ABSTRACT

SACHS, CHRISTINA E. Using Selective Breeding Methods to Develop a Strain of Yeast for Ethanol Fermentation of Duckweed Feedstock. (Under the direction of Dr. Ryan Sartor).

Duckweeds have the potential to be used as a biomass feedstock for fermentation due to their rapid growth rates, starch accumulation properties, and lack of competition with food crops. Fermenting new feedstocks requires optimized processes, including microbe strain development. Current methods for microbe strain development utilize evolutionary engineering or genetic engineering methodology, which are either simple but very limited in selection capability, or involve complex genetic modifications, respectively. In this study, we propose to use selective breeding as an alternative method for strain development of the yeast *saccharomyces cerevisiae,* with the intention of improving existing wild strains to be more efficient at fermenting duckweed feedstock. In the selective breeding method, individuals from the starting population are phenotyped for a trait of interest, and the best individuals are selected and crossed to form a new generation. *S. cerevisiae* has a well-characterized breeding cycle, with a unique ability to undergo meiosis during the sporulation process, which allows for crossing of two or more individuals at a microbial scale. In this study, wild strains of *s. cerevisiae* were selected from different geographical regions and the two strains with the best sporulation efficiencies were chosen as the parents for this study. Additionally, an artificial laboratory evolution (ALE) breeding cycle was carried out in parallel to the selective breeding cycle, and a domesticated yeast was used as a control. After obtaining spores for the parents, a spore isolation treatment was optimized with exposure to Y-PER protein extraction reagent and heat shock at 60°C. simultaneously with an incubation time of 40 minutes. Dilution plating was performed to isolate 96 individual colonies, forming a new population. For selecting the top individuals, growth rate

was used as a proxy for ethanol production. A high-throughput phenotyping method was developed to quantify growth rate using a spectrophotometric reading. Additionally, small-scale fermentations were performed in duckweed hydrolysate and YPD media. Quantification of ethanol requires an expensive assay or advanced techniques like GC-MS, while growth rate is much more efficient to quantify on a large population of individuals. For the initial small-scale fermentations, domesticated control yeast outperformed the wild yeast for ethanol production when fermented in a sucrose media, while the wild yeast outperformed the domesticated yeast in duckweed. The top 10 individuals based on growth rate were bulk crossed to form the next generation, and this process was repeated for a total of 3 selection cycles. The top lines from the final generation, the ALE, and the control yeast were fermented in duckweed hydrolysate and ethanol was quantified with GC-MS. Final tests were inconclusive due to contamination of the duckweed hydrolysate. Future work would include testing the developed strains in a fresh duckweed media. It has been shown in previous studies that fermenting duckweed biomass can yield high ethanol concentrations. In addition, the selective breeding of the final lines could be continued for further generations to yield greater genetic improvement.

Using Selective Breeding Methods to Develop a Strain of Yeast for Ethanol Fermentation of Duckweed Feedstock

by
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BIOGRAPHY

Christina Sachs is a student of Biological Engineering at NC State University. She worked as a laboratory technician before taking on more complex research projects for the department. Upon completion of her bachelor's degree, she decided to pursuit her Master of Science in the same program.

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AUTHORSHIP STATEMENT

This thesis is the sole work of the student; there are no original contributions from others aside from those captured through citations. There was no use of Generative Artificial Intelligence in the making of this document.

CHAPTER 1: LITERATURE REVIEW

1.1 Current Ethanol Production

Most gasoline for vehicles in the United States is classified as either E10 or E15 fuel, which contains 10 or 15% ethanol (renewable energy) and 90 - 85% gasoline (derived from fossil fuels), respectively (U.S. Energy Information Administration, 2024). Using ethanol as a fuel additive is common practice in the US and other countries like Brazil. In the US there are federal regulations such as the Renewable Fuel Standard that include requirements to meet a minimum volume of renewable fuels such as bio-based ethanol. In the US, 94% of this ethanol is produced from maize starch, mostly grown in the Midwest. This crop is water intensive, uses significant land area, and requires significant post-processing for conversion to ethanol (USDA; Barboza Devos & Colla, 2022). Figure 1 shows (AFDC) how much land area of corn is dedicated to ethanol vs. food production in the United States. Currently, about 40% of the maize grown in the US is used for ethanol production, accounting for over 10% of the total cultivated cropland in the country.

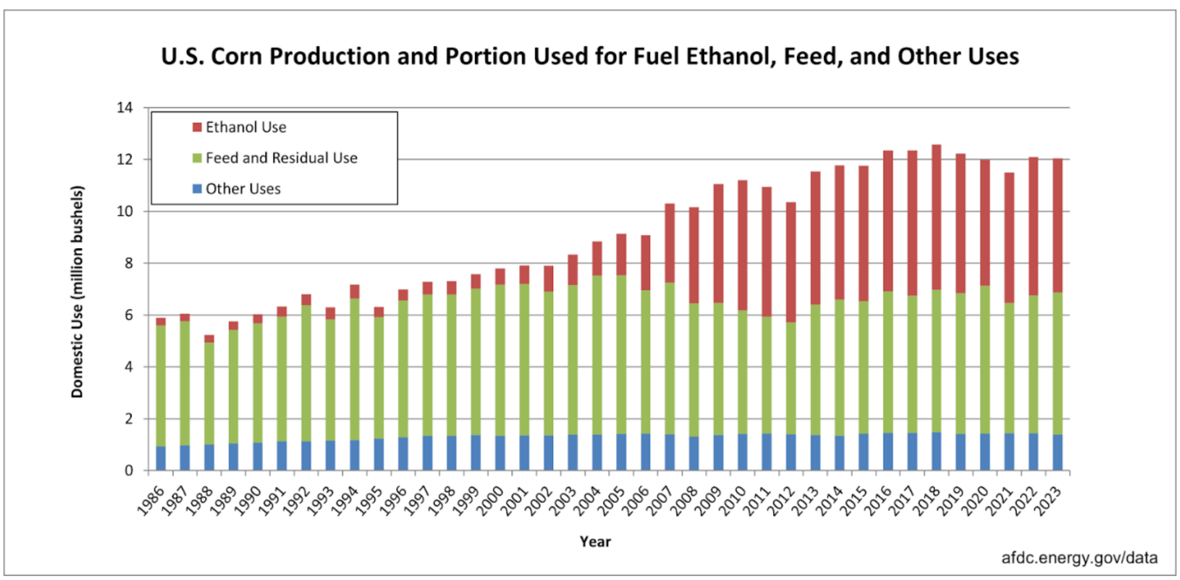

Figure 1: land area of corn dedicated to ethanol has increased significantly in recent years.

The original U.S. Renewable Fuel Standard Program (below figure 2) capped corn ethanol at 15 billion gallons/year (National Research Council, 2011) due to the competition this would create with food production. In addition, corn is a relatively unsustainable crop and in the early 2000s, it appeared that better alternative fuel feedstocks were just on the horizon. The original intent with the RFS was to begin ramping up cellulosic ethanol production around 2011. It was clear, even 20 years ago, that alternative feedstocks would be essential for producing bio-based ethanol in order to meet the energy demand in the US, while using sustainable production methods.

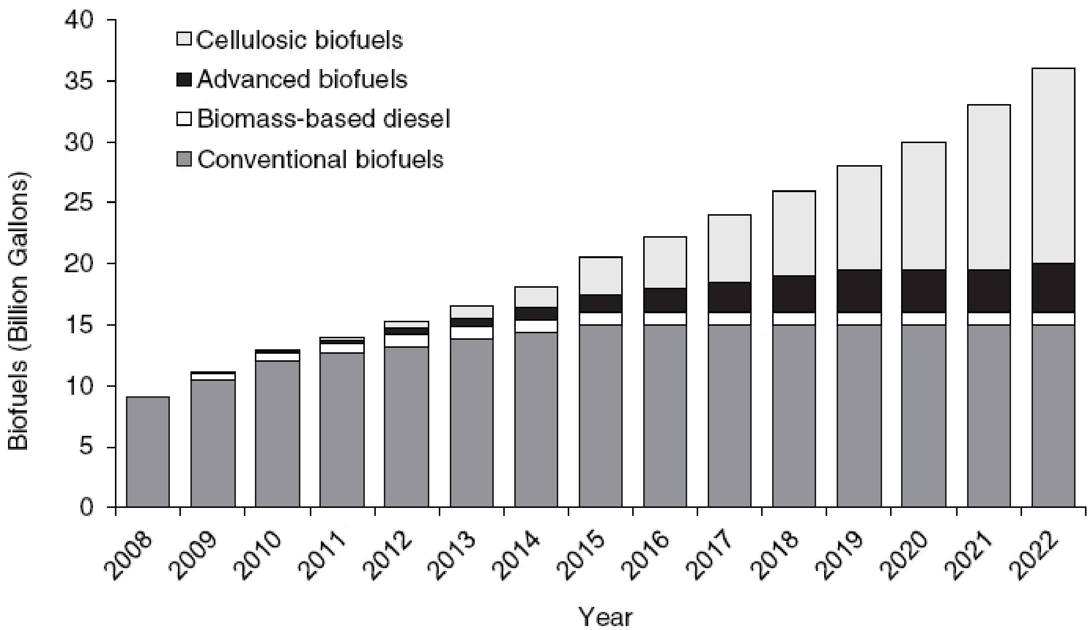

Figure 2: biofuels production in the US, RFS prediction (2011).

Alternative feedstocks, such as switchgrass or miscanthus, have been explored for ethanol production. These crops have lower water and fertilizer requirements compared to maize, but mainly produce cellulose instead of starch (AFDC). Cellulosic ethanol feedstocks contain cellulose, hemicellulose, and lignin. Due to this complex composition, additional processing is required to release the sugars for ethanol conversion. Despite extensive research that has gone into developing methods to convert cellulosic biomass to ethanol, an economically feasible route of converting cellulosic biomass to simple sugars (figure 3) has yet to be found. Cellulosic biomass was never able to fulfill the promise of a sustainable, productive biofuel feedstock. The last functioning commercial cellulosic ethanol plant in the United States was shut down as of 2022 (US Energy Information Administration, 2024). As a result, nearly all ethanol production today uses starch- and sugar-based feedstocks, such as corn or sugar cane (AFDC). With these feedstocks, the starches and sugars are more readily available, making ethanol conversion more efficient and economically viable.

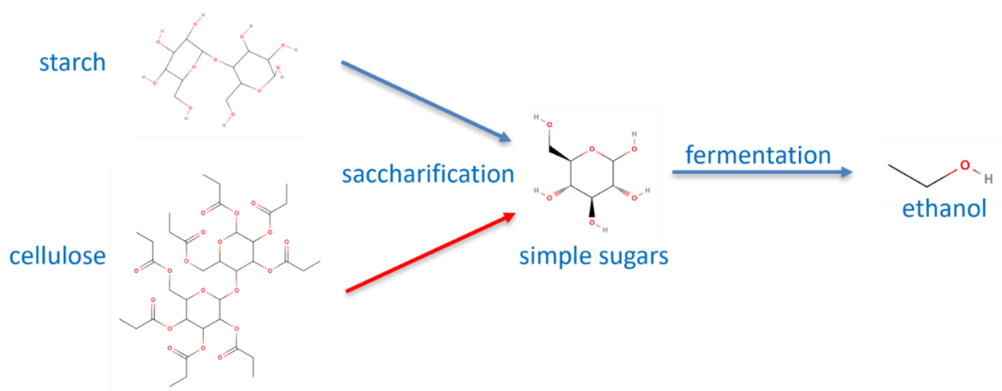

Figure 3: ethanol conversion pathway; conversion of cellulose to simple sugars (arrow in red) has been a limiting factor in implementation of cellulosic ethanol feedstocks.

Any future alternative biomass ethanol feedstock would need to meet several criteria:

- Low input (fertilizer, pesticide, herbicide, water, labor)
- High output (similar or greater yields compared to corn)
- Not complete with food crops for arable land
- Produce high starch or high sugar biomass

1.2 Duckweed as a potential feedstock for ethanol fermentation

Lamiaceae, or duckweed, is a small, freshwater aquatic plant (figure 4) that grows and reproduces mostly through continual clonal propagation. It can achieve rapid growth rates when grown in nutrient-rich wastewater (Zhou et al., 2023). Duckweed is naturally high in protein, ranging from 15-45% dry weight (Xu et al., 2012). In optimal growth conditions, starch content is moderate (10-20%), but nitrogen limitation is known to result in starch accumulation (Xu et al., 2011). Its rapid growth rate and ability to accumulate starch make it a promising candidate as a feedstock for ethanol fermentation.

Figure 4: duckweed (pictured lemna aequinoctialis, Valez-Gavilan, 2019) is an aquatic plant known for rapid growth and nutrient absorption.

Past studies suggest that duckweed could be used as a potential feedstock for ethanol production (Cheng & Stomp, 2009; Ge at al., 2012; Xu et al., 2011). Duckweed boasts many benefits compared to other alternative feedstocks:

1) It can yield high biomass per unit area, with higher growth rates compared to maize and other land-based crops (Ziegler at al., 2014). Studies routinely find yields of 12 MT/hectare/year or more (Cui & Cheng, 2014; Xu et al., 2011).

2) It can grow rapidly in waste water and absorb nutrients, serving as a natural wastewater remediation system by removing excess nitrogen and phosphate (Cheng et al., 2002; Coughlan et al., 2022; Zhou et al., 2023). It also requires no synthetic fertilizer, pesticide or herbicide inputs (Zhou et al., 2023). Its potential to be implemented on existing farms or municipal sites as a wastewater treatment system has been demonstrated (Mohedano et al., 2012; Zhou et al., 2018).

3) It produces high quality biomass that is naturally high in protein (Appenroth et al., 2017), and starch accumulation can occur through nitrogen starvation, resulting in a high-starch feedstock (Xu et al., 2011).

Nutrient starvation is currently the most viable method for cultivation of high-starch duckweed on a large scale (Xu et al., 2011). Duckweed is first grown using wastewater as the nutrient source. During this growth phase, the plant will hyperaccumulate nutrients, including nitrogen in the form of protein. The duckweed is then transferred to well water containing very low nutrients. During this time, the duckweed utilizes its stored proteins and other stored nutrients for growth and the starch content increases significantly. Using these methods, Xu et. al conducted a 2011 pilot study for cultivation of high-starch duckweed on a commercial swine farm in North Carolina. Results showed that after the nutrient starvation step, starch content increased nearly doubled from 17 to 31%.

Duckweed also has many sustainability benefits. Since duckweed is an aquatic plant, it does not compete with food for arable land and requires significantly less land compared to any current terrestrial crop because of its exceptionally high yield potential. (Coughlan et al., 2022) In addition, the plant is able to utilize the nutrients from wastewater, negating the use of synthetic fertilizers. Once a culture is established, it is resilient to biotic and abiotic stress and requires no herbicides or pesticides (Sree & Appenroth, 2024). By growing in the wastewater, it can serve a role in wastewater remediation, eliminating fertilizer runoff and leaching, and therefore reducing nutrient pollution.

Duckweed cultivation can be implemented on existing farms to provide wastewater treatment, and the biomass could then be harvested for conversion to ethanol or other biofuels, providing for a circular, cost-effective, and environmentally beneficial method for producing biofuel.

Methods for conversion of duckweed biomass to ethanol have been developed (Xu et al., 2011). However, several aspects of duckweed ethanol production have not been explored. Two main research topics include developing a strain of duckweed that is optimized for use as a fermentation feedstock, and developing a strain of microbe that is optimized for fermenting this duckweed. Any new feedstock represents potential complications for an existing process. When corn is used as a feedstock, the grain is harvested, dried and ground to expose starch. Corn grain is mostly starch and contains relatively low amounts of any potential microbial inhibitors. In the case of duckweed, the whole plant is harvested, dried and ground to expose starch. This creates a much more complex molecular cocktail of plant metabolites, including potential microbial inhibitors. In preliminary experiments (Figure 5 below), it was observed that two wild strains of yeast had improved ethanol production from duckweed fermentation compared to a control strain of yeast that was optimized for grain fermentation. Therefore, **in order to ferment a new feedstock, such as duckweed, a strain of microbe needs to be developed that is optimized to work with the new feedstock**. This study will focus on the second topic of developing a microbe for fermentation of duckweed feedstock. Specifically, we will explore using selective breeding methodology to create a new strain of yeast for optimized fermentation of duckweed.

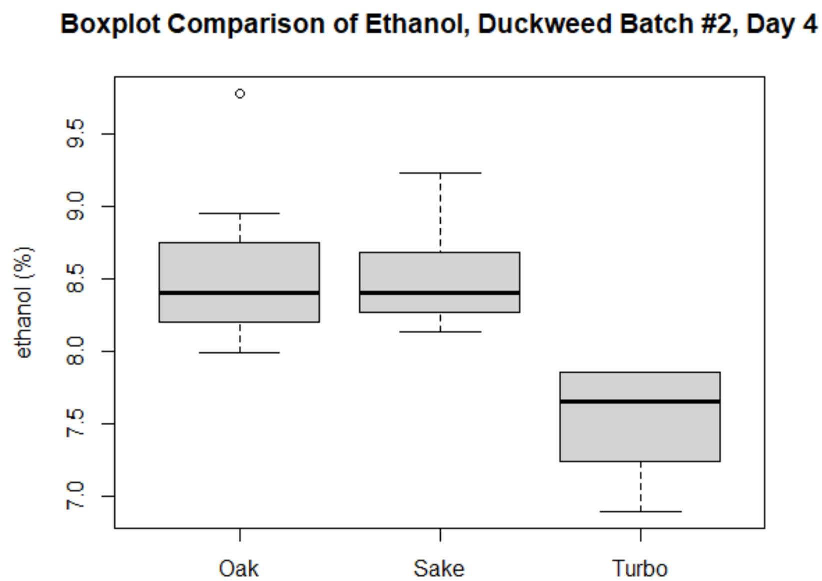

Figure 5: boxplot comparing mean ethanol concentrations (%) from duckweed fermentations; wild strains (Oak and Sake) outperform the domesticated strain (Turbo).

1.3 Breeding Methods

The yeast *Saccharomyces cerevisiae* is a common model organism for microbial research due to its rapid breeding cycles and ability to maintain large population sizes at a small scale (Vanderwaeren et al., 2022). It is the most commonly used microbe for industrial ethanol fermentations due to its survivability in various growth conditions, such as high ethanol concentrations or ability to grow in a wide pH range (Azhar et al., 2017). This yeast has benefitted from thousands of years of strain development and process optimization from the brewing and baking industries (Bai et al., 2022), and in more recent years has been explored for its potential in developing biotechnology like biofuels, chemicals, and pharmaceuticals (Borodina & Nielsen, 2014).

In addition to these benefits, the yeast *s. cerevisiae* has a well characterized breeding cycle which allows it to undergo sexual reproduction through sporulation (Walker, 2009). Therefore, selective breeding practices (sometimes called artificial evolution) can be used to quickly develop new varieties that could produce desired compounds for the bioproducts industry. Because microbes are typically propagated asexually, selective breeding on the microbial scale is a relatively underexplored concept that warrants further investigation. Currently, most research involving sexual reproduction in yeast focuses on yeast evolutionary biology and not on potential applications for improved yeast varieties.

The process of selective breeding has been extensively applied to nearly all plants and animals that we use in our modern society. It is a relatively simple but powerful way to develop new and improved breeds or varieties. These improved varieties can significantly increase production, increase quality, decrease input costs or produce new products all together. The value of selective breeding has been demonstrated over centuries of agriculture, in which breeders have produced desirable traits such as larger size, increased density tolerance or resistance to disease by combining parent strains with desirable characteristics to improve the next generation of crops. For example, Figure 6 below shows results from a long-term selective breeding study in maize (Rocheford, 2009) where one selection cycle per year was carried out. In the study, both high and low grain oil content was selected for and within 80 cycles, grain oil content increased 4-fold. This demonstrates the power of this approach. With corn, selective breeding took 80 years but a microbe can theoretically go through a sexual reproductive cycle in a matter of days, making this process much faster.

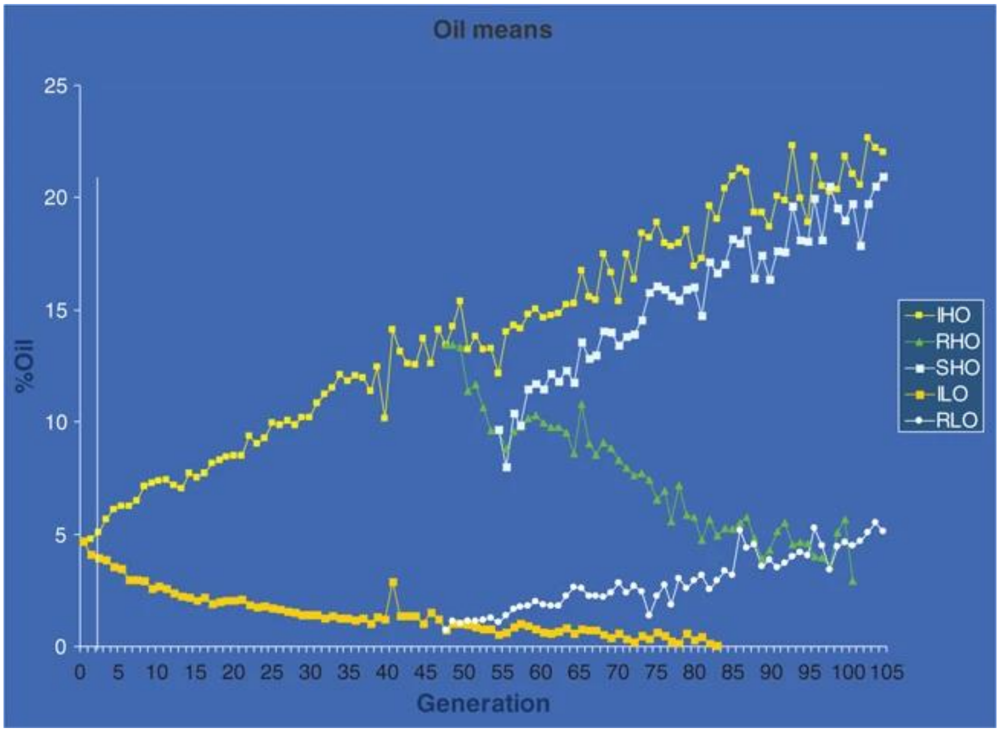

Figure 6: grain oil content increased 4-fold within 80 cycles (Rocheford, 2009).

A common method of selective breeding is recurrent selection. The goal of this process is to improve the performance of future populations for one or more characteristics. In this process, a population is phenotyped, in other words, observable traits are measured. The superior individuals based on the phenotyping are crossed to form the next generation. The process can be repeated cyclically in future generations to continue genetic improvement over time (Fehr, 1991) The process of selection enriches desirable genes in the population and depletes undesirable genes. These genes recombine through the cellular sexual reproduction process of meiosis, resulting in novel genotypes and individuals with improved traits.

In the yeast *S. Cerevisiae* most reproduction occurs asexually where a yeast cell buds off a genetically identical daughter cell. However, yeast also has the ability to reproduce sexually, providing the opportunity to explore breeding methods that are routinely used in higher organisms. Meiosis occurs in yeast when sporulation is induced under stressful environmental conditions (Sherman, 1997). Harnessing this microbial sexual reproduction cycle through selective breeding has the potential to become a powerful tool of microbial strain development to produce compounds for use in the bioproducts industry to manufacture specific chemicals or materials. There are a few current publications on selective breeding of yeast but, to our knowledge, all of them rely on a specific stressor in the media to drive selection. This process limits the traits that can be improved upon. For example, selection studies for salt and ethanol resistance are possible (Li et al., 2023; Fiedurek et al., 2011) but selecting for the production of a high-value metabolite is not possible. For this, the selection process would require the isolation and phenotyping of specific strains. In this study, we seek to determine if this process will work.

A study by Burke et al. (2014) demonstrated replicability of selection experiments across independent replicate populations of s. cerevisiae yeast, which reassures the feasibility of selective breeding of yeast for future experimentation. The workflow for the selective breeding process involved sporulation, spore isolation, diploid recovery, and diploid growth. However, the methodology for the individual steps in the workflow requires further investigation.

Burke et al. (2014) demonstrated a method for selection of *S. cerivisiae* yeast to benefit evolve-and-resequence (E&R) experiments. Taking advantage of the sexual reproduction phase of this yeast could greatly benefit E&R experimentation by enabling selection for desired traits on a smaller and more efficient scale. The objective of this study was to show replicability of selection experiments across independent replicate populations to confirm the feasibility of this method for future E&R experimentation.

E&R experiments have been used to select for desired traits and discover methods of adaptation in both microbes and eukaryotes. E&R can be conducted efficiently on microbes; large populations can be easily maintained and many generations can be produced quickly. While convenient for experimental purposes, most microbes lack the ability to recombine genotypes through sexual reproduction. As such, de novo mutations contribute to genetic changes in the population, and these mutations cannot be reproduced for an independent replicate population. In higher eukaryotes, adaptation is driven from pre-existing genetic variation as opposed to de novo mutations, and therefore selection can be replicated for independent populations. However, experimentation on these species is more difficult than working with the convenience of small-scale microbes with short breeding cycles.

While the yeast *s. Cerevisiae* tends to produce asexually, sexual reproduction can occur under exposure to certain conditions, enabling genotype shuffling via sexual recombination. Taking advantage of this unique property of yeast could greatly benefit future E&R experiments. Burke et al. (2014) used diploids from a 4-way cross of S. cerevisiae strains with high sporulation efficiency as the base population for experimentation. Random spore analysis was conducted to induce sexual reproduction of the yeast. Populations were transferred to potassium acetate media to induce sporulation. After incubation for approximately 60 hours, the cultures were exposed to Y-PER protein extraction reagent and mechanically agitated to kill the remaining diploid cells. The spores were germinated and haploid cells were randomly mated and grown for ~48 hours, after which diploids were recovered in YPD media. The diploids were grown in 24-well culture plates to allow competition among the newly created diploid lines and then this new generation was transferred to potassium acetate sporulation media to begin a new cycle. This breeding process was repeated for 18 weeks on 12 independent replicate populations.

DNA was isolated at week 0, week 6, week 12, and week 18. Single nucleotide polymorphisms (SNPs) were tested to determine genetic variation at the different time points, and the genome was scanned for de novo mutations. De novo mutations did not impact selection, and the authors did not find changes in allele frequencies that were unique to independent replicate populations, indicating that standing genetic variation in the starting populations was the primary driver behind evolution in this experiment

Bahalul, et al. (2010) presented a method, the Ether–zymolyase (EZ) ascospore isolation procedure, for efficiently isolating yeast ascospores from Saccharomyces cerevisiae yeast. This procedure was developed by combining two previously established ascospore isolation protocols: diethyl ether treatment and zymolyase treatment. By combining these treatments, spores can be isolated from yeast cultures with low sporulation levels. The objective of this research was to increase the efficiency of ascospore isolation of S. cerevisiae yeast strains to enable improved genetic research and selective breeding of these yeast strains.

Ether treatment has been used for ascospore isolation of yeast cultures that contain both spores and polyploid cells. In this procedure, the yeast are exposed to diethyl ether, which causes cell membrane destruction in the diploid cells, leaving the ascospores isolated due to their resistance to the ether. Another procedure used for isolation of ascospores is treatment with zymolyase, an enzyme that releases ascospores by destroying the ascus wall.

Bahalul et al. (2010) followed standard procedures to grow different strains of yeast (heterothallic diploids (MATa/α) numbers 9, 10, 57 and 58). Afterwards, sporulation was induced by moving the yeast to a YPA medium in which it was grown to stationary phase before being transferred to sporulation medium (SM). These SM cultures were divided into two groups for low and medium sporulation, which were incubated at 30deg C for 24 and 48 hours, respectively. Finally, these cultures were transferred to a potassium acetate buffer and sporulation levels were determined by visual inspection through a microscope.

These sporulation cultures were then treated with three different treatments: diethyl ether (E), zymolyase (Z), and a combination of both (EZ). In the E treatment, cultures were put in diethyl ether and then mechanically agitated to destroy cell walls of the vegetative cells. In the Z group, zymolyase-100T was added to the cultures and incubated for 2 hours before being emulsified with glass beads and water. For the EZ group, the E treatment described above was carried out, followed by the Z treatment. For each sample described above, log reduction of CFU was determined as a measure of lethality. Sporulation efficiency was determined via microscope analysis. A lab strain of yeast, Y103, was used as a control to determine how many vegetative cells survived all three treatments. The EZ ascospore isolation procedure log reduction was significantly larger than the E, Z, and control groups.

Both Bahalul et al. (2010) and Burke et al. (2014) used potassium acetate to induce sporulation in yeast, confirming its reliability for use as a sporulation media for *s. Cerevisiae*. While the study by Bahalul et al. (2010) demonstrated a new and efficient spore isolation procedure, some methods used referenced old articles with limited detail on methodology, putting the replicability of this isolation procedure into question. In addition, diethyl ether is a hazardous chemical and not used much in practice today. Burke et al. (2014) mentioned exposing cultures to Y-PER protein extraction reagent and mechanical agitation, while also referencing the spore isolation procedure described by Bahalul et al. (2010). However, duration of exposure to

Y-PER and type of agitation were not specified. The spore isolation process needs to be tested and optimized for future research.

1.4 The Yeast Breeding Cycle

S. cerevisiae is a budding yeast whose vegetative cells are either haploid (1 set of chromosomes) or diploid (2 sets of chromosomes) and reproduce asexually through budding, utilizing the process of mitosis. Haploid cells can be one of two mating types: MATa or MATα (mating type a or alpha). Opposite haploid mating types conjugate into diploid cells, containing two sets of chromosomes and characterized as MATa/MATα (Sherman, 1997). Figure 7 below depicts this conjugation:

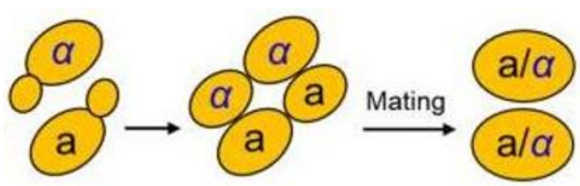

Figure 7: haploid cells of opposite mating types (MATa or MATα) conjugate to form diploid cells (MATa/MATα) (modified from Bai et al., 2022).

Sporulation in yeast occurs when vegetative cells are starved of essential resources such as nutrients. When yeast sporulates, diploid vegetative cells undergo meiosis, during which genetic material is recombined as a single diploid cell turns into four haploid spores (figure 8). The individual spores are contained in an ascus wall, forming a spore tetrad (Sherman, 1997). The cells have very low metabolic activity and go into a dormant state. The individual spores are surrounded by an extremely tough cell wall that acts as a shield, forming an almost impenetrable barrier. This dormant spore stage acts as a survival mechanism to protect the yeast from environmental stress. At the same time, when sporulation occurs, genes are recombined to form haploids that are genetically diverse from the parental diploid cells (Sakai et al., 2025). This process ultimately results in sexual reproduction where new strains of yeast are formed that may be genetically better equipped to deal with the stress of a changing environment. When When the stress is removed and proper nutrients are available, the spores germinate by transitioning to a vegetative state where the cell wall is lost and the cell resumes normal growth, resulting in a population of haploids. The haploids then mate with haploids of the opposite mating type, forming diploids (Sherman, 1997). After spores germinate, there are opportunities for either inbreeding or outbreeding. (Bai et al., 2022) When inbreeding occurs, the new generation will have similar genetics as the parental diploid generation. Inbreeding is either caused by haploids mating with their siblings or through intra-tetrad mating. Outbreeding is the necessary path to maximize genetic variability (our desired outcome), and occurs when the released spores mate with spores from a separate yeast parent line. The complete yeast breeding cycle is summarized in detail in figure 9 below:

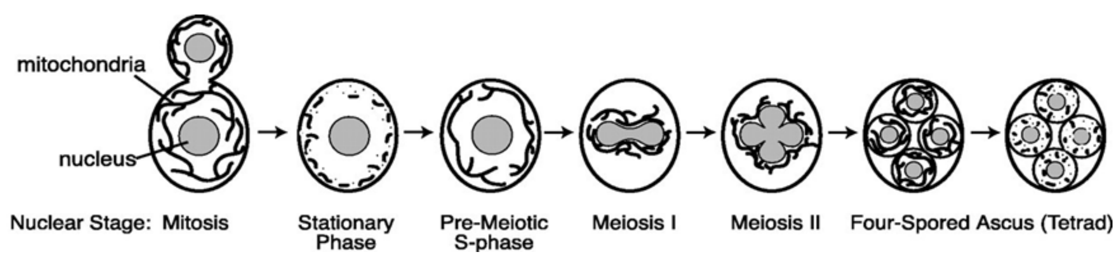

Figure 8: meiosis during sporulation of budding yeast (Gorsich & Shaw, 2004).

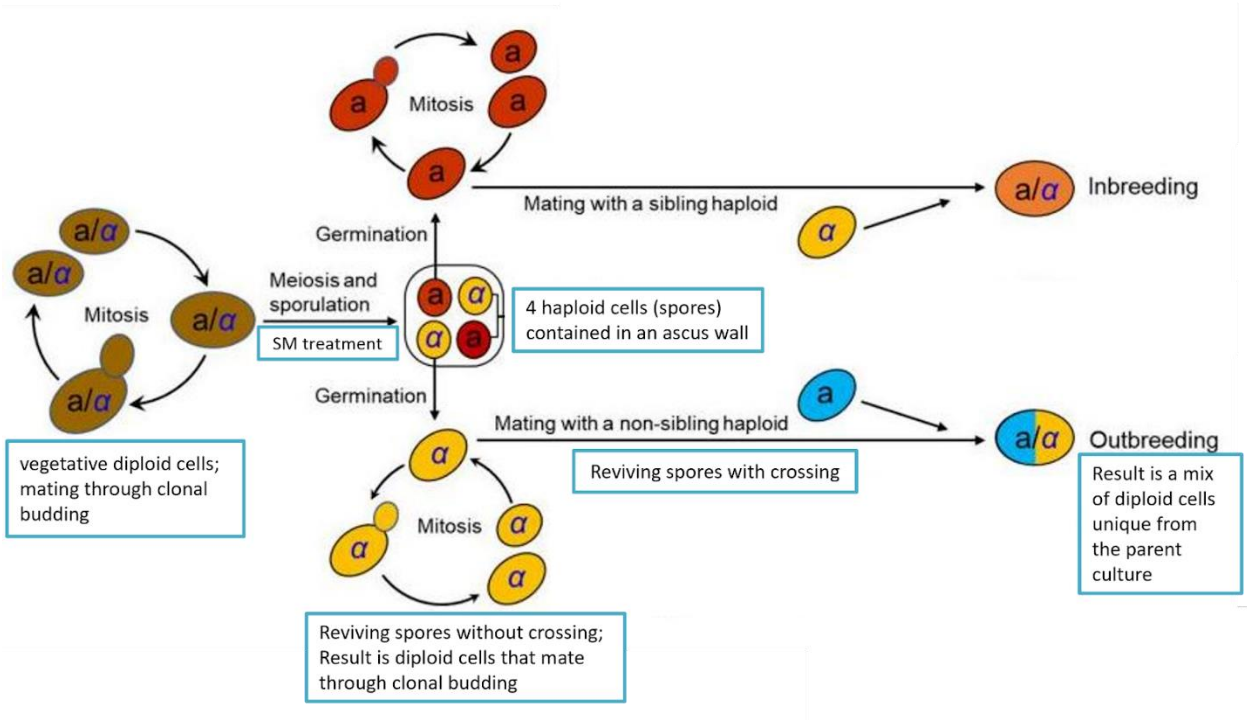

Figure 9: yeast breeding cycle (modified from Bai et al., 2022).

1.4.1 Wild vs. Domesticated Yeast Strains

Nearly all experimental or applied microbiology work on yeast utilizes strains that have been propagated in the laboratory for hundreds or thousands of generations (Bai et al., 2022). These domesticated strains have been inadvertently selected on and evolved into varieties that thrive in the artificial laboratory environment. Similarly, commercial strains of yeast used to produce alcoholic beverages or fuel ethanol have been selected to efficiently ferment specific feedstocks and to generate high concentrations of ethanol.

Thousands of wild strains of *S. cerevisiae* exist on the planet and hundreds of them have been collected over the years and are available to researchers through the National Collection of Yeast Cultures. These wild yeast represent a vast reservoir of genetic variation that can be used to develop novel yeast strains with new traits of interest.

In crop breeding, we have analogous examples of domesticated vs. wild varieties. For many decades crop researchers and breeders have exploited these wild reserves of genetic material and introgressed wild genes into domesticated cultivars to generate improved varieties with higher pest and stress tolerance (Hajjar & Hodgkin, 2007). Here we propose to do the same with yeast.

In theory, if a haploid cell is isolated and allowed to propagate asexually, a culture of one single mating type can be grown. These cells will lack the opposite mating-type and therefore cannot mate to form diploids. Such a system would be advantageous for research since every cell will be a genetic clone and researchers do not have to worry about the population changing over time. However, evolution has found a way around this. In yeast, the presence of the HO gene causes haploid cells to switch mating types, such that a MATa haploid will bud forming a MATα haploid. This allows the haploids to mate, forming MATa/MATα diploids in a process called "haplo-selfing" (Bai et al., 2022). Over the years, scientists have found genetic tricks to prevent this from happening and to maintain isogenic, haploid cell lines indefinitely. In domesticated strains, the HO gene can be disrupted to prevent mate switching (Breeden & Nasmyth, 1985).

Altering genes for a specific purpose can prove advantageous like in the case of the HO gene, but determining the genetic targets for mutation is not trivial and requires detailed understanding of the pathways of interest. In addition, working with transgenic strains requires specialized laboratory spaces and permitting. Contrarily, wild strains can be used in any lab with simple equipment and no additional permitting. Wild yeast strains are inexpensive and readily available from various sources in the wild (oak, fig, etc.). Due to their variety in source and lack of previous genetic modification, the genetic diversity of wild strains is significantly greater than that of domesticated strains (Liti et al., 2009).

Wild strains were chosen as the parents for this study due to the increased genetic variability. The selection methods used in this study were minimally affected by the starting cell type due to the methods of inducing sporulation and isolating spores described below.

1.4.2 Current Methods of Microbe Strain Development

Development of new strains of microbes for production of fuels and chemicals often involves genetic engineering, evolutionary engineering, or a combination of both methods. Additional studies, using phenotyping and genome sequencing, can identify mutations contributing to the phenotype and allow new, further improved strains to be engineered with this knowledge. Figure 10 below shows this process of strain development:

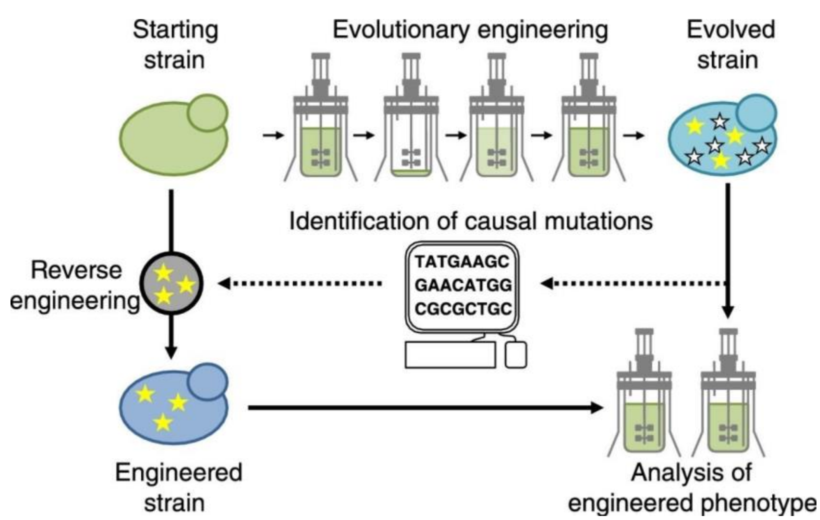

Figure 10: microbe strain development methods (Mans & Pronk, 2018).

Using these methods to develop strains has required knowledge of necessary genetic alterations to achieve a shift in the phenotype, which can be a complex task if unknown. While effective, genetic engineering is a complicated, time consuming, and expensive process. In addition, genetic alterations can have inadvertent effects on the evolved strain, such as inducing stressed cellular states (Sandberg et al., 2019). Alternative methods for yeast strain development have also been used that implement only evolutionary engineering principles. Common alternative methods include the Adaptive Laboratory Evolution (ALE) and the ALE with crossing. In this study, we propose using selective breeding as an alternative strain development method.

The Adaptive Laboratory Evolution (ALE) method can be used with any type of microbe. In this method, a microbial culture is started in the desired cultivation conditions (for example, duckweed hydrolysate media). It is then transferred to fresh media at a given time interval for a given number of cycles. With each transfer, a cycle is completed, and the process relies on random mutations in the population over time as the mechanism of genetic improvement. A small subset of these mutations make individual microbes more fit in the specific culture conditions and these are more likely to clonally reproduce. Over time, these advantageous mutations are selected for, resulting in a population that can grow in the specified media. This method is often used when developing strains for growth in certain conditions, such as resistance to different pH ranges or concentrations of specific chemicals. The ALE method is shown in figure 11 below:

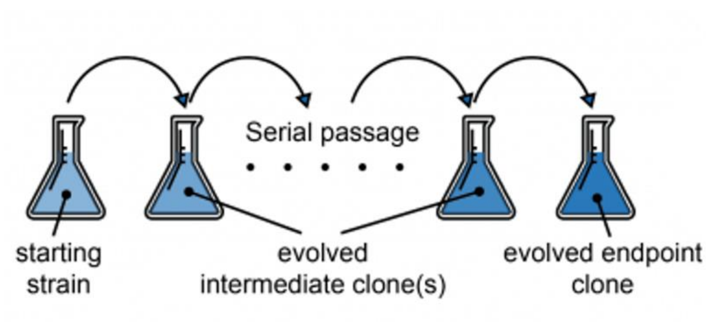

Figure 11: ALE laboratory method: starting strain is serially transferred to fresh media over a given time interval to produce the final strain. (*ALE*, 2025).

The ALE methodology is more simple, easy to perform, and fast to complete when compared to the genetic engineering methods. It works with any microbes and is a well documented and effective method for strain development. However, it has limitations that it only works to develop lines that are more resistant to some condition, in other words to grow in a specified media. For this same reasoning, the ALE method cannot be used to target specific chemical production, which is a large disadvantage compared to selective breeding.

A small number of research groups are experimenting with a modified approach where a crossing (sexual reproduction) step is added to the ALE method. While yeast is a eukaryotic microbe and tends to reproduce asexually, sexual reproduction can occur under exposure to certain conditions, enabling genotype shuffling via sexual recombination. Burke et. al (2014) demonstrated a breeding program that introduced a forced outcrossing scheme to the ALE model. Adding a sexual reproduction step at the end of each cycle improves the genetic variability.

The ALE with crossing method has been shown to improve strain development. A study (Kosheleva & Desai, 2017) demonstrated improvement in ALE with crossing compared to the ALE without crossing as seen in Figure 12 below:

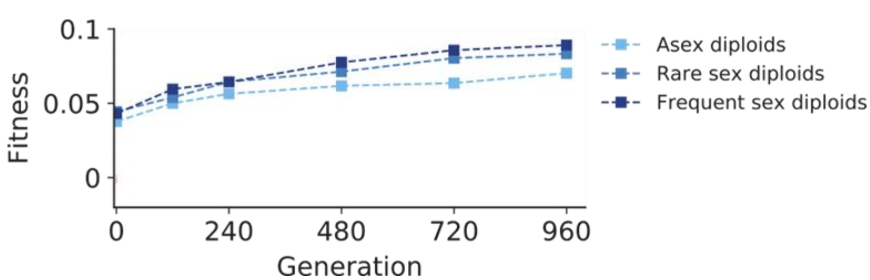

Figure 12: fitness improvement over time for ALE (Asex) vs. ALE with crossing (rare and frequent sex diploids); greater phenotypic improvement from ALE with crossing method vs. ALE alone (Koshevela & Desai, 2017).

The ALE with crossing method is still faster and more efficient than genetic engineering methods, but adding a crossing step increases the breeding time compared to the ALE alone. The introduction of the crossing step forces genetic recombination to take place, greatly increasing the genetic variability. This method no longer relies only on random mutations, but also on forced crossing which results in a greater phenotypic change.

In selective breeding, an additional phenotyping step takes place between each cycle. In other words, specific traits are quantified and only a top percentage of individuals with optimum traits are selected and then crossed. This greatly improves the selection intensity and variability. An optimized phenotyping protocol (in our case, ethanol quantification) would result in a high selection accuracy. Although there is a large improvement in the selection intensity, accuracy, and variability, the methods are more complex and the generation interval (L) is increased compared to both ALE and ALE with crossing. This increased generation time needs to be compared to the potential phenotypic improvements in the next generation.

Selective breeding is not currently used on microbes due to the complexity of the additional phenotyping step, when ALE has proven to be an effective method to produce new strains. However, these strains are limited to their growth media, while selective breeding could be used to target specific products with broader applications. In this study we will compare selective breeding to the ALE evolutionary engineering methods.

1.4.3 The Breeder's Equation

This study will use the breeder's equation as a framework to justify the use of selective breeding to improve yeast varieties when compared to other breeding methods. The breeder's equation describes the potential genetic improvement over time for a given breeding method:

$$\Delta G = \frac{ir\sigma}{L}$$

Where:

ΔG = genetic improvement over time
i = selection intensity
r = the accuracy of the selection
σ = genetic standard deviation of the trait under selection
L = generation interval

This equation can be used as a model to guide breeding decisions, where the selection intensity, accuracy, and standard deviation are directly proportional to the genetic improvement over time (ΔG). *Selection intensity (i)* is a measure of how selective the cross is (*what proportion of the top individuals are crossed*). *Selection accuracy (r)* describes how accurate the phenotyping protocol is at quantifying the trait of interest. The *genetic standard deviation (σ)* represents the genetic variability, or the difference in genetics within or between populations (Beedanagari & Mahadevan, 2014). The generation interval (L) represents the time to complete one breeding cycle, or produce the next generation. Adding a crossing step significantly increases the genetic variability. A comparison for the different breeding methods is summarized in the table 1 below:

Table 1: Breeder's equation parameter comparison.

Breeding method	**Selection intensity (i)**	**Selection accuracy (r)**	**Genetic variability (σ)**	**Generation interval (L)**
ALE	*difficult to control and quantify*	*difficult to control and quantify*	*low*	*low*
ALE + crossing	*difficult to control and quantify*	*difficult to control and quantify*	*high*	*medium*
Selective breeding	*high*	*high*	*high*	*high*

In the ALE method, the breeder's equation is not optimized since the genetic standard deviation is very low due to relying on random genetic mutations to obtain all genetic variability in the population. In addition, it is very hard to control or quantify the number of individuals that survive and are passed to the next cycle, potentially resulting in low selection intensity and accuracy. The major advantage of ALE, however, is the low generation interval, and with enough time, the desired genetic improvement can be achieved.

In the ALE with crossing method, the genetic variability is higher by forcing genetic recombination during the crossing step, but the selection intensity and accuracy remain low. Also, the generation time is slightly increased compared to ALE alone due to the additional crossing step.

With selective breeding (our proposed method), the genetic variability, selection intensity and selection accuracy are all optimized. Selective breeding includes a crossing step, which increases the genetic variability. Additionally, a phenotyping step is introduced prior to crossing during which the trait of interest is quantified and used to select the top n individuals to cross to the next generation. With an optimized trait quantification and low n, the selection accuracy and intensity are high compared to the other methods. However, this method has the longest generation interval, decreasing potential improvement over time. In this study, we aim to compare all 3 methods in an experimental trial to determine their relative efficacies.

1.5 Hypothesis and Objectives

Hypothesis: Applying established methods of selective breeding to introgress wild genetic material into eukaryotic microbes can result in improved strain development compared to traditional methods of artificial laboratory evolution.

Objectives:

1. Identify wild strains of yeast that can be used for crossing
2. Test these wild strains for ethanol production using duckweed feedstock
3. Develop methods for spore induction, spore isolation and mating of wild yeast
4. Develop methods for high-throughput phenotyping of yeast
5. Perform a comparative breeding trial using selective breeding vs. ALE
6. Evaluate new yeast line performance on duckweed hydrolysate and YPD

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CHAPTER 2: MATERIALS AND METHODS

2.1: Introduction

In this study, wild yeast strains were acquired and used to generate new strains through selective breeding. A domesticated variety that produces high ethanol concentrations was used for comparison. Two initial wild-type parents were selected and crossed to form an initial population, which we denote as generation zero (gen0). Crossing the two wild type strains results in a genetically diverse population of individuals. From this population, phenotyping was performed to select the top 10 ethanol producers, which were crossed to form new generations. Briefly, phenotyping involves measuring the traits of interest (ethanol production), selecting the top individuals based on this trait, and crossing them to increase the genetic variability of the next generation and shift the average population phenotype towards the traits of interest. The breeding process (figure 13) was repeated for 3 generations, and 2 final lines (top ethanol producers) from the third generation (“gen3”) were selected for ethanol fermentation analysis.

To perform selective breeding of yeast, the general workflow involves choosing the parents, inducing sporulation, isolating spores, and combination (germinating spores, mating and regrowth) (Burke et al., 2014). The methods for these steps are described in the sections below. Figure 13 shows the implementation of these methods within the breeding scheme:

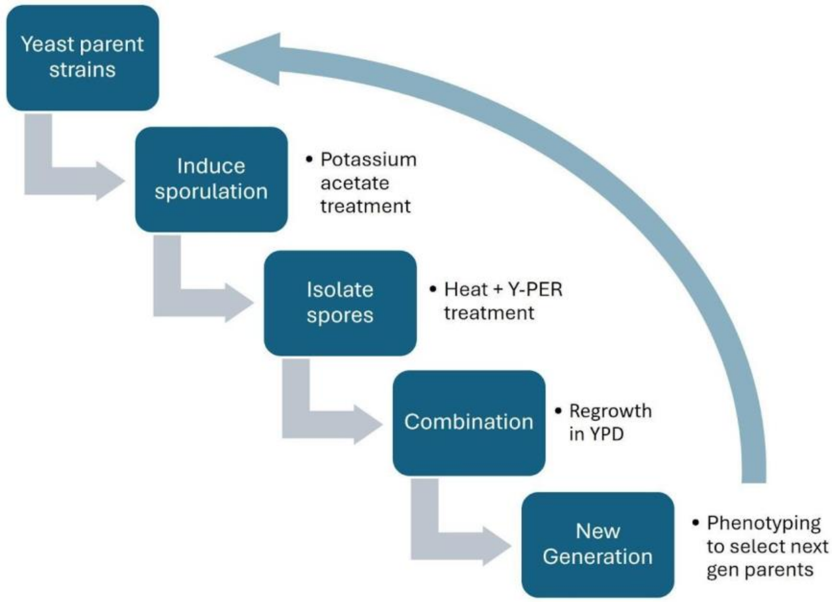

Figure 13: selective breeding with crossing methodology; the process can be repeated cyclically on subsequent new generations.

2.2 Yeast Crossing

2.2.1 Selecting the parents

To obtain an initial yeast population, six wild-type varieties of yeast were selected from different geographical regions. Varieties were selected from different branches of the s. Cerevisiae phylogenetic tree (figure 14) to maximize the genetic variability in the starting population. Each of the six wild-type varieties (table 2) were treated with potassium acetate to induce sporulation according to the procedures of Bahalul et al. (2010). Samples were analyzed in a microscope throughout a three-week period to confirm presence of spores. Two strains (Oak and Sake) were found to have sporulated after one week, whereas all other varieties took two-to-three weeks to sporulate. Oak and Sake were selected as the parents for the study due to faster sporulation rates, which would allow for more rapid breeding cycles. In addition to selecting the initial wild type varieties, a domesticated yeast (Turbo) was chosen for use as a control throughout the study. The turbo yeast is commercially available for fermenting spirits, and can theoretically yield up to 14% ethanol by volume.

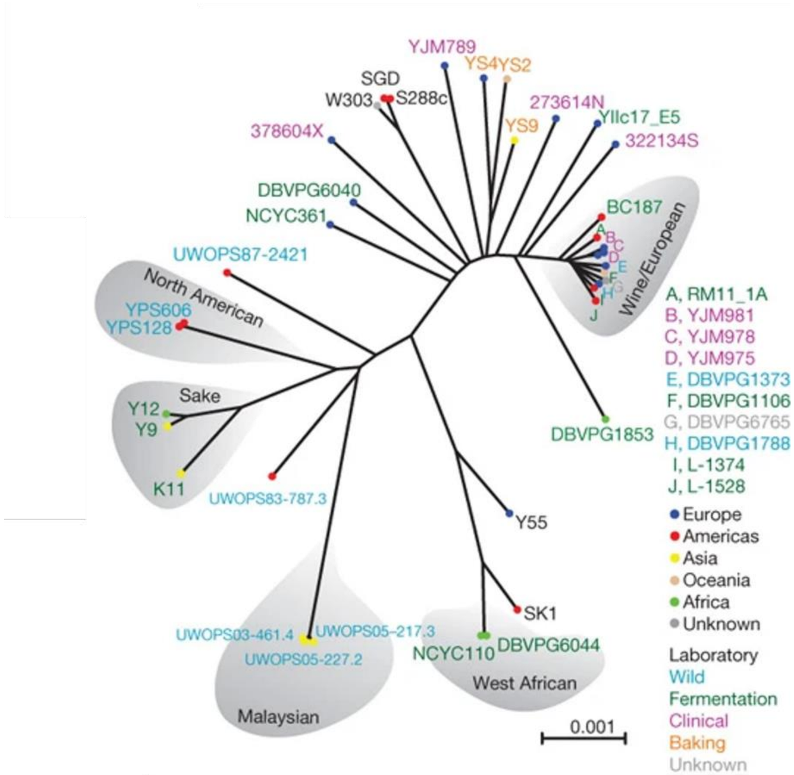

Figure 14: phylogenetic tree of s. Cerevisiae (adapted from Liti et al., 2009).

Table 2: Initial wild strains and control strain with sporulation times.

Strain	**Location**	**NCYC Code**	**Name**	**Sporulation Time (weeks)**
DBVPG6044	*West Africa*	*NCYC 3290*	*Bili*	*3*
UWOPS03-461.4	*Malaysia*	*NCYC 3461*	*Nectar*	*2*
Y12	*Japan/Sake*	*NCYC 3445*	*Sake*	*1*
YPS128	*North America*	*NCYC 3284*	*Oak*	*1*
DBVPG1788	*European*	*NCYC 3311*	*Turku*	*2*
S288C	*North America*	*NCYC 3466*	*Fig*	*3*
control	*North America*	*n/a*	*Turbo*	*n/a*

2.2.2 Inducing Sporulation

To induce sporulation, yeast cultures were grown in YPD media for 4 days and then transferred to potassium acetate sporulation media (SM) as described by Bahalul et. al. (2010). Samples were incubated in SM at 30°C for several weeks and checked weekly for spores. Samples were analyzed with a microscope to visually confirm the presence of spore tetrads (figure 15). Spores appeared after one week of growth in SM for the Oak and Sake parent cultures, while spores took 2-3 weeks of incubation for the other varieties. Therefore, Oak and Sake were chosen as the two initial parents for this study.

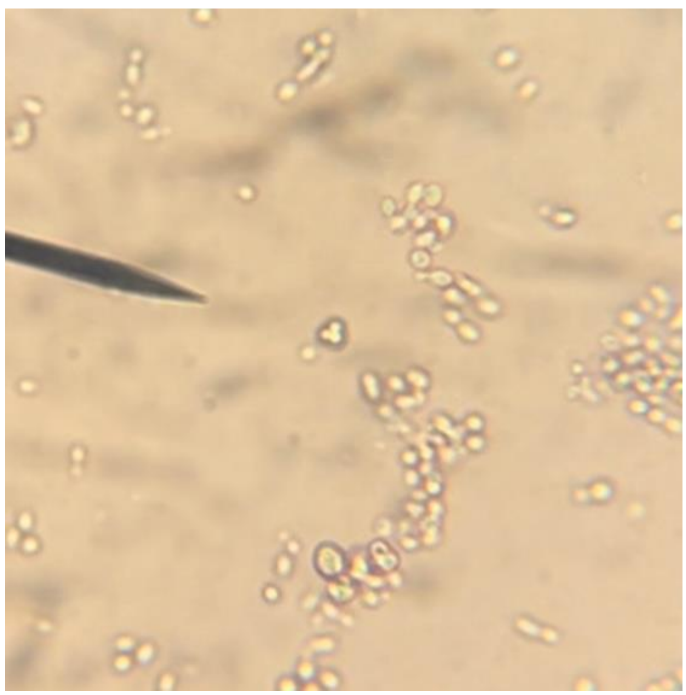

Figure 15: spore tetrad visualized in microscope at 40x magnification (3 spores are seen at a time from a 2-D plane; the spore tetrad can be seen rotating in real time).

2.2.3 Spore Isolation

In a sporulated yeast culture, not all vegetative cells will undergo sporulation. The resulting culture is a mixture of spore tetrads and vegetative cells that did not sporulate. The diploid vegetative cells cannot undergo mating and should be eliminated to isolate the genetically diverse spore tetrads (Bahalul et al., 2010). Spore tetrads need to be isolated from the mixture before germinating and releasing the haploids to ensure maximum genetic variability in the next generation. This spore isolation is an essential step to ensure that the resulting progeny is a product of mating and not just passing on parental vegetative cells to the next generation.

To fully isolate haploid spores, a protocol is needed to remove vegetative cells that failed to sporulate, and additionally disrupt the ascus wall of the spore tetrads to release the haploid cells that were contained within. According to Bahalul et. al (2010), treatment with a combination of diethyl ether and zymolyase can be used to kill vegetative cells and isolate spores from a culture. However, this method is time-consuming, ether is a dangerous chemical and not used often in practice anymore, and zymolyase is very expensive. A method briefly mentioned in the Burke et. al (2014) study was to expose yeast cultures to Y-PER protein extraction reagent to kill vegetative cells and mechanical agitation to disrupt the ascus wall. However, the type of mechanical agitation and duration of exposure to Y-PER were not specified. In addition, these methods tend to be effective at killing the vegetative cells, but are not as replicable in their ability to disrupt the ascus wall of spores. Burke found that adding a heat shock step to the protocol improved spore isolation results and increased genetic diversity for the surviving cells. However, the ideal treatment duration and temperature differ across strains (Burke et al., 2020).

2.2.4 Spore Isolation: Heat and Y-PER experiment

Initially, several iterations of exposure to Y-PER protein extraction reagent along with mechanical agitation treatment were attempted with varying treatment durations and using glass beads in a shaker for mechanical agitation. After treatment, samples (treatment and a no-treatment control) were plated with a small volume (250uL) to use visual growth of colonies as a measure of the treatment success. In addition, a culture containing only vegitative cells, without spores was used. A successful spore isolation protocol would result in growth on spore control and treatment plates, with growth on vegetative control plates but no growth on vegetative treatment plates, indicating survival of spores and killing off of vegetative cells. However, the results were unsuccessful, with all the vegetative cells surviving and spores were not isolated.

Further experiments were attempted in which samples were incubated in Y-PER protein extraction reagent for a varying duration (20, 40mins), after which the Y-PER was rinsed and samples were resuspended in the original media (YPD or SM). The heat treatment was performed immediately after the Y-PER incubation, during which samples were incubated at 55°C in a water bath for varying times (20, 40mins). After treatment, treated samples and controls (initial cultures without any treatment) were plated in a similar manner to measure the treatment success. Initial experiments using these methods were performed on Oak vegetative (YPD) and spore (SM) cultures. However, using a separate Y-PER treatment followed by heat incubation was unsuccessful, with similar growth on all plates, including controls, indicating that no vegetative cells were killed and spores were not isolated.

Treatment of exposure to both Y-PER and heat simultaneously was conducted next on both Oak and Sake vegetative (YPD) and spore (SM) cultures. Samples from the initial cultures were centrifuged for 1 minute at 3000 rpm to remove the culture media and resuspended in 0.2mL of Y-PER, vortexed, and incubated in a water bath at 60°C while still exposed to the Y-PER. Samples were removed from the treatment after 10, 20, 30, and 40 minute incubation times. Immediately after removal from the water bath, samples were centrifuged (1min, 3000rpm) to remove the Y-PER, rinsed with their original media (YPD or SM, depending on the sample), and resuspended in the original media to end the treatment. Treated samples were vortexed for 30 seconds and streaked onto plates.

2.2.5 Mating and regrowth

After spores are isolated, crosses of yeast cultures can be carried out by simply adding equal concentrations of spores from each parent in YPD media and incubating at 30°C for at least 6 hours (Sherman, 1997). This allows isolated spores to germinate and haploid cells to mate, forming a new generation of diploid cells that are genetically diverse from the parents.

The isolated spore samples resulting from the Y-PER and heat treatment from Oak and Sake were crossed based on these methods, resulting in the first cross, gen0. In addition, isolated Oak spores were crossed with themselves, and similarly for Sake, to allow for comparison of outbreeding vs. inbreeding (discussed in the phenotyping chapter). The crosses were grown for 24 hours on a shaker plate in an incubator at 30°C, after which budding cells were confirmed in the microscope and dilution plating was performed to isolate individual colonies. Individuals were collected with autoclaved toothpicks and suspended in YPD media in individual wells of an autoclaved deep-well 96-well plate for storage. 56 individuals from the gen0 cross were collected, as well as 24 oak selves and 24 sake selves.

2.3 Phenotyping

2.3.1 Introduction

A phenotype is any observable trait of an organism. Phenotyping is simply the act of measuring that trait (NHGRI). In our case, the phenotype of interest is the ethanol production rate using duckweed feedstock. The objective of this study is to utilize selective breeding methods that are well established in plants and animals to generate an improved variety of yeast. These methods require hundreds of phenotypic measurements to be carried out and are usually limited by the time and expense of the phenotyping. Quantification of ethanol for phenotyping would require setting up many small-scale fermentations and sophisticated assays or detection equipment like GC-MS or HPLC, all of which are labor intensive, time consuming, and expensive to perform in large quantities. Therefore, we decided to use growth rate as an alternative phenotype of interest under the assumption that the yeasts with optimal growth in a specific media will correlate with optimal ethanol production rates. Yeast growth rates can be easily detected with a spectrophotometer by measuring the optical density at wavelength 600nm (OD600) over time (IMPLEN). 96 samples can be run at a time, and samples do not require any additional reagents, lending to a high-throughput and cost-effective method of phenotype quantification.

To conduct selective breeding, after quantification of a phenotype for the initial population, the top n individuals are selected as parents. These parents with ideal traits are then crossed to form the next generation. The two parents, Oak and Sake, were crossed as described in a previous chapter to form an initial genetically diverse population for phenotyping, gen0. Phenotyping was performed on this population to select the top ethanol producers, which were crossed to form the next generation, “gen1”. The phenotyping process was repeated on gen1, resulting in “gen2”, and repeated again on gen2 to form the final generation, “gen3”. This process can be repeated cyclically for many generations, but was stopped after 3 breeding cycles due to limitation of time and resources. The third generation was phenotyped to select our final line (the top ethanol producer). The overall selective breeding scheme is shown in figure 16 below.

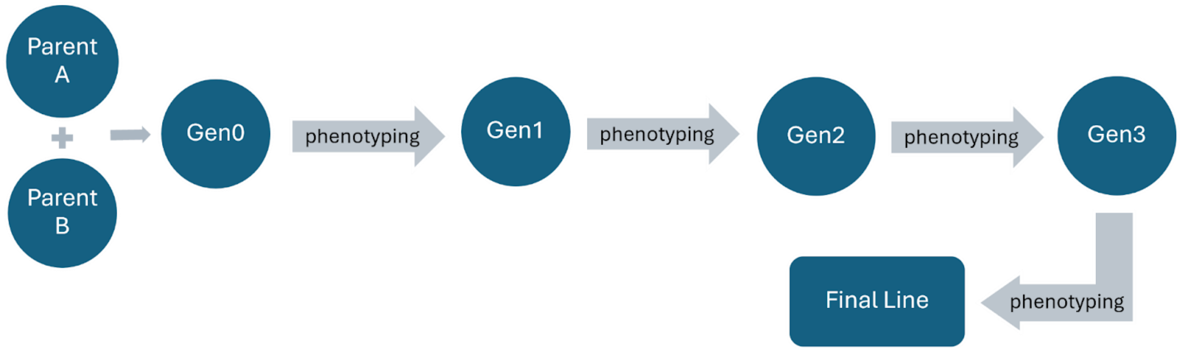

Figure 16: overall selective breeding scheme; phenotyping is the process of choosing the top individuals and crossing them to form the next generation.

2.3.2 Growth rate assay on YPD cultures

One Oak self, one Sake self, and three cross lines from gen0 were chosen at random to test the growth rate assay protocol. Briefly, a 1:100 dilution of each culture was performed using YPD media within a clear 96-well plate. In this iteration, no plate cover was used. The plate was run through a spectrophotometer, scanning OD600 every 30 minutes for 2 days. There were 6 technical replicates per sample, yielding 6 growth rate curves for each sample. The growthcurver package in R (Sprouffske, 2020) was used to fit a model to the growth rate curves and extract the model parameter r (maximum growth rate) for each curve.

2.3.2.1 Wild vs. domesticated yeast

To compare growth rates of wild vs domesticated yeast in YPD, another growth rate assay was performed on Oak, Sake, (wild) and Turbo (domesticated) with 4 biological replicates per variety, in other words, 4 individual cultures tubes for each variety were used to inoculate the 96-well plate. This time and for all future growth rate assays, an optical adhesive film (Applied Biosystems™, Cat# 4313663) was used on the 96-well plate to prevent evaporation during the assay. Similarly, samples were prepared in a 1:100 dilution of YPD media and the OD600 was measured every 30 minutes for 2 days to create growth curves, from which the model parameter r was extracted. Tukey multiple comparisons of means with 95% confidence level was performed on the averages of the biological replicates to compare.

2.3.2.2 Gen0 cross growth rate variability

A growth rate assay was performed on the initial gen0 cross. 40 individuals from the total population of 96 were chosen at random for the assay. 80 wells of a 96-well plate were filled with 1:100 dilution of the samples, at 2 reps per sample, and 2 identical plates were run, for a total of 4 technical replicates for each sample. Models were constructed for each sample as described above and the maximum relative growth rate (r) for each model was extracted.

2.3.2.3 Cross vs. selves

To confirm that the cross was successful, gen0 cross was compared to Oak and Sake selves by performing a growth rate assay on these lines using the previously described methods. Selves were controls created from spores of one parent combined with themselves, in other words, a control inbred line was obtained from each of the parent lines. The cross is a result of outbreeding and is expected to outperform the selves, being inbred lines.

2.3.3 Growth rate assay on duckweed cultures

Duckweed hydrolysate was centrifuged at 15000 rpm for 5 minutes. The resulting supernatant was collected and centrifuged in the same manner, resulting in a final duckweed media free of particulates for use in the spectrophotometric assay. A 1:100 dilution of Oak, Sake, and Turbo cultures was made in this duckweed media in a clear 96-well plate. A cover was used on the plate to prevent evaporation and the growth rate assay was run in the same manner, scanning the OD600 every 30 minutes for 2 days.

2.3.4 Applying phenotyping to the selective breeding cycle

Using the parent cultures Oak and Sake, spores were induced, isolated, and combined to form the first generation cross ("gen0"). Briefly, spores were induced for Oak and Sake by transferring yeast cultures to SM media for 1 week. After confirmation of spore tetrads in the microscope, the heat and y-PER treatment was conducted to isolate spores. Spores isolated from Oak and Sake were combined in a fresh YPD media culture tube forming the gen0 cross. In addition, controls were created where spores from Oak were combined with themselves, and similarly for Sake, to compare the results of outbreeding (cross) vs. inbreeding (self). The resulting yeast cultures were plated on YPD agar plates using dilution plating techniques to isolate individual colonies. Individual colonies were then selected from the plates with an autoclaved toothpick and grown in YPD media in a sterile deep-well 96-well plate with cover, which was stored at 5°C as the gen0 storage plate. Figure 17 below depicts this process of crossing:

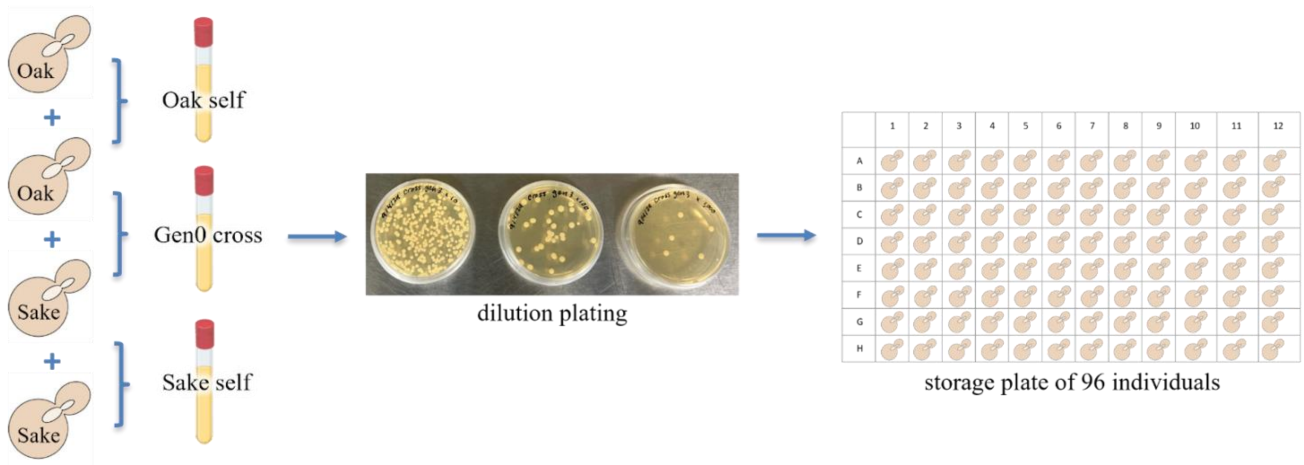

Figure 17: selection cycle process; crossing oak and sake results in the gen0 cross, from which 96 individuals were selected; alternately, oak and sake can be crossed with themselves

After obtaining a storage plate with 96 individual lines of yeast, the phenotyping protocol was carried out: A growth rate assay was performed using duckweed hydrolysate as the growth media, with samples run through a spectrophotometer at wavelength 600 nm, scanning every 30 minutes for 2 days, with a 1:100 dilution of the sample into the same respective growth media. 3 identical 96-well plates were made and run to obtain growth rate data in triplicate. This data was used to produce growth rate curves in R, and the maximum relative growth rate (r) was quantified using the "growthcurver" package. This package fits a logarithmic growth curve to empirical data. Various growth parameters can be extracted from this model including the relative growth rate ('r'). This growth rate was used to determine the best growing lines.

When conducting breeding cycles, the top 10 fastest growing lines from the growth rate phenotyping were selected based on median r value and then crossed to form the next generation. To do so, these top selected lines were grown in YPD media for 2 days, after which sporulation was induced by transferring to SM and incubating for 1 week. After one week of growth and confirmation of spores in the microscope, the heat and y-PER treatment was applied to these SM cultures to isolate spores. To perform a cross, cell densities of the isolated spore cultures for the parents (top individuals from the previous generation) were measured with a single OD600 measurement and used to standardize cell counts for inoculation of each line so that all varieties had roughly equal spore counts. A single YPD culture was inoculated with each of the top 10 individual yeast lines and grown on a shaker plate in an incubator at 30℃ for 2 days to allow for germination and combining of haploid cells, resulting in the next generation. Once budding cells were confirmed via microscope, dilution plating was performed to isolate individual colonies from the new generation, and 96 individual colonies were collected and stored in YPD media in a deep-well 96 well plate with cover. These 96 individuals compromise the next generation, gen1. This process of phenotyping for top individuals and crossing them was repeated on gen1, resulting in gen2, and again on gen2 resulting in gen3. The selective breeding cycle was completed with a total of 3 total rounds of selection. The figure 18 below summarizes the selective breeding process with phenotyping:

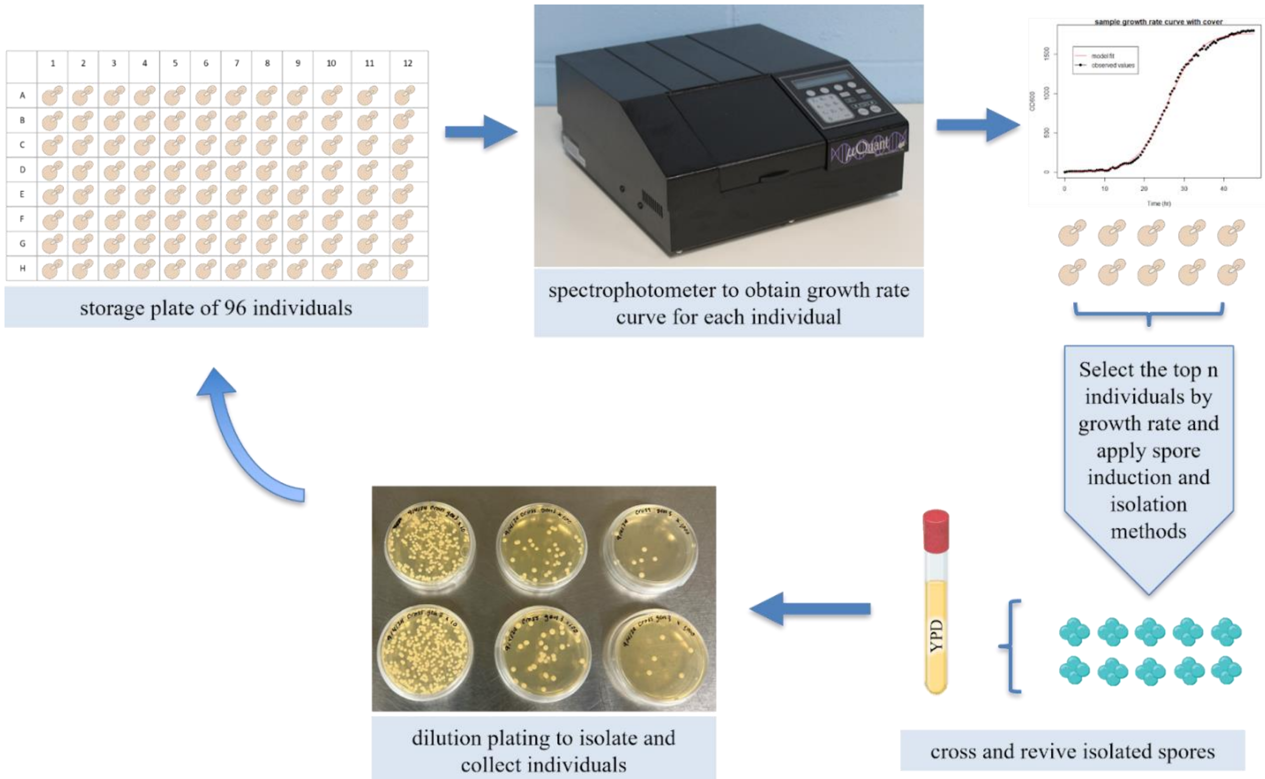

Figure 18: selective breeding cycle with phenotyping method implemented; the cycle can be repeated on subsequent generations

2.3.5 Obtaining the final lines of yeast

After completion of the selective breeding process, the final population (gen3) of 96 individuals was phenotyped for growth rate. The top 2 median growth rate individuals were selected as the final yeast lines for analysis: gen3-32 and gen3-56.

In addition to selective breeding, an artificial laboratory evolution (ALE) was performed in parallel. Equal cell densities of the parents, Oak and Sake, were combined in 10mL of duckweed hydrolysate and incubated on a shaker plate at 30°C for one week, after which 1mL was collected and used to inoculate a fresh duckweed hydrolysate tube. The new tube was incubated under the same conditions for 1 week and a new culture was inoculated. This method was carried out for 9 weeks to match the amount of time spent on 3 generations of selective breeding (1 cycle every 3 weeks), resulting in a final line, “ALE”.

These final lines (gen3-32, gen3-56, ALE) were compared to the parents (Oak and Sake) and the control yeast (Turbo) by performing fermentations and quantifying ethanol as described below.

2.4 Duckweed hydrolysis

Duckweed was collected from an agricultural pond in Granville county, NC. The genus is Lemna, likely Lemna aequinoctialis. This species is known for being naturally high in starch and having fast growth rates (Ma et al., 2018). The duckweed was grown in adequate nutrients and then transferred to nutrient-free well water for 1 week for nutrient deprivation to induce starch accumulation. Duckweed was then harvested and dried in an oven overnight. Enzymatic hydrolysis of the duckweed was performed according to the procedures of Xu et al. (2011) to convert starch into available sugars for fermentation. Four separate hydrolysis batches took place, the differences for which are outlined in table 3 and described in the sections below.

Table 3: Hydrolysis batch differences summary

Batch No.	volume	Solids content *(g dried duckweed/L buffer)*	Incubation #1 details	Incubation #2 details	Incubation #3 details
1	500mL	50g/L	Enzyme: 3.75×10^4 units alpha-amylase from *Bacillus licheniformis* Temperature: 90°C Duration: 45 minutes	Enzyme: 5.00×10^3 units pullulanase Temperature: 60°C Duration: 30 minutes	Enzyme: 2.50×10^2 units amyloglucosidase Temperature: 50°C Duration: 4 hours
2	1.25L	150g/L *(left as slurry)*	Enzyme: 2.25×10^5 units alpha-amylase from *Bacillus licheniformis* Temperature: 90°C Duration: 45 minutes	Enzyme: 3.00×10^4 units pullulanase Temperature: 60°C Duration: 30 minutes	Enzyme: 1.50×10^4 units amyloglucosidase *(correction from previous batch)* Temperature: 50°C Duration: 4 hours

Table 3: Hydrolysis batch differences summary, continued

3	5L	100g/L	Enzyme: 1.50 x $10^{6.}$ units alpha-amylase from porcine pancreas Temperature: 70°C Duration: 45 minutes	Enzyme: 2.00 x 10^{5} units pullulanase Temperature: 60°C Duration: 30 minutes	Enzyme: 1.00 x 10^{5} units amyloglucosidase Temperature: 50°C Duration: 4 hours
4	5L	100g/L	Enzyme: 1.50 x 10^{6} units alpha-amylase from porcine pancreas Temperature: 50°C *(reduction from previous batch)* Duration: 45 minutes	Enzyme: 2.00 x 10^{5} units of pullulanase Temperature: 60°C Duration: 30 minutes	Enzyme: 1.00 x 10^{5} units amyloglucosidase Temperature: 50°C Duration: 4 hours

2.4.1 Duckweed batch #1 (small scale)

An initial small batch (0.5L) duckweed hydrolysis was performed using 50g dried duckweed/liter of hydrolysate. The hydrolysis was carried out in a beaker on a hot plate. Duckweed was dried and ground with mortar and pestle. 25g (dry weight) ground duckweed was added to 250mL mops buffer. 3.75 x 10^{4} units of alpha-amylase from Bacillus licheniformis was added (sigma, A3403) and this mixture was incubated at 90°C for 45min. The pH was adjusted to 4.5 with addition of glacial acetic acid. 5.00 x 10^{3} units pullulanase (sigma, E2412) was added and the hydrolysate was incubated at 60°C for 30min. Heating was stopped to drop the temperature to 50°C, and 2.50 x 10^2 units of amyloglucosidase (sigma, 10115-1G-F) was added. This mixture was incubated for 4 hours at 50°C. DI water was added to bring the final volume to 500mL. Following incubation, the mixture was filtered using 250um Nitex Nylon mesh filter and pH was adjusted to 7 with addition of KOH. The mixture was autoclaved before inoculating with yeast cultures.

2.4.2 Duckweed batch #2 (small scale)

Duckweed was oven dried overnight and ground into a fine powder using an MF 10 basic grinder drive and passed through two sieves of decreasing mesh size: 2mm and 1mm. The hydrolysis was carried out in a New Brunswick Scientific Bioreactor with a heating coil for temperature regulation and agitation control. An agitator speed of 150rpm was continued throughout the duration of the hydrolysis. 150g (dry weight) ground duckweed was added to 1L mops buffer. 2.25 x 10^5 units alpha-amylase from Bacillus licheniformis was added (sigma, A3403) and this mixture was incubated at 90°C for 45min. The pH was adjusted to 4.5 with addition of glacial acetic acid. 3.00 x 10^4 units pullulanase (sigma, E2412) was added and the hydrolysate was incubated at 60°C for 30min. Heating was stopped to drop the temperature to 50°C, and 1.50 x 10^4 units amyloglucosidase (sigma, 10115-1G-F) was added (a correction from batch #1). This mixture was incubated for 4h at 50°C. DI water was added to bring the final volume to 1.25L. Following incubation, the pH was adjusted to 7 with addition of KOH and the hydrolysate was autoclaved as a slurry before inoculating with yeast cultures.

2.4.3 Duckweed batch #3 (large scale)

A 5L batch of duckweed hydrolysis was performed in a New Brunswick bioreactor with agitation control and temperature regulation via heating coil. Duckweed was oven dried overnight and ground into a fine powder using an MF 10 basic grinder drive and was passed through three sieves of decreasing mesh size: 2mm, 1mm, 0.25mm. Agitation was maintained at 600 rpm throughout the duration of the hydrolysis. 500g dried, ground duckweed was added to 4L MOPS buffer. 1.50 x 106 units alpha-amylase from porcine pancreas (Sigma, A3176) was added and incubated at 70°C for 45 minutes, after which pH was reduced to 4.5 with glacial acetic acid and temperature was brought down to 60°C. 2.00 x 105 units pullulanase (Sigma, E2412) was added and the mixture was incubated at 60°C for 30 minutes. The temperature was reduced to 50°C and 1.00 x 105 units amyloglucosidase (Sigma, 10115) was added, followed by a final 4 hours incubation. Upon completion, DI water was added to bring the total volume to 5L per reactor and pH was adjusted to 7 with addition of KOH. The hydrolysis was completed as a slurry at 10% solids, but an additional dilution with DI water was performed to reduce the final solids content to 5%, for a total 10L batch. The diluted hydrolysate was autoclaved in 500mL jars.

2.4.4 Duckweed batch #4 (large scale)

Two 5L batches of duckweed hydrolysis were performed simultaneously for a total 10L batch in two New Brunswick bioreactors with agitation control and temperature regulation via heating coil. Duckweed was oven dried overnight and ground into a fine powder using an MF 10 basic grinder drive and was passed through two sieves of decreasing mesh size: 2mm, and 1mm.

Agitation was maintained at 600 rpm throughout the duration of the hydrolysis. 500g dried, ground duckweed was added to each reactor with 4L MOPS buffer. 1.50 x 106 units alpha-amylase from porcine pancreas (Sigma, A3176) was added and incubated at 50°C for 45 minutes, after which pH was reduced to 4.5 with glacial acetic acid and temperature was brought down to 60°C. 2.00 x 105 units of pullulanase (Sigma, E2412) was added to each reactor and incubated at 60°C for 30 minutes. The temperature was reduced to 50°C and 1.00 x 105 units amyloglucosidase (Sigma, 10115) was added to each reactor and incubated for 4 hours. Upon completion, DI water was added to bring the total volume to 5L per reactor and pH was adjusted to 5 with addition of KOH. The hydrolysate was left as a slurry (100g/L) for fermentation without further dilution.

2.5 Quantification of Ethanol with GC-MS

Gas Chromatography Mass-Spectrometry (GC-MS) was used to quantify ethanol for all large scale ethanol fermentation analyses. A GC-MS method for detection of ethanol was developed, based on modified procedures of Jones (2017) and Sriariyanun et al. (2019). The method is highly sensitive, and can also detect other fermentation byproducts.

Sample preparation: Duckweed fermentation samples were centrifuged and the resulting supernatant was transferred to a fresh 1mL tube, from which two ten-fold serial dilutions were performed. For samples with very low ethanol, original samples with no additional sucrose spike, the 1:10 dilution was used for analysis. For 15% sucrose samples, the 1:100 final dilution was used for analysis. Samples were thoroughly vortexed for 1 minute between each dilution step and prior to GC-MS injection.

GC-MS protocol: All analyses were performed using a Gas Chromatograph (Agilent 7890A) equipped with Mass Spectrometer (Agilent G3172A) with Agilent DB-5MS Ultra Inert column (122-5562UI, 60mx0.250mmx0.25um) and autosampler (G4513A). 5uL of sample was injected in the back inlet at an initial temperature of 35°C, followed by a temperature ramp of 20°C/min to 250°C and held for 5 minutes. An additional 3 minute cool-down period was added to reduce injector temperature back down to 35°C prior to injection of the next sample, for a total runtime of 15.75 minutes. DI water was used to wash the syringe between samples. The following GC-MS parameters were used: split inlet with a split ratio of 50:1, CFT flow rate of 1.5mL/min, MS gain factor of 1.00, energy of 70eV, with the scanning range 40-450 m/z.

Areas under the curve of the chromatogram were quantified using the MSD Chemstation software autointegration tool. Manual integration was performed periodically to confirm results. Ethanol standards ranging from 1 to 0.0325% ethanol by volume were made in DI water and run using the GC-MS method described above to produce a standard curve.

2.6 Small scale fermentations

To compare lines of yeast, fermentations were performed in both duckweed hydrolysate and YPD media. Larger scale fermentations were performed for a more accurate ethanol quantification, but limited resources mean fewer replicates. Small-scale fermentations were also performed for increased replicability.

Cell densities of yeast cultures were approximated by measuring the optical density at wavelength 600nm (OD600) using a spectrophotometer. This is an efficient and common method for approximating cell densities of microbial cultures (Millipore Sigma). The OD600 of yeast starter cultures was measured by spectrophotometer with a clear 96 well plate to estimate cell densities. Based on these results, fermentation inoculation volumes were determined so that biological replicates were inoculated with roughly the same starting cell density.

Small scale batch fermentations were set up with a total volume of 20mL in a glass culture tube with a one-way flow cap to support anaerobic conditions (figure 19). Samples were incubated at 30°C for varying incubation times on a shaker plate. Fermentation samples were collected and frozen for later analysis. Ethanol concentrations were determined using the bioassay systems ethanol assay kit (DIET-500), a spectrophotometric method for rapid ethanol detection in a 96 well plate. Standard curves were plotted with each run and used to calculate the ethanol concentration as a percentage of total culture volume. To determine statistical significance of differences in ethanol concentrations between yeast varieties, a Tukey multiple comparisons of means with 95% confidence level was performed using R software.

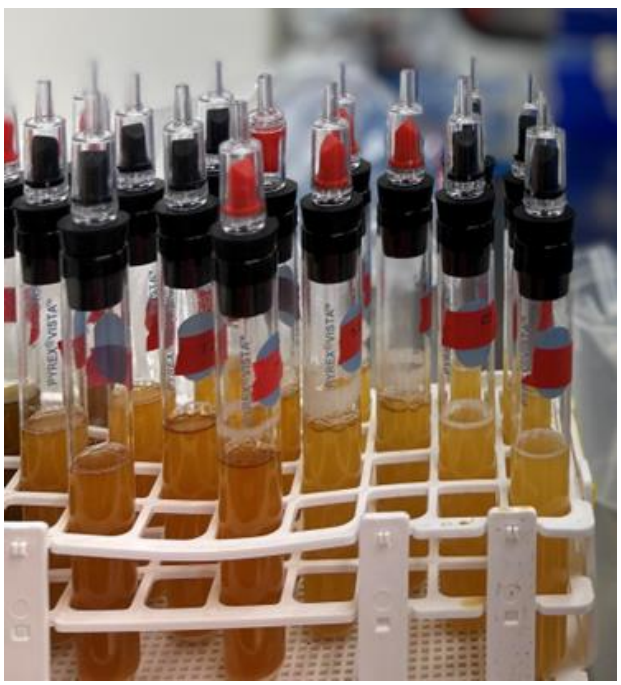

Figure 19: small-scale fermentation setup with one-way flow caps.

2.6.1 wild vs. domesticated in sucrose YPD

To test out the mini-fermentation setup, samples were first prepared using Yeast extract, Peptone Dextrose (YPD) media spiked with 15% (150 g/L) sucrose as the feedstock. Yeast varieties Oak, Sake, and Turbo were compared, with Turbo serving as a control yeast compared to the wild parent strains, Oak and Sake. An initial fermentation was carried out on these varieties with a 7-day incubation time and 3 biological replicates per variety, with 8 technical replicates each for ethanol analysis. The Turbo yeast is expected to perform better in the sucrose YPD media compared to the parents, as it has been specifically developed for optimal ethanol conversion of sucrose.

To determine the best incubation duration, a mini-fermentation experiment was carried out on Oak, Sake, and Turbo in 15% sucrose YPD media with samples taken at the following timepoints: day 0, 1, 2, 3, 4, and 7. There were 5 biological replicates per sample.

2.6.2 Wild vs. domesticated in duckweed batch #1 with and without sucrose

Using the duckweed hydrolysate #1 as the feedstock, mini fermentations were set up for Oak, Sake, and Turbo with 5 biological replicates each and an incubation time of 6 days. The hydrolysate was fermented again both with and without addition of 15% sucrose. Oak, Sake, and Turbo were compared with an incubation time of 2 days.

2.6.3 Wild vs. domesticated in duckweed hydrolysis #2

Small scale fermentations were performed on Oak, Sake, and Turbo using duckweed hydrolysis batch #2 as the feedstock. The fermentation volume was reduced from 20mL to 10mL to conserve duckweed media, and there were 6 biological replicates per sample. Samples were collected on day 3 and 4 of incubation.

2.6.4 Final lines in duckweed batch #3 and YPD

Mini-fermentations were conducted according to the same methods on duckweed hydrolysate #3 as well as duckweed hydrolysate #3 and YPD both spiked with 15% sucrose. Fermentations were performed on all 6 final yeast lines: Oak, Sake, Turbo, ALE, gen3-32, gen3-56.

2.6.5 Final lines in duckweed batch #4

To test the final yeast lines (Oak, Sake, Turbo, ALE, gen3-32, gen3-56), small-scale (10mL) fermentations were set up using the same methods described previously, using duckweed hydrolysate #4 as the feedstock, with 3-5 replicates each.

2.6.6 Final lines in sucrose spiked YPD

As a control, mini fermentations were performed on the final yeast lines (Oak, Sake, Turbo, ALE, gen3-32, gen3-56) using the same methods as previously described, but with YPD media spiked with 15% sucrose as the

2.7 Large scale fermentations

The resulting duckweed hydrolysate was transferred to 500mL fermentation bottles and autoclaved. 10mL YPD cultures for all 6 strains listed in table __ below were inoculated and grown on a shaker plate at 30°C. After 2 days of growth, the cell densities were estimated for each strain by measuring the optical density at a wavelength of 600nm (as described in the previous fermentation chapter). Cell densities were used to determine the fermentation inoculation volume to ensure equal starting cell densities in each bottle, and therefore a fair comparison of ethanol production across the 6 strains. Based on this, each duckweed hydrolysate bottle was inoculated with the same starting cell density. 500mL fermentations were carried out in jars fitted with one way flow devices (figure __) to support anaerobic conditions, on a shaker plate at 30°C. Samples were collected on day 3, 4, and 6 of incubation and frozen for analysis.

2.7.1 Final lines in duckweed batch #3

The resulting duckweed hydrolysate was transferred to 500mL fermentation bottles and autoclaved. 10mL YPD cultures for all 6 strains listed in table 4 below were inoculated and grown on a shaker plate at 30°C. After 2 days of growth, the cell densities were estimated for each strain by measuring the optical density at a wavelength of 600nm (as described in the previous fermentation chapter). Cell densities were used to determine the fermentation inoculation volume to ensure equal starting cell densities in each bottle, and therefore a fair comparison of ethanol production across the 6 strains. Based on this, each duckweed hydrolysate bottle was inoculated with the same starting cell density. 500mL fermentations were carried out in jars fitted with one way flow devices (figure 20) to support anaerobic conditions, on a shaker plate at 30°C. Samples were collected on day 3, 4, and 6 of incubation and frozen for analysis.

Table 4: yeast strains used for final ethanol comparison

Strain	**Description**
Oak	*Original parent; wild strain*
Sake	*Original parent; wild strain*
Turbo	*Control yeast; domesticated strain*
Gen3-32	*Final line resulting from selective breeding; top ethanol producer from gen3*
Gen3-56	*Final line resulting from selective breeding; second-best ethanol producer from gen3*
ALE	*Final line resulting from artificial laboratory evolution*

Figure 20: large-scale fermentation setup with one-way flow cap.

2.7.2 Final lines in duckweed batch #4

Large scale fermentations (500mL) were set up with the same methods as above on the same yeast varieties (table 4), using duckweed hydrolysate #4 as the feedstock. Additionally, small-scale (10mL) fermentations were set up using the same methods described previously, using duckweed hydrolysate #4 as the feedstock without a sucrose spike, with 3-5 replicates each.

Samples were analyzed at a 1:10 dilution using the GC-MS method described. Resulting ethanol areas and concentration calculations for the large- and small-scale fermentations were quantified.

2.8 References

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CHAPTER 3: RESULTS AND DISCUSSION

3.1 Inducing sporulation

For the spore induction, the previously published method involving exposure to potassium acetate media (SM) was sufficient. After growth in the SM media for 1 week, the presence of spore tetrads was confirmed in the microscope.

3.2 Spore Isolation - heat and y-PER experiment results

The spore isolation step following the methods in the literature was unsuccessful. However, a successful spore isolation method was developed using a combined treatment of exposure to both y-PER and heat.

Growth on both vegetative and spore control plates for all treatment times (10, 20, 30, 40 minutes) was observed, as to be expected. The treated spore samples showed growth for all incubation times, indicating that spores survived the treatment regardless of duration. For the treated vegetative samples, there was some growth for the shorter incubation times (10, 20 minutes), with no growth for the longer incubation times (30, 40 minutes). Growth on the spore treatment plate with no growth on the vegetative treatment plate is indicative of a successful spore isolation treatment, showing that vegetative cells were killed while spores survived the treatment. In addition, growth on the spore treatment plates appeared around 24 hours later than on the control plates, indicating that haploid cells were successfully germinated from the spores and vegetative growth resumed the initial 24 hour period after the isolation treatment.

Based on these results, the final spore isolation treatment was optimized with exposure to Y-PER protein extraction reagent and heat shock at 60°C simultaneously with an incubation time of 40 minutes. It is important to note that the Y-PER must be rinsed at the conclusion of the incubation time to end the treatment. Figure 21 shows a sample successful spore isolation treatment result for Oak:

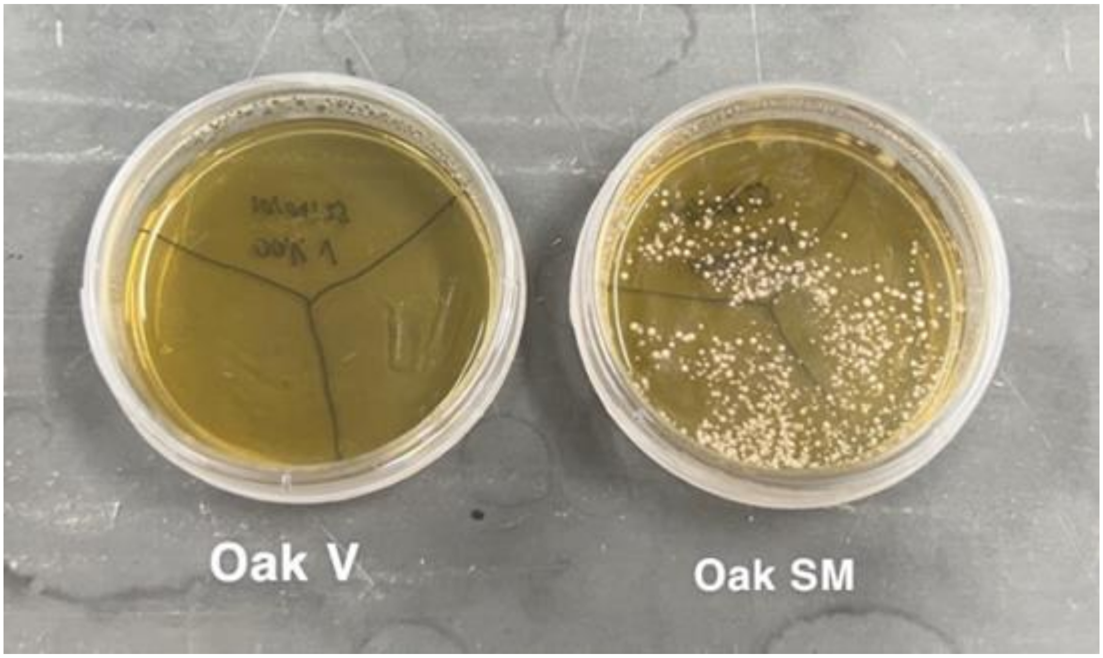

Figure 21: heat + y-PER treatment results on Oak vegetative (V) vs. SM cultures (3 treatment reps per plate); growth on SM with no growth on V indicates a successful spore isolation treatment.

3.3 Phenotyping

3.3.1 Growth rate assay on YPD cultures - plate cover comparison

The growthcurver package in R was used to fit a model to the growth rate curves. However, the growth rate curves did not appear as expected, with a sharp decline occurring after around the 12 hour mark, followed by a plateau. Figure 22 shows a sample growth rate curve with model fit from the initial growth curve run. The model fit was poor, as it was designed to fit a logistic S-curve, typical of microbial growth. After removing the well plate from the spectrophotometer, it was found that the wells had evaporated at some point during the duration of the time series scan, and likely contributed to the unexpected growth rate curves with poor model fits.

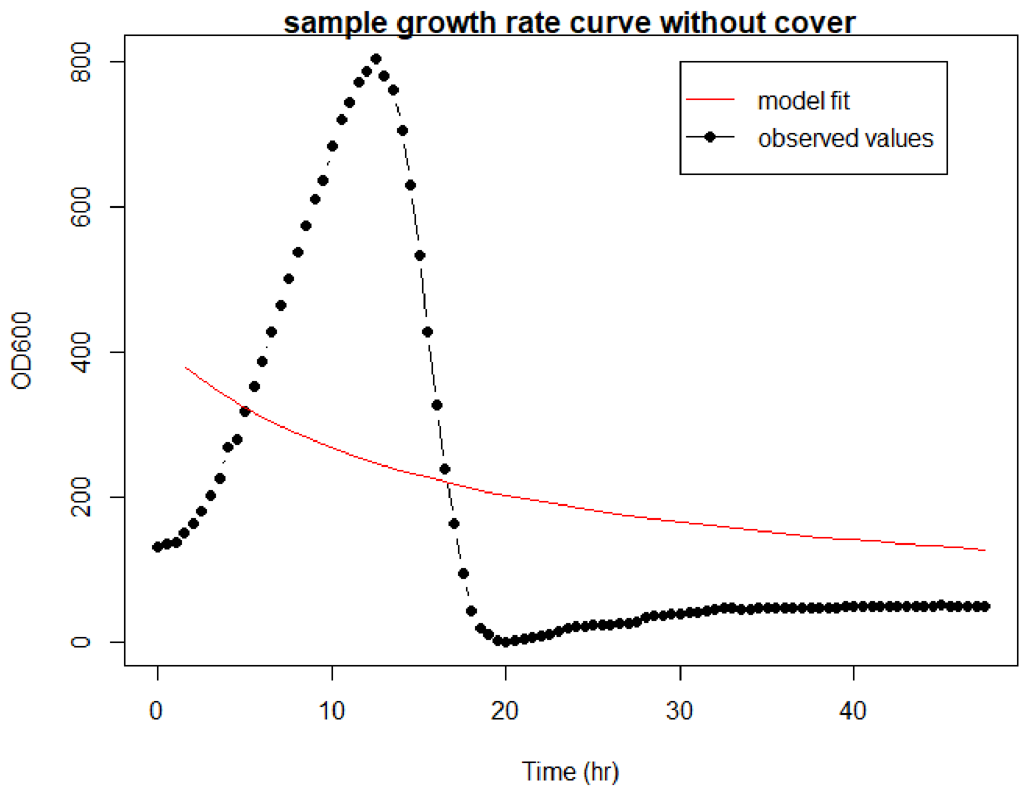

Figure 22: Sample growth rate curve from plate without cover; the initial log growth phase does not carry through to completion and is followed by a rapid decline and plateau.

A sample growth rate curve in figure 23 below shows the difference with using an optically transparent plate cover. With the cover, growth rate curves followed the typical microbial growth behavior as expected, and the models performed well with low residual standard errors. As such, all further growth rate experiments utilized the clear plate cover prior to running the assay.

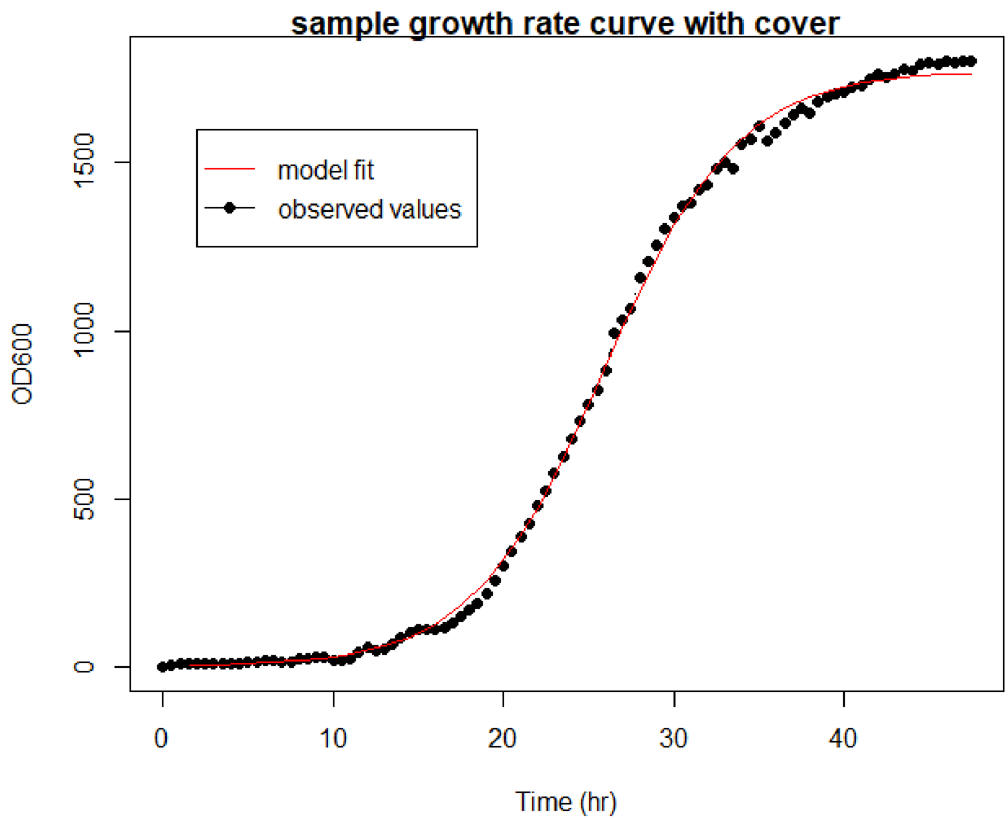

Figure 23: Sample growth rate with cover; the model fit is good with a low RSE of 23.8.

3.3.2 Wild vs. domesticated yeast growth in YPD

The maximum relative growth rate (r) model parameter was extracted from all models and used to compare yeast growth. Figure 24 below shows a boxplot comparison of growth rates for the original wild parents, Oak and Sake, compared to the domesticated control strain, Turbo, when grown in YPD media. Tukey multiple comparisons of means with 95% confidence level was performed on the averages of the biological replicates to compare Oak, Sake, and Turbo (Table 5).

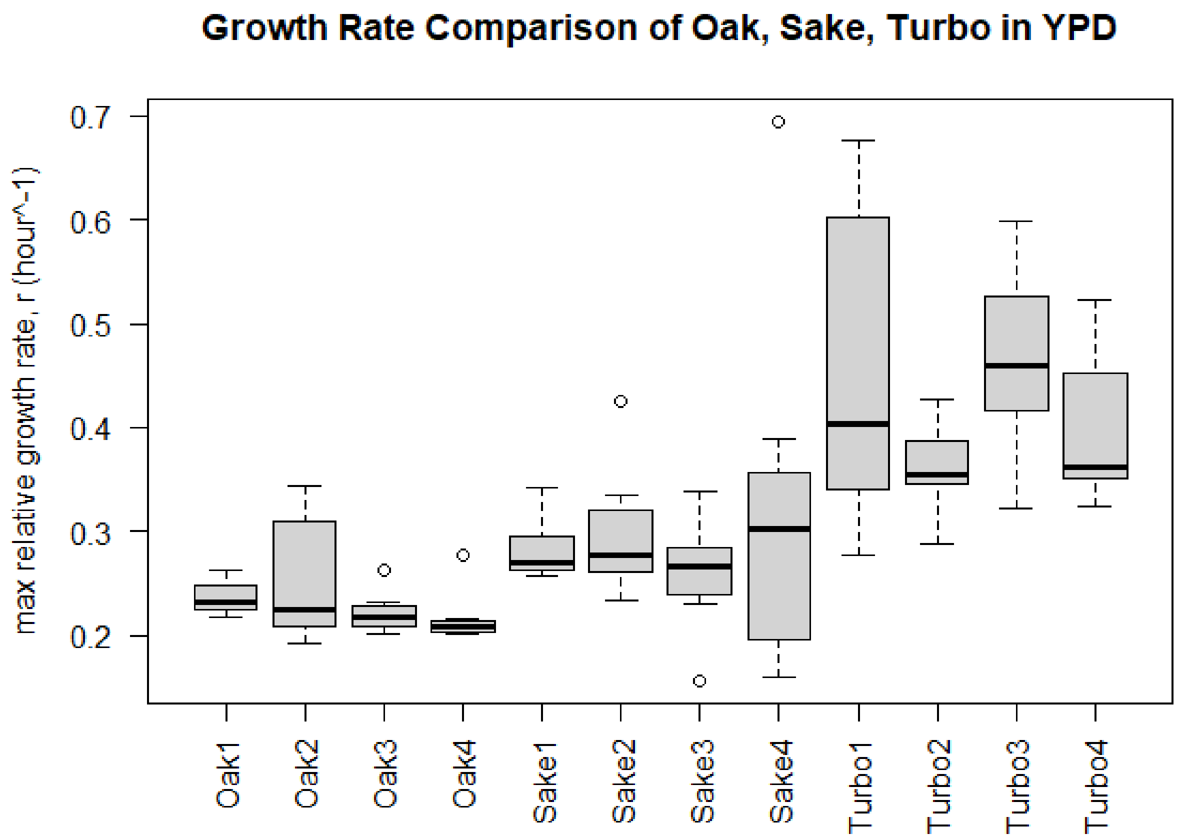

Figure 24: boxplot comparison of growth rates for parents vs. control; each tick mark represents
a yeast culture (4 biological replicates each)

Table 5: p-values for comparison of Oak, Sake, Turbo in YPD

Interaction	**p-value**
Sake-Oak	*0.0167*
Turbo-Oak	*0.0000*
Turbo-Sake	*0.0000*

While there is some variation within the biological replicates for each yeast variety, there was a significant difference across the yeast varieties, with the Turbo yeast having overall higher growth rates compared to the parents (p=0.0000), and the difference in growth rate of the parents was also statistically significant (p=0.0167). The domesticated yeast outperformed the wild yeast when grown in YPD media.

3.3.3 Gen0 cross growth rate variability in YPD

After confirming the feasibility of our growth rate assay on the parents and control yeast in YPD, another growth rate assay was performed on 40 randomly selected individuals from gen0 to get an estimate of the genetic variability of the initial population. Figure 25 below shows a boxplot comparing the r value across the 40 individuals.

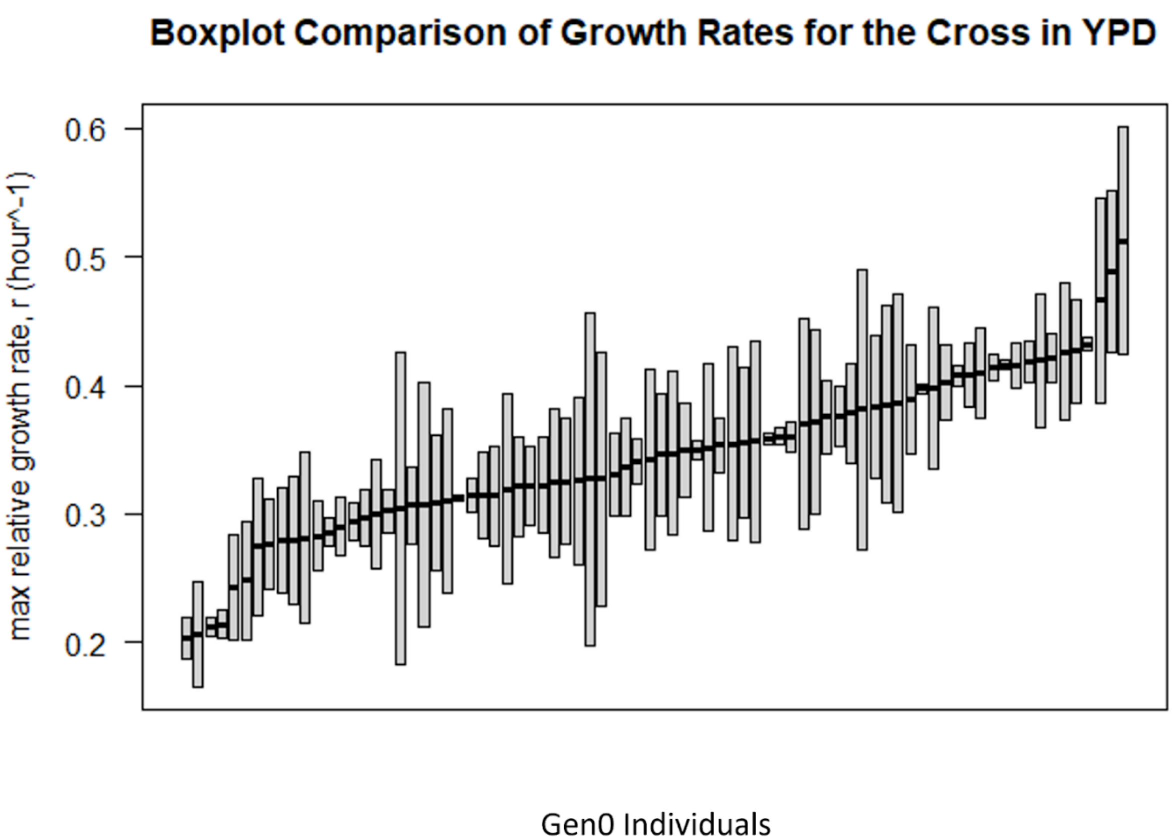

Figure 25: boxplot comparison of max relative growth rate (r) for 40 randomly selected individuals from the gen0 cross; each boxplot represents an individual from the gen0 cross population, and samples are ordered by median r value, increasing from left to right

The results show clear variability across the individuals, with median r values ranging from around 0.2 up to 0.5 hour^{-1}. However, the variability within each individual is inconsistent. That being said, the top median r value individuals overall outperform the individuals with lower growth rates. Having high variability across the individuals from the cross is expected and helps to reassure that the cross was successful, as an increase in genetic variability in the next generation would be expected from crossing two individuals.

3.3.4 Cross vs. selves in YPD

A boxplot comparison of the growth rates for the cross vs. selves is shown in figure 26 below. Accompanying p-values from Tukey multiple comparisons of means with 95% confidence level are shown in Table 6.

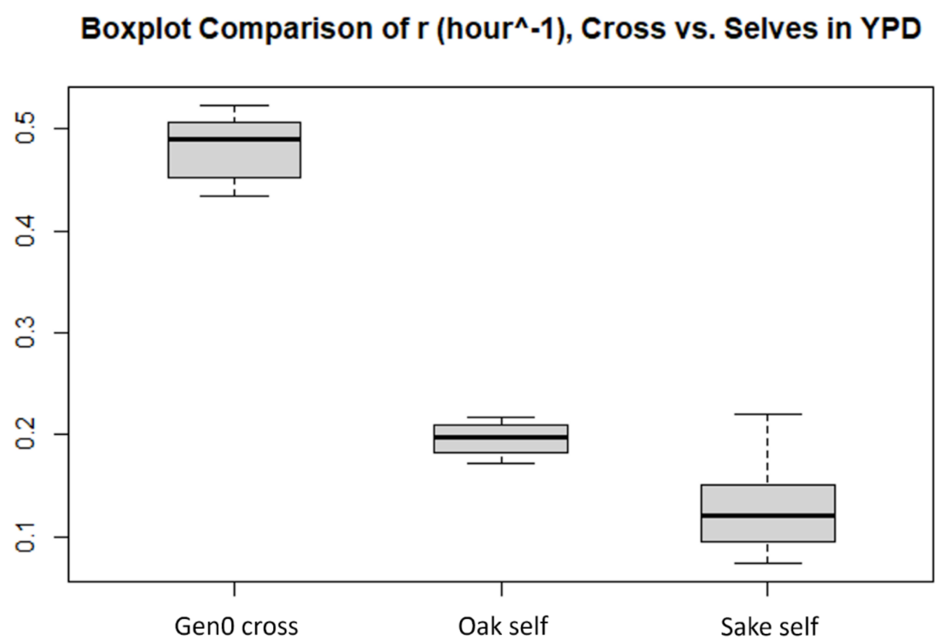

Figure 26: boxplot comparison of growth rates for cross vs. selves in YPD.

Table 6: p-values for comparison of gen0 cross with oak and sake selves in YPD

Interaction	p-value
Oak self - Gen0 cross	*0.0000*
Sake self - Gen0 cross	*0.0000*
Sake self - Oak self	*0.0246*

Results show that both selved lines had significantly lower growth rates compared to the gen0 cross (p=0.0000) in YPD. This is further evidence that the cross was successful, as the results of outcrossing would be expected to produce a genetically diverse generation, which leads to better potential for growth, while the inbred lines could be limited in their growth potential due to low genetic variability. This supports the hypothesis that performing the crossing step increases the genetic variability.

3.3.5 Growth rate assay on duckweed cultures

In the duckweed media, the growth rate curves followed the expected logarithmic growth pattern. The models tended to have higher residual standard errors compared to the growth rate curves in YPD media, possibly due to interference of the duckweed background with the spectrophotometer reading. A sample model for a growth rate curve in duckweed media is seen in figure 27 below.

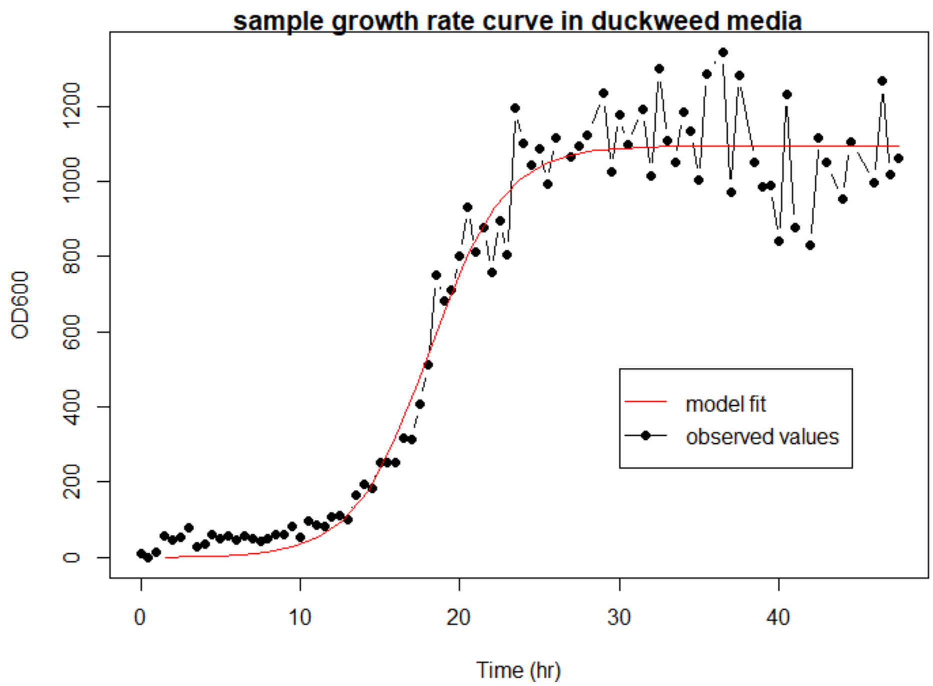

Figure 27: sample growth rate curve in duckweed media.

3.3.6 wild vs. domesticated yeast growth in duckweed

Figure 28 below shows a boxplot comparison of growth rates for Oak, Sake, and Turbo when grown in duckweed media. The maximum relative growth rate (r) was extracted from all models and used to compare yeast growth. Associated p-values from Tukey's significance test are in Table 7. The relative growth rate of the control Turbo yeast was significantly lower than that of both Oak and Sake (p=0.00), while there was no significant difference between Oak and Sake (p=0.13). The results indicate that the wild strains outperformed the domesticated yeast when grown in the duckweed media, contrary to results when grown in the YPD media. This supports the hypothesis that the wild yeast strains may contain genetic variants that are beneficial for in the bioprocessing of novel feedstocks like duckweed.

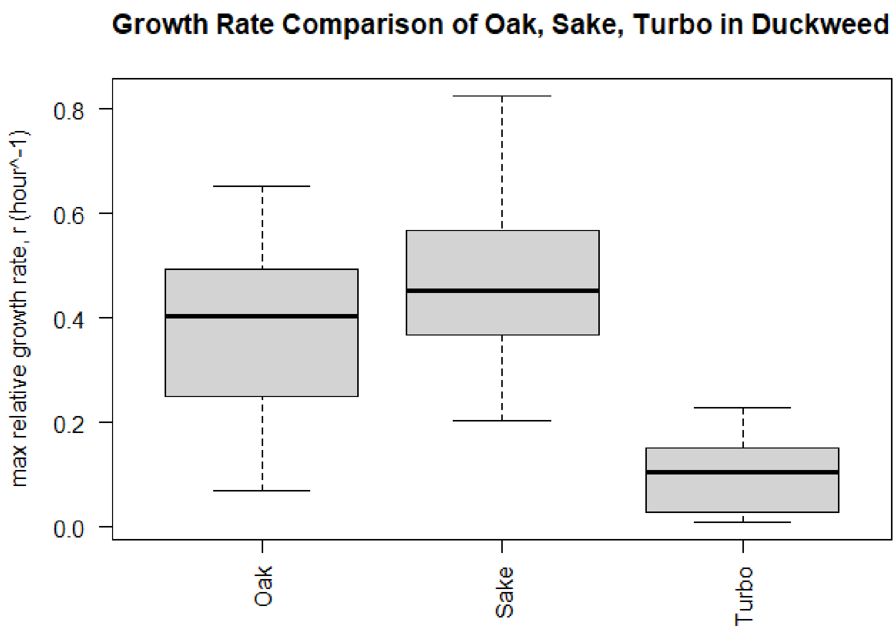

Figure 28: boxplot growth rate comparison of wild vs domesticated in duckweed.

Table 7: p-values for comparison of wild vs. domesticated yeast in duckweed

Interaction	**p-value**
Sake-Oak	*0.1314*
Turbo-Oak	*0.0009*
Turbo-Sake	*0.0000*

3.3.7 Applying phenotyping to the selective breeding cycle

After confirming the feasibility of the growth rate assay using duckweed hydrolysate as the background media, the growth rate phenotyping protocol was carried out as described in the phenotyping materials and methods using duckweed hydrolysate as the growth media. Models were constructed for each growth rate curve and the model parameter maximum relative growth rate (r) was extracted to compare individuals. The boxplot in figure 29 below shows the distribution of r values for each individual in gen1. Table 8 below contains the top 10 r value lines.

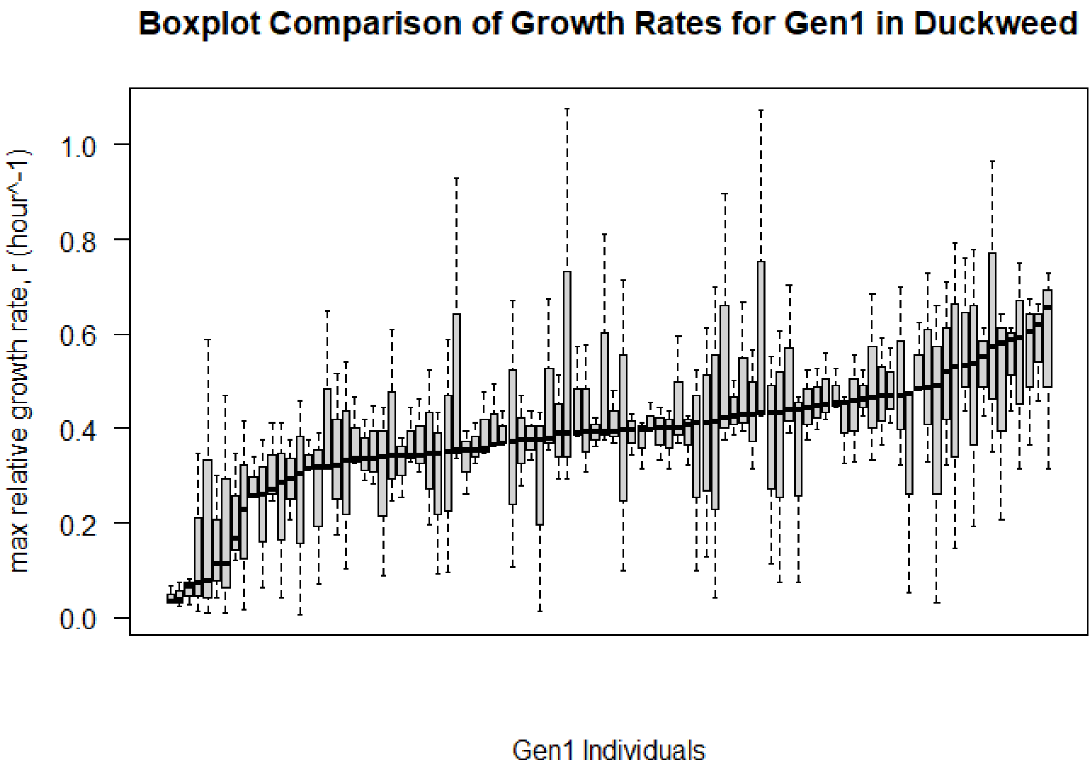

Figure 29: boxplot variability of r for gen1 in duckweed, sorted by median r value increasing from left to right; each boxplot represents an individual from gen1.

Table 8: top 10 gen1 lines by median r value.

Gen1 line	**Median r value (hour^{-1})**
gen1-01	0.656
gen1-50	0.607
gen1-73	0.591
gen1-85	0.589
gen1-69	0.583
gen1-72	0.575
gen1-89	0.552

Table 8: top 10 gen1 lines by median r value, continued

gen1-90	0.539
gen1-92	0.534
gen1-57	0.532

These top 10 r value individuals were chosen for bulk crossing to combine and form the next generation as described in the materials and methods section. This resulted in a new population of 96 individuals, gen2.

A growth rate assay was performed on gen2 using the same methods described above. Models were constructed and the parameter r was extracted. Figure 30 below shows the boxplot variability of r for each individual in gen2, and the top 10 individuals by median r value are shown in table 9.

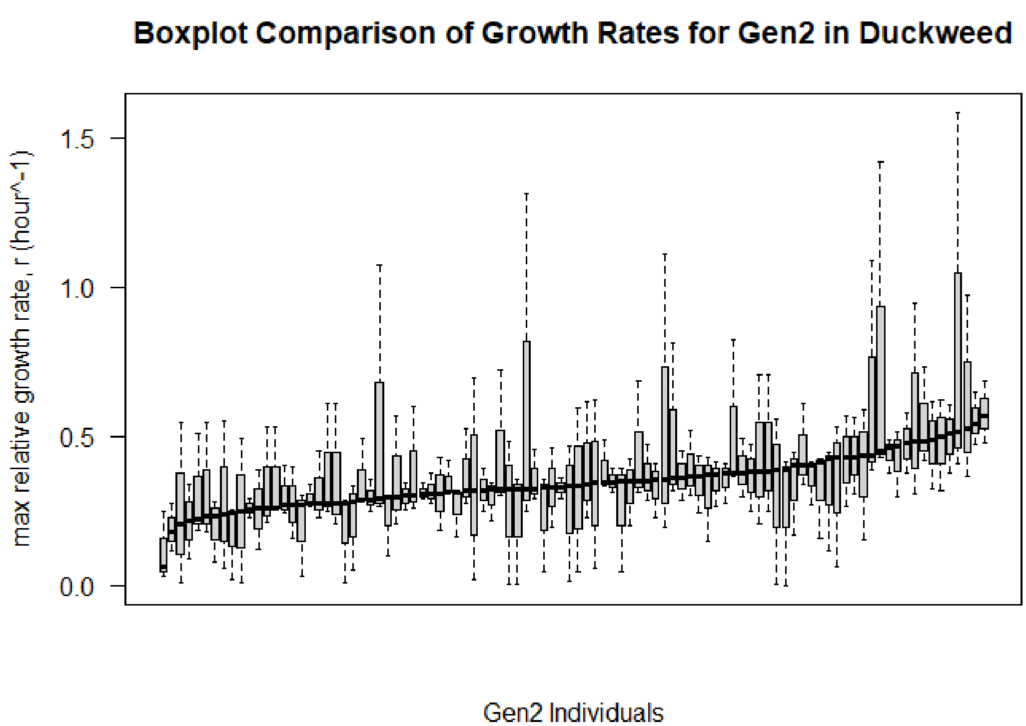

Figure 30: boxplot variability of r for gen2 in duckweed, sorted by median r value increasing from left to right. Each boxplot represents an individual from gen2

Table 9: top 10 gen2 lines median r value

Gen2 Line	Median r value (hour-1)
gen2-41	*0.570*
gen2-10	*0.542*
gen2-73	*0.525*
gen2-82	*0.514*
gen2-79	*0.507*
gen2-59	*0.500*
gen2-42	*0.491*
gen2-26	*0.484*
gen2-66	*0.481*
gen2-94	*0.475*

These top 10 individuals from gen2 were crossed using the same methods as above, forming the final generation, gen3. Similarly, the growth rate assay was performed on gen3 to generate growth rate curves, models were fitted, and r values were extracted. Figure 31 below shows the boxplot variability of r across the individuals from gen3, and table 10 shows the top 2 lines. These top 2 lines from gen3 (gen3-32 and gen3-56) were chosen as the final lines for further fermentation analysis.

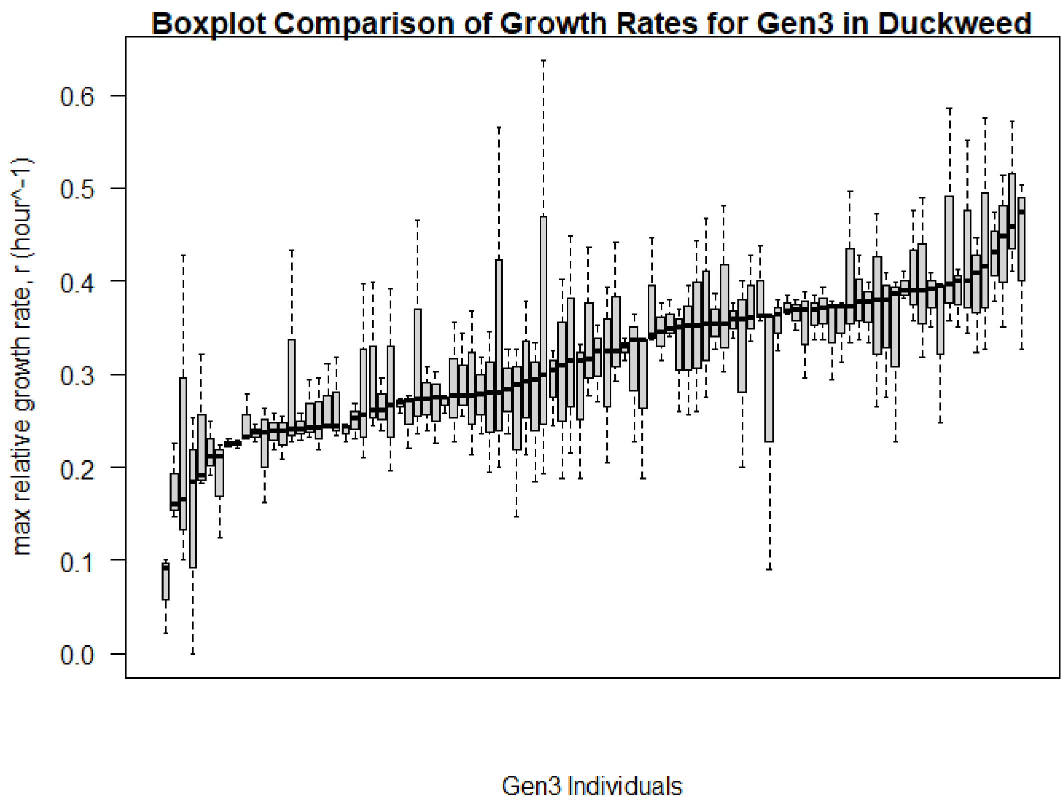

Figure 31: boxplot variability of r for gen3 in duckweed, sorted by median r value increasing from left to right; each boxplot represents an individual from gen3

Table 10: top 2 lines from gen3 by median r value

Gen3 Line	**Median r value (hour^{-1})**
gen3-32	*0.474*
gen3-56	*0.459*

3.4 Quantification of ethanol with GC-MS

An overlaid chromatogram of ethanol standards at concentrations ranging from 0-1% is shown in figure 32 below:

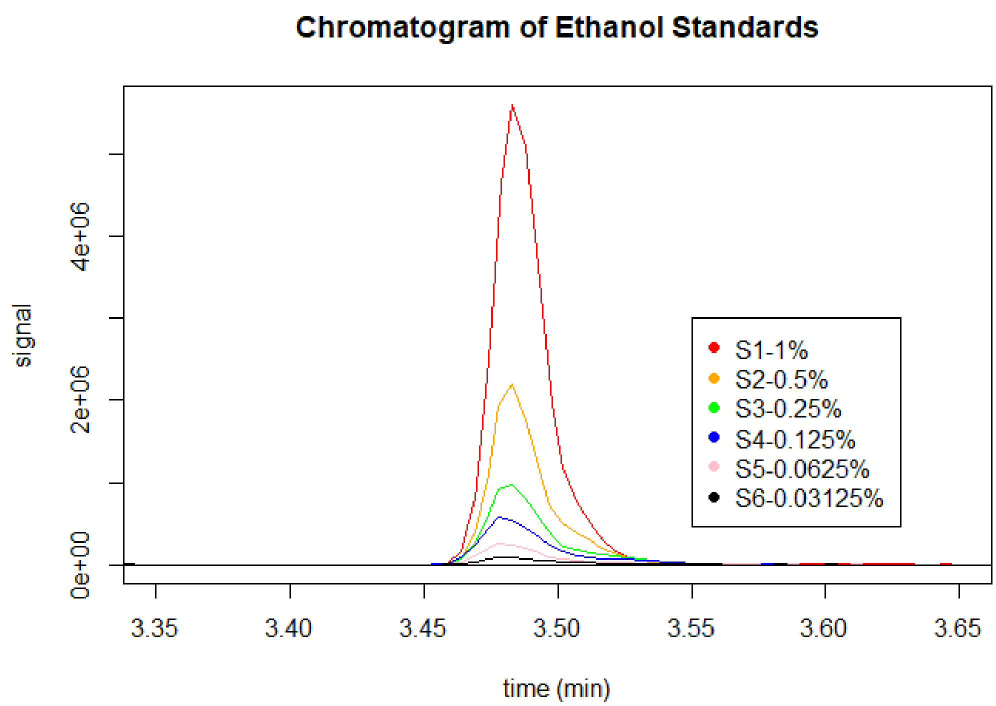

Figure 32: overlaid chromatogram of ethanol standards

The ethanol peaks for the standards eluted with consistent retention times (RT) around 3.48 minutes, and the mass spectrometer identified this compound as ethanol. Areas under the curve were quantified and plotted against known ethanol concentrations to produce standard curves. Areas under the curve followed a linear relationship with known concentrations, as expected, and a linear equation was fitted to the standard curve and used to calculate ethanol concentrations in the samples (figure 33).

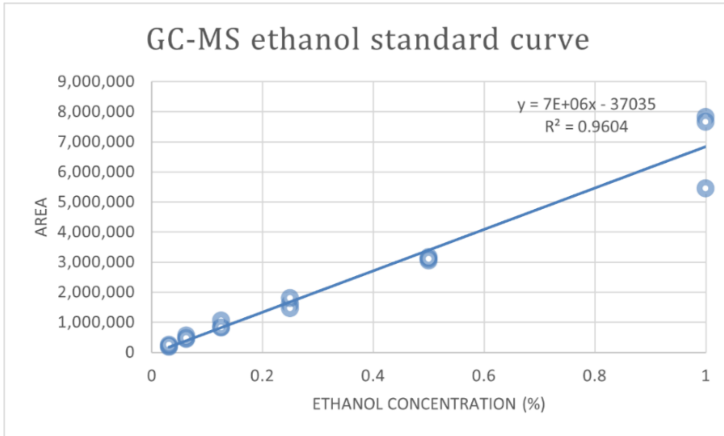

Figure 33: GC-MS ethanol standard curve with linear fit

3.5 Small-scale fermentations

3.5.1 Wild vs. domesticated in sucrose YPD

Results show ethanol percentages ranging from 8.9 - 10.3%. The turbo performed better in the 15% sucrose YPD media compared to Oak and Sake (Figure 34). Results show the difference between Oak and Sake vs. Turbo was not statistically significant with a p-value of 0.064 (table 11).

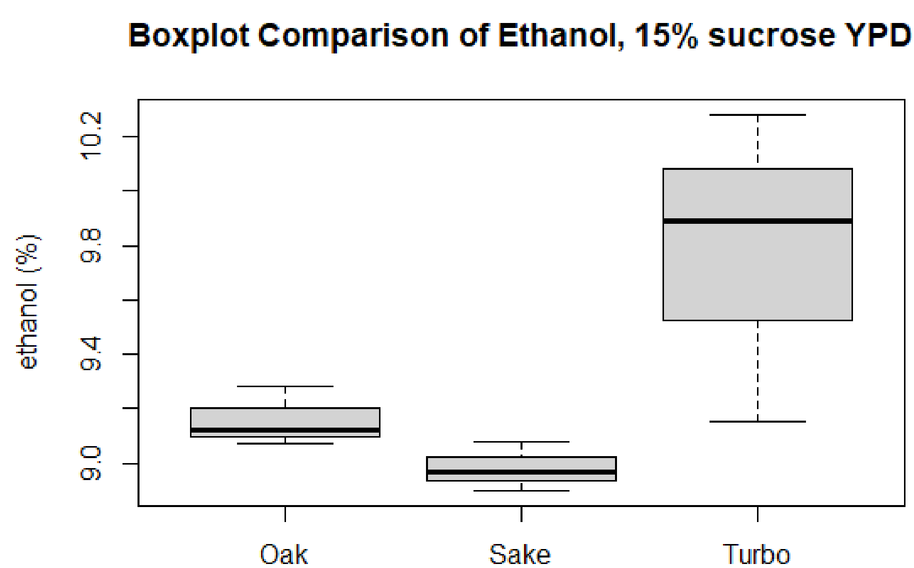

Figure 34: boxplot comparison of ethanol concentrations, wild (Oak and Sake) vs. domesticated (Turbo)

Table 11: p-values, wild vs. domesticated in 15% sucrose YPD

interaction	**p-value**
sake-oak	*0.8099*
turbo-oak	*0.1450*
turbo-sake	*0.0652*

The general comparison between the wild parents and the domesticated control was as expected for the YPD media, with turbo appearing to perform better than the wild strains.

3.5.2 Ideal fermentation time experiment

Figure 35 below shows the ethanol concentration results at different timepoints:

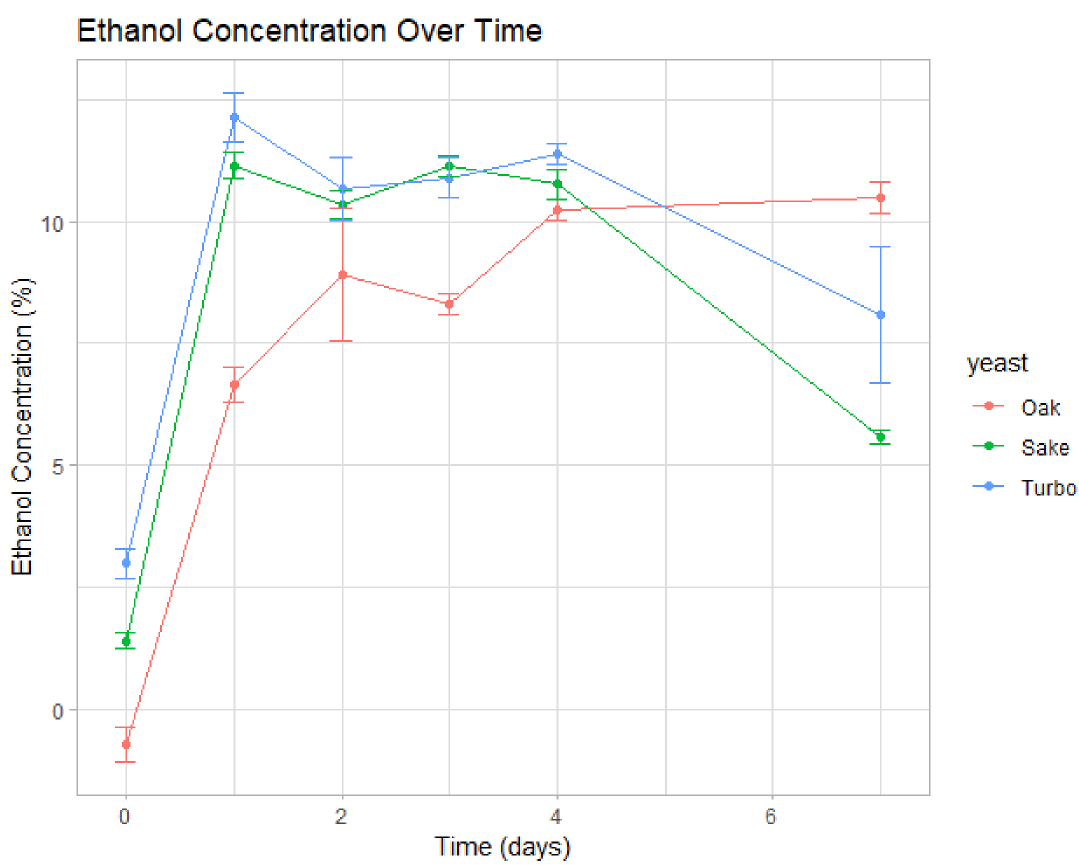

Figure 35: Fermentation time series of average ethanol concentrations for Oak, Sake, and Turbo (the error bars represent plus or minus one standard error from the mean).

In 15% sucrose YPD media small scale fermentations, for all three yeast varieties, there was a sharp increase in ethanol concentration from day 0 to day 1. From day 1-3, Sake and Turbo remained relatively constant, while Oak kept increasing up until day 4. At the day 4 time point, ethanol concentrations had stabilized for all three varieties, in other words, Oak had reached its maximum and Sake and Turbo had not started to decline yet. After day 4, Sake and Turbo began to decline while Oak remained constant. The results indicate that 4 days is the optimal incubation period for these yeast strains.

3.5.3 Wild vs. domesticated in duckweed batch #1 with and without sucrose

Results from the initial small scale duckweed hydrolysis show that the wild-type parents Oak and Sake tend to perform better than the Turbo control in the duckweed media, both with and without the additional 15% sucrose spike. Ethanol concentrations were low, ranging from 0-1.8%, with higher values for the sucrose spiked samples. There does appear to be a difference between the turbo yeast and the parents, with the parents producing higher ethanol concentrations in the duckweed media compared to the turbo control regardless of sucrose spike. Tukey's significance test was performed for both sucrose and non-sucrose samples (table 12). In the non-sucrose samples, there were no statistically significant differences in means of the yeast varieties (figure 36). For the sucrose-spiked samples, results showed a significant difference between turbo and sake ($p = 0.0007$) and between turbo and oak ($p = 0.0102$) (figure 37).This supports the hypothesis that the wild-type yeast may be better able to ferment duckweed compared to the turbo control yeast.

Table 12: p-values from duckweed batch #1, with and without sucrose spike

interaction	p-value, no sucrose spike	p-value, with sucrose spike
sake-oak	*0.9414*	*0.0496*
turbo-oak	*0.2029*	*0.0102*
turbo-sake	*0.1297*	*0.0007*

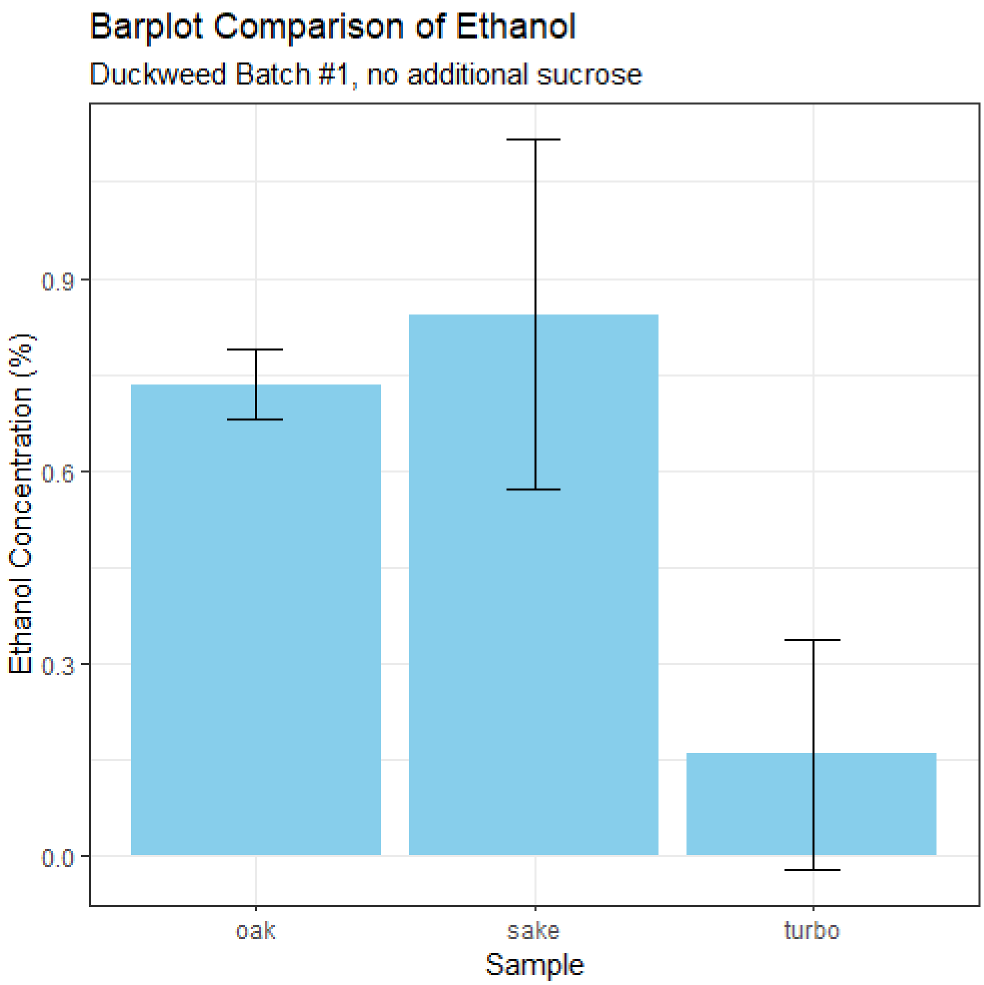

Figure 36: barplot comparing the average ethanol concentrations (%) across oak, sake, and turbo in duckweed batch #1 with no sucrose supplementation (the error bars represent plus or minus one standard error); the differences between the strains were not found to be statistically significant.

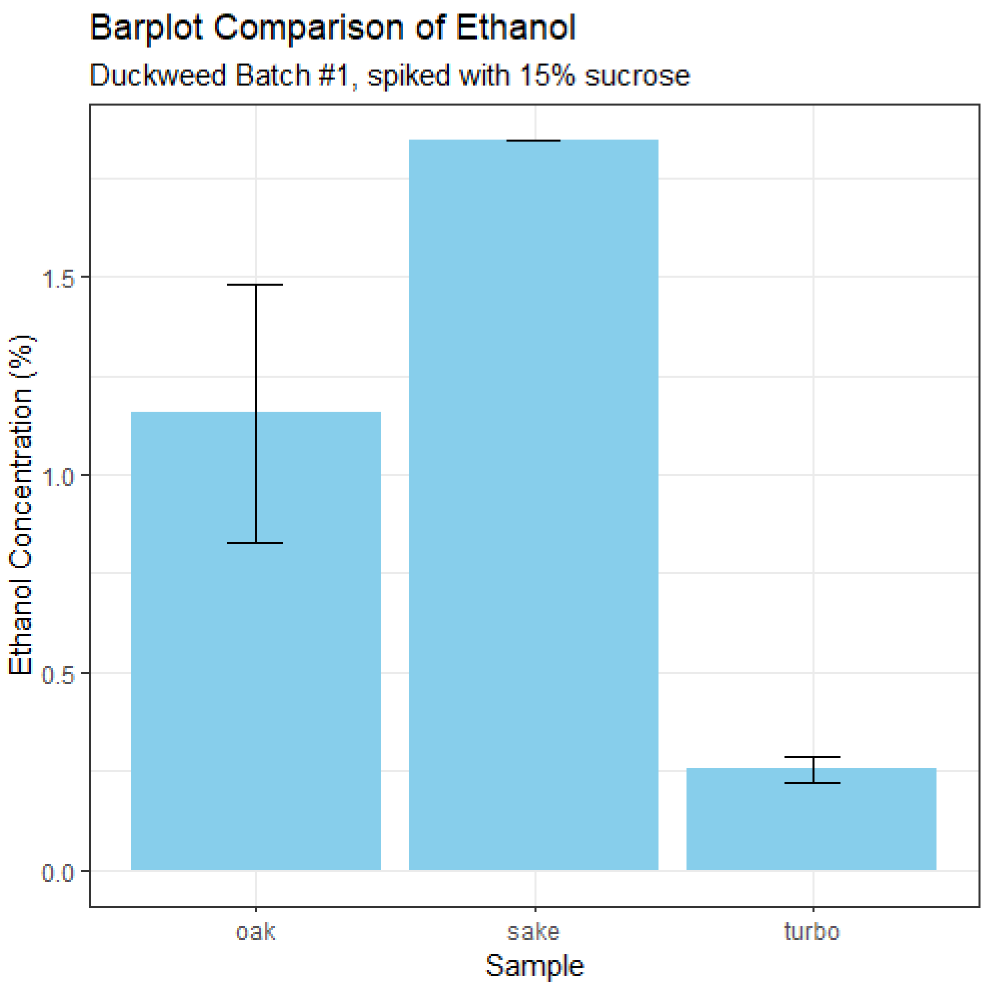

Figure 37: barplot comparing the average ethanol concentrations (%) across oak, sake, and turbo in duckweed batch #1 spiked with 15% sucrose (the error bars represent plus or minus one standard error); Turbo's ethanol concentration was found to be significantly lower than that of both oak and sake.

The results from the first duckweed hydrolysate revealed lower ethanol concentrations than expected. It is well documented in the literature that duckweed hydrolysate can be used as an effective fermentation feedstock (Cheng et al., 2002; Cheng & Stomp, 2009; Cui & Cheng, 2014; Ge et al., 2012). However, interestingly, a significant increase in performance from both wild yeast strains compared to the domesticated strain when using duckweed feedstock was observed, indicating that these wild varieties may be promising starting points for an optimal strain of yeast that ferments duckweed.

One reason for low ethanol production could be that very little sugar was present either due to low starch concentrations in the duckweed or failed conversion of starch to sugar. Upon further inspection, the amount of amyloglucosidase in the first hydrolysis was mistakenly reduced by a factor of 10. We theorize that this was a large cause of the low ethanol concentrations. Another hydrolysis was performed to conduct further fermentation experiments, with key differences being a higher concentration of amyloglucosidase and no filtration of the hydrolysate prior to fermentations.

3.5.4 Wild vs. domesticated in duckweed batch #2

Results from the second hydrolysis batch show ethanol concentrations ranging from 7-9%, with the Turbo control yeast tending to have lower concentrations compared to the wild strains. A Tukey's test was performed to compare differences in the mean ethanol concentrations (table 13). On day 3, there was a significant difference between Turbo and Oak, with a p-value of 0.0005, while on day 4 there was a significant difference between both turbo and Oak, and Turbo and Sake, with p-values of 0.0000 for both. The boxplot in figure 38 visualizes the difference distributions of ethanol concentrations on day 4 fermentation samples. These results support the hypothesis that the wild-type parents will perform better in the duckweed media compared to the turbo control. In addition, with ethanol concentrations being around 8%, we are led to believe that adjusting the enzyme concentration in the hydrolysis protocol helped with improving starch conversion, and the reduced concentration in batch #1 likely resulted in the low ethanol concentrations. We also theorize that by leaving the hydrolysate as a slurry, there will be more starch available for the yeast to consume from the solids content, which may improve the ethanol production.

Table 13: p-values from duckweed batch #2, day 3 and 4

interaction	**p-value, day 3**	**p-value, day 4**
sake-oak	*0.1826*	*0.9032*
turbo-oak	*0.0005*	*0.0000*
turbo-sake	*0.0564*	*0.0000*

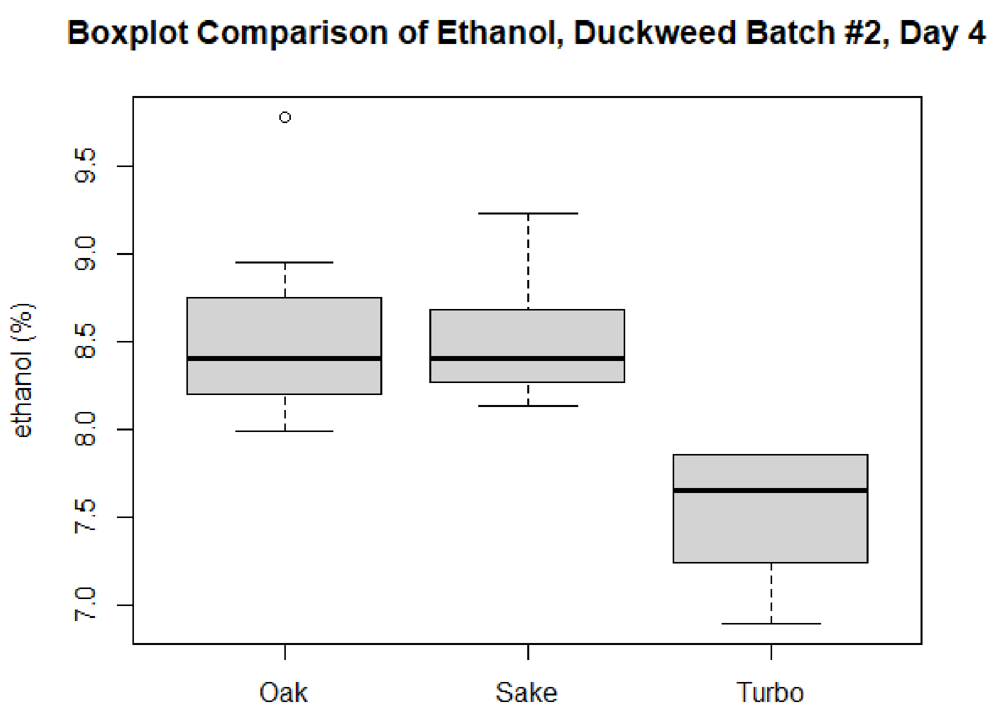

Figure 38: boxplot comparing mean ethanol concentrations (%) on day 4 fermentations in duckweed hydrolysate batch #2 (6 replicates per strain); the difference in turbo vs. both oak and sake is statistically significant.

3.5.5 Final lines in duckweed batch #3 and YPD with sucrose spike

Mini-fermentations were conducted on the final lines using duckweed hydrolysate #3, as a feedstock, however almost no ethanol was detected, so the hydrolysate was spiked with sucrose and fermented again. Ethanol was confirmed using the GC-MS ethanol quantification method for the small scale fermentations on the final yeast lines with addition of sucrose to the duckweed media from hydrolysis #3 and YPD media. The table 14 below summarizes the GC-MS ethanol quantification results:

Table 14: 15% sucrose fermentation ethanol results

Sample	**Feedstock**	**GC-MS ethanol peak area**	**Calculated ethanol concentration (%)**
Oak	*Duckweed, 15% sucrose*	*19,934,286.00*	*4.79*
Sake	*Duckweed, 15% sucrose*	*18,466,545.00*	*4.42*
Turbo	*Duckweed, 15% sucrose*	*363,324.00*	*0.00*
ALE	*Duckweed, 15% sucrose*	*18,210,414.00*	*4.35*
Gen3-32	*Duckweed, 15% sucrose*	*22,109,386.00*	*5.33*
Gen3-32 replicate	*Duckweed, 15% sucrose*	*21,810,548.00*	*5.25*
Gen3-56	*Duckweed, 15% sucrose*	*19,282,540.00*	*4.62*
Oak	*YPD, 15% sucrose*	*19,903,288.00*	*4.78*
Sake	*YPD, 15% sucrose*	*17,269,307.00*	*4.12*
Turbo	*YPD, 15% sucrose*	*1,611.00*	*0.00*
ALE	*YPD, 15% sucrose*	*21,689,766.00*	*5.22*
Gen3-32	*YPD, 15% sucrose*	*19,899,139.00*	*4.78*
Gen3-32 replicate	*YPD, 15% sucrose*	*16,950,039.00*	*4.04*
Gen3-56	*YPD, 15% sucrose*	*18,354,593.00*	*4.39*

In the 15% sucrose fermentations, there was no ethanol for Turbo in either the duckweed or YPD feedstock. Turbo is expected to produce high ethanol in YPD with sucrose, so there was likely something wrong with the starting culture (such as contamination) and therefore comparisons cannot be made to the Turbo yeast for this round of fermentations.

3.5.6 final lines in duckweed batch#4

For the large and small scale fermentations in duckweed hydrolysate #4, the GC-MS ethanol quantification protocol was used to calculate and extract the ethanol areas and concentrations. Interestingly, large acetic acid peaks were also observed and quantified. Ethanol and acetic acid quantification for the small scale duckweed fermentations from hydrolysate #4 are summarized in the barplot and table (figures 39 and table 15) below:

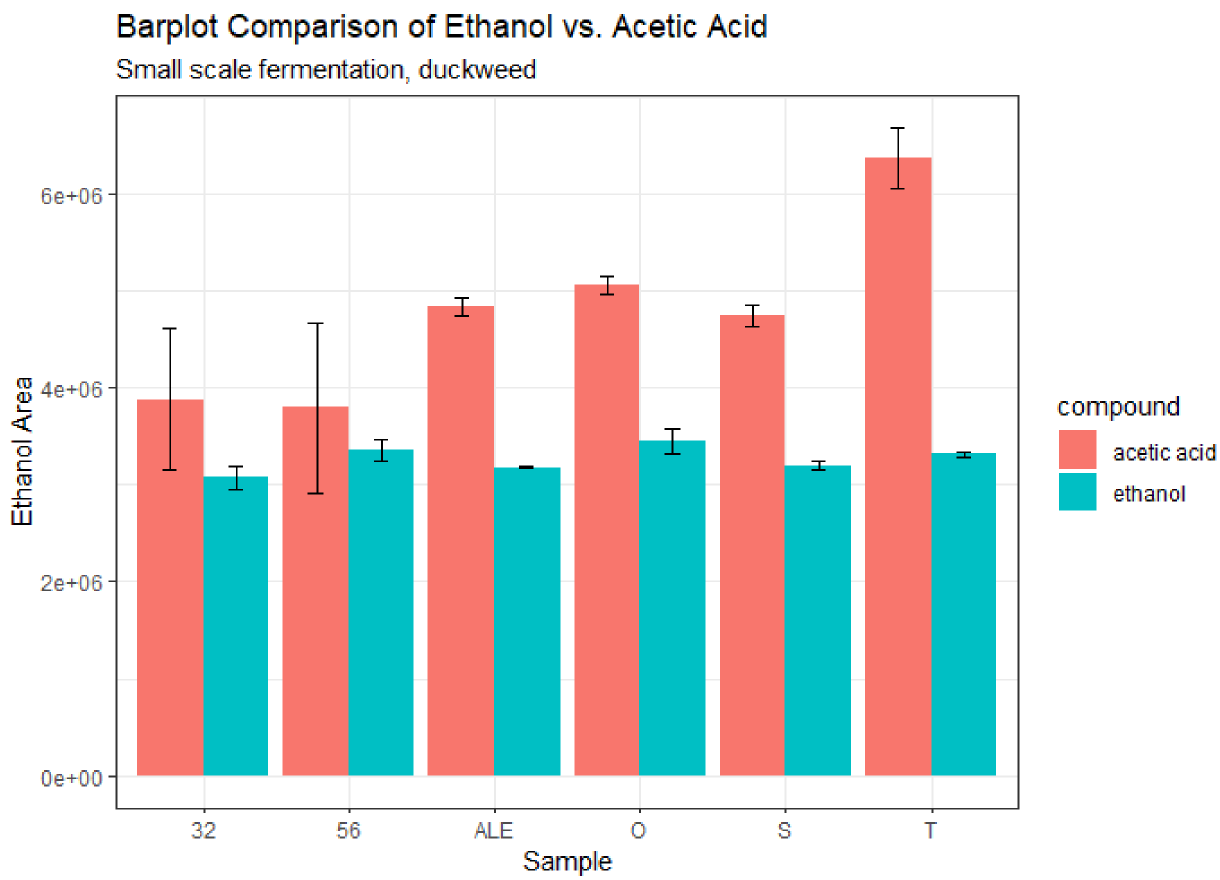

Figure 39: barplot comparison of ethanol and acetic acid areas under the curve for gen3-32, gen3-56, ALE, Oak, Sake, and Turbo yeast for small scale fermentations in duckweed hydrolysate #4.

Table 15: small scale GC-MS ethanol quantification (1:10 dilution, duckweed batch #4, 3-5 reps each).

Sample	**Ethanol peak area, average**	**Ethanol peak area, standard deviation**	**Calculated ethanol concentration, avg (%)**	**Acetic acid peak area, average**	**Acetic acid peak area, standard deviation**
Oak	*3457422*	*288678*	*1.70*	*5052643*	*210243*
Sake	*3200632*	*87307*	*2.30*	*4738057*	*253435*
Turbo	*3313171*	*48702*	*2.31*	*6374052*	*542661*
ALE	*3178702*	*21400*	*2.70*	*4840708*	*167631*
Gen3-32	*3071855*	*217165*	*2.87*	*3879418*	*1260312*
Gen3-56	*3357061*	*231647*	*2.42*	*3800144*	*1963890*

A tukey's significance test was performed on the ethanol and acetic acid peak areas.

Resulting p-values are shown in the table 16 below:

Table 16: p-values from tukey's significance test for small scale fermentations, duckweed hydrolysis batch #4.

interaction	**p-value, ethanol**	**p-value, acetic acid**
gen3-32 - ALE	*0.9827*	*0.8643*
gen3-56 - ALE	*0.8029*	*0.7476*
oak - ALE	*0.3976*	*0.9997*
sake - ALE	*0.9999*	*0.9999*
turbo - ALE	*0.9539*	*0.4922*
gen3-56 - gen3-32	*0.3738*	*0.9999*
oak - gen3-32	*0.1195*	*0.6470*
sake - gen3-32	*0.9394*	*0.8650*
turbo - gen3-32	*0.6553*	*0.0821*
oak - gen3-56	*0.9605*	*0.4366*
sake - gen3-56	*0.7948*	*0.7163*
turbo - gen3-56	*0.9995*	***0.0340***

Table 16: p-values from tukey's significance test for small scale fermentations, duckweed hydrolysis batch #4, continued.

sake - oak	*0.3345*	*0.9964*
turbo - oak	*0.9061*	*0.5307*
turbo - sake	*0.9650*	*0.3106*

For the small-scale fermentations, there were no significant differences across the varieties for ethanol production. The control Turbo yeast produced significantly more acetic acid compared to the developed line, gen3-56 (p=0.03).

3.5.7 final lines in YPD with sucrose

High acetic acid concentrations were detected in all fermentations using duckweed hydrolysate #4. This indicates bacterial contamination and is likely the reason for low ethanol yields. As a control, the final lines were also fermented in 15% sucrose YPD media. Ethanol and acetic acid peak areas were quantified and are shown in the figures below (figure 40):

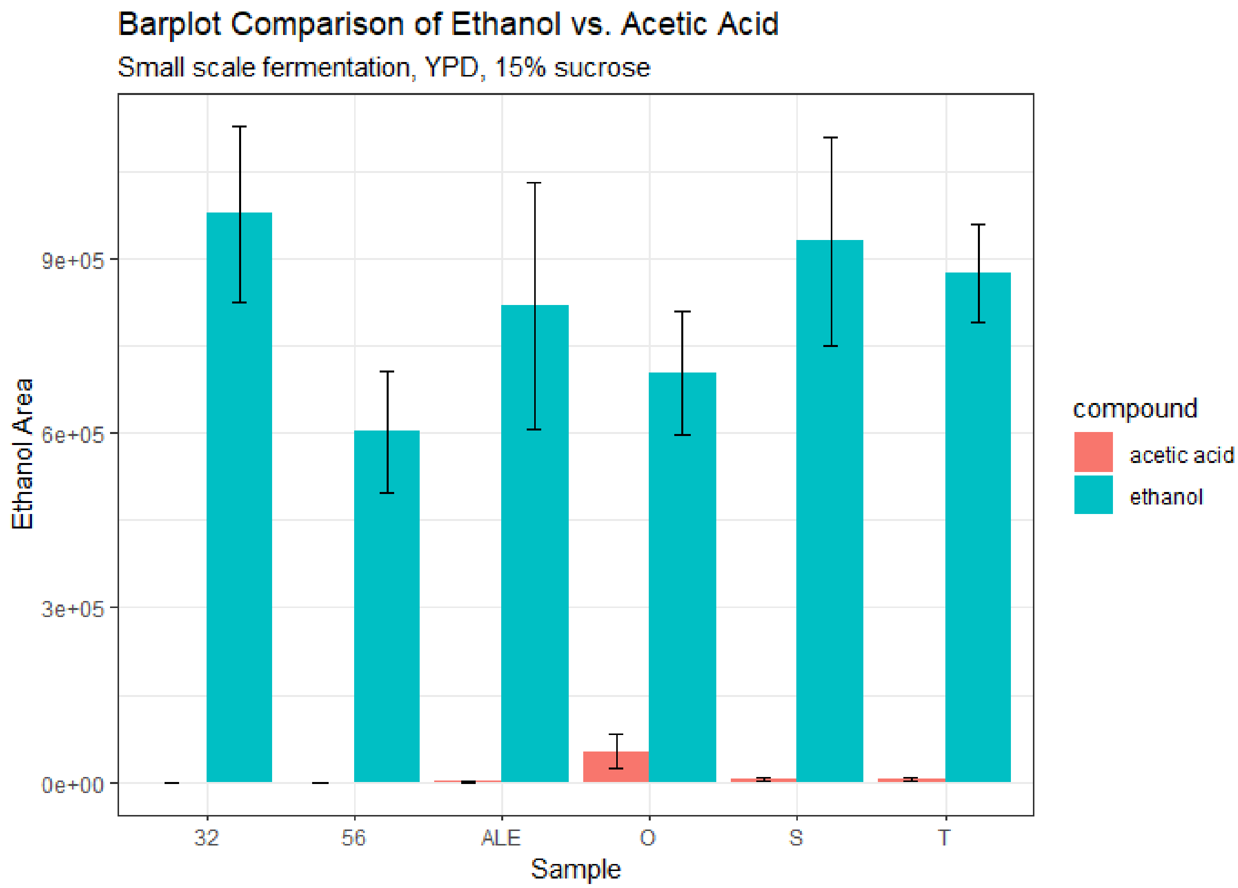

Figure 40: barplot comparison of ethanol and acetic acid areas under the curve for gen3-32, gen3-56, ALE, Oak, Sake, and Turbo yeast small scale fermentation in YPD supplemented with sucrose

Table 17: GC-MS ethanol quantification (YPD media, small scale, sucrose); average of 3 biological reps, samples diluted 1:100 prior to GC-MS injection

Sample	Ethanol peak area, average	Ethanol peak area, standard deviation	Calculated ethanol concentration (%)	Acetic acid peak area, average	Acetic acid peak area, standard deviation
Oak	*703,179*	*256787*	*10.77*	*53198*	*28614*
Sake	*929,947*	*438648*	*14.08*	*5592*	*3750*
Turbo	*874,753*	*207454*	*13.27*	*5359*	*3050*
ALE	*818,949*	*520560*	*12.46*	*1846*	*1846*
Gen3-32	*976,390*	*370084*	*14.75*	*0*	*0*
Gen3-56	*602,344*	*257810*	*9.31*	*0*	*0*

Areas under the curve were averaged across the technical reps, and then the biological replicates were compared using a Tukey's significance test. Resulting p-values are shown in table 18 below:

Table 18: p-values from tukey's significance test for small scale fermentations, YPD media with 15% sucrose spike

interaction	**p-value**
gen3-32 - ALE	*0.9050*
gen3-56 - ALE	*0.9196*
oak - ALE	*0.9944*
sake - ALE	*0.9954*
turbo-ALE	*0.9998*
gen3-56-gen3-32	*0.4050*
oak - gen3-32	*0.6536*
sake - gen3-32	*0.9944*
turbo - gen3-32	*0.9688*
oak - gen3-56	*0.9970*
sake - gen3-56	*0.6910*
turbo - gen3-56	*0.8206*
sake - oak	*0.9046*
turbo - oak	*0.9682*
turbo - sake	*0.9998*

For the small scale fermentations in 15% sucrose YPD, there were no significant differences across any of the yeast varieties. However, ethanol concentrations were higher compared to the duckweed fermentations, averaging around 12% ethanol, which would be expected with a sucrose spike. Acetic acid concentration was negligible in the YPD media feedstock samples compared to the duckweed samples.

3.6 Large scale fermentations

3.6.1 Duckweed batch #3

The GC-MS protocol allows for highly sensitive ethanol detection limits. Despite this, no ethanol peaks were detected in any of the large scale fermentation samples from duckweed hydrolysis #3. Something likely went wrong with the hydrolysis. The previous hydrolysate #2 resulted in around 7-9% ethanol, so the results from the large-scale hydrolysis were not as expected. Small scale fermentations with additional sucrose (15%) were performed which would theoretically yield high ethanol.

Comparing hydrolysis #3 to the previous batches, the alpha-amylase enzymes came from different sources. Batch #2 used alpha-amylase sourced from *Bacillus licheniformis*, which has a higher optimum temperature and higher temperature tolerance compared to the alpha-amylase from porcine pancreas used in batch #3. The protocol was not altered to account for the different enzyme and it’s likely that the hydrolysis failed due to inactivation of the porcine alpha-amylase enzyme at 70°C . In addition, performing a dilution after the completion of the hydrolysis reduced the available sugar. Still, 5% solids should have been sufficient for detectable ethanol production based on previous studies.

3.6.2 Duckweed hydrolysis #4

Ethanol and acetic acid quantification for the large scale duckweed fermentations are summarized in the barplot (Figure 41) and table (Table 19) below:

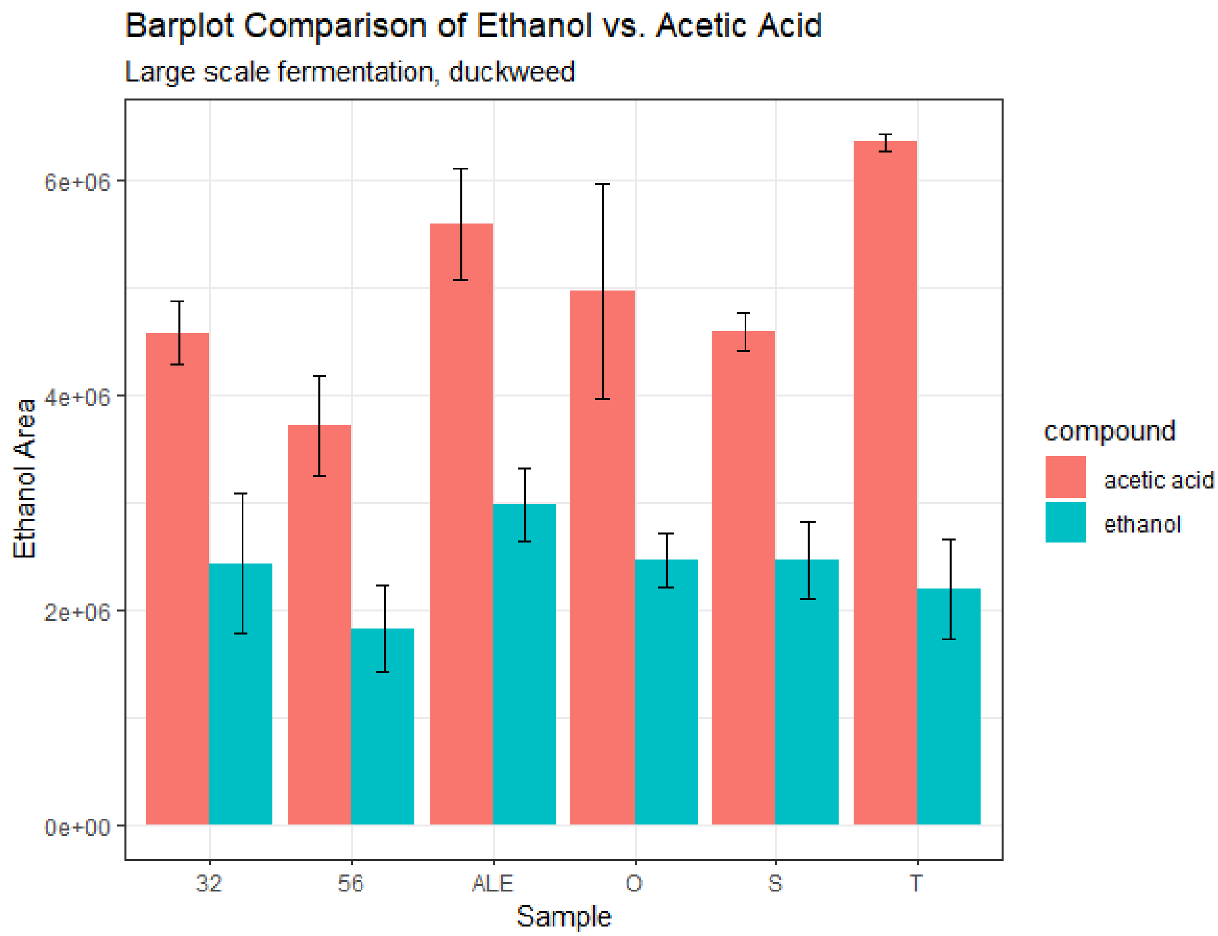

Figure 41: barplot comparison of ethanol and acetic acid areas under the curve for gen3-32, gen3-56, ALE, Oak, Sake, and Turbo yeast for large scale fermentations in duckweed hydrolysate #4.

Table 19: large scale GC-MS ethanol quantification (1:10 dilution, duckweed batch #4, 2-4 reps each)

Sample	**Ethanol peak area, average**	**Ethanol peak area, standard deviation**	**Calculated ethanol concentration (%)**	**Acetic acid peak area, average**	**Acetic acid peak area, standard deviation**
Oak	*2459282*	*548106*	*3.57*	*4963493*	*2230832*
Sake	*2458828*	*798574*	*3.57*	*4579281*	*398965*
Turbo	*2194905*	*1044686*	*3.19*	*6349471*	*177843*
ALE	*2983404*	*1083127*	*4.31*	*5579735*	*1631521*
Gen3-32	*2432252*	*1452499*	*3.53*	*4575279*	*642245*
Gen3-56	*1822401*	*1257202*	*2.66*	*3713307*	*1463920*

A tukey's significance test was performed on the ethanol and acetic acid peak areas. Resulting p-values are shown in the table 20 below:

Table 20: p-values from tukey's significance test for large- scale fermentations, duckweed hydrolysis batch #4; significant p-values are bolded.

interaction	p-value, ethanol	p-value, acetic acid
gen3-32 - ALE	*0.9406*	*0.7717*
gen3-56 - ALE	*0.2011*	*0.0515*
oak - ALE	*0.9516*	*0.9636*
sake - ALE	*0.9514*	*0.7746*
turbo - ALE	*0.7799*	*0.9107*
gen3-56 - gen3-32	*0.9115*	*0.8640*
oak - gen3-32	*1.000*	*0.9977*
sake - gen3-32	*1.000*	*1.0000*
turbo - gen3-32	*0.9993*	*0.3525*
oak - gen3-56	*0.8956*	*0.5766*
sake - gen3-56	*0.8959*	*0.8618*
turbo - gen3-56	*0.9890*	***0.0167***
sake - oak	*1.000*	*0.9978*
turbo - oak	*0.9989*	*0.6179*
turbo - sake	*0.9989*	*0.3549*

For the large scale duckweed fermentations, ethanol concentrations were low and there were no significant differences in ethanol production across the final yeast lines. The turbo yeast produced significantly more acetic acid than gen3-56.

Although ethanol concentrations were low, the hydrolysis #4 was more successful than the previous batch. Changes were made to reduce the initial incubation temperature to optimize activity of the alpha-amylase from porcine pancreas. In addition, the hydrolysate was left as a slurry (10% solids) compared to the previous batch, which was diluted, to maximize available starch. Further, the final pH of the hydrolysate was brought to 5, rather than 7, for optimal yeast growth.

That being said, duckweed fermentation samples consistently showed low ethanol concentrations and had large acetic acid peaks. This is likely due to bacterial contamination of the samples, or possibly contamination of the GC-MS column. However, acetic acid did not appear in the standard or blank chromatograms or in YPD + sucrose samples, ruling out contamination of the column. It is likely that contamination occurred during the hydrolysis step prior to fermentation. The hydrolysate was transferred to the 500mL jars and autoclaved for a 30 minute sterilization cycle. This cycle was likely insufficient to fully sterilize the larger volume of media. With the previous hydrolysis, the higher incubation temperature of 70°C during an incubation period was probably sufficient to pasteurize the media, sterilizing it during the hydrolysis. The change to a lower incubation temperature (50 °C) seemed to be sufficient for enzyme activation and starch hydrolysis but may have had the negative side effect of increasing contamination risk.

While this experiment was inconclusive in determining if ethanol production differed between lines, an interesting result was observed with acetic acid production. The domesticated line generally had higher acetic acid production and significantly higher than one of the selected lines. Acetic acid is produced by bacteria in the fermentation process. While the interaction between bacteria and yeast is complex, lower acetic acid production may indicate that the gen3-56 selected line may have a competitive advantage over bacteria compared to the domesticated yeast (turbo). This may point to another potential advantage for incorporating wild yeast into industrial fermentation processes.

3.7 Conclusions and Future Work

In this study, we developed methods to efficiently induce and isolate spores in wild yeast strains. We also developed an automated high throughput phenotyping method for quantifying yeast growth rate. This protocol can continue to be used in the lab in the future by using the automated python script developed for the growth rate phenotyping. By combining the spore induction and isolation with phenotyping, we were able to complete three cycles of selective breeding and developed two new wild type strains of yeast (gen3-32 and gen3-56). An alternative laboratory evolution was also completed in parallel (ALE).

While the framework for comparison of the final lines was meant to be the final fermentations in duckweed, there were technical issues (contamination) that prevented the hydrolysis from being a success. Limitations in time and resources did not allow for the hydrolysis to be repeated within the timeframe of the project. Despite this, there were some promising initial results:

1. In our initial small scale hydrolysis (batch #2), we yielded around 8.5% ethanol from duckweed fermentation from the original wild type parents, and past studies have also shown even higher ethanol yields from fermentation of duckweed, suggesting that duckweed fermentation does have potential to yield high ethanol
2. ***The domesticated control yeast (Turbo) outperformed the wild-type parents (Oak and Sake) in YPD media, but the wild-type parents outperformed Turbo when using duckweed hydrolysate as a feedstock. This supports our hypothesis that the wild-type***

yeast may be better able to ferment duckweed feedstock compared to domesticated varieties.

3. In YPD media, the growth rate of the first cross was around double that of the selved lines. This result is indicative of a successful cross.

With these preliminary results and full development of two new yeast strains, retesting these newly developed lines in a fresh duckweed fermentation would be a top priority for future work.

3.8 References

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