Overexpression of *OYE2* in *Saccharomyces cerevisiae* for Improved Tolerance against Fermentation Inhibitors

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Abstract

Alternatives to fossil fuels are necessary to combat the rising concerns of the harmful effects of fossil fuel emissions on the environment and to accommodate current energy supply demands. The pressure to find an alternative fuel source that is both cost effective and environmentally friendly has increased in recent years. One alternative is bioethanol, a type of biofuel, which can be produced using yeast, specifically *Saccharomyces cerevisiae*, to break down many different substrates. Currently, bioethanol is fermented from sugar cane or cornstarch by releasing sugars, but this poses a problem. In the United States, the amount of cornstarch needed to produce enough bioethanol to meet market demand is more than the economy can afford to take from other industries that rely on the use of corn. Corn is used in too many different ways to take a vast majority of it to use solely for the purpose of bioethanol production. Therefore, researchers have been trying to develop new ways to ferment bioethanol from yeast. However, the replacement substrates, such as biomass waste, produce multiple growth inhibitors during the fermentation process that prevents the yeast from producing bioethanol. To detoxify these inhibitors the cellular reducing agent, NADPH, is needed. The specific purpose of NADPH is unclear, but is believed to be needed as a cofactor for enzymes that function in cellular stress protection. The enzyme encoded by *OYE2* is a possible target, as it is a NADPH specific oxioreductase linked to stress tolerance. I hypothesize that the overexpression of *OYE2* will increase stress tolerance in yeast exposed to fermentation inhibitors such as furfural and thus prove to be a cost effective method to produce bioethanol.

Introduction

Alternatives to fossil fuels are necessary to combat the rising concerns of the harmful effects of fossil fuel emissions on the environment, to accommodate current energy demands, and lessen our dependency on foreign oil. The pressure to find an alternative fuel source that is both cost effective and environmentally friendly has increased in recent years (Hahn-Hagerdal B, Galbe M, Gorwa-Grauslund MF, Liden and G, Zacchi G, 2006). One alternative is bioethanol, which is fermented using yeast, specifically *Saccharomyces cerevisiae*. Currently, most bioethanol is fermented from sugar cane or cornstarch by releasing sugars using liquefaction and hydrolysis steps. The primary sugar released is glucose, which is readily fermented into bioethanol. However, using glucose obtained from agricultural products poses a problem. The amount of cornstarch substrates needed to produce enough bioethanol to meet market demand is more than the economy can afford to take from other industries. In truth, there are not enough cornstarch substrates in existence to accommodate the amount necessary to replace fossil fuels. Because there is a limited supply of corn, it is not a cost effective method (Hahn- Hagerdal et al. 2006).

Alternatives to cornstarch are needed in order for a bioethanol fuel economy to be fully realized. One such option is converting what is currently considered as waste into bioethanol, such as lignocellulosic biomass waste from agriculture, municipalities, and industry. However, breaking down lignocellulose into fermentable sugars is not cost effective. In addition, breaking down the waste products generates many growth and fermentation inhibitors that prevent yeast from making optimal levels of bioethanol (Almeida J, Modig T, Petersson A, Hahn-Hagerdal B, Liden G, Gorwa-Grauslund MF, 2006; Groom MJ, Gray EM and Townsend PA, 2007).

Developing strategies that use lignocellulosic waste products will eliminate the need to dispose of them and will also give what was once garbage, a use.

Though inefficient, yeast can produce bioethanol from lignocellulosic biomass, such as wood pulp or shavings, or waste from the corn industry (Modig T, Almeida J RM, Gorwa-Grauslund M, Liden G, 2008). This is done by releasing fermentable monosaccharide's by size reduction and pretreatment of the biomass. The pretreatments consist of breaking down the cellulose and hemicellulose polymers, into monomeric sugars using enzymes or acids. These sugars are then fermented by yeast (Hahn- Hagerdal et al. 2006). In addition to generating fermentable sugars the pretreatment step also generates inhibitors, which then limit yeast growth and fermentation. Several inhibitors are produced during the pretreatment process and can cause the yeast to die earlier than expected. 2-furaldehyde (furfural) and 5-hydroxymethyl-2-furaldehyde (HMF) are the most common byproducts, but others include different acids and phenolic compounds, such as vanillin (Almeida et al. 2006). Modig et al. (2008) believe that the current strains of yeast being used to produce bioethanol work reasonably well, but the development of more robust strains can aid in the process and yield more bioethanol. This may lead to the transition from fossil fuels to bio fuels. Figure 1 shows where the inhibitors, furfural, HMF and phenolic compounds, attack the cell and how it is possible for these inhibitors to cause cell death or in the very least, inhibit fermentation.

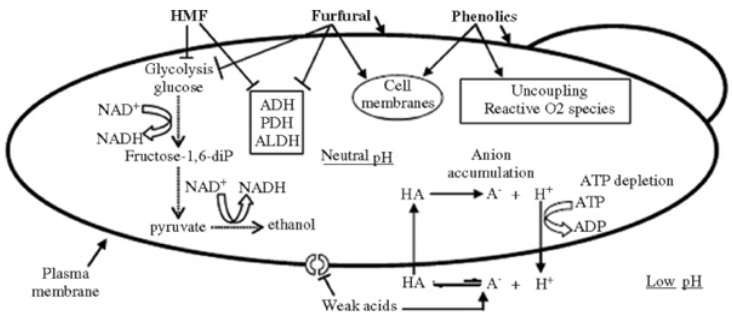

Figure 1:
Schematic of where the fermentation inhibitors created during the pretreatment processes of bioethanol production attack a yeast cell. This figure specifically shows how these inhibitors cause programmed cell death in yeast (Almeida et al. 2007).

Furfural and HMF are fermentation inhibitors. Therefore, they do not allow yeast to produce bioethanol. *S. cerevisiae* is capable of detoxifying low concentrations of furfural and HMF during anaerobic growth by reducing the inhibitors to their less toxic alcohol derivatives. Unfortunately, yeast cannot detoxify these inhibitors at concentrations found during industrial fermentation of biomass. Limited success has been found that increases resistance to furfural and HMF by a process of mutation and selection (Gorsich SW, Dien BS, Nichols NN, Slininger PJ, Liu ZL, Skory CD, 2006; Liu, ZL and Slininger, PJ, 2005; Petersson A and J.R. Almeida, 2006). By making the yeast more stress tolerant, the yeast will survive longer which can produce higher yields of bioethanol.

Increased stress tolerance can lead to the more effective use of yeast to produce bio fuels. In an article by Swan and Watson (1999), stress tolerance was studied using a yeast lipid mutagen to influence the yeasts' tolerance of heat and bioethanol. Mutant forms of genes can be used to enhance the effectiveness of yeast (Almeida et al. 2006). Stress tolerance can be increased by: altering the genes that control the breakdown of harmful byproducts, reducing the amount of mutated genes that code for decreased stress tolerance, and increasing the amount of genes that code for NADPH production. Gorsich et al. (2006) identified more than 62 different genes associated with sensitivity to furfural. This study indicated that genes needed for protection against oxidative damage were critical (Gorsich et al., 2006).

One gene that protects the cell against oxidative damage is *OYE2*, which encodes an enzyme needed to catalyze the production of $NADP^+$ in the cytosol and breakdown oxidative free radicals (Niino YS, Chakraborty S, Brown BJ, Massey V, 1995). Several studies have shown the protective role of old yellow enzymes against oxidative stress. One such study found that the overexpression of *OYE2* allowed cells to recover more quickly than control cells in the

presence of hydrogen peroxide, which is a standard inducer of programmed cell death in yeast. This article also found that the accumulation of reactive oxygen species (ROS) in vivo was higher in *oye2p* knock out cells than in wild type cells (Odat O, Matta S, Khalil H, Kampranis SC, Pfua R, Tsichlis PN, 2007). This further confirms that *OYE2* is an antioxidant gene and is involved in stress tolerance. *OYE2* codes for the production of old yellow enzyme (Oye2p), which catalyzes the reaction: NADPH + H^+ + quinone acceptor = $NADP^+$ + hydroquinone. NADPH is an essential reducing chemical when fermentation inhibitors (*e.g.* furfural) are present. Since Oye2p is an NADPH dependent oxioreductase linked to inhibitor tolerance (Niino YS, Chakraborty S, Brown BJ, Massey V, 1995), it is reasonable to think Oye2p functions in furfural tolerance. I predict that the overexpression of *OYE2* should allow yeast to grow better in the presence of furfural. This increased survival should allow the yeast to be able to produce more bioethanol. What needs to be discovered is what effect the overexpression of *OYE2* will have on the yeast ability to survive in the presences of fermentation inhibitors produced in the pretreatments that allow yeast fermentation of biomass, such as furfural and HMF. I propose that overexpressing *OYE2* will increase the tolerance of yeast to fermentation inhibitors such as furfural. Determining if the overexpression of *OYE2* causes increased stress tolerance may allow for a more cost effective production method.

Methodology

Subcloning of OYE2 into an expression vector

Yeast *OYE2* will be amplified by performing a Polymerase Chain Reaction (PCR). Forward and reverse PCR primers were designed using sequence information from www.yeastgenome.org. The forward primer used was (GGGG- Spe1-GACGATAATATAG

TATCGA) and the reverse primer used was (GGGG- Xho1-AATGGTGCTACAAAGTACGG). Primers were ordered from Integrated DNA Technologies. The amplified product contained restriction enzyme sights Spe1 and Xho1 at the ends of *OYE2*. The multi cloning site of pRS425-MET25 expression vector contained reciprocal Spe1 and Xho1 restriction sites.

In order to determine the optimal annealing temperature for PCR, a temperature gradient was performed. Twelve different temperatures were checked for optimal annealing capability and then these samples were run out on a 0.7% agarose gel with ethidium bromide to visualize the DNA with UV light. The optimal annealing temperature was found to be approximately 55^{o} C because at this temperature the amplified product shown on the gel was the brightest. Using the ideal annealing temperature, PCR was performed, product purified using QIAquick spin columns, the size was verified on a 0.7% agarose gel, and its concentration was determined.

OYE2 was subcloned into the multi cloning site of the pRS425-Met25 expression vector. The MET25 plasmid contains an ampicillin resistant gene which allows for easy maintenance in bacteria on LB plates supplemented with ampicillin. The MET25 plasmid was isolated using a Qiagen mini prep spin column. The purified MET25 vector and *OYE2* PCR product were digested with restriction enzymes Spe1 and Xho1 in 50µL reactions at 37^{o}C for approximately 24 hours. Reactions were heat inactivated at 65^{o}C for 20 minutes. Digestions were verified and concentrations determined using a 0.7% agarose gel. DNA from the gel was recovered using the Zymoclean Gel DNA recovery kit.

The purified digested MET25 vector and *OYE2* PCR product were ligated together in 10 µL reactions with T4 DNA ligase. Controls were used to verify that there was no contamination. After the ligation, 1µL of the plasmid was transformed into electorocompetent bacteria using an electroporator. Transformed bacteria containing putative clones were selected on LB + ampicillin

plates. Putative *MET25-OYE2* clones were grown overnight in LB+ amp liquid media at 37^{o}C and the plasmid isolated using a Qiagen miniprep column.

Sixteen different putative clones were tested to verify they contained *OYE2* in the correct orientation. Samples of these colonies were digested with the original cloning enzymes (Spe1 and Xho1) as well as Xba1. Correct subclones have unique banding patterns compared to the original vector. One of the putative subclones (plasmid #9), was verified as containing *OYE2*. To verify *OYE2* did not have mutations it was sequenced using an Applied Biolsystems 3130 Genetic Analyzer and analyzed using the software program, Sequencher.

Plasmid #9 was transformed into 2 different strains of yeast, SGY110 (haploid) and SGY114 (diploid) using a standard lithium acetate transformation technique (Gietz RD, Woods RA, 2002). Transformants were grown for 3-5 days at 30^{o}C on synthetic dextrose media without leucine (SD-LEU). Four colonies were chosen from each plate of transformed SGY110 and SGY114 and verified to contain the correct *Met25-OYE2*. These colonies were added to 30µL of 0.2% SDS and the plasmid isolated using the standard procedure for running a single colony PCR procedure using Promega Taq Green PCR Master Mix. The resultant solutions were run out on a 1.0% agarose gel along with a 1kb ladder, the empty vector (MET25) and the plasmid (#9) alone to identify colonies in which the transformation was successful. Colonies 1, 2, 4 and 8 were all recognized as containing plasmid #9 and were therefore used to assess growth in the presence of Furfural.

Growth Analysis of Yeast

Wild-type yeast and over expressed *OYE2* yeast (colonies 1, 2, 4 and 8) were grown in synthetic dextrose media complete and without leucine, respectively (Gorsich et al. 2006). Yeast were grown to the exponential phase and then supplemented with either no inhibitor or with 25

m*M* or 50 m*M* furfural. Cell density was monitored using a spectrophotometer set at 600 nm absorbance. Measurements were taken until they reached saturated growth, usually before the end of the 72 hour span. Experiments were performed 3 times and averages determined. Growth curves were generated using Microsoft Excel 2007 to document yeast tolerance to furfural with the over expressed *OYE2* gene as well as wild-type cells of the original strain.

Results

Overexpressed OYE2 provides increased furfural tolerance

OYE2 was successfully subcloned into the pRS425-MET25 expression vector. This vector is a multi-copy vector that activates gene expression at a high rate due to the MET25 promoter. After the gene was sequenced and verified to contain no mutations it was transformed into yeast strain, SGY110. The transformed strain was grown to exponential phase and then treated with no inhibitor, 25 m*M* furfural, or 50 m*M* furfural. All strains grown exited lag phase rapidly when no inhibitor was present (Figure 2a). However in the presence of furfural there was a difference in growth depending upon whether or not overexpressed *OYE2* was present. In the presence of 25 m*M* furfural (non-lethal concentration), yeast cultures overexpressing *OYE2* escaped lag phase quicker and grew to a higher density when compared to yeast cultures not overexpressing *OYE2* (Figure 2b). In the presence of a lethal concentration of furfural (50 m*M*) yeast not overexpressing *OYE2* did not grow. However, yeast overexpressing *OYE2* were moderately protected in the presence of 50 m*M* furfural (Figure 2c). To summarize, yeast with the over expressed gene had greater cell densities after the addition of either 25 m*M* or 50 m*M* furfural and continued to show increased cell densities throughout the entire 72 hour period.

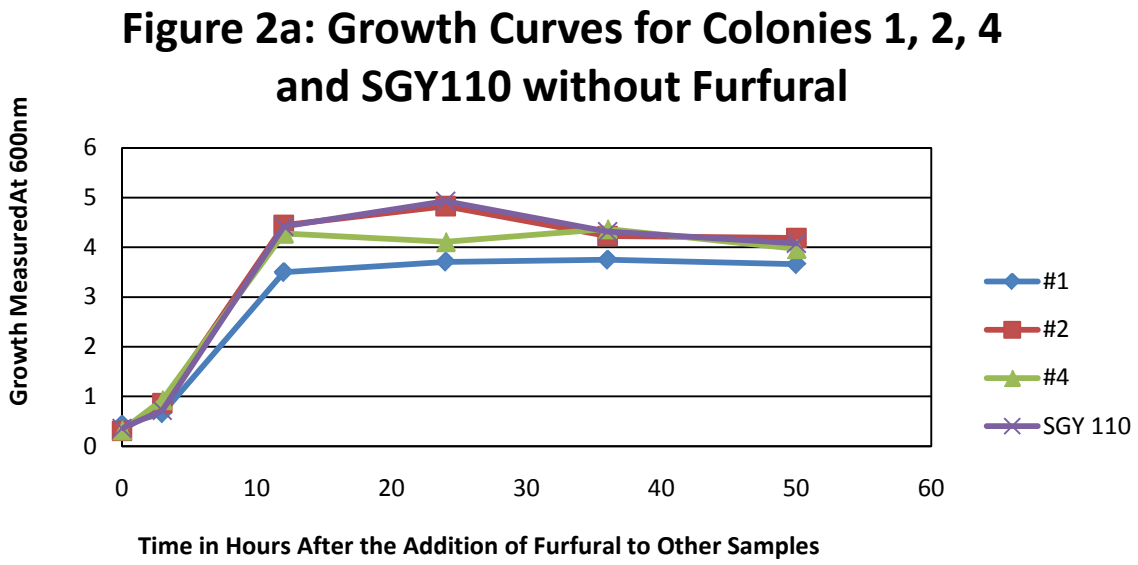

Figure 2a: Graph showing the cell densities of 4 different strains of yeast over a 50 hour period in the absence of Furfural. All colonies are growing at approximately equal rates. This shows that under normal circumstances the overexpressed *OYE2* cells do not have any advantage over the wild-type cells.

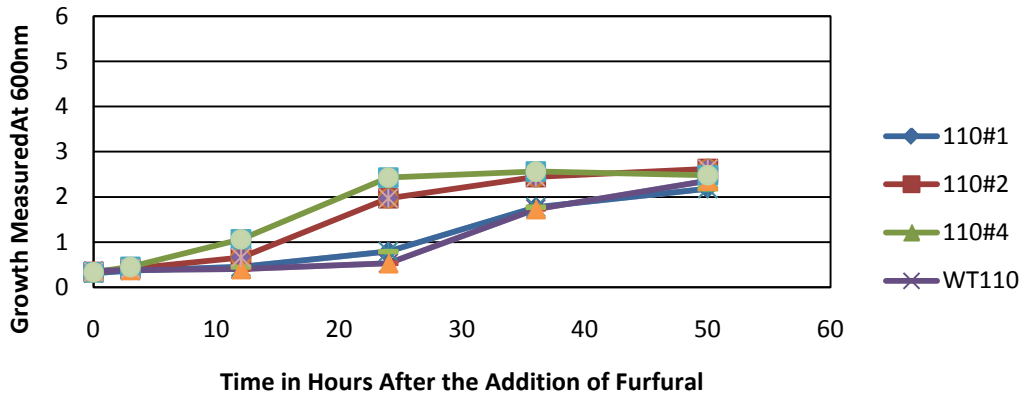

Figure 2b: Graph showing the cell densities of 4 different strains of yeast over a 50 hour period in the presence of 25mM Furfural. Colonies 1 and 2 both show increased cell densities when compared to the wild type parent strain.

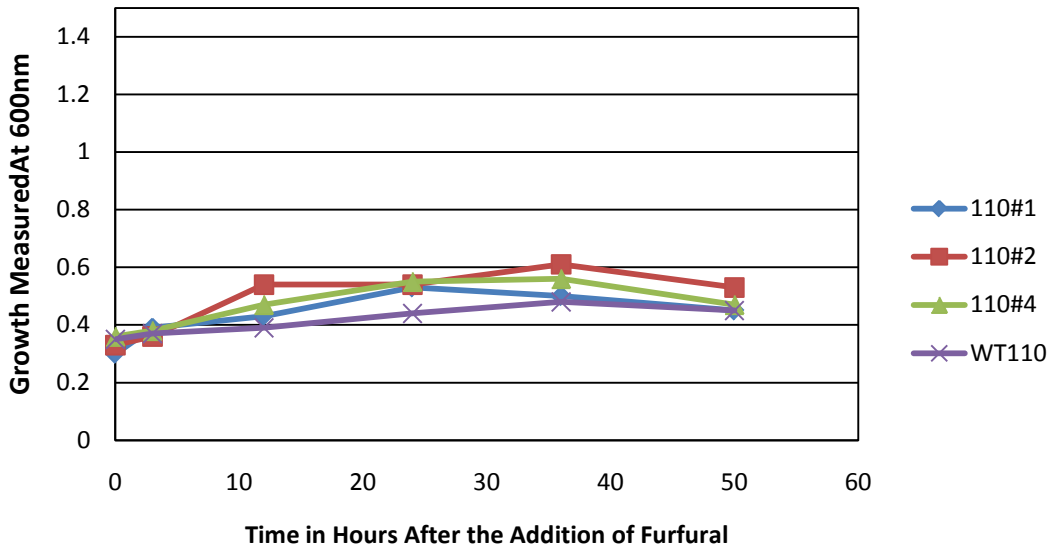

Figure **2c: Graph showing the cell densities of 4 different strains of yeast over a 50 hour period in the presence of 50mM Furfural.** Colonies 1, 2 and 4 all show increased cell densities when compared to the wild type parent strain.

Discussion

From the results of the growth analysis, the overexpression of *OYE2* did aid in the yeasts' ability to survive and reproduce when one of the fermentation inhibitors, furfural, was present. This can be seen in the increased cell density at both 25mM and 50mM of furfural when compared to the parent stain not overexpressing *OYE2*. One of the overexpressed strains, #4, was able to enter the log phase twice as fast as the parent strain. This shows that the altered yeast was able to tolerate the furfural approximately twice as well and is, therefore, more effective than the parent yeast. However, just knowing that the strain is able to tolerate furfural better is not enough. From here it is important to discover what exactly the overexpression of *OYE2* is doing

for the cell. A cellular damage analysis is needed to assess what makes the altered strain more effective. With additional information it becomes possible to understand why the increased *OYE2* expression is able to detoxify furfural faster and may give way to new lines of research. Without knowing what specifically is enhanced there is no way to further increase the effects.

To test for cellular damage aliquots of cells either overexpressing or not overexpressing *OYE2* will be analyzed for cellular damage using 3 different assays. First the accumulation of reactive oxygen species (ROS) will be measured using the ROS-specific dye, dihydroethidium. This dye only fluoresces when cellular ROS accumulates and interacts with it. ROS is known to damage cellular structure. Second, to analyze membrane damage mitochondrial and vacuole membrane structures will be analyzed with the targeted-GFP (mito-GFP) and vacuole membrane dye, FM 4-64, respectively. Third, nuclear chromatin will be analyzed using the DNA specific dye, DAPI. With this information it will be possible to fully assess the effectiveness of over expressing *OYE2.*

The overall goal of this experiment was to construct a yeast strain that is robust enough to tolerate inhibitors present during industrial fermentation of biomass. This alternation to the yeast genome is a step in the right direction. Further experimentation is needed to confirm the usefulness of the over expressed *OYE2* gene, however, the tolerance it has shown to inhibitors could lead to an efficient commercial production of bioethanol from alternative substrates. If found effective in the presence of other inhibitors, the use of cells over expressing *OYE2* may be able to supplement or replace the use of corn to make bioethanol and possibly render useful something that would otherwise just be thrown away. Ultimately, increasing the productivity of yeast in the presence of fermentation inhibitors can lead to decreased use of fossil fuels in the transportation network of the United States. The economy could also be improved. Bioethanol

can be produced in the United States by local farmers. With the help of rural America there would be no need to rely on foreign countries for oil, thereby increasing available jobs, the unemployment rate could be reduced and fewer people could be living below the poverty line. Finding an alternative fuel source is of the utmost importance because with it comes sustainability and with sustainability, the nation can continue to thrive.

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