Designing a Novel Yeast Brewing Strain Using CRISPR-Cas9 Genome Engineering

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How do specific genes in yeast contribute to flavor profiles?

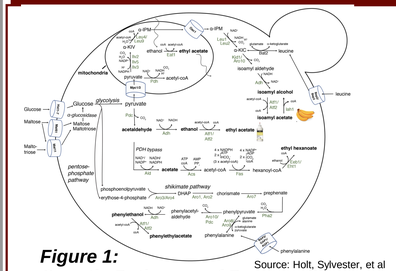


Figure 1: Genetic Pathways of Brewing Process

Source: Holt, Sylvester, et al

- During fermentation, yeast produce a number of aromatic compounds that contribute to beer flavor.
- Isoamyl alcohol is catalyzed by alcohol acetyl transferase (ATF1/ATF2) to form Isoamyl acetate, an ester with a banana aroma. Isoamyl Acetate Hydrolyzing Esterase (IAH1) drives the reaction backwards.
- Yeast strains for particular beer styles have evolved to produce specific, desirable flavors. High levels of isoamyl acetate is a hallmark of a great Hefeweizen, while being undesirable in many other styles.

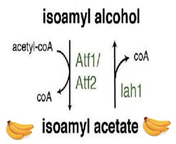


Figure 2: Isoamyl Acetate Process

- Took samples over the course of 4 days for 4 types of beer: Hefeweizen, Belgian Saison, California Ale, and San Diego Super to understand gene expression.
- Ran mRNA extractions to determine gene expression and an overhead GC to determine the isoamyl acetate levels.

Yeast strains and their flavor profile

Strain	Flavor Profile
WLP090 - San Diego Super Ale	Low Ester Producing/ Neutral
WLP001 - California Ale	Clean/Neutral
WLP565 - Belgian Saison Ale	Earthy/Spicy/Fruity
WLP300- Hefeweizen Ale	Banana and Clove

Figure 3: Beer Strains Flavor Profile

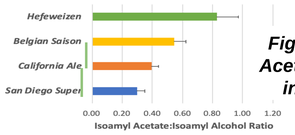


Figure 4: Isoamyl Acetate Production in Each Strain

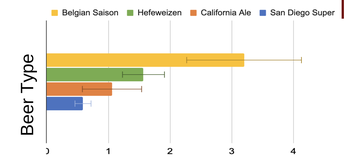


Figure 5: Relative ATF1 Gene expression

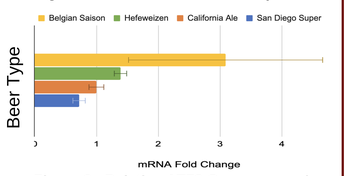


Figure 6: Relative ATF2 Gene expression

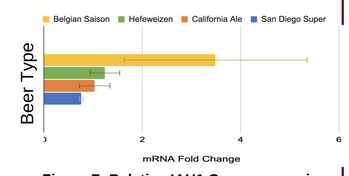


Figure 7: Relative IAH1 Gene expression

- The Hefeweizen strain has a significantly higher isoamyl acetate to isoamyl alcohol ratio
- mRNA expression fold calculated using $\Delta\Delta$Cq method.
- *ACT1* was used as a reference gene compared to California Ale as the control strain.
- **Figure 5**: There was significant difference between Belgian Saison and every other type of beer.
- **Figure 6**: There was significant difference between beer types.
- **Figure 7**: There was significant difference between Belgian Saison and every other type of beer.

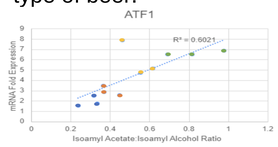


Figure 8: *ATF1* mRNA fold expression vs Isoamyl Acetate:Isoamyl Alcohol

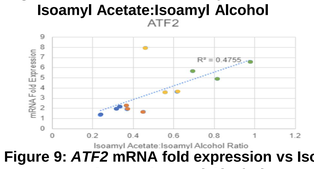


Figure 9: *ATF2* mRNA fold expression vs Isoamyl Acetate:Isoamyl Alcohol

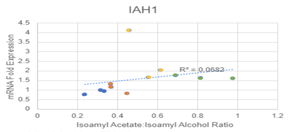


Figure 10: *IAH1* mRNA fold expression vs Isoamyl Acetate:Isoamyl Alcohol

- The figures 8, 9, and 10 represent XY scatter plots comparing mRNA fold expression to isoamyl acetate:isoamyl alcohol ratio.
- There is not a strong linear correlation when plotting any individual gene's expression vs. isoamyl acetate:isoamyl alcohol ratio.
  - ATF1 does have the strongest correlation for any individual gene.

How do ATF1, ATF2, and IAH1 work together?

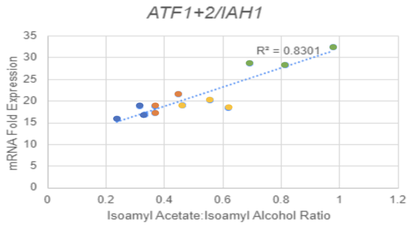


Figure 11: (*ATF1+ATF2)/IAH1* mRNA fold expression vs Isoamyl Acetate:Isoamyl Alcohol

- When factoring in the balance of *ATF1* & *ATF2* driving the forward reaction, and *IAH1* driving the reverse reaction, there is a strong linear correlation (r = 0.8301) when comparing (*ATF1+2*):*IAH1* ratio vs. isoamyl acetate:isoamyl alcohol ratio.
  - ATF1 has a relative good correlation of r=0.6021, but not as good as the gene fold expression vs isoamyl acetate:isoamyl alcohol ratio.

This means…..

1)The expression of any one gene is not indicative of isoamyl acetate production.

2) The relative expression of all three genes factored together is indicative of isoamyl acetate production. There is a strong correlation between ATF1&ATF2:IAH1 expression and Isoamyl acetate production.

Hypothesis:

By lowering (or completely removing) IAH1 production, there will be an increase in isoamyl acetate.

Modifying the Genes

- Now knowing this gene ratio, we aim to modify isoamyl acetate production by genetically modifying IAH1.
- **Goal**: Increase the amount of isoamyl acetate in beer by knocking out IAH1 via the use of CRISPR-Cas9.

Source: Wikipedia

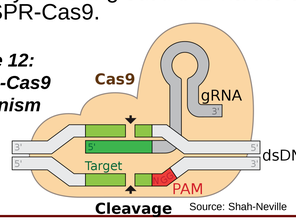


Figure 12: CRISPR-Cas9 Mechanism

Source: Shah-Neville

- CRISPR-Cas9 is a novel engineering technique that is able to change genes on a base precision level.
- It is independent of copy number variations.
- It will insert a stop codon and an mRuby gene into the IAH1 gene preventing the translation.
- This will prevent the transformation of isoamyl acetate to isoamyl alcohol.

Plasmid Design

- **Step 1: Designing sgRNA site**
  - Using E-Crisp, design the site that would be inserted into Cas9 in order to show the target site.
  - Using the web protocol, the sgRNA was designed to avoid the mutation found within the IAH1 sequence within our genome.
  - New primer was designed in order to work around the mutations found in:
    - 23T (ATT>ACT) missense mutation
    - A81A (GCC>GCT) silent mutation
    - K163N (AAA>AAT) missense mutation
  - Identified mRuby gene in one of the plasmids ordered in order to add to our sgRNA.
  - The following oligos were created:
    - Forward oligo: oJM120 – 5'-GACTTTGGCAAAGATCAGTATGCTCT-3'
    - Reverse oligo: 0JM121 – 5'-AAACAGAGCATACTGATCTTTGCCAA -3'
    - Annealed oligos
      - 5'-GACTTTGGCAAAGATCAGTATGCTCT-3'
      - 3'-AACCGTTTCTAGTCATACGAGACAAA-5'
  - The resulting sgRNA plasmid was added to benchling for the next steps.
  - Run a virtual digest to confirm and compare to actual digest.

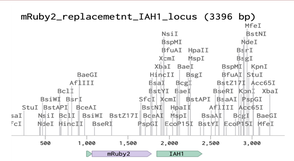


Figure 13: Plasmid for Insertion (linear)

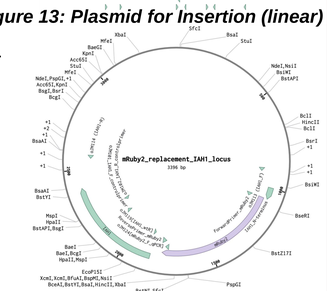


Figure 14: Plasmid for Insertion

Creating Plasmid for Insertion

- **Step 2: Prepping for CRISPR editing**
  - Prepare plasmids:
    - pYTK050, pYTK003, pYTK068, pYTK095, pYTK-DN1, pYTK-DN2, pYTK-DN4, and pYTK-053
  - Prepare Plates and Media
    - Grow up E. Coli and confirm competent cells
- **Step 3: Anneal sgRNA oligos**
  - Prepare 100 uM stock of oligos in molecular grade ddH2O
  - Mix 10 uL of forward and 10 uL of reverse oligo in PCR tube.
  - Anneal the oligos using the thermal cycler under the following conditions
    - 95 C for 5 min
    - 55 C for 15 min
    - 25 C for 15 min
    - 4 C forever

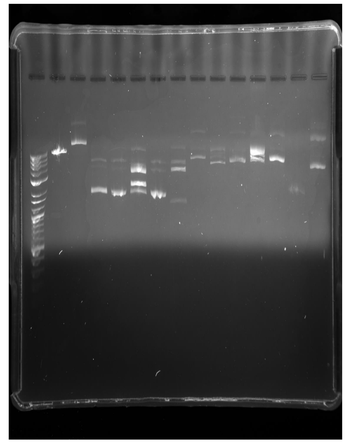


Figure 15: Confirmation of Plasmid on Gel

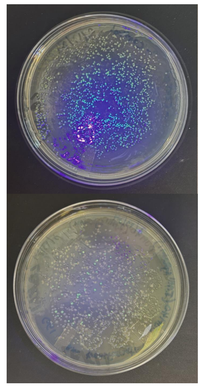
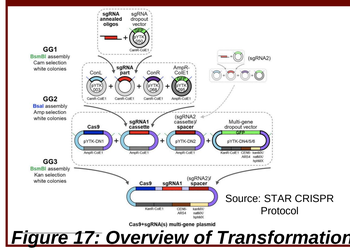


Figure 16: (a) negative control, (b) Golden Gate 1 Transformation product

- **Step 4: Run 1st golden gate reaction**
  - Create 2 reaction mixes: a negative control and the golden gate.
    - Normal reaction has the sgRNA
  - In each one, add
    - 1 uL 10x T4 DNA ligase Buffer
    - 0.5 uL T7 DNA ligase
    - 0.5 uL BsmBI-V2
  - Ran in thermal cycler under the following conditions:
    - 42 C for 1 hr
    - 16 C for 1 hr *(ligation step not included in NEB protocol for 1 insert, but can't hurt)
    - 60 C for 10 min
    - 80 C for 10 min
    - 4 C forever
  - Plate samples and preform extraction to see if the first editing work
  - Run a mini prep to confirm the knockout of GFP gene

Future Steps



Source: STAR CRISPR Protocol

Figure 17: Overview of Transformation Steps

- Run second and third golden gate reactions to insert plasmid into the bacteria
- Transform the plasmid into the yeast after confirmation it works via plate
- Brew beer and test to see if the gene of interest was seen
- Test Isoamyl Acetate levels and gene expression

Acknowledgements and Works Cited

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Scan above for references