BIOTRANSFORMATION OF NITRILES AND ALCOHOLS TO CARBOXYLIC ACIDS USING INDUSTRIAL ENZYMES

THESIS

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San Marcos, Texas

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DEDICATION

To Guide Pomeyie, Cynthia Agbley, Kaicy Agbley, Kylee Agbley and Lawrence Agbley.

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First, I thank the almighty God for the gift of life and guidance that have brought me thus far.

My sincere gratitude goes to Dr. Linette M. Watkins, my advisor and friend, for intellectually inspiring me and allowing me to conduct this research in her Lab.
I appreciate my committee members for all their constructive criticisms and intellectual discourse pertaining to this project. I extend this acknowledgement to all the Professors at the Department of Biochemistry, University of Ghana, especially my undergraduate advisor, Dr. Samuel Sackey, for providing me with a solid background in theoretical Biochemistry, which helped me immensely during my graduate studies at Texas State University-San Marcos. Finally, I am forever grateful to my mother, Guide Pomeyie for her steadfast love and daily prayers, Lawrence Agbley, my father for financially sponsoring my pursuit of higher education in the United States, Cynthia, Kylee, and Kaicy Agbley for their encouragement.

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ABSTRACT

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by

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Texas State University-San Marcos

May 2013

SUPERVISING PROFESSOR: LINETTE M. WATKINS

Nitrilases (EC 3.5.5.1) hydrolyze nitriles to corresponding carboxylic acids and ammonia while alcohol oxidases (EC 1.1.3.13) and alcohol dehydrogenases (EC 1.1.1.1) oxidize alcohols to aldehydes using molecular oxygen and NAD^+ as oxidants, respectively. In this study, the enzyme activity of nitrile and alcohol substrates from Huntsman LLC, The Woodlands, TX was investigated. Alcohol dehydrogenase from both yeast and equine liver did not react with Huntsman's alcohols L1 and L2. The alcohol oxidase, however, showed increasing activity for the alcohols A3, A2 and A1, respectively.

The nitrilase reaction turned over 150 μM of products in 4 hours at the optimum pH (7.5) and temperature (25 °C) of the enzyme. The effects of temperature and pH on the stability of the nitrilase as well as catalysis in organic solvents were also studied. The enzyme exhibited high thermal stability between 25 °C- 40 °C. At 50 °C, activity decreased to 50% with complete loss of activity occurring at temperatures above 60 °C. Slightly alkaline and neutral pH environments also improved enzyme stability. These pH ranges are consistent with values reported in the literature. The incorporation of the organic solvents, methanol and ethanol at 80% (v/v), into the reaction denatures the enzyme and little or no activity was observed. However, low ethanol concentrations (10% v/v) increased the enzyme activity to 120% relative to the standard reaction (no organic solvent) by increasing the solubility of the hydrophobic nitrile. In addition, the enzyme appears to be more stable in ethanol than methanol. A comparison of enzyme activity for benzonitrile and Huntsman's nitrile (aliphatic) suggests that the enzyme is more efficient in hydrolyzing aromatic nitriles than aliphatic nitriles.

CHAPTER I

INTRODUCTION

Nitriles are organic compounds with cyano functional groups (R-CN). They are produced naturally by plants, animals and fungi as cyanoglycosides, cyanolipids, ricinine (1, 2-dihydro-4-methoxy-2-oxo-3-pyridinecarbonitrile) and phenylacetonitriles [1]. Chemically, they are synthesized by the Strecker reaction, the Sandmeyer reaction and the reaction of alkyl halides with cyanide ion [2]. In spite of the toxicity, mutagenicity and carcinogenicity of most nitriles due to the cyano group, they are important intermediates/precursors in the synthesis of other organic compounds like carboxylic acids, amides, amines, esters and carbonyls and are used extensively in the pharmaceutical and polymer industries [1, 3].

Chemical hydrolysis of nitriles to carboxylic acids requires harsh reaction conditions like high temperature and pressure, using strong acids as catalysts and often results in low yields [4]. The formation of undesired by-products means that one or more separation techniques are needed to purify the desired products [5]. The generation of organic wastes (by-products) on an industrial scale is a further drawback. An estimated 18 billion liters of nitrile wastes are generated per annum in the United States [6].For complex nitriles containing labile functional groups, additional protective chemical

groups are needed to prevent the undesired transformations of these functional groups. For instance, the protection of the carbonyl functional groups by transforming them into ketals and acetals using diols is commonplace in organic synthetic reactions.

Biotransformation, which is the use of microorganisms, cell extracts and enzymes (free or immobilized) to modify or convert substrates into desired products, has piqued the interest of scientists in both academia and industry in recent times because it offers a safer, milder and "greener" alternative to chemical synthesis with high yield and substrate selectivity [7, 8, 9]. There are no requirements for additional protective groups as compared to chemical synthesis since enzymes are specific [10]. Furthermore, there is an advantage of chemo-, regio-, stereo- and/or enantioselectivity in products. Such selectivities are usually absent in most chemical syntheses. For example, chemical hydrolysis of dinitriles produces dicarboxylic acids. This reaction is not regioselective; however, enzyme catalyzed hydrolysis of the same dinitriles yields cyanocarboxylic acids, where only one of the two cyano groups is hydrolyzed to carboxylic acid [11].

Currently, microbial nitrile catabolism is known to occur via two distinct pathways: the nitrilase route or the nitrile hydratase and amidase route (figure 1). Most nitrilase producing organisms isolated and characterized thus far possess one of the two pathways. However, in 2001, Piotrowski et al. reported that nitrilase from *Arabidopsis thaliana* possesses an amidase activity as well [12]. Nitrilases (EC 3.5.5.1) catalyze the direct hydrolysis of nitriles to their corresponding carboxylic acids and ammonia.

However, the two enzyme route of nitrile hydratases (EC 4.2.1.84) and amidases (EC3.5.1.4) achieves the same products but via an amide intermediate [13].

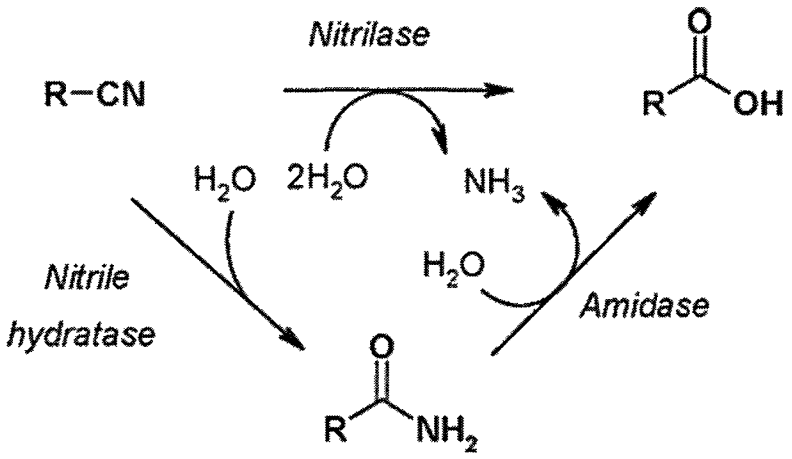

Figure 1. Generic reaction scheme of nitrile catabolism. Nitrilase hydrolyzes nitriles to carboxylic acids and ammonia in one step while the two enzyme system of nitrile hydratase and amidase achieves the same transformation but via an amide intermediate in two steps [14].

Nitrilases act on a broad spectrum of nitriles and they are classified as aromatic, aliphatic and arylacetonitrilases. This classification is based on substrate specificity. Aromatic nitrilases act on aromatic or heterocyclic nitriles (i.e. benzonitrile), aliphatic nitrilases hydrolyze aliphatic (straight chain) nitriles (i.e. propionitrile) and arylacetonitriles (i.e. phenylacetonitrile) are transformed by arylacetonitrilases [15]. A myriad of plants, fungi and bacterial species with the nitrilase and nitrile hydrolyzing

enzymes have been identified and applied industrially in the synthesis of various compounds (figure 2) [15, 16, 17]. Notable among these are the synthesis of 3-indole acetic acid, a plant hormone, from 3-indoleacetonitrile [18, 19], (R)-2-chloromandelic acid (precursor for the synthesis of Clopidogel®, a platelet aggregation inhibitor) from 2-chloromandelonitrile [20], nicotinic acid (Vitamin B_3) and isonicotinic acid from 3-cyanopyridine [3, 21, 22]. The highest yield of 172 g/L of nicotinic acid was obtained from *Rhodococcus rhodochrous* J1 with 100% conversion of 3-cyanopyridine [23]. Nitrilase from *Arabidopsis thaliana* has been utilized to synthesize the amino acids aspartic acid and asparagine with a high enantiomeric excess using β-cyano-L-alanine as substrate [24]. Annually, about 40,000 tons of acrylamide is produced from acrylonitrile using nitrile hydrolyzing enzymes from *Rhodococcus rhodochrous* J1 [25]. Glycolic acid, the simplest α-hydroxycarboxylic acid with extensive use in household cleaners, cosmetics, industrial and electronic elements, has been successfully synthesized using immobilized *Alcaligenes* sp. and glycolonitrile as substrate [26]. Most nitrile hydrolyzing enzymes reported in the literature focus on product synthesis. A few have been utilized in surface modification of polymers particularly polyacrylonitrile fibers, bioremediation and biodegradation [27].

Table 1. Selected organisms from which nitrile hydrolyzing enzymes have been isolated and characterized

Bacteria	Plants	Fungi
Rhodobacter sphaeroids LHS-305[28]	*Zea mays*[41]	*Fusarium oxysporum* [45]
Rhodococcus rhodochrous J1 and K22 [29]	*Bradyrhizobium japonicum*[42]	*Fusarium solani* [46]
Alcaligenes faecalis JM3[30]	*Arabidopsis thaliana*[43]	*Penicillium multicolor*[47]
Klebsiella pneumoniae ssp.[31]	Chinese cabbage[44]	*Aspergillus niger* [48]
Nocardia globerula[32]		
Pseudomonas fluorescens [33]		
Pseudomonas putida 10145 [34]		
Bacillus subtilis ZJB-063 [35]		
Bacillus pallidus [36]		
Cyanobacterium synechocystic sp.[37]		
Comamonas testosterone [38]		
Acinetobacter sp.[39]		
Acidovorax facilis[40]		

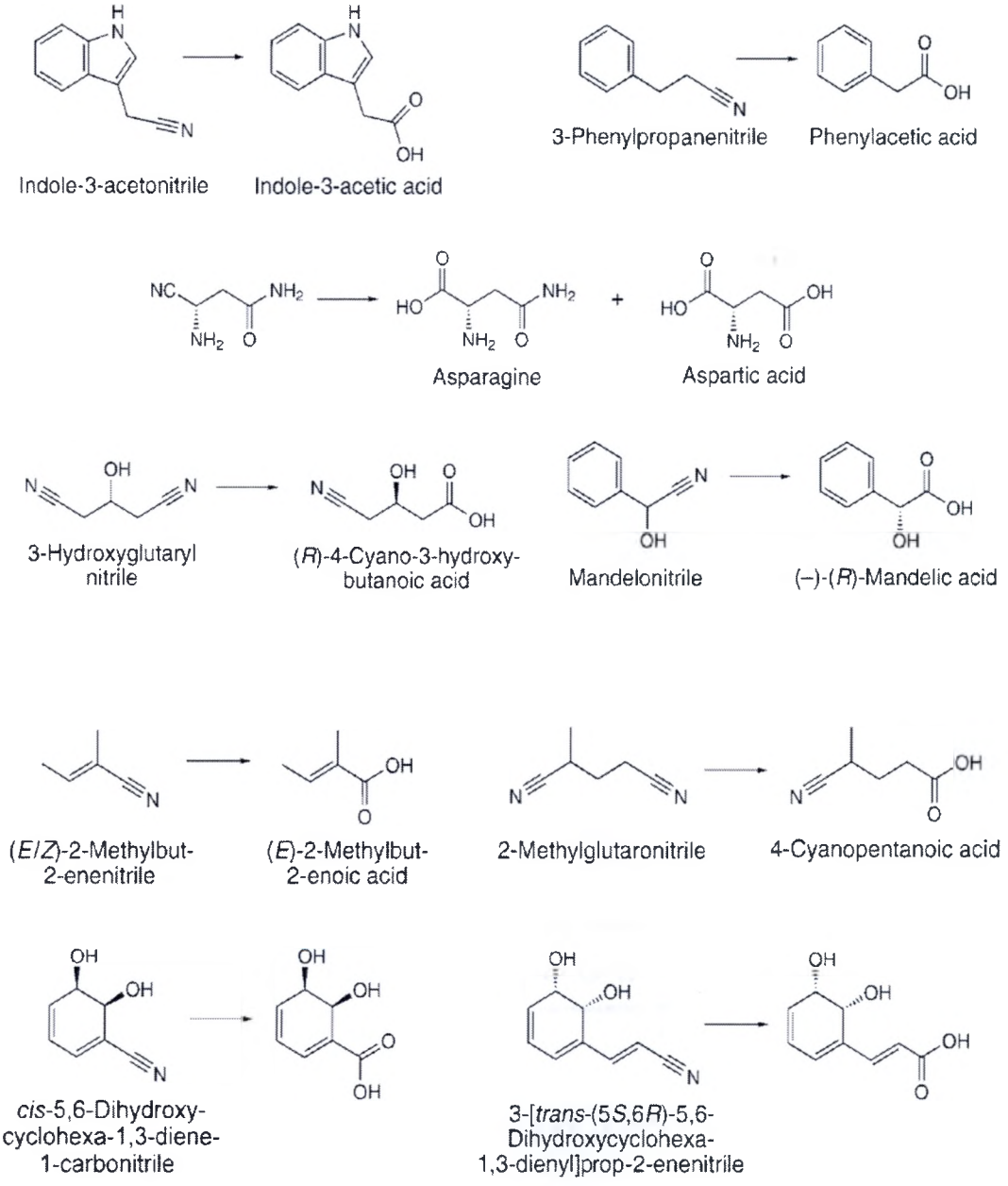

Figure 2. Synthetic applications of nitrile hydrolyzing enzymes; enzyme catalyzed reactions showing substrates and products. Singh, *et al*. 2006[1].

In spite of their numerous advantages and extensive industrial applications, there has still been an increased demand for new nitrilases since all the enzymes characterized thus far exhibit low thermal stability and low activity [49].

The physiological role of nitrilases is unclear. However, it is an important component of metabolic pathways in plants and animals. In plants, it is an integral part of the biosynthetic pathway of indole-3- acetic acid from tryptophan [50]. It is also involved in the degradation of glucosinolates and aldoximes (figure 3) [51, 52]. Cyanide is a known potent inhibitor of cytochrome c oxidase, the fourth complex of the electron transport chain. For this reason, nitrile hydrolyzing enzymes are implicated in the detoxification of cyano group releasing compounds, usually metal cyanides.

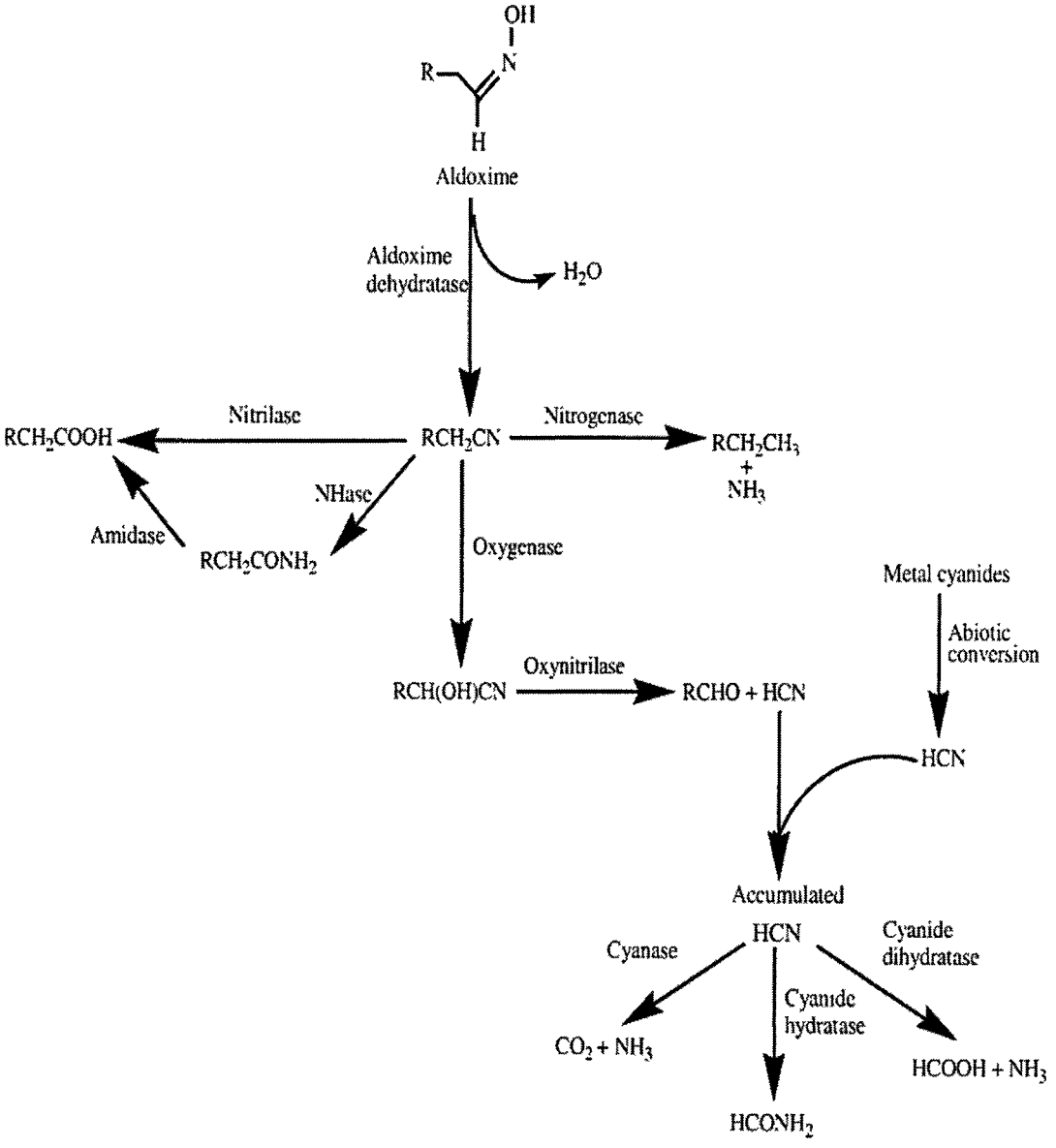

Figure 3. Nitrile metabolic pathways showing nitrilase, nitrile hydratase and amidase reactions [52].

Nitrilases belong to the Nitrilase superfamily. Members of this group are thiol enzymes that possess a characteristic α-β-β-α structural motif and are involved in nitrile hydrolysis to carboxylic acids and ammonia via a single or multiple steps [53]. Constituents of this group appear to be widespread and are subdivided into 13 branches based on structure, sequence similarity and the presence of additional domains [54]. The branches include: *i)* nitrilases, *ii*) aliphatic amidases. *iii*) amino-terminal-amidases, *iv)* biotinidases, *v*) β-ureidopropionases and *vi*) carbamylases. The rest are vii) prokaryote NAD synthetases, *viii)* eukaryotic NAD synthetases, *ix*) apolipoprotein N-acyltransferases, *x*) Nit and NitFhit, *xi*) NB11, xii) NB12 and *xiii*) nonfused outliers [54]. Of the 13 branches, only branch one has a true nitrilase activity whereas seven branches of the family have amidase or amide condensing activity [55].

Nitrilases are cysteine hydrolases with a conserved Cys-Glu-Lys catalytic triad at their active site (figure 6) [56]. The sulfhydryl group of cysteine acts as a nucleophile, a glutamate residue is involved in a general acid base catalysis and a lysine residue in an electrophilic catalysis by stabilizing the tetrahedral intermediate formed as a result of the nucleophilic attack on the electrophilic cyano carbon [57, 58, 59]. Two molecules of water play a crucial role in the nitrilase mechanism. The first molecule converts the imine functional group formed as a result of the nucleophilic attack on the nitrile carbon to a corresponding ketone (Schiff base hydrolysis), releasing ammonia in the process. The resulting acyl-enzyme is then hydrolyzed by a second water molecule to liberate the carboxylic acid and regenerate the enzyme for further catalysis (figure 5). While the crystal structure of nitrilases have not yet been solved [60], structural and mechanistic information of the nitrilase branch of the superfamily have been proposed based on other

members of the superfamily particularly N-carbamoyl-D-amino-acid amidohydrolase (D-NCAase), whose crystal structure has been elucidated [61]. Evidence that an active site cysteine is involved in catalysis was reported by Layh *et al.,* 1992. In *Pseudomonas fluorescens* DSM 7155, thiol-complexing reagents inhibit the activity of nitrilase while enhanced activity was observed with thiol reducing agent agents in *Alcaligenes faecalis* JM3 and *Rhodococcus rhodochrous* J1[62].

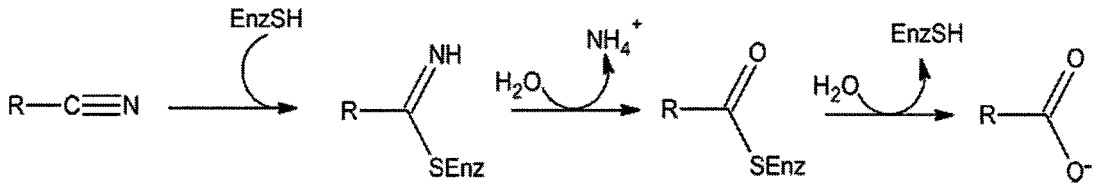

Figure 4. A scheme of nitrile hydrolysis by nitrilase [63].

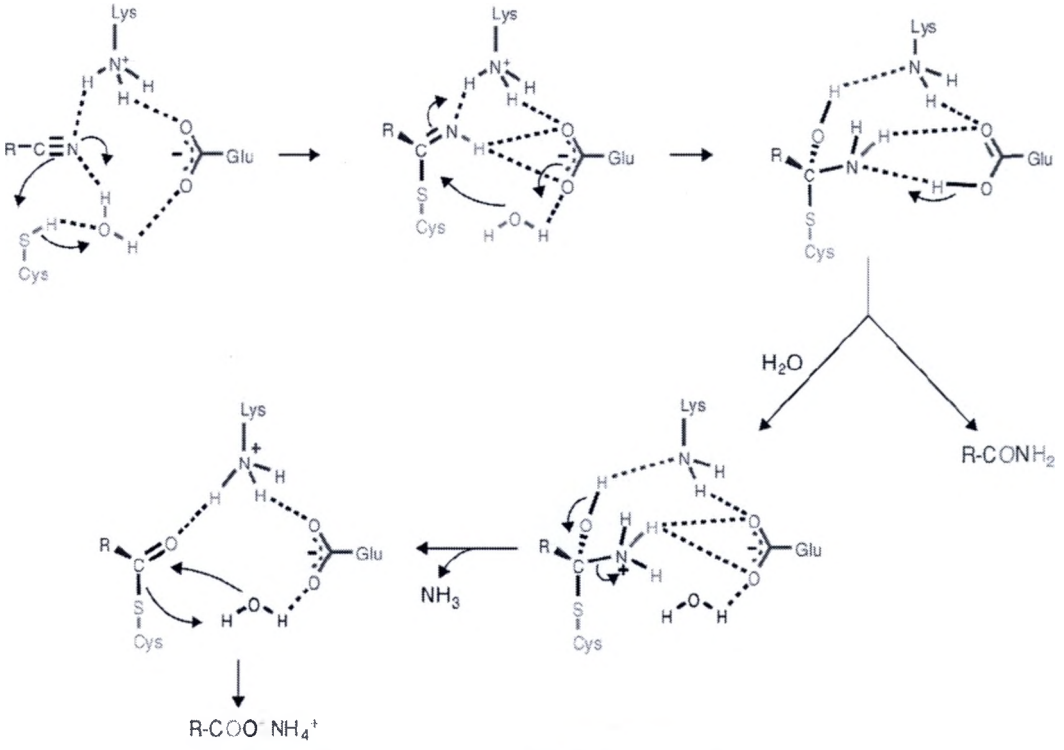

Figure 5. Proposed reaction mechanism for nitrilase [64**].** The sulfhydryl group of cysteine nucleophilic attacks the partially positive cyano carbon of the nitrile to form a covalent enzyme-thioimidate intermediate. The resulting imine is hydrolyzed to a tetrahedral intermediate with release of ammonia as by-product.

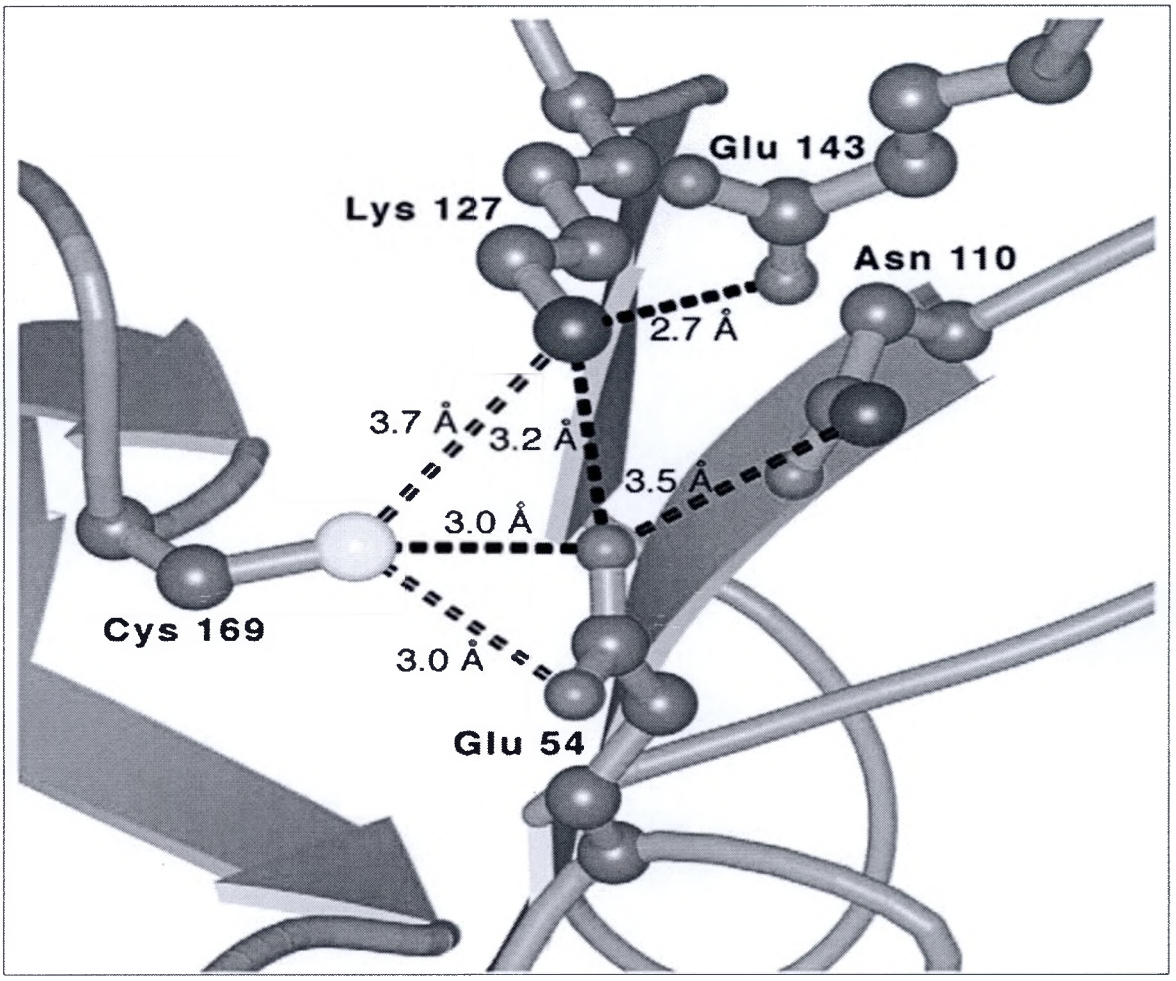

Figure 6. Putative active site of nitrilase. The catalytic triad of Glu54, Lys127 and Cys169 is conserved in the nitrilase superfamily [65].

Alcohol dehydrogenases (ADH) (EC 1.1.1.1) catalyze the reversible oxidation of alcohols to aldehydes using NAD $(P)^+$ as an electron acceptor. This class of enzyme utilizes a wide variety of substrates including primary alcohols, secondary alcohols, aldehydes, ketones and hemicetals [66, 67] and is responsible for the metabolism and detoxification of ethanol and other alcohols. ADHs occur widely in nature with ADH

from *Saccharomyces cerevisiae* showing the highest activity towards ethanol of all the ADHs characterized and reported in the literature [68]. Substrate specificity studies on yeast and equine liver ADH, two of the most studied isoforms of ADH, shows that yeast ADH has a more narrow specificity than the equine ADH. It is most active towards ethanol with its activity decreasing with increasing size of the alcohol while the equine enzyme prefers long chain alcohol but not short chain alcohols [69]. The inversion of substrate specificity of both enzymes is attributed to the smaller size of the substrate binding pocket of the yeast ADH which excludes bulky/long chain alcohols from the active site. Enlarging the binding pocket of the yeast enzyme via site-directed mutagenesis enhances activity towards long chain alcohols [70]. Structural, kinetic and mechanistic studies show that equine liver ADH reaction is a compulsory ordered reaction. The cofactor, NAD^+ binds first, followed by the alcohol substrate, and then the release of the product and finally the dissociation of the reduced cofactor from the enzyme [71]. The active site of ADH consists of zinc atom coordinated by residues Cys-46, Cys-174, and His-67 (not shown in figure 7) as well as the cofactor NAD^+ and Ser-48. In the molecular mechanism (figure 7), NAD^+ bind first followed by the alcohol which is coordinated to the active site zinc. The deprotonation of the alcohol leads to the formation of zinc-bound alkoxide. Hydride transfer from the alkoxide to NAD^+ leads the formation of NADH and zinc bound product (aldehyde). The product is then released followed by the dissociation of NADH from the active site [72].

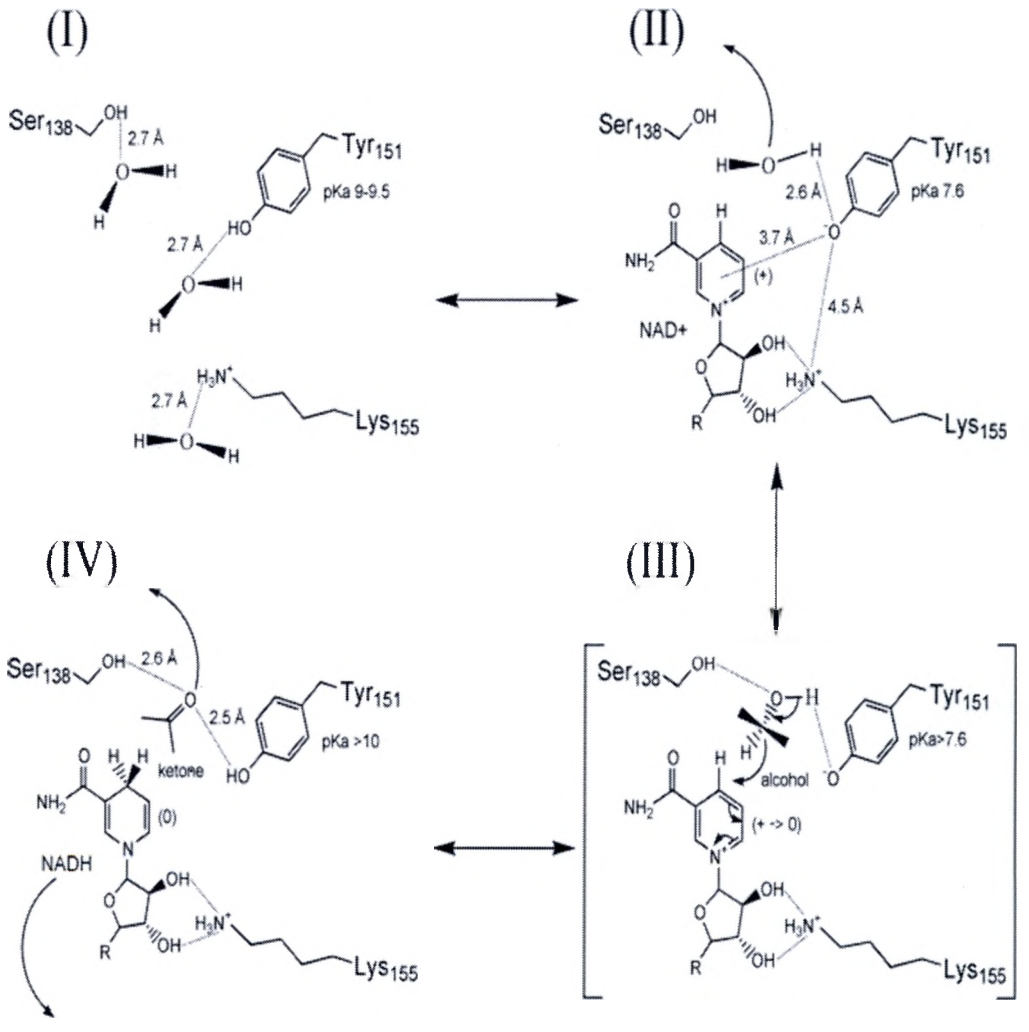

Figure 7. Proposed reaction mechanism of alcohol dehydrogenase [73].

Alcohol oxidase (AOX) (EC 1.1.3.13) catalyzes the oxidation of short chain, primary, aliphatic alcohols to hydrogen peroxide and the corresponding aldehyde using molecular oxygen (O_2) as the electron acceptor (figure 8). The AOX reaction is irreversible due to the strong oxidizing character of O_2 [73]. AOX has the highest affinity for methanol than any other alcohol with affinity decreasing with increasing size of the alcohol substrate [73]. Like alcohol dehydrogenase, alcohol oxidase is involved in the oxidation of methanol. In contrast, the reaction mechanism, the products and the choice of cofactor used by both enzymes are different.

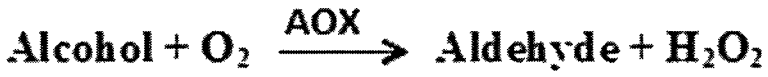

Figure 8. Reaction catalyzed by alcohol oxidase.

The objective of this thesis is to test proprietary nitrile and alcohol compounds from Huntsman LLC, The Woodlands, TX for enzyme activity and optimize conditions for high product output.

CHAPTER II

MATERIALS

Nitrile and alcohol substrates were provided by Huntsman LLC, The Woodlands, TX. All commercially available enzymes: nitrilase, alcohol dehydrogenase, aldehyde dehydrogenase, alcohol oxidase, horseradish peroxidase and glutamate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored under conditions specified by the manufacturer. Reagents used in the Berthelot reaction (phenol-hypochlorite method) such as phenol, sodium nitroprusside, sodium hypochlorite and sodium hydroxide were also obtained from Sigma-Aldrich. NADH and NAD^+ were procured from AMERSCO LLC (Solon, OH, USA). All chemicals/reagents unless otherwise stated were of analytical or molecular biology grade.

The nitrilase reactions were carried out in 1.5 ml Eppendorf tubes and incubated on Thermomixer R from Eppendorf (Germany). All other enzyme reactions were performed in microtiter plates. Spectrophotometric analyses (end-point) were done on DU 730 series, UV/ VIS spectrophotometer from Beckman Coulter, Inc. (Brea, CA, USA). The oxidation of alcohols using NAD^+ as the oxidant was monitored on SpectraMax® 190 Absorbance Plate Reader from Molecular Devices, LLC (Sunnyvale, CA, USA). Data from the standard curve,

catalysis in organic solvent and time dependent hydrolysis curves were analyzed with GraphPad prism 5 from GraphPad Software, Inc. (La Jolla, CA).

CHAPTER III

METHODS

Standard Curve and Berthelot Reaction

The nitrilase reaction was monitored by following the release of ammonia using the Berthelot reaction [74]. The standard was prepared using ammonium chloride. Different concentrations of ammonium chloride solution (0-100 μM) were added to 200 μL of reagent A (100 mM phenol and 190 μM sodium nitroprusside), followed by the addition of 200 μL reagent B (125 mM sodium hydroxide and 13 mM sodium hypochlorite). The absorbance of the resulting blue colored solution was measured at 640 nm after heating the solution to 95 °C for 5 minutes on Thermomixer R (Eppendorf, Germany). A calibration curve, a plot of absorbance versus concentration, was constructed with GraphPad prism 5 from GraphPad Software, Inc. (La Jolla, CA). Reagents A and B were freshly prepared and stored at 4 °C for up to a month away from light. Reactions were performed in duplicate.

Nitrilase Assay

The activity of the nitrilase was monitored spectrophotometrically by the Berthelot reaction. The standard assay was carried out by mixing the substrate, 3-methoxypropionitrile (2 mM) and 0.1 mg/ml enzyme in 80 mM potassium phosphate buffer (pH 7.5). The reaction (final volume of 500 µL) was incubated at 25 °C with shaking at 800 rpm on Thermomixer R. At 30 min time intervals, an aliquot of the enzyme reaction was taken and the reaction quenched by adding HCl (100 µL, 2 M). The amount of ammonia liberated in the reaction was quantified as follows; freshly prepared, 250 µL reagent A (100 mM phenol and 190 µM sodium nitroprusside) and 250 µL reagent B (125 mM sodium hydroxide and 13 mM sodium hypochlorite) were added to the stopped reactions, respectively. The absorbance at 640 nm was recorded after heating for 5 minutes at 90 °C. The concentration of ammonia formed was extrapolated from the standard curve. The control reaction (blank) was carried out in the absence of the substrate. One unit of enzyme activity is defined as the micromoles of ammonia produced per hour by 1 mg of the enzyme under conditions stated above.

Time Course Hydrolysis of Benzonitrile and Huntsman's Nitrile

The enzyme activity for benzonitrile and Huntsman's nitrile was determined as described for 3-methoxypropionitrile. Reaction conditions were the same as the standard enzyme assay (reaction temperature, final volume, Berthelot reagents and buffer) except that the hydrophobic benzonitrile and Huntsman's nitrile were dissolved in 100% ethanol.

The Huntsman's nitrile was diluted 10,000 fold while the final concentration of the benzonitrile in the assay was 20 mM. The benzonitrile reaction was separate from the Huntsman's nitrile reaction. Both reactions were performed in duplicate.

Thermal Stability of Nitrilase

The effect of temperature on the stability of the enzyme was studied by pre-incubating the enzyme at different temperatures (25 °C, 40 °C, 50 °C and 70 °C) for 30 minutes. Before the reaction was started, all 4 samples were maintained at the optimum temperature of the enzyme (25 °C) for 30 min. The total reaction volume of 500 μL comprised 3-methoxypropionitrile (2 mM), 0.1 mg/ml nitrilase and 80 mM potassium phosphate buffer (pH (7.5). At fixed time intervals, aliquots of the reaction were stopped with HCl (100 μL, 2 M). The amount of ammonia produced at the various time intervals was determined by the Berthelot reaction as described for the standard assay. The Berthelot reagents were freshly prepared. The 25 °C reaction is the control reaction and the blank reaction lacked the substrate.

pH Stability of Nitrilase

The stability of nitrilase was determined in 100 mM potassium phosphate buffers at different pH values (4, 6, 7.5 and 8) using 3-methoxypropionitrile as substrate. The pH of the buffers was adjusted with 1M HCl or potassium pyrophosphate. The enzyme was prepared in the buffers of different pH values to a concentration of 1 mg/ml. After pre-incubating the enzymes in their respective buffers for an hour at the optimum temperature

of 25 °C, a reaction was started by mixing 0.1 mg/ml enzyme solution with 2 mM substrate in 80 mM potassium phosphate buffer pH 7.5, final volume 500 µL. At specific time intervals, the reaction was stopped and the amount of ammonia produced in each reaction was quantified by the Berthelot reaction.

Enzyme Catalysis in Organic Solvents

To assess the tolerance of the enzyme for organic solvents, the hydrolysis of 3-methoxypropionitrile was carried out in different concentrations of methanol and ethanol (0-80% v/v). The 5% reaction mixture contained 0.1 mg/ml nitrilase, 75 mM potassium phosphate buffer, pH 7.5 and 25 µL of absolute methanol or ethanol. This mixture was incubated at 25 °C for 10 minutes and the reaction initiated by adding the substrate 3-methoxypropionitrile (2 mM). At specific time intervals, the reaction was stopped and the amount of ammonia produced was quantified by the Berthelot reaction and the standard curve in the same manner as the standard nitrilase assay.

Coupled Enzyme Reaction

To shift the equilibrium of the nitrilase reaction towards the products, the nitrilase reaction was coupled with the reduction of α-ketoglutarate to glutamate using NADH as the reducing agent. The nitrilase was assayed by monitoring the disappearance of NADH spectrophotometrically and kinetically at 340 nm. The enzyme coupled reaction, final volume of 300 µL, contained 4 mM NADH, 33 mM α-ketoglutarate, 50 µL Huntsman's nitrile (diluted 1000 fold in absolute ethanol), 0.17 mg/ml nitrilase, 0.17 mg/ml glutamate

dehydrogenase and 16 mM potassium phosphate buffer (pH 7.5). The reaction was carried out in a microplate and monitored on SpectraMax 190 spectrophotometer using a blank that has the same constituents as the reaction but for the substrate (Huntsman's nitrile).

Alcohol Oxidase Assay

The standard alcohol oxidase assay was determined by monitoring the oxidation of 2, 2'-Azino-bis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) or ABTS spectrophotometrically at 405 nm at 25 °C. Immediately before the assay, molecular oxygen was bubbled through the 2 mM ABTS solution from an oxygen tank for 10 minutes. Molecular oxygen serves as an oxidant. A 300 µL reaction was started by mixing 1 mM ABTS, 16 mM potassium phosphate buffer (pH 7.5), 50 µL methanol (1% v/v), 0.02 units/ml alcohol oxidase and 41 units/ml horseradish peroxidase. Both enzymes were prepared in 100 mM potassium phosphate buffer (pH 7.5).The blank reaction lacks methanol (substrate).

Oxidation of Alcohols

Under the same conditions as the standard alcohol oxidase assay, the enzyme was used to oxidize the following alcohol substrates, A1, A2, A3 and methanol as standard. The final concentration of the substrates in a 300 µL reaction was 167 mM each.

CHAPTER IV

RESULTS AND DISCUSSION

The research in this thesis describes the testing of various long chain proprietary nitrile and alcohol compounds for enzyme activity and the conditions for enzyme stability. The nitrilase reaction produces equimolar amounts of carboxylic acid and ammonia. The enzyme assay is therefore based on the analysis of either carboxylic acid or ammonia. Techniques such HPLC, mass spectrometry, gas chromatography and NMR have been used to assay nitrilase from *Pseudomonas putida, Rhodococcus rhodochrous, Bacillus subtilis* and *Pseudomonas fluorescens* [75]. These carboxylic acid based assays are more rigorous and require further sample preparation before analysis. Ammonia based assays, like Berthelot reaction [76], Nessler reaction and $CoCl_2$ assay [77], however, are direct, simpler and much easier to interpret. All nitrilase assays in this thesis were based on the Berthelot reaction [78]. This reaction, widely used in the detection of ammonia and paracetamol by spectrometry [79], is based on the formation of blue-colored indophenol from ammonia, phenol, hypochlorite and catalytic amounts of sodium nitroprusside (figure 9) [80]. Though the detailed mechanism of this reaction is unknown, the reaction is known to occur in two steps; the first step is the oxidation of ammonia to monochloroamine by sodium hypochlorite and the second step is the reaction of phenol

with the chloroamine to form indophenol [81]. A reproducible, linear standard curve was obtained from this reaction (figure 10).

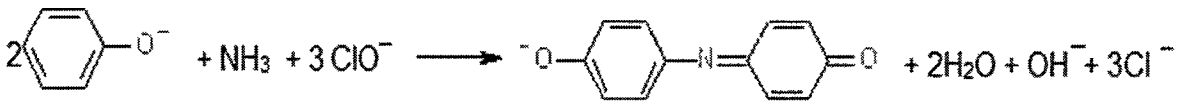

Figure 9. The Berthelot reaction.

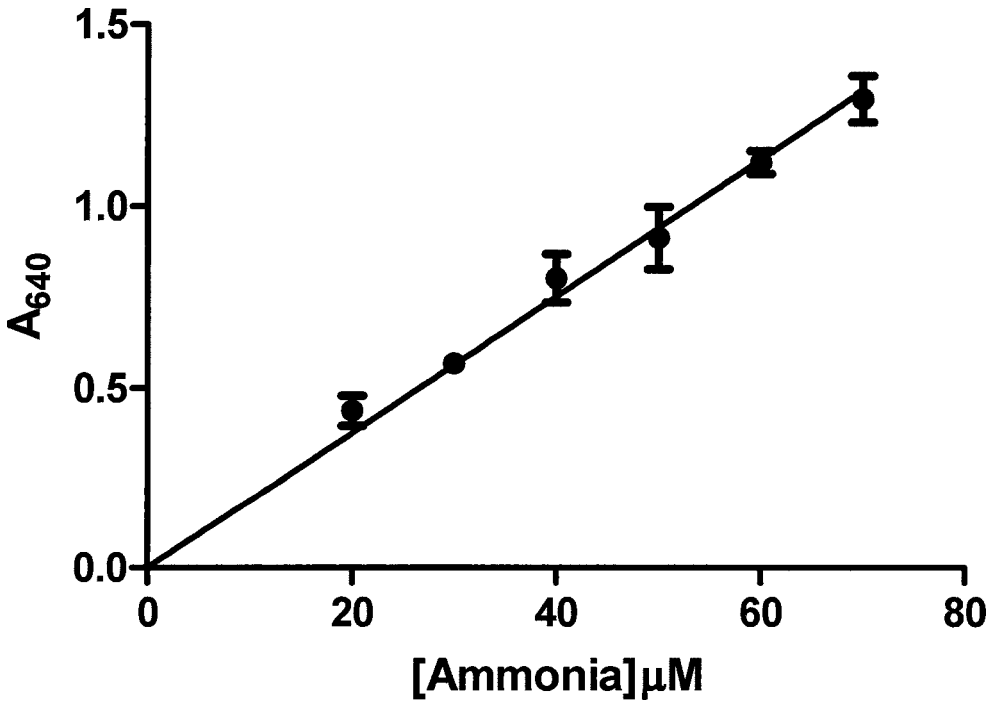

Figure 10. Calibration curve for ammonia. Different concentrations of ammonium chloride prepared in 100 mM Potassium phosphate buffer pH 7.5 were monitored spectrophotometrically using the Berthelot reaction.

The ability of the nitrilase to catalyze the hydrolysis of different nitriles especially Huntsman's nitrile; one of the main goals of this project, was examined (figures 11, 12, 13). The amounts of products formed when the enzyme-catalyzed hydrolysis reaction reached equilibrium were 300 μM benzoic acid, 250 μM 3-methoxypropanoic acid and 150 μM Huntsman's carboxylic acid. Though 3-methoxypropionitrile and Huntsman's nitrile are structurally similar aliphatic nitriles, the observed activity for Huntsman's nitrile was lower. This is very likely due to the longer alkyl-, hydrophobic group on Huntsman's nitrile. Of the three substrates tested, the enzyme had the highest preference for benzonitrile, suggesting that the enzyme is an aromatic nitrilase.

To drive the equilibrium of the nitrilase reaction towards the products based on Le Chatelier's principle, the reaction was coupled with the glutamate dehydrogenase reaction (figure 14). Glutamate dehydrogenase catalyzes the reduction of α-ketoglutarate to glutamate and NAD^+ using NADH. When coupled with the nitrilase reaction, the glutamate dehydrogenase draws ammonia from the reaction, decreasing the concentrations of both ammonia and NADH. The coupled assay therefore monitors the decrease in NADH concentration at 340 nm. The activity of the nitrilase for the Hunstman's nitrile was found to be 50 μM per hour. In comparison to the Berthelot assay (fixed time assay, the results did not only show that 50 μM more of the product is formed in the coupled assay (continuous assay) but also the reaction reached equilibrium much faster.

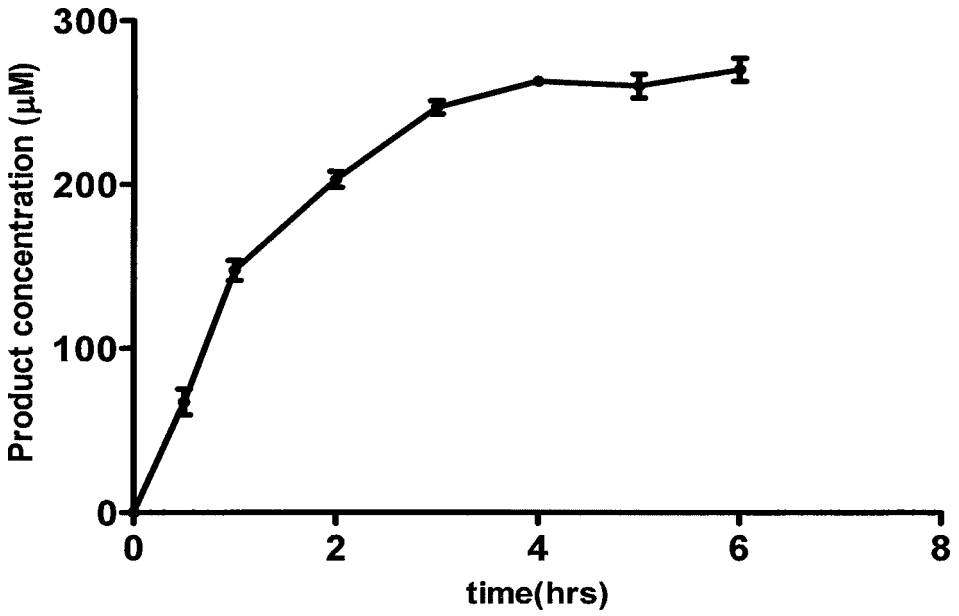

Figure 11. Time course hydrolysis of 3-methoxypropionitrile (standard). The reaction was carried out at 25 °C in a 100 mM potassium phosphate buffer (pH 7.5). The total reaction volume of 500 µL contained 3-methoxypropionitrile (2 mM), 0.1 mg/ml nitrilase and 80 mM potassium phosphate buffer (pH 7.5). Product formation was monitored spectrophotometrically at 640 nm as described above.

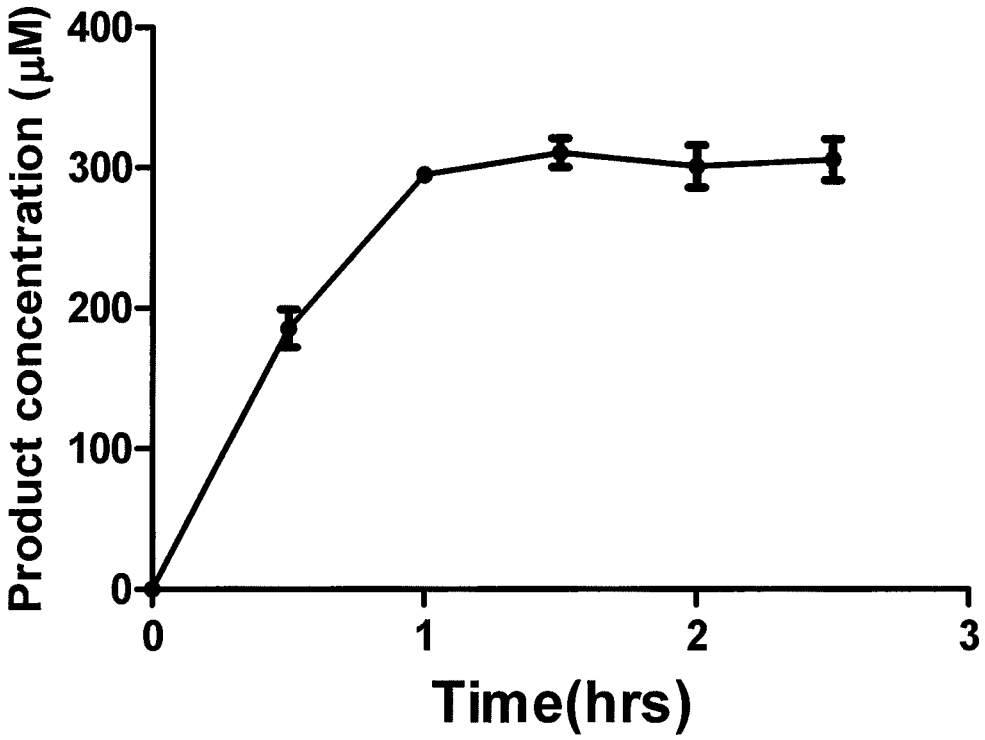

Figure 12. Time course hydrolysis of benzonitrile. The hydrolysis reaction is essentially the same as the standard nitrilase reaction except that the hydrophobic benzonitrile was dissolved in 100% methanol to a final concentration of benzonitrile 20 mM.

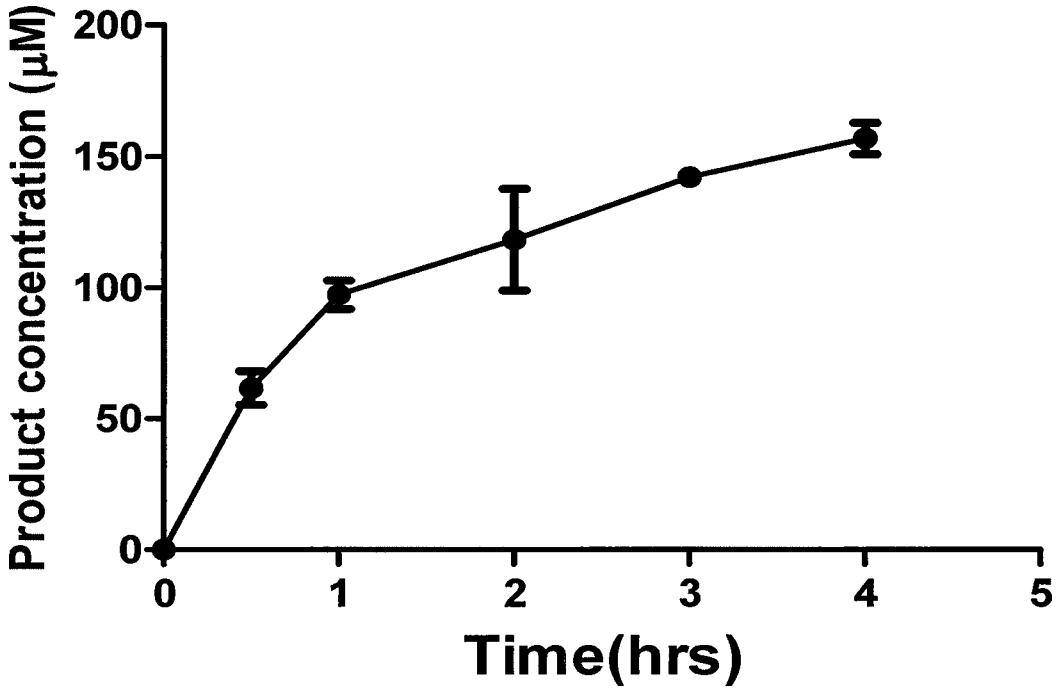

Figure 13. Time course hydrolysis of Huntsman's nitrile. Enzyme reaction was performed at 35 °C, final volume of 500 µL, in a 100 mM potassium phosphate buffer pH (7.5). The reaction contained 50 µL nitrile, 0.1 mg/ml enzyme and 80 mM buffer. Huntsman's nitrile was diluted a 1000 fold in 100% ethanol.

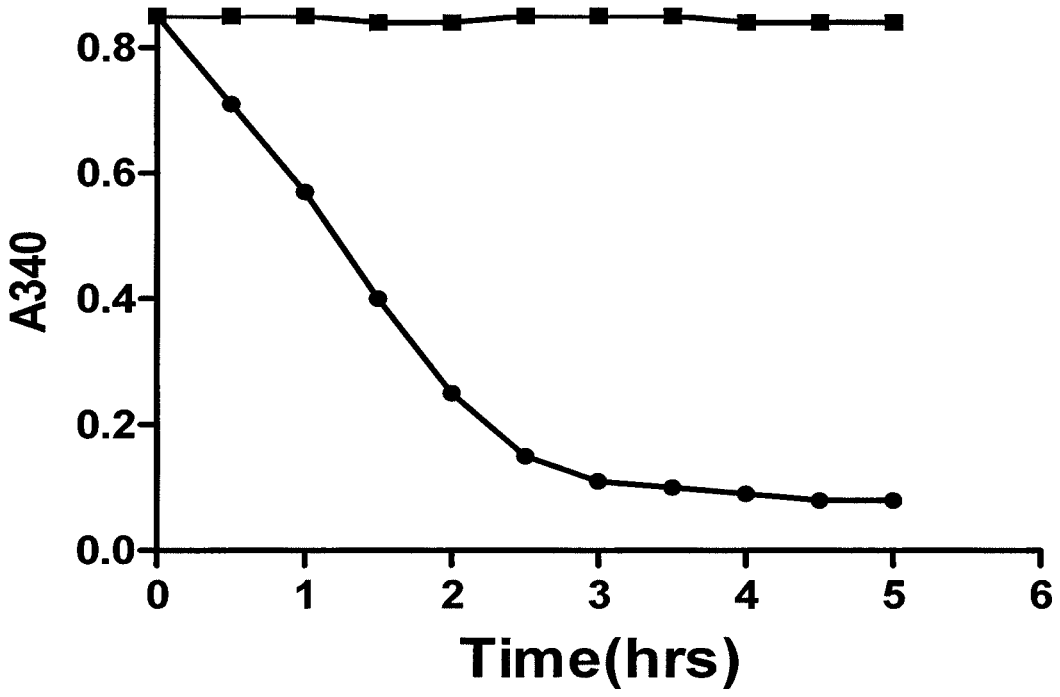

Figure 14. Hydrolysis of Huntsman's nitrile coupled with glutamate dehydrogenase reaction. The decrease in NADH concentration was monitored at 340 nm. The control reaction (solid squares) lacked the nitrile substrate. The enzyme reaction (solid circle) contained NADH (4 mM), α-ketoglutarate (33 mM), nitrile (diluted 10,000 fold), nitrilase (0.17 mg/ml), glutamate dehydrogenase (0.17 mg/ml) and buffer (16 mM).

The water soluble Huntsman's alcohols (L1 and L2) did not react with both alcohol oxidase and alcohol dehydrogenase (data not shown). This is likely due to the size of the alcohol. Both L1 and L2 are aliphatic and contain more than 10 alkyl groups. It was demonstrated however, that the standard (ethanol) was oxidized sequentially from ethanol to ethanal and from ethanal to ethanoic acid using alcohol dehydrogenase and aldehyde dehydrogenase, respectively. The reduction of 2 equivalent amounts of NAD^+ is evidence that the reaction produced ethanoic acid. Since both L1 and L2 have surfactant properties, it was initially theorized that enzyme denaturation might have been responsible for the lack of reactivity of L1 and L2. This was, however, debunked since spiking the alcohol dehydrogenase reaction with higher concentrations of L1 and L2 (twice as high as those used in their respective assays) did not affect the activity of the enzyme. Structurally similar to L1 and L2 but of smaller size (short chain aliphatic alcohols or diols), the Huntsman's alcohols A1, A2 and A3 did react with alcohol oxidase. The enzyme showed the highest activity for A1, followed by A2 and then A3 (figure 15).

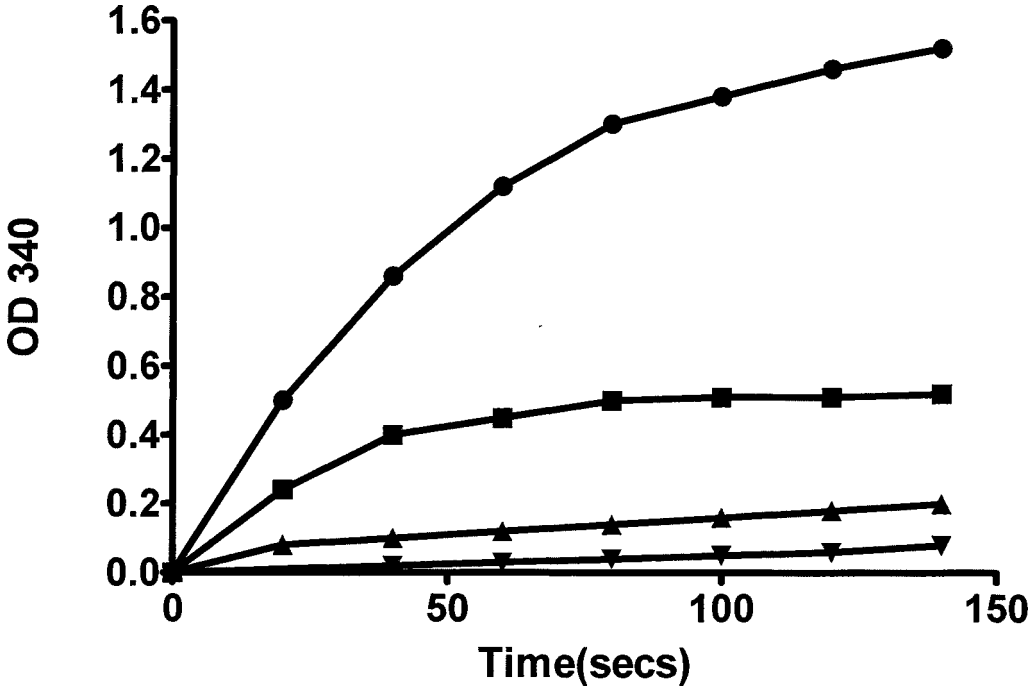

Figure 15. Oxidation of Huntsman's alcohols using alcohol oxidase from *Pichia pastoris*. The alcohol oxidase assay was determined by monitoring the oxidation of 2, 2'-Azino-bis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) or ABTS as described above. The final concentrations of alcohol substrates in the reaction were ● 0.017% methanol ■ 167 mM A1 ▲ 167 mM A2 ▼ 167 mM A3

Most nitrilases reported in the literature exhibit low stability and become quickly inactivated under extreme conditions. The stability of nitrilase from *Pichia pastoris* was therefore assessed varying 3 different conditions: pH, temperature and organic solvents. The pH stability was determined at various pH values ranging from 4 to 9 at the optimum temperature 25 °C. The enzyme showed the highest stability at pH 7.5, producing 150 μM of products in 2.5 hours (Figure 16). As the pH changes above and below the 7.5, stability decreases with no activity observed outside the pH range 6-9. At pH 6, the enzyme retained 50% activity after 2.5 hours. The nitrilase used in this work has similar pH stability profile and range (6-9) to aromatic nitrilase from *Bacillus pallidus Dac521* [82] and arylacetonitrilase from *P. putida* [83].

The thermal stability of the enzyme was examined by incubating the enzyme at various temperatures (25 °C, 40 °C, 50 °C and 70 °C) for 30 minutes at pH 7.5. Temperature changes between 25 °C - 40 °C appear to have no significant effect on the stability of the enzyme (figure 17). At these temperatures, the enzyme is most stable, producing 140 μM of product in 2 hours. Like most nitrilases, enzyme denaturation occurred at temperatures above 70 °C with little or no activity observed. The thermophilic nitrilase from *P. abyssi*, an exception, is stable at 100 °C [84]. While temperature is a crucial factor that influences the rate of any chemical reaction owing to the fact that activation energies of reactants are increased with increasing temperature (activation energy theory) and/ or an increase in effective collisions (collision theory), extreme temperatures lead to protein denaturation and thus loss of enzyme activity.

The tolerance of the enzyme for organic solvents was investigated by incorporating different concentrations of methanol and ethanol into the enzyme reaction (figure 18 and 19). It was observed that at low concentrations of ethanol (10% v/v), the activity of the enzyme increased from 100% (no ethanol) to 120%. Even at 20% and 40% ethanol, the enzyme retained 100% and 75% of initial activity, respectively. No activity was observed for both methanol and ethanol at concentrations greater than 80% (v/v) due to the denaturation of the enzyme. Hydrophobic interactions, one of the stabilizing forces of the tertiary structure of proteins, are likely the most affected at high organic solvent concentrations. Despite the similar solvent stability profiles of the nitrilase in both solvents, the data shows that the enzyme is more stable in ethanol than methanol especially at 20% (v/v). In 2003, Heinemann et al. reported that nitrilase from *Synechocystis sp.* showed increased activity for hydrophobic aliphatic nitriles with the inclusion of 10-20% (v/v) methanol in the reaction [85]. More than half of the initial activity was retained in the presence of 50% n-hexane and 50% hexadecane in *F. solani* O1and *Pseudomonas sp.*DSM 11387 [86]. Nitriles, especially those with long chain R groups, exhibit poor solubility in aqueous environments thus incorporation of organic solvents at low concentrations increases the solubility of these substrate (bioavailability) hence the enhanced activity.

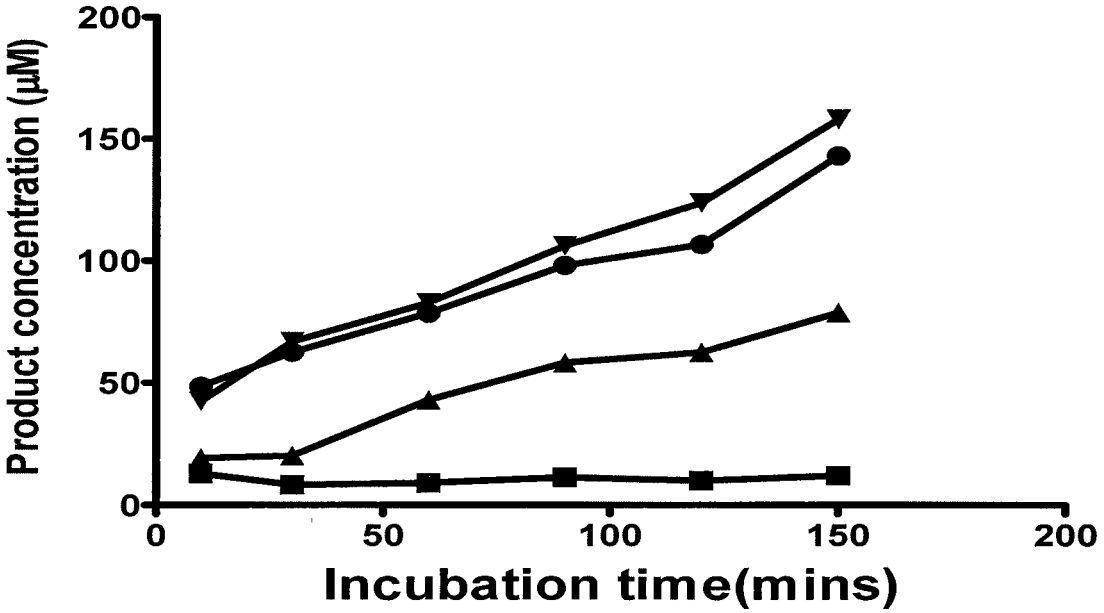

Figure 16. pH stability profile of nitrilase. The stability of the enzyme was assessed by pre incubating the nitrilase in potassium phosphate buffer (100 mM) at different pH values (■ 4, ▲ 6, ●7.5 and ▼8). After 30 minutes of incubation, the enzyme was assayed under the same conditions as the standard nitrilase assay.

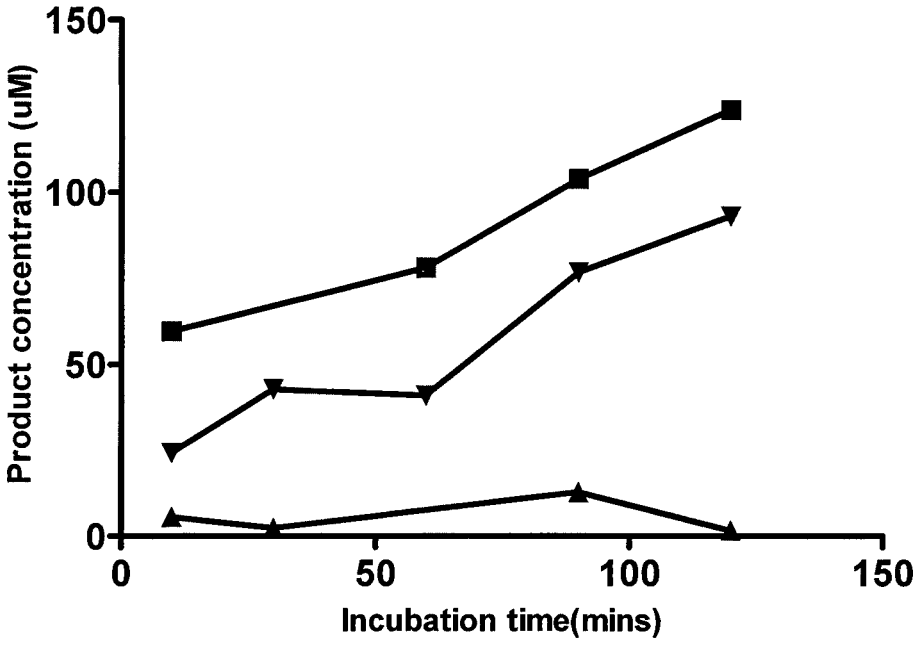

Figure 17. Thermal Stability of Nitrilase. The enzyme was pre incubated at different temperatures, ■ 25 °C, 40 °C (data not shown), ▼50 °C and ▲ 70 °C) for 30 minutes. A reaction was set up and the amount of ammonia liberated was determined as described for the nitrilase assay.

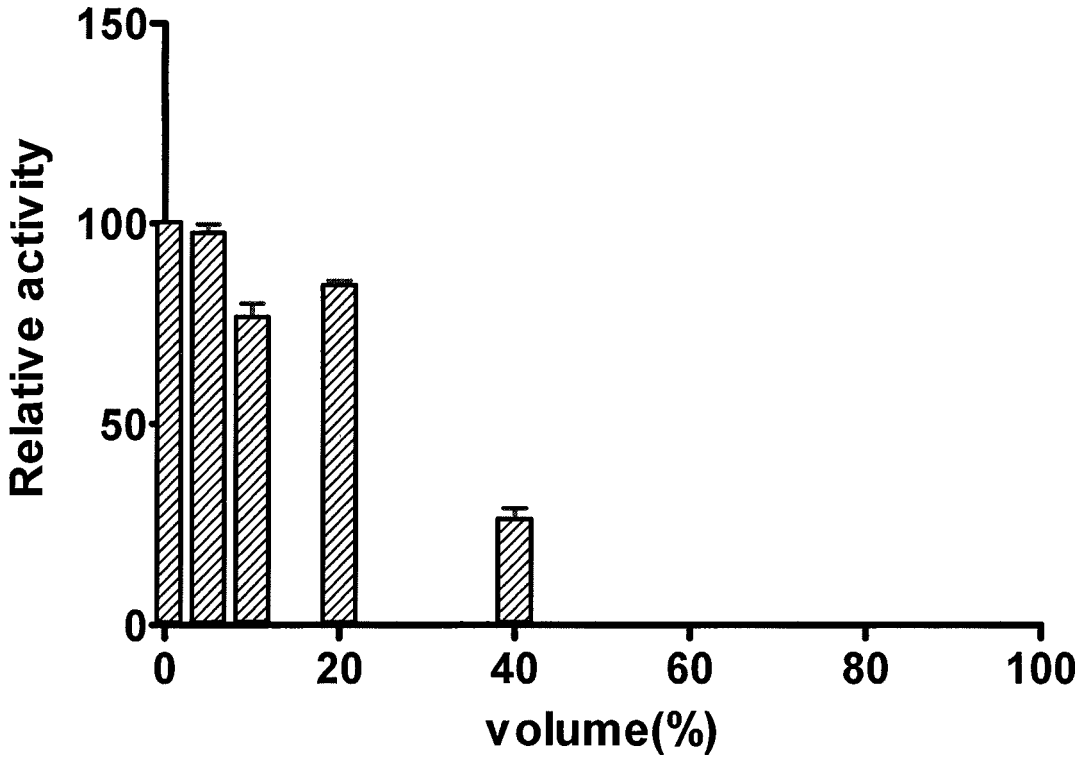

Figure 18. Hydrolysis of 3-methoxypropionitrile by nitrilase in the presence of different concentrations of methanol. The 5% (v/v) reaction mixture contained 0.1 mg/ml nitrilase, 75 mM potassium phosphate buffer (pH 7.5) and 25 μL of 100% methanol. The mixture was incubated at 25 °C for 10 minutes and the reaction initiated by adding the substrate 3-methoxypropionitrile (2 mM). After 2 hours, the reaction was quenched and the amount of ammonia produced was quantified by the Berthelot reaction.

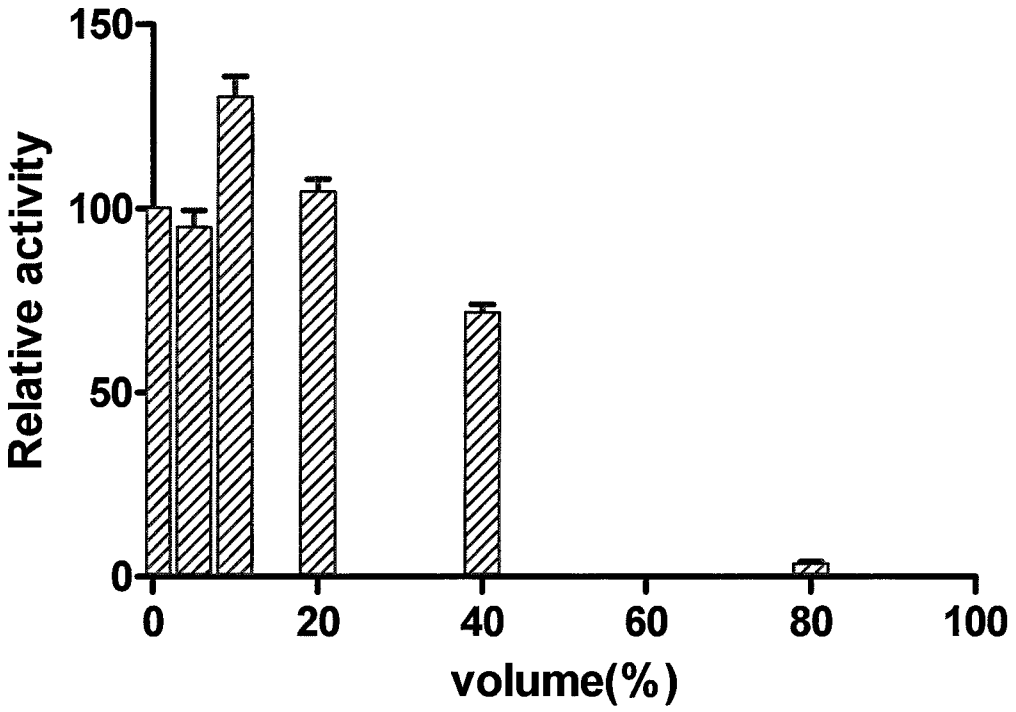

Figure 19. Hydrolysis of 3-methoxypropionitrile by nitrilase in the presence of different concentrations of ethanol. The 5% (v/v) reaction mixture contained 0.1 mg/ml nitrilase, 75 mM potassium phosphate buffer (pH 7.5) and 25 μL of 100% ethanol. The mixture was incubated at 25 °C for 10 minutes and the reaction initiated by adding the substrate 3-methoxypropionitrile (2 mM). After 2 hours, the reaction was quenched and the amount of ammonia produced was quantified by the Berthelot reaction.

Conclusions and future experiment

Currently, nitrilase research efforts are focused on discovering novel nitrilases with enhanced biocatalytic properties and /or improving the enzymatic properties such as activity, catalysis in non-polar environments (nitrile compound are poorly soluble in water) and stability of the about 300 known nitrilases [87]. The immobilization of nitrilases by entrapment, cross-linking or adsorption, has been reported to enhance the properties of the enzyme.

In summary, the mesophillic nitrilase used in this work is more stable in low concentrations of ethanol than methanol at neutral to slightly alkaline pH environments. This enzyme appears to be more specific for aromatic nitriles than aliphatic nitriles. At the expense of low activity, the enzyme is ideal for the biocatalysis of long chain Huntsman's nitrile. Alcohol dehydrogenase from yeast and equine as well as alcohol oxidase from *Pichia pastoris* did not react with Huntsman's alcohols L1 and L2. However, the alcohol oxidase converted the Huntsman's alcohols or hydroxyl containing compounds: A1, A2 and A3 to the corresponding aldehyde, showing increasing activity for A3, A2 and A1, respectively.

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VITA

Gustav Agbley was born in Ghana, West Africa on December 10, 1983 to Guide Pomeyie and Lawrence Agbley. Having completed his secondary school education at Achimota School, Accra, Ghana in 2001, he entered the University of Ghana, Legon in 2002 to pursue a Bachelor of Science degree. In August 2006, he was awarded B.Sc. (hons) Biochemistry. After serving a mandatory one year term as National Service Personnel in his country and a semester into his graduate studies in Forensic science at the University of Amsterdam, Netherlands, he moved to the United States where he entered the Graduate College of Texas State University- San Marcos in January 2010.

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