1-12-2007

Enhanced Stabilization of Nitrile Hydratase Enzyme From Rhodococcus Sp. DAP 96253 and Rhodococcus

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ENHANCED STABILIZATION OF NITRILE HYDRATASE ENZYME FROM *RHODOCOCCUS SP.* DAP 96253 AND *RHODOCOCCUS RHODOCHROUS* DAP 96622

by

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Under the Direction of George E. Pierce

ABSTRACT:

Treatment of industrial wastewaters contaminated with toxic and hazardous organics can be a costly process. In the case of acrylonitrile production, due to highly volatile and toxic nature of the contaminant organics, production wastewaters are currently disposed by deepwell injection without treatment. Under the terms granting deepwell injection of the waste, alternative treatments must be investigated, and an effective treatment identified. Cells of two Gram-positive bacteria, *Rhodococcus sp*. DAP 96253 and *R. rhodochrous* DAP 96622 were evaluated for their potential as biocatalysts for detoxification of acrylonitrile production wastewaters. *Rhodococcus sp*. DAP 96253 and *R. rhodochrous* DAP 96622 when multiply induced, are capable of utilizing the hazardous nitrile and amide components present in the wastewater as sole carbon and/or nitrogen sources, employing a 2-step enzymatic system involving nitrile hydratase (NHase) and amidase enzymes. There is a significant potential for overproduction of NHase upon multiple induction. However, high-level multiple induction required the presence of highly toxic nitriles and/or amides in the growth

medium. Asparagine and glutamine were identified as potent inducers with overexpression at 40% of total soluble cellular protein as NHase.

In native form (either cell free enzymes or whole cells) the desired NHase is very labile. In order to develop a practical catalyst to detoxify acrylonitrile production wastewaters, it is necessary to significantly improve and enhance the stability of NHase. Stabilization of desired NHase activity was achieved over a broad range of thermal and pH conditions using simultaneous immobilization and chemical stabilization. Previously where 100% of NHase activity was lost in 24 hours in the non-stabilized cells, retention of 20% of initial activity was retained over 260 days when maintained at 50-55 C, and for over 570 days for selected catalyst formulations maintained at proposed temperature of the biodetoxification process.

In addition, NHase and amidase enzymes from *Rhodococcus sp*. DAP 96253 were purified. Cell free NHase was characterized for its substrate range and effect of common enzyme inhibitors and was compared to available information for NHase from other organisms.

As a result of this research a practical alternative to the deepwell injection of acrylonitrile production wastewaters is closer to reality.

INDEX WORDS: *Rhodococcus*, Nitrile hydratase, Amidase, Acrylonitrile, Wastewater treatment, Biocatalyst

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2005

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Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
December 2005

ACKNOWLEDGEMENTS

First, I wish to acknowledge my advisor, Dr. George E. Pierce, for the guidance leading to the completion of this dissertation. Thank you for inspiring me, motivating me and supporting me through this endeavor. I would also like to thank Dr. Sidney A. Crow, for all the help and assistance given to me over the entire duration of my curriculum. You were the only person who I knew the name of, as I left my home to join the program at Georgia State University, thank you for being there always. I extend my gratitude to Dr. Eric S. Gilbert, for agreeing to be my committee member and for all your valuable help throughout the years. Very special thanks to Trudy Tucker, Jennifer Hooker, Jie Zhang, Samandra Demons, Rollin Dennard, Gene Drago, Emad Hussein and Anthony Jones for being my friends and putting up with me. Finally, I would like to thank my mum, dad and sister for believing me and always supporting me. Dad I know how hard it was for you to let me go when I left home, I appreciate and love you for that. Mum, you have always inspired me and have been my role model; thank you for making me believe that I can.

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LIST OF ABBREVIATIONS

AN……..	Acrylonitrile
AC……..	Acetonitrile
SN……...	Succinonitrile
AMD……...	Acrylamide
Asn…….....	Asparagine
Gln…….	Glutamine
NHase…….....	Nitrile hydratase
H-NHase…….....	High molecular weight nitrile hydratase
L-NHase…….....	Low molecular weight nitrile hydratase
Co-NHase……...	Cobalt containing nitrile hydratase
Fe-NHase…….....	Iron containing nitrile hydratase
AMDase……..	Amidase
ASNase……...	Asparaginase
NSB…….....	Net stripper bottoms
WWCB…….....	Waste water column bottoms
Ca-alginate……..	Calcium alginate
PEI…….....	Polyethyleneimine
PAM…….....	Polyacrylamide
GA……..	Glutaraldehyde
MWCO…….....	Molecular weight cut off

cdw…….	Cell dry weight
cww…….	Packed cell wet weight
SEM ……...	Scanning electron microscopy
TEM ……...	Transmission electron microscopy
PAGE……..	Polyacrylamide gel electrophoresis
C.I……	Confidence Interval

INTRODUCTION

Background:

Nitriles, which are common in nature as plant, algal or fungal products, have a wide variety of uses in industrial chemistry and commerce (Cowan et al. 1998). They can be used in the synthesis of a wide variety of compounds, which include amines, amides, amidines, carboxylic acids, esters, aldehydes, ketones, imines, and heterocyclics. Nitriles are also used as herbicides, to synthesize detergents, and antiseptics. Examples of the more important commercial nitriles include acetonitrile (AC) and acrylonitrile (AN). Acetonitrile is most commonly used as a solvent, whereas acrylonitrile is used to make acrylamide (AMD), acrylic acid (AA), acrylic fibers, copolymer resins and nitrile rubbers (Pierce 1999).

AN became industrially important around 1930 first in Germany and then in the United States. In 1996, the world production capacity for AN was reported to be 4.3 x 10^6 tons per year. Of this, 1.4 x 10^6 tons per year was manufactured in the USA (Weissermel and Arpe 1997).

The current method employed globally for the production of AN is the Sohio/BP process. In the Sohio process, AN is produced by the direct ammoxidation of propylene by ammonia vapors in air (Weissermel and Arpe 1997). The catalyst involved in AN production used to be bismuth molybdate, but this has been supplanted by the uranyl antimonite catalyst. In this process, stoichiometric amounts of propylene and ammonia are reacted with a slightly higher amount of air and water, at 450 C and 1.5 bar. The heat

released in the process (160 kcal/mol) is used to generate superheated steam. The AN obtained in this process is of greater than 99% purity. However, for every 1000 kg AN produced, 30-40 kg AC and 140-180 kg of HCN also are produced. These byproducts are often burned as waste when the proper additional equipment and funds required for processing these byproducts are unavailable. The wastewater from the Sohio process contains a complex mixture of organic nitriles, amides and acids including AN and the byproducts. The major organic constituents in AN effluent wastewater are succinonitrile (SN), fumaronitrile, 3-cyanopyridine and AN, together with AMD, maleimide, acrolein, acrylic acid and acetic acid. Table 1 shows the range of concentration and major organic components of effluent streams from two typical AN manufacturing plants. Most of these compounds are neurotoxic, mutagenic, teratogenic, and/or suspect carcinogens (Léonard et al. 1999).

This wastewater effluent cannot be released into the environment due to its toxicity and high concentration of reduced organic materials. In some countries, including China and India, this wastewater is diluted and subjected to wet air oxidation. This "treatment" results in a very large wastewater volume, and the volatile toxic contaminants are removed from the liquid phase and are released into the atmosphere. In the US, under a treatability exemption, this wastewater is disposed of by deep well injection into subsurface formations without treatment (Pierce 1999).

As an alternative to disposal of untreated wastewater, and to save on high energy consumption and costs of traditional chemical treatment processes; biodetoxification of AN wastewater has been gaining importance over the past few years (Aitken 1993, Ebb

Table 1. Range of concentration of compounds present in typical wastewater samples [Pierce 1999[1], Wyatt and Knowles 1995[2]].

Name	Fortier Waste[1]		Monsanto Waste[2]	
	NSB* (ppm)	WWCB* (ppm)	Stream 1 (ppm)	Stream 2 (ppm)
Acrolein	10-100	50-1200	52	36
Acrylamide (AMD)	10-130	1100-1500	821	49
Proprionitrile (PN)	20	10-150	NR	NR
Acetonitrile (AC)	0-3000	20-4500	NR	NR
Acrylonitrile (AN)	5-180	10-1250	NR	NR
Succinonitrile (SN)	300-40,000	50-5000	231	2380
Fumaronitrile (FN)	100	20-1500	794	66

*NSB = Net Stripper Bottom, WWCB = Waste Water Column Bottom

NR= Not Reported

2004, Ramakrishna et al. 1989, Wyatt and Knowles 1995a, 1995b). Wyatt and Knowles (1995a, 1995b) and Wyatt and Linton (1988) proposed the concept of biodegradation for treatment of AN production wastewater. Their strategy was to analyze the wastewater and identify the major organic components, followed by isolation of microorganisms capable of or aiding in biodegrading/biotransforming each of the components. All these isolates were combined to prepare inocula for a continuous culture, which was operated initially on a mixture of the most easily biodegradable compounds. This was done to adopt the cultures to the toxic components and to obtain a stable mixed culture, which could then be used to degrade the actual waste in an activated sludge system. The mixed culture showed gradual detoxification of some of the components present in the wastewater. This proved that biodetoxification of AN production wastewater may be a practical option and since then the microbial metabolism of nitriles has been investigated in detail.

Microorganisms which can utilize a nitrile compound:

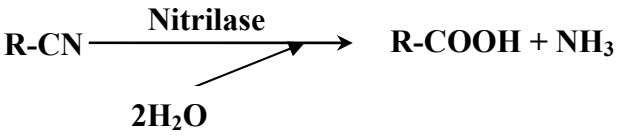
It is well known that in spite of the toxicity of nitrile compounds to humans, selected bacteria and fungi can utilize these chemicals as the sole source of carbon and/or nitrogen (Asano et al.1982a, 1982b; Harper 1977a, 1977b; Hook and Robinson 1964; Kobayashi et al. 1992; Yamada et al. 1979). Among the most studied nitrile utilizing bacteria are: *Acinetobacter* (Yamamoto et al. 1990), *Corynebacterium* (Martinkova et al. 1992), *Bacillus* (Cramp and Cowan 1999, Graham et al. 2000), *Arthrobacter* (Asano 1982a), *Pseudomonas* (Nawaz et al. 1989, 1993), *Klebsiella* (Nawaz et al. 1992), *Nocardia* (Harper 1985) and *Rhodococcus* (Watanabe 1987a, Wyatt and Knowles 1995a and b).

Biotransformation of nitriles takes place by one of two well established mechanisms: either nitriles are transformed to the corresponding carboxylic acid and ammonia by a one-step process involving the enzyme nitrilase; or they are converted via a two step process involving two enzymes. In the first step of the two-step process, nitrile hydratase (NHase) converts the nitrile to its corresponding amide and in the second step amidase (AMDase) converts the amide to its corresponding carboxylic acid and ammonia. Figure 1 shows the reaction mechanisms involved in nitrile metabolism. Aliphatic nitriles are usually catabolized via the two step process, while aromatic nitriles are transformed by nitrilase in the one step process by all organisms including the rhodococci (Kobayashi et al 1992, Nagasawa and Yamada 1990). Due to their ability to transform and utilize nitriles and amides, members of the genus *Rhodococcus* are promising candidates for catalyst formation for the purpose of treating the wastewater generated by the AN production industry.

Rhodococcus

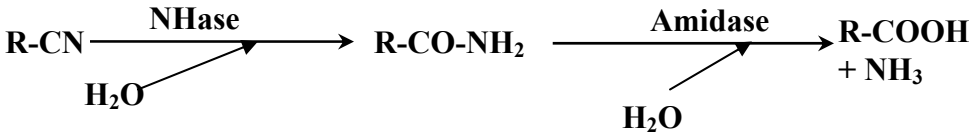
The rhodococci are Gram-positive, aerobic and nonmotile nocardioform actinomycetes. They are ubiquitous in nature and their growth cycle ranges from cocci to short rods to more complex growth phases. In complex growth phases, these organisms can form filaments with short projections, elementary branching, or, in some species, extremely branched hyphae (Goodfellow 1986).

The rhodococci possess a wide metabolic diversity and exhibit an array of novel enzymatic capabilities for the transformation and degradation of diverse classes of substrates (Finnerty 1992). Some of these transformations can be exploited for industrial and commercial use; and though rather limitedly, rhodococci are now being employed in



Here, R=generally phenyl or α,β-alkenyl group

Or,



Here, R=generally an alkyl group

Figure 1. Biotransformation pathways of nitriles
(Nagasawa and Yamada, 1990).

commercial applications (Wyatt and Linton 1998). Due to the robust and diverse metabolic nature of these organisms, a broad range of basic research is being carried out on rhodococci and related bacteria. The research is also being rapidly translated into practical applications.

The use of *Rhodococcus* in the production of AMD and acrylic acid from AN in Japan, Korea, China, and Germany etc. leads all other industrial applications of the rhodococci (Weissermel and Arpe 1997). These organisms also have been reported to be used in processes for steroid modifications, production of hydroxy and keto fatty acids, and decomposition of cholesterol in fat and oil.

In the present dissertation study, two strains of rhodococci, *Rhodococcus sp*. DAP 96253 and *R. rhodochrous* DAP 96622 were evaluated to develop a biological based system for the treatment of hazardous organics present in AN production wastewater. Both of the organisms evaluated, use NHase-AMDase to metabolize aliphatic nitriles. Under certain growth conditions these organisms also exhibited asparaginase (ASNase) activity on L-asparagine.

Nitrile Hydratase

NHase is a soluble metalloenzyme catalyzing the hydrolysis of aliphatic nitriles to their corresponding amides. To date, NHase has been extensively studied in two bacterial genera: *Pseudomonas* and *Rhodococcus* (Yamada et al. 2001, Leonova et al. 2000). NHase enzyme has been used commercially for the production of AMD (Yamada and Kobayashi 1996). The basic NHase enzyme is a heteromer of equal numbers of two subunits, α and β. At the catalytic center of the enzyme either a non-heme iron (Sugiura et al. 1988) or a non-corrinoid cobalt (Kobayashi and Shimizu 1998) ion is present. The

enzyme structure can be generalized as $\alpha_n\beta_nM_1$, where, M=Fe^{3+} or Co^{3+} and n=number of subunits, usually from 2 to 9 (Noriji et al. 2000). Kobayashi and Shimizu (1998) described two types of NHases based on the molecular mass of the enzyme: light (L-NHase) and heavy (H-NHase), depending on the number of subunits present; 4 subunits: 2 of each α and β for L-NHase and 18-20 subunits: 9-10 of each α and β for H-NHase.

Kobayashi et al. in 1992 reported the two subunits (α and β) to have almost the same molecular mass of 23kDa; but later evidence shows the subunits are different (22 to 27kDa) in molecular mass for L and the H-NHases respectively (Leonova-Pogorelova et al. 2000, Komeda et al. 1996a, 1996b, Stevens et al. 2003). NHase may be produced constitutively, but in selected rhodococci and related bacteria it is inducible and it has been reported to be overproduced to more than 50% of all soluble proteins in *R. rhodochrous* J1 (Kobayashi et al. 1992, Komeda et al. 1996 a, 1996b). Biosynthetic regulation of the NHases has been reported by Kobayashi and Shimizu (1998) as shown in figure 2. The H-NHase genes include *nhh*A, *nhh*B, *nhh*C, *nhh*D, *nhh*E, *nhh*F and *nhh*G; of which *nhh*A and *nhh*B code for the α and β subunits respectively and the genes *nhh*C and *nhh*D code for regulatory proteins. In the L-NHase gene cluster, the structural genes are called *nhl*A and *nhl*B. The other genes present in the H-NHase cluster are also present in the L-NHase cluster. There is also the presence of the gene coding for amidase (*amd*A) in the L-NHase cluster, though this gene is absent in the H-NHase cluster. The *nhh*F and *nhl*F genes code for a membrane protein dedicated to cobalt uptake from the environment (Komeda et al. 1997). It is also reported that post translational modifications (PTMs) are required for the nascent polypeptide to become active (Hashimoto et al. 2002, Hourai et al. 2003, Huang et al. 1997, Miyanaga et al. 2001,

2004, Murakami et al. 2000, Noriji et al. 2000, Odaka et al. 2001, Stevens et al. 2003). The PTMs are in cystein residues of the α subunit; where C112 is oxidized to a sulfinic acid, C114 is modified to a sulfenic acid, and C109 remains in the reduced form in the native protein. Of these, the modification of C112 to C112-SOOH is thought to be essential for the catalytic activity of the Fe-type NHase (Odaka et al. 2001). C109 is thought to be the axial ligand. Studies on cobalt substituted Fe-Type NHase showed that stabilization of C112-SOOH was essential for activity. Oxidation of C114 to C114-SOOH also is thought to be responsible for the catalytic activity of the enzyme. Cobalt type NHases are generally thought to be of the high molecular weight type, and do not exhibit any photoreactivity, unlike the light sensitive Fe-type enzyme (Endo et al. 1999). NHases from *R. rhodochrous* J1 have been shown to have one trivalent cobalt ion in each α subunit, which is responsible for water activation, CN-triple-bond hydration and protein folding (Kobayashi and Shimizu 1998, Shearer et al. 2001).

It is unknown if the cobalt center of NHase is the site for nitrile-binding, and the hydrolytic cleavage of CN triple bond in the substrate (Kobayashi and Shimizu 1999). Leonova et al. (2000) using *R. rhodochrous* M8 and Kobayashi (1998) using *R. rhodochrous* J1 showed that addition of cobalt ions to the culture medium is necessary for both synthesis and catalytic activity of NHase. They also reported that no other metal can substitute cobalt to bring about the same effect. Kobayashi et al. (1992), Kobayashi and Shimizu (1998), and Yamada and Kobayashi (1996) further reported that the regulation of NHase at the transcription level in *R. rhodochrous* J1 is under the influence of the reaction product (amide).

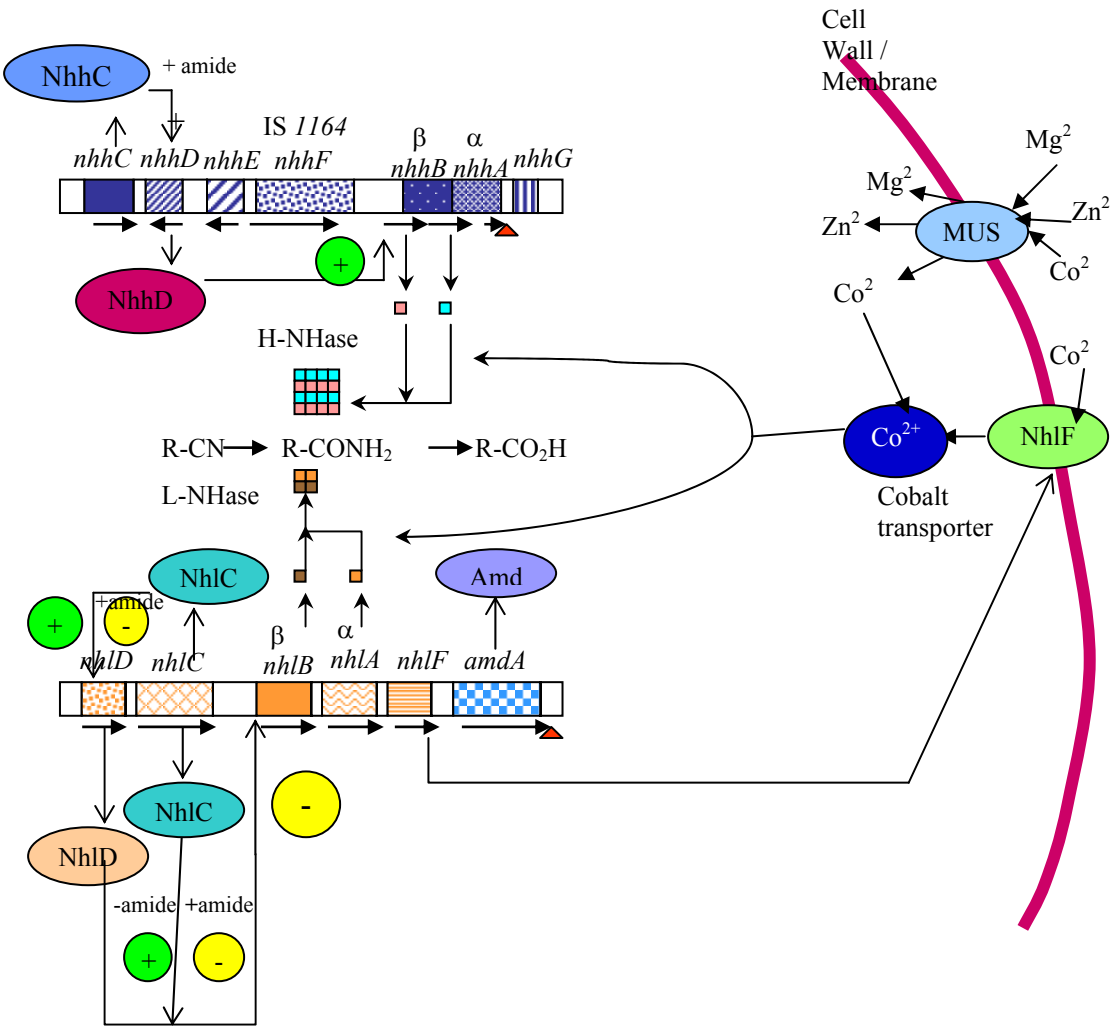


Figure 2. Genetic organization of NHase (Pierce modified Kobayashi and Shimizu 1998).

In the current dissertation study, two *Rhodococcus* strains exhibiting NHase activity were induced for higher NHase expression using the methods of Pierce (1999, 2000) and the NHases were stabilized using immobilization and chemical stabilization techniques to generate a robust catalyst for the biodetoxification of wastewater generated in the AN production industry.

Amidase

AMDase (amidohydrolase) is the second enzyme involved in the aliphatic nitrile degrading pathway, and catalyzes the transformation of carboxylic amides to free carboxylic acids and ammonia. These enzymes are involved in nitrogen metabolism in cells and are widely distributed in nature. Often found in members of nocardia-like actinomycetes, this AMDase is essential for the growth of the organisms showing NHase activity. This is due to the fact that the amides produced by NHase activity, are toxic to the system and have to be converted into the corresponding acid and ammonia (Kobayashi et al. 1993, Kotlova et al. 1999). AMDases have been isolated from various sources and characterized by distinct substrate specificities (Asano et al.1982c, Kobayashi et al. 1993, Mayaux et al. 1990, Maestracci et al. 1988).

In bacteria, AMDases from *Brevibacterium* sp. R312, *Rhodococcus rhodochrous* J1 and *Pseudomonas aeruginosa* have been the most studied. Native AMDase consist of identical subunits. Though the number and size of subunits may vary depending on the microorganism the AMDases were obtained from, most of these enzymes have been shown to have an even number of subunits. Independent studies reported that AMDase from *R rhodochrous* M8 was a homo-tetramer with a molecular mass of 42±2 kDa (Kotlova et al. 1999), that of *P. aeruginosa* consisted of six subunits with 38.4 kDa total

mass (Maestracci et al. 1988), *P. chlororaphis* B23 AMDase is a homodimer with a subunit molecular mass of 54 kDa (Ciskanik et al. 1995) and *Brevibacterium* sp. R312 AMDase is made up of 4 subunits of 43 kDa molecular mass each (Thiery et al. 1986). Ciskanik et al. (1995) showed that AMDase does not require any metal ions but requires a cysteine residue for activity (indicated by inactivation in presence of thiol reagent and not affected by EDTA). Kobayashi et al. (1993) reported the maximum production of AMDase by *R. rhodochrous* J1 was only 8% of total soluble protein, and this was a homodimer of 55 kDa subunits. It was also suggested that this enzyme is coupled with the L-NHase produced by the organism and both the enzymes are regulated similarly, being induced by the same inducers; and they cooperate in degradation of their substrates. The position of the AMDase gene has been reported to be downstream of the α NHase gene in *R. rhodochrous* J1, but in *P. chlororaphis* B23 it has been reported in the same orientation, but upstream of the NHase structural genes. Though AMDase levels of the organisms studied thus far have been reported to be lower by comparison with NHase enzyme levels, the presence of AMDase is essential to prevent toxicity. In the present study two rhodococci strains were induced for higher AMDase production. This will help in production of a catalyst, which when stabilized, is capable of detoxifying the AN production wastewater completely, without addition of external sources of AMDase.

Asparaginase

In past, rhodococci have not been reported to possess any ASNase activity. In the current research the two strains of rhodococci (DAP 96253 and DAP 96622) induced for NHase production with L-Asn exhibited ASNase activity. The mode of action was not similar to the ASNase reported, i.e. addition of external AMDase was required to release

ammonia from L-Asn. This ASNase activity of *Rhodococcus* cells can be of major importance to the treatment of lymphoblastic leukemia. This enzyme with ASNase activity can serve as an alternate source of treatment, having the advantage of absence of toxins and immune response initiation associated with traditional bacterial ASNases in use.

10-25% of paediatric acute lymphoblastic leukemia patients have relapses quite early despite successful treatment (Avramis and Panosyan 2005). This is most likely due to acquired drug resistance, manifested either as leukaemic blast or host resistance. ASNase, which catalyzes the hydrolysis of L-Asn to L-aspartic acid and ammonia (and to a lesser extent the formation of L-glutamate from L-glutamine), is one of the drugs used in the treatment process. In the 1960s tumor-inhibitory properties of ASNase was first reported by Broome (1961, 1963). Soon afterwards, ASNase of bacterial origin were identified and isolated (Schwartz et al. 1966, Campbell et al. 1967, Cedar and Schwartz 1968).

Lymphoblastic leukemia cells lack the ability to synthesize Asn, and are dependent on Asn present in the serum for protein synthesis and survival. Administration of ASNase to the patients depletes the sera of all Asn and protein synthesis is inhibited in the leukemia cells. This makes ASNase an effective drug in the treatment of childhood acute lymphoblastic leukemia.

There are two major bacterial sources of ASNase which are in use as treatment drugs: *E. coli* and *Erwinia*. However, ASNase based therapy often produces side effects, including hypersensitivity reactions, leucopenia, neurological seizures, pancreatitis and hyperglycemia (Wikman et al. 2005). The toxicity of the ASNase is partially attributed

to an intrinsic glutaminase activity of the enzymes, resulting in L-glutamine depletion in the blood. It was also found that bacterial ASNase, especially from *E. coli*, induce anti-ASNase antibodies in a significantly large portion (44-60%) of patients, thus resulting in failure of the target amino acid deamination in serum (Avramis and Panosyan 2005). Erwinase (ASNase from *Erwinia*) did not induce the same response in patients and showed lower L-glutaminase activity; but this enzyme appears to be less effective in the treatment process compared to the *E. coli* derived enzyme. Currently, the use of bacterial ASNase in the treatment process involves administration of less immunogenic form of the enzymes (polyethylene glycol linked), instead of the native enzyme; and after antibody development in the patients, switching them to the non-cross-reacting erwinase. Research is in progress to screen for and isolate alternative sources, and to prepare a purer protein, rather than the existing fermentation products, which may be contaminated with immunogenic protein fragments and bacterial toxins.

Since the discovery of ASNase in *E. coli* and *Erwinia*, the medical need of a large quantity of L-ASNase, has driven studies in other bacteria: *Serratia marcescens* (Heinemann and Howard 1969), *Rhodopseudomonas* (Tchan et al. 1971), *Pseudomonas* (Nikolojev et al. 1971, Foda et al. 1974), *Mycobacterium phlei* (Pastuszak and Szymona, 1975), *Pyrococcus horikoshii* (Yao et al. 2005). Zedan et al. (1981) screened 64 cultures (40 bacteria and 24 yeasts) and reported Asnase activity in *Bacillus* strains along with *Erwinia* and *Arthrobacter*.

L-ASNases from several bacterial sources has been shown to be composed of four identical subunits (Shifrin and Grochowski, 1972; Cammack et al. 1972; Tosa et al. 1973); or it has been reported, in *E. coli*, as a heterotetramer $(\alpha\beta)_2$ of molecular masses

of 23kDa and 14kDa respectively (Borek et al. 2004). On the other hand, the ASNase from *Erwinia* has been reported to be a homotetramer with a MW of 34kDa per subunit (Wikman et al. 2005). Along with differences reported in the subunit mass and compositions of the two ASNases used in therapy, there is a difference in the isoelectric points reported for ASNase from *E. coli* (pH 4.35-5.2) and that from *Erwinia* (pH 8.4±0.2).

Although there is extensively published information on L-ASNases in literature, hardly any of those enzymes have been applied in cancer therapy. This is due to a combined effect of low yields, fast clearance of these enzymes from blood, and difference in the affinity of the enzyme for its substrate.

Thus induction of increased ASNase activity in the two strains of *Rhodococcus* studied and purification of this novel enzyme can potentially result in an alternate source of ASNase for the treatment of acute lymphoblastic leukemia.

Biocatalysts

In strict sense biocatalysts are catalysts of cell metabolism, i.e. enzymes. In broader terms, any biological entity capable of catalyzing the conversion of a substrate into product is a biocatalyst. Accordingly, biocatalysts can be either cellular (growing, resting or non-living cells), or they can be non-cellular (enzymes that have been extracted from the cells producing them). Biocatalysts have always competed with conventional chemical catalysts. Biocatalysts have the advantage of having high specificity, high activity under mild environmental conditions and being biodegradable. Thus screening for an enzyme that catalyzes a particular process of interest, isolation of the organisms producing it, and inducing higher production of the enzyme in the cells can result in a

biocatalyst which is highly competitive with the traditional catalysts. Drawbacks of biocatalysts are their complex molecular structure that makes them costly to produce and intrinsically unstable. Biocatalyst stability, i.e. the capacity to retain activity through time, is undoubtedly the limiting factor in most bioprocesses. This makes biocatalyst stabilization a central issue of biotechnology. In fact, biocatalyst operational stability determines to a large extent, the viability of the process (Illanes 1999). Quite reasonably, a significant effort in research in the field of biocatalyst development is devoted to different aspects of enzyme stabilization. The main areas of focus in enzyme stability are stability of shelf life of the catalyst and stability under operation conditions. Biocatalysts are often produced at the manufacturers' convenience and stored till the need to use them arises. Stability of activity while in storage (shelf-life) thus is of importance to the industry employing the biocatalyst in its processes. Biocatalysts are also often made to perform in an environment quite different from its natural ideal conditions which favors inactivation of the enzymes. Therefore, biocatalyst stabilization under operation conditions is also an important issue.

Several strategies are available to increase stability of biocatalysts. They include use of stabilizing additives, chemical modification of enzyme structure, derivatization, immobilization, crystallization and medium engineering (Cao et al. 2003, Bickerstaff 1997). Use of stabilizing additives is a customary practice in enhancing the shelf-life of the enzymes, whereas immobilization is a common method to bring about operational stability and reduction of physical loss of catalysts during downstream processing. Immobilization also helps to achieve thermal stability of the enzyme as a result of molecular rigidity (Armitage and Kullar 2005).

In this dissertation work two processes of immobilization were employed to achieve stability of NHase and AMDase: entrapment and crosslinking. The entrapment methods were: ionotropic gelation of whole cells with multivalent cation alginate to form beads; and organic polymerization by chemical reaction with acrylamide to form cubes. In the crosslinking method of immobilization, cells were linked to each other by reacting with glutaraldehyde.

Objectives:

The aim of the current research was to investigate the potential of NHase and AMDase based biocatalytic systems for their ability to detoxify wastewater generated in the AN production industry. For this purpose, the NHase-AMDase systems from two Gram-positive organisms, *Rhodococcus sp*. DAP 96253 and *R*. *rhodochrous* DAP 96622 were evaluated. It was hypothesized that increase in cellular yield of the NHase enzyme can be achieved by manipulating the growth medium of the organisms. It was also hypothesized that considerable stability can be attained by simultaneous immobilization and chemical stabilization of the catalysts.

To accomplish these objectives, cells were propagated in media supplemented with nutrients and/or chemicals known to induce enzyme production. Enzyme activity was then evaluated for each media type to determine the best composition. The enzymes were also evaluated for their affinity for all the chemicals present in the AN production wastewater. The catalysts were evaluated for stability under mild conditions (ideal natural conditions) and application conditions in both free cells and cells immobilized in different matrices. Retention of enzyme activity for a prolonged period reflects achievement of stability in a particular catalyst formulation. As stability of catalyst

formulations increased, the time required to evaluate the effect of a particular stabilizer increased proportionally. To overcome this, a rapid assay was developed in which the catalyst formulations were exposed to restrictive conditions accelerating the rate of nitrile hydratase degeneration. This assay permitted accurate evaluations of specific nitrile hydratase activity in the catalyst formulations in a fraction of the time. The enzymes were isolated and purified and evaluated for stability in a cell free system.

MATERIALS AND METHODS

Microorganisms:

All strains were obtained from American Type Culture Collection (ATCC, Vienna, VA), were revived and initially maintained according to the methods recommended by ATCC (nutrient agar, or yeast extract malt extract agar) and maintained at the recommended temperatures of 26 C or 30 C. Six microorganisms were initially screened for NHase and AMDase activity, of which: three were rhodococci, *Rhodococcus* sp. strain DAP 96253 (ATCC 55899), *R. rhodochrous* strain DAP 96622 (ATCC 55898), and *R. erythropolis* (ATCC 47072), two were pesudomonads, *Pseudomonas chlororaphis* (ATCC 43051) and *P. chlororaphis* (ATCC 13985); and the last was *Brevibacterium ketoglutamicum* (ATCC 21533). DAP 96253, *P. chlororaphis* (43051) and *B. ketoglutamicum* (21533) were propagated at 30 C, while DAP 96622, *P. chlororaphis* 13985 and *R. erythropolis* (47072) were maintained at 26 C.

After the cultures were revived, they were maintained either on R2A medium (BBL, Sparks, MD) or yeast extract malt extract medium (YEMEA) (Dietz and Thayer 1980).

Preparation of buffers:

All buffers used were prepared according to the formulae in Methods for General and Molecular Bacteriology (Breznak and Costillow 1994) (see Table 2).

Table 2. Formulae for biological buffers used. (Final molarity 0.1 M)

pH	Acetate Buffer[a]	Citrate-Phosphate Buffer[b]	Phospate Buffer[c]	Tris-Hydrochloride Buffer[d]
4.6	25.5	26.7		
4.8	20	25.2		
5.4	8.8	22.2		
5.88		19.7	46	
6.4		15.4	36.75	
6.6		13.6	31.25	
7.0		6.5	19.5	
7.2			14	44.2
7.4			9.5	41.4
7.8			4.25	32.5
8.2				21.9
8.6				12.2
9.0				5

[a]**Formula 1:** x ml of 0.2 M of Acetic Acid + (50 – x) ml of 0.2 M of Sodium acetate diluted to 100 ml.

[b]**Formula 2:** x ml of 0.1 M citric acid + (50 – x) ml of 0.2 M of Na_2HPO_4 diluted to 100 ml [c]**Formula 3:** x ml of 0.2 M $NaHPO_4$ + (50 – x) ml of 0.2 M of Na_2HPO_4 diluted to 100 ml

[d]**Formula 4:** 50 ml of 0.2 M TRIS + x ml of 0.2 M of HCl, diluted to 100 ml

Induction of NHase:

NHase was induced by supplementing the media (either YEMEA or R2A) with urea (7.5 g/l), $CoCl_2$ (10 mg/l), and with either AN and AC (both at 500 mg/l), or with asparagine (Asn) and glutamine (Gln) (both at 500 mg/l). The six selected strains were grown on solidified media for 5-6 days, after which cells were harvested from the plates using sterile 50 mM phosphate buffer, pH 7.2. The harvests were then centrifuged for 10 min (10,000 rpm, Beckman J2, GSA rotor, 4 C), and the cell pellets were pooled for each cell type, was washed with sterile phosphate buffer (3X) and the final cell pellet resuspended in sterile phosphate buffer to achieve a cell density of 1.00 (OD_{600}). Cells were evaluated for their NHase, AMDase and ASNase activities immediately following harvesting and were cast into catalyst particles. Cells not used immediately were flash frozen (dry ice and acetone) and stored at -80 C. The frozen cells were thawed to ambient temperature, and their enzyme (NHase, AMDase and ASNase) activities were evaluated before they were used. When preparing frozen stocks, the cells were suspended in a sterile 10% glycerol solution in 50 mM phosphate buffer, pH 7.2, and stored at -80 C.

Determination of Enzyme Activity

<u>NHase:</u>

One unit of NHase is defined as the conversion of 1 μM of AN per minute per mg dry weight of cells at 30 C, pH 7. NHase activity was determined by adding 1.5-2.0 mg dry weight of cells (suspended in 50 mM phosphate buffer, pH 7.0) to 9 ml of solution containing 1000 μg/ml AN (in sterile 50 mM phosphate buffer, pH 7.0). Upon termination of the reaction by acidifying the sample to pH 2.0 with 2 N H_2SO_4, the cells

were then removed by filtration (0.2 μ filter, Pall, Ann Arbor, MI), and then the cell-free sample was pH neutralized. AN and AMD concentrations were determined by gas-liquid chromatography (GLC) (Perkin Elmer Autosystem XL, Shelton, CT) using the following conditions: injector 200 C, detector 275 C, with the following temperature program: initial oven temperature 40 C for 2 min, temperature increased at 15 C/min till 220 C; a final hold for 5 min. The column employed was a Phenomenex (Torrence, CA) ZB-WAX glass column (30 m X 0.53 mm ID). Alternatively, the cell-free fraction was readjusted to neutrality and was incubated at 37 C in presence of an excess amount of amidase (Sigma#A6691, St.Louis, MO). External amidase was added to convert all amides present to their corresponding acids and ammonia. Ammonia was measured by a modification of method of Fawcett and Scott (1960) (see following section).

Amidase:

One amidase unit is defined as the conversion of one μM/min of AMD to one μ mole of acrylic acid and ammonia per mg dry weight of cells at 30 C, pH 7. Amidase activity was determined by the production of ammonia from the amide using the modified method of Fawcett and Scott (1960), in which one ml of the catalyst (with comparable cell wet weights) was added to 9 ml of 1000 ppm AMD solution (in 50 mM phosphate buffer, pH 7.0). After two minutes, duplicate 1 ml samples of the reaction were removed and filtered through a 0.2 μ filter (Pall, Ann Arbor, MI) to remove the catalyst. To each 1 ml of the filtered sample (also cell/catalyst-free samples from NHase evaluation reactions), 2 ml of sodium phenate, 3 ml of sodium nitroprusside and 3 ml of 0.02 N sodium hypochlorite solutions were added in quick succession and the mixture was vortexed. The samples were then incubated in the dark at 27 C for 30 min, after

which the absorbance was read at 630 nm using a microplate reader (Wallac Victor$^{2™}$ 1420 multilabel counter, Perkim Elmer Life Sceinces, Shelton, CT). Each sample was read in triplicate and the data averaged.

Asparaginase I:

One unit of ASNase is defined as the conversion of 1 μM of Asn per minute per mg dry weight of cells at 30 C, pH 7. ASNase activity was determined by adding 0.5-2.0 mg dry weight of cells (suspended in 1 ml 50 mM phosphate buffer, pH 7.0) to 9 ml of solution containing 1000 μg/ml Asn (in sterile 50 mM phosphate buffer, pH 7.0). Upon termination of the reaction by acidifying the sample to pH 2.0 with 2 N H_2SO_4, the cells were then removed by filtration (0.2 μ filter, Pall, Ann Arbor, MI). The cell-free fraction was readjusted to neutrality and was incubated at 37 C in presence of an excess amount of amidase [Sigma#A6691, St.Louis, MO]. External amidase was added to convert all amides present to their corresponding acids and ammonia. Ammonia was measured by a modification of method of Fawcett and Scott (1960) (see previous section).

Enzyme production during growth:

DAP 96253 and DAP 96622 were grown on YEMEA broth supplemented with Co and urea at their respective temperatures (30 C or 26 C). The inoculum was either grown on uninduced (YEMEA with no Co, urea or nitriles) or induced for elevated NHase production. By the addition of a mixture of AN and AC (both at 500 ppm) or Asn and Gln (both at 500 ppm) at 55 h of growth. Samples were taken out periodically and tested for NHase and AMDase levels to correlate cell growth and enzyme production. Three replicate 500 ml flasks with 200 ml YEMEA broth were inoculated with equivalent amounts of the two strains of *Rhodococcus* spp. (DAP 96253 and DAP 96622), were

incubated at 30 C and 26 C, in a New Brunswick gyratory water bath shaker (150 rpm), and monitored for cell density at OD 600 nm for 96 hours.

Cell Immobilization:

1. Calcium alginate and hardened Ca-Alginate:

A) Calcium alginate immobilization:

Cells with previously determined NHase and amidase specific activities were separated into 3 equal aliquots: a control aliquot which was not immobilized, one aliquot which was immobilized in Ca-alginate (alginic acid-sodium salt, Sigma, St. Louis, MO), and the last aliquot which was immobilized in Ca-alginate and then was hardened by cross-linking with polyethyleneimine (PEI) (Sigma, St. Louis, MO). Cells immobilized in Ca-alginate beads (2-3 mm in dia.) were prepared according to a modification of the method of Wu and Li (2002). Cells (5 g packed wet wt) were resuspended in 50 ml of 50 mM TRIS, pH 7.2, mixed thoroughly with a 25 g of 4% sodium alginate (in 50 mM DI water) and the mixture was passed through a hypodermic needle (27 gauge) drop wise into a solution of 0.1 M calcium chloride. The beads were cured in the calcium chloride solution for 60 min, washed and stored at 4 C until used.

B)Hardened Calcium alginate:

Hardened Ca-alginate beads were prepared by adding 50% w/w PEI (in 1 M $CaCl_2$ solution, to achieve 0.5% solution) to previously prepared ca-alginate beads. The beads were incubated in the PEI solution for 24 h at room temperature, washed once with DI water and stored in 50 mM TRIS buffer, pH 7.2 at 4 C untill used.

2. Polyacrylamide (PAM) Immobilization:

Unless otherwise specified, all chemicals were obtained from Sigma (St. Louis, MO). 10 g (packed wet weight) cells were suspended in 40 ml DI H_2O and thoroughly mixed with 40 ml of acrylamide-bisacrylamide solution (4.5 g AMD and 0.5 g N-methyl-bisacrylamide in DI water) on ice. To the 80 ml of admixed solution, first 5 ml of 3-dimethylaminoproprionitrile and then 10 ml of 2.5% potassium persulfate solution were added in rapid succession with vigorous mixing on ice, and then quickly poured between two glass plates to form a gel slab of 2 mm thickness. The gel was allowed to incubate at room temp for 60 min for complete polymerization. After complete polymerization, the gel was cut into thin strips and then further cut into small cubes of 2-3 mm. The cubes were washed 3 X with DI H_2O to remove residual monomers and stored at 4 C in DI H_2O till used.

3. Glutaraldehyde Immobilization:

Cells 10 g (packed wet weight) were suspended in 100 ml DI H_2O, to which a 25% solution (in DI water) of glutaraldehyde (GA) was added with constant stirring, to achieve a 0.5% final concentration of GA. The cell mixture was left stirring for 60 min at room temperature, at which time 400 ml DI H_2O was added to the mixture with stirring, followed by the slow addition of PEI solution (50% w/v in DI water). PEI was added until flocculation was achieved. The flocculated material was harvested by filtering (0.45 μ nalgene bottletop filter, Nalge Nunc International, Rochester, NY). The packed flocculate was extruded through a syringe and dried for 24 hours. It was cut into small pieces (2-3 mm length) and stored at room temperature till used.

Electron Microscopy:

Whole cells grown on agar plates were analyzed for cell structure; and catalysts made as described above were analyzed for distribution of cells throughout the catalyst particles. Samples were prepared as per standard protocol (Bozzola and Russell 1999). Samples in sodium cacodylate buffer (Electron Microscopy Science, Ft. Washington, PA) were fixed using GA (3% in 0.1 M cacodylate buffer), followed by an overnight exposure to OsO_4 (1% in 0.1 M cacodylate buffer) and subsequent dehydration using ethanol. Propylene oxide was used as a transitional solvent prior to infiltration with epoxy resin (Embed 812 Araldite Epoxy Resin, Electron Microscopy Science, Ft. Washington, PA). After curing of the resin, samples were trimmed and sectioned. Sectioned samples were then stained using uranyl acetate and lead citrate. Specimens were imaged using a Leo 906E TEM.

SEM samples were dehydrated in ethanol, incubated in pure hexamethyldisilizane (Electron Microscopy Science, Ft. Washington, PA) and dried, mounted on aluminum stubs and sputter coated with a gold/palladium alloy, and then imaged using a Leo 1450 VP SEM.

Chemical Stabilizers:

The effect of isobutyric acid, acrylic acid, AN, AC, succinonitrile (SN), Asn, and Gln on NHase stability was evaluated by adding these chemicals (alone or in combinations) to the catalyst formulations. In all cases total concentration of the stabilizer was maintained at 2 g/L.

Comparison of NHase stability: (in free cells, cells immobilized in Ca-alginate and cells immobilized in hardened Ca-alginate with and without stabilizers)

Equivalent amounts (40 mg cell wet weight/ 1 ml) of free-cells, Ca-alginate immobilized cells, hardened ca-alginate cells, and GA immobilized cells, all in 50 mM Tris buffer, pH 7.2, were incubated either at 30 C or at 50-55 C (for accelerated testing of NHase). At prescribed times, an entire aliquot of equivalent cells of each catalyst type was removed from incubation and tested for NHase activity. Mixed substrate (AC and AN both at 1000 ppm) was added and conversion to acetamide and AMD were determined by the method described above.

pH range of immobilized NHase

Immobilized catalysts (calcium alginate, hardened calcium alginate and glutaraldehyde) were maintained at the appropriate optimal temperature (either 30 C or 26 C depending on the organism) in buffers of different pHs ranging from 4.6 to 9.0. Four different buffers were used: acetate (pHs 4.6, 4.8 and 5.4), citrate-phosphate (pHs 5.8, 6.3, 6.8 and 7.0), phosphate (pHs 7.4 and 7.8), and TRIS-HCl (pHs 8.2, 8.5 and 9.0). Equivalent aliquots (1 ml packed volume) of catalysts were removed at prescribed times and subjected to the NHase assay described above to evaluate residual NHase activity. Reduction of activity over time for each pH condition was compared and the pH optimum was determined.

Substrate Specificity

NHase from the three *Rhodococcus* (*Rhodococcus sp*. DAP 96253, *R. rhodochrous* DAP 96622, and *R. erythropolis*) strains and *B. ketoglutamicum* were compared for their substrate affinity and range. Equal amounts of appropriately grown

cells (~40 mg wet weight) were incubated with substrate stock solutions (all at 1000 ppm concentrations). Both aliphatic and aromatic nitriles were tested as substrates. For a list of nitriles tested as substrates please see Table 3a and 3b. Activity of NHase from *Rhodococcus sp*. DAP 96253 against acetonitrile (AC) was considered as 100% and all data was normalized against this.

Effect of Inhibitors on NHase

NHase as non-stabilized and non immobilized in whole cells, calcium alginate, hardened calcium alginate, GA immobilized, and cell-free purified enzyme was subjected to inhibition by the chemicals listed in Table 4 at 1000 ppm concentration. Duplicate samples were run for each sample set, where the catalysts were exposed to the inhibitor solution for 15 min. At the end of the incubation time, one set was tested for residual NHase activity in presence of the inhibitor, and in the other set, the inhibitor was removed, the catalyst washed with DI H_2O and then tested for residual NHase activity using the assay described previously.

Amidase Stability:

AMDase stability was evaluated in the immobilized catalysts (calcium alginate, hardened Ca-alginate, PAM and GA) by maintaining the catalysts in a 50 mM phosphate buffer (pH 7.2) environment at 4 C, 26 C and 30 C. At defined times equivalent aliquots were tested for residual AMDase activity using the assay described above.

Purification of NHase and AMDase:

Cells (60 g packed wet weight) grown on YEMEA supplemented with Co, urea and Asn were suspended in 50 mM phosphate buffer, pH 7.2, and dismembrated in presence of glass beads (0.15-0.25 mm) by passing twice through the Dyno Mill™ (type

Table 3a. List of aliphatic nitriles used as substrates of NHase.

	Name	Formula
1	Isobutyronitrile	CH_3CHCN CH_3
2	Succinonitrile	NCCHCHCN
3	Isovaleronitrile	CH_3CHCH_2CN CH_3
4	$_{DL}$-Lactonitrile	$CH_3CH(OH)CN$
5	Fumaronitrile	$NCCH_2CH_2CN$
6	Glycolonitrile	$HOCH_2CN$
7	Acetonitrile	CH_3CN
8	Acrylonitrile	CH_2CHCN
9	Adiponitrile	$C_6H_8N_2$

Table 3b. List of aromatic nitriles used as substrates of NHase

	Name	Formula
1	Benzonitrile	C_6H_5CN
2	Cinnamonitrile	$C_6H_5CHCHCN$

Note: All chemicals were obtained from Sigma (St. Louis, MO)

Table 4. List of chemicals evaluated for their inhibitory effect on NHase

	Name	**Concentration (mM)**
1	Zinc chloride	7.35
2	Magnesium chloride	4.92
3	Ammonium persulfate	4.38
4	Silver nitrate	5.89
5	Hydrogen peroxide	29.41
6	2-mercaptoethanol	12.8
7	Iodoacetamide	5.41
8	DTT	6.49
9	Sodium azide	15.38
10	Ferrous sulfate	3.6
11	Cupric sulfate	4.01
12	Cobalt chloride	4.2
13	EGTA	2.63

Note: All chemicals were obtained from Sigma (St. Louis, MO)

KDL, Helmut Claus, WAB, Germany) at maximal power at 0 C. Cell debris was removed by centrifugation 2 x at 13,000 rpm for 30 min at 4 C. NHase and AMDase activity of the lysate were evaluated and recorded. The lysate (in 50 ml aliquots) was then dialysed (10,000 molecular weight cut off) against 2 L of 10 mM phosphate buffer pH 7.2, at 4 C overnight. The dialyzed protein solution was evaluated for NHase and AMDase activity. 50 ml of the dialyzed sample was then applied to a 16 x 100 mm DEAE-Sephacel (Amersham Biosciences, Piscataway, NJ) (anion exchange) column on an ÄKTA™ FPLC™ system (Amersham Biosciences, Piscataway, NJ) at 4 C. The bound proteins were eluted with 50 mM TRIS-HCl buffer, pH 7.5, and collected using a Frac-950™ fraction collector (Amersham Biosciences, Piscataway, NJ). 1 M NaCl was used to create the gradient. The fractions showing the presence of protein (detected at 280 nm, by the fraction collector) were tested for NHase and AMDase activity. The active fractions were pooled and run through the same column again after regenerating the resin according to manufacturer's instructions. The fractions showing NHase and AMDase activity were again pooled and concentrated (all protein were precipitated, and re-suspended in 1 ml of 150 mM phosphate buffer, pH 7.5) before loading onto a 10 mm x 100 cm Sephacryl S-200 (Amersham Biosciences, Piscataway, NJ) column. Proteins were eluted with the same buffer and evaluated for NHase and AMDase activity. At each chromatographic step, the fractions with highest enzyme activity were subjected to SDS-PAGE for visualization of purity. The gels consisted of 12.5% total monomer (AMD and bisacrylamide) (Sigma, St. Louis, MO), 5 µg protein was loaded in each well and electrophoresis was carried out at 12.5 mA per gel at 10 C. The gels were stained with silver stain for imaging. Gels from the final chromatography step were stained with

Coomassie Brilliant Blue (PhastGel™ Blue R, Amersham Biosciences, Piscataway, NJ) and bands were excised to extract the protein. The gel fragments were subjected to trypsin digestion, and the fragments were extracted from the gel plugs according to the manufacturers' instructions using the ProteoProfile™ Trypsin In-Gel Digest Kit (# PP0100, Sigma, St. Louis, MO). The samples were then concentrated with a ZipTip® (# ZTC18M096, Millipore, Billerica, MA) before spotting onto a MALDI target. MALDI-TOF-TOF was carried out for identification of the proteins purified at the Microchemical Facility of School of Medicine, Emory University. The amino acid sequences of the proteins were determined from the mass spectrometry data using the Mascot™ software at the above facility.

RESULTS

Morphology:

Rhodococcus sp. DAP 96253 and *R*. *rhodochrous* DAP 96622 when grown on solid medium, formed distinct colonies, which were salmon pink in color, dry, slightly raised and wrinkled. Figure 3 and 4 are SEM images showing short rods typical of rhodococci on solid medium. Both strains showed substrate mycelium formation when grown on solid medium and no aerial mycelium, which is a distinct characteristic feature of rhodococci (Goodfellow 1986). Figure 4 shows substrate (and no aerial) mycelia forming by strain DAP 96253.

Growth curve:

Figure 5 shows the increase in turbidity over time for strains DAP 96253 and DAP 96622 respectively. Both organisms exhibit regular sigmoid growth patterns. Doubling time for DAP 96253 was 60 hours and that for DAP 96622 was 65 hours.

Propagation and Induction of enzymes on different media:

The six organisms (listed in Table 3) were propagated on YEMEA medium. The medium was either supplemented with cobalt, urea, and an inducer package; or contained no added inducer(s) for NHase. The inducer package contained either AN and/or AMD (at 500 ppm each); or it contained L-Asn and/or L-Gln (at 500 ppm). Growth on each medium is shown in Table 5. Microorganisms showing growth on each media (un-induced, or induced) were evaluated for NHase and AMDase levels. Apart from *R*. *erythropolis*, which appears to be constitutive with respect to NHase and AMDase,

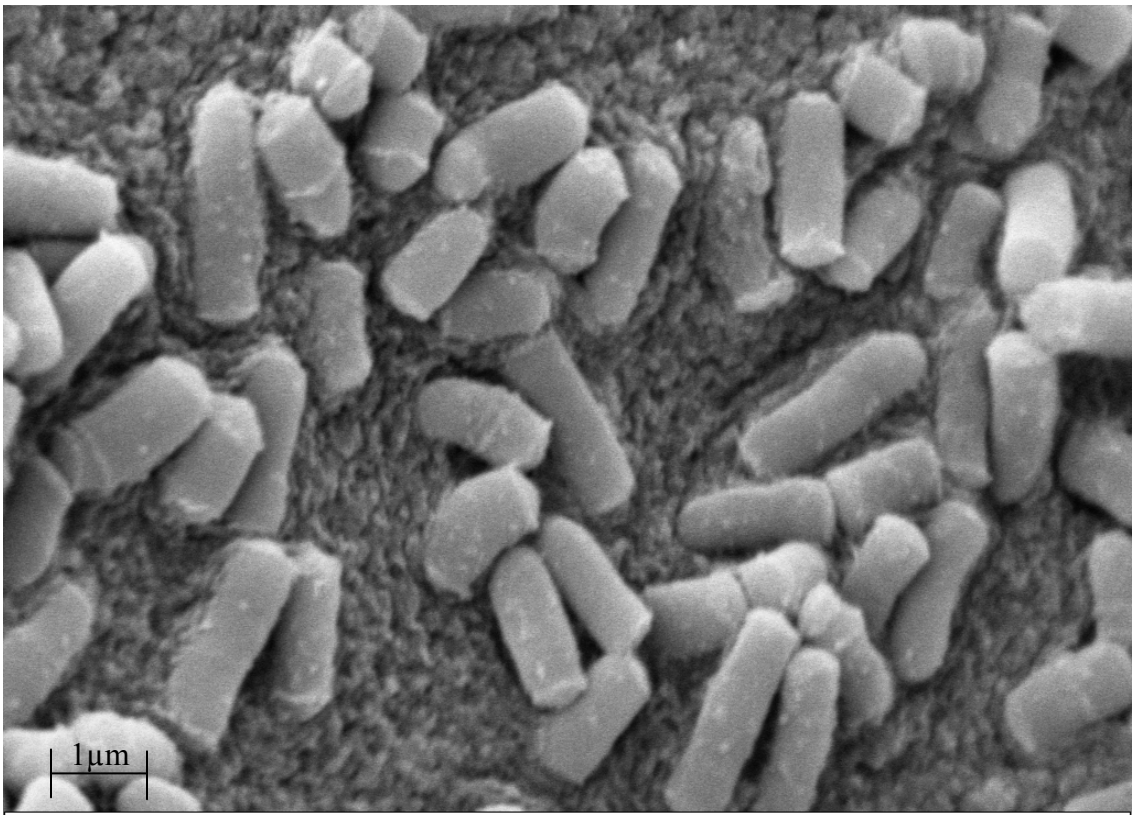


Figure 3. SEM of *Rhodococcus* grown on solid YEMEA medium. Magnification: 25,050 X

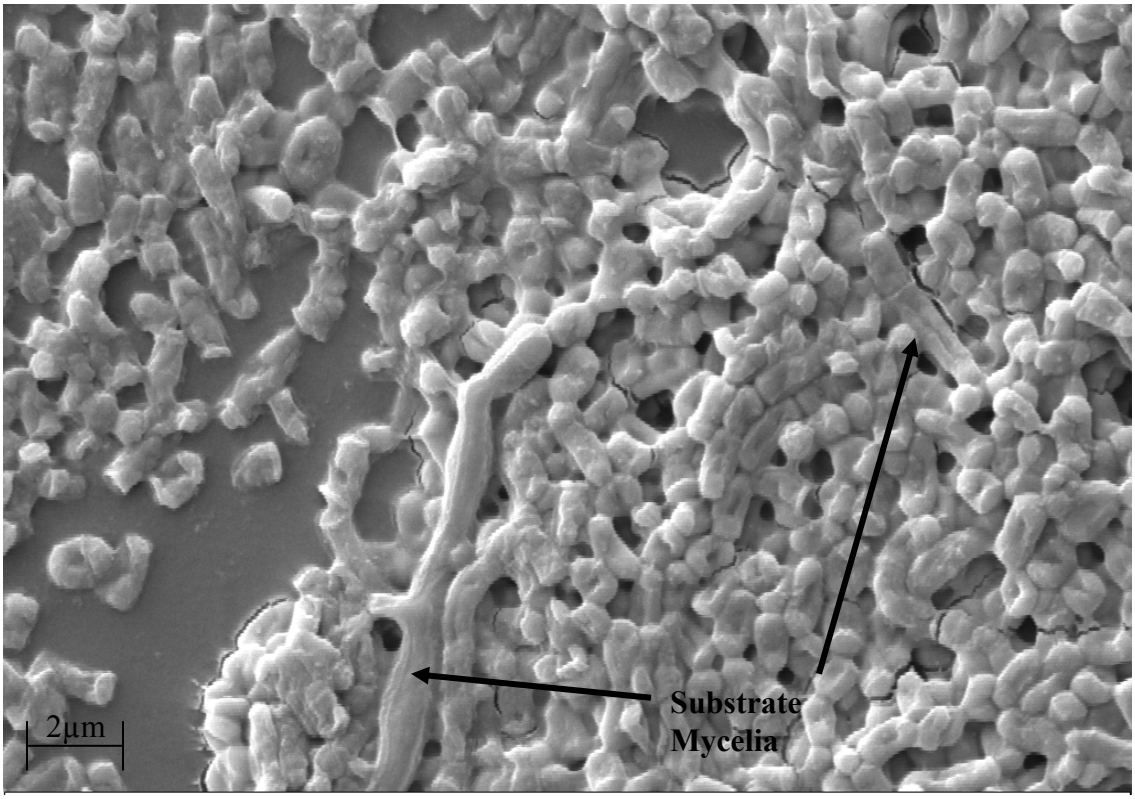


Figure 4. Substrate mycelium formation by *Rhodococcus sp*. DAP 96253.
Magnification: 10,000 X

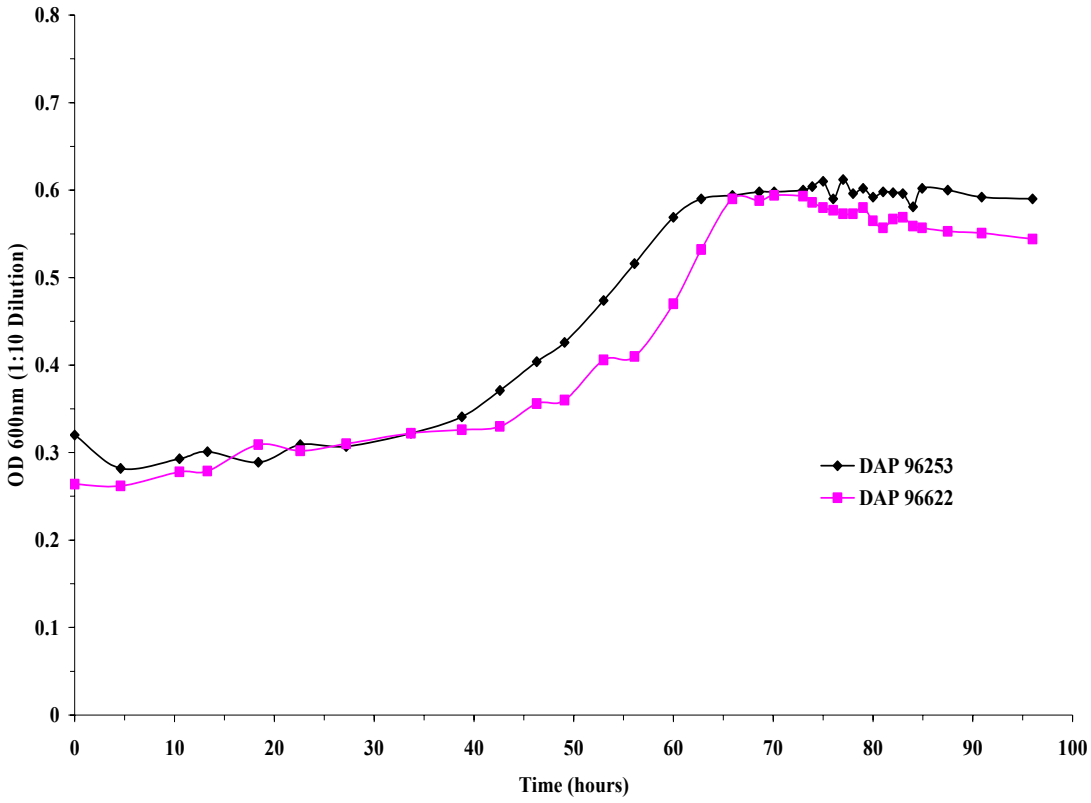


Figure 5. Growth of *Rhodococcus sp*. DAP 96253 and *R. rhodochrous* DAP 96622 on liquid yeast extract malt extract medium.

the microorganisms evaluated showed significantly higher levels of NHase induced with AN (see Table 6 and 7). In comparison, AMDases were expressed at a higher level when induced with amino acids. Table 7 summarizes AN specific activity when the cells were induced by traditional nitrile and amide inducers compared to the non-toxic amino acids, while Table 8 summarizes the AMD specific activities of the cells when induced with traditional nitrile and amide inducers and also the non-toxic amino acid.

In all cases, *Rhodococcus sp.* DAP 96253 and *R. rhodochrous* DAP 96622 showed the highest levels of inducible NHase amongst all six organisms tested. Hence these two organisms were selected for further studies directed towards stabilizing the NHase and AMDase with the goal of production of a robust, stable catalyst for detoxification of AN production wastewaters. Enzyme production as a function of time during growth was evaluated in the two *Rhodococcus* strains (DAP96253 and DAP 96622) (Figure 6). In liquid culture, both the strains showed a decline in NHase activity in absence of any inducers, but this was rapidly reversed when the inducers (e.g., AC and AN) were added to the liquid culture medium. AMD specific activity also showed a similar trend.

Asparaginase I activity in rhodococci:

When induced with Asn (500 ppm), the cells of *Rhodococcus sp*. DAP 96253 and *R. rhodochrous* DAP 96622 showed ASNase I activity. Strain DAP 96253 showed an average activity of 12.5 U/mg cell dry weight, while strain DAP 96622 showed slightly lower activity of 12 U/mg cell dry weight. A unit of specific activity is defined as one μmole L-asparagine converted per minute per mg cell dry weight at 30 C, pH 7.0. There

Table 5. Growth of organisms on various induction media.

Strain name	ATCC #	Maintenance Temperature (C)	Growth Medium						
			Y-U	Y-AN	Y-AMD	Y-AN +AMD	Y-Asn	Y-Gln	Y-Asn +Gln
Pseudomonas chlororaphis	43051	30	++	-	-	-	+	-	+
Pseudomonas chlororaphis	13985	26	++	+	+	+	+	+	++
Brevibacterium ketoglutamicum	21533	30	++	+	+	+	+	+	+
Rhodococcus erythropolis	47072	26	++	+/-	+	+	++	++	+++
Rhodococcus sp. DAP 96253	55899	30	++++	++++	++++	++++	++++	++++	++++
Rhodococcus rhodochrous DAP 96622	55898	26	++++	++++	++++	++++	++++	++++	++++

Y-U: Yeast extract malt extract agar (YEMEA) without any inducers
Y-AN: YEMEA supplemented with cobalt, urea (7.5 g/L), and AN (500 ppm)
Y-AMD: YEMEA supplemented with cobalt, urea (7.5 g/L), and AMD (500 ppm)
Y-AN+AMD: YEMEA supplemented with cobalt, urea (7.5 g/L), AN (500 ppm) and AMD (500 ppm)
Y-Asn: YEMEA supplemented with cobalt, urea (7.5 g/L), and L-Asn (500 ppm)
Y-Gln: YEMEA supplemented with cobalt, urea (7.5 g/L), and L-Gln (500 ppm)
Y-Asn+Gln: YEMEA supplemented with cobalt, urea (7.5 g/L), L-Asn (500 ppm) and L-Gln (500 ppm)
- No detectable growth
+/- Marginal growth
+ Little growth
++ Good growth
+++ Very good growth
++++ Excellent growth

Table 6. Induction of NHase by AN.

Organism	ATCC #	Medium			
		Y-U		Y-AN	
		NHase (U/mg cdw)	AMDase (U/mg cdw)	NHase (U/mg cdw)	AMDase (U/mg cdw)
Pseudomonas chlororaphis	43051	49	0	ND	ND
Pseudomonas chlororaphis	13985	30	4	75	1
Brevibacterium ketoglutamicum	21533	34	2	75	2
Rhodococcus erythropolis	47072	55	2	ND	ND
Rhodococcus sp.	55899	82	4	172	8
Rhodococcus rhodochrous	55898	63	5	155	9

Units of specific activity defined as: μmoles converted/min/mg cdw at 30 C, pH 7.0.

ND: No detectable growth,

Y-U: YEMEA media without cobalt, urea, or additional N-source

Y-AN: YEMEA with cobalt, urea, and AN (500 ppm)

Table 7. Induction of NHase using traditional and non-toxic chemicals.

Strain	ATCC #	Average NHase (U/mg cdw)					
		Media					
		Y-AN	Y-AMD	Y-AN+AMD	Y-Asn	Y-Gln	Y-Asn+Gln
Pseudomonas chlororaphis	43051	ND	ND	ND	28	ND	45
Pseudomonas chlororaphis	13985	1	6	11	14	0	8
Brevibacterium ketoglutamicum	21533	73	32	88	30	37	42
Rhodococcus erythropolis	47072	ND	43	50	48	42	55
Rhodococcus sp. DAP 96253	55899	172	152	175	155	135	152
Rhodococcus rhodochrous DAP 96622	55898	155	138	162	158	160	170

Units of specific activity defined as: µmoles converted/min/mg cdw at 30 C, pH 7.0.

Y-AN: YEMEA w cobalt, urea and AN (500 ppm)
Y-AMD: YEMEA w cobalt, urea and AMD (500 ppm)
Y-AN+AMD: YEMEA w cobalt, urea, AN (500 ppm) and AMD (500 ppm)
Y-Asn: YEMEA w cobalt, urea and L-Asn (500 ppm)
Y-Gln: YEMEA w cobalt, urea and L-Gln (500 ppm)
Y-Asn+Gln: YEMEA w cobalt, urea, L-Asn (500 ppm) and L-Gln (500 ppm)
ND: No detectable growth

Note: *R. erythropolis* levels are constitutive (see Table 6)

Table 8. Induction of AMDase using traditional and non-toxic chemicals.

Strain	ATCC #	Average AMDase (U/mg cdw)					
		Media					
		Y-AN	Y-AMD	Y-AN+AMD	Y-Asn	Y-Gln	Y-Asn+Gln
Pseudomonas chlororaphis	43051	ND	ND	ND	0	ND	0
Pseudomonas chlororaphis	13985	1	6	11	14	0	8
Brevibacterium ketoglutamicum	21533	2	8	10	0	0	3
Rhodococcus erythropolis	47072	ND	12	10	9	14	6
Rhodococcus sp. DAP 96253	55899	8	10	9	13	7	10
Rhodococcus rhodochrous DAP 96622	55898	9	11	10	10	6	12

Units of specific activity defined as: µmoles converted/min/mg cdw at 30 C, pH 7.0.

Y-AN: YEMEA w cobalt, urea and AN (500 ppm)

Y-AMD: YEMEA w cobalt, urea and AMD (500 ppm)

Y-AN+AMD: YEMEA w cobalt, urea, AN (500 ppm) and AMD (500 ppm)

Y-Asn: YEMEA w cobalt, urea and L-Asn (500 ppm)

Y-Gln: YEMEA w cobalt, urea and L-Gln (500 ppm)

Y-Asn+Gln: YEMEA w cobalt, urea, L-Asn (500 ppm) and L-Gln (500 ppm)

ND: No detectable growth

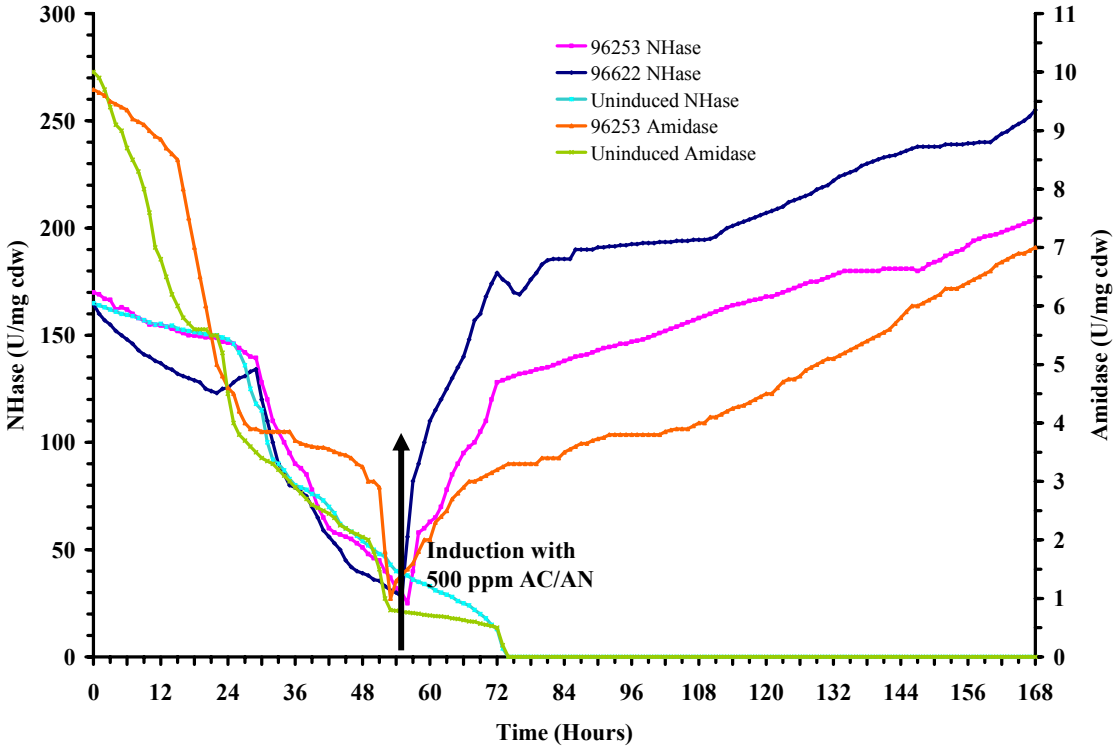


Figure 6. Production of NHase and AMDase in *Rhodococcus sp*. DAP 96253 and *R. rhodochrous* DAP 96622 during growth.

are no previous reports in the peer reviewed literature of ASNase activity by rhodococci. Table 9 summarizes average ASNase values for *Rhodococcus sp.* DAP 96253, and *R. rhodochrous* DAP 96622 and other selected strains, when induced with either nitriles/amides.

Immobilization of whole cells:

Cells of *Rhodococcus sp*. DAP 96253 and *R. rhodochrous* DAP 96622 were immobilized in Ca-alginate, polyacrylamide (PAM), and glutaraldehyde (GA) matrices. SEM and TEM images of the prepared catalysts clearly show the distribution of cells in the catalyst, are shown in Figures 7-14. The Ca-alginate, either as is or PEI cross linked Ca-alginate catalysts were cast directly as 2-3 mm diameter beads, while the PAM immobilized catalysts were cast as cubes (2 mm^3) and the GA immobilized catalysts were first cast and then made into irregularly particles by attrition.

All catalyst types were evaluated for diffusional limitations of the different immobilization matrices. Table 10 shows the NHase activity for Ca-alginate, PAM and GA immobilized cell catalysts when exposed to an increasing concentration of the substrate (AN). In all cases, the increase in activity was proportional to the increase in enzyme amount (cell loading) and increase in substrate concentration. Changes in enzyme amount were achieved by altering the amount of cells (packed wet wt) immobilized per ml of the catalyst (for each catalyst type).

Substrate specificity of NHase:

The NHase of three strains of rhodococci (*Rhodococcus sp*. DAP 96253, *R. rhodochrous* DAP 96622, and *R. erythropolis*) along with that of *B. ketoglutamicum* were evaluated for specificity towards selected aliphatic and aromatic nitriles. The results of

Table 9. Induction of ASNase using traditional and non-toxic chemicals.

Strain	ATCC #	Average ASNase (U/mg cdw)					
		Media					
		Y-AN	Y-AMD	Y-AN+ AMD	Y-Asn	Y-Gln	Y-Asn+ Gln
Pseudomonas chlororaphis	43051	ND	ND	ND	18.4	ND	18.73
Pseudomonas chlororaphis	13985	2	0	3	0	0	1
Brevibacterium ketoglutamicum	21533	14.62	15.43	13.6	19.1	20.3	17.8
Rhodococcus erythropolis	47072	ND	0	0	1	2	0
Rhodococcus sp. DAP 96253	55899	7.8	2	7.4	12.5	11.1	13.9
Rhodococcus rhodochrous DAP 96622	55898	8.2	7.85	10.1	12.3	10	13.8

Units of specific activity defined as: µmoles converted/min/mg cdw at 30 C, pH 7.0.
Y-AN: YEMEA w cobalt, urea and AN (500 ppm)
Y-AMD: YEMEA w cobalt, urea and AMD (500 ppm)
Y-AN+AMD: YEMEA w cobalt, urea, AN (500 ppm) and AMD (500 ppm)
Y-Asn: YEMEA w cobalt, urea and L-Asn (500 ppm)
Y-Gln: YEMEA w cobalt, urea and L-Gln (500 ppm)
Y-Asn+Gln: YEMEA w cobalt, urea, L-Asn (500 ppm) and L-Gln (500 ppm)
ND: No detectable growth

Figure 7. SEM of non-hardened calcium alginate beads. Magnification: 100 X

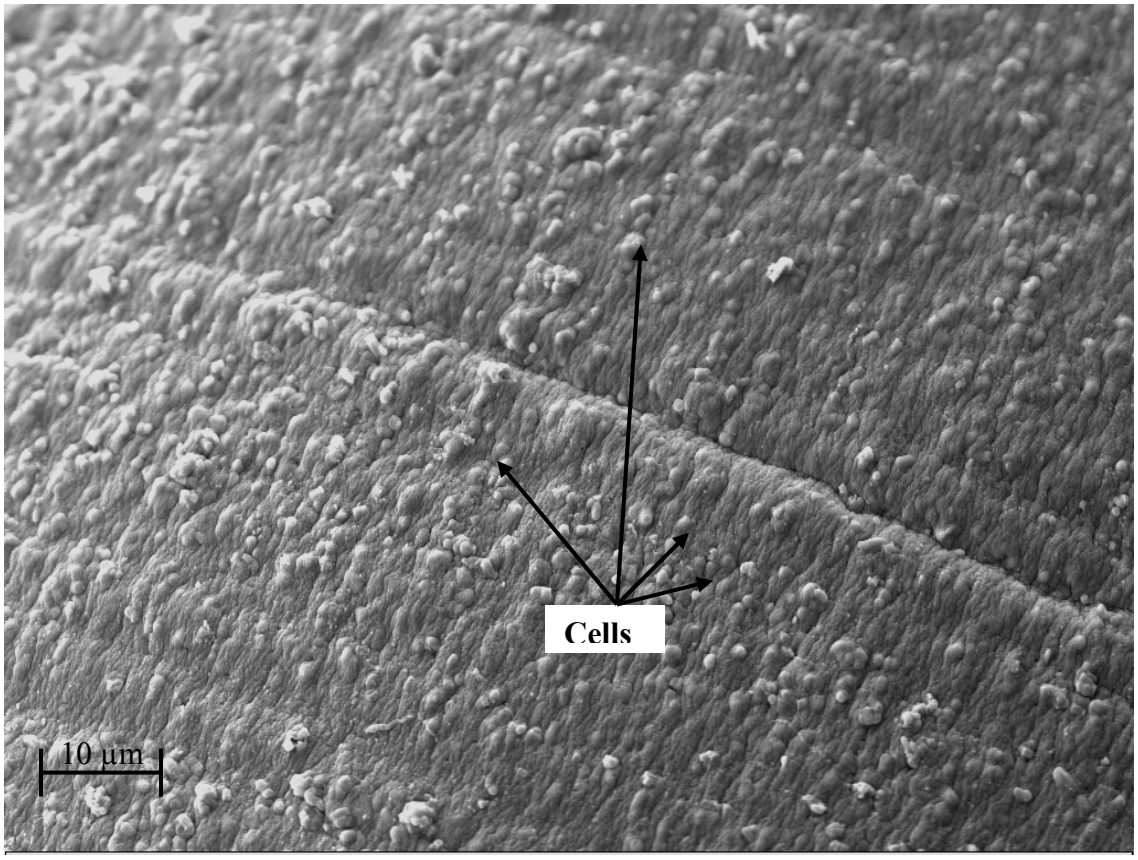


Figure 8. SEM of calcium alginate bead surface showing *Rhodococcus* cells.
Magnification: 2,500 X

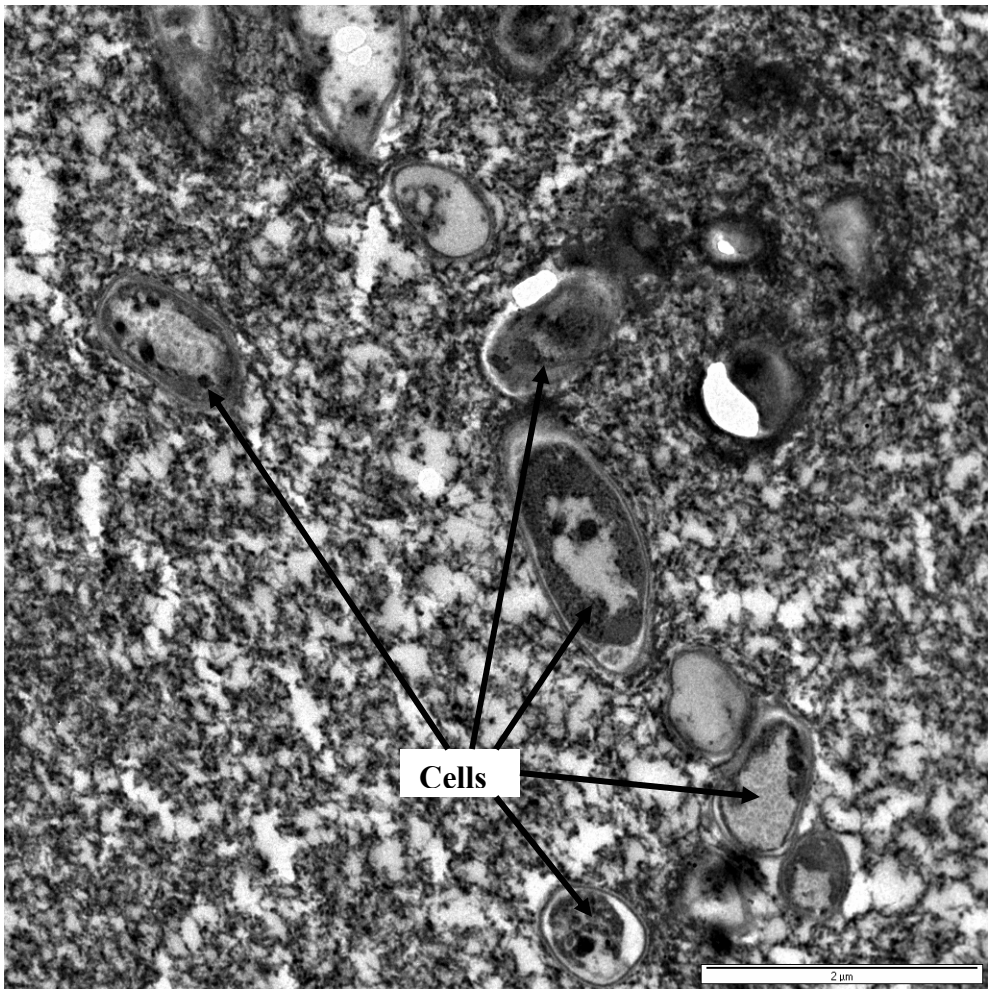


Figure 9. TEM of calcium alginate beads showing *Rhodococcus* cells distributed throughout the catalyst particle. Magnification 5,000 X

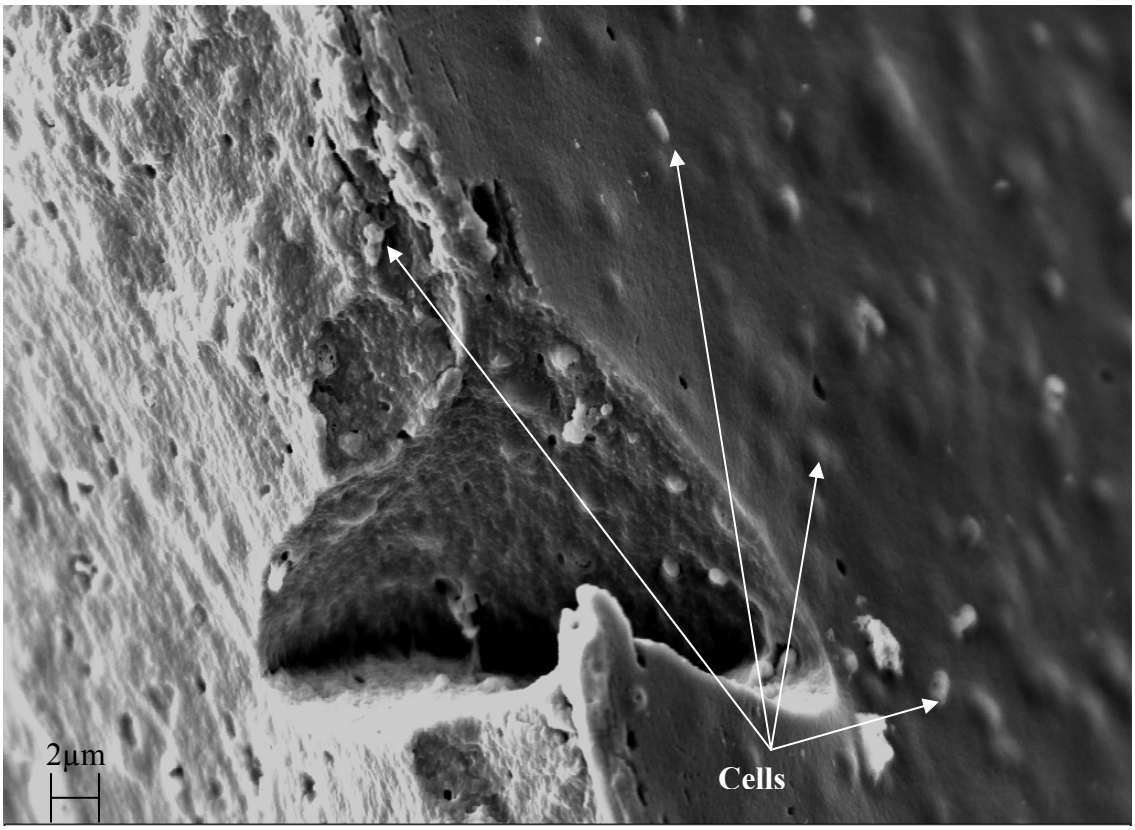


Figure 10. Cut edge of a PAM catalyst cube. Magnification: 5,000 X

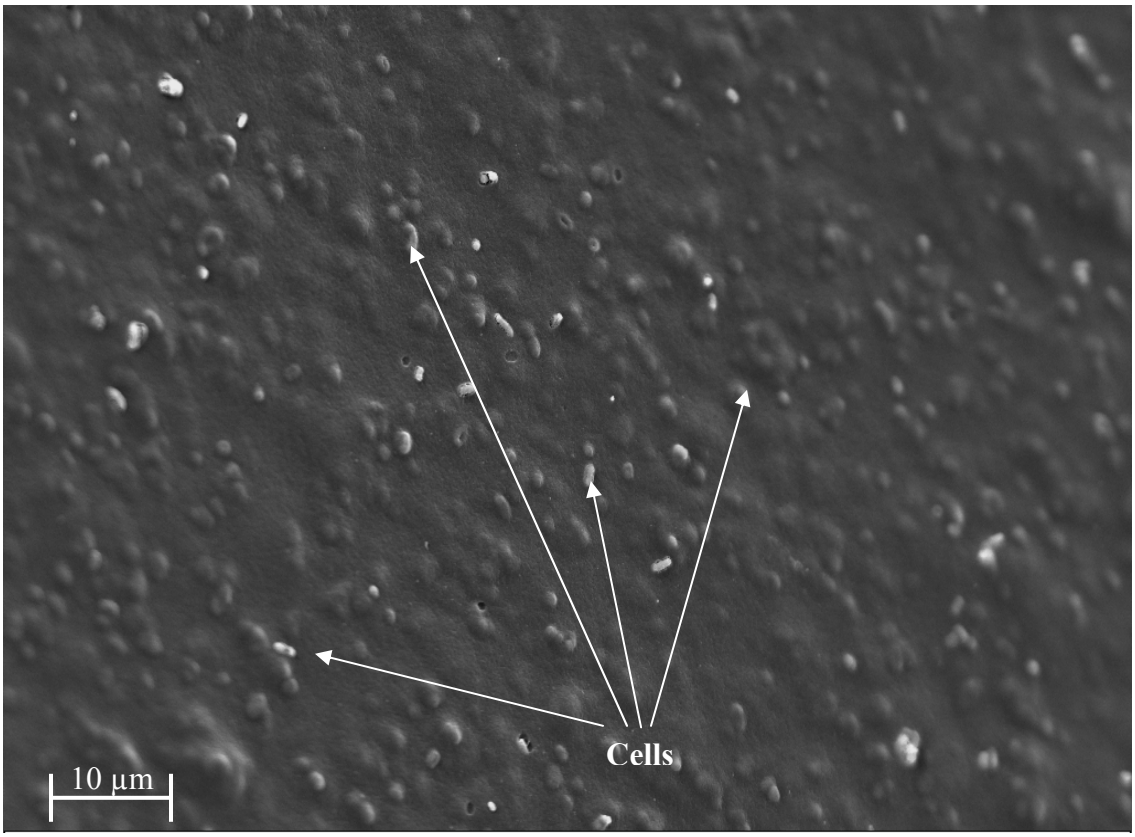


Figure 11. SEM of surface: PAM catalyst particle, showing *Rhodococcus* cells.
Magnification: 2,500 X.

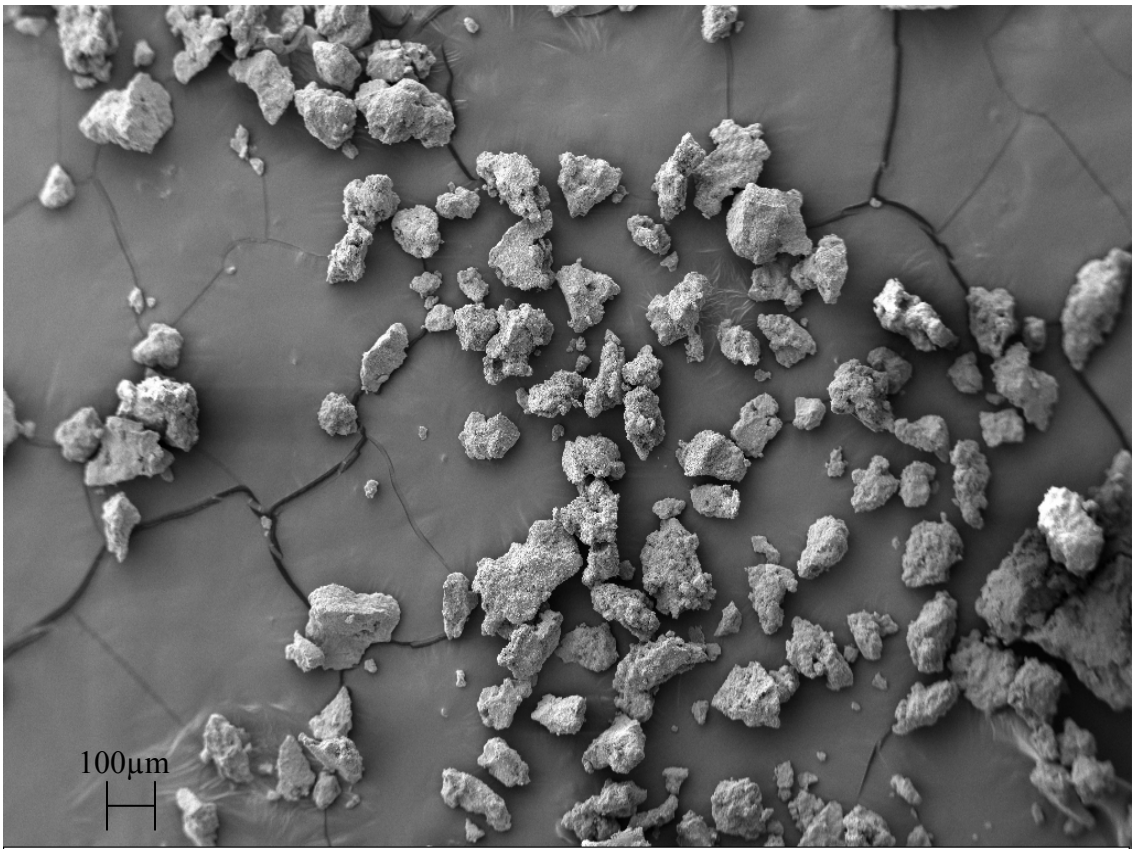


Figure 12. SEM of glutaraldehyde immobilized *Rhodococcus* cells. Magnification: 50 X

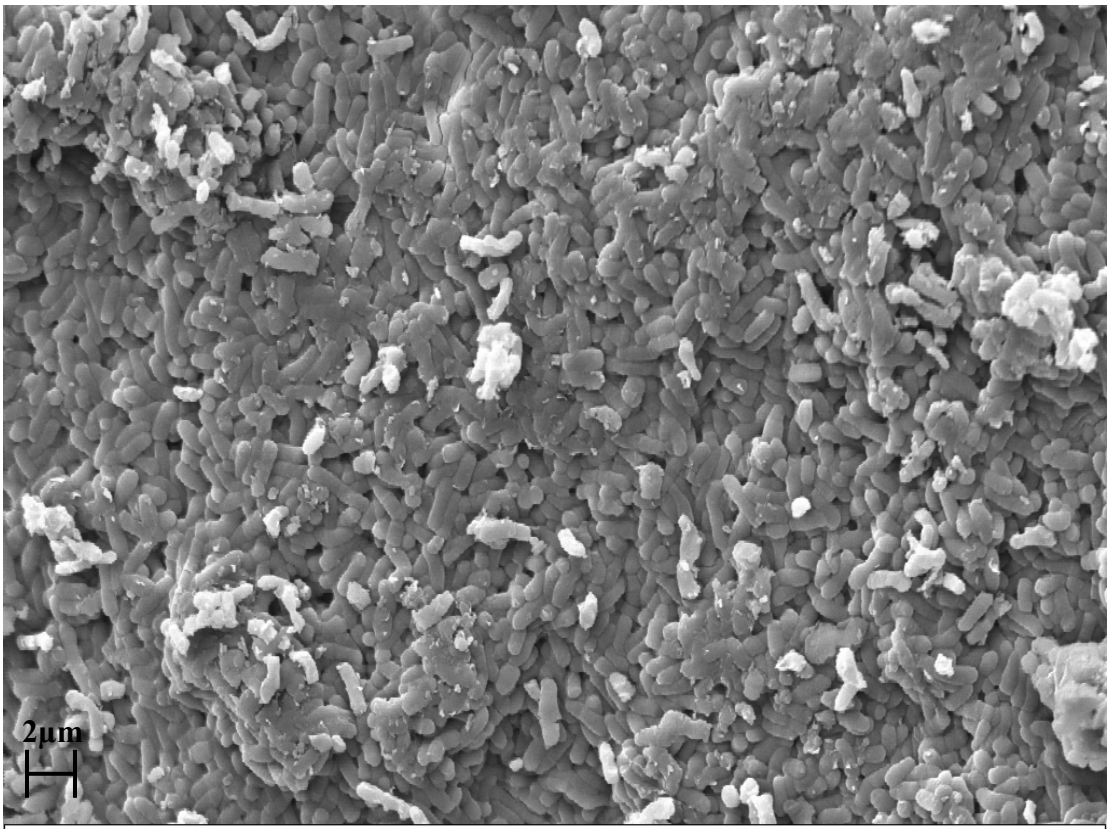


Figure 13. SEM of glutaraldehyde catalyst particles showing *Rhodococcus* cells.
Magnification 5,000 X

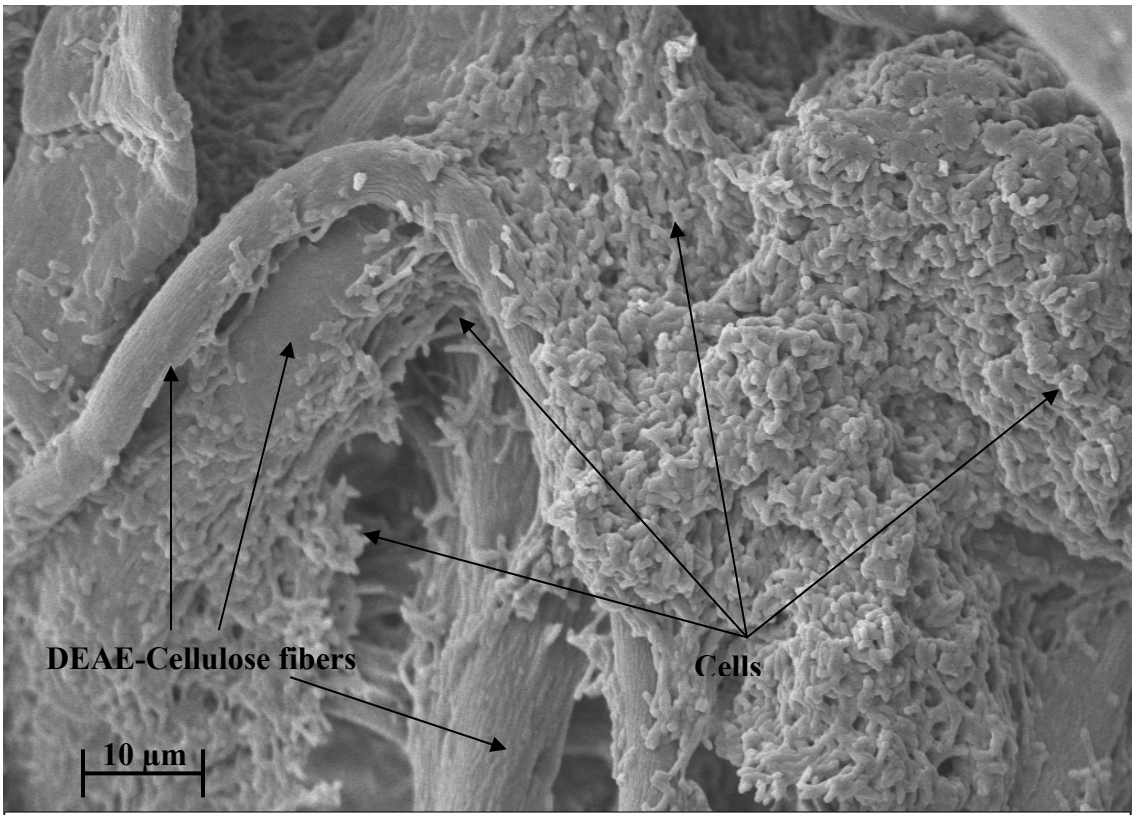


Figure 14. SEM of glutaraldehyde immobilized catalyst particles showing *Rhodococcus* cells cross linked to supporting DEAE cellulose particles. Magnification: 2,500 X.

Table 10. Evaluation of diffusional limitation of catalyst formulations.

Catalyst type	Cell Load (g/50ml catalyst packed vol)	AN concentration in the stock reaction solution (ppm)			
		500	1000	1500	2000
		NHase (U/ml)	NHase (U/ml)	NHase (U/ml)	NHase (U/ml)
Calcium Alginate	1	145	302	491	593
	2	287	479	588	920
	3	350	620	1105	1257
	4	605	1248	1867	2192
PAM	1	165	342	504	695
	2	327	650	1009	1135
	3	420	789	1235	1507
	4	665	1283	1970	2448
Glutaraldehyde	1	115	234	367	478
	2	230	419	650	796
	3	289	532	802	1025
	4	455	837	1354	1788

Units of AN specific activity defined as: μmoles converted/min/ml catalyst packed volume at 30 C, pH 7.0.

this comparison are shown in Tables 11a and 11b. NHase from both DAP 96253 and DAP 96622 showed good activity against all of the nitriles present in wastewater generated in the AN production industry. This precludes the need for additional NHase(s) from other sources, or other processes for complete biodetoxification of the AN production wastewater.

Evaluation of thermal stability of NHase:

When equivalent amounts, of NHase (mg packed wet weight of non-immobilized and immobilized), were incubated at 50-55 C, the immobilized NHase showed a higher initial activity compared to the non-immobilized catalyst (see Figure 15). Non-immobilized NHase showed 10% loss of activity in about 2 hours and complete loss of activity in 23 hours at this restrictive temperature. Even when maintained at optimum temperature for each *Rhodococcus* strain (30 C or 26 C), the non-immobilized cells rapidly lost all activity, compared to the immobilized cells. Immobilization in Ca-alginate matrix helped to stabilize NHase to some extent, and it took 34 hours to reach complete inactivation when maintained at 50-55 C, pH 7.0.

Figures 16-27 show the effect of simultaneous immobilization and chemical stabilization (i.e. chemical stabilizer added during immobilization) on the thermal stability (at 55 C) of NHase from *Rhodococcus sp*. DAP 96253 and *R. rhodococcus* DAP 96622. The effects of various nitriles, amides and carboxylic acids upon the AN specific NHase stability from these two strains of *Rhodococcus* was evaluated using actual waste, simulated waste and individual compounds. Actual waste contains considerable amounts of free cyanide, which has been reported to be detrimental to NHase stability in other reported organisms. Effect of free cyanide on NHase in the two DAP strains was tested

Table 11a. Relative activity of NHase(s) in induced cells of selected strains against different aliphatic nitriles[a,b] .

Substrate (1000ppm)	*Rhodococcus sp.* DAP 96253	*Rhodococcus rhodochrous* DAP 96622	*Rhodococcus erythropolis* *	*Brevibacterium ketoglutamicum*
Isobutyronitrile	66	36	50	51
Succinonitrile	65	28	53	55
Isovaleronitrile	45	16	53	67
DL-Lactonitrile	14	11	11	8
Fumaronitrile	46	56	95	74
Glycolonitrile	30	8	10	6
Acetonitrile	100	79	55	60
Acrylonitrile	89	77	92	51
Adiponitrile	60	46	49	111

* Not induced

[a] All activities compared to *Rhodococcus sp*. 39484 against acetonitrile

[b] All assays were performed using the same amount of cells, and all cells were grown up in the same induction medium.

Table 11b. Relative activity of NHase(s) in induced cells of selected strains against different aromatic nitriles[a,b]

Substrate (1000ppm)	*Rhodococcus sp.* DAP 96253	*Rhodococcus rhodochrous* DAP 96622	*Rhodococcus erythropolis* *	*Brevibacterium ketoglutamicum*
Benzonitrile	51	24	56	85
Cinnamonitrile	51	25	62	60

* Not induced

[a] All activities compared to *Rhodococcus sp*. 39484 against acetonitrile

[b] All assays were performed using the same amount of cells, and all cells were grown up in the same induction medium.

Note: Activity (Units/min) by DAP 96253 vs. AC was set as 100% and all other activities compared to this level.

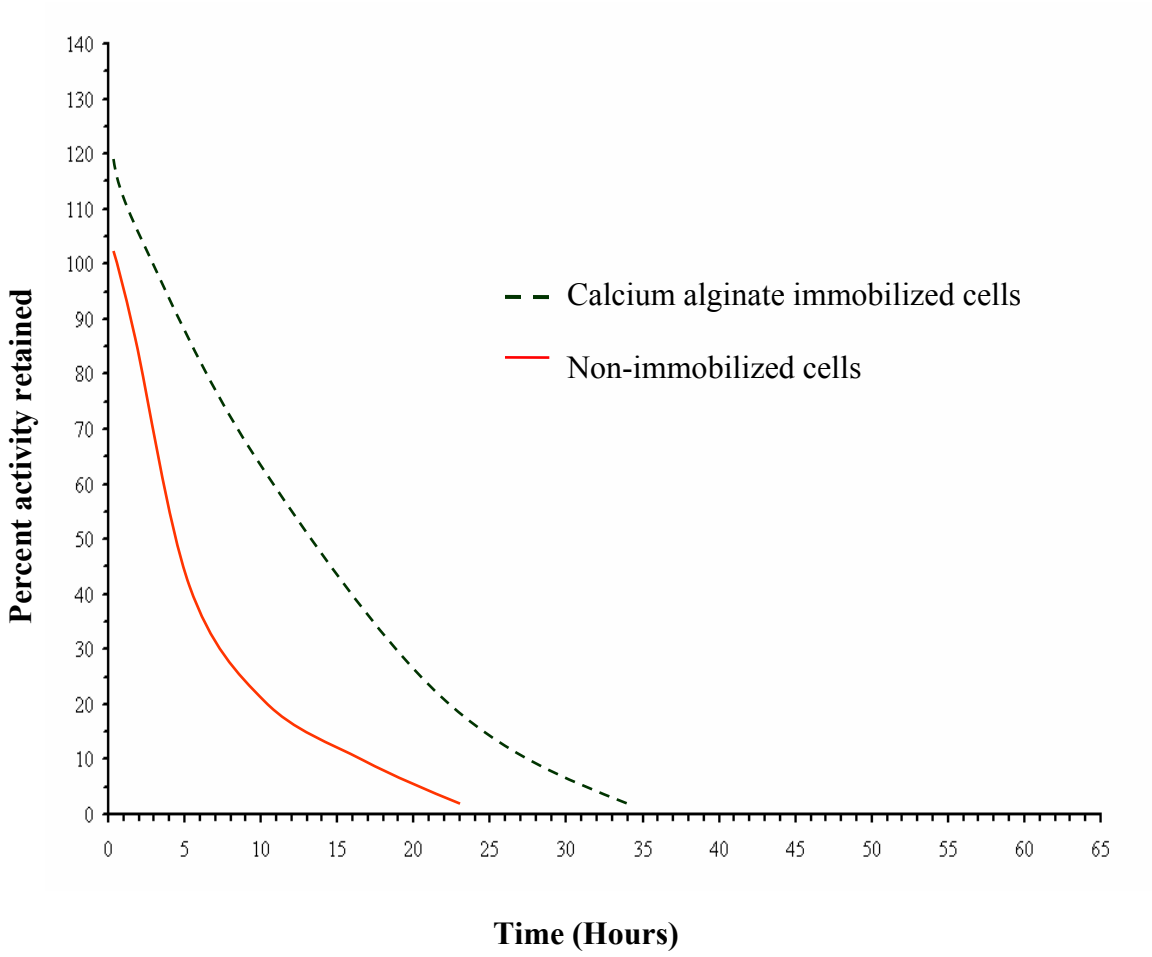


Figure 15. Effect of immobilization on NHase stability at 50-55 C. *Rhodococcus* cells grown on YEMEA supplemented with cobalt, urea and AN. 100% activity corresponds to 150 U/mg cdw at t=0. Standard deviation ± 0.02-1.2.

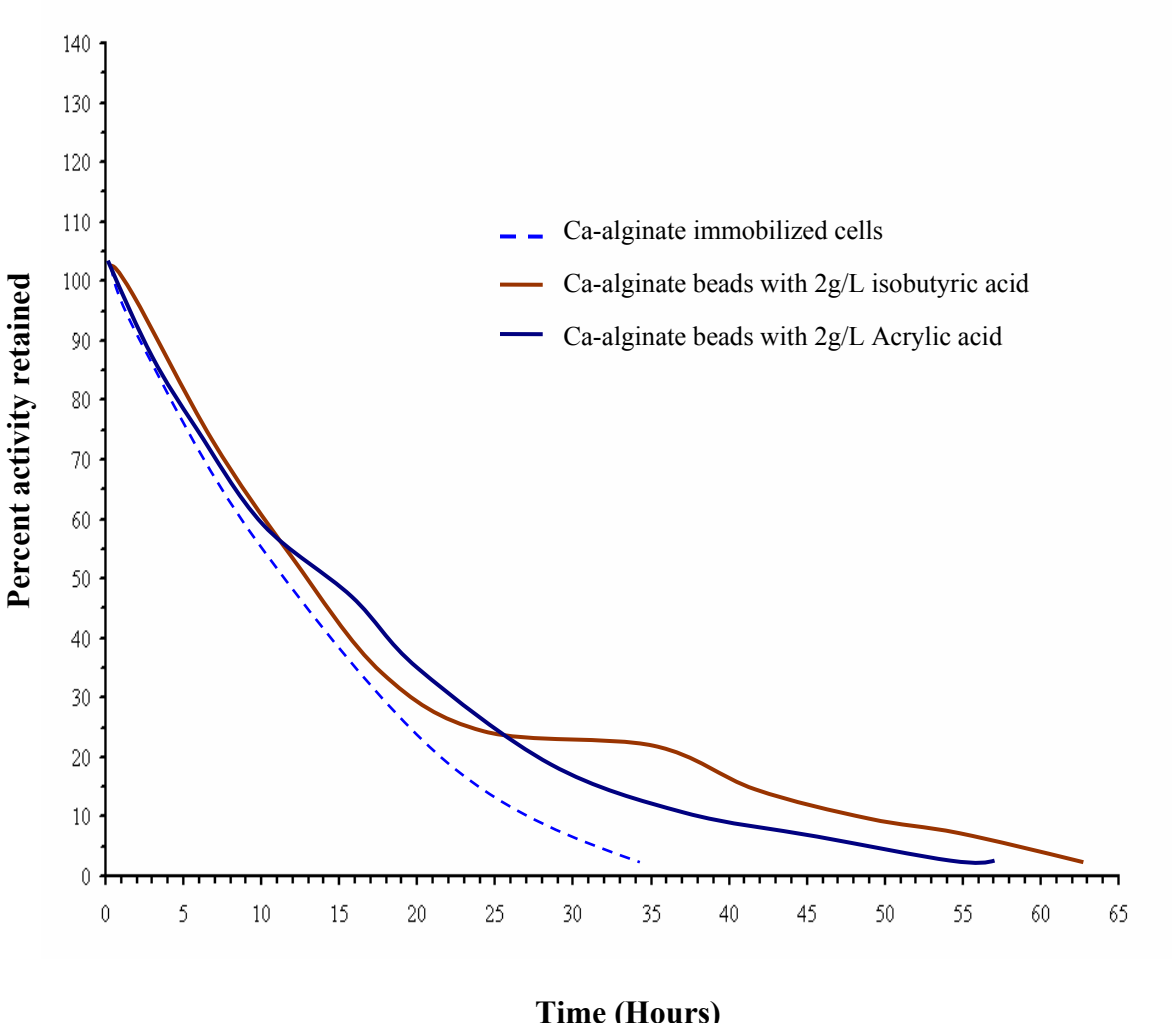


Figure 16. Effect of simultaneous immobilization and chemical stabilization on NHase stability. Cells were grown on solid YEMEA medium with cobalt, urea and AN supplementation. The Ca-alginate catalyst formulations were maintained at 50-55 C. Standard deviation ± 0.04-0.5.

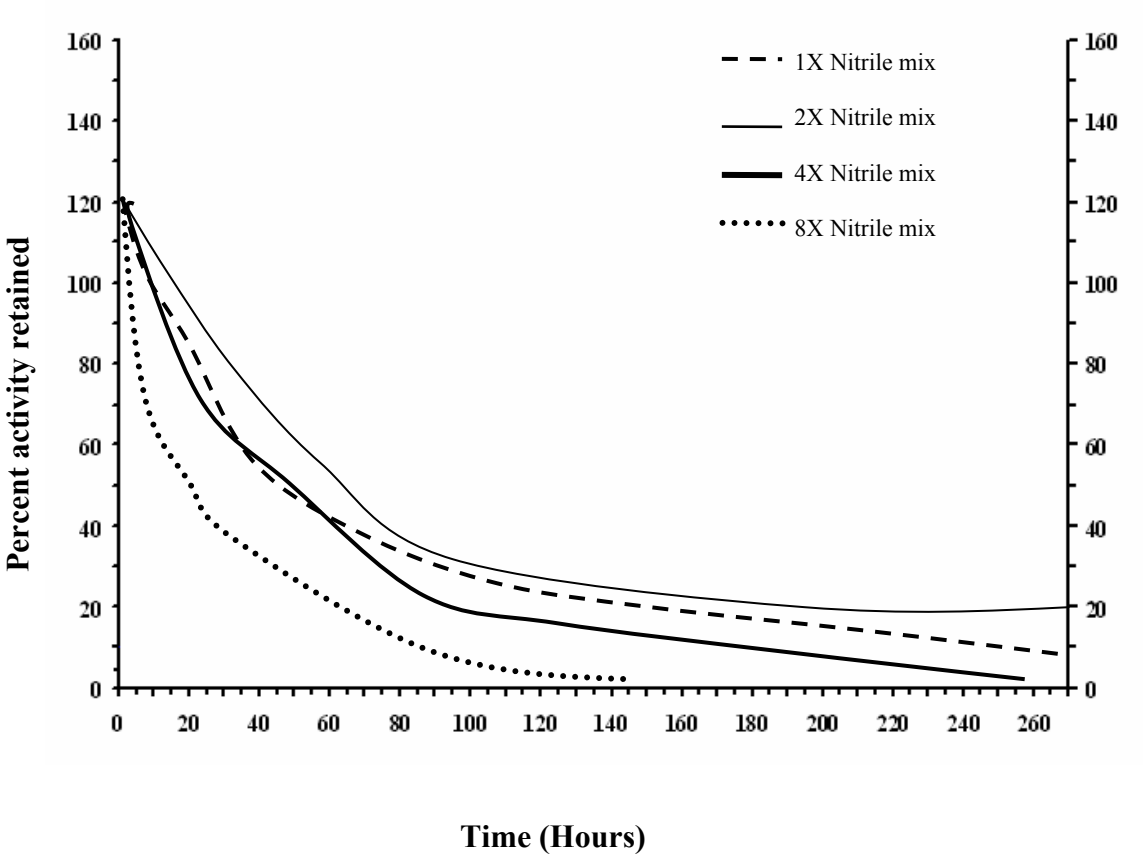


Figure 17a. Effect of nitrile mixture on NHase stability in *Rhodococcus sp.* DAP 96253. Cells were immobilized in calcium alginate matrix and stabilized with a mixture.

Nitrile mixture is AN:AC:SN=3:3:1.

1X= 2 g/L total nitriles, 2X= 4 g/L total nitriles, 4X=8 g/L total nitriles, 8X= 16 g/L total nitriles. Catalysts maintained at 50-55 C. Standard deviation ±0.07-0.9.

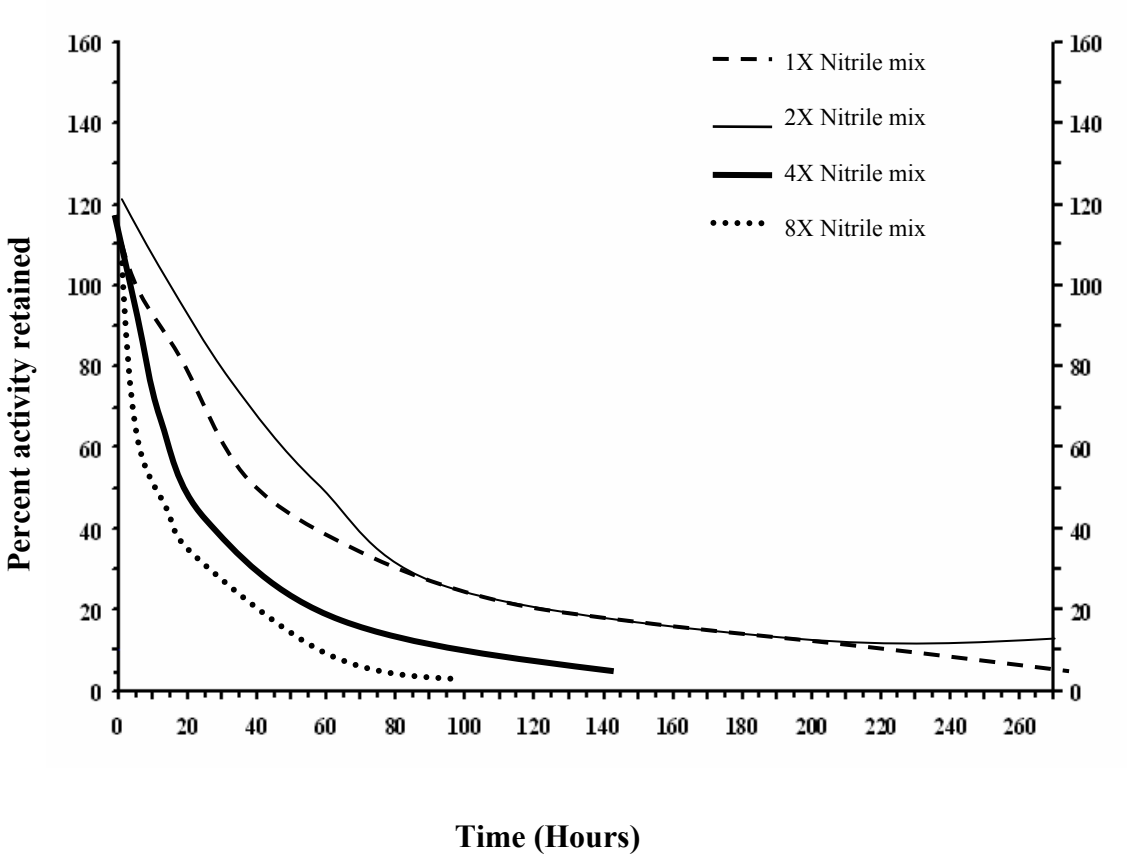


Figure 17b. Effect of nitrile mixture on NHase stability in *R. rhodochrous sp.* DAP 96622. Cells were immobilized in calcium alginate matrix and stabilized with a mixture of AN, AC and SN. 1X= 2 g/L total nitriles, 2X= 4 g/L total nitriles, 4X=8 g/L total nitriles, 8X= 16 g/L total nitriles. Catalysts maintained at 50-55 C. Standard deviation ±0.1-0.6.

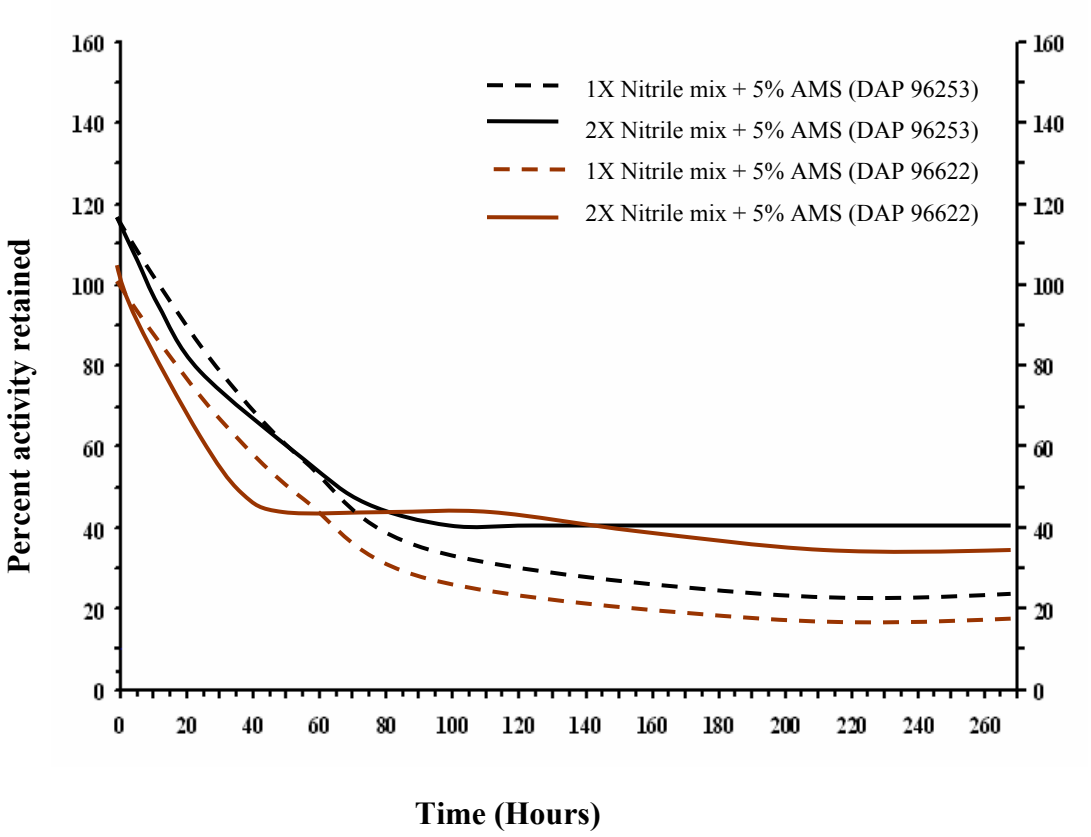


Figure 18. Effect of ammonium sulfate (AMS) on *Rhodococcus* NHase stabilized with nitrile mix (2 g/L). Catalysts maintained at 50-55 C. Standard deviation ± 0.9-1.2.

by exposing the immobilized cell catalyst to ≥300 ppm of cyanide concentrations. The NHase from the two DAP strains did not show significant cyanide susceptibility when the catalyst was stabilized with mixtures of AC, AN and SN. The tolerance to HCN enhances the feasibility of these NHases as biocatalysts for waste detoxification purposes, where the wastewater contains elevated amounts of free cyanide.

The chemicals present in the actual waste showed significant stabilization of the NHases in both DAP strains, and particularly when a mixture of wastewater samples from net stripper bottom and wastewater column bottom was used in the ratio of 1:4. The level of stability achieved for NHase formulations when the non-toxic inducer (L-Asn) was used, was slightly lower than that achieved by the nitriles and amides. But when the factors of toxicity and risk posed by the traditional nitrile stabilizers are considered, L-Asn represents a good, safe stabilizer. While GA immobilization reduced the initial activity 20% compared to Ca-alginate immobilized NHase, the stability of the GA catalyst, even without stabilizers was far superior.

Traditional techniques for GA immobilization require a large amount of cells per unit volume of catalyst. To reduce the amount of cells needed, an inert "filler" material (DEAE-cellulose) was added to increase the volume without requiring large amounts of cells and without compromising the quality of the catalyst. Addition of DEAE-cellulose fibers to the GA catalyst formulation during casting resulted in an increase in catalyst volume with fewer cells (wet wt) being used to achieve a certain volume. DEAE-cellulose, GA-immobilized cells showed comparable NHase stability to that of the GA immobilized cells.

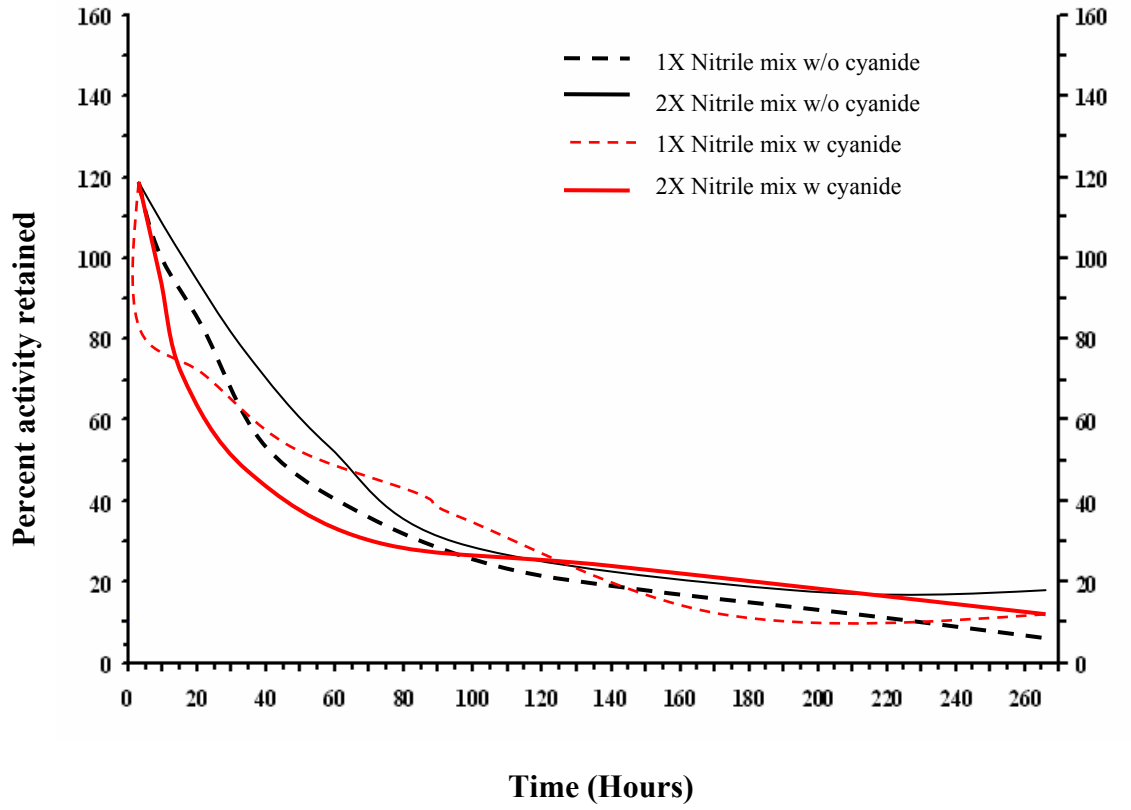


Figure 19a. Effect of free cyanide on *Rhodococcus sp*. DAP 96253 NHase. Cells immobilized in calcium alginate matrix and stabilized with AN, AC and SN; and maintained at 50-55 C. Standard deviation ± 0.3 – 0.8.

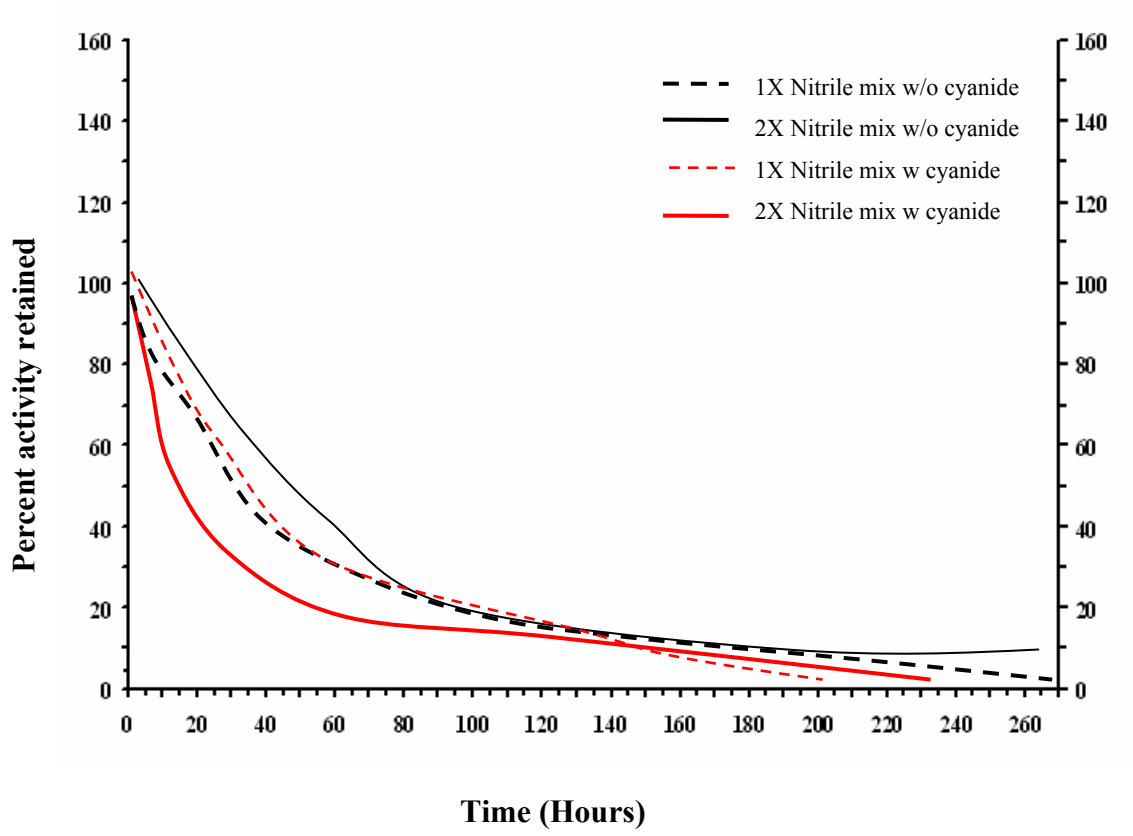


Figure 19b. Effect of free cyanide on NHase from *R. rhodochrous* DAP 96622. Cells immobilized in calcium alginate matrix and stabilized with AN, AC and SN; and maintained at 50-55 C. Standard deviation ± 0.2-1.1.

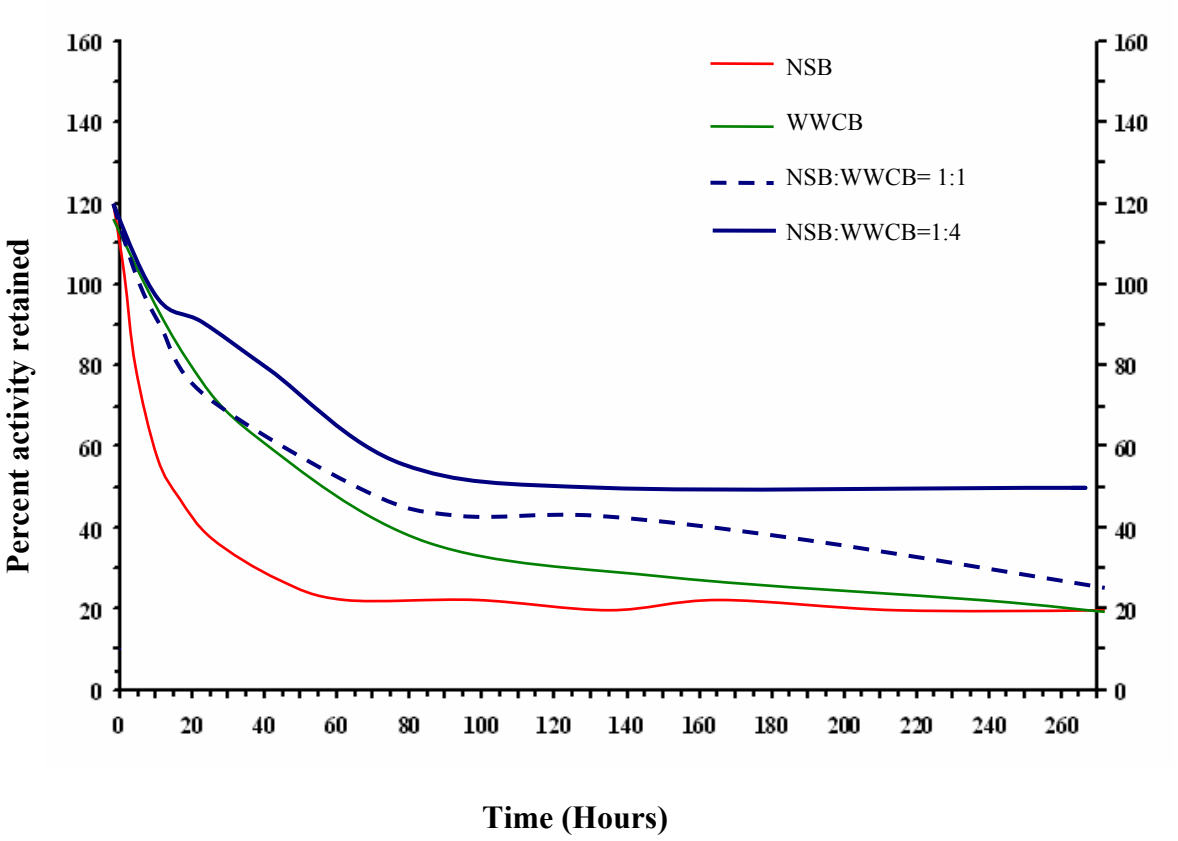


Figure 20a. Evaluation of actual waste chemicals on *Rhodococcus sp*. DAP 96253 NHase stability. Cells immobilized in PEI cross linked Ca-alginate matrix, maintained at 50-55 C. NSB=Net Stripper Bottom, WWCB=Waste Water Column Bottom. Standard deviation ± 0.75-1.5.

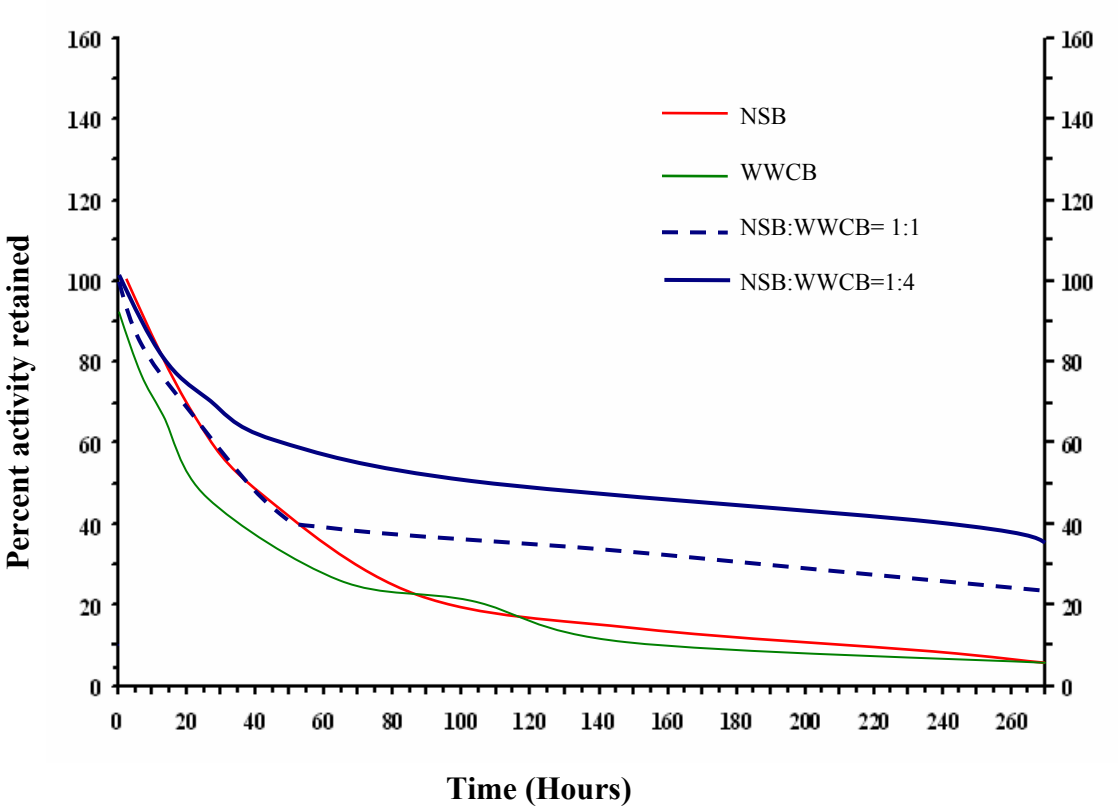


Figure 20b. Evaluation of actual waste chemicals on *R. rhodochrous sp.* DAP 96622 NHase stability. Cells immobilized in PEI cross linked Ca-alginate matrix, maintained at 50-55 C. NSB=Net Stripper Bottom, WWCB=Waste Water Column Bottom. Standard deviation ± 0.6-0.8.

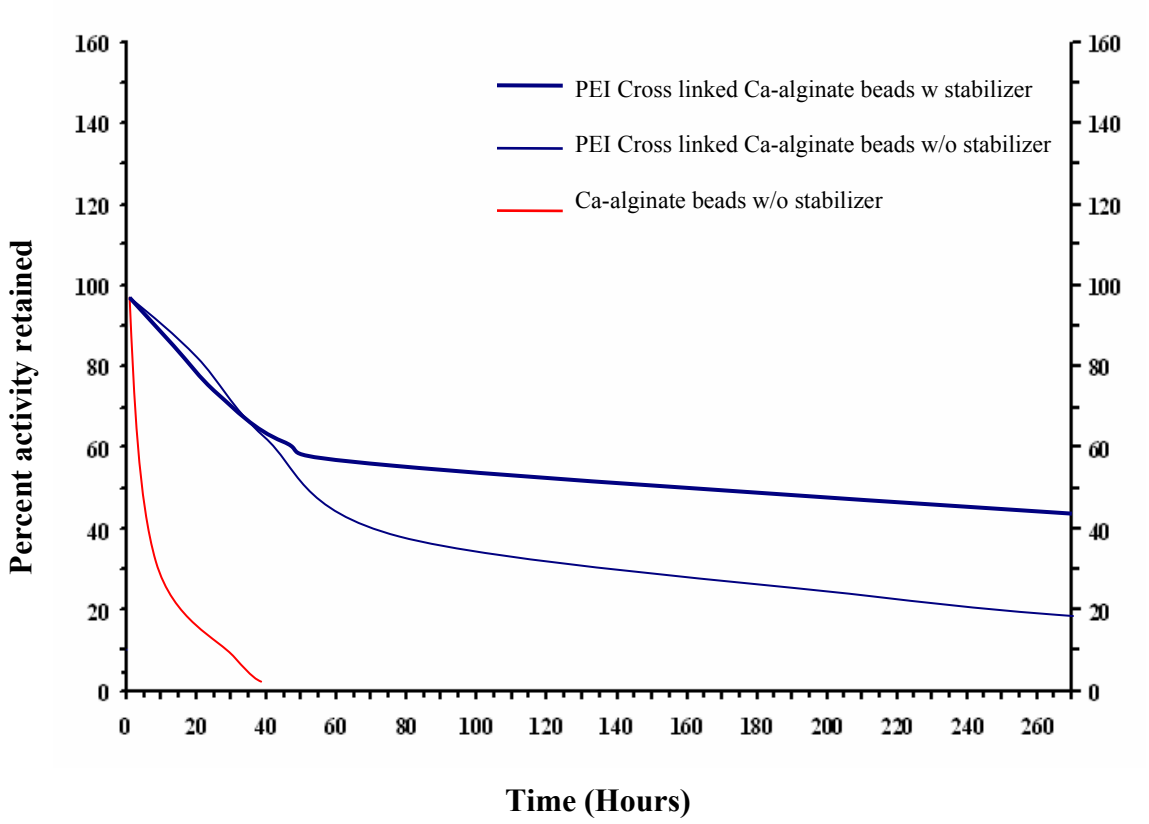


Figure 21. Comparison of *Rhodococcus sp*. DAP 96253 NHase stability in calcium alginate immobilized and polyethyleneimine (PEI) crosslinked calcium alginate beads. The catalyst formulations were maintained at 50-55 C. When used, nitrile mix (AN:AC:SN=3:3:1) with 5% AMS was the stabilizer. Standard deviation ± 0.8-1.4.

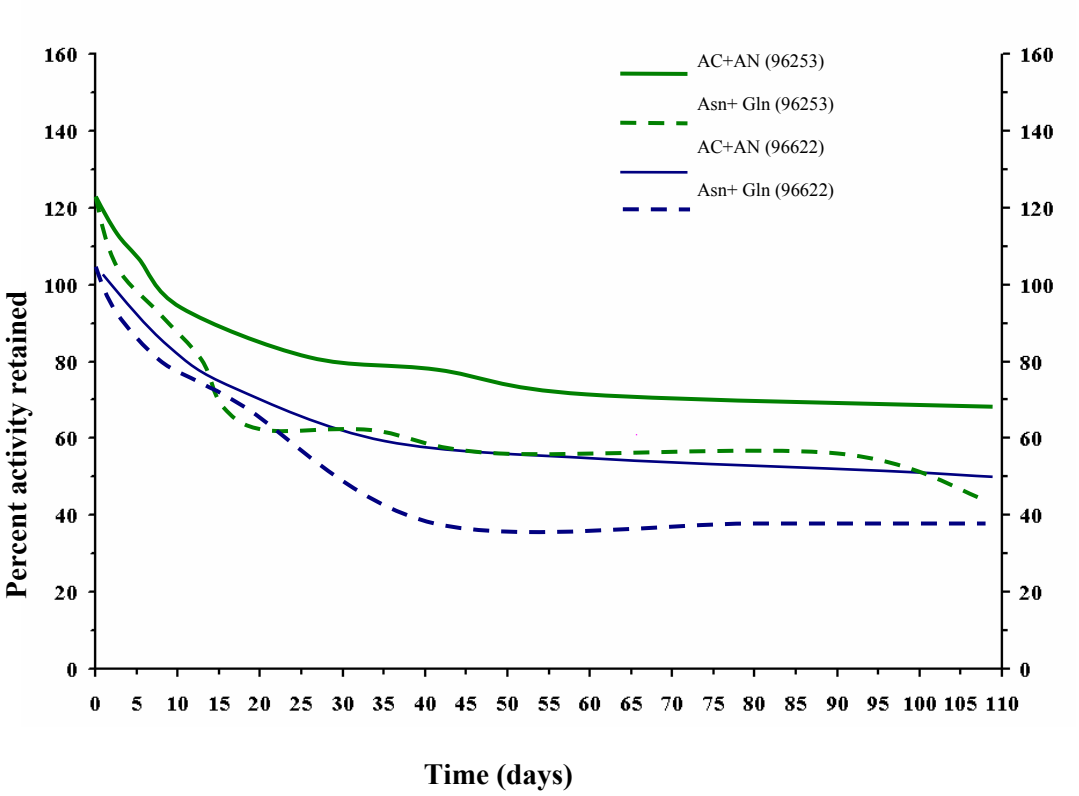


Figure 22. Evaluation of stabilizing effects of non toxic amino acids L-Asn and L-Gln on NHase. Cells immobilized in PEI cross linked calcium alginate beads, maintained at 50-55 C. Each amino acid and nitrile added in equal amounts to reach a final concentration of 2 g/L. Standard deviation ± 0.6-0.85.

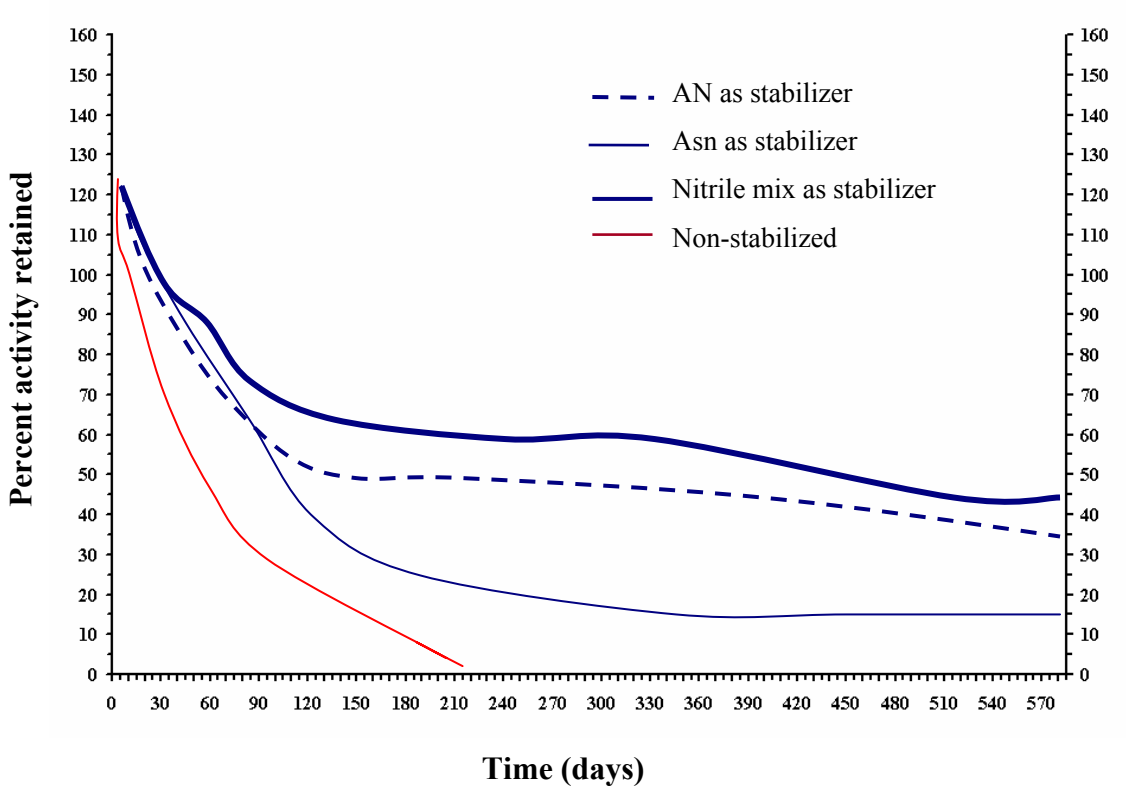


Figure 23a. PAM immobilization of *Rhodococcus sp*. DAP 96253 cells. Catalyst cubes (2 mm^3) were maintained at 30 C, pH 7.0. Standard deviation ± 0.2-1.8.

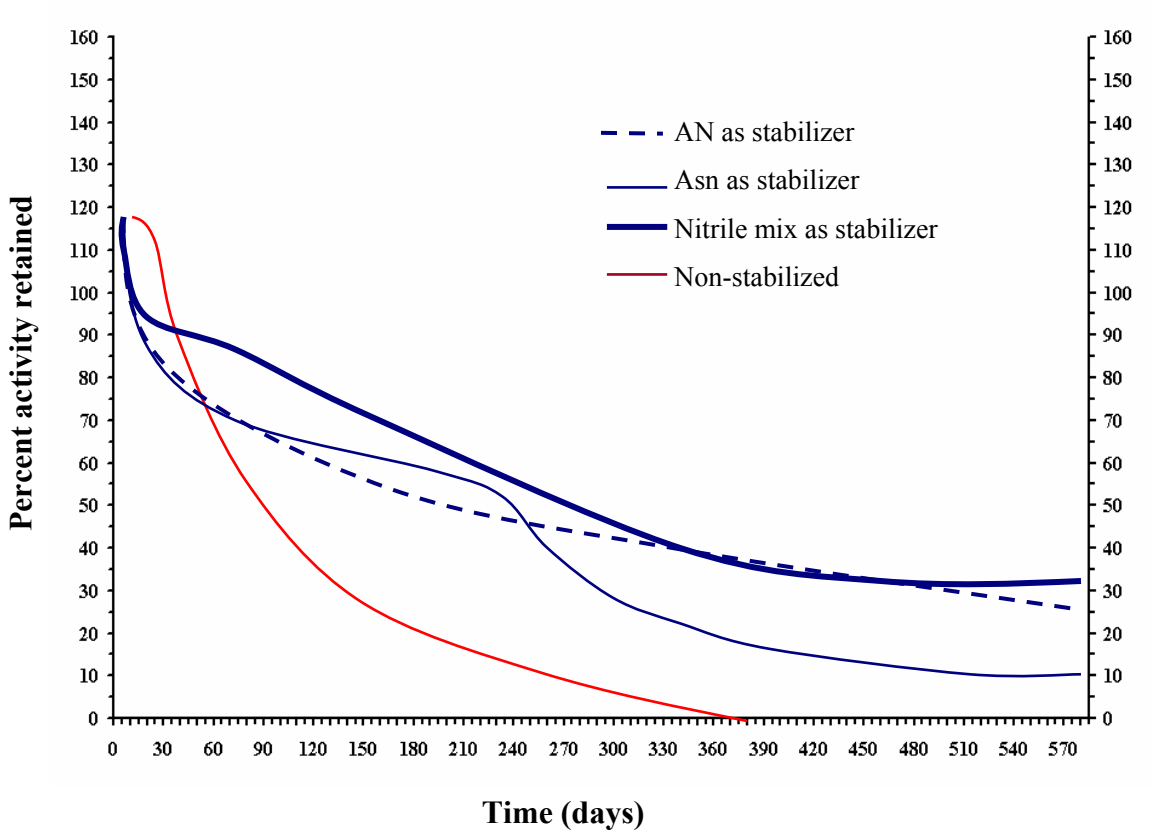


Figure 23b. PAM immobilization of *R. rhodochrous* DAP 96622 cells. Catalyst cubes (2 mm^3) were maintained at 30 C, pH 7.0. Standard deviation ± 0.45-0.9.

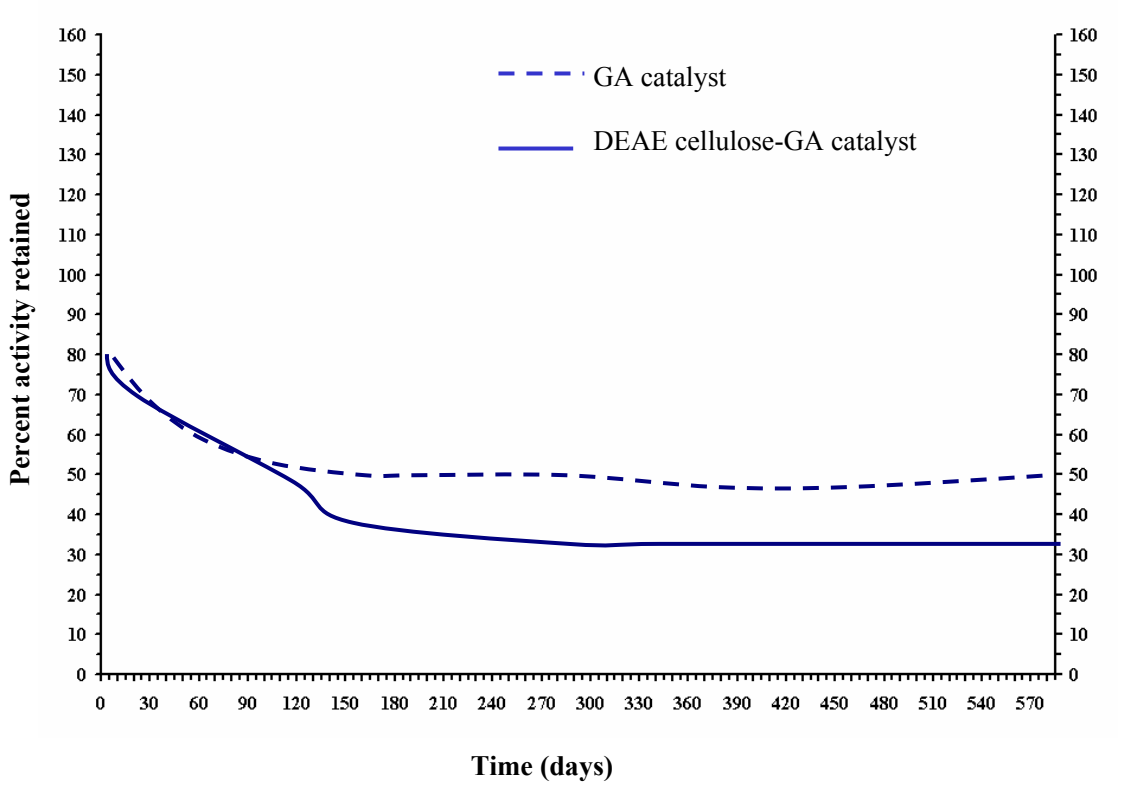


Figure 24a. Effect of glutaraldehyde (GA) immobilization on stability of intracellular NHase from *Rhodococcus sp*. DAP 96253. Catalyst particles maintained at 30 C, pH 7.0. Standard deviation ± 0.08-0.4.

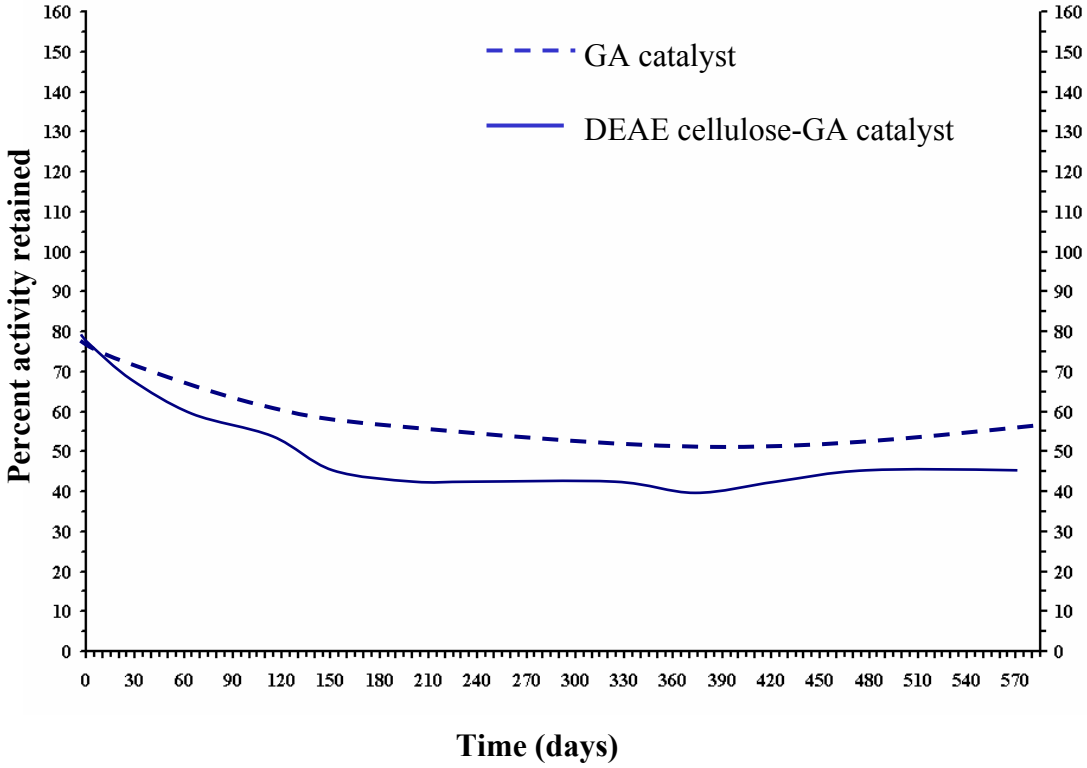


Figure 24b. Effect of glutaraldehyde (GA) immobilization on stability of intracellular NHase from *R. rhodochrous sp*. DAP 96622. Catalyst particles maintained at 30 C, pH 7.0. Standard deviation ± 0.04-0.2.

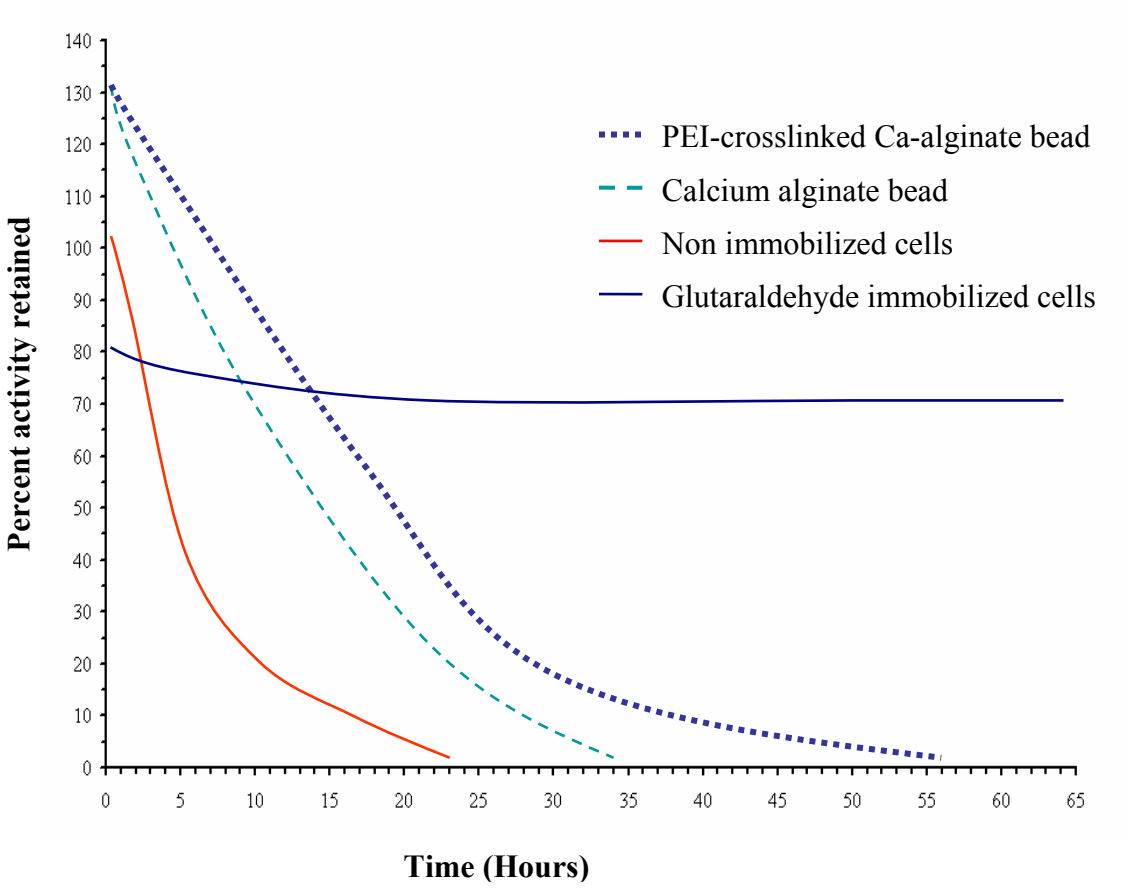


Figure25. Comparison of different immobilization techniques on NHase from *Rhodococcus sp*. DAP 96253 at 50-55 C. Standard deviation ± 0.5-1.4.

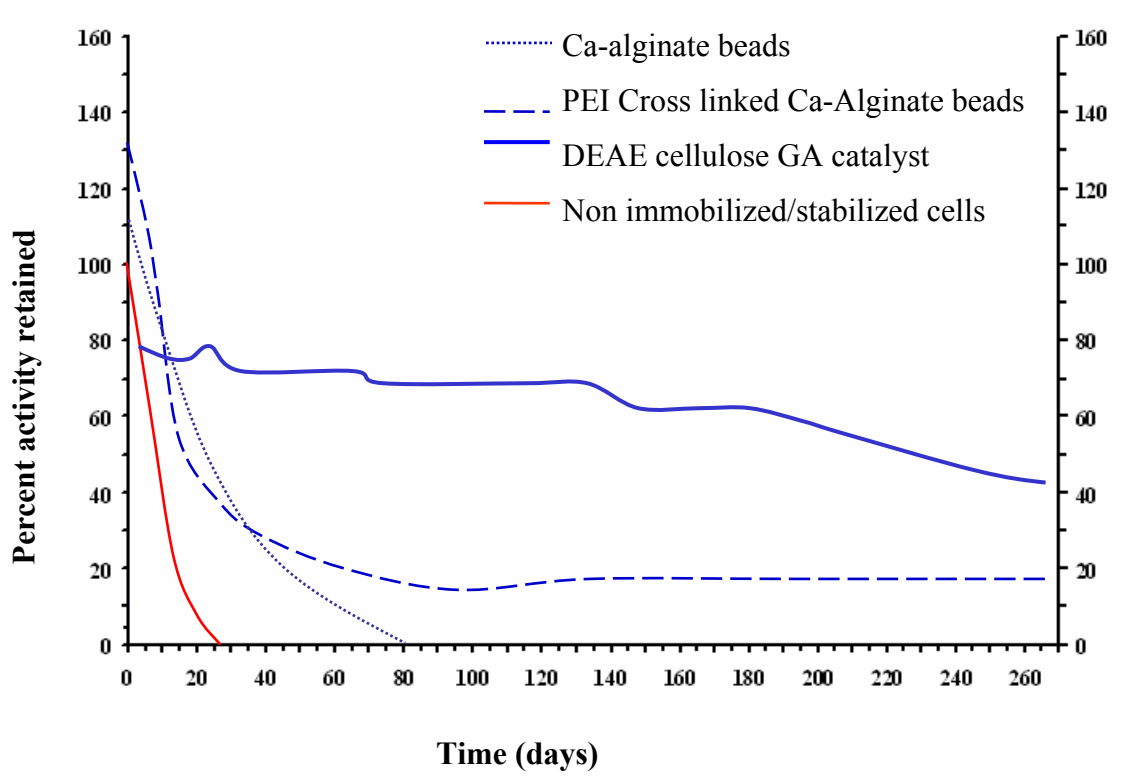


Figure 26a. Comparison of NHase stability in calcium alginate immobilized *Rhodococcus sp.* DAP 96253 maintained at 50 C. Standard deviation ± 0.2-2.05.

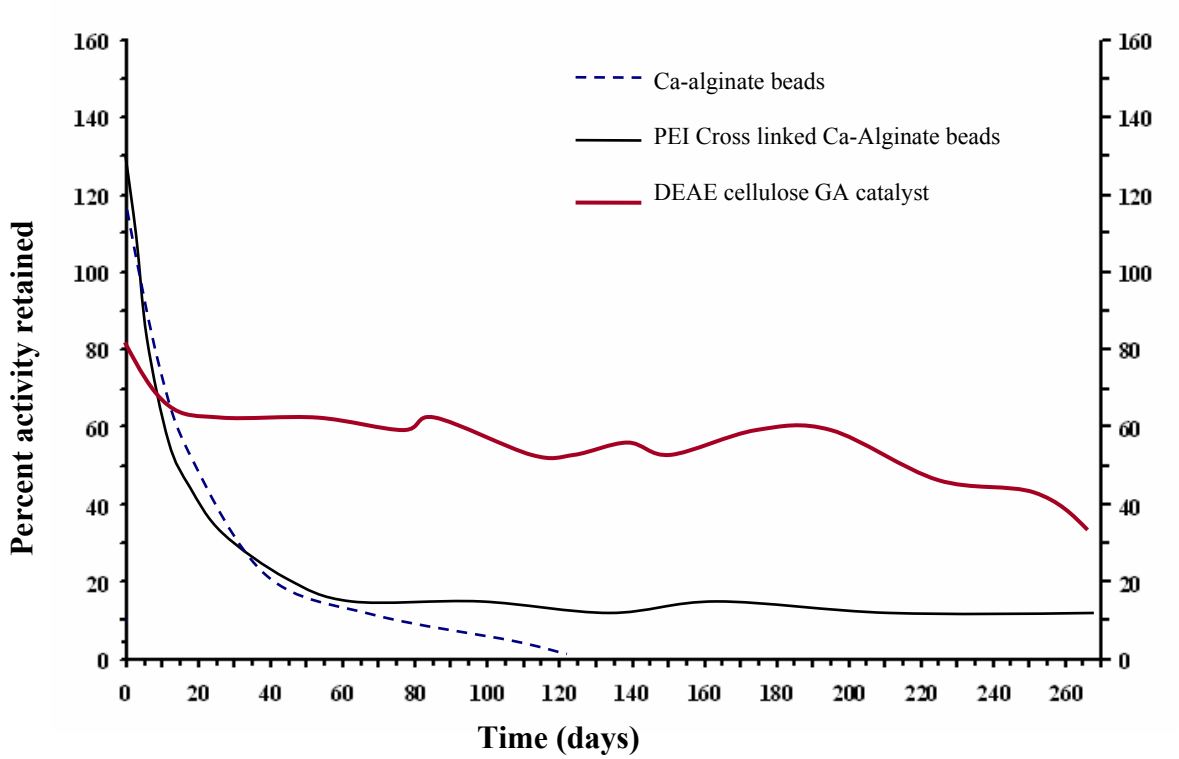


Figure 26b. Comparison of NHase stability in calcium alginate immobilized *Rhodococcus sp.* DAP 96622 maintained at 50 C. Standard deviation ± 0.45-0.7.

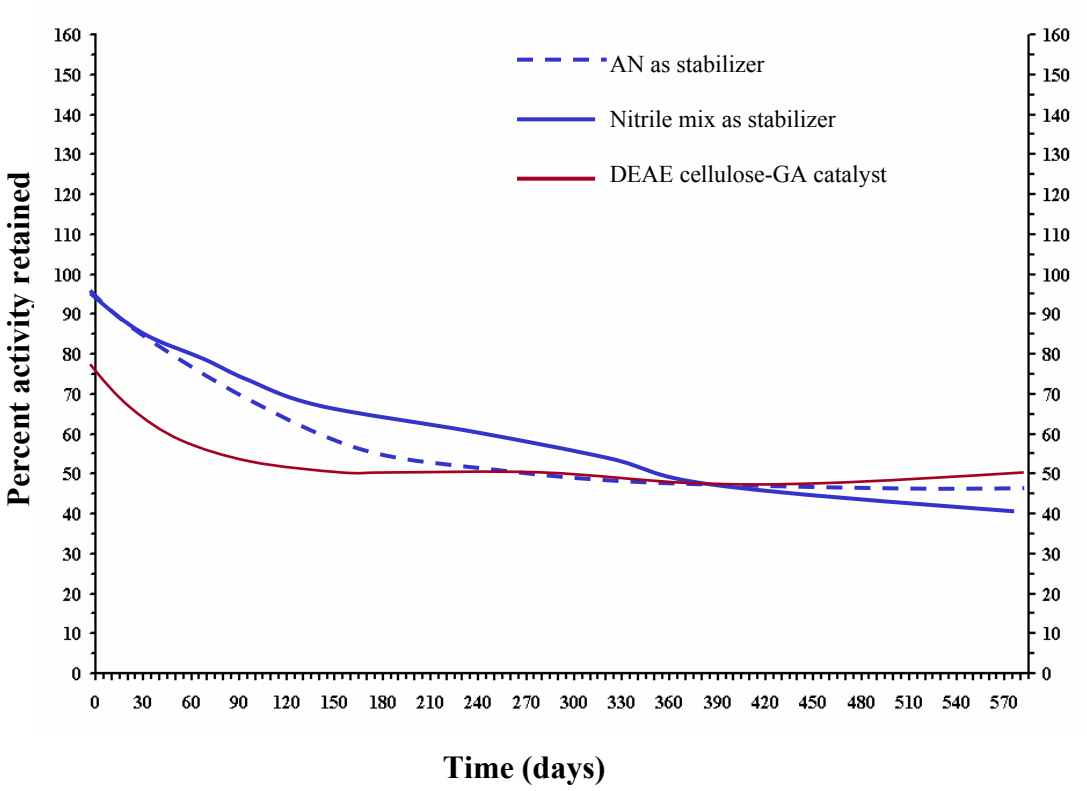


Figure 27a. Effect of chemical stabilization on PEI cross-linked Ca-alginate immobilized *Rhodococcus sp*. DAP 96253 NHase maintained at 30 C, compared to DEAE cellulose GA-catalyst. Standard deviation ± 0.5-2.1.

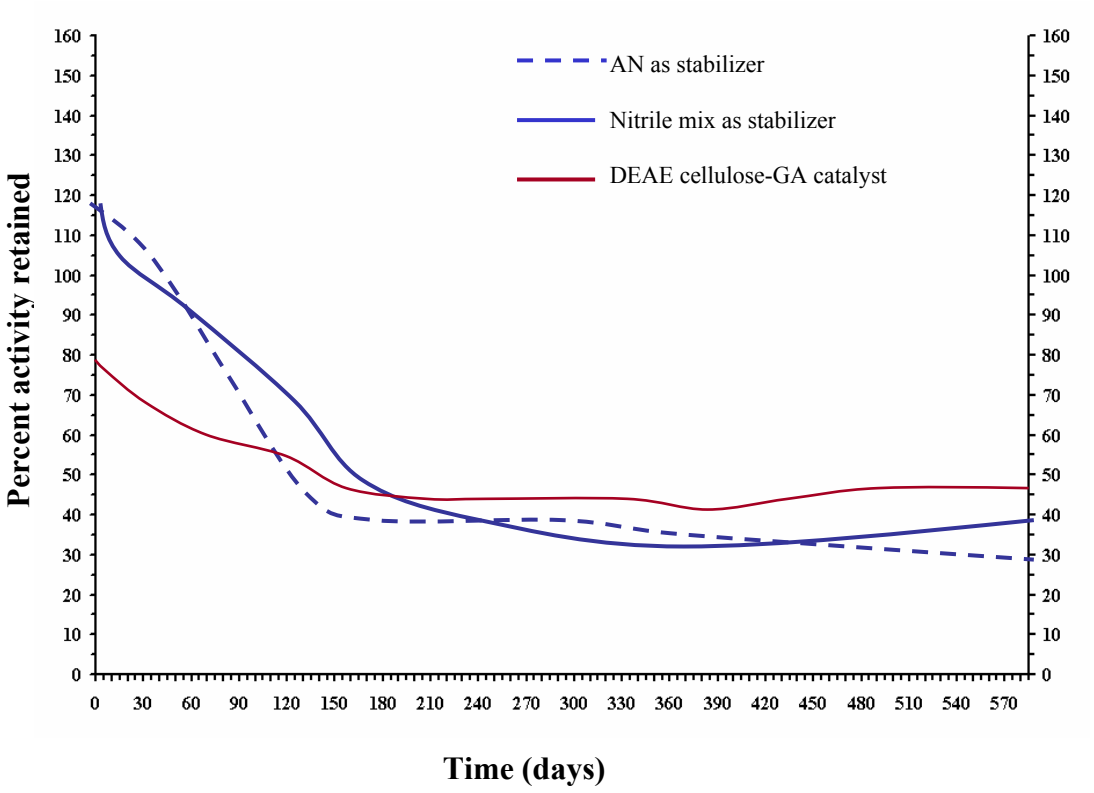


Figure 27b. Effect of chemical stabilization on PEI cross-linked Ca-alginate immobilized *Rhodococcus rhodochrous* DAP 96622 NHase maintained at 30 C, compared to DEAE cellulose GA catalyst. Standard deviation ± 0.3-1.0.

pH stability of NHase immobilized in DEAE cellulose-GA matrix:

The pH stability of the DEAE cellulose, GA-immobilized catalyst was evaluated by exposing catalyst to various pH conditions in the following buffers: acetate (pH 4, 4.5, 5.0, 5.5), citrate-phosphate (pH 6, 6.5), phosphate (7, 7.5, 8), TRIS-HCl (8.5, 9). In Figure 28 AN specific NHase activities of immobilized *Rhodococcus sp*. DAP 96253 are compared to that of immobilized cells of *R. rhodochrous* DAP 96622. In both cases the NHases showed a slightly acidic pH optimum, with DAP 96253 NHase showed highest activity at pH 6.3, while DAP 96622 NHase has a pH optima of 6.8.

Catalyst formulations were maintained at various pH environments and the loss of activity over time was monitored. Effect of various pH conditions on NHase from DAP 96253 is represented in Figure 29a, while that of DAP 96622 is represented in Figure 29b. For DAP 96253, pH conditions of 6.5 to 7 achieved best NHase stability, and for 96622, maintaining at pH 6 resulted in the highest stability of NHase.

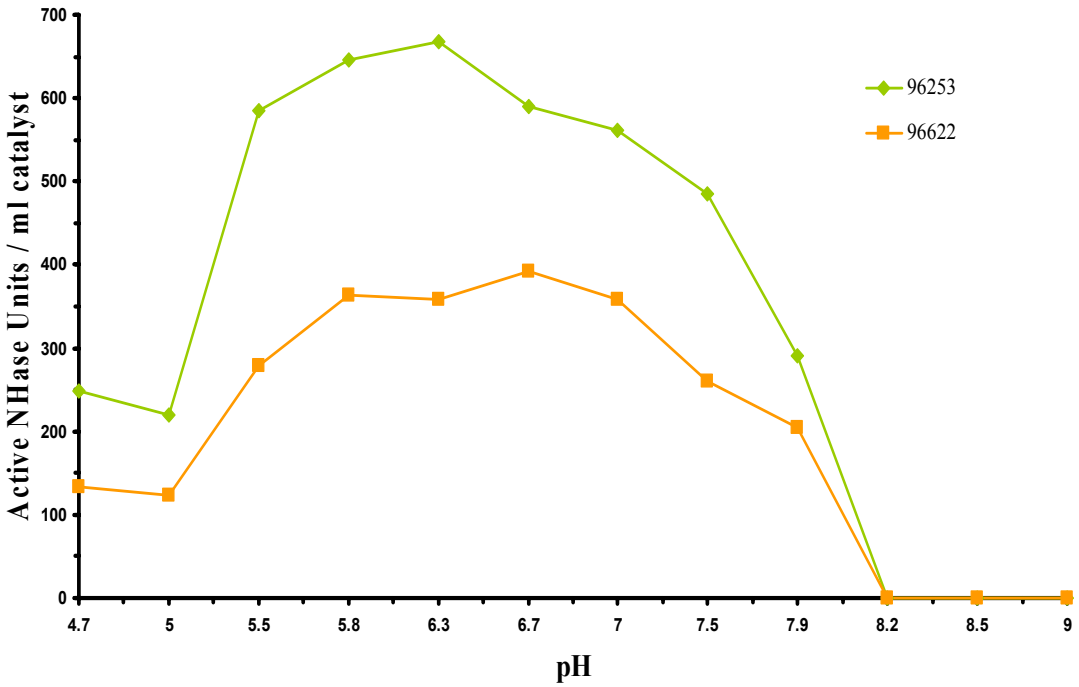


Figure 28. pH stability of glutaraldehyde immobilized NHase from *Rhodococcus*. Catalyst particles maintained at 30 C for DAP 96253 and 26 C for DAP 96622. Standard deviation ± 0.2-0.6.

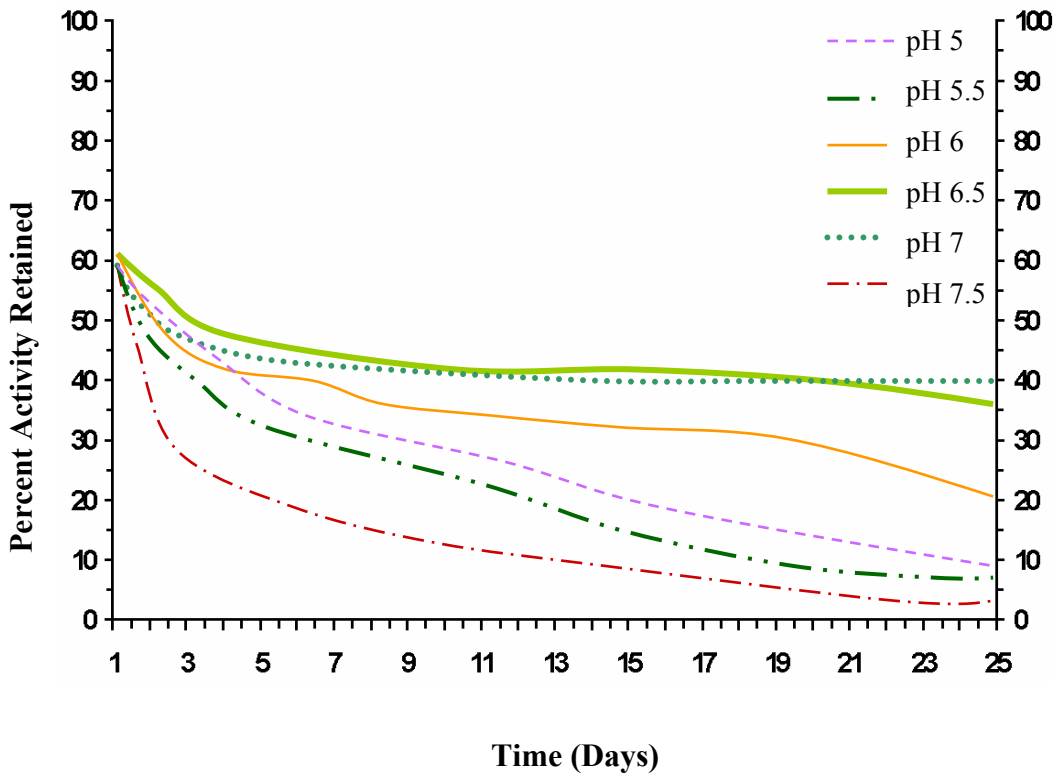


Figure 29a. Effect of selected pHs on *Rhodococcus* 96253 NHase stability in DEAE-cellulose-glutaraldehyde catalyst. Standard deviation ± 0.05-0.2.

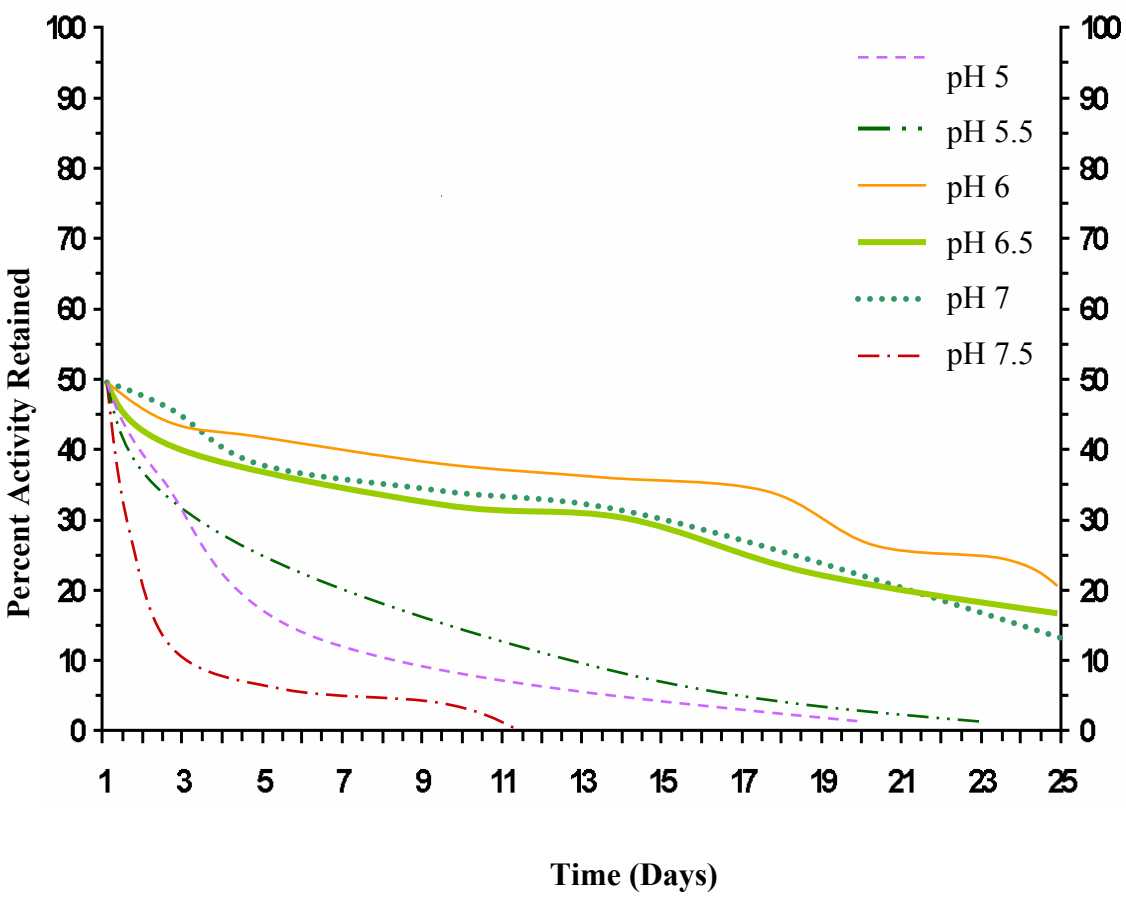


Figure 29b. Effect of selected pHs on *Rhodococcus* 96622 NHase stability in DEAE-cellulose-glutaraldehyde catalyst. Standard deviation ± 0.08-0.3.

Amidase stability during storage:

AMD specific activity in rhodococci cells were evaluated immediately following immobilization. The residual AMDase activity in each catalyst formulation was determined after prolonged storage at 4 C. Table 12 summarizes AMDase storage stability for various immobilization and stabilization techniques used. Stability was higher than all situations tested. In addition AMDase stability was achieved without the use of stabilizers.

Chemical inhibition of NHase activity:

Equivalent amounts of NHase (whole cells, PEI cross-linked Ca-alginate beads, and GA catalysts) were exposed to various inhibitor chemicals for 15 min, and the residual NHase activity against AN was tested either in presence of the inhibitor, or after the removal of the inhibitor. In all cases, immobilized NHase showed a higher tolerance to the inhibitors tested when compared to the non-immobilized NHase. Table 13 summarizes the effect of inhibitors on NHase from *Rhodococcus sp*. DAP 96253.

Table 12. Summary of AMDase stability in immobilized *Rhodococcus sp*. DAP 96253 and *R. rhodococcus* DAP 96622, when maintained at 4 C. All cells grown up on plates of respective media; Y= Yeast extract malt extract agar with glucose; AN= Acrylonitrile (500 ppm); Asn= Asparagine(500 ppm); Iba= Isobutyric acid (2 g/L)
Hardened Ca-alginate beads= PEI cross-linked Ca-alginate beads, PAM= Polyacrylamide cubes

	Cell Type	Growth Medium	Type of catalyst	Stabilizer added	Initial AMDase (U/mg dry cell wt)	Residual Amidase (U/mg dry cell wt)	Percent retained	Months at 4C
1	96622	Y-Asn	Glutaraldehyde		11	2.56	23.3	1
2	96253	Y-AN	Glutaraldehyde		8	1.08	13.5	2
3	96253	Y-AN	Glutaraldehyde		6	1.79	29.8	11
4	96253	Y-Asn	Glutaraldehyde		12	2.28	19	11
5	96622	Y	Hardened Ca-alginate Beads	NSB: WWCB =1:4	5	2.37	47.4	13
6	96622	Y-Asn	Hardened Ca-alginate Beads		14	5.66	40.4	13
7	96622	Y-AN	PAM	Asn	16.8	10.14	60.4	13
8	96253	Y-AN	PAM		9	5.58	62	13
9	96253	Y-Asn	PAM	AN	10	7.01	70.1	13
10	96253	Y	Hardened Ca-alginate Beads		8.7	7.64	87.8	4
11	96253	Y-AN	Hardened Ca-alginate Beads	Iba	6	2.97	49.5	29
12	96253	Y-AN	Hardened Ca-alginate Beads		5.4	1.72	31.9	28
13	96253	Y-AN	PAM	Asn	14.2	10.59	74.6	25
14	96253	Y-Asn	Hardened Ca-alginate Beads	Asn	10	5.91	59.1	19

Table 13. Effect of inhibitors on Intracellular NHase (immobilized and non-immobilized) *Rhodococcus sp*. DAP 96253 NHase.

Inhibitors	Free cells with inhibitor	Free cells after inhibition	Hardened calcium alginate with inhibitor	Hardened calcium alginate after inhibiton	Glutaraldehyde with inhibitor	Glutaraldehyde after inhibiton
$ZnCl_2$	2.25	2.97	34.91	51.62	62.22	79.28
$MgCl_2$	1.93	15.56	43.42	61.59	70.63	86.56
Ammonium Persulfate	25.29	27.38	66.72	77.52	90.15	92.28
EDTA	2.50	4.68	26.38	43.38	56.08	72.20
$AgNO_3$	3.22	5.44	35.22	54.61	41.14	56.30
H_2O_2	1.48	4.65	26.10	58.16	50.15	62.20
Mercaptoethanol	0.06	6.17	0.00	8.39	0.00	11.67
Iodoacetamide	1.07	5.65	9.14	22.02	69.97	85.67
DTT	2.56	9.65	0.00	8.63	0.00	10.51
Sodium Azide	19.50	31.90	18.24	52.23	40.90	51.17
$FeSO_4$	2.12	11.91	26.77	42.19	61.15	69.35
$CuSO_4$	1.22	5.20	21.41	34.40	80.05	88.21
$CoCl_2$	1.94	3.09	25.44	58.96	73.07	80.65
EGTA	5.68	7.32	39.35	68.57	84.97	92.08
Initial NHase	100.00		100.00		100.00	

Purification of NHase and AMDase from *Rhodococccus sp*. DAP 96253:

Rhodococcus sp. DAP 96253 grown on YEMEA media, and induced with cobalt, urea and Asn for enhanced NHase (168 U/mg cdw NHase, 14 U/mg cdw AMDase) were used as the source for the isolation and purification of NHase and AMDase. The NHase and AMDase activity of the fractions used in each step as compared to that of the initial sample is summarized in Tables 14a and 14b. PAGE of the cell lysate, the dialyzed sample and the various fractions with highest activity after each chromatography step is shown in Figures 30-33. Consecutive reduction in number of proteins is visual confirmation of purification. In each lane 5 μg of protein is loaded, and increase in band sizes indicate presence of that protein in a higher amount in latter steps of purification process. The proteins from the gels were excised and identified with mass spectrometry. Similarity in sequence data of alpha and beta subunits of NHase and AMDase to that from the protein database is represented in Figures 36-38. Sequence data obtained (see Table 15) from mass spectrometry studies shows that the β-subunit of NHase from DAP 96253 has highest similarity with the same of *Nocardia* sp. JBR, while the α-subunit is closest to that from *R*. *rhodochrous* in amino acid composition.

Table 14a. NHase (AN specific) and AMDase (AMD specific) activity from cells of *Rhodococcus sp*. DAP 96253. Activity represented as Units/100µl sample volume.

	NHase	AMDase
Whole cells	168 (U/mg cdw)	14 (U/mg cdw)
Cell lysate	14	2
Dialyzed lysate	6.5	1.0

Table 14b. Summary of purification of NHase and AMDase from *Rhodococcus sp*. DAP 96253 cells.

	NHase (U/mg protein)	**Purification compared to initial (fold)**	**Amidase (U/mg protein)**	**Purification compared to initial (fold)**
First anion exchange	56.4	8.7	10.6	10.6
Second anion exchange	193	28	24	24
Gel filtration	388	60	58	58
Total Purification	14,616		14,755	

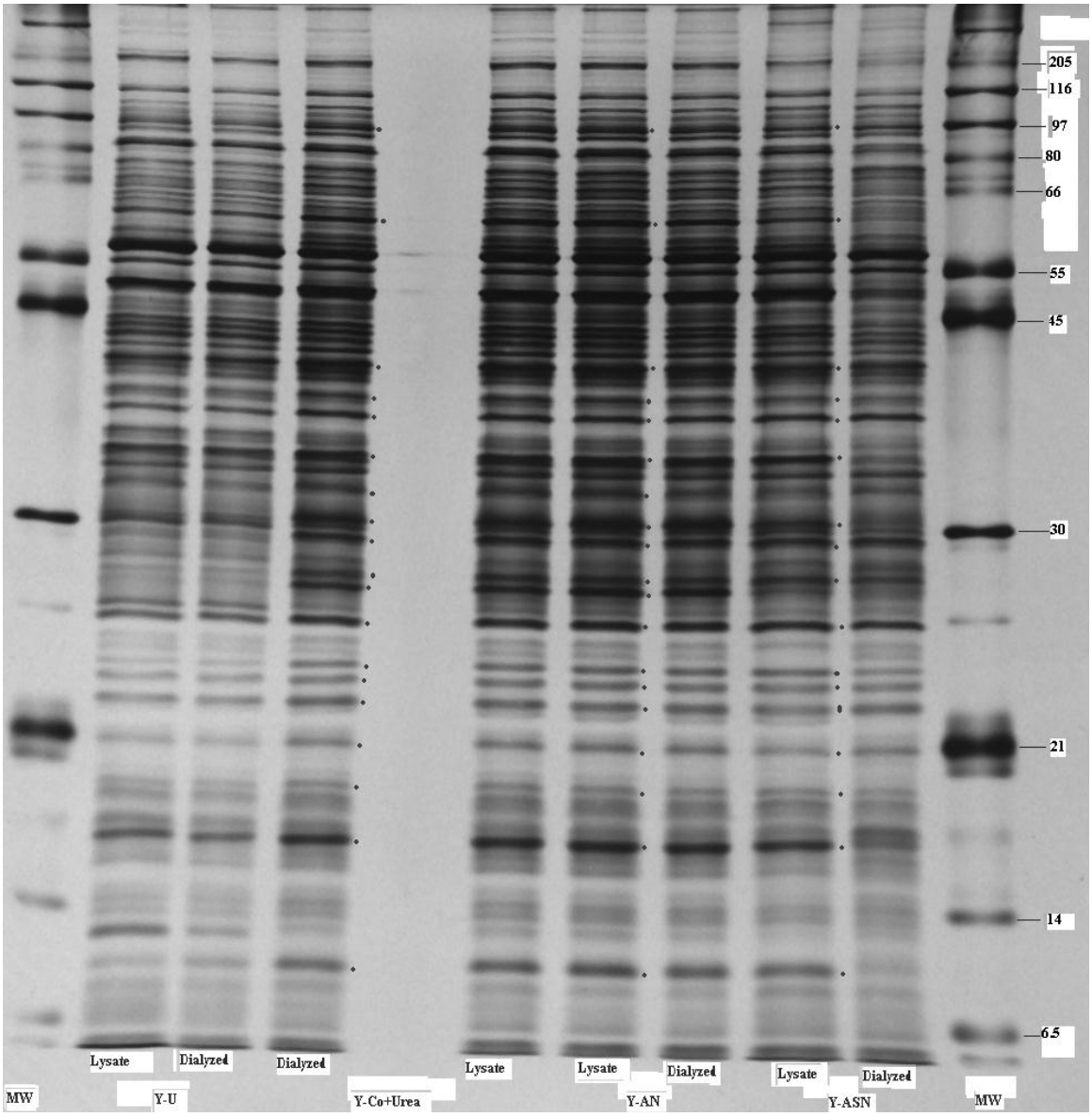


Figure 30. PAGE of cell lysate and dialyzed (10000 mwco) protein samples from *Rhodococcus* cells grown on YEMEA medium. The medium either was uninduced for NHase production, or was supplemented with cobalt and urea, and in the third set with cobalt, urea and AN (500 ppm).

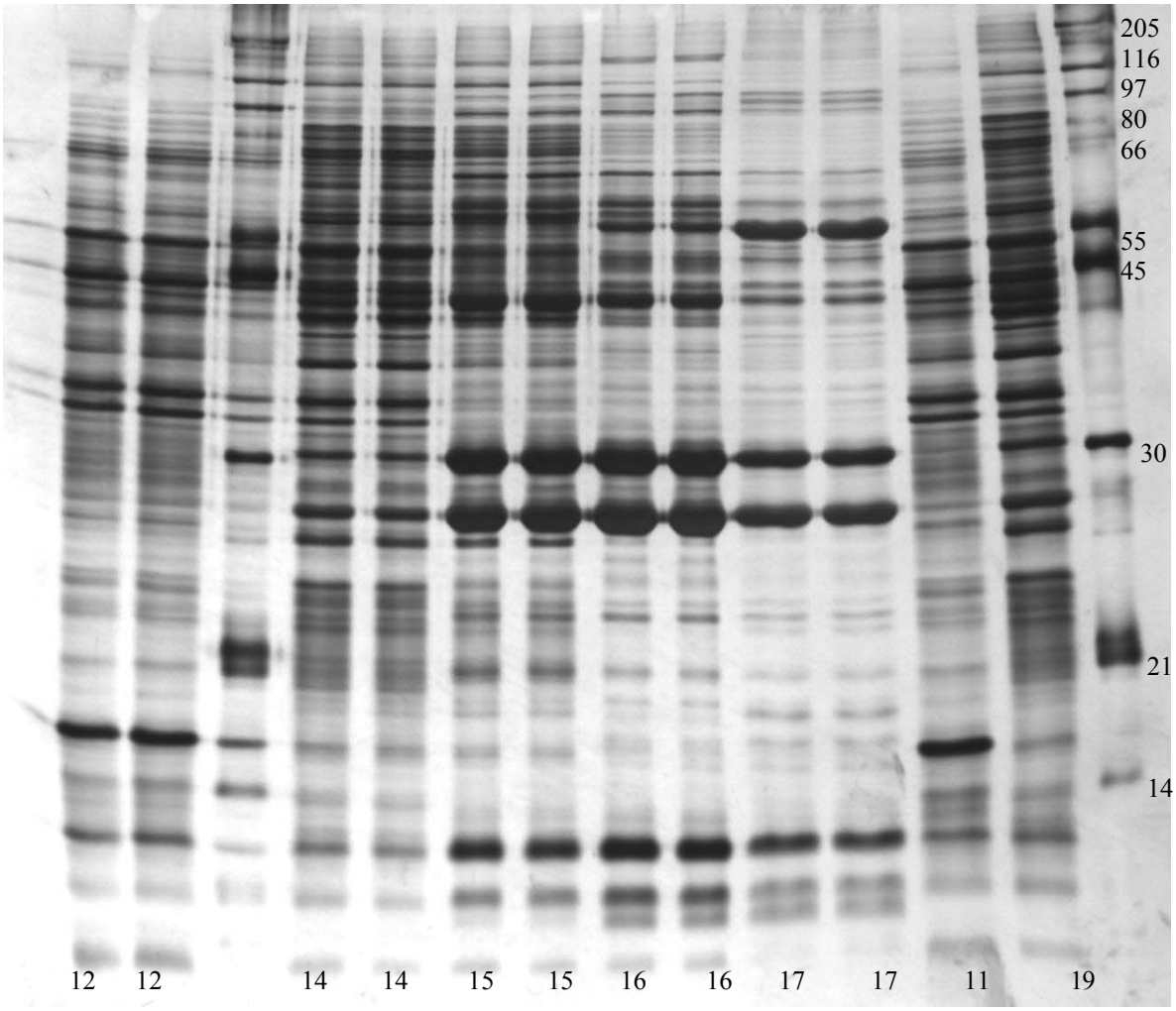


Figure 31. PAGE of fractions with highest AN and AMD specific activity collected after first anion exchange chromatography.

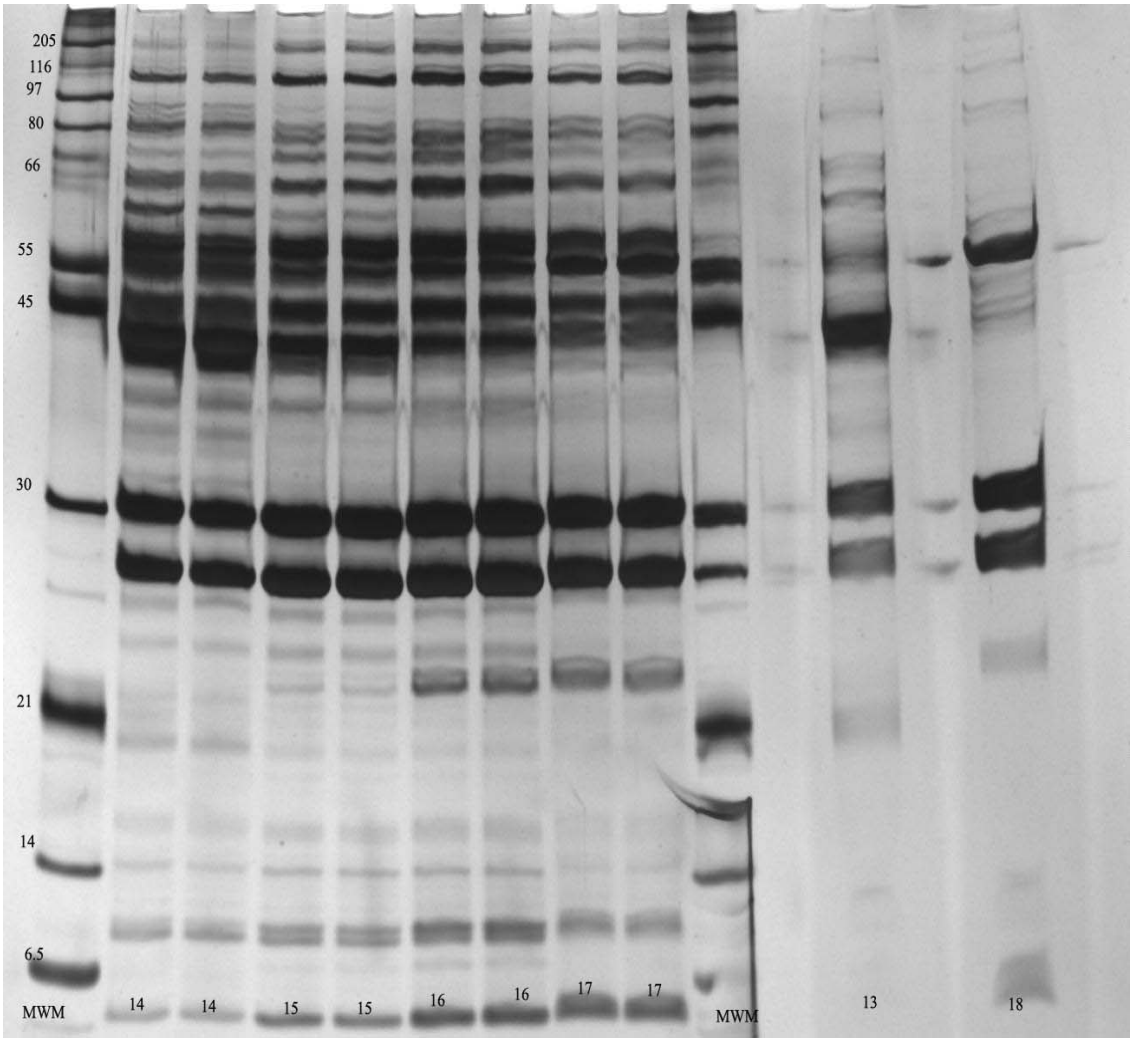


Figure 32. PAGE of fractions with highest AN and AMD specific activities collected after second anion exchange chromatography.

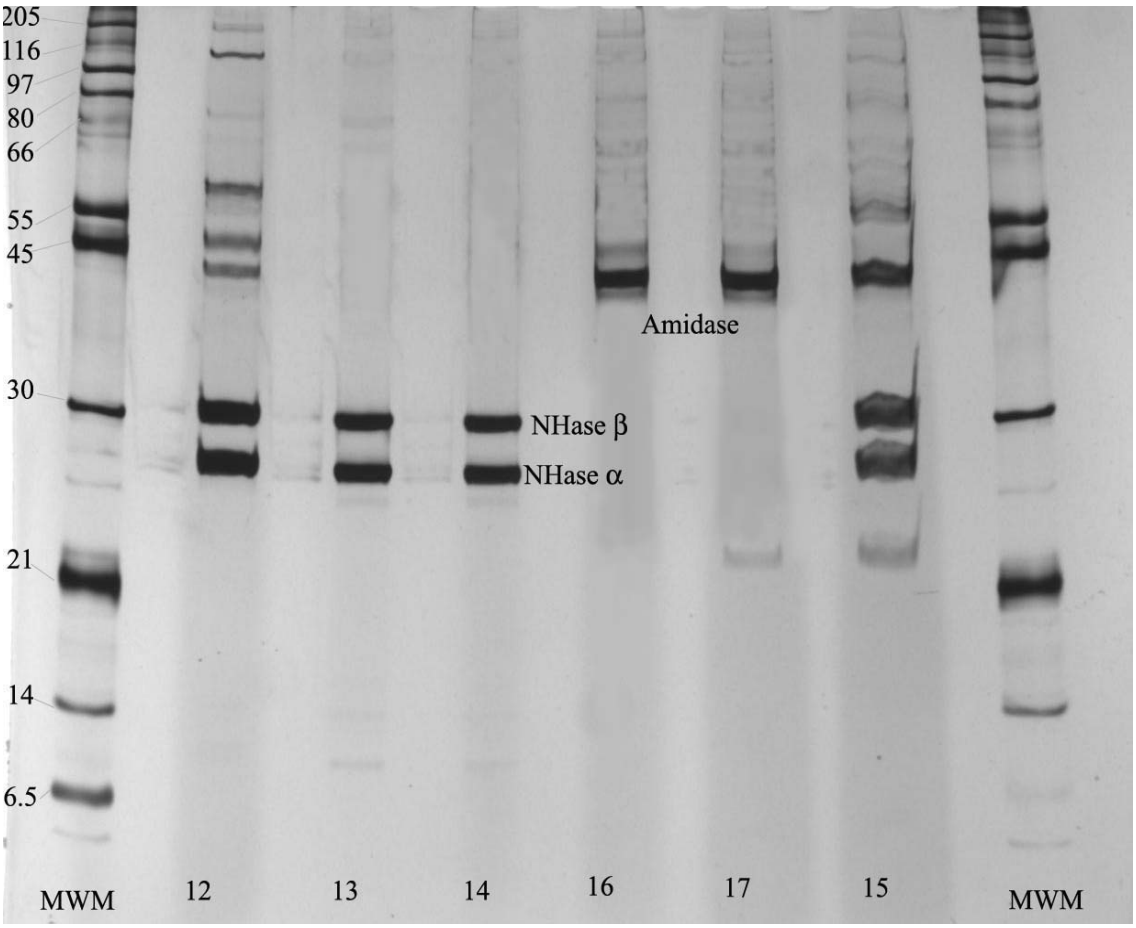


Figure 33. PAGE of fractions showing highest NHase and AMDase activity collected after gel filtration chromatography.

Table 15. Overview of mass spectroscopy identification of the proteins isolated. Only protein C I % score of above 94% were tabulated.

	Protein score C. I.%	ID	Accession #	MW (Da)
1	100	Nitrile hydratase [*Rhodococcus sp*.]	806580	26276.9
2	100	Nitrile hydratase beta subunit [*Nocardia* sp. JBRs]	27261874	26258.9
3	100	Nitrile hydratase [*Rhodococcus rhodochrous*]	49058	26305
4	100	Nitrile hydratase beta chain [uncultured bacterium BD2]	27657379	19344.4
5	100	Nitrile hydratase [*Rhodococcus rhodochrous*]	581528	22820.3
6	100	Nitrile hydratase alpha chain [uncultured bacterium SP1]	27657369	20309.1
7	100	Nitrile hydratase [*Rhodococcus sp*.]	806581	22776.3
8	100	Aliphatic amidase [*Rhodococcus rhodochrous*]	62461692	38026.4
9	100	Putative aliphatic amidase [*Nocardia farcinica* IFM 10152]	54022723	38158.5
10	99.6	ALIPHATIC AMIDASE [*Helicobacter pylori* J99]	15611349	37688.7
11	98.3	Aliphatic amidase [*Pseudomonas aeruginosa* PAO1]	15598562	38469.7
12	97.7	Aliphatic amidase (aimE) [*Helicobacter pylori* 26695]	2313392	37688.7
13	94	Aliphatic amidase [*Pseudomonas aeruginosa*]	150980	38481.7

Nitrile hydratase from *Rhodococcus sp*. (Accession# 806580)

```
1   mdgihdtggm tgygpvpyqk depffhyewe grtlsiltwm hlkgmswwdk srffresmgn
61  enyvneirns yythwlsaae rilvadkiit eeerkhrvqe ilegrytdrn psrkfdpaei
121 ekaierlhep hslalpgaep sfslgdkvkv knmnplghtr cpkyvrnkig eivtshgcqi
181 ypesssaglg ddprplytva fsaqelwgdd gngkdvvcvd lwepylisa
```

Nitrile hydratase beta subunit from *Nocardia* sp. JBRs (Accession# 27261874)

```
1   mdgihdtggm tgygpvpyqk depffhyewe grtlsiltwm hlkgiswwdk srffresmgn
61  enyvneirns yythwlsaae rilvadkiit eeerkhrvqe ilegrytdrn psrkfdpaei
121 ekaierlhep hslalpgaep sfslgdkvkv knmnplghtr cpkyvrnkig eivtshgcqi
181 ypesssaglg ddprplytva fsaqelwgdd gngkdvvcvd lwepylisa
```

Nitrile hydratase from *Rhodococcus rhodochrous* (Accession# 49058)

```
1   mdgihdtggm tgygpvpyqk depffhyewe grtlsiltwm hlkgiswwdk srffresmgn
61  enyvneirns yythwlsaae rilvadkiit eeerkhrvqe ilegrytdrk psrkfdpaqi
121 ekaierlhep hslalpgaep sfslgdkikv ksmnplghtr cpkyvrnkig eivayhgcqi
181 ypesssaglg ddprplytva fsaqelwgdd gngkdvvcvd lwepylisa
```

Nitrile hydratase beta subunit from uncultured bacterium BD2 (Accession# 27657379)

```
1   mdgihdtggm tgygpvpyqk depffhyewe grtlsiltwm hlkgiswwdk srffresmgn
61  enyvdeirns yythwlsaae rilvadkiit eeerkhrvqe ilegrytdrk psrkfdpaqi
121 ekaierlhep hslalpgaep sfslgdknqs eeyepagtht vpeica
```

Figure 34. Alignment of mass spectromerty data sequence of ß subunit of NHase from *Rhodococcus sp*. DAP 96253 with that of other organisms from the database. Green letters represent fraction sequence obtained from mass spectrometry data.

Nitrile hydratase from *Rhodococcus sp*. (Accession# 806581)

```
1   msehvnkyte yeartkaiet llyerglitp aavdrvvsyy eneigpmgga kvvakswvdp
61  eyrkwleeda taamaslgya geqahqisav fndsqthhvv vctlcscypw pvlglppawy
121 ksmeyrsrvv adprgvlkrd fgfdipdeve vrvwdsssei ryiviperpa gtdgwsedel
181 aklvsrdsmi gvsnaltpqe viv
```

Nitrile hydratase alpha chain from *Rhodococcus rhodochrous* (Accession# 581528)

```
1   msehvnkyte yeartkaiet llyerglitp aavdrvvsyy eneigpmgga kvvakswvdp
61  eyrkwleeda taamaslgya geqahqisav fndsqthhvv vctlcscypw pvlglppawy
121 ksmeyrsrvv adprgvlkrd fgfdipdeve vrvwdsssei ryiviperpa gtdgwseeel
181 tklvsrdsmi gvsnaltpqe viv
```

Nitrile hydratase alpha chain from uncultured bacterium SP1 (Accession# 27657369)

```
1   msehvnkyte yeartkavet llyerglitp aavdrvvsyy eneigpmgga kvvakswvdp
61  eyrkwleeda taamaslgya geqahhvvvc tlcscypwpv lglppawyks meyrsrvvad
121 prgvlkrdfg fdipdevevr vwdssseiry iviperpagt dgwseeeltk lvsrdsiigv
```

Figure 35. Alignment of mass spectrometry data sequence of α subunit of NHase from *Rhodococcus sp*. DAP 96253 with that of other organisms from the database. Green letters represent fraction sequence obtained from mass spectrometry data.

Aliphatic amidase from *Rhodococcus rhodochrous* (Accession# 62461692)

```
1   mrhgdisssp dtvgvavvny kmprlhtkad vlenaraiak mvvgmkaglp gmdlvvfpey
61  stmgimydnd emyataatip gdetdifaqa crdaktwgvf sitgerhedh pnkppyntlv
121 lindqgeivq kyrkilpwtp iegwypggqt yvtdgpkglk isliicddgn ypeiwrdcam
181 kgaelivrpq gymypskeqq vlmakamawa nncyvavana tgfdgvysyf ghsaiigfdg
241 rtlgecgeed ygvqyaqlsl stirdarand qsqnhlfkll hrgytgvfag gdgdkgvadc
301 pfdfyrnwvn daeatqkave aitretigva dcpvydlpse ktmda
```

Aliphatic amidase from *Nocardia farcinica* IFM 10152 (Accession# 54022723)

```
1   mrhgdisssp dtvgvavvny kmprlhtkae vldncrriad mlvgmksglp gmdlvvfpey
61  stqgimydeq emydtaatvp geetaifsaa creagvwgvf sitgeqhedh prkppyntlv
121 liddhgeivq kyrkilpwcp iegwypgdtt yvtegpkglk islivcddgn ypeiwrdcam
181 kgaelivrcq gymypskdqq vlmakamawa nncyvavana agfdgvysyf ghsaligfdg
241 rtlgetgeee ygiqyaqlsi sairdarahd qsqnhlfkll hrgysgvhaa gdgdrgvadc
301 pfefyklwvt daqqarerve aitrdtvgva dcrvgslpve qtlea
```

Aliphatic amidase from *Pseudomonas aeruginosa* PAO1(Accession# 15598562)

```
1   mrhgdisssn dtvgvavvny kmprlhtaae vldnarkiae mivgmkqglp gmdlvvfpey
61  slqgimydpa emmetavaip geeteifsra crkanvwgvf sltgerheeh prkapyntlv
121 lidnngeivq kyrkiipwcp iegwypggqt yvsegpkgmk isliicddgn ypeiwrdcam
181 kgaelivrcq gymypakdqq vmmakamawa nncyvavana agfdgvysyf ghsaiigfdg
241 rtlgecgeee mgiqyaqlsl sqirdarand qsqnhlfkil hrgysglqas gdgdrglaec
301 pfefyrtwvt daekarenve rltrsttgva qcpvgrlpye glekea
```

Aliphatic amidase from *Helicobacter pylori* J99 (Acession# 15611349)

```
1   mrhgdisssp dtvgvavvny kmprlhtkne vlencrniak viggvkqglp gldliifpey
61  sthgimydrq emfdtaasvp geetailaea ckknkvwgvf sltgekheqa kknpyntlil
121 vndkgeivqk yrkilpwcpi ecwypgdkty vvdgpkglkv sliicddgny peiwrdcamr
181 gaelivrcqg ymypakeqqi aivkamawan qcyvavanat gfdgvysyfg hssiigfdgh
241 tlgecgeeen glqyaqlsvq qirdarkydq sqnqlfkllh rgysgvfasg dgdkgvaecp
301 fefyktwvnd pkkaqenvek ftrpsvgvaa cpvgdlptk
```

Aliphatic amidase (aimE) from *Helicobacter pylori* 26695 (Accession# 2313392)

```
1   mrhgdisssp dtvgvavvny kmprlhtkne vlencrniak viggvkqglp gldliifpey
61  sthgimydrq emfdtaasvp geetaifaea ckknkvwgvf sltgekheqa kknpyntlil
121 vndkgeivqk yrkilpwcpi ecwypgdkty vvdgpkglkv sliicddgny peiwrdcamr
181 gaelivrcqg ymypakeqqi aivkamawan qcyvavanat gfdgvysyfg hssiigfdgh
241 tlgecgeeen glqyaqlsvq qirdarkydq sqnqlfkllh rgysgvfasg dgdkgvaecp
301 fefyktwvnd pkkaqenvek itrpsvgvaa cpvgdlptk
```

Aliphatic amidase from *Pseudomonas aeruginosa* (Accession# 150980)

```
1   mrhgdisssn dtvgvavvny kmprlhtaae vldnarkiad mivgmkqglp gmdlvvfpey
61  slqgimydpa emmetavaip geeteifsra crkanvwgvf sltgerheeh prkapyntlv
121 lidnngeivq kyrkiipwcp iegwypggqt yvsegpkgmk isliicddpn ypeiwrdcam
181 kgaelivrcq gymypakdqq vmmakamawa nncyvavana agfdgvysyf ghsaiigfdg
241 rtlgecgeee mgiqyaqlsl sqirdarand qsqnhlfkil hrgysglqas gdgdrglaec
301 pfefyrtwvt daekardnve rltrsttgva qcpvgrlpye glekea
```

Figure 36. Alignment of mass spectrometry data sequence of aliphatic amidase from *Rhodococcus sp*. DAP 96253 with that of other organisms from the database. Green letters represent fraction sequence obtained from mass spectrometry data.

This indicates that the enzyme is a "hybrid" when amino acid sequence similarity is considered.

Characterization of purified NHase:

Purified NHase fractions were evaluated for their substrate specificity against both aliphatic and aromatic nitriles generally found in AN production wastewaters (tables 18a and 18b). The substrate specificity of the purified enzyme is similar to the catalyst from whole cells. This enzyme shows good affinity for all the nitriles present in AN production wastewater. The purified enzyme showed less specificity for aromatic nitriles compared to intracellular NHase (see Table 11b).

The purified NHase was tested using the same inhibitors previously used on the cells containing NHase activity. The results of the inhibition assay are summarized in table 16. NHase from *Rhodococcus sp*. DAP 96253 was compared for the effect of the inhibitors with published data from other organisms and is shown in Table 17. The comparison indicated that the NHase from DAP 96253 is affected similarly to the NHases reported by certain inhibitors, while shows higher susceptibility to reducing agents, such as β-mercaptoethanol and dithiothreitol. This clearly establishes that the isolated NHase from *Rhodococcus sp*. DAP 96253 is a unique enzyme, similar to NHases from other organisms only to a certain extent.

Table 16. Effect of inhibitors on purified NHase

Inhibitors used (all inhibitors were used at a concentration of 1000 ppm). 288 units of NHase were exposed to each inhibitor for 15 minutes, the substrate (AN) added and active NHase units retained was measured.

	Inhibitor	Concentration (mM)	NHase units after 15 min exposure	Percent NHase units retained
1	Zinc chloride	7.35	63.9±0.25	22.2
2	Magneseum chloride	4.92	22.3±0.16	7.74
3	Ammonium persulfate	4.38	188.5±0.4	65.5
4	EDTA	2.69	21±0.5	7.3
5	Silver nitrate	5.89	17.2±0.15	6
6	Hydrogen peroxide	29.41	21.2±0.05	7.4
7	Mercaptoethanol	12.8	0±0	0
8	Iodoacetamide	5.41	17.6±0.35	6.1
9	DTT	6.49	0.6±0.02	0.2
10	Sodium azide	15.38	18.3±0.2	6.4
11	Ferrous sulfate	3.6	16±0.05	5.6
12	Cupric sulfate	4.01	10.8±0.35	3.8
13	Cobalt chloride	4.2	30.7±0.25	10.7
14	EGTA	2.63	26.4±0.06	9.2

Table 17. Comaprison of effect of inhibitors on purified NHase from *Rhodococci sp*. DAP 96253 with published results on NHase from other micro-organisms (Percent of AN specific activity retained after 15 min exposure to each inhibitor at same concentration)**.** NR= Not Reported
(Compiled from Nagasawa et al. 1987[3], 1991[1], Takashima et al. 1998[2], Pereira et al. 1998[4])

	Rhodococcus sp. DAP 96253	*R. rhodochrous* J1 (high mw) [1]	*Bacillus smithii* [2]	*Pseudomonas chlororaphis* B23 [3]		*Bacillus* sp. RAPc8 [4]
$ZnCl_2$	98	95-105	NR	90-100	NR	110
$MgCl_2$	85	95-105	NR	90-100	NR	116
Ammonium Persulfate	120	100	106	NR	NR	NR
EDTA	150	97-100	101	97	91	100
$AgNO_3$	20	0	6.3	1	0	0
H_2O_2	75	81	NR	0	0	NR
Mercaptoethanol	0	97	97	NR	NR	NR
Iodoacetamide	35	NR	NR	NR	NR	0
DTT	5	98	101	NR	NR	NR
Sodium Azide	50	NR	NR	NR	NR	NR
$FeSO_4$	62	95-105	NR	71	116	112
$CuSO_4$	47	91	40	19	NR	0
$CoCl_2$	87	95-105	NR	90-100	NR	114
EGTA	9.2	NR	NR	NR	NR	NR

Table 18a. Relative activity of purified NHase(s) against different aliphatic nitriles[a,b]

Substrate (1000ppm)	NHase from *Rhodococcus sp*. DAP 96253	
	Units	% Activity
Isobutyronitrile	26.85±0.05	9.3
Isovaleronitrile	20.65±0.08	7.2
DL-Lactonitrile	0±0	0
Fumaronitrile	5.45±0.06	29
Glycolonitrile	0.4±0.03	0.14
Acetonitrile	203.55±0.25	70.7
Acrylonitrile	288±0.3	100
Adiponitrile	11.4±0.05	4

Table 18b. Relative activity of purified NHase(s) against different aromatic nitriles[a,b]

Substrate (1000ppm)	NHase from *Rhodococcus sp*. DAP 96253	
	Units/mg cell dry weight	% Activity
Benzonitrile	13.55±0.2	4.7
Cinnamonitrile	17.5±0.15	6.1

[a] All % activities compared to *Rhodococcus sp*. DAP 96253 against acrylonitrile
[b] All assays were performed using the same amount enzyme (288 Units), and all cells were grown up in the same induction medium (YEMEA with cobalt, urea and asparagine).

DISCUSSION

An important practical consideration, in the use of an enzyme or a biological based catalyst, is the effective longevity over which the catalyst activity can be maintained. For some applications, where catalyst cost is a serious consideration, enzyme longevity is critical. For those applications that require extended enzyme life, the evaluation and testing of the stability of enzyme preparations and formulations can be quite lengthy. The practical use of NHase based enzyme systems and particularly the use of NHase in the treatment of waste nitriles and cyanohydrins would benefit from NHase formulations which exhibited extended stability and activity. In the current research, methods were developed and employed to improve the stability of NHase, resulting in biological catalysts with extended, practical activity. In addition, methods were developed which greatly shortened the time period required to assess practical stability.

Although microorganisms which utilize nitrile compounds have been described previously, these microorganisms have been shown to be limited in their ability to degrade nitriles and as such are inappropriate candidates to treat the mixed nitriles/amides present in many industrial wastestreams. This is particularly true for AN production wastewater as the ability to degrade AN is the least common trait. Processes reported so far for the treatment of AN production wastewaters also required mixed cultures, rather than a pure one making it difficult to maintain the right composition of organisms in the biodetoxification system. This is due to changes in conditions as a result of ongoing biotransformation of the wastewater. This change greatly influences the growth of

certain organisms over others and, over time the characteristics of the mixed culture change and can result in decrease of degradation.

AN production wastewater on an average contains in excess of 300 ppm of hydrogen cyanide (HCN). The AN degrading organisms reported in scientific literature are sensitive to presence of HCN and fail to detoxify the wastewater at such elevated – CN concentrations. Wyatt and Knowles (1995a) reported the biotransformation of AN production wastewaters by a mixed bacterial culture, but they first had to dilute (> 100 X) the waste for this degradation to occur. This resulted in greater than a 100 fold increase in the volume handled by the treatment system and also enhanced the problem of disposal of huge quantity of wastewater.

The lack of an efficient process for treatment of AN production wastewater necessitated the study into methods of detoxification of mixed nitrile and/or amide containing wastewater using pure cultures. In the current research, pure cultures of two strains of *Rhodococcus*, DAP 96253 and DAP 96622 were utilized for the purpose of generating an efficient, efficacious, and economic biocatalyst for treatment of AN production wastewater. These organisms have previously been shown to utilize nitriles as their nitrogen or carbon and nitrogen source (Pierce 1999, 2000). Cells from these two organisms were induced for elevated NHase and AMDase production, and thereby increased nitrile and amide degradation/transformation ability. The NHase and AMDase enzymes from these organisms were then stabilized to create stable and practical biocatalysts for treatment of AN production wastewater. Implementation of this catalyst in the industry will result in disposal of treated AN production wastewater rather than the untreated; as is the current practice.

Both strains of *Rhodococcus*, DAP 96253 and 96622 are short rods (see figure 3 and 4) and showed similar growth patterns (figure 5). Both organisms are capable of producing an inducible aliphatic NHase (table 4 and 5). When subjected to multiple induction (with cobalt, urea and either a mix of nitriles and amides, or with amino acids), the two strains significantly overproduce NHase to a level comprising 40% of the total soluble protein. When compared to other organisms reported for high NHase activity, as well as another *Rhodococcus* strain (*R. erythropolis*), these two *Rhodococcus* strains both showed superior degradation of nitrile and amide mixtures, a broader substrate diversity, and a much higher tolerance to cyanides.

Inducible Co-NHase activity has been shown in the literature to be dependant upon the addition of nitriles, amides or their acids. As these inducer chemicals are toxic (some quite highly toxic), the use of these inducers introduces significant risks in production of this NHase. In the current study, production of NHase and AMDase during 72 hours of growth in a liquid medium (represented in figure 6) decreased when in the absence of inducer(s) in the growth medium, even when the organisms were pre-induced and had a high NHase and AMDase activity at the time of inoculation. This is rapidly recovered when the inducers (AC and AN) are introduced at 55 hours of growth. This shows that induction during growth is necessary to yield biomass with consistent high NHase and AMDase activity. The two *Rhodococcus* strains were shown to be capable of high levels of Co-NHase induction by the addition of asparagines and/or glutamine. This is the first report of high-level Co-NHase induction with non-toxic compounds. Furthermore, the addition of asparagine and glutamine resulted in higher biomass. While AC and AN can be potent inducers of NHase, these compounds can also inhibit growth.

The two DAP strains show higher levels of tolerance to AN and AC than other known NHase producing strains evaluated in the course of this study. For example, *Rhodococcus* sp DAP 96253 and *R. rhodochrous* DAP 96622 are unaffected by the presence of AN or AMD (at or below 500 ppm) in the growth medium. *Pseudomonas chlororaphis* (ATCC 43051) showed no visible growth in presence of AN and/or AMD at 500 ppm each, but grew in presence of Asn in the medium (table 5). Table 5 also illustrates that even when the organisms could grow on AN and/or AMD, the organisms tested showed higher growth when propagated in a medium containing Asn and/or Gln.

With respect to the NHase producing strains evaluated, *Rhodococcus* sp DAP 96253 and *R. rhodochrous* DAP 96622 showed consistently higher activity against all nitriles tested. These two microorganisms also showed activity against selected aromatic nitriles suggesting the presence of more than one NHase. Or that the NHase from these two microorganisms is active against both aliphatic and aromatic nitriles. Interestingly, the NHase(s) from *Brevibacterium ketoglutamicum* showed highest affinity for adiponitrile.

This research showed that, with proper induction the NHase activity of the two DAP strains can be significantly enhanced with respect to the ability to detoxify the components of concern present in AN production wastewater.

In this study the practical active life of NHases from *Rhodococcus* sp. DAP 96253 and *R. rhodochrous* DAP 96622 was stabilized using immobilization technology and through the use of stabilizers. With improvements in retention of practical activity of the NHase formulations, the time required for evaluating and assessing the effect of a particular stabilizer increased proportionally. The development of accelerated methods to

measure NHase stability significantly shortened the time needed to assess stability improvements. This in turn shortened the time required to develop a practical catalyst.

Immobilization enhanced AN specific NHase activity and immobilized cells show an improved NHase stability (see Figure 15). When both immobilized and chemically stabilized (e.g. with isobutyric acid), NHase stability and practical activity was further enhanced. Chemical stabilization prodided additional stability/practical activity benefits to Ca-alginate, hardened Ca-alginate and PAM immobilized catalysts, indicating that NHase formulations can benefit from simultaneous immobilization and chemical stabilization.

The nitriles present in AN production wastewater proved to be far superior than carboxylic acids [isobutyric acid (Figure 16) and/or acrylic acid)] in the stabilization of AN specific NHase in the multiply induced *Rhodococcus* DAP strains. Furthermore, Asn and/or Gln were better chemical stabilizers than either isobutyric acid or acrylic acid. A "Model" waste consisting of the three major nitrile components present in AN production wastewater (AN, AC and SN) enhanced the stability of NHase and retention (20%) of activity could be recorded for over 260 hours (Figure 17 a and b) even when maintained at 50-55 C. This was a >10 fold increase in stability of NHase activity (i.e. An specific Nhase activity in multiply induced *Rhodococcus* AP strains) compared to use of carboxylic acids as stabilizers. The best stabilizing effects were achieved by using a synthetic nitrile mixture with total nitrile concentration of 4 g/L of catalyst formulation. When higher nitrile concentrations (either 8 g/L or 16 g/L) were used, the activity was retained for only 140 hours and the rate of loss of activity was much faster compared to either 4 g/L or 2 g/L of nitriles.

When the nitrile mixture used above was supplemented with AMS at 5% (generally present in AN production wastewaters at 4-8%) even greater stability was achieved for both DAP 96253 and DAP 96622 AN specific NHase activity. DAP 96253 retained 40% activity over 260 hours at 50-55 C, while DAP 96622 retained 34% activity for the same duration (Figure 18). The chemicals present in actual AN production wastewater have great potential for stabilizing NHase from *Rhodococcus* DAP strain 96253 and DAP 96622 which have been immobilized in Ca-alginate matrix.

Of the NHases (Fe-type and Co-type) described in the literature, all have been reported to be sensitive to free cyanide. As the AN production wastewater usually consists of in excess of 300 ppm free cyanide, none of these NHases represent a practical catalyst for treatment of such wastewater. DAP 96253 or DAP 96622 derived immobilized catalysts was significantly resistant to the presence of cyanide in the environment, when in presence of nitrile mix as chemical stabilizers (Figure 19a and 19b).

Ca-alginate immobilization proved to be of great value for evaluating chemicals present in the wastewater for their ability to stabilize NHase and also to entrap the cells so that there was no loss of catalyst volume when the catalysts were used to degrade wastewater (model as well as actual wastewater). This immobilization matrix was also very gentle on the cells, which remained viable throughout the immobilization process. When the catalyst beads were chemically dissolved (by incubating in phosphate buffer) about 40% recovery of live cells was achieved. The practical drawback of the Ca-alginate matrix is the lack of physical hardness and rigidity of the catalyst particles, making them unsuitable for scale-up.

Other more practical immobilization matrices were investigated. Three practical immobilization matrices evaluated were:

1. Ca-alginate cross linked with PEI,

2. PAM and

3. GA.

PEI cross linked beads when maintained at 50-55 C, even without any additional chemical stabilizers maintained activity for a longer time compared to plain Ca-alginate beads. At this temperature range, the Ca-alginate beads without additional stabilizers showed complete loss of activity in about 40 hours, while the PEI-cross linked beads still retained 40% activity at that time point and showed complete inactivation at 145 hours (Figure 21). When additionally supplemented with the stabilizer package of nitrile mixture and AMS, PEI cross linked beads lost 40% of their initial activity over 40 hours and then maintained this activity over a long period of time and the rate of decline in activity was greatly reduced, which resulted in about 43% activity retained over 260 hours of incubation at 50-55 C.

Actual wastewater samples (obtained from Cytec Industries, Fortier, LA) from the two wastewater streams, net stripper bottoms (NSB) and waste water column bottoms (WWCB) were used separately or together (in various ratios) as chemical stabilizer in conjunction with PEI cross-linked Ca-alginate immobilization on multiply-induced cells of the two *Rhodococcus* DAP strains. It was seen that a combination of NSB and WWCB (1:4) provided highest stability of NHase from both DAP strains (figure 20 a and b). Notably, this is the ratio of the two streams in the actual wastewater. After 270 hours at 50-55 C, the retention of AN specific NHase of PEI cross-linked Ca-alginate cells of

DAP DAP 96253 catalyst stabilized with: a) NSB or WWCB, or b) NSB:WWCB (1:1), or c) NSB:WWCB (1:4) was 20%, 25% and 50% respectively. A similar but lower trend was observed for AN-specific NHase of DAP 96622.

The need for additional chemicals, especially the toxic nitriles, for stabilization of NHase activity in both the *Rhodococcus* DAP strains, raises the same concerns the use of nitriles involved in induction of this enzyme does. To solve the problems of toxicity, the non toxic inducers (amino acids) were evaluated for their ability to stabilize NHase. The effects of simultaneous use of Asn and Gln (each 1 g/L) as stabilizer on both 96253 and 96622 NHase as compared to that of simultaneous use of AC and AN (each 1 g/L) over 110 days is summarized in Figure 22. On one hand, it was observed that for both strains, use of the nitriles as stabilizer achieved better stability (20% better for 96253, 10% for 96622), but the use of amino acids as stabilizer does offer stabilizing effects on NHase as compared to non-stabilized catalysts (see Figures 21 and 22). The amino acids on the other hand have the added advantage of being safe in handling and use. When considering both the stabilizing effects of and the safety in handling of the amino acids, they become stabilizer of choice over the toxic nitriles, similar to the case of inducers. This is the first known report of using amino acids for induction and stabilization of NHase activities in *Rhodococcus* sp. DAP 96253 and *R. rhodochrous* DAP 96622.

With the ongoing search for a more practical immobilization matrix, PAM immobilization of cells of *Rhodococcus* sp. DAP 96253 and *R. rhodochrous* DAP 96622 were carried out. The PAM immobilized catalyst showed significant increase in stability of AN specific NHase over both Ca-alginate and PEI cross linked Ca-alginate immobilized catalysts. When maintained at 30 C, for 96253 NHase, PAM

immobilization alone (without any additional stabilizer) achieved considerable stability and complete loss of activity occurred in 216 days (Figure 23a). This enhancement of stability is thought to be due to the presence of residual AMD monomer in PAM formulations. When additional stabilizers were used an enhancement in retention of activity was observed. There was 15% residual activity when Asn was used, 35% when AN was used and 45% when nitrile mixture (AN, AC and SN) was used as stabilizer over 580 days.

DAP 96622 derived, PAM immobilized NHase catalyst formulations showed even better retention of AN specific NHase activity without any added stabilizers, when compared to that of DAP 96253, and retained detectable activity for 368 days at 30 C (Figure 23b). When the same chemicals stabilizers (as that used on DAP 96253) were used, DAP 96622 derived NHase showed better retention of AN specific activity compared to the non stabilized formulation, though percent activity retained was less compared to that by DAP 96253 derived NHase.

GA immobilization resulted in a 20% decline in the initial AN specific NHase activity of the catalyst. GA immobilization resulted in loss of cell viability. Despite the 20% reduction in activity, GA immobilization resulted in the greatest stability achieved of NHase in either DAP 96253 or DAP 96622 (Figure 24 a and b). For DAP 96253, even without the use of any additional chemical stabilizer, greater than 62% AN specific NHase activity was retained over 580 days of incubation at 30 C (Figure 24a). When the GA immobilization process was supplemented with DEAE cellulose fibers to enhance catalyst volume, a similar level of activity retention was observed, (with 43.8% AN specific NHase activity maintained). This reduction in percent activity retained at 580

hours is explained by the lower number of cells required to make up comparable catalyst (with DEAE cellulose fibers incorporated) volume that was tested (all data is based on AN specific activity of 1 ml catalyst volume).

As seen in Figure 24b, DAP 96622 derived NHase retained a significant 68% activity (initial activity being 80% of Ca-alginate immobilized catalyst) in the GA immobilized catalyst formulations; and 56% activity was retained for the GA catalyst formulations supplemented with DEAE cellulose. This was due to fewer quantity of NHase being present per unit volume of the catalyst incorporating DEAE cellulose fibers as compared to the plain GA immobilized particle. When the activities were calculated on cell weight basis and compared for GA immobilized catalysts with and without DEAE cellulose added, both the catalysts showed similar retention of activity.

Figures 25, 26 (a and b) and 27 (a and b) show when various immobilization techniques were compared for their effect on NHase stability, GA (both with and without use of DEAE cellulose) proved to be the best option, followed by PAM immobilization and PEI cross linked Ca-alginate. Ca-alginate, PEI cross linked Ca-alginate and PAM immobilization were ideal for evaluation of various chemicals stabilizers, but due to the lack of physical rigidity these catalyst formulations are unsuitable for scale up implementation for wastewater treatment. GA catalyst formulations (both with and without DEAE cellulose) on the other hand had the rigidity required for use in the wasterwater treatment process thus making GA immobilization the immobilization technique of choice for making a practical catalyst. When chemical stabilizers used were compared, taking into consideration toxicity issues and stability achieved for a particular catalyst composition, use of amino acids are preferred over traditional nitriles.

As seen from Figure 28, AN specific NHases from both DAP 96253 and DAP 96622 had slightly acidic pH optimas (6.5 for DAP 96253 and 6.85 for DAP 96622). Best retention of activity was achieved when maintained at between pH 6.5 through pH 7.0 for DAP 96253 AN specific NHase (Figure 29a) and pH 6.0 through pH 7.0 for DAP 96622 AN specific NHase (Figure 29b). Even though DAP 96622 derived NHase showed lower retention of AN specific activity, the range of pH conditions it can be maintained at is broader than NHase derived from DAP 96352.

In all catalyst formulations evaluated, DAP 96253 derived NHase thus showed a better thermal and pH stability (when maintained at selected pH conditions) compared to that from DAP 96622. For all three rhodococci evaluated, the level of AMDase produced was much lower than that of NHase produced. This initially suggested that additional sources of AMDases may be required for a AN production wastewater biodetoxification catalyst to achieve rapid and complete detoxification of the nitriles and amides present to their respective non-toxic acid salts. When AMDase stability in the various catalyst formulations tested was evaluated, it was seen that the AMDase from both DAP 96253 and DAP 96622, are considerably more stable over long periods compared to NHase, and this stability was seen both in presence and absence of stabilizers used to achieve NHase stability. While the initial levels of *Rhodococcus* DAP strains was significantly less than the amount of NHase produced, the inherent stability of the amidase indicated that over time, this level of AMDase was sufficient to ensure the complete conversion of Nhase generated amides to their respective acids.

When the effects of traditional NHase inhibitors were evaluated, it was observed that immobilization resulted in a mitigation of the effects of certain inhibitors as

compared to the whole cells. When compared to the reported effect of the inhibitors on NHases from other organisms, purified NHase from DAP 96253 showed higher sensitivity to certain inhibitors, while remaining almost unaffected by others (Table 17). *R. rhodochrous* J1 and *Bacillus smithii* NHase showed a level of insensitivity to 2-mercapethanol and DTT, which was not seen with the NHase from *Rhodococcus sp.* DAP 96253. *Rhodococcus sp.* DAP 96253 derived NHase on the other hand showed significant tolerance to silver nitrate, which was not seen with *R. rhodochrous* J1, *B. smithii*, *P. chlororaphis*, or *Bacillus* sp. RAPc8. This suggested that the AN specific NHase from these organisms may be different. The amino acid sequences of α and β NHase subunits obtained from mass spectrometry studies on DAP 96253 NHase were compared to those of three known NHase sequences: 1) *R. rhodochrous*, 2) *Nocardia* sp JBRs, and 3) an uncultured bacterium. Based on the amino acid sequences of the fractions generated by MS studies, the α subunit of NHase from DAP 96253 had highest homology with that of the *R. rhodochrous*, whereas the β subunit from the DAP 96253 derived NHase showed highest homology to the β subunit of H-NHase of *Nocardia* sp. JBRs. This further suggests that the Co-NHase derived from *Rhodococcus sp*. DAP 96253 was somewhat different from and potentially uniquely different from both these organisms.

The purified NHase was evaluated for its substrate range and showed similar preferences to the aliphatic nitriles as induced cells (Table 18a). The purified enzyme also showed activity for aromatic nitriles (Table 18b) indicating that the aliphatic NHase produced by the cells of DAP 96253 possessed the ability to also hydrolyze aromatic nitriles. The decrease in activity against aromatic nitriles by the purified NHase

compared to the intracellular (Table 11b) may be explained by possible production of more than one NHases by cells of DAP 96253. These NHases could not be isolated during the purification process as they may not have been induced and produced in high quantities.

When the amino acid sequence information of the aliphatic AMDase purified was compared to the known AMDase sequences from the database, the highest homology was found to be with AMDase from *R*. *rhodochrous*, followed by that from *Nocardia farcinica* IFM 10152 and *P*. *aeruginosa* PAO1.

The goal of the current research was to develop a NHase based biocatalyst for the treatment of wastewater generated in the AN production industry. The methodologies developed in the current study permitted a higher number of stabilization experiments to be run in a shorter time period and also allowed more replicates to be run during this period. Evaluation of *Rhodococcus sp*. DAP 96253 and *R*. *rhodochrous* DAP 96622 in the present study showed beyond doubt that the production of NHase can be induced in these organisms. Also significant stability of NHase activity in both of the organisms could be attained by simultaneous immobilization and chemical stabilization. The AMDase produced by these organisms showed significant stability when immobilized even without the aid of any additional stabilizers. This qualified the catalyst formulations generated using the organisms *Rhodococcus* sp. DAP 96253 and *R*. *rhodochrous* DAP 96622 as practical biocatalysts for the treatment of nitrile containing wastewater.

Future studies should be concentrated on implementing the catalysts generated in this study on treating actual waste in a scaled-up pilot facility and determining the parameters of ideal operation. Since the NHases from both the *Rhodococcus* DAP strains

showed ability to transform aromatic nitriles, the potential as a biocatalyst of these two NHases should be investigated for treating wastewater containing a mixture of not only aliphatic nitriles but aromatic nitriles as well. Studies should also be carried out to inducing higher AMDase production by these organisms.

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