Drew University

College of Liberal Arts

Yeast Alcohol Dehydrogenase Catalyzed Reduction of Nitriles to Amines

A Thesis in Biochemistry

by

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Abstract

The role of yeast alcohol dehydrogenase (YADH) in producing ethanol during alcoholic fermentation is well known thanks to the prominence of the brewing industry and scientific interest in enzymology. However, its possible laboratory and industrial applications have not been thoroughly explored. YADH naturally reduces acetaldehyde to ethanol via hydride attack, a mechanism shared by powerful but environmentally adverse reducing agents such as $LiAlH_4$. Because of its chemically green nature and recyclability, YADH has been investigated in the past to perform commercially useful reductions outside of its natural scope. One such reduction that would be of interest but has not yet been studied is the reduction of nitriles to amines. The conversion of nitriles to amines is a critical process with industrial, pharmaceutical, and environmental relevance. In this investigation, we explored the potential of YADH to catalyze the reduction of nitriles into amines. The nitriles acetonitrile, propionitrile, and butyronitrile were converted into their corresponding amines by YADH. The reaction kinetics were measured spectrophotometrically, and the detection of the amines was confirmed by gas chromatography–mass spectrometry analysis.

Table of Contents

Chapter 1: Introduction

1.1 Environmental Impact

In recent years, greater attention has been drawn to the environmental impact of the large industries responsible for nitrile and amine production. As the dangers of climate change become increasingly apparent, researchers have consistently looked to alternative methods to supplement or replace the environmentally costly processes that sustain modern society. Chemical catalysis involving metallic reagents poses an increasing threat to the environment. For example, the Sohio process is currently used on a large scale to synthesize acrylonitrile for later conversion to plastics or other polymers and produces toxic acetonitrile and hydrogen cyanide as byproducts (Figure 1).[1] Because of the large quantity of byproduct created, these cyano-compounds are burned off, in turn producing copious amounts of NO_x and CO_x.[2] The ramifications of this process were made clear during the 2014 Olympics in Beijing, where clouds of gaseous pollutants could be seen; a sight which is becoming more common in the manufacturing districts of mainland China.[3]

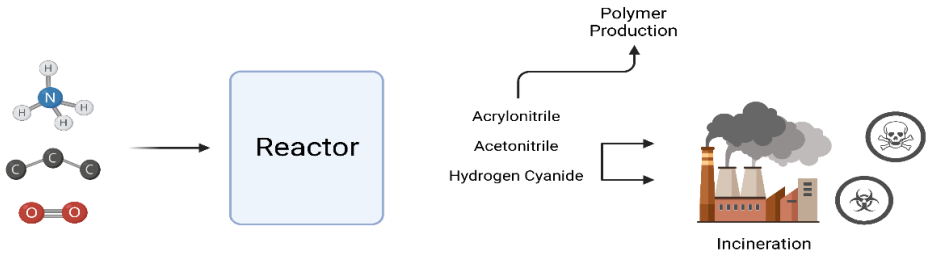

Figure 1. Graphical depiction of the Sohio process.

Conversion or recycling of the excess nitriles to a more useful product would be a preferred alternative, extending the synthetic life of the nitrile in a “green” way, but only if the process itself is carbon neutral. This puts increased pressure on the scientific community to

develop a safer and greener process, perhaps through the continued investigation and modification of biocatalysts.

1.2 Significance of Nitrile-Derived Amine Production

Nitriles are common a functional group present in everything from rubber to antidepressants.[4] As synthetic precursors to aldehydes, alcohols, and amines, nitriles are a useful tool in organic synthesis.[5] Their relative stability and potential for biological activity make nitriles valuable in their own right, but through conversion of a nitrile to an amine, chemists retain a valuable nitrogen-containing substituent and gain access to a wealth of synthetic potential. Amines are crucial to life as we know it: amino acids, painkillers, pesticides, decongestants, and even cleaning products all have this functional group in common.[6–7] Notably, the pharmaceuticals morphine, Demerol, diphenhydramine, and Novacaine all contain amine functional groups. Biogenic amines such as the neurotransmitters serotonin (5-hydroxytryptamine) and dopamine are also of pharmacological interest as we improve upon our synthetic mimicry of these molecules to treat disease (Figure 2).[8]

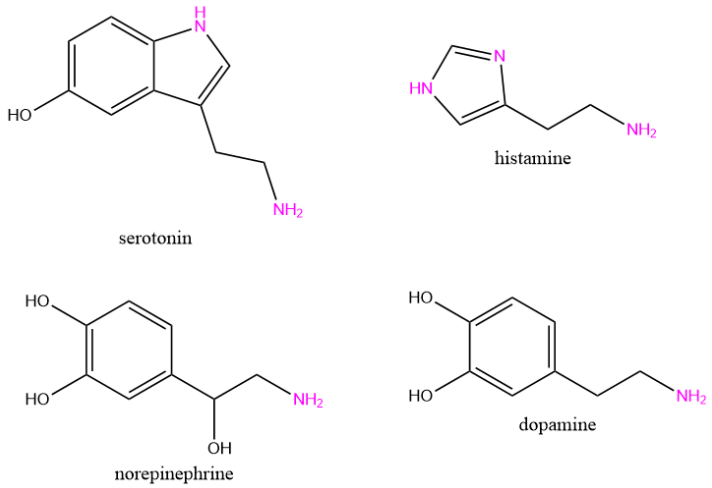

Figure 2. Biogenic amines: starting from amino acid precursors, naturally occurring amines in our bodies play key roles as neurotransmitters and signaling molecules.

The production of primary amines is particularly important, owing to their basicity and nucleophilicity. These chemical properties allow primary amines to form subsequent N–C bonds, which makes them more flexible synthetic precursors. Primary amines are most easily produced by direct alkylation of ammonia with haloalkanes or the reduction of nitrile or nitro-containing functional groups.[9] An abundance of multistep techniques also exists, but these involve more complicated starting products and reagents. They also have additional disadvantages in that they may involve several reaction intermediates (reducing atom economy) and require multiple sets of chemical conditions.[10]

Direct alkylation, while capable of mass-producing amines, also produces undesired side products. The lone pair electrons of nitrogen in combination with reactive haloalkanes make for an SN2-rich environment; this environment makes it difficult to regulate the degree of alkylation that occurs due to the inherent nucleophilicity of the nitrogen. The various primary, secondary, and tertiary amines must then be separated out before a final pure product is acquired. Following workup, the halide leaving groups form their associated acids – further complicating the mixture.

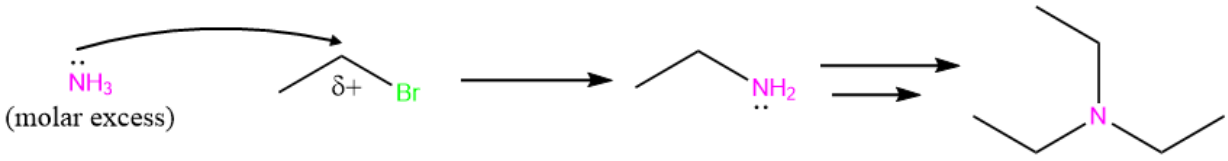

Figure 3. Amine synthesis via direct alkylation. Excess ammonia and alkyl halide yield primary alkyl amines. Because of their increased nucleophilicity compared to ammonia, these primary amines continue to react until fully alkylated.

1.3 Chemical Catalysis

By comparison, reduction of nitrogen-containing functional groups to yield the corresponding amines is a less complicated process. These reductions are generally one-reagent reactions that afford few side products. Reduction of nitriles and nitro compounds can be accomplished chemically, electrochemically, or through enzyme-driven biocatalysis.

The most common chemical techniques involve hydrogenation over a metal catalyst or nucleophilic hydride attack. The former technique is considered to be the most economical, though use of more expensive metals such as palladium and platinum are preferred as they permit more easily attainable reaction conditions.[11] If hydrogenation is instead achieved via more affordable metal catalysts such as nickel or cobalt, much higher pressures and temperatures are required for the reaction to proceed favorably, and these are harder to achieve.[12] An advantage of this method is that the catalyst and reaction conditions can be fine-tuned to reduce a molecule of choice.

Hydrides, such as lithium aluminum hydride ($LiAlH_4$) or sodium borohydride ($NaBH_4$), are another powerful tool employed to perform chemical reductions of nitriles and other C–X functional groups.[13,14] While effective at fully reducing just about anything, $LiAlH_4$ is not necessarily efficient, suffering from extreme reactivity and limited selectivity. Especially in the case of $LiAlH_4$, caution must be taken to avoid even ambient amounts of atmospheric water for risk of a dangerous exothermic reaction. Use of copious organic solvent and inert conditions are required for its large-scale application. $NaBH_4$ is milder by comparison and is an improvement upon catalytic hydrogenation in that it will not reduce conjugated C=C bonds. However, this decrease in reactivity also narrows its scope to reducing aldehydes, imines, and ketones, making it ineffective for quick reduction of nitrile compounds because another reagent would be required

to perform the nitrile to imine conversion. Both reagents are health hazards and must be handled accordingly, making their frequent laboratory use unadvisable. Mechanistically, hydride reagents all behave similarly. A hydride anion is donated to an electrophilic atom on the target molecule, forming a new C–H bond that replaces the previous C–X bond in SN2 fashion. Regardless of the catalyst used, multifunctional or unsaturated molecules are not always well tolerated due to the potential for alternative reductions to occur.[15]

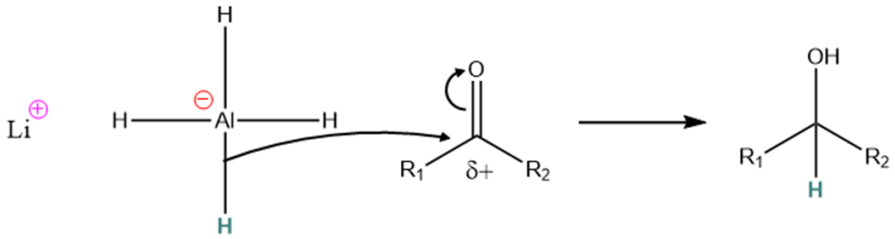

Figure 4. Metallic hydride reduction. Metal hydrides, such as lithium aluminum hydride, are a source of nucleophilic hydrogen commonly employed in reduction reactions.

In an attempt to address the safety and reactivity concerns of the aforementioned techniques, electrochemical methods for reducing nitriles, oximes, and nitro compounds have been developed.[16,17] By taking advantage of the voltage difference in an electrochemical cell, electrons can be ferried to the electrophilic carbon of the target molecule at the anode, thereby reducing it. A recent study by Xia et al. demonstrated the reduction of acetonitrile to ethylamine with 90% selectivity, using water as a hydrogen source. Other researchers have accomplished similar feats with oximes and NO_x functionalities. As a result, electrochemical reduction is becoming a more feasible alternative to harsher chemical methods. Despite this, it too has its complications. Expensive metal catalysts are still required for the electrochemical cell, and

electric current needs to be maintained for the duration of the reaction. The authors acknowledge this drawback with the hope that renewable energy will be used to drive the reduction.[16] At a larger scale and without a renewable energy source, driving the reaction may no longer be environmentally green due to the associated electrical cost.

1.4 Biocatalysis

Recent advances in biochemistry have allowed scientists to transform naturally occurring enzymes into potent synthetic tools with increasing industrial potential. Replacement of conventional chemical reagents with enzymes is becoming more commonplace due to their increased accessibility and customizability.[18] Enzymes also offer the advantage of being usable at standard conditions (1 atm, 298 K) and simple to dispose of, in contrast to many chemical catalytic methods.[19] These traits have made them successful in many applications, especially pharmaceutical synthesis.[20]

A landmark example of successful implementation of biocatalysts is that of halogenase enzymes, capable of adding useful halogen functionalities to a variety of substrates. Halogen functionalities are present in over one-third of modern pharmaceuticals.[20] However, their addition to complex molecules can be synthetically challenging. The discovery and later bioengineering of halogenases has allowed medicinal chemists to add these important functionalities in a precise and consistent way, thanks to the inherent specificity of enzymes.

1.4.1 QueF

Because of the similar importance of amine moieties, which are found in up to 85% of pharmaceutical compounds, researchers have investigated how they might be synthesized enzymatically from nitriles. The category of enzymes first expected to be most suited for this task are known as nitrile reductases, of which the bacterial *QueF* nitrile reductases are the only known.[21] Much work has been done to elucidate the structure and specific mechanism of this enzyme in hopes of using it as a greener alternative to chemically catalyzed reductions.[22]

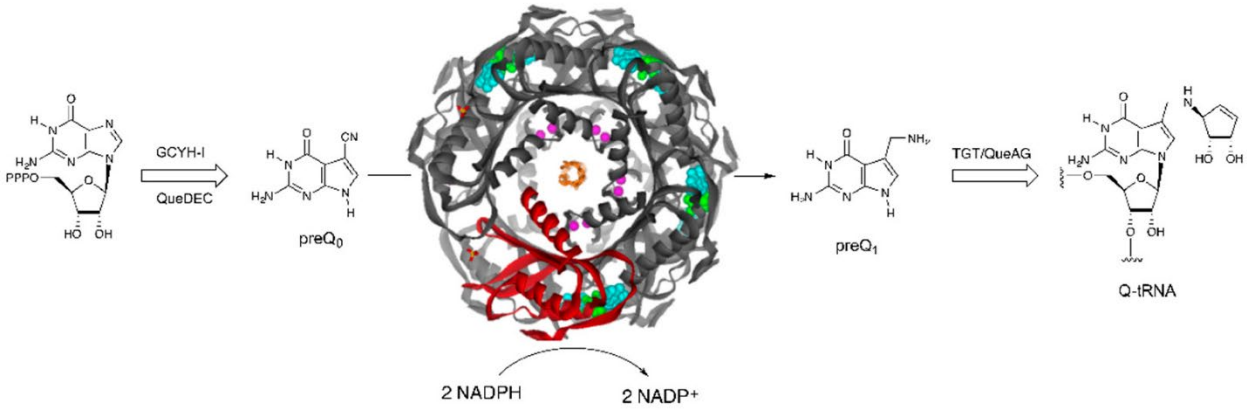

Figure 5. QueF reduction mechanism. QueF, a nitrile reductase, catalyzes the reduction of nucleoside nitriles to the corresponding amines. Image reprinted from ref 22.

QueF typically catalyzes the reduction of its substrate nucleoside 7-cyano-7-deazaguanine (preQ0) to 7-aminoethyl-7-deazaguanine (preQ1), using the cofactor NADPH as a hydride donor. It is important to note that the fundamental hydride transfer mechanism utilized by QueF and other reductase enzymes is identical to that of metal hydride reagents. In the case of nitrile substrates, two hydride reductions are required to yield the corresponding amine.

However, the enzyme–substrate interactions required for this reaction to occur complicate the process.

Most critically, QueF shows extreme specificity/selectivity for its natural substrate preQ0 or other molecules like it. The active site of QueF is well suited for nucleosides, with several ring-stacking and coordination interactions involving the indole bases and nitrogen atoms.[21] Additionally, positioning preQ0 for reduction in the active site requires a temporary covalent bondage between the nitrile carbon and a cysteine residue. These factors all contribute a high free-energy barrier (~21 kcal/mol) to the imine to amine reduction, meaning that QueF in its natural state is unsuitable for industrial applications.[23]

1.4.2 Flavoprotein Reductase

Another NADPH-dependent reductase, flavoprotein reductase, was also investigated for this task. Flavoprotein reductase belongs to a group of enzymes commonly referred to as "nitro-reductases" and is used by plants, fungi, and bacteria to metabolize nitroaromatic compounds.[24] Seeking to produce the corresponding amines, researchers introduced novel nitroalkane substrates to the enzyme in ideal reaction conditions. However, instead of amines, the reaction yielded various carbonyls and ammonia, both products of imine hydrolysis. Following the conversion of the oxime intermediate to an imine, the imine seemed to quickly hydrolyze in the aqueous reaction media, leaving no traces of imine or amine to be detected.[24]

1.4.3 Alcohol Dehydrogenases

Many useful *reductases* exist, but *dehydrogenase* enzymes are also a viable choice due to their similar functionality and prevalence in nature. Alcohol dehydrogenases (ADHs) are highly conserved and present in many organisms, such as those found in the livers of horses and even those in our own bodies.[25] In addition, they can easily recycle their hydride source when paired with an appropriate system to do so.[26] As one of the most studied groups of enzymes, our familiarity with ADHs could prove beneficial to applying them in new ways.

Thousands of years of evolution-directed improvements have allowed ADHs to become adept at performing a one key step: the reversible production of ethanol from acetaldehyde.[25] This is only possible due to the overall common structure of these enzymes, which contain several motifs that aid in stabilizing their shape and facilitating reactions. Importantly, ADHs contain coordinating amino acids and zinc (II) ions in their active sites. Because of their cationic nature, the zinc ions are thought to stabilize the ADH reaction mechanism by coordinating with the electron-dense oxygen in acetaldehyde or ethanol (Figure 5).[25]

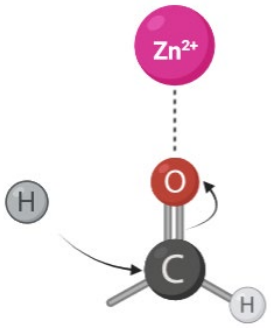

Figure 6. Zinc ion–substrate coordination. Alcohol dehydrogenases make use of a Zn^{2+} ion to position substrates for catalysis.

Once the substrate has been positioned by the zinc and amino acid residues in the active site, an additional molecule, nicotinamide adenine dinucleotide (NAD(P)), is required for the reaction to take place.[27] In eukaryotes, NAD(P) and its derivatives act as electron carriers for oxidation–reduction reactions. This cofactor therefore gives the greatest clue to, and serves the most important role in, specific function of ADHs, that of an oxidoreductase. The presence of NAD(P) in the form of electron-carrying NAD(P)H or electron-deficient $NAD(P)^+$ allows ADHs to carry out redox reactions without the need for oxygen.[28]

At anaerobic conditions, a reduction by hydride transfer is favorable, with the hydride anion (H^-) provided by NAD(P)H.[29] The reduction normally proceeds via nucleophilic attack of the hydride anion at the electrophilic carbonyl carbon of acetaldehyde, producing ethanol and $NAD(P)^+$ (Figure 7).[30] ADHs, like any other catalyst, are not consumed in the process and can be reused to catalyze further reactions.

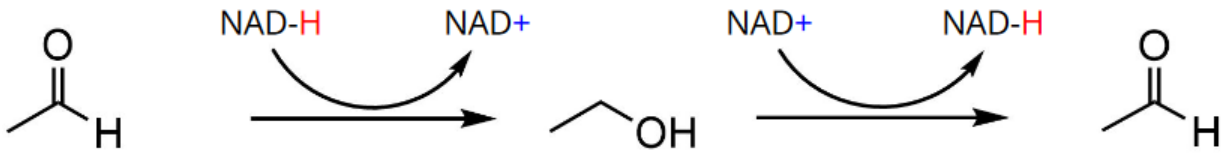

Figure 7. Reversible reductase/dehydrogenase hydride transfer. Enzymatic reductions are mechanistically identical to those performed with metal hydrides, except for use of nicotinamide adenine dinucleotide (NAD) derivatives as a hydride source. A separate reaction can be performed to recycle this hydride source or recover the original aldehyde.

Use of ADHs with a large number of alternative substrates is well-documented, including those with pharmaceutical or industrial relevance.[31] Notably, yeast alcohol dehydrogenase

(YADH) has been found to have some affinity for larger carbonyl compounds such as acetophenone and acetyl pyrimidines, as well as substituted cinnamaldehydes.[32] A great deal of work has been done to test the reduction potential of various ADHs on carbonyl compounds with different associated motifs and structures,[33 34] and their propensity to reduce alternative functional groups such as those with nitrogen-containing moieties has also been investigated; most notably those with nitro and/or oxime substituents.

Krautil demonstrated the ability of ADHs to convert phenyl acetaldoxime to the corresponding alcohol.[35] The proposed mechanism involves a hydride attack at the nitrogen atom of the oxime forming an unstable imine, which is quickly hydrolyzed to an aldehyde. In both cases, the instability of the imine intermediate in aqueous media seems to prevent a second hydride attack; which would reduce the imine to an amine.[24, 35] While the authors were unsuccessful in creating the desired amine products, their work showcased the chemical promiscuity of ADH and other reductive enzymes.

1.5 Yeast Alcohol Dehydrogenase

YADH, an enzyme found in the baker's yeast *Saccharomyces cerevisiae*, has been an asset to humanity for thousands of years. By helping metabolize many of the sugars found in nature through a process called fermentation, the biochemical action of YADH has given rise to consumer products such as sourdough bread and alcoholic beverages. Apart from its enjoyable byproducts, fermentation plays a key role in generating chemical energy for yeast and is a small part of a metabolic system that has been fine-tuned over many millennia.

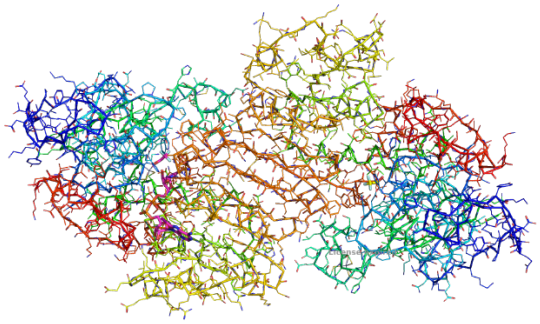

Figure 8. Yeast alcohol dehydrogenase crystal structure of YADH with colored secondary structural motifs. Image generated using PyMOL.

YADH is a tetrameric protein consisting of four subunits.[25] These four subunits are structurally similar and further arranged as a pair of dimers, "AB" and/or "CD", with each individual subunit containing a cofactor binding site and a catalytic binding site or the "active" site.[36] The pairing of these dimers is in a back-to-back arrangement, with two AB subunits or two CD subunits being paired together to form the overall quaternary structure. This back-to-back arrangement is ideal as it conveniently allows potential substrate molecules to more easily access the catalytic site.[36] The subunits also rely heavily on the presence of zinc(II) cofactors, with a total of 8 zinc ions found throughout the enzyme - one structural and one catalytic per subunit.

Structural zinc ions are associated with the sulfur atoms of cysteine residues in each subunit, namely Cys97, Cys100, Cys107, and Cys111.[37] Catalytic zinc ions are also coordinated with cysteine residues (Cys46 and Cys174) as well as the nitrogen atom of one histidine residue (His67), which overall form the active site of YADH.[37]

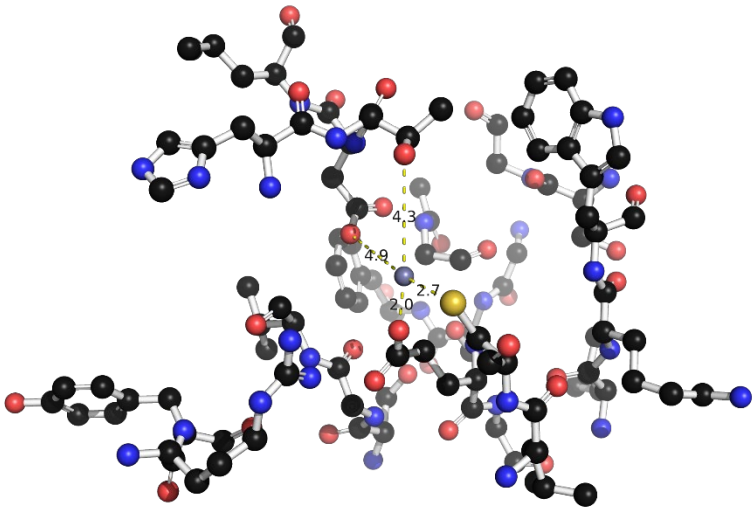

Figure 9. Zoomed-in image of the YADH active site shows a small substrate binding pocket and amino acid residues coordinated to the catalytic zinc ion. Image generated using PyMOL.

The ability of the active site of YADH to position an electrophilic carbon by zinc coordination with the electron-withdrawing substituent (i.e., the oxygen of a carbonyl group) suggests the possibility of exchanging acetaldehyde for another molecule with similar chemical properties. Bulk dried yeast has already shown promise in reducing *N*-benzylidinemethylamine to *N*-methylbenzylamine when properly stabilized and given sufficient time to do so, but a single responsible enzyme, if there is one, has not been elucidated.[38]

Because the reduction of nitrile can be performed by hydride transfers, using YADH may be a viable, and recyclable, alternative. Barriers previously preventing large-scale enzymatic

catalysis such as extreme specificity or sensitive reaction conditions now have the potential to be overcome by simply modifying the reaction medium. Now more than ever, both scientists and society at large are becoming increasingly conscious of our environmental footprint and looking for innovative ways of reducing it. The potential to perform a commonplace reaction in a novel, greener way with YADH offers a compelling reason to continue its study. In this work, the potential application of YADH to catalyze the reduction of nitriles was investigated. The concentration-dependent reactivities of the alkyl nitriles acetonitrile, propionitrile, and butyronitrile as substrates for YADH were analyzed via UV–visible (UV–vis) spectroscopy. Once the kinetic parameters were obtained, product formation was determined by ninhydrin testing and by gas chromatography–mass spectrometry (GC-MS) analysis following chloroformate derivatization and extraction. To our knowledge, this work represents the first known conversion of nitriles to the corresponding amines catalyzed by YADH.

Chapter 2: Materials and Methods

2.1 Materials

Lyophilized YADH and nicotinamide adenine dinucleotide salt hydrate (NADH) were obtained from Sigma Aldrich (MO, USA). All amines, nitriles, isobutyl chloroformate (IBCF), and ninhydrin were also supplied by Sigma Aldrich. Dichloromethane and toluene were obtained from Fisher Chemical (MA, USA).

YADH was redissolved in sodium pyrophosphate buffer (0.06 *M*, pH 8.5) to a final concentration of 1 mg/mL, aliquoted, and frozen at –20 °C. A fresh aliquot was used for each assay to prevent loss of catalytic activity from repeated freezing and thawing. A primary stock solution of 0.1 *M* NADH in Millipore water, used for most reactions, was created weekly and

stored at 0 °C to prevent degradation of the coenzyme. Working stock solutions of 0.01 *M* NADH were prepared as necessary for kinetic assays. Nitrile solutions (1 *M*) were made as needed, with the exception of butyronitrile, of which a 0.4 *M* solution was made due to poor solubility in water.

2.2 Kinetics

Several 1 mL reaction aliquots with varying final concentrations (100–1000 m*M*) of nitrile substrate were prepared in a 1.5 mL cuvette with the following reaction components: 720 µL of nitrile solution in Millipore water, 170 µL of 0.06 *M* pH 8.5 sodium pyrophosphate buffer, 100 µL of 0.01 *M* NADH, and 20 µL of 1 mg/mL YADH.

The Cary 60 UV–vis spectrophotometer was set to measure sample absorbance at 340 nm every 5 s, for a total of 90 s. Substrate, buffer, and enzyme were added first and served as a blank for each run. NADH was then added and mixed thoroughly to start the reaction. Following data collection, the absorption values at each timepoint were converted to NADH concentration using Beer's Law. The decrease in NADH concentration over time was plotted to determine the reaction velocity for each substrate concentration.

2.3 Ninhydrin Assay

A 2% (w/v) solution of ninhydrin was prepared using acetone as a solvent. A reaction mixture with a final nitrile concentration of 720 m*M* was mixed in a test tube with buffer, enzyme, and coenzyme and allowed to sit for 30 min. Meanwhile, a water bath was heated to 100 °C. After 30 min, the reaction mixture was transferred to a 15 kDa filter centrifuge tube and centrifuged for 5 min at 3500 rpm to remove the enzyme. The filtrate was transferred to a new

test tube, and a few drops of ninhydrin reagent were added using a Pasteur pipette until the solution was visibly tinted yellow. The test tube was lowered into the water bath using a clamp and heated for 5 min to observe a color change.[39]

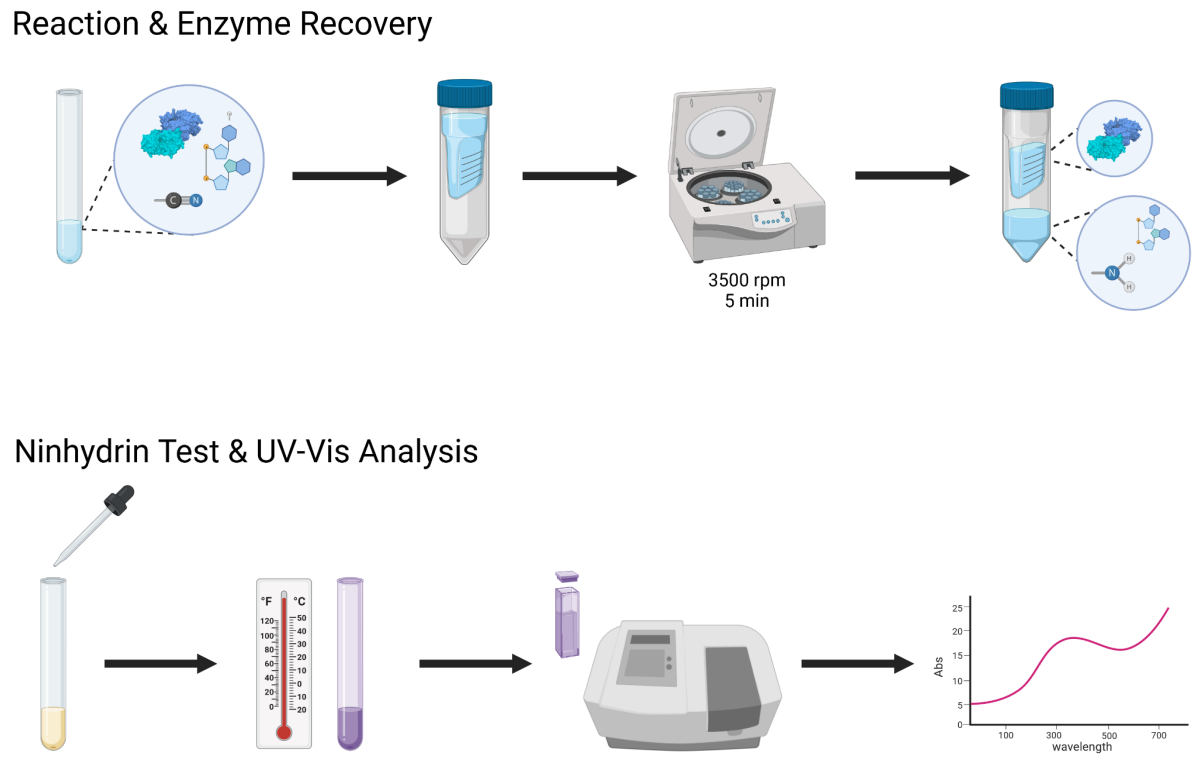

Figure 10. Ninhydrin assay methodology.

2.4 Functionalization

This functionalization procedure was adopted from Cunha et al.[40] Reaction mixtures were prepared at the same scale as the kinetic and ninhydrin assays, with each reaction allowed to sit for 30 min. The mixture was added to a screw-capped vial containing 1 mL of toluene. A 1 mL portion of 0.5 *M* pyrophosphate buffer (pH 12) followed by 25 µL of IBCF were added. After shaking for 10 min, the mixture was centrifuged at 3500 rpm for 5 min. A 500 µL portion

of the upper organic layer was transferred to a new vial, to which 500 µL of alkaline methanol was added. The vial was shaken for 5 min, and 1.5 mL of 5 *M* NaOH was added before shaking again for another 5 min. Following a second centrifugation at 3500 rpm for 5 min, the sample was analyzed via GC-MS. Functionalized amine standards were prepared by adding 50 µL of each amine solution to 5 mL of deionized water and then functionalizing a 1 mL aliquot of the mixture.

2.5 Gas Chromatography–Mass Spectrometry Parameters

The oven was programmed for an initial temperature of 50 °C, held for 2 min before ramping up to 300 °C at a rate of 15 °C/min. All samples were run on an HP-5MS 5% phenyl methyl silox column, with the column inlet set to 250 °C and a 3 min solvent delay. Electrospray ionization was used for charged fragment generation, with a triplex quadrupole mass separator.

Chapter 3: Results and Discussion

3.1 Ninhydrin Assay

Ninhydrin (1,2,3-indantrione monohydrate) is a commonly used reagent in the qualitative identification of proteins or amino acids in solution due to its availability, ease of use, and sensitivity.[41] When excess ninhydrin reagent is reacted with primary (“free”) amino groups or ammonia gas, a redox reaction occurs that results in the formation of a color complex known as Ruhemann’s purple. Secondary amines, such as those found in the amino acids proline and hydroxyproline, can yield a yellow or red color depending on reaction temperature. Because of ninhydrin’s sensitivity and ability to detect the presence of free amines, it was employed in this

work as a screening method to attempt to qualitatively detect the formation of the primary amine ethylamine following reduction of acetonitrile by YADH.

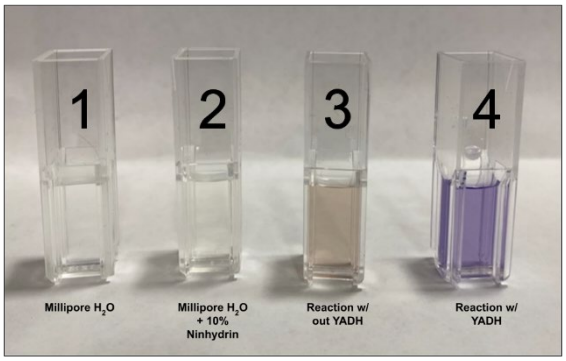

Figure 11: Ninhydrin assay showing the purple solution color following reaction with YADH (4). Controls 1–3 show no purple color.

Ninhydrin assays resulted in formation of purple and red solutions, corresponding to the solutions with and without enzyme present in the reaction mixture respectively (Figure 11, cuvettes 3 and 4). The red hue seen in sample 3 of Figure 11 is thought to be a result of ninhydrin partially reacting at high temperatures with nitrogen atoms in the adenine or nicotinamide portion of NAD, which the literature describes as normally unreactive.[42] A sample of ninhydrin added to Millipore water was also prepared to demonstrate the difference between a positive and negative result (Figure 11, cuvette 2).

When scanned for absorbance in the visible spectrum (400–800 nm), the purple sample had a λ_{max} peak at ~570nm. The NADH control sample showed a λ_{max} peak at ~510 nm, and the ninhydrin control absorbed at ~420 nm. These absorption values correspond to purple, red, and yellow as expected and serve as a more definitive verification of the colors seen by the naked eye (data not shown).

Formation of a purple compound absorbing at ~570 nm following reaction with ninhydrin indicates the presence of a primary amine or ammonia in solution.[41] The reaction solutions analyzed were filtrates, with the ninhydrin assay being performed after removal of enzyme, to account for the potential of exposed amino side chains in YADH to react and skew the results. However, the potential for hydrolysis of the proposed imine intermediate introduces some ambiguity due to ninhydrin's reactivity with both free amines and ammonia; both of which result in Ruhemann's purple (Figure 12).

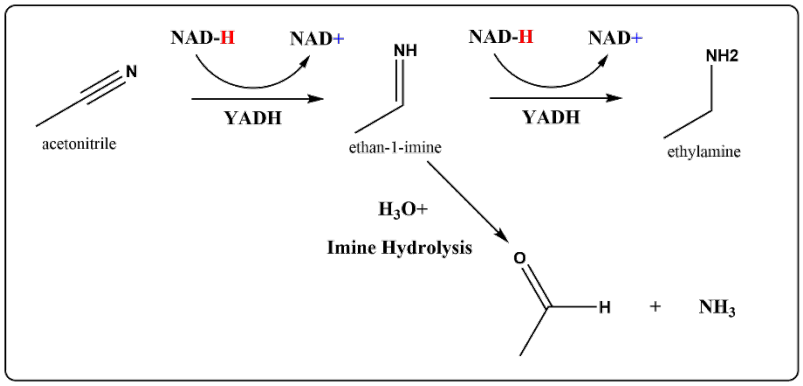

Figure 12. Proposed reaction scheme starting with the nitrile. YADH must perform two hydride transfers to yield the corresponding amine. The imine intermediate is hypothesized to undergo hydrolysis in the aqueous reaction environment.

Additionally, multiple trials of this assay demonstrated that the color formation seemed to have a sensitive dependence on the duration and intensity of heat provided, with NADH-only samples turning either dark yellow, orange, or red, and full reaction samples occasionally turning a dark red-purple or even light blue. Use of a boiling water bath in a medium-sized beaker provided the most consistent results, but use of a heating element was also investigated and did

not provide an improvement. Because of this, it was not possible to determine product formation or percent conversion from Beer's Law using this approach.

Although the ninhydrin assay did not confirm (or preclude) amine formation, it did indicate at least one enzyme-catalyzed reduction of the nitrile substrate to an imine which could either hydrolyze or be further reduced and therefore served its purpose as an effective screening method. However, if only the hydrolysis products are formed, then YADH would suffer a similar fate to other reductases investigated for this purpose, necessitating further investigation with a more definitive assay.

3.2 Functionalization and Gas Chromatography–Mass Spectrometry Analysis

Because of the ambivalent and qualitative nature of the ninhydrin assay results, a better method of quantitatively visualizing amine formation was required. Separation by chromatography followed by mass spectroscopy was a natural choice owing to the relatively unambiguous results provided by mass spectra as well as availability of LCMS and GC-MS equipment in the laboratory. Because shorter alkyl amines are volatile and we were attempting to identify ethylamine, GC-MS was chosen.

The volatility of ethylamine combined with the solvent delay of the GC-MS presented an initial challenge in that ethylamine alone could not be seen on the chromatogram. To overcome this, an amine derivatization procedure utilizing IBCF was adopted to increase the chromatographic visibility of the ethylamine-derived molecule (Figure 13). By performing such a functionalization after the enzymatic reaction is allowed to occur, any amines present in solution react with IBCF to form a higher molecular weight product. The heavier product has a higher boiling point, which increases its retention time beyond the solvent delay. This

functionalization takes advantage of the nucleophilicity of amines and is therefore additive in nature, maintaining the integrity of the original alkylamine and still allowing it to be distinguished from others.

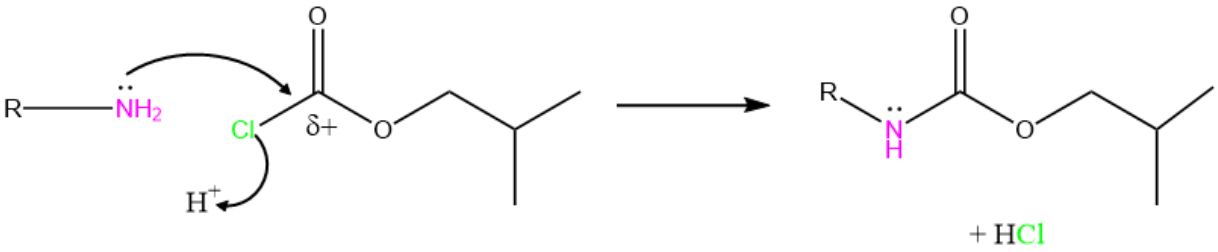

Figure 13. Amine-functionalization reaction. In the presence of nucleophilic amines, isobutyl chloroformate reacts to form a more chromatographically visible carbamate derivative.

Before testing enzymatic reactions, pure amines were functionalized to verify the practicality of the method. The fragment sizes and relative abundances of the pure functionalized amine solutions were found to be nearly identical to those cited in the literature despite different GC-MS parameters, lending confidence to their use as a standards to which YADH-catalyzed reductions can be compared.[40]

YADH was first reacted with acetonitrile, yielding what at first seemed to be GC peaks corresponding to a successful functionalization of ethylamine. However, upon closer analysis, it was noticed that both the ethylamine standard and isobutyl chloroformate elute at the same time; and that the fragment sizes noted in the acetonitrile reaction correspond to those seen in the IBCF mass spectrum. The elution times of both substances remained the same even when alternative GC methods were used. Attempts were also made to fully react or otherwise convert the excess isobutyl chloroformate to elucidate if it was masking the desired ethylamine derivative, but these were unsuccessful. If ethylamine was in fact produced from the enzymatic reaction and reacted

successfully with IBCF, the relative abundance of this product was too small to be seen compared to the IBCF peak (Figure 14).

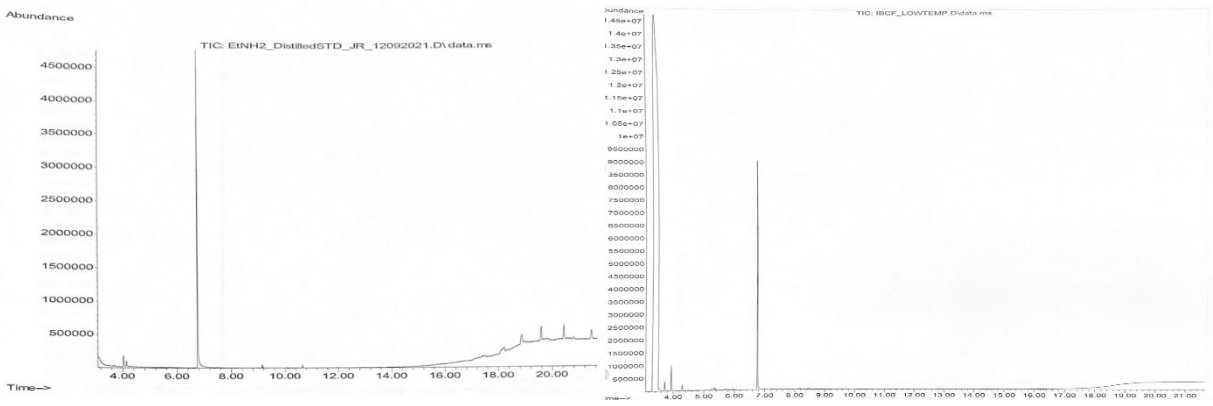

Figure 14. Gas chromatographs of functionalized ethylamine standard (left) and unreacted isobutyl chloroformate (right).

In the interest of time and procuring conclusive results, the experiment was expanded to include longer alkyl nitriles, namely propionitrile and butyronitrile, as potential substrates. Pure functionalized standards of the corresponding amines had increased retention times with no peak overlaps.

The YADH-catalyzed propionitrile reaction had a peak retention time and mass fragment sizes directly corresponding to the functionalized propylamine standard (Figure 15). Method controls were conducted without presence of enzyme and/or NADH, in which no formation of functionalized propylamine was noted (see Appendix). Butyronitrile reactions and controls yielded similar results, despite difficulty solubilizing the butyronitrile in the aqueous reaction solution (Figure 16).

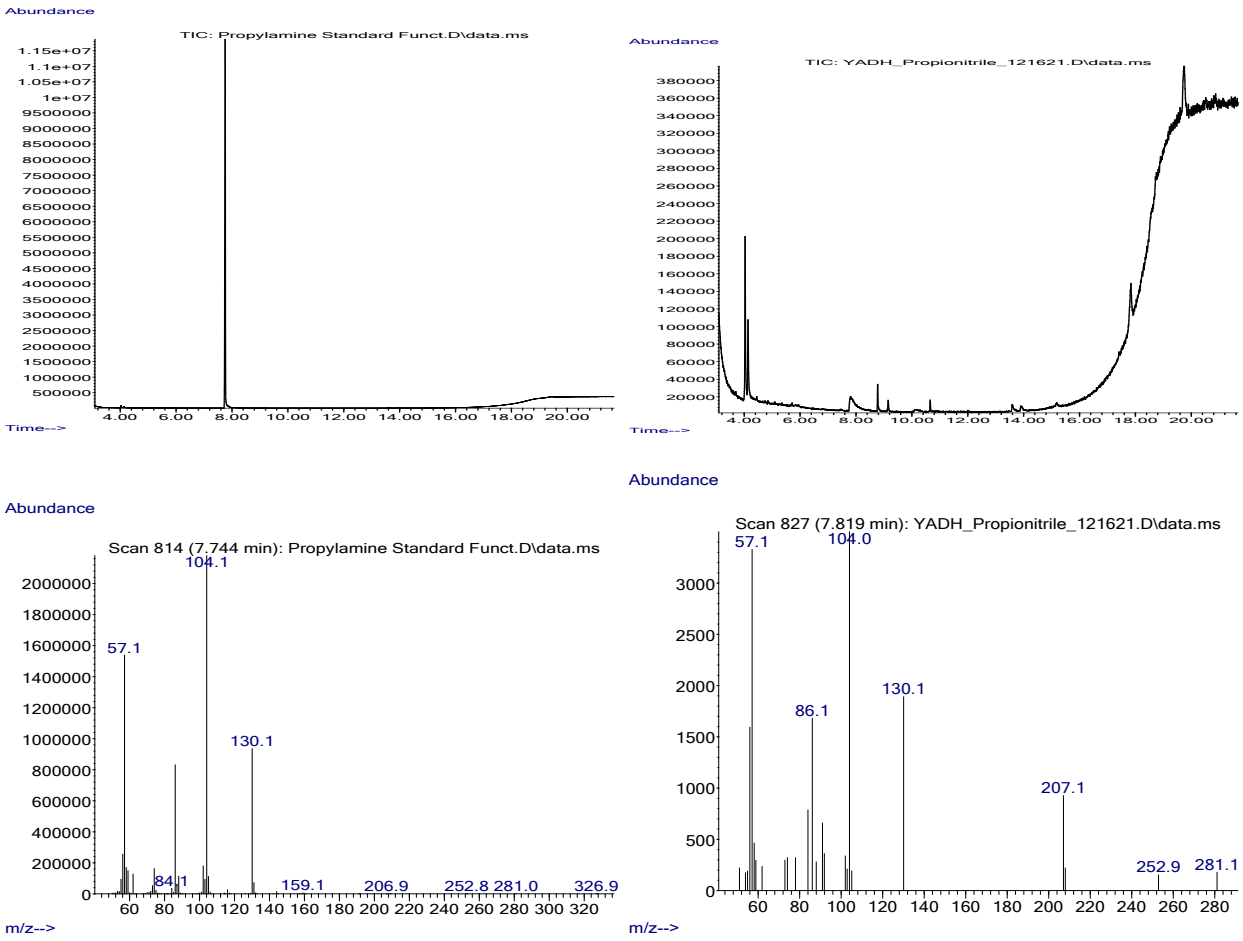

Figure 15. Gas chromatograms (top) and mass spectra (bottom) of functionalized propylamine standard (left) and YADH-catalyzed propionitrile reaction (right) showing consistent retention times and mass spectra fragmentation peaks.

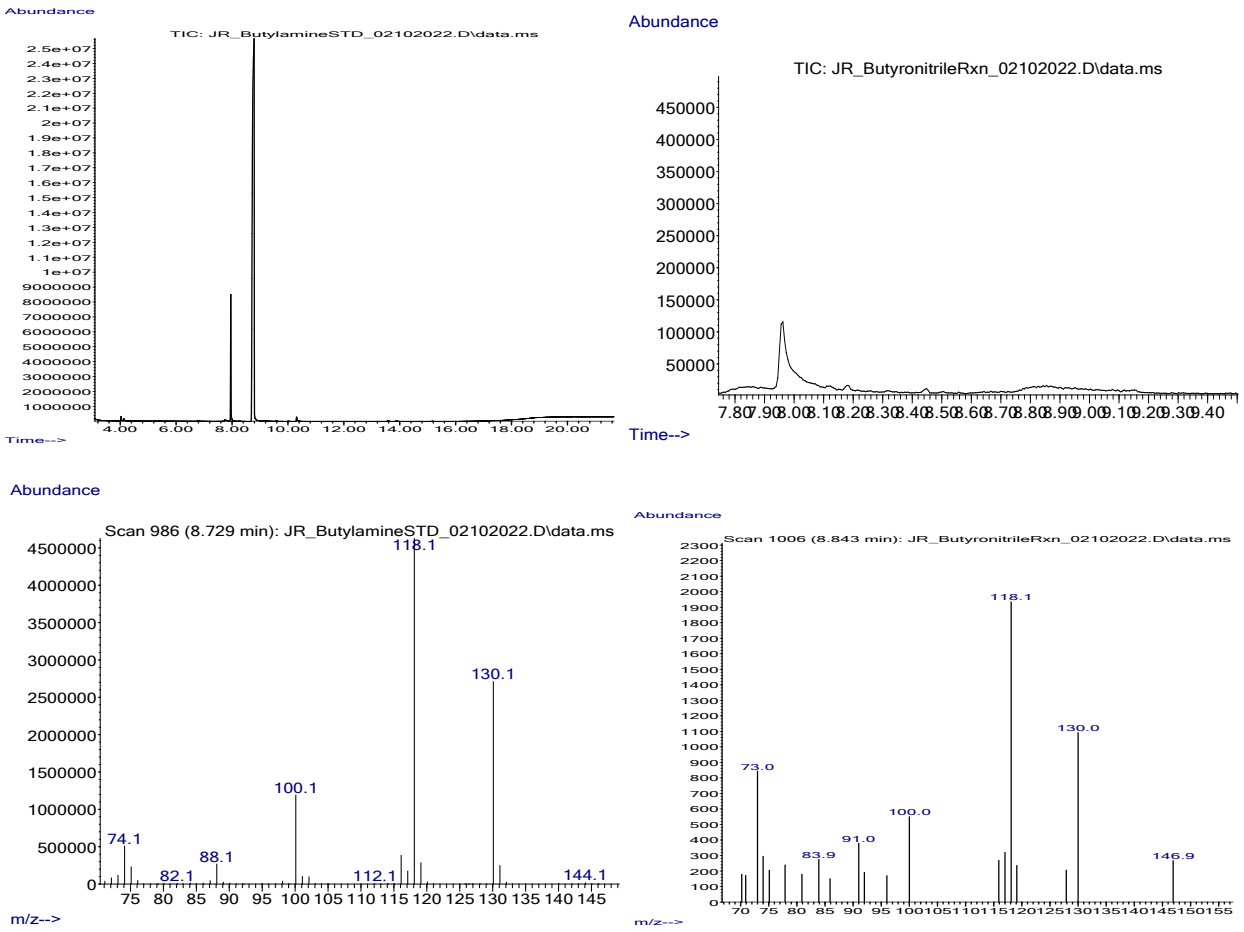

Figure 16. Gas chromatograms (top) and mass spectra (bottom) of functionalized butylamine standard (left) and YADH-catalyzed butylamine reaction (right) showing consistent retention times and mass spectra fragmentation peaks.

These data lend strong support to a full YADH-catalyzed reduction of nitriles to the corresponding amines, which has not been previously demonstrated. While significant, this raises the question of why YADH is able to catalyze such a reaction when other, similar enzymes were not able to overcome hydrolysis of the imine intermediate. One potential hypothesis involves the way YADH binds its substrate and cofactor. For the reduction of aldehydes to alcohols, the reaction rate is limited by the speed at which NAD^+ dissociates from the product–enzyme–NAD^+

complex.[33, 36] This allows the possibility of a new molecule of NADH to enter the active site following the first (nitrile to imine) reduction, while the imine remains coordinated with and stabilized by the catalytic zinc ion. A second reduction to the amine can occur if the NAD^+ dissociates and a new NADH binds before the substrate is released (Figure 17). However, the cited study also notes that different substrates affect the speed of dissociation, such as methanol slowing it down but cyclohexanol speeding it up, so further investigation of the reaction kinetics is necessary to ascertain the true cause.

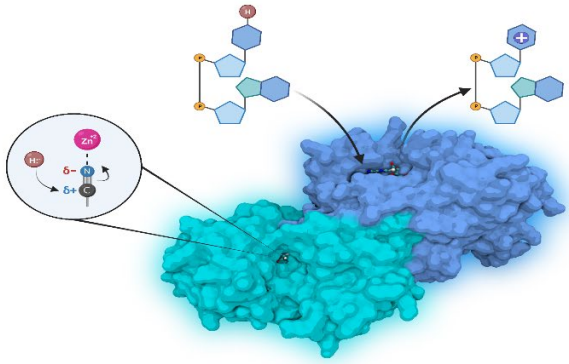

Figure 17. Reaction processivity. A second, new molecule of NADH may be able to enter the active site following NAD^+ dissociation, allowing a second reduction to occur before dissociation of the imine intermediate.

3.3 Reaction Kinetics

Promising GC-MS results urged further investigation of the reaction and how it behaves kinetically. Establishing kinetic parameters (K_M, V_{max}) for each substrate was of high priority, as these would provide insight into how well the reduction is catalyzed and result in a baseline to which subsequent reactions under different conditions could be compared. Ultimately, the behavior of YADH with acetonitrile was evaluated at substrate concentrations from 72 to 720

m*M*. Due to solubility issues, propionitrile and butyronitrile could not be investigated at the time of this work.

Reaction velocity (V_0, m*M*/s) for acetonitrile demonstrated a linear dependence on substrate concentration (Figure 18). However, the respective reaction velocities did not level off to a maximum (V_{max}) within the range of substrate concentrations tested, and thus a true Michaelis–Menten curve fit was not possible.

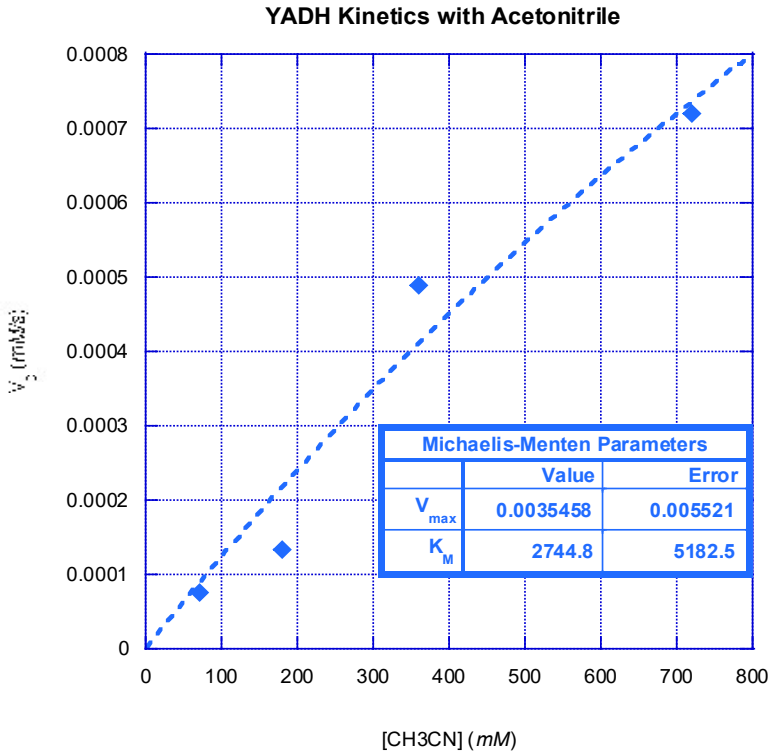

Figure 18. Michaelis-Menten plot of YADH reaction velocity vs. substrate concentration. Image generated using KaleidaGraph software.

The linearity of the V_0 versus [S] plot indicates that the substrate concentration at which the reaction goes most quickly (K_M) has also not been reached.

$$V_0 = \frac{V_{max}[S]}{K_M + [S]}$$

Standard Michaelis– Menten equation

$$V_0 = \frac{V_{max}}{K_M}[S]$$

If $K_M >> [S]$, the equation adopts the form $y = mx$ and becomes effectively linear.

While consistent values for these parameters were not obtained, the preliminary data allow inference of a large K_M, indicating that nitriles are not good substrates for YADH when compared to the corresponding aldehydes. The predicted K_M for acetonitrile as calculated by KaleidaGraph is approximately 2745 m*M* (with a large associated error), compared to a K_M value of 0.22 for YADH's native substrate acetaldehyde.[43] Since the V_{max} and K_M values remained elusive, further kinetic tests can be performed to confirm the enzyme dependence of the reaction by modifying the total enzyme concentration while maintaining substrate concentration.

$$V_{max} = k_2[E]_T$$

Maximum reaction velocity as a function of total enzyme concentration, where k_2 represents the catalytic rate constant of the reaction.

$$V_0 = \frac{k_2[S]}{K_M + [S]}[E]_T$$

When substituted into the standard equation

$$V_0 = \frac{k_2[E]_T}{K_M}[S]$$

If again $K_M >> [S]$, and $[S]$ is constant, reaction velocity is shown to be dependent on the total enzyme concentration in a linear fashion.

On the basis of the above equations, an enzyme-catalyzed reaction exhibiting Michaelis–Menten kinetics would have a hyperbolic relationship between velocity and substrate concentration as well as a linear relationship between velocity and total enzyme concentration. Further kinetic data is expected to indicate that the behavior of YADH with the acetonitrile substrate follows the rules of and can be predicted by steady-state kinetics.

Chapter 4: Conclusions and Future Works

To our knowledge, this body of work is the first to conduct and report on an in-depth analysis of YADH specificity with aliphatic nitriles. YADH-driven formation of propylamine from propionitrile and butylamine from butyronitrile were qualitatively confirmed via GC-MS. Additionally, acetonitrile exhibited substrate and enzyme concentration dependent reaction kinetics, suggesting standard steady-state behavior. Now that successful reactivity with these novel substrates has been demonstrated, YADH should be further investigated as an alternative reducing agent for nitrile compounds.

Future experiments are required to fully understand and improve upon the reactivity of YADH with aliphatic nitriles. Of primary importance is the quantitative assessment of product formation to serve as a baseline for subsequent experimentation. A key limitation of this work

was our inability to obtain pure functionalized amine standards within the project timeframe, which would allow calculation of percent conversion and percent yield when paired with an internal standard.

Another general limitation is the small size of the YADH binding site, which obstructs bulkier molecules (such as those with longer R chains or attached phenyl groups) from properly binding and coordinating, resulting in a decreased conversion rate,[44] which limited our investigation to smaller alkyl nitriles. One approach to opening up the active site to larger substrates is through site-directed mutagenesis, whereby changes in the primary amino acid sequence of the enzyme can modify the overall structure and result in a larger binding area.[45] While effective, this method is costly and time consuming, requiring an efficient way of producing and extracting the modified enzyme for use at a reasonable scale.[46]

An alternative workaround is to simply increase the thermal energy available in the reaction system by adding heat. This partially compensates for the decreased conversion rate, but the excess heat quickly leads to denaturation through unfolding of the enzyme, resulting in a dramatic loss of function.[47] Heat-tolerant ADHs from other organisms do exist, but they are not as commonly available or economic as those produced at large scale from yeast.

Several methods for stabilizing YADH in nonstandard conditions have been developed. It was discovered that YADH and its NAD cofactor can be encapsulated together in liposomes, thereby increasing the stability and efficiency of the complex in performing various reactions with alternate substrates when heated.[28] Another study examined YADH stabilized on microscaffolds, which could be used to oxidize alcohols of varying hydrocarbon chain lengths into carbonyl compounds useful to the production of detergents.[44] These works demonstrated

that YADH has potential be successfully employed in the laboratory as a redox catalyst under the right conditions.

In addition to mechanical stabilization methods, it has also been found that solvation of lyophilized YADH in organic media allows for a higher temperature tolerance and resistance to denaturation, resulting in a higher rate of catalysis (Figure 19).[47] This is thought to be a result of decreased flexibility in organic solvents when compared to water, preventing heat-based denaturation due to the rigidity of the enzyme in solution.[48] Protein folding is primarily driven by the "hydrophobic effect", where Gibb's free energy is provided from freeing up water molecules to hydrogen bond with each other instead of interacting hydrophobic regions of the protein. This effect is not as prominent in an aprotic organic solvent system, as the hydrogen bonding between protein and solvent would be much decreased in comparison.[49]

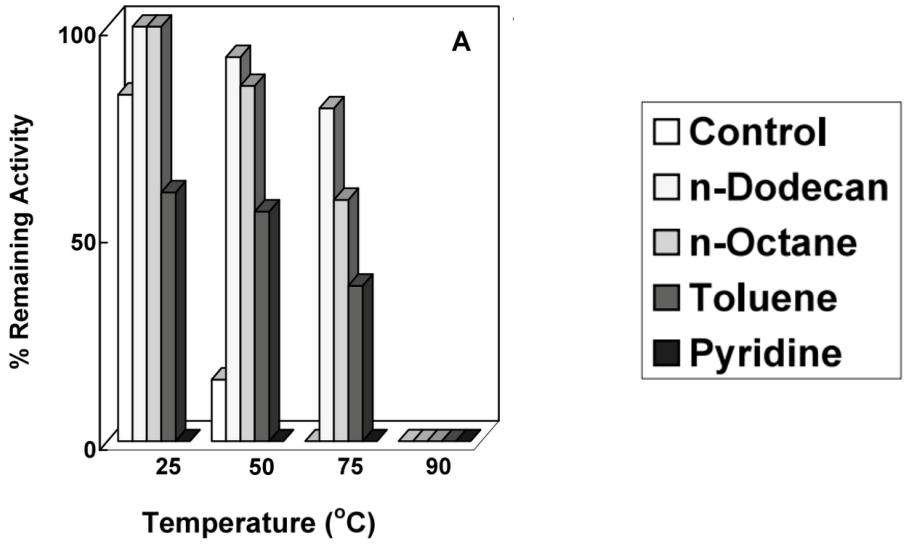

Figure 19. Organic Solvent Stabilization of ADH Activity (Modified and reprinted from ref 47.)

Aprotic environments are atypical for enzymes, which require aqueous environments for proper folding and a narrow pH range for optimal function. However, if properly isolated from the aqueous environments of cells and freeze-dried, many enzymes retain a "molecular memory" that allows them to maintain the structure and protonation state at the pH of their last protic environment.[50] This allows the enzyme to function at its optimal protonation state and structure, even outside of aqueous media.

Although further research is necessary, YADH demonstrated a previously undiscovered propensity for reducing nitriles to amines in an environmentally friendly way. With further investigation and modification of the reaction conditions, YADH could earn a place among other biocatalysts in the toolbox of pharmaceutical and industrial chemists alike.

References

(1) Rezaie, F.; Pirouzfar, V.; Alihosseini, A. Technical and economic analysis of acrylonitrile production from polypropylene. *Therm. Sci. Eng. Prog.* **2020**, *16*, 100463.

(2) Deepa, H. A.; Anusha, R.; Asha, P. Evaluation of Production and Economics of Acrylonitrile by Sohio Process. *Int. J. Eng. Res. Sci. Technol.* **2016**, *4* (Special Issue 21).

(3) Li, L.; Chen, Y.; Zeng, L.; Shao, M.; Xie, S.; Chen, W.; Lu, S.; Wu, Y.; Cao, W. Biomass burning contribution to ambient volatile organic compounds (VOCs) in the Chengdu–Chongqing Region (CCR), China. *Atmos. Environ.* **2014**, *99*, 403-410.

(4) Kennepohl, D.; Farmer, S.; Reusch, W.; Morsch, L. *Chemistry of Nitriles*. LibreTexts, 2021. https://chem.libretexts.org/Bookshelves/Organic_Chemistry/Organic_Chemistry_(McMurry)/20%3A_Carboxylic_Acids_and_Nitriles/20.07%3A_Chemistry_of_Nitriles

(5) Fleming, F. F.; Yao, L.; Ravikumar, P. C.; Funk, L.; Shook, B. C. Nitrile-Containing Pharmaceuticals: Efficacious Roles of the Nitrile Pharmacophore. *Int. J. Med. Chem.* **2010**, *53* (22), 7902-7917. DOI: 10.1021/jm100762r

(6) Sánchez-Jiménez, F.; Ruiz-Pérez, M. V.; Urdiales, J. L.; Medina, M. A. Pharmacological potential of biogenic amine-polyamine interactions beyond neurotransmission. *Br. J. Pharmacol.* **2013**, *170* (1), 4-16. DOI: 10.1111/bph.12109

(7) Kennedy, G. Ethylamine. In *Encyclopedia of Toxicology*, Company, D. Ed.; Elsevier, 2014.

(8) Artini, D.; Buttinoni, A.; Dradi, E.; Logemann, W.; Mandelli, V.; Melloni, P.; Tommasini, R.; Tosolini, G.; Vita, G. Amides and amines with analgesic and anti-inflammatory activity. *Arzneimittel-Forschung* **1971**, *21* (1), 30-36. PubMed.

(9) Smith, P. A. S. Occurrence and sources of amines. In *Encyclopedia Brittanica*.

(10) Kennepohl, D. *Preparation of Amines*. Lumen Learning, https://courses.lumenlearning.com/suny-potsdam-organicchemistry2/chapter/23-2-preparation-of-amines/

(11) Eller, K.; Henkes, E.; Rossbacher, R. Aliphatic Amines: General Production Methods. In *Ullmann's Encyclopedia of Industrial Chemistry*, 2000.

(12) Mukherjee, A.; Srimani, D.; Chakraborty, S.; Ben-David, Y.; Milstein, D. Selective Hydrogenation of Nitriles to Primary Amines Catalyzed by a Cobalt Pincer Complex. *J. Am. Chem. Soc.* **2015**, *137* (28), 8888-8891. DOI: 10.1021/jacs.5b04879

(13) Amundsen, L. H.; Nelson, L. S. Reduction of Nitriles to Primary Amines with Lithium Aluminum Hydride1. *J. Am. Chem. Soc.* **1951**, *73* (1), 242-244. DOI: 10.1021/ja01145a082

(14) Billman, J. H.; Diesing, A. C. Reduction of Schiff Bases with Sodium Borohydride. *Tetrahedron Lett.* **1957**, *22* (9), 1068-1070. DOI: 10.1021/jo01360a019

(15) Sweeting, L. M. Reducing Agents. 1998.

(16) Xia, R.; Tian, D.; Kattel, S.; Hasa, B.; Shin, H.; Ma, X.; Chen, J. G.; Jiao, F. Electrochemical reduction of acetonitrile to ethylamine. *Nat. Commun.* **2021**, *12* (1), 1949. DOI: 10.1038/s41467-021-22291-0.

(17) Wirtanen, T.; Rodrigo, E.; Waldvogel, S. R. Recent Advances in the Electrochemical Reduction of Substrates Involving N−O Bonds. *Adv. Synth. Catal.* **2020**, *362* (11), 2088-2101. DOI: 10.1002/adsc.202000349

(18) Truppo, M. D. Biocatalysis in the Pharmaceutical Industry: The Need for Speed. *ACS Med. Chem. Lett.* **2017**, *8* (5), 476-480. DOI: 10.1021/acsmedchemlett.7b00114

(19) Devine, P. N.; Howard, R. M.; Kumar, R.; Thompson, M. P.; Truppo, M. D.; Turner, N. J. Extending the application of biocatalysis to meet the challenges of drug development. *Nat. Rev. Chem.* **2018**, *2* (12), 409-421. DOI: 10.1038/s41570-018-0055-1

(20) Fraley, A. E.; Sherman, D. H. Halogenase engineering and its utility in medicinal chemistry. *Bioorg. Med. Chem. Lett.* **2018**, *28* (11), 1992-1999. DOI: 10.1016/j.bmcl.2018.04.066

(21) Kim, Y.; Zhou, M.; Moy, S.; Morales, J.; Cunningham, M. A.; Joachimiak, A. High-Resolution Structure of the Nitrile Reductase QueF Combined with Molecular Simulations Provide Insight into Enzyme Mechanism. *J. Mol. Biol.* **2010**, *404* (1), 127-137. DOI: 10.1016/j.jmb.2010.09.042

(22) Ribeiro, A. J. M.; Yang, L.; Ramos, M. J.; Fernandes, P. A.; Liang, Z.-X.; Hirao, H. Insight into Enzymatic Nitrile Reduction: QM/MM Study of the Catalytic Mechanism of QueF Nitrile Reductase. *ACS Catal.* **2015**, *5* (6), 3740-3751. DOI: 10.1021/acscatal.5b00528

(23) Yanga, L.; Koha, S. L.; Suttonb, P. W.; Liang, Z.-X. Nitrile Reductase as Biocatalyst: Opportunity and Challenge.

(24) Durchschein, K.; Ferreira-Da Silva, B.; Wallner, S.; Macheroux, P.; Kroutil, W.; Glueck, S. M.; Faber, K. The flavoprotein-catalyzed reduction of aliphatic nitro-compounds represents a biocatalytic equivalent to the Nef-reaction. *Green Chem.* **2010**, *12* (4), 616. DOI: 10.1039/b922691e

(25) de Smidt, O.; du Preez, J. C.; Albertyn, J. The alcohol dehydrogenases of Saccharomyces cerevisiae: a comprehensive review. *FEMS Yeast Res.* **2008**, *8* (7), 967-978. DOI: 10.1111/j.1567-1364.2008.00387.x.

(26) Tauber, K.; Hall, M.; Kroutil, W.; Fabian, W. M. F.; Faber, K.; Glueck, S. M. A highly efficient ADH-coupled NADH-recycling system for the asymmetric bioreduction of carbon-

carbon double bonds using enoate reductases. *Biotechnol. Bioeng.* **2011**, *108* (6), 1462-1467. DOI: 10.1002/bit.23078

(27) Klinman, J. P. The Mechanism of Enzyme-catalyzed Reduced Nicotinamide Adenine Dinucleotide-dependent Reductions. *J. Biol. Chem.* **1972**, *247* (24), 7977-7987. DOI: 10.1016/s0021-9258(20)81798-0

(28) Yoshimoto, M.; Sato, M.; Yoshimoto, N.; Nakao, K. Liposomal Encapsulation of Yeast Alcohol Dehydrogenase with Cofactor for Stabilization of the Enzyme Structure and Activity. *Biotechnol. Prog.* **2008**, *24* (3), 576-582. DOI: 10.1021/bp070392e

(29) Leskovac, V.; Triviä□, S.; Periä□In, D. The three zinc-containing alcohol dehydrogenases from baker's yeast,Saccharomyces cerevisiae. *FEMS Yeast Res.* **2002**, *2* (4), 481-494. DOI: 10.1111/j.1567-1364.2002.tb00116.x

(30) Dauben, W. G.; Fonken, G. J.; Noyce, D. S. The Stereochemistry of Hydride Reductions. *J. Am. Chem. Soc.* **1956**, *78* (11), 2579-2582. DOI: 10.1021/ja01592a069

(31) Patel, R. Biocatalytic Synthesis of Chiral Alcohols and Amino Acids for Development of Pharmaceuticals. *Biomolecules* **2013**, *3* (4), 741-777. DOI: 10.3390/biom3040741

(32) Carnahan, M.; Cox, K.; Espinosa, J.; Feaster, R.; Hirsch, R.; Lam, A.; Roller, S.; Steadman, B.; Toth, J.; Lee, M.; et al. Probing the Active Site of Yeast Alcohol Dehydrogenase through Microscale Yeast-Mediated Reductions of Acetophenone and Acetylpyridines. A Collaborative and Research-Based Advanced Bioorganic Chemistry Laboratory Project. *J. Chem. Educ.* **2000**, *77* (3), 363. DOI: 10.1021/ed077p363

(33) Dickinson, F. M.; Monger, G. P. A study of the kinetics and mechanism of yeast alcohol dehydrogenase with a variety of substrates. *Biochem. J.* **1973**, *131* (2), 261-270. DOI: 10.1042/bj1310261 (acccessed 2021-12-02T00:12:55).

(34) Madden, K.; Todd, P. M. T.; Urata, K.; Russell, A.; Vincent, K.; Reeve, H. A Pharmacophore-Based Approach to Demonstrating the Scope of Alcohol Dehydrogenases. American Chemical Society (ACS): 2020.

(35) Kroutil, W. Chemo-promiscuity of alcohol dehydrogenases: reduction of phenylacetaldoxime to the alcohol. Tetrahedron: 2010; Vol. 66, pp 3410-3414.

(36) Raj, S. B.; Ramaswamy, S.; Plapp, B. V. Yeast Alcohol Dehydrogenase Structure and Catalysis. *Biochemistry* **2014**, *53* (36), 5791-5803. DOI: 10.1021/bi5006442

(37) Men, L.; Wang, Y. The Oxidation of Yeast Alcohol Dehydrogenase-1 by Hydrogen Peroxide in Vitro. *J. Proteome Res.* **2007**, *6* (1), 216-225. DOI: 10.1021/pr0603809

(38) Chimni, S.; Singh, R. Bioreduction of a carbon-nitrogen double bond using immobilized baker's yeast - a first report. *World J. Microbiol. Biotechnol.* **1998**, *14*, 247-250.

(39) Lennard, C. Fingerprint detection: current capabilities. *Aust. J. Forensic Sci.* **2007**, *39* (2), 55-71. DOI: 10.1080/00450610701650021.

(40) Cunha, S. C.; Faria, M. A.; Fernandes, J. O. Gas Chromatography-Mass Spectrometry Assessment of Amines in Port Wine and Grape Juice after Fast Chloroformate Extraction/Derivatization. *J. Agric. Food Chem.* **2011**, *59* (16), 8742-8753. DOI: 10.1021/jf201379x.

(41) Yemm, E.; Cocking, E.; Ricketts, R. The Determination of Amino-Acids with Ninhydrin. *Analyst* **1955**, *80*. DOI: 10.1039/an9558000209.

(42) John Harding, V.; M. MacLean, R. The Ninhydrin Reaction with Amines and Amides. *J. Biol. Chem.* **1916**.

(43) Laadan, B.; Wallace-Salinas, V.; Carlsson, Å. J.; Almeida, J. R.; Rådström, P.; Gorwa-Grauslund, M. F. Furaldehyde substrate specificity and kinetics of Saccharomyces cerevisiae

alcohol dehydrogenase 1 variants. *Microb. Cell Fact.* **2014**, *13* (1). DOI: 10.1186/s12934-014-0112-5

(44) Ottone, C.; Bernal, C.; Serna, N.; Illanes, A.; Wilson, L. Enhanced long-chain fatty alcohol oxidation by immobilization of alcohol dehydrogenase from S. cerevisiae. *Appl. Microbiol. Biotechnol.* **2018**, *102* (1), 237-247. DOI: 10.1007/s00253-017-8598-5.

(45) Nealon, C. M.; Musa, M. M.; Patel, J. M.; Phillips, R. S. Controlling substrate specificity and stereospecificity of alcohol dehydrogenases. *ACS Catal.* **2015**, *5* (4), 2100-2114. DOI: 10.1021/cs501457v.

(46) Shimada, A. PCR-based site-directed mutagenesis. *In Vitro Mutagenesis Protocols* **1996**, 157-165.

(47) Miroliaei, M.; Nemat-Gorgani, M. Effect of organic solvents on stability and activity of two related alcohol dehydrogenases: a comparative study. *Int. J. Biochem. Cell Biol.* **2002**, *34* (2), 169-175. DOI: 10.1016/s1357-2725(01)00109-1.

(48) Klibanov, A. M. Improving enzymes by using them in organic solvents. *Nature (London)* **2001**, *409* (6817), 241-246. DOI: 10.1038/35051719.

(49) Tesfai, B. T.; Wu, D.; Chen, S.; Chen, J.; Wu, J. Effect of organic solvents on the yield and specificity of cyclodextrins by recombinant cyclodextrin glucanotransferase (CGTase) from Anaerobranca gottschalkii. *J. Inclusion Phenom. Macrocyclic Chem.* **2013**, *77* (1-4), 147-153. DOI: 10.1007/s10847-012-0225-6.

(50) Sheldon, R. A. Reactions in non-conventional media for sustainable organic synthesis. *NATO Sci. Ser., II* **2008**, *246*, 1-28.

Appendix

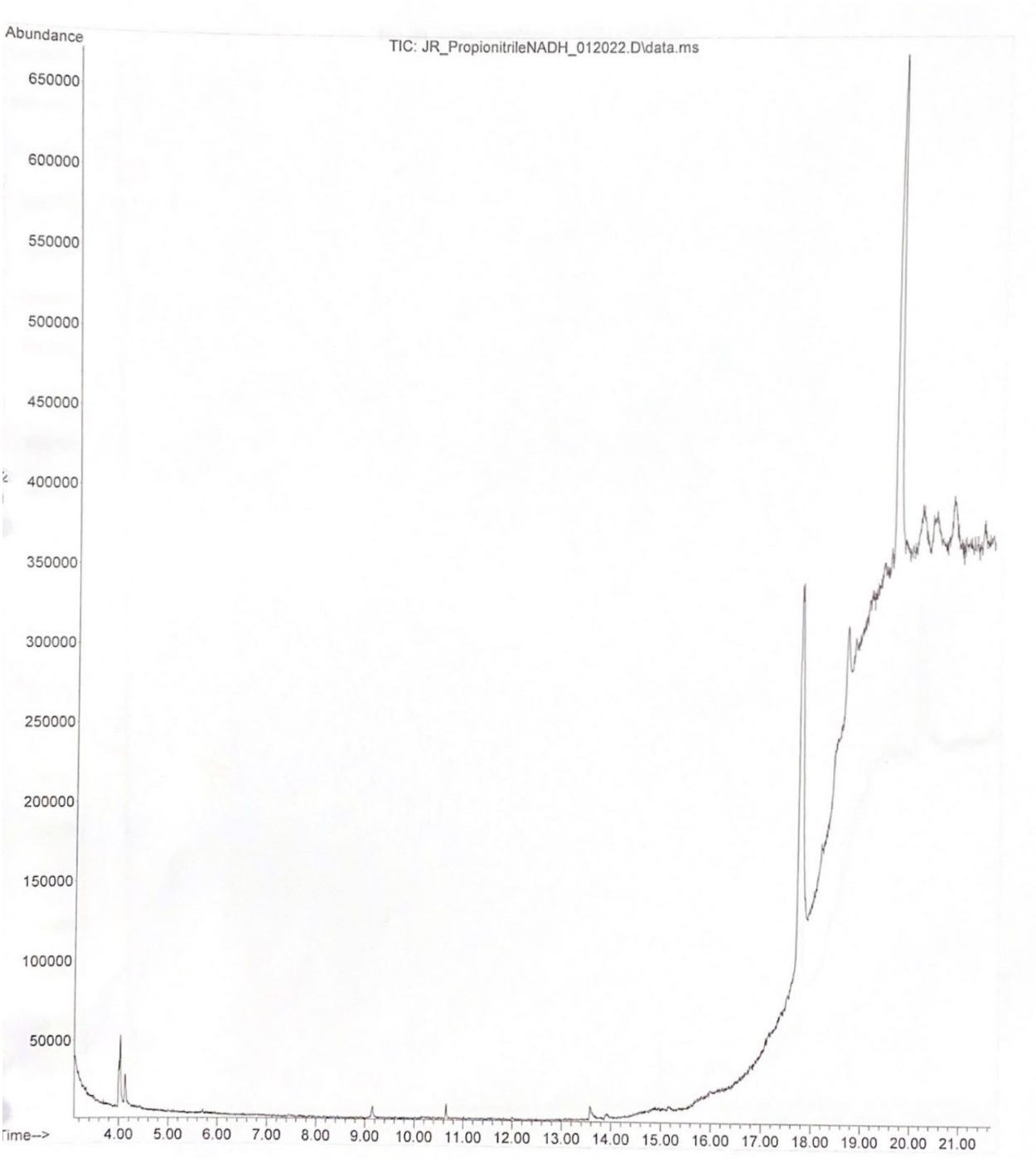

Figure A1. Propionitrile reaction control for NADH.

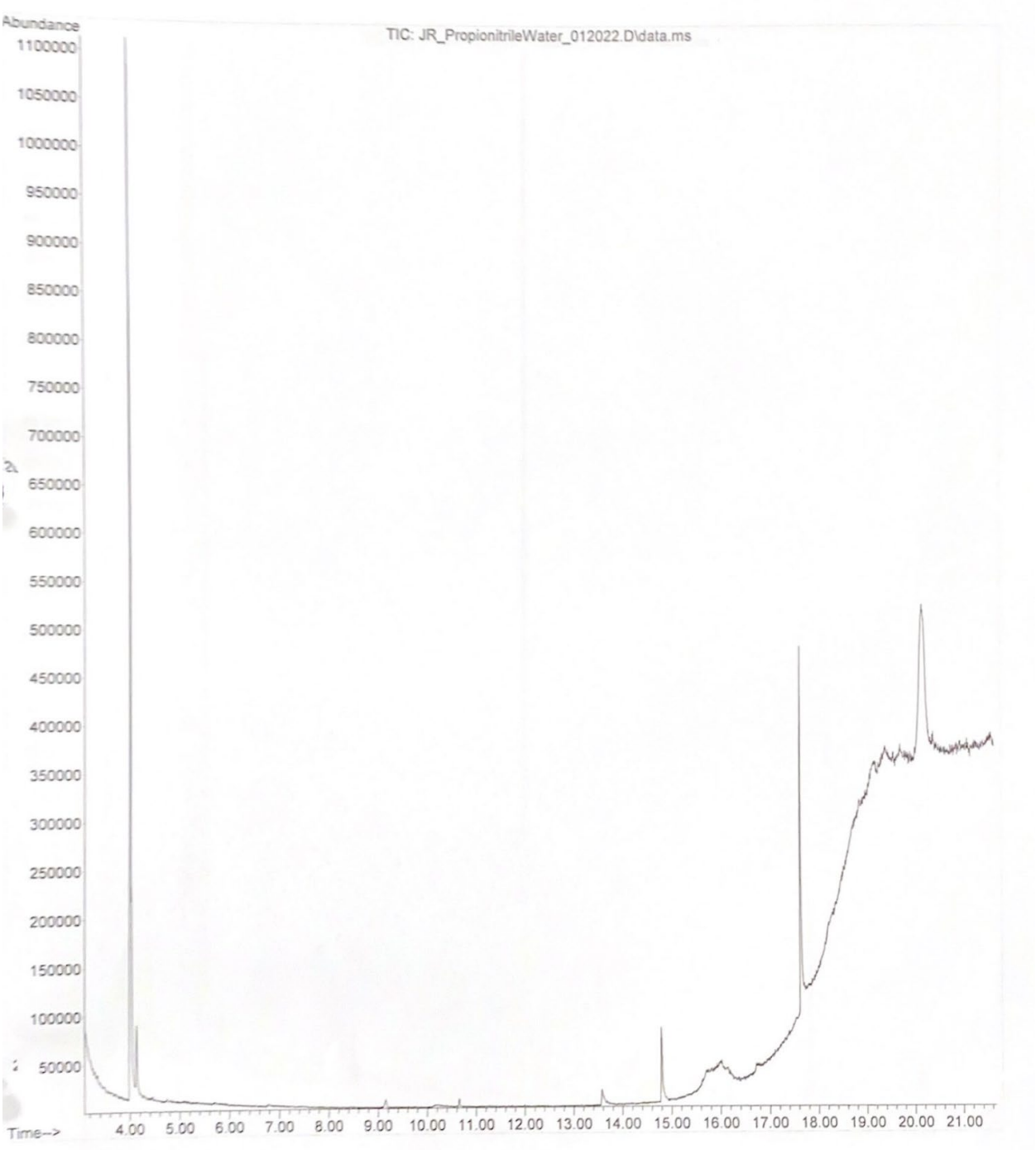

Figure A2. Propionitrile reaction control for aqueous reaction medium.

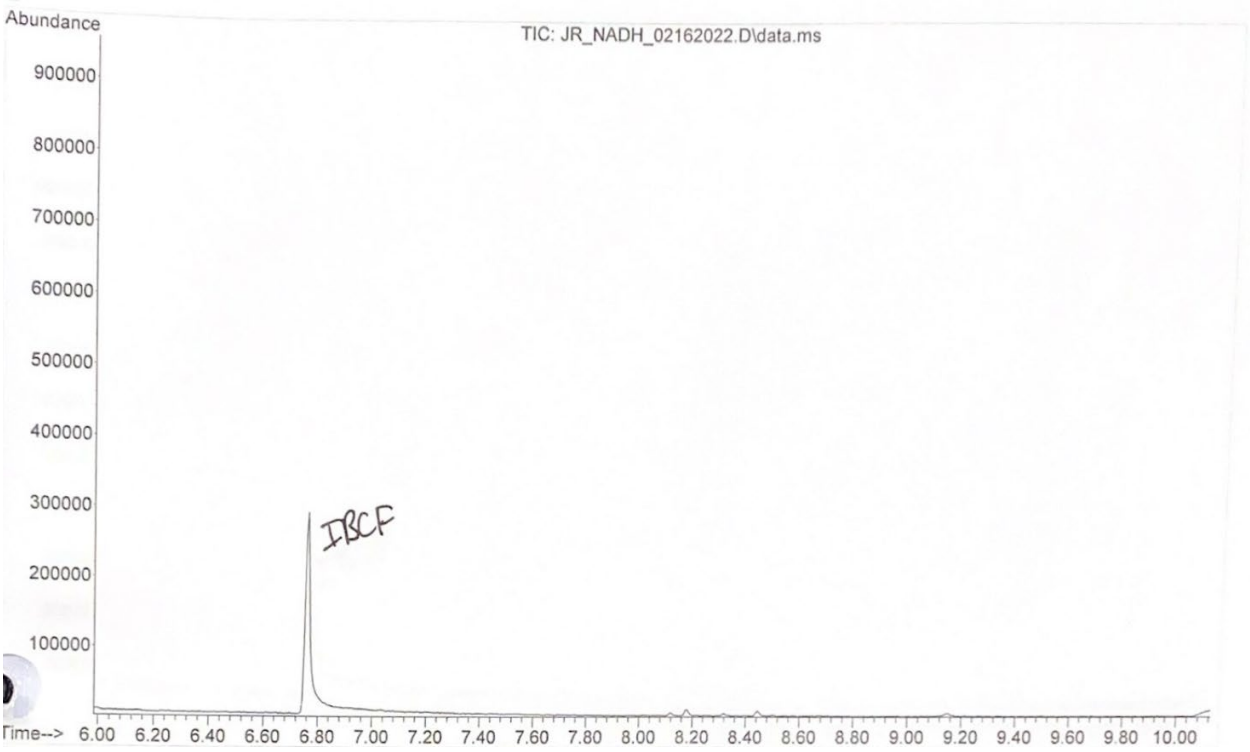

Figure A3. Butyronitrile reaction control for NADH.

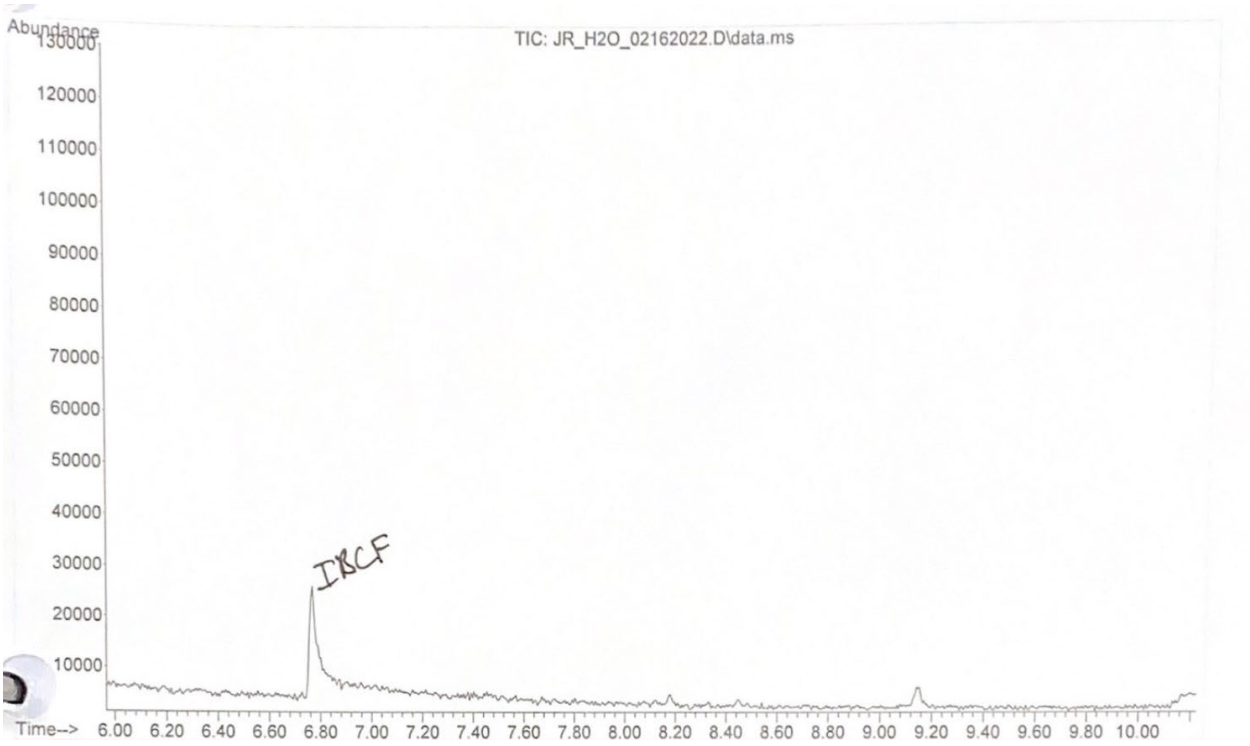

Figure A4. Butyronitrile reaction control for aqueous reaction medium.