites of inhibitory compounds. This analysis showed that the environment is not static, and that the concentration of inhibitors changes as the fermentation proceeds.

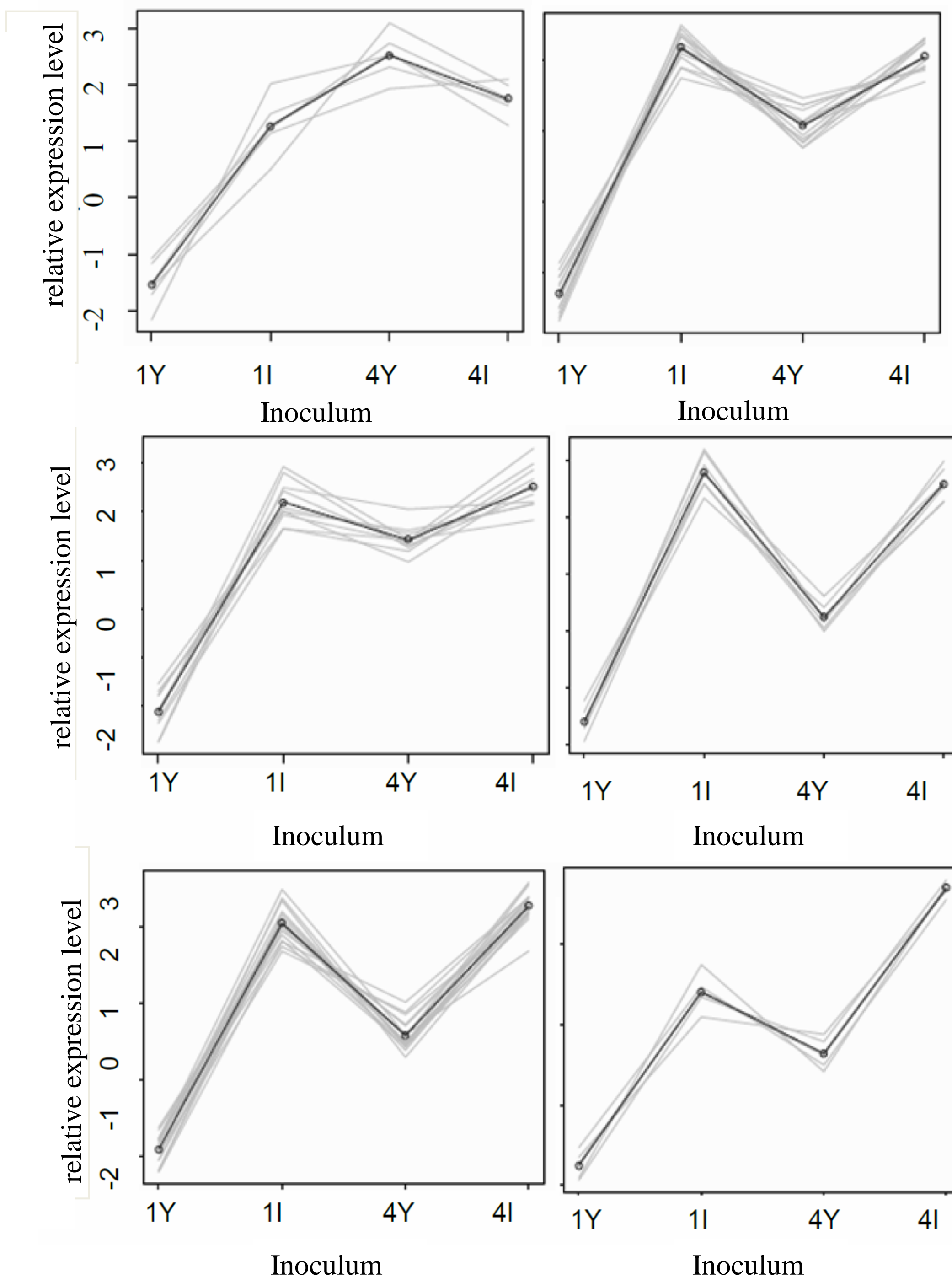


**Figure 4.3.** Clusters of transcripts that displayed the pattern of being highly expressed in performing inocula, GHP1 grown in YPDI (1I), and GHP4 grown in YPD (4Y) or YPDI (4I) relative to nonperforming inocula GHP1 grown in YPDI (1Y). The bold line shows the average expression level for the sequences in a given cluster, while the lighter grey lines represent the expression level for each single sequence in a cluster. The sequences in all clusters above were pooled and analyzed using BLAST and the *Saccharomyces* Genome Database

**Table 4.3.** Genes overexpressed in performing inocula

| Gene | Description | Fold Change[a] |
|---|---|---|
| *Fatty Acid Metabolism* | | |
| *TES1* | peroxisomal acyl-CoA thioesterase, involved in fatty acid oxidation | 5.0 |
| *PXA2* | peroxisomal ATP-binding cassette transporter | 2.7 |
| *OLE1* | fatty acid desaturase, required for proper mitochondrial functioning | 2.6 |
| *ETR1* | thioester reductase, required for proper mitochondrial functioning | 2.2 |
| *General Cellular Metabolism* | | |
| *TDH3* | glyceraldehyde-3-phosphate dehydrogenase | 8.2 |
| *DFR1* | dihydrofolate reductase, involved in tetrahydrofolate synthesis | 4.3 |
| *ALD3/ALD2* | aldehyde dehydrogenase | 4.2 |
| *PDC6/PDC1* | pyruvate decarboxylase | 4.0 |
| *FDH1* | formate dehydrogenase | 3.8 |
| *TKL1/TKL2* | transketolase | 3.6 |
| *ASN1* | asparagine synthetase | 3.1 |
| *ARO80* | transcriptional activator for aromatic amino acid catabolism. | 2.9 |
| *ADH1* | alcohol dehydrogenase | 2.9 |
| *Membrane/Cell wall associated* | | |
| *PUN1* | plasma membrane protein | 5.2 |
| *HES1* | implied regulator of ergosterol synthesis | 4.3 |
| *PIR1* | glycosylated cell wall protein, required for Apn1p mitochondrial translocation | 2.3 |
| *Transport* | | |
| *SEC27* | protein in the COPI coatomer | 4.6 |
| *ATG20* | sorting nexin required for cytoplasm to vacuole targeting | 4.4 |
| *CHS5* | exomer complex component | 4.4 |
| *CCC2* | copper transporting P-type ATPase | 3.5 |
| *FLC1* | putative FAD transporter | 3.4 |
| *PCA1* | cadmium transporting P-type ATPase | 2.9 |
| *SUL1* | high affinity sulfate permease | 2.9 |
| *SEC22* | R-SNARE protein | 2.6 |
| *GYP7* | GTPase-activating protein | 2.6 |
| *PDR10* | ATP-binding cassette (ABC) transporter, multidrug transporter | 2.6 |
| *SGE1* | plasma membrane multidrug transporter | 1.9 |
| *Mitochondria associated* | | |
| *MRM2* | mitochondrial O-ribose methyltransferase | 4.6 |
| *YNL195C* | unknown function, part of the mitochondrial proteome | 4.1 |
| *RPO41* | mitochondrial RNA polymerase | 3.7 |
| *FMP48* | unknown function, part of the mitochondrial proteome | 3.7 |

| Gene | Description | Fold Change[a] |
|---|---|---|
| *MSH1* | MutS Homologue involved in mitochondrial DNA repair | 3.4 |
| *AEP2* | mitochondrial protein involved in translation of OLI1 mRNA | 3.4 |
| *YKL187C* | unknown function, part of the mitochondrial proteome | 2.8 |
| *YNL208W* | unknown function, part of the mitochondrial proteome | 2.8 |
| *MKS1* | pleiotropic transcriptional regulator, involved in retrograde signaling | 2.7 |
| *APJ1* | chaperone protein involved in SUMO-mediated protein degradation | 2.7 |
| *MIP1* | mitochondrial DNA polymerase | 2.6 |
| *MSC1* | unknown function, part of the mitochondrial proteome | 2.5 |
| *PUT1* | proline oxidase | 2.5 |
| *FMP16* | unknown function, part of the mitochondrial proteome | 2.5 |
| *CYB2* | cytochrome b2 | 2.3 |
| *YNL200C* | NADHX epimerase | 2.0 |
| *DLD3* | D-lactate dehydrogenase, part of the retrograde regulon | 2.0 |
| *DNA Stress Response* | | |
| *IWR1* | RNA Pol II transport factor, relocates to cytoplasm upon DNA stress | 4.4 |
| *ENO1* | Enolase I, converts 2-phosphoglycerate to phosphoenolpyruvate | 2.7 |
| *TFS1* | inhibits carboxypeptidase Y and Ira2p | 2.4 |
| *TPS2* | phosphatase involved in synthesis of trehalose | 2.0 |
| *Oxidative Stress Response* | | |
| *CTT1* | cytosolic catalase T | 3.7 |
| *GTT1* | glutathione transferase | 2.7 |
| *GAD1* | glutamate decarboxylase | 2.1 |
| *AHP1* | thiol-specific peroxiredoxin | 2.1 |

[a]average expression of performing inocula compared to nonperforming

The similar performance of GHP1 and GHP4 after growth in rich media in model fermentation media supplemented with inhibitory compounds suggests that GHP1's failure to produce ethanol in 17.5% dry w/v pine fermentations was not due to a loss of inhibitor tolerance and implies that inhibitor tolerance alone is insufficient for fermentation of high dry weights of pine. Model fermentation media may not sufficiently mimic all the stressors present in a pine wood fermentation. The medium lacks any solid material and has a reduced number of inhibitory chemicals relative to pinewood fermentations. Comparison of the transcriptomes of performing

inocula and the nonperforming inoculum suggested that the performing inocula were overexpressing at least six genes known to be involved in inhibitor tolerance from previous work in the field. Our performing inocula also displayed increased expression of 46 genes not previously associated with a response to biomass derived inhibitors.

Previous work has shown the importance of alcohol and aldehyde dehydrogenases, such as *ADH1* and *ALD2/3*, in the tolerance of aldehyde inhibitors, such as furans, found in biomass fermentations [115, 118, 124]. The heightened expression of these genes may be responsible for the more rapid decrease in furfural, hydroxymethylfurfural, and vanillin observed in the performing inocula fermentations. Transketolase (*TKL1*) plays a role in the pentose phosphate pathway; overexpression of genes in this pathway have been shown to enhance the performance of *Saccharomyces* in biomass fermentations possibly by altering the NADH/NADPH levels inside the cell [118, 120]. *FDH1,* formate dehydrogenase, may be responsible for protecting cells from formate, an inhibitory aliphatic acid known to be produced during biomass pretreatment [76]. Overexpression of formate dehydrogenase has been shown to improve fermentation performance in the presence of high concentrations of formic acid in engineered *Saccharomyces* strains [181, 182]. *ARO80*, combined with *PUT1*, may alter the amino acid biosynthetic pathways in the cell and allow for more rapid ATP regeneration via the TCA cycle [115]. *TDH3* may allow for more rapid NAD/NADH cofactor regeneration in the performing inocula [118]. The overexpression of multidrug transporters has been shown to help *Saccharomyces* survive a variety of chemical stressors, including biomass derived inhibitors [115, 123]. The performing inocula displayed heightened expression of two such genes, *SGE1* and *PDR10*.

Cell walls and membranes are important for cells under stress from biomass derived inhibitors as these target and disrupt these structures [102]. Cellular transport has been shown in previous studies to be important for resisting the effects of hydroxymethylfurfural [115]. *CHS5, FLC1, PIR1,* and *PUN1* are important for cell wall biosynthesis and functioning [183-187]. *HES1*, an assumed regulator of ergosterol biosynthesis, was overexpressed in performing cell inocula. Ergosterol has been shown to be important for the tolerance of various inhibitory compounds, particularly vanillin [113, 188, 189].

The mitochondrion is an important organelle in eukaryotic cells that fulfills a number of roles in *Saccharomyces* including housing a variety of metabolic activities, bioenergetics, and involvement in apoptosis [190-192]. Inhibitory chemicals found in biomass fermentations, including furfural, damage the mitochondria [141]. Seventeen sequences found in our study are connected with this organelle, suggesting an important role in the fermentation of high solids loadings of pine wood biomass. The increased expression of both the mitochondrial DNA and RNA polymerases suggests a role for mitochondrial replication and gene synthesis in the fermentation of high pine solids. *MSH1* showed increased expression in performing inocula. This gene is one of six MutS homologs in *Saccharomyces* and is the only one that is involved in the repair and protection of mitochondrial DNA. This gene is essential for the maintenance and functioning of the mitochondria [193]. *DLD3* and *MKS1*are part of the retrograde regulon which mediates signaling between the mitochondria and the nuclear genome and is expressed when the mitochondria are damaged [194]. In addition to their roles in fatty acid synthesis, *ETR1* and *OLE1* are important for proper formation and functioning of the mitochondria [195, 196]. *PIR1*is required for the localization of Apn1p to the mitochondria, where it functions in DNA repair and

maintenance [197]. The increased expression of these numerous genes associated with the mitochondrion suggests a prominent role for this organelle in high biomass solids fermentations.

When exposed to biomass derived inhibitors, cells experience both DNA damage and oxidative stress; eight genes associated with the response to these stressors displayed increased expression in performing inocula [141]. Oxidative damage can be particularly severe to the mitochondrial DNA and causes a number of defects including loss of mitochondrial DNA and the mitochondrion itself [198]. Both catalase and glutathione have been shown to protect cells from reactive oxygen species [199-201]. The overexpression of glutathione transferase (*GTT1*) may increase the available glutathione pool in performing inocula, while the heightened expression of cytosoloic catalase (*CTT1*) may increase the cells resistance to reactive oxygen species. Contrary to our findings, *CTT1* had decreased expression upon exposure to hydroxymethylfurfural, acetic acid, or hardwood spent sulfite liquor in microarray studies using *Saccharomyces cerevisiae* T2, a strain adapted for high performance in lignocellulosic biomass fermentations [169]. *AHP1*, a peroxiredoxin, has also been shown to protect cells from oxidative damage by the reduction of hydroperoxides [202].

Clonal populations isolated from evolved yeast AJP50 displayed differing phenotypes in pine wood fermentations after growth in rich liquid media without any selective pressure. This suggests that AJP50 is not a single strain of uniform genotype but is rather a mixed population of yeast with varying genetic content. Transcriptomic analysis of our isolates revealed a number of genes that show heightened expression in performing inocula. Some of these genes have already been shown in the literature to be involved in inhibitor tolerance and to allow for increased performance of those strains. Prominent among these are the increased expression of genes with

aldehyde dehydrogenase activities. These genes may be responsible for the more rapid removal of aldehyde inhibitors observed in fermentations with the performing inocula.

We also found heightened expression of 46 genes not previously linked to *Saccharomyces'* ability to withstand inhibitor challenge. Among these are a number of genes associated with the mitochondrion, suggesting this organelle may be vital for performance in fermentations of high concentrations of pretreated pine wood biomass. These include a number of genes of unknown function, but part of the mitochondrial proteome; genes associated with regular mitochondria functioning such as *RPO41* and *MIP1*; and ones associated with mitochondria damage and repair, *MSH1* and *MKS1*. *CTT1* and *GTT1*, which may allow our strains to withstand the reactive oxygen species that can be caused by the chemical inhibitors released during biomass pretreatment were also increased in expression in the performing inocula. Our performing inocula display unique gene expression patterns which combine genes known to provide inhibitor tolerance with genes not previously known to play a role in these processes. These expression patterns may allow for the cells to better withstand some of the toxic effects of inhibitory compounds found in pretreated biomass fermentations (Figure 4.4).

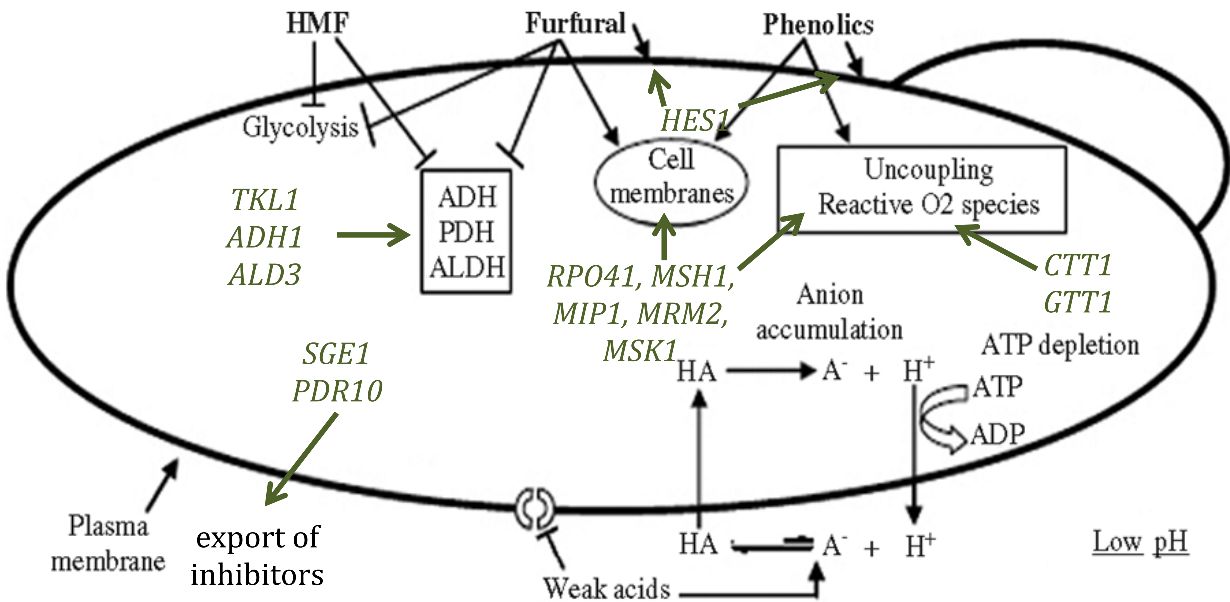


**Figure 4.4.** Potential mechanisms by which the differential expression in performing inocula may aid fermentation performance in high pine wood biomass. In brief: *TKL1, ADH1, ALD3* may enhance aldehyde dehydrogenase activity which relieves stress caused by furfural and HMF. *SGE1, PDR10* efflux pumps may remove inhibitory compounds from the cytosol before they can have deleterious effects. *RPO41, MSH1, MIP1, MRM2, MKS1* may enhance mitochondrial activity, this organelle suffers both membrane and reactive oxygen damage upon exposure to furfural and other inhibitors. *HES1* may assist in cell wall damage caused by inhibitory compounds. *CTT1, GTT1* remove reactive oxygen species and prevent this damage from occurring during fermentation [78 ,99].

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# CHAPTER 5

# CONCLUSIONS

The increasing desire to move toward sustainable fuels necessitates the development of new technologies that will enable the use of a broader suite of fuel feedstocks than what is implemented in the current economy, which is dominated by corn in the US and sugarcane in Brazil. Pine and other softwoods represent one of many potential feedstocks broadly categorized as lignocellulosic biomass. These trees have the advantage of being grown and harvested currently for both lumber and paper; this type of woody biomass also possesses very low concentrations of five carbon sugars, which cannot be fermented by wild-type *Saccharomyces* strains. They are however lower in sugar than corn and sugarcane, and the sugars present are more difficult to access. This necessitates the use of a pretreatment process which also creates inhibitory compounds from the biomass; these compounds are one factor that confounds the fermentation of high solids which is required for the process to be economically viable.

For a pine ethanol fermentation industry to flourish, biocatalytic strains must be developed that can withstand these compounds while efficiently converting the sugars liberated from the feedstock. AJP50, a *Saccharomyces cerevisiae* yeast capable of producing ethanol from high concentrations of pretreated pine wood, has been developed and described here (Chapter 2). AJP50 was developed via adaptation and directed evolution from an industrial strain of yeast used in corn ethanol fermentations, XR122N. This industrial yeast was evolutionarily adapted by constant transfer into batch fermentations of increasing concentrations of pretreated pine wood. AJP50 is capable of producing ethanol from 17.5% and greater dry weight loadings of pine wood, much higher than what the parent strain is able to withstand. With a proper pretreatment,

this strain will produce sufficient ethanol titers for cost effective distillation, enabling the process to be economically viable. The ability of AJP50 to survive and ferment in these conditions may be due at least in part to its heightened ability to tolerate biomass derived inhibitory compounds and survive and grow in media where the growth of the parent strain is severely inhibited.

Single colony isolates from AJP50 are able to be cultured on inhibitor containing media and when inocula of isolates are prepared in this fashion they always retain the phenotype of being able to ferment high dry weights of pine wood (Chapter 3). Large concentrations of the yeast may be propagated, and when inoculated at the industrially relevant level of 2 g/L, pine solids loadings of up to 22.5% dry weight/v can be fermented with high yields. These isolates can also be cultured on rich liquid media typically used for *Saccharomyces* growth. When grown in this media, the isolates display differing phenotypes, with certain isolates retaining the high solids fermentation phenotype and others losing this ability and displaying a fermentation phenotype similar to the original parent strain (Chapter 4).

This divergence in phenotype was used to determine what gene expression differences existed between performing and nonperforming inocula by sequencing the transcriptomes of these yeast inocula. Certain differences, such as heightened expression of alcohol and aldehyde dehydrogenases, have been previously shown in the literature to provide heightened performance of *Saccharomyces* in biomass fermentations. Numerous genes, including many which are associated with the mitochondria, were also found to have higher expression in inocula capable of fermenting high biomass loadings. Further study of these isolates will continue to provide insight into the mechanisms behind these strains' robust performance.

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