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Accelerating Fermentation in Brewer's Yeast

Safraz Bacchus
LIU Post, safraz.bacchus@my.liu.edu

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Accelerating Fermentation in Brewer's Yeast

An Honors College Thesis

By

Safraz Bacchus

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Advisor Theodore J. Brummel

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Reader Daniel Hanley

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I. *<u>Abstract</u>*

Domestication of organisms was one of the key strategies that led to the dominance of humans on the planet. *Saccharomyces cerevisiae*, brewer's yeast, was one of the first organisms to be deliberately cultivated. This organism has been used in the production of alcohol since before written history(White, 2010). When *S. cerevisiae* runs out of oxygen, it undergoes anaerobic fermentation, which generates alcohol as a byproduct. Currently, various strains of this yeast are used in the production of the multi-billion dollar beer industry.

The goal of this project is to generate a strain of yeast that has an accelerated fermentation rate without compromising the flavor profile of the resulting beer. Using conventional chemical mutagenesis, hundreds of independent strains of yeast have been developed and tested. We describe the methodology utilized to generate and characterize rapid fermentation strains and provide an approach to ultimately reduce production time in the brewing process.

II. *Introduction*

    Saccharomyces cerevisiae is the organism responsible for the generation of the alcohol content of beer. Fermentation is the rate limiting step in the brewing process and acceleration of this would benefit the ability to meet the growing demand for craft beer around the world by reducing total production time. If the rate of fermentation is genetically linked, then random mutations to the yeast's DNA should affect fermentation time. While most of these mutations are likely to be detrimental, the chance exists that some of these cells will enjoy an increased metabolic rate. The number of individuals in laboratory yeast population permits that random mutagenesis is likely to produce some viable cell with mutant DNA. Selection for the fastest fermenters from these mutants should be possible by assaying for a product of the reaction: carbon dioxide. CO_2 is a gaseous byproduct that can be captured as it is produced to quantify the rate of ethanol production(Theodorou, 1994). Mutations that benefit alcohol production hold the potential to also affect the perceptible flavor and aroma of the fermented solution. This carries crucial implications if faster fermenting yeast are to be utilized in the production of beer for consumption. If the genes for flavor and fermentation are linked then selected mutants will inevitably yield an altered flavor relative to our precursor strain. If these gene are not linked then selecting for an accelerated mutant of the same flavor as the original strain should be possible.

    The fundamental hypothesis of this experiment is that a gene on the yeast chromosome controls the rate of fermentation in our strain of yeast. Furthermore, modification of this gene has the potential to improve the metabolic rate at which the yeast performs under anaerobic conditions. Assuming modification of the yeast could accelerate the rate of fermentation, it must be possible to perform this without drastically altering the flavor. Our second hypothesis is that the gene(s) for rate of fermentation and the gene(s) for flavor are independent on the chromosome and can be

separated. While it was not quantified by this study, flavor is a critical aspect if faster fermenting mutants are to be used for brewing purposes.

III. *Background*

Brewer's yeast was among the first species to form a mutually symbiotic relationship with man. Though it was unknown that yeast were a living organism, they have been found in recipes dating back to Sumeria(White, 2010). *Saccharomyces cerevisiae* is product of thousands of years of selective pressures placed on the wild strains of yeast that grew on our crops and found its way into the brewing process. By reusing fermentation vats and selecting for the brews that tasted favorable, mankind inadvertently began to domesticate this single-celled microbe(White, 2010). The process by which these vats came to contain alcohol was thought to be mediated by the deities to which they prayed. Yeast were first observed under a microscope in 1680 by Anton van Leeuwenoek(Huxley, 1871). In 1793, Antoine-Laurent Lavoisier's studies divulged the chemical substituents that permitted alcoholic fermentation. Still, the dots between chemistry and biology had not yet been connected(Lavoiser M, 1793).

It was not until Louis Pasteur, considered the "father of microbiology", that yeast was recognized as a living microorganism (Martini, 1993). Pasteur disproved the concept of spontaneous generation in the mid-1860s, which previously considered yeast as a *byproduct* of fermentation(White, 2010). Yeast were finally recognized for the fundamental role they played in the brewing process. Pasteur further went on to explore the cause of poor flavor and spoilage in beer. He theorized that bacteria and other yeast strains were the culprit, a hypothesis that he would prove to be true . Pasteur's work brought the process of fermentation from the realm of the

whimsical into a scientific context. His work directly founded the study of brewing that has grown so rapidly in the past 200 years (White, 2010).

The discovery and understanding of yeast led to the practice of culturing pure strains yeast, free of contaminants. At the time of Pasteur, two strains of yeast were known. *Saccharomyces cerevisiae* was known as "ale yeast" or top-fermenting yeast, which had long been the common active ingredient among most brews. *S. cerevisiae* fermented best at relatively warm temperatures (White, 2010). In 1883, Carlsberg Laboratories introduced a new "lager" yeast they named *Saccharomyces carlsbergensis*. This strain is a bottom-fermenting yeast that prefers colder temperatures, an attribute that was uncommon to many bacteria and wild yeast(White, 2010). Lager beers were less likely to be victims of contaminants, thus tasted better and quickly grew in popularity. As would be expected from the low fermentation temperature, *S. carlsbergensis* took a longer time to ferment than its top-fermenting cousins. *Saccharomyces carlsbergensis* was later renamed to *Saccharomyces pastorius*(White, 2010).

Many wild yeast have since been domesticated, with many more yet to be found. Today, there are hundreds, if not thousands of strains used for brewing. Each strain is unique in its optimized growth conditions, characteristic flavor profile and metabolic rate. Despite its origins in necessity, brewing has grown to extraordinary heights in popularity today(Baker, 2018). "Craft" beer is one of the fastest growing and most competitive industries, due in large part to the technological advancements made in the past 150 years. This increase in demand has placed strong selective forces on yeast that taste favorable and brew quickly. Mass produced beers with low alcohol content are losing market share to their more potent and flavorful competitors (Baker, 2018).

In order for a brewer to enter this volatile market, it is essential to understand the science and mechanism by which fermentation occurs. The process of fermentation from a chemical perspective is relatively simple. In the presence of glucose and absence of oxygen, yeast will produce carbon dioxide and ethanol. It has been shown by the work of Karl Balling that yeast convert 46.3% of the material they digest into carbon dioxide, 48.4% into ethanol, and 5.3% into new yeast cells (White, 2010). While these calculations add up to a whole solution, it fails to account for the array of secondary metabolites that may accumulate as byproducts of the reaction. These metabolites have been shown to make up less than 1% of the compounds present in the solution, yet they are perceptible in the taste and smell of the brew (Romano, et al. 1996). There are a large number of volatile organic compounds that may form during fermentation and they vary in species and concentration to comprise the flavors we commonly know (White, 2010).

Each of these individual compounds can be associated with a characteristic taste and smell. Yeast can produce many different metabolites as they ferment. The concentration and volatile compounds produced can be affected by environmental conditions such as temperature or available starches (Romano, et al. 1996). In the context of this study, genetic changes are also likely to contribute as mutagenesis could interfere with the fermentation pathway at a number of stages. This can lead to an accumulation or depletion of a compound in one's brew. Many of these compounds can be favorable for taste while others can "spoil" your brew. These perceptible compounds include many esters, organic acids, alcohols, and phenolic compounds. In order to quantify the study of flavor, gas chromatography is an industry standard (Romano, et al. 1996). It can be used to detect differences in the concentration or species of organic compounds present, which can be quite telling as to what is occurring during your brew.

Mutation is a naturally occurring phenomenon in cellular DNA. Mutation is the mechanism by which evolution occurs by causing either beneficial or detrimental changes in the genome of an organism (Alberts B., et al. 2002). The practice of mutagenizing DNA for scientific study was first pioneered by Hermann Muller who found that high temperatures could induce mutation (Gleason K, 1926). He later utilized an x-ray machine to mutagenize fruit flies, observing chromosome rearrangements that suggested mutation to be the cause of cancer (Muller H.J., 1927). In 1947, Charlotte Auerbach and J.M Robson found that mustard gas could also cause mutations in fruit flies (Auerbach, C., et al. 1947). While yeast naturally incur mutations at a low rate, methods such as chemical and UV mutagenesis are used to increase the frequency of mutation for laboratory studies (Winston, 2008). The changes these mutagens install in the genome are somewhat predictable and chemical mutagens generally only install a single mutation per single strand of DNA. Ethyl methanesulfonate(EMS) has been shown to cause primarily GC to AT transversions, while UV mutagenesis behaves less predictably (Winston, 2008). A similar mutagen, methyl methanesulfonate(MMS) was used for this study. It is thought that MMS works by introducing double stranded breaks, which is implied by pulse field gel electrophoresis studies of mutagenized DNA (Lundin et al. 2012). The mechanism by which MMS induces these double stranded breaks has not been demonstrated, however repair of this mutation requires the ability of a cell to perform homologous recombination (Lundin et al. 2012). Mutagenic chemicals impart mutations on the chromosome randomly at chemically compatible sites. These undirected mutations permit us to affect the fermentation of the yeast without knowing which or how many genes are being affected.

IV. ***Materials/Methodology***

Mutagenesis

Random chemical mutagenesis is performed in an effort to produce mutant strains of yeast. The chemical methyl methanesulfonate (MMS) is added to the control strain of yeast, which then induces changes in the genetic material of the cells, resulting in mutants. Each mutant colony that results from the mutagenesis is now biologically different and unique. The goal of mutagenizing the control strain is to produce at least one mutant that will have the gene(s) for fermentation altered in a positive way.

To develop the mutants, the control strain of yeast is grown to approximately 200 million cells per milliliter overnight in a 5 mL YPD culture; 1 mL of that being transferred into a sterile micro-centrifuge tube (Winston, 2008). Pellet the yeast in a micro-centrifuge tube in a centrifuge and pour off the supernatant. Re-suspend the pellet in 1mL of water and repeat. Add 50 microliters of 0.2% MMS to the cells and dispersed by vortexing. The micro-centrifuge tube is placed on a rotating platform so the cells and the MMS can mix (Winston, 2008). Take 0.2 mL of the yeast/MMS mix to 8 mL of 5% sodium thiosulfate that inactivates MMS and further stops the mutagenesis (Winston, 2008). The mutagenized cells can now be plated at a 1/1000 dilution to obtain isolated colonies that can be picked.

Carbon Dioxide Assays

Carbon dioxide is produced by yeast during anaerobic fermentation as a gaseous byproduct. The strain of yeast that produces the greatest amount of CO_2 in a given period of time will also be the faster fermenter. The amount of CO_2 produced was measured using a Gas Production System. The Gas Production System (GPS) is a series of gas pressure sensors with a transmitter that is screwed on to the top of five 250 mL bottles that broadcasts information back to a computer

containing software that manages the data. The GPS tells us the temperature of the bottle and the amount of pressure released through the vent on the module and graphs it in real time.   For this study, the bottles are filled with 100 mL of yeast peptone dextrose(YPD) media. The media is then inoculated via sterile loop from a 5mL liquid YPD culture of approximately 200 million cells per milliliter. Usually one bottle contains the control strain while the other four bottles contain mutant strains. The bottles are placed in a water bath at 30 degrees Celsius to ensure optimal temperature for the yeast to grow in.

Brewing

By analyzing the data from the Gas Production System, we determine which mutant strains of yeast are faster fermenters. These strains of yeast are then grown up to at least 400 billion cells. The strain is then sent to the brewery to be used in a 30-gallon batch. This brew is taste tested and the time required to reach the desired alcohol by volume(ABV) is recorded.

Microscopy

Brewer's yeast can exhibit a number of potential ploidies, meaning the number of copies of their chromosome that they have can vary. They also have the potential to form multiple nuclei. This is important when attempting to alter the yeast genome in some way. A gene knockout would be difficult to execute if the yeast have multiple copies of a gene, stored in multiple locations. For these reasons we examine our yeast under a microscope. A simple benefit to microscopy is that it permits us to visually verify that the culture is only yeast and contains no contaminants or secondary flora. DAPI is a commonly used stain in microbiology with an affinity for the nuclei of cells. Culturing yeast with DAPI permits the uptake of the fluorescent stain into the cell, which can then be viewed with a high energy light source under a microscope. Using DAPI we can

visually observe the number of nuclei in a yeast cell and with enough magnification we can potentially even observe the number of copies of the chromosome within a nucleus.

Examining yeast cells with stain begins by starting a 2 mL YPD culture with your desired strain. Allow the culture to incubate for 4-8 hours to ensure cells are in mid-log phase and are actively dividing. Add 1uL of 5 mg/mL DAPI stain to the culture and allow it to incubate for an additional half hour. Then transfer 1 mL of the culture to a micro centrifuge tube and the remaining 1 mL to another micro centrifuge tube. Pellet the cells and pour off the YPD. Resuspend each pellet in 1 mL of PBS and then re-pellet. Discard the PBS and then add an additional 25-50 uL of PBS and resuspend. Using a glass pipet, deposit some solution onto a microscope slide and lay a coverslip over it. Use a vacuum to clean any excess and the slide is now ready to be viewed. Observe the cells under brightfield and DAPI filters at 400x and 1000x magnification. Record images of each to be overlayed for visualization.

Auxotrophic Screening for Yeast Mating

While the process of mating organisms to each other requires very little, mating cells provides a slight hurdle. The microscopic size of each cell makes it impossible to monitor and determine which cells could be the product of mating between two desired parents. The best way to overcome this is through the use of a selectable factor in conjunction with replica plating. If yeast have the potential to grow on one media but not another, then the factor preventing growth can be utilized to determine if a strain has undergone an exchange of genetic information. An event like this requires that a parent with the selectable factor exchange genetic

material with a cell missing the selectable factor. The product of these events should have genetic material from both parents and should have acquired traits from both parents. If the correct genes

are inherited the cell should regain the ability to grow on the media. Cells that fit this description should be identifiable through the use of replica plating.

The most difficult step to this process is identifying a selectable factor that can be used. Though one may be installed through the addition or knockout of a gene, it was preferable for our experiment to determine if one already exists. Each organism carries the genes to create some of the amino acids on their chromosome while others cannot be synthesized. Growth is possible without some amino acids present however some are required. The amino acids required for growth are considered "essential". If an essential amino acid cannot be made by an organism then it must find this amino acid in its diet to grow. The amino acids essential to yeast can vary by strain, therefore knowing the deficiencies of a strain has value.

If a strain is unable to make a certain amino acid, it is considered "auxotrophic" for that amino acid. Through the use of protein free ingredients, we are able to completely control all amino acids in a media. Creating medias lacking an individual amino acid and observing yeast growth allows for us to identify what the cells can and cannot make.

The first step to conducting an auxotrophic cassette requires making minimal media, containing no proteins. This media should have all components necessary for cellular growth with the exception of any amino acids. This ensures any proteins available to the yeast are limited to what we supply. The media is then supplemented with a blend of all amino acids, excluding six. The six amino acids are histidine, methionine, tryptophan, leucine, isoleucine, and valine. These are essential to many yeast strains, meaning a deficiency in the ability to synthesize of any of these should prevent growth on media lacking that amino acid. Seven groups of media must be prepared for each strain to be screened. The groups should each be

missing a single amino acid as seen in table 1 below. Note the name of the media denotes which amino acid is missing.

Group	1	2	3	4	5	6	7
Missing Amino	None	All	Met	His	Trp	Iso/Val	Leu

Table 1. Each experimental group in our study will be deficient for a single amino acid in an effort to test our strains abilities to synthesize this amino acid. Our first group will serve as a positive control by containing a blend of all amino acids. Our second group will contain none of the essential amino acids and will serve as a negative control.

Media missing only the six amino acids may be used as a base to construct the rest of the amino acid blends needed to conduct the screen. The cassette may be done on plates or in liquid culture. The rest of the missing amino acids must be added to each trial excluding the amino acid that the media will lack. Refer to table 2.

| Group	1	2	3	4	5	6	7
Amino Acids	-All	-None	-Met	-Trp	-Leu	-Iso/Val	-His
Met		x		x	x	x	x
Trp		x	x		x	x	x
Leu		x	x	x		x	x
Iso/Val		x	x	x	x		x
His		x	x	x	x	x	

Table 2. The media for each group in the auxotrophic cassette is missing a single amino acid. Growing our yeast on this cassette will allow us to determine which essential amino acids the cells are able to produce. Failure to grow will indicate an inability of the strain to produce the relevant amino acids. Group one represents our negative control which will contain none of the amino acids being considered in this trial. None of our strains should show growth in this trial. Group two will contain all amino acids and will serve as a positive control for our study. All trials should show growth in this column. The remaining groups will each be deficient for a single amino acid, revealing the characteristics performance of our strain.

Once the amino acids have been added to the media, the yeast may now be inocculated by streaking. Incubate the trials at 30C and return in two to three days to observe growth.

IV. **Results**

Carbon Dioxide Assays

Random mutagenesis of the yeast has allowed for the identification of more than eight strains that consistently ferment faster than the initial strain. More than 150 mutants have been streaked and the strains that grew the fastest were assayed. Among the mutants picked, more than 45 strains were tested. Most of the cells that underwent mutagenesis accumulated fatal mutations. Greater than 90% of cells died in the process however a portion of the cells retain the ability to grow and divide. Among these, a small fraction carry beneficial mutations for the rate of fermentation. Based on these results approximately 5% of surviving yeast are able to ferment faster as a result of mutation. This study was carried out with glass aliquots prior to the availability of the gas production system(GPS). This manual assay identified mutant B4 as fermenting 2-3 times faster and A6 as just slightly faster. When these strains were tested on the GPS the results showed that strain B4 approached four times the speed at which the control fermented. Meanwhile, strain A6 held a small lead over our parent strain at the 18-hour mark of our trial (Graph 1). This outcome was accurately predicted by our manual assays.
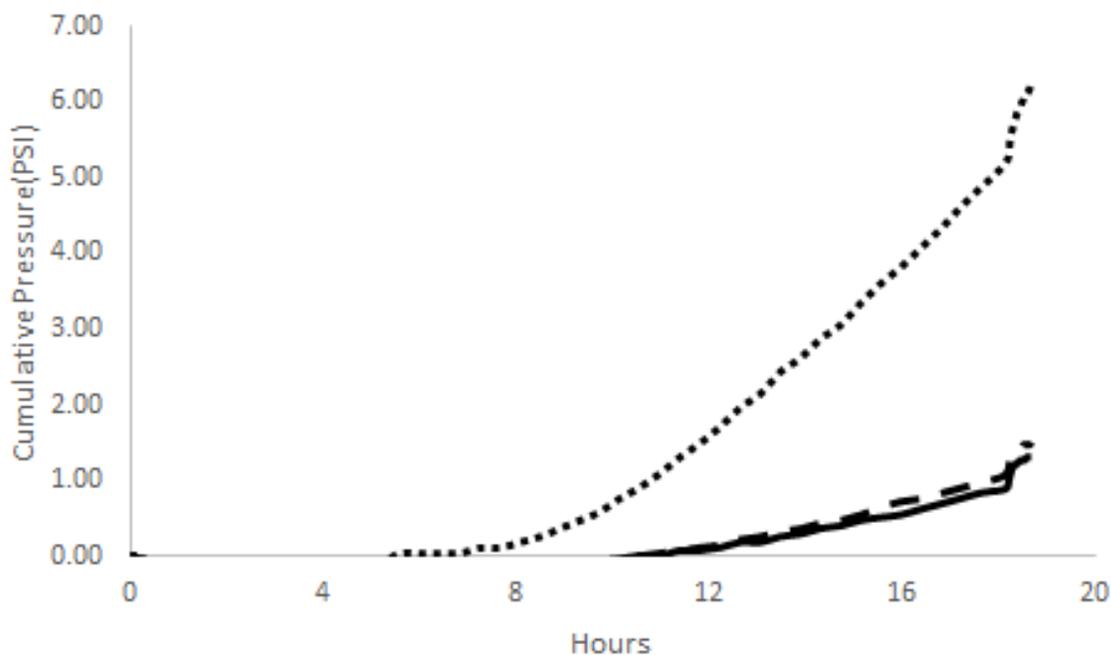
Graph 1. The control strain (represented by the solid line) fermented to about 1 PSI. Strain A6(dashed line) performed just slightly better than our control strain which is exactly the results we predicted based on our manual carbon dioxide assays. Meanwhile, strain B4(dotted line) fermented 4.8 times as fast as its parent strain. This strain was predicted to ferment at about 3 times the speed.

The uncharacterized viable yeast strains we generated were plated onto YPD and isolated into monocultures with eight mutant strains growing per plate. Sixteen plates of eight were generated and each mutant was screened. The GPS found that many strains picked were slower than the control or failed to ferment at all. This was to be expected, however it was found that some of the mutants fermented faster. As seen in Graph 2, the mutants in the trial varied in the degree to which they were faster. Mutant θ2 found the greatest success and reached almost three times the pressure of the control in the 24-hour period. Mutant θ6 was only twice as fast, however it climbed PSI aggressively early in the trial. In culture they each had slight variance in olfaction and some had minor visual differences between colonies. This likely suggests they will

each taste different from each other and certainly should differ from the strain that we began with. Graph 3 shows the results of a secondary screening of high performing mutants. Star mutants achieved some of the highest pressures observed in any variants of this strain. While this may be significant, it is significant to note that in this trial the control strain also fermented to a greater cumulative PSI than in other 24-hour trials.
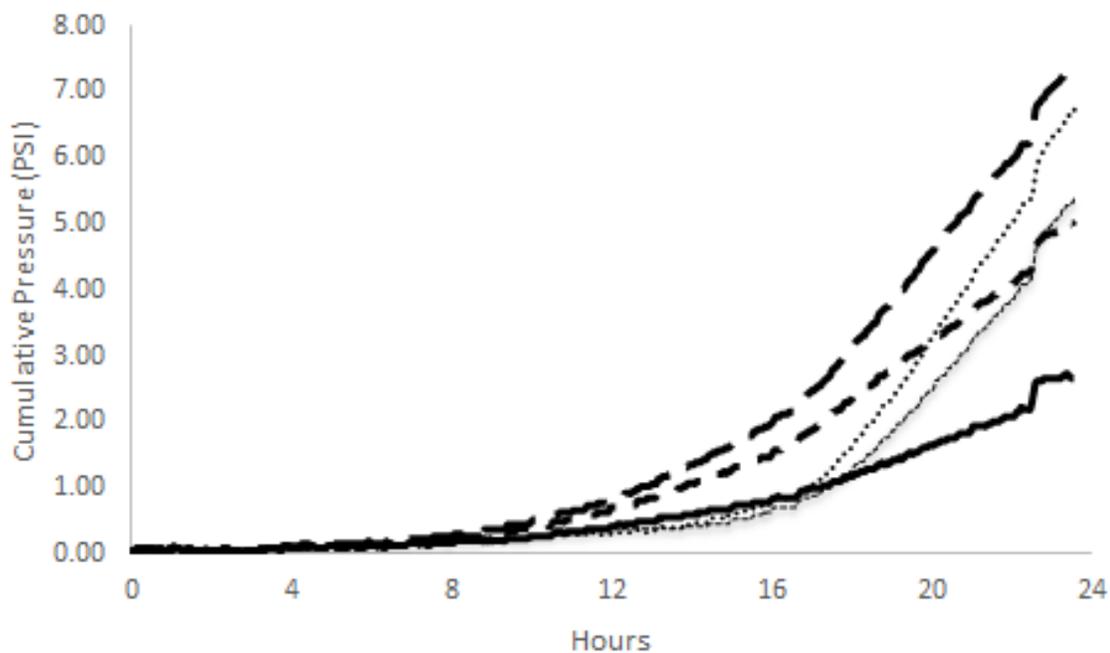
Graph 2. The parent strain of our study is represented by the solid line. Strain θ2(long dashed line) fermented to 2.8 times the cumulative PSI. Similar performance was exhibited by strain θ3(dotted line) which fermented to 2.5 times the cumulative PSI relative to the control. Strains θ4(small dashed line) and θ6(medium dashed line) performed similarly, fermenting to 2.0 and 1.8 times the cumulative pressure.
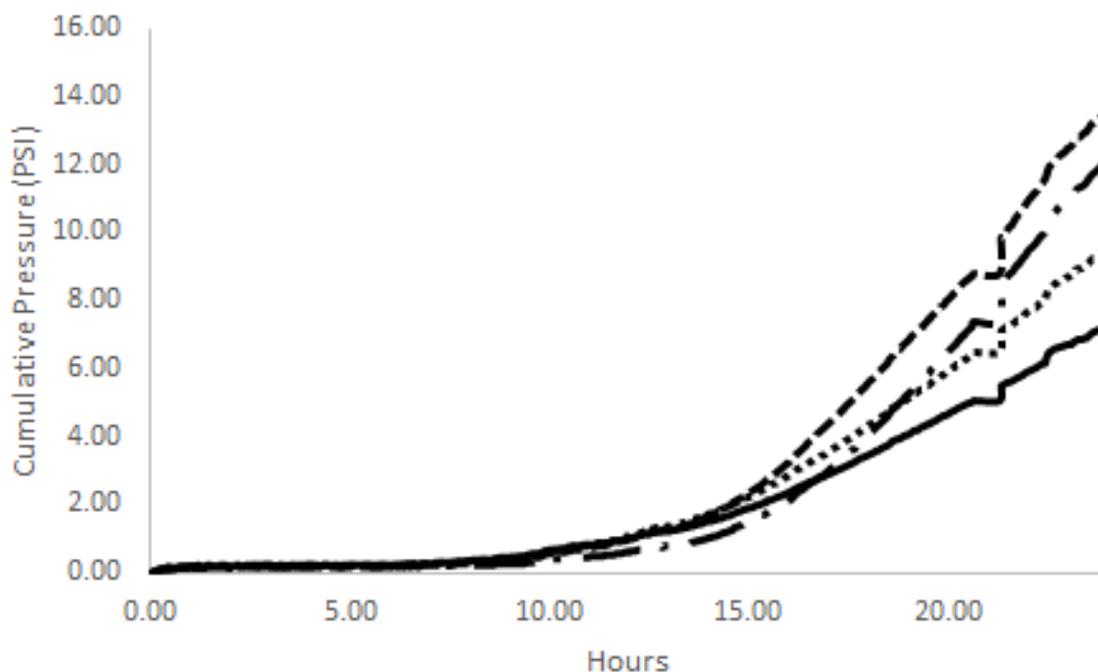
Graph 3. Strain N2(dotted line), Star 1(medium dashed line), and Star 2(dashed-dotted line) all fermented to cumulative pressures far greater than that of other mutants. The control strain (solid line) of this trial also fermented to a greater pressure than normal, making it difficult to determine if this is significant.

Auxotrophic Cassettes

Characterizing the amino acids that our strain of *S. cerevisiae* can produce allow us to mate the strains to each other. We opted to do this on agar plates initially however encountered difficulty. Upon first attempt at creating the amino acid solutions associated with each group, we failed to fully suspend the dry amino acids. The heat of an autoclave would degrade some amino acids therefore we chose to filter sterilize the solutions. As a result, we filtered out the least soluble amino acids to be added to the first few trials. The lack of growth in the positive control indicated the error. It was discovered that heating the solutions allowed all substrates to suspend

and be sterilized. A second attempt at the trial found that this time the negative control showed growth. Unsure of how to locate our fault, we switched to lab strain F729 with known auxotrophies. We hoped that the results we found would match how we expected the strain to perform. Still we found discrepancies as F729 is proven to be unable to grow on -Leu media however in our trial it still grew. Presently we believe the protocol is allowing for cross contamination of amino acids between plates as the solutions are spread on the plate for even drying. To remedy this, an identical trial can be executed in liquid culture, removing the potential for contamination by removing the step of plating the amino acid solution. These trials must be reconducted in aliquots to determine if the experimental protocol requires further troubleshooting. The predicted outcomes and design of the trials can be seen in Table 3. A trial with growth on both the positive and negative control can be seen in image 1, indicated the failure of our effort to control all amino acid substituents in the media.

Group	1	2	3	4	5	6	7
	-All	-None	-Met	-Leu	-Trp	-Iso/Val	-His
Strain							
F729	No Growth	Growth	No Growth	No Growth	Growth	Growth	No Growth
Mutant	No Growth	Growth	?	?	?	?	?

Table 3. In an effort troubleshoot inconsistencies in our auxotrophic studies, we tested a lab strain whose auxotrophic characteristics are known. Strain F729 is known to be unable to produce methionine, leucine, or histidine. If our auxotrophic media is being produced correctly, the lab strain should be unable to grow on the media deficient in these amino acids.
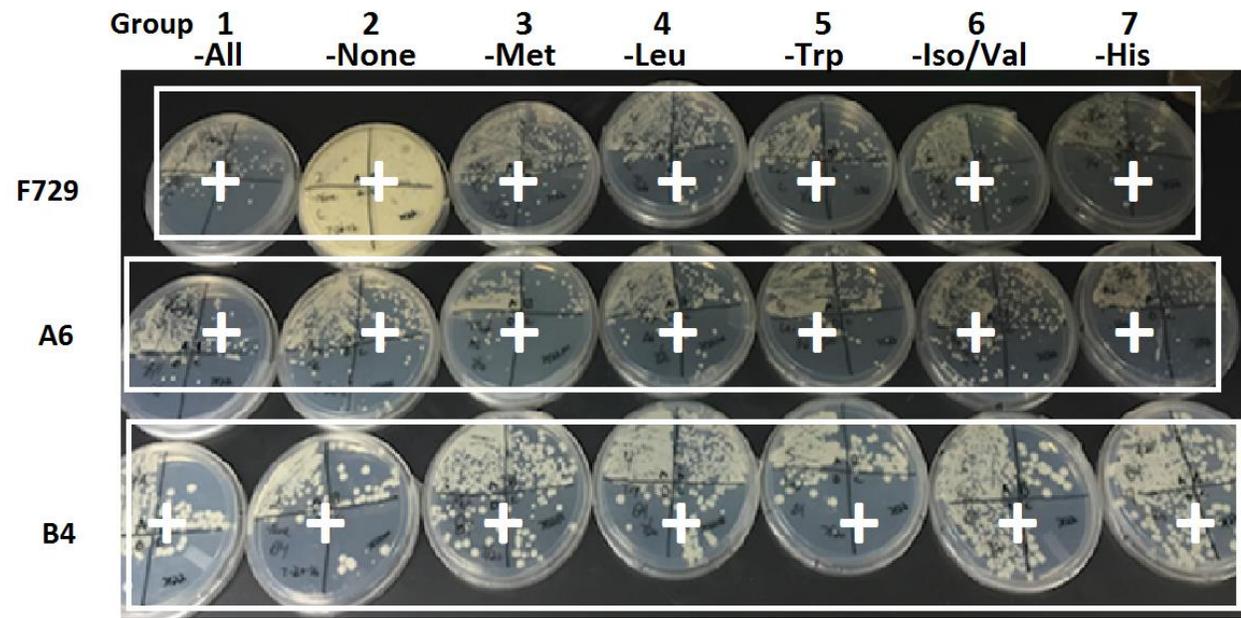
Image 1. We observed growth on all of our plates in this trial, indicating an error in our protocol. The growth in our negative control was most telling, indicating to us that our method of plating amino acids on the media was causing cross contamination between plates.

Microscopy

Development of fluorescent microscopy in this experiment has allowed for the confirmation that this microbe is in fact *Saccharomyces cerevisiae*. Some brewers utilize two or more strains of yeast in brewing while foreign or unwanted microbes like bacteria or wild fungi can sometimes contaminate cultures and alter the characteristics of yeast. Examining them under a bright field microscope has allowed for visual verification that the culture of study is a single strain. Furthermore, the use of DNA-philic DAPI stain allows for the locations of DNA in the cell to be seen. DNA resides in the nucleus of the cell therefore under high energy light and a filter the nuclei of the cells can be seen. This allows us to determine the number of nuclei in each

cell. Some strains of brewer's yeast are aneuploid, meaning they have multiple nuclei and multiple copies of each gene. This is valuable for understanding how we might be able to alter a gene through both targeted and untargeted means.

Examining our strain of yeast with stain has led us to believe that it is diploid or haploid, containing all genetic material in a single nucleus. Further examination is necessary under stronger magnification. Images 3, and 4 below show the brightfield view of the cells on the right with the same cells under a DAPI filter on the right.

<div align="center">

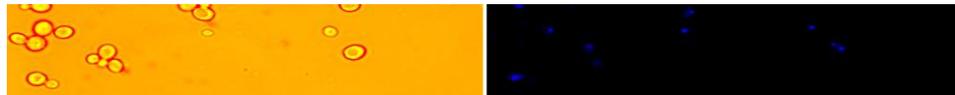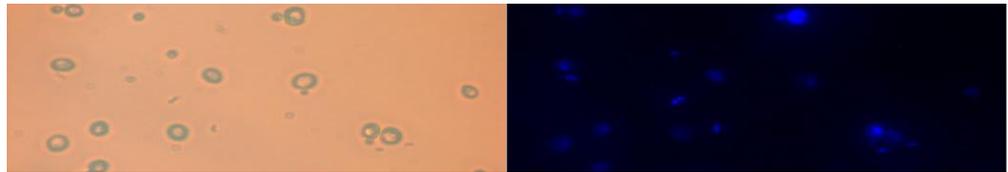<u>Bright Field</u>   <u>DAPI Stained</u>

</div>

Image 3 & 4. Yeast cells under brightfield microscope (left) and with DAPI stain (right). DAPI binds the nucleus of the cell and can be used to determine the ploidy of a culture. Though stain was not taken up by all of the cells, we are able to determine that there is approximately one nucleus in each of the cells. The nuclei can be seen on the right stained in blue. Microscopy further reveals to us that our strain is a monoculture, consisting of a single yeast strain.

V. **Discussion**

The primary objective of this experiment is to develop a faster fermenting strain of yeast without perceptibly altering the flavor the yeast contribute to the brew. We have found that the development of faster fermenting mutants is relatively simple through mutagenesis and generate a strain of yeast that ferments faster without altering the flavor they contribute to a brew. Use of these faster strains for brewing has resulted in off-flavor batches, each unique to the mutant. The flavor of each strain generated has yet to be tested however past results indicate that mutant flavor has the potential to vary from the control at an unpredictable range. This has two potential explanations. The mutation affecting rate of fermentation could also be altering the flavor produced by the yeast. Alternatively, the genes controlling flavor are separate from those controlling fermentation and chemical mutagenesis caused secondary mutations in these flavor genes. The means of mutagenesis was methyl methanesulfonate(MMS), a mutagen that methylates DNA and its bases. This means that a methyl group containing a carbon and three hydrogens is attached to available sites on the DNA. This was previously believed to directly cause double stranded DNA breaks however mechanism by which this occurs is yet unknown. Use of chemical mutagenesis forfeits the ability to limit where and how many mutations form on the chromosome without sequencing. This is an advantage if it is unknown which gene you would like to target when seeking to make a phenotypic change.

In the pursuit of understanding why the yeast had a change in flavor, it is necessary to understand what causes flavors to differ. In order for the yeast to taste differently, they must change the concentrations at which they produce the many metabolites they actively generate and cycle. This is most easily detected through smelling or tasting the yeast solution however this fails to

quantify the shift and limits the ability to identify the compound responsible for the flavor. It is for this purpose that we seek to develop gas chromatography as a means of detecting changes in flavor. Many of the compounds yeast contribute to beer include esters and aldehydes, both of which are volatile and therefore best sampled with gas chromatography over liquid chromatography.

One of the gaps in knowledge directly affecting the experiment is our understanding of how flavor and rate of fermentation are inherited and their linkage to each other. For this reason, it would be of great benefit to conduct two concurrent studies in supplement to this experiment. For the first study, unmodified strains of *Saccharomyces cerevisiae* with assorted distinct flavor should be mated to each other and the inheritance of flavor genes may be observed. The characteristics of the offspring will provide clues as to how many genes control for flavor. A second study may be ran alongside the first, this time studying the inheritance of rate of fermentation. Fast fermenting strains of unmodified yeast may be mated to slow fermenting strains and the performance of the offspring will give clues as to how fermentation is linked to the chromosome. The first requisite to this study is that a functional yeast mating protocol be developed. Secondly it will require the ability to test the yeast flavor in a cheap and time efficient manner. Additionally, a cheap and accurate assay for fermentation will be required however this has already been developed in the study.

In this experiment, the auxotrophic studies were never successfully executed. The goal of this protocol was to determine if our yeast strain is able to synthesize all of the essential amino acids we tested for. We repeatedly found inconsistent results for trial, and were unable to reproduce any results we found. With the exception of the negative control, none of our trials gave us conclusive evidence towards prototrophy or auxotrophy. In an effort to troubleshoot this, a laboratory strain of yeast with known auxotrophic characteristics was procured and tested in

parallel with our primary strain of study. Known as F729, this strain was purported to be unable to synthesize methionine, leucine, and histidine. Using a strain with a predictable outcome allows us to determine if our protocol for auxotrophic media was the source of error in our experiments. Unfortunately, the results of this experiment remained inconsistent, with some strains failing to grow on the synthetic complete media. Further, the laboratory strain failed to grow in the pattern as predicted by the information that came with it. Possible causes for this may include some form of cross contamination between amino acid mixes, insuffcient concentration of amino acids present, or some form of contamination of the laboratory stocks. Further troubleshooting of this procedure will be critical to understanding about our more about our yeast without employing advanced molecular techniques. The identification of a selectable factor suited to this yeast strain would allow for us to perform some simple tests to divulge the nature of the relationship between the flavor and fermentation of a brew. Once we have isolated a mutant that is confirmed as a faster fermenter, we may use outcrossing to determine if a faster fermenting offspring with conserved flavor occurs. This would require the introduction of yeast mating factor, artificially instigating the mating of the yeast. This is possible however without a selectable factor, it will be impossible to differentiate the offspring from the parent cells. If we can confirm that our cells are prototrophic, we could potentially use next generation molecular techniques to intentionally break this strain's ability to produce an amino acid of our choosing and install a selectable factor.

If enough is known about the functions of the genes on the yeast chromosome, we may also use more specific molecular techniques to modify the performance of our strain with targeted changes to the chromosome. The development of techniques like the CRISPR/Cas9 system in combination with high-throughput sequencing allows for expansive possibilities in these applications. For example, if we know the sequence of our parent strain and the sequence of our

accelerated mutant, identifying the difference becomes a matter of alignment and analysis. If we know the nature of our mutation, we may synthetically reproduce it using enzymes and oligonucleotides in order to study it.

This experiment carries heavy implications for the multi-billion-dollar brewing industry. Countless strains of yeast are actively utilized for the production of beers and wines, each with their own flavor and metabolic rate. New strains are developed and enter the market consistently. The ability to reduce the brewing time for popular beverages would give an edge to non-corporate craft brewers, whom must budget limited production space. The demand for craft beer has been on a consistent climb since 2012. An increase in supply would allow craft beers the opportunity to price their products more competitively and expand distribution.

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