

University of Arkansas, Fayetteville

ScholarWorks@UARK

---

Biological Sciences Undergraduate Honors  
Theses

Biological Sciences

---

5-2021

## How Acetylation Regulates Metabolic Enzyme Function During Environmental Shifts

Jared Canonigo

Follow this and additional works at: <https://scholarworks.uark.edu/biscuht>



Part of the [Biology Commons](#), [Microbial Physiology Commons](#), and the [Organismal Biological Physiology Commons](#)

---

### Citation

Canonigo, J. (2021). How Acetylation Regulates Metabolic Enzyme Function During Environmental Shifts. *Biological Sciences Undergraduate Honors Theses* Retrieved from <https://scholarworks.uark.edu/biscuht/38>

This Thesis is brought to you for free and open access by the Biological Sciences at ScholarWorks@UARK. It has been accepted for inclusion in Biological Sciences Undergraduate Honors Theses by an authorized administrator of ScholarWorks@UARK. For more information, please contact [scholar@uark.edu](mailto:scholar@uark.edu).

# **How Acetylation Regulates Metabolic Enzyme Function During Environmental Shifts**

An Honors Thesis submitted in partial  
fulfillment of the requirements for  
Honors Studies in Biology

By

Jared Canonigo

Spring 2021

Department of Biological Sciences  
J. William Fulbright College of Arts and  
Sciences  
**The University of Arkansas**

## ACKNOWLEDGMENTS

First and foremost I would like to extend my gratitude to Dr. Lewis and Dr. Steucker for welcoming me into the Lewis lab with open arms and allowing me to remain in it for the past two years. Without their constant support and guidance, my work and success in my undergraduate years would not have been possible. I could not have asked for better mentors.

I would also like to thank all of the graduate students, past and present, and lab personnel who have helped me along the way including Crystal Crook, Kala Downey, Rebecca Hardman, Stephanie Hood, Julio Molina Pineda, and Amanda Scholes. Each of them have taken time out of their busy days to help me understand complex biological concepts, help me run experiments, and helped fill me with laughter when I did not think I could continue going on with my project. They helped me grow academically, professionally, and personally, and for that I will be forever thankful.

I would also like to thank my fellow undergraduate peers Wejeia Shi and Savannah Washburn for being a constant source of support throughout this entire process.

In addition to those in the Lewis Lab, I would also like to thank Dr. Dan Lessner and Dr. Chenguang Fan for being on my honors thesis defense committee. Several people within the department of Biological Sciences were also generous along the way, allowing me to use their equipment and offer advice along my project.

Lastly, I would like to thank my friends and family for their unwavering support that catalyzed me to keep going through my time in undergrad.

## TABLE OF CONTENTS

<b>Acknowledgements.....</b>	<b>1</b>
<b>Introduction.....</b>	<b>3</b>
<b>Methods and Materials.....</b>	<b>11</b>
Testing for a Phenotype in <i>ADH1</i> and <i>ADH2</i> .....	11
Amplifying <i>ADH1</i> and <i>ADH2</i> for Cloning into pAG36.....	11
Cloning <i>ADH1</i> and <i>ADH2</i> into the yeast expression vector pAG36.....	15
Complementation Growth Curve for Adh1p.....	16
Site-directed mutagenesis of <i>ADH1</i> .....	17
Mimetic Growth Analysis.....	18
<b>Results.....</b>	<b>19</b>
Determining if ADH deletion strains have growth defects in Glucose and Ethanol..	19
Cloning of <i>ADH1</i> and <i>ADH2</i> into a Yeast Expression Vector.....	21
Complementation Growth Curve for Adh1p.....	24
Site-directed Mutagenesis of <i>ADH1</i> .....	26
Mimetic Mutation Growth Analysis Under Heat Shock.....	28
<b>Discussion and Future Directions.....</b>	<b>30</b>
Discussion.....	30
Future Directions.....	33
<b>References.....</b>	<b>36</b>

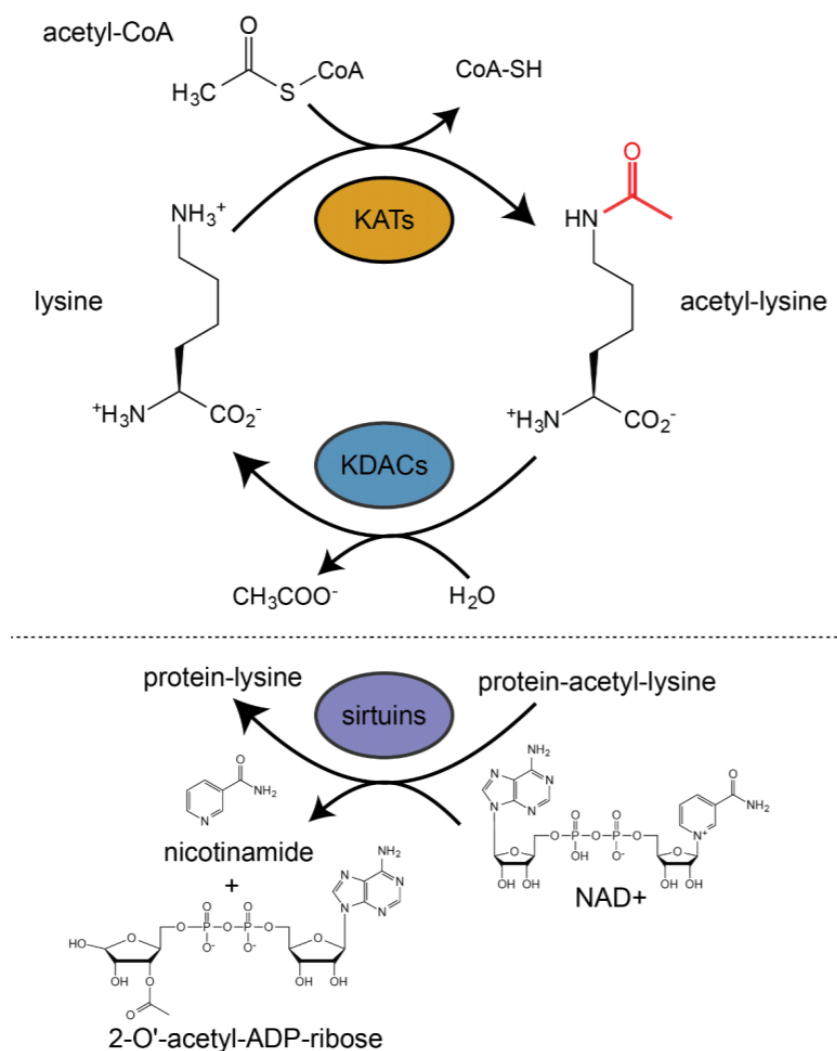
## INTRODUCTION

Organisms such as *Saccharomyces cerevisiae* can regulate the mechanisms of proteins through post-translational modification. Posttranslational modification utilizes enzymes to target pre-existing proteins by altering their chemical properties with the covalent addition or removal of a variety of chemical groups (Rhee et al. 2005). These modifications play a vital role in functional proteomic activity because they can regulate protein activity, localization, and interaction with other cellular molecules (Hardman 2019). Some examples of posttranslational modifications are phosphorylation, methylation, and the focus of this thesis, acetylation.

Protein acetylation was first discovered on histones nearly four decades ago (Norris et al. 2009). The research established that histone acetylation plays an important role in chromatin remodeling that is important for gene transcription (Norris et al. 2009). This is carried out through a specific type of acetylation known as lysine acetylation. Recent discoveries have been made to reveal that histone proteins are not the only proteins that get acetylated at lysine residues. Non-nuclear proteins, proteins that shuttle from the nucleus to the cytoplasm, and even heat shock proteins can be acetylated, showcasing that acetylation has a multiplicity of cellular targets that it may potentially regulate. (Kouzarides 2000 & Ohn and Anderson 2010).

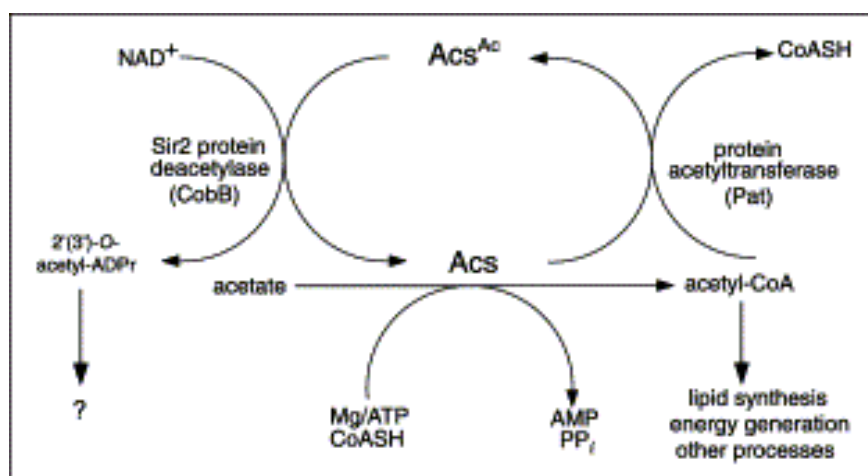
Lysine acetylation is the process in which Lysine Acetyltransferases facilitate the transfer of an acetyl from acetyl-CoA to an amino group in lysine (Ali, Ibraheem, et al. 2018). The acetylation cancels out the positive charge of lysine, which can have many consequences to the protein's function (Ali, Ibraheem, et al. 2018). For example, those who suffer from chronic lung disease have increased KAT expression and increased acetylation level, favoring increased inflammatory gene expression (Bhavsar,Ahmad, Adcock 2008). Acetyl Deacetylases can then remove the acetyl group via Acetyl Deacetylases (KDACS) (Ali, Ibraheem, et al. 2018). Sirtuins

can also remove acetyl groups from acetylated proteins, using NAD<sup>+</sup> as a cofactor [Reviewed in (Menzies et al. 2016)].



**Figure 1 Schematic of regulation by reversible lysine acetylation.** Lysine acetylation is a post-translational modification where acetyltransferases (KATs) add an acetyl group to lysine, deacetylases (KDACs), and sirtuins can remove acetyl groups from lysine. (Figure by Hardman 2019)

Additionally, researchers found that many proteins modified by acetylation, including intermediate metabolic enzymes, were conserved in different species and were heavily enriched for cellular metabolism (Choudhary et al. 2009, Henriksen et al. 2012). This could imply that acetylation may play some sort of regulatory role in metabolism. Notably, within *Salmonella enterica*, the activity of the central metabolic enzyme Acetyl-CoA Synthetase is regulated by lysine acetylation (Starai & Escalante-Semerena 2004). Sirtuin protein activity is necessary to deacetylate Acetyl-CoA Synthetase, which activates it to synthesize Acetyl-CoA from acetate (Starai & Escalante-Semerena 2004). The newly formed Acetyl-CoA can then be shunted to energy generation, lipid synthesis, and other processes. Protein acetyltransferase (Pat proteins) can then mediate the transfer of an acetyl group from Acetyl-CoA onto lysine residue 609 of Acetyl-CoA Synthetase to inactivate its activity when Acetyl-CoA concentrations are low (Starai & Escalante-Semerena 2004). The regulation Acetyl-CoA Synthetase showcases how metabolic processes within cells can be regulated through activation or inactivation of metabolic proteins via acetylation and deacetylation activity.

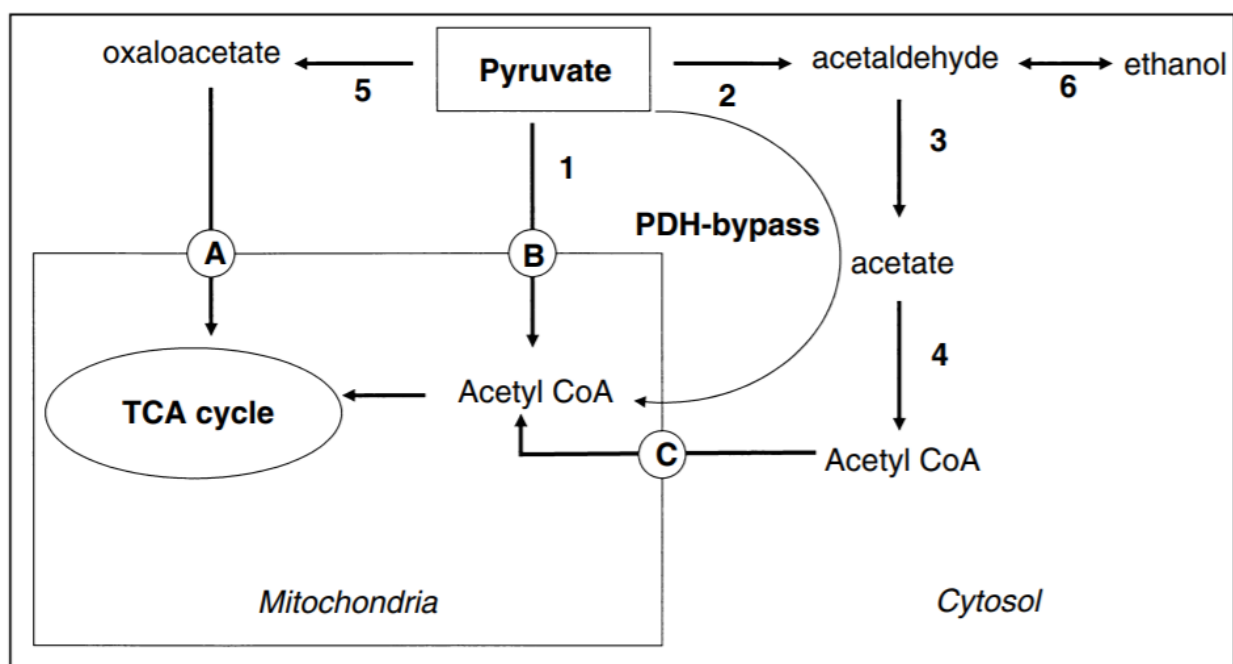


**Figure 2:** Depiction of the Post-Translational regulation of Acs activity by Pat/Sir2 Acetylation And Deacetylation (Figure by Starai & Escalante-Semerena)

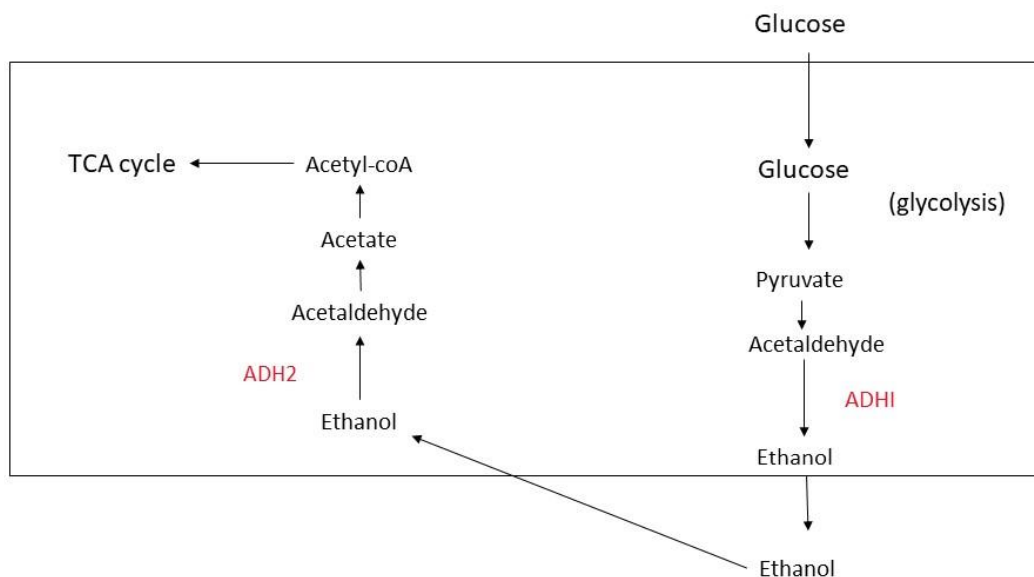
Global mass spectrometry studies have been conducted and found that many enzymes in *Saccharomyces cerevisiae* involved in metabolism are acetylated (Henriksen et al. 2012). A potential system that may follow a similar pattern of regulation via acetylation and deacetylation of metabolic enzymes may be seen within *Saccharomyces cerevisiae*. Recall that the central parts of carbon metabolism are glycolysis and the TCA cycle. Glycolysis utilizes glucose and converts it into Pyruvate through the Glycolysis pathway (Rodrigues et al. 2006). That pyruvate can then be used to go down two possible different pathways depending on the conditions the cell finds itself in: (1) it can get converted into ethanol and exported from the cell via fermentation, or (2) it can get converted into Acetyl-CoA via the multienzyme complex pyruvate dehydrogenase and enter the TCA cycle and oxidative phosphorylation through respiration (Rodrigues et al. 2006). Generally speaking, most microbes undergo respiration in the presence of oxygen because more ATP is acquired out of respiration than fermentation.

Yeast are unique because they have the ability to ferment pyruvate into ethanol and excrete the ethanol into the medium even when oxygen is present (Smidt et al. 2012). Adh1p is responsible for carrying out this reaction by catalysing the reduction of acetaldehyde to produce ethanol as seen in figure 4 (Denis et al. 1983). From an evolutionary standpoint, this is advantageous to yeast because they often grow in glucose-rich environments, such as the leaves of fruit trees, where competition from other microbes is high (SlÁviková et al. 2009). Additionally, yeast also have higher ethanol tolerance than other microbes by being able to alter its cellular lipid composition in response to ethanol exposure (Beaven et al. 1982). As such, by excreting out ethanol, yeast are able to kill off their competitors to have more glucose for itself. However, whenever glucose starts to run out, they switch their metabolism in what is known as the diauxic shift (Galdieri et al. 2010). The diauxic shift is characterized by decreased growth

and by switching its carbon source to aerobic utilization of ethanol (Galdieri et al. 2010). This is when Adh2p comes into play by oxidizing ethanol into acetaldehyde, which can be directed to the TCA cycle and the electron transport chain for energy synthesis as seen in figure 4 (Beier *et al.*, 1985; Young & Pilgrim, 1985)

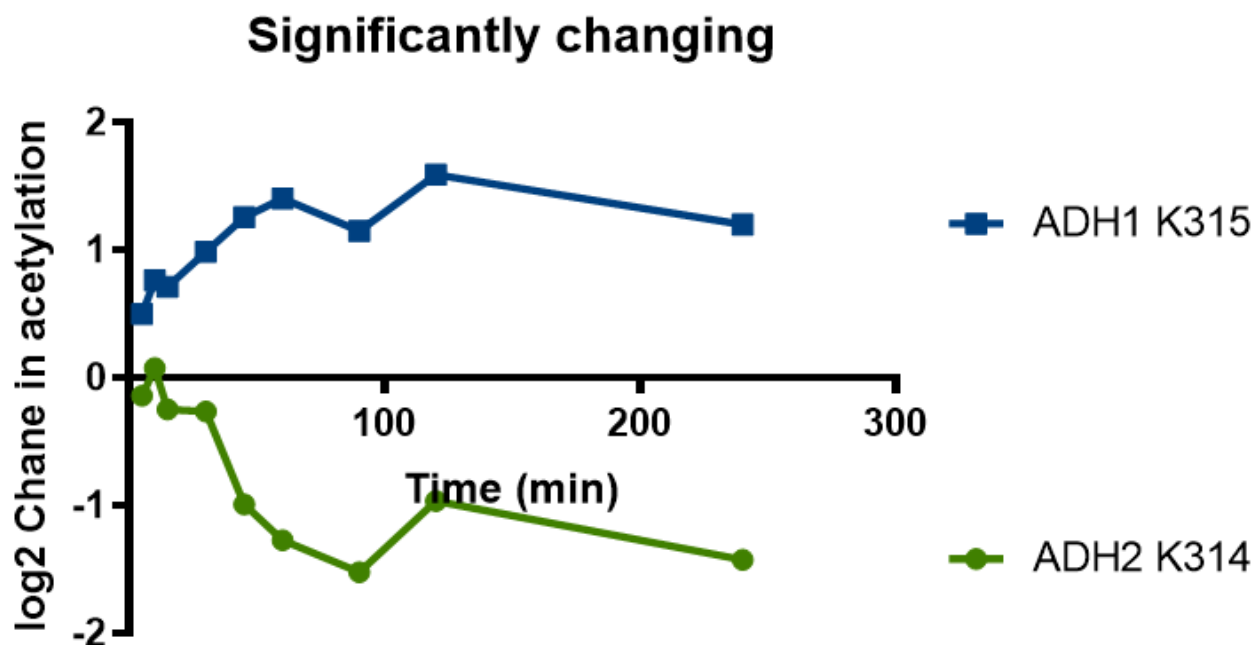


**Figure 3** Schematic representation of pyruvate pathways. Pyruvate synthesized in glycolysis is converted to acetyl-CoA or oxaloacetate and is directed to the TCA Cycle. Pyruvate can also synthesize acetyl-CoA via the PDH-bypass system (Figure by Rodrigues et al. 2006).



**Figure 4:** Metabolism of Glucose and the role of Adh1p and Adh2p (Figure by Canonigo).

The metabolic mechanisms of yeast became of keen interest to our lab because our lab noticed many stress defense proteins were being acetylated during stress heat shock. Notably, Adh1p and Adh2p showed both an increase and a decrease in acetylation at two lysine residues (K315 and K314) overtime during heat shock respectively. This type of regulation could be similar to the Acs system present in *S. enterica*, though the exact purpose of this pattern of acetylation exhibited is unknown. As such, the goal of this project is to determine the effect acetylation has on Adh protein activity during heat stress.



**Figure 5:** Acetylation of *ADH1* K315 and *ADH2* K314 overtime during heat shock. Unpublished Data by Rebecca Sides. The data has also been normalized to protein abundance.

To determine the effect acetylation has on Adhp activity, I used site directed mutagenesis to introduce mutations in the genome at lysine residue K315 in *ADH1* and K314 in *ADH2* by replacing the lysine in three different ways. I replaced lysine with glutamine because glutamine mimics acetylated lysine. I replaced lysine to arginine because arginine mimics un-acetylatable lysine. For the final mutation, I replaced lysine to alanine to test whether these lysine residues are even essential for Adh1p and Adh2p function. This was accomplished by carrying out a series of *E. coli* transformations and yeast transformations. I then tested these mutant yeast strains against the wild-type strain under normal growth conditions, 30°C, and growth under high temperature conditions, 40°C.

Ultimately, the goal of this thesis was to understand what role acetylation plays during environmental shifts. By having a better understanding of this, my project may provide novel

insights into how lysine acetylation affects the capability of yeast to adapt to heat shock.

Moreover, understanding this mechanism can lay an important foundation to study other types of stress such as oxidative stress, ethanol stress, salt stress, etc. The Adh enzyme is also conserved in other species such as human beings (Cederbaum 2012). Adh is found in the liver and stomach, which aids in the conversion of ethanol into carbon dioxide and water (Cederbaum 2012). As a result, discovering new information about the Adh enzymes has implications for human disease and even pathogenesis.

## METHODS AND MATERIALS

### *Testing for a Phenotype in ADH1 and ADH2*

Yeast diploid strains JL1188 (*ADH1* deletion), J11363 (*ADH2* deletion), and JL2 (BY4742 wild-type) were streaked onto YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates. Four isolated colonies from each plate were inoculated in 2-mL cultures of YPD and were grown overnight at 30°C. A growth curve in a 24 well plate was then conducted. 1mL of YP 2% Glucose and YP 20% Ethanol were placed in each well, and each strain was placed in each medium in biological triplicate. The growth curve was grown in the EON plate reader with the following conditions: 30°C, continuous orbital shaking at max speed for 48 hours with readings taken every 15 minutes. Data from the growth curves were plotted using Graphpad Prism software. The data was plotted by taking the mean of the triplicate cultures with error bars representing the standard deviation.

### *Amplifying ADH1 and ADH2 for Cloning into pAG36*

Since a phenotypic difference where growth was dependent on Adh1p function was determined, a PCR and gel electrophoresis analysis was done to amplify *ADH1* for future cloning. Though a phenotypic difference where growth was dependent on Adh2p function was not determined, it was still amplified for future cloning. The first attempt to amplify *ADH1* and *ADH2* was done with the high fidelity polymerase known as KOD. *ADH1* and *ADH2* primers were utilized. Description of these primers can be seen in Table 1. The following materials were obtained to conduct a PCR and a gel electrophoresis analysis: PCR water, Buffer (10X), dNTPS (2 mM each), *ADH1* & *ADH2* pAG36 F/R primers, MgSO<sub>4</sub>, and KOD polymerase. Two

mastermixes were made with the following components: 92.4 uL water, 15 uL buffer, 15 uL dNTPs, 4.8 uL F primer, 4.8 uL R primer, 9 uL MgSO<sub>4</sub>, and 3 uL of KOD. Genomic DNA of *ADH1* and *ADH2* was extracted using the method of Looke (Looke et al. 2011). I collected 2-4 colonies, added LiOAc lysis buffer, incubated at 70°C for 15 min, washed with EtOH, spun in the microfuge, resuspended the pellet with TE, then spun again to acquire DNA in the supernatant. Six uL of template DNA of *ADH2*::kan for MM1 and *ADH1*::kan for MM2 was collected using fresh Lithium acetate DNA prep. Twenty-five uL of each mastermix was divided into 6 tubes and placed into the PCR machine with the following cycling parameters: initial denaturation at 95°C x 2min, followed by 40 cycles of denaturation 95°C x 20sec, annealing at a gradient of 45-60°C x 10s, elongation at 70°C x 1 min, and incubation at 70°C x 5min.

Since the PCR using KOD was unsuccessful, a new polymerase known as Taq with a lower fidelity will be used for the second trial. For the Taq polymerase PCR and gel analysis, the mastermix components were 93.6-ul water, 24-ul of 5x buffer, 12-ul of 2mM dNTPs, 6-uL of 10uM primer, and 0.4-ul of Taq polymerase. DNA of *ADH2* and *ADH2* deletion strains were extracted using Looke's method (Looke et al. 2011). Nine uL of mastermix and 1-uL of template DNA were divided into 6 PCR tubes. The PCR was then set with the following cycling parameters: initial denaturation at 94°C x 4 min, followed by 30 cycles of denaturation at 94°C x 30 sec, annealing on a temperature gradient x 45 sec, elongation at 72°C x 2.5min, and incubation at 72°C x 5 min. The temperature gradient for *ADH1* was 48-58°C and the temperature gradient for *ADH2* was 45-55°C.

Since Taq was successful, a higher fidelity polymerase than Taq but lower one than KOD polymerase known as Herculase was conducted. The final PCR and gel electrophoresis using Herculase polymerase contained the following mastermix components: 28.22-uL water, 10-uL

buffer, 6.26-uL dNTPs, 1.26-uL primers, and 1-uL Herculase. DNA of *ADH1* and *ADH2* deletion strains were extracted using the same method as above. Forty-eight uL of mastermix with 2-uL of template DNA were divided into 6 PCR tubes. The cycling parameters for this PCR were an initial denaturation at 95°C x 2 min, followed by 35 cycles of denaturation at 95°C x 20 sec, annealing at 50°C x 20 sec, elongation at 72°C x 1.5min, and incubation at 72°C x 5 min.

Primer Name	Sequence	Purpose
<i>ADH1</i> pAG36 F	AAACGAGCTCGAATTCAGACAGG TACATACAACACTGGAAAT	Pairs with <i>ADH1</i> pAG36 R to amplify 2,124 bp fragments from S288c. Product includes <i>ADH1</i> gene + 929 bp upstream + 148 bp downstream flanking sequence.
<i>ADH1</i> pAG36 R	CGCATAGGCCACTAGTCCTGAGA AAGCAACCTGACCTACAG	Pairs with <i>ADH1</i> pAG36 F to amplify 2,124 bp fragment from S288c. Product includes <i>ADH1</i> gene + 929 bp upstream + 148 bp downstream flanking sequence.
<i>ADH2</i> pAG36 F	AAACGAGCTCGAATTCTGAAGAG ACTAATCAAAGAATCGT	Pairs with <i>ADH2</i> pAG36 R to amplify 2,334 bp fragment from S288c. Product includes <i>ADH2</i> gene + 641 bp upstream + 646 bp downstream flanking sequence.
<i>ADH2</i> pAG36 R	CGCATAGGCCACTAGTTACTTCCT GTATAGGCCCGC	Pairs with <i>ADH2</i> pAG36 F to amplify 2,334 bp fragment from S288c. Product includes <i>ADH2</i> gene + 641 bp upstream + 646 bp downstream flanking sequence.
<i>ADH</i> SEQ 234 F	AAACGTTAAGGGCTGGAAGATCG	Sequencing <i>ADH1</i> or <i>ADH2</i> on the leading strand
<i>ADH</i> SEQ 389 R	TTGAACAGCGTCAGCGGTAG	Sequencing <i>ADH1</i> or <i>ADH2</i> on the lagging strand
T7 Promoter	TAA TAC GAC TCA CTA TAG GG	To sequence DNA in vectors containing the T7 Promoter
<i>ADH1</i> K315A F	CTTCGCCAGAGGTTTGGTCGCGTC TCCAATCAAGGTTGTC	Site directed mutagenesis of Lysine 315 to Alanine
<i>ADH1</i> K315A R	GACAACCTTGATTGGAGACGCGA CCAAACCTCTGGCGAAG	Site directed mutagenesis of Lysine 315 to Alanine
<i>ADH1</i> K315R F	CTTCGCCAGAGGTTTGGTCAGGT CTCCAATCAAGGTTGTC	Site directed mutagenesis of Lysine 315 to Arginine
<i>ADH1</i> K315R R	GACAACCTTGATTGGAGACCTGA CCAAACCTCTGGCGAAG	Site directed mutagenesis of Lysine 315 to Arginine
<i>ADH1</i> K315Q F	CTTCGCCAGAGGTTTGGTCCAGTC TCCAATCAAGGTTG	Site directed mutagenesis of Lysine 315 to Glutamine
<i>ADH1</i> K315Q R	CAACCTTGATTGGAGACTGGACC AAACCTCTGGCGAAG	Site directed mutagenesis of Lysine 315 to Glutamine
<i>ADH</i> 540 SEQ F	GGTGGTCTAGGTTCTTTGGC	Used to verify presence of site directed mutations

**Table 1:** List of primers, sequences, and purpose that were used for this thesis

### *Cloning ADH1 and ADH2 into the yeast expression vector pAG36*

I carried out a plasmid preparation using the Midi-Prep kit (Zymo Research) to purify pAG36. I then eluted the DNA with water and calculated its concentration using a Nanodrop machine. The concentration of my plasmid product was 151.4 ng/ul. From here I carried out a DNA digest using 3-uL of the restriction enzymes EcoRI and SepI and 30-uL of pAG36 DNA at 37°C to incubate overnight. The digest was then placed into the PCR machine at 37°C overnight to incubate. Once completed, I purified the linearized vector using the Zymo DNA Clean and Concentrator kit. I then cloned the *ADH1* and *ADH2* gene into it using the In Fusion Cloning Kit. Details can be found in table 2. Reactions were incubated overnight at 37°C.

	Vector			Insert				Reaction Conditions (uL)			
	Name	ng/uL	ng to add	Name	ng/ul	ng to add		Vector	Insert	Water	Enzyme
1	pAG36	364.8	100	<i>ADH1</i>	99.5	69.4	1	0.27	0.70	3.03	1
2	pAG36	364.8	100	<i>ADH2</i>	278.8	76.25	2	0.27	0.27	3.45	1
3	pAG36	364.8	100	None	0	0	3	0.27	0.00	3.73	1

**Table 2:** In-Fusion Cloning Calculations for placing *ADH1* and *ADH2* gene into pAG36

The cloning reactions were then transformed directly into *E. coli* and plated onto LB (Lysogeny broth) plates containing ampicillin. From here, ten clones (5 *ADH1* and 5 *ADH2* clones) were purified using the Promega purification kit and sequenced using Sanger sequencing to verify the inserts. Sanger sequencing using *ADH* SEQ 234 F, *ADH* SEQ 389 R, and the T7 promoter primers was then performed to verify that the *ADH1* and *ADH2* genes were inserted correctly into the plasmid (table 1). The resulting plasmids were named pADH1-1 for *ADH1* into pAG36 and pADH2-1 for *ADH2* into pAG36.

### *Complementation Growth Curve for Adh1p*

Complementation analysis was conducted to determine whether expressing wildtype Adh1p in the *ADH1* deletion strain rescued the growth defect. pADH1-1 (pAG36 expressing wildtype Adh1p) pAG36/*ADH1* was then transformed into the yeast *ADH1* deletion strain. This transformation resulted in *ADH1* deletion/pADH1-1 (WT). Additionally, pAG36 was transformed into the wild type strain and the *ADH1* deletion strain using the same technique to create strains to serve as positive and negative controls respectively during growth curve analysis. These strains were plated onto SD (Synthetic Minimal Glucose Medium) Ura- plates (Sherman 2002). Three isolated colonies from each strain were inoculated into 2-mL cultures of SD Ura- to select for the plasmid. They were then grown overnight. After, each strain was placed in a 24-well-plate in triplicate fashion with SD Ura- 2% Glucose serving as the media. This media was chosen because we have to use minimal media (SD) lacking uracil in order to maintain selection for our plasmids. The growth curve was carried out at 30°C, continuous orbital shaking at max speed for 48 hours with readings taken every 15 minutes. Data from the growth curves were plotted using Graphpad Prism software. The data was plotted by taking the mean of the triplicate cultures with error bars representing the standard deviation.

### *Site-directed mutagenesis of ADH1*

Site-directed mutagenesis of *ADH1* was carried out to introduce mutations that mimic acetylated lysine (glutamine) or unacetylatable lysine (arginine) at lysine 315 in *ADH1* gene. Additionally, I created a control strain by introducing an alanine mutation to assess if this lysine residue is important for Adh1p function. I carried out site directed mutagenesis using the Stratagene Quikchange Kit with primers shown in table 1. The plasmid I mutated was pADH1-1. The PCR conditions were 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 10 s, and elongation at 70°C for 5 mins. One uL of DpnI was added to each reaction to digest the original template plasmid. The mutated plasmids were then transformed into chemically competent *E. coli* cells and plated onto selective plates. Sanger sequencing using *ADH* 540 Seq F was used to verify the presence of the mutations (table 1). After verifying the addition of these mutations, I transformed the mimetic plasmids I created into the ADH1 deletion yeast strain.

Strain Name Synthesized	Description
pADH1-2 (K315A)	Yeast strain with an Alanine mutation introduced at K315. The purpose of this mutation is to assess if this lysine residue is important for Adh1p function.
pADH1-3 (K315R)	Yeast strain with an Arginine mutation introduced at K315. The purpose of this mutation is to mimic un-acetyltable lysine due to its structure and charge.
pADH1-4 (K315Q)	Yeast strain with a Glutamine mutation introduced at K315. The purpose of this mutation is to mimic acetylation due to its charge and structure.

**Table 3:** Strains Synthesized from Yeast Transformations

### *Mimetic Growth Analysis*

With the successful introduction of the mimetic mutations, growth curve analysis was carried out to analyze whether acetylation has an impact on metabolism when yeast are subjected to heat stress. Growth curves will be carried out at normal temperature conditions of 30°C and heat shock conditions of 40 °C.

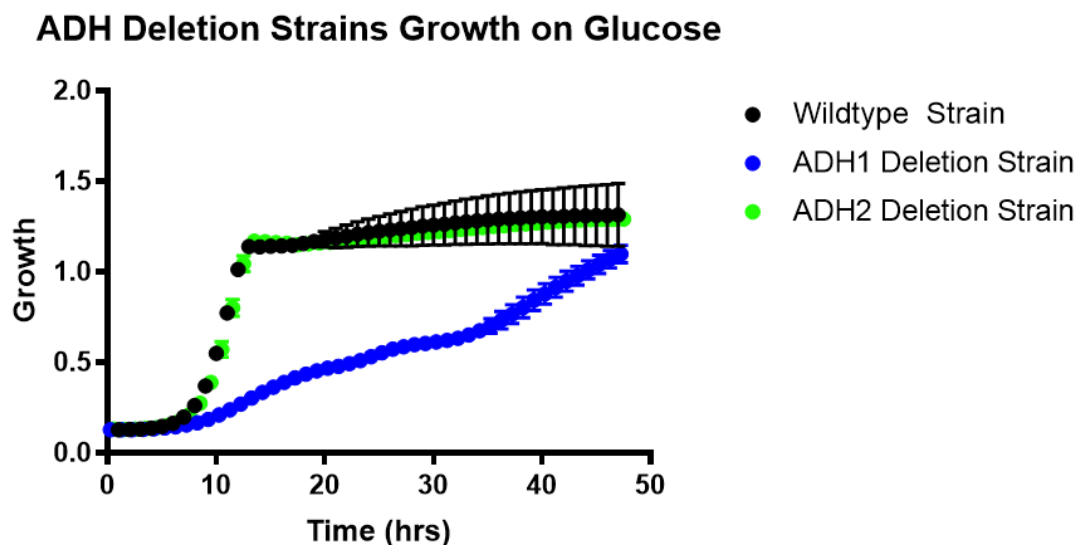
For my 30°C growth curve, I prepared 2-mL cultures of the mimetic strains and control strains in YPD +Nat. Samples were placed in the 30°C incubator to grow. The overnight samples were then put into a 24-well-plate in biological triplicate in SD Ura- 2% Glucose Media. The growth curve was carried out for 48 hours at 30°C with shaking in the EON plate reader. I conducted a second growth curve to test these strains when subjected to heat stress. The same conditions were used when preparing the samples, but I carried out the growth curve for 48 hours at 40°C. Each growth curve data was plotted using prism software. The data was plotted by taking the average of the triplicate cultures with error bars representing the standard deviation.

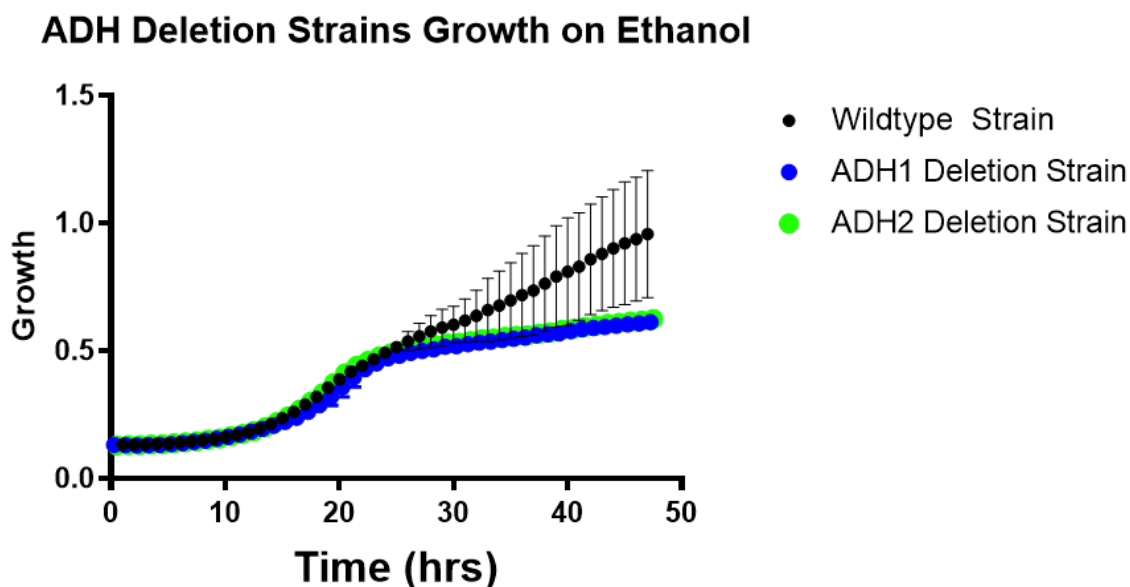
## RESULTS

### *Determining if ADH deletion strains have growth defects in Glucose and Ethanol*

The purpose of these initial growth studies was to find a growth phenotype where growth was dependent upon Adh function. We needed this as a tool to do mimetic experiments later on to look at the effects of acetylation state of Adh on its function. Thus, establishing a growth phenotype was needed in order to do mimetic growth curves.

For this experiment, the *ADH1* deletion strain, *ADH2* deletion strain, and the wild type strain were grown on both YP 2% Glucose and YP 2% Ethanol. Glucose was selected because glucose is needed for Adh1p function since it converts acetaldehyde into ethanol during fermentation. Ethanol was selected because Adh2p converts ethanol back into acetaldehyde to use the TCA cycle. Results of these growth curves are depicted in Fig.6.





**Figure 6:** Growth of the *ADH1* deletion (blue circles) and *ADH2* deletion (green circles) in comparison to wild type *S. cerevisiae* (black circles) in YPD (top) and YPE (bottom). Glucose and Ethanol carbon sources were present at a final concentration of 2% (w/v). All growth curves were performed at 30°C in biological triplicate.

YP 2% glucose growth (Fig.6, top panel) shows that the *ADH2* deletion strain grows in a similar fashion to the wildtype strain, whereas the *ADH1* deletion strain presents a growth defect because it is growing slower than the wildtype strain. This showcases that growth is dependent upon Adh1p function when grown in glucose. Growth is not dependent on *ADH2* in this medium. YP 2% ethanol growth showcases that both the *ADH1* and *ADH2* deletion strains show a very minimal phenotypic difference. For Adh2p, the difference in growth between the wildtype and *ADH2* deletion was not great enough to be an effective condition to use for the mimetic growth analysis. This could be due to other *ADH* genes compensating for the *ADH2* deletion.

In conclusion, it appears that I found a growth condition where growth is dependent on Adh1p as evidenced by lack of growth when deleted in glucose. No growth dependence on Adh2p is clear in both media since the difference between growth of the *ADH2* deletion and wild

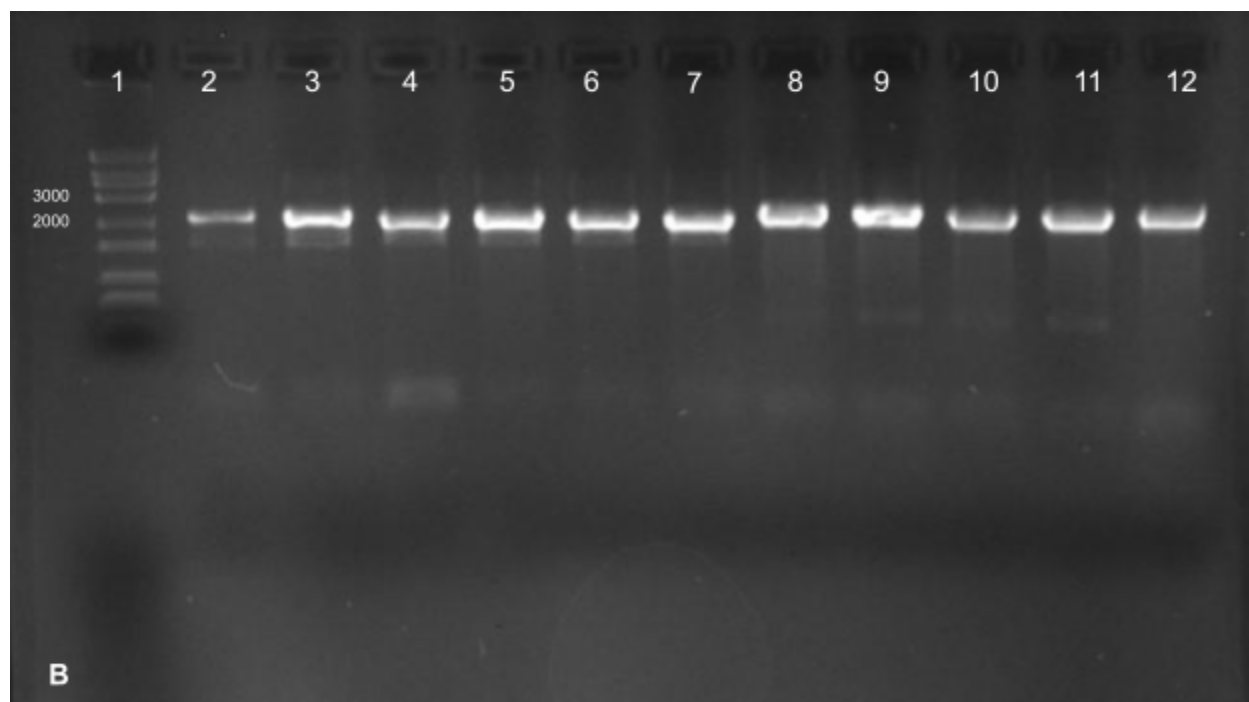
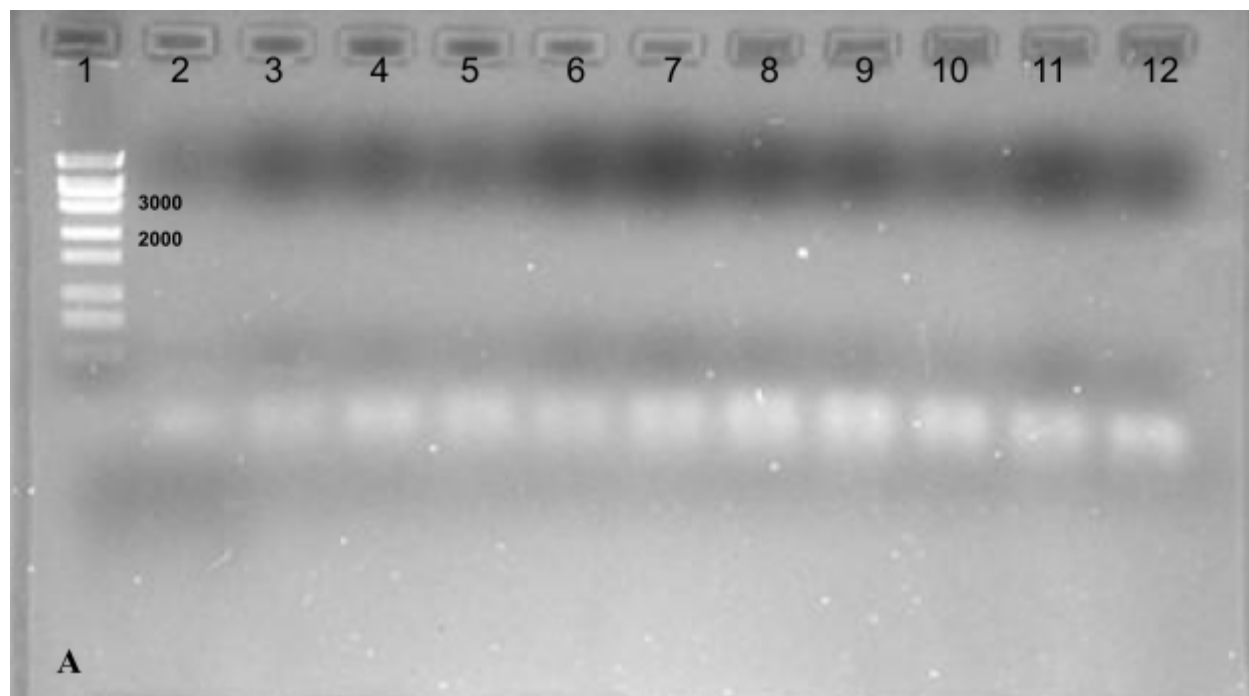
type was minimal. Since a phenotype is apparent in *ADH1*, cloning experiments with *ADH1* may now be conducted. Even though minimal phenotype was seen with the *ADH2* deletion strain, *ADH2* was still used for cloning experiments.

### *Cloning of ADH1 and ADH2 into a Yeast Expression Vector*

To conduct further research on the Adh1p and Adh2p enzymes, the *ADH1* and *ADH2* genes needed to be amplified for cloning into a plasmid, then reintroduced into the deleted strain's genome. This is carried out to see if the wild-type phenotype can be recovered. If successful, this strain can be used to introduce mimetic mutations for further experimentation. I conducted three separate trials for amplifying these gene segments using different polymerases with different fidelities for each.

The first trial gel analysis utilized KOD polymerase and *ADH1* pAG36 F/R and *ADH1* pAG36 F/R primers (table 1). KOD polymerase was chosen because it is a high fidelity polymerase. I chose a high fidelity polymerase because it is important that I cloned the *ADH* genes without any mutation introduced by PCR. As evidenced in figure 7, KOD polymerase was not successful because no bands are present. I used Taq polymerase in my second trial and the same primers. Taq polymerase was used because I could not get KOD polymerase to work. The primers as well as Taq polymerase were effective because bands appeared for both the *ADH1* and *ADH2* gene with their respective expected product size. However, Taq polymerase is not the ideal polymerase choice because it has low fidelity and it introduces mutations at an average rate of 1 mutation per kb. I then decided to try one more high fidelity polymerase (Herculase) for my third and final trial. The primers for *ADH2* worked as evidenced by the presence of bands but not *ADH1*. In the end, I had to go with *ADH1* with Taq polymerase for cloning because I could not

get any of the other polymerases to work and *ADH2* with KOD polymerase. These samples were then cloned into the yeast expression vector pAG36.





**Figure 7:** KOD Gel Analysis (A), Taq Gel Analysis (B), and Herculase Gel Analysis (C). A 5kb ladder was placed in well 1 for each trial. For KOD (A), lanes 2-6 were filled with *ADH1* and lanes 7-12 were filled with *ADH2*. For Taq (B), lanes 2-6 were filled with *ADH1* and lanes 7-12 were filled with *ADH2*. For Herculase (C), lanes 2-4 were filled with *ADH1* and lanes 5-7 were filled with *ADH2*. Each trial used more than one lane for each PCR because a temperature gradient was used in each trial. The expected product size for *ADH1* is 2.214 kb and 2.334 for *ADH2*.

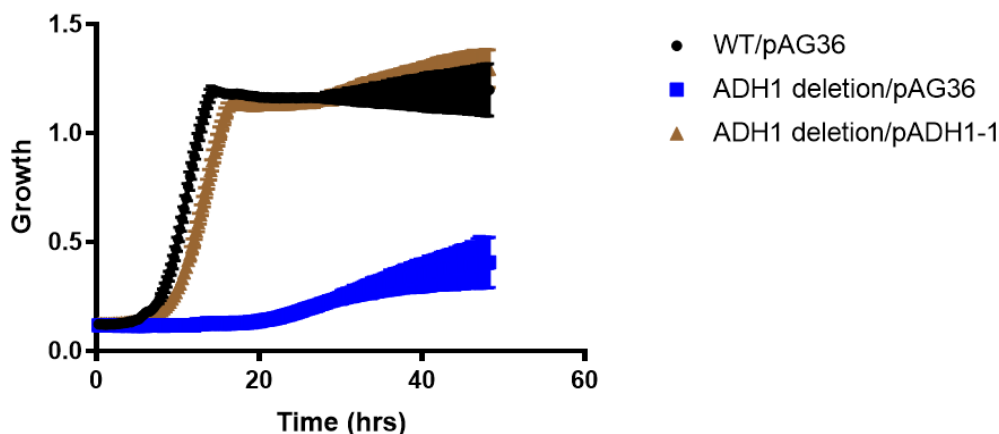
From here I then transformed directly into competent *E. coli* cells and plated onto ampicillin resistant plates. I took five distinct colonies from the *ADH1* transformation and five distinct colonies from the *ADH2* transformation and carried out Sanger sequencing to check that the *ADH1* and *ADH2* genes were inserted correctly into the plasmid. Upon analyzing the sequences, *ADH1* had no issues, only two silent mutations being added; however, sequencing for *ADH2* was not successful due to the introduction of unwanted mutations. From this point forward, we decided to just study Adh1p because it had a more prominent phenotype in regards

to metabolic growth when compared to Adh2p against the wildtype strain (Figure 6). Because all of the initial transformations were done with *E. coli* cells, they needed to be transformed into yeast cells to be able to conduct more growth curve analysis. I transformed the plasmid I created, *ADH1* into pAG36, into the yeast *ADH1* deletion strain. I also synthesized a yeast strain that expressed wild type in phenotype into the yeast expression vector and the *ADH1* deletion strain into the yeast expression vector to act as positive and negative controls respectively when conducting growth curve experiments.

#### *Complementation Growth Curve for ADH1*

Prior to the introduction of mutations, I conducted complementation analysis to assess if expressing wildtype *ADH1* in the *ADH1* deletion strain resulted in the wild type phenotype. I did this complementation analysis in SD Ura- 2% Glucose to see if SD Ura- is a good media to use for growth curve experiments later on. SD Ura- 2% Glucose was chosen because the original experiments of analyzing growth curves were done in a rich medium containing glucose (top panel Figure 6). Minimal medium (SD) lacking uracil needs to be used in order to maintain selection for the plasmids I created. Additionally, I wanted to see if the growth phenotype exhibited in rich media would be similar in minimal media.

### Positive and Negative Control Growth and pADH1-1 Growth in SD Ura- 2% Glucose

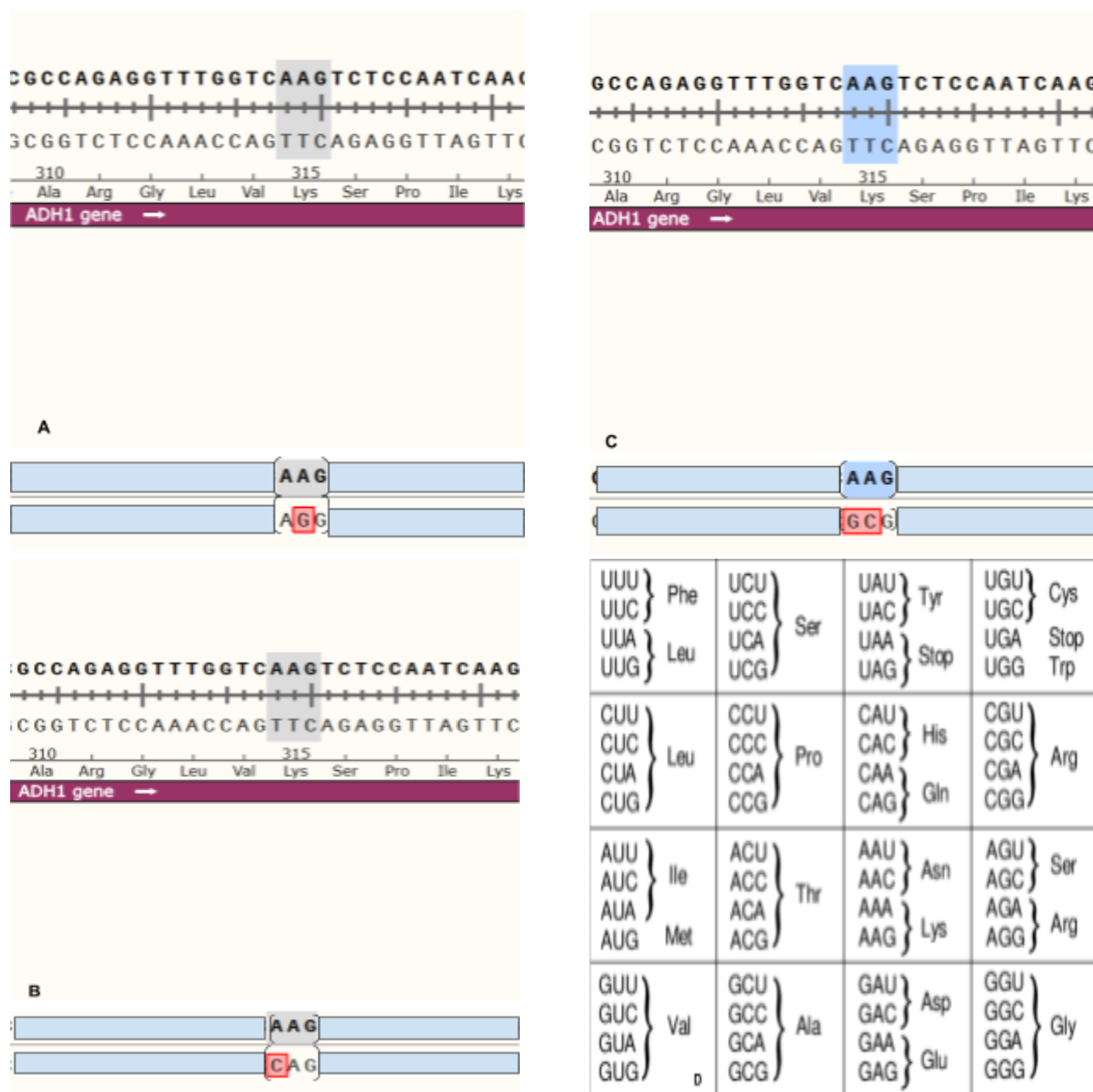


**Figure 8:** Complementation Growth Analysis in SD Ura- 2% Glucose. Wild type strain introduced into yeast expression vector (black), *ADH1* deletion strain in pAG36 (blue), and *ADH1* deletion strain with yeast expression vector expressing *ADH1* (brown). Glucose was present at a final concentration of 2% (w/v). All growth curves were performed at 30°C in biological triplicate.

The growth curve demonstrated that the wild-type phenotype was successfully rescued when the *ADH1* expression vector was introduced into the *ADH1* deletion strain (brown). This can be seen because the *ADH1* deletion/pADH1-1 strain is growing in similar fashion to WT/pAG36. The fact that expressing wildtype Adh1p in the *ADH1* deletion strain rescues the growth defect seen in the top panel of figure 6 means that the cause of the growth defect is the lack of Adh1p activity. The growth curve also demonstrates that minimal media lacking uracil is a good medium to use going forward as each strain grew in the way they were predicted to. Since I verified that Adh1p is required for growth, and SD Ura- 2% Glucose was deemed as a good media, I can now introduce mimetic mutations that mimic acetylation and deacetylation.

### *Site-directed mutagenesis of ADHI*

Now that *ADHI* has been sequenced, verified, and introduced successfully into the yeast expression vector, I can now introduce mimetic mutations at a specific lysine residue to test their phenotypes against the wild type strains. The chosen lysine residue for creating these mimetic mutations was K315. K315 was specifically chosen because as seen in figure 5, K315 showed an increase in acetylation overtime during heat shock. Because of this, we wanted to know if acetylation at K315 is affecting Adh1p activity in any way. The specific mutations that were introduced at K315 were Lysine to Arginine, Lysine to Glutamine, and Lysine to Alanine. Arginine mimics unacetylated Lysine because it has the same charge as Lysine, and it cannot be acetylated. Glutamine mimics acetyl-lysine because of charge and structure similarity. Lastly, Alanine was introduced because it mimics the loss of the lysine side chain. Each mimetic strain was constructed by site-directed mutagenesis using the Stratagene Quikchange method. Sanger sequencing was then completed to verify the introduction of the mimetic mutations into the *ADHI* gene. The plasmids containing the arginine and alanine mutations were implemented successfully; however, the glutamine mutation primers did introduce a silent mutation at Serine97. Since the mutation is silent and did not alter the amino acid, the plasmids were then transformed into *E. coli* cells for further experimentation.

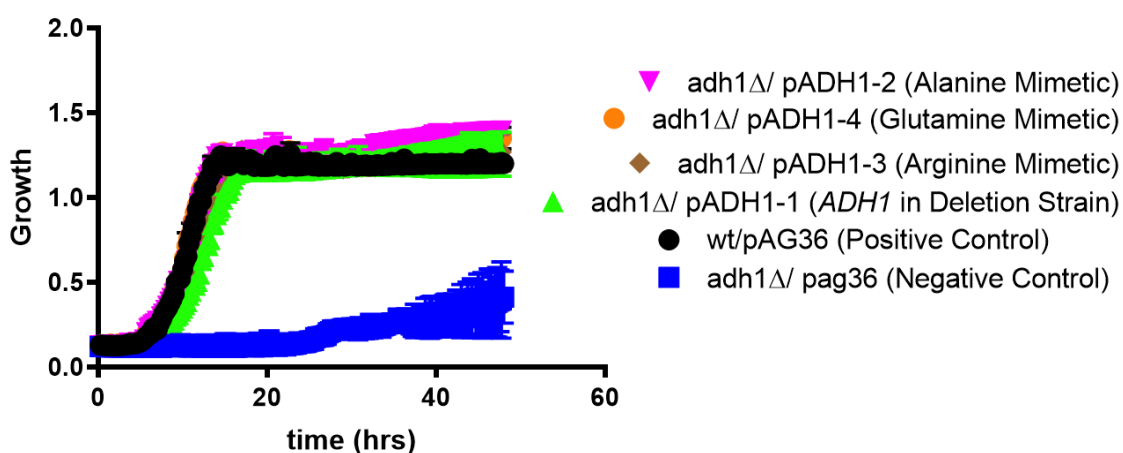


**Figure 9:** Introduction of Mimetic Mutations. For each sequencing image, the top line of sequence of the bottom half represents the wild type genomic sequence, and the bottom half with the red highlight indicates the introduction of a mutation. A codon table in panel D is provided to show the mutation was introduced. In panel A, Lysine is successfully mutated into Arginine as indicated by a codon change from AAG to AGG. In panel B, Lysine is successfully mutated into Glutamine as indicated by a codon change from AAG to CAG. In panel C, Lysine is successfully mutated into Alanine as indicated by a codon change from AAG to GCG.

### *Mimetic Mutation Growth Analysis Under Heat Shock*

The purpose of introducing the mimetic mutations was to introduce the ability to mimic acetylation and unacetylation so that growth curve analysis can be carried out to see if altering Adh1p's acetylation state would impact its activity. Prior to conducting growth curve analysis, I took the *E. coli* transformations with the mimetic mutation plasmids and transformed them into yeast (Table 4). Next, I carried out a growth curve under normal conditions of 30°C to see if any phenotypic difference is apparent.

#### **Mimetic Mutation Growth at 30°C in SD Ura- 2% Glucose**

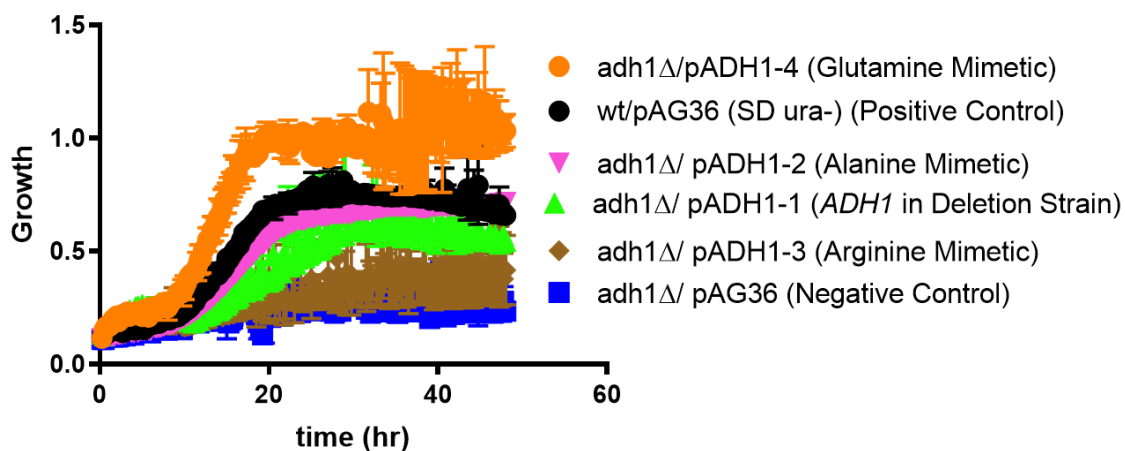


**Figure 10:** Mimetic Mutations Growth Curve at 30°C in SD Ura- 2% Glucose. Glucose was present at a final concentration of 2% (w/v). All growth curves were performed at 30°C in biological triplicate.

The results show that at 30°C, all of the mimetic versions grew similar to wild type as indicated by the black line. Thus, under standard growth conditions, it appears that acetylation state at Lysine 315 is not affecting Adh1p function. I wanted to see if the same results would occur during elevated temperatures because previous data showed that acetylation increases

during heat shock (figure 5). To test this, I conducted another growth curve analysis experiment at 40°C.

### Mimetic Mutation Growth at 40°C in SD Ura- 2% Glucose



**Figure 11:** Mimetic Mutation Growth at 40°C in SD Ura- 2% Glucose. Glucose was present at a final concentration of 2% (w/v). All growth curves were performed at 30°C in biological triplicate.

Under heat shock conditions, it appears that the mutation mimicking acetylation (orange) grew better than the wild type strain (black), whereas the unacetylated strain (brown) was growing slower than the wild type strain. Additional experimentation, such as trying a growth curve at 37°C to see if they still have the phenotype needs to be conducted to get a clearer result of this phenomenon.

## DISCUSSION AND FUTURE DIRECTIONS

### *Discussion*

Ultimately, the aim of this study was to assess whether changing the acetylation state of Adh1p impacted *Saccharomyces cerevisiae*'s growth behaviors when subjected to different temperatures. The first temperature I chose to analyze growth behavior was 30°C because it is the standard temperature of growth for *Saccharomyces cerevisiae*. This investigation showed that at 30°C, the strain that is mimicking acetylated Adh1p did not show improvements in growth in comparison to the wild type (Figure 10). Similarly, the strain mimicking unacetylated Adh1p did not show improvement in its growth (Figure 10). As such, it appears that the mimetic versions are growing similar to wildtype, so under standard growth conditions, the acetylation state at lysine 315 is not affecting Adh1p function.

I then analyzed the growth behavior at 40°C. This temperature was chosen because previous data collected in our lab demonstrated that Adh1p experienced an increase in acetylation when subjected to heat shock (Figure 5). I wanted to see if acetylation of Adh1p at this temperature would lead to any changes in its growth behavior. Interestingly, it appeared that the unacetylated version grew slower than the wild type strain, while the acetylated strain grew better than the wild-type (Figure 11). This may be due to acetylation altering the activity of the Adh1p. Acetylation may turn off the activity of Adh1p to make the cell stop producing ethanol, and the ethanol already produced can be detoxified by using Adh2p to convert it back into acetaldehyde (Figure 4). Then it can enter the TCA cycle to be used to generate more ATP. Additionally, the alanine mimetic mutation was indistinguishable from the wild-type strain.

Recall that yeast can take two different pathways of metabolism after Pyruvate synthesis in Glycolysis: it can convert pyruvate into ethanol via fermentation and export out the cell, or pyruvate can be converted into acetyl-CoA and enter the TCA cycle and the electron transport chain for ATP generation in a process called respiration (Rodrigues et al. 2006). A precise understanding of what is happening during yeast's metabolic pathways during heat shock is not known. There has been some research conducted in the past that shows yeast actually switching to respiration during stress, resulting in higher activation of some respiration genes during stress (Timón-Gómez et al. 2013). If yeast are still growing on glucose during that stress, it is likely that they would be bypassing all of the Adh proteins and taking pyruvate synthesized from glycolysis directly into the TCA cycle instead of converting into ethanol. However, this bodes a problem for all the ethanol yeast have already made and that has accumulated in the medium. Both ethanol stress and heat stress have similar effects on the cell, such that they can damage mitochondrial DNA, inactivate some enzymes, and impact the fluidity of the cell membrane (Kyung Man You 2003 & Galdieri et. al 2010). So, having both heat stress and ethanol stress may be additive in the damage it is doing to the cell. It is probably beneficial to detoxify the ethanol created in the medium by using Adh2p to convert ethanol back into acetaldehyde. Past studies have reported that Adh2p has a high affinity, thus a low  $K_m$ , for ethanol (Bakker et. al 2000). The acetaldehyde made from the ethanol can then enter the TCA cycle and be utilized to generate more ATP. This is a win-win for the cell since it is both detoxifying itself and synthesizing more energy.

Given all of this, I hypothesize that the acetylation of Adh1p turns off its activity. Previous lab data has shown an increase in Adh1p acetylation and a decrease of Adh2p acetylation (Figure 5). If the cell is trying to stop producing ethanol, turn off Adh1p activity, and

detoxify the ethanol that has already been made by directing it back into the TCA cycle, turning Adh2p on, it would seem more plausible to say acetylation turns off Adh1p activity during heat shock. Additionally, I would hypothesize that heat shock also eventually turns off Adh2p activity after all the ethanol has been detoxified because all Adh proteins have the same active site architecture and the acetylation of both Adh1p and Adh2p occurred at Lysine 315 (Ganzhorn et. al 1987). Though I cannot confirm this is the exact mechanism occurring in the cell, so further research is needed.

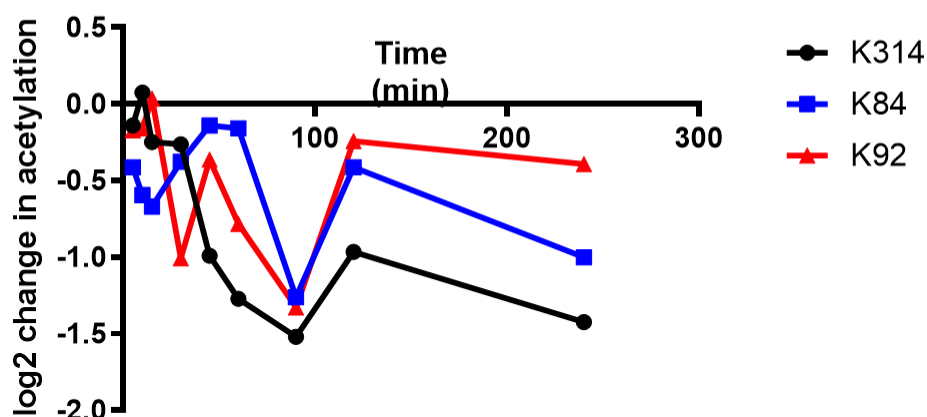
### *Future Directions*

Ultimately, numerous steps were taken to understand the role acetylation plays on Adh1p and metabolism. My data shows promise that acetylation may play an important role in turning off Adh1p function in heat shock to lead to more efficient metabolism. The next steps from here would be to see if my results are reproducible. I would redo the growth curves at 30°C and 40°C. Additionally, I was going to try these growth curves at 37°C to see if I could potentially get a clearer result of the acetylated form growing better than the wildtype and the unacetylated form growing slower than the wildtype.

While I was in the process of redoing my heat shock growth curve experiments, the lab shut down due to COVID-19, and I was unfortunately unable to continue experiments. Nevertheless, this project will hopefully be continued in the future once COVID-19 restrictions have been lifted. If the results are reproducible, then the next step would be to purify the Adh1p in *E. Coli* with a yeast tag. A purified acetylated version and a purified unacetylated version would need to be obtained. Then, a biochemical analysis would then be conducted to assay the activity of the protein to confirm that acetylation is actually turning on the activity of the protein. If this were to be true, it would provide novel insights for the organism's ability to metabolize under heat shock conditions.

In regards to Adh2p, I did not find a phenotype where growth was dependent on Adh2p activity (Figure 6). I can conduct a heat shock growth analysis of the *ADH2* deletion strain to see if growth is dependent on Adh2p activity. It may be the case that other *ADH* genes are compensating for the *ADH2* deletion, so I may have to delete several other *ADH* genes to assess Adh2p function in vivo.

### ADH2 deacetylation pattern during heat shock



**Figure 12:** Pattern of Deacetylation experienced by *ADH2*. Black represents Lysine 314, blue represents Lysine 84, and red represents Lysine 92. The data have been normalized to protein abundance

It may also prove beneficial to analyze other lysine residues that experience a similar pattern of deacetylation when undergoing heat shock. In the lab, steps have been taken to introduce mimetic mutations of acetylation and unacetylation in *ADH2* at lysine residues 84 and 92. These mimetic mutations were introduced successfully, and were successfully transformed into *E. coli*, but again further experiments were not able to be conducted due to COVID-19. Once the lab re-opens, these transformations can then be introduced into yeast and used for growth curve analysis at 30°C, 37°C, and 40°C. If a unique phenotype is apparent, such as evidence of acetylation or unacetylation turning on or off the activity of Adh2p, then they can also be purified and assayed to confirm the role of acetylation on the activity of the protein.

Adh proteins are conserved in other species such as human beings. Adh1p is found in the liver and stomach, and it is the primary enzyme involved in alcohol metabolism. Previous studies have found that variants (i.e. alleles) of *ADH1* within humans are associated with altered kinetic

properties. These allelic variants arise from post-transcriptional or post-translational modifications. For example, different Adh1p variants encode for different activities of the Adh1 enzyme, which can result in more rapid conversion of alcohol to acetaldehyde (Edenberg 2007). These variants have been shown to influence a person's drinking ability, and, consequently, the risk of developing alcohol abuse or dependence (Edenberg 2007). As a result, discovering new information about acetylation's role on the Adh enzymes may have implications for studying human diseases, such as finding new ways to metabolize ethanol more efficiently, and even pathogenesis. If my findings could not be replicated or the purified acetylated version of the protein does not turn on the activity of the protein, then it will show that acetylation of Adh1p alone is not the underlying mechanism that affects improved cellular metabolism during heat shock and that other pathways need to be explored to further investigate this phenomenon. For example, it may prove beneficial to analyze different post-translational modifications like phosphorylation or methylation and see if they play a role in impacting metabolism during heat shock. Additionally, looking at whether acetylation increases during other environmental stresses such as oxidative stress or cold shock could be potential avenues to investigate further.

## REFERENCES

- Alan J. Wolfe, The Acetate Switch, *Microbiology and Molecular Biology Reviews* Mar 2005, 69 (1) 12-50.
- Ali, Ibraheem, et al. "Lysine acetylation goes global: from epigenetics to metabolism and therapeutics." *Chemical reviews* 118.3 (2018): 1216-1252.
- Bakker BM Bro C Kotter P Luttik MA van Dijken JP Pronk JT (2000) The mitochondrial alcohol dehydrogenase Adh3p is involved in a redox shuttle in *Saccharomyces cerevisiae*. *J Bacteriol* 182: 4730–4737.
- Beaven, M. J., C. Charpentier, and A. H. Rose. 1982. Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC. *J. Gen. Microbiol.* 128:1447-1455.
- Beier DR Sledziewski A Young ET (1985) Deletion analysis identifies a region, upstream of the *ADH2* gene of *Saccharomyces cerevisiae*, which is required for *ADR1*-mediated derepression. *Mol Cell Biol* 5: 1743–1749.
- Bhavsar, Pankaj, Tehireem Ahmad, and Ian M. Adcock. "The role of histone deacetylases in asthma and allergic diseases." *Journal of Allergy and Clinical Immunology* 121.3 (2008): 580-584.
- Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY and Allis CD (1996) *Tetrahymena* histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell*, 84, 843–851.
- Cederbaum, Arthur I. "Alcohol metabolism." *Clinics in liver disease* 16.4 (2012): 667-685.

- Choudhary, C., C. Kumar, F. Gnädig, M. L. Nielsen, M. Rehman, T. C. Walther, J. V. Olsen & M. Mann (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*, 325, 834-40
- Denis, Clyde L., J. Ferguson, and E. T. Young. "mRNA levels for the fermentative alcohol dehydrogenase of *Saccharomyces cerevisiae* decrease upon growth on a nonfermentable carbon source." *Journal of Biological Chemistry* 258.2 (1983): 1165-1171.
- Edenberg, Howard J. "The genetics of alcohol metabolism: role of alcohol dehydrogenase and aldehyde dehydrogenase variants." *Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism* vol. 30,1 (2007): 5-13.
- Galdieri, Luciano et al. "Transcriptional regulation in yeast during diauxic shift and stationary phase." *Omics : a journal of integrative biology* vol. 14,6 (2010): 629-38.  
doi:10.1089/omi.2010.0069
- Ganzhorn AJ Green DW Hershey AD Gould RM Plapp BV (1987) Kinetic characterization of yeast alcohol dehydrogenases. Amino acid residue. 294 and substrate specificity. *J Biol Chem* 262: 3754–3761.
- Hardman, R. E. (2019). Global Acetylation Dynamics in the Heat Shock Response of *Saccharomyces cerevisiae*. Theses and Dissertations Retrieved from <https://scholarworks.uark.edu/etd/3489>
- Henriksen, P., S. A. Wagner, B. T. Weinert, S. Sharma, G. Bacinskaja, M. Rehman, A. H. Juffer, T. C. Walther, M. Lisby & C. Choudhary (2012) Proteome-wide analysis of lysine acetylation suggests its broad regulatory scope in *Saccharomyces cerevisiae*. *Mol Cell Proteomics*, 11, 1510-22.
- Kouzarides, T. (2000). Acetylation: a regulatory modification to rival phosphorylation? *The*

- EMBO J*, 19(6), 1176–1179.
- Lööke, Marko, Kersti Kristjuhan, and Arnold Kristjuhan. "Extraction of genomic DNA from yeasts for PCR-based applications." *Biotechniques* 50.5 (2011): 325-328.
- Menzies, K. J., H. Zhang, E. Katsyuba & J. Auwerx (2016) Protein acetylation in metabolism - metabolites and cofactors. *Nat Rev Endocrinol*, 12, 43-60.
- Norris, Kristi L., Joo-Yong Lee, and Tso-Pang Yao. "Acetylation goes global: the emergence of acetylation biology." *Science signaling* 2.97 (2009): pe76-pe76.
- Olga de Smidt, James C. du Preez, Jacobus Albertyn, Molecular and physiological aspects of alcohol dehydrogenases in the ethanol metabolism of *Saccharomyces cerevisiae*, *FEMS Yeast Research*, Volume 12, Issue 1, February 2012, Pages 33–47
- Rodrigues, Fernando & Ludovico, Paula. (2006). Sugar Metabolism in Yeasts: an Overview of Aerobic and Anaerobic Glucose Catabolism. 10.1007/3-540-30985-3\_6.
- Sherman, Fred. "Getting started with yeast." *Methods in enzymology* 350 (2002): 3-41.
- SlÁviková, Elena, Renata Vadkertiová, and Dana Vránová. "Yeasts colonizing the leaves of fruit trees." *Annals of microbiology* 59.3 (2009): 419-424.
- Taunton J, Hassig CA and Schreiber SL (1996) A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science*, 272, 408–411.
- Timón-Gómez, Alba, Markus Proft, and Amparo Pascual-Ahuir. "Differential regulation of mitochondrial pyruvate carrier genes modulates respiratory capacity and stress tolerance in yeast." *PLoS One* 8.11 (2013): e79405.
- Vincent J Starai, Jorge C Escalante-Semerena, Identification of the Protein Acetyltransferase (Pat) Enzyme that Acetylates Acetyl-CoA Synthetase in *Salmonella enterica*, *Journal of*

Molecular Biology, Volume 340, Issue 5, 2004, Pages 1005-1012

You, Kyung Man, Claire-Lise Rosenfield, and Douglas C. Knipple. "Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content." *Applied and environmental microbiology* 69.3 (2003): 1499-1503.

Young ET Pilgrim D (1985) Isolation and DNA sequence of *ADH3*, a nuclear gene encoding the mitochondrial isozyme of alcohol dehydrogenase in *Saccharomyces cerevisiae*. *Mol Cell Biol* 5: 3024–3034.





