

## **ABSTRACT**

HECKMANN, BENJAMIN DAVID. Genetic and Phenotypic Characterization of Wild Yeast Native to North Carolina. (Under the direction of Dr. John Sheppard and Dr Rodolphe Barrangou).

Yeast have a myriad of commercial applications encompassing brewing, bioenergy production, biologic processing, and pharmaceutical manufacturing. The exploration of wild yeast strains from diverse environments offers significant potential for enhancing biotechnological applications by introducing new traits that are not encoded by traditional yeast. In brewing applications these traits can materialize as novel flavor components, aromatic compounds, or attenuation of sugars. While these novel phenotypes may aid in fermentation operations, they may also lead to undesirable flavor compounds, create a poor ethanol tolerance, or otherwise render the wild strains unsuitable for fermentation.

Two strains of wild yeast were isolated from the natural environment in and around Raleigh, NC and were cultivated from environmental samples that were collected. Samples spanned diverse sources, including flowers, feathers, and berries. Microbes from the surface of samples were selectively cultivated to allow for isolation of wild yeast strains. These wild strains were characterized genotypically through PacBio whole genome sequencing (WGS), assembly, and comparative genomics. Wild type strains were tested phenotypically, specifically for aptitude in brewing applications.

The objective of this research was to characterize these wild strains through genomic analysis and phenotypic testing to determine their suitability in and potential for brewing applications. Genomic analysis led to identification of genes related to fermentation as well as comparisons with fully characterized reference strains. These comparisons indicated genetic differences that could be functionally substantiated through experiments in fermentation but

were not clearly observed. Phenotypic testing involved screening yeast for tolerance to brewing related stress, such as ethanol and hops, as well as ability to reach quality attributes relevant to beer brewing, such as maltose attenuation and ethanol production. The potential of wild yeast is vast and untapped with exciting applications across food, biofuel, and brewing industries. The unique genetic diversity and metabolic capabilities can provide novel applications for wild strains.

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Genetic and Phenotypic Characterization of Wild Yeast Native to North Carolina.

by  
Benjamin Heckmann

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## DEDICATION

To my family and friends, for allowing me to pursue this journey

## **BIOGRAPHY**

Ben Heckmann was born in Leverkusen, Germany. He completed his undergraduate education at North Carolina State University earning a Bachelor of Science degree in Bioprocessing in 2023. During his undergraduate studies, Ben Heckmann developed an interest in microbiology and genetics, which led to pursuing graduate studies in Food Science at North Carolina State University.

As a master's student, Ben conducted research on characterizing wild yeast under the supervision of Dr. John Sheppard and Dr. Rodolphe Barrangou. His work focused on combining genetic and microbiological techniques to characterize wild yeast and determine suitability for brewing.

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## TABLE OF CONTENTS

LIST OF FIGURES .....	vi
LIST OF TABLES .....	vii
Research Objectives .....	viii
<b>Chapter 1: Literature Review</b> .....	<b>1</b>
1.1 Introduction .....	1
1.2 History of Yeast .....	2
1.3 Genetics of Wild Yeast populations .....	5
1.4 Modern Brewing .....	10
1.5 Applications for Wild Yeast .....	13
References .....	17
<b>Chapter 2: Genetic Assembly and Analysis of novel yeast</b> .....	<b>24</b>
2.1 Introduction .....	24
2.2 Materials and Methods .....	25
2.3 Results .....	29
2.4 Discussion .....	36
2.5 Future Work .....	39
2.6 Conclusion .....	40
References .....	41
<b>Chapter 3: Phenotypic Characterization</b> .....	<b>43</b>
3.1 Introduction .....	43
3.2 Materials and Methods .....	44
3.3 Results .....	49
3.4 Discussion .....	63
3.5 Future Work .....	74
3.6 Conclusion .....	75
References .....	77

**LIST OF FIGURES**

Figure 1.3.1 Genetic similarities of yeast species.....	10
Figure 2.3.1 Assembly of Feather reads into Contigs.....	30
Figure 2.3.2 Assembly of Flower reads into Contigs .....	32
Figure 3.3.1 HPLC analysis of dried malt extract substrate Fermentation Profiles of Yeast Strains .....	51
Figure 3.3.2 Impact of Temperature on Alcohol by Volume (ABV) and Gravity During Fermentation of Wild and Control Yeast strains.....	53
Figure 3.3.3 Effect of Initial Sugar Concentration on Fermentation Dynamics Across Yeast Strains.....	54
Figure 3.3.4 Flocculation and sedimentation of cells .....	58
Figure 3.3.5 Ethanol Inhibition of Final Cell Count in Three Yeast Strains at 0% and 10% Ethanol.....	59
Figure 3.3.6 Effect of Ethanol on Final Gravity in Three Yeast Strains at 0% and 10% Ethanol... .....	60
Figure 3.3.7 Cedex analysis of Glucose, Ethanol, Glycerol, Pyruvate, Acetate, Lactate, and Formate during anaerobic fermentation of wild yeast.....	62

**LIST OF TABLES**

Table 2.3.1	Assembly of Feather reads into Contigs.....	30
Table 2.3.2	Assembly of Flower reads into Contigs .....	31
Table 2.3.3	Data from Contig BLAST queries.....	33
Table 2.3.4	Comparison of Gene Presence Across Wild and Control Yeast Strains .....	34
Table 3.3.1	Jmp Connecting Letter report of final specific gravity by strain and temperature ...	52
Table 3.3.2	Jmp Connecting Letter report of final alcohol by volume (ABV) by strain and starting DME concentration .....	55
Table 3.3.3	Biolog FF microplate result comparison .....	56
Table 3.3.4	Ethanol Inhibition of Final Cell Count in Three Yeast Strains at 0% and 10% Ethanol.....	60
Table 3.3.5	Connecting letter report of overall likeability.....	61

## Research Objectives

This study aims to characterize the genetic and metabolic differences between two wild yeast isolates and one domesticated strain through the lens of brewing applications.

Characterization began with comparisons of the size and structure of genomes of both wild isolates and domesticated reference strains: S288C, EC1118, and Kyokai K7. Genes relevant to brewing are also identified to compare suitability to industrial applications. Phenotypic comparisons of carbohydrate metabolism, ethanol tolerance and production, and flocculation behavior were measured as a means of differentiating wild isolates and a commercial brewing benchmark strain, *S. cerevisiae* Nottingham. Further experimentation into the effects of growth conditions, like temperature and specific gravity, elucidated similarities and differences in fermentation performance between wild isolates and a commercially available brewing yeast. These findings may contribute to a growing body of literature that suggests potential industrial applications for bioprospected strains of wild yeast, especially brewing. While these findings may be further evidence of the high levels of genetic diversity within wild yeast species that genetic diversity was not necessarily reflected in functional differences under the conditions tested.

## CHAPTER 1: Literature Review

### 1.1 Introduction

Yeast are unicellular, eukaryotic, fungi that predominantly reproduce through budding. Industrial fermentation with yeast is responsible for billions of dollars in biofuel, biopharmaceutical, and food sales annually. Yeast have played an important role in human civilization for millennia, driving the fermentation of bread, beer, and wine since ancient times. Their natural ability to convert sugars into alcohol and carbon dioxide has made them an important factor in food preservation, nutrition, and cultural traditions worldwide. Yeast also play an important role ecologically contributing to soil health, carbon cycling, and even gut health. Wild yeast refers to budding fungus that were isolated from the natural environment and non-fermentative niches; this includes both *Saccharomyces* and non-*Saccharomyces* strains, like *Torulasporea delbrueckii* and *Lachancea thermotolerans*. Wild yeast, like many microorganisms, are ubiquitous in the environment. Samples taken from sugar-rich sources like berries, low-sugar sources like soil, highly mobile sources like feathers, and completely immobile sources like tree bark can all be used to cultivate wild yeast strains of both *Saccharomyces* and non-*Saccharomyces* varieties (Deak, 2006).

Through long processes of domestication, yeast strains have evolved and been isolated, purified, and characterized. This process has caused fundamental changes in these strains of yeast leading them to be considered domesticated. These strains, usually kept for the purpose of brewing, feature an improved alcohol tolerance, maltose utilization, and temperature tolerance that lends to their ability to create consistent products and achieve certain quality attributes. Wild yeast differs from conventional domesticated strains of yeast in a few key areas. Firstly, wild yeast strains are not well characterized and can exhibit starkly different performance in

fermentation. These differences in fermentation characteristics can be of interest in optimizing bioprocesses for unique products. Wild yeast strains, typically, are associated with slower fermentations (Molinet & Cubillos, 2020). Unlike domesticated strains, the wild type yeast are not optimized for rapid utilization of specific sugars but can grow on a wide range of sugar sources. Wild yeast also produce many unknown flavor and aroma compounds during fermentation due to their metabolic diversity. Secondly, some wild strains are able to mate and sexually reproduce, an ability that domesticated strains have lost (Stelkens & Bendixsen, 2022). This function creates very high levels of genetic diversity within populations of wild yeast as these strains exhibit more complex pathways of gene transfer than domesticated strains that only exhibit vertical gene transfer. Additionally, some wild strains maintain the ability to sporulate to allow a higher resistance to environmental stresses like temperature or nutrient depletion; this ability has also been lost in many domesticated strains (Freese et al., 1982; Nikulin et al., 2020).

## **1.2 History of Yeast**

Yeast has been utilized in human processes for thousands of years. The history of brewing is wrought with examples of wild yeast being used in fermentation- from French wine to Belgian ales. Historically, fermented beverages provided a safe alternative to the contaminated water that was prevalent before the rise of modern sanitation since the acidity, ethanol, and competition of the yeast created an environment that was not conducive to the growth of spoilage organisms. Additionally, beer wort was usually boiled, killing pathogens that were present in the water. The rise of brewing systems likely coadapted with forced domestication of wild yeast. Much like many other fauna, the impact of human civilization on wild yeast is significant. However, the current brewing yeast has been domesticated beyond its ability to produce relevant

ethanol yields and has lost functionality that is present in wild strains (De Chiara et al., 2020). The introduction of hops into beer brewing also benefited the antimicrobial properties and shelf life of the beer.

Ancient and medieval brewers were reliant on climate and ambient temperatures to encourage the growth of wild microorganisms. These spontaneously fermented products were highly variable and highly susceptible to infection from souring bacteria. In fact, some infections from wild yeast would cause an equally undrinkable beverage. Spurred on by the variability in spontaneously fermented beer, brewers began using leftover “sediment” from previous batches to ferment new beer, a practice referred to as back-slopping; a direct increase in quality was observed. In 1516 the Reinheitsgebot (German Beer Purity Law) became one of the first national food safety laws and ensured that beer was made only from water, barley, and hops, since the role of yeast in fermentations was unknown at the time; this law was pre-dated by the local Munich Purity Law. Later, yeast was accepted as one of the key ingredients in the process. This legislation ensured safety, quality, and authenticity in German beer.

The birth of microbiology is closely linked to the development of microscopy in the late 17th century. In 1676, Antonie van Leeuwenhoek, using handcrafted microscopes, became the first to observe and describe bacteria and protozoa, which he referred to as "animalcules" (Kutschera, 2023). His meticulous documentation of these microscopic organisms unveiled an unseen microbial world, challenging prevailing scientific beliefs. In 1857, Pasteur helped to determine the role of yeast in fermentation, rebuking the idea that it was purely a chemical process (University of Alabama at Birmingham, n.d.). Building upon these discoveries, in the 19th century, Louis Pasteur and Robert Koch conducted foundational research that established the germ theory of disease, linking specific microorganisms to particular diseases and further

solidifying microbiology as a scientific discipline. In the late 1800's the role of yeast as a biocatalyst in these processes was elucidated, leading to efforts to cultivate and domesticate yeast strains, especially in large brewery settings. As location-specific strains were pitched, repitched, and cultivated, breweries shifted to using pure cultures of increasingly predictable individual yeast strains. In the 19th century Robert Koch and Christian Hansen, separately, began propagating yeast under laboratory conditions (Raihofer et al., 2022).

At the end of the 1800's and into the following century fermentative metabolism was unraveled, and the effects of process parameters such as temperature, oxygen, or pH were elucidated. Modern brewing methods often focus on minimizing variability and maximizing quality control of the final product. This focus requires that process parameters be highly controlled and that yeast strains function reliably. As such, most commercial brewing yeast strains are thoroughly domesticated.

*S. cerevisiae* is a unicellular organism. This renders it relatively simple to cultivate, compared to other eukaryotes, and it became a valuable system for understanding eukaryotic biology. *S. cerevisiae* also grows faster than many other eukaryotes. Additionally, *S. cerevisiae* is important for a number of industrial applications. The traits of *S. cerevisiae*, along with its industrial importance, leads to its significance as a model organism (Parapouli et al, 2020). In 1996, *Saccharomyces cerevisiae* was the first eukaryotic organism to be fully sequenced. Since then, hundreds of additional yeast strains from a variety of sources have been sequenced and annotated. *S. cerevisiae* is a model organism for studying eukaryotic cell biology, including processes like cell division, metabolism, and gene regulation, The genome encodes about 6,000 genes, with around 5,800 of them being protein-coding genes. The *Saccharomyces* Genome Database is a resource for yeast genetic and molecular biology information, including access to

genomic sequences, annotations, functional data, and genetic information. Genome editing technologies, particularly CRISPR-Cas9, have revolutionized genetic modifications in yeast and other microorganisms. This technology enables optimization of metabolic pathways, improved stress tolerance, and the synthesis of compounds with speed and accuracy (Rainha et al., 2021). Complementing genome editing advancements, artificial intelligence (AI) and machine learning have emerged as transformative forces in strain characterization and design. These technologies facilitate the analysis of large genomic datasets, predict gene-editing outcomes, and optimize fermentation conditions in real time, significantly reducing the trial-and-error nature of traditional strain development (Jervis et al., 2019). The prevalence of new technologies, from CRISPR-Cas9 to AI, present unrivaled opportunities for strain characterization and engineering.

### **1.3 Genetics of Wild Yeast populations**

The distribution of wild yeast is affected by a number of intrinsic and extrinsic factors, relating both to the species of wild yeast as well as environmental factors. Sugar sources and water activity are both important factors in the variety of wild strains and species that one may find in a given environment. Yeast, like many other microorganisms, may require specific sugars to grow as well as an ideal range of water activity. Environmental factors such as temperature, pH, and ambient moisture are also important. The species *S. paradoxus* isolation is typically associated with a European summer temperature, with highest chances of isolation at intermediate temperatures (Robinson et al., 2016). Latitude is an important factor in determining the types of wild yeast that are most prevalent in the environment due to its impact on temperature and weather conditions. A strong correlation between trunk girth and *S. paradoxus*

prevalence was also established when collecting bark samples of oak trees, indicating that older, and larger, trees carry more yeast.

The genetic content of yeast strains can be influenced by their environment whether in a natural environment or laboratory setting. Yeast populations are susceptible to adaptive evolution in response to environmental stimuli such as temperature, light/solar radiation, pH, nutrient availability, and the presence of toxins (Deak, 2006). Genetic variants that confer survival advantages under specific conditions are favored and more likely to reproduce under those conditions, increasing genetic diversity within the population and shifting the genetics of a population to become more suited to their environment (Robinson et al., 2016). In laboratory settings, yeast are subjected to similar selective pressures as in natural environments, albeit often under experimental control, and can cause gradual changes in the genetics of a yeast population, known as directed evolution or adaptive lab evolution (Sniegowski, 2001). This practice yields similar, albeit more controlled, effects on the population of yeast to back-slopping. The selection of yeast by humans encouraged the chosen strains to develop characteristics to increase their utility; leading to significant, heritable, genetic changes: and the domestication of the species. This process can reveal insights into the mechanisms of genetic diversity and the evolution of specific traits.

Wild yeast populations exhibit a high level of diversity since their genomes are susceptible to many modes of alterations that are often not applicable to domesticated strains. Wild yeast are subject to sexual reproduction, horizontal gene transfer, mutations, rearrangement, and hybridization while domesticated laboratory strains have highly conserved genomes that experience much less alteration since they do not readily sexually reproduce (Fukuda, 2020). The genetic diversity of wild yeast is a reflection of the diverse environments

and ecological niches that they occupy since the genetics are shaped by the environmental pressures of their habitats. Additionally, since domesticated yeast, especially brewing strains, are often stored in pure cultures via modern preservation techniques there are fewer opportunities for gene transfer to occur than in wild populations. The domestication of yeast leads to a specialized and simplified genome and populations that excel in specific brewing conditions. The specialization and simplification of domesticated strains is a stark contrast to the genetic and functional diversity of their wild counterparts. However, there are cases of domesticated strains forming hybrids with other strains, much like the *Saccharomyces cerevisiae* × *Saccharomyces eubayanus* hybrid yeast that is responsible for lager beer production. The hybrid nature of the aforementioned strain allows for improved temperature tolerance, maltotriose utilization, and the formation of sensory compounds that create a product that could not be mimicked by either of the non-hybrid yeast strains. The hybridization of yeast provides both opportunities for the creation of new yeast species as well as key insights into the evolutionary history of yeast and invokes the broad spectrum of genetic diversity within yeast populations.

Sexual reproduction is a key process undergone in eukaryotic systems. Sexual reproduction involves the exchange and combination of genetic material from two genetically different parent cells to create offspring that contain a combination of genetic material from both parents. While many domestic yeast strains have lost this ability, it is maintained in wild populations (Krogerus et al., 2016). This sharing and transfer of genetic information creates genetic diversity within populations as DNA is modified and passed on.

Horizontal gene transfer is the act of transferring genetic material between two nearby cells. While this is very common in prokaryotes it is less common in eukaryotic systems. However, this is evidence of this occurring in yeast. *FSY1*, a gene coding for a fructose

transporter in *Saccharomyces cerevisiae* strain EC1118 and other wine strains is evidence of horizontal gene transfer (Galeote et al., 2010). Passage of genetic information between strains that share proximity cultivates genetic diversity within local populations and allows for new gene functions to be adopted (Tapia et al., 2023).

Mutations are an important factor in driving evolution, adaptation, and diversity within a population. The rate at which mutations naturally occur as well as the susceptibility to mutations caused by environmental stresses both contribute to genetic changes. Despite the importance of mutations, estimating a mutation rate can be difficult due to lack of uniformity in mutation rates throughout the genome (Lang & Murray, 2008). However, if mutations are not favorable for survival so the mutants, often, they do not persist.

During cell division, it is crucial for cells to maintain stability in their genome to ensure that daughter cells continue to grow and divide without detrimental mutations. Yeast genomes are susceptible to a myriad of potential rearrangements, and structural changes in the chromosomes, including inversions, translocations, duplications, deletions, and even changes in ploidy. These changes all lead to increases in genetic diversity within populations as well as lead to increased speciation.

During mitotic replication, both crossover (CO) and non-crossover (NCO) rearrangements can occur during the division and unification of the chromosome. While both events represent rearrangements it is only the CO that results in phenotypic variation (Schwarzkopf et al., 2024). The rate of both CO and NCO events varies by organism, strain, and even location on the chromosome with certain locations deemed as CO hotspots (Srivatsan et al., 2018). These hotspots lead to the hotspot paradox as the locations are prone to self-destruction.

Wild strains have much higher rates of CO events while maintaining a similar amount of NCO events (Liu et al., 2018).

Unlike domesticated strains, wild populations of yeast maintain the ability to mate. As a result of this, hybrid yeast species are prevalent in natural environments. Wild populations are subject to sexual reproduction, mutations, transposons, changes in ploidy, horizontal gene transfer, and recombination events (between homologous and nonhomologous loci). All of these mechanisms contribute to the vast genetic diversity of wild populations. The ability to mate, as well as reproduce asexually, provides a unique opportunity for yeast to cultivate substantial biodiversity. Mating of yeast strains follow three tracts: cell-to-cell, spore-to-cell, and spore-to-spore mating. The practicality of any of these approaches depends on the sexual cycle of the potential strains.

Hybridizing yeast strains provides opportunities similar to selective breeding in agricultural settings. Hybrid strains can gain the ability to tolerate higher ethanol levels, improve fermentation rates, produce a wide variety of flavor compounds, or a reduction in unwanted byproducts like diacetyl. Hybridization of yeast strains allows for the combination of multiple phenotypes of interest.

The variety of means of propagating genetic diversity in populations of wild yeast leads to both genetic diversity as well as genetic similarities. *S. pastorianus*, *S. cerevisiae*, and *S. bayanus* are considered domesticated species (Libkind et al., 2011). *S. paradoxus* is commonly considered a wild strain, having been isolated from tree bark. However, *S. cerevisiae* is over 50% similar to *S. paradoxus* and *S. pastorianus* while *S. pastorianus* is 72% similar to *S. bayanus* and 23% similar to *S. paradoxus* (Oda and Ouchie, 2004). The long history of hybridization and genetic diversity within yeast species, especially *Saccharomyces*, blurs the lines that divide

species and strains, creating a complex evolutionary landscape that requires genomic sciences to make clearer (Gabaldón, 2020; Casaregola, Weiss, and Morel, 2011)). Through natural and human-driven processes, yeast species have exchanged genetic material, resulting in hybrids that exhibit traits from multiple parental lineages. Consequently, defining clear taxonomic boundaries becomes challenging, as many strains share overlapping phenotypic and genetic characteristics, reflecting a continuum rather than distinct categories (Peris et al, 2023).

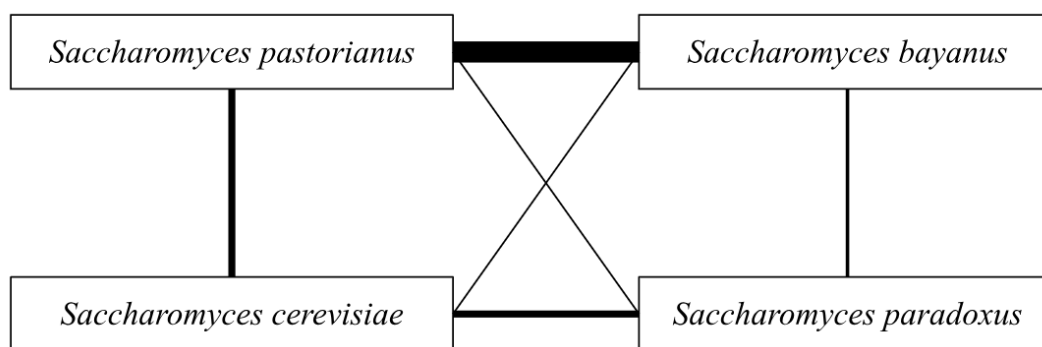


Figure 1.3.1. Genetic similarities of yeast species. Line thickness is proportional to percent similarity. Adapted from Encyclopedia of Food Microbiology (Oda and Ouchie, 2004)

#### 1.4 Modern Brewing

As aforementioned, modern brewing is usually based on the use of domesticated yeast strains. Within the framework of domesticated commercial brewing yeast there is still some variability. The two main species of domesticated yeasts used for brewing are *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* (Araujo Piraine et al., 2021). These two species are both domesticated for beer production but provide different fermentation attributes.

*Saccharomyces cerevisiae*, primarily Top-fermenting yeasts, are used in ale production. They ferment at warmer temperatures, typically between 15°C and 24°C (59°F to 75°F), and rise to the

surface during fermentation, forming a thick, frothy layer. This yeast imparts fruity and complex flavors, making it ideal for creating ales with rich aromatic profiles. In contrast, bottom-fermenting yeasts, mainly *Saccharomyces pastorianus*, are used for lager fermentation. These yeasts work best at cooler temperatures, around 7°C to 13°C (45°F to 55°F), and settle at the bottom of the fermentation vessel. They produce cleaner, crisper flavors with fewer fruity esters, resulting in the characteristic taste of lagers (Stratford and Carter, 1997).

These domesticated species contain many individual strains that can be deployed when brewing specific styles. Effective strain collection and preservation are necessary to ensure yeast strains, and resulting beer styles, be maintained for long term storage. Cryopreservation and freeze drying both represent gold standards for long term banking of yeast strains, while storing yeast in liquid cultures at 4 °C is an effective method for short term storage in laboratory settings.

Even while utilizing domesticated strains of *Saccharomyces pastorianus* and *Saccharomyces cerevisiae* brewers are able to introduce variety into modern beer fermentations by altering process conditions, recipe, and fermentation techniques. Fermentation conditions, including temperature and pitching rate, play crucial roles in determining the outcome of the fermentation process. Adjusting these parameters can alter the production of flavor compounds (Gibson et al, 2017). For example, higher fermentation temperatures can increase the production of esters, leading to more pronounced fruity flavors, while lower temperatures may result in cleaner profiles. Changes to the recipe such as altering the mash bill (quantity and/or variety of grains) or adding fruits can also impact the final product and can change the profile of fermentable sugars. Finally, fermentation techniques like spontaneous fermentation or co-cultured fermentation allow for diverse products. These methods involve multiple yeast species

and bacteria, contributing to complex flavor profiles and increased microbial diversity in the final product. Sour beers, for instance, are often the result of fermentation with brewing yeast as well as lactic acid bacteria to create unique aroma, flavor, and mouthfeel.

Currently, wild yeasts are used for some novel experimental batches as the variability in flavor and aroma likely discourages large breweries from brewing with uncharacterized wild yeast that could create phenols or other flavor compounds that could render the product undrinkable. Despite the risk, some brewers are now interested in using wild strains of yeast found in the environment for fermentation because of the different flavor and aroma profiles that the wild yeast can produce. Using local wild yeast can also be a unique selling point to differentiate a product from others on the market.

While wild yeast strains may provide novel flavor profiles, they often exhibit reduced fermentation performance by measurable standards like sugar utilization, notably maltose. Maltose transporter gene MAL31, maltase genes MAL12 and MAL32, and a MALx1 and MALx2 are duplicated in most domesticated strains, sometimes two-fold greater than in wild strains (Duan et al, 2018). The effect of this genetic difference is stark. The growth rate of domestic yeast in maltose media is often significantly higher than that of most wild isolates, with some isolates lacking the ability to utilize the maltose. Wild yeast isolated from fruits and milk show significantly higher maltose utilization rates compared to many other wild isolates, but still a slightly slower average rate than domesticated strains (Duan et al, 2018). While this may cause some strains of wild yeast to be ineffective in brewing, others can at least partially ferment grain-based worts.

Advances in sequencing technologies allow for the capability to analyze yeast genetics. Whole-genome sequencing (WGS) enables the identification of genes linked to industrially

relevant traits, while transcriptomics reveals gene expression patterns under different conditions. These technologies allow scientists and brewers to parse the fundamental differences between strains that manifest into flavor, aroma, and fermentation profile. Understanding the genomes of specific strains can, in theory, allow brewers and scientists alike to optimize fermentation performance, capitalize on unique strain attributes, and craft new products. Genetic insight into these strains also allows for opportunities to genetically engineer strains with superior performance metrics, as well as the removal of unwanted byproducts (Jung et al, 2023). Applications of the CRISPR-Cas9 system range from simple gene knockouts to complex modifications, such as integrating artificial pathways and regulating transcription (Rainha et al 2021).

With the rise of new technologies, the understanding of genetics has become increasingly significant. The convergence of genome modification technologies, like CRISPR, artificial intelligence, and synthetic biology has unlocked new opportunities for precise efficient modifications of genetic material that can lead to unprecedented advancements in various fields (Ansori et al, 2023). In yeast research, CRISPR techniques have been honed to allow for more efficient edits and an expansion of the toolkit which scientists can deploy (Zhao, Zhang, & Nielsen, 2022). AI is also playing a growing role in genetic research. AI is instrumental in predicting outcomes of CRISPR edits and allows for rapid annotation of novel genomes (Zhao, Zhang, & Nielsen, 2023). The increased speed of annotation fundamentally changes the ability of researchers to compare genes and predict functionality. Advancements in synthetic biology complements these technologies and expands the potential applications of yeast in industry. Collectively, new technologies are reshaping the way microorganisms are studied and paves the way for innovative solutions across multiple industries.

## 1.5 Applications for Wild Yeast

The genetic biodiversity that exists within populations of wild yeast creates the opportunity for bioprospecting of new strains to fit industrial niches. For example, hearty wild yeast may exhibit enhanced tolerance to extreme temperatures, pH, or osmotic pressure. All these traits would render the wild strain effective in conditions that model strains would traditionally struggle in; yeasts that thrive at high temperatures can have more effective fermentations and reduce cooling costs in bioethanol production (Steensels et al., 2014).

Numerous strains of wild yeast have the potential to perform well in industry applications such as use in the biomass, biofuel, baking, or brewing applications. Some studies have investigated wild yeast and how they respond to fermentation in brewing (Araujo Piraine et al., 2021). Often wild yeast is used in conjunction with traditional strains to provide both novel flavors and aromas while also reaching target ethanol content since wild yeasts are not always thought to be optimal for achieving all quality attributes in brewing (Molinet & Cubillos, 2020). While the shortcomings of non-conventional yeast in some industries make them an undesirable alternative to domesticated strains, some of the unique aspects of wild strains lend well toward other industries. Higher stress tolerance, for instance, lends well toward use in biofuel production using; where pretreated biomass has high concentrations of inhibitory compounds (Radecka et al., 2015).

Bioprospecting wild *Saccharomyces* and non-*Saccharomyces* strains provides potential opportunities in a variety of areas, from brewing to pharmaceuticals to bioethanol production. Whole genome sequencing methods allow scientists to rapidly gather genotypic information, these technologies allow for high throughput and rapid genetic analysis. Since wild yeast is

ubiquitous in the environment, rapid sequencing technologies alleviate a bottleneck to new strain discovery and characterization.

Strains of wild yeast recovered from bioethanol facilities exhibit highly beneficial traits in bioethanol production. *M. caribbica* MJTm3 displays tolerance to high heat (45 C), low pH (2), high sugar content (50%), and high ethanol content (18%). These traits all allow for better ethanol production than some laboratory strains. Whole genome sequencing could allow for better insight into the genetic basis for these traits, which in turn could allow for further optimization of both this strain and other microbes that could be modified to express *M. caribbica* MJTm3 genes (Hawaz et al., 2022).

Additionally, due to the wide range of substrates that can be consumed by wild yeast utilizing mixed cultures is an area of future research. In fact, the use of yeast for bioremediation of heavy metals has seen potential as yeast cells are able to remove dangerous metal ions through multiple mechanisms of action (Perera et al., 2023), including biosorption, bioaccumulation, and bioreduction. Additionally, yeast cells are able to release acids that can interact with metal ions and result in insoluble complexes, which pose a smaller risk to humans (García-Béjar et al., 2020). Yeast strains (*Kluyveromyces marxianus* CMGBP16 (P1), *Saccharomyces cerevisiae* S228C (P2), *Saccharomyces cerevisiae* CM6B70 (P3), *Saccharomyces cerevisiae* CMGB234 (P4), and *Pichia anomala* CMGB88) also show the ability to be used for bioremediation of wastewater. In addition to remediating heavy metals they maintain a capability to process other ions (COD,  $\text{NO}_3^-$ ,  $\text{NO}_2$ ,  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$ ,  $\text{Pb}_2^+$ ,  $\text{Cd}_2^+$ ) with removal efficiency of 70-93% of COD, nitrate, nitrite, phosphate, and sulfate (Nicula et al., 2023).

Wild yeast, like many microorganisms, can be used in a number of applications, from fermentation to bioremediation. The genetic diversity of wild yeast is so stark that further

research is necessary to bioprospect new strains that may be suited for specific industrial applications as well as to elucidate the ideal process conditions to allow for wild yeast to thrive in biomanufacturing, biofuel, fermentation, and bioremediation situations. Genetic engineering technologies and a growing understanding of the role of genetics will play a critical role in helping to tailor wild yeast for improved functionality in industrial applications. CRISPR-Cas9 and other genome editing tools allow precise modifications to overcome some of the limitations in wild strains, inefficient sugar utilization or by-product formation. Engineering of strains can also improve synthesis of high value compounds like biofuels, aroma compounds, or pharmaceuticals. Advances in synthetic biology also allows for opportunities to create hybrid strains that combine the robust characteristics of *S. cerevisiae* with favorable attributes from wild strains. Additionally, exploring microbial interactions in mixed fermentations could also yield novel insights and innovative applications for wild yeast strains. Future work will be crucial in harnessing the full potential of wild yeast strains for diverse applications across food, energy, and pharmaceuticals.

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## CHAPTER 2: Genetic Assembly and Analysis of novel yeast

### 2.1 Introduction

Yeast have been man's industrial partner for millennia playing a crucial role in brewing, baking, and pharmaceutical production. This long history of utilization has led to the domestication of certain strains, particularly *Saccharomyces cerevisiae*, resulting in significant genetic changes to enhance fermentation efficiency, flavor profile, and stress tolerance. These genetic changes represent the environments where commercial yeast are used, and the selective pressures imposed upon them. Wild yeast are a diverse group of microorganisms that are ubiquitous in the natural environment and can be isolated from sources ranging from flora to fauna. Their diverse habits influence populations to maintain diversity and unique phenotypic traits to allow wild strains to thrive in unique and varied ecological niches. Their diversity also makes wild yeast valuable when studying environmental pressures, evolutionary adaptations, and potential novel traits revealing untapped metabolic pathways that may be useful in brewing, biofuel production, and pharmaceuticals.

Advancements in genetic analysis technologies have revolutionized the study of microorganisms. Next-generation sequencing (NGS) allows for rapid, high resolution genomic data and detailed investigation of genetic variation within species, strains, and populations. Additionally, long-read sequencing technologies, such as PacBio, improve the accuracy in which researchers can assemble complex genomes. These technologies, deployed alongside genome assembly and annotation tools, pave the way for deeper understanding of fundamental processes.

Genome annotation also plays a critical role in understanding genetic and phenotypic diversity within wild yeast. Accurate genome annotations identify coding sequences, non-coding RNAs, and regulatory elements enabling the characterization of genomic data. These annotations

can be fundamental for comparative genomics highlighting gene conservation and adaptation. Comparisons between wild and model yeast can illuminate unique genes, pathways, and traits that contribute to survival in diverse ecological niches and potential industrial novelty.

Understanding the genetic landscape of wild yeast is essential for uncovering their potential in various industrial applications and gaining insights into their evolutionary adaptability. This chapter focuses on the assembly and analysis of the wild yeast genome, leveraging long-read sequencing technologies to produce high-quality contigs. Comparative genomic techniques, including BLAST analysis and gene annotation, are employed to identify key genetic features and their divergence from model yeast species such as *Saccharomyces cerevisiae*. Analysis will be conducted on both wild yeast isolates, hereby referred to as Feather and Flower based on their source of isolation.

## **2.2 Materials and Methods**

### **2.2.1 Yeast Collection**

Prior to the beginning of this project, nine samples were collected from natural environments in Raleigh, NC. Sample sources included rocks, feathers, and flowers. Samples (whole rocks, flowers, feathers, etc.) were initially cultured in YPD broth held within 500 mL Erlenmeyer flasks incubated at 25 °C and shaken at 100 rpm for 72 hours. The cultures were then streaked onto YPD agar plates containing chloramphenicol to inhibit bacterial growth. Each sample was applied to a single plate. Individual colonies grown on YPD agar plates containing chloramphenicol were selected and streaked onto YPD agar plates to isolate pure cultures of yeast. Once pure cultures were isolated, they were observed under a microscope using slides for the cell counter to determine whether the yeast cells were budding. The selected budding yeast

were identified by conducting rRNA (16S/ITS rRNA) sequencing through an external company (Genewiz). The data from the yeast isolates was then entered into the BLAST system allowing for a comparison between the identified strains and previously documented strains.

### 2.2.2 DNA Extraction

To begin this project DNA extraction was completed using the Zymo Research YeaStar Genomic DNA Kit (catalogue number d2002)

Extraction began by centrifuging 1-1.5 ml of cells briefly or at 500 g for 2 minutes, then supernatant was completely removed. 120  $\mu$ l of YD Digestion Buffer and 5  $\mu$ l of R-Zymolyase<sup>TM</sup>1 (RNase A + Zymolyase<sup>TM</sup>) was added to the pellet, and it was resuspended by vortexing, and incubated at 37°C for 40-60 minutes. Next, 120  $\mu$ l of YD Lysis Buffer was incorporated and mixed by gently vortexing. It was recommended to vortex vigorously for 10-20 seconds after adding the YD Lysis Buffer to enhance DNA recovery, although this may result in shorter genomic DNA fragments (20-35 kb), with most DNA remaining above 35 kb. The mixture was centrifuged at >10,000 rpm for 2 minutes and the supernatant was loaded onto the Zymo-Spin<sup>TM</sup> III Column, then centrifuged again at >10,000 rpm for 1 minute. To wash, 300  $\mu$ l of DNA Wash Buffer was added and tubes were centrifuged for 1 minute at  $\geq$ 10,000 rpm, the wash was repeated with another 300  $\mu$ l of DNA Wash Buffer and another minute of centrifugation. Finally, the Zymo-Spin<sup>TM</sup> III Column was transferred to a new 1.5 ml centrifuge tube, with the addition of 60  $\mu$ l of water or TE directly onto the membrane. After one minute, the tube was centrifuged for 10 seconds to elute the DNA.

### 2.2.3 Nanodrop DNA Quantification

After the pedestal was cleaned with DI water a small amount (2  $\mu$ L) of water was added to blank the system. After measurement, the pedestal was cleaned with Kimwipe. 2  $\mu$ L of sample was added to the pedestal for measurement. The concentration of DNA, A260/A280, and A260/A230 were recorded.

### 2.2.4 Agarose Electrophoresis Gel

Agarose Gel was constructed with 0.8 g agarose fully dissolved in 100 mL of Tris Acetic acid EDTA (TAE) buffer and cast with a comb. DNA samples were mixed with ethidium bromide to a final concentration of 1x EtBr. Samples, as well as a DNA ladder, were loaded into the wells of the gel. The gel was submerged in a TAE buffer and runs between 80-120V until the dye has migrated two-thirds down the gel. Once run, the gel was removed from the buffer and visualized using transillumination to confirm that isolated DNA is high quality.

### 2.2.5 DNA sequencing

Isolated DNA was sent to the NC State Genomic Sciences Laboratory for Pacbio sequencing. <https://research.ncsu.edu/gsl/>

### 2.2.6 Assembly

PacBio reads were assembled into contigs using the Flye plugin in Geneious Prime (version 2024.0.7). Flye is a *de novo* assembler designed for long-read sequencing data, making it well-suited for high-error-rate reads typical of PacBio platforms. Default parameters were used for assembly, which included error correction and contig polishing steps to improve sequence

accuracy. After assembly, contigs were visually inspected within Geneious for structural integrity and compared against reference sequences for validation.

### 2.2.7 Alignments with Mauve

To generate Mauve alignments, Geneious Prime (2024.0.7) (Biomatters Ltd.) was used. DNA sequences were imported into the software in FASTA format. Each sequence was carefully checked for quality, ensuring no gaps or ambiguities that could interfere with alignment. Mauve alignments were created using the "Align with Mauve" plugin, selecting the progressive alignment algorithm. Default parameters were employed unless otherwise specified, including the setting to preserve collinear blocks and allow rearrangements. For visual clarity, local collinear blocks (LCBs) were color-coded and scaled according to their relative sequence similarity. The final alignments were exported in [specific format, e.g., XMFA] for further comparative genomic analysis and visualization.

### 2.2.8 Annotations

Wild yeast genomes were annotated using the Yeast Genome Annotation Pipeline from the Wolfe Laboratory, University College Dublin. <http://wolfe.ucd.ie/annotation/>

### 2.2.9 Genomic analysis

To identify chromosomal matches between wild yeast genomic contigs and *Saccharomyces cerevisiae*, portions of assembled contigs ranging in size from 50,000 to 100,000 base pairs were selected for BLAST analysis. The sequences were extracted and formatted in FASTA files using Geneious Prime (version 2024.0). Each contig was queried using the NCBI

BLASTn algorithm with default parameters. High-confidence matches were determined based on sequence identity percentages ( $\geq 90\%$ ) and alignment coverage across the length of the contig. Chromosome assignments for matching regions were recorded along with associated statistical metrics, including bit scores and E-values. Results were further validated by visual inspection of alignments to ensure biological relevance.

### **2.3 Results**

During the initial phase of characterization, it was important to isolate DNA from both wild yeast strains. Isolated DNA could then be used for NGS and assembly. Obtaining genetic data relating to the tested wild strains allowed for insight into the genotypes of the wild yeast as well as allowed for observed characteristics to be compared to underlying genes. DNA extraction, electrophoresis gel was used to ensure quality of isolated DNA. Gel was used to ensure integrity of isolated DNA before sequencing.

Table 2.3.1: Assembly of Feather reads into Contigs. Figure contains statistics relating to quality of reads, length of reads and Contigs assembled in Geneious 2024.0 using Flye.

Nucleotide Statistics		Nucleotide Statistics	
Length (mean)	6812 bp	Length (mean)	541,134 bp
Sequences	1,132,778	Sequences	21
Mean	3811.5	Mean	541134.2
Std Dev	3496.5	Std Dev	380494.2
Confidence Mean	85.4	Freq	%
Expected Errors	7,874,474	A: 3,505,825	30.9
At least Q20	99.2%	C: 2,175,230	19.1
At least Q30	98.1%	G: 2,175,230	19.1
At least Q40	96.3%	T: 3,505,825	30.9

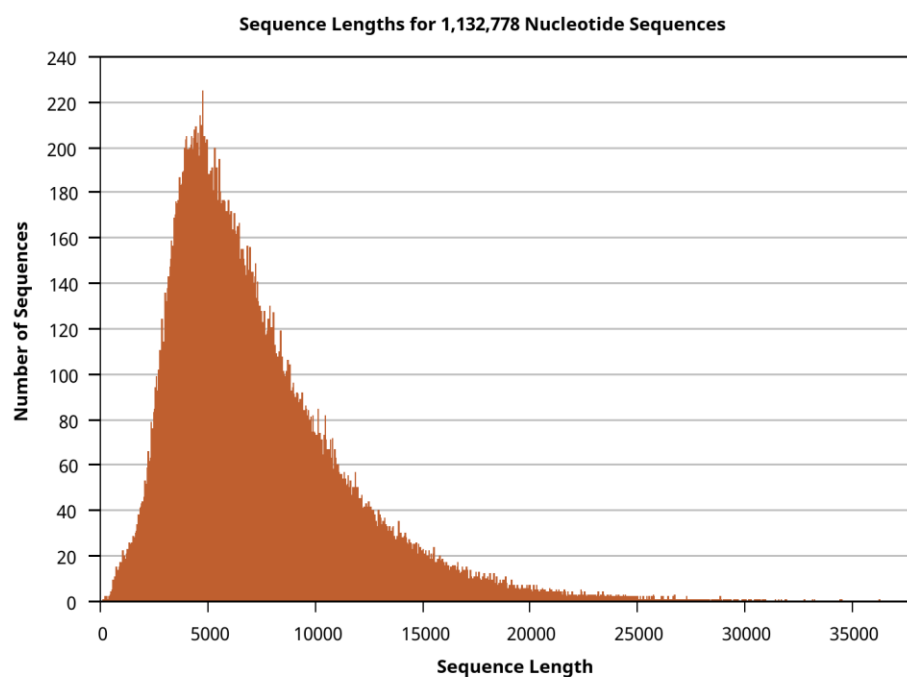


Figure 2.3.1: Assembly of Feather reads into Contigs. Figure contains statistics relating to quality of reads, length of reads and Contigs assembled in Geneious 2024.0 using Flye.

A lower base call accuracy of 99% (Q20) will have an incorrect base call probability of 1 in 100, meaning that every 100 bp sequencing read will likely contain an error.

Table 2.3.2: Assembly of Flower reads into Contigs. Figure contains statistics relating to quality of reads, length of reads and Contigs assembled in Geneious 2024.0 using Flye.

Nucleotide Statistics			Nucleotide Statistics	
Length (mean)	6867 bp		Length (mean)	673,979 bp
Sequences	1,120,652		Sequences	12
Mean	6866.7		Mean	673979.0
Std Dev	3575.5		Std Dev	378009.2
Confidence Mean	85.5		Freq	%
Expected Errors	7,605,746		A: 2,502,836	30.9
At least Q20	99.2%		C: 1,548,840	19.2
At least Q30	98.1%		G: 1,540,472	19.0
At least Q40	96.4%		T: 2,495,600	30.9

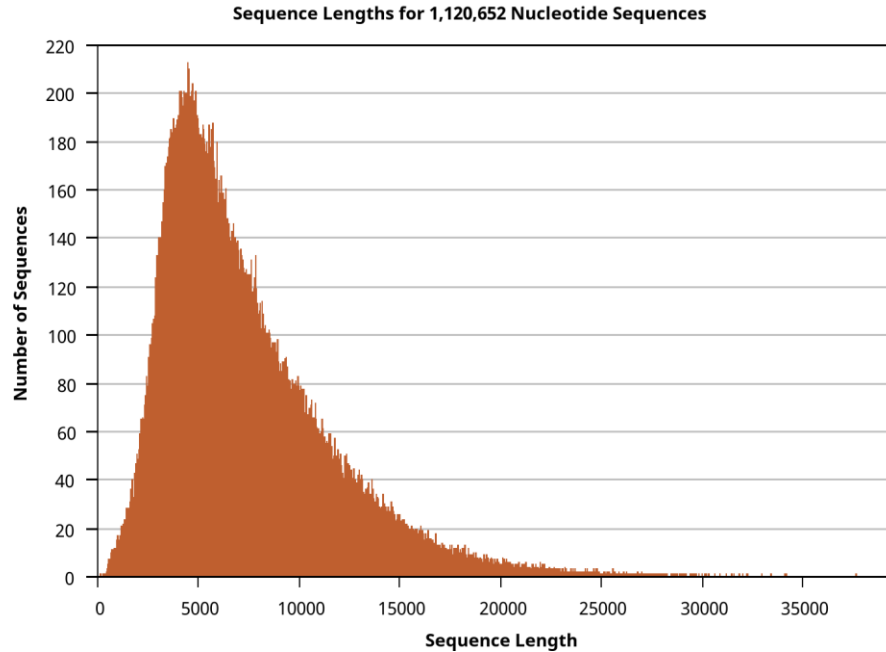


Figure 2.3.2: Assembly of Flower reads into Contigs. Figure contains statistics relating to quality of reads, length of reads and Contigs assembled in Geneious 2024.0 using Flye. A lower base call accuracy of 99% (Q20) will have an incorrect base call probability of 1 in 100, meaning that every 100 bp sequencing read will likely contain an error.

Table 2.3.3: Data from Contig BLAST queries. Data compares assembled contigs to *S. cerevisiae* chromosomes of best fit.

Chromosome #	# of Matching Contigs (Feather)	# of Matching Contigs (Flower)
1	0	0
2	1	0
3	1	1
4	1	1
5	1	1
6	1	1
7	1	1
8	1	1
9	1	0
10	1	1
11	1	1
12	4	2
13	1	0
14	3	0
15	1	1
16	0	1

Once DNA was sequenced, a number of key brewing genes were identified. Key genes included alcohol dehydrogenases, flocculation genes, heat shock genes, and many others. Genes were identified in both wild yeast strains and to reference strains, s288c, and EC1118: a wine yeast. The presence of genes provides insight into suitability of wild yeast strains for brewing applications. Absences of genes was used to steer future assays and elucidate novel characteristics of wild yeast strains.

Table 2.3.4: Comparison of Gene Presence Across Wild and Control Yeast Strains. The chart highlights genetic differences between two wild yeast strains, S288C, and EC1118. ✓

indicates the presence of a gene, while X indicates its absence, showcasing variability in genetic composition across strains.

Gene	Function	FW	FT	s288c	EC1118
CHA1	Catabolic L-serine (L-threonine) deaminase; catalyzes the degradation of both L-serine and L-threonine; required to use serine or threonine as the sole nitrogen source,	✓	✓	✓	✓
ILV (1, 3, 5, 6)	Isoleucine Biosynthesis	✓	✓	✓	✓
PDC	pyruvate decarboxylase	✓	✓	✓	✓
THI3	activates thiamine biosynthesis transcription factors	✓	✓	✓	✓
ADH	Alcohol Dehydrogenase	✓	✓	✓	✓
SFA1	Bifunctional alcohol dehydrogenase and formaldehyde dehydrogenase	✓	✓	✓	✓
BAT (1, 2 paralogs)	preferentially involved in BCAA biosynthesis	✓	✓	✓	✓
ARO10	Phenylpyruvate decarboxylase; catalyzes decarboxylation of phenylpyruvate to phenylacetaldehyde	✓	✓	✓	✓
LEU (1, 2, 4, 9 paralogs)	Alpha-isopropylmalate synthase (2-isopropylmalate synthase); the main isozyme responsible for the first step in the leucine biosynthesis pathway	✓	✓	✓	✓
IRA1	GTPase-activating protein; negatively regulates RAS by converting it from GTP- to the GDP-bound inactive form, required for reducing cAMP levels under nutrient limiting conditions	✓	✓	✓	✓
EHT	Octanoyl-CoA: ethanol acyltransferase; also functions as thioesterase; plays a minor role in medium-chain fatty acid	✓	✓	✓	✓
MDH	Malate dehydrogenase	✓	✓	✓	✓
MPH3	Alpha-glucoside permease; transports maltose, maltotriose, alpha-methylglucoside, and turanose	✓	X	✓	X
MPH2	Alpha-glucoside permease; transports maltose, maltotriose, alpha-methylglucoside, and turanose	X	✓	✓	✓

Table 2.3.4: (Continued)

GDH	NADP(+)-dependent glutamate dehydrogenase	✓	✓	✓	✓
GLO1	Monomeric glyoxalase I; catalyzes the detoxification of methylglyoxal (a by-product of glycolysis)	X	✓	✓	✓
MSN2	Stress-responsive transcriptional activator; activated in stochastic pulses of nuclear localization in response to various stress conditions	✓	✓	✓	✓
HSP12	Heat Shock Protein	✓	✓	✓	✓
PMA1	Plasma membrane P2-type H <sup>+</sup> -ATPase; pumps protons out of cell; major regulator of cytoplasmic pH and plasma membrane potential	✓	✓	✓	✓
FLO 1	Flocculation	X	X	✓	✓
FLO 5	Flocculation	X	X	✓	✓
FLO 8	Flocculation	✓	✓	✓	✓
FLO 11	Flocculation	X	X	✓	X
TPS 1	Regulatory subunit of trehalose-6-phosphate synthase/phosphatase	X	✓	✓	✓
TPS 2	Regulatory subunit of trehalose-6-phosphate synthase/phosphatase	✓	✓	✓	✓
HXK	Hexokinase isoenzyme 1; a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism	✓	✓	✓	✓
imd2	Inosine monophosphate dehydrogenase; catalyzes the rate-limiting step in GTP biosynthesis,	✓	X	✓	✓
Pho12	One of three repressible acid phosphatases;	X	X	✓	✓
COS12	Endosomal protein involved in turnover of plasma membrane proteins	X	X	✓	X
Ipp1	Cytoplasmic inorganic pyrophosphatase (PPase)	✓	X	✓	✓
Matalpha2	Homeobox-domain protein; with Mcm1p	X	X	✓	X
ENA 1, 5, 2	ATPase sodium pumps	✓	X	✓	X
NUM1	Protein required for nuclear migration; component of the mitochondria-ER-cortex-anchor (MECA)	✓	X	✓	✓
AAD15	Putative aryl-alcohol dehydrogenase; similar to P. chrysosporium aryl-alcohol dehydrogenase;	X	✓	✓	X
CUP 1, 2	Copper resistance	X	✓	✓	✓
SNO3	Expression is induced before the diauxic shift	X	✓	✓	✓

## 2.4 Discussion

### 2.4.1 DNA isolation

Ensuring quality of isolated DNA was an important quality control step to ensure successful genome sequencing. Agarose gel electrophoresis was used to determine if the isolated DNA was of sufficiently high molecular weight for sequencing. Nucleotide statistics in Table 2.3.1 and 2.3.2 provided similar data into the quality of reads and assembly. Q20 data indicates the probability of an incorrect base per 100 bases read. For both genomes this value is over 99%, indicating less than 1% error rate, and high quality reads.

### 2.4.2 Differences in genomic content

Isolation and annotation of DNA from both tested wild yeast strains allowed for analysis of a number of genes and comparisons between conventional *S. cerevisiae* strains to be made. *S. cerevisiae* S288C genome is 12.15 million bases, a common size for *S. cerevisiae* genomes, while Feather and Flower wild yeast genomes were 11.36 and 8.08 million bases, respectively. This discrepancy in size could be due to issues in isolation, assembly, or due to fundamental differences in the species. It may also explain the absence of genes from the wild strains. However, smaller genomes are not typical of wild strains; and analysis of 1,011 isolates did not find wild genomes to be significantly smaller (Peter et al., 2018). Many *S. cerevisiae* genomes are around 12,000,000 bases, so it is likely that some content is missing from the assemblies. The tested wild strains had many brewing relevant genes. These, along with phenotypic data, indicate that these wild strains are capable of thriving in media, fermenting maltose, producing alcohol, and creating palatable final products. Although domesticated yeast are subject to stark genetic

changes, this is consistent with the idea that domestication may cause more changes in transcriptional regulation than genomic alterations (Siddiq and Wittkopp, 2022). However, some notable genetic differences were identified. FLO 1, 5, 8, and 11 are important genes for flocculation. Other than FLO 8 they are absent in both wild type yeast, as seen in Table 2.3.4. While this distinction is manifested in an observed trend of decreased flocculation speed the difference is not statistically significant and cells were still capable of sedimentation in a similar time course, as seen in Figure 3.3.4. Despite this difference, many genes that are important for brewing are present in both wild strains. This ranges from maltose utilization, to flavor compound creation, to heat shock. While differences are present between the tested wild strains and the reference genome, the similarity is more extensive and significant when assessing genes relevant for beer brewing.

Maltose utilization is also an important attribute in brewing yeast. Many wild strains lack this ability, and domesticated strains often exhibit duplicated or upregulated maltose transporter genes (Baker & Hittinger, 2019). In contrast, wild strains are typically associated with worse maltose utilization, since maltose is not prevalent in natural habitats. Variations in the MAL gene cluster are largely responsible for this distinction (Gallone et al, 2016). While there were observed differences in maltose utilization, tested wild strains were able to utilize the substrate, albeit at a decreased efficiency to their domestic counterparts, as seen in Figure 3.3.1.

Comparing contigs of wild genomes to chromosomes of the model *S. cerevisiae* allowed for comparisons in genomic content. In Table 2.3.3, it is shown that some chromosomes had multiple matching contigs. For instance, chromosome 12 of *S. cerevisiae* matches to four assembled contigs from the Feather wild yeast strain genome and two from the Flower wild yeast strain genome while chromosome 1 does not match any contigs. This may provide insight into

the cause of the wild genomes being smaller than the reference genome- missing chromosomes. While this is not definitive evidence of aneuploidy it does indicate genetic flexibility within populations and species of wild yeast, especially since it is theorized that wild strains have a higher tolerance for aneuploidy (Hose et al, 2020). However, since wild yeast have a higher tolerance for the loss of specific genes and chromosomes further work could identify what content was missing in the tested wild strains (Hose et al, 2024). This genetic flexibility is important because, despite the large difference in genetic content, there were more minute differences in observed sugar utilization, alcohol production, and growth characteristics of the strains alluding to the robust nature of wild yeast and the murky landscape of wild yeast genetics.

In addition to S288C, wild genomes were also compared to Kyokai K7 (a sake yeast isolated from Nagano Prefecture, Japan) and EC1118 (a yeast strain used in wine) in instances in which wild genome contigs aligned poorly to S288C. In these instances, Kyokai K7 or EC1118 were seen to be better matches, although EC1118 has a complex lineage (Novo et al, 2009). Similarities with Asian yeast strains allude to potential common ancestry as well as the importance of Asian yeast in the evolutionary history of the wild yeast (Duan et al, 2018). The contrast between wild strains and S288C was explored as a potential source of novelty in wild strains. These alignments may suggest ancestry or gene transfer events between different clades of brewing yeast. It may also suggest potential fermentation traits from flavor compounds to sugar utilization that aren't present in typical wild yeast. Finally, this may allude to a higher level of homogeneity and mixing in yeast populations than previously expected. Wild yeast populations are thought to be genetically clustered to continents, with Asian strains being more similar to one another than to European strains (Gayevskiy & Goddard, 2012). If wild yeast in southeastern United States contains high degrees of genetic similarities to EC1118 (a diploid

French wine strain of *Saccharomyces cerevisiae* with high degrees of dissimilarity from S288C due to gene transfer events), Kyokai K7 (a Japanese sake strain of *Saccharomyces cerevisiae*), and the model strain of *Saccharomyces cerevisiae* there must be high levels of similarity between many wild and domesticated yeast strains, and high levels of gene transfer between these populations as well as within populations of wild yeast (Novo et al, 2009). Despite some genetic differences and differences in genome size no major phenotypic differences were observed that would indicate the absence of significant genes relevant to brewing nor differences that would indicate the tested wild strains are superior to commercial counterparts.

## **2.5 Future work**

While genomic information provides valuable insight into the capability and function of wild yeast it does not indicate the differential expression of the genes in various environments. Insight into expression and RNA would paint a fuller picture of how wild yeast strains function in brewing media, in laboratory environments, and through propagation. Additionally, further genetic analysis could illuminate the basis for flavor and aroma compounds. Novel flavor and aroma differences would provide an inherent value to wild yeast in brewing context and would allow for creation of novel products that cannot be readily mimicked with domesticated yeast.

Future work could also be done into hybridizing and engineering wild strains for superior performance. Addition of genes could be used to improve alcohol production, sugar utilization, or flocculation. The removal of genes could have similarly potent effects on fermentation. Creating hybrid strains with wild yeast could also allow researchers to capitalize on the novel effects of wild yeast while maintaining robust and precise fermentations. Preliminary genetic characterization of these wild isolates provides some valuable insight into their functionality,

however, further work would be necessary to ensure an accurate and complete genome assembly. Finally, further work could be done to parse the lineage of these tested wild yeast strains as well as their place in the evolutionary history of the species. Extensive phylogenetic analysis could paint a fuller picture of the genetic landscape of wild yeast, domesticated yeast, and the similarities therein.

## **2.6 Conclusion**

Genome sequencing and annotation offered valuable insights into the genetic relationships between wild and domesticated yeast strains. Although notable differences exist, the genetic similarities are striking and both highlight evolutionary parallels as well as reflect fundamental functional similarities. The wild yeast strains analyzed in this study show a high degree of similarity to *S. cerevisiae* S288C, the widely used laboratory reference strain, as well as industrial strains such as Kyokai K7, a prominent sake yeast, and EC1118, a renowned wine yeast. These similarities suggest a complex and interconnected evolutionary history, where genetic material appears to have been exchanged or conserved through diverse mechanisms, including hybridization, horizontal gene transfer, and selective environmental pressures. This obscures clear genetic delineation between wild and domesticated yeast populations, challenging traditional notions of yeast classification and emphasizing the fluidity of their evolutionary paths.

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## CHAPTER 3: Phenotypic Characterization

### 3.1 Introduction

Wild yeast are naturally occurring microorganisms that are ubiquitous in the environment. The diverse habitats they thrive in reflect genetic and phenotypic variation within populations and species of wild yeast. Domesticated strains, on the other hand, have been adapted to specific stresses of their industrial application. These adaptations lead to commercial domesticated strains exhibiting predictable fermentation performance and characteristics. Conversely, wild yeast strains exhibit diversity in their growth rates, metabolic pathways, stress tolerance, and fermentation characteristics. Studying these phenotypes is essential to understanding the adaptive mechanisms in yeast populations as well as discovering strains with desirable traits for industrial applications.

Phenotypic analysis elucidates the fundamental link between the genetic diversity of yeast populations and the functional expression of these differences. By examining the growth, fermentation performance, stress tolerance, and characteristics of wild yeast, researchers can link genetic variation to observable phenotypes. Additionally, the phenotypic diversity can help paint the picture of how yeast adapt and thrive in diverse wild environments.

This chapter will explore various methods of analyzing phenotypes of wild yeast. High-performance liquid chromatography (HPLC) is utilized to monitor the concentration of growth media components, allowing insight into metabolic activity and specific sugar utilization patterns. Fermentation experiments with varied temperatures or sugar concentrations help to assess stress tolerance, alcohol production, flavor compounds, and other attributes relevant to brewing fermentation. Flocculation assays indicate aggregation and sedimentation behavior of yeast, relevant properties in brewing and bioprocessing applications. Similarly, testing of ethanol

tolerance provides insight into how wild strains cope with this specific stress. Finally, Cedex Bio analysis helps to quantify metabolic byproducts, offering a glimpse into the complex metabolic processes and pathways involved in fermentation.

This chapter seeks to investigate the phenotypic diversity of wild yeast and to systematically compare specific wild and domesticated strains. By utilizing fermentation experiments and advanced analytical techniques the research aims to uncover correlations between genetic and phenotypic diversity and to elucidate the functional variability and similarities between wild and cultivated strains. Finally, this chapter will highlight the potential of wild yeast for industrial fermentation applications.

## **3.2 Materials and Methods**

### **3.2.1 Media Preparation (Liquid and Plate)**

Liquid media was prepared using dried malt extract (DME) and deionized water (DI H<sub>2</sub>O). First, DME was weighed and added to a clean flask. DI H<sub>2</sub>O was then added. Control medium was made with 14% DME by weight. The mixture was placed on a magnetic stir plate to mix before being allotted into Erlenmeyer flasks. The flasks were sterilized through a 20-minute autoclave cycle at 121 °C.

To produce YPD media for agar plating, in a clean beaker yeast extract (10g/L), peptone (20g/L), glucose (20g/L), and agar powder (15g/L) were added to water. The mixture was stirred and heated until all components were fully dissolved. The mixture was then added to the Erlenmeyer flask(s) and autoclaved for sterilization for 30 minutes at 121 °C. After removing from the autoclave, flasks were placed in a biosafety cabinet to cool to approximately 50-55°C.

A thin layer was poured into petri dishes ensuring the bottom was covered and it was free from air bubbles. Plates were allowed to cool in the biosafety cabinet until solid. Plates were refrigerated at 4°C until use.

### 3.2.2 Aerobic Growth Parameters, Propagation, and seed expansion

Seed flasks, which contain a smaller volume than experimental flasks, were inoculated from plate cultures. Seed flasks were placed in an incubator at 26 °C and 100 rpm. After 24-48 hours of growth, cell counts were taken, and a measured portion was used to inoculate experimental flasks to obtain a specific cell count,  $10^6$  cells/mL.

### 3.2.3 Growth Media Sample Collection

Experimental flasks were carefully placed in a biosafety cabinet. 10 mL samples were aseptically collected at discrete time points throughout fermentation and transferred to 15 mL conical tubes for future analysis.

### 3.2.4 Specific Gravity and Alcohol Analyses

The beverage samples to be analyzed were prepared by ensuring they were free from any visible particles or bubbles that could interfere with the measurement. This was done first by centrifuging, using Fisher Scientific Model 225 Centrifuge at speed setting 7, 10 mL conical tubes containing samples for 5 minutes, samples were then transferred to a beaker and placed in a vacuum to be degassed for 5 to 10 minutes. Using a syringe, approximately 10 mL of the prepared beverage sample was transferred into the sample chamber of the Anton Paar density meter and alcoholizer, ensuring the chamber was free from air bubbles. The instrument utilizes

oscillating U-tube technology to measure the density of the sample while the AlcoLyzer measures alcohol content by Near Infra-red absorption. Both values were recorded.

### 3.2.5 Carbohydrate Analyses

#### Sample Storage

Samples for future analysis of maltose and glucose by HPLC were collected during fermentation trials and centrifuged for 5 minutes at moderate to high speed. 1 mL of supernatant was then pipetted into a 1.5 mL microcentrifuge tube, labeled, and frozen (-18 °C).

#### Autosampler Loading

Frozen HPLC samples were allowed to thaw at ambient temperature. Samples were filtered through 0.22 µM filters and transferred to glass vials. 1.2 mL Glass vials were capped and loaded into the HPLC autosampler wheel.

#### HPLC parameters

After loading the autosampler, the pump (Waters 1525 Binary) was set to operate at a flow rate of 0.5 mL/min for 30 minutes, with a 1-minute buffer between samples to minimize carryover. The mobile phase used was 0.5 mM sulfuric acid, running through a Bio-Rad Aminex HPX-87H column. Concentrations of maltose and glucose were measured using a Waters 2414 Refractive Index Detector, with both the column and detector maintained at 40°C. Standard curves for each compound were developed for accurate comparison.

#### Data Collection and Analysis

The HPLC was run with a set of standards for maltose and glucose at known concentrations (5-200 g/L). HPLC data from the standards was used to create a standard curve

comparing peak areas and known concentration. This standard curve was used to calculate the concentration of glucose and maltose in experimental samples.

### 3.2.6 Cell Count

Samples were collected from flasks of growth medium. Flasks were heavily agitated to ensure even mixing of yeast cells. A small amount, 5 mL, was taken using a sterile pipette and allocated to a smaller flask. If necessary, this sample may have been diluted with deionized water 2, 5, or 10x. 20  $\mu$ L was then added to a clean well in a Cellometer SD100 cell counting chamber ensuring the chamber was evenly filled with no air bubbles. The chamber was then inserted into the Nexcelom cellometer vision automated cell counter. The Cellometer utilizes bright-field microscopy to identify and quantify the number of cells in 6 separate viewing fields and then calculates the cell concentration in the sample based on the dilution factor (number per mL).

### 3.2.7 Mid-Scale Fermentation

For mid-scale fermentation trials, 6 liters of simple “wort” was prepared with 12% DME (w/w) and DI water. Components were heated and stirred rapidly until dissolved. Tettnanger Hop pellets were added to a concentration of 8 g/L. Media was sterilized by autoclave using a 45 minute cycle at 121 °C to allow for isomerization of hop compounds. 10 L stainless steel conical fermenters were also sterilized by autoclave using a 30 minute cycle at 121 °C. Sterile media was then added to the fermenters and inoculation was performed to a starting cell count of  $10^6$  per mL. Vessels were allowed to ferment at ambient temperature (21 °C) for 7 days before being moved to a cold room (2 °C) for 7 more days. “Beer” was siphoned to separate product from yeast sediment.

### 3.2.8 Brewing Wort Preparation

Mashing was performed using a Sabco Brew Magic system, with 10 kg of Epiphany Modern Pilsner barley malt combined with approximately 40 liters of water. The mash was held for 40 minutes at 66 °C and was subsequently boiled for 30 minutes with the addition of 56 grams of Solange hops. The resulting wort was cooled and density was measured at 12.5 °Plato. After boiling, the wort was transferred into sterilized containers under aseptic conditions in preparation for fermentation. Inoculation was performed to a starting cell count of  $10^6$  per mL. Vessels were allowed to ferment at ambient temperature (21 °C) for 7 days before being moved to a cold room (2 °C) for 7 more days.

### 3.2.9 Sensory Evaluation

“Beer” from mid-scale fermentation was chilled at refrigerated temperature prior to sensory evaluation. Samples were allocated into plastic cups labelled A–E. Participants were provided each sample blind to its contents. Attributes (Sweetness, Bitterness, Floralness, Fruitiness, Earthiness, and Overall Likeability) were rated from 0-5; with 5 being the highest score. Sensory evaluation was repeated using beer made from brewing wort, with each evaluation including samples brewed with both tested wild yeast isolates and Nottingham *S. cerevisiae*.

### 3.2.10 Flocculation Assay

Yeast cells were grown aerobically until stationary phase and harvested. Flocculation was measured using a spectrophotometer at 600 nm to assess changes in optical density (OD). A

blank was made from spent media after centrifugation removes solid debris. Media with cells were added to a cuvette to measure  $t=0$  OD value. 0.5 mL of 100mM  $\text{CaCl}_2$  was added to each sample to induce flocculation. OD was measured and recorded at distinct time intervals to determine the extent of flocculation.

### 3.2.11 Cedex Analysis

10 mL daily samples collected from anaerobic growth media with each wild strain (14% DME + 8 g/L Tettnanger hops) were collected and centrifuged. 1 mL of supernatant was then collected in microcentrifuge tubes and frozen for later analysis. Samples, once thawed, were loaded into Roche Cedex for analysis of Glucose, Ethanol, Glycerol, Pyruvate, Acetate, Lactate, and Formate.

### 3.2.12 Biolog FF (Filamentous Fungus) Microplate

Overnight seed cultures of each wild strain and Nottingham *S. cerevisiae* were grown, and optical density measurements (600 nm) were taken with spent media as a blank. Samples were diluted with sterile water to .3 OD. 100 microliters were added to each well and plates were incubated at  $\sim 27$  °C. Plates were visually inspected at 24 and 48 hours and color changes (beginning opaque to turning pink) were recorded.

## 3.3 Results

### 3.3.1 Effect of Temperature on Fermentation

In analyzing the effect of temperature on fermentation performance, cells were inoculated into 14% (w/w) DME media. Both tested wild strains as well as Nottingham control strain were

tested in triplicate at 21, 26, 31, and 36 °C, although only 21-, 26-, and 31-degree samples were included in HPLC quantification of glucose and maltose attenuation. Daily samples were taken for HPLC and/or Anton Paar analysis. HPLC analysis indicated that both wild strains were capable of readily utilizing maltose, albeit not to the extent of the control strain. The tables below, Figure 3.3.1, report HPLC data of glucose and maltose attenuation of DME media during fermentation. Starting maltose concentrations ranged from 50-70 g/L while starting glucose concentrations were 20 +/-3 g/L. Maltose was utilized gradually over the course of fermentation and more quickly at higher temperatures. During the first four days of fermentation, for the feather wild yeast strain, maltose utilization was (on average) 12.68 g/L/day at 21°C, 14.8 g/L/day at 26°C and 15.02 g/L/day at 31 °C, 18% faster at 31 than 21°C. For flower wild yeast strain maltose utilization (on average) was 11.22, 9.21, and 15.28 g/L/day at 21, 26, and 31 degrees Celsius, respectively, 36% faster at 31°C than 21°C. For the control strain, maltose utilization (on average) during the first four days was 9.11, 10.32, and 15.27 g/L/day at 21, 26, and 31 °C, respectively, 67% faster at 31 than 21°C. The stark increase for the control strain is partly due to utilization being only 9.11 g/L/day. Glucose, on the other hand, was fully utilized

within the first 2 days of fermentation at all temperature

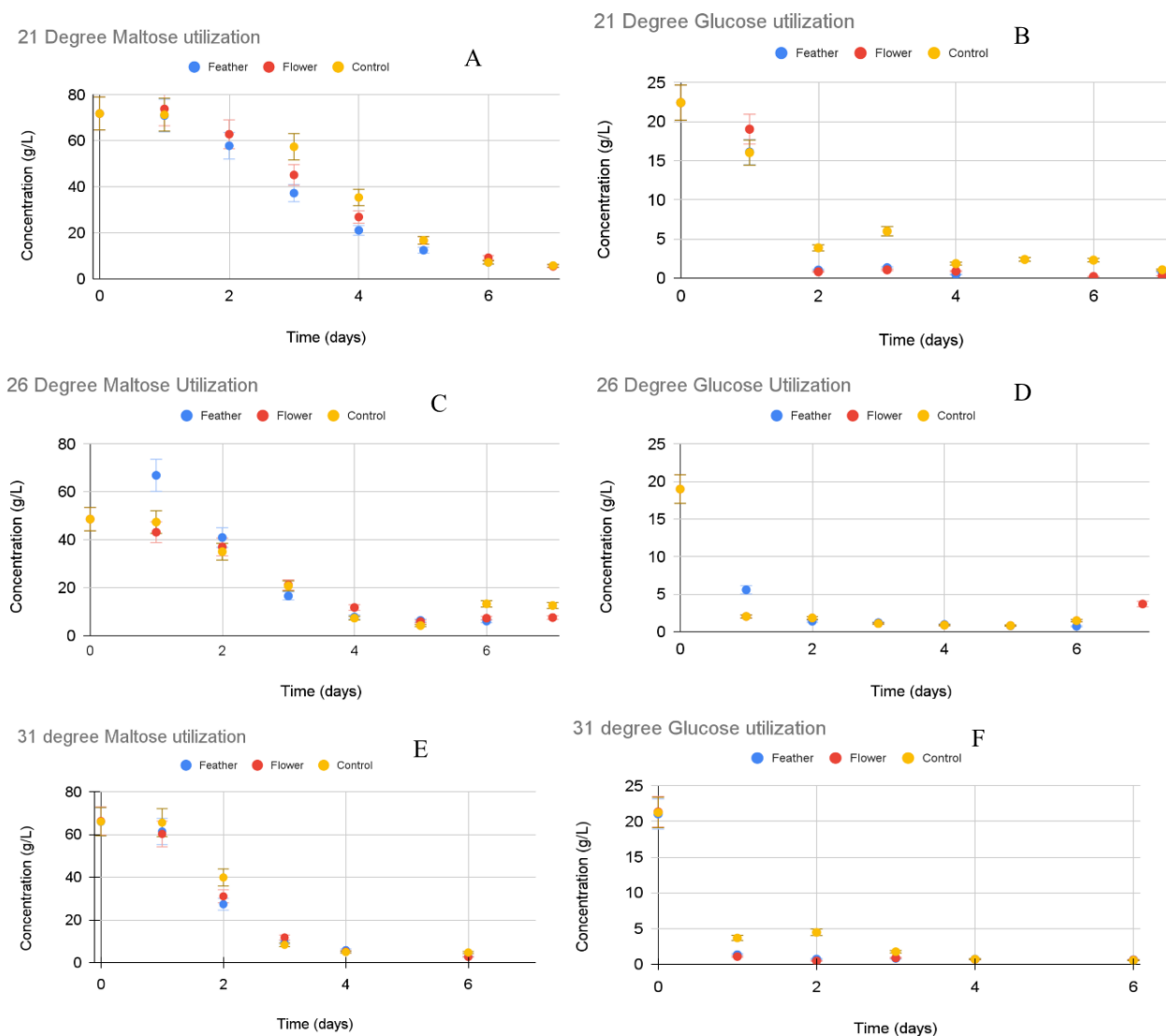


Figure 3.3.1 A-F: HPLC analysis of dried malt extract substrate Fermentation Profiles of Yeast Strains: Maltose and Glucose Utilization at 21°C, 26°C, and 31°C

This multi-panel Figure 3.3.1 illustrates the fermentation data for three yeast strains, highlighting the utilization of maltose and glucose at three different temperatures (21°C, 26°C,

and 31°C). Each panel represents the sugar consumption dynamics over time, emphasizing differences in strain performance and temperature-dependent substrate utilization.

Table 3.3.1: Jmp Connecting Letter report of final specific gravity by strain and temperature. Entries connected by the same letter do not show statistically significant differences. Control (CTRL) samples reached significantly different final gravity at 26°C. Feather (FT) and Flower (FW) wild yeast strains were significantly different at 36°C compared to other tested temperatures

Sample						Mean	
36 Feather	A					1.023	
36 Flower		B				1.0196	
21 Flower			C			1.0173	
21 Feather			C	D		1.0163	
31 Feather			C	D		1.0160	
31 Flower			C	D		1.0160	
26 Flower			C	D		1.0156	
26 Feather				D		1.0153	
21 Control					E	1.0060	
36 Control					E	1.0056	
31 Control					E	F	1.0046
26 Control						F	1.0033

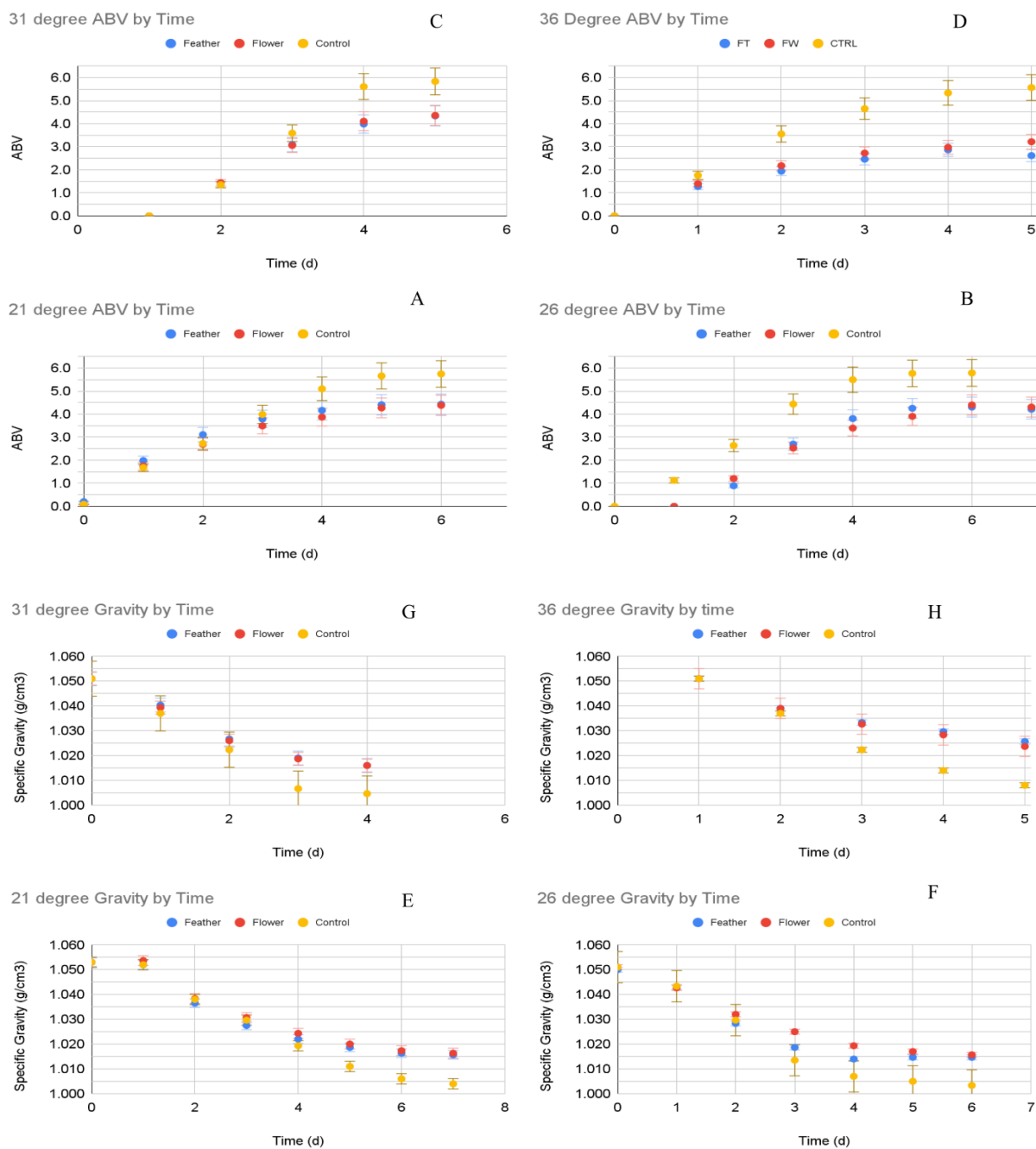


Figure 3.3.2 A-H: Impact of Temperature on % Alcohol by Volume (%ABV) and Specific Gravity During Fermentation of Wild and Control Yeast strains. This multi-panel figure illustrates the changes in alcohol by volume (ABV) and gravity over the course of fermentation in 14% DME for three yeast strains at varying temperatures analyzed with the Anton Paar. Panels A-D show the progression of ABV for the strains, across temperature conditions (e.g.,

21°C, 26°C, 31 °C, and 36°C). Panels D-H depict the corresponding changes in gravity for the same strains under the same conditions.

### 3.3.2 Fermentation with Varied sugar concentration

Next, tests comparing the performance of wild and commercial yeast strains during fermentation at varying starting sugar concentrations were performed. Key brewing metrics such as % alcohol by volume (%ABV) and gravity were monitored over time by analyzing samples with the Anton Paar alcolyzer to assess the fermentation performance of each yeast type. These findings indicate the capability of wild yeast to ferment well in high sugar media and produce higher final ABV (7%). This data also exacerbates the difference in alcohol production and attenuation between wild and domesticated commercial yeast as starting sugar content increases.

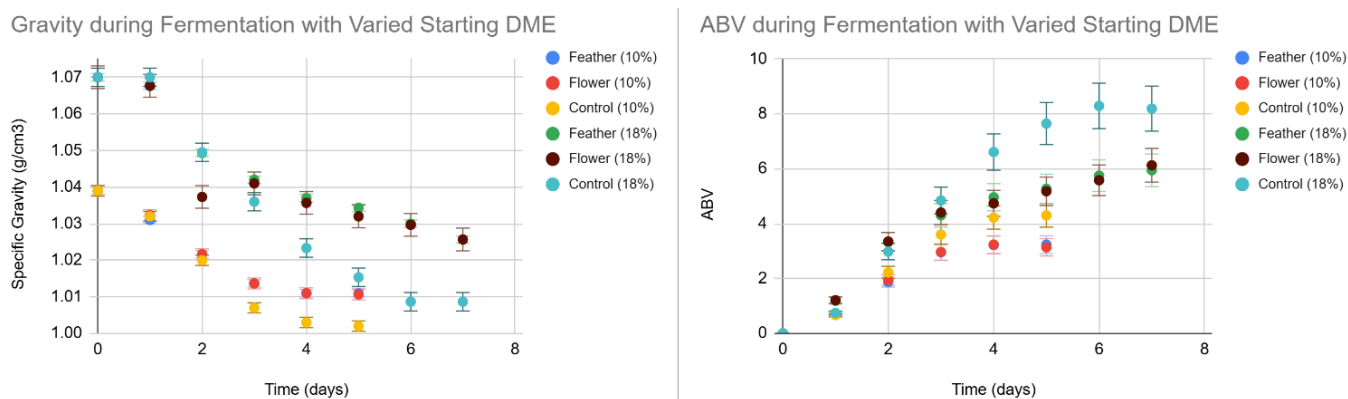


Figure 3.3.3 A-B: Effect of Initial Sugar Concentration on Fermentation Dynamics

Across Yeast Strains. This multipaneled figure compares the impact of varying initial sugar concentrations on fermentation metrics for three yeast strains. Panel A shows the change in gravity over time for low and high starting sugar concentrations. Panel B illustrates the corresponding changes in alcohol by volume (ABV) for the same conditions. The data highlights the influence of initial sugar concentration on fermentation performance, showcasing strain-specific differences in sugar utilization and ethanol production efficiency.

Table 3.3.2: Jmp Connecting Letter report of final alcohol by volume (ABV) by strain and starting DME concentration. Control (CTRL) strain produces a higher ethanol concentration than either wild strain at each tested starting gravity. Increases in starting gravity yielded significantly different final ABV, and Feather (FT) wild yeast functioned the same as Flower (FW) strain.

Sample				Mean
Control 18%	A			8.19
Flower 18%		B		6.12
Feather 18%		B		5.94
Control 14%		B		5.79
Feather 14%			C	4.41
Flower 14%			C	4.36
Control 10%			C	4.30
Feather 10%			D	3.23
Flower 10%			D	3.13

### 3.3.3 Biolog Plates

Utilization of substrates can be used to identify and compare yeast strains. Biolog FF microplates were used to generate a substrate utilization profile and compare wild yeast strains with a commercial control. The control strain was able to use a range of substrates that the wild strains were not.

After testing the three yeast strains on Biolog FF microplates, a number of genes were identified that correlate to substrates that were utilized. In many instances, genetic data reinforced the observed phenotypes presented by the microplates. However, in some instances genetic and phenotypic data differed. For instance, both wild strains were unable to utilize the maltose well (C11) despite the fact that maltose genes were present and that HPLC data confirmed maltose utilization during fermentation of DME in flasks.

Table 3.3.3: Biolog FF microplate result comparison. Figure includes well number and substrate as well as comparison of strain specific results. ✓ indicate that a substrate was utilized by at least one tested strain and color change was observed. X indicate that a well was not utilized. Substrates that were not utilized by any tested strain were omitted. Genes correlating to metabolized substrates are also included with ✓ or X indicating that the gene was present or absent.

Well #	Phenotypes	Feather	Flower	Control	Genes	Feather	Flower	S288C
A1	Water	X	X	✓				
A2	Tween 80	✓	✓	X				
A3	N-Acetyl - D-Galactosamine	✓	✓	X				
A4	N-Acetyl-D Glucosamine	✓	✓	✓	GNA1	✓	✓	✓
A5	N Acetyl Mannosamine	X	X	✓				
A7	Amygdalin	X	X	✓	EGH1	X	X	✓
A8	D-Arabinose	X	X	✓	ARA1	✓	X	✓
A9	L-Arabinose	X	X	✓	ARAA	✓	✓	✓
A10	D Arabitol	X	X	✓	ARD	✓	✓	✓
A11	Arbutin	X	X	✓	DSE4	X	X	✓

Table 3.3.3: (Continued)

A12	D-Cellobiose	X	X	✓	CDT-1	X	X	✓
B1	$\alpha$ Cyclodextrin	X	X	✓				
B2	$\beta$ Cyclodextrin	X	X	✓				
B3	Dextrin	✓	X	X	STA1	X	X	✓
B5	D Fructose	X	X	✓	HXT3	✓	✓	✓
B6	L Fructose	✓	✓	✓	FSY	✓	✓	✓
B7	D Galactose	X	X	✓	GAL1	✓	✓	✓
B8	D Galacturonic acid	✓	✓	✓	GCY1	✓	✓	✓
B9	Gentiobiose ( $\beta$ 1,6 Glucose Disaccharide)	✓	✓	✓				
B10	D Gluconic Acid	✓	✓	✓				
B11	D Glucosamine	X	X	✓	AGT1	X	X	✓
B12	$\alpha$ -d-Glucose	✓	X	✓	HXT	✓	✓	✓
C1	Glucose-1-phosphate	X	X	✓	UGP1	✓	✓	✓
C2	Glucuronamide	X	✓	X				
C3	D-Glucuronic acid	✓	✓	X	UGD1	X	X	X
C4	Glycerol	✓	X	X	GUT-GCY1	✓	✓	✓
C5	Glycogen	X	X	✓	GPH1, GDB1	✓	✓	✓
C6	m-inositol	X	X	✓	MIO1	X	X	✓
C7	2-keto-d-Gluconic acid	X	X	✓	kguK	X	X	X
C8	$\alpha$ -d-lactose	X	X	✓				
C9	Lactulose	X	X	✓				
C10	Maltitol	X	X	✓				
C11	Maltose	X	X	✓	Mal	✓	✓	✓
C12	Maltotriose	X	X	✓	MTT1	✓	X	✓

### 3.3.4 Flocculation

Flocculation, measured as the percentage of yeast cells forming aggregates over time, and sedimentation, observed as the rate at which these aggregates settle, were analyzed to evaluate strain-specific differences. Within this trait there are observed genetic differences between wild and domesticated yeast. These genetic differences were compared to phenotypic variance to substantiate the effect of genetic variance in the tested wild yeast. The graph displays variations in the onset, intensity, and stability of these processes, providing insight into how each yeast strain performs in terms of aggregation and settling efficiency.

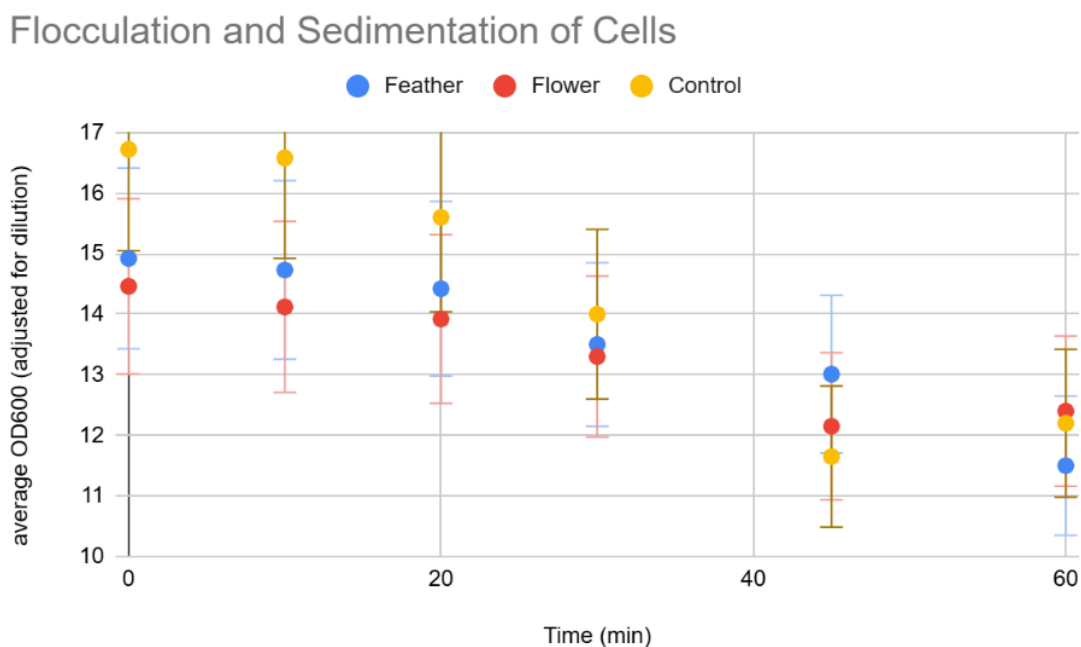


Figure 3.3.4: Flocculation and sedimentation of cells. Stationary phase cells were harvested and diluted into a range (0.2-0.8 AU) of spectrophotometers.  $OD_{600}$  was measured over time. Slope of lines approximates degree of flocculation of cells. The slope of Feather strain: -0.057, slope of Flower strain: -0.034, and slope of Control strain: -0.075

### 3.3.5 Ethanol tolerance

Ethanol stress is common in brewing and suitable brewing strains must be able to handle environments with this stress. An important line of inquiry is comparing the ethanol tolerance of three yeast strains by analyzing their final cell counts and sugar attenuation in media composed of dry malt extract (DME) mixed with 95% ethanol to achieve 0% and 10% ethanol concentrations. The data reveals strain-specific differences in cell growth under ethanol stress, providing insight into their ability to ferment in high-alcohol environments and grow despite ethanol stress. By contrasting the final cell counts and final SG at both ethanol levels, the graphs illustrate each strain's resilience and potential performance in ethanol-rich fermentation processes.

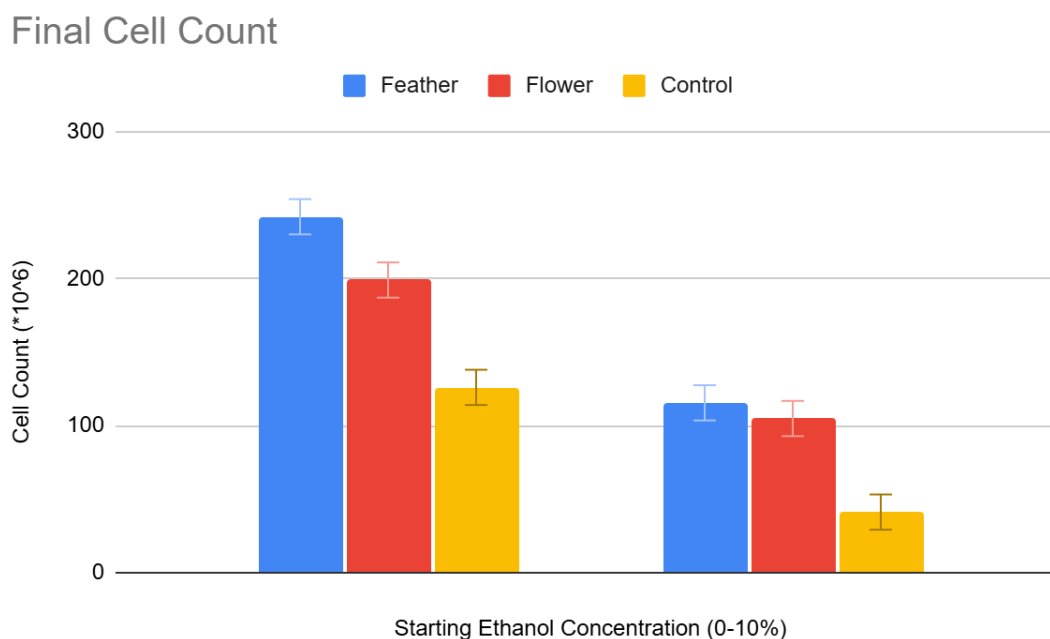


Figure 3.3.5: Ethanol Inhibition of Final Cell Count in Three Yeast Strains at 0% and 10% Ethanol. Comparison of final cell counts for three yeast strains, inoculated to  $10^6$  cells/mL, grown under conditions of 0% (left columns) and 10% ethanol (right columns), highlighting the impact of ethanol on cellular proliferation.

Feather, flower, and control strains all produced fewer cells under ethanol stress with final cell counts being 47.7%, 52.6% and 32.6% of the unstressed cells, respectively. Statistically significant differences depicted.

Table 3.3.4: Ethanol Inhibition of Final Cell Count in Three Yeast Strains at 0% and 10% Ethanol.

Sample				Mean (*10 <sup>6</sup> )
0% Feather	A			242
0% Flower	A	B		199
0% Control		B	C	126
10% Feather			C	115.5
10% Flower			C	104.8
10% Control			D	41.2

### Ethanol Inhibition of Final Fermentation Gravity

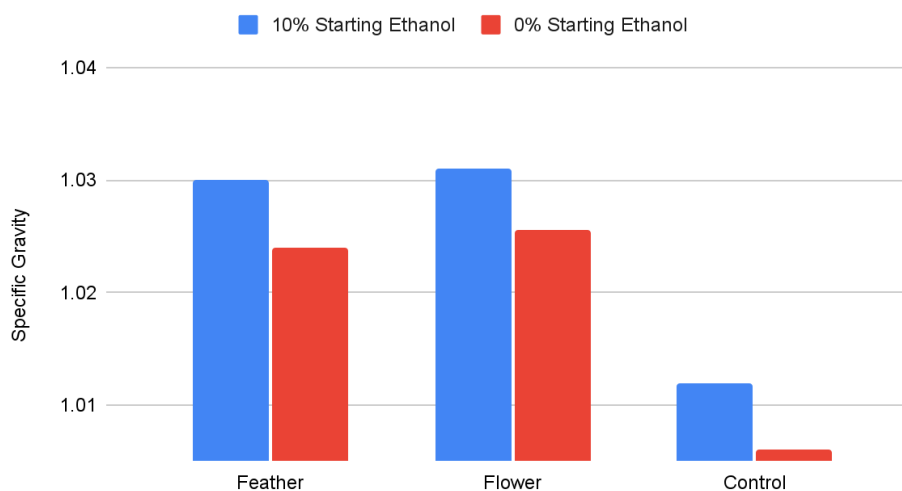


Figure 3.3.6: Effect of Ethanol on Final Gravity in Three Yeast Strains at 0% and 10% Ethanol.

Analysis of final gravity measurements for three yeast strains grown at 0% and 10% ethanol, demonstrating the influence of ethanol on sugar utilization and fermentation efficiency. At 0% ethanol final gravity reached was 1.024, 1.0255, and 1.006 for Feather strain, flower strain, and control strain, respectively. At 10% ethanol the final gravity reached was 1.030, 1.031, and 1.02 for Feather strain, flower strain, and control strain, respectively. With a starting gravity of 1.050, and unstressed conditions, Feather strain had an attenuation of 52%, Flower strain 49%, and control strain 88%. Ethanol stress decreased the attenuation of all samples. Under stressed conditions, Feather strain attenuation was 76.9% of that when ethanol was not initially present, Flower strain was 77.5%. Control strain attenuation under ethanol stress was 86.3% of without the ethanol present.

### 3.3.6 Sensory Evaluation

Table 3.3.5(A-B): Connecting letter report of overall likeability for batches brewing in 10 L stainless steel fermenters with 8 g/L Tettnanger Hops, 12% DME (1.035)(A), and batches brewed with wort and carbonated. During second trial (B) Feather, Flower, and Nottingham, were compared. The first sensory trial (A) featured 15 participants while second (B) featured 18.

Sample	Trial A		Mean
Flower Beer	A		3.428
Nottingham Beer	A	B	2.714
Feather Beer		B	2.285

Samples	Trial B	Mean
Flower Beer	A	2.222
Feather Beer	A	2.111
Nottingham Beer	A	2.055

### 3.3.7 Analysis of Metabolic Byproducts

Cedex Bio allowed for analysis of a number of fermentation byproducts. Samples were taken during anaerobic growth of both wild yeast strains throughout seven-day fermentation. Glucose, Ethanol, Glycerol, Pyruvate, Acetate, Lactate, and Formate were measured to provide insight into the fermentation profiles of the wild yeast strains

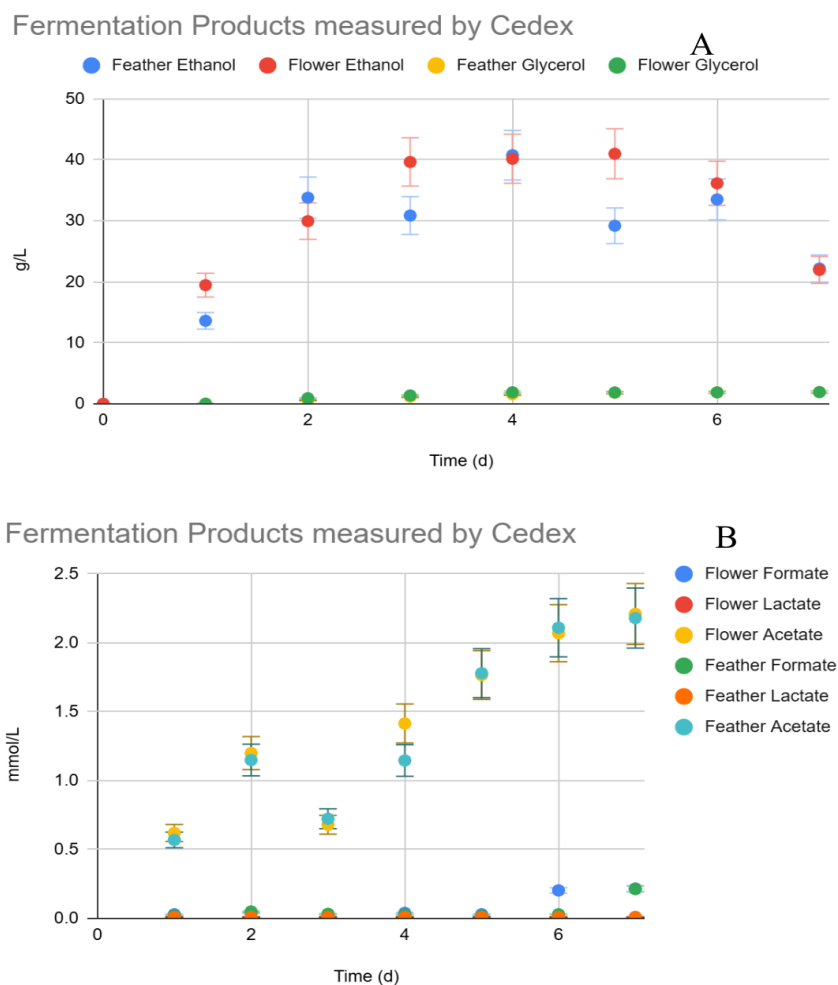


Figure 3.3.7(A-B): Cedex analysis of Ethanol, Glycerol, Acetate, Lactate, and Formate during anaerobic fermentation by wild yeast. Pyruvate production was also measured but maintained 0 mg/L readings.

### 3.4 Discussion

#### 3.4.1 Effect of Temperature on Fermentation

The control strain exhibited higher ethanol production across all tested temperatures compared to the wild strains. Sugar utilization was more rapid at elevated temperatures between 21 and 31°C, for all samples, with the control strain demonstrating a consistent ability to reduce final gravity to 1.004, while the wild strains rarely achieved a final gravity below 1.011, regardless of temperature, indicating a significant difference in maximum attenuation. The control strain consistently utilized more sugar at all tested temperatures compared to the wild strains. At 21°C, the control strain was able to reach a lower specific gravity than wild type yeast at any temperature that was tested. However, at 21°C, wild strains showed relatively faster sugar utilization, with more glucose and maltose consumed on days 2, 3, and 4 when compared to the domesticated *S. cerevisiae*. Growth of the wild strains was not inhibited at 31°C, and even at 36°C, fermentation persisted, albeit at a reduced rate compared to 31°C. Notably, the wild strains contained the HSP12 gene (heat shock protein) at the same copy number as the control strains S288C and EC118 (one copy), as seen in Table 2.3.4. This may indicate that wild strains experience most rapid fermentation between 30 and 36 °C.

Both the control and wild strains exhibited minimal differences between fermentation at 26°C and 31°C, with 21°C, shown in Figures 3.3.1 and 3.3.2. Overall, wild strains demonstrated a more detrimental effect on attenuation at high temperature (36°C) than the control strain. At 36°C the wild strains had a significantly higher final gravity than at lower tested temperatures, indicated more sugars remaining in the media. This was not the case for the control yeast. When testing ethanol stress, it was seen that the added stress decreased attenuation of wild yeast more

than the control, however, cell counts were higher in the wild samples. This may indicate a trade-off between stress tolerance and fermentation efficiency.

Fermentation at low temperatures is affected by the FLO1 gene. Strains that lack this gene exhibit slower fermentation at lower temperatures (Deed, Gardner, and Fedrizzi, 2017). Importantly, both tested wild strains lack this gene. This highlights the important genetic basis of observed traits and exhibits an opportunity for genetic engineering to improve wild type strains. Additionally, this demonstrates the importance of evolutionary lineage when bioprospecting wild strains for industrial applications. While the origin of these strains is unknown, the lineage of wild strains heavily impacts their functionality due to the diverse and specific evolutionary pressures of their habitat. Wild yeast strains seem to adopt specific temperature preferences and tolerances based on the environment they have become adapted to. These tolerances are reflected in fermentation rates and capacity (Salvadó et al. 2011). Screening for these differences would be essential when bioprospecting natural strains for industrial applications.

#### 3.4.2 Fermentation with varied sugar concentration

The control strain demonstrated significantly higher production of alcohol at all tested sugar levels, shown in Table 3.3.2. In fact, 4% increases in starting concentration of DME resulted in significant changes in final alcohol concentration with each increasing starting gravity level producing a significantly higher ABV than the last in both wild and commercial strains, seen in Figure 3.3.3. Additionally, the control strain was able to produce the same ethanol level at lower starting gravity than the wild yeast strain. Finally, the two wild strains did not produce significantly different ABV from one another in this experiment, also seen in Table 3.3.2. This data indicates that the tested wild strains are significantly worse ethanol producers than

domesticated control strains at all tested starting gravity levels but not different from one other when compared using this metric.

While both tested wild yeast strains are worse alcohol producers than their commercial counterparts, they are still viable brewing strains. Many commercial beers are sold between 4-6% ABV, which wild strains have demonstrated ability to produce. When inoculated into a media or brewers wort with a starting gravity between 1.05 (12 degrees Plato) and 1.08 (17 degrees Plato) both wild strains are able to produce a final product of 4-6% ABV, indicating that they are viable for brewing operations.

The relationship between yeast fermentation activity and starting sugar level is complex. With more available substrate the culture is able to produce more ethanol. However, the relationship is more nuanced. Increasing sugar concentration applies increasing osmotic stress to the yeast, which can present problems during fermentation (Horváth, et al 2020). Additionally, wild yeast from floral nectar and honey have indicated a decrease in cell growth with increased sugar concentration. This may allude to specific optimal sugar concentrations pertaining to wild yeast strains (Canché-Collí et al., 2021). Once more this shows the importance of genetics and evolutionary lineage when utilizing wild yeast as the optimal sugar concentration is likely shaped by the ecological niche and habitat of the wild yeast strain. Genetic engineering provides opportunity to override the natural genetic makeup of individual wild isolates, however, since wild yeast populations owe their genetic diversity to their diverse habitats, researchers could bioprospect for targets with traits, such as tolerance to high osmotic stress, which match their intended application.

### 3.4.3 Carbohydrate data

Data on carbohydrate utilization obtained by HPLC analyses was used to confirm and measure strain-specific utilization of glucose and maltose, a disaccharide of glucose. The ability to metabolize maltose is an essential characteristic in brewing strains, and commonly thought to be absent in many populations of wild yeast (Nikulin et al, 2020). Fermentation samples were collected from cultures grown anaerobically at temperatures 21-31 °C. HPLC data of maltose attenuation indicated a steady decrease of maltose concentration throughout fermentation corresponding to the growth of cells and production of ethanol, shown in Figure 3.3.1. This data is reinforced by the presence of genes for maltose metabolism being present in the genome of both tested wild yeast strains, illustrated in Figure 3.3.3. Wild strains did not have the ability to attenuate to the level of the commercial *S. cerevisiae* but were still able to utilize the maltose present. The diminished utilization speed of the wild yeast strains has a genetic basis and highlights opportunities for genetic engineering of wild yeast strains. Domesticated yeast owe their superior maltose attenuation to increased copy number of maltose transporters and higher expression of those genes (Duval et al, 2010). Genetic engineering of wild yeast strains to include additional copies could alleviate the discrepancy between wild strains and their domesticated counterparts.

Utilization of glucose compared more similarly between the wild strains and the control in this experiment. In all tested strains, glucose concentration of the media was decreased below 5 g/L within the first three days of fermentation, as shown in Figure 3.3.1. Glucose utilization was more rapid than maltose indicating a hierarchy of preferred sugar consumption, as typical of yeast fermentations (Saleema-Oom et al, 2005).

In all tested strains it was observed that utilization of sugars was more rapid at higher temperatures, until 36 degrees, at which point, utilization slowed in comparison to 31°C. Additionally, utilization of sugars was quicker in the commercial *S. cerevisiae* as opposed to its wild counterparts in this experiment, Figure 3.3.1.

#### 3.4.4 Biolog Plates

To better understand the metabolic capabilities of wild and domesticated yeast strains, strains were grown in Biolog FF metabolic plates to assess their ability to utilize a diverse array of substrates. This comparison provides insights into the metabolic profile of wild and domesticated yeast, albeit under specific conditions. Domesticated Nottingham yeast were capable of utilizing a wide array of substrates that wild yeast are unable to metabolize under static atmospheric conditions. Notably, Biolog plates indicate that tested wild strains were unable to utilize maltose, an important sugar in brewing environments. However, maltose utilization was seen experimentally in flasks and confirmed by HPLC, seen in Figure 3.3.1. Additionally, strains of yeast unable to utilize maltose typically are unable to effectively ferment brewers' wort- which both tested wild strains were able to do. Finally, genotypic data from annotated wild yeast DNA indicates the presence of a number of genes relevant to maltose utilization, Table 3.3.3. These findings confirm that, despite the result from Biolog FF metabolic plates, both tested wild yeast strains can grow in maltose media. This discrepancy raises questions about the results from other wells on the plate and leaves the door open for the possibility that wild yeast strains, including but not limited to the two tested strains, are able to utilize an array of substrates that were not indicated during experimentation. This may be due to environmental adaptation. Certain lactic acid bacteria do not produce many of their own amino acids, since they are adapted

for an environment that is rich in proteins that can be imported (Christiansen et al, 2008). The tested wild strains of yeast may exhibit the opposite trait in which they lack transporters for substrates that are not normally present in their natural environment. Additionally, the tested wild strains may require other present substrates to activate the relevant metabolic genes. Since the wells were pure substrate they would not be able to activate the genes for metabolism in the Biolog wells, but may be able to utilize the substrates in complex media. It is possible that, under correct growth conditions, the differences in substrate utilization between wild and cultivated strains could be minimized. While the observed phenotypic differences as well as the genetic differences may seem to allude to an important contrast between domesticated and wild type yeast, when grown in complex media the distinctions did not manifest themselves into diminished cell growth.

Substrate metabolism in wild yeast strains is thought to be substantially more diverse than with domesticated yeast, despite the more limited observed substrate usage. While domestication has allowed for enhanced utilization of substrates relevant to industrial applications, different wild isolates will feature stark differences in their metabolism between due to the wide range of environments that wild yeast inhabit (Bell, Higgins, and Attfield, 2001). Additionally, wild yeast strains will be suited for metabolism of substrates relevant to the environment they inhabit. It is possible that the tested wild yeast isolates are evolved for environments that do not require the transport and metabolism of some of the compounds tested. The tested wild yeast may, for instance, be more suited for creation of their own amino acids rather than uptake of amino acids from its environment if its natural habitat does not feature many free amino acids. Finally, regulatory systems for metabolism of substrates can be complex,

rendering the tested wild isolates unable to efficiently metabolize some of the pure substrates in the Biolog FF microplate wells without the presence of other substrates.

### 3.4.5 Flocculation

Flocculation data was collected experimentally and can be compared to genetic data. Experimental flocculation assays indicated that wild yeast strains were not able to flocculate as well as commercial control. Slope of OD<sub>600</sub> gives an approximation of flocculation and sedimentation rates. Control strain slope was 2.19 times higher than flower strain while feather strain was 1.66 times higher than the wild flower yeast alternative. Control strain had a slope that was 1.32 times higher than wild feather yeast, all shown in Figure 3.3.4. These results indicate that control strain may experience more rapid flocculation and sedimentation than wild alternatives, although the difference was not statistically significant and the wild strains could sediment in a similar time course. Cell size may effect sedimentation speed. It is possible the wild strains flocculate less, but sediment more quickly. This data is supported by genetic analysis of wild yeast strains when compared to S288C (model *S. cerevisiae*). FLO 1, 5, 8, and 11 are genes identified for flocculation. S288C has all four flocculation genes, while the tested wild strains only have FLO 8: shown in Table 2.3.4. While this gene may be sufficient for some flocculation, both genetic and experimental data indicate that wild yeast strains lack the flocculation efficacy of domesticated brewing strains. Flocculation is an important trait when brewing, but far from the most important. The lack of genes for flocculation may indicate that the tested wild yeast strains may be bottom fermenting. While some wild yeast may exhibit rapid flocculation others may not (Westman et al, 2014). This contrasts the predictable nature of commercial yeast that in which flocculation characteristics are much more uniform (Soares,

2011). Like with many traits, the genetic diversity of wild yeast leads to high levels of variation in observed traits. Strain selection for specific beer styles should prioritize flocculation characteristics that align with desired clarity, attenuation, and maturation timelines. The addition of adjuncts such as calcium salts or modifications to fermentation conditions can further optimize flocculation behavior. Ultimately, beer can be filtered if yeast lack the ability to readily flocculate.

#### 3.4.6 Ethanol tolerance

Wild and control strains were compared at 0% and 10% starting ethanol levels in 14% DME media. Effects of ethanol stress were seen in both final cell count as well as attenuation. Wild yeast strains showed less reduction in final cell count compared to the domesticated strain. Wild yeasts under ethanol stress produced a final cell count of 47% and 52% of unstressed cells, while the domesticated strain under ethanol stress only produced 32% as many cells as when unstressed, seen in Figure 3.3.5. While the tested wild yeast strains may have had an observed higher tolerance to ethanol stress than the domesticated strain, this difference was not found to be statistically significant. Ethanol tolerance is an important factor in bioethanol production and brewing, although brewing operations require a lower ethanol tolerance than bioethanol. Wild yeast often have high tolerance to stresses, although domesticated yeast strains are uniquely adapted to brewing environments due to decades of adaptation. Additionally, while domesticated yeast will function predictably during fermentation, wild strains may not. Wild strains exhibit highly variable ethanol tolerance between species and strains (Haas et al, 2019). While some wild yeast are capable of fermenting well over 10% ABV, others may not (Xu et al, 2017). This highlights the importance of structured methods for bioprospecting strains with desirable

attributes, to ensure that wild yeast isolates possess traits relevant to their intended process, and potential opportunities for genetic engineering, to mold wild strains to their application.

One of the few significant phenotypic differences observed between wild type and commercial yeast was maximum attenuation. The commercial ale strain was readily able to lower the specific gravity to 1.004, while the gravity when fermenting with the wild strains never went below 1.010. Introducing ethanol stress exacerbated this distinction. Under stressed conditions the control strain utilized 76% of available sugars, while wild strains Feather and Flower utilized 39.9% and 37.9% respectively; seen in Figure 3.3.9. This data indicates that the control strain attenuation is less effected by ethanol stress than its wild counterparts, although further replication is necessary to ensure statistically relevant data on ethanol tolerance. While these wild strains may have a formidable ethanol stress tolerance, they lack in fermentation efficiency compared to the control; a difference made more distinct when ethanol stress was introduced. The different outcomes seen when measuring attenuation and final cell count under ethanol stress may indicate a trade-off between stress tolerance and fermentation efficiency. Domesticated strains have been selected for ethanol tolerance and have adapted to environments with ethanol stress. Wild strains, on the other hand, are from diverse environments in which high ethanol titers are often absent. Some wild strains may be more suited for high ethanol fermentations than others (Molinet & Cubillos, 2020). In fact, the tested wild strains seem to have an ethanol tolerance competitive with the domesticated control. This ethanol tolerance corresponds with the ethanol production displayed in previous tests and displayed in Figure 3.3.3. Strains lacking ethanol tolerance may lead to issues in propagating wild yeast harvested after experiencing an elevated ethanol levels and may render some wild strains less effective for producing wine, mead, or high alcohol beers. For many years it has been assumed that

domesticated brewing strains had a special ability to tolerate brewing stresses, like ethanol, that was not present in wild populations. This data counters that assumption and alludes to the viability of wild yeast strains being utilized efficiently in brewing environments because of their ability to tolerate ethanol stress.

### 3.4.7 Sensory Evaluation

An initial sensory test of beer made from DME wort and 8 g/L hops provided inconclusive results. 10 liter fermenters indicated that wild yeast strains may have been bottom fermenting. While the control strain had a thick layer of yeast on top of the bulk liquid, wild strains did not. Sensory evaluation was completed by 15 untrained panelists in which Feather and Flower wild yeast were compared to a control commercial strain (*S. cerevisiae* Nottingham). Two varieties of Kombucha were also included in the sensory test. Sweetness, sourness, floral, bitterness, fruitiness, earthiness, and overall likeability were all assessed on a five point scale. In all categories other than overall likeability the beer samples were not ranked as being significantly different. This may indicate that panelists were not adequately trained to elucidate differences between the samples. However, when overall likeability is compared, the beer made with the Flower wild yeast (3.54) was significantly more liked than the Feather wild yeast (2.28), as seen in Table 3.3.5. However, this data is inconclusive since the panel size was likely too small as observed differences did not have statistical significance, the beer was uncarbonated, and not made from a proper wort. When beer was made using wort, and carbonated before sampling, no significant differences were observed in any measured category (Overall impression, sweetness, bitterness, sourness, grainy taste, body, or earthiness). While this does not do much to characterize the beer made with the tested wild strains, it does indicate it is free from

undesirable flavors. Additionally, without GC analysis of flavor and aroma compounds it is unknown what difference exists. When fermenting with wild strains of yeast one may or may not try to highlight novel flavors produced by the wild strain. Choice of yeast strain can be used to add novel flavors to wine and cider fermentation (Way et al, 2022). Wild yeast strains can also be used to add novel flavors during baking. Further research is needed to elucidate the genetic basis of flavor and construct a methodology to bioprospect strains with desirable flavor profiles.

#### 3.4.8 Cedex

Analysis with Cedex Bio provided insight into a number of fermentation byproducts. Genetically, the two tested wild yeast strains differed in both gene content and genome size, highlighting their distinct evolutionary paths and adaptations. Despite these genetic differences, their metabolic profiles for key compounds such as ethanol, glycerol, pyruvate, acetate, lactate, and formate were remarkably similar, as seen in Figure 3.3.7. As was observed from the HPLC analysis, Figure 3.3.1, glucose was fully utilized within the first 2 days of fermentation. The findings underscore the complexity of genotype-phenotype relationships in yeast and the potential for diverse strains to fulfill similar functional roles in fermentation environments. However, the production of byproducts during fermentation of wild yeast is highly variable due to the diversity that exists between wild yeast. Production of byproducts is important during brewing and can lead to strain-to-strain and batch-to-batch variability. Further work is needed to delve deeper into the identification and quantification of byproducts produced by wild strains when using them in industrial fermentation since this area lacks data. However, due to the high levels of genetic diversity of wild yeast, byproducts produced during fermentation are likely to be highly variable between strains. Refining strains with genetic engineering techniques like

CRISPR is an important area of research both to minimize unwanted byproducts and to add genes for products of interest (Henningesen et al, 2015).

### **3.5 Future Work**

While the results demonstrate both the viability of wild yeast in brewing applications as well as the similarities between wild and domesticated yeast strains, further investigation is needed to add replicates to make the data more robust and to explore the use of wild yeast strains in large scale brewing environments as well as potential novelty flavors that may be provided with wild yeast strains. Additionally, it would be valuable for future work to include further replication of tests done in this study, especially flocculation and ethanol tolerance, to bolster their accuracy and statistical relevance. Due to constraints on the project, volatile analysis and GC were excluded. These assays would allow much greater insight into the compounds produced during fermentation and their effect on the final product. These potential flavor compounds would allow wild yeast strains to be differentiated from domesticated strains and elucidate potential benefits of utilizing wild strains. Future work into optimization of brewing conditions would be necessary to make these wild yeast strains practical for large scale or commercial brewing environments. Finally, the scope of these tests was limited and while the preliminary characterization of these wild yeast isolates is important, further testing would be necessary to adequately determine their functionality and distinguish them from domesticated yeast.

### 3.6 Conclusion

Brewing yeast represents specific species of yeast domesticated through hundreds of years of propagation and brewing. It is commonly believed that brewing strains of yeast possess attributes and characteristics that are not present in wild strains. These traits, from flavor compounds to ethanol tolerance to maltose attenuation, are desirable for efficient and high quality brewing. Wild strains have often been avoided in brewing due to potential undesirable flavor compounds, inability to fully metabolize maltose, or poor ethanol tolerance. New technologies, like CRISPR systems, as well as the vast genetic diversity within wild yeast may help researchers circumvent these weaknesses traditionally ascribed to wild yeast. Scientists can bioprospect wild yeast strains with novel traits, such as increased stress tolerances, suited for industrial purpose and further refine them into superior strains. Moreover, researchers continuing to explore the vast diversity of wild yeast may uncover metabolic pathways, stress tolerance mechanisms, and regulatory systems that can be harnessed for industrial practices.

This research indicates that wild *S. cerevisiae* strains are prevalent in the natural environment, relatively easy to isolate, and can be used to brew comparable products to domesticated yeast strains. Wild yeast exhibited comparable fermentation efficiency, with the ability to adapt to varying sugar concentrations, produce desirable alcohol levels, and ferment at a range of applicable temperatures. While this study does not provide evidence of any novel flavor characteristics, sensory testing indicates that products brewed with wild strains were free from obviously undesirable flavors. Genome editing technologies present opportunities for flavor pathways to be added to wild strains as well as off-flavors to be removed. Furthermore, the natural variability of wild yeast presents opportunities for enhancing flavor complexity and creating specialty brews that distinguish themselves in an increasingly competitive market.

Isolation of microbes from diverse environments can allow researchers to harness their capabilities across various industries. While isolation of microbes, and wild yeast, is relatively simple, screening isolates and domesticating them for industrial purposes is more complex. Doing so efficiently would require selection and examination of genes relevant to the application. By applying these techniques and utilizing new technologies, like AI, for annotation and genetic comparison, researchers can accelerate identification of key traits in wild yeast enabling more efficient strain development. AI will also allow more rapid identification of novel traits in bio-prospected strains allowing a more efficient pipeline for strains to be isolated from nature for industrial purposes, varying from bioremediation and biopharmaceutical to food and cosmetic (Chen et al, 2024).

However, challenges such as consistency, strain stability, and contamination risks must be carefully managed. With further research and strain optimization, wild yeast holds significant promise for expanding creative possibilities and enriching the brewing landscape.

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