THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

ASSESSING GENE EXPRESSION OF NAD^+-DEPENDENT PATHWAYS IN NON-MODEL YEAST FOR BIOFUEL PRODUCTION

LILY MARIE WILLIAMS
SPRING 2026

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry & Molecular Biology
with honors in Biochemistry & Molecular Biology

Reviewed and approved* by the following:

Dr. Melanie McReynolds
Associate Professor of Biochemistry & Molecular Biology
Thesis Supervisor

Dr. Wendy Hanna-Rose
Professor of Biochemistry & Molecular Biology
Thesis Honors Adviser

* Electronic approvals are on file.

ABSTRACT

The burning of fossil fuels continues to accelerate climate change, underscoring the urgent need for renewable alternatives such as biofuels. While most biofuels are currently derived from crops that require substantial resources to cultivate, microbes like yeast provide a cost-effective and efficient platform for biofuel production. Oleaginous yeasts, such as *Rhodotorula toruloides*, provide an excellent alternative due to their ability to produce high levels of lipids and fatty alcohols, which serve as additives in biofuels and other bioproducts. However, as a non-model organism, it remains understudied. Lipid and fatty alcohol biosynthesis depend on NADP(H) and its precursor NAD^+, which is known to decline over time in most species. However, the intricacies contributing to this decline remain poorly understood, particularly in *R. toruloides*. This raises the central question: How do NAD^+-dependent pathways change during the growth of this non-model yeast? Understanding these changes is critical to optimizing bioproduct yields. We hypothesize that there is decreased expression of NAD^+ biosynthetic genes over time, coupled with increased expression of degradation pathway genes. Using quantitative reverse transcription PCR, we profiled expression of genes involved in NAD^+ recycling over time in three nutrient conditions: rich media, minimal media, and nitrogen limitation. Utilizing these different conditions highlights how nutrient availability can alter the pathways of the NAD metabolome. This experiment highlights vulnerabilities in NAD^+ biosynthesis that may alter lipid and fatty alcohol production. Changes in gene expression were shown between the different growth conditions tested. Identifying and addressing critical bottlenecks in a variety of environmental conditions with different nutrient sources helps to inform strategies for engineering *R. toruloides* into a more robust and sustainable platform for biofuel and bioproduct generation.

TABLE OF CONTENTS

LIST OF FIGURES iii

LIST OF TABLES iv

ACKNOWLEDGEMENTS v

Chapter 1 Introduction 6

- The Climate Crisis 6
- Novel Biofuel Production using Yeast 8
- NAD^+: An Essential Molecule 9
- NAD^+ Biosynthesis in Yeast 12
- Impacts of Nutrient Source on Growth of *R. toruloides* 14
- Assessing NAD^+ Biosynthesis Gene Expression to Optimize Biofuel Production 15

Chapter 2 Materials and Methods 16

- Media Preparation 16
- Sample collection 17
- RNA Extraction 18
- cDNA Synthesis 18
- Primer Design and Validation 19
- Quantitative Reverse Transcription PCR (qRT-PCR) 20
- Data Analysis 20

Chapter 3 Results 21

- NA/NAM Salvage Pathway Changes Dynamically with Nutrient Rich, Minimal, and Nitrogen Limited Growth Conditions Over Time 21
- NR Salvage Pathway Highly Expresses Genes to Degrade NAD^+ 24

Chapter 4 Discussion 29

Chapter 5 Future Directions 32

Appendix A SYBR Green Primer Sequences 34

BIBLIOGRAPHY 35

LIST OF FIGURES

Figure 1. United States primary energy consumption 1949-2025. 6

Figure 2. Global average surface temperature 1880-2024. 7

Figure 3. United States Domestic Corn Use (USDA). 8

Figure 4. Triacylglycerol synthesis from glucose. 10

Figure 5. Fatty alcohol synthesis. 11

Figure 6. NAD^+ biosynthesis pathways in yeast. 12

Figure 7. Expression of *NPT1* in *R. toruloides* with different media types. 21

Figure 8. Expression of *NMA1* in *R. toruloides* with different media types. 22

Figure 9. Expression of *QNS1* in *R. toruloides* with different media types. 23

Figure 10. Expression of *SIR2* in *R. toruloides* with different media types. 24

Figure 11. Expression of *PHO8* in *R. toruloides* with different media types. 25

Figure 12. Expression of *NRK1* in *R. toruloides* with different media types. 26

Figure 13. Expression of *MEU1* in *R. toruloides* with different media types. 27

Figure 14. Expression of *PNP1* in *R. toruloides* with different media types. 28

Figure 15. Expression of *URH1* in *R. toruloides* with different media types. 29

LIST OF TABLES

Table 1. Composition of Yeast Peptone Dextrose (YPD) pH 5.3. 16

Table 2. Composition of Yeast Nitrogen Base (YNB). 17

Table 3. Composition of Nitrogen Limited Media (C/N100). 17

Table 4. cDNA Synthesis Master Mix. 18

Table 5. Thermal Cycler Setting for cDNA Synthesis. 19

Table 6. qRT-PCR Master Mix. 20

Table 7. Settings for Comparative CT qRT-PCR Run Cycle. 20

Table 8. SYBR Green Primer Specifications for qRT-PCR. 34

ACKNOWLEDGEMENTS

I would like to thank the members of the McReynolds lab, who have created a welcoming and supportive environment for me to strive for success over the past 3 and a half years. Thank you to Dr. Melanie McReynolds for seeing my passion and drive for research and supporting me in traveling to conferences to expand my reach as a scientist. Thank you to my mentor and close friend, Alexandria Murphy, for allowing me to join her team, having full faith in my abilities from the start, and so many wonderful moments throughout this experience. I would not be the person and scientist I am without her guidance. I would like to thank Julia Mudryk, Praveena Prasad, and Mahmoud Yahia for their help and support with method development and brainstorming. Thank you to the many undergraduate students of the McReynolds lab, especially Anant Pothakamury, Blanca Lizanne, Garret Diven, Donovan Mauney, and Cooper Johnson for their friendship and making this the best research journey.

I could not have succeeded in this process without the complete support of my family, friends, and boyfriend. I hope I continue to make you all proud and earn your support every day. Thank you especially to my dear friend, Hannah Bucher, for going through this process with me all four years within the Schreyer Honors College. I feel very fortunate to have found a great community here at Penn State, which I will take with me as I continue onto new opportunities.

This work would not be possible without the DOE-funded Center for Advanced Bioenergy and Bioproducts Innovation (Grant #500000031193 awarded to MRM). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the author and do not necessarily reflect the views of the Center for Advanced Bioenergy and Bioproducts Innovation.

Chapter 1

Introduction

The Climate Crisis

A key contributor to the climate crisis our society is facing is our reliance on fossil fuels for energy. Fossil fuels are a limited resource which we heavily rely on for power. According to the United States Energy Information Administration (US EIA), fossil fuels have remained the largest source of energy consumed in the United States since 1949. While coal usage has declined steadily since 2008, natural gas and petroleum usage have increased (Figure 1). (December 2025 Monthly Energy Review, 2025). Usage of renewable energy sources has slowly increased over time but cannot currently replace our reliance on fossil fuels.

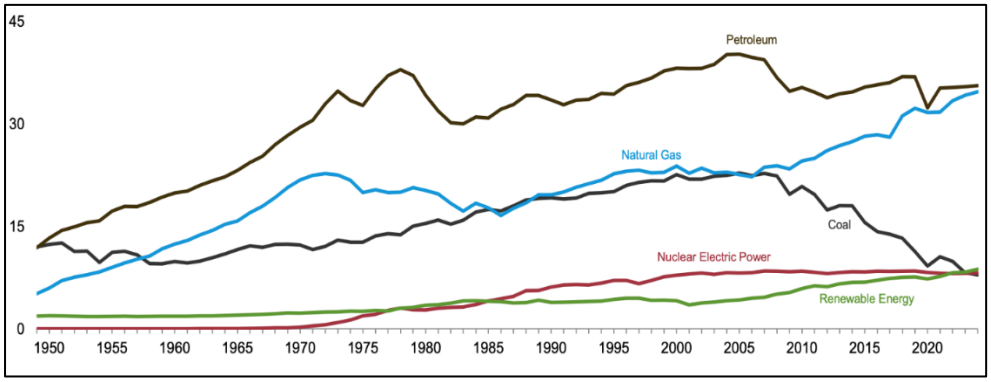

Figure 1. United States primary energy consumption 1949-2025. Changes in energy consumption by source measured in quadrillion British Thermal Units (BTU). Petroleum (brown), natural gas (blue), and coal (black) are all fossil fuels and are consumed at higher amounts than renewable energy sources (December 2025 Monthly Energy Review, 2025).

Fossil fuels produce high levels of greenhouse gases (GHG), like cardon dioxide, that get trapped in our atmosphere, drastically increasing global temperatures. In figure 2, the National Oceanic and Atmospheric Administration shows the average global surface temperature for each

year from 1880 to 2024. Beginning around 1940, most years had a higher-than-average surface temperature. There is also a steady increase seen starting in the mid-1970s and continuing to present day. These increases are consistent with the usage of fossil fuels (Figure 1), suggesting that the burning of fossil fuels and high emissions of greenhouse gases plays a significant role in the warming of our planet. Although other greenhouse gases trap heat more effectively than carbon dioxide (CO_2), it accounts for 76% of total greenhouse gases in the atmosphere (Shivanna, 2022). Our increasing reliance on these limited and detrimental resources creates a need for alternate fuel sources that are renewable and produce less greenhouse gases. The use of biofuels provides a solution to this issue.

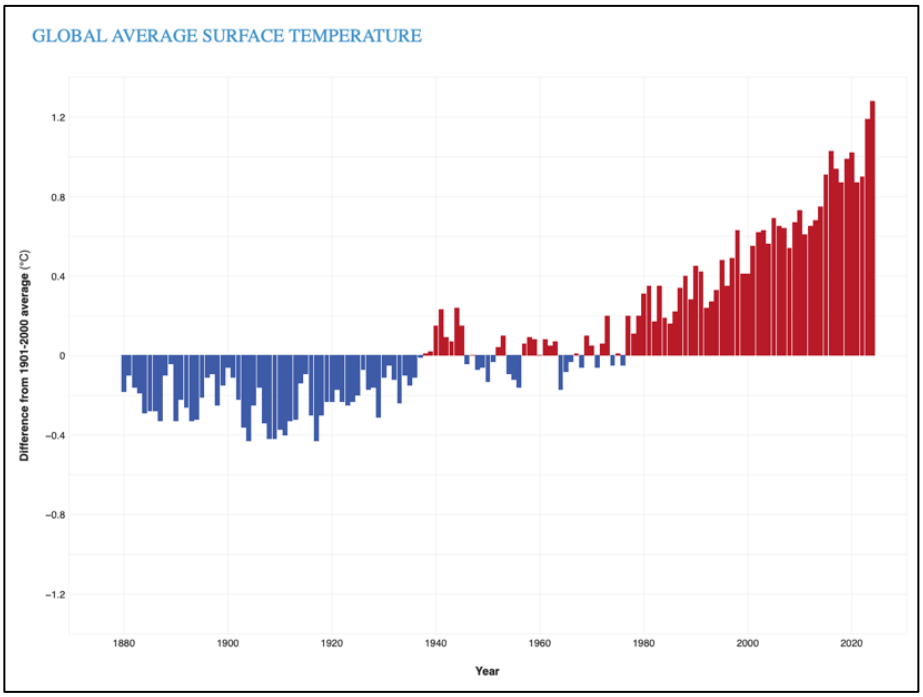

Figure 2. Global average surface temperature 1880-2024. Yearly surface temperature from 1880–2024 compared to the 20th-century average (1901-2000). Blue bars indicate cooler-than-average years; red bars show warmer-than-average years (*Climate Change*, 2025).

Novel Biofuel Production using Yeast

Biofuels are conventionally made using crops, like corn, sorghum, or sugarcane, but there are many drawbacks to using these as substrates. Crops take time to grow and require large amounts of space and nutrients, making biofuel production a costly process (Karimi et al., 2025). These crops are also utilized as a food resource, so growing them for biofuel production diverts key resources away from an essential food supply for many people. Using corn to produce biofuels through ethanol has increased significantly from the early 2000s. Figure 3 from the United State Department of Agriculture (USDA) shows how corn is used in the United States (USDA, 2022). Corn is utilized as a critical food source for humans and livestock. Although we rely on corn in many ways, its cultivation results in high emissions of greenhouse gases like ammonia, sulfur oxides, and nitrogen oxides (Hill et al., 2019). While replacing corn as a food source is implausible, new methods of producing biofuels could help to offset the greenhouse gas emissions of corn cultivation.

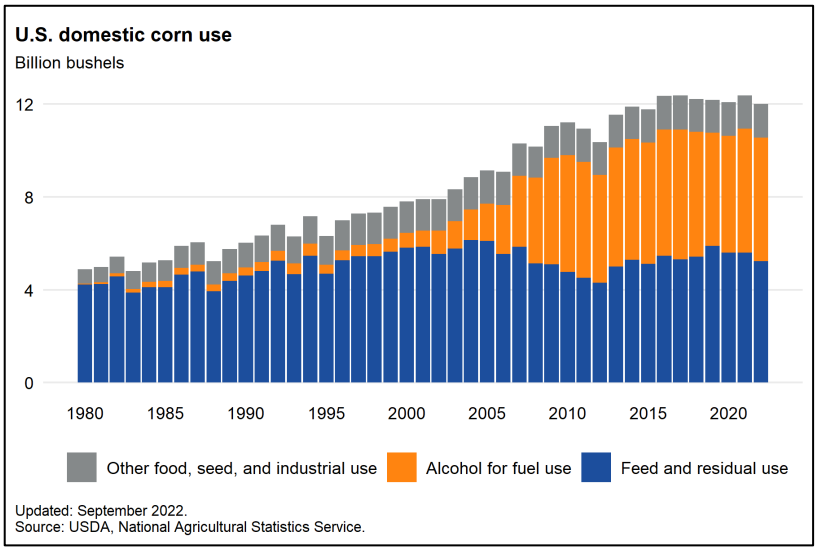

Figure 3. United States Domestic Corn Use (USDA). Changes in corn usage between 1980 and 2022. Corn usage increases gradually but is primarily used for animal feed across this period. After 2000, corn is used more consistently for fuel production (USDA, 2022).

Microbes, including yeast, provide an exciting new approach for the synthesis of biofuel by serving as microbial biofactories. They require less time, space, and resources for growth, making them more cost effective (Wen et al., 2020). Oleaginous yeast species, like *Rhodotorula toruloides IFO0880* (*RT88)*, produce high amounts of lipids and fatty alcohols which can be used as additives in biofuels and other bioproducts, like pharmaceuticals and cosmetics (Spagnuolo et al., 2019). One example of this uses triacylglycerol (TAG), a key lipid produced by *R. toruloides*, which undergoes transesterification with alcohol to produce fatty acid alkyl esters (FAAEs) to create a renewable biodiesel (J. Wang et al., 2022).

R. toruloides is set apart from other oleaginous yeast species because it can produce roughly three times the amount of lipids compared to other oleaginous yeasts (Sunder et al., 2024). A larger abundance of lipids creates the potential for more bioproducts to be created from *R. toruloides*. Another benefit of using this species for biofuel production is its ability to thrive in a variety of environments and use different nutrient sources (Wen et al., 2020). These characteristics make *R. toruloides* a promising source for bioproduct output, but the production of the major substrates relies on nicotinamide adenine dinucleotide, which has a complex role in the cell and has not been thoroughly studied in this species.

NAD^+: An Essential Molecule

Nicotinamide adenine dinucleotide (NAD^+) is an essential co-substrate and cofactor in many biological processes of all organisms. Production of lipids, fatty alcohols, and sugar alcohols in *R. toruloides* requires both NAD^+ and its related metabolites, especially NADPH. One example of the importance of NAD^+ in lipid synthesis is the production of triacylglycerols

(TAG). The synthesis of TAG from one molecule of glucose requires 6 molecules of NAD^+ and 2 molecules of NADPH (Figure 4). Similarly, the synthesis of one molecule of fatty alcohol requires 2 molecules of NADPH (Figure 5). A prior study of two genetically modified strains of *R. toruloides* showed that when multiple genes linked to the production of NADPH were upregulated, it led to greater lipid production (Guo et al., 2019). This emphasizes the importance of the NAD metabolome and the genes associated with it in the production of lipids, especially for this species.

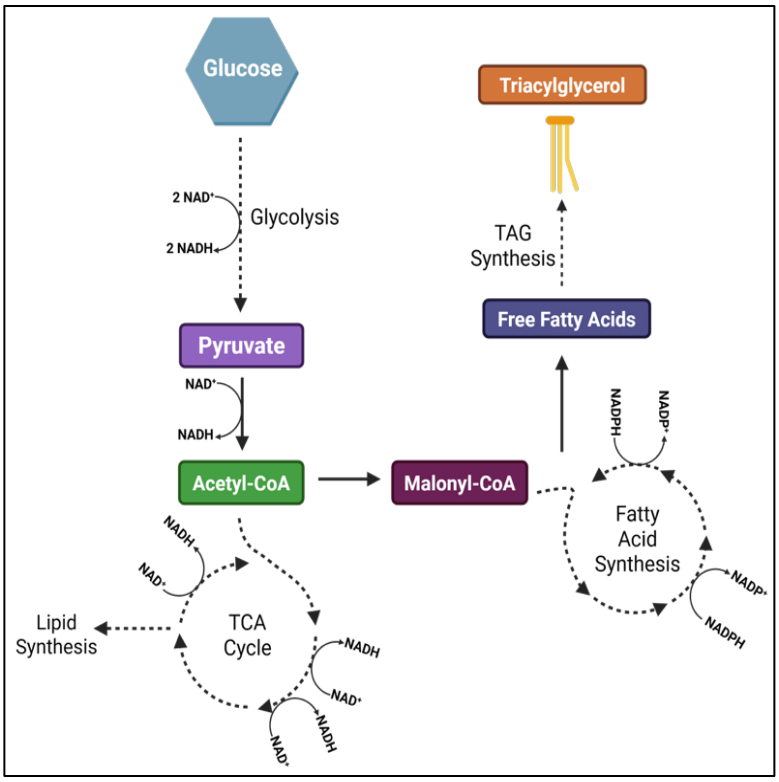

Figure 4. Triacylglycerol synthesis from glucose. The synthesis of triacylglycerol (TAG) from glucose requires 6 molecules of NAD^+ and 2 molecules of NADPH (Priyadarshini & Kataria, 2025).

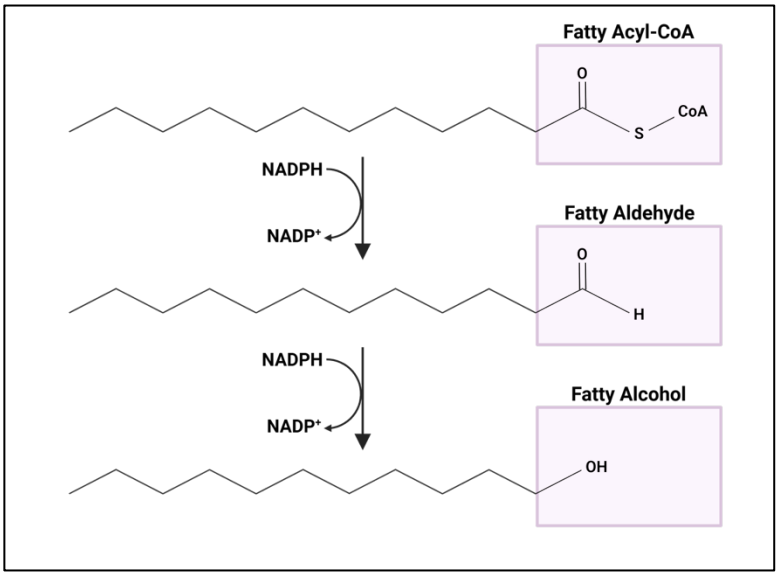

Figure 5. Fatty alcohol synthesis. The synthesis of fatty alcohol from fatty acyl-CoA requires two molecules of NADPH.

While *R. toruloides* is a very promising source of bioproducts, it is highly understudied because it is a non-model species. One key point is how the NAD metabolome, more specifically the genes which are directly involved in synthesis and degradation of NAD^+, changes over time in this organism. A decline of NAD^+ has been documented in all species (McReynolds et al., 2020) so changes in the NAD metabolome over time will similarly impact production of the desired bioproducts from *R. toruloides*. This decline is seen in the model yeast species, *Saccharomyces cerevisiae* (Kato & Lin, 2014), but has not yet been characterized in *R. toruloides*. Since the NAD metabolome changes overtime as the yeast grows, we must understand this process, the obstacles that emerge over time, and the potential impact on production of our key products.

NAD^+ Biosynthesis in Yeast

NAD^+ is synthesized through three main pathways in yeast: the *de novo* synthesis pathway from tryptophan, the nicotinic acid (NA)/ nicotinamide (NAM) salvage pathway, and the nicotinamide riboside (NR) salvage pathway (Croft et al., 2020). Because *R. toruloides* is understudied, the pathways have been documented in *S. cerevisiae* but are assumed to be conserved between the species. These pathways and genes associated with each conversion can be seen in Figure 6. NAD^+ appears to be synthesized primarily through the NA/NAM salvage pathways in the model species, but the precursors for both salvage pathways are readily exchanged between the media and cells (Kato & Lin, 2014). This study focuses on the salvage pathway genes and the NAD^+ kinase gene because of its vital role in producing $NADP^+$ and NADPH which are required for synthesis of lipids and fatty alcohols.

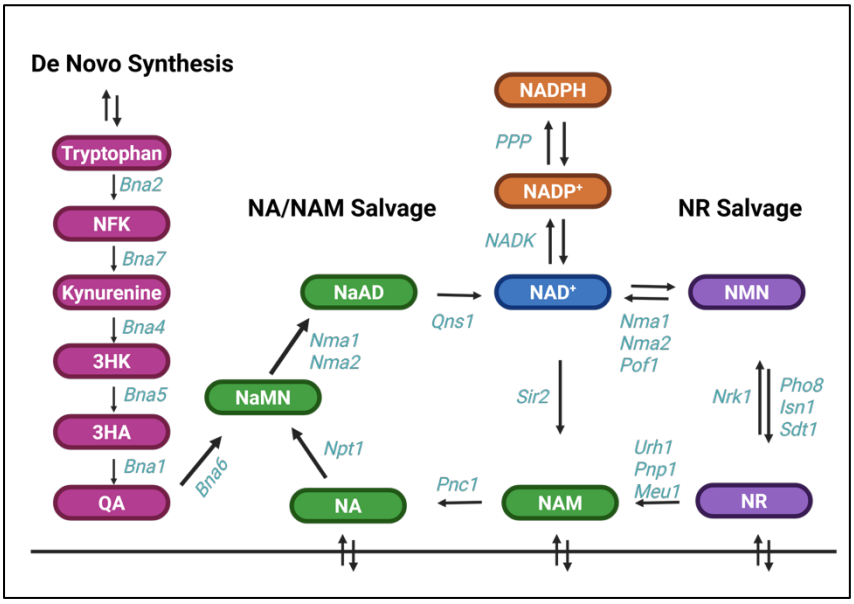

Figure 6. NAD^+ biosynthesis pathways in yeast. The three major NAD^+ pathways in yeast: *de novo* synthesis from tryptophan, nicotinic acid/nicotinamide salvage, and nicotinamide riboside salvage. The intermediate metabolites and the genes associated with the conversion between them are shown.

This study focuses on 5 genes from the nicotinic acid/nicotinamide salvage pathway seen in Figure 6: *NPT1*, *NMA1*, *NMA2*, *QNS1*, and *SIR2*. The first gene of interest is nicotinate phosphoribosyl transferase (*NPT1*). This gene is involved in the conversion of nicotinic acid to nicotinic acid mononucleotide (NaMN). It is linked to rDNA gene silencing and determining NAD^+ concentrations intracellularly (Smith et al., 2000). There are two paralog genes involved in the transition from NaMN to nicotinic acid adenine dinucleotide (NaAD): nicotinamide mononucleotide adenylyl transferase (*NMA1* and *NMA2*). Similar to *NPT1*, both *NMA* genes are linked to silencing rDNA and telomeres (Anderson et al., 2002). *QNS1* or glutamine (Q)-dependent NAD^+ synthetase, is directly involved in the conversion of NaAD to NAD^+ (Bieganowski et al., 2003). The final gene of this salvage pathway is *SIR2,* silent informer regulator, which is a member of the highly conserved, NAD^+-dependent Sirtuin family. This gene also functions in silencing of rDNA and telomeres but additionally functions in histone deacetylation (Landry et al., 2000).

There are 7 genes from the nicotinamide riboside salvage pathway that are investigated in this study and are seen in Figure 6: *NMA1*, *NMA2*, *PHO8*, *NRK1*, *MEU1*, *PNP1*, and *URH1*. This pathway works in both directions to both synthesize and degrade NAD^+. *NMA1* and *NMA2* function similarly in this pathway as they did in the NA/NAM salvage pathway. The primary difference is it works as an adenylyl transferase for NMN directly to NAD^+ but in both pathways the ribose-5-phosphate moiety is replaced with an ADP-ribose moiety that is donated from ATP. One of the genes responsible for the synthesis of NR from NMN is *PHO8,* phosphate metabolism, which is a phosphatase. This gene can work in response to changes in phosphate levels. In times of limited inorganic phosphate, it can increase removal of phosphate from NMN, therefore producing more NR (Lu & Lin, 2011). This gene also responds to a decrease of NaMN

in the NA/NAM salvage pathway which helps to provide a compensatory pathway for NAD^+ metabolism (Lu & Lin, 2011). In contrast to this gene, nicotinamide riboside kinase (*NRK1*) acts to generate NMN from NR. This was one of the first genes in this pathway to be discovered and is conserved in other eukaryotes, including humans, highlighting its importance in NAD^+ biosynthesis (Bieganowski & Brenner, 2004). The final three genes, *MEU1, PNP1,* and *URH1*, all work to synthesize NAM from NR and link the NR salvage pathway into the NA/NAM salvage pathway for NAD^+ recycling (Belenky et al., 2007). *MEU1*, multicopy enhancer of UAS2, was initially discovered as part of the methionine salvage pathway but acts as a nucleosidase in this context (Croft et al., 2020). *PNP1*, purine nucleoside phosphorylase, and *URH1,* uridine hydrolase, both work to hydrolyze NR into NAM and ribosyl to be recycled via the NA/NAM salvage pathway (Tempel et al., 2007). All the genes that are investigated in this paper were originally annotated in the model species, *Saccharomyces cerevisiae*.

Impacts of Nutrient Source on Growth of *R. toruloides*

R. toruloides can grow in a variety of conditions, which is one reason it is so desirable for industrial biofuel production. It can utilize different sources of carbon including glucose and xylose (Jagtap et al., 2021). Additionally, limiting nitrogen has been shown to increase its production of lipids, which is especially exciting for generating bioproducts. Previous studies have documented this phenomenon with an increase of lipids from 18% of the total biomass to 52% of the total biomass with nitrogen limited in the media (Evans & Ratledge, 1984). Since this initial observation, nitrogen limitation has been studied as a possible method of increasing lipid output from *R. toruloides* for biofuel production. Different ratios of carbon to nitrogen (C/N) can

be used to have varying outputs with higher ratios (C/N100+) resulting in a higher output of lipids. One drawback of nitrogen limitation is a decrease in other pathways including phospholipid synthesis, amino acid metabolism, and both transcription and translation (Mishra et al., 2024). This increase in lipid production must rely on a higher usage of NAD^+ and its related metabolites due to the high importance of these molecules for lipid synthesis. Assessing how different conditions alter the expression of the NAD^+ biosynthesis pathway genes can provide novel insight into the best growth conditions for *R. toruloides* in an industrial setting for high lipid production without impairing its natural growth.

Assessing NAD^+ Biosynthesis Gene Expression to Optimize Biofuel Production

This study aims to elucidate the intricacies in the NAD^+ biosynthesis pathway genes of *R. toruloides* to optimize their growth for bioproduct output. The central question being addressed is: How do NAD^+-dependent pathways change during the growth of this non-model yeast when grown in different conditions? To do this, *R. toruloides* was grown over a span of 72 hours. Samples were collected every three hours for the first 24 hours of growth and additionally at 48 and 72 hours. 6 biological replicates were collected for each time point. Three media types were utilized to assess the best condition for growth to optimize the desired products. Yeast peptone dextrose (YPD) was used as a rich, crude media to assess gene expression under basal conditions. Yeast nitrogen base (YNB) was the minimal nutrient media to assess what changes occur when fewer nutrients are given. Finally, a nitrogen limited media with a carbon: nitrogen ratio of 100 was used to assess how the biosynthesis pathways change in conditions of higher lipid production. RNA was then extracted from these samples using an organic extraction

method with TriZol. The Quant Bio cDNA synthesis kit was then used for cDNA synthesis for qRT-PCR. qRT-PCR using SYBR green was completed after primers were designed for each gene of interest in both of the NAD^+ salvage pathways. These primers were created using a Protein: Protein blast to the model species, *S. cerevisiae*, and were validated using serial dilution prior to use for experimental analysis. Since there was no control group, Ct was utilized to compare the amplification of genes of interest to the housekeeping genes. This results in a comparison of mRNA expression between timepoints and different media types. Using this approach, I hypothesized seeing a general decrease in expression across timepoints for YNB and C/N100 conditions compared to the rich media, YPD.

Chapter 2

Materials and Methods

Media Preparation

Preparation of the three media types for this experiment is completed following the ratios seen in the tables below. The composition of YPD is shown in Table 1, which must be adjusted to pH 5.3 and autoclaved. The composition for YNB is shown in Table 2 that is then filter sterilized. The composition for the nitrogen limited media with a carbon to nitrogen ratio of 100 (C/N 100) is shown in Table 3.

Table 1. Composition of Yeast Peptone Dextrose (YPD) pH 5.3.

Component	Amount
Water	~ 500 mL
Yeast Extract	5 g
Peptone	10 g
Dextrose	10 g

Total volume	500 mL

Table 2. Composition of Yeast Nitrogen Base (YNB).

Component	Amount
Water	~500 mL
YNB (-AA)	3.4 g
Dextrose	10 g
Total volume	500 mL

Table 3. Composition of Nitrogen Limited Media (C/N100).

Component	Working g/L	Stock	Volume (mL)
Water	N/A	N/A	685
YNB (-AA-AS)	1.7	17	100
$(NH_4)_2SO_4$	0.75	50	15
Phosphate Buffer	100	1000	100
D-Glucose	40	40%	100
Total volume			1000

Sample collection

A 5 mL overnight culture of *RT88* in specified media is started at least 12 hours prior to sample collection. A 1:50 dilution (overnight culture: specified media) is created in baffled flasks. The flasks are incubated at 30°C and shaking at 230 RPM. Samples are collected at 3, 6, 9, 12, 15, 18, 21, 24, 48, and 72 hours. At the 3- and 6-hour time points, 10 mL of culture is pelleted. At all other time points, 5 mL of culture is pelleted. For the nitrogen-limited condition, an additional 5 mL of culture is pelleted for each sample to ensure the pellet is large for RNA extraction. Cell count is measured using Countess. Samples are flash frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA Extraction

Cells are lysed using TriZol and homogenized using a Bead Mill. Chloroform is used to separate the sample into distinct phases— the organic phase, interphase (DNA), and aqueous phase (RNA). The aqueous phase is removed, and the RNA is precipitated. The sample is washed using ethanol. The concentration and purity of the RNA sample is measured using a Nanodrop. Based on the purity reading from Nanodrop, the RNA is cleaned using Phenol: Chloroform: Isoamyl alcohol and subsequent ethanol washes before measuring the new concentration and purity of the sample.

cDNA Synthesis

The qScript cDNA Synthesis kit is utilized to synthesize cDNA for qRT-PCR. A master mix is made in a 1.7 mL tube on ice following the amounts in table 4. To thin-walled 0.2 mL PCR tubes, 20 µL of master mix is added, along with 20 µL RNA diluted to 1 µg with nuclease-free water (Calculated using concentration obtained using NanoDrop). The PCR tubes are briefly microcentrigfuged and then run in a thermal cycler using the settings outlined in table 5. The cDNA is transferred to 1.7mL tubes and diluted by adding 120 µL nuclease-free water.

Table 4. cDNA Synthesis Master Mix.

Reagent	µL* number of samples + 4
Nuclease-Free Water	10
qScript Reaction Mix (5X)	8
qScript RT	2

Table 5. Thermal Cycler Setting for cDNA Synthesis.

Temperature (°C)	Time (Min.)	Number of Cycles
22	5	1
42	30	1
85	5	1
4	Infinite hold	

Primer Design and Validation

The sequence of each desired protein in the model species, *S. cerevisiae*, is found on the Saccharomyces Genome Database. Using the NCBI Protein: Protein Blast tool, the protein is found in *R. toruloides*. The gene sequence of this protein is used to design primers with the following parameters:

- Product size:100-200 base pairs (closer to 100 if possible)
- Melting temperature: 50-60°C
- GC Content: 40-60%
- Avoid triple G or C at the ends
- If possible, have primer at a splice junction/ span exon-exon junction

The primers are validated using a serial dilution in triplicate. The No RT control is also serially diluted. Three housekeeping genes (ACTIN, HPT1, and TAF10) were designed and utilized for comparison to the genes of interest. The primer specifications for each gene of interest are in appendix A.

Quantitative Reverse Transcription PCR (qRT-PCR)

For each gene of interest, SYBR Green, forward and reverse primers, and nuclease-free water are mixed following the ratio in table 4 to create a master mix. To set up the 96-well plate, 8 µL of master mix is added to each well according to each gene of interest and 3 µL of cDNA. Each sample and gene are run in triplicate, including a No RT control for each gene. The plate is run using QuantStudio3 with the settings outlined in table 5.

Table 6. qRT-PCR Master Mix.

Reagent	µL * number of wells + 4
SYBR Green	5
Forward Primer	0.5
Reverse Primer	0.5
Nuclease Free Water	2

Table 7. Settings for Comparative CT qRT-PCR Run Cycle.

Step	Temp. (°C)	Time	Cycles	Ramp Rate (°C/sec)
Enzyme activation	95		1	
Anneal/extend	60		40	
Denature	95			
Melt Curve	95	15 sec	1	1.6
	60	1 min		1.6
	95	15 sec	1	0.075

Data Analysis

The amplification of each sample is recorded, and the average of the triplicates is calculated. For the housekeeping genes, a geometric mean was calculated to use a baseline comparison for the genes of interest. ΔCt, which represents changes in mRNA expression, was

calculated by subtracting the geometric mean of the housekeeping genes from the average amplification of the gene of interest.

Chapter 3

Results

NA/NAM Salvage Pathway Changes Dynamically with Nutrient Rich, Minimal, and Nitrogen Limited Growth Conditions Over Time

Generally, there is a decrease in expression of all genes when *R. toruloides* is grown in the minimal media compared to the nutrient rich media. YNB also results in more consistent expression trends than YPD, likely due to the constant composition compared to the crude make up of YPD. Expression of all genes in this pathway is generally downregulated across time points in C/N 100 compared to YPD and YNB (Figures 7-10).

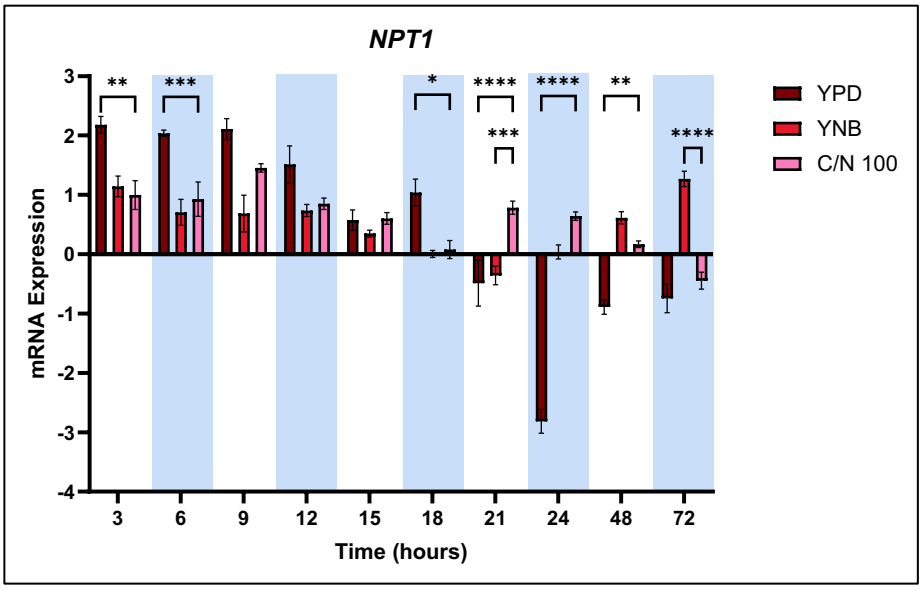

Figure 7. Expression of *NPT1* in *R. toruloides* with different media types.

There is a general downward trend seen in *NPT1* expression for YPD. Expression of this gene is significantly altered when *R. toruloides* is grown in YNB at almost all time points. The downward trend observed in YPD is seen again until roughly 21 hours before expression increases slightly. When grown under nitrogen starvation conditions, we see a change in expression in comparison to YPD at most time points. From 3 to 18 hours, there is a decrease in expression. After 18 hours, *NPT1* expression in C/N 100 is higher than in YPD (Figure 7).

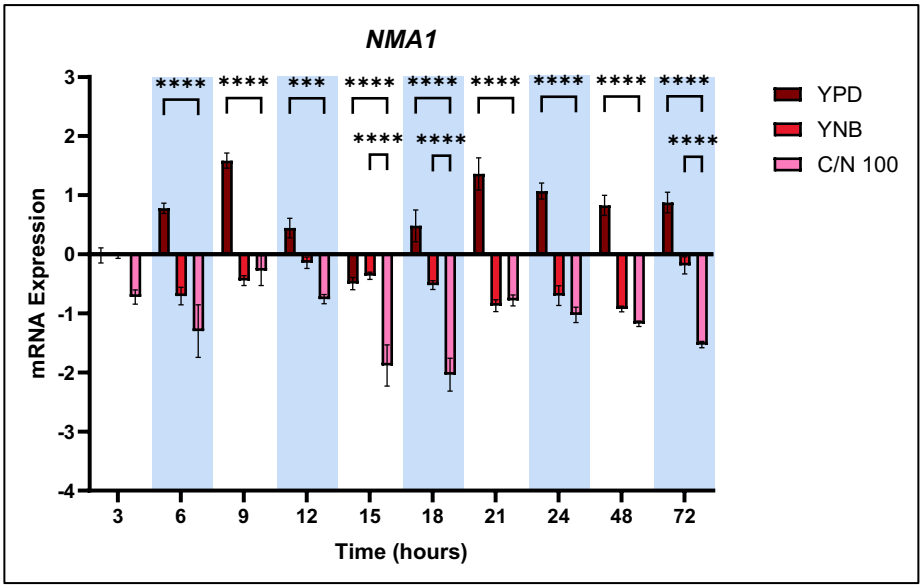

Figure 8. Expression of *NMA1* in *R. toruloides* with different media types.

The next gene in the pathway, *NMA1*, follows a different trend compared to *NPT1*. In YPD, expression is down between the 12- and 18-hour timepoints but is generally up. Conversely, expression in YNB is consistently down. Similar to expression in YNB, expression of *NMA1* in C/N 100 is consistently down but to a higher degree at 15, 18, and 72 hours (Figure 8).

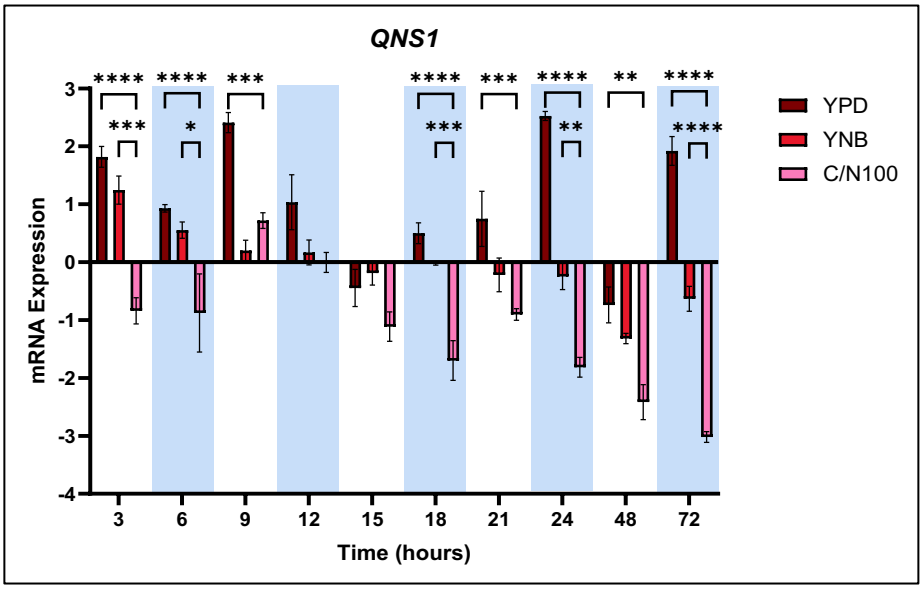

Figure 9. Expression of *QNS1* in *R. toruloides* with different media types.

Expression of *QNS1* in YPD is highly variable, likely due to the crude nature of the media. This gene is directly responsible for synthesis of NAD^+ from NaAD so this variable expression may be due to changes in needs for NAD^+ within the cell. In YNB, the expression of *QNS1* linearly decreases steadily until 48 hours with a slight increase at 72 hours. In C/N 100, expression is down at every time point other than 9 hours. There are significant decreases in expression seen at most time points for C/N 100 compared to both YPD and YNB. This would suggest a decrease in conversion of NaAD to NAD^+ (Figure 9).

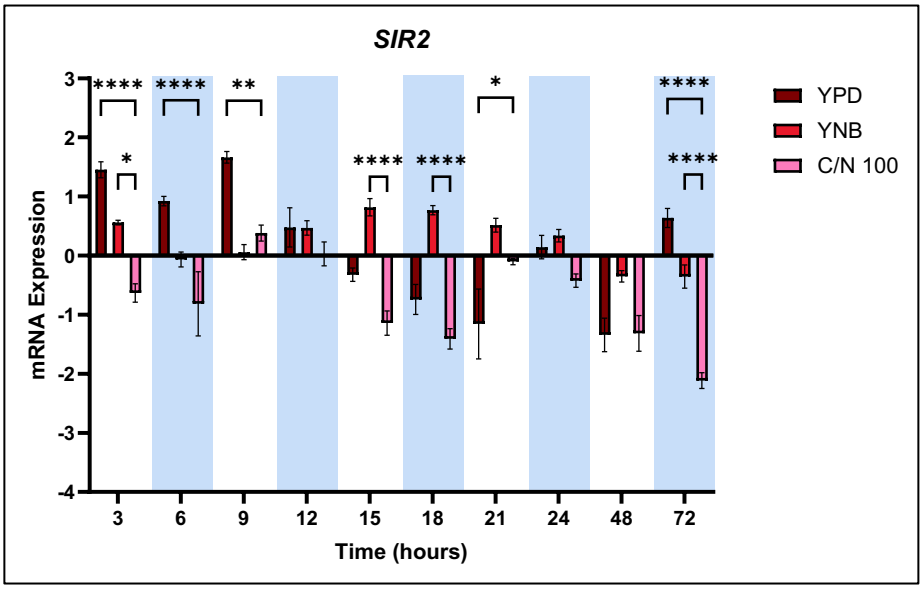

Figure 10. Expression of *SIR2* in *R. toruloides* with different media types.

In YPD, *SIR2* follows a similar trend to *QNS1* but with higher levels of downregulation at the 15-, 18-, and 21-hour timepoints. This suggests that NAD^+ is being degraded at a similar rate as it is being synthesized. Expression of *SIR2* in YNB is minimally expressed at most timepoints and becomes downregulated at the later timepoints during the stationary phase. Once again, expression in C/N 100 follows a very similar trend to *QNS1* with expression of *SIR2* being down at most timepoints other than the 9-hour time point (Figure 10).

NR Salvage Pathway Highly Expresses Genes to Degrade NAD^+

The genes involved in degradation of NAD^+ in this pathway are much more highly expressed than all other biosynthesis genes. Conversely, *NRK1*, which is involved with synthesis, is minimally expressed and even downregulated at all timepoints in all conditions. This suggests that the NR salvage pathway is more actively involved in the degradation of NAD^+ than

synthesis compared to the NA/NAM salvage pathway. This is further supported in the expression seen under nitrogen-limited conditions (Figures 11-15).

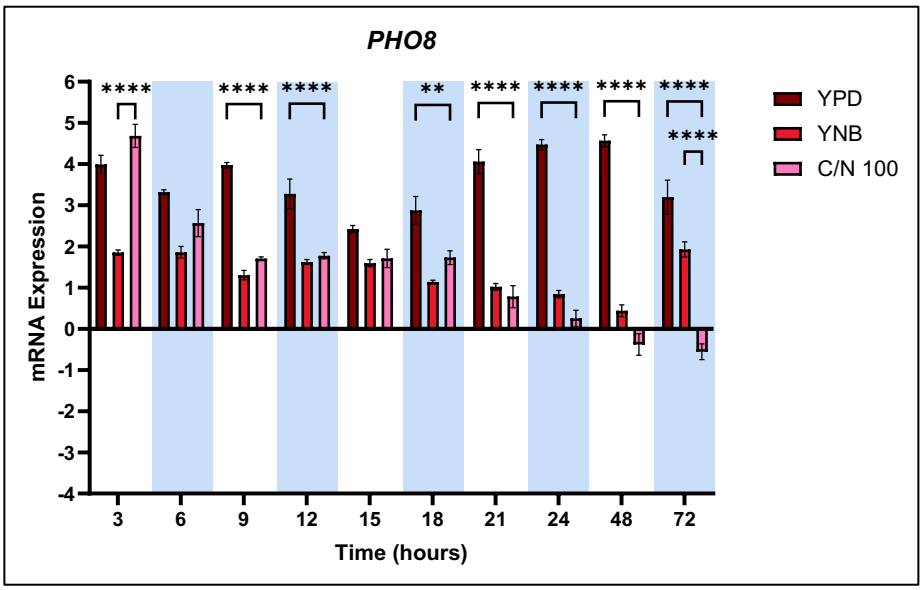

Figure 11. Expression of *PHO8* in *R. toruloides* with different media types.

PHO8 is highly expressed at all time points when *R. toruloides* is grown in YPD. There is a slight decrease in expression at the 15-, 18-, and 72-hour timepoints. While there is significant downregulation of this gene in YNB compared to YPD, it is still expressed more in comparison to the genes of the NA/NAM salvage pathway genes in YNB. There is also a more linear decrease over time with a sharp increase at 72 hours. Expression of this gene in nitrogen limited media is up at all time points until 48 hours, where expression is minimally decreased. This is a variation from the trends seen in both YPD and YNB. There is also a very linear decrease seen over time, similar to the pattern of expression in YNB. At the 3-hour timepoint, there is significantly higher expression of this gene under nitrogen limited conditions (Figure 11).

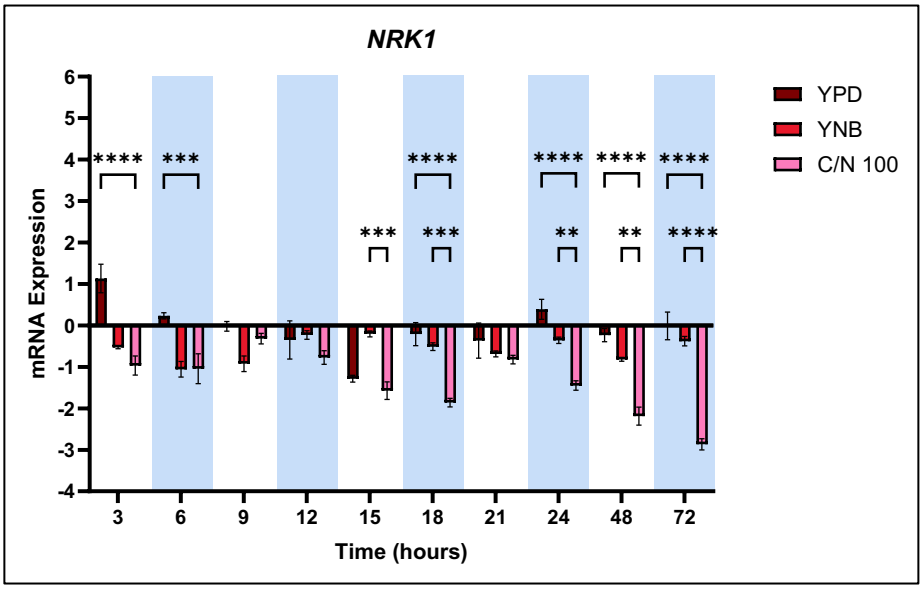

Figure 12. Expression of *NRK1* in *R. toruloides* with different media types.

NRK1 is down regulated at almost all time points in YPD and at all time points in YNB. As this gene is directly contrasting PHO8, which is highly expressed at all time points, this pattern was expected and suggests that the NR salvage pathway is more active in degrading NAD^+. When *R. toruloides* is grown in C/N 100, expression of this gene is downregulated across all time points. This further confirms that this pathway is more active for degrading NAD^+ than synthesizing it (Figure 12).

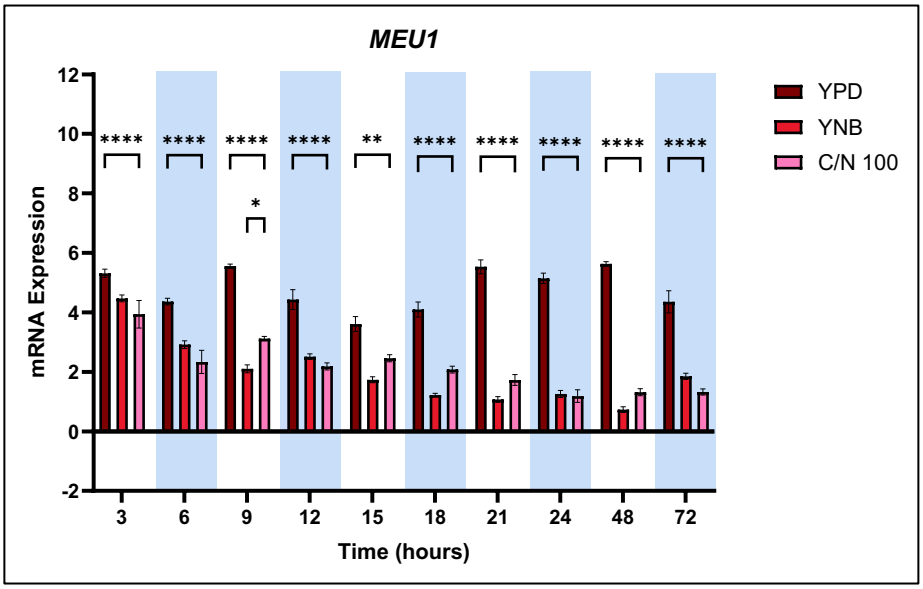

Figure 13. Expression of *MEU1* in *R. toruloides* with different media types.

Similar to *PHO8*, *MEU1* is very highly expressed in YPD across all time points but shows a less distinct trend. Furthermore, expression in YNB is significantly down regulated in YNB at all time points compared to YPD. This downregulation is prominent with expression decreasing up to fivefold in YNB compared to YPD. C/N100 expression is also significantly decreased at all timepoints when compared to YPD. Expression in YNB is very similar to C/N 100 at all timepoints (Figure 13).

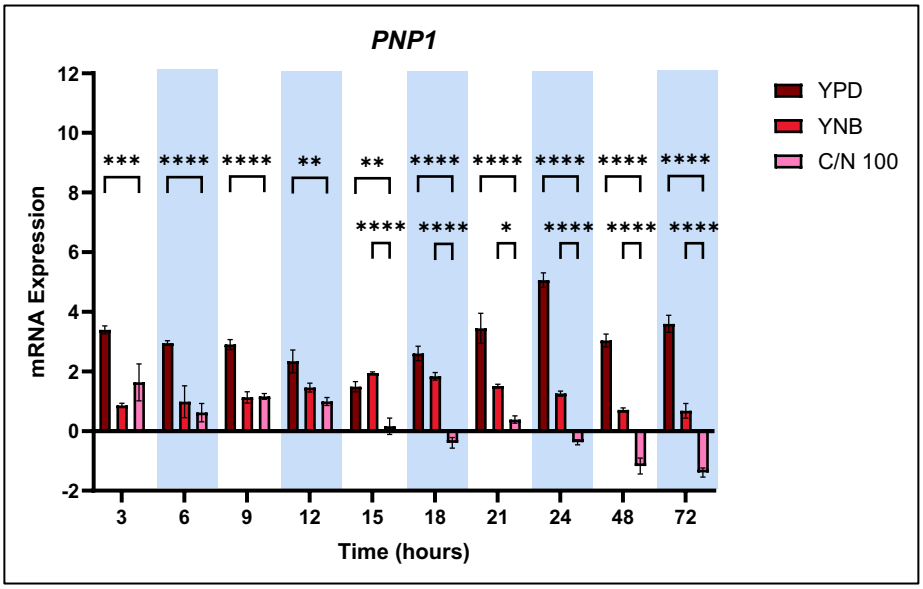

Figure 14. Expression of *PNP1* in *R. toruloides* with different media types.

PNP1 follows a similar trend to *MEU1,* but *PNP1* has less expression in comparison to *MEU1*. There is a significant decrease in expression in YNB compared to YPD for most timepoints. Under nitrogen limited conditions, expression of this gene is generally decreased compared to the other media types and is even downregulated at the 18-, 24-, 48-, and 72-hour timepoints (Figure 14).

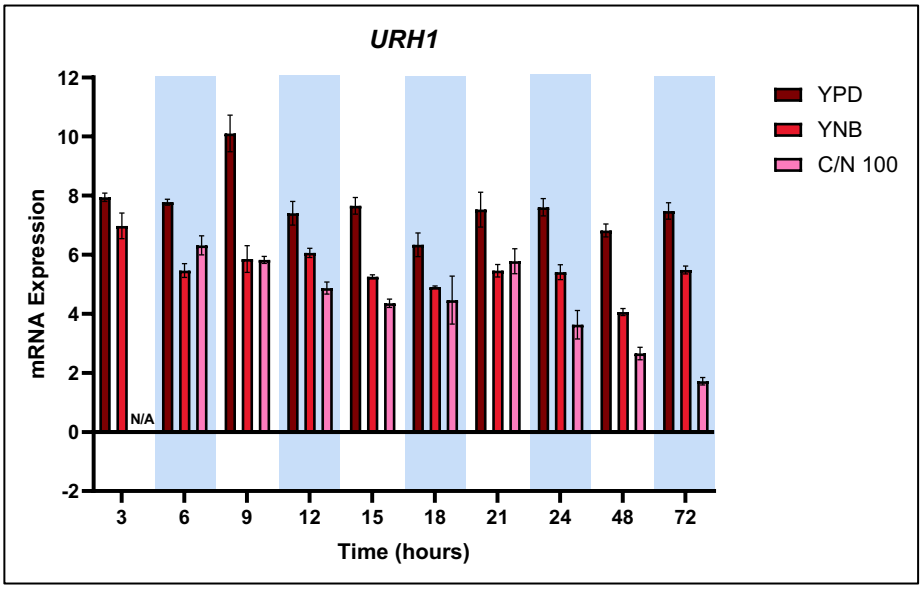

Figure 15. Expression of *URH1* in *R. toruloides* with different media types.

URH1 is the most highly expressed gene which is involved in the conversion of NR to NAM across timepoints and media types. There is decreased expression of *URH1* in YNB compared to YPD. Expression of this gene in C/N100 follows a similar trend when compared to the other two media types. There were no 3-hour timepoint samples for this condition (Figure 15).

Chapter 4

Discussion

The purpose of this study was to assess how different nutrient compositions affect the expression of the NAD^+ recycling pathways in *R. toruloides*. Overall, there are clear differences seen in expression of both pathways when comparing all three media types. Expression in rich media is greater overall than in the other, less nutrient dense media types. This was expected due to the greater availability of nutrients. The variations across time points and genes that are seen

may be due to the crude nature of this media. The minimal media, YNB, resulted in generally decreased expression across timepoints in comparison to YPD and more consistent trends seen over the growth period. While the reduction in nutrient availability contributed to lower gene expression of this pathway, the more consistent results could be more favorable for an industrial setting to achieve consistent and more predictable bioproduct output. Under high lipid producing conditions, expression is decreased similarly to minimal media, occasionally to a high degree. There was one instance of expression under nitrogen limited conditions being higher than the other media types: *PHO8* during the 3-hour timepoint (Figure 11). This could suggest a higher reliance on phosphate at the early stages of growth under nitrogen limitation. Further investigation of this gene under other stress conditions would be beneficial for assessing its role. The combination of high lipid output and gene expression similar to YNB could make C/N100 the most optimal media for industrial bioproduction. There are drawbacks to using this media industrially; firstly, this media is more complex to prepare and has more components while containing fewer nutrients than the rich media. Second, there may be other negative effects on the growth of *R. toruloides* under nitrogen-limited conditions. These potential consequences need to be more thoroughly assessed in order to deem this as the most optimal condition for industrial growth.

Additionally, there are some trends seen within each of the salvage pathways. Firstly, the NA/NAM salvage pathway is generally expressed to a lower degree overall in comparison to the NR salvage pathway. The only exception to this is the expression of *NRK1* (Figure 12), which in *S. cerevisiae* is believed to be more active when NA or NAM is not available in the media or if there is a supplementation of NR. The same study also found that *PNP1* and *MEU1* were less necessary for NAD^+ synthesis from NR than *URH1* and *NRK1* (Belenky et al., 2007). The results

of this experiment showed consistent expression of all three genes linked to the cleavage of NR to NAM (*PNP1, MEU1,* and *URH1*) and low expression of *NRK1*. Expression of *URH1* (Figure 15) was higher than *PNP1* (Figure 14) and *MEU1* (Figure 13), similar to the findings in the model species, but there is a clear decrease in the importance of *NRK1* in *R. toruloides*. The trend of continuous high expression seen with *PHO8*, *MEU1*, *PNP1*, and *URH1* strongly supports that the NR salvage is highly active in degrading NAD^+. Overall, the NR salvage pathway appears to be more active in recycling NAD^+ through the NA/NAM salvage pathway than it is in synthesizing NAD^+ directly from NR. The NA/NAM salvage pathway also appears to be downregulated to a higher degree under nitrogen-limited conditions. Apart from *NPT1* (Figure 7), this pathway shows more downregulation across time points than the other two media types. This suggests that NAD^+ recycling is slower under these conditions. This is unexpected due to the high reliance on NAD^+ and its related metabolites to produce lipids. Assessing the expression of other genes related to NAD^+, like NAD^+ kinase, could elucidate how NAD^+ is utilized and recycled under high lipid producing conditions.

There is not a clear trend to suggest that NAD^+ production is declining, or degradation is increasing overtime in either pathway. This was unexpected because NAD^+ levels are believed to decrease overtime. There are a few possibilities that could contribute to this. The first is other pathways which rely on NAD^+, like glycolysis, TCA, or pentose phosphate, are more active and utilize more NAD^+ overtime. Another possibility is that NAD^+ levels are not declining in this species overtime, contrary to all other model organisms.

The results of this study provide a baseline understanding of how different growth conditions alter gene expression of the NAD^+ recycling pathways of *R. toruloides*. This is the first step of many towards optimizing the growth of this non-model species for industrial

bioproduction. While nitrogen limiting conditions are known to increase production of lipids, the genes involved with NAD^+ recycling are altered, which could impact the long-term growth of this species. Investigating the effect of other intensities of nitrogen limitation on the expression of the NAD^+ biosynthesis pathways should be conducted to have a full picture of how this stress impacts this species.

Chapter 5

Future Directions

The next experiment that should be completed to continue to explore the impacts of nitrogen limitation on the expression of NAD^+ biosynthesis pathway genes in *R. toruloides* would be to test various ratios of carbon to nitrogen. There have been other ratios tested in literature: Mishra et al. tested the effects of C/N 5, 100, and 150 on lipid accumulation using a multi-omic approach. Other studies have investigated how C/N 60, 80, 100, and 120, in combination with varying other conditions, alter lipid production to optimize this output for industrial purposes (Lopes et al., 2020). Testing these different ratios of carbon to nitrogen to find the ideal for lipid production by looking at NAD^+ biosynthesis gene expression could allow *R. toruloides* to be more easily used in an industrial setting.

Furthermore, exploring how different carbon sources affect the growth and gene expression of *R. toruloides* could provide alternate, more economic, and environmentally friendly options for turning this yeast into a bio factory. *R. toruloides* is naturally able to grow on xylose, unlike the model species, which presents another carbon source. Growth on xylose can also increase lipid production (Wiebe et al., 2012) but can alter its growth rate (Tiukova et al.,

2019). Still, the ability for this species to naturally use xylose for growth creates the possibility for using hydrolysates from plant waste, making the production of biofuels from this species more environmentally friendly.

Another possible way to increase lipid production is through phosphate limitation. Wang et al. found that limiting phosphate for *R. toruloides* greatly increased lipid production but did not increase cell mass. They also found that TAG synthesis in particular is upregulated (Y. Wang et al., 2018) which is promising as this is what is the most useful for biofuel production. Investigating changes in gene expression of the NAD^+ biosynthesis pathways of the species under phosphate limited conditions could provide more insights into the best environment for growth of this yeast for bioproduction purposes.

Finally, under any of the conditions tested or mentioned above for future experiments, other pathways that rely on NAD^+ or its related metabolites should be assessed for changes in gene expression overtime. Key pathways for analysis include *de novo* NAD^+ synthesis, glycolysis, pentose phosphate, the TCA cycle, and ß-oxidation. Assessing the expression of the genes involved in these pathways can provide insights on how NAD^+ is utilized in *R. toruloides* overtime. This information could allow for further optimization of the growth of this species for producing lipids and fatty alcohols to use for biofuel production.

Appendix A

SYBR Green Primer Sequences

Table 8. SYBR Green Primer Specifications for qRT-PCR.

Gene	Sequence 5'-3'	Direction
ACTIN	GCTGTCTTCCCCTCGATTGT	Forward
	GGGTCAGGATACCACGCTTC	Reverse
HPT1	CGTCCACGATGGTCACAAGA	Forward
	TTCAGGAAGGTGCGGAGAAC	Reverse
TAF10	ACGTCCGAGTAAAACGCCTC	Forward
	AAAGGCGTCGGCTGAGATTG	Reverse
NPT1	CAAGGCGAATGAGGAATGCG	Forward
	CGCCAAATCGTACTTCTGCG	Reverse
NMA1	TACCGGCACAACATCCACAT	Forward
	CAGGTACTTGACGCTCATGC	Reverse
QNS1	GGATGTACGCGATCAACCG	Forward
	GAGGAAAGGCCTGAGGTCAA	Reverse
SIR2	GCTTGCAGGAGAAGGCAAC	Forward
	AGTGTACTGATGCTGCTCGG	Reverse
PHO8	GCGAGCGATCACATGGAGTA	Forward
	GGCGTGAGTAGCCATTTCCT	Reverse
NRK1	AGAAGCGAAGATACGAGCGG	Forward
	TTGTCCCAGTAATCAGGCGG	Reverse
MEU1	CGGCTTGCTGTGTCTTTCC	Forward
	GGTTGTAGCCCGGTTCCAT	Reverse
PNP1	AGGAGGTGGCGAATCATCAG	Forward
	CAACACAATATCCCCATGCTCC	Reverse
URH1	CTGATGGCTTTGCATCTGCC	Forward
	GGTGTTGAAAAGCGAAGCGT	Reverse

BIBLIOGRAPHY

Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O., Cohen, H., Lin, S. S., Manchester, J. K., Gordon, J. I., & Sinclair, D. A. (2002). Manipulation of a Nuclear NAD+ Salvage Pathway Delays Aging without Altering Steady-state NAD+ Levels. *Journal of Biological Chemistry*, *277*(21), 18881–18890. https://doi.org/10.1074/jbc.M111773200

Belenky, P., Racette, F. G., Bogan, K. L., McClure, J. M., Smith, J. S., & Brenner, C. (2007). Nicotinamide Riboside Promotes Sir2 Silencing and Extends Lifespan via Nrk and Urh1/Pnp1/Meu1 Pathways to NAD+. *Cell*, *129*(3), 473–484. https://doi.org/10.1016/j.cell.2007.03.024

Bieganowski, P., & Brenner, C. (2004). Discoveries of Nicotinamide Riboside as a Nutrient and Conserved NRK Genes Establish a Preiss-Handler Independent Route to NAD+ in Fungi and Humans. *Cell*, *117*(4), 495–502. https://doi.org/10.1016/S0092-8674(04)00416-7

Bieganowski, P., Pace, H. C., & Brenner, C. (2003). Eukaryotic NAD+ Synthetase Qns1 Contains an Essential, Obligate Intramolecular Thiol Glutamine Amidotransferase Domain Related to Nitrilase. *Journal of Biological Chemistry*, *278*(35), 33049–33055. https://doi.org/10.1074/jbc.M302257200

Climate change: Global temperature | NOAA Climate.gov. (2025, May 29). https://www.climate.gov/news-features/understanding-climate/climate-change-global-temperature

Croft, T., Venkatakrishnan, P., & Lin, S.-J. (2020a). NAD+ Metabolism and Regulation: Lessons From Yeast. *Biomolecules*, *10*(2), 330. https://doi.org/10.3390/biom10020330

Croft, T., Venkatakrishnan, P., & Lin, S.-J. (2020b). NAD+ Metabolism and Regulation: Lessons From Yeast. *Biomolecules*, *10*(2), 330. https://doi.org/10.3390/biom10020330

December 2025 Monthly Energy Review. (n.d.). Retrieved January 3, 2026, from https://www.eia.gov/totalenergy/data/monthly/pdf/mer.pdf

Evans, C. T., & Ratledge, C. (1984). Influence of Nitrogen Metabolism on Lipid Accumulation by Rhodosporidium toruloides CBS 14. *Microbiology*, *130*(7), 1705–1710. https://doi.org/10.1099/00221287-130-7-1705

Guo, M., Cheng, S., Chen, G., & Chen, J. (2019). Improvement of lipid production in oleaginous yeast Rhodosporidium toruloides by ultraviolet mutagenesis. *Engineering in Life Sciences*, *19*(8), 548–556. https://doi.org/10.1002/elsc.201800203

Hill, J., Goodkind, A., Tessum, C., Thakrar, S., Tilman, D., Polasky, S., Smith, T., Hunt, N., Mullins, K., Clark, M., & Marshall, J. (2019). Air-quality-related health damages of maize. *Nature Sustainability*, *2*(5), 397–403. https://doi.org/10.1038/s41893-019-0261-y

Jagtap, S. S., Deewan, A., Liu, J.-J., Walukiewicz, H. E., Yun, E. J., Jin, Y.-S., & Rao, C. V. (2021). Integrating transcriptomic and metabolomic analysis of the oleaginous yeast Rhodosporidium toruloides IFO0880 during growth under different carbon sources. *Applied Microbiology and Biotechnology*, *105*(19), 7411–7425. https://doi.org/10.1007/s00253-021-11549-8

Karimi, M., Simsek, H., & Kheiralipour, K. (2025). Advanced biofuel production: A comprehensive techno-economic review of pathways and costs. *Energy Conversion and Management: X*, *25*, 100863. https://doi.org/10.1016/j.ecmx.2024.100863

Kato, M., & Lin, S.-J. (2014). Regulation of NAD+ metabolism, signaling and compartmentalization in the yeast Saccharomyces cerevisiae. *DNA Repair*, *0*, 49–58. https://doi.org/10.1016/j.dnarep.2014.07.009

Landry, J., Sutton, A., Tafrov, S. T., Heller, R. C., Stebbins, J., Pillus, L., & Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proceedings of the National Academy of Sciences*, *97*(11), 5807–5811. https://doi.org/10.1073/pnas.110148297

Lopes, H. J. S., Bonturi, N., Kerkhoven, E. J., Miranda, E. A., & Lahtvee, P.-J. (2020). C/N ratio and carbon source-dependent lipid production profiling in Rhodotorula toruloides. *Applied Microbiology and Biotechnology*, *104*(6), 2639–2649. https://doi.org/10.1007/s00253-020-10386-5

Lu, S.-P., & Lin, S.-J. (2011). Phosphate-responsive Signaling Pathway Is a Novel Component of NAD+ Metabolism in Saccharomyces cerevisiae. *The Journal of Biological Chemistry*, *286*(16), 14271–14281. https://doi.org/10.1074/jbc.M110.217885

McReynolds, M. R., Chellappa, K., & Baur, J. A. (2020). Age-related NAD+ decline. *Experimental Gerontology*, *134*, 110888. https://doi.org/10.1016/j.exger.2020.110888

Mishra, S., Deewan, A., Zhao, H., & Rao, C. V. (2024). Nitrogen starvation causes lipid remodeling in Rhodotorula toruloides. *Microbial Cell Factories*, *23*, 141. https://doi.org/10.1186/s12934-024-02414-0

Priyadarshini, L. A. S., & Kataria, R. (2025). Microbial synthesis and extraction of value-added metabolites by Rhodotorula toruloides from waste stream: A sustainable approach. *Microbial Cell Factories*, *24*, 134. https://doi.org/10.1186/s12934-025-02752-7

Shivanna, K. R. (2022). Climate change and its impact on biodiversity and human welfare. *Proceedings of the Indian National Science Academy*, *88*(2), 160–171. https://doi.org/10.1007/s43538-022-00073-6

Smith, J. S., Brachmann, C. B., Celic, I., Kenna, M. A., Muhammad, S., Starai, V. J., Avalos, J. L., Escalante-Semerena, J. C., Grubmeyer, C., Wolberger, C., & Boeke, J. D. (2000). A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(12), 6658–6663. https://doi.org/10.1073/pnas.97.12.6658

Spagnuolo, M., Yaguchi, A., & Blenner, M. (2019). Oleaginous yeast for biofuel and oleochemical production. *Current Opinion in Biotechnology*, *57*, 73–81. https://doi.org/10.1016/j.copbio.2019.02.011

Sunder, S., Gupta, A., Kataria, R., & Ruhal, R. (2024). Potential of Rhodosporidium toruloides for Fatty Acids Production Using Lignocellulose Biomass. *Applied Biochemistry and Biotechnology*, *196*(5), 2881–2900. https://doi.org/10.1007/s12010-023-04681-w

Tempel, W., Rabeh, W. M., Bogan, K. L., Belenky, P., Wojcik, M., Seidle, H. F., Nedyalkova, L., Yang, T., Sauve, A. A., Park, H.-W., & Brenner, C. (2007). Nicotinamide Riboside Kinase Structures Reveal New Pathways to NAD+. *PLoS Biology*, *5*(10), e263. https://doi.org/10.1371/journal.pbio.0050263

Tiukova, I. A., Brandenburg, J., Blomqvist, J., Sampels, S., Mikkelsen, N., Skaugen, M., Arntzen, M. Ø., Nielsen, J., Sandgren, M., & Kerkhoven, E. J. (2019). Proteome analysis of xylose metabolism in Rhodotorula toruloides during lipid production. *Biotechnology for Biofuels*, *12*, 137. https://doi.org/10.1186/s13068-019-1478-8

USDA. (2022, September). *Corn and Other Feed Grains—Feed Grains Sector at a Glance | Economic Research Service*. https://www.ers.usda.gov/topics/crops/corn-and-other-feed-grains/feed-grains-sector-at-a-glance

Wang, J., Singer, S. D., Souto, B. A., Asomaning, J., Ullah, A., Bressler, D. C., & Chen, G. (2022). Current progress in lipid-based biofuels: Feedstocks and production technologies. *Bioresource Technology*, *351*, 127020. https://doi.org/10.1016/j.biortech.2022.127020

Wang, Y., Zhang, S., Zhu, Z., Shen, H., Lin, X., Jin, X., Jiao, X., & Zhao, Z. K. (2018). Systems analysis of phosphate-limitation-induced lipid accumulation by the oleaginous yeast Rhodosporidium toruloides. *Biotechnology for Biofuels*, *11*, 148. https://doi.org/10.1186/s13068-018-1134-8

Wen, Z., Zhang, S., Odoh, C. K., Jin, M., & Zhao, Z. K. (2020). Rhodosporidium toruloides—A potential red yeast chassis for lipids and beyond. *FEMS Yeast Research*, *20*(5), foaa038. https://doi.org/10.1093/femsyr/foaa038

Wiebe, M. G., Koivuranta, K., Penttilä, M., & Ruohonen, L. (2012). Lipid production in batch and fed-batch cultures of Rhodosporidium toruloides from 5 and 6 carbon carbohydrates. *BMC Biotechnology*, *12*, 26. https://doi.org/10.1186/1472-6750-12-26