Ryan Moriarty

Lab Section 505

3/23/15

Mining diversity in *Saccharomyces* genus for biofuel improvement

Abstract

As the demand for fuel grows globally while the supply decreases, alternative fuel source must be discovered. Bioethanol provides a much cleaner source of fuel than current petroleum fuels and can be derived from yeasts such as *Saccharomyces cerevisiae* that ferment sugars present in AFEX (Ammonia fiber expansion) treated Corn Stover Hydrolysate (ACSH) in order to produce ethanol. The two current chassis strains, an aerobically and anaerobically evolved *Saccharomyces cerevisiae,* GLBRCY73 and GLBRCY128, respectively, have been evolved for biofuel purposes. The exploration of biodiversity could uncover genes in other species that would be useful for the production of biofuels.. This experiment analyzes biomass, xylose consumption, and ethanol production of wild and artificial hybrid strains, and how tolerance of these traits are affected by the most important lignotoxins. Statistically significant values were found for the wild and engineered strains during the comparison of xylose consumption by media and the comparison of each strain in ACSH. The hybrid strains also provided statistically significant results during the comparison of xylose consumption in ACSH and ethanol production across medias.

Keywords: ACSH, lignotoxin, yeast, biofuel, *Saccharomyces* genus

Introduction

Because the demand for energy is increasing globally faster than natural energy resources are being replenished, alternatives must be found. The fossil fuels that are available currently tend to be concentrated in a small section of the globe, which can cause great issues with world economics and politics. The addition of more available renewable energies can help increase energy supply stability and security (Coelho 2005). Although biofuel production has been evolving for years, it still has much room for improvement. The biorefineries dealing with this biomass are tasked with engineering creative ways to gain energy, such as biofuels, from biological materials such as wheat, sugarcane, and biomass (Hong 2012). Low levels of ethanol tolerance, low variance of sugar consumption, low ethanol production in comparison to sugar consumption by microbes, and reactions to the methods of biomass degradation stand in the way of a better biofuel

Current production of biofuel from bioethanol is extremely stressful to the microbes used to convert sugars into ethanol, due to the highly stressful environment involving an enzymatic degradation of the pretreated biomass (Jin 2013). The pretreatment used on the biomass creates highly chemically diverse lignocelluolosic hydrolysate (LCH) inhibitors that reduce the biofuel yield through the inhibition of various cellular processes. The cells must partition their resources in order to survive these conditions, which can cause negative effects such as membrane, protein, and nucleic acid damage, enzyme inhibition, reduced NADH/NADPH pools, DNA

mutagenesis, and induced apoptosis. While it would be optimal to remove these harmful molecules, this can be extremely difficult and expensive, and remove a large amount of the fermentable sugars (Piotrowski 2014). The lignotoxins tested in this experiment are a result of degradation of the cellulose, hemicellulose, and lignin present in the lignocellulosic feedstocks. 5-hydroxymethylfurfural (HMF) is an aliphatic acid formed by the dehydration of hexose sugars. Two of its degradation products, formic and levulinic acid, have been found to inhibit yeast at concentrations higher than 100mM, while the degradation of these products also causes a drop in intracellular pH, and therefore induced cell death (Jönsson 2013). Feruloyl amide (FA) is a phenolic with similar cellular effects as HMF, and is a result of the use of high-pressure/temperature ammonia, which alters the cellulose matrix and allows hydrolysis by cellulases, causing the production of the amide version of ferulic acid (Piotrowski 2014). P-Coumaric (PC) acid is a main component of lignin and is a hydroxyl derivative of cinnamic acid, which is present in biomass. The sodium acetate (SA) is the sodium salt of acetic acid. The acetic acid is derived from lignocellulose and interferes with macromolecule biosynthesis and DNA synthesis and repair (Piotrowski 2014). The effects of these compounds must be studied further in order to fully understand their effect on xylose consumption and ethanol production in yeast species.

Saccharomyces cerevisiae is often used as a model species due to its extensively studied and easily manipulated genome. In optimal scenarios involving this species, ethanol is produced quite efficiently through the conversion of glucose and xylose. However, the industrial version of this strain must be pushed further in order to maximize its stress tolerance, therefore creating more ethanol. At this time, a series of yeast strains

have been genetically engineered, many by the Great Lakes Bioenergy Resource Center (GLBRC) in Madison, WI to contain xylose fermentation pathways. These strains have been selected because of their thermotolerance in ACSH, ethanol tolerance, and xylose consumption (Jin 2013). Because many natural *Saccharomyces* species lack the ability to ferment xylose, this engineering has taken specific genes from other genera in order to improve tolerance to these conditions. The GLBRC has engineered two reference *Saccharomyces cerevisiae* strains engineered to ferment xylose in aerobic and anaerobic conditions, GLBRCY73, and GLBRCY128, respectively.

The utilization of biodiversity in the biofuel industry is an undeniable asset to the improvement of these microbes. Various wild *Saccharomyces* strains may contain useful traits for increased ethanol tolerance and xylose consumption. There are currently seven distinct species within the genus, with exceptionally diverse traits and protein sequences. While it was originally used for brewing wines and beers, *Saccharomyces* strains and various hybrids have been engineered for other purposes, such as medicine and biotechnology (Hittinger 2013). This experiment studies all members of the genus, as well as common hybrids.

The hybridization of these wild isolates could also prove very beneficial to the improvement of biofuels. The engineering of strains is a long and more complicated process compared to hybridization. By placing two genomes together, a new strain may be derived with an intermediate set of traits. Only the strains tolerant to ethanol survived, allowing for the selection of ethanol-resistance traits. The genomes that survived the hybridization process, yHDPN1 and yHDPN5 in this study, are then evolved and compared. This process is further explained in the materials and methods section.

This study aims to discover beneficial traits through the exploration of biodiversity and hybridization, in hopes of finding genetic predispositions to xylose consumption, increased ethanol production, and improved lignotoxin tolerance. Wild *Saccharomyces* strains previously selected due to their high performance in ACSH were screened and compared to current chassis strains. The exploration of fermentative properties of previously generated hybrids allowed for further understanding of the hybridization capabilities and trait combination of two different species. Through a series of fermentation experiments coupled with molecular analysis of end products, these aims can be further understood.

Materials and Methods

Strain cultures

The strains used during this experiment are listed below in Table 1. Stocks of each strain have been cryogenically preserved and samples were grown on a YPD (2% glucose, 2% peptone, 1% yeast extract) plate sealed with parafilm at room temperature. Once colonies could be seen on the plate, the plate was placed in a fridge so it could be used for up to 3-6 months.

Artificial hybrid generations

Two artificial hybrids and their evolved strains (Table 1) were selected from a previous study to test. The fermentative properties of these strains in ACSH, as well as the combination of important traits from different species through hybridization, was studied in order to improve bioethanol production and tolerance to lignotoxins. Artificial hybrids were generated by mass-mating of haploid versions of *S. cerevisiae* engineered

strains with haploid non-*Saccharomyces* strains engineered with drug markers (Navarro). Crosses are detailed in Table 1.

Artificial hybrids were evolved in triplicate for 50 generations (G) at 30ºC in ACSH. Briefly, evolution assays required consisted of serial dilutions in ACSH at 30ºC every 14 days. In these conditions, it was expected that cells able to consume xylose will expand in population and survive the bottleneck of the serial dilutions due to the absence of glucose after 48 hours. In addition, cells with higher growth rates and increased tolerance to lignotoxins and ethanol will expand more rapidly in the population. Every 14 days, each culture was serial diluted once again into a new ACSH medium with a starting OD_{600} 0.1 of the previous ACSH culture. Only two replicates of yHDPN1 and one replicate yHDPN5 reached the 50G. The best-growing colony from each evolved yHDPN1 and yHDPN5 in a screening of 10 colonies was selected for each replicate in Synthetic Minimal + Xylose (2%Xyl, 6.7g Yeast Nitrogren Base without amino acids) (Navarro). Evolved strains are described in Figure 1.

Fermentations

Each of the YPDX+ individual lignotoxin media contained YP (1% Yeast Extract, 2% bactopeptone), glucose (40%) and xylose (40%) to create the fimal YPDX (6% glucose+ 3% xylose). The following lignotoxins were added to create the individual lignotoxin medias: sodium acetate (2.65g, 32mM), p-Coumaric (344.4mg, 2.10mM), feruloyl amide (1007.5mg, 5.5mM), and HMF (138.6mg, 1.1mM). The YPDX+All lignotoxins contained the previous chemicals as well as acetamide (4.7428g, 60mM), ferulic acid (139.7mg, .71mM), and coumaroyl amide (896.5mn, 5.5mM). The final pH was 5.2 in order to mimic the pH of ACSH.

Metabolically active strains were obtained by growing them in 5mL of Synthetic Complete media (1.7g Yeast Nitrogen Base without amino acids, 5g Ammonium Sulfate, 2g Drop-out Mix Complete without Yeast Nitrogen Base) in culture tubes with a small cluster of cells from the plates. Saturated cultures were placed on a wheel set at full speed for at least 48 hours.

Once the strains have been pre-cultured, an optical density (OD_{600}) measurement was taken using a 600nm spectrometer, ensuring each fermentation flask had an equal amount of cells and an OD_{600} of 0.2. Pre-culture cells were collected by centrifugation of 1 minute at 15000 rpm. The supernatant was removed and discarded, and cells were resuspended with 1 mL of double distilled milliQ water.

Sterilized UV flasks were coupled with a rubber stopper and an airlock to create a microaerobic environment. The stopper was placed with one hole covered by the airlock and the other with a small rubber piece with tape so that samples can be taken at each time point. The working media volume was 50mL; 49mL of media and 1mL of sample. A negative control (media with 1mL of milliQ and no cells added) was added to ensure no contamination occurred. In order to avoid contaminations, all sample measurements were taken in a sterilized UV hood. 1 mL of each cell sample mixture was then added to its respective flask. Once all samples were added to the media, they were mixed and 600 μL of each sample was taken. The rubber stoppers were then sealed onto the flasks using a strip of parafilm.

The samples taken were then vortexted until completely homogenous. For the initial time point, 200 μL of each sample was added to its respective culture tube containing 1800 μL of water. The optical density was then recorded, using the negative

control as a “blank”. The remaining 400 µL were then centrifuged to separate the cells and media. The supernatant was then placed into a tube and frozen at -80C until it was sent to the HPLC.

The number of informative time points across a 14 day fermentation was previously explored. There are five total time points: 0 hours (T_0), 46.5hours (T_1), 166.5 hours (T_2), 262.5 hours (T_3) and 334.5 hours (T_4). At each time point, 600µl of each sample is taken.

HPLC data analysis

Concentrations of glucose, xylose, and ethanol in hydrolysates and fermentation samples for each tested media were determined by high-performance liquid chromatography (HPLC; Agilent 1260 infinity) using a quaternary pump, chilled (4 ºC) autosampler, vacuum degasser, and refractive index detector (Agilent Technologies, Inc., Palo Alto, CA). The HPLC column consisted of an Aminex HPX-87H column (Bio-Rad) operating at 50 °C, a mobile phase of 0.02N H2SO4, a flow rate of 0.5 ml/min (Navarro).

Statistical analysis

A one-way ANOVA test was used to compare glucose consumption, xylose consumption, and ethanol production across medias. Individual strains were analyzed using one-way ANOVA for ACSH. Kruskal-Wallis tests were conducted to provide p-values between medias, and a Tukey HSD test was used to compare strains in ACSH.

Results

Biomass of Wild and Engineered Strains

Biomass data was collected by using the OD measurements collected during the fermentation process. The flocculant strains were discarded because of the high probability of error within the measurements, as an improper amount of cells could be taken. The strain with the highest biomass out of all wild and engineered strains is yHCT77 in most conditions, except for ACSH. The wild and engineered strains with the lowest biomass is as follows: GLBRCY128 in YPDX, YPDX+PC, YPDX+SA, YPDX+ALT and ACSH, and yHAB335 in YPDX+FA and YPDX+HMF. See Table 1. There was a nonsignificant p-value calculated between the seven medias overall when using a Kruskall-Wallis test. However, a Wilcoxon Rank Sum identified a significant p-value during the comparison of the following: ACSH and YPDX+SA ($p=0.018$), ACSH and YPDX ($p=0.028$), and YPDX+All lignotoxins and YPDX ($p=0.029$). YPDX+HMF had the highest number of flocculant strains.

Xylose Consumption in Wild and Engineered Strains

The examination of the xylose consumption in the wild and engineered strains (Table 3) showed that the most xylose consumed was mainly when the strains were in YPDX (Figure 1). The comparison of xylose consumption across medias provided two significant p-values. The comparison of ACSH and YPDX, as well as the comparison of ACSH and YPDX+SA, provided a p-value of 0.033. When in ACSH, GLBRCY73 consumed the largest amount of xylose. A Tukey HSD test identified three homogenous groups in ACSH: Group A consisted of yHAB413, yHAB407, yHAB94, yHCT77, GLBRCY128, yHAB335, and yHDPN14; group B of yHCT77, GLBRCY128, yHAB335, yHDPN14, and yHAB336; and group C of only GLBRCY73.

Ethanol Production of Wild and Engineered Strains

In order to see the effects of sugar consumption on ethanol production, a series of scatterplots were made to show any correlation between the two variables (Figure 2). It could be expected that a higher amount of sugar consumed leads to a higher ethanol production. A scatterplot was created for each media. When compared to ACSH, all the individual lignotoxins had a higher correlation between total sugar consumed and ethanol produced. The correlation between ethanol production and percent sugar consumed was higher in the medias containing one lignotoxin than in ACSH. While no distinction was made between hybrid, wild, and engineered strains, the data is still accurate. These correlations are consistent with the other data found.

The production of ethanol of wild and engineered strains was tested across a variety of medias and compared to sugar consumption. All wild and engineered strains produced more ethanol in (g/L) in ACSH than in other medias, aside from GLBRCY128, which produced the most ethanol in YPDX+HMF (Figure 3). Further triplicate analyses will be done later this year to ensure accuracy. The ethanol produced was also compared to the theoretical yield for each strain. The final ethanol production of wild strains was lower than that of hybrid strains when compared to the theoretical yields. A significant p-value was found in the comparison of ACSH and YPDX. A Tukey HSD test identified three homogenous groups: group A consisted of yHDPN14, yHAB336, yHAB413, yHAB94, yHCT77, yHAB407, and GLBRCY128; group B consisted of yHAB336, yHAB413, yHAB94, yHCT77, yHAB407, GLBRCY128, and GLBRCY73; and group C consisted of yHAB335, yHDPN14, yHAB336, and yHAB413. Group B produced the most ethanol in ACSH, followed by group A, then group C.

Since many yeast species also consume ethanol, it can be difficult to determine the exact amount of ethanol produced. In almost all HPLC samples taken, the ethanol amount in grams per liter is higher at the first time point than at nearly any other point. These can be seen in the graphs that compare the total ethanol in each HPLC time point in each media by species.

Hybrids

Many of the hybrid strains were flocculant. YPDX had three flocculant strains, YPDX+HMF had all flocculant hybrid strains, and YPDX+All lignotoxins had two flocculant strains. See Table 1. The hybrid strain with the lowest biomass in each media are as follows: yHDPN5 in YPDX+PC, YPDX+FA, and ACSH; yHDPN399 in YPDX+SA; and yHDPN1 in YPDX+All lignotoxins. The highest biomass was attributed to the following: yHDPN1 in YPDX+PC, YPDX+SA, and YPDX+FA; yHDPN379 in YPDX+All lignotoxins; and yHDPN399 in ACSH. A Kruskal-Wallis test identified no significance among the six medias containing non-flocculant strains.

The xylose consumption of four hybrids in a 30^0C incubator was compared (Figure 1, Table 3). All of the hybrids produced approximately the same amount of ethanol, but the evolved hybrids consumed drastically more xylose than the ancestral hybrids when in ACSH. When these strains were immersed in different medias containing an individual toxin that is present in ACSH, the xylose amounts decreased. An ANOVA test comparing the medias identified a significant decrease in xylose consumption in the ancestral hybrids. Significant p-values were found during the comparison of ACSH and YPDX, YPDX+FA, and YPDX+SA as well as the comparison

of YPDX+ all lignotoxins and YPDX+FA. The comparison of YPDX+ all lignotoxins also produced two p-values slightly above 0.05; YPDX and YPDX+SA.

Ethanol production of hybrid strains was also analyzed in a variety of medias (Figure 3). This graph shows that the ethanol production of ethanol in (g/L) is increased in ACSH for all hybrids except for yHDPN399, in which the largest ethanol production in (g/L) is in YPDX+SA. Triplicate analysis will be completed on the YPDX+ individual lignotoxins data later this year so further statistical analysis can be completed. A Kruskal-Wallis test provided a statistically significant value ($p = 0.01$) during the comparison of ethanol production across medias, with strains producing the most ethanol in ACSH. As seen in Figure 4, the consumption of xylose production of the evolved hybrids is much higher than its ancestor hybrid, while the ethanol production is approximately equal to that of the parent GLBRCY73.

Discussion

The observations within this study are consistent with previous works. This study identified a decrease in xylose consumption but an increase of ethanol production with the addition of more lignotoxins. The average biomass (excluding flocculant strains, but including hybrdis) also appeared to drop with the addition of more lignotoxins. The cellular effects of lignocellulose derived LCH inhibitors and process derived LCH inhibitors, and their diversity, were previously described (Piotrowski 2014). These inhibitors have been known to deplete cellular energy resources by channeling them into detoxification of the cell and repair rather than growth or sugar conversion. They also damage key enzymes of fermentation pathways, which can greatly reduce the ethanol

production by the microbes. The furans derived by the lignocellulose, such as HMF, reduce NADH/NADPH pools, which are necessary for the detoxification of the cell, sulfur assimilation, and fermentation. The cell's main focus is survival, so in the presence of these furans, it uses its NADH/NADPH pools for detoxification rather than the production of ethanol.

The analysis of xylose consumption in engineered and wild strains did produce some negative xylose consumption values in the HPLC data. However, *Saccharomyces* yeasts are incapable of producing xylose, and these negative numbers are likely the effect of inaccurate measuring by the machine. In this data analysis, they were counted as zero xylose consumption. There is a significant difference between the consumption of xylose in YPDX+ all lignotoxins and ACSH when compared to other medias. This could be a result of the stress the cell undergoes as a result of the large amount of toxins in the medias. As the cell attempts to filter through the toxin-rich media in search of food, it potentially uptakes xylose. The phenolics present in the medias decrease the cellular ATP, which is necessary for the transportation of toxic molecules out of the cell. This depletion of ATP affects xylose conversion due to the lower cellular energy produced per molecule of xylose transported. These LCH inhibitors that affect coping mechanisms requiring ATP directly reduce the conversion of xylose (Piotrowski 2014). The strains that consumed xylose well in the presence of ACSH (GLBRCY73, yHDPN399, yHDPN379) and YPDX+ all lignotoxins (GLBRCY73, GLBRCY128, yHDPN399, yHDPN379) will be analyzed further for their biofuel potential. Through engineering and hybridization the traits that allowed these strains to consume xylose well can be combined and analyzed for their potential in the biofuel industry.

Ethanol production in all strains and medias was also analyzed. Because the comparison is done by (g/L), in many cases, it appears strains in ACSH produce the most ethanol. However, this is not necessarily contingent with the correlation data in Figure 3. Further analysis will be required. There appears to be no statistically significant change in ethanol production in the hybrid strains. The final ethanol production was also compared to the theoretical ethanol production. This low ethanol production, as discussed before, is likely a result of the furan and phenolic derivatives from lignocellulosic material pretreatment. These furans and phenolic derivatives act synergistically to inhibit fermentation, likely due to imbalances of ATP and NADH, which are required for fermentation and detoxification (Piotrowski 2014). The production of ethanol is dependent on efficient utilization of D-xylose, which although *S. cerevisiae* contains all the enzymes required for a full xylose metabolic pathway, produce a considerably lower amount of ethanol than glucose (Matsushika 2009). While improvements have been made regarding the ethanol yield from *Saccharomyces* strains, there are still major by-producs, such as xylitol, that are produced. The reduction of xylitol production has also been shown to accompany higher ethanol production (Matsushika 2009).

The biomass data obtained provides an idea as to how quickly a species is growing and adapting in a specific media. The cell must expend its energy on detoxification, due to the presence of furans and phenolics. As a result, NADH and NADPH that would be used for fermentation must be used for cell repair (Piotrowski 2014), which can hinder growth. The growth of the cell, while occasionally correlated with xylose consumption and ethanol production, cannot be used alone to determine the success of a species in a particular media. The flocculation of the strain as well interferes

with the biomass calculation. Highly flocculant strains are often more difficult to accurately measure because too many or two few cells may be taken with a sample. Because YPDX+HMF has the highest number of flocculant strains, the statistical data including this media may be slightly inaccurate. A nonsignificant p-value was calculated during the comparison of biomass in all medias, indicating that there was no significant difference in the way the strains adapted to each media overall. However, looking media by media, significance was found when ACSH was compared to YPDX+SA ($p=0.018$) and YPDX ($p=0.028$), as well as when YPDX+All lignotoxins was compared to YPDX ($p=0.029$). These differences indicate the media contains compounds that interfere with cellular growth. Further analysis will be needed to determine the differences in resource partitioning.

The xylose consumption of hybrid yeasts was also analyzed. The significant p-values found indicate a notable difference in xylose consumption of the strains when they are in YPDX+ all lignotoxins and ACSH. The graphic comparison of these hybrids also displays this data. These hybrids are vital to the improvement of biofuels because they provide a less complex way of combining traits of two species for desired traits. Figure 4 displays how these hybrids produce ethanol almost as efficiently as the engineered GLBRCY73, as well as consume xylose far better than its ancestral hybrid.

The analysis of ethanol production in the hybridized strains indicated that there was a general increase of ethanol production with an increase of lignotoxins, such as when in ACSH or YPDX+All lignotoxins. A potential explanation could be the cell filtering out toxins present in the media, which also transports whatever ethanol or xylose is in the media. This result needs to be further investigated.

Conclusion

Because a main goal of this project is to increase ethanol yield and tolerance along with increasing xylose consumption across the *Saccharomyces* genus, these data will need to be further analyzed in order to determine further projects that will optimize the characteristics desired, such as resilience to the conditions present during biofuel production. These further projects will enhance our understanding of the *Saccharomces* genus and how its diversity can be utilized to benefit bioethanol production on an industrial scale.

The presence of lignotoxins in the environment was shown to inhibit xylose consumption while also increasing ethanol production in wild, engineered, and hybridized strains. This relationship must be further explored in order to understand the cellular processes. The analysis of by products not discussed in this paper, such as glycerol, succinate, xylitol, and acetate, may help uncover new ways to improve the production of ethanol through the conversion of sugars.

Since we already know that *Saccharomyces* yeasts are capable of producing ethanol on an industrial scale for beer, wine, and ciders, we must utilize this knowledge so it can be incorporated into the biofuel industry. These industrialized strains for alcoholic beverage production are already tolerant to many stressors present in the production of bioethanol, such as ethanol. These natural hybrids are likely exceptionally important to the understanding of tolerance to industrial stressors. Through the exploration of biodiversity within the *Saccharomyces* genus, more strains with the ability to consume and ferment xylose can be uncovered and hybridized.

Acknowledgments

I thank William G. Alexander, Mario Sardi, Lucas Parreiras, Alex Reau, Yaoping Zhang, and Trey Sato for strains, as well as Yaoping Zhang, David Benton and HPLC service from GLBRC facilities. I would like to thank David Peris and Chris Todd Hittinger for their mentorship. This material is based upon work supported by the National Science Foundation under Grant No. DEB-1253634 and funded in part by the DOE Great Lakes Bioenergy Research Center (DOE Office of Science BER DEFC02-07ER64494).

References

Almeida, Pedro *et al.* 2014. A Gondwanan imprint on global diversity and domestication of wine and cider yeast *Saccharomyces uvarum*. Nature Communications. 5:4044. doi:10.1038/ncomms5044

Coelho, Suani Teixeira. (2005) Biofuels- Advantages and Trade Barriers. United Nations Conference on Trade and Development.

Hittinger, Chris Todd. (2013) *Saccharomyces* diversity and evolution: a budding model genus. *Trends in Genetics* Vol. 29.5

Hittinger, Chris Todd. 425 Henry Mall. University of Wisconsin-Madison, Madison, WI 53706. Pers. Comm.

Hong, Kuku-Ki, and Jens Nielson.(2012) Metabolic engineering of Saccharomyces cerevisiae: a key cell factory platform for future biorefineries. *Cellular and Molecular Life Sciences.*.

Jin, Mingjie and Trey Sato *et al*. (2013) Phenotypic selection of a wild *Saccharomyces cerevisiae* strain for simultaneous saccharification and co-fermentation of AFEX pretreated corn stover. *Biotechnology for Biofuels.* 6:108.

Jönsson, Leif J *et al*. (2013) Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnology for Biofuels*. 6:16.

Matsushika, A. (2009) Ethanol production from xylose in engineered *Sacchromyces cerevisiae* strains: current state and perspectives. *Applied Microbiology and Biotechnology*. 84:37.

Navarro, David Peris. 425 Henry Mall. University of Wisconsin-Madison, Madison, WI 53706. Pers. Comm.

Parreiras LS, Breuer RJ, Avanasi Narasimhan R, Higbee AJ, La Reau A, et al. (2014) Engineering and Two-Stage Evolution of a Lignocellulosic Hydrolysate-Tolerant *Saccharomyces cerevisiae* Strain for Anaerobic Fermentation of Xylose from AFEX Pretreated Corn Stover. PLoS ONE 9(9): e107499. doi: 10.1371/journal.pone.0107499

Piotrowski JS *et al.* (2014) Death by a thousand cuts: the challenges and diverse landscape of lignocellulosic hydrolysate inhibitors. *Front. Microbiol.* ***5****:90.*

Wohlbach, Dana J. *et al*. (2014) Comparative genomics of *Saccharomyces cerevisiae* natural isolates for bioenergy production. *Genome Biology and Evolution*.

Figure legends

Table 1. Strains Used in this Study. A list of all strains used for the duration of this study and reference strains, including synonyms and species, the papers and studies they debuted in, population, and origin.

Table 2. Strain Biomass by Media. Yellow, green and red indicates flocculation, the highest and lowest biomass producer, respectively

Table 3. Xylose Consumption by Media. The xylose consumption (g/L) of each strain is numerically compared across all medias tested with raw values, means, and standard deviations.

Figure 1. Through these graphs, the consumption of xylose in various strains is compared. The wild yeasts were grown in room temperature, while the hybrid strains were grown in a 30^0 incubator. In the majority of cases, the highest xylose consumption is in YPDX. However, this contains no lignotoxins and is an unrealistic expectation for biofuels. ACSH closely resembles the solution the yeasts are exposed to. The negative values of xylose consumption are impossible, because yeasts are incapable of producing xylose, and therefore that data is likely inaccurate due to measuring or sampling errors. ACSH is also the only media tested in triplicate, so the average value is displayed. The other medias will have further analysis done later this year. These data show that so far, the best three strains consuming xylose in ACSH are GLBRCY73, yHDPN399, and yHDPN379.

Figure 2. The ethanol production by percent sugar consumed is compared in various medias. The correlation was then calculated to determine strength of relationship. Glucose and xylose were combined into a total sugar consumed. All strains used in this study are included in these graphs. No distinction is made between hybrid and wild strains. (A) In YPDX, there is a strong, positive correlation between the ethanol produced in (g/L) and the percentage of sugar consumed, indicating that higher amounts of sugar consumed is related to a higher ethanol production. (B) The correlation between ethanol produced and sugar consumed in YPDX+PC is high and positive, indicating an increase of ethanol production with an increase of sugar consumption. (C) Ethanol production in (g/L) is strong and positively correlated with the total percentage of sugar consumed in the media YPDX+SA. Higher percentages of sugar consumption were associated with higher ethanol production. (D) A strong positive correlation is found between the ethanol

production and sugar consumption of wild and hybrid species in YPDX+FA. (E) The comparison of ethanol production and total sugar consumed produced a strong positive correlation in YPDX+HMF. (F) A mild positive correlation between ethanol production and sugar consumption is found in ACSH. Triplicate analysis was used.

Figure 3. Final Ethanol Production of Yeasts in Various Medias. The final ethanol production in (g/L) of wild and engineered strains in room temperature is shown in 3A, while the final ethanol production in (g/L) of hybrid strains in 30^{o}C is shown in 3B. Ethanol is also consumed by yeasts, causing the earlier time points to have higher ethanol concentrations than the final time point. Further analysis will be done.

Figure 4. Comparison of ethanol production and xylose consumption in hybrids. Ethanol production and xylose consumption is analyzed. Shades of green represent *Saccharomyces cerevisie* and *Saccharomyces mikatae* crosses, and shades of purple represent *Saccharomyces cerevisiae* and *Saccharomyces kudriazevii* crosses. Dark shades are evolved strains. This figure displays how evolution over 50G allowed for xylose consumption larger that of the ancestor hybrid and approximately equal to that of the parent *S. cerevisiae* (GLBRCY73) while producing an approximately equal amount of ethanol.

Tables and Figures