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An Analysis of Novel Yeasts and Their Brewing Potential

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Introduction

Within the brewing community, there is a race to discover new ways to brew beers to create new flavor and smell profiles. Scientists across the globe are searching for novel species of yeasts which have the potential to enhance the flavor and smell of their beer. While most beers are brewed using one of a handful of domesticated yeast strains -- also known as pitching yeasts -- finding “wild” yeasts is one way to change fermentation products. Experimentation has been started on this front due to the wide array of properties that a wild yeast can change in a brew. For example, wild beers like the Belgium Lambic are often called sours due to a distinctive tart taste. This is achieved by adding bacteria, most commonly *Lactobacillus,* to the fermentation process to create lactic acid. Common flavors are fruity notes, maltiness, woodiness, and less common ones are citrus and peppery flavors. Yeasts can also determine the dryness of a beer. Many yeasts will also accentuate pre-existing flavors like honey and caramel and citrus. Discovery of new yeast strains adds variety to the brewing industry and partners the science industry with the commercial beverage industry.

In choosing the ideal strain for industrial usage, we are evaluating the potential strains with respect to temperature tolerance, pH tolerance, and ethanol tolerance. Fermentation temperature is easily standardized by the temperature of the surroundings around the fermentation vessel. Generally, brewing temperatures are within 9-14℃ for lagers and 15-26℃ for ales.[1,2,3] These temperature ranges are conserved across a wide variety of the commonly used yeasts. Temperature parameters will determine the base flavor profiles which each strain produces. pH also works to influence potential flavor. The pH of beers change dynamically during fermentation; all ferments start at around pH 5 and changes are monitored over time.[4,5,6] Since pH drops largely during fermentation, the final pH determines the flavor profile of the final beer. Lastly, ethanol tolerance is an indicator for the amount of final alcohol that the yeast produces. During the fermentation process, yeasts consume sugars in the ferment and produce carbon dioxide and alcohol. Fermentation will end and the alcohol concentration will be capped

when the alcohol concentration exceeds the yeasts tolerance. A yeast which is able to tolerate a higher concentration of alcohol will continue to ferment for longer periods.

To discover new yeasts that might be appropriate for brewing, local fruits were harvested from Illinois Wesleyan University campus. The fruits collected were ground and strained. Cultures were isolation-streaked and grown on Yeast Peptone Dextrose (YPD) media with chloramphenicol at 30℃ for three days. The plates were then examined using colony morphology features which are consistent with known yeast. Once they were confirmed as yeasts, the cultures were analyzed using genetic tests and light microscopy imaging. Three isolates, which were confirmed as yeasts, were chosen for further work based on genetic identification and lab safety.

Methods

1.1 Collection and Isolation

Yeasts were collected[7] from various fruit trees and fruits on campus. Fruits were harvested from three sources. A Sycamore (*Platanus occidentalis*) seed pod, a Red Chokeberry (*Aronia arbutifolia*) berry, and a banana from Sodexo. These fruits were mashed and strained, and a solution was created with the supernatant removed. This solution was vortexed, and 150µL of lysozyme was added to achieve a solution concentration of 10mg/mL. This mixture was incubated for five minutes, spread on a YPD agar plate, and the plates were incubated for two days at 25℃. Representative colonies were then streaked for isolation on fresh YPD plates and grown at 25℃.

1.2 Genetic Testing

Selected colonies were grown in liquid culture in YPD media overnight at 25℃. One colony was suspended in 100µL of water and was lysed using a thermocycler at 98℃ for 20 mins. Lysate was used as template for 18S PCR using forward primer Fungi V9D (5’ TTAAGTCCCTGCCCTTTGTA 3’) and reverse primer Fungi LS266 (5’ GCATTCCC AAACAACTCGACT 3’). PCR products were confirmed by gel electrophoresis and sent for

Sanger sequencing at Eton BioScience using the forward primer listed above. Results were interpreted using BLAST (GenBank).

1.3 Temperature tolerance

Colonies of each of the three yeast strains were inoculated in YPD liquid media and master cultures were grown at 20℃ for two days. These cultures were then used to inoculate three fresh replicates. Initial measurements of optical density were collected using a spectrophotometer. Experimental temperatures were chosen to be 20°C, 25℃, and 30℃. Over the course of 36 hours, sequential measurements were taken to observe the optical density. Prior to analysis, the tubes were vortexed until the particulates were fully resuspended and then a measurement was taken. During the incubation time, the tubes were held statically at the experimental temperatures. A graph was generated from the final daily measurements to analyze trend lines. Three replicates were performed and analyzed per temperature.

1.4 pH tolerance

Master cultures of the three isolates were started as described above. The pH of liquid YPD media was altered to pH 3, 5, or 7 by the addition of HCl. Three tubes of each pH were then inoculated with cells from each master culture. Upon inoculation, the original culture optical density was measured. The cultures were grown at 25℃ and optical density was measured again at the end of five days. Prior to analysis, the tubes were vortexed until the particulates were fully resuspended and then a measurement was taken. A graph was generated from the final density and averaged from the three replicates. An ANOVA test with a Tukey's b test was run to determine statistical significance. Three replicates were performed and analyzed per pH amount.

1.5 Ethanol Tolerance

Master cultures of the three isolates were started as described above. Liquid YPD media was prepared with ethanol added to 3, 5, and 7 percent concentration of solution respectively. Three tubes of each ethanol concentration were then inoculated with cells from each master culture.

Upon inoculation, the original culture's optical density was measured. The cultures were grown at 25℃ and optical density was measured at the end of five days. Prior to analysis, the tubes were vortexed until the particulates were fully resuspended and then a measurement was taken. A graph was generated from the final density and averaged from the three replicates. An ANOVA test with a Tukey's b test was run to determine statistical significance. Three replicates were performed and analyzed per percent ethanol.

1.6 Microscopy

One colony of cells were grown in liquid YPD culture at 25℃ for 2 days. Cultures were vortexed and 1 mL of cell cultures were removed and then 100nL of Mitoview 633 and 100nL of 4′,6-diamidino-2-phenylindole (DAPI) were added. The mixture was incubated at room temperature for 15 minutes and then slides were prepared using poly-L-Lysine-treated glass coverslips. Photos were captured under 1000X oil immersion microscopy with a red light filter (mCherry/Texas Red) for the Mitoview, a blue light filter (DAPI) for the DAPI, and a white light blank to create images. The excitation filters of each were 562/40 for the mCherry and 356/30 for the DAPI filter. These individual images were layered to create the final figure. The microscope using light filters allowed for images to be collected and then layered over each other to obtain the final figure.

Results

1.1 Isolation and Molecular Identification

Upon first plating of environmental samples (Fig. 1), a variety of colonies were observed with different morphologies. Nine representative colonies were streak plated to create axenic cultures. Among those 9 plates there were 11 colonies which exhibited characteristics of interest such as color variations and colony size differences. Two additional colonies on the edge of the isolation plates were identified and chosen to be tested further as well. After confirming yeast morphology by 400X light microscopy, eight samples were chosen to identify at the genus level by 18S rDNA sequencing. Of those eight samples, seven seven produced interpretable sequence results.

Yeasts which posed a possible threat to human health, such as a fungal infectious agent or parasitic agent, were discarded. The three remaining isolates were therefore chosen for continued analysis, and the genus with the highest percentage similarity was chosen as the nomenclature for future reference. The three samples chosen were a *Taphrina* sp., a *Curvibasidium* sp., and a *Metschnikowia* sp.. The *Metschnikowia* sp. had a 67% coverage. The *Curvibasidium* sp. had a 85% coverage. The *Taphrina* sp. had a 66% coverage.

Figure 1. Red circles indicate the locations of where the three initial fruits which were used for testing were collected.

1.2 Temperature Tolerance

During fermentation, temperature fluctuation will determine the types of fermentation that the yeast can undergo and flavor compounds which it may create. To assess the temperatures typically used in brewing which each isolate could tolerate, we conducted three trials to determine tolerance. We used YPD media to grow each of the yeast cultures at the experimental temperatures of 20℃, 25℃, 30℃. For each of the temperatures, the rate of optical density increase of *Metschnikowia* (*Met*) and *Taphrina (Tap)* were similar (Fig. 2). This was consistent with growth plates where *Curvibasidium (Curv)* lagged behind in growth rate (not shown). Through this analysis, it was observed that the *Met* and *Tap* samples grew fastest and to the highest maximum density at temperatures at and over 25℃. *Met* and *Tap* did not grow at the same rate for the 20℃ trial and while they reached the same absorbance in the end, they didn't

follow the same logistic curve as in the 25℃ and 30℃. *Curv* followed a different pattern than the other two species; it is a slower growing species, and this can be seen by the fact that it never reached an absorbance of three. It had a higher growth rate at 25 degrees celsius and had higher densities at the colder temperatures. At 30 degrees it barely grew over the five day period. One fact to note is that the 25 and 30 degree trials of figure two shows six day trials, this is due to a lack of data from day three and thus it was made up in the sixth day data point. The trends which we are able to observe due to this in those two trials but not the 20 degree trial may affect the conclusions.

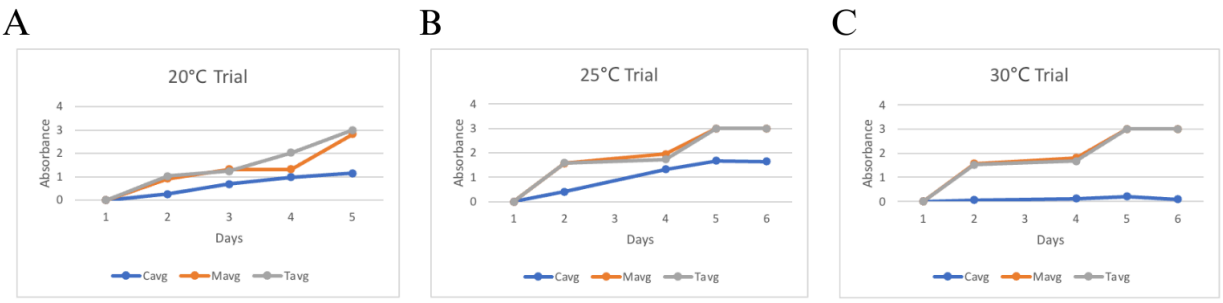

Figure 2. Average Daily Optical Densities at Different Temperatures. These graphs all represent the growth curves of each species over the course of five days. Averages of three trials is represented here and the trends are depicted. C is *Curvibasidium*, M is *Metschinkowia*, T is *Taphrina.*

1.3 pH Tolerance

The pH is another important factor to test for brewing so we used important brewing pH values and observed how the yeast species responded to them. Each of the tubes had the pH manipulated to reach each of the experimental conditions and then was incubated at 25℃. As seen with the error bars, the trials have incredibly similar results even with drastic shifts in pH. This consistency is compounded by the graphs being averages of three trials. YPD has a pH around 7 which is why we see similar results to the temperature graphs for all of the species except for *Tap*. *Tap* did not grow to the absorbance of 3 which we see in the temperature trials, but rather gets to slightly above one in the more acidic environments which indicates that it is not an acidophile. In contrast, *Curv* grew to its highest density in the pH 3 condition, which indicates that it is an acidophile. *Met* reached the highest density in all three conditions which indicates

that it grows well in a variety of environments. There was no statistically significant difference between the means of the three conditions (df_1=2, df_{total}=8) for the *Met* sp. (F=1, p<.422) or the *Tap* sp. (F=0.256, p<0.782). It can be concluded that there is a difference in the *Curv* sp. though. The means of the pH 3 trial and the pH 7 trial were significantly different, but the pH 5 trial was not significantly different from the other two conditions (F=6.912, p<0.028). This shows that while we can observe that there is a difference, we can only conclude that the means actually differ in the *Curv* trials and this is between the most extremes being tested. The other two isolates cannot be considered to have a difference in absorbance for the three conditions.

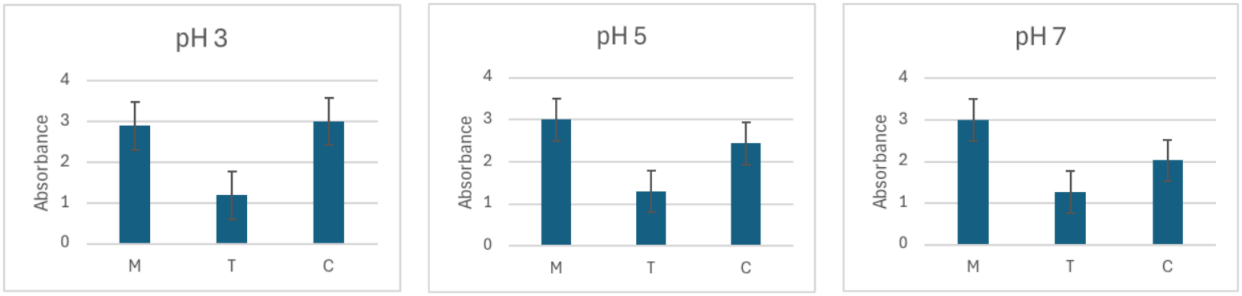

Figure 3. End Point Assay of pH Differed Media. Graphs show the average of three trials of growth for each species at three different pH levels. The end point was taken after six days of growth at 25 degrees Celsius and measured by optical density of the sample. Error bars were added to represent the standard error of each sample.

1.4 Ethanol Tolerance

These trials all take important brewing ethanol concentrations and replicate them to determine the ability of each species to be a brewing yeast. Each of the tubes had the ethanol concentration manipulated to reach each of the experimental conditions and then was incubated at 25℃. As seen in figure 4 there is little statistical difference between the ethanol concentrations for each of the species as the ethanol concentration increases. Numerically, the average endpoint optical density decreases as the ethanol concentration increases. There was no data which showed the max amount of ethanol each species could tolerate, but there is evidence that *Metschnikowia* is the most tolerant of ethanol and *Taphrina* is the least due to examination again the temperature trials where the growth was reaching the highest optical density we could observe. There was no statistically significant difference between the means of the three conditions (df_1=2, df_{total}=8) for

the *Tap* sp. (F=1.291, p<0.342). It can be concluded that there is a difference in the *Curv* sp. and the *Met* sp. The means of the *Curv* sp. for all three conditions were significantly different. (F=21.364, p<0.002). For the *Met* sp., there was a significant difference between the 3% and 5% conditions, but the 7% condition was not significantly different from its other two conditions (F=5.532, p<0.043). This shows that while we can observe that there is a difference, we can only conclude that the means actually differ in the *Curv* and the *Met* trials. In the *Curv* trials, there is a significant difference between each of the different conditions and therefore there is a decline in ethanol tolerance as the concentration increases. For the *Met* trials, we can observe that there is a difference between the 3% and 5% trials, but not the 7% trial. This means that there was significantly less growth from the 3% to the 5% but the mean in the 7% trial increased and was not significantly different. The *Tap* isolates cannot be considered to have a difference in absorbance for the three conditions therefore it has to be assumed that no change in growth was observed; we can see in the graphs though that growth was already minimal which indicates a very low tolerance across all conditions.

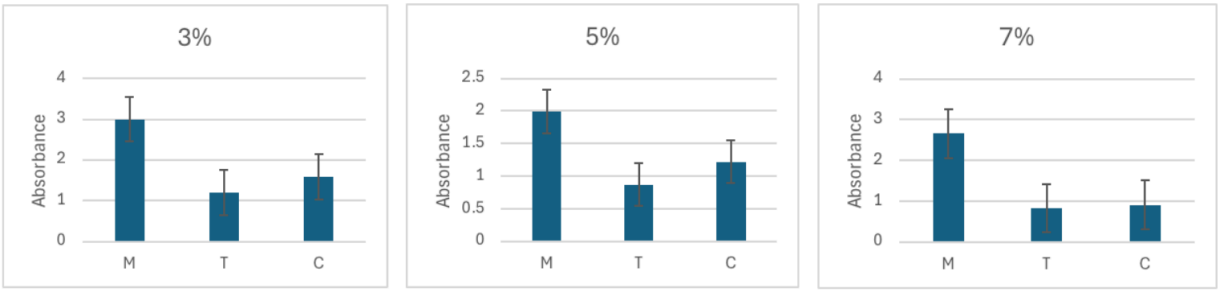

Figure 4. End Point Assay of Ethanol Treated Media. Graphs show the average of three trials of growth for each species at three different pH levels. The end point was taken after six days of growth at 25 degrees Celsius and measured by optical density of the sample. Error bars were added to represent the standard error of each sample.

1.5 Microscopy

The images collected all depict the cell wall in red and the DNA (nucleus) in blue. The observed morphology of the three isolates showed two common yeast characteristics: budding reproduction and a nucleus. This is consistent with the genetic testing which was conducted. Concentration of cells in culture can also be observed and while not easily seen from these

images, both *Met* and *Tap* were dense cultures while *Curv* was a sparse culture and this grouping of cells was largely alone on the slide.

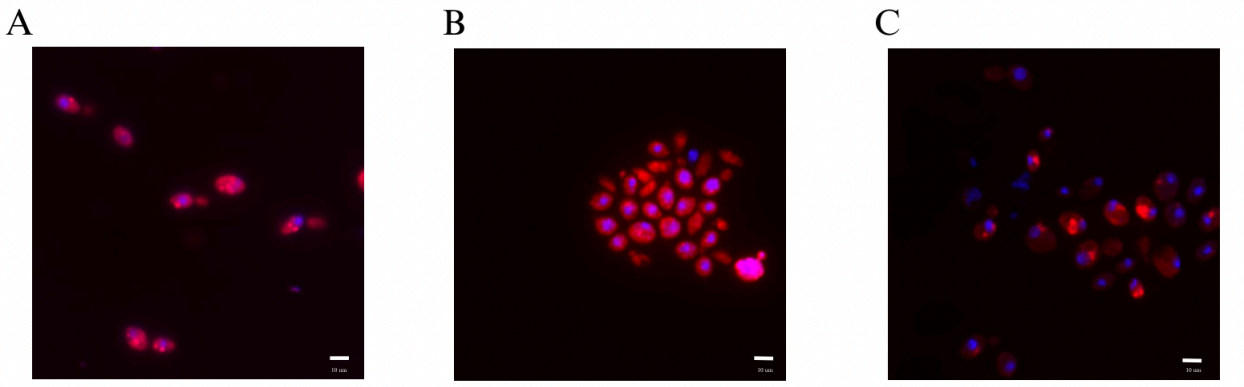

Figure 5. Images show A) *Metschnikowia sp*. B) *Curvibasidium sp*. C) *Taphrina sp*. under fluorescence microscopy using Mitoview 633 and DAPI dyes.

Discussion

The yeasts which I investigated all had different properties which were characteristic of their genus. *Metschnikowia* is a species which has already been investigated for wine making.[8] Previous studies have illuminated its abilities which include moderate fermentation and interesting enzymatic activities which allow it to be considered an alternative. *Taphrina*[9,10] has been cited to cause plant diseases such as leaf curl and survive cold conditions but has not been studied outside of these conditions. *Curvibasidium*[11] has not been studied extensively and while it has been isolated from wine grapes, not much is known about its ecological niche or its abilities and traits. Overall, the yeast culture collected in this trial which shows the most promise for brewing is the *Metschnikowia* sp. due to its temperature, pH, and ethanol tolerance. It shows promise for being a brewing yeast for multiple types of beer. *Taphrina* sp. does not seem to have any promise for brewing. This species is not tolerant of the conditions which we tested and therefore would not tolerate the brewing conditions well. *Curvibasidium* sp. also shows promise as a brewing yeast. It was not a quick-growing yeast and it did not reach a high density overall, but it is an acidophile. Since it thrives in acidic conditions and can tolerate pH well, it would be worth conducting further testing on its brewing potential. In the future, research could be conducted to determine the max pH and ethanol tolerance each species can tolerate. It would also

be important to test the hops tolerance each isolate has and the flavor and smell compounds each produces during fermentation.

Citations

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