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Bolstering the Cell Membrane of S. cerevisiae for Efficient Bioethanol Production from Lignocellulosic Biomass

Conary Meyer
Santa Clara Univeristy

Matt Kubit
Santa Clara Univeristy

SANTA CLARA UNIVERSITY

Department of Bioengineering

I HEREBY RECOMMEND THAT THE THESIS PREPARED
UNDER MY SUPERVISION BY

Conary Meyer & Matt Kubit

ENTITLED

Bolstering the Cell Membrane of *S. cerevisiae* for Efficient Bioethanol Production from Lignocellulosic Biomass

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By

Conary Meyer, Matt Kubit

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ABSTRACT

With the need for alternatives to fossil fuels becoming more prevalent, biofuels has become an increasingly attractive alternative. Traditional biofuel production was quickly halted as a result of its ethical complications, leading to the development of second-generation biofuels. This system utilizes plant waste instead of food as its starting material, allowing for rapid recycling of this widely available and cheap carbon source. This switch was, however, coupled with complications. Of those, the most prominent is the inevitable release of acetic acid resulting from the breakdown of the lignocellulosic waste. This acetic acid is challenging to neutralize or extract in a scalable manner, leaving it in high concentrations in the substrate fed to the yeast, greatly decreasing their efficiency. To combat that problem, we are implementing an acid resistance system endogenous to *E. coli* inside of the yeast to impart a similar resistance. The system functions on a cyclopropanation mechanism that decreases the permeability of the membrane to slow the diffusion of the acid into the cell. With this system, in conjunction with other complementary modifications, we look to increase the efficiency of second-generation biofuel production bringing it another step closer to playing a prominent role in our energy economy.

Acknowledgements

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List of Abbreviations

- **CFA**-Cyclopropane Fatty Acid
- **UFA**-Unsaturated Fatty Acid
- **PCR**-Polymerase Chain Reaction
- **Acetyl-CoA**-Acetyl Coenzyme A
- **BLAST**-Basic Local Alignment Search Tool
- **DNA**-DeoxyriboNucleic Acids
- **GFP**-Green Fluorescent Protein
- **SAM**-S-Adenosyl Methionine
- **SAH**-S-Adenosyl Homocysteine
- **ATCC**-American Type Culture Collection
- **MUSCLE**-Multiple Sequence Comparison by Log-Expectation

Introduction and Significance

Background

Alternatives to fossil fuels have been highly sought after for many years and though first generation biofuels offered promise, the detrimental impact that it would have on the food supply if implemented at large scale superseded its potential benefits. To compensate for this issue, industry has moved toward second-generation biofuels, specifically bioethanol, that uses organic waste instead of food as its substrate.

However, there are still numerous obstacles that must be surmounted to ensure its economic feasibility. The primary bottle-neck of this potentially lucrative industry is that the digestion of the lignocellulosic waste generates inhibitors, specifically weak acids, which lead to a decrease in the overall metabolic yield possible from the yeast *S. cerevisiae* that is responsible for the fermentation. (Loow et al., 2015)

The most detrimental of these weak acids is acetic acid, which results from the unlinking of the acetyl groups that attaches the hemicellulose in the biomass. This hydrolysis is necessary to generate the simple sugars that the yeast requires for fermentation and so acetic acid generation is inevitable (Doğan, Demirci, Aytekin, & Şahin, 2014). The acetic acid, as it is lipophilic, can pass through the lipid bilayer of the yeast and, once inside the cell, it dissociates and causes detrimental effects on the cell. There are internal mechanisms designed to handle the anion and accompanying proton but this process is metabolically taxing on the cell and is bounded by the export rate of the transporters. If the acetic acid levels exceed certain levels it begins to dramatically slow the growth kinetics and will eventually lead to apoptosis. As a result of this inherent issue, more resilient yeast must be engineered to properly make use of this abundant raw material. Developing tolerant strains of yeast is what our senior project is designed to do.

Review of Field

There are methods that have been employed to impart greater acetic acid resistance such as the modifications disclosed in patent US2015/0079652. This patent includes genetic modifications, specifically the alcohol/aldehyde dehydrogenase and the acetyl-CoA synthetase, which allows for the conversion of acetic acid into acetyl-CoA and then acetyl-CoA into ethanol. This serves to handle the acetate anion that results from the dissociation of acetic acid to alleviate its deleterious effects, but is unable to compensate for the free protons that cause the decrease in cytosolic pH. (Angeles, 2015)

Instead of targeting the acetate directly, Zheng et al. 2013 turned to modifying the membrane to limit the passive diffusion of inhibitors. They utilized the elongase gene that leads to an alteration in the lipid biosynthetic pathway resulting in an extension of the fatty acid tails. This increased length creates a thicker membrane leading to a longer path length for incoming inhibitors, such as ethanol or acetic acid. The increased distance through the membrane made its entry less thermodynamically favorable. This modification leads to a marginal increase in ethanol production and raises concerns for the other molecular effects it could have on proper protein integration into the membrane (Zheng et al., 2013).

Utilizing a similar approach, the teaching in WO20151/03002 makes use of several other lipid modifications to introduce isobutanol resistance to yeast. They also utilized the elongase gene as well as the genes encoding the fatty acid desaturase. All of these genes serve the function of decreasing the permeability, while also decreasing the fluidity of the membrane. This much like the previous setup, leads to issues of protein integration (Dyk, 2015).

Aside from the direct lipid modifications there have been studies specifically looking for other forms of transport that allows for the entry of acetic acid. There have been reports of an aquaglyceroporin, encoded by the Fps1 gene, that allows for the passive diffusion of acetic acid into the cell. Mallapour et al. 2007 preformed gene knock-outs to elucidate the Fps1 gene's role in acetic acid tolerance and found that it was the cause for the initial influx of acetic acid (Mollapour & Piper, 2007). They also showed how the mitogen-activated protein kinase (Hog1) will rapidly phosphorylate the porin leading to its ubiquitination and eventual degradation. It was

at first suspected that the elimination of this porin would render cells resistant to acetic acid but it was later discovered that it only imparts initial resistance because the acetic acid is still able to pass through the membrane making it an undesirable gene target for tolerance engineering, as discussed in Lindberg et el. 2013 (Lindberg, Santos, Riezman, Olsson, & Bettiga, 2013).

Project Goals, Objectives, and Expected Results

Herein, we describe the use of a cyclopropanation mechanism that can be implemented as a means to decrease permeability of yeast to impart resilience to acetic. This decreased permeability could then allow for an increase in efficiency of second-generation biofuels. The initial goal of our research is to prove that we are capable of imparting acetic acid resistance in any organism using this mechanism to confirm that we can reproduce the results found in other studies. To conduct this initial set of experiments we chose to engineer *E. coli* as it endogenously expresses the required enzyme and is easy to engineer. We will extract the gene required to carry out this reaction and then over express it and test for acetic acid tolerance. Once this is confirmed to be effective, we will move to expression in the yeast *S. cerevisiae*, as it is the prevalently used yeast for biofuel production. This will require the construction of a codon-optimized sequence of this same gene being cloned into a yeast expression vector. This plasmid will then be used to transform into the yeast and then tested in acetic acid. If once again successful, we will shift to scale up our cultures and begin analyzes on the effect of the modification at the large scale.

Backup Plan

As this project requires the use of an enzyme not endogenously expressed inside of *S. cerevisiae*, it is possible for the protein to have numerous auxiliary, and potentially deleterious effects. For this reason, we want to establish other modifications that could be employed at a later time if it is found that the modification yields negative results. The modifications that we could implement have already been discussed in the early section. We could shift to the other known lipid modifications, such as the elongase and desaturase, and create a dual expression system to minimize the permeability of the membrane. If this too, fails to succeed, we could then move to implementing the knockout of the aquaglyceroporin, Fps1, to achieve the resilience and study its effect on acetic acid resistance, as well as on ethanol production. (Zheng et al., 2013)

Significance

The progression toward a future powered by biofuels has been a series of small incremental steps that has allowed it to reach its current state. These steps of increased efficiency, though apparently small, have monumental impacts on the field due to the scale that it must be implemented at. We see this modification as one of those pivotal steps that will bring green energy that much closer to the hands of the world. With the ability to decompose lignocellulosic waste without having to try and neutralize the released inhibitors, it will open the door to cheaper biofuels for the world making it a stronger contestant against fossil fuels. (Loow et al., 2015)

Team and Management

Our team is comprised of two students: Matt Kubit and Conary Meyer. Matt is a bioengineering senior who has conducted several years of research on plants, giving him insight into lignocellulose and its degradation pathways. Conary Meyer is also a bioengineering senior, with numerous years of research experience, specifically working with bacteria and cloning. This skill set has equipped him with the necessary knowledge to construct and use the necessary plasmids.

Our management is made up of two professors: Drs. Tracy Ruscetti and Maryam Mobed. Dr. Ruscetti functioned as the mentor for the molecular biology side of the project. She worked to make sure that we remained on track with specific emphasis on the intentionality behind experiments to limit superfluous activity by the team. Dr. Mobed was the mentor for the eventual scale up and simulation aspect of the project. Though the project did not reach this point, she assisted in the further understanding of the diffusion modification that we were implementing.

Budget

Our team received $1,000 from the School of Engineering, as well as $2,000 more from the Roelandt's Grant. This money was used to purchase the necessary strains, plasmids, and reagents required to carry out this project. The specifics of the budget can be found in the Appendix.

Timeline

1. Fall Quarter

1. Extract the cyclopropane fatty acid synthase gene from *E. coli* genome
2. Clone the CFA synthase gene downstream of an inducible high expression promoter within a plasmid backbone
3. Transform into T7 protein expression *E. coli* cells
4. Assess toxicity of the over expression
5. Develop and run an acetic acid shock assay
6. Run a gradient of acetic acid concentration to determine baseline tolerance
7. Conduct acetic acid shock assay on the recombinant bacterium and assess whether or not it has improved growth kinetics compared to the control

2. Winter Quarter
    1. Screen potential *S. cerevisiae* strains to choose the optimal strain
    2. Run a gradient of acetic acid concentration on the chosen strain to establish a baseline tolerance
    3. Build an *E. coli/S. cerevisiae* shuttle vector with the CFA synthase downstream of a constitutive high expression promoter.
    4. Create competent yeast and then transform with the recombinant plasmid
    5. Run the acetic acid tolerance assay on the transformants
    6. Analyze data and assess use in scale up applications
3. Spring Quarter
    1. Troubleshoot potential issues CFA expression
    2. Transition into larger scale experiments

Chapter 1 – Proof of Principle in *E. coli*

Introduction

Review of possible membrane modifications

After extensively reviewing the literature we compiled a list of potential membrane modifications that result in the desired decrease in permeability. A detailed description of the strong contenders was described earlier in the review of the field. The strongest contenders from our search were the elongase and desaturase but both of these modifications can have dramatic ramifications on the membrane. The primary consequence is the decreased ability for protein integration into the membrane. By increasing the length of the fatty acid tails in the membrane, it increases the length of the hydrophobic region that can result in that region exceeding the length of the hydrophobic region of transmembrane proteins. This will lead to the instability of that protein in the membrane and potentially disrupt its function (Zheng et al., 2013). Desaturases also result in decreased integration of proteins as they greatly decrease the fluidity of the membrane. This decreased fluidity can result in the improper assimilation of protein bound vesicles targeted to the membrane as well as improper dispersion of those proteins (Bogdanov, Aboulwafa, & Saier, 2013; Dyk, 2015). To avoid these issues, we turned our to attention to a unique modification known as membrane cyclopropanation.

Cyclopropanation Mechanism

Cyclopropanation reactions have been observed in a variety of bacteria, but has been clearly elucidated in *E. coli* (Chang & Cronan, 1999). The reaction specifically converts an unsaturated fatty acid into a cyclopropane fatty acid through the addition of a carbon at the double bond within the fatty acid tail. This carbon is pulled from the methyl donor S-adenosyl methionine to generate a cyclopropane ring protruding from the fatty acid chain. The reaction and its mechanism are displayed in Figure 1.1.

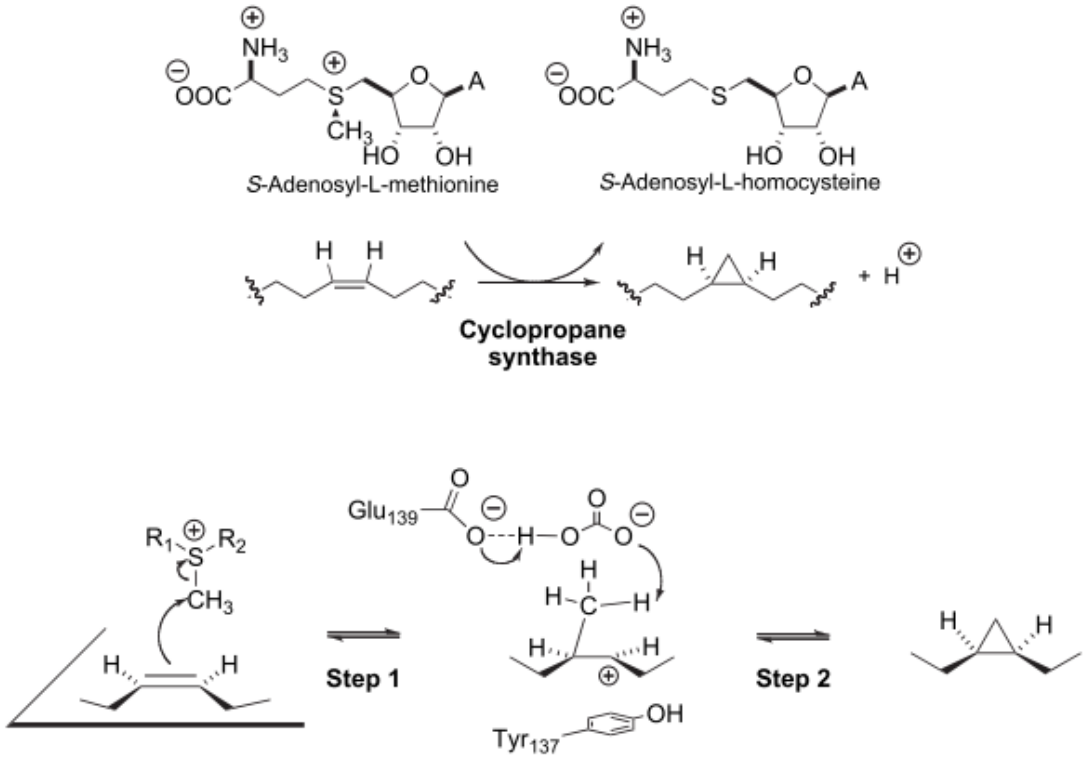

Figure 1.1: Cyclopropanation reaction of *E. coli* cyclopropane synthase (Guangqi, Lesage, & Ploux, 2010)**.**

The addition of this carbon results in several favorable conditions that were, until recently, viewed as antithetical. In a recent computational study of the dynamics of cyclopropane lipids, Poger et al. elucidated the unique effects of this cyclopropanation. It was found that the formation of this strained cyclopropane group resulted in the maintenance of the kink in fatty acid tail of the unsaturated fatty acid. Though the angle was found to be consistent, the ring structure caused the tail to remain fixed opposed to the "flexible" double bond that was there previously. It was discussed that it was the flexing of the unsaturated fatty acid tails that served as the primary entry way for small chemical species, like acetic acid. The strained ring locks the tail in place and limits entry. It is this phenomenon that explains the perceived decrease in permeability that we were interested in (Poger & Mark, 2015).

This fixed kink also maintains the area per lipid that the previous unsaturated fatty acid had. This allows for the easy passage of lipids around one another in the lipid bilayer. The membrane fluidity allows proteins to integrate into the membrane and migrate as needed (Figure 1.2).

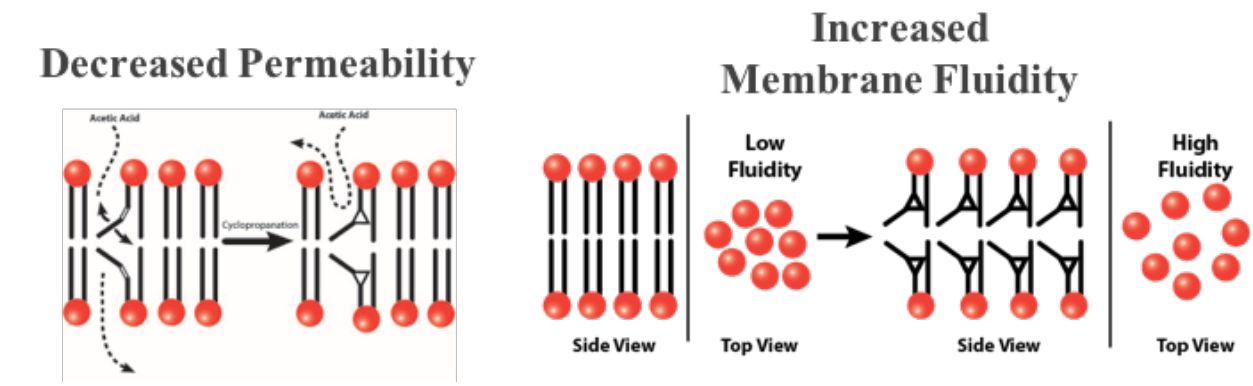

Figure 1.2: Diagram depicting the effects of a cyclopropanated membrane.

The protein required to cyclopropanate the membrane is an enzyme known as the cyclopropane fatty acid (CFA) synthase. This is a cytosolic protein has a lipophilic domain that allows its association with the membrane and subsequent catalysis of the cyclopropane reaction detailed above (Figure 1.2, Chang & Cronan, 1999). We chose the overexpression of this protein to increase cyclopropanation of the membrane and impart the acid resistant phenotype (Figure 1.3).

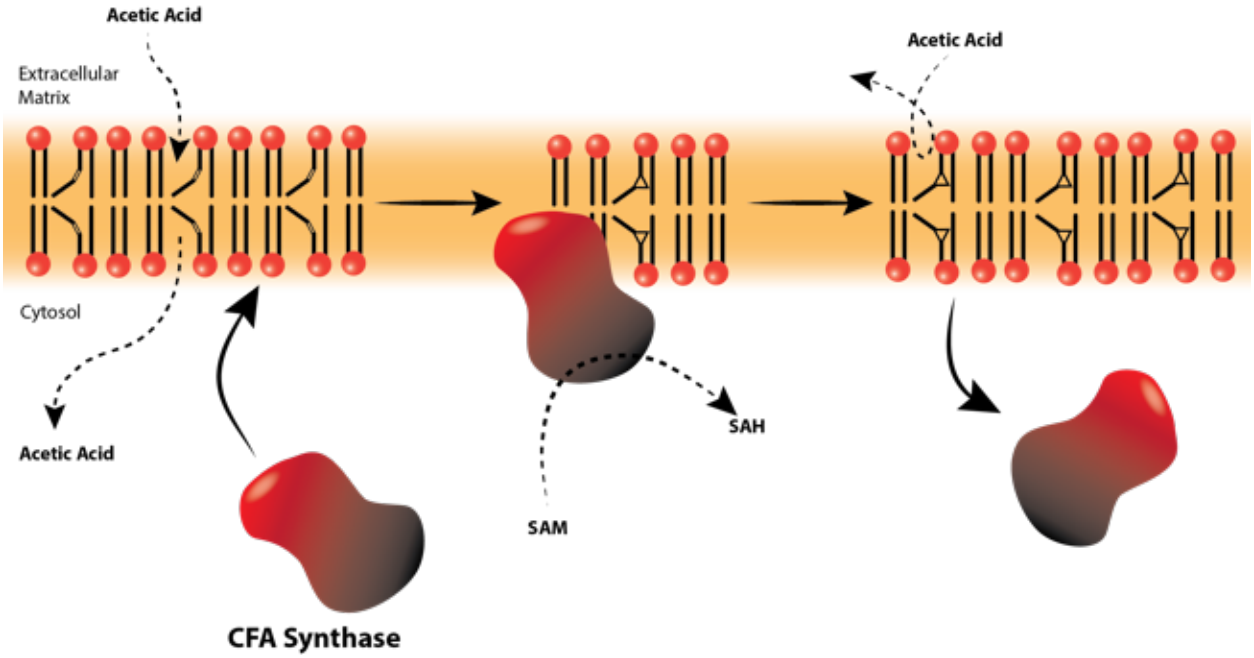

Figure 1.3: Diagram depicting CFA synthase mediated cyclopropanation of the cell membrane.

Utilization of CFA

With a comprehensive understanding of the CFA mechanism and the requirements of the system we shifted our attention to the steps required to utilize it. Our first step was to determine if the overexpression of the CFA synthase would be toxic. We performed this proof-of-principle experiment by overexpressing CFA within the genetically malleable and readily available *E. coli* to validate our design.

Under acid stress conditions, *E. coli* will express CFA. We can validate our system by continuously overexpressing CFA to test toxicity in *E. coli*. In this phase of our project, our goals included testing the toxicity of overexpressing CFA and increasing the tolerance of *E. coli* by overexpression of CFA.

In the second phase of our project, we will express CFA within *S. cerevisiae.* Currently, existing technologies to mitigate the impact of acetic acid on biofuel production of *S. cerevisiae* use pretreatment methods and the addition of base into the bioreactor to deal with the excess acid. These alternative technologies come with drawbacks that reduce efficiency of biofuel production. We focused on engineering the organism to increase acid tolerance and therefore increase efficiency. Our desire is to modify the plasma membrane with CFA to increase its tolerance. Other genes also alter the plasma membrane such as the desaturase and elongase enzymes (Zheng 2013). These gene targets have already been researched and patented but we believe that CFA has a possibility of playing an important role in acidic tolerance due to its steric hindrance on acidic molecules.

Design Description

The process of overexpressing CFA within *E. coli* involves gene expression. The gene, CFA, was cloned into a bacterial vector. The vector contained a bacterial-specific inducible promoter which up-regulates expression of the gene of interest. The vector also contained sites such as an origin of replication, selectable marker, and transcription terminator. The origin of replication ensured that the replication of the plasmid occurs to allow the continual expression of the gene while passing on the plasmid to the next generation of cells. The selectable marker allowed for the selection of cells only containing the plasmid containing CFA. The transcription terminator stopped the RNA polymerase from transcribing anything past the CFA gene.

The process of transformation allows foreign DNA into the cell of an organism. For the focus of overexpression of our gene, the goal was to allow the engineered plasmid into cells. Antibiotic resistance encoded on the plasmid selected for those cells that successfully incorporated the plasmid DNA. We confirmed transformation by running a colony-PCR. This process amplified the CFA region within the plasmid using primers complementary to the vector. A positive result from the colony-PCR yielded an amplicon the size of the gene, meaning the CFA gene was successfully cloned into the vector. A negative result from the colony-PCR would have yielded an amplicon much smaller than the CFA gene, meaning the gene was not within the plasmid.

We tested acetic acid tolerance by exposing *E. coli* harboring the plasmid containing the CFA gene to an acetic acid. We determined the optimal concentration of acetic acid, one that effects the growth rate yet allows the culture to go through the growth cycle. Once the concentration had been determined, the acetic acid growth assay was devised.

The acetic acid growth assay will involve continuously inducing the expressing of CFA in the engineered E coli strain along with control E coli strains. The cultures will contain 3mL of LB media and inoculated with *E. coli* containing CFA or a control GFP plasmid at similar starting concentrations (measured by OD_{600}). After an hour of growth shaking at 37 degrees Celsius, a 1:2 dilution was done to make the final concentration of acetic acid as experimentally determined. Optical density was measured at a wavelength of 600nm using the Genesys 20 every 30 minutes to determine the growth of the cultures. The Genesys 20 ensures sterility due to its ability to read the optical density of the inoculation tubes that our used in our growth assay.

Supporting Analyses

Initial experiments testing our *E. coli* with CFA and with a control plasmid showed almost uniform growth patterns. Testing under no acetic acid conditions expressed the addition of IPTG, which induces transcription of CFA or GFP in our *E. coli*, showed a 3% decrease in growth and saturation level compared to uninduced cultures.

Expected Results

We expected the growth of *E. coli* with the overexpression of CFA under acetic acid conditions to have a shorter lag phase, increased growth rate, and a higher final saturation level CFA's ability to inhibit acetic acid from passing through the plasma membrane. We measured these parameters by analyzing the OD600 values taken from our acetic acid growth assay. Lag phase was determined by analyzing the timeframe where the cultures had only minimal doubling time, in comparison to the rest of the growth cycle. Log phase and growth rate was measured by doubling time, calculated by using the OD600 values and converting to cells per milliliter. With an OD600 value of 1.0, there are 8×10^8 cells of *E. coli* per milliliter. The culture saturates when nutrients run out or waste products build up. Saturation occurs when the slope between two time points approaches zero. The concentration at which the slope approaches zero is considered the saturation level.

Materials and Methods

Materials

LB (Luria Bertani, 10g/l tryptone, 5g/l yeast extract, 10g/l NaCl, pH = 7.5) broth was the media we used to grow E coli. 2% agar was added to make agar plates. Kanamycin sulfate, the selectable marker, from was ordered from Sigma-Aldrich. One Shot Top10 Chemically Competent *E. coli* was used for the transformation and used to extract the genomic DNA of *E. coli*. A bacterial genomic mini-prep, Pure-Link Genomic DNA Prep from Invitrogen was used to extract out genome of *E. coli*. The extraction of CFA from the genomic DNA required Taq Polymerase 2X (Thermofisher), Barnstead Ultra-Pure Water and Forward_CFA (ATTCGAAATCCGTACATCCA) and Reverse_CFA (CTACTATTACTTATCTACT) primers were ordered from IDT. Agarose was purchased from Sigma-Aldrich. The Electra Cloning Kit was ordered from DNA2.0 with the pD411.mal vector used for the cloning process. S.O.C. media was ordered from Thermofisher. The following confirmation primers were used Forward_CFA_Plasmid (CTTAAGCGGTATCGATCG) and Reverse_CFA_Plasmid (GTACGTAGGACCTAAACG) from IDT. IPTG and Glacial Acetic Acid was ordered from Sigma-Aldrich. The Genesys 20 Visible Spectrometer from Thermofisher was used along with 15mm test tubes for OD600 measurements.

Methods

The CFA gene was cloned out of *E. coli* genome by first performing a genomic prep on cultured *E. coli*. TOP10 *E. coli* was grown overnight in 3 milliliters of LB media. The following day, a genomic mini-prep (Pure-Link Genomic DNA Prep from Invitrogen) was performed on the saturated culture. Using the genomic DNA as the template, a polymerase chain reaction was performed to amplify the CFA gene. The amplification primers were homologous to the CFA coding sequence overhangs to clone into the vector. Template DNA was added for a total of 20ng. Primers were added for a final concentration of 1 micromolar. The reaction was run at 95 degrees Celsius, then 30 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 60 seconds at 72°C, before a final extension of 7 minutes at 72°C and then holding at 12°C. A 1% agarose TAE gel was run to analyze the amplicon of the polymerase chain reaction to ensure the reaction completed as expected.

For the cloning procedure, make a cloning mixture to add into the competent *E. coli.* The cloning mixture will be 20ng of DNA, 1 mL of Electra Buffer, 1mL of vector, 1mL of Electra Enzyme, and 15mL of Water. Add in 1 mL of the cloning mixture into 20uL of chemically competent *E. coli*. We incubated the mixture on ice for 30 minutes, heat shocked at 42°C for 30 seconds, then placed on ice for 5 minutes. The cells were recovered in 950mL of S.O.C. media into the mixture. And shake at 250rpm at 37°C for 30 minutes. In LB + Kan (50ug/mL) plates, plate the transformed cells in 10-fold dilutions. Incubate at 37°C for 24 hours and check for transformants.

From 0 mM to 100 mM of acetic acid within LB was tested in increments of 5 mM in 3 mL cultures. Cultures were inoculated with the same volume of overnight *E. coli*. The OD600 values were measured every 30 minutes to determine growth. 10mM of acetic acid was determined to be our experimental concentration.

The acid shock assay involved incubating both *E. coli* with CFA and *E. coli* with GFP (same plasmid substituting GFP) in 10 mL of LB + Kan for 24 hours within a 250-mL Erlenmeyer flask at 37°C shaking at 225rpm. Basing off the OD600, we calculated the volume of the culture needed to make a starting OD600 of 0.100 for 3 mL and inoculated triplicates for each variant. Each culture was grown in 3 mL of LB + Kan with the addition of 1mM of IPTG. Again shaking

at 225rpm at 37°C, the cultures were taken out every 30 minutes to measure their OD600. After 60 minutes, the cultures were diluted 1:2 in 3mL of 20mM acetic acid + LB + Kan for an acetic acid shock at a final concentration of 10mM. To remain at a culture of 3mL, 3mL of the now diluted culture was taken out to maintain consistency. Time points every 30 minutes for OD600 readings were taken until the cultures had reached saturation.

Results

The PCR reaction to extract out the CFA gene resulted in a 2% gel with a band at roughly 1200 base pairs in comparison to a 100 bp ladder from Invitrogen**.** This band is the correct size considering the CFA gene is 1152 base pairs.

The acetic acid gradient experiment can be seen in the data presented in **Figure A1.** At 10mM acetic acid, E coli growth is inhibited (**Figure 1.1**). In comparison to the 5 mM culture, the 10mM is growing 2% decrease in growth rate. But in comparison to 20mM and higher acetic acid concentration cultures, the 10mM is growing at a faster rate while showing the effects of hindrance of acetic acid.

Once the experimental concentration of acetic acid was chosen, the acetic acid growth assay was planned to test the effect of CFA within *E. coli*. The IPTG was added with the LB + Kan before inoculation to induce CFA expression immediately. After the acetic acid shock test, **Figure 1.2** shows the overall growth cycle of the cultures. **Table 1.1** shows the lag time, log phase, and saturation level with statistical significance.

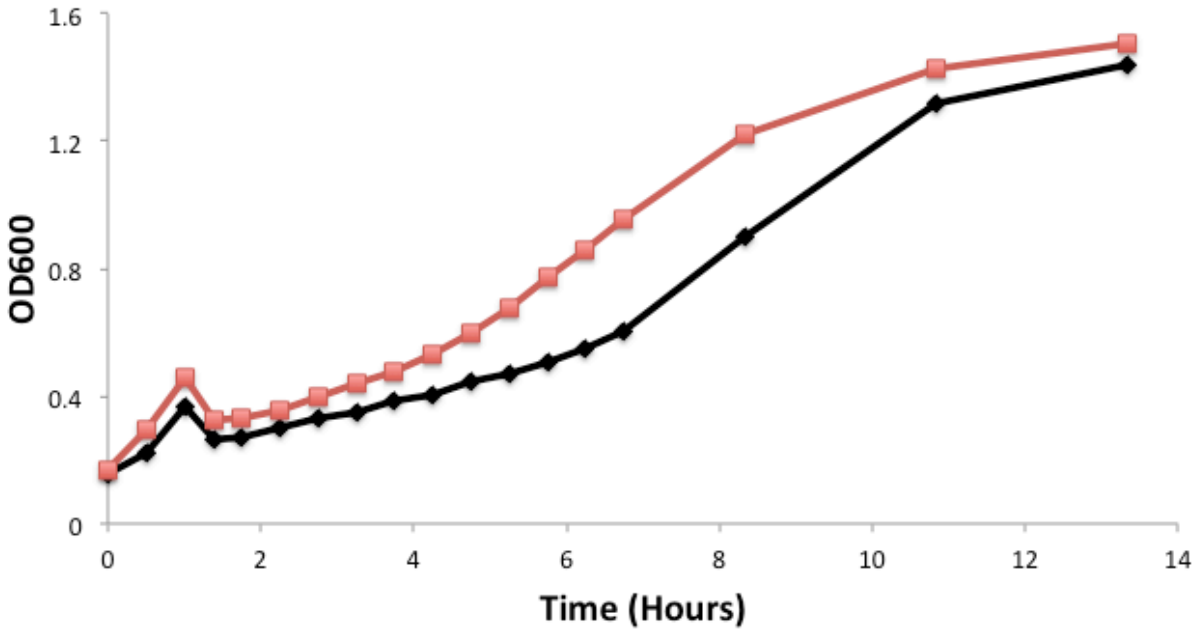

Figure 1.2: *E. coli* acetic acid shock assay. Red Squares represent the CFA mutants and black diamonds represent the control culture. (n=3)

Table 1.1: Growth kinetics analysis of *E. coli* acetic acid shock assay.

	10mM Acetic Acid		
	Lag Time (Hours)	Growth Rate (Cells/mL*hour)	Saturation (OD600)
CFA	4.25	1.36 E+08	1.51
Control	6.75	1.42 E+08	1.44
Percent Difference	-37.0	-4.2	4.8
p-Value	0.044	0.490	0.020

The drop in the OD600 at time 60 minutes is due to the dilution to add in the acetic acid. There is an overall 37% decrease in lag time and 4.8% increase in saturation level of CFA in comparison to the control. The lag time of CFA within this experiment is 4.25 hours in comparison to the control's 6.75 hours with a p-value of 0.044, a difference of 2.50 hours. The growth rates between the two are 4.2% different with CFA at $1.36x10^8$ cells per mL hour in comparison to the control's $1.42x10^8$ cells per mL hour, with a p-value of 0.490. CFA had a slightly higher saturation level with an OD600 of 1.51 in comparison to the control's 1.44, a 4.8% difference.

Discussion

Our results suggest that the expression of CFA has a beneficial effect on the growth parameters in the presence of acetic acid. The most significant impact on E coli growth kinetics was observed in the decreased lag time. By decreasing lag time by 37%, the overall growth cycle is shortened, which can increase the efficiency of bioreactors and thereby increase profit margins. The overall growth rate is not effected by CFA expression suggesting little in the way of negative effects.

Wild type *E. coli* expresses CFA under acetic acid stress but constitutive overexpression of CFA improves the growth cycle of *E. coli* within acetic acid conditions. Increased levels of CFA increases the organism's tolerance of the negative effects of the acidic conditions. The eventual overlap of the control's growth rate shows that *E. coli* is able to survive within these acetic acid conditions, but the early expression of CFA improves its growth cycle by minimizing the lag time.

Summary & Conclusion

The goals for the first phase of our project were successfully completed. The expected growth of *E. coli* with the overexpression of CFA under acetic acid conditions had a shorter lag phase and higher final saturation level, but fell short of our expectation of having an increased growth rate. Although the increased growth rate within the control may have been due to the endogenous expression of CFA improving its growth while our engineered *E. coli* consistently overexpressed CFA, possibly creating a metabolic tax on the system.

The successful decrease in 37% of lag time for the overexpressed CFA construct while maintaining a similar growth rate is promising. This proves the possible beneficial effects CFA can have on an organism's ability to tolerate acidic environments.

To continue on with our project, we will be moving to the next phase where we will be implementing CFA into the yeast *S. cerevisiae* to focus on bioethanol production.

Chapter 2 – Transition to Yeast Expression

Introduction

As stated before, yeast is an effective producer of ethanol. Yeast is also oftentimes used in bioreactors to create biofuels such as bioethanol. This process involves taking simple sugars from a biomass, and harnessing the energy to create bioethanol. This process has been used for centuries, possibly longer, for baking and creating wine and beer.

As we observed in E coli during phase 1 of our project, we wanted to use *S. cerevisiae* to overexpress CFA to increase its tolerance to acetic acid. This required many of the same experiments as done in *E. coli* to test the effects of CFA on yeast. In doing so, we ran a similar growth assay on yeast with and without CFA, in conditions involving acetic acid and no acetic acid. These experiments will test the effects CFA has on the organism as a whole, and to see if it has any effect on the organism's growth cycle within acetic acid conditions.

Design Description

Our process of overexpressing CFA within *S. cerevisiae* was done in the same manner as overexpressing CFA within *E. coli*. We cloned a plasmid to overexpress CFA and then transformed into the organism. In this case, the vector will contain a eukaryotic-specific constitutive promoter, which will constitutively overexpress CFA in yeast. The vector will still contain the origin of replication, multiple selectable markers, and a transcription terminator.

The process of cloning again followed the Electra Cloning System. Then, the transformation was first done within *E. coli* using the same method as before. Then, the successful transformed colonies were cultured and the plasmid was extracted. Once purified, the plasmids were verified by restriction digest. The restriction digest gel expressed the correct bands, meaning the plasmid was transformed into yeast using a transformation kit specifically designed for yeast. The transformed yeast was tested with a colony-PCR, to confirm the plasmid contained the CFA gene.

Like Phase I of our project, the yeast underwent an acetic acid gradient growth assay to determine a viable acetic acid concentration to test our construct at. Once the acetic acid concentration was determined, the acetic acid growth assay was performed.

In a very similar manner to the *E. coli* growth assay, OD600 was used to measure the number of cells within a culture and time points were determine growth rates under acetic acid and non-acetic acid presence. The acetic acid was present at the start of the growth assay this time around, as CFA was constitutively expressed. The volume of the cultures was kept the same (3 mL), the media was Synthetic Complete Medium, leucine was left out in order to maintain selection, and the temperature was changed to 30°C.

Expected Results

After analyzing the *E. coli* acetic acid shock assay, the expectations for yeast were similar. We expected yeast with CFA to have a decrease in lag time, similar growth rate, and a slightly higher saturation level in comparison to yeast without CFA. The yeast-CFA construct may perform better than the *E. coli*-CFA construct, considering *E. coli* endogenously expressed CFA. These results were determined in the same manner as the previous growth assays in Phase 1 of the project. The OD600 values were taken at intervals to measure the growth of each culture and the lag phase, growth rate and saturation levels were determined based upon the analysis of these raw values. With an OD600 value of 1.0, there are 1.75×10^7 cells of *S. cerevisiae* per milliliter.

Materials and Methods

Materials

For *S. cerevisiae*, a strain was ordered on ATCC catalog #BY4741. In order to grow this strain, Synthetic Complete Media was made without Leucine for future selection. This was made up of 1.7g/liter of yeast nitrogen base (Sunrise Science), 5g/liter of ammonium sulfate(Sigma-Aldrich), 20g/liter of dextrose (Sigma-Aldrich), and all essential amino acids except leucine. Media was filter sterilized.

A similar acetic acid gradient assay was run with the wild-type *S. cerevisiae*, we ran 3mL tubes of inoculated cultures with a gradient of 0 mM to 300 mM acetic acid, increasing by increments

of 25 mM. Taking OD600 readings every 60 minutes, we selected a concentration of 60mM to use as our growth assay.

Using the same gene extraction PCR product, the cloning proceeded in a very similar manner as previously with the Electra Cloning Kit (DNA2.0). This time, vector pD1211 was purchased which contains the TEF1 promoter, Leu2 expression marker, and *E. coli* high copy number with kanamycin selection.

The cloning process used Top10 Chemically Competent *E. coli* Cells again. The Spin MiniPrep Kit (Qiagen) was used to extract and purify the plasmids from the transformed *E. coli* cultures. To ensure the plasmid was successfully within the *E. coli*, a colony-PCR was performed on individual colonies. The primers were designed such that the forward primer would bind within the sequence encoding CFA while the reverse primer was designed to fit on the vector. This way we ensure that CFA is both present within the cell and oriented appropriately inside the plasmid.

The transformation was done through the Frozen-EZ Yeast Transformation II Kit (Zymo Research). Frozen chemically competent *S. cerevisiae* cells and the transformation was accomplished following the protocol available.

The Genesys 20 Visible Spectrometer from Thermofisher was used again along with 15mm test tubes for OD600 measurements.

For further growth assay tests, Hydrogen Chloride (Sigma-Aldrich) and Sodium Acetate (Sigma-Aldrich) were used to alter the pH of the initial cultures and the free acetate available. Matlab Version 2015b was used for our data analysis program.

Methods

For the cloning procedure, we made a cloning mixture to add into the competent *E.* coli. The cloning mixture was 20ng of DNA, 1 mL of Electra Buffer, 1mL of vector, 1mL of Electra Enzyme, and 15mL of Water. We added in 1 mL of the cloning mixture into 20uL of chemically competent *E. coli*. We placed mixture on ice for 30 minutes and heat shocked at 42°C for 30

seconds. After placing on ice, we added 950mL of S.O.C. media into the mixture and shook at 250rpm at 37°C for 30 minutes. In LB + Kan (50ug/mL) plates, we plated the transformed cells in 10-fold dilutions. We incubated at 37°C for 24 hours and checked for transformants.

After growing up the transformed cultures within LB+Kan media, a plasmid extraction was performed. Following the protocol of the Spin MiniPrep Kit (Qiagen), we nanodropped a microliter and found a concentration of 150 nanograms per microliter.

We then made competent *S. cerevisiae* cells with the Frozen-EZ Yeast Transformation II Kit (Zymo Research) before transforming the cells based upon the protocol given. The cells were plated on SC-Leu plates for selection.

To ensure the plasmid was successfully within *S. cerevisiae*, a colony-PCR was performed on individual colonies. The same primers and protocol was used as the colony-PCR as in *E. coli*.

From 0 mM to 300 mM of acetic acid within SC-Leu was tested in increments of 25 mM in 3 mL cultures were tested to determine a hindering, yet survivable concentration to run our growth assay with. Cultures were all inoculated with the same volume of an overnight culture of *S. cerevisiae*. The OD600 values were measured every 60 minutes to determine growth. 60mM of acetic acid was determined to be our experimental concentration.

The acetic acid growth assay was performed by inoculating 10mL cultures for 48 hours at 30°C shaken at 225rpm with a single colony of either transformed or untransformed *S. cerevisiae*. After checking the OD600 of the 48 hour cultures, we inoculated 3mL of SC-Leu with acetic acid and SC-Leu without acetic acid with enough of the 48 hour cultures to reach an OD600 value of 0.100. Each parameter had a triplicate and was then inserted into a shaker at 30°C while shaken at 225rpm. OD600 readings were taken every 30 minutes, and were lightly vortexed before.

The pH gradient growth assay was completed by testing the *S. cerevisiae* growth cycle from pH 3 to 7 with increments of 1. Both transformed and untransformed *S. cerevisiae* were tested in

triplicates. Hydrogen chloride and potassium phosphate was used to change the pH of SC-Leu. The same 3mL cultures were used with 48-hour cultures. OD600 readings were taken every 30 minutes, and were lightly vortexed before.

The methionine acetic acid growth assay was completed by testing the *S. cerevisiae* growth cycle with 2mM and 5mM methionine addition in the presence of acetic acid and without. The 3mL cultures of SC-Leu, with and without the addition of methionine, and with and without the addition of acetic acid were tested in triplicates and were inoculated with 48-hour 10mL cultures. OD600 readings were taken every 30 minutes, and were lightly vortexed before.

The incubation dependent growth assay was completed by testing the *S. cerevisiae* growth cycle with 48 and 72-hour 10mL cultures. The 3mL cultures of SC-Leu and acetic acid were tested in triplicates. OD600 readings were taken every 30 minutes, and were lightly vortexed before.

The analyzation program was created on Matlab without the need for additional libraries. Importing the raw OD600 values from our excel files, the program will parse out the data to create individual time courses associated with the speficied string used to identify each tube. Once the individual matrices are determined, the program calculates the rate of change between each time point and then converts that into doubling time. These values were compared within each own's triplicate to ensure statistical significance. Then, the log phase was inferred by the comparison of doubling times. The maximum doubling time and the additional time points plus or minus 20% of the maximum doubling time were categorized as the log phase. The time points before were categorized as the lag phase. The time points after the log phase were categorized as the stationary phase. The program then gave out a graph to visualize the overall growth cycle per construct. A table was included to state statistical significance and give overall values for each phase.

Results

The initial transformation of the CFA-construct into *E. coli* was successful, as colonies were present on the LB+Kan plates. The plasmid extraction from *E. coli* colonies grown up resulted in 124.1 ng/uL of plasmid DNA. The restriction digest showed that EcoRI resulted in two

distinct bands of expected size (700 and 400 base pairs) that were shown on an agarose gel. The transformation of the plasmid into *S. cerevisiae* was shown to be successful by the growth of yeast colonies on the SC-LEU plates.

The acetic acid gradient growth assay was tested for 0 mM to 300 mM acetic acid, with increments of 25 mM. **Figure A1** shows the overall growth of the cultures grown within the varying acetic acid grown with 3 mL of SC-Leu, measured by OD600. 60 mM acetic acid was chosen due to its growth yet, obvious hindrance by acetic acid.

The acetic acid growth assay where CFA was overexpressed within *S. cerevisiae* in the presence of 60mM acetic acid produced a 36% decrease in lag time, in comparison to the control, as seen by **Table 2.1**. We also observed a 30% decrease in growth rate in the engineered CFA cells. **Figure 2.1** also shows the overall growth kinetics of the *S. cerevisiae* grown in the presence of acetic acid. The yeast expressing CFA versus the control yeast grown in SC-Leu without acetic acid showed lag times with 18% difference at a p-value of 0.374 and growth rates, only 10% different with a p-value of 0.005.

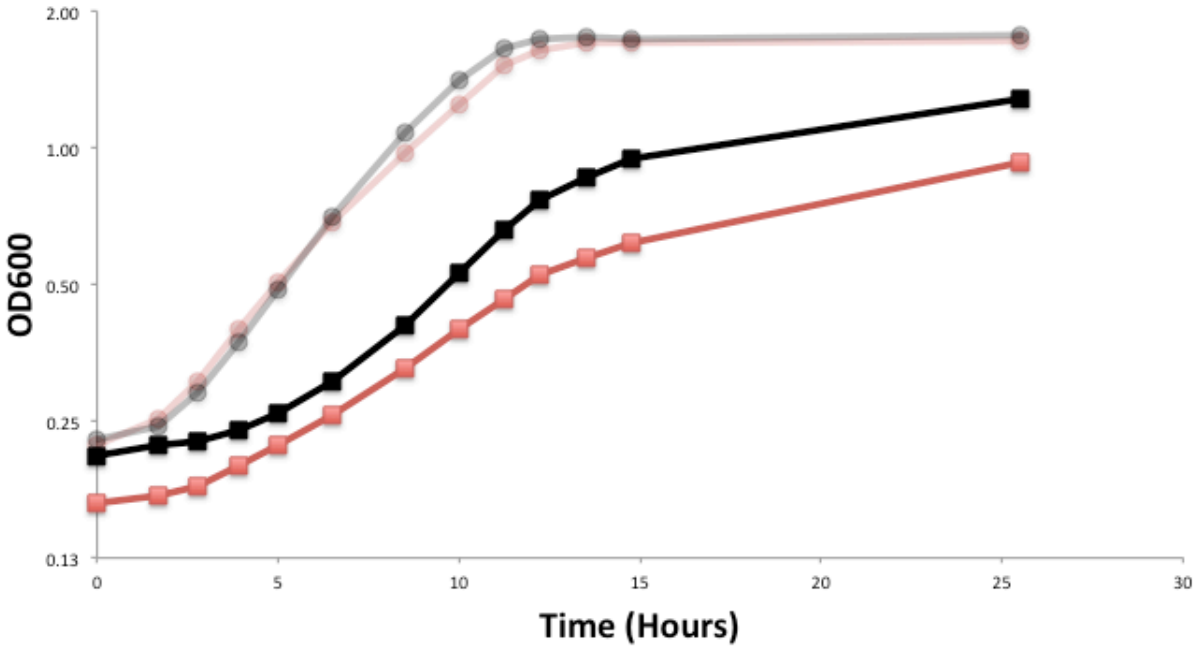

Figure 2.1: Acetic acid tolerance assay conducted with CFA mutant *S. cerevisiae.* (n=3)

Table 2.1: Growth kinetics analysis of acetic acid tolerance assay of *S. cerevisiae.*

	No Acetic Acid				60mM Acetic Acid			
	Lag Time	Growth Rate	Log Time	Saturation	Lag Time	Growth Rate	Log Time	Saturation
CFA	1.67	3.21	8.33	1.72	3.53	5.44	7.72	0.93
dasherGFP	2.03	2.91	6.47	1.76	5.50	4.17	6.08	1.28
Percent Diff	-18	10	29	-2	-36	30	27	-27
p-Value	0.3739	0.0052	0.0067	0.0075	0.0357	0.0001	0.0179	0.0007

The pH gradient growth assay showed the effect of a lower pH on the system. The growth rates in the pH 3 cultures for CFA versus control showed a 4.6 percent difference. The data is shown in **Figure A2**.

Running all the previous data collected on the Matlab Program expressed no change in analysis of our data. The analysis was standardized, yet the analysis showed no change in conclusion from any experiments.

Discussion

As seen by the results, the CFA-yeast construct was successfully engineered. Yet, our acetic acid growth assay is resulting in different results in comparison to our data collected with the *E. coli*-CFA construct. The overall decrease in lag phase is still present, but it is accompanied with a 30% decrease in growth rate. The non-acetic acid cultures had very only a 10% and 18% difference in growth rate and lag time with p-values of 0.005 and 0.374, respectively. This would suggest the large change of growth kinetics is the direct result of growing in the presence of acetic acid.

Summary & Conclusion

With the overall goal of this project to increase efficiency within bioethanol production in yeast, the growth rate correlates directly with ethanol production. The lag phase decrease should be highlighted as this will shorten the length of the overall cell cycle, but the decrease in growth rate must be changed. After running numerous tests to identify the issue resulting in the decreased growth rate, we believe that it must be an unknown biological mechanism. More research must be done to better understand what pathways are being hindered for us to be able to determine a solution to the slow in growth rate.

Chapter 3 – Addressing Growth Defect

Introduction

In this chapter we will describe the methodology that we employed to further characterize the potential causes leading to the slowed growth kinetics seen in our CFA synthase. After exhausting all of the parameters that appeared to be involved in the expression of the CFA synthase we determined that the cause of the change in growth rate must be resultant of a more complex biological phenomenon. The hypothesis was that if we could identify the root cause of the slowed growth we could create another genetic modification to alleviate that problem. We could then take a compensatory gene and clone it into our CFA expression vector so that we could co-express the two proteins to retain the decrease in lag time while restoring the growth rate and saturation point. Our approach to doing this was to conduct a literature search to determine potential cause of the growth perturbation then build them into an expression vector to test for toxicity, and then engineer them into our CFA expression vector to co-express them and then rerun the mutant through the acetic acid tolerance assay

Literature Review

To avoid embarking on a blind search through endless publications, we established a set of criteria that we gathered through our experimentation. From our control conditions we found that there was little to no growth perturbation from the expression of CFA when no acetic acid was added. This told us that the toxicity was not resultant from the CFA synthase expression itself. This told us that it is likely not the protein but possibly the substrate that it uses. The methyl donor utilized in the cyclopropanation reaction is S-adenosyl methionine, or SAM, and functions as our link between the CFA synthase and the larger metabolic pathways of *S. cerevisiae.* We also know that the problem only arises when acetic acid is present in high concentrations. So in summary what we were looking for was something in the S-adenosyl methionine biosynthetic pathway that would be inhibited when acetic acid is present.

Our first step in our search was to identify potential targets in S-adenosyl methionine pathway that acetic acid could interact with. While it is possible for the acetic acid to interact directly with the metabolites involved in the cycle, we figured that it would be far more likely that it is

inhibiting one of the proteins responsible for catalyzing the reactions. To being our search we went to the Kyoto Encyclopedia of Genes and Genomes, KEGG, which has a comprehensive metabolic pathway for *S. cerevisiae*. The pathway obtained from KEGG is shown in Figure __.

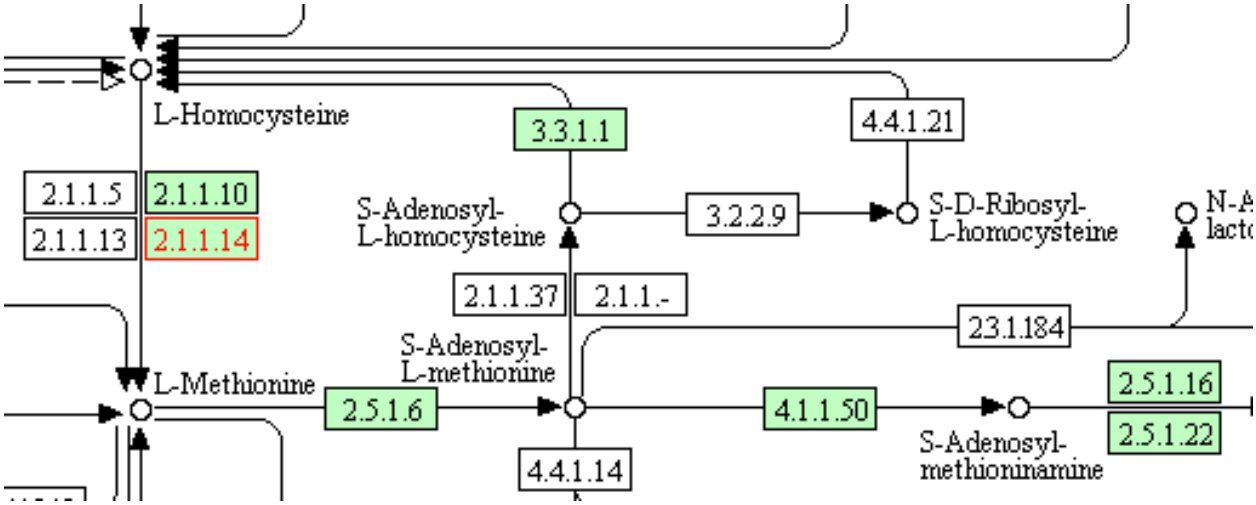

Figure 3.1: KEGG S-adenosyl methionine biosynthetic pathway.

From this pathway we figured the most logical method to screen the proteins involved would be to work backward from our cyclopropanation mechanism that converts S-adenosyl methionine into S-adenosyl homocysteine. This lead us to the enzyme S-adenosylmethionine synthetase, SAM2, which catalyzes the conversion of methionine to S-adenosyl methionine. We ran an literature search on this protein with any association with acetic acid or acetate. Our searches through Web of Science, PubMed and Google Scholar came back with no results that disclosed any clear relationship between its activity and acetic acid concentrations. After scanning through all of the potential articles we decided to take another step backward in pathway, the conversion of homocysteine to methionine.

The protein responsible for converting homocysteine to methionine in *S. cerevisiae* is the homocysteine methyltransferase, Met6. We first went to the protein's flatfile on NCBI to look through its references. After screening those as well as those citing them, we moved to the same searching method employed for SAM2. Unfortunately, we still returned nothing that indicated that Met6 should be inhibited by acetic acid or acetate. We decided to then broaden the search a bit more and came up with a review paper covering the effects of carboxylic acids on

biocatalysts in *S. cerevisiae*. This paper described many of the mechanisms that we were already familiar with but in one of the last paragraphs, it stated: "Accumulation of carboxylate anions could increase the ionic strength of the cell interior, potentially inhibiting the activity of enzymes such as homocysteine transmethylase." (Jarboe, Royce, & Liu, 2013) This statement was backed by two citations that we investigated further.

The first publication which was referenced was from many years ago but they noted that there was inhibition due to acetic acid but did not elaborate much further (Whitfield, Steers, & Weissbach, 1970). The next publication elaborated much further on this observed inhibition, however it was all in reference to the *E. coli* protein responsible for catalyzing the same reaction as Met6. The paper discusses the growth inhibition seen by disruption of the methionine biosynthetic pathway specifically referring to the buildup of homocysteine in the cells. This accumulation of homocysteine upon the exposure to acetic acid was then traced to the activity of homocysteine methyltransferase MetE in *E. coli* (Roe, O'Byrne, McLaggan, & Booth, 2002). In order to glean an insight into the possibility of the *S. cerevisiae* homocysteine methyltransferase also being inhibited, we conducted a bioinformatics study.

Bioinformatic Assessment of Homology

The workflow that we sought to employ to determine the relationship was to acquire the necessary sequence, construct a global alignment, locate catalytic residues, and assess identity at those sites. To locate and extract the desired sequences we searched through the Entrez Life Science Database. By searching for the protein and organism name, the sequences for both *E. coli* and *S. cerevisiae* were relatively easy to find. The accession numbers that we used were YP_002414981.1 for MetE and NP_011015.3 for Met6. Both sequences are RefSeqs, meaning that they are verified sequences.

Once we had our sequences, we ran a blastp suite-2 sequence analysis on them. The goal behind running this analysis was to a local alignment of our sequences to quickly assess whether the two sequences were homologous. The results of the Blast strongly indicated homology between the two polypeptide sequences as the reported Expect value was zero over a 98% query coverage.

Confident that we had homologous sequences we went ahead and created an alignment (Altschul, Gish, Miller, Myers, & Lipman, 1990).

To generate our global alignment we utilized the program Geneious. We uploaded our GenBank files for the above sequences and then ran a MUSCLE alignment. With stringent gap and extension costs we were able to generate a strong alignment (Kearse et al., 2012).

Armed with the alignment, we proceeded to track down the catalytic and binding residues to determine the likelihood that Met6 would also be inhibited by the presence of the acetate anion. We searched the flat files of the two sequences to determine where and what residues made up the sites. We determined that there were 20 significant residues in both proteins and that they shared 100% identity across those sites, except for one residue in the THF binding site. There is a mutation from a Tyrosine to a Cysteine in the *S. cerevisiae* Met6 protein. According to the Blosum matrix, which assess the similarity in chemistry between amino acids, this conversion was rated a -2 (Goffeau et al., 1996; Touchon et al., 2009).

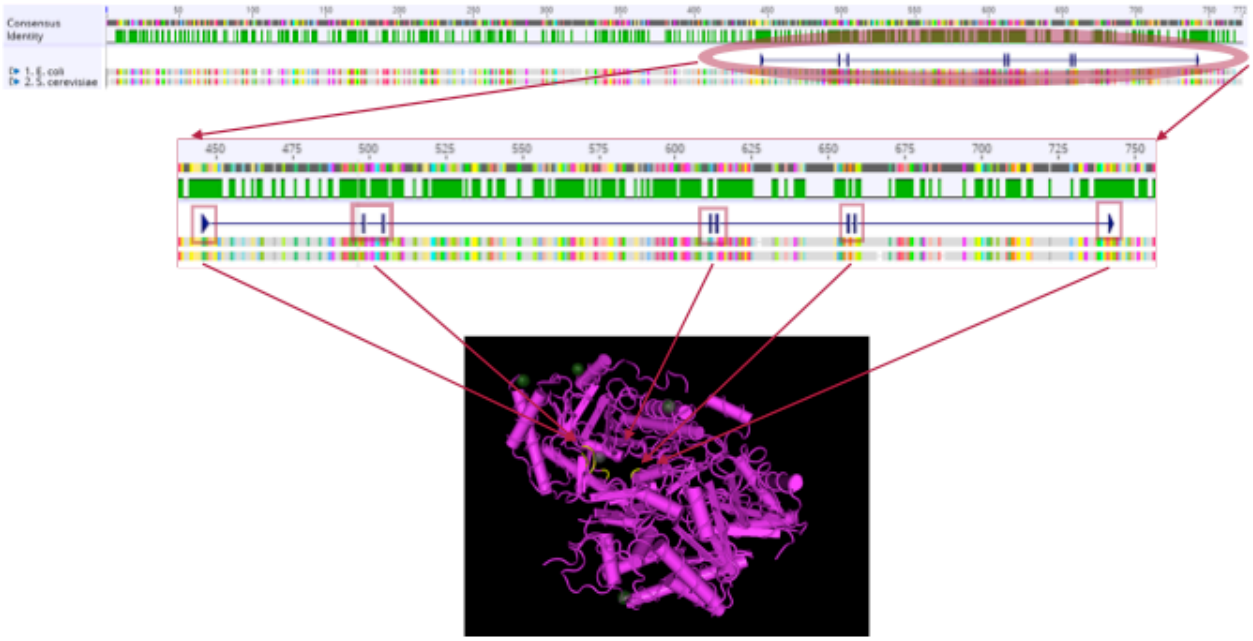

Figure 3.2: Homocysteine methyltransferase alignment, generated on Geneious, with catalytic residues highlighted and indicated on protein structure visualized on Cn3D.

The very similar identity that we see at these prominent loci indicates that they likely use very similar mechanisms. Therefore, if MetE were inhibited by acetate, it would be reasonable to assume that the Met6 protein would also be inhibited. Feeling comfortable with that conclusion, we decided to shift back to wet lab and design experiments to directly assess whether or not this hypothesis is true.

Experimentation

Addressing the Methionine Deficiency

To assess the validity of our hypothesis that the *S. cerevisiae* methyltransferase Met6 is inhibited by acetate, we determined what would be the direct result of the inhibition as well as what parameters could actually be observed given our resources. To understand its larger effect we created a clearer depiction of the biosynthetic pathway that allowed us to better understand the effects. This figure is shown in Figure 3.3. From this figure it is evident what the direct consequences of the inhibition are that pertain to our cyclopropanation modification: decrease in methionine, S-adenosyl methionine, and S-adenosyl homocysteine concentrations, with an increase in homocysteine concentration.

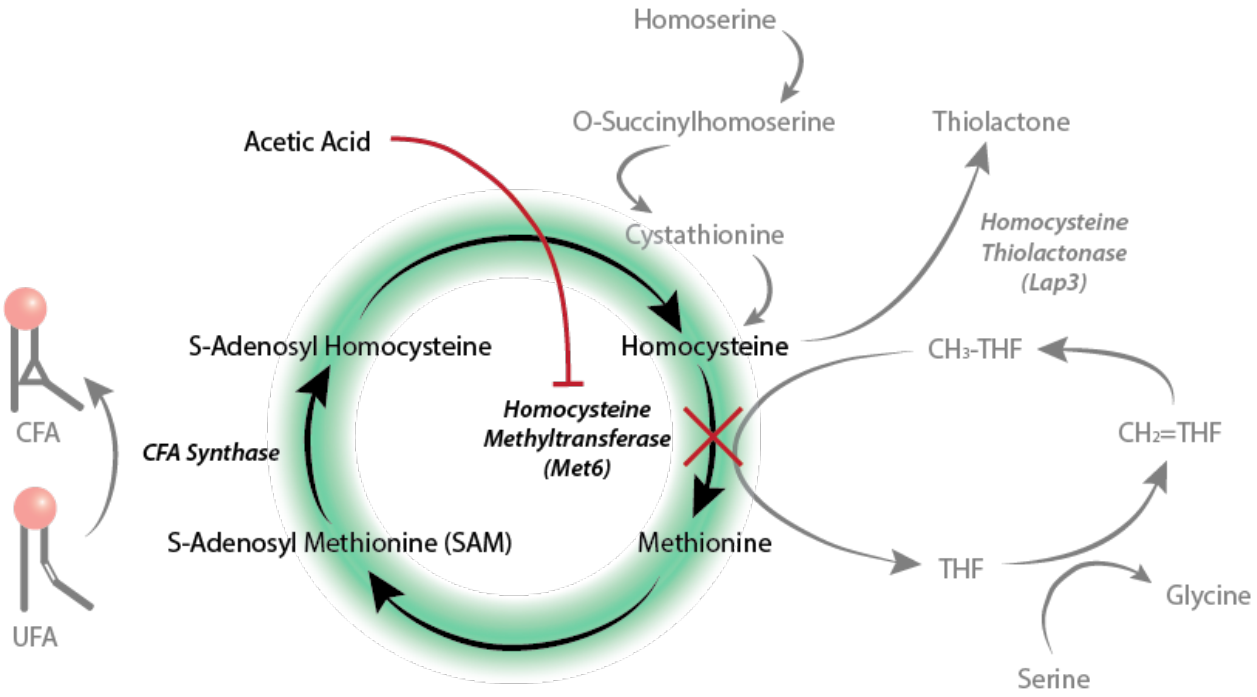

Figure 3.3: Diagram depicting the methionine biosynthetic pathway with acetic acid inhibition.

Of these potential effects, the one that was the easiest to test was the decrease in methionine and its subsequent metabolites. To address this deficiency we decided to rerun the acetic acid tolerance assay but this time supplement with methionine. Based on protocols for producing media for methionine auxotrophs, we gathered that the required concentration for methionine is 2 mM. We then ran the experiment yielding the growth curve seen in Figure 3.4 as well as the results from our MatLab analysis code in Table 3.1.

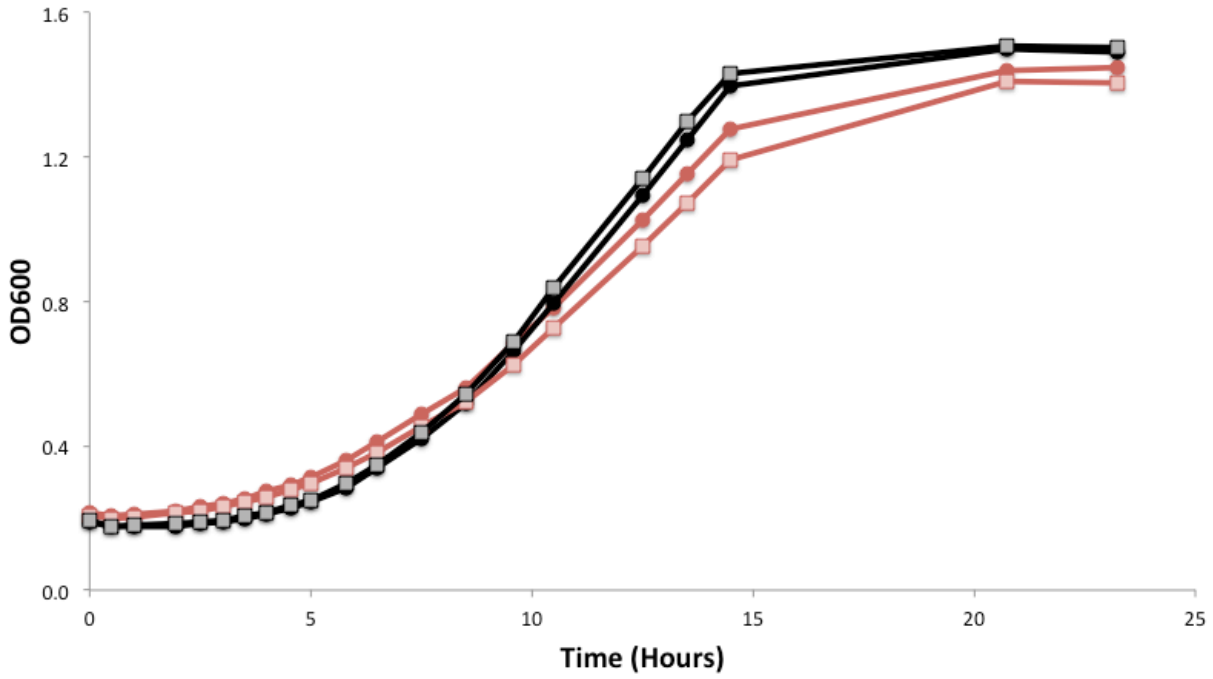

Figure 3.4: Acetic acid tolerance assay with methionine supplementation. The red lines represent the CFA mutant while the black lines represent the control. The faded boxes represent samples with 2 mM methionine, and the filled represent samples without methionine. (n=3)

Table 3.1: Growth kinetics analysis of methionine supplemented acetic acid tolerance assay.

	CFA				dasherGFP			
	Lag Time	Growth Rate	Log Time	Saturation	Lag Time	Growth Rate	Log Time	Saturation
No Met	3.85	3.82	7.32	1.45	4.85	2.98	5.35	1.49
2mM Met	4.17	3.97	7.67	1.41	4.85	2.99	5.05	1.50
Percent Diff	-8	-4	-5	3	0	0	6	-1
p-Value	0.604	0.541	0.817	0.234	1.000	0.932	0.561	0.739

Though the growth curve portrays the CFA mutant preforming worse in the presence of methionine, our deeper growth analysis indicated that it was not significant. We found this result to be inconclusive because we were unable to differentiate whether the decrease in methionine was the cause of the decreased growth kinetics seen in acetic acid conditions or if the concentration of methionine was not high enough inside of the cells. We proceeded to replicate the assay, however this time we ran a gradient of methionine concentrations, ranging from 5 mM to 20 mM. The growth curve as well as our analysis can be found in the **Appendix**. This assay

revealed that levels of 5 mM methionine and above leads to toxicity and decreased growth. With the addition of methionine yielding no data indicating a positive result we moved to our second possibility, which was to address the accumulation of homocysteine.

Prior to pursuing our hypothesis that homocysteine was the root cause of the decreased growth we turned to the literature to determine whether homocysteine is toxic in high concentrations. Based on the papers that we read, the consensus is that the accumulation of homocysteine leads to the formation of homocysteine-thiolactone. Homocysteine-thiolactone then initiates an error prone pathway, which leads to the use of homocysteine, instead of methionine, during protein translation. This results in dysfunctional proteins and decreased cell vitality (Zimny, Sikora, Guranowski, & Jakubowski, 2006).

Confident in our findings we decided to move forward with testing. To evaluate the intracellular concentration of homocysteine would require HPLC and other sophisticated analytics which we did not have access to, so instead we decided that the best way to evaluate this hypothesis was to create other gene constructs that would be capable of alleviating the homocysteine stress. We decided to create two new gene constructs, one to over express the homocysteine methyltransferase, Met6, and the other to express the homocysteine thiolactonase, Lap3. Met6 was chosen because it is the most direct way to recover the inhibition. If the acetic acid concentration that we are utilizing is capable of inhibiting a significant portion of the Met6 present in the cell, we could over express the protein to accommodate for the decreased flux through the pathway. This however might not be able to adequately compensate for the inhibition. To employ a potentially more efficacious modification we turned to the endogenous homocysteine stress relief mechanism. This mechanism utilizes Lap3 to hydrolyze homocysteine-thiolactone back into homocysteine and thiolactone, rendering them inert.

To construct these new gene constructs, we decided to follow the same workflow as we did to generate our CFA expression mechanism. We extracted and purified the *S. cerevisiae* genome and designed the necessary primers to be able to PCR the Met6 and Lap3 genes from the purified genome. Once we had the primers we began running the necessary PCR reactions.

Fps1 Knockout

While running the cloning procedure for the other constructs we also decided to begin developing our other gene target from our backup plan. We chose to work on the Fps1 knockout due to its simplicity and connection to our growth defect. These porins are degraded rapidly in the presence of high acetic acid concentrations, but that only occurs once the acetic acid has entered. Our hypothesis is that the initial diffusion through these porins compromises the membrane, irregardless of whether it is cyclopropanated. It was originally suspected that the acetate anion is exported through the carboxylic acid ABC transporter Pdr12 (Piper et al., 1998), but it was later proven that Pdr12 is only responsible for longer chain carboxylic acid extrusion (Nygård et al., 2014). With this disproven, we were unable to find any other acetate transporters for yeast potentially meaning that there are transporters for acetate. This would leave diffusion or conversion as its only method to leave the cell. If diffusion is in fact the primary method, this would be greatly limited in our CFA mutants, as the cyclopropanation slows diffusion both in and out of the cell. This trapping of acetate inside the cell might also be contributing to the decreased growth rate.

To test this hypothesis, we were granted access to a yeast knockout library. From this library we selected the Fps1 knockout and attempted to grow it up. Over the course of several weeks we tried to culture it with no success, so Dr. Ruscetti contacted another lab with a similar library. They, too, were unable to culture the knockout. We hypothesized that the cause could be from the deletion of the Fps1 knockout itself. As this porin also allows glycerol through, without it, glycerol might not be able to enter the cell and effectively protect it from internal crystallization, leading to its death in a -80C freezer (Mollapour & Piper, 2007).

Characterizing CFA Mutants

During the time that we were developing all of these other constructs we decided to reanimate our CFA mutant and rerun our acetic acid tolerance assay to make sure that we had the system up and running prior to testing our new mutants. However, upon use of these reanimated CFA mutants we did not see the decrease in lag time that we had seen previously**.** Confused by the result, we decided to rerun the experiment but this time picked numerous colonies from our plate and ran a colony PCR to be sure they all still contained the plasmid. This run also yielded no

results resembling that of our previous experiments. Having kept all of the controllable parameters constant we decided that it was possibly a result of the freeze-thaw procedure, we decided to remake our CFA mutant. We pulled from the same stock of competent yeast that we made and transformed with the CFA plasmid that we created. The transformation was successful and so we once again conducted the acetic acid tolerance assay with colony PCR. Unfortunately, our transformants showed to evidence of a decrease in lag time.

With the cause of this variability unclear we looked for any possible source of variation. Upon revision we found that between our assays there were as high as 12-hour differences in the incubation time allotted for culturing the samples used to inoculate the cultures for the assay. We hypothesized that this could have resulted differences between the growth stages of our CFA compared to our control. CFA mutants have shown to grow slower than our control and so it might not reach death phase. Pulling cells from an early growth phase could potentially lead to more favorable growth kinetics in the inoculated cultures. To see if our early runs were a result of differential pre-culture incubation times we conducted yet another acetic acid tolerance assay with a gradient of incubation times. The data is seen below in Figure 3.5.

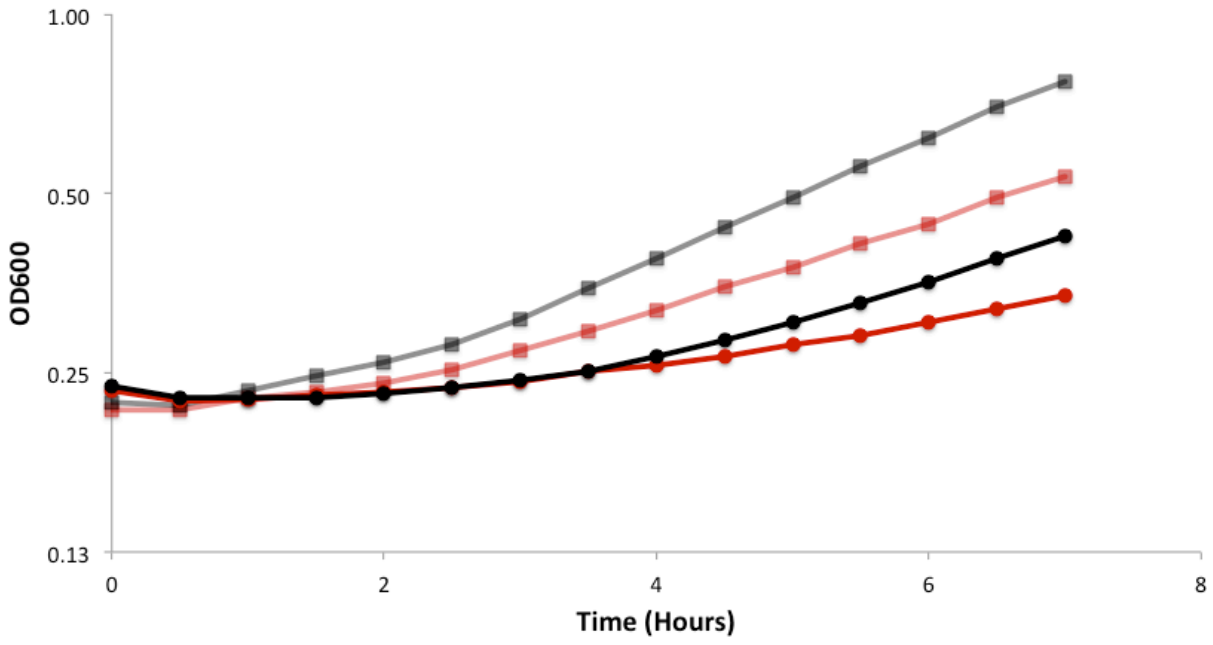

Figure 3.5: Growth curve of acetic acid tolerance assay with variable pre-culture incubation time. Red lines represent CFA mutants and black lines represent the control.

Transparent squares represent cultures inoculated after 48 hours of incubation. Solid circles represent cultures inoculated after 72 hours of incubation. (n=3)

From this data we concluded that the growth kinetics are impacted by the incubation of the cultures used to inoculate. However even though there is a change between the cultures incubated for 48 hours compared to 72 hours, our CFA mutant preformed unfavorably in both cases. We once again saw no significant decrease in our lag time resulting from our modification.

After this assay, we were unsure what other tests we could run as we had examined all of the potential variants from our original assays that demonstrated the decrease in lag time. The next logic step would be to ensure that our protein was actually functioning as we intended. To do so we would need to conduct a lipidomics study to determine the percentage of the membrane that is cyclopropanated to ensure that the CFA synthase is in fact active. Many studies that we found running lipidomic analyses utilized HPLC to identify the concentrations of lipids. Due to the inaccessibility to HPLC, we looked for other means. The only alternative that we found was to outsource the analysis to a company. There were several companies capable of conducting the required profiling for *S. cerevisiae* but it was also estimated to take three weeks to receive the results. As we were nearing the end of the quarter we recognized that we would not be able to get the results from the company in time.

Future Perspectives

If allotted more time, we would start by sending our samples out for the lipidomics analysis. From this result we would either see the presence of cyclopropane fatty acids or not, informing us on the activity of the inserted gene. If the results returned that there was in fact cyclopropane fatty acids, that would tell us that it was possible that something was incorrect with our original assays that revealed the decrease in lag time. If the results, however, showed no cyclopropanation, this would not confirm that our prior results were correct but it would indicate that the perturbation in growth is not specifically due to the cyclopropanation and that the lack of decrease in lag time is not there because the cyclopropanated membrane is unable to slow the diffusion of acetic acid. The inactive CFA synthase could then be explained through a variety of

ways, ranging from the original transformation, the transcription of the plasmid, translation of the CFA encoding mRNA, to the final folding of the protein, etc. This is where we would recommend a future team to begin their project, if someone decided to continue it. We also recommend the further investigate the other gene constructs that we identified, with specific emphasis on the homocysteine methyltransferase inhibition. This acetate meditated inhibition has never been recorded in *S. cerevisiae* and so if found to be valid, it could inform future studies looking to impart acetic acid resistant.

Engineering Standards and Realistic Constraints

Economic

The economic considerations played a considerable role in the initial project ideation process. We knew that we wanted to improve upon biofuel technology, and we knew that the major issue currently standing in the way of this technology's scalability is its cost. Currently, fossil fuels are still significantly less expensive and so it is constantly out competed. In order to get this sustainable technology to reach a state we need to address every possible attribute of the process and reduce its cost and increase efficiency. For our specific project we wanted to develop a system that could work within the existing infrastructure, as the machinery required to produce biofuels at large scale are both complex and very expensive. To do this we decided upon implementing a genetic modification because after the initial investment in development, the cost required to use this system is no different than the previous system. However, it would be slightly more efficient, reducing its cost.

Environmental

The primary goal surrounding the advancement of biofuel technology is the pursuit of a more environmentally friendly solution to our energy demands. This technology works to aid the environment both in its production and it is use. The refinement of fossil fuels into usable fuel, requires excessive energy input and the release of harmful toxins. The production of biofuels only requires the breakdown of plant waste and its formulation in a bioreactor. Fossil fuels also release enormous amounts of carbon dioxide upon use compared to the clean emissions of the biofuel. This drive toward a more environmentally conscious future was the driving force behind our project and therefore it persisted through the design process by ensuring that our system would be able to work within their system so as to not disrupt tis environmentally friendly process. Our idea to decrease the susceptibility would actually make the process better for the environment because it wouldn't require the use of excessive amounts of weak base to neutralize the acetic acid in the environment, because our modified cells would be resilient.

Sustainability

The other goal of biofuel production is the pursuit of a fuel source that will not deplete. It has become increasingly more apparent that there is a limited supply of fossil fuels and at the rate that we are consuming it, we will need alternatives for the generations to come. By switching to biofuels over fossil fuels we turn to a sustainable solution because it offers the possibility to continually replenish our supplies through the ongoing processes that we already employ, such as farming. Within the context of our specific project within biofuels, ours fits well within this system because the further implementation of our system will not require the any continually input to implement. Once the stable modification has been made, the industrial yeast can be continually cultured and used to make biofuels.

Manufacturability

Large-scale manufacturability was the Achilles heel of first generation biofuels. The original concept for biofuel production required the use of food, such as corn, to produce its fuel. This process was not scalable, as the scaling of its production would continually pull from the food supply leaving many people hungry around the world. To shift to a substrate more readily available, scientists turned to bio-waste, one of the most abundant carbon sources. This change has allowed this process to become scalable due to the availability of this material. Our modification is then capable of easily scaling with this technology. By placing the modified yeast into a larger bioreactor it will readily scale to occupy the space allotted provided ample substrate and growth conditions.

Ethical

Our desire to create an ethically conscientious addition to an already ethically driven pursuit was present throughout the entirety of our project. The primary ethical consideration that drove our project was our obligation to society to help bring it a better future. Through the process of making biofuel production more economically feasible, we bring this technology closer to the hands of the public and therefore carry out our obligations to society.

Appendix

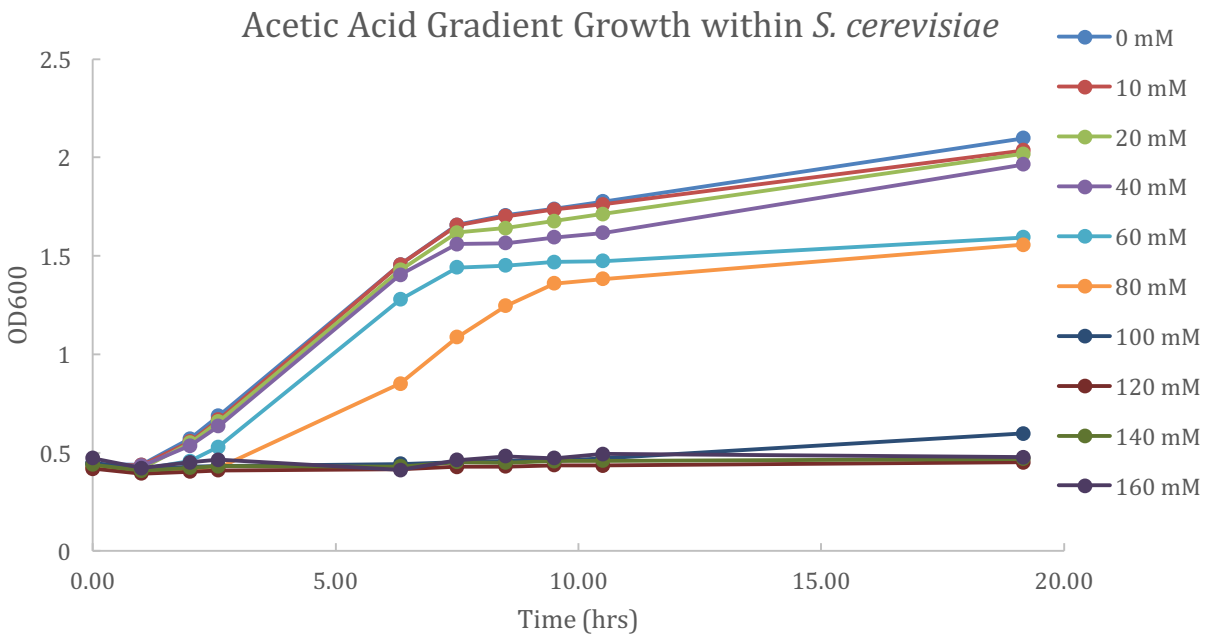

Figure A1: Acetic acid gradient assay. (n=3)

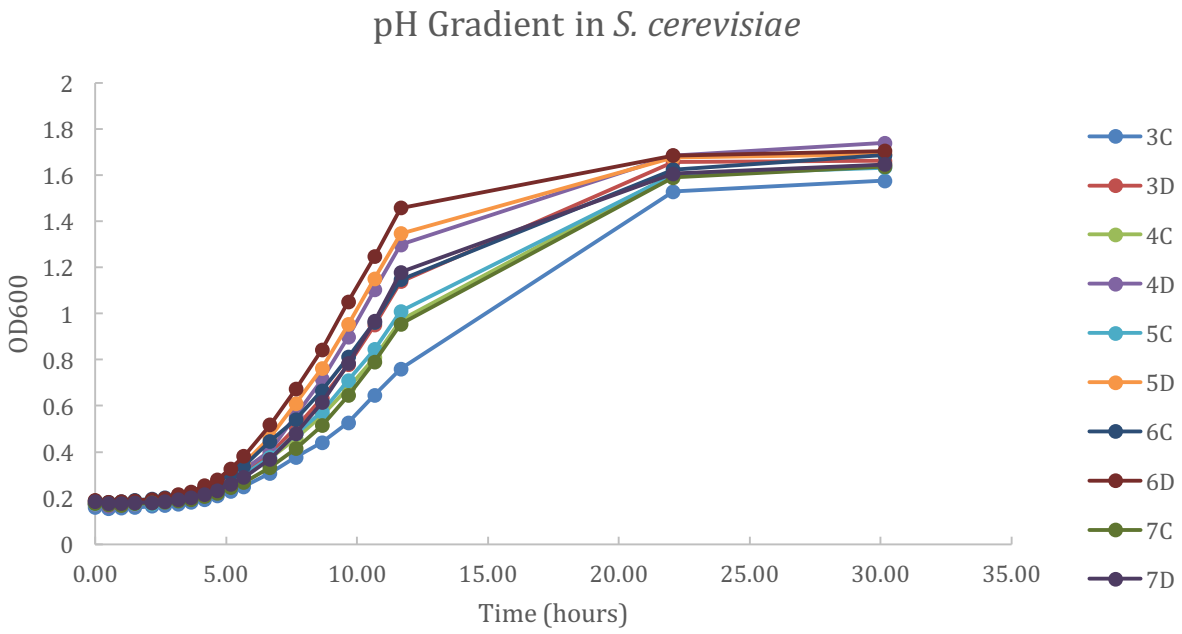

Figure A2: pH gradient tolerance assay of *S. cerevisiae* utilizing hydrochloric acid.

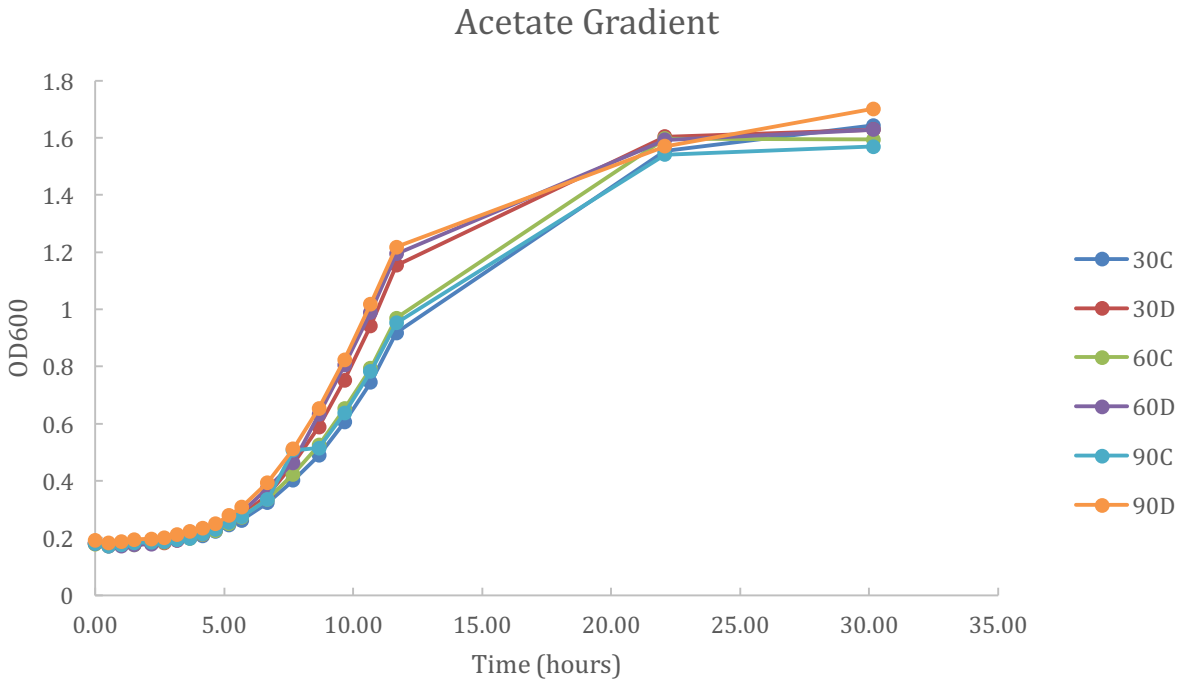

Figure A3: Acetate gradient tolerance assay of *S. cerevisiae* using sodium acetate.

Bibliography

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–10. http://doi.org/10.1016/S0022-2836(05)80360-2

Bogdanov, M., Aboulwafa, M., & Saier, M. H. (2013). Subcellular localization and logistics of integral membrane protein biogenesis in Escherichia coli. *Journal of Molecular Microbiology and Biotechnology*, *23*(1-2), 24–34. http://doi.org/10.1159/000346517

Chang, Y.-Y., & Cronan, J. E. (1999). Membrane cyclopropane fatty acid content is a major factor in acid resistance of Escherichia coli. *Molecular Microbiology*, *33*(2), 249–259. http://doi.org/10.1046/j.1365-2958.1999.01456.x

Doğan, A., Demirci, S., Aytekin, A. Ö., & Şahin, F. (2014). Improvements of tolerance to stress conditions by genetic engineering in Saccharomyces cerevisiae during ethanol production. *Applied Biochemistry and Biotechnology*, *174*(1), 28–42. http://doi.org/10.1007/s12010-014-1006-z

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., … Oliver, S. G. (1996). Life with 6000 genes. *Science (New York, N.Y.)*, *274*(5287), 546, 563–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8849441

Guangqi, E., Lesage, D., & Ploux, O. (2010). Insight into the reaction mechanism of the Escherichia coli cyclopropane fatty acid synthase: isotope exchange and kinetic isotope effects. *Biochimie*, *92*(10), 1454–7. http://doi.org/10.1016/j.biochi.2010.05.019

Jarboe, L. R., Royce, L. A., & Liu, P. (2013). Understanding biocatalyst inhibition by carboxylic acids. *Frontiers in Microbiology*, *4*, 272. http://doi.org/10.3389/fmicb.2013.00272

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., … Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics (Oxford, England)*, *28*(12), 1647–9. http://doi.org/10.1093/bioinformatics/bts199

Lindberg, L., Santos, A. X., Riezman, H., Olsson, L., & Bettiga, M. (2013). Lipidomic profiling of Saccharomyces cerevisiae and Zygosaccharomyces bailii reveals critical

changes in lipid composition in response to acetic acid stress. *PloS One*, *8*(9), e73936. http://doi.org/10.1371/journal.pone.0073936

Loow, Y.-L., Wu, T. Y., Tan, K. A., Lim, Y. S., Siow, L. F., Jahim, J. M., … Teoh, W. H. (2015). Recent Advances in the Application of Inorganic Salt Pretreatment for Transforming Lignocellulosic Biomass into Reducing Sugars. *Journal of Agricultural and Food Chemistry*, *63*(38), 8349–63. http://doi.org/10.1021/acs.jafc.5b01813

Mollapour, M., & Piper, P. W. (2007). Hog1 mitogen-activated protein kinase phosphorylation targets the yeast Fps1 aquaglyceroporin for endocytosis, thereby rendering cells resistant to acetic acid. *Molecular and Cellular Biology*, *27*(18), 6446–56. http://doi.org/10.1128/MCB.02205-06

Nygård, Y., Mojzita, D., Toivari, M., Penttilä, M., Wiebe, M. G., & Ruohonen, L. (2014). The diverse role of Pdr12 in resistance to weak organic acids. *Yeast (Chichester, England)*, *31*(6), 219–32. http://doi.org/10.1002/yea.3011

Piper, P., Mahé, Y., Thompson, S., Pandjaitan, R., Holyoak, C., Egner, R., … Kuchler, K. (1998). The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *The EMBO Journal*, *17*(15), 4257–65. http://doi.org/10.1093/emboj/17.15.4257

Poger, D., & Mark, A. E. (2015). A ring to rule them all: the effect of cyclopropane Fatty acids on the fluidity of lipid bilayers. *The Journal of Physical Chemistry. B*, *119*(17), 5487–95. http://doi.org/10.1021/acs.jpcb.5b00958

Roe, A. J., O'Byrne, C., McLaggan, D., & Booth, I. R. (2002). Inhibition of Escherichia coli growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity. *Microbiology (Reading, England)*, *148*(Pt 7), 2215–22. http://doi.org/10.1099/00221287-148-7-2215

Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., … Denamur, E. (2009). Organised genome dynamics in the Escherichia coli species results in highly diverse adaptive paths. *PLoS Genetics*, *5*(1), e1000344. http://doi.org/10.1371/journal.pgen.1000344

Whitfield, C. D., Steers, E. J., & Weissbach, H. (1970). Purification and properties of 5-methyltetrahydropteroyltriglutamate-homocysteine transmethylase. *The Journal of*

Biological Chemistry, *245*(2), 390–401. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4904482

Zheng, D.-Q., Liu, T.-Z., Chen, J., Zhang, K., Li, O., Zhu, L., … Wang, P.-M. (2013). Comparative functional genomics to reveal the molecular basis of phenotypic diversities and guide the genetic breeding of industrial yeast strains. *Applied Microbiology and Biotechnology*, *97*(5), 2067–76. http://doi.org/10.1007/s00253-013-4698-z

Zimny, J., Sikora, M., Guranowski, A., & Jakubowski, H. (2006). Protective mechanisms against homocysteine toxicity: the role of bleomycin hydrolase. *The Journal of Biological Chemistry*, *281*(32), 22485–92. http://doi.org/10.1074/jbc.M603656200