Educational/Professional Partnership in the Development of a Novel Strain of S-11 Saccharomyces cerevisiae (*Brewing Yeast)*

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Background Information

Craft beers have revolutionized the beer industry. In 2018, the craft beer market made up 20% of the sales of beer in the United States, totaling over 26 billion dollars and experiencing significant growth during a period when many traditional breweries are reporting losses or shrinkage in their market values (Gaille, 2019). Survival in an increasingly competitive market is reliant on the ability to develop novel products that match the ever-evolving tastes of consumers. I will work with the Three Roads Brewing Company of Farmville and Lynchburg, Virginia to meet this goal using molecular biology. My institution has a preexisting relationship with Three Roads Brewery, as my research advisor has been doing similar but less intensive projects to teach students about the fundamentals of genetic engineering. The Brewmaster uses a strain of baker's yeast (Saccharomyces cerevisiae) called S-11 to generate a French Saison beer, a highly carbonated pale ale. My research will be working on modifying the S-11 strain to generate a French Saison beer that lacks fusel alcohols while still maintaining the complex flavor profile of the rest of the beer. Because fusel alcohols are "key contributors to the intoxicating effect [of alcohol products]," this request is very common in the brewing community (Xie, 2018). By removing these fusel alcohols, the Brewmaster will have a product that he feels will be more enjoyable for his consumers to drink.

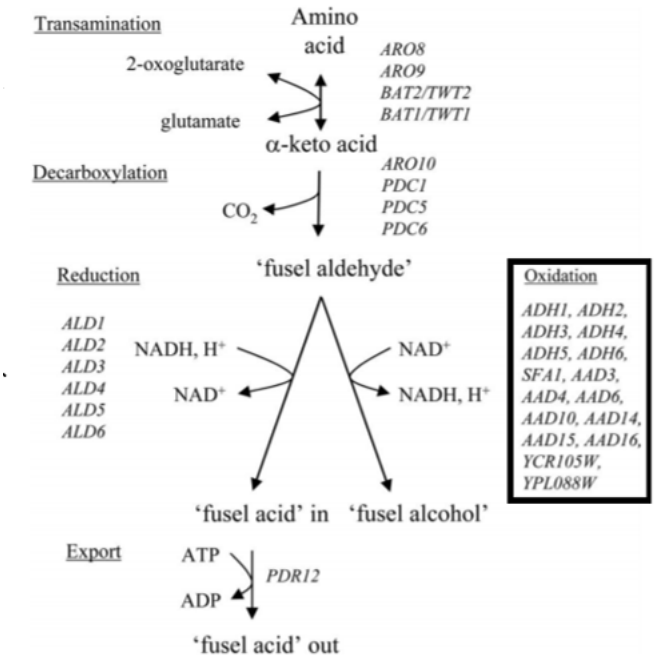

Figure 1. A simplified diagram of the Ehrlich Pathway. Enzymes participating in the Oxidation fork (boxed in black) convert fusel aldehydes to fusel alcohols. These genes will be the targets for my knockout procedures.

During the fermentation process, fusel alcohols are produced through a process called the Ehrlich pathway, which is normally responsible for the catabolism of amino acids (Hazelwood, 2008). There is a key divergence point within the Ehrlich pathway that is especially relevant to brewers during which the fusel aldehyde is either oxidized into a fusel alcohol or reduced into a fusel acid (see Figure 1, Hazelwood, 2018). Using molecular genetics to disrupt key genes in this pathway, I can eliminate relevant proteins in this process in order to force reduction as opposed to oxidation, and thereby eliminate all fusel alcohols.

Methods

PCR workup of homologous recombination dsDNA.
10 mL Master Mix
3 mL Forward primer
3 mL Backwards primer
1 mL Yeast template DNA (C9)
3 mL Sterile water

1) 95C for 2:00
2) 95C for 0:30
3) 55C for 0:30
4) 68C for 1:30
5) GOTO step 2, 34x
6) 68C for 10:00
7) 4C for ∞

Transformation and recombination procedure.

1) Generate an overnight culture of yeast cells, grow overnight with shaking
2) Pellet cells gently using a centrifuge
3) Resuspend in 7-8mL of a 10mM Tris-HCl, 1mM EDTA, and .1 M Lithium acetate solution
4) Add to 10µg of salmon testes DNA to each tube that will be used for transformation
5) Add yeast dsDNA prepared using the last procedure
6) Add 100µL of the competent yeast cells
7) Add 600µL of a 40% Polyethylene Glycol, 10mM Tris-HCl, 1mM EDTA, and .1M Lithium Acetate solution, incubate for 30 mins with shaking
8) Plate onto G418 plates.

SPME Fiber Insertion and Removal.

1) Unscrew the little screw using a Jewler's screwdriver
2) Then remove the metal end piece
3) Remove the black plastic piece from the metal holder, then take the needle of interest and thread it through the proper side on the black plastic piece
4) Insert the complex into the metal holder.
    a. Be exceptionally sure you do not move the plunger (the colored plastic piece) towards or away from the brass ring, lest you damage the fiber!!!!!!
5) Load the complex by putting the indented end of the plastic piece through the side of the metal piece where you unscrewed the metal stopper as shown below
6) Push the complex in until the brass ring on the fiber is flush with the metallic casing
    a. Again, do not move the plunger and the brass piece
7) Reattach the metal end casing to the end that now has a protruding needle
8) Reinsert the screw into the hole in the black plastic piece:

To remove the fiber, follow the steps exactly, except for step 3, where you should unscrew the fiber from the black plunger.

SPME Procedure.

1) Condition the fiber in the 250C oven for 1-2.5 hours. Use a funnel or similar object to hold the fiber upright.
2) Immediately insert into the solution to be tested (no headspace testing was done during this procedure)
3) Retract the fiber and remove from the sample vial.
4) Start the GC-MS batch file
5) When the timer has begun to count down insert the needle into the septum and press the plunger until it is in line with the *o* in the Supelco logo
6) Hold the fiber apparatus as still as possible until the timer reaches .5 minutes
7) Retract the fiber and remove the fiber apparatus from the GC-MS instrument entirely.

Data

Sample:	Weighted Reduction	Raw Reduction
AAD4P	-0.2628	-8.280855363
AAD4P.2	-0.0692	-9.142953359
AAD4P.3	-0.8686	-28.90385587
ADH1P	0.5557	-11.1904361
ADH1P.2	0.5492	-6.246842903
ADH1P.3	0.9692	-8.429028456
ADH2P	1.1527	-7.324465398
ADH2P.2	1.3509	-2.758040074
ADH2P.3	0.9917	-1.343660549
ADH3P	0.6220	6.159286075
ADH3P.2	0.4438	4.731436269
ADH3P.3	0.3643	-0.629735646
ADH4P	-0.3516	-5.977437279
ADH4P.2	-0.0893	-0.79137902
ADH4P.3	-0.8575	-9.277656171
ADH4P.4	-1.0379	-31.36891733
ADH6P	1.1239	-0.11786496
ADH6P.2	1.7337	-0.602795083
ADH6P.3	0.8403	-0.266038054

Results

The primary data collected during this procedure were readouts from the GC-MS. A table of GC-MS readouts and corresponding data analysis. Each yeast strain knockout was evaluated at least three times, with each sample being evaluated as soon as it was taken. The weighted reduction tab was calculated by taking the concentration readout for each relevant fusel alcohol from the GC-MS run on each sample, subtracting it by the readout for original samples, then divided by the readout for the original sample. The formula used is listed below:

$$\sum \frac{E - S}{S}$$

The unweighted column was acquired by the summation of all the fusel alcohol readouts and subtracted the sum of the fusel alcohols in the S11 strain. The reason the two columns were included is to demonstrate that the raw summation is not necessarily representative of an actual decrease in fusel alcohol concentration from the yeast waste product. The raw decrease in fusel alcohol concentration is not necessarily representative of a true decrease in fusel alcohol concentration, because the SPME fiber preferentially picks up large non-polar molecules to small polar molecules. For example, Phenylethyl Alcohol is one of the primary fusel alcohols observed in solution, it is a large polar molecule, and the raw data suggests there are solutions in which the Phenylethyl Alcohol concentration is higher than the ethanol concentration. However, it is much more likely that the ethanol concentration is about 100x the Phenylethyl Alcohol concentration in most solutions, but the SPME fiber preferentially adsorbs Phenylethyl Alcohol molecules.

While the expected fusel alcohols were isoamyl alcohol, 2-methyl-1-butanol, isobutyl alcohol,

and 1-Propanol (Windholz, 1983), but in reality the primary fusel alcohols I found were 2-Methyl-Propanol (or isobutyl alcohol), Butanol, 2-Methyl-Butanol, 3-Methyl-Butanol, and Phenylethyl Alcohol. Their retention times during the protocol used are listed below:

Alcohol	Retention Time (mins)	Average in S11
2-Methyl-Propanol	2.06	0.99
Butanol	2.30	6.50
2-Methyl-Butanol	3.00	26.96
3-Methyl-Butanol	2.96	18.38
Phenylethyl Alcohol	8.71	21.41

These alcohols appeared in varying concentrations. The average fusel alcohol readouts in the original (S11) strain is also included. It is important to note that the readouts are not a concentration readout, it is merely a readout of the relative concentration that gets adsorbed onto the SPME fiber.

The two strains that performed the best were the S11ΔAAD4 and S11ΔADH4. Both strains resulted in decreases in fusel alcohol production from the original strain. My next steps will involve the brewing of a Saison to see whether the beer produced from these strains is good for drinking!

REFERENCES

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