DEVELOPMENT OF NITRILE METABOLIZING ENZYME FOR SURFACE MODIFICATION OF POLYACRYLONITRILE

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **DEVELOPMENT OF NITRILE METABOLIZING ENZYME FOR SURFACE MODIFICATION OF POLYACRYLONITRILE** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from Jan, 2006 to December, 2010 under the supervision of Dr. Bijan Choudhury, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Vikash

(VIKASH BABU)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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The Ph.D. Viva-Voce Examination of **Mr. Vikash Babu,** Research Scholar, has been held on.....30th May 2011.....

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ABSTRACT

Polyacrylonitrile (PAN) is a synthetic polymer and widely used in the textile industries and biomedical field. The main disadvantage with PAN is its poor hydrophilicity which affects the processability of fibers, restricting the application of finishing compounds such as dyes and other coloring agents in the textile industry and reduces the biocompatibility for use as biomaterials. Low hydrophilicity of PAN is due to the presence of –CN groups on the surfaces. Nitrile metabolizing enzymes has opened a new route for the transformation –CN to –COOH functional groups with many advantages over chemical methods of surface modification of polyacrylonitrile.

During isolation and screening of nitrile metabolizing enzyme producing isolate, total seven isolates were obtained and among those, one isolate (No.6/b) was releasing ammonia from polyacrylonitrile powder. Therefore, this strain was selected for this research work.

Adiponitrile was found to be the most efficient inducer or nitrogen source for the production of nitrile metabolizing enzyme which showed maximum activity with PMA (co-polymer of PAN).

Isolated strain found to have rounded configuration, convex elevations, and rough surface with white and creamy yellow pigmentation. Gram staining of isolated strain showed gram positive reaction and single/Y shaped morphology on visualization at 100X with oil immersion in a compound microscope. Isolated strain was identified by 16S rDNA phylogenetic analysis. Due to the maximum similarity with other *Amycolatopsis* strains, isolated strain named as *Amycolatopsis* sp.IITR215.

Whole cells and cell free extract of *Amycolatopsis* sp.IITR215 showed activity towards aliphatic and aromatic nitriles as well as with arylacetonitriles and acrylamide. Thus, it indicates the presence of amidase and probably nitrilase and nitrile hydratase. Similar specificity was found with the cell free extract but specific activity was less as compare to whole cells activity. In presence of DEPA (an amidase inhibitor), no ammonia was released with acrylonitrile, isobutyronitrile, valeronitrile, propionitrile, acetonitrile, butyronitrile, isovaleronitrile, 3- hydroxypropionitrile, 4-cyanopyridine, 3-hydroxyglutaronitrile, malononitrile. Henceforth, it was concluded that ammonia released from these nitriles was

solely due to nitrile hydratase and amidase. This was further confirmed by checking the presence of acrylamide in the reaction mixture when cells were incubated with 10 mM acrylonitrile along with 10 mM DEPA. In case of hexanenitrile, the presence of the inhibitor did not affect ammonia production. It was therefore concluded that ammonia released from hexanenitrile was probably due to nitrilase or another set of nitrile hydratase and amidase that was not affected by DEPA. For nitrile hydratase and amidase activities, acrylonitrile and acrylamide, respectively, were used as substrates.

Further study on the nature of enzyme responsible for hexanenitrile hydrolysis was attempted by studying the effects of various reported inhibitors on enzymes activities with acrylonitrile, acrylamide and hexanenitrile respectively. At 1 mM N-bromosuccimide concentration, no activity was detected with acrylonitrile and 5% and 36% activities were retained with acrylamide and hexanenitrile, respectively. This result indicates high inhibitory effects of N-bromosuccimide on nitrile hydratase, amidase and less inhibition on enzyme involved in hexanenitrile hydrolysis. With 5 mM N-ethylmaleimide, enzyme with acrylonitrile and acrylamide showed 15% and 31% activities respectively and 56% activity was observed with hexanenitrile, which confirms that N-ethylmaleimide had a greater inhibitory effect on amidase and nitrile hydratase as compared to the enzyme involved in hexanenitrile hydrolysis.

Amidase of the isolated strain was highly active for isobutyramide, propionamide, benzamide and hexanamide while acyl-transferase activity was maximum for hexanamide. To study the optimum conditions of nitrile hydratase, 10 mM acrylonitrile with DEPA was used as a substrate. The amidase and nitrile hydratase found to have an optimum temperature of 45°C. The optimum temperature for hexanenitrile hydrolysis was found to be 55°C.

Optimum pH for amidase and nitrile hydratase was found to be 7.0 in 50 mM phosphate buffer while it was 5.8 for hexanenitrile metabolizing enzyme in 50 mM acetate buffer. Ferric salts were found to have no effect on hexanenitrile hydrolysis but affected the nitrile hydratase/amidase pathway marginally. Salts of Barium and Nickel were found to affect hexanenitrile hydrolysis but not the nitrile hydratase/amidase pathway. Di-thiobisnitrobenzoic was also observed to inhibit both hexanenitrile hydrolysis and the nitrile hydratase/amidase. In absence of nitriles and amides in the MB media, *Amycolatopsis*

sp.IITR215 strain also produced nitrile metabolizing enzymes in media containing 1 g/l yeast extract and 1 g/l NH_4Cl as sole nitrogen source which confirms the constitutive nature of all nitrile-metabolizing enzymes of *Amycolatopsis* sp. IITR215

The half life of hexanenitrile metabolizing enzyme was found to be 252 min at 40°C, pH 5.8. Different stabilizers were checked to increase the stability. Maximum stability of hexanenitrile metabolizing enzyme was found in 100 mM NaCl, 50% activity was retained after 420 min. in 100 mM NaCl salt concentration at 40°C, pH 5.8.

The whole cell enzyme for hexanenitrile hydrolysis from *Amycolatopsis* sp.IITR215 was highly active in 50% (v/v) alcohols. Relative activities of 29%, 50% and 14% were detected in 50 % (v/v) of ethyl alcohol, methyl alcohol and isopropyl alcohol respectively.

For the separation of nitrile metabolizing enzymes from *Amycolatopsis* sp.IITR215, cells were lysed by using 2 g/l lysozyme. Proteins in cell free extract were precipitated by 30% sodium sulphate and loaded onto the anion exchange (Q-sepharose) column. From the elution profile, total eight peaks were obtained on the basis of protein O.D. Active fractions were clubbed and used for activity determination. First three factions were of amidase (named amidase 1, 2 and 3) and next three fractions were active on nitrile thus confirmed as nitrile hydratase (named nitrile hydratase 1, 2 and 3). Substrate specificity pattern of these amidases were similar whereas maximum activity for nitrile hydratase 1 was found with acetonitrile and this nitrile hydratase was highly active for butyronitrile, valeronitrile, propionitrile and adiponitrile while nitrile hydratase 2 was showing totally different profile from nitrile hydratase 1. Maximum activity was found with butyronitrile and it was highly active for hexanenitrile, acrylonitrile, isobutyronitrile, adiponitrile, propionitrile and methacrylonitrile. Moreover, maximum activity for nitrile hydratase 3 was found with hexanenitrile and it was highly active for long chain nitrile such as propionitrile, butyronitrile and dinitrile (adiponitrile). On the basis of protein band position in native-PAGE analysis and zymogram, presence of one amidase was confirmed. Substrate specificity and native-PAGE analysis of nitrile hydratases supports the presence of three probable nitrile hydratase fractions but this needs to be further confirmed after subsequent purification of these three active fractions.

Cell free extract of *Amycolatopsis* sp.IITR215 was used to treat PAN powder at 30, 37 and 45°C. No ammonia was detected at 30°C and maximum was at 37°C after 12 hours of polymer treatment. Protein adsorption was higher at pH 7.0 as compared to pH 5.8. Nearly 4283 and 7962 µM ammonia was detected at pH 7.0 and 5.8 respectively after 30 hour of polymer treatment. Enzyme treated polymer of different pH was subjected to FTIR analysis. In FTIR spectra, a new peak was detected at 1547 cm^{-1} which corresponds to the formation of carboxylate group whereas peak at 1644 cm^{-1} gets broadened which supports the formation of amide group on PAN.

Carboxyl groups on the enzyme treated polymer was quantified by Rhodamine 6G by decrease in absorbance at 480 nm. Untreated PAN was taken as the control. The carboxyl group formation was maximum at pH 5.8. At pH 5.8, 106 µmole/g polymer and 456 µmole/g polymer of carboxyl group were found in control and enzyme treated PAN respectively. At pH 7.0, 117 µmoles/g polymer and 227 µmoles/g polymer of carboxyl group were found in control and enzyme treated PAN respectively. On the basis of protein adsorption, ammonia release and formation of new peak at 1541 cm^{-1}, 15 g/l of PAN found to be the optimum concentration for surface modification of PAN.

Protein adsorption and ammonia release on other PAN co-polymers such as PMA, PAN-co-butadiene-co-styrene and PAN-co-methacrylate were also studied at pH 7.0. Protein adsorption was higher on PMA. Nearly 60% proteins were adsorbed on PMA after 36 hour of polymer treatment while protein adsorption process was slow on PAN-co-butadine-co-styrene. In FTIR spectra, no distinct peak for carboxylate group was detected with enzyme treated PAN-co-butadiene-co-styrene, PMA and PAN-co-methacrylate. Similarly, no ammonia was detected with PAN-co-butadiene-co-styrene, PMA and PAN-co-methacrylate which shows that nitrile metabolizing enzyme from *Amycolatopsis* sp.IITR did not convert –CN groups present on these PAN co-polymers to carboxylic acid. In FTIR spectra, no distinct peak was observed but peak at 1638 cm^{-1} get broader with these polymers which supports the formation of amide groups on these polymers.

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VIKASH BABU

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ABBREVIATIONS

ATR-FTIR	Attenuated total reflectance fourier transform spectroscopy
AU	Arbitrary unit
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CTAB	Cetyl trimethylammonium bromide
DEPA	Di-ethylphosphoramidate
DMA	Di-methyl acetamide
DMF	Di-methyl formamide
DNA	Deoxy ribonucleic acid
dNTP	deoxyribonucleotide triphophate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
et al	and others
etc	etcetera
ETP	Effluent Treatment Plant
Fig	Figure
FTIR	Fourier transform infra-red spectroscopy
GC	Gas chromatography
g	Gram
h	Hour
HDPE	High density polyethylene
HPLC	High performance liquid chromatography
IAA	Indoleacetic acid
IAN	Indoleacetonitrile
I.D.	Inner diameter
IU	International Unit
kb	Kilobase
kDa	Kilodalton
kHz	kilo Hertz

l	Litre
LB	Luria Bertani
LDPE	Low density polyethylene
LLDPE	Linear low density polyethylene
MB	Mineral base
M	Molar
mM	Millimolar
mg	Milligram
min	Minutes
ml	Millilitre
N	Normal
NCBI	National Center for Biotechnology Information
NHase	Nitrile hydratase
NO	Nitrogen oxide
OD	Optical density
PAGE	Poly-acrylamide Gel Electrophoresis
pH	Potential of hydrogen
PAN	Poly-acrylonitrile
PBO	Poly-*p*-phenylene-2,6-benzobisthiazole
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Polyethylene
PEG	Polyethylene glycol
PET	Polyethylene terepthalate
PHS	Polyhydroxystyrene
PMA	Poly-acrylonitrile-co-methacrylonitrile
RNA	Ribo-nucleic acid
rpm	Rotations per minute
r-RNA	ribosomal- Ribonucleic acid
TBE	Tris-borate-EDTA
TBO	Toludine blue

TE	Tris-EDTA
TEMED	N, N, N', N'- tetramethylenediamine
UV	Ultra Violate
v/v	Volume per volume
w/v	Weight per volume
XPS	X-ray photoelectron spectroscopy
µg	Microgram
µl	Micolitre
µM	Micromolar

Chapter 1
Introduction

1. INTRODUCTION

Biocatalysis had long been a key focus area in biotechnology. It involves the use of enzymes and microbes to carry out chemical reactions. Enzymes increase the rate of reaction by lowering the activation energy without affecting the reaction equilibrium. Application of enzymes is well established in the production of pharmaceuticals, food ingredients, agrochemicals, fine chemicals and for detection purposes (Gautam and Kumar 2008). Enzymes are also widely used in the production of fine chemicals and optically active compounds of industrial interest (Table.1.1).

Over the last decade, efforts were made to modify the surface of synthetic polymers. Synthetic polymers contribute a major portion of textile materials and biomedical materials besides its usual applications as technical materials. Synthetic polymers have unique properties, such as high uniformity, mechanical strength and resistance against chemicals to impart skin comfort and antimicrobial activity for textile material. Effective production of polymers and improving fibers properties are necessary for further application. Surface of the synthetic polymers can be modified by chemical, physical and enzymatic methods (Fig.1.1). Chemical modification requires harsh reaction due to which strength properties of polymers get affected. Chemical treatments are difficult to control and have negative impact on the environment. Physical methods include plasma treatment, UV irradiation, corona discharge and flame treatment. Among these methods plasma treatment is widely used for the surface modification of synthetic polymers. Plasma can be obtained by exciting gases into energetic state by radio frequency, microwave, or electrons from a hot filament discharge. Generation of plasma requires a vacuum which cause many complications for continuous operation in a large scale industrial process (Inagaki 1996). Moreover, plasma treatments are difficult to repeat, require complicated machinery and difficult to optimize. The main disadvantage of plasma treatments is the formation of heterogeneous undesired functional groups on the surfaces. To overcome these problems, enzyme treatments can be chosen as it offers many advantages over chemical and physical methods which includes: (i) they can act at very low concentration of catalyst (Menger 1993) (ii) they can act under moderate reaction conditions

Table 1.1: Industrial applications of some important enzymes (Kirk *et al.*, 2002)

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Color clarification, anti-redeposition
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Pullulinase	Saccharification
	Xylanase	Viscosity reduction (fuel and starch)
	Cyclodextrin glucosyl-transferase	Cyclodextrin production
	Protease	Protease (yeast nutrition-fuel)
Food	Protease	Milk clotting, infant formulas, flavor
	Trypsin	To predigest baby foods
	Lipase	Cheese flavor
	Lactase	Lactose removal
	Pectinase	Fruit based products
Baking	Amylase	Bread softness and volume
	Xylanase	Dough conditioning
	Glucose oxidase	Dough strengthening
	Protease	Biscuits, cookies
Animal feed	Phytase	Phytate digestibility
	β-Glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Acetolatate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer)
Textile	Cellualase	Denim finishing, cotton softening
	Amylase	De-sizing
	Catalase	Bleach termination

Industry	Enzyme class	Application
Pulp and paper	Lipase	Pitch control
	Protease	Biofilm removal
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	**Synthesis of enantiopure carboxylic acids**
	Nitrile hydratase	**Synthesis of useful amides**
	Amidase	**Synthesis of carboxylic acids**
Leather	Protease	Unhearing, bating
	Lipase	De-pickling
	Tannase	Tanin removal
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, Anti-microbial
	Peroxidase	Antimicrobial
Rubber industry	Catalase	To convert latex into foam rubber
Molecular Biology	Restriction enzymes	To manipulate DNA
	DNA ligase	To ligate digested DNA strands
	Polymerases	In polymerase chain reaction

which leads to minimal damage to strength properties of synthetic polymers (iii) they display selectivity: such as (a) chemo- selectivity (act on a single type of functional group) (b) regioselectivity (enzymes can distinguish between same functional groups based on position) (c) enantioselectivity (specificity for specific enantiomer) (Loughlin 2000) (iv) they can act

on synthetic substrate (v) they can work in non- aqueous environment, although some activity can be lost (vi) enzymatic surface modification limits to the surfaces of polymers.

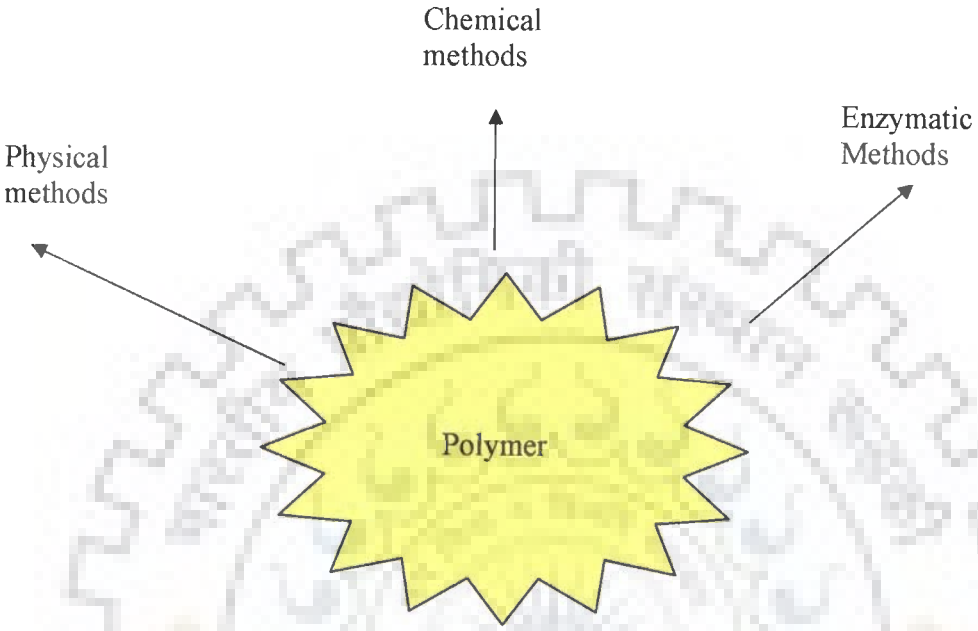


Fig.1.1: Methods of surface modification of synthetic polymer

Polyacrylonitrile (PAN) is a polymer of acrylonitrile monomers. Worldwide 2.73 million tons of polyacrylonitrile are produced per year, of which over 98% are processed as filament yarn serving as material in the textile industry (Tauber *et al.*, 2000). PAN usually has a molecular weight of 55000-70000 g mol^{-1} and is most commonly a copolymer produced by radical polymerization from acrylonitrile, 5-10mol% vinyl acetate (or similar non-ionic co-monomers) to disrupt the regularity and crystallinity, and ionic co-monomers, such as sulphuric or sulphonic acid salts (Saurer 2004). Due to the presence of –CN groups, PAN shows less hydrophilicity which affects the processability of the fibers. The surface is not easily wetted, thus impeding the application of finishing compounds and coloring agent and also hinders water from penetrating into the pores of fabric. So, for many applications surface modification is required to make it more hydrophilic. Nitrile metabolizing enzymes can be used for the surface modification of polyacrylonitrile with many advantages over chemical and physical methods. Thus, there is a need to develop nitrile metabolizing enzyme which

will be able to modify the –CN groups present on the surfaces of polyacrylonitrile. This bioconversion will lead to a polyacrylonitrile with improved surface properties.

In earlier reported literature, nitrile hydratase from *Brevibacterium imperiale* and *Corynebactrium nitrophilus* was used to convert nitrile groups to amide groups in PAN polymer which resulted in increase in hydrophilicity (Battistel *et al.*, 2001). Similarly, nitrile metabolizing enzymes from *A. tumefaciens* BST05 were able to incorporate amide groups in PAN powder as assessed by ATR-FTIR and after prolong treatments, only traces of ammonia was detected which showed the formation of carboxyl groups which was further confirmed by K/S values after staining with Methylene blue. (Fischer-Colbrie *et al.*, 2006). However, Tauber *et al.* 2000 found that nitrile hydratase and amidase from *Rhodococcus rhodochrous* NCIMB 11216 were able to partially convert –CN groups of granular PAN (40 kDa) and PAN190 (kDa) to the corresponding acid while nitrile groups of acrylic fibers were only hydrolyzed to the amide (Tauber *et al.*, 2000). Fischer-Colbrie et al. 2007, isolated *Micrococcus luteus* BST20 for the production of membrane-bound nitrile hydrolyzing enzymes which was able to convert nitrile groups on PAN powder surface to the corresponding acids. They confirmed formation of carboxylic acid by determining the NH_3 release from PAN powder, measuring the depth of shade of enzyme treated fabric after dyeing with a basic dye and by solid state NMR (Fischer-Colbrie *et al.*, 2007). The surface of an acrylic fiber was also modified with commercial nitrilase. Matama *et al.* (2007) investigated the enzymatic conversion of nitrile groups into the corresponding carboxylic groups by commercial nitrilase (Matama *et al.*, 2007) In all these reports, there is a lack of data on mechanism of enzyme action and efficiency with other nitrile co-polymers. All these reported literature on enzymatic surface modification of nitrile polymers concentrated on polymer characterization and there are lacks of data on the enzyme actions on the polymer. Keeping in view of this available information, present research work entitled "Development of nitrile metabolizing enzyme for surface modification of polyacrylonitrile" was undertaken with following objectives:

1. Isolation and screening of micro-organism for the production of nitrile metabolizing enzyme having specificity for cyano group of polyacrylonitrile.
2. Characterization of polyacrylonitrile hydrolyzing enzyme.

3. Treatment of polyacrylonitrile with enzyme and optimization of its treatment conditions.
4. Surface characterization of polyacrylonitrile by FTIR and quantification of functional groups.

Chapter 2
Review Literature

2. LITERATURE REVIEW

Enzymatic surface modification of natural polymers has already been reported (Jingchan *et al.,* 2005). Surface modification of cellulose fibers by cellulases has already been demonstrated in the industry (Gübitz and Paulo 2003). Synthetic polymers have wider application in biomedical field and textile industries where surface properties play an important role. Thus, interest on modifying surface properties of polymers by adopting greener route has increased.

Synthetic polymers such as polyamides, polyethylene, poly (methyl methacrylate), polyacrylonitrile, polyethyleneterephthalate are widely used polymers as biomaterials in medical field for making sutures, joint replacement, dental implants, haemodialysis membrane, vascular prosthesis catheters, surgical thread etc. For an ideal biomaterial, biocompatibility is the key requirement. When a foreign material is implanted in the body, it is being attacked by microorganisms which are present in the body fluid and protein adsorption is also a main problem with the biomaterial (Shmack *et al.,* 2000). Likewise, proteins in the form of coagulation factor are also adsorbed on the hydrophobic polymeric surface. Above mentioned synthetic polymers are hydrophobic or less hydrophilic in nature. Therefore, special treatments are required for making the ideal biomaterial by transforming hydrophobic surface into hydrophilic hemocompatible surface.

Synthetic polymers are also a major contributor in the textile industry. Synthetic fibers show excellent strength properties, chemical resistance, wrinkle resistance and abrasion resistance. These fibers also show some undesired properties such as hydrophobicity due to which they show wearing discomfort as the perspiration can not penetrate the fabric and low reactivity with chemical agents which act as barrier to other finishing agents. These problems are due to the presence of hydrophobic groups on the surface of synthetic polymers. Therefore, surface modification of synthetic polymers is carried out with the major objective of making textile fabric more hydrophilic without affecting bulk properties of polymer fiber.

2.1 Enzymes for surface modification of polymers

Few reports are available for the enzymatic surface modification of synthetic fibers. Peroxidase, lipase, cutinase, nitrilase, nitrile hydratase, amidase, protease and hydrolases have been reported for surface modification of synthetic polymers (Table 2.1)

Table 2.1: Enzymes used for the surface modification of synthetic polymers

Enzyme	Source	Polymeric Substrate	Application of polymer	Reference
Peroxidase	Soyabean peroxidase	High-density polyethylene	Haemofilteration membranes, sutures	Zhao *et al.*, 2004
	Horseradish	Poly-p-phenylene-2,6-benzobisthiazole	In aerospace and civil industry	Wang *et al.*, 2007
Lipase	*T. lanuginosus*	PET	Textile fibers, vascular prosthesis	Brueckner *et al.*, 2008
	Rhizopus miehei	poly (methyl acrylate)	Textile fibers	Inprakhon *et al.*, 2007
	Commercial Lipase	PET	Textile fibers, vascular prosthesis	Kim and song *et al.*, 2006
Cutinase	*Fusarium solani pisi*	PET	Textile fibers, vascular prosthesis	Araujo *et al.*, 2007
Nitrilase,	*Micrococcus luteus*	PAN (with 5% vinyl acetate)	Textile industry, haemodialysis membrane	Fischer-colbrie *et al.*, 2007
	Commercial nitrilase	PAN (with 7% vinyl acetate)	Textile industry, haemodialysis membrane	Matama *et al.*, 2007
Nitrile Hydratase	*Rhodococcus rhodochrous*	PAN 40, PAN 190 (with 7% vinyl acetate groups)	Textile industry, haemodialysis membrane	Tauber *et al.*, 2000
	Brevibacterium imperiale	PAN (with 10% acetate groups)	Textile industry, haemodialysis membrane	Battistel *et al.*, 2001
	Corynebacterium nitrilophilus	PAN (with 10% acetate groups)	Textile industry, haemodialysis membrane	Battistel *et al.*, 2001
	Agrobacterium tumefaciens	polyacrylonitrile	Textile industry, haemodialysis membrane	Fischer-colbrie *et al.*, 2006
Amidase	*Nocardia farcinica*	Polyamide 6	Textile industry, sutures	Heumann *et al.*, 2009

Protease	*Bacillus subtilis*	Nylon 6.6	Textile industry, haemofilteration membranes, sutures	Parvinzadeh *et al.*, 2009
Hydrolase	*Thermonospora fusca*	polyethylene terephthalate	Textile industry, haemodialysis membrane	Alisch-Mark *et al.*, 2006
	Fusarium solani f. sp. pisi	polyethylene terephthalate	Textile industry, haemodialysis membrane	Alisch-Mark *et al.*, 2006
	Fusarium oxysporum strain (LCH 1)	polyethylene terephthalate	Textile industry, haemodialysis membrane	Nimchua *et al.*, 2007

2.1.1 Peroxidases

Peroxidases belong to the class of oxidoreductases contain iron (III), and protoporphyrin IX as the prosthetic groups. Peroxidase catalyzes the reduction of peroxides with simultaneous oxidation of many organic and inorganic compounds. These enzymes are widely used for the removal of phenolic compounds, decolorization of synthetic dyes, and deodorization of swine manure, in enzyme immunoassays, for biofuel production and organic compound and polymer synthesis (Hamid *et al.*, 2009). Peroxidases have also been used for the surface modification of poly-*p*-phenylene-2,6-benzobisthiazole (PBO) (Wang *et al.*, 2007), polyethylene (Zhao *et al.*, 2003) and grafting of acrylamide onto kevlar fibers.

Poly-*p*-phenylene-2,6-benzobisthiazole (PBO) fibers are widely used in aerospace industries and civil industries for its excellent properties such as good chemical and fire resistance, light weight and good toughness (Wang *et al.*, 2007). These fibers possess poor adhesive properties due to its hydrophobicity. Therefore, surface modification of these fibers is required. Wang *et al.* modified these fibers by horseradish peroxidase so that they became hydrophilic and acrylic acid was grafted onto the modified PBO fibers which were confirmed by FTIR and XPS analysis (Wang *et al.*, 2007).

Polyethylene is widely used inexpensive and versatile polymer due to its abundant supply, good chemical resistance, good process ability and low energy demand for processing. Polyethylene exists in low density polyethylene (LDPE), linear low density polyethylene (LLDPE) and high density polyethylene (HDPE) forms. HDPE has very poor

adhesion properties to other materials because of PE's low surface energy, which limits its applications in gluing, painting and printing. Peroxidase from soybean was used to modify high density polyethylene (HDPE) at the expense of hydrogen peroxide. Due to the incorporation of –OH and –CO- functional groups, the hydrophilicity and dyeing ability of water soluble dyes was enhanced which was confirmed by water contact angle measurement and XPS analysis (Zhao *et al.,* 2004). Horseradish peroxidase was also used to graft acrylamide onto the Kevlar fibers for their surface modification. Modified fibers were analyzed by scanning electron microscopy and elemental analysis (Zhao *et al.,* 2005)

2.1.2 Lipases

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are hydrolytic enzymes, which act on the carboxyl ester bonds present in acylglycerols to liberate fatty acids and glycerol (Joshi *et al.,* 2006, Fernandez *et al.,* 2008). Lipases show chemo-, regio- and enantio-selectivities (Saxena *et al.,* 2003). A lot of work has been done on the surface modification of PET fibers using lipases (Fig.2.1). Kim and Song (2006) analyzed the effect of nine commercial lipases on PET fabrics. Moisture regain of PET fibers was improved by 2.4 times as compared to alkaline treatment and also the carboxyl and hydroxyl groups were introduced (Kim and Song *et al.,* 2006). Similarly, Lipase from *T. lanuginosus* was used to increase the hydrophilicity of PET fabrics by introducing hydroxyl and carboxyl functional groups (Brueckner *et al.,* 2008) whereas lipase from *Rhizopus miehei* was used to introduce regioselective modification in a telomere of poly (methyl acrylate) by modifying the ester group (Inprakhon *et al.*, 2007). Hydrolases from *Thermomonospora (Thermobifida) fusca* and *Fusarium solani f. sp. pisi* were used to increase hydrophilicity of the PET fibers. Due to increase in hydroxyl groups, reactive dye on polymer showed more intense color which was confirmed by reflectance spectroscopy and their water absorption ability was also enhanced (Araujo *et al.,* 2007).

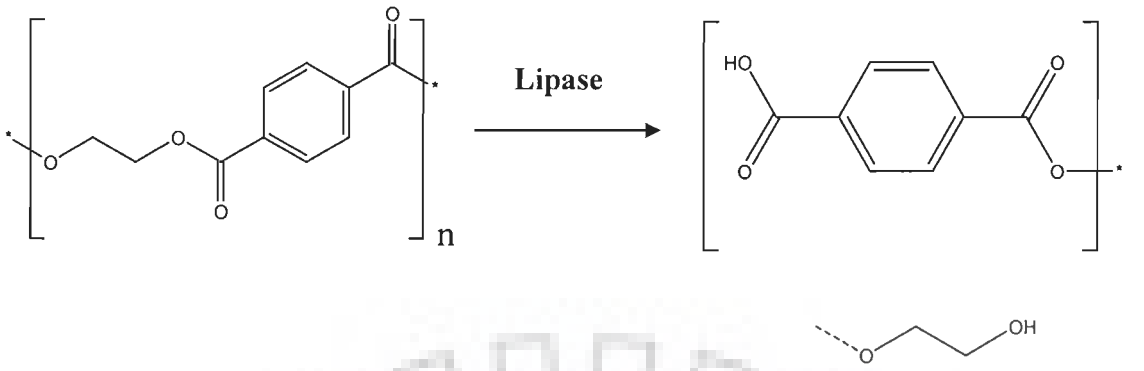


Fig. 2.1. Hydrolysis of PET fibers by lipase.

2.1.3 Cutinases

Cutinases are hydrolytic serine esterases that degrade cutin which is polyester of hydroxy and epoxy fatty acids (Purdy and Kolattukudy 1975, Ronkvist *et al.,* 2009) and specific for primary alcohol esters (Murphy *et al.,* 1996). The fatty acids of cutin are usually *n*-C16 and *n*-C18 oxygenated hydroxyacids (contain one to three hydroxyl groups). Cutins are lipid based polymer of plants and ester bonds dominates in the cutins. Therefore, cutinases are considered as esterase and they can be used for the hydrolysis of ester groups present in the synthetic fibers such as PET. There are many reports on the surface modification of polyester, polyamide 6,6 and acrylic fibers using cutinases. Cutinase from *Fusarium solani pisi* was able to hydrolyze polyester groups to modify the surface of polyester, polyamide and acrylic fibers (introduce hydroxyl groups in case of polyester and co-monomer of acrylic fiber, amino group in polyamide). This cutinase had higher activity on polyamide than on polyester (Silva *et al.,* 2005) (Fig.2.2). Later on, this cutinase was genetically modified near the active site, by site-directed mutagenesis, to enhance its activity towards polyethylene terephthalate (PET) and polyamide 6,6 (PA 6,6) fibers by increasing the size of active site in order to fit a larger polymer chain (Araujo *et al.,* 2007).

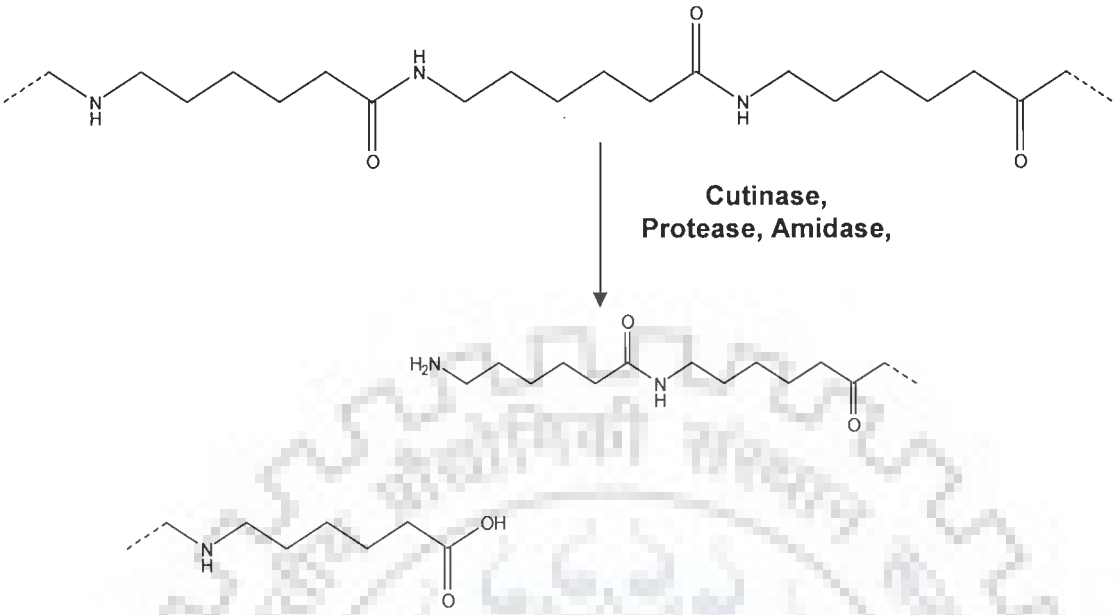


Fig. 2.2. Mechanism of action of cutinase, protease and amidase on polyamide.

2.1.4 Proteases and tyrosinases

Proteases are hydrolytic enzymes that catalyze the hydrolysis of peptide bond. Alkaline proteases are most commonly used in textile industry as additives of detergents (Gupta *et al.,* 2002) and can be used as for the surface modification of Nylon 6-6 fibers. Nylon 6, 6 is a copolymer of hexamethylene diamine and adipic acid. Many proteases such as protex Gentle L, protex 40L, protex multiplus L and protex 50FP were used to investigate changes in the nylon 6,6 polymer. Protease treatment of Nylon 6, 6 fibers showed significant decrease in thermal degradation temperature whereas reactive and acid dyes showed higher dye bath exhaustion on the protease treated polymer (Parvinzadeh *et al.,* 2009).

Tyrosinase catalyzes the oxidation of phenols. These enzymes are widespread in fungi, plants and animals. Polyhydroxystyrene (PHS) is a phenol containing polymer used as the excellent polymer matrix due to its good coating properties. Phenol moieties of PHS can be oxidized by tyrosinase. Mushroom tyrosinase was observed to catalyze the oxidation of 1-2% phenolic moieties of the synthetic polymer, poly (4-hydroxystyrene) (PHS) (Shao *et al.,* 1999) (Fig.2.3).

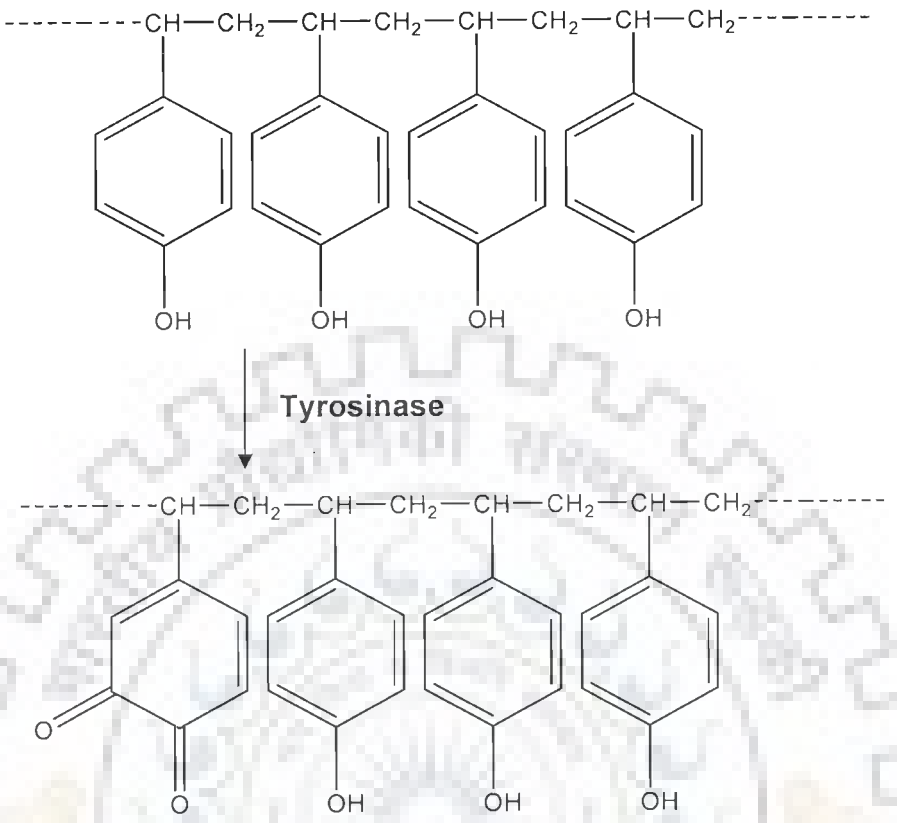


Fig. 2.3. Oxidation of Poly(4-Hydroxystyrene) by tyrosinase

2.5 Nitrile metabolizing enzymes

Nitrile metabolizing enzymes such as nitrilase, nitrile hydratase and amidase can be used for the hydrolysis of polyacrylonitrile and polyamide (Fig. 2.4). This bioconversion will lead to improved surface properties with minimal impact on environment.

Nitrile metabolizing enzymes are such biocatalyst which finds widespread applications in the fine chemical industry. Nitrile metabolizing enzymes consist of nitrilase and nitrile hydratase/amidase. Nitrile metabolizing enzymes belong to the class of hydrolases which catalyze the hydrolysis reactions. These enzymes convert –CN group to –COOH by two pathways. Nitrilase (EC 3.5.5.1) which belong to the class hydrolase catalyzes the direct cleavage of nitriles to the corresponding acids and ammonia. In the second pathway, nitriles are catabolized in two stages, via conversion to the corresponding amides by nitrile hydratase (EC 4.2.1.84) and then the acids plus ammonia by amidase (EC 3.5.1.4). These nitrile-converting enzymes are expected to have great potential as catalysts in organic chemical

processing because of the mild reaction conditions, quantitative yields, and absence of by-products and in some cases enantio- or regio-selectivity.

Twenty six years ago, change in properties of filament of PAN was reported due the attack of fungal enzyme of *Cladosporium cladosporioides* (Sato 1984a, b). Thereafter, *Rhodococcus rhodochrous* was used for the hydrolysis of both granular PAN and acrylic fibers by nitrile hydratase and amidase (Tauber *et al.*, 2000). Battistel *et al.*, (2001) used nitrile hydratase from commercially available *Brevibacterium imperiale*, *Corynebacterium nitrilophilus* for the conversion of nitrile groups into the corresponding amide as assessed by XPS analysis (Battistel *et al.*, 2001). Similarly, *Agrobacterium tumefaciens* (BST05) was found to convert polyacrylonitrile to polyacrylic acid by nitrile hydratase and amidase (Fischer-Colbrie *et al.*, 2006). Nitrilase was also used for the surface hydrolysis of polyacrylonitrile from *Micrococcus luteus* BST20 (Fischer-Colbrie *et al.*, 2007). Matamá *et al.* modifies acrylic fiber by using commercially available nitrilase (Matama *et al.*, 2007). However, Polyamidase from *Nocardia farcinica* leads to an increase of polar groups on the surface of polyamide which was measured by tensiometry. (Heumann *et al.*, 2009). Most of theses studies reported on treatment conditions with enzyme and characterization of treated polymers. Rarely any study concentrated on mechanism of enzyme action on these polymers.

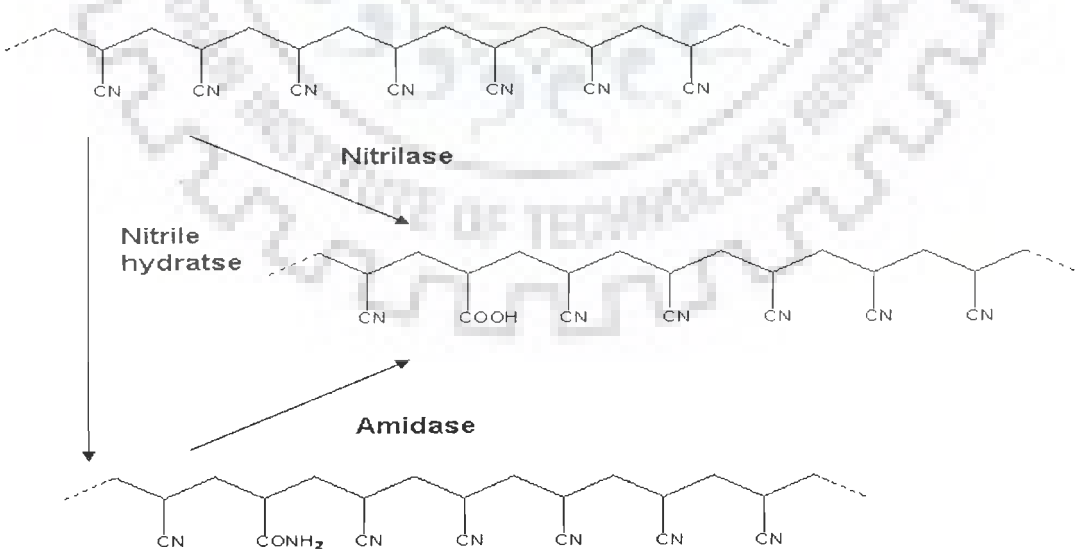


Fig. 2.4. Mechanism of nitrilase, nitrile hydratase and amidase on polyacrylonitrile.

2.5.1 Historical Background of nitrile metabolizing enzymes

Nitrilases were first reported by Thimann and Mahadevan in 1964 in Gramineae (grasses), Cruciferae (cabbage group and radish), and Musaceae (banana family) (Thimann and Mahadevan, 1964). This enzyme was partially purified from barley leaves. In the same year, another nitrilase was purified from ricine (Robinson 1964). Nitrile hydratase discovered two decades later. (Asano *et al.,* 1980). Yamada and Glazy in 1992 reported the enzymatic production of acrylamide from acrylonitrile using nitrile-hydrating whole cells of *Rhodococcus* sp. N-774, *Pseudomonas chlororaphis* B23 and *Rhodococcus rhodochrous* J1 (Yamada H and Kobayashi M. 1996).

2.5.2 Distribution of nitrile-metabolizing enzyme

Nitrile metabolism is found in 3 of 21 plant families (Gramineae, Cruciferae, and Musaceae) (Thimann and Mahadevan 1964), also reported in some fungi such as *Kluveromyces thermotolerans* MGBY37 (Prasad *et al.,* 2005), *Fusarium, Aspergillus and penicillium* (Harper.a 1977). Existence of nitrile metabolizing enzyme is very common in bacteria. A number of bacteria such as *Bacillus pallidus* Dac521, *Pseudomonas putida, Alcaligenes faecalis, Arthobacter nitroguajacolicus ZJUTB06-99, Klebsiella pneumoniae subsp. Ozaena, Bacillus subtilis ZJB-063,* (Almatawah *et al.,* 1999, Banerjee *et al.,* 2006, Singh *et al.,* 2005, Shen *et al.,* 2009, McBride *et al.,* 1986, Zheng *et. al.,* 2008) has been isolated for the metabolism of nitriles as carbon and nitrogen source. The physiological role of nitrile metabolism is still unclear. In plants, nitrile metabolism has been reported for the degradation of glucosinolates (Bestwick *et al.,* 1993), detoxification of cyanides (Piotrowski *et al.,* 2001) and in the production of indole-3- acetic acid (Bartel and Fink 1994).

2.5.3 Enzymology of nitrile metabolizing enzymes

Three types of biochemical reactions eliminate the –CN group of nitriles: hydrolysis, oxidation and reduction.

- **Oxidation-** Nitriles are oxidized to cyanohydrins by oxygenase enzymes presents in some plants and insects. Cyanohydrins can further be decomposed to aldehyde and HCN by the action of other enzymes such as oxynitrilase or hydroxyl nitrile lyase (Ramakrishan *et al.,* 1999).

- **Reduction**- Nitriles are reduced to hydrocarbon and ammonia by nitrogenase present in nitrogen fixing bacteria (Ramakrishnan *et al.,* 1999).
- **Hydrolysis**- This is the most common pathway of nitrile metabolism. It proceeds via formation of carboxylic acid or amides and ammonia. Enzymes that catalyze nitrile hydrolysis are Nitrilase and nitrile hydratase (NHase) that are present in bacteria, fungi and plants (Ramakrishnan *et al.,* 1999).

Hydrolysis is the most common pathway for the microbial metabolism of nitriles. Nitrilases catalyze the conversion of organic nitriles to corresponding acids and NH_3, while NHases catalyze the formation of amides from nitriles, which are subsequently converted to acids and NH_3 by amidases.

2.5.4 Nitrilase

Nitrilases (EC 3.5.5.1) are also known as α/β hydrolases belong to the nitrilase super-family. Nitrilases have immense potential to transform broad range of nitriles. Nitrilase is the first nitrile metabolizing enzyme to be discovered and first described by Thimann and Mahadevan (1964) in barley during growth on the naturally occurring nitrile, ricinine (N-methyl-3-cyano-4-methoxy-2- pyridone), as a sole carbon source. Leaves, catalyzing the conversion of indoleacetonitrile (IAN) to indoleacetic acid (IAA) found to contain enzyme, initially called as indoleacetonitrilase. Thereafter, the enzymes had shown to have 26 nitriles as substrates and thus because of this broad range of substrates specificity, the enzyme was renamed as "Nitrilase" (Thimann and Mahadevan 1964). The first bacterial nitrilase was isolated from a soil bacterium (possibly a *Pseudomonas* species) by growth on selective media that contain the naturally occurring nitrile, ricinine (N-methyl-3-cyano-4-methoxy-2-pyridone), as a sole carbon source (Hook and Robinson 1964; Robinson and Hook 1964). The biotechnological potential of nitrilases has led to the isolation of a range of bacteria and fungi capable of hydrolyzing nitriles. Most of these were isolated by using a particular nitrile as a carbon and/or nitrogen source. Production media of nitrilases has been optimized by several co-workers. Media composition of some of the nitrilase producing bacteria and fungi are shown in Table. 2.2.

Till date, more than 20 nitrilases have been purified and characterized. Based on their substrate specificity, microbial nitrilases are differentiated into three categories. They are categorized as aromatic, aliphatic and arylaliphatic nitrilases. Most of them are inducible enzymes composed of one or two types of subunits. These subunits associate to convert an enzyme to active form in the presence of substrate, i.e. nitrile (Banerjee *et. al.,* 2002). Substrate specificity, optimum pH, temp., molecular weight, no. of subunits and nature of some of the nitrilases have been summarized in (Table.2.3).

Table.2.2: Production media for some nitrilases from different micro-organisms

Organism	Production media Composition (g/l)	Inducer/Nitrogen source	pH	Temp (°C)	Cultivation Time (h)	Reference:
Arthbacter nitroguajacolicus ZJUTB06-99	Glucose-10, peptone-5, K_2HPO_4-0.5; $MgSO_4 \cdot 7H_2O$-0.5, and 0.75, monosodium Glutamate-0.75.	ε-caprolactam-0.4	7.0	30	72	Shen *et al.*, 2009
Pseudomonas Putida	Starch-5, Soy peptone-5, sodium chloride-5, yeast extract 1.5, malt extract-1.5	Acrylonitrile	7.5	30	12	Banerjee *et al.*, 2006
Klebsiella pneumoniae subsp. *Ozaenae*	K_2HPO_4 -3.5 g, KH_2PO_4 -1.5, of $MgSO_4 \cdot 7H_2O$-0.1, glucose -2.0, 3,5-dibromo-4-hydroxybenzonitrile sodium salt -0.5	3,5-dibromo-4-hydroxybenzonitrile (bromoxynil)	7.0	30	24	McBride *et al.*, 1986
Bacillus pallidus strain Dac521	KH_2PO_4 -2, NaC1-1, $MgSO_4 \cdot 7H_2O$ -0.2, $FeSO_4 \cdot 7H_20$ -0.03	Benzonitrile	7.2	50	20	Almatawah *et al.*, 1999
Acidovorax facilis strain 72W (ATCC 55746)	5x basal medium supplemented with glucose -10, Adipamide -4 fed at 0 h and 24 h, respectively.	Adipamide	7.2	30	48-65	Gavagan *et al.*, 1999
Bacillus subtilis ZJB-063	Glucose-13.5, yeast extract-5, $(NH4)_2SO_4$ -5, K_2HPO_4 -0.66, KH_2PO_4 -0.5, $MgSO_4 \cdot 7H_2O$ -0.5 and $FeSO_4 \cdot 7H_2O$ -0.01	Nil	7.0	30	5 days	Zheng *et al.*, 2008
Fusarium solani O1	modified Czapek-Dox agar (sucrose -30, K_2HPO_4 -1.0, $MgSO_4 \cdot 7H_2O$ -0.5, KCl -0.5, $FeSO_4 \cdot 7H_2O$ -0.01, $CoCl_2 \cdot 6H_2O$ -0.001, $ZnSO_4 \cdot 7H_2O$ -0.0067,	3-cyanopyridine NaNO3	7.5	28	2-6days	Kaplan *et al.*, 2006
Alcaligenes faecalis MTCC 126	ammonium acetate -10, peptone -5, yeast extract -5, dipotassium hydrogen phosphate -5, magnesium sulphate -0.2, ferrous sulphate -0.03, sodium chloride -1	*n*-butyronitrile	7.2	30	20h	Kaul and Banerjee, 2008
Streptomyces sp.MTCC 7546	yeast extract -1, glycerol -10, trisodium citrate -0.2, 5X mineral base (NaCl -5, KH_2PO_4 -1.35, $MgSO_4 \cdot 7H_2O$ -1, K_2HPO_4 -0.87, $CaCl_2$ - 0.05, $FeCl_3$ -1.25 mg L− 1) and trace element solution (H_3BO_3 -0.3, $CoCl_2 \cdot 6H_2O$ -0.2, $ZnSO_4 \cdot 7H_2O$ -0.1, $MnCl_2 \cdot 4H_2O$ -0.03,$Na_2MoO_4 \cdot H_2O$ -0.03, $NiCl_2 \cdot 6H_2O$ -0.02, and $CuCl_2 \cdot 2H_2O$ - 0.01)	Benzonitrile	7.0	45	24h	Khandelwal *et al.*, 2007

Table 2.3: Characteristics of some purified nitrilases from different micro-organisms

Micro-organism	Nature	Molecular mass		Optimum Temp.	Optimum pH	Substrate specificity	Reference
		Native	No of subunit and its Mwt (kDa)				
Alcaligenes faecalis	Inducible	32		7.5	40-45	Arylacetonitrile	Yamamoto *et al.*, 1992
Bacillus pallidus Dac 521	Inducible	600		7.6	65	Aromatic nitriles	Almatawah *et al.*, 1999
Rhodococcus rhodochrous J1	Constitutive	78	2(41.5)	7.5	45	Aliphatic and aromatic nitriles	Kobayashi *et al.*, 1989
Acidovorax facilis 72W		40	14(570)			Aliphatic nitriles	Chauhan *et al.*, 2003
Rhodococcus sp. ATCC 39484	Inducible	560	_40	7.5	30	Aromatic nitriles	Stevenson *et al.*, 1992
Nocardia sp.	Inducible	560	45	8	30	Aromatic nitrile	
Pseudomonas fluorescens	Inducible		40, 38	9.0	55	Arylacetonitriles	Layh *et al.*, 1998
Rhodococcus rhodochrous J1	Constitutive	78	2 (41.5)	7.5	45	Aliphatic and aromatic nitriles	Kobayashi *et al.*, 1989
Pyrococcus abyssi		60	2 (30)	7.4	80	Aliphatic nitriles	Mueller *et al.*, 2006
F. solani IMI196840		40	550	8	45	benzonitrile and 4-cyanopyridine	Vejvoda. 2010
F. solani O1		40	580	8	40-45	Aromatic nitriles	Vejvoda *et al.*, 2008
F. oxysporum f. sp. melonis		37	550	6-11	40	Arylaromatic nitriles	Goldlust *et al.*, 1989

2.5.4.1 Mechanism of nitrilase catalysis

A possible mechanism for the nitrilase-catalyzed reaction indicates a nucleophilic attack by a thiol group on the carbon atom of the nitrile with concomitant protonation of nitrogen to form a tetrahedral thiomidate intermediate. Thereafter, further steps involve attack of two water molecules and protonation of the nitrogen atom, which is lost as ammonia (Fig.2.5). In some cases the tetrahedral intermediate formed can break down anomalously to produce amide instead of the normal acid product (Banerjee *et al.*, 2002).

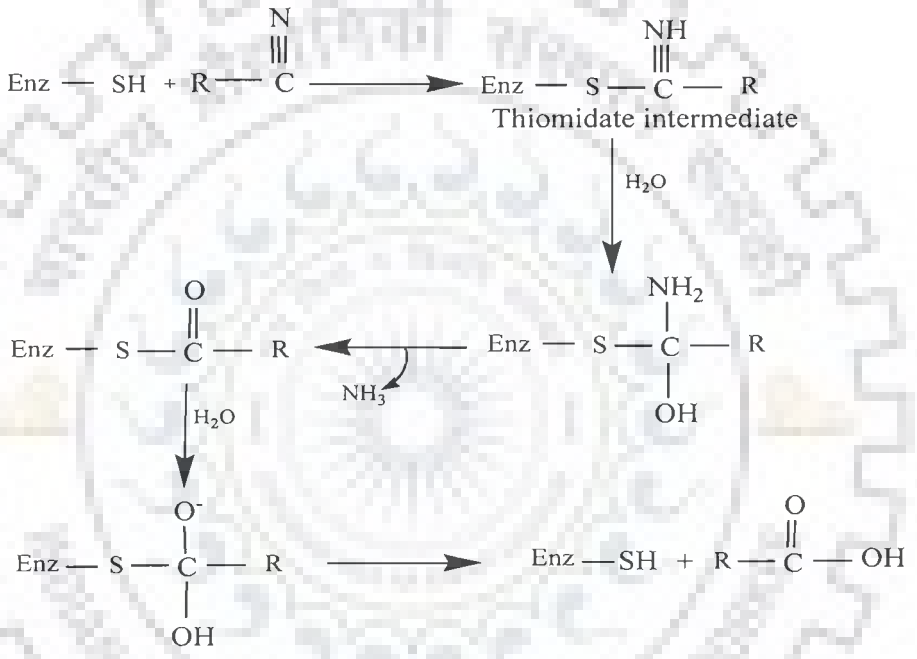


Fig.2.5. Mechanism of nitrilase catalysis.

2.5.4.2 Structure of nitrilase

Till date, no crystal structure was available but recently Raczynska and co-workers solved crystal structures of a thermoactive nitrilase from *Pyrococcus abyssi*. This enzyme belongs to the nitrilase super-family exhibiting true nitrilase activity. It is the only identified microbial nitrilase active as dimer and not as a larger assembly (Raczynska *et al.*, 2010).

In both crystal forms, a dimer possessing 2-fold molecular symmetry is present in the asymmetric unit. Each subunit contains 262 residues and has a αββα sandwich fold, same

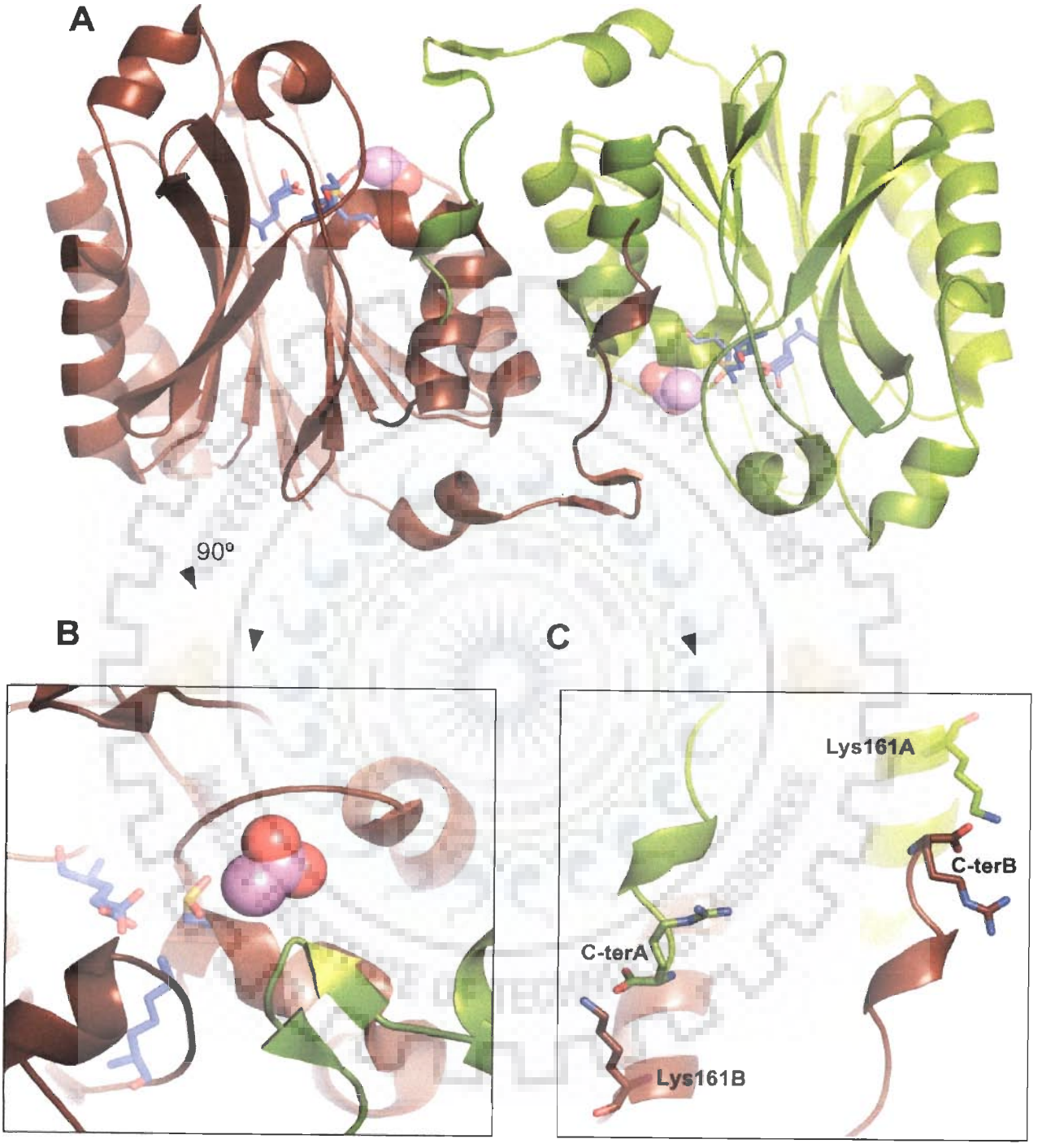


Fig. 2.6. Overall structure nitrilase (a) Nitrilase dimer shown as a cartoon model, view along the non-crystallographic two fold axis. The bound acetate ions are shown as spheres while the catalytic residues (Cys146, Glu42, Lys113) as sticks. (B) A close-up view of the binding site, the scene is rotated 90°downwards compared to A. (C) A detailed view of the C-termini, interacting residues are shown as sticks.

structure was described earlier for the nitrilase super-family members (Nakai *et al.,* 2000, Pace *et al.,* 2000). When the two subunits associate, a 'super-sandwich' (αββαααββα) structure is formed (Fig. 2.6a). The C-terminal part of each subunit extends away from the core and interacts with the other subunit thus playing an important role in protein dimerization (Fig. 2.6). In all but one structures the carboxyl end, forms a salt bridge with Lys161 of the other chain. The exception is the Nit–Br structure where the carboxylate group of Arg262A interacts with Arg255B. The dimer interface contains hydrophobic as well as charged residues. Salt bridges between arginine and glutamate residues constitute a considerable part of interactions responsible for the dimer formation (Raczynska *et al.,* 2010).

2.5.5 Nitrile hydratase

Nitrile hydratases are soluble metalloenzyme (Sugiura *et. al* 1987). These enzymes convert nitriles to its corresponding amides and have been reported in *Bradyrhizobium* (Vega-Hernández *et al.,* 2002), *Acidovorax* (Gavagan *et al.,* 1999), *Agrobacterium* (O'Grady and Pembroke, 1994), *Bacillus* (Kim and Oriel, 2000, Takashima *et al.,* 2000), *Comanonas* (Petrillo *et al.,* 2005), *Klebsiella* (Nawaz *et al.,* 1996), *Mezorhizobium* (Feng *et al.,* 2008), *Pseudomonas* (Yanase *et al.,* 1985; Masutomo *et al.,* 1995, *Rhizobium* (Kobayashi *et al.,* 1996), *Rhodopseudomonas* (Ramakrishna and Desai, 1993), *Brevibacterium* (Lee *et al.,* 1993, Alfani *et al.,* 2001), *Corynebacterium* (Tani *et al.,* 1989), *Nocardia* (Bhalla and Kumar, 2005), *Pseudonocardia* (Miyanaga *et al.*, 2001), *Rhodococcus* (Prasad *et al.,* 2004). Production media for nitrile hydratases has been optimized by many researchers (Table 4). Generally, this enzyme has α and β subunits in equimolar amounts with a basic stoichiometry of α, β and M (M, Fe or Co). On the basis of the metal ion present, NHases can be classified into two broad groups based on the presence of metal ions in their active site: ferric NHases and cobalt NHases. These metal ions may act as either good catalysts for -CN hydration or required for the folding of enzyme. In addition to their function in the active center, the metal ions may play a role in enhancing the folding or the stabilization of the subunit polypeptides of the enzyme (Banerjee *et. al., 2002*). The primary sequences are well conserved among all known NHases while there is no apparent homology between two subunits (Kobayashi *et al.,* 1992). Co-factor, optimum pH, temperature and substrate specificity of purified nitrile hydratases is summarized in (Table 4).

Table.2.4: Production media for some nitrile hydratases from different micro-organisms

Microorganism	Media (g/l)	Inducer	pH	Temp.	Cultivation time (h)	References
Nitrile hydratase gene of *Rhodococcus erythropolis* cloned in E.coli	LB medium , antibiotics (100µg amplicillin /ml; 20µg kanamycin/ml and 20µg chloramphenicol /ml)	IPTG	8.0	25	overnight	Song *et al.*, 2008
Rhodococcus pyridinovorans MW.3	Tryptone -7.5, yeast extract -1, K2HPO4 -0.5, KH2PO4 -0.5, CoCl2.6H2O -0.01, FeSO4.6H2O -0.01, MgSO4 -0.5.	Acrylamide, acronitrile and ure	7.2	28	48 h	Precigou *et al.*, 2004
Nitrile hydratase gene of *Rhodococcus equi* TG328-2 cloned in E. coli	Yeast extract -5 , peptone -10 and NaCl -10 .	IPTG	N.A	30	22h	Rzeznicka *et al,*. 2010
Rhodococcus equi A4	Beef extract -3, peptone -10, NaCl 5.	Methacrylamide a	7 .0	30	24	Pr˘epechalová *et al.*, 2001.
Mesorhizobium sp. F28	R2A media: yeast extract -0.5, peptone -0.5, glucose -0.5, soluble starch -0.5, K2HPO4 -0.3, MgSO4.7H2O -0.05, pyruvate -0.3, agar -15.	Acrylonitrile	7.0 -7.5	37 -45	48	Feng *et al.*, 2008
Pseudomonas putida NRRL-18668	Glucose -20, ferric citrate -0.18, cobaltic citrate -0.1, NaHSO4 -2.2, MgSO4 -2.0, MnCl2 -0.8, boric acid -0.8, NiSO4 -0.2, CuSO4 -0.2, ZnSO4 -0.2, NaMoO4 -0.08, KBr -0.08, KI -0.06, yeast extract -0.05	Butyronitrile	7.2	28	70	Payne *et al.*, 1997
Bacillus smithii SC- JO51	Glycerol -20, Polypepton -3.64, yeast extract -2.18, malt extract -2.18, KH2P04 -6, K2HP04 -4, FeS04.7H20 -0.01, ZnSO4.7H20 -0.01, MnS04.7H20 -0.01 and CoC12.6H20 -0.01.	Acrylonitrile	6.5	45	50	Takashima *et al.*, 2000
Rhodococcus sp. SHZ-1	Glucose -20, yeast extract -5, NaCl -2, KH2PO4 -0.5, K2HPO4·3H2O -0.5, MgSO4·7H2O -0.2.	Acrylonitrile and chloride	7.2	30	48	Chao *et. al*, 2007
Nitrile hydratase gene of *Nocardia* sp cloned in *E.coli.*	LB culture medium supplemented with ampicillin-0.1	IPTG	N.A.	28	20	Shi *et al.*, 2004
Rhodococcus sp. N-771	LB medium: ampicillin (150 µg/ml) and kanamycin (100 µg/ml), ferric citrate -0.4, cobalt chloride -0.4,	IPTG	N.A	27	24	Nojiri *et al.*, 2000

Table.2.5: Characteristics of some purified nitrile hydratases from different micro-organisms

Micro-organism	Nature	Cofactor	Molecular mass		Optimum Temp.	Optimum pH	Substrate specificity	Reference
			Native	No Of subunit and its Mwt (kDa)				
Rhodococcus equi A4	Inducible		60	2 (α: 25) (β: 25)			Aliphatic nitriles	Prepechalova *et. al.*, 2001
Brevibacterium imperalis CBS489–74	Constitutive					6.0	Acrylonitrile	Alfani *et al.*, 2001
Pseudonocardia thermophila JCM 3095		Co		α-23.0 β-26.6		7.0-7.5	Aliphatic nitriles	Yamaki *et al., 1997*
Mesorhizobium sp. F28				(α: 22.66) (β: 28.66)	45	7.0-7.5	Acrylonitrile, propionitrile, isobutyronitrile, and benzonitrile	Feng 2008
Rhodococcus erythropolis N'4	Inducible		92.3	4 (α: 27) (β: 29)	25	7.5	Butyronitrile, Propionitrile	Choi *et al.*, 2008
Rhodococcus sp. AJ270				(α: 22.975) (β: 23.493)	25	7.6	Aliphatic, heterocyclic and aromatic nitriles	Song *et al.*, 2007
Rhodococcus sp. RHA1		Co, Cu and Zn	430	6 (α: 63.0) (β: 56.0)		5.5-10.0	acetonitrile, propionitrile, acrylonitrile, butyronitrile	Okamoto and Eltis 2007
R. rhodochrous PA-34		Co	86	3 (α: 25.04) (β: 30.6)	40	8.0		Prasad *et al.*, 2009
R. equi A4b			74	α-25.0 β-25.0	32-35	7.5	aromatic, heterocyclic and arylaliphatic nitriles	Prepechalova *et al.*, 2001

2.5.5.1 Mechanism(s) of nitrile hydration by NHases

The mechanism of catalysis by NHases remains unresolved at this time. Nelson and coworkers have suggested three plausible mechanisms of catalysis which are shown in Fig.2.7. In the first postulated mechanism (Fig. 2.7a), the nitrile substrate displaces a hydroxide ligand from the coordination sphere of the M (III) center and the metal-bound nitrile undergoes hydrolysis by a water molecule. The reaction generates a metal bound imminol intermediate that rearranges to the amide product and then the amide is released. This is known as the inner-sphere mechanism. The presence of a molecule of iodoacetonitrile in close proximity of the iron (III) center noted in crystallographic structure by Nelson and coworkers provides some support to this mechanism. There is also precedence for platinum (II) and rhodium(II) complexes that bind nitriles and catalyze their hydrolysis via an inner-sphere mechanism (Mascharak 2002).

The second postulated mechanism (Fig. 2.7b), known as the outer-sphere mechanism, involves a nucleophilic attack of the metal-bound hydroxide on the nitrile substrate nested at the active site pocket. The reaction generates a transient imminolate species that is O-bonded to the metal. This species then rearranges to amide and is released as product. In the third postulated mechanism (Fig. 2.7c), the metal-bound hydroxide causes deprotonation of a free water molecule near the active site. It is the newly generated hydroxide that carries out the hydrolysis of the nitrile substrate. The eventual rearrangement of the hydrolyzed intermediate leads to the amide product. This mechanism is known as the second outer-sphere mechanism of catalysis. It is to be noted that in this mechanism, no substitution occurs at the coordination sphere of the M (III) center.

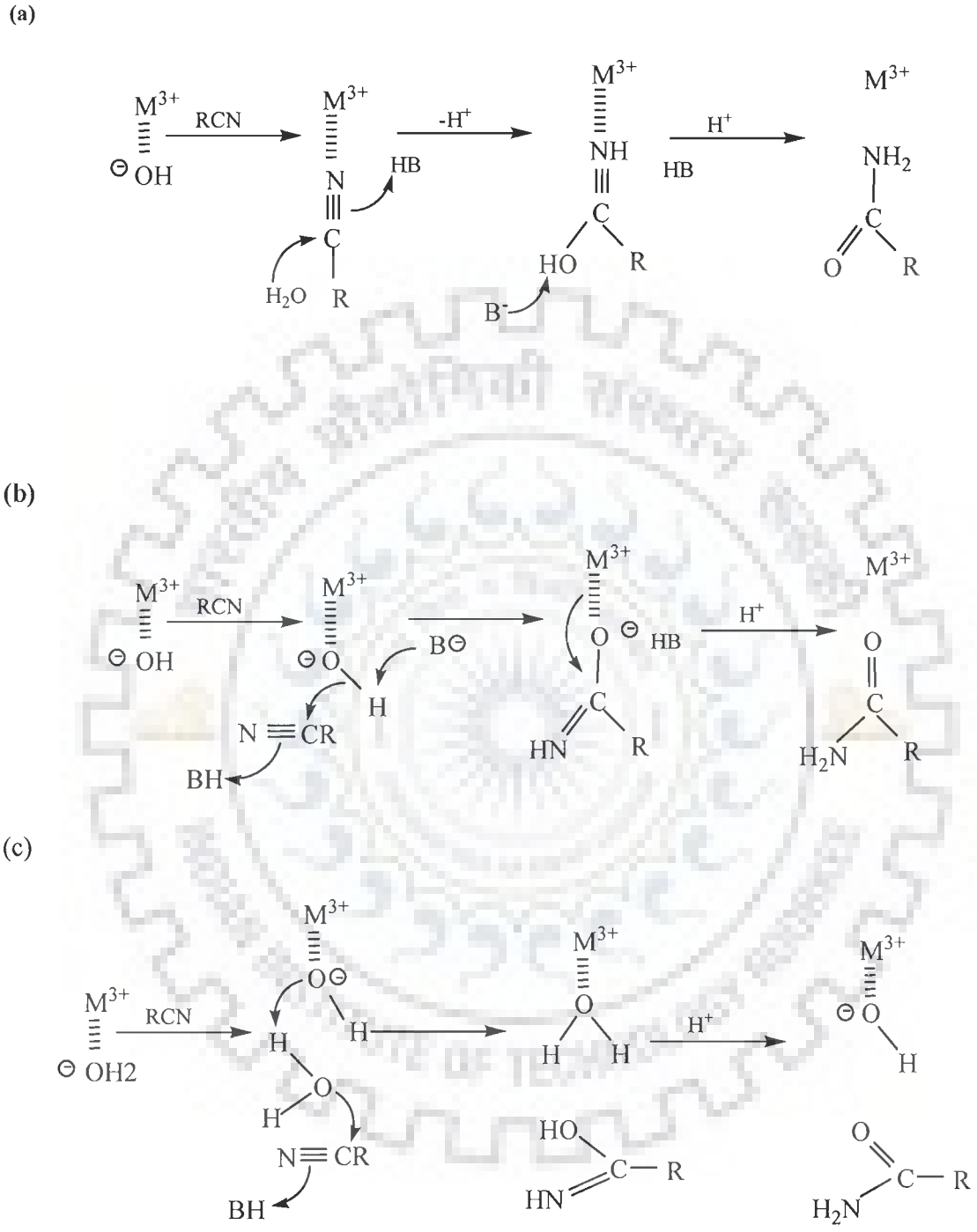


Fig.2.7. Possible mechanism of nitrile hydratase (Mascharak 2002).

2.5.5.2 Ferric NHase

Ferric NHases have been characterized in more detail than the Co-type because of its unique photoreactivity (Endo *et al.,* 1999 and Nagamune *et al.,* 1990) and are more interesting due to this property. The enzyme activity lost during aerobic incubation in the dark and recovery upon light irradiation (photoreactivation) was probed by FTIR spectrum which was measured before and after the photoreactivation of nitrile hydratase. These spectra indicate that NO with Fe^{3+} in β subunit of enzyme complex is responsible for activation and deactivation of NHase. Endogenous NO molecule which is bound to non-heme iron center in the inactive nitrile hydratase dissociate during the light irradiation (Noguchi *et al.,* 1995). This dissociation of NO molecule leads to active enzyme complex for the conversion of –CN to $–CONH_2$ (Fig. 2.8). The Ferric NHases are reported to be inactivated by N^{3-}, CN^- and by some metal ions like Hg^{2+} and Ag^+ (Nagasawa *et al., 1987*).

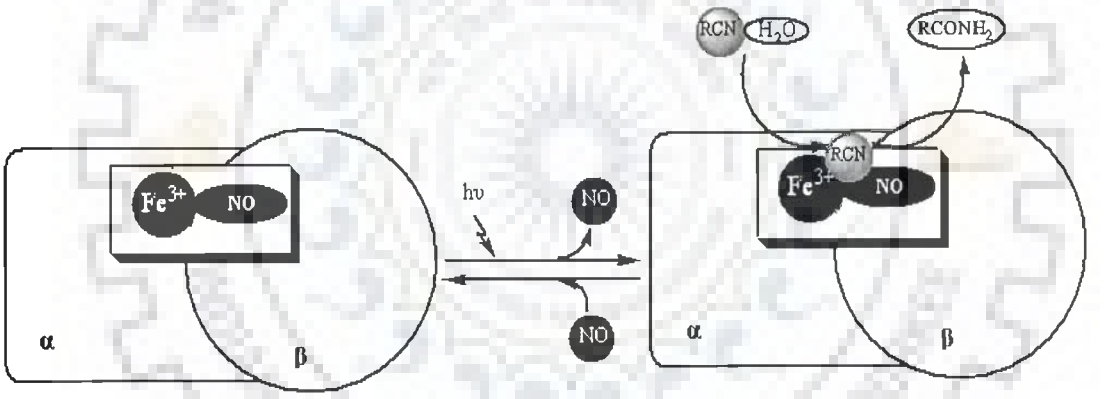


Fig. 2.8. Mechanism of nitrile hydratase catalysis.

2.5.5.3 Cobalt NHases

Cobalt NHases have non-corrinoid cobalt atom and belong to a small group of cobalt dependant enzymes in nature (Kobayashi *et al.,* 1999). Co-type NHase is more stable and efficient for acrylamide production than Fe-type and has been used for the industrial process (Kobayashi *et al.,* 2000 and Yamada *et al.,* 1996). They are also the first example of enzymes that incorporate a non-corrinoid cobalt (III) center in their structure (Mascharak 2002). The cobalt-containing NHase contains threonine and tyrosine as third and eighth amino acid residues of metal binding domain (Payne *et al.,* 1997). Crystal structure of nitrile hydratase

from *Pseudonocardia thermophila* JCM 3095 revealed presence of an $(\alpha\beta)_2$ heterotetramer and noncorrin cobalt at the catalytic center (Fig.2.9). In this nitrile hydratase, two cysteine residues (αCys111 and αCys113) coordinated to the cobalt were post-translationally modified to cysteine-sulfinic acid and to cysteine-sulfenic acid, respectively (Miyanaga *et al.*, 2001).

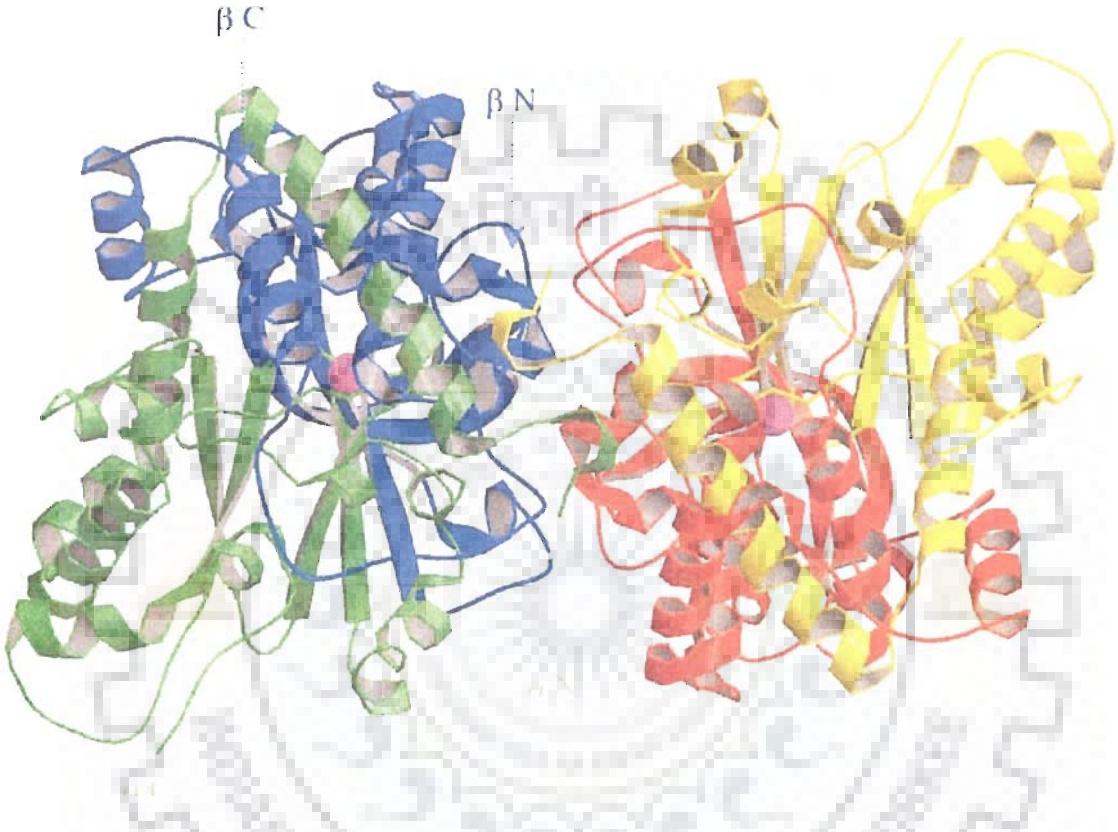


Fig.2.9. A ribbon drawing diagram of the (αβ)2 heterotetramer of NHase from *P. thermophila*. The α subunit is in green and yellow, and the β subunit in blue and red. The magenta spheres are cobalt atoms of the active center. The green and blue ones represent the α and β subunits of one heterodimer, and the other two represent another heterodimer, respectively (Miyanaga *et al.*, 2001).

2.5.6 Amidase

Amidases (E.C 3.5.1.4) in also known as amidohydrolases, belongs to the nitrilase superfamily (Pace and Brenner 2001). It catalyzes the hydrolysis of amides to free carboxylic acids and ammonia. Therefore, these enzymes are widely used for the production of industrially important organic acids such as acrylic acid, nicotinic acid etc. These enzymes are involved in nitrogen metabolism in both prokaryotic and eukaryotic cells. Amidases have

been reported in many of bacterial genera such as *Delftia acidovorans* strain 16 (Hongpattarakere *et al.,* 2005), Pseudomonas *chlororaphis* B23 (Ciskanik *et al.,* 1995) and fungal strains such as *Kluyveromyces thermotolerans* MGBY (Prasad *et al.*, 2005), *Klebsiella pneumoniae* NCTR 1 (Nawaz et al. 1996). Amidases usually have wide range of substrate specificity whereas some are specific for aliphatic amides (Asano *et al., 1982b*), others cleave amides of aromatic acids or aryl or aryloxypropionamide (Mayaux *et al.,* 1991). Some amidases were also able to hydrolyze amides of α- or ω-amino acids and aromatic amides (Stelkes-Ritter *et al.,* 1995, Hirrlinger *et al.,* 1996). Stereo-selectivity, an important property of amidases has been reported to be generally associated with the amidases in the bi-enzymatic pathway. Production media composition for amidases and characteristics of some of reported amidases is summarized in table 6 and 7 respectively.

2.5.6.1 Mechanism and Structure of amidases

The carbonyl group of amide undergoes a nucleophilic attack, resulting in the formation of a tetrahedral intermediate, which is converted to acyl-enzyme with the removal of ammonia and subsequently hydrolyzed to acid (Benerjee *et al.,* 2002) (Fig.2.10).

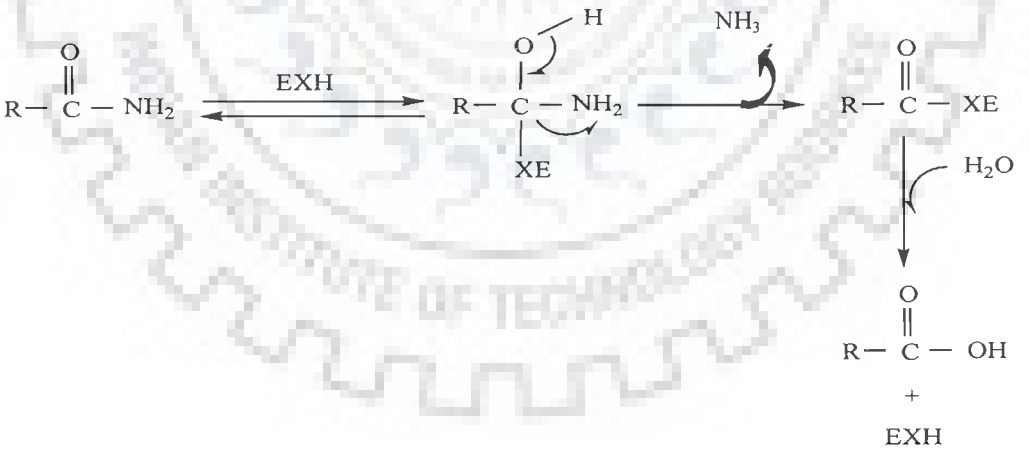


Fig 2.10. Mechanism of amidase catalysis.

Table.2.6: Production media for some amidases from different micro-organisms (Sharma *et al.*, 2005)

Microorganism	Media composition (g/l)	Inducer	pH	Temp.	Cultivation time (h)
Alcaligenes sp.	Yeast extract-10, peptone-10, beef extract-5, NaCl-2.5, phenyl acetic acid-1	Phenyl acetic acid	7.2	28	36
Corynebacterium nitrilophilus	Nutrient broth medium (Oxoid)	Acetamide	7.2	30	N.A
Klebsiella pneumoniae NCTR 1	Glucose-10, K_2HPO_4-17.5, KH_2PO_4-13.4, $MgSO_4$-0.5, Micronutrient solution-10 ml	Acryalmide	7.2	30	48
Kluyveromyces thermotolerans MGBY 37	Yeast extract-3, malt extract -3, peptone -5, dextrose -10	Propionitrile	6.2	30	24
Mycobacterium neoaurum ATCC 25795	Yeast carbon base Difco-10, urea -4, nitrilotriacetic acid -4.8	N.A	7.2	37	48
Pseudomonas aeruginosa 8602/A	Sodium succinate-5, K_2HPO_4-12.5, KH_2PO_4-3.8, $(NH_4)_2SO_4$-1, $MgSO_4 7H_2O$-0.1, trace element solution-5 ml	Acetamide	N.A	37	N.A
Pseudomonas chlororaphis B 23	KH_2PO_4 -8.6, yeast extract-0.01, glucose-10, citric acid- 0.24, $MgSO_4 7H_2O$-0.5, $FeSO_4 H_2O$-0.05, SL-7 trace metal solution-1 ml	Butyronitrile	70	25	17.5
Pseudonocardia thermophila	Yeast extract-5, soluble starch-4, KH_2PO_4 -0.3, Na_2HPO_4 -0.6, $MgSO_4 7H_2O$-0.1, NaCl-5, sodium formate-0.5, $CoCl_2 6H_2O$-0.01	Methacrylamide	7.5	55	72
Rhodococcus sp.	K_2HPO_4- 1.0, KH_2PO_4 -1.0, $MgSO_4 7H_2O$-0.2, $CaCl_2 2H_2O$-0.02, Glucose- 1.0 and trace element solution -10 ml	Acrylamide	7.5	30	N.A
Rhodococcus rhodochrous J1	Glycerol-10, KH_2PO_4- 0.5, K_2HPO_4-0.5; $MgSO_4 7H_2O$- 0.1, yeast extract-1; polypeptone-5	Propionamide	7.2	26.5	48
Variovorax paradoxus	KH_2PO_4-3.3, K_2HPO_4-0.8, NaCl-1.0, $CaCl_2 2H_2O$-0.05, $MgSO_4 7H_2O$-0.3, vitamin solution-2.5 ml, trace element solution-0.8 ml, glucose-4.5, trace element solution-0.8 ml, vitamin solution-2.5 ml	DL- tert-leucine amide	N.A	30	92
Xanthobacter agilis	Peptone-12.5, yeast extract-3, beef extract-5, NaCl-5	Phthalic acid	8.0	30	24

Table 2.7: Characteristics of some purified amidases from different micro-organisms

Micro-organism	Nature	Molecular mass		Optimum Temp.	Optimum pH	Substrate specificity	Reference
		Native (kDa)	No Of subunit and its Mwt (kDa)				
Brevibacillus borstelensis BCS-1		199	30 (6)	85	9.0	D-amino acid amides	Baek *et al.*, 2003
Brevundimonas diminuta TPU 5720		288	53 (6)	50	7.5	L-amino acid amides	Komeda *et al.*, 2006
Klebsiella pneumoniae NCTR 1	Inducible	62	(1)	65	7.0	Aliphatic amides	Nawaz *et al.*, 1996
Geobacillus pallidus RAPc8		218	38 (6)	50	7.0	Aliphatic and unsaturated amides	Makhongela *et al.*, 2007
Pseudonocardia thermophila	Constitutive	108	52 (2)	70	7.0	aliphatic organic acids	Egorova *et al.*, *2004*
Sulfolobus solfataricus		55	(1)	95	7.5	aliphatic or aromatic amides	d'Abusco *et al.*, 2001
Sulfolobus tokodaii strain 7		43.7	(1)	75	7.0–8.0.	S-2-phenyl-propionamide	Suzuki and Ohta 2006
Ochrobactrum anthropi SV3		38	(1)	45	8.5-9.5	d- or l-amino-acid amides	Komeda and Asano 2000
Brevibacterium iodinum		290	29 (10)	35	7.2	d-amino acid amides	Komeda and Asano 2008
Agrobacterium tumefaciens d3		490	63 (8)			2-phenylpropionamide	Trott *et al.*, 2001
Delftia acidovorans		50	N.D	40	8.5	L & D-amino acid amide	Hongpattarakere 2005

2.5.6.2 Structure of amidase

Unlike nitrile hydratases, amidases are non metal containing enzyme except amidase of *Brevundimonas diminuta* TPU 5720 (Komeda *et al.*, 2006), *Klebsiella pneumoniae NCTR 1* (Nawaz *et al.*, 1996), *Rhodococcus sp.* (Nawaz *et al.*, 1994) which contain Co^{+} and/or $Fe3^{+}$ ion in their active site (Sharma *et al.*, 2009). *Rhodococcus* sp. R312 was reported to have wide spectrum of amidases; an α-amino acid amidase specific for L-α-amino amides, an aliphatic amidase, an enantioselective amidase hydrolyzing aryloxy propionamides, a novel amidase hydrolyzing dimities. These amidases contain Asp 191 and Ser 195 in the active site in place of the more familiar cysteine residues (Novo *et al.*, 1995).

2.5.6.3 Acyl-transferase activity of amidase

Acyl transfer activity of amide-hydrolysing enzymes has been described long since. Grossowicz and coworkers reported in 1950 the formation of hydroxamic acids (RCONHOH) by the enzyme-catalysed replacement of the amide groups of glutamine and asparagine with hydroxylamine. They also reported that the enzyme, prepared from cell-free extracts of *Proteus vulgaris* X-19, could split hydroxamic acids (Grossowicz *et al.*, 1950). Later, in 1959, Kimura showed that an enzyme preparation purified 19-fold from *Mycobacterium avium,* catalysed hydroxamate formation from butyric or valeric acid and hydroxylamine (Kimura 1959). Thereafter, acyl-transferase activity of amidase has widely studied in *Rhodococcus sp.* R312 which was cloned, over-expressed in an *E. coli* strain (Fournand *et al.*, 1998), *Geobacillus pallidus* (Makhongela *et al.*, 2007). Acyl-transferase activity was found to involve a 'bi-bi-ping-pong' mechanism (Maestracci 1986). These studies thus allowed the drawing of the general reaction pathway shown in Fig.2.11. One substrate A (acyl donor) reacts with the enzyme to give an acyl-enzyme complex E *, which then transfers the acyl group to the second substrate B (acyl acceptor).

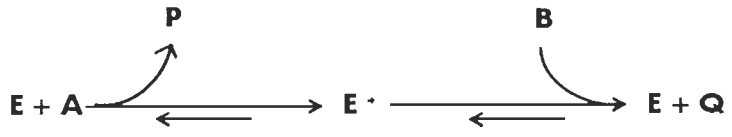


Fig.2.11. Enzyme-substituted mechanism. For example, A = amide, B = hydroxylamine, P = ammonia, and Q = hydroxamic acid. E = enzyme and E * = acyl-enzyme complex. (Fournand et. al 1998).

2.5.7 Properties of nitrile metabolizing enzymes

Enzymes have the potential to produce wide variety of chemical, started from bulk to fine chemicals. Therefore, enantioselectivity, regioselectivity and chemoselectivity are important steps towards the production of fine chemicals. Nitrile metabolizing enzymes posses these properties:

2.5.7.1 Enantioselectivity

The past decade has seen tremendous development of biotransformations using microbes and isolated enzymes in organic synthesis. Since most of the reactions are highly chemo-, regio- and stereo-selective and are performed in mild conditions, biotransformations offer many opportunities for the synthesis of compounds which are sometimes not readily obtainable by conventional chemical methods. In contrast to these extensively investigated hydrolytic enzymes, nitrilases in organic synthesis have remained largely unexplored until recently, despite of the fact that nitriles are very important compounds commercially and the bioconversion of nitriles into the corresponding carboxylic acids has been known for decades. Pure enantiomers of chiral compounds have wide applications in the pharmaceutical industries. Nitrilases from *Bradyrhizobium japonicum* strain USDA110 catalyzed the enantioselective hydrolysis of β-hydroxy nitriles to (S)-enriched β-hydroxy carboxylic acids (Kamila *et al.,* 2006). Dinitriles were selectively converted to cyanocarboxylic acids by the heat-treated resting cells of *Acidovorax facilis* 72W (Gavagan *et al., 1998*). Effenberger and Osswald have demonstrated that a nitrilase from *Arabidopsis thaliana* catalyzed the selective hydrolysis of α, ώ -dinitriles to ώ-cyanocarboxylic acids (Effenberger *et al.,* 2001). Selectivity depends on chain length of aliphatic dinitriles which was demonstrated by chain length-dependent selectivity of nitrilases from *Synechocystis sp.* strain PCC 6803 toward the hydrolysis of aliphatic dinitriles (Mukherjee 2006). Enantioselectivity has also been reported with nitrile hydratase and amidases in many strains. Li et al. have reported the enantioselective hydrolysis of α,α-disubstituted malononitriles by *Rhodococcus* sp. CGMCC 0497 strain having both nitrile hydratase and amidase activity to give (R)- α,α-disubstituted malonamic acids (Wu *et al.,* 2003). Similarly, nitrile hydratase and amidase from *Rhodococcus erythropolis* ATCC 25544 was used to convert racemic 100 mM 2,2-dimethylcyclopropane carbonitrile to 45mM (*S*)-2,2-dimethylcyclopropane carboxylic acid at

pH 7.0 and at 20°C with 45% yield and an 81.8% enantiomeric excess after 64 h (Yeom *et al.,* 2007).

2.5.7.2 Regioselectivity

Nitrile metabolizing enzymes has been used for the regioselective hydrolysis of dinitriles for the production of cyanocarboxylic acids and cyanoamides. Five types of products could be prepared in high yields by applying regioselective nitrile metabolizing enzymes while only two products, i.e. diamide and diacid are possible with chemical synthesis. Precursors for the nylon 6 and nylon 66 polymers, adipic acid and caprolactum can be synthesized from adiponitrile by combining both chemical and enzymatic conversion (Ramakrishna *et al.,* 1999). Nitrile hydratase from *P. putida* was used for the regioselectivity by DuPont in the preparation of 5-cyanopentanamide from adiponitrile.

2.5.8 Applications

2.5.8.1 Synthetic applications

Nitrile metabolizing enzymes have been used for the production of important organic compounds such as p-aminobenzoic acid, acrylamide, indole acetic acid, nicotinic acid, benzamide, thiophenamide, pyrazinoic acid etc. from microbial cells and in the production of some important drugs. Tranexamic acid, a homeostatic drug, is obtained by selective mono-hydrolysis of trans 1, 4-dicyano cyclohexane by *Acremonium* sp. (Nishise *et al.,* 1987).

Some strains of *Rhodococcus* and *Nocardia* have been more extensively studied for the production of acrylamide, nicotinamide, butyramide, pyrizanamide, isonicotinamide, picolinamide, 3-indoleacetmide, benzamide, indole-3-acetamide and 5-cyanovaleramide etc (Prasad *et al.,* 2009). NHases also shows enantio and regioselectivity. Enantioselectivity of NHase was used to convert phenylglycine nitrile to phenylglycine, a precursor for semisynthetic cephalosporins and penicillins (Wegman *et al.,* 2000). Moreover, many NHases have been used for the synthesis of enantiopure compounds such as α-arylaliphatic amides, cyclopropanecarboxylic acids, amide derivatives, oxirane carboxamides, beta-amino acids, etc. (Preiml *et al.,* 2003, Wang, 2005).

Acyltranferase activity of amidase is useful in the production of several hydroxamic acids. Amidase transfer acyl group of some short chain amides to hydroxylamine and

produce corresponding useful hydroxamic acid. Hydroxamic acids have been used to treat ureaplasma, anemia and as potent inhibitors of several metalloproteases. These enzymes are involved in tissue remodeling and ubiquitous in human diseases such as osteoarthritis and rheumatoid arthritis. Moreover, some hydroxamic acids have been investigated as anti-HIV agents through combined action with AZT and DDI drugs (Fournand 1997).

2.5.8.2 Waste treatment

Nitrile compounds are widely used by the chemical industries for the synthesis of various chemicals and products of petrochemical industries. These compounds are highly neurotoxic, carcinogenic in nature and considered as environmental pollutants (Ramakrishna *et al.,* 1999). Therefore, remediation of these toxic compounds is necessary for pollution free environment. Nitrile metabolizing enzymes could be the solution for removal of these compounds from the environment.

2,6-dichlorobenzonitrile (dichlobenil) and bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) are widely used herbicides. Dichlobenil is mainly degraded to 2,6-dichlorobenzamide (BAM) and leached to ground water which causes toxicity (Holtze 2006). Nitrile-metabolizing enzymes efficiently degrade these cyano group-containing herbicides and prevent them from entering the food chain. *Rhizobium* sp. 11401, *P. fluorescens* 11387 and *P. putida* 11388 known to degrade aliphatic nitriles by nitrilases (Layh *et al.,* 1997) *Agrobacterium radiobacter*, a bromoxynil-degrading soil bacterium, is used for the degradation of the herbicide under nonsterile batch and continuous conditions. The bromoxynil concentration in a column reactor decreases to 65% after 5 days. The efficacy of degradation is enhanced by addition of ferrous, cobaltous or cupric ions (Muller and Gabriel 1999). Similarly, other nitrile-degrading enzymes could also be employed for the degradation of these herbicides.

In this work nitrile metabolizing enzyme was explored for its ability to modify surface of polyacrylonitrile which has rarely been studied in detail.

Chapter 3

Materials and Methods

3.1 Materials

Samples for the isolation and screening of nitrile metabolizing enzyme producing microbes were collected from different chemical, pharmaceutical manufacturing industries, organic manure and hot springs of different regions of Chandigarh and Himachal Pradesh in the form of soil and water (Table.3.1). Polyacrylonitrile, polyacrylonitrile-co-methacrylonitrile, polyacrylonitrile-co-butadiene-co-styrene and other nitrile substrates were obtained from Sigma-Aldrich (USA). Different media components and solvents were obtained from S.D. Fine Chem. (India) and were of analytical grade. All other chemicals were purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India.

Table.3.1: Collection of samples from different sources

S.No.	Place	Description of Source	Sample Type
1.	Chandigarh	Activated sludge of Ranbaxy	Water
2.	Chandigarh	Activated sludge of nectar life science	Water
3	Chandigarh	Soil near the ETP of Ranbaxy	Soil
4.	Chandigarh	Soil near the ETP of nectar life science	Soil
5.	Mohali	Sewage	Water
6.	Mohali	Sewage	Soil
7.	Tatta pani (H.P)	Hot spring (temp.60-65°C)	Water
8.	Tatta pani (H.P)	Hot spring (temp.60-65°C)	Soil
9.	Vashishtha (Manali, H.P)	Hot spring (temp.45°C)	Water
10	Vashishtha (Manali, H.P)	Hot spring (temp.45°C)	Soil
11.	Shimla	Organic manure	Soil

3.2 Isolation and screening of nitrile metabolizing micro-organism

Isolation of nitrile metabolizing isolates was carried out with various soils (0.4 g/l) and water samples (4% v/v) using mineral base (MB) media. Mineral base (10% v/v) and trace elements solution (0.1% v/v) were added into the medium after sterilization (Khandelwal *et al.,* 2007). Soil and water samples were added to 50 ml of the MB medium in 250 ml flask and incubated at 45°C and 160 rpm. After 2 weeks of incubation, 1 ml of broth was transferred again in 50 ml of same medium and incubated for 1 week at 45°C and 160 rpm. After 1 week of incubation, 50 µl of sample was taken and spreaded to mineral base agar plates containing 20 mM acrylonitrile. Individual colonies growing on these plates were purified on 20 mM acrylonitrile plates and stored at 4°C. The purity of isolate was further confirmed from microscopic study at 100 X with oil immersion.

3.2.1 Mineral base medium

The composition of mineral based (MB) medium used for maintaining the culture is given below:

Glycerol	5 g/l
Tri sodium citrate	0.2 g/l
KH_2PO_4	0.27 g/l
K_2HPO_4	0.174 g/l
Trace element solution	0.1% (v/v)
Mineral base solution (10X)	1% (v/v)

Mineral base and trace element solution were added into the medium after sterilization.

3.2.2 Composition of trace element solution

H_3BO_3	0.3 g/l
$CoCl_2.6H_2O$	0.2 g/l
$ZnSO_4.7H_2O$	0.1 g/l
$MnCl_2.4H_2O$	0.03 g/l
$Na_2MoO_4.H_2O$	0.03 g/l
$NiCl_2.H_2O$	0.02 g/l
$CuCl_2.2H_2O$	0.01 g/l

3.2.3 Composition of mineral base solution (10X)

NaCl	10 g/l
$MgSO_4.7H_2O$	2 g/l
$CaCl_2$	0.1 g/l

One loopful of culture from agar plate was transferred to 50 ml MB media in 250 ml flasks containing different nitriles as the sole nitrogen source and incubated at 45°C, 160 rpm for 36 hours. Cells were harvested from the culture broth by centrifugation at 16000 x g for 10 min. at 4°C and washed twice with 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA. Finally cell pellet was suspended in the same buffer and the cell suspension was used for enzyme assay with acrylonitrile (10 mM), PAN and PMA by ammonia estimation using Bertholet method (Weatherburn 1967). Ammonia was estimated from a calibration curve of ammonium chloride solution. In terms of enzyme activity and growth on polyacrylonitrile plates, one culture (6/b) from soil sample of sewage, Mohali, was selected as the best strain.

3.3 Enzyme assay

3.3.1 Ammonia estimation

The activity of the enzymes was determined following a standard method. The basic principle of activity determination is quantification of product (ammonia) formed due to enzymes action. The steps followed for enzyme assay are as follows:

- 0.1 ml of cell/enzyme suspension was prepared in 100 mM phosphate buffer of pH 7.0 and mixed with 0.4 ml of buffer solution of pH 7.0 containing nitrile or amide (acrylonitrile, acrylamide and hexanenitrile) as substrate. The concentration of nitrile in the reaction mixture was 10 mM and generally acrylonitrile, acrylamide and hexanenitrile were used as substrate for different enzyme assay.
- The suspension was mixed properly and incubated at 45°C for 30 minutes in shaking condition.
- Reaction was stopped after 30 min. of incubation by adding one drop of 1 N HCl solution.

- The cells were separated from the suspension by centrifuging at 16000 x g for 10 minutes.
- The supernatant obtained was subjected to ammonia estimation by Bertholet method and calculating the enzyme activity with the help of a standard plot.
- An enzyme blank was also prepared by adding 0.1 ml of cell/enzyme suspension in 400 µl buffer and thereafter method followed was same as substrate.

*NOTE: One International unit (IU) of enzymes activity has been defined as amount of enzyme required to release 1 µmole of ammonia per minute.

3.3.1.1 Reagents for Ammonia estimation

The reagents used were prepared as given in the table 3.2

Table 3.2: Compositions of reagents used

Reagent	**Concentration**	**Constitution**	
		Component	**Amount**
Sodium Phenolate	0.33 M	Phenol	7.76 g
		Sodium hydroxide	3.3 g
		Distilled water	250 ml
Sodium Hypochlorite	0.02 M	Sodium hypochlorite solution	4 ml
		Distilled water	96 ml
Sodium Nitroprusside	0.01 %	Sodium nitroprusside	0.025 g
		Distilled water	250 ml

3.3.1.2 Standard plot for ammonia estimation

The activity of the enzyme in aqueous phase was studied by measuring ammonia released during the reaction using Bertholet method. This method converts ammonium ions to a colored complex. Absorbance at 640 nm gives the measure of ammonia released. A standard plot of ammonia concentration vs. O.D (640 nm) was plotted to calculate ammonia concentration in the reaction mixture. The steps for standard curve are as follows:

- A series of ammonium chloride solutions of different concentrations were prepared in distilled water from a stock solution of 0.1% (w/v).

- 0.2 ml of each of the NH_4Cl solution was taken in a separate test tube and 0.2 ml each of 0.33 M Sodium Phenolate, 0.01% Sodium Nitroprusside and 0.02 M, Sodium Hypochlorite solutions were added to it.
- The mixture was heated in a boiling water bath for two minutes and then cooled in ice bath for two minutes.
- 1.2 ml of distilled water was then added to each test tube.
- A blue colored solution was obtained. Its absorbance at 640 nm was measured using UV spectrophotometer.

In case of enzyme assay, instead of ammonium chloride solution, 0.2 ml of reaction mixture was used.

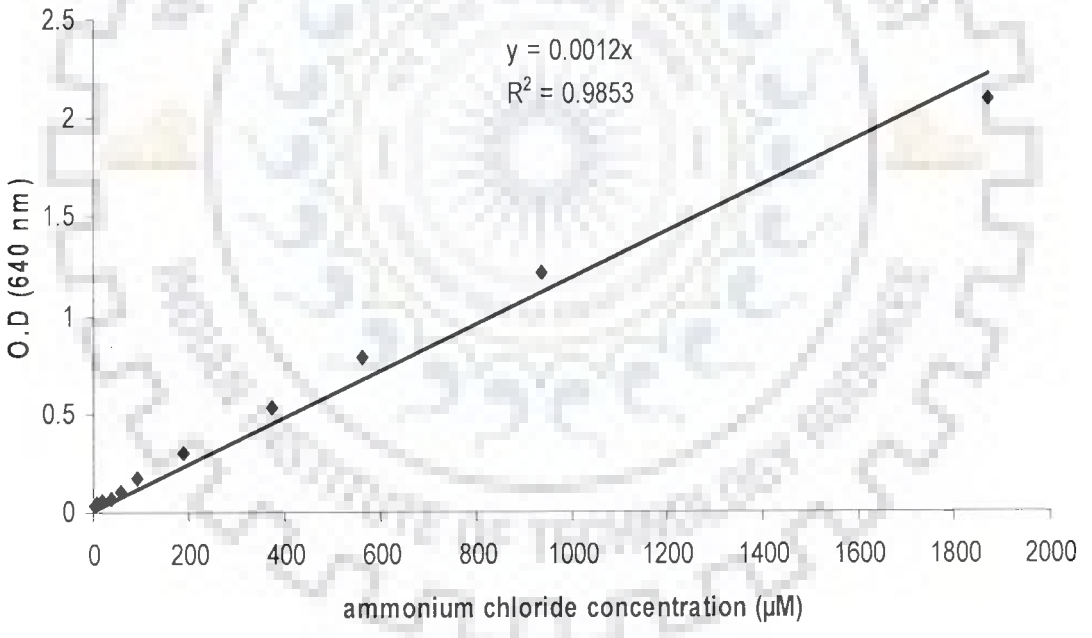


Fig.3.1. Standard plot of ammonium chloride using Bertholet method

3.3.2 Acyl-tranferase activity of amidase

Different amides (isobutyramide, benzamide, adipamide, thioacetamide, propionamide, acetamide, acrylamide and hexanamide) were prepared in 100 mM phosphate buffer (pH 7.0). Lyophilized cells at concentration of 20 g/l incubated with 2 g/l lysozyme

for 1 h at 30°C (pH 7.0). Cell debris was separated by centrifugation at 16000 x g for 10 min. Supernatant was diluted 5 times for estimating acyl-tranferase activity. For acyl acceptor, 1.5 M hydroxylamine was prepared in phosphate buffer pH 7.0 and pH was adjusted with 10 M NaOH. 250 µl of 1.5 M hydroxylamine was added in the 500 µl reaction mixture of enzyme and substrate containing 100 µl 5 times of diluted supernatant. This mixture was incubated for 30 min. at 45°C. For quantification of hydroxamic acid in the reaction mixture, 335 mM $FeCl_3$ in 0.6 N HCl was prepared and 1 ml of $FeCl_3$ solution was added in the reaction mixture. On addition of 335 mM $FeCl_3$, colour of the reaction mixture turned to deep brown and the optical density of the mixture determined at 500 nm. Acyl-transferase activity was calculated from optical density by using $A = €cl$ (where, A- absorbance, c-concentration and l is path length) where molar extinction coefficient (€) taken as 10.16×10^2 (Fournand et. al 1997b). One unit of activity denotes amount of enzyme necessary for production of 1 µmol of hydroxamic acid formed per minute under reaction conditions (structure of amides and their corresponding hydroxamic acids is given in appendix II).

3.3.3 Gas chromatography analysis

The amount of acrylamide formed in the reaction mixture was determined by gas chromatography with a forte capillary column (0.32 mm I.D, 30 m length and 0.5µm film thickness) packed with SOLGEL-WAX™ equipped with a flame ionization detector. The operational conditions were: column temperature, 100 to 200 °C @ 15°C/min with 2 min hold at 200°C; injector and detector temperature, 260°C; and carrier gas N_2 at a flow rate of 2 ml/ min. One unit of nitrile hydratase was defined as the amount of enzyme which catalyzed the formation of 1 µmol of acrylamide per min. Maximum activity was assumed as 100% and rest of the activities were with respect to its maximum activities.

3.3.4 HPLC analysis

Formation of aromatic, aliphatic and unsaturated amides due to nitrile hydratase was determined by Waters HPLC with a Waters spherisorb® 10µm ODS2, 4.6 X 250 mm column. 2.5 mM H_2SO_4/acetonitrile (9:1, v/v) was used as mobile phase at a flow rate of 1 mL/min and monitored at 210 nm in UV detector. One unit of nitrile hydratase was defined as the amount of enzyme which catalyzed the hydrolysis of nitrile to form 1 µmol of amide per min under the assay conditions.

3.3.5 Protein estimation by modified Lowry method (Lowry *et al.*, 1951)

Reagents Required

1. BSA stock solution (1mg/ml),

2. Analytical reagents:

Solution A. 50 ml of 2% sodium carbonate was mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water)

Solution B. 10 ml of 1.56% copper sulphate solution was mixed with 10 ml of 2.37% sodium potassium tartarate solution.

Solution C. 2 ml of solution B mixed with 100 ml of solution A.

Solution D. Folin - Ciocalteau reagent solution (1N). Diluted commercial reagent (2N) with an equal volume of water on the day of use (2 ml of commercial reagent + 2 ml distilled water)

Procedure

Two milliliter of solution C was added in 200 µl of protein sample. This mixture was kept at room temperature for 10 min. To this solution, two hundred microlitre of solution D was added with vigorous shaking and incubated for 30 min. at room temperature. Optical density was taken at 660 nm. A standard curve was prepared by using various concentration of BSA (1 g/l of BSA to 0.05 g/l in water) (Fig. 3.2).

3.3.6 Protein estimation by modified Bradford method (Bradford 1976)

Reagents Required

5X Bradford Reagent:

1) Coomassie Brilliant Blue **G250**	0.05 % (w/v)
2) Ethanol	25 % (v/v)
3) Phosphoric acid	42.5 % (v/v)

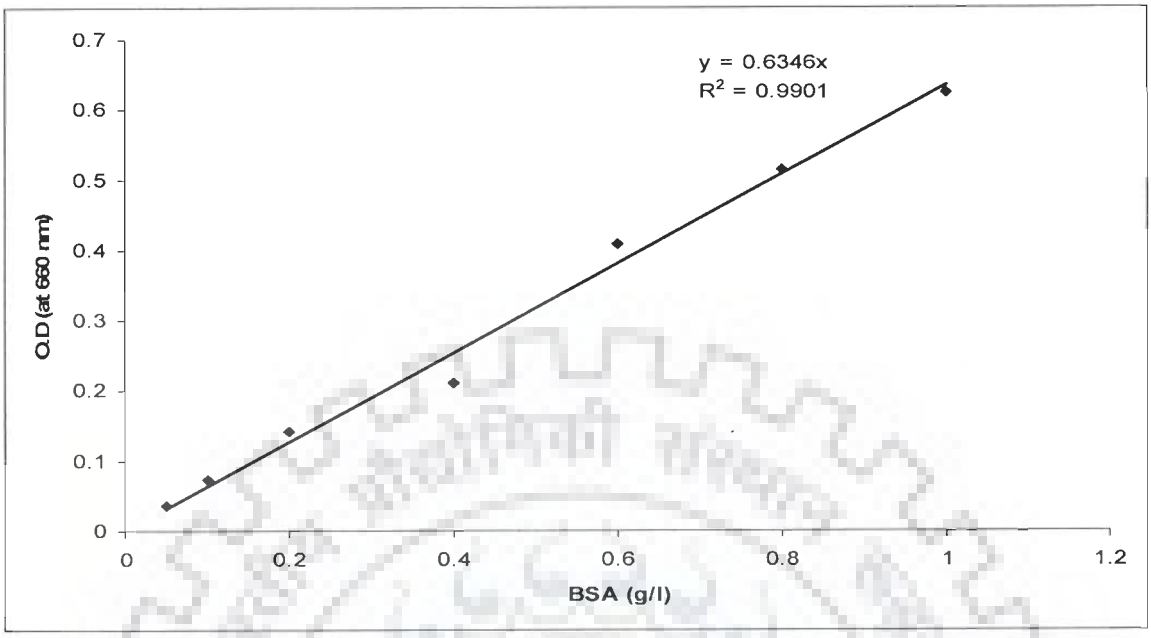


Fig. 3.2. Standard plot of BSA by Lowry method

All these solutions were made with MilliQ water and filtered through a Whatman Filter Paper No. 2. Filtered solution stored in "Amber Bottle" and kept it in 4°C.

Solution used for the calibration curve

Standards: 1 mg/ml BSA stock and various dilutions of stock

Preparation of solution for the calibration curve:

Procedure:

1. Standard curve was prepared by diluting 1 µg/µl of BSA to 10 µg/µl in water and bring volume up to 800 µl with water.

2) Added 200 µl of 5X Bradford reagent and incubate at room temperature for 5 minutes.

3) Absorbance for the samples measured at 595 nm (Fig. 3.3).

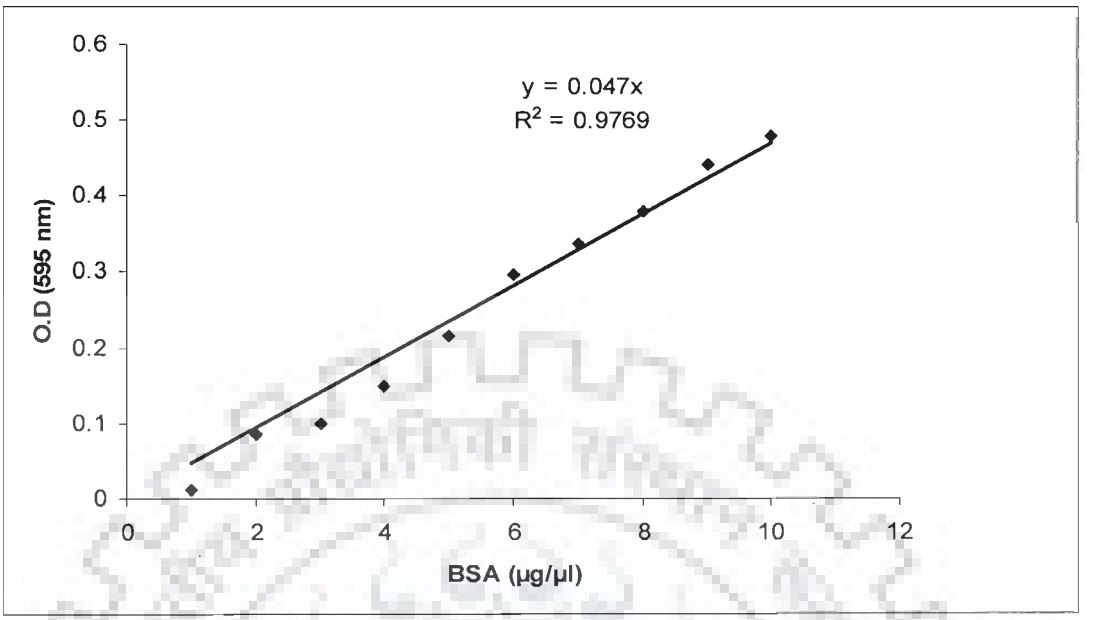


Fig. 3.3. Standard plot of BSA by Bradford method.

3.4 Effect of inducers/nitrogen source on ammonia release from polyacrylonitrile-co-methacrylonitrile (PMA)

Polyacrylonitrile-co-methacrylonitrile (PMA) is a co-polymer of polyacrylonitrile. PMA has molecular weight lower than PAN. Therefore, there was a possibility to hydrolyze PMA using nitrile metabolizing enzyme of the isolated strain. Some of the reported inducers/nitrogen sources (valeronitrile, butyronitrile, adiponitrile, propionitrile, phenylacetonitrile, isobutyronitrile, 2-cyanopyridine, benzonitrile, acetonitrile, €-caprolactam) were used for the production of nitrile metabolizing enzyme in the growth media. These nitrogen sources were added in MB media at a concentration of 10 mM after sterilization. Cells of *Amycolatopsis* sp.IITR215 were inoculated and allowed to grow at 45 °C, 200 rpm for 36 hours. Cells were harvested by centrifugation at 16000 x g and 4°C for 10 min. Media components were removed by two times washing with 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA. Cell pellet was suspended in 100 mM phosphate buffer of pH 7.0. Whole cells suspension was incubated with 10 g/l PMA at 45 °C and samples were taken after 3 and 12 h for ammonia estimation.

3.5 Identification and characterization of isolated strain

Cells were harvested from media containing 10 mM adiponitrile after 30 hours of growth and centrifuged at 16000 x g and 4°C, followed by two times washing with TE buffer for genomic DNA isolation by modified CTAB-NaCl protocol (Conn and Franco 2004).

3.6 Isolation of total genomic DNA by modified CTAB-NaCl protocol

Cell pellet was suspended in 500 µl solution containing 1g/l lysozyme and incubated for 1 h at 37°C. 10 µl of proteinase K and 33.5 µl of 10 % sodium dodecyl sulphate were added and incubated for another 1 h at 55°C. After 1 h, 100 µl of 5M NaCl and 65 µl of CTAB-NaCl (700 mM NaCl, 275 mM CTAB) were also added to the mixture and incubated for further 10 min. The lysate was centrifuged at 20000 x g for 15 min. to separate the cell debris. Supernatant was collected and chloroform-isoamyl alcohol (24:1) were added to precipitate the proteins and kept for 30 min. at room temperature with intermittent shaking. After centrifugation, DNA was precipitated from the supernatant with 3 volume of absolute ethanol and 0.1 volume of 3 M sodium acetate. Precipitated DNA was washed with 70% (v/v) ethanol with spin at 20000 x g for 10 min. Finally, DNA was suspended in 100 µl of nuclease free water and stored at -20 till further use.

3.7 16S rRNA gene amplification and DNA sequencing

Homology based on 16S rRNA gene is a valuable tool for phylogenetic classification of an organism. The 16S rRNA gene was amplified using polymerase chain reaction (PCR). The PCR amplification of 16S ribosomal DNA was carried out with forward primers p16S-8 (5-'AGAGTTTGATCCTGGCTCAG-3') and reverse primer p16S-1541 (5'-AAGGAGGTGAT CCAGCCGCA-3') (Zheng *et al.*, 2007). A volume of 25 µl of PCR reaction mixture consisting of following components:

PCR assay buffer (10X)	2.5 µl
dNTP mix (10 mM)	2 µl
Forward primer (20 pmol/µl)	1 µl
Reverse primer (20 pmol/µl)	1 µl
Template DNA (10 ng/µl)	0.5 µl
Taq polymerase (5 unit/µl)	1 µl
Milli 'Q' water	17 µl

Genomic DNA was visualized on 0.8% (w/v) agarose containing EtBr by gel documentation system. PCR reaction mixture was mixed vigorously and subjected to PTC-100 thermocycler (MJ Research) and following programme was used for PCR amplification:

Step 1	Initial denaturation	95°C	5 min
Step 2	Denaturation	95°C	40 s
Step 3	Annealing	53°C	60 s
Step 4	Elongation	72°C	2 min
Step 5	Repetition from step 2 to step 4 for 30 cycles		
Step 6	Final elongation	72°C	10 min
Step 7	End		

3.8 Agarose gel electrophoresis for genomic DNA and amplified product

Isolated DNA and amplified PCR product was analyzed by agarose gel electrophoresis as mentioned earlier.

3.8.1 Tris-Borate-EDTA (TBE) Buffer: A 5x stock solution of TBE was prepared by dissolving 54 g of Tris base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA (pH 8.0) in 1 liter of distilled water.

3.8.2 EtBr stock solution (10 mg/ml): 100 mg of EtBr (HiMedia) was dissolved in 10 ml of distilled water to prepare the stock solution of ethidium bromide.

3.8.3 Gel loading dye: 30% glycerol (v/v), 0.25% (w/v) bromophenol blue.

Procedure: 0.8% (w/v) agarose gel was prepared by dissolving appropriate amount of agarose in 50 ml of 1.0X TBE buffer and heated in microwave oven until the solution became transparent. The solution was cooled to about 50°C and 2.5 µl of EtBr stock solution was added to get a final concentration of 5µg/ml. The warm agarose was mixed and poured into the horizontal gel casting tray containing a gel comb at the proper place. The gel was allowed to solidify at room temperature. The gel comb was removed carefully and the gel tray along with gel was submerged in tank buffer (TBE). The DNA samples were prepared by adding 1 µl of gel loading dye to 5 µl of DNA solution and were loaded into the well

along with the marker DNA to determine the size of amplified fragment. The electrodes present at two sides of the tank were connected to power supply and 50V direct current was applied. When the dye migrated to 2/3rd of the gel, the electric current was turned off and DNA was visualized under gel documentation system (BIORAD).

3.9 Sequencing and molecular phylogenetic analysis

The amplified PCR product was sequenced by Ocimum biosolutions, Hyderabad (India). Forward and reverse 16S rRNA gene sequences obtained from Ocimum biosolutions were subjected to similarity search through nucleotide BLAST programme provided by NCBI (http://www.ncbi.nlm.gov/Blast.cgi) Genebank database. Most similar sequences were selected and along with the query sequences were aligned using the multiple alignment tool CLUSTAL W from Megalign software. Terminal nucleotides not common to all sequences were removed. Alignment file was saved as .MEGA. Phylogenetic analysis was carried out using Megalign verson 4.0 and consensus tree was drawn by neighborhood joining method. Evaluation of tree was determined by bootstrap analysis.

3.9.1 Nucleotide sequence accession number

The partial 16S rRNA gene sequence of strain *Amycolatopsis* sp.IITR215 determined in this study has been deposited in GenBank under the accession number FJ744759.

3.10 Substrate specificity of whole cells and cell free extract

The substrate specificities of whole cells were investigated using cells of *Amycolatopsis sp.*IITR215 grown in MB medium containing 10 mM adiponitrile as the sole nitrogen source for 36 hours. The cells were harvested by centrifugation at 16000 x g and 4°C for 10 min. and washed twice with 0.1 M phosphate buffer (pH 7.0) containing 1 mM EDTA. Finally cell pellet was suspended in the same buffer and the cell suspension was used to check the biotransformation ability of enzyme with 10 mM of different substrates (benzonitrile, phenylacetonitrile, valeronitrile, propionitrile, isobutyronitrile, adiponitrile, acrylonitrile, acetonitrile, butyronitrile, isovaleronitrile, glutaronitrile, 3-hydroxypropionitrile, methacrylonitrile, hexanenitrile, cyclohexanecarbonitrile, 2-cyanopyridine, 4-hydroxybenzonitrile, 4-aminobenzylcyanide, 4-phenylbutyronitrile, 3-cyanopyridine, 4-cyanopyridine, 3-hydroxyglutaronitrile and acrylamide in 100 mM

phosphate buffer (pH 7.0 containing 1 mM EDTA). While phenoxyacetonitrile, phenylthioacetonitrile, hydrocinnamonitrile, mandelonitrile, malononitrile and indole-3-acetonitrile were dissolved in 5% (v/v) methanol in 100 mM potassium phosphate buffer of pH 7.0 (due to their low solubility in aqueous phase). In the reaction mixture, 400 µl substrate and 100 µl whole cell suspension was used. This reaction mixture was incubated at 45°C in an incubator shaker at 200 rpm. The substrate specificity of cell free extract was also determined using lysate obtained by lysis of cells using 2 g/l lysozyme in the cell suspension and incubated at 30°C for 1 h. Cell debris after lysis was separated by the centrifugation at 20000 x g and 4°C for 10 min. and the supernatant was designated as the cell free extract for determining the substrate specificity (structure of nitriles is given in appendix I). Total protein, in the cell free extract was estimated by Lowry method with bovine serum albumin (BSA) as a protein standard. The optical density of the cell suspension was determined at 660 nm and the dry weight of cell was determined from a standard curve.

3.11 Determination of nature of enzyme

From the substrate specificity data it was observed that *Amycolatopsis* sp.IITR215 cells might contain more than one nitrile metabolizing enzyme. Therefore, some of reported inhibitors (Di-ethyl phosphoramidate, N-bromosuccinimide and N-ethylmaleimide) for nitrilase, nitrile hydratase and amidase were used for determining extent of inhibition of enzymes. Experiments with inhibitors were carried out by preparing 10 mM substrates in presence and absence of 10 mM Di-ethyl phosphoramidate (DEPA), an amidase inhibitor (Bauer *et al.*, 1998) and different concentrations of N-bromosuccinimide (0.1, 0.5, 1.0, 2.0, 3.0 and 5.0 mM) and N-ethylmaleimide (0.05, 0.1, 0.5, 1.0 mM) were prepared in 100 mM phosphate buffer of pH 7.0 containing 1 mM EDTA. *Amycolatopsis* sp.IITR215 cells were harvested from MB media containing adiponitrile as the sole nitrogen source and harvested cells were incubated at 45°C with different substrates in the incubator shaker and reaction conditions were same as the enzyme assay method.

3.12 Effects of lyoprotectants

Lyophilization is required for long term storage of microbial cells. During lyophlization, whole cell catalyst loses enzyme activity. Therefore, addition of different lyoprotectants is desirable for preventing the loss of enzyme activity. One loopful of culture

was inoculated in 100 ml MB medium with adiponitrile as the sole nitrogen source in 500 ml of flask. The cells were harvested after 36 hours of growth by centrifugation at 16000 x g and 4°C for 10 min. and washed twice with 100 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. Finally cell pellet was suspended in the 5 ml of same buffer and divided in five centrifuge tubes containing 5% (w/v) of different lyoprotectants such as trehalose, sorbitol, sucrose and PEG, and among these, one centrifuge tube was taken as a control where no lyoprotectant was added. Enzyme activity was determined before and after the lyophilization. Enzyme activity before lyophilization was considered as 100 % and residual activity obtained after lyophilization was represented with respect to 100 % activity. Lyophilized cells of this experiment were used in all the further experiments.

3.13 Effect of temperature

The optimum temperature for the nitrile metabolizing enzymes was determined by carrying out enzyme assay at temperatures ranges from 30-60°C in 50 mM phosphate buffer with 1 mM EDTA (pH 7.0) with whole cell suspension. Reaction mixture was incubated for 30 min. Reaction was stopped by adding 10 µl of HCl and cells were separated by centrifugation at 20000 x g. Supernatant was used for measuring ammonia released and formation of acrylamide by GC analysis due to enzymes activity.

3.14 Effect of pH

The effect of pH on nitrile metabolizing enzymes was studied in different pH buffers (50 mM) {acetate buffer (pH 4.0-5.8), phosphate buffer (pH 5.8-8.0), borate buffer (pH 8.0-9.2), carbonate buffer (pH 9.2-10.0)} containing 1 mM EDTA with 10 mM hexanenitrile, 10 mM acrylonitrile with DEPA and 10 mM acrylamide as substrates in the reaction for determining the nitrile metabolizing enzyme activities. The optimization of pH was carried out with whole cell suspension. Formation of acrylamide at different pH was determined by GC analysis due to nitrile hydratase activity. In case of amidase and hexanenitrile hydrolyzing activities, ammonia estimation was carried out using Bertholet method as described earlier.

Maximum activity was assumed as 100% and rest of the activities were with respect to its maximum activities. In all future studies, during enzyme assay with hexanenitrile,

acrylonitrile and acrylamide optimum temperature of 55°C, 45°C, 45°C and optimum pH of 5.8, 7.0, 7.0 respectively maintained during the reaction.

3.15 Effect of metal ions and different compounds on enzyme activity

Adiponitrile grown cells was harvested and incubated with acrylonitrile and hexanenitrile with different concentrations of metal ions and compounds {$CaCl_2$(10 mM), $CuSO_4$(10 mM), $CuCl_2$(10 mM), $FeCl_3$(5 mM), $FeSO_4$(5 mM), $MnCl_2$ (1 mM), $NiCl_2$ (1 mM), $BaCl_2$ (0.5 mM), EDTA (1 mM), H_2O_2 (1 mM), n-Butyric acid (10 mM), Hydroxylamine (1 mM), Di-Thiobisnitrobenzoic acid (1 mM), Iodoacetamide (1 mM)} in the 100 mM phosphate buffer under optimized conditions for determining nitrile metabolizing enzyme activities. Enzyme activity was determined by ammonia release and relative activity was expressed as a percentage of the activity obtained in the absence of any test compound.

3.16 Effect of nitrogen sources to determine constitutive nature of enzymes

One loopful culture of *Amycolatopsis* sp.IITR215 was inoculated in the MB media containing 1 g/l yeast extract and 1g/l NH_4Cl as nitrogen sources and allowed to grow at 45°C. Samples of broth were taken at different time intervals and cells were harvested by centrifugation at 20000 x g and 4°C for 10 min. Cells were washed with 100 mM phosphate buffer (pH 7.0). Washed cells were used for determining the enzyme activity with different substrates under optimized conditions. The cell OD was also determined using the method as mentioned earlier. Cell concentration in media was calculated by using standard plot of section 3.16.1.

3.16.1 Determination of cell concentration

Lyophilized cells at a concentration of 3.3 g/l were dissolved in 100 mM phosphate buffer (pH 7.0) and this suspension was used to make various dilutions. Different dilutions were subjected to O.D at 600 nm and a standard curve was plotted O.D v/s cell concentration (Fig.3.4)

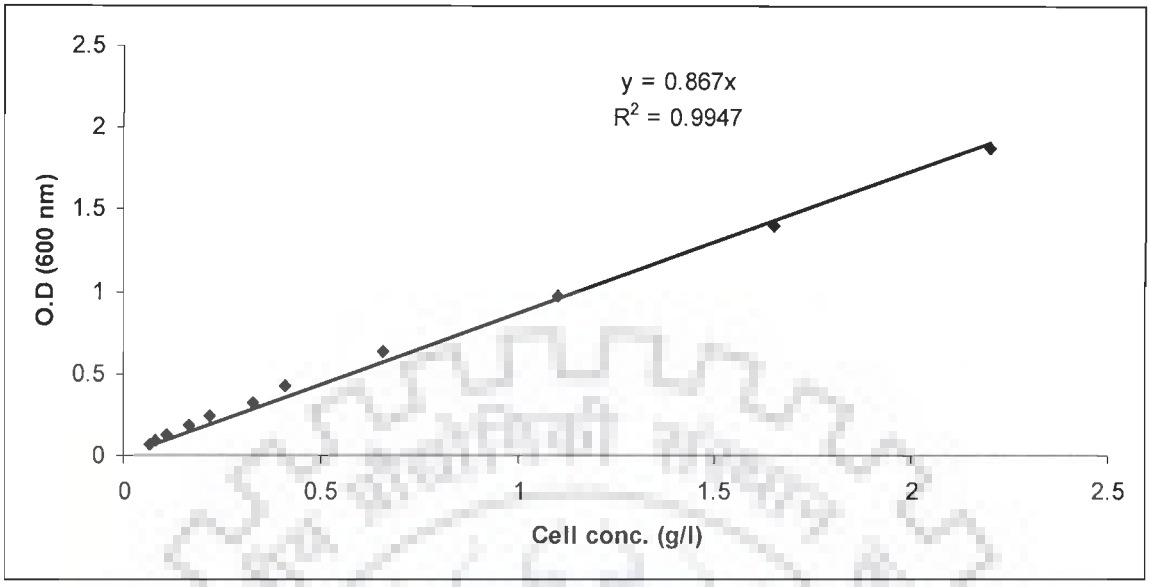


Fig 3.4. Standard Plot for cell concentration.

3.17 Enzyme stability at pH 7.0

Cells of *Amycolatopsis sp.*IITR215 were grown in the MB medium containing 10 mM adiponitrile at 45°C, 200 rpm for 40 hours. Cells were harvested by centrifugation at 20,000 x g and 4°C for 10 min. with two times washing with 100 mM phosphate buffer containing 1 mM EDTA (pH 7.0) and suspended in the same buffer. Cell suspension was incubated at 35°Cand 45°C and samples were taken at 2, 4 and 8 hours for measuring the residual activities with acrylonitrile, hexanenitrile and acrylamide respectively under optimized conditions.

3.18 Enzyme stability at pH 5.8 and effect of different stabilizers

Half life of hexanenitrile metabolizing enzyme was determined by using 3.3 g/l lyophilized cells in 2 ml of 0.1 M phosphate buffer with 1 mM EDTA and cells were incubated at 40 °C, pH 5.8. Cell suspension of 200 µl was taken after every one hour to determine residual activity till 6 hours. Half life of enzyme was determined using these data assuming first order deactivation kinetics.

Effects of some reported stabilizers were studied on the stability of hexanenitrile metabolizing enzyme. Lyophilized cells suspension in 100 mM phosphate buffer (pH 7.0) containing glycerol (30 % v/v), sucrose (5 mg/ml), NaCl (100 mM), tween 20 (3 % v/v) and

EDTA (1mM) as stabilizers. Cell suspension without any additive was taken as a control and activity was determined under optimized conditions.

3.19 Effect of sodium chloride concentration on stability on hexanenitrile metabolizing enzyme

The effect of different salt (NaCl) concentration was studied on hexanenitrile metabolizing enzyme stability. Lyophilized cell at a concentration of 3.3 g/l were incubated in 100 mM phosphate buffer of pH 7.0 with 1mM EDTA at 40°C containing 25, 50, 100, 200 mM NaCl. Samples were taken after every 3 h to study the effect of different NaCl concentrations on the stability of nitrile metabolizing enzyme. Enzyme assay was done under optimized conditions.

3.20 Effect of other additives and protease inhibitors on hexanenitrile metabolizing enzyme

Lyophilized cells in the concentration of 4 g/l lysozyme were dissolved in 100 mM phosphate buffer and lysed by 2 g/l lysozyme for 1 h at 30°C. Cell debris was removed by centrifugation at 16000 x g for 10 min. Cell free extract was used to study the effect of PMSF, DTT, Protease inhibitor Cocktail, β-Mercaptoethanol, Butyric acid, Cystine, Sodium thiocyanate, Sodium thioglycolate, Sodium acetate, Glutathione, L-cysteine, BSA and Casein hydrolaysate on hexanenitrile metabolizing enzyme at 45°C and pH 7.0.

3.21 Organic solvent compatibility

The tolerance of the hexanenitrile metabolizing enzyme with whole cell against several solvents (Di-methylformamide, Di-methylsulphoxide, N-methyl pyrollidone, ethyl alcohol, methyl alcohol, isopropyl alcohol and acetone) in 100 mM phosphate buffer at pH 7.0 was also tested at 20 % (v/v) and 50 % (v/v) of organic solvent. Enzyme activity was determined by ammonia release and relative activity was expressed as a percentage of the activity obtained in the absence of any solvent.

3.22 Cell lysis for the recovery of nitrile metabolizing enzyme

Following methods of cell lysis were tested to obtain the maximum recovery of nitrile metabolizing enzymes:

3.22.1 Enzymatic method

Cell suspension of lyophilized cells (3.3 g/l) in 100 mM phosphate buffer containing 1 mM EDTA were incubated with lysozyme at a concentration of 0.5, 1.0, 2.0 and 5.0 g/l at 30°C for 1 hour in an incubated shaker at 200 rpm for lysis of *Amycolatopsis* sp.IITR215 cells. Cell debris was removed by centrifugation at 16000 x g and 4°C for 10 min. Supernatant was taken for the analysis of enzyme activity using hexanenitrile as substrate. Cell debris was again suspended in the same amount of 100 mM phosphate buffer for determining the residual activity.

3.22.2 Mechanical methods

Sonication: The cells suspension as mentioned in section 3.21.1 was sonicated on ice in 5 ml glass tubes using a hielscher ultrasonic processor UP100H (100W, 30 kHz) for a total sonication time of 2, 4, 6 and 8 min. (30s pulse-on and 30s pulse-off). Supernatant and pellet were separated by centrifugation and used for enzyme assay with hexanenitrile and for determining the recovery of enzyme in the supernatant and residual activity in the pellet respectively.

Vortexing with glass beads: Lyophilized cell suspension was vortexed with 10 mg/ml glass beads (100-200 µm) in 25 ml glass test tube at highest speed for the shearing of cell wall. Vortexing was done for 2, 4, 6, 8 and 10 min. with 1 min break after every minute and kept on ice. Recovery of enzyme was determined from enzyme assay data.

3.23 Cell growth and preparation of cell free extract for protein purification

Cells of *Amycolatopsis* sp.IITR215 were grown in MB media containing 10 mM adiponitrile. Cells were harvested after 38 hours of growth by centrifugation at 16000 X g for 10 min. Cells pellet was lyophilized for 6-8 hours to remove the water content. These lyophilized cells were used in all the further experiments. Cells were lysed by using 2 g/l of lysozyme in 20g/l of lyophilized cell suspension in 50 mM phosphate buffer (pH 7.0) and incubated at 30°C for 1 hour at 200 rpm. Cell debris was removed by centrifugation at 16000 x g and 4°C for 10 min.

3.24 Protein precipitation

3.24.1 Precipitation by ammonium sulphate:

Lyophilized cells of *Amycolatopsis* sp.IITR215 cells at a concentration of 10 g/l were lysed with 2 g/l lysozyme at pH 7.0, 30°C for 1h. Cell debris was removed by centrifugation at 16000 x g and 4°C for 10 min. Proteins in the supernatant were serially precipitated by different concentrations of ammonium sulphate and various concentrations of ammonium sulphate were prepared by using the following formula:

Amount of ammonium sulphate (in grams) = $533\ (S_2-S_1)/100-0.3\ S_2$

S_2 = Final % saturation

S_1 = Initial % saturation

Proteins in the pellet were used for checking the nitrile hydratase, amidase and hexanenitrile metabolizing activities. Proteins were estimated by Lowry method. Precipitation was carried out at 4°C.

3.24.2 Precipitation by organic solvents

10 g/l lyophilized cells of *Amycolatopsis* sp.IITR215 cells were lysed by 2 g/l lysozyme at pH 7.0, 30°C for 1h. Cell debris was removed by centrifugation at 16000 x g and 4°C for 10 min. Proteins in the supernatant were precipitated by different concentrations (% v/v) of acetone and ethanol serially. Proteins in the pellet were used for checking nitrile metabolizing enzyme activity with hexanenitrile. Proteins were estimated by Lowry method (Lowry *et al.*, 1976)

3.24.3 Sodium sulphate precipitation

Cell free extract was precipitated by 10, 20, 30 and 40 % of sodium sulphate (w/v). Precipitated proteins were separated by centrifugation at 16000 x g and 4°C for 10 min. Supernatant and pellet obtained after centrifugation was used to determine the enzyme activity by taking hexanenitrile as a substrate and proteins by Lowry method.

3.25 Ion exchange chromatography

Protein pellet obtained after sodium sulphate precipitation was dissolved in 1 ml of 20 mM phosphate buffer (pH 7.0) and the protein suspension was loaded onto Q-sepharose ion-exchange column (1.5 cm × 30 cm) which was pre-equilibrated with 20 mM phosphate buffer (pH 7.0). Unbound proteins were washed with 20 mM phosphate buffer at flow rate of 1 ml/min. A step gradient of 0.2-0.5 M NaCl was used to elute bound proteins. Fractions were collected with fraction volume of 2.5 ml and used for measuring protein O.D at 280 nm and enzyme assay. Activity of nitrile hydratase in the cell free extract and the protein pellet suspension obtained after sodium sulphate precipitation was determined by using acrylonitrile as a substrate with an amidase inhibitor, Di-ethylphosphoramidate (Babu *et al,*. 2010) by HPLC and activity of amidase was determined by using hexanamide as a substrate. Amidases in active fraction 2, 3 and 4 were clubbed and designated as amidase 1, 2 and 3 respectively. Similarly, nitrile hydratases in active fraction 5, 6 and 7 were clubbed and designated as nitrile hydratase 1, 2 and 3 respectively. The activity of amidase active fractions was determined using the method as described earlier. The activity of nitrile hydratase fractions was determined using acrylonitrile as substrate and product was analyzed by using HPLC. Due to the presence of high salt concentration, proteins were estimated by Bradford method as mentioned in section 3.3.6. Same experiment was repeated by removing sodium sulphate from the precipitated protein suspension by using amicon®ultra centrifugal filters (10 kDa cutt-off). Desalted protein was bring to the final volume of 1 ml and loaded in the ion-exchange column.

3.26 Native-PAGE

Clubbed protein fractions were concentrated using amicon®ultra centrifugal filters (10kDa cutt-off). Concentrated protein samples and gel loading buffer (5X) in the ratio of 40:10 was mixed and loaded on the gel for native-polyacrylamide gel electrophoresis (native-PAGE). Native-PAGE was performed at 100 Volt for 4 h using a Bio-red mini polyacrylamide gel system electrophoresis unit with 0.75 mm 10 % native-PAGE resolving gels and a 5 % acrylamide stacking gel.

Reagents:

Solution A: A stock solution of 29% (w/v) acrylamide solution and 1% (w/v) N, N'-methylene-bis-acrylamide was prepared in deionized warm water.

Solution B: Resolving buffer, 1.5 M Tris-HCl, pH 8.8.

Solution C: Stacking buffer, 1.0 M Tris-HCl, pH 6.8

Solution D: 10 % (w/v) ammonium per sulphate (freshly prepared)

Solution E: TEMED (N, N, N', N'- tetramethylenediamine)

Table 3.3: Solutions for preparing resolving and stacking gels

Reagents	Resolving gel (10 %) (ml)	Stacking gel (5 %) (ml)
Solution A	6.7	0.83
Solution B	5.0	–
Solution C	–	0.63
Solution D	0.2	0.05
Solution E	0.008	0.005
Water	8.1	3.58
Total Volume (ml)	**20**	**5**

Casting of gel

A sandwich of glass plates (8 X 10 cm) was assembled by plastic spacers and plates were held together by clamps. Resolving gel was prepared by mixing all the components followed by addition of ammonium persulphate and TEMED. The mixture was gently mixed and poured between the plates by 1 ml micro-pipette and leaving the adequate space at the top for pouring the stacking gel after polymerization. Resolving gel was overlaid by distilled water to prevent oxygen from diffusing into the gel and inhibiting polymerization. After completion of polymerization, overlay was poured-off and washed the top of gel several times with deionized water to remove any un-polymerized acrylamide. Stacking gel was poured similarly as the resolving gel and comb was immediately inserted into the mixture to form the wells. After polymerization of stacking gel, comb was carefully removed and

formed wells were rinsed with de-ionized water and polymerized gel was used for electrophoresis.

Sample preparation:

Gel-loading Buffer (5X): 50 mM Tris-HCl (pH 6.8), 0.1 % bromophenol blue, 10 % (v/v) glycerol.

Clubbed protein fractions were concentrated in amicon®ultra centrifugal filters (10kDa cutt-off). Concentrated protein samples and gel loading buffer in the ratio of 40:10 were mixed and loaded on the gel for electrophoresis.

Electrophoresis

Tris-glycine buffer (1X): 25 mM Tris, 250 mM glycine (electrophoresis grade) (pH 8.3)

Vertical electrophoresis chamber was filled with Tris-glycine buffer and chamber at the bottom of glass plates was filled with Tris-glycine buffer. Total 25 µl of sample was loaded on the gel using micropipette. Electrophoresis was carried out at a constant voltage of 100 Volt for 4 h using a Bio-red mini polyacrylamide gel system unit until the tracking dye reached to the bottom. Gel after electrophoresis was removed and stained overnight with gentle shaking in 0.25 % Coomassie Brilliant blue R-250 (w/v) in methanol: glacial acetic acid: water (50:10:40) at room temperature. Gel was then destained by washing in methanol: glacial acetic acid: water without the dye on a slowly rocking platform for 4-6 hours. This destaining procedure was repeated many times so as to get the stained bands over a clear background.

3.27 Zymogram of amidase

Amidase activity in polyacrylamide gels was examined using a zymogram staining technique (Egorova *et al.,* 2002). Active fractions of anion-exchange chromatography were clubbed and concentrated using amicon®ultra centrifugal filters (10 kDa cutt-off). Concentrated fractions were subjected to native PAGE. To detect bands with amidase activity, the gel was incubated at 45°C for 30 min. in 20 mM phosphate buffer (pH 7.0) containing 70 mM hexanamide and 0.7 M hydroxylamine hydrochloride (the pH was adjusted to 7.0 with 10 M NaOH). The gels were washed in water and covered with an acid

solution of iron chloride (0.1 M $FeCl_3$ in 0.5 M HCl). Iron reacts with hydroxamic acid and forms a red-brown band, which corresponds to amidase activity (Egorova *et al.*, 2004).

3.28 Substrate specificity of purified nitrile hydratase and amidase fractions

Relative activities of different active fractions of nitrile hydratase and amidase were measured by quantification of ammonia released from amides and formation of amides from nitriles which was determined by HPLC. A reaction mixture containing 400 µl of 10 mM amides (isobutyramide, benzamide, adipamide, propionamide, acetamide, acrylamide, and hexanamide) with 100 µl of fraction was incubated at 45°C, pH 7.0 for 30 min for the determination of amidase activity whereas different nitriles (isobutyronitrile, benzonitrile, adiponitrile, propionitrile, acetonitrile, acrylonitrile, 3-cyanopyridne, butyronitrile, hexanenitrile, cyclohexanecarbonitrile and methacrylonitrile) with 100 µl of fraction was incubated at 45°C, pH 7.0 for 30 min. for determining nitrile hydratase activity. Reaction was stopped by adding 10 µl of 2 N HCl. Ammonia release from amides was calculated by Bertholet method and formation of amides from different nitrile was determined by HPLC as mentioned in section 3.3.

3.29 Inhibitor study with purified amidase and nitrile hydratase

Di-ethyl phosphoramidate (DEPA), an amidase inhibitor was used at a concentration of 10 mM with hexanamide and acrylamide for amidase and acrylonitrile for nitrile hydratase. Hexanamide, acrylamide and acrylonitrile were prepared in 100 mM phosphate buffer (pH 7.0) and incubated at 45°C for 30 min. with clubbed fractions. Amide formation and ammonia release were determined as mentioned earlier.

3.30 Temperature optimization for polymer treatment

Lyophilized cells at a concentration of 25 g/l lysed using 2 g/l of lysozyme by incubating for 1 h at 30°C and 200 rpm in 50 ml oakridge tube at pH 7.0. Cell debris was removed by centrifugation at 16000 x g and 4°C for 10 min. Nitrile metabolizing enzymes in the supernatant was used to treat 10 g/l PAN at 30°C, 37°C and 45°C at pH 7.0 for 12 hours. Released ammonia was estimated to determine the polymer hydrolysis. Ammonia released in blank sample (enzymatic solution without polymer) was subtracted from ammonia released

from enzyme treated polymer sample for determining ammonia release due to enzyme action. A control sample was also taken where polymer suspension at same concentration as enzyme sample were taken without any addition of enzyme and incubated along with enzyme sample. Ammonia released in control sample was insignificant as compared to blank and enzyme samples.

3.31 Protein adsorption and ammonia release from polyacrylonitrile during enzymatic surface modification at different pH

Cells of *Amycolatopsis* sp.IITR215 at a concentration of 20 g/l were dissolved in 6 ml of 50 mM phosphate buffer (pH 7.0) and subjected to cell lysis using 2g/l lysozyme by incubating for 1 h at 30°C and 200 rpm in 50 ml oakridge tube at pH 7.0. Cell debris was removed by centrifugation at 16000 x g and 4°C for 10 min. Proteins in supernatant were divided into two parts of 3 ml each and precipitated by 30 % sodium sulphate (w/v) to remove some of the undesired proteins. Precipitated proteins were re-suspended in 4 ml of 100 mM acetate buffer of pH 5.8 and 100 mM phosphate buffer of pH 7.0. This protein suspension was used to treat 10 g/l polyacrylonitrile at 37°C in an incubator shaker at 200 rpm and samples were taken after every 6 hours to determine residual protein in the supernatant by Lowry method and ammonia released in the reaction mixture. A blank sample was also incubated at similar experimental conditions where only enzyme at same concentration was taken without any polymer. Ammonia released due to the enzyme action was calculated after subtracting the ammonia release in the blank sample from the ammonia release in enzyme treated polymer sample. A control sample was also taken where polymer suspension at same concentration as enzyme sample were taken without any addition of enzyme and incubated along with enzyme sample. Ammonia released in control sample was insignificant as compared to blank and enzyme sample.

3.32 FTIR analysis

After 48 hours of enzymatic treatment of polymer, enzyme treated polymers of pH 5.8 and 7.0 were washed thrice with 50 ml of water followed by 1.5 % (w/v) sodium carbonate solution and water (Fischer-colbrie *et al.*, 2006) to remove the adsorbed proteins. After every washing stage centrifugation method was followed for recovery of washed

polymer. The washed samples were subjected to FTIR analysis. FTIR (Fourier transform Infra-Red) spectroscopy was carried out using a Thermo Nicolet Nexus FT-IR spectrometer using KBr technique.

3.33 XPS Analysis

Both the control and enzyme treated samples of pH 7.0 were probed by X-ray photo-electron spectroscopy (XPS) for the confirmation of surfacial functional groups. X-ray photoelectron spectroscopy (XPS) is a powerful technique for the identification of elements, percentage composition and oxidation states. XPS measurements were carried out in an ultra-high vacuum chamber (PHI1257) with base pressure of 4×10^{-10} Torr. The XPS spectrometer was equipped with a high-resolution hemispherical electron energy analyzer (279.4 mm diameter) with 25 meV resolution, and a dual anode Mg/Al K_α X-ray source. The source used for the present study was the Al (K_α) x-ray excitation of energy 1486.6 eV with pass energy of 100 eV for survey scan and 40 eV for core level spectra of each element. All binding energies (BE) were calibrated by the BE (284.6 eV) of C1s, which gave BE values within an accuracy of 0.1 eV.

3.34 Nitrile hydratase and amidase adsorption on PAN surfaces during enzymatic treatment of polymer

The adsorption of nitrile hydratase and amidase on the polyacrylonitrile surfaces was studied indirectly by determining the residual activities of theses two enzymes in the reaction mixture at regular interval of time. Cell lysis and protein precipitation was performed as mentioned in the previous sections. Precipitated protein suspension was used to treat 10 g/l polyacrylonitrile at pH 5.8 and 7.0. Initially, sample was taken after 1 hour and then samples were taken after every 6 hours for analysis of unbound enzyme activities. Activity of amidase in reaction mixture was determined using acrylamide as substrates whereas activity of nitrile hydratase was determined using acrylonitrile with DEPA (amidase inhibitor) as substrate. Enzyme assay methods were same as described in previous sections. In this experiment also a blank sample was taken where only enzyme at same concentration was added without any polymer and incubated at similar experimental conditions. The nitrile hydratase and amidase activities of blank samples at various time intervals were determined using methods as

described in previous sections. The protein concentrations of enzyme samples and blank samples were determined by Lowry method. A control sample was also taken where polymer suspension at same concentration as enzyme sample was taken without any addition of enzyme and incubated along with enzyme sample. Ammonia released in control sample was insignificant as compared to blank and enzyme samples.

3.35 Quantification of carboxyl groups in enzyme treated polymer

Rhodamine 6G (4 mg) was dissolved in a phosphate buffer saline solution (PBS, pH 12, 4 ml) and then immediately extracted with 100 ml of benzene through vigorous shaking in stoppered Erlenmeyer flask. The extract exhibited a yellow color and was used in further experiments as the dye reagent. In this study for standard plot, acrylic acid was used standard. As acrylic acids were increasingly added to the dye reagent, the absorbance at 530 nm, based on the complex of Rhodamine and carboxylic acid, increased, yet at 486 nm, based on only free Rhodamine itself, the absorbance decreased. 0.2 g/l of enzyme treated PAN powder was soaked in the dye reagent for 2 h and the decrease in the absorbance at 486 nm was measured (Kim *et. al.,* 2000). PAN powder without enzymatic treatment was taken as a control. The concentration of the carboxylic acid groups on the polymer surfaces were calculated from the calibration curve between the decrease in absorbance at 486 nm and the concentration of acrylic acid in the dye reagent (Fig. 3.5).

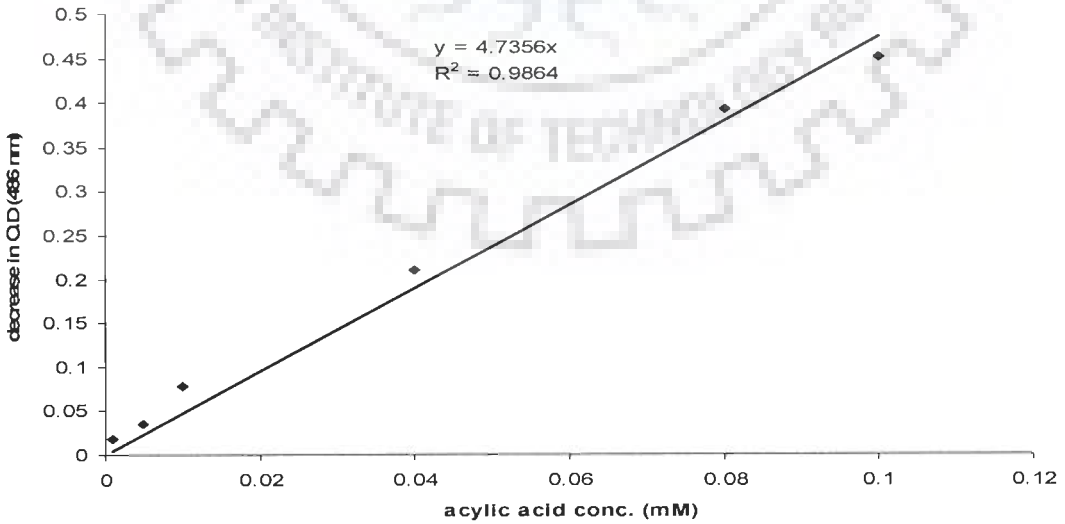


Fig.3.5. Standard plot of acrylic acid.

3.36 Protein adsorption and ammonia release from polyacrylonitrile-co-polymers

Cells of *Amycolatopsis* sp.IITR215 at a concentration of 15 g/l were suspended in 6 ml of 50 mM phosphate buffer (pH 7.0) and subjected to cell lysis using 2 g/l lysozyme by incubating for 1 h at 30°C and 200 rpm in 50 ml oakridge tube at pH 7.0. Cell debris was removed by centrifugation at 16000 x g and 4°C for 10 min. Proteins in supernatant precipitated by 30 % sodium sulphate and re-suspend in 100 mM phosphate buffer of pH 7.0. This protein suspension was used to treat 10 g/l polyacrylonitrile-co-methacrylonitrile, polyacrylonitrile-co-butadiene-styrene and polyacrylonitrile-co-methacrylate. Samples were taken after every 6 hours to determine unbound protein in the supernatant and ammonia release from polymers samples and control samples (without polymer). Final ammonia released due to enzymatic treatment of polymer was determined by subtracting the ammonia release data of control sample from the ammonia released data of enzyme sample.

3.37 Effect of polymer concentration in enzymatic treatment of PAN

Precipitated protein solution was prepared according to the section 3.30 in acetate buffer of pH 5.8. This protein suspension was used to treat 5, 10, 15, 20 and 25 g/l polyacrylonitrile at 37°C in an incubator shaker at 200 rpm for 48 hours to determine residual protein in the supernatant by Lowry method and ammonia released in the reaction mixture. A blank sample was also incubated at similar experimental conditions where only enzyme at same concentration was taken without any polymer. Ammonia released due to the enzyme action was calculated after subtracting the ammonia release in the blank sample from the ammonia release in enzyme treated polymer sample. A control sample was also taken where polymer suspension at same concentration as enzyme sample were taken without any addition of enzyme and incubated along with enzyme sample. Ammonia released in control sample was insignificant as compared to blank and enzyme sample.

Chapter 4
Results & Discussion

4. RESULTS AND DISCUSSION

Results and discussions of this study were presented in three sections:

- **Section 4.1** Isolation, characterization of nitrile metabolizing enzyme producing strain and enzyme characterization.
- **Section 4.2** Partial Purification of nitrile metabolizing enzymes
- **Section 4.3** Enzymatic treatment of polymer and characterization of treated polymer

Section 4.1

4.1.1 Isolation and screening of nitrile metabolizing producing micro-organism

As number of nitrile metabolizing enzymes having specificity for polyacrylonitrile are limited, it was felt to isolate a culture having ability to produce nitrile metabolizing enzyme with specificity for PAN (polyacrylonitrile). In this work, eleven different soil and water samples collected from various parts of Punjab and Shimla used as source of organism (Table 4.1). The media used for isolation of culture having nitrile metabolizing enzyme was made selective by addition of polyacrylonitrile powder as sole nitrogen source in mineral base(MB) media. The culturing carried out in shake flask with different soil and water samples for two generations before spreading on the mineral base media agar plates containing acrylonitrile as sole nitrogen source. Total seven isolates were obtained and all isolates were grown in MB medium containing various nitriles (benzonitrile, isovaleronitrile, phenylacetonitrile, acrylonitrile and adiponitrile) as an inducer and sole nitrogen source. Cells grown on these inducers were used for activity checking with acrylonitrile, polyacrylonitrile (PAN) and polyacrylonitrile-co-methacrylonitrile (PMA). Among these seven isolates, one of the isolate (no.6/b) found to produce ammonia when whole cell incubated with acrylonitrile, polyacrylonitrile (PAN) and polyacrylonitrile-co-methacrylonitrile (PMA). This isolate was also able to grow on agar plates (Fig 4.1) containing polyacrylonitrile powder. Thus isolate no. 6/b was selected as the best strain for further research work (Table 4.1).

Fig4.1. Growth of isolate (6/b) on agar plates.

Fig.4.2. Gram staining of isolate no. 6/b.

Table 4.1: Enzyme activity of different isolates grown in different inducers with three nitrile substrates (with whole cells at 45°C, pH 7.0).

Culture No.	Inducer/Nitrogen source	Ammonia release with 10g/l PAN in 12 hr. (μM/min)	Ammonia release with 10mM acrylonitirile in 1 hr (μM/min)	Ammonia release with 10 g/l (PMA) (PMA) in 12 hr. (μM/min)
1/a & 1/b	Phenylacetonitrile	N.D	N.D	**N.D**
	Isovaleronitrile	N.D	N.D	**N.D**
	Benzonitrile	N.D	N.D	**N.D**
	Acrylonitrile	N.D	N.D	**N.D**
3/a	Phenylacetonitrile	N.D	N.D	**N.D**
	Isovaleronitrile	N.D	N.D	**N.D**
	Benzonitrile	N.D	N.D	**N.D**
	Acrylonitrile	N.D	N.D	**N.D**
4/a	Phenylacetonitrile	N.D	10.43	**0.25**
	Isovaleronitrile	N.D	15.73	**0.11**
	Benzonitrile	N.D	1.72	**N.D**
	Acrylonitrile	N.D	N.D	**N.D**
6/a	Phenylacetonitrile	N.D	12.16	**N.D**
	Isovaleronitrile	N.D	15.05	**N.D**
	Benzonitrile	N.D	9.58	**N.D**
	Acrylonitrile	N.D	N.D	**N.D**
6/b	Phenylacetonitrile	0.19	13.01	**0.82**
	Isovaleronitrile	ND	23.32	**0.05**
	Benzonitrile	N.D	11.74	**0.13**
	Acrylonitrile	N.D	N.D	**N.D**
6/c	Isovaleronitrile	N.D	N.D	**N.D**
	Benzonitrile	N.D	N.D	**N.D**
	Acrylonitrile	N.D	N.D	**N.D**

*No growth was detected in the sample no.2,5,7,8,9,10,11

4.1.2 Selection of suitable inducers/nitrogen source in the growth media for enzyme production

Isolate 6/b was selected as best strain in terms of ammonia release from PAN and its co-polymer (PMA). From Table 4.1, it was concluded that isolate 6/b when grown in mineral base media containing phenylacetonitrile, produced nitrile metabolizing enzyme having specificity for PAN and PMA. So, an experiment was planned where isolate 6/b grown in media containing different inducer/nitrogen sources and grown cells were incubated with PMA. Based on the ammonia release rate data, the suitable inducer/nitrogen source was selected. No growth was detected in €-caprolactam and 2-cyanopyridine containing media while adiponitrile was found to be the most efficient inducer or nitrogen source for the production of nitrile metabolizing enzyme which showed maximum activity with PMA (Table 4.2). All future experiments were carried out with adiponitrile as an inducer.

Activities with PAN and PMA indicate that either enzyme is located on cell membrane or part of polymer is soluble in aqueous phase. Nitrile metabolizing enzymes are generally inducible in nature. So, to determine the reason for whole cell enzyme activity from insoluble polymer, a study was conducted with aqueous phase (obtained after separation of polymer powder) saturated with polymer for 24 hour. It was observed that activity with polymer suspension and aqueous phase saturated with polymer gave same activity under similar experimental conditions (Table 4.2). Thus, it indicates that part of polymer is soluble due to the presence of low molecular weight oligomers and they are responsible for ammonia release from whole cell and thus it can also be concluded that enzyme is intra-cellularly located. In all future studies, polymer after washing with distilled water was used to avoid soluble oligomers fractions.

4.1.3 Morphological characterization of isolated strain

Isolated strain had rounded configuration, convex elevations, rough surface, white and creamy yellow pigmentation. Gram staining of isolated strain showed gram positive reaction (Table 4.3) and visualized at 100X with oil immersion which was showing the single/Y shaped morphology (Fig. 4.2). Further characterization of isolate was conducted with respect to growth temperatures and growth pH (Table 4.3).

Table 4.2: Effect of different inducers on ammonia release from PMA (with whole cells at 45°C, pH 7.0)

Inducer/Nitrogen Source	Ammonia release from PMA (μM/min)	
	After 3 hrs	After 12 hrs
Valeronitrile	0.09	0.27
Butyronitrile	N.D	0.08
Adiponitrile	1.42	0.90
		0.90 (aqueous phase saturated with PMA)
Propionitrile	0.65	0.15
Phenylacetonitrile	N.D	0.68
Isobutyronitrile	N.D	0.08
2-cyanopyridine	N.D	N.D
Benzonitrile	N.D	0.16
Acetonitrile	N.D	0.10
Є-caprolactam	N.D	N.D

Table 4.3: Morphological and growth characterization of isolated strain

Property	Test	Result
Colony morphology	Configuration	Round
	Margin	Entire
	Elevations	Convex
	Surface	Rough
	Density	Opaque
	Pigments	White & creamy yellow
Gram's reaction		+
Spore		-
Motility		-
Fluorescence		-
Growth at temperature	37 °C	+
	45 °C	+
	55 °C	-
Growth at pH	pH 5.7	+
	pH 6.8	+
	PH 7.0	+
	pH 8.0	+

4.1.4 Isolation of genomic DNA and PCR amplification of 16S rDNA

Isolated DNA was loaded on agarose gel and visualized in gel documentation system (Fig. 4.3a) and further 16S rDNA was amplified by using universal primers. The result of PCR amplification is shown in Fig.4.3b. Amplified fragment correspond to 1492 bp. The amplified fragment was sequenced by Ocimum Biosolutions Pvt. Ltd, Hyderabad, India.

4.1.5 Phylogenetic analysis

Alignment of FASTA sequences of 16S rDNA of the isolated strain was showing 100% homology with *Amycolatopsis* sp. ATCC 39116 (Accession no. AM263203.1), *Amycolatopsis thermoflava* strain 173573 (Accession no. EU570741.1), *Amycolatopsis sp.* (Accession no. AJ000285) and *Amycolatopsis methanolica.* (Accession no. AJ249135) (Table 4.4) and thus this isolate was placed at the same position of phylogenetic tree (Fig.4.4). Now onwards, this isolated strain will be referred as *Amycolatopsis* sp.IITR215. Lechevalier *et al.* (1986) proposed genus *Amycolatopsis* for amycolate, nocardioform actinomycetes. Members of this genus produce branched, fragmenting aerial and substrate mycelia (Lechevalier, *et al.,* 1986). Genus *Amycolatopsis* contains belongs to the family of *Pseudonocardiaceae*. Species of this genus have potential applications in the production of many antibiotics such as epoxyquinomicins, balhimycin, rifamycin and vancomycin etc. (Dhingra *et al.,* 2003). In this genus, very few reports are available on nitrile metabolizing enzymes. However, Robin and Nagasawa used this genus for the preparation of amides (Robin and Nagasawa, 1999).

Fig.4.3 (a) Genomic DNA from isolated culture (b) amplified 16S rDNA (lane 1 & 2), Marker DNA ranges from 1000-5000bp (lane 3).

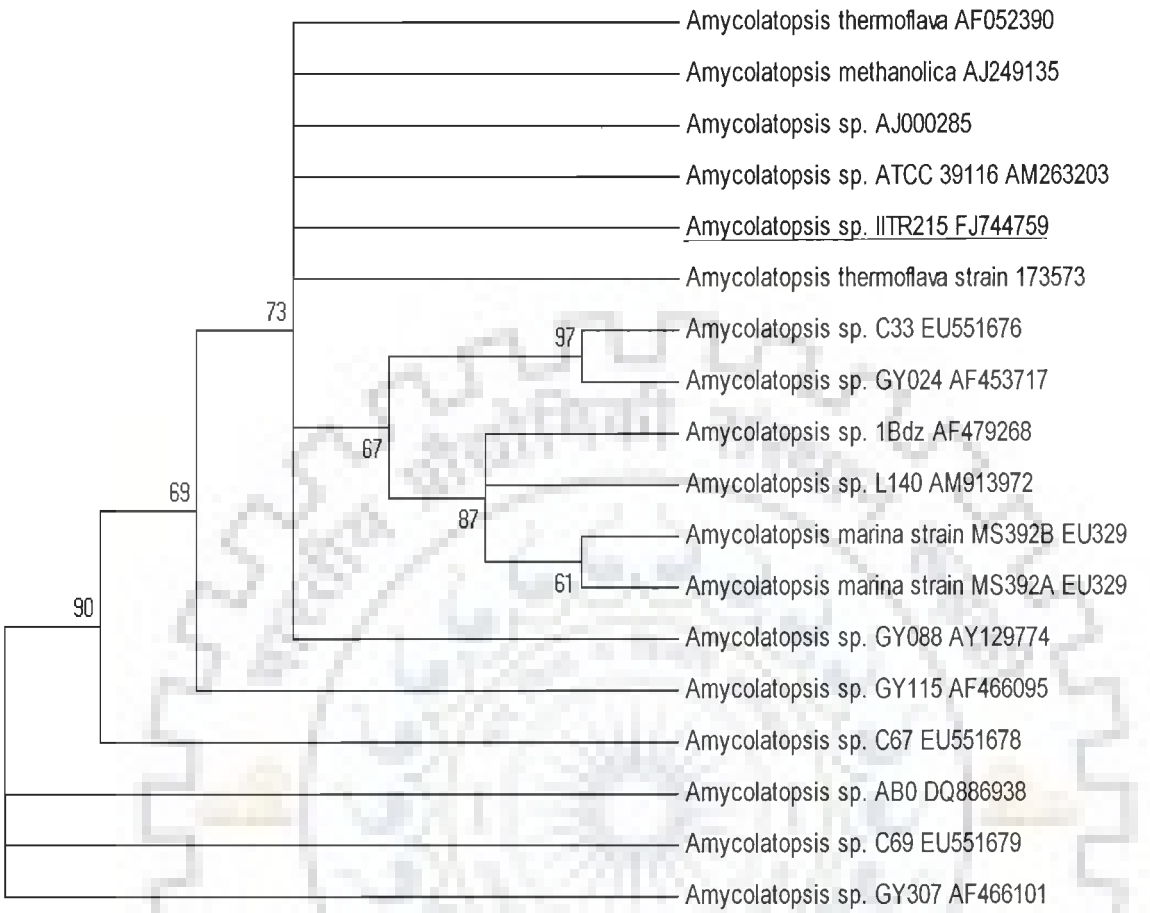


Fig.4.4. Phylogentic tree of *Amycolatopsis* sp.IITR215 and related strains based on the 16S rDNA sequence. Numbers after the name of organisms are accession numbers of published sequences.

Table 4.4: Homology of 16 S rDNA of isolated strain from other strains (data obtained from http://blast.ncbi.nlm.nih.gov/Blast.cgi)

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
AM263203	*Amycolatopsis* sp. ATCC 39116 16S rRNA gene, strain ATCC 39116, gene 2	1356	1356	100%	0.0	100%
EU570741	*Amycolatopsis thermoflava* strain 173573 16S ribosomal RNA gene, partial sequence	1356	1356	100%	0.0	100%
AJ000285	*Amycolatopsis* sp. DNA for 16S ribosomal RNA	1356	1356	100%	0.0	100%
AF052390	*Amycolatopsis thermoflava* 16S ribosomal RNA gene, partial sequence	1351	1351	100%	0.0	99%
AJ249135	*Amycolatopsis methanolica* 16S rRNA gene, strain IMSNU 20055T	1351	1351	100%	0.0	99%
DQ886938	*Amycolatopsis* sp. AB0 16S ribosomal RNA gene, partial sequence	1345	1345	100%	0.0	99%
EU551679	*Amycolatopsis* sp. C69 16S ribosomal RNA gene, partial sequence	1336	1336	100%	0.0	99%
AY129774	*Amycolatopsis* sp. GY088 16S ribosomal RNA gene, partial sequence	1327	1327	100%	0.0	99%
EU551678	*Amycolatopsis* sp. C67 16S ribosomal RNA gene, partial sequence	1315	1315	98%	0.0	99%
EU551676	*Amycolatopsis* sp. C33 16S ribosomal RNA gene, partial sequence	1314	1314	100%	0.0	99%
AF466095	*Amycolatopsis* sp. GY115 16S ribosomal RNA gene, partial sequence	1314	1314	99%	0.0	99%
AF453717	*Amycolatopsis* sp. GY024 16S ribosomal RNA gene, partial sequence	1314	1314	100%	0.0	99%
AF479268	*Amycolatopsis* sp. 1Bdz 16S ribosomal RNA gene, partial sequence	1303	1303	100%	0.0	98%
AM913972	*Amycolatopsis* sp. L140 partial 16S rRNA gene, isolate L140	1303	1303	100%	0.0	98%
EU329846	*Amycolatopsis marina* strain MS392B 16S ribosomal RNA gene, partial sequence	1297	1297	100%	0.0	98%
EU329845	*Amycolatopsis marina* strain MS392A 16S ribosomal RNA gene, partial sequence	1291	1291	100%	0.0	98%

4.1.6 Substrate specificity

Amycolatopsis sp. IITR215 cells grown in MB medium containing 10 mM adiponitrile, found to produce nitrile metabolizing enzyme having wide range of substrate specificity (Table 4.5). Maximum activity was detected with isobutyramide with whole cells as well as with the cell free extract. Among nitriles, enzyme system of *Amycolatopsis sp.*IITR215 was exhibiting specificity with aliphatic, aromatic as well as with arylacetonitriles except mandelonitrile. Similar specificity was found with the cell free extract but specific activity was less as compare to the whole cells (Table 4.5). Lower specific activity in the cell free extract might be due to incomplete lysis of cells with lysozyme and toxic effects of nitrile towards soluble enzyme. The enzyme system of *Amycolatopsis sp.*IITR215 showed activity towards amide and nitrile. Thus, it indicates the presence of amidase and probably nitrilase and nitrile hydratase. From this study, it can be concluded that nitrile metabolizing enzymes from the isolated strain had higher specificity towards aliphatic nitriles and amides. This study also indicated that *Amycolatopsis* sp.IITR215 had probably multiple enzyme system for the nitrile metabolism. In the earlier reported multiple enzyme systems, nitrilase of *Bacillus subtilis* ZJB-063 was specific for arylacetonitriles whereas nitrile hydratase/amidase of the same strain exhibited higher activity towards *para*-substituted phenylacetonitrile (Zheng *et al.,* 2008). Bhalla *et al.* had also reported nitrilase, nitrile hydratase and two amidases in the *Nocardia globerula* NHB-2. Nitrilase of their isolate was specific for the aromatic, unsaturated and saturated nitriles whereas nitrile hydratase was specific for the saturated aliphatic nitriles and two amidases of this strain were specific for the aliphatic and aromatic amides (Bhalla and Kumar 2005).

Table 4.5: Activities of enzyme with different nitriles and amides substrates with whole cells at 45°C, pH 7.0

S.No.	Substrate	Activity with whole cells (IU/g)	Activity with cell free extract (IU/g)
1.	3-hydroxypropionitrile	12.31	8.01
2.	Valeronitrile	30.01	25.98
3.	Acrylonitrile	11.01	6.24
4.	Acetonitrile	8.36	4.93
5.	Phenoxyacetonitrile	28.50	22.39
6.	Propionitrile	24.56	23.01
7.	Mandelonitrile	0.43	N.D
8.	Adiponitrile	2.53	N.D
9.	Isobutyronitrile	28.38	23.71
10.	3-Cyanopyridine	12.63	6.71
11.	Methylacrylonitrile	10.66	6.94
12.	Hydricinnamonitrile	3.03	1.54
13.	Cyclohexanecarbonitrile	11.29	6.21
14.	Malononitrile	9.27	1.87
15.	Glutaronitrile	22.82	12.91
16.	4-Phenylbutyronitrile	6.57	1.47
17.	Indole-3-acetonitrile	1.49	N.D
18.	Benzonitrile	11.31	6.21
19.	4-Hydroxybenzonitrile	5.65	2.29
20.	Phenylacetonitrile	18.81	12.46
21.	Isovaleronitrile	10.25	4.81
22.	Hexanenitrile	12.19	5.42
23.	4-Aminobenzylcyanide	6.24	2.30
24.	Phenylthioacetonitrile	8.92	4.96
25.	Butyronitrile	29.09	21.40
26.	4-Cyanopyridine	4.27	1.01
27.	2-Cyanopyridine	2.16	1.00
28.	Adipamide	6.68	2.73
29.	Acetamide	13.68	6.09
30.	Benzamide	9.86	7.38
31.	Propionamide	28.83	15.97
32.	Isobutyramide	30.51	27.61
33.	Acrylamide	11.73	5.04

•Sp. activity in cell free extract was calculated by taking the concentration of whole cell. However, protein content was 0.536 g/l in reaction mixture.

4.1.7 Determination of nature of enzyme

In the study of substrate specificity, it was observed that enzymes consist of amidase and probably nitrilase or nitrile hydratase, or both. An effort was therefore made to differentiate the presence of nitrile hydratase from nitrilase. Ammonia released from nitrile compounds can be due to the action of either nitrile hydratase/amidase or nitrilase or both. To differentiate the presence of nitrile hydratase/amidase and nitrilase, we used an amidase inhibitor along with various nitriles. The effectiveness of the amidase inhibitor was also confirmed by incubating whole cells with the amidase inhibitor along with acrylamide (Table 4.6). From these results, it can be concluded that ammonia released from some nitrile compounds (acrylonitrile, isobutyronitrile, valeronitrile, propionitrile, acetonitrile, butyronitrile, isovaleronitrile, 3-hydroxypropionitrile, 4-cyanopyridine, 3-hydroxyglutaronitrile, malononitrile) was solely due to nitrile hydratase and amidase, because with the added inhibitor (DEPA), there was no release of ammonia. This was further confirmed by determining the presence of acrylamide in the reaction mixture when cells were incubated with 10 mM acrylonitrile along with 10 mM DEPA. In the case of hexanenitrile, the presence of the inhibitor did not affect ammonia production (Table 4.6). It was therefore concluded that ammonia released from hexanenitrile was probably due to nitrilase or the combined action of nitrile hydratase plus a second amidase that was not affected by DEPA. The enzyme involved in the hydrolysis of hexanenitrile is most likely a different enzyme than the one catalyzing acrylonitrile hydrolysis. For nitrile hydratase and amidase activities, acrylonitrile and acrylamide, respectively, were used as substrates.

Moreau et al. reported 100% inhibition of nitrile hydratase with 0.005 mM N-bromosuccimide and 68% inhibition with 0.05 mM. ethylmaleimide in *Brevibacterium* sp. (Moreau *et al.,* 1993), whereas Harper reported 100% inhibition of nitrilase with 0.005 mM N-ethylmaleimide in *Nocardia* sp. (Harper 1977b). To confirm the nature of the enzyme involved in hexanenitrile hydrolysis, a new experiment was carried out with the previously reported nitrile hydratase and nitrilase inhibitors (N-bromosuccimide and N-ethylmaleimide). The effects of inhibitors were assessed by determining relative activities as compared to those without inhibitor using hexanenitrile, acrylonitrile and acrylamide as substrates. At 0.1 mM concentration of N-bromosuccimide, 80% activity was detected with acrylonitrile

whereas it was 90% and 93% for acrylamide and hexanenitrile, respectively (Fig.4.5). At 1 mM N-bromosuccimide concentration, no activity was detected with acrylonitrile and 5% and 36% activities were retained with acrylamide and hexanenitrile, respectively. This result indicates that higher concentration of N-bromosuccimide was inhibitory to nitrile hydratase and amidase and lesser inhibition was observed with enzymes involved in hexanenitrile hydrolysis (Fig.4.5). At 0.5 mM N-ethylmaleimide concentration, 42% and 36% activities were found with acrylamide and acrylonitrile, respectively, whereas 81% activity was found with hexanenitrile, which confirms that N-ethylmaleimide had a greater inhibitory effect on amidase and nitrile hydratase as compared to the enzyme involved in hexanenitrile hydrolysis (Fig.4.6). At a higher concentration of 5 mM N-ethylmaleimide, acrylonitrile and acrylamide showed 15% and 31% activities, respectively, and 56% activity was observed with hexanenitrile, which again confirms the higher inhibitory effect of N-ethylmaleimide on nitrile hydratase as well as on amidase (Fig.4.6). Thus from this study, it was observed that the effects of inhibitors were different on hexanenitrile, acrylonitrile and acrylamide hydrolysis, and thus we concluded that multiple nitrile-metabolizing enzymes are present in whole cells grown in the media containing adiponitrile as sole nitrogen source. Furthermore, it was concluded that hydrolysis of acrylonitrile was due to nitrile hydratase and amidase, and that this amidase is inhibited in the presence of DEPA. In the case of hexanenitrile hydrolysis, it was either due to the action of nitrilase or the action of a second set of nitrile hydratase/amidase enzymes. The nature of the enzyme involved in hexanenitrile hydrolysis was difficult to pinpoint, so in order to differentiate it from the nitrile hydratase and amidase involved in acrylonitrile hydrolysis, enzyme activities were monitored using acrylonitrile, acrylamide and hexanenitrile as substrates.

It was also concluded that the nitrile hydratase of *Amycolatopsis* sp. IITR215 had exclusive specificity for propionitrile, valeronitrile, isobutyronitrile, acrylonitrile, acetonitrile, butyronitrile, isovaleronitrile, 4-cyanopyridine, 3-hydroxyglutaronitrile and malononitrile. Other enzymes from this isolate had exclusive specificity for hexanenitrile, cyclohexanecarbonitrile and indole-3-acetonitrile.

Further confirmation about the nature of nitrile metabolizing enzymes of *Amycolatopsis sp* IITR215 was carried out after partial purification of enzymes using ion-exchange chromatography (Section II).

Table 4.6: Effect of DEPA on enzyme activities with various nitrile substrates (with whole cells at 45°C, pH 7.0).

S.No.	Substrate	Activity Without Inhibitor (IU/g)	Activity With amidase Inhibitor (DEPA) (IU/g)
1.	Benzonitrile	8.88	4.34
2.	Phenylacetonitrile	12.88	3.90
3.	Valeronitrile	13.06	N.D
4.	Propionitrile	15.05	0.19
5.	Isobutyronitrile	13.63	N.D
6.	Adiponitrile	5.63	1.67
7.	Acrylonitrile	9.10	0.02
8.	Acetonitrile	11.17	N.D
9.	Butyronitrile	14.09	0.05
10.	Isovaleronitrile	7.57	0.01
11.	Glutaronitrile	14.79	2.08
12.	3-Hydroxypropionitrile	11.98	0.06
13.	Methacrylonitrile	2.30	0.02
14.	Hexanenitrile	11.26	12.59
15.	Cyclohexanecarbonitrile	0.58	0.57
16.	2-Cyanopyridine	4.44	0.77
17.	4-Hydroxybenzonitrile	5.18	1.46
18.	4-Aminobenzylcyanide	5.20	0.56
19.	Phenoxyacetonitrile	13.13	2.25
20.	Phenylthioacetonitrile	5.28	2.57
21.	Hydrocinnamonitrile	13.34	4.97
22.	4-Phenylbutyronitrile	10.96	1.91
23.	3-Cyanopyridine	9.07	0.90
24.	4-Cyanopyridine	3.08	0.21
25.	3-Hydroxyglutaronitrile	2.85	0.29
26.	Mandelonitrile	0.03	N.D
27.	Malononitrile	4.83	0.19
28.	Indole-3-acetonitrile	2.29	1.82
29.	Acrylamide	10.04	N.D

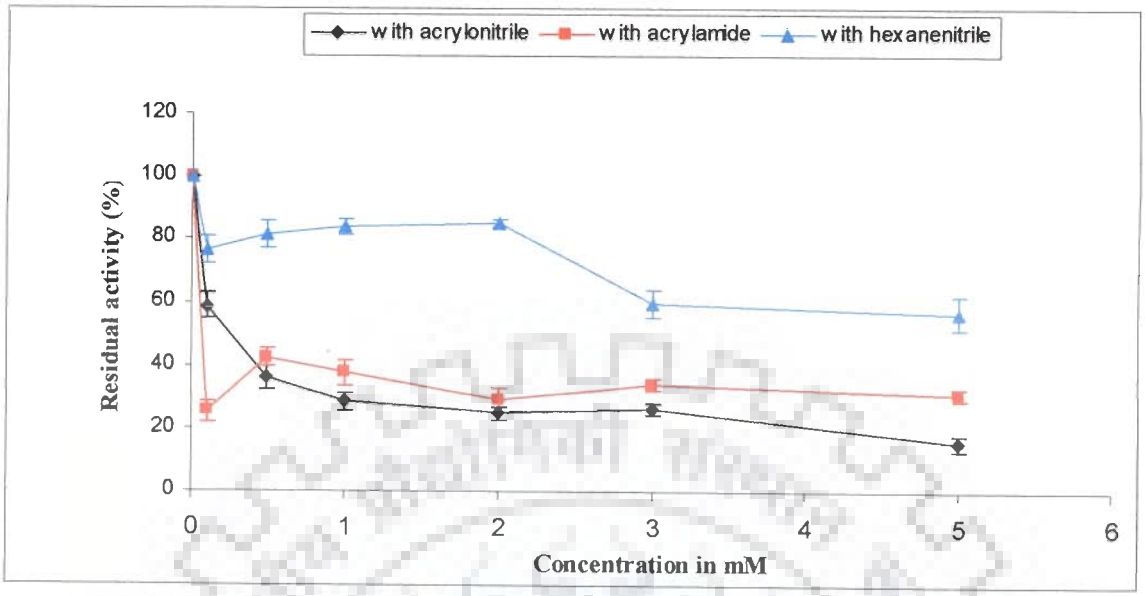


Fig.4.5. Effect of N-Bromosuccinimide on nitrile metabolizing enzyme activity with hexanenitrile (triangle), nitrile hydratase activity with acrylonitrile (square), amidase activity with acrylamide (circle) (with whole cells at 45°C, pH 7.0).

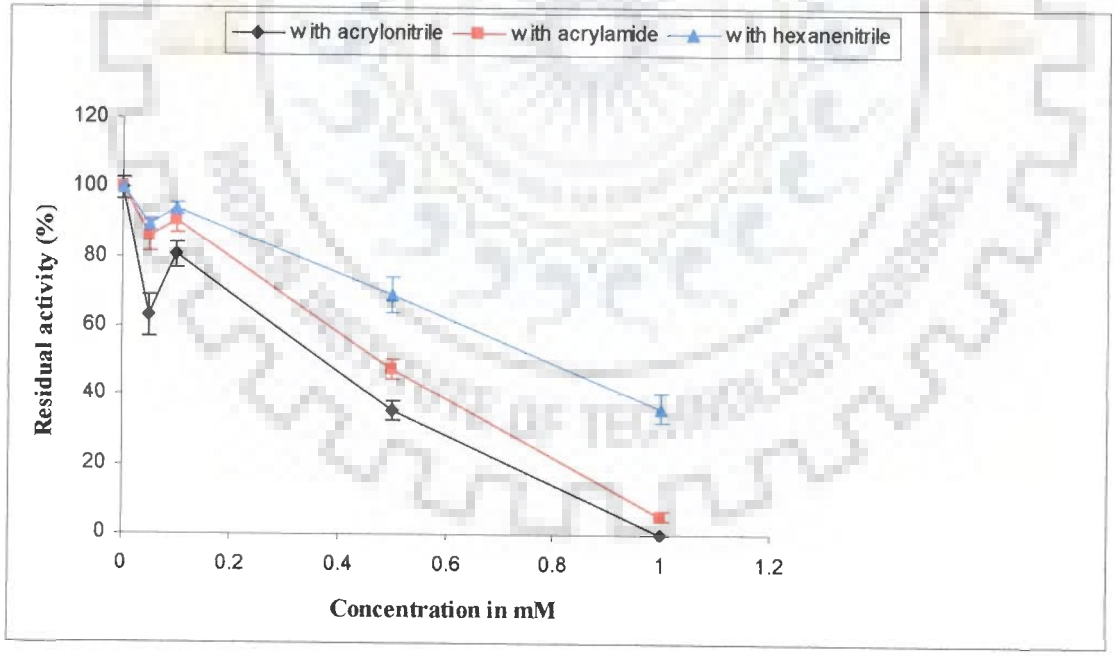


Fig.4.6. Effect of N-Ethylmaleimide on nitrile metabolizing enzyme activity with hexanenitrile (triangle), nitrile hydratase activity with acrylonitrile (square), amidase activity with acrylamide (circle) (with whole cells at 45°C, pH 7.0).

4.1.8 Acyl-tranferase activity of amidase

Along with hydrolysis of amides, there is a considerable interest in enzymatic transfer of an acyl group on external nucleophile. Hydroxylamine was generally used as external nucleophile and used as acyl acceptor. Acyl-tranferase activity of amidase is necessary for the production of important hydroxamic acids. In this study, acyl-transferase activity was determined with eight amides (isobutyramide, benzamide, adipamide, thioacetamide, propionamide, acetamide, acrylamide and hexanamide) by using cell free extract of *Amycolatopsis* sp. IITR215. Hydrolyzing activity of amidase was maximum for isobutyramide whereas acyl group transfer activity was maximum with hexanamide and negligible acyl-transferase activity was detected with acetamide and no activity was detected with acrylamide. From these results, it can be concluded that amidase from *Amycolatopsis* sp.IITR215 had higher acyl-tranferase activity with long chain aliphatic amides (Table 4.7) whereas very-short chain (C_2-C_3) aliphatic amides were found to be efficient substrates for an amidase from *Rhodococcus sp.* R312 which was cloned, over-expressed in an *E. coli* strain (Fournand *et al.*, 1998). Similarly short chain amides were preferred substrates for a thermophilic amidase from *Geobacillus pallidus* but maximum acyl-tranferase activity was detected with isobutyramide (Makhongela *et al.*, 2007)

Table 4.7: Acyl-transferase activity of amidase from *Amycolatopsis* sp.IITR215 (from cell free extract at 45°C, pH 7.0).

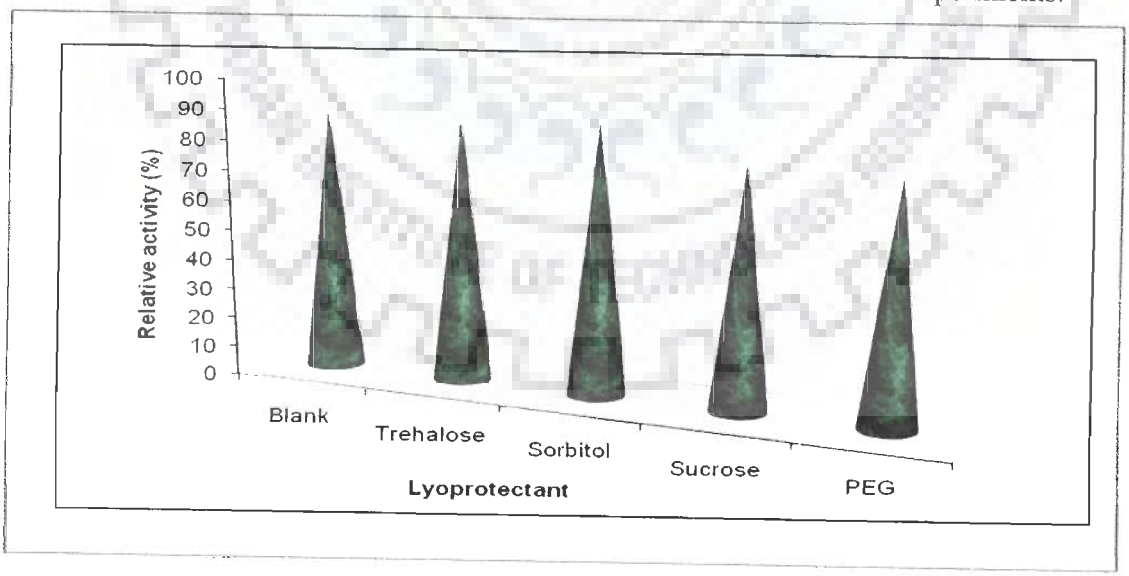
Substrate	Ammmonia release from amidase (Relative activity %)	Acyl tranferase activity (Relative activity %)
Isobutyramide	100	67.73
Benzamide	65.34	35.27
Adipamide	10.30	3.49
Thioacetamide	0	0
Propionamide	33.91	32.26
Acetamide	4.50	0.77
Acrylamide	5.10	0
Hexanamide	54.30	100

* 100% amidase activity corresponds to 397.11 IU/g.
* 100% acyl-transferase activity corresponds to 618.65 IU/g.

4.1.9 Effects of lyoprotectants

Lyophilization is a useful technique for long term storage of whole cell enzyme activity. However, during lyophilization enzyme gets deactivated and to reduce the extent of deactivation, lyoprotectants are generally used. In general, lyoprotectants such as sugars and polyols stabilize the enzyme structure by replacing the water molecules around them. Therefore, addition of lyprotectants is desirable. The effects of reported lyoprotectants were studied on hexanenitrile metabolizing enzyme activity. The cells were lyophilized in presence of 5% (w/v) of different lyoprotectants. The Activity of enzyme before lyophilization was assumed as 100 %. After lyophilization, 88% of nitrile metabolizing activity was retained in cells in absence of any lyoprotectant. Sorbitol proved to be a marginally better lyoprotectant but sucrose and PEG had negative impact as lyoprotectant (Fig.4.7).

In reported literature, sucrose shown to be an effective lyoprotectant for nitrile hydratase of *Agrobacterium tumifaciens* (Fischer–Colbrie *et al.*, 2006) but in this study, there was no major advantage of using sucrose as lyoprotectants was observed. Similarly, with other lyoprotectants no significant advantage was observed with whole cell enzyme activity. Therefore, lyophilized cells without lyoprotectant were used in all further experiments.



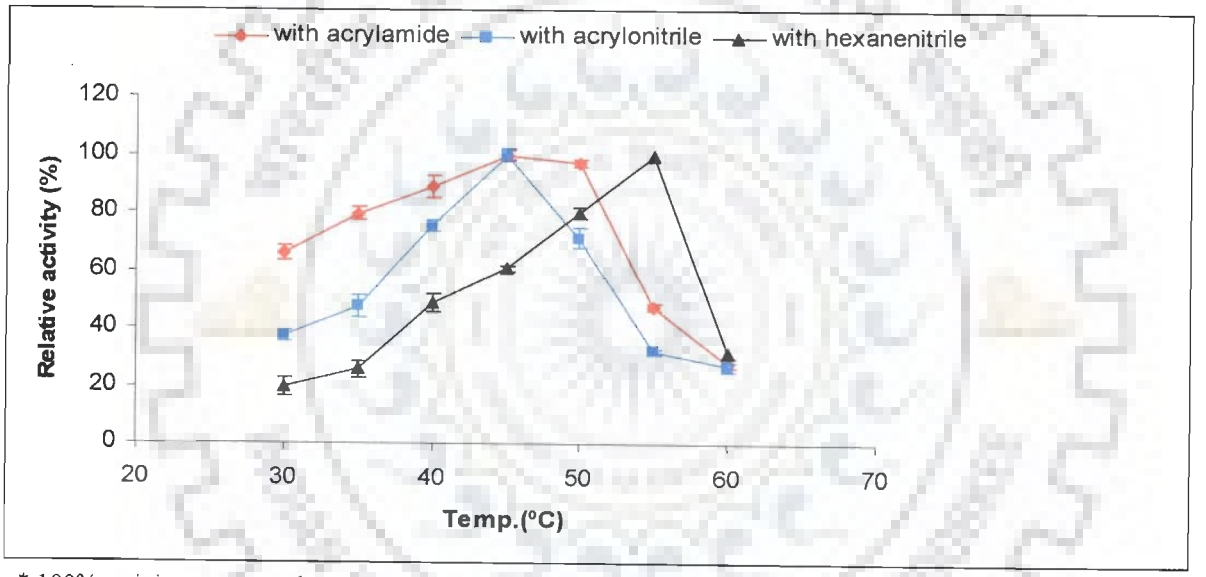
* 100% activity correseponds to 70.21 IU/g

Fig.4.7. Effect of lyoprotectants on hexanenitrile hydrolyzing activity (with whole cells at 45°C, pH 7.0).

4.1.10 Effect of temperature

Amycolatopsis sp. IITR 215 was isolated at 45°C and till now most of the enzyme activities were determined at 45°C. So, an effort was made to find out the optimum temperature of enzyme activity. It was very difficult to determine the optimum temperature due to the presence of multiple enzymes in this isolate. The enzyme for hexanenitrile hydrolysis was active in the temperature range of 30–60°C. Nearly 60% and 80% relative activities for hexanenitrile hydrolysis were found at 45°C and 50°C, respectively. The optimum temperature for hexanenitrile hydrolysis was found to be 55°C (Fig. 4.8).

* 100% activity corresponds to 87.53 IU/g for hexanenitrile



* 100% activity corresponds to 28.57 IU/g for acrylamide

* 100 % activity corresponds to 27.1 IU/g for acrylonitrile

Fig.4.8. Effects of temperature on enzyme activity with hexanenitrile, nitrile hydratase activity with acrylonitrile and amidase activity with acrylamide (with whole cells at pH 7.0).

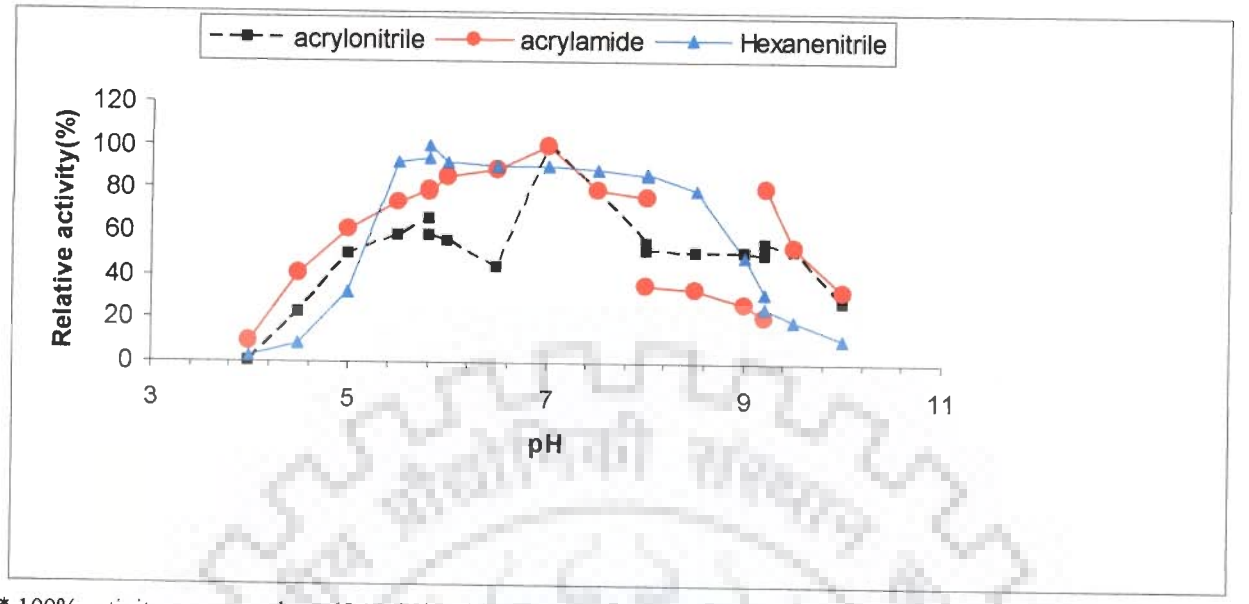
Very few nitrile metabolizing enzymes reported to have optimum temperatures in this range. The nitrilase of *Bacillus pallidus* DAC521 (Almatawah *et al.*, 1999), *Pseudomonas* sp.13 (Yanase *et al.*, 1983) and *Aspergillus niger* K10 (Kaplan *et al.*, 2006) were reported to have optimum temperatures of 60°C, 55°C and 45°C, respectively. To determine the optimum temperature for amidase activity, acrylamide was employed as a substrate. The amidase was found to be highly active in the temperature range of 35–50°C. Relative activities of 80% and 90% were found at 35°C and 40°C, respectively, whereas 97% activity

was found at 50°C (Fig. 4.8). The amidase found to have an optimum temperature of 45°C. As the hydrolysis of acrylonitrile was accomplished by subsequent action of nitrile hydratase and amidase, 10 mM acrylonitrile with DEPA (an inhibitor of amidase) in 50 mM of phosphate buffer (pH 7.0) was used as a substrate to study the effects of temperature on acrylamide formation by nitrile hydratase. The optimized temperature for nitrile hydratase was found to be 45°C (Fig. 4.8). Similar optimum temperature was also reported for the nitrile hydratase of *Mesorhizobium* sp. F28 (Feng *et al.,* 2008) and *Rhodococcus* sp. (Kaakeh *et al.,* 1991).

4.1.11 Effect of pH

Kobayashi et al. reported an optimum pH of 5.5 for purified nitrilase from *Rhodococcus rhodochrous* K22 (Kobayashi *et al.,* 1990), whereas the nitrilase from *Aspergillus niger* K10 had an optimum pH in the basic pH range of 8.0 (Kaplan *et al.,* 2006). Nitrile-metabolizing enzymes from *Amycolatopsis* sp. IITR215 were highly active in the pH range of 5.5–8.5. Relative activities of 91% and 79% were detected at pH 5.5 and 8.5, respectively, with hexanenitrile and the optimum pH was found to be 5.8 for hexanenitrile hydrolysis (Fig. 4.9). A similar optimum pH was reported for nitrilase of *Arthrobacter nitroguajacolicus* (Shen *et al.,* 2009).

The amidase was highly active at pH 7.0 but had 78% and 75% relative activities at pH 6.0 and 8.0, respectively (Fig. 4.9). A similar optimum pH was reported with the constitutive amidase from *Rhodococcus rhodochrous* M8 (Kotlova *et al.,* 1999). As mentioned earlier, the hydrolysis of acrylonitrile takes places by nitrile hydratase/amidase, so we attempted to optimize the pH for nitrile hydratase using 10 mM acrylonitrile with DEPA in 50 mM of various buffers. From the pH profile (Fig. 4.9) it was observed that there are two optimum peaks at two pH values (5.8 and 7). From this result, it can be concluded that the optimum pH for nitrile hydratase was 7.0, and the other peak was probably due the non-uniform effect of pH on DEPA. Nitrile hydratase of some of the reported strains such as *Arthrobacter* sp. J1 (Asano *et al.,* 1982), *Bacillus* RAPc8 (Pereira *et al.,* 1998), *Agrobacterium tumefaciences* d3 (Bauer *et al.,* 1998) exhibited the same optimum pH. All future enzyme assays with acrylonitrile, acrylamide and hexanenitrile were carried out at 45°C, 45°C and 55°C respectively and respective pH was 7.0, 7.0 and 5.8 respectively.



* 100% activity corresponds to 65.15 IU/g with heaxanenitrile
* 100% activity corresponds to 27.97 IU/g with acrylamide
* 100% activity corresponds to 26.11 IU/g with acrylonitrile

Fig.4.9. Effects of pH on enzyme activity with hexanenitrile, nitrile hydratase activity with acrylonitrile and amidase activity with acrylamide (with whole cells at 45°C for acrylonitrile and acrylamide, 55°C for hexanenitrile).

4.1.12 Effect of different metal ions and compounds

To further differentiate these enzymes, effects of different metal ions and other compounds were also evaluated. Salts of Cu^{++} were found to be strong inhibitors for the enzyme involved in hexanenitrile hydrolysis as well as for the nitrile hydratase/amidase pathway which indicates that thiol groups may play an important role in the function of the active site (Kobayashi *et al.*, 1989) of both enzymes (Table 4.8). Similar results were also reported by Banerjee *et al.* and Layh *et al.* for *Pseudomonas putida* and *Pseudomonas fluorescens* DSM175, respectively (Banerjee *et al.*, 2006, Layh *et al.*, 1998). Salts of Fe^{+++} were found to have no effect on hexanenitrile hydrolysis and effects on nitrile hydratase/amidase pathway were marginal. Salts of Ba^{++} and Ni^{++} were found to affect hexanenitrile hydrolysis but insignificant effect on the nitrile hydratase/amidase pathway. Addition of Di-thiobisnitrobenzoic was also observed to inhibit both hexanenitrile hydrolysis and the nitrile hydratase/amidase. Interestingly, Bhalla *et al.* have reported on complete inhibitory effect of di-thiobisnitrobenzoic acid on nitrilase (Bhalla *et al.*, 1992). From this study, it can be concluded that salts of Ni^{++} and Ba^{++} had higher inhibitory effect on

hexanenitrile metabolizing enzyme as compared to acrylonitrile metabolizing enzyme while Di-Thiobisnitrobenzoic acid and salts of Cu^{++} found to have significant inhibitory effect on both acrylonitrile metabolizing enzyme and hexanenitrile metabolizing enzyme.

Table 4.8: Effect of metal ions and compounds on enzyme activity

S. No.	Inhibitor	Residual activity with Acrylonitrile (%)	Residual activity with Hexanenitrile (%)
1.	None	100	100
2.	$CaCl_2$(10mM)	108.53	104.28
3.	$CuSO_4$(10mM)	6.24	4.51
4.	$CuCl_2$(10mM)	8.95	8.21
5.	$FeCl_3$(5mM)	94.37	106.18
6.	$FeSO_4$(5mM)	82.09	85.61
7.	$MnCl_2$(1mM)	97.44	91.54
8.	$NiCl_2$(1mM)	98.65	78.17
9.	$BaCl_2$(0.5mM)	101.33	74.45
10.	EDTA(1mM)	83.12	73.20
11.	H_2O_2(1mM)	74.59	85.88
12.	n-Butyric acid(10mM)	66.28	71.59
12.	Hydroxylamine(1mM)	64.31	67.05
14.	Di-Thiobisnitrobenzoic acid(1mM)	18.11	28.65
15.	Iodoacetamide(1mM)	56.34	37.21

4.1.13 Effect of nitrogen sources in growth media to determine constitutive nature of enzymes

Most of reported literature on nitrile metabolizing enzymes showed inducible nature of nitrilase, nitrile hydratase and amidase (Stevenson *et al.*, 1992, Layh *et al.*, 1998, Almatawah *et al.*, 1999, Yamamoto *et al.*, 1992, Nawaz *et al.*, 1996, Prepechalova *et al.*, 2001). Few reports confirmed the presence of constitutive nitrile metabolizing enzymes (Alfani et al., 2001, Kobayashi *et al.*, 1989, Egorova et al., 2004). To confirm the inducible or constitutive nature of enzymes, ammonium chloride and yeast extract had been used as

sole nitrogen source in growth media during enzyme production. In the absence of nitriles and amides from MB media, *Amycolatopsis* sp. IITR215 strain also produced acrylonitrile, acrylamide and hexanenitrile metabolizing enzymes in media containing 1 g/l yeast extract and 1 g/l NH_4Cl as sole nitrogen source. However, the enzyme activity for hexanenitrile hydrolysis was high when compared to the acrylonitrile and acrylamide metabolizing enzymes. This result thus confirms the constitutive nature of all nitrile-metabolizing enzymes of *Amycolatopsis* sp. IITR215 (Fig. 4.10 and Fig. 4.11). In the earlier reported literature, constitutive amidase had been reported in *Delftia acidovorans* strain 16, *Kluyveromyces thermotolerans* MGBY 37, *Pseudomonas aeruginosa* mutant PACIII (C0 I), *Pseudomonas putida* PPW-3, *Pseudonocardia thermophila*, *Rhodococcus sp* (Hongpattarakere *et al.*, 2005, Prasad *et al.*, 2005, Farin and Clarke 1978, Wyndham and Slater 1986, Egorova *et al.*, 2004, Nawaz *et al.*, 1994) while constitutive nitrile hydratases had been reported in *Rhodococcus* sp.N774, *Rhodococcus* sp.N771, *Pseudonocardia thermophila*, *Brevibacterium* sp. R 312,, *Pseudomonas chlororaphis* B23, *Pseudomonas putida* NRRL-18668, *Brevibacterium imperalis* CBS489–74, (Endo and Watanabe 1989, Yamada and Kobayashi 1996, Yamaki *et al.*, 1997, Nagasawa *et al.*, 1986, Nagasawa *et al.*, 1987, Fallon *et al.*, 1997, Alfani *et al.*, 2001).

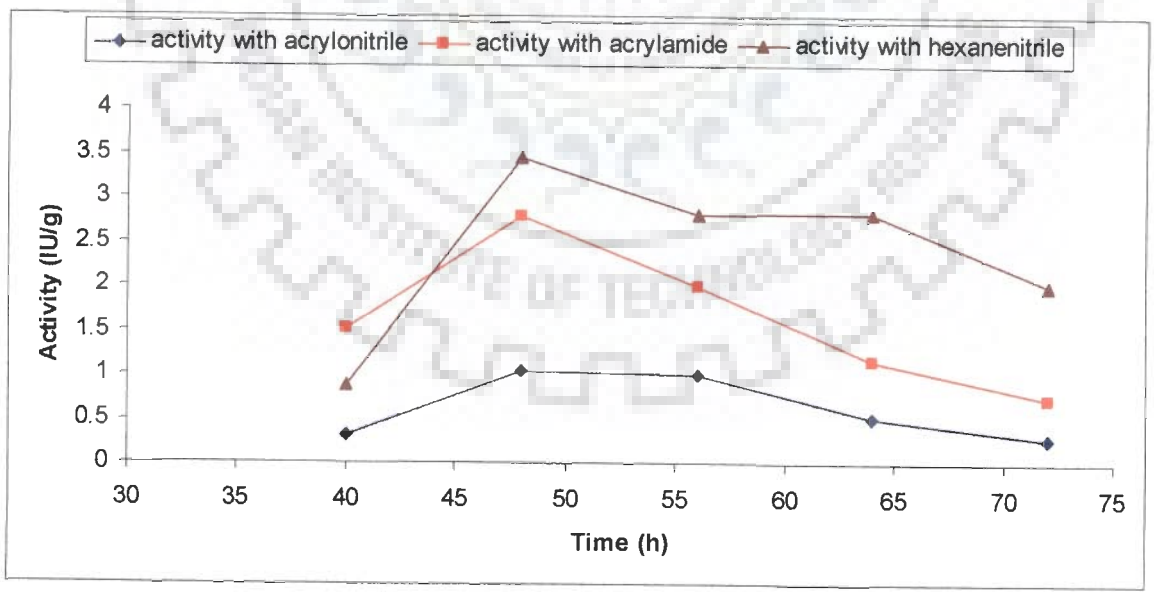


Fig.4.10. Enzyme activities of whole cells grown in media containing 10 mM ammonium chloride as sole nitrogen source.

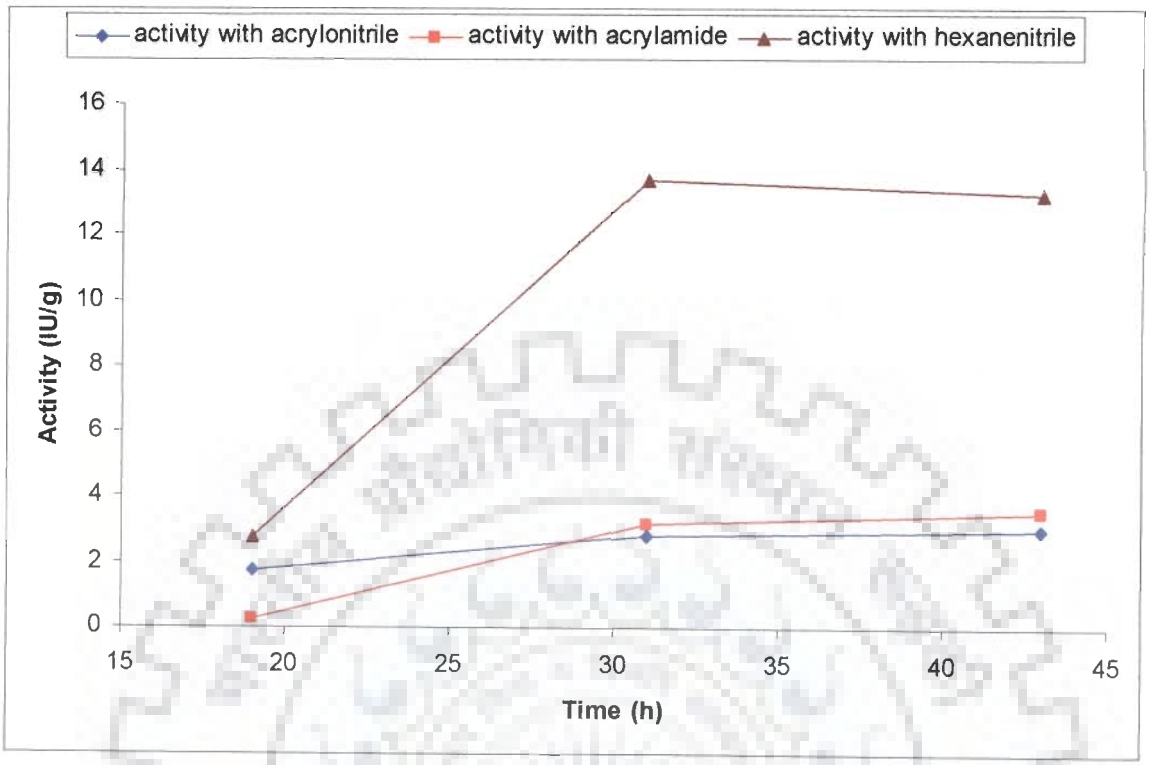


Fig.4.11. Enzyme activities of whole cells grown in media containing yeast as sole nitrogen source.

4.1.14 Enzyme stability at pH 7.0

The operational stability of an enzyme determines its suitability for commercial applications. Based on the stability results (Table 4.9), it can be concluded that the stability of enzymes involved in hexanenitrile hydrolysis was higher than those of the nitrile hydratase and amidase at 35°C. Nearly 68% residual activity was found after 8 h with hexanenitrile, whereas 42% and 46% residual activities were found with acrylonitrile and acrylamide, respectively, at 35°C. Thus, the enzyme for hexanenitrile hydrolysis was more stable than nitrile hydratase and amidase under similar experimental conditions. Nearly same results were obtained at 45°C. Residual activities of 18% and 34% were found with acrylonitrile and acrylamide, respectively, at 45°C, which showed that the thermal stability of amidase was higher than that of nitrile hydratase, but the stabilities of both were lower than that of the enzyme for hexanenitrile hydrolysis (Table 4.9). This study again indicates the involvement of two different nitrile-metabolizing enzymes for hexanenitrile and acrylonitrile hydrolysis.

Table 4.9: Enzyme stability of Nitrile hydratase, amidase and hexanenitrile hydrolyzing enzyme at pH 7.0.

Time (in hours)	Temperature (°C)	Residual activity of enzyme for acrylonitrile (%)	Residual activity of enzyme for acrylamide (%)	Residual activity of enzyme for hexanenitrile (%)
2	35	97	100	100
	45	57	83	100
4	35	96	100	96
	45	39	62	48
8	35	42	46	68
	45	18	34	39

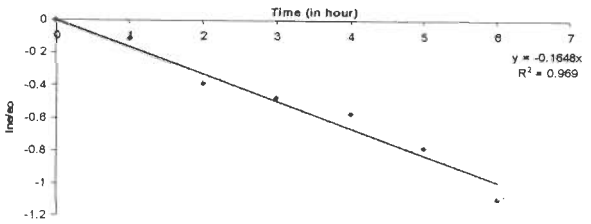
* 0 hour activity corresponds to 100 % activity

4.1.15 Enzyme stability at pH 5.8 and effect of different stabilizers

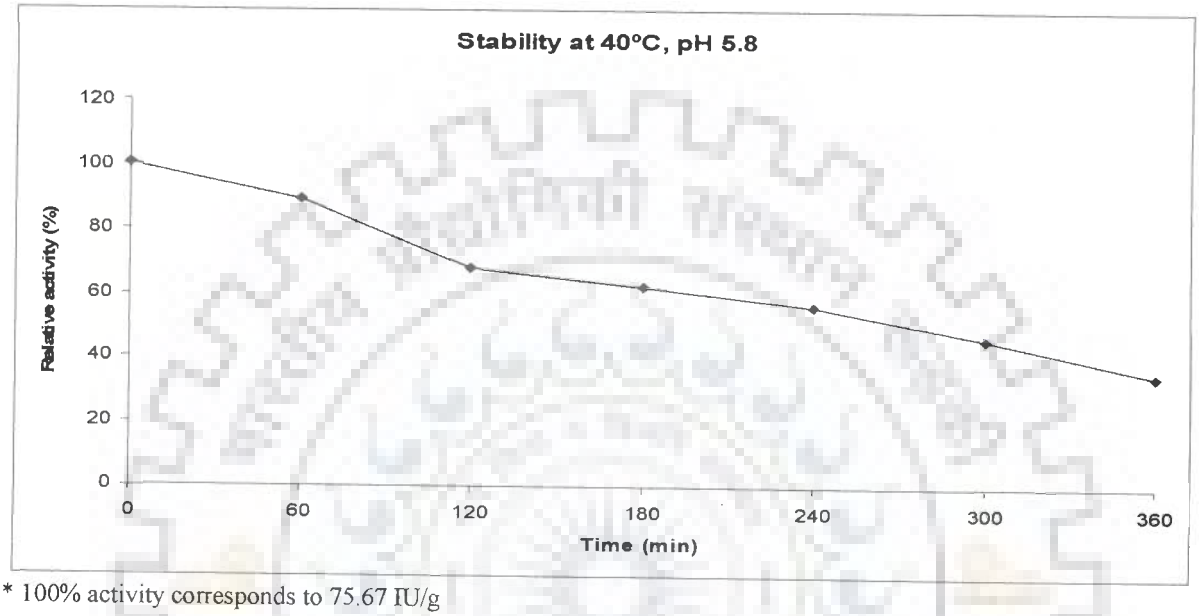
The stability of nitrile metabolizing enzymes is a necessity for successful commercialization. Therefore, half life of the hexanenitrile metabolizing enzyme in whole cells was determined and it was found to be 252 min. at 40°C and pH 5.8 (Fig. 4.12a). The half-life was calculated by $t_{1/2}$=0.693/ K_d, where K_d was calculated by plotting a graph (lne/e0 v/s time, Fig.10.b), where e is the specific activity at time t and e0 is the initial specific activity. Nearly 35% activity was detected after 360 min. In this study, pH 5.8 was chosen as the optimum pH for hexanenitrile metabolizing enzyme is 5.8.

An attempt was made to study the effects of various reported stabilizers on hexanenitrile metabolizing enzyme activity in whole cells. Various stabilizers were used under similar experimental condition. Enzyme stability was decreased in 30% glycerol, 3% tween 20 and 1mM EDTA whereas it was slightly increased with 0.5% sucrose. The maximum stability of enzyme was found in 100 mM NaCl, 50% activity was retained after 420 min. in 100 mM NaCl salt concentration (Fig 4.13). This increase in stability might be due to protein stabilization by controlling the water activity around the enzyme (Chaniotakis 2004). Sugars and polyols stabilize the hydrophobic interactions between non-polar amino acids which minimize the thermo-deactivation (Iyer and Ananthanarayan 2008) but no such increase in stability of enzyme from *Amycolatopsis* sp.IITR215 was detected.

(b)

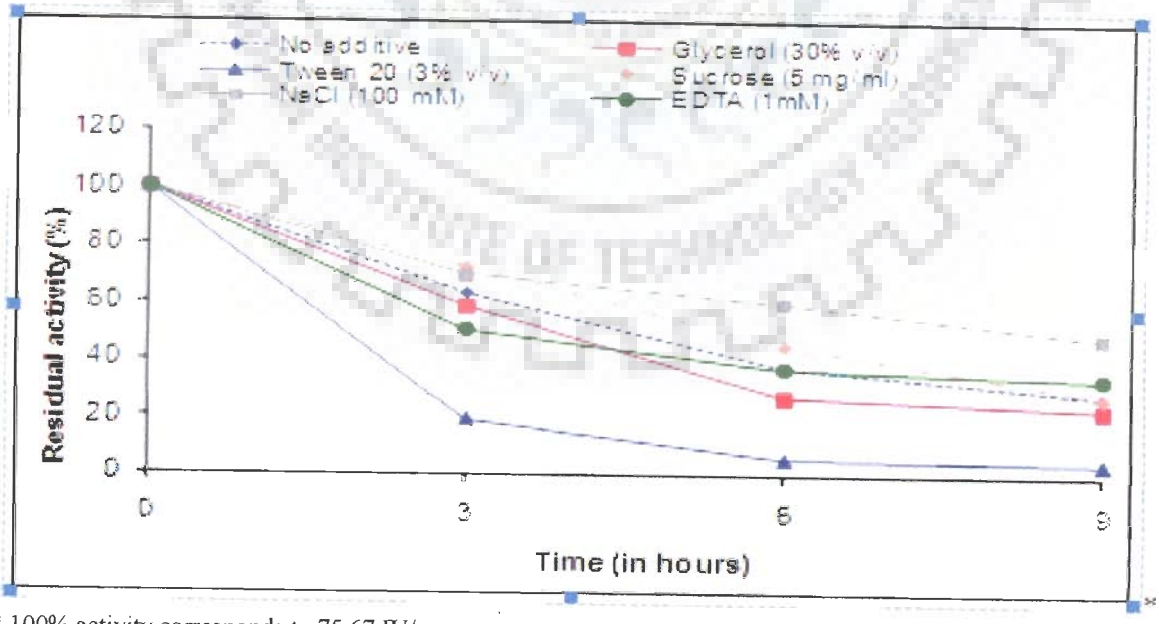


(a)



* 100% activity corresponds to 75.67 IU/g

Fig.4.12. (a) Stability of hexanenitrile metabolizing enzyme (b) ln e/e0 v/s time (t) plot for the calculation of half life.



* 100% activity corresponds to 75.67 IU/g

Fig.4.13. Effect of stabilizers on hexanenitrile metabolizing stability.

4.1.16 Effect of sodium chloride concentration on stability on hexanenitrile metabolizing enzyme

In the previous section, maximum stability was found with 100 mM NaCl and so an effort was made to study the effect of NaCl concentrations on hexanenitrile metabolizing enzyme stability. In this study, stability increased till 100mM concentration but thereafter it decreased gradually (Table 4.10). Nearly 47.8% activity remained after 540 min with 100 mM NaCl concentration at 40°C, pH 5.8 but in 200 mM NaCl concentration, 35.31% residual activity was detected in same time period. So, it can be concluded that 100 mM NaCl concentration was effective for the stabilization of hexanenitrile metabolizing enzyme.

Table 4.10: Effect of different concentration of NaCl on hexanenitrile metabolizing enzyme (at 55°C, pH 5.8)

NaCl (conc.)	Time (hour)	Residual activity (%)
None	0	100
	3	62.98
	6	43.28
	9	37.79
25mM	0	100
	3	43.79
	6	31.6
	9	27.52
50mM	0	100
	3	71.27
	6	53.9
	9	40.28
100mM	0	100
	3	69.12
	6	59.64
	9	47.8
200mM	0	100
	3	58.44
	6	45.85
	9	35.31

* 100% activity correspond to 58.78 IU/g

4.1.17 Organic solvent compatibility

Some nitriles are insoluble in water. They are soluble in the presence of some organic solvents. Therefore, the organic solvent compatibility of enzymes for the hydrolysis of hexanenitrile was determined. The enzyme for hexanenitrile hydrolysis from whole cells of *Amycolatopsis sp.*IITR215 was active in 50 % (v/v) alcohols (Fig. 4.14). Relative activities of 29 %, 50 % and 14 % were detected in 50 % (v/v) of ethyl alcohol, methyl alcohol and isopropyl alcohol respectively. This is the highest organic solvent tolerance of nitrile metabolizing enzyme has been reported so far. No activity was found in 50 % alcohols with purified nitrilase of *Fusarium solani* O1 (Vejvoda et al., 2008). Nearly similar results were reported with the purified nitrilase of *Pseudomonas putida* (Banerjee *et al.*, 2006) in aliphatic alcohols. In case of nitrile metabolizing enzyme of *Amycoatopsis sp.*IITR215, no activity was detected with 80 % organic solvent (v/v).

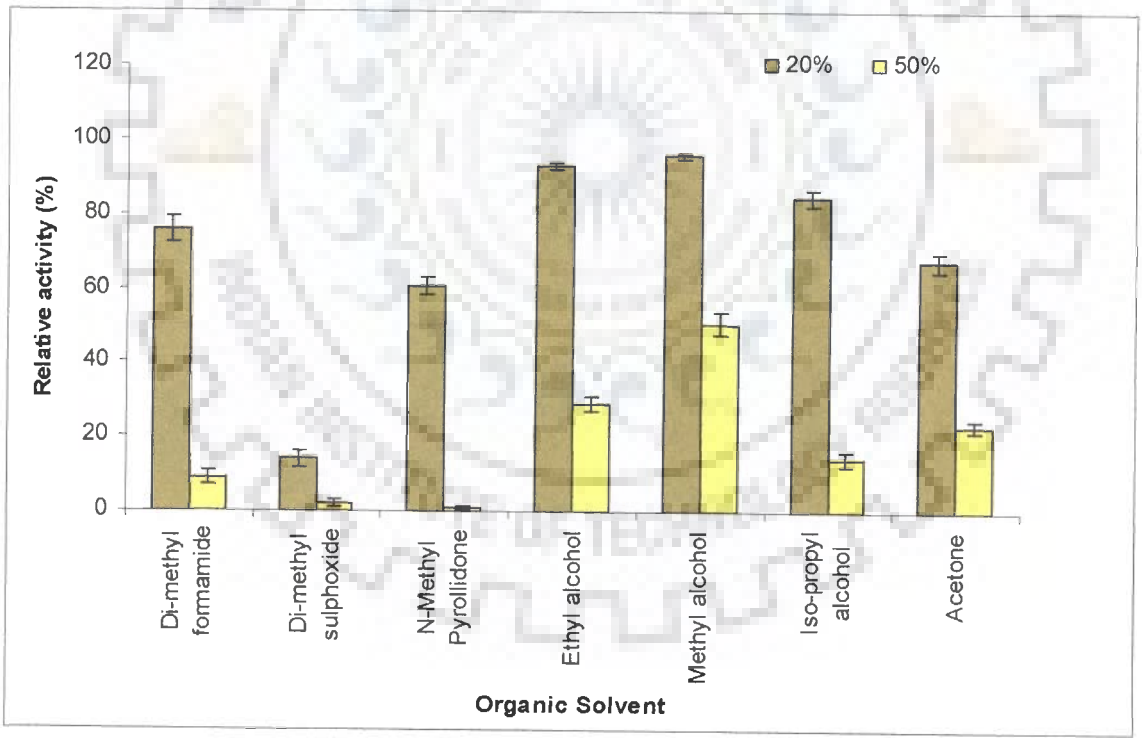


Fig.4.14. Effect of organic solvents on hexanenitrile metabolizing enzyme.

4.1.18 Effect of other additives and protease inhibitors on hexanenitrile metabolizing enzyme

Natural environment of enzymes inside a cell is very different from cell free environment. Inside the cell, enzymes are protected by many stabilizers (Scopes 2005). Low oxygen tension and reducing compounds are responsible for enhanced stability of intracellular enzymes inside the cell. On disruption of cell, many proteolytic enzymes are also released along with the desired enzyme which affects enzyme activity and stability. The active site of some enzymes contains abnormal reactive amino acid residues which are responsible for enzyme catalytic ability (Scopes 2005). These residues are more prone to modification in oxygenated environment outside the cell. Cysteine, a sulfhydryl residue which is common among nitrile metabolizing enzymes such as nitrilase is more prone to oxidation. Therefore, some of the sulfahydryl protective agents can be used to prevent the oxidation of cysteine residues. It is also well known that the very dilute enzyme solution loses enzyme activity very quickly. This loss can be prevented by using another protein like bovine serum albumin (BSA) or casein. This may be due to the fact that dilute proteins are more prone to dissociation into subunits. Therefore, presence of other proteins such as BSA and casein can act as stabilizer by preventing dissociation of enzyme into subunits (Iyer and Ananthanarayan 2008).

Keeping in mind of these problems, some of the commercially available cocktail of protease inhibitors, sulfahydryl protective agents and other protective agents were checked to study the effects of these compounds on enzyme activity in cell free extract.

Hexanenitrile metabolizing enzyme activity was found to be inhibited by these protease inhibitors. Reducing agent DTT dissolved in 0.5 mM PMSF found to have any effect on enzyme relative activity (Table 4.11) suggesting that the cysteine residues are maintained in a reduced state at the active site.

Among, reducing agents, maximum relative activity was detected with sodium thiocyanate at 2 mM concentration. Activity was completely inhibited by glutathione at 10 mM concentration.

Different concentrations of BSA and casein were taken to study the effect on hexanenitrile metabolizing enzyme. Enzyme activity was higher at 2 mg/ml of BSA and casein but with further increase in BSA and casein concentrations, enzyme activity decreased (Table 4.11). Significant increase in half life of catalase of *Bacillus* sp. was detected with casein and BSA (Costa *et al.*, 2002). From these results, it can be concluded that BSA and casein at concentration higher than 2 mg/ml may not have any beneficial effect on the hexanenitrile metabolizing enzyme in cell free extract.

Thus from this study it can be concluded that sodium thiocyanate, BSA and casein at concentration of 2 mg/ml can be tested for its ability to improve storage stability of hexanenitrile metabolizing enzyme.

Table 4.11: Effect of other additives and protease inhibitors on hexanenitrile metabolizing enzyme (with cell free extract at 45°C, pH 7.0)

Additive	Concentration	Relative activity (%)
PMSF	1mM	23
Protease inhibitor Cocktail	10µl	42
DTT in 0.5mM PMSF	1mM	105
β-Mercaptoethanol in 0.5mM PMSF	1mM	87
Butyric acid in 0.5mM PMSF	1mM	98
Cystine in 0.5 mM PMSF	1mM	54
PMSF in DMSO	0.5mM	0
Sodium thiocyanate	2mM	127
Sodium thioglycolate	10mM	53
EDTA	5mM	75
B-Mercaptoethanol	10mM	47
Sodium acetate	100mM	82
Glutathione	10mM	0
L-cysteine	100mM	28
BSA	2mg/ml	131
	10mg/ml	99
	20mg/ml	74
	50mg/ml	50
Casein hydrolaysate	2mg/ml	129
	10mg/ml	38
	20mg/ml	13

In this section, isolation, characterization of isolate, characterization of nitrile metabolizing enzyme were reported. From these studies, it was concluded that *Amycolatopsis* sp.IITR215 harbors multiple nitrile metabolizing enzymes with higher specificity for aliphatic nitriles and amides. Enzymes grown in MB media containing 10 mM adiponitrile as sole source of nitrogen was able to produce nitrile metabolizing enzymes with specificity for cyano group of PAN and PMA. It was also observed that there are specific enzymes involved for hydrolysis of acrylonitrile and probably different from enzyme involved in hexanenitrile hydrolysis. The optimum temperature and pH of enzymes involved in hydrolysis of acrylonitrile and hexanenitrile were determined and it was found to different in case of acrylonitrile and hexanenitrile hydrolysis. The operational stability of enzymes was determined with whole cell and it was found that enzyme responsible for hexanenitrile hydrolysis was more stable at 100 mM NaCl concentration. Thus it was concluded that 100mM NaCl has some stabilizing effect on hexanenitrile metabolizing enzyme. As from these studies it was difficult to pinpoint the nature of all enzymes, it was planned to partially purify enzymes with the objective to ascertain the nature of enzymes.

Section 4.2

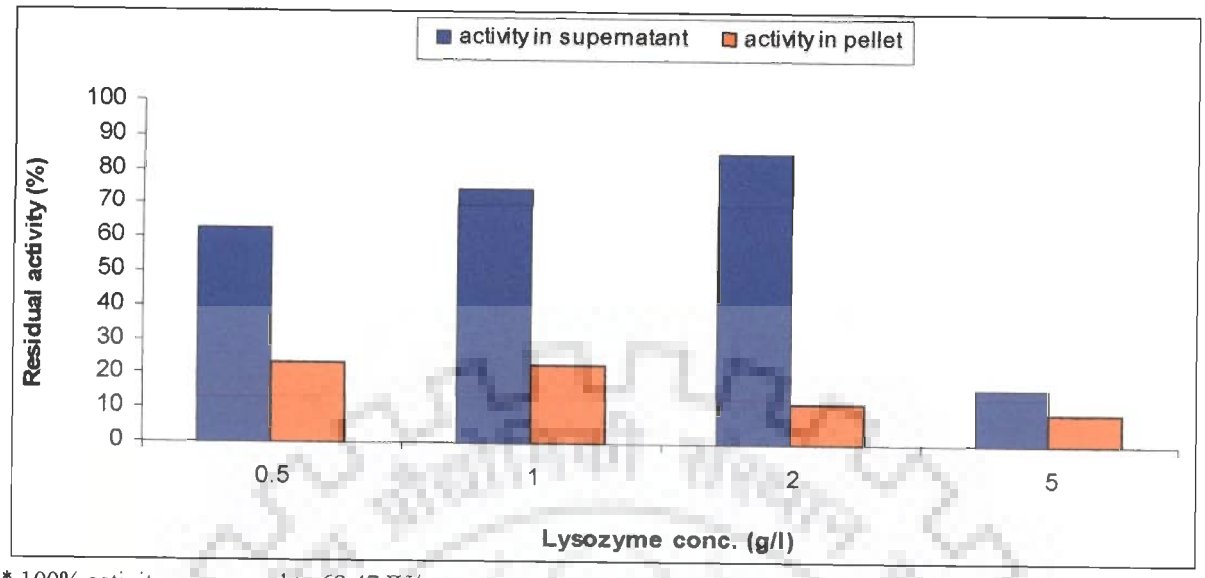
4.2.1 Methods of cell lysis for recovery of nitrile metabolizing enzyme

Nitrile polymers are insoluble in water and nitrile metabolizing enzymes are intracellular in nature which restricts their application in the surface modification of synthetic fibers with whole cells. Therefore, lysis of cells is an important step for the recovery of nitrile metabolizing enzyme for surface catalysis of nitrile polymers. Besides this, lysis is also an important step of protein purification for intracellular enzymes. Henceforth, cheap and efficient method of cell lysis is required for application of these enzymes.

Lysozyme has been widely used for the lysis of Gram positive bacteria (Mahalanabis *et al.*, 2009). In the present study, efficiency of recovery of nitrile metabolizing enzymes was determined by comparing hexanenitrile hydrolyzing activity of whole cell and supernatant. In the enzymatic lysis, activity of enzyme increased as the lysozyme concentration increased upto 2 g/l. Nearly 85% and 12% activities were recovered in supernatant and pellet respectively at 2 g/l lysozyme concentration (Fig. 4.15a) whereas in sonication, nearly 30% activity was recovered in the supernatant till 6 minutes of sonication. Further increase in sonication time to 8 minutes resulted in decrease in enzyme activity (Fig. 4.15b). Same results were found with the disruption of cells by vortexing with glass beads. Nearly 30% activity was recovered in the supernatant (Fig. 4.15c).

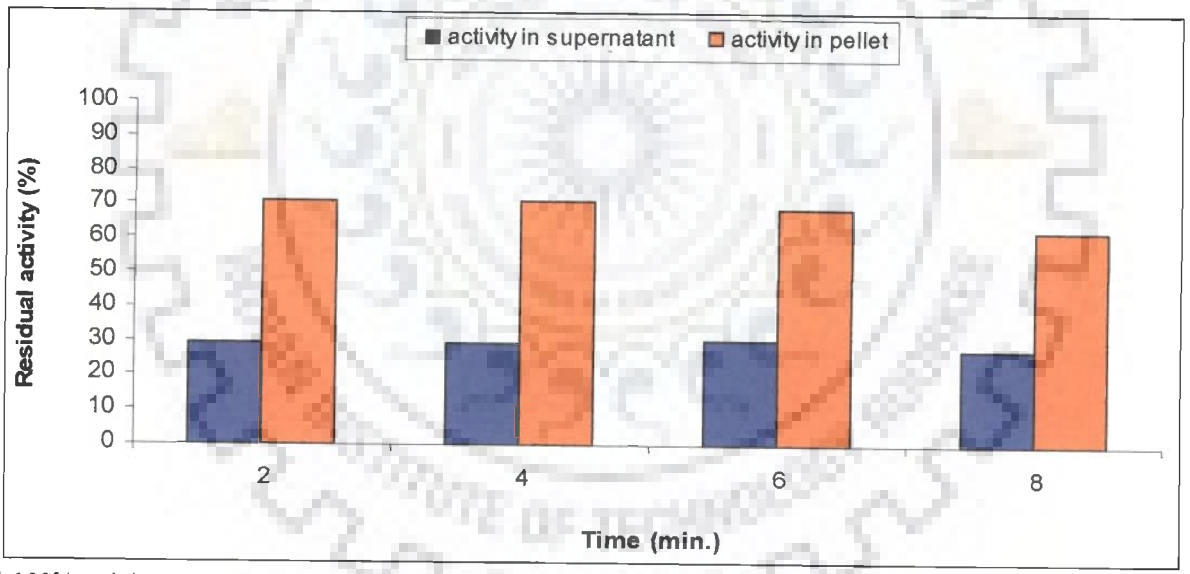
In the earlier report of cell lysis for the recovery of nitrile metabolizing enzyme, high pressure homogenizer and sonication were found to be efficient methods for the recovery of nitrilase from *Alcaligenes faecalis* MTCC 126 (Singh *et al.*, 2005) but due to the presence of complex cell wall, sonication methods restricts the cell lysis of *Amycolatopsis* sp.IITR215. Enzymatic cell lysis by lysozyme proved to be an effective method of cell lysis and recovery of intracellular enzymes.

(a)



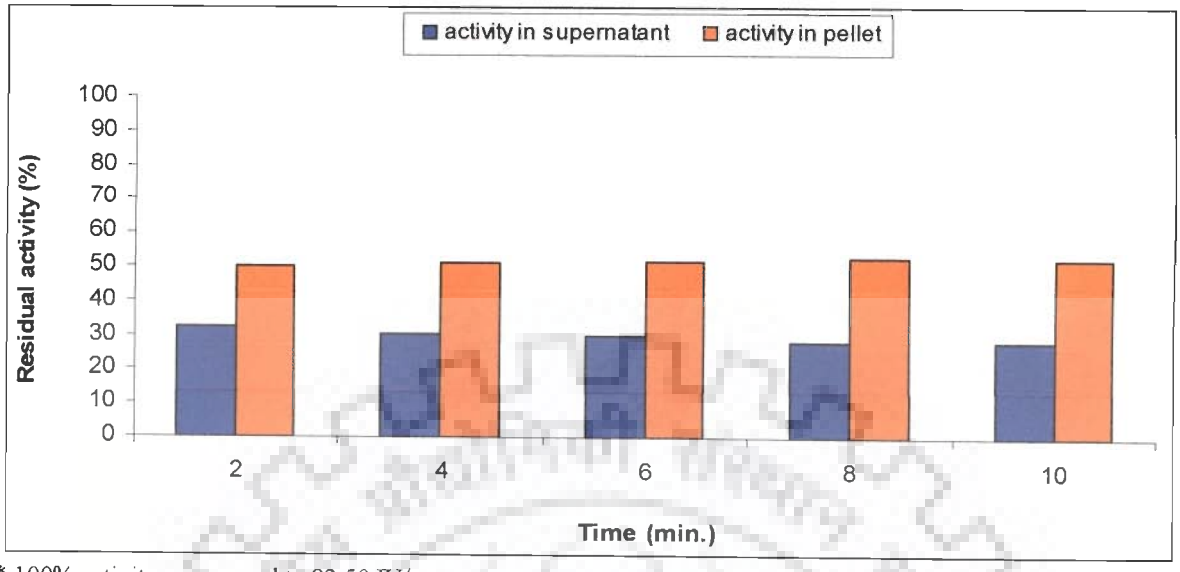
* 100% activity correspond to 69.47 IU/g

(b)



* 100% activity correspond to 82.80 IU/g

(c)



* 100% activity correspond to 82.50 IU/g

Fig.4.15. Methods of cell lysis for the recovery of nitrile metabolizing enzyme (a) Effect of lysozyme (b) Effect of sonication (c) Lysis by vortexing (enzyme assay with hexanenitrile at 55°C, pH 5.8).

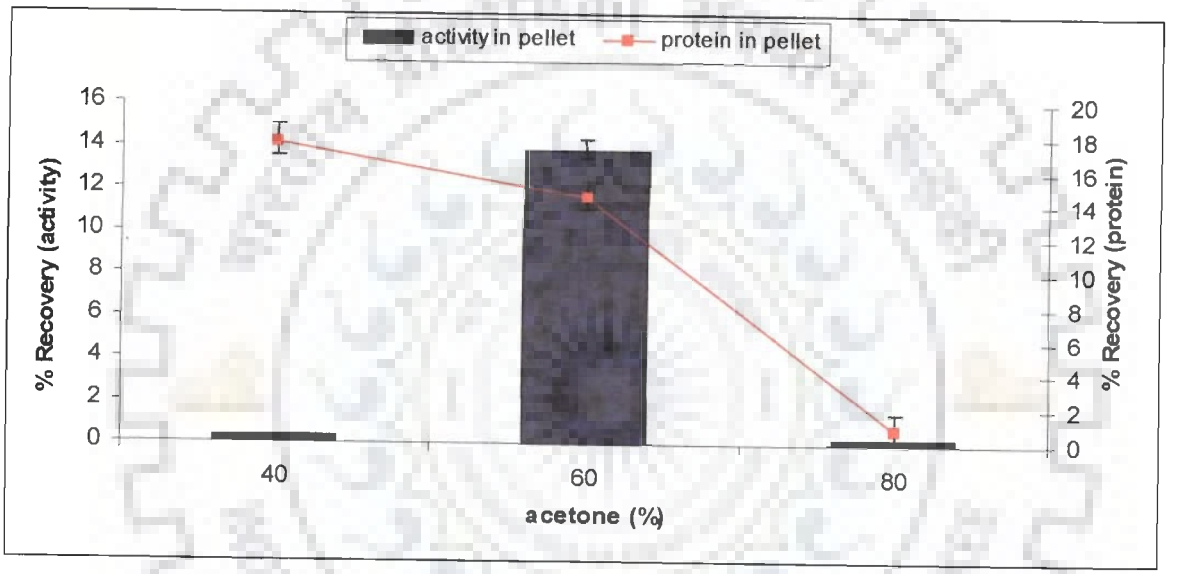
4.2.2 Protein precipitation

After lysis of cells, it was necessary to concentrate protein for future use in enzymatic treatment of polymer and partial purification of enzymes. In this study, existing protein precipitation methods by using ammonium sulphate, acetone and ethanol were used (Curling 1980). Ammonium sulphate precipitation was interfering in ammonia estimation which is the end product of nitrile metabolizing enzyme catalyzed reaction. Almatawah *et al.*, (1999) precipitated nitrilase with 50% (v/v) acetone with 55% and 20% recovery of proteins and activity respectively (Almatawah *et al.*, 1999). In our study, 13.8% and 5% activities were recovered with 60% (v/v) of acetone and 90% (v/v) of ethanol precipitation respectively (Fig.4.16 a & b). Thus, this proved that organic solvent at higher concentration was not effective as precipitating agent of nitrile metabolizing enzymes of *Amycolatopsis* sp.IITR215. Therefore, there was a need to develop other method of protein precipitation. Sodium sulphate proved to be the most effective method for the protein precipitation from cell free extract of *Amycolatopsis* sp.IITR215.

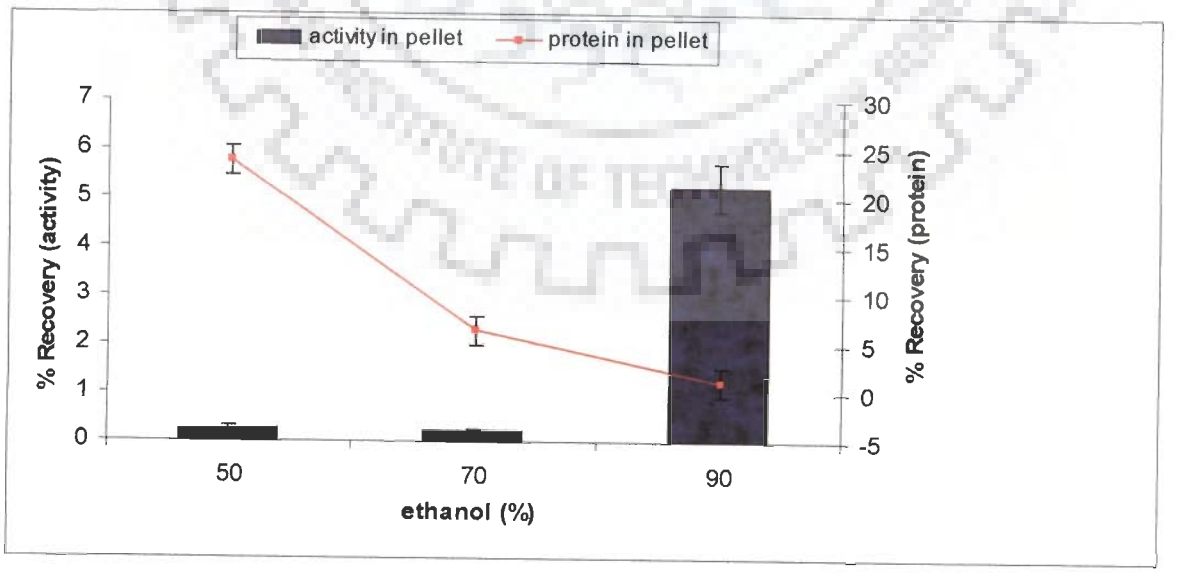
Different concentrations of sodium sulphate were used to precipitate proteins from cell free extract. Precipitation of proteins increased linearly with increase in sodium sulphate from 10 to 30 % and thereafter there was a marginal increase in protein precipitation with

increase in sodium sulphate concentration. Similarly, Nitrile hydrolyzing enzyme activity was also increased with increase in sodium sulphate concentration from 10 to 30% and further increase in concentration of sodium sulphate didn't improve precipitation of enzyme. Nearly 7% and 5% activity was remained in supernatant whereas 86 and 90 % activities were recovered in precipitated proteins at 30% and 40% sodium sulphate respectively (Fig.4.16c). Therefore, to minimize the effect of salt in subsequent purification process and for optimal enzyme activity recovery, 30% of sodium sulphate was used to precipitate proteins which were further used for anion exchange chromatography.

(a)



(b)



(c)

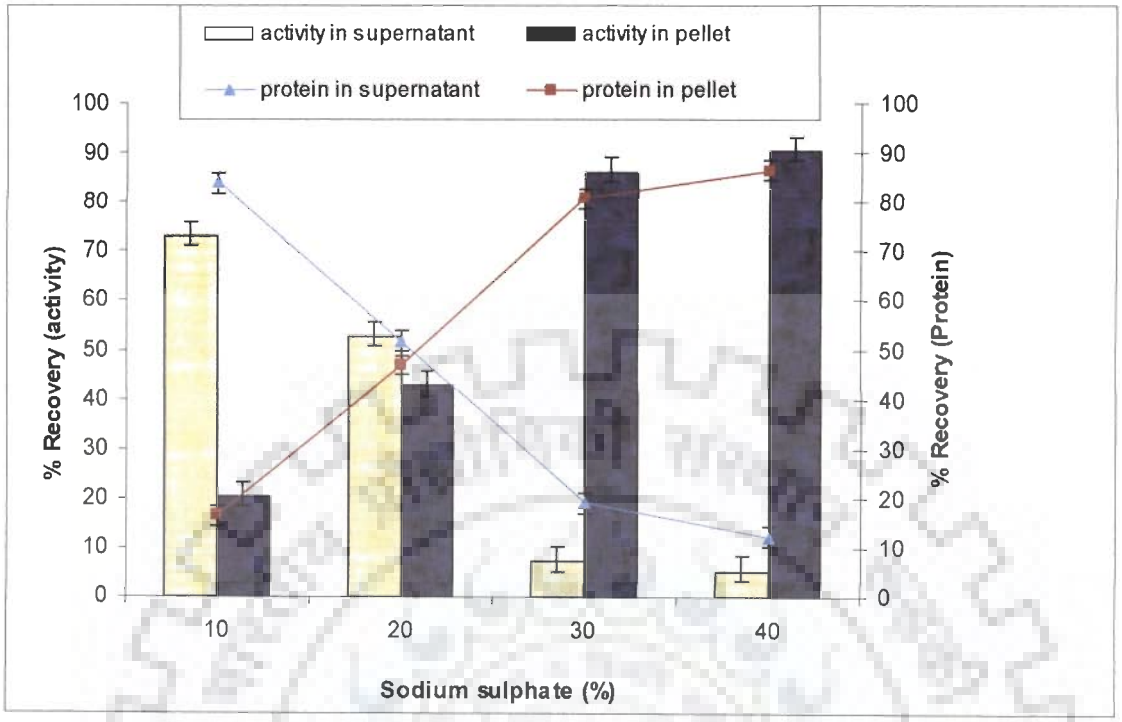


Fig.4.16. Protein precipitation methods with (a) acetone (b) ethanol (c) sodium sulphate (enzyme assay with hexanenitrile at 55°C, pH 5.8).

4.2.3 Anion-exchange chromatography

In the existing literature, Q-sepharose has been widely used as an strong anion exchanger for the purification of amidases of *Xanthobacter agilis* (Briggs *et al.*, 1996), *Variovorax paradoxus* (Krieg *et al.*, 2002), *Providencia rettgeri* (Sevo *et al.*, 2002) and nitrile hydratases of *Agrbacterium tumefaciences d3* (Bauer *et al.*, 1998), *Rhodococcus equi A4* (Pr˘epechalová *et al.*, 2001) in the ion-exchange chromatography. Protein precipitated by sodium sulphate dissolved in 20 mM phosphate buffer (pH 7.0) and loaded in a column containing Q-sepharose. Collected fractions from ion-exchange chromatography were subjected to protein O.D at 280 nm, total eight peaks were obtained. Elution of proteins was higher in fraction no. 36-47 at 300 mM NaCl concentration and most of the protein was eluted out till 475 mM NaCl concentration (Fig.4.17). Fractions corresponding to each peak were clubbed and used for activity determination. In S. No. 1 and 8, neither amidase activity nor nitrile hydratase activity was detected whereas amidase activity was detected in S. No. 2, 3 and 4 and nitrile hydratase was present in S. No. 5, 6 and 7 (Table 14.12). No nitrilase activity was detected in any of the clubbed fractions. Overall 12.2 fold purification for

amidase and 17.5 fold purification for nitrile hydratase were obtained (Table 14.13 & 14.14). Three amidase and three nitrile hydratase fractions were further used for native-PAGE analysis. Substrate specificity of these fractions was also carried out to characterize these fractions.

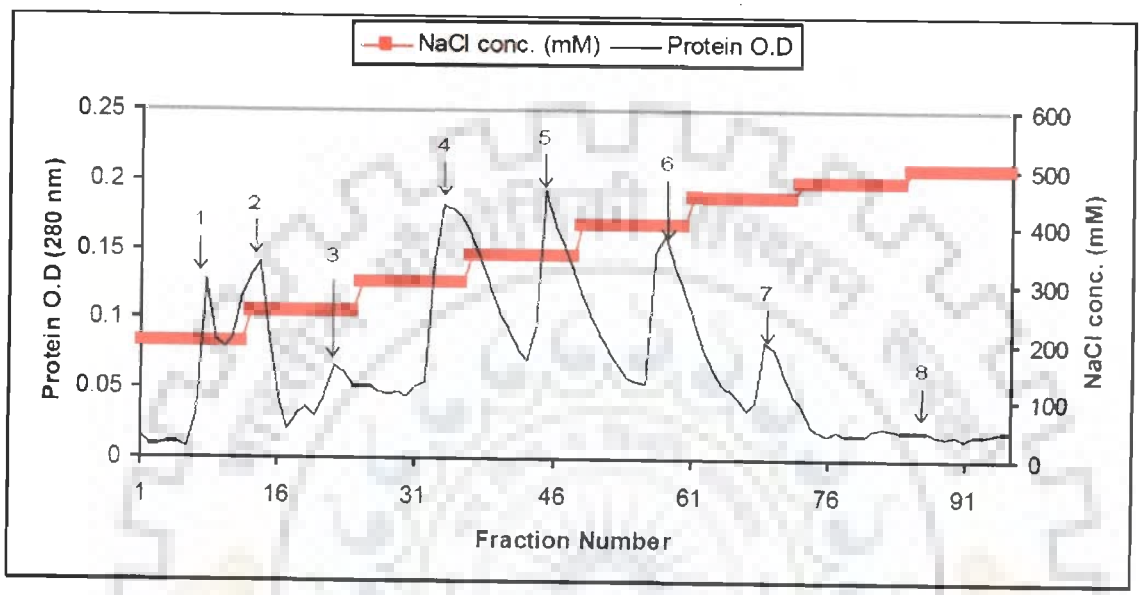


Fig.4.17. Fractions of ion-exchange chromatography.

Table 4.12: Clubbed fractions of ion-exchange chromatography

Peak (S.No.)	NaCl concentration (mM)	Fraction No.	Type of activity
1	200	10 - 16	N.D
2	200 – 250	17 – 22	Amidase
3	250 – 300	24 – 31	Amidase
4	300 – 350	36 – 47	Amidase
5	350 – 400	49 – 58	Nitrile hydratase
6	400 – 450	62 – 68	Nitrile hydratase
7	450 – 475	72 – 79	Nitrile hydratase
8	475	86 – 90	N.D

Table 4.13: Purification of amidase

Step	Specific activity with hexanamide (IU)/g	Purification (-fold)
Cell free extract	788.19	1
Sodium sulphate	834.76	1.06
Amidase 1	1690.51	
Amidase 2	378.47	12.2
Amidase 3	7553.10	

Table 4.14: Purification of nitrile hydratase

Step	Specific activity (IU)/g	Purification (-fold)
Cell free extract	1772.83	1
Sodium sulphate	1498.31	0.84
Nitrile hydratase 1	1778.28	
Nitrile hydratase 2	16631.49	17.5
Nitrile hydratase 3	12600.96	

4.2.4 Native-PAGE

All clubbed fractions with activity, concentrated using amicon®ultra centrifugal filters (10kDa cutt-off) and concentrated proteins used for further study. Concentrated fractions were loaded into native-gel and after destaining, the gel was photographed (Fig.4.18). From figure 4.18 it was concluded that active fractions for amidases were exhibiting nearly same band pattern while active fractions for nitrile hydratases showed somewhat different band pattern. On the basis of these results, it was difficult to differentiate them with respect to molecular weight. From the band pattern of nitrile hydratase active fractions it can be concluded that nitrile hydratase 3 has minimum impurity and position of major protein bands are distinctly different from other two nitrile hydratases. As from native-gel elctrophoresis it was difficult to differentiate amidases and nitrile hydratases so it was planned to study substrate specificity of these enzymes.

Fig.4.18. Native-PAGE of various fractions. *Lane.1.* crude, *Lane 2*- 30% sodium sulphate precipitation, *Lane 3*- nitrile hydratase 1, *Lane 4*- nitrile hydratase 2, *Lane 5*- nitrile hydratase 3, *Lane 6*- amidase 1, *Lane 7*-amidase 2, *Lane 8*-amidase3, *Lane 9*- protein marker (from left to right).

4.2.5 Substrate specificity of amidases

It was observed that all these amidases had maximum activity with isobutyramide followed by propionamide and hexanamide. Similar relative activity was also observed with whole cell enzyme and cell free extract. Relative activity patterns of all amidases were nearly similar. It was concluded that amidase system of *Amycolatopsis* sp.IITR215 was highly active for aliphatic amides such as isobutyramide, propionamide and hexanamide (Table.4.15) which is comparable to amidases from *Bacillus* sp. BR449 (Kim and Oriel 2000), *Helicobacter pylori* (Skouloubris *et al.*, 1997), *Klebsiella pneumoniae* NCTR 1 (Nawaz *et al.*, 1996), *Rhodococcus* sp. strain NCTR 4 (Nawaz *et al.*, 1994), *Sulfolobus solfataricus* (d'Abusco *et al.*, 2001). From this result it can also be concluded that all the amidases might be of same nature or identically similar. Further, a zymogram was carried out to differentiate these amidases based on the band position in native-PAGE. .

Table 4.15: Substrate Specificity of amidases (at 45°C, pH 7.0)

Substrate	Relative activity of Amidase 1 (%)	Relative activity of Amidase 2 (%)	Relative activity of Amidase 3 (%)
Isobutyramide	100	100	100
Benzamide	8.50	8.62	17.73
Adipamide	4.85	1.62	8.88
Propionamide	45.61	46.50	61.65
Acetamide	13.14	7.18	15.37
Acrylamide	10.68	12.75	13.17
Hexanamide	43.35	39.85	60.43

* 100% activity corresponds to 1996.35 IU/g for amidase 1
* 100% activity corresponds to 967.01 IU/g for amidase 2
* 100% activity corresponds to 14709.97 IU/g for amidase 3

4.2.6 Zymogram

Zymogram is an efficient technique to detect hydrolytic enzymes following electrophoresis in gel matrices (Lantz and Ciborowski 1994). Amidase is a hydrolytic enzyme and was detected by zymogram analysis in partially purified fractions. Bands of amidases in all three fractions were found at the same position in the zymogram and compared with Comassie stained gel. Although, molecular weight of any protein band was

difficult to predict from native-PAGE but here an approximate effort was made to identify the position of amidase band from molecular weight of protein marker. Approximate molecular weight for amidase seems to be nearly 33 kDa (Fig 4.19). From the zymogram it can be concluded that the amidases which were eluted at different NaCl concentration are probably identical with respect to molecular weight and substrate specificity. However, further confirmation needs to be carried out by purifying these fractions and determining their sequences.

4.2.7 Substrate specificity of nitrile hydratases

In the native-PAGE (Fig. 4.18) of all the eluted fractions, band pattern for nitrile hydratase fractions was somewhat different from each other. To study different nitrile hydratase fractions, activities were determined with various nitriles and quantifying the amide formation using HPLC. It was found that the activity for nitrile hydratase 1 was maximum with acetonitrile and this nitrile hydratase was highly active for butyronitrile, valeronitrile, propionitrile and adiponitrile while nitrile hydratase 2 was showing totally different profile from nitrile hydratase 1. Maximum activity was found with butyronitrile and it was highly active for hexanenitrile, acrylonitrile, propionitrile and methacrylonitrile. Moreover, maximum activity for nitrile hydratase 3 was found with hexanenitrile and it was highly active for long chain nitrile such as propionitrile, valeronitrile, butyronitrile and dinitrile (adiponitrile) (Table 6). Therefore, it can be concluded that nitrile hydratases from *Amycolatopsis* sp.HTR215 have specificity towards aliphatic nitriles which is comparable to many earlier reported constitutive nitrile hydratase from *Brevibacterium* sp. R312 (Nagasawa *et al.*, 1986) and *Rhodococcus* sp. N771 (Yamada and Kobayashi 1996).

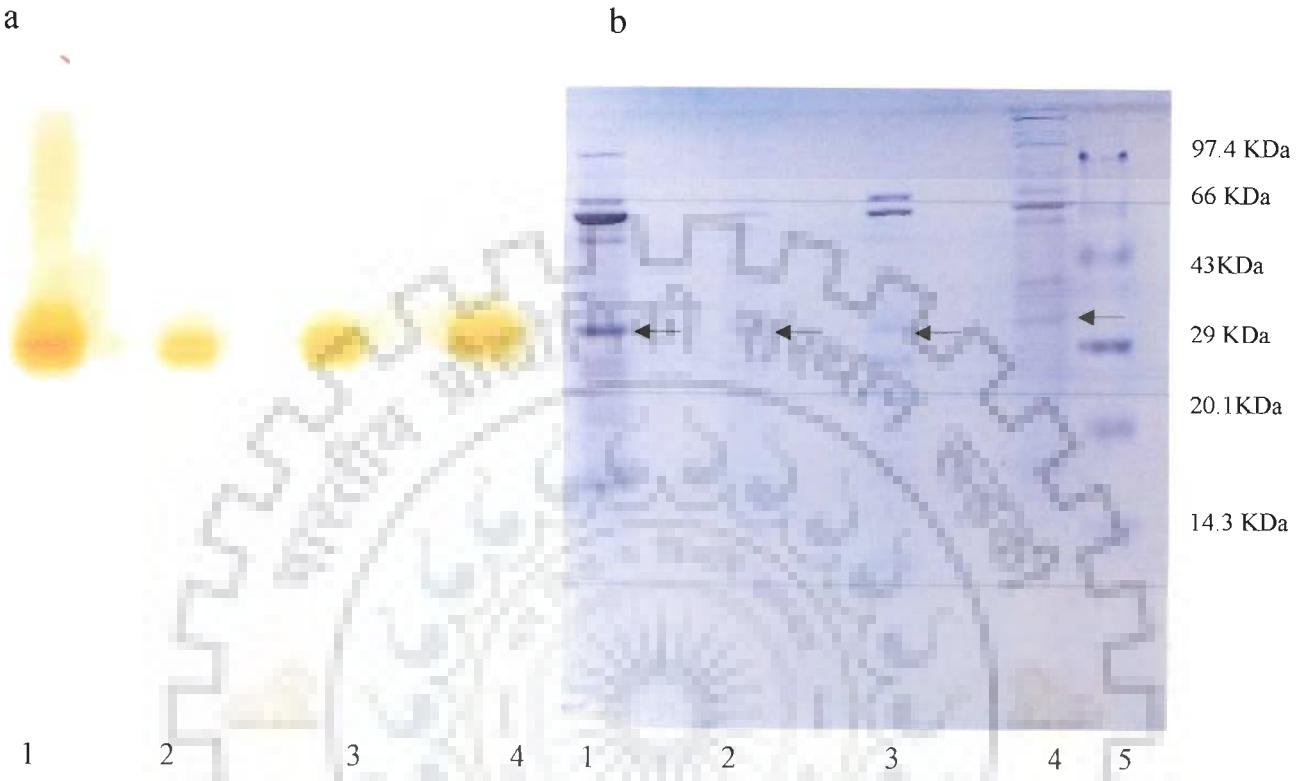


Fig. 4.19. (a) Zymogram of amidase active fractions ; *Lane 1* amidase 3, *Lane 2* amidase 2, *Lane 3* amidase 1, *Lane 4* crude; **(b) native-PAGE;** *Lane 1* amidase 3, *Lane 2* amidase 2, *Lane 3* amidase 1, *Lane 4* crude *Lane 5* protein marker; (arrow indicates the band of amidase).

Table 4.16: Substrate specificities of nitrile hydratase active fractions (at 45°C, pH 7.0)

Substrate	Relative activity for nitrile hydratase 1 (%)	Relative activity for nitrile hydratase 2 (%)	Relative activity for nitrile hydratase 3 (%)
Isobutyronitrile	13.32	34.13	37.79
Benzonitrile	12.44	17.36	32.28
Adiponitrile	40.70	5.98	64.04
Acetonitrile	100	0.59	2.88
Acrylonitrile	13.49	53.01	58.26
Hexanenitrile	45.21	69.46	100
3-Cyanopyridine	15.16	31.13	54.85
Butyronitrile	71.35	100	64.82
Cyclohexanecarbonitrile	29.57	19.76	46.58
Propionitrile	76.03	59.88	64.17
Valeronitrile	65.02	8.38	63.25
Methacrylonitrile	8.63	43.71	21.91

* For acetonitrile- 100% activity corresponds to 2599.21 IU/g for nitrile hydratase 1.
* For butyronitrile - 100 activity corresponds to 3162.879 IU/g for nitrile hydratase 2.
* For hexanenitrile- 100% activity corresponds to 21896.55 IU/g. for nitrile hydratase 3

4.2.8 Native-PAGE for nitrile hydratases

All active fractions of nitrile hydratases showed different substrate specificity pattern. Thus, it was planned to study the native-PAGE analysis for only three nitrile hydratases separately. Band pattern for the active fractions of nitrile hydratase 3 was different from other two nitrile hydratases. Band pattern of nitrile hydratase 2 and 3 was totally different but band pattern of nitrile hydratase 1 is more or less similar to nitrile hydratase 2 except a major band at 21 kDa (Fig 4.20). From these results, it can be concluded that nitrile metabolizing enzyme system of *Amycolatopsis* sp. IITR215 exhibit two nitrile hydratases and differentiation of nitrile hydratase 1 and 2 needs to be further explored after further purification of these two fractions.

In earlier reports, multiple nitrile hydrtases have been reported in *Rhodococcus rhodochrous* J1. This strain produced low-molecular weight NHase (L–NHase) and high-molecular weight NHase (H–NHase). L–NHase which was induced by

cyclohexanecarboxamide and showed higher activity with aromatic and heterocyclic while H–NHase which was induced by urea and exhibited higher specificity for aliphatic nitriles (Wieser *et al.,* 1997; Nagasawa *et al.,* 1991). There are very few reports on multiple nitrile hydratases in any specific organisms.

4.2.9 Inhibitor study with purified amidase and nitrile hydratase

In section 1, nature of enzyme was ascertained by using an amidase inhibitor (DEPA) in nitrile substrates. It was observed that with acrylonitrile complete inhibition was obtained whereas with hexanenitrile no inhibition of enzyme activity was observed. So, to further understand the inhibition pattern of partially purified amidase, an experiment was planned to study the effect of amidase inhibitor (DEPA) on partially purified amidase(all three fractions clubbed) and nitrile hydratases using acrylamide and hexanamide as substrates for amidase and acrylonitrile for nitrile hydratase. It was observed that no activity was detected with acrylamide. However, 70% activity was detected with hexanamide (Fig. 4.21). This indicates that DEPA probably not effective inhibitor in case of hexanamide. In case of nitrile hydratase, with acrylonitrile it was found to have significant inhibitory effect on nitrile hydratase 3 (72% inhibition) and minimum effect on nitrile hydratase 1 (Fig.4.21). Thus, with respect to our previous study (section 4.1.7) it can be concluded that in case of acrylonitrile, inhibition was not only due to amidase but also due to nitrile hydratase 3 and 2.

From all these studies it can be concluded that all amidase fractions had almost similar properties with respect to substrate specificity and native-PAGE band pattern. As far as nitrile hydratases are concerned, substrate specificity and inhibition pattern indicates three distinct nitrile hydratases are present but this cannot be conclusively ascertained from Native-PAGE.

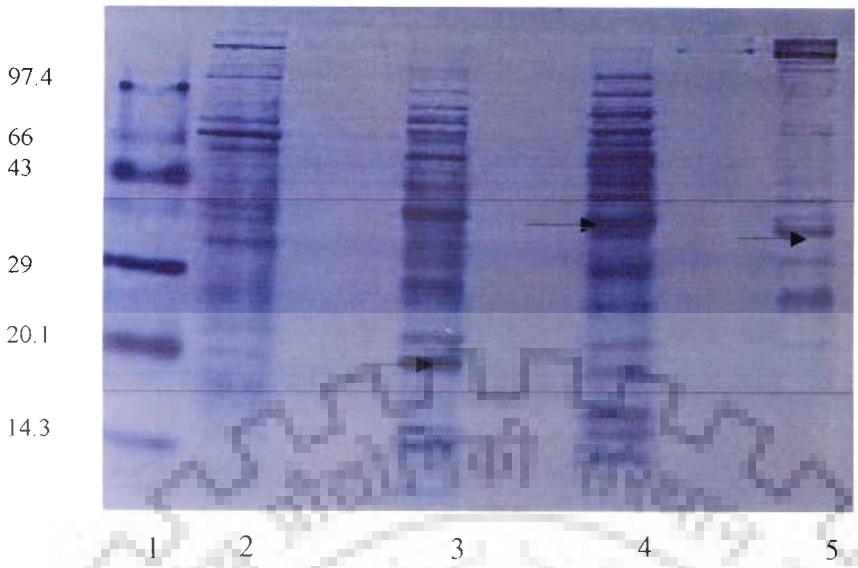


Fig 4.20 Native-PAGE of nitrile hydratase active fractions *Lane 1* protein marker, *Lane 2* crude, *Lane3* nitrile hydratase 1, *Lane 4* nitrile hydratase 2, *Lane 5* nitrile hydratase 3.

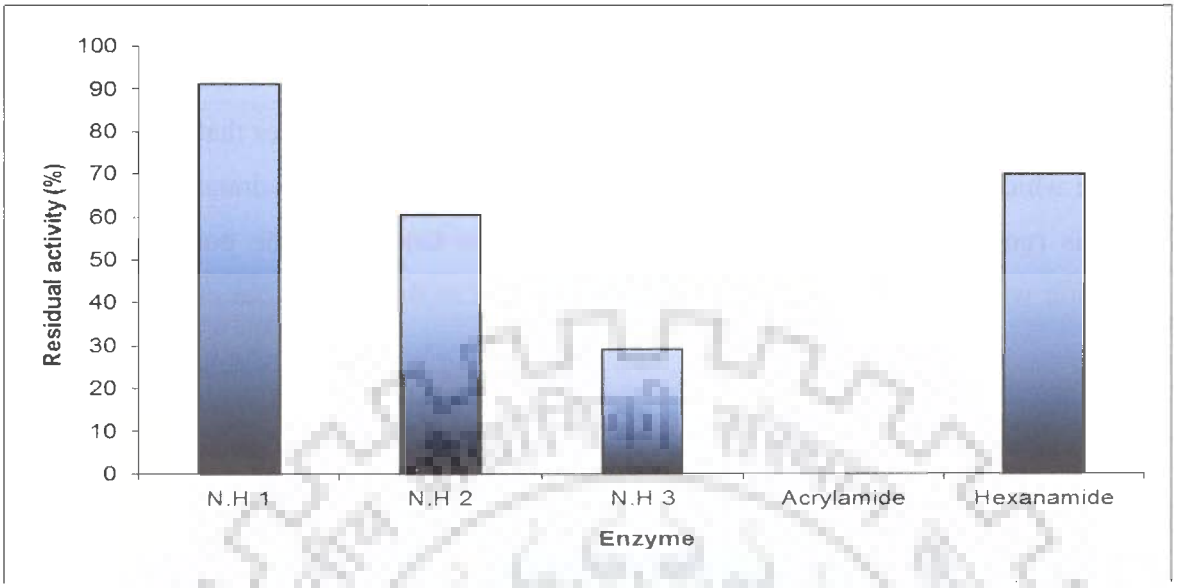


Fig. 4.21. Effect of DEPA(amidase inhibitor) on purified amidase and nitrile hydratases (N.H 1,N.H 2, N.H 3).

4.2.10 Purification by sodium sulphate removal

In previous section (section 4.2.3), precipitated proteins (by sodium sulphate) were dissolved in 20 mM phosphate buffer (pH 7) without any buffer exchange and loaded onto the anion-exchange column. It was observed that three distinct peaks based on amidase activity and protein O.D were obtained after elution with NaCl. However, native page band pattern and substrate specificity data indicates that these three amidases are probably identical in nature. So, a new experiment on ion-exhange chromatography was performed with same column under similar experimental condition except desalted precipitated proteins solution (achieved using amicon® ultra centrifugal filters) were loaded on the column. Again, nearly similar peak pattern was obtained (Fig.4.22) and active fractions were clubbed and used for the activity determination. In the earlier result, amidase activity was detected in the peak no. 2, 3 and 4 but in this experiment amidase activity was obtained only in the peak no. 4 which supports the presence of a single amidase, while nitrile hydratase activity was detected in the peak no. 6 and 7 and no nitrile hydratase activity was detected in peak no.5. From these results, it can be concluded that sodium sulphate was probably interferring with the elution of amidase in the ealier experiments. In this case, two peaks of nitrile hydratases were obtained which also supports the presence of two nitrile hydratases.

Native-PAGE analysis was also performed after the salt removal. Again, different band pattern for nitrile hydratases was obtained (Fig.4.23) which again confirms the presence of two nitrile hydratases. However, careful inspection of lane 4 indicates that protein band at 21 kDa which was the differentiating nitrile hydratase 1 and nitrile hydratase 2 obtained in previous run (section 4.2.8) was also present in this lane. Thus the possibility of low resolution with respect to nitrile hydratases separation in the present run couldn't be ruled out.

Thus from this study, it can be concluded that there are one amidase and two distinct nitrile hydratases in cell free extract of *Amycolatopsis* sp.IITR215. The probability of another distinct nitrile hydratase needs to be further investigated after subsequent purification of all the active fractions of nitrile hydratases obtained in the previous run. However, the major objective of partial purification of enzymes of *Amycolatopsis sp.* IITR215 was to determine the nature of nitrle metabolzing enzymes. Here it can be confirmed that only nitrile hydratase and amidase were present .

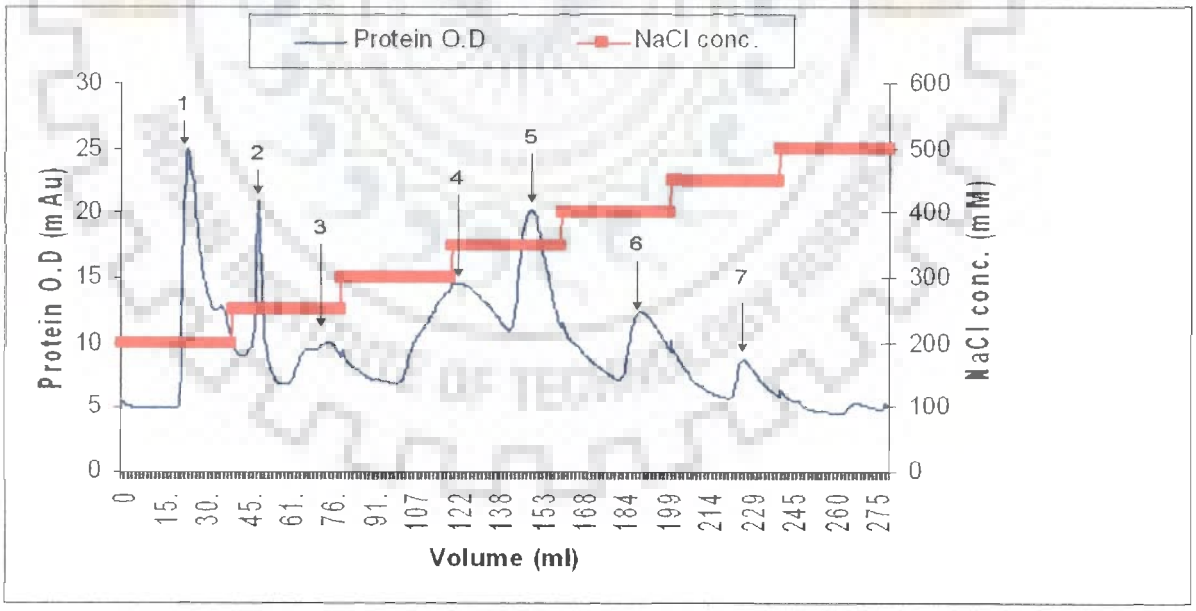


Fig.4.22. Fractions of ion-exchange chromatography with desalted protein solution.

The cell free extract was further explored for enzymatic surface modification of polyacrylonitrile and other co-polymers. As enzyme system consist of nitrile hydratase and amidase, so it is expected to have amide and carboxylic acid on enzymatically modified polymer.

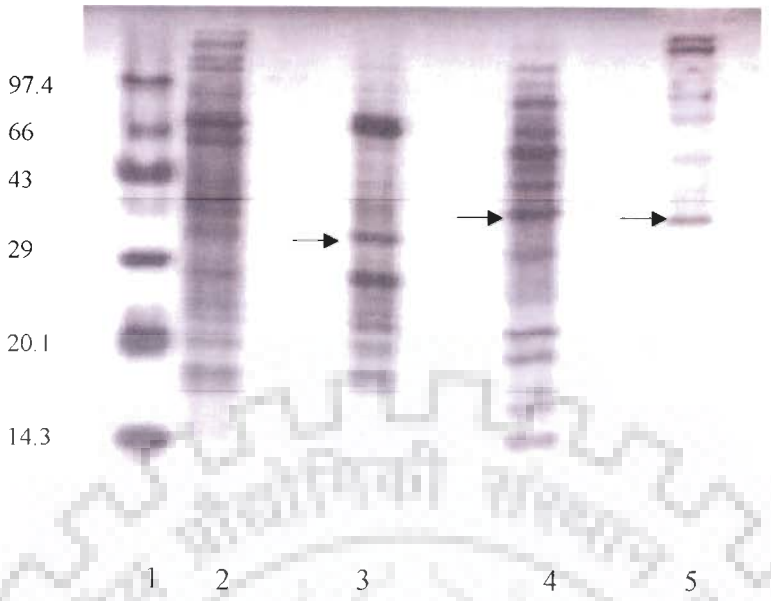


Fig.4.23. Native-PAGE of active fractions obtained from ion-exchange chromatography with desalted protein solution *Lane1*- Protein Marker. *Lane 2*-30% sodium sulphate precipitation 1, *Lane 3*- active fraction of amidase (arrows shows band of amidase), *Lane 4*- acive fraction of nitrile hydratase, *Lane 5*- 2nd acive fraction of nitrile hydratase (from left to right).

Section 4.3

4.3.1 Effect of temperature on ammonia release from PAN

Low temperature of polymer treatment with enzyme favors protein adsorption while nitrile metabolizing enzymes from *Amycolatopsis* sp.IITR215 were optimally active at 45°C (Babu *et al.*, 2010). Therefore, PAN at a concentration of 10 g/l was treated with cell free extract of *Amycolatopsis* sp.IITR215 for 12 hours at 30°C, 37°C and 45°C. Cell free extract of *Amycolatopsis* sp.IITR215 was also incubated at same reaction conditions without polymers which is defined as blank sample. Some amount of ammonia was detected in blank sample but ammonia in enzyme treated PAN was comparatively higher. Total ammonia release due enzyme action on polymer was calculated by subtracting ammonia release in blank sample from ammonia release in enzyme treated polymer. Traces of ammonia were also detected from enzyme treated PAN sample at 45°C but significant amount of ammonia was detected at 37°C. Nearly 428 µM and 10 µM ammonia were released at 37°C and 45°C after 12 hours of polymer treatment respectively. However, no ammonia was detected at 30°C (Fig.4.24). Therefore, 37°C temperature as chosen as the best temperature for the catalysis of –CN groups present on the surface of polyacrylonitrile.

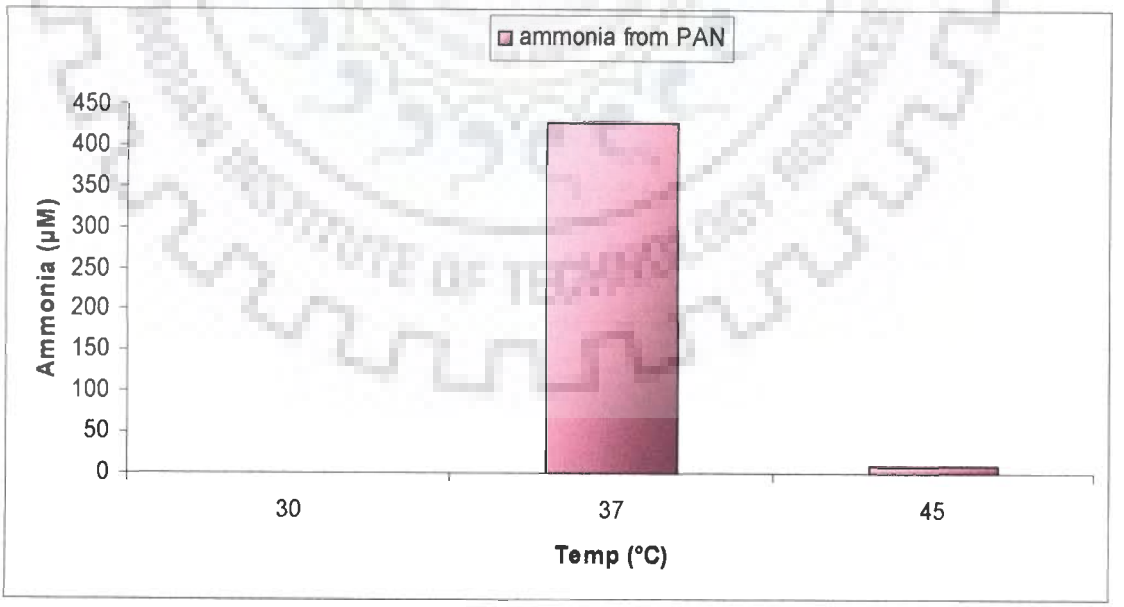


Fig.4.24. Effect of temperature on ammonia release from PAN (pH 7.0).

From these results, it can be concluded that 37°C is the optimum temperature for the conversion of –CN to –COOH from cell free extract and longer incubation time may be advantageous for this biotransformation. During enzymatic treatment of polymer, long incubation time is necessary thus stability of enzyme also plays an important role along with adsorption of enzyme at low temperature. In all further studies, enzymatic treatment of polymer was carried out at 37°C.

4.3.2 Enzymatic treatment of polyacrylonitrile

In the previous experiment, PAN hydrolysis was found to be maximum at 37°C. Therefore, enzymatic treatment of polyacrylonitrile performed at this temperature and pH of 7.0 with solution of precipitated proteins obtained from cell free extract and the progress of reaction monitored for longer period of time with respect to ammonia release along with determination of unbound protein in the reaction mixture. It was observed that after 6 hours of enzymatic treatment of PAN, 37% of protein seems to be adsorbed on the polymer and during this period, amount of ammonia release was negligible. After 6 hours of enzymatic treatment, ammonia release increased significantly and monitored till 36 hours (Fig.4.25). Nearly 75% proteins adsorbed on the PAN and 4283 µM ammonia was released after 36 hour of PAN treatment. Enzyme treated sample was subjected to FTIR and XPS analysis. Polymer without any enzyme treatment was taken as control. FTIR spectra showed formation of a new peak at 1547 cm^{-1} and broadening of another peak at 1644 cm^{-1} (Fig. 26). Among these peaks, peak at 1644 cm^{-1} corresponds to the stretching of the carbonyl group of the amide while the peak at 1547 cm^{-1} strongly supports the presence of carboxylate groups. Zhang *et al.,* (2009) hydrolyzed polyacrylonitrile membrane with KOH, NaOH and LiOH, and observed peaks at 1568 cm^{-1} and 1405 cm^{-1} provided strong evidence of occurrence of carboxyl groups obtained primarily via alkaline hydrolysis. They also concluded that carboxyl functionality was in the form of $–COO\text{-}K^+$, $–COO\text{-}Na^+$ and $–COO\text{-}Li^+$ (Zhang et. al 2009). In their study, the presence of carboxylate groups was further confirmed by XPS analysis. Similarly, in our experiments a new peak at 1547 cm^{-1} and ammonia release indicates formation of $–COO^-$ groups on the surface which was further confirmed by XPS analysis.

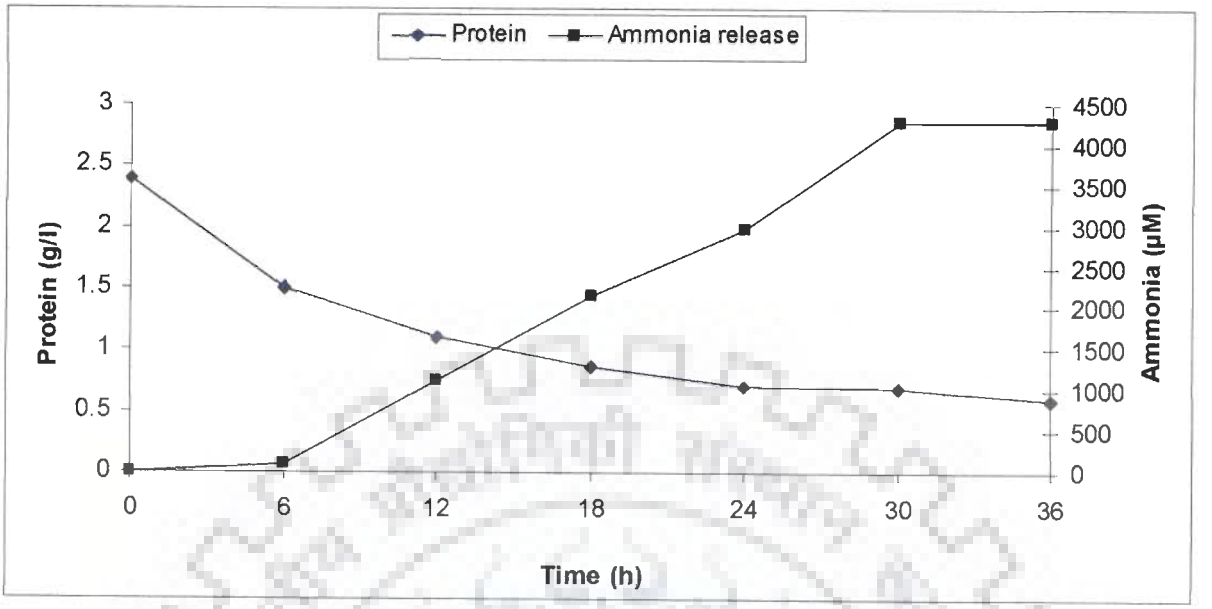


Fig.4.25. Protein adsorption and ammonia release during enzymatic treatment of PAN at 37°C, pH 7.0.

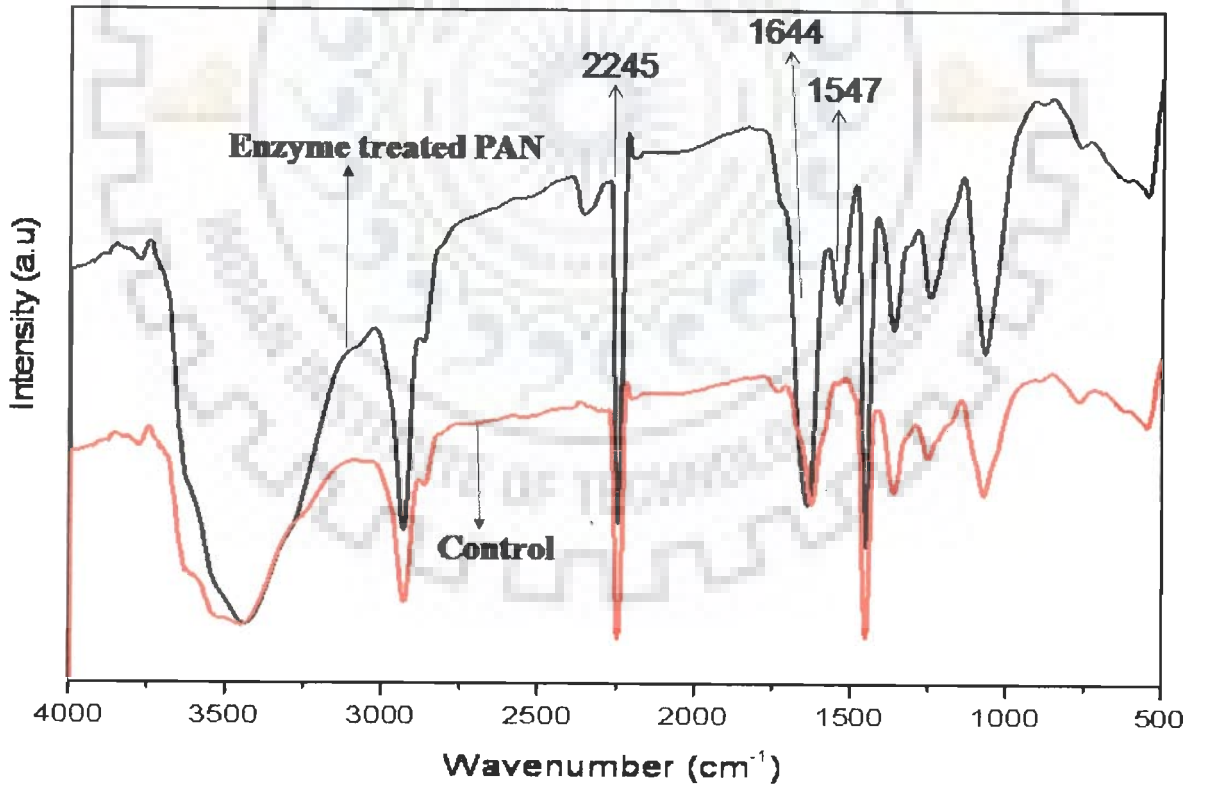


Fig.4.26. FTIR spectra of enzyme treated PAN and control PAN (from 37°C, pH 7.0).

4.3.3 XPS analysis

In earlier literature reports, XPS analysis used as a powerful technique to study surfaces of modified polyacrylonitrile after treatment with nitrile metabolizing enzyme system of *Rhodococcus rhodochrous*, *Arthrobacter sp*, *Brevibacterium imperiale*, *Corynebacterium nitrilophilus* and *Agrobacterium tumefaciens* (Tauber *et al.*, 2000, Battistel *et al.*, 2001, Fischer-Colbrie *et al.*, 2006). Therefore, control and enzyme treated samples of PAN were analyzed using XPS and the survey scans of both the samples are shown in Fig.4.27. The survey spectrum showed the presence of carbon, nitrogen and oxygen elements in both samples. It can be observed from XPS spectra that there was a significant change in carbon and oxygen contents of the control and enzyme treated samples. The elemental composition of both the samples was specified in table 4.17, indicating a decrease of approximately 4% in carbon and an increase of 4 % in oxygen content of enzyme treated sample as compared to controlled sample. A small decrease in the nitrogen content of enzyme treated sample was also observed. The C1s spectra for both the samples were deconvoluted and details shown in Figure 4.28 (a) and (b) respectively. The deconvoluted spectra clearly indicated the presence of C-C, C-OH, C-O, $CONH_2$ (amide) and O-C=O (carboxylic) components in both the samples at binding energy of 284.6 eV, 285.5 eV, 286.6 eV, 288 eV and 289.05 eV respectively. Enzymatic treatment resulted in decrease in the amide component at 288 eV while the carboxyl component at 289.05 eV increased, indicating the presence of carboxylic acid group in enzyme treated PAN. The decrease in nitrogen content of enzyme treated sample is also evident from comparative spectra of N(1s), shown in figure 4.29.

Table 4.17: Elemental composition of carbon, oxygen and nitrogen in controlled and enzyme treated samples.

Elemental composition	**Carbon**	**Nitrogen**	**Oxygen**
Controlled Sample	84.5	13.0	2.5
Enzyme treated Sample	80.6	12.6	6.8

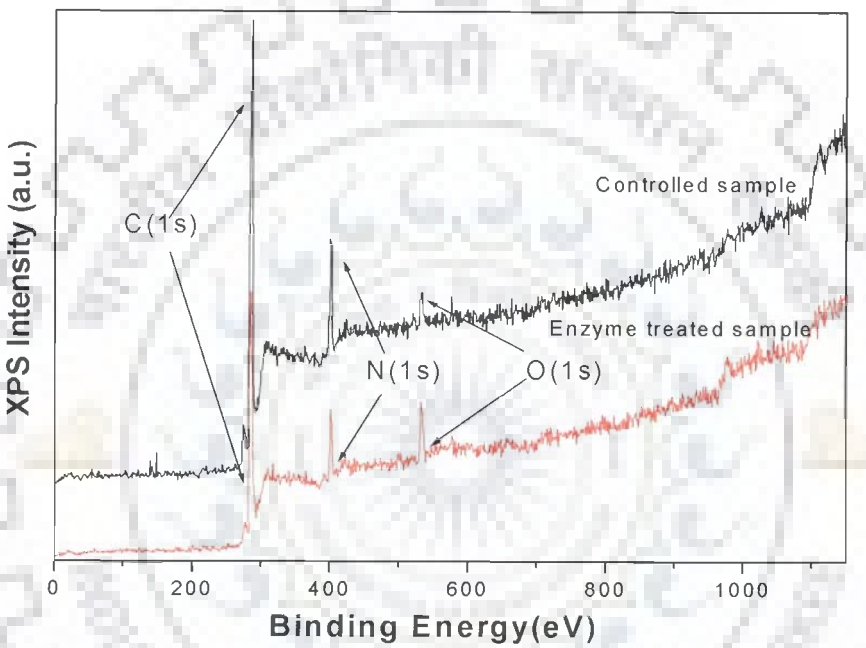
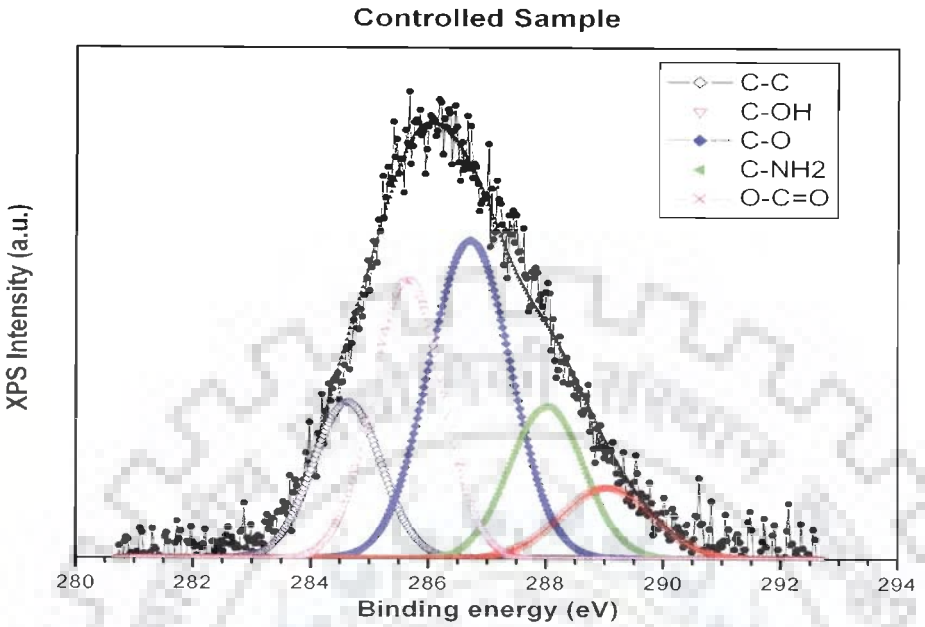


Fig.4.27. XPS survey scan of control and enzyme treated samples.

(a)



(b)

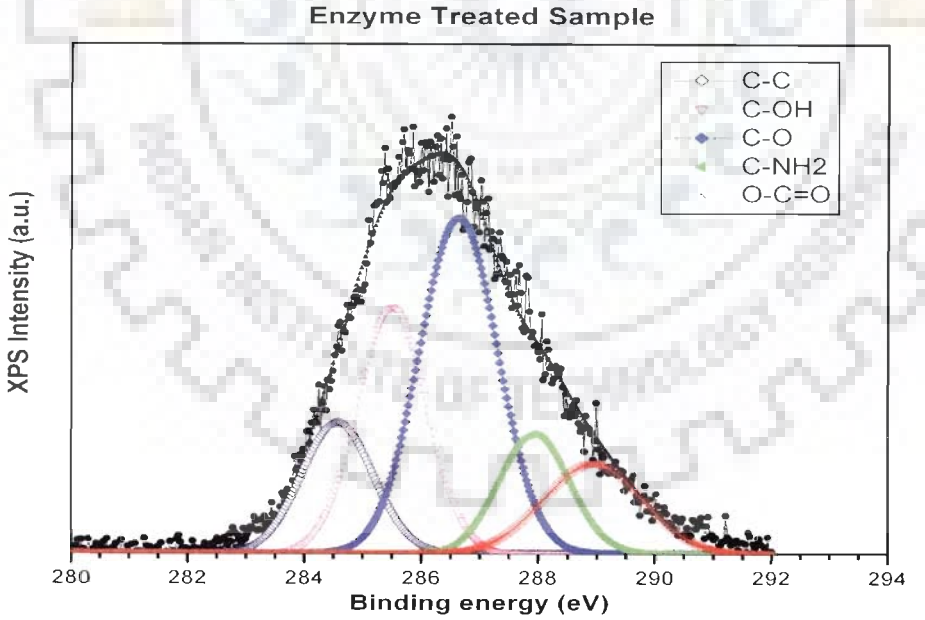


Fig.4.28. Deconvoluted carbon (1s) spectra of (a) control and (b) enzyme treated samples.

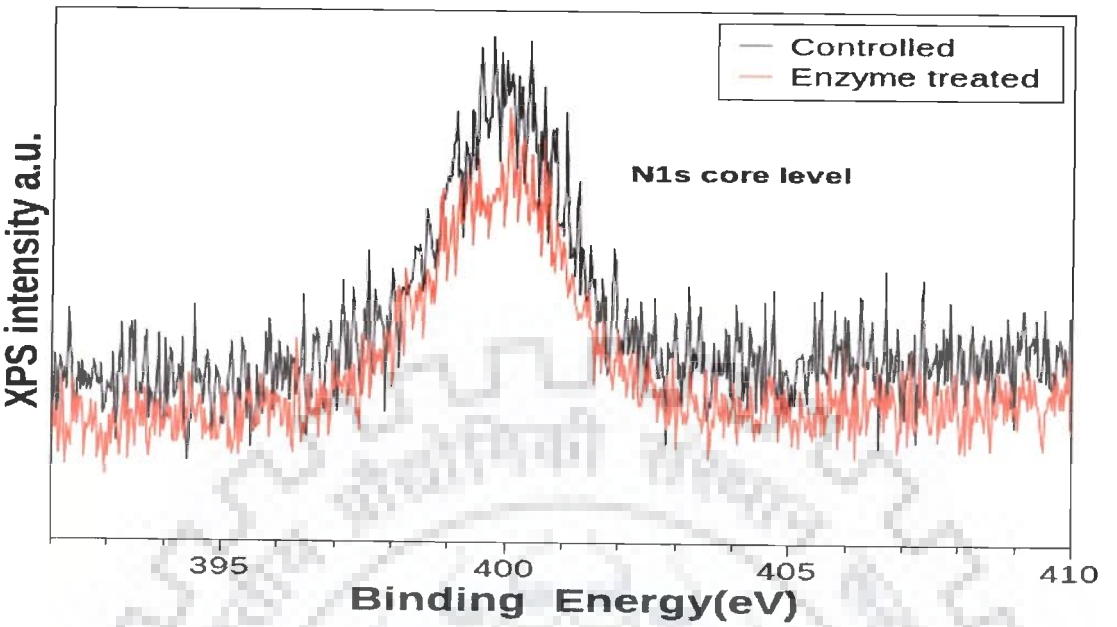


Fig.4.29. N1s core level spectra of control and enzyme treated sample.

4.3.4 Effect of pH on enzymatic treatment of polyacrylonitrile

FTIR and XPS analysis of modified polyacrylonitrile indicated the presence of higher carboxyl groups in the enzyme treated polyacrylonitrile as compared to the control sample. Moreover, ammonia release from polyacrylonitrile strongly confirms the transformation of –CN groups to –COOH groups. In previous section (section 4.1.11), it was reported that hexanenitrile hydrolysis with enzyme of *Amycolatopsis* sp. IITR 215 was maximum at pH 5.8 and the optimum pH for amidase and nitrile hydratase was 7.0. Therefore, we planned to study the enzymatic treatment of PAN at pH 5.8 with respect to protein adsorption and ammonia release. The results were compared with enzymatic treatment of PAN at pH 7.0. The protein adsorption was assessed by monitoring the unbound protein concentration in the reaction mixture (Fig.4.30). In the first 6 hours of treatment, there was a sharp decrease in protein content in the treatment solution at both pH values but without any release of ammonia. After 6 hours, protein adsorption was higher at pH 7.0 as compared to pH 5.8. At pH 7.0 and 5.8, 24% and 34.8% of protein respectively remained as unbound protein after 36 hours of treatment. In case of ammonia release, it was observed that at pH 5.8, release of ammonia started only after 18 hours whereas in case of pH 7.0, ammonia release started after 6 hours. After 30 hours of treatment, protein adsorption and ammonia release pattern didn't

change significantly. Final ammonia release was lower at pH 7.0 as compared to pH 5.8 and at pH 5.8, final ammonia release was 1.8 times higher as compared to pH 7.0. Although final protein adsorption was higher at pH 7.0 but conversion of cyano group to carboxylic acid was less as compared to enzymatic treatment of PAN at pH 5.8. This indicates that binding of specific enzyme probably plays an important role in conversion of pendent –CN group to –COOH group with simultaneous release of ammonia. However, it should be noted that in this study no effort was made to determine the amide group on enzymatically treated polymer. Possibility of higher amide group formation at pH 7 on PAN can not be ruled out.

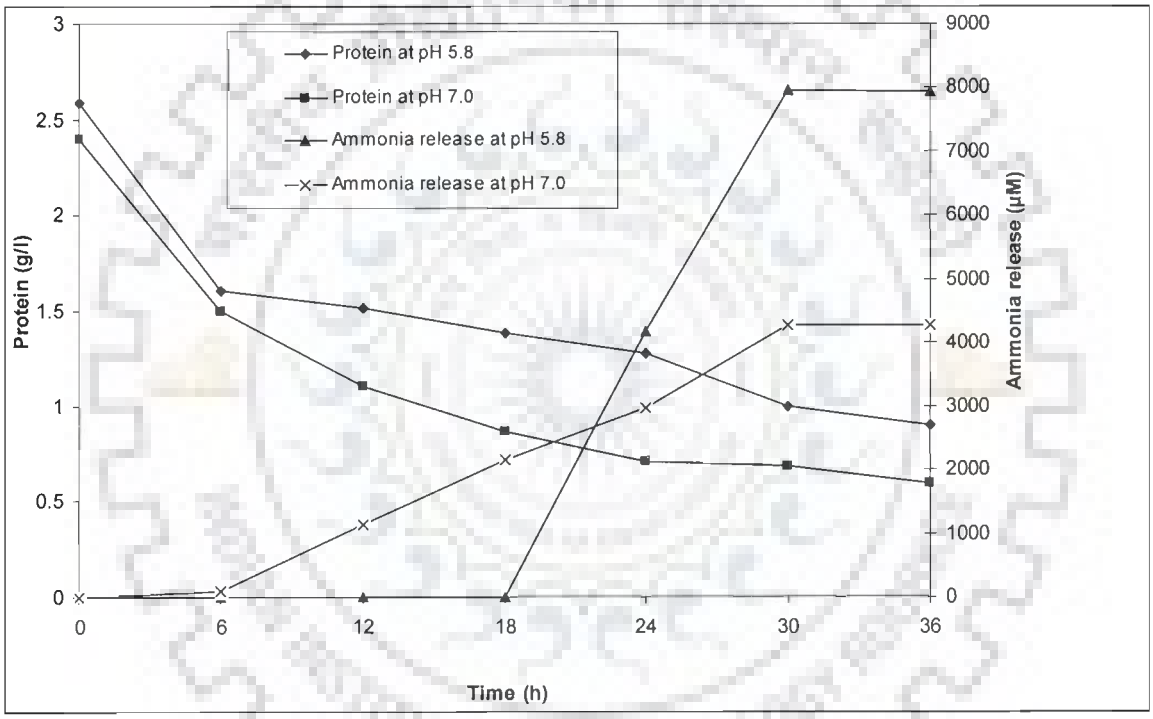


Fig.4.30. Protein adsorption and ammonia release during enzymatic treatment of PAN at different pH and 37°C.

4.3.5 FTIR analysis

Samples of enzymatically treated polymer at pH 5.8 and 7.0 were subjected to FTIR analysis. Polymer samples without any enzyme treatment were taken as controls. FTIR spectra of both samples showed formation of a new peak at 1547 cm^{-1} and broadening of another peak at 1644 cm^{-1} (Fig.4.26 and 4.31). Among these peaks, peak at 1644 cm^{-1} corresponds to the stretching of the carbonyl group of the amide while the peak at 1547 cm^{-1} strongly supports the presence of carboxylate groups. Zhang *et al.* (2009) reported new peaks

at 1568 cm^{-1} and 1405 cm^{-1} which strongly supported the formation of carboxyl groups (Zhang *et al.*, 2009). Fischer-Colbrie et. al reported the formation of amide group by treating PAN powder with nitrile hydratase/amidase system of *Agrobacterium tumifaciens* BST05 (Fischer-Colbrie *et al.*, 2006). Moreover, the presence of carboxylate groups in the present study was further confirmed by dye (Rhodamine 6G) binding assay.

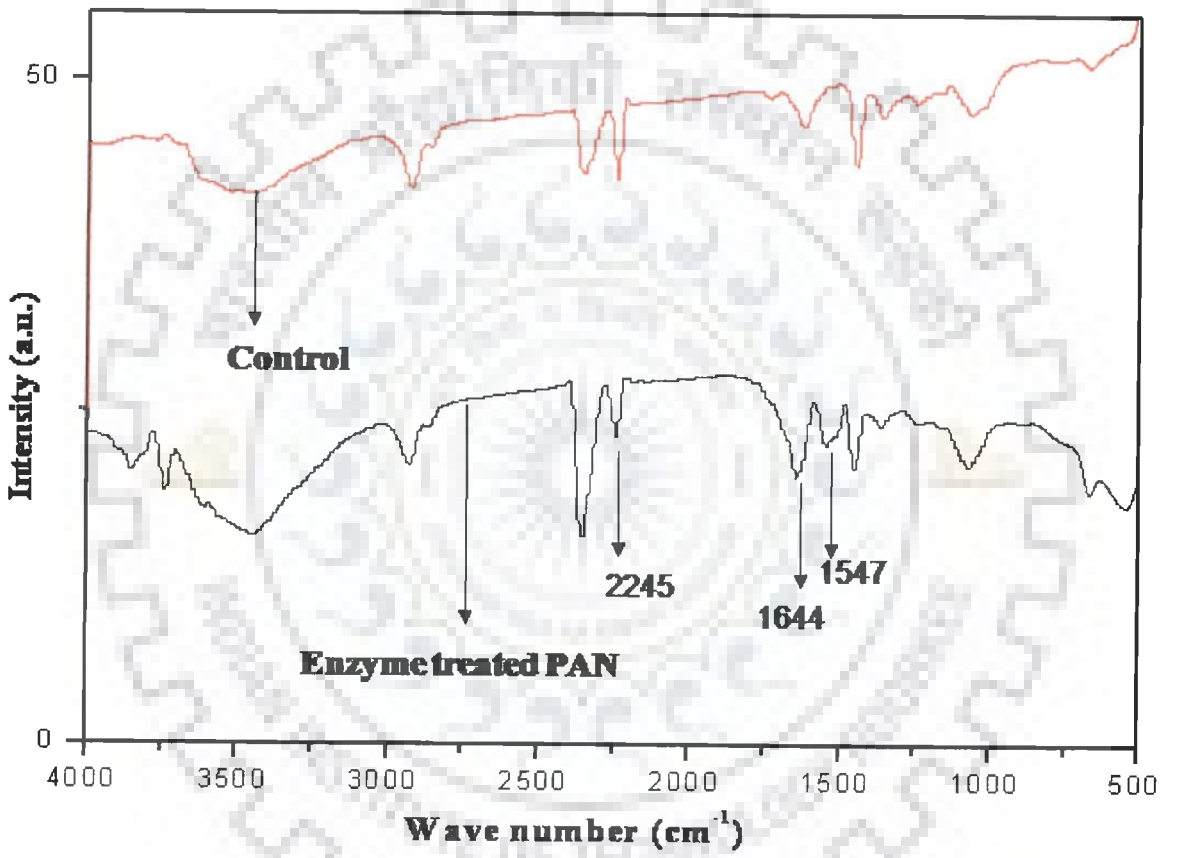


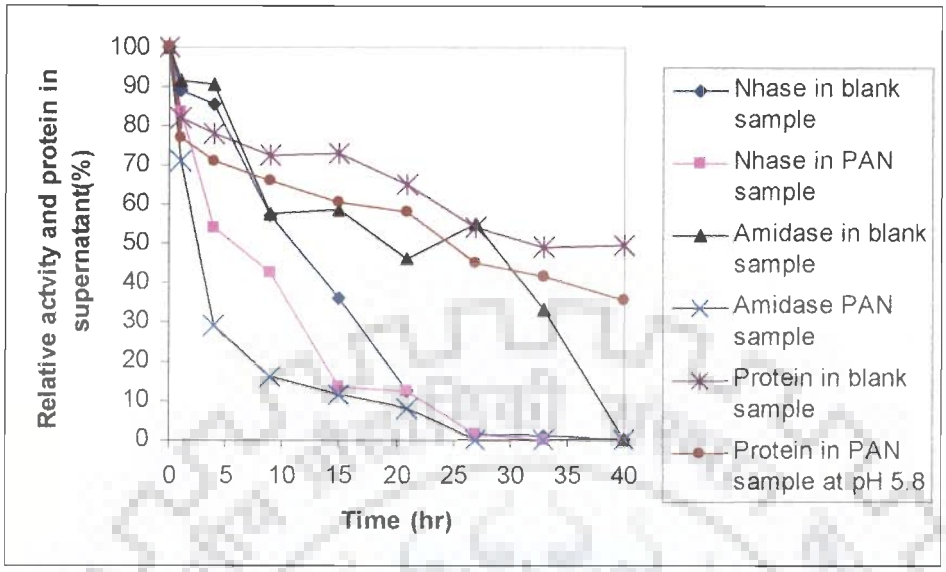
Fig.4.31. FTIR spectra of enzyme treated PAN and control PAN (from 37°C, pH 5.8).

4.3.6 Specific adsorption of nitrile hydratase and amidase during enzymatic treatment of PAN surfaces at two pH values

In previous section it was observed that at pH 5.8 higher conversion of –CN to carboxylic acid was achieved during enzymatic treatment of PAN. So, to understand the basic mechanism responsible for higher conversion of cyano group to carboxylic acid group, adsorption of nitrile hydratase and amidase on polymer surface during enzymatic treatment

of PAN was monitored. During enzymatic treatment, a blank sample was also used where precipitated protein solution (without polymer) that has been used to treat polymer was also kept at 37°C to determine the rate of deactivation of enzymes. At pH 5.8, it was observed that nitrile hydratase didn't show any significant adsorption on PAN surface after 1 hour of polymer treatment but amidase showed higher rate of adsorption at this time (Fig 4.32a). Similar pattern was also observed at pH 7.0 during 1 hour of polymer treatment (Fig. 4.32b). Most striking feature was significant deactivation of nitrile hydratase at pH 7 as compared to pH 5.8. As far as amidase stability is concerned, at pH 5.8, stability was marginally better than at pH 7. Further analysis of specific activities of bound nitrile hydratase and amidase at two pH values were determined by calculating bound nitrile hydratase and amidase activities and bound protein concentrations. Bound enzyme activity was calculated by subtracting enzyme activity of blank sample from corresponding enzyme activity of supernatant of enzyme treated polymer sample. Similarly bound protein concentration was also calculated. From the bound specific activity profiles (Fig. 4.32c) of nitrile hydratase and amidase it can be concluded that specific activity of nitrile hydratase was higher than bound specific activity of amidase at both pH values. Another striking feature was specific activity of bound nitrile hydratase was higher at pH 5.8 as compared to pH 7.0. This indicates that more specific binding of nitrile hydratase occurred at pH 5.8 as compared to pH 7.0. However, when comparison was made between specific activity values of bound amidase at these two pH values it was observed that there was no significant difference, except at pH 7.0, optimum binding of amidase occurred at a later stage (24 h). In case of pH 5.8, optimum binding of amidase was obtained within 5 h and this optimum binding profile was also similar with nitrile hydratase. Bound amidase was more stable at pH 5.8 as compared to pH 7.0 and it was stable till 33 h. From theses observations it can be concluded that due to higher specific binding of nitrile hydratase at pH 5.8, higher conversion of –CN to amide functional group was achieved at pH 5.8 as compared to pH 7. At pH 5.8, this newly formed amide functional group was further converted to carboxylic acid group at higher rate as compared to pH 7.0 due to higher stability of bound amidase at this pH value. Thus formation of higher ammonia at pH 5.8 during enzymatic treatment of PAN was due to higher specific binding of nitrile hydratase and stable bound amidase. In literature, there are no such studies on binding profiles of enzyme on polymer during enzymatic treatment of polymer.

(a)



(b)

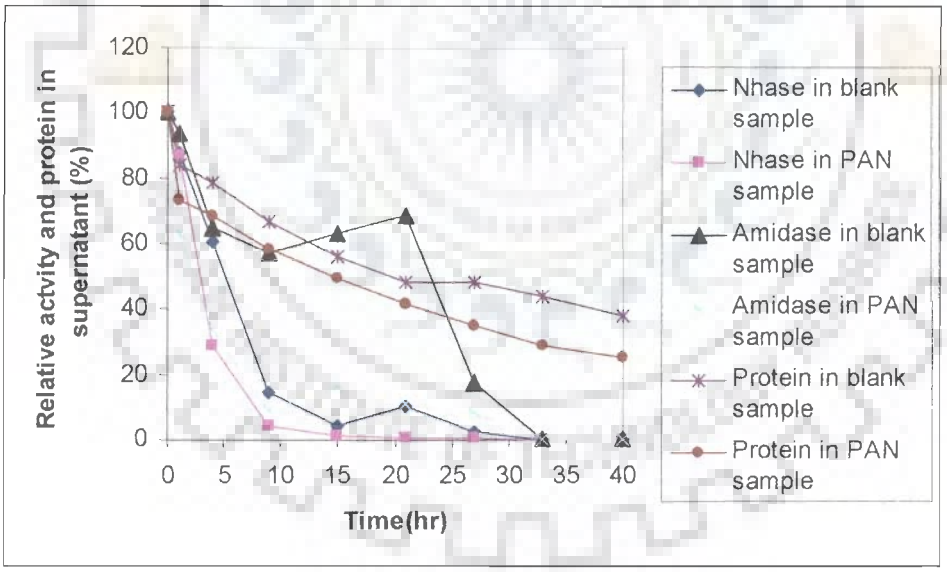


Fig.4.32. Nitrile hydratase, amidase and protein profile in supernatant during enzymatic treatment of PAN at (a) pH 5.8 (b) pH 7.0, at 37°C.

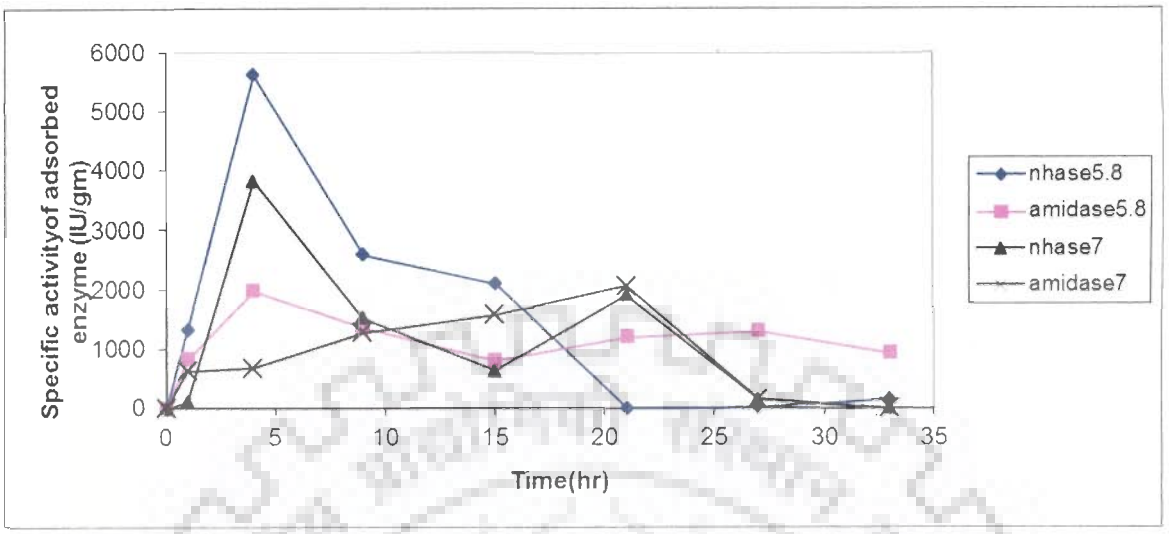


Fig.4.33. Profile of specific activity of bound nitrile hydratase and amidase on PAN at pH 5.8 and 7.0, at 37°C.

4.3.7 Quantification of carboxyl groups

Formation of carboxyl groups on polymer surface can be quantified by using basic dyes such as Basic Blue 9, Toluidine blue and Rhodamine 6G. These dyes have cationic groups which can form ionic bonds with anionic groups on the polymer (Matamá *et al.*, 2007, Ma *et al.*, 2007). The carboxyl group formed on the enzyme treated polymer was quantified by Rhodamine 6G due to its high sensitivity as mentioned in various literature reports (Liu et. al 2005, Kim et. al 2000, Bae et. al 1999). The decrease in absorbance at 486 nm was determined with enzyme treated polymer at pH 5.8 and 7.0. Untreated PAN was taken as the control. The carboxyl group formation was found to be maximum at pH 5.8. At pH 5.8, 106 µmoles carboxyl groups /g was found in control and it was 456 µmoles/g in enzyme treated PAN whereas at pH 7.0, 117 µmoles/g carboxyl groups was found in control and it was 227 µmoles/g for enzyme treated PAN (Fig.4.34). Thus this data also confirms that at pH 5.8, carboxylic acid group formation was 3 times higher than at pH 7. However, corresponding ammonia release at pH 5.8 was 1.38 times higher than at pH 7.0 during enzymatic treatment of PAN (Fig.4.34).

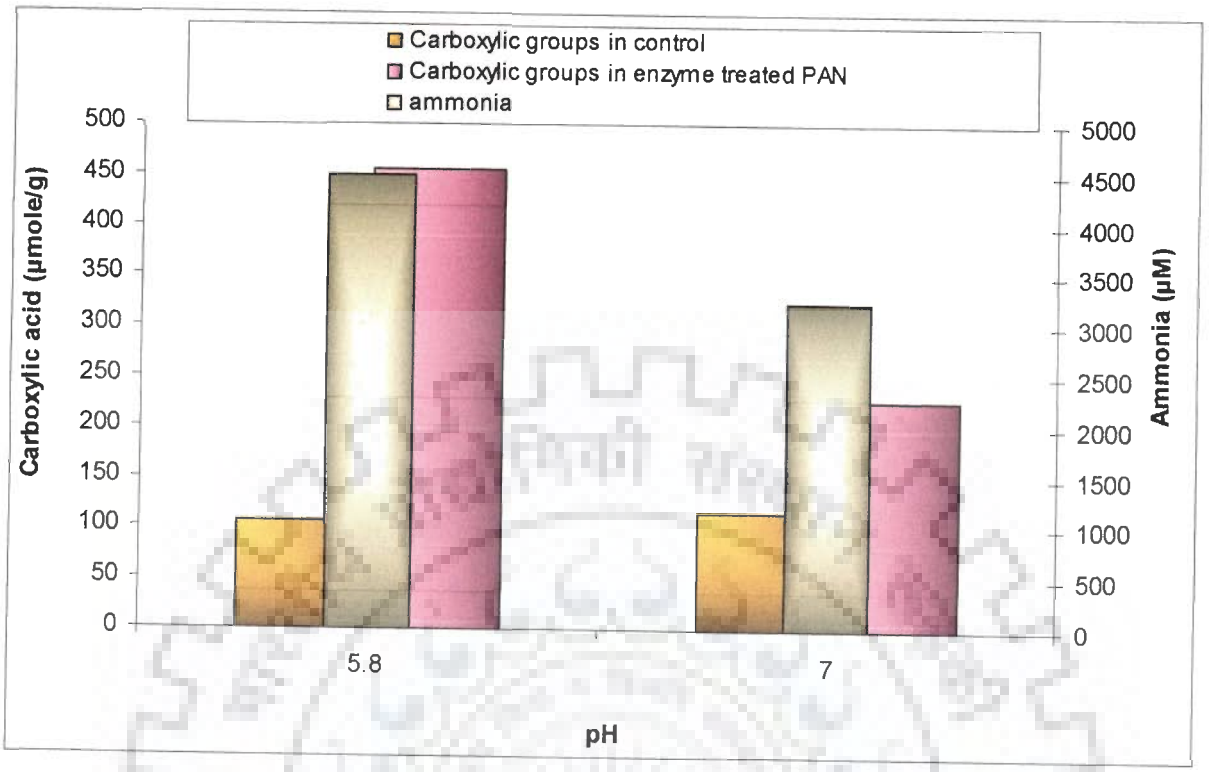


Fig.4.34. Quantification of carboxyl groups on enzyme treated PAN at two pH values by Rhodamine 6G.

4.3.8 Effect of polymer concentration and enzyme loading in enzymatic treatment of PAN

The effect of polyacrylonitrile concentration was also studied to determine the optimum polymer concentration for the enzymatic treatment of PAN using precipitated protein solution at pH 5.8 and 37°C. The protein adsorption was indirectly assessed by monitoring the unbound protein concentration in the treatment solution. At 5 g/l of polyacrylonitrile, finally 62% protein was present as unbound protein whereas higher protein adsorption was detected at 15 g/l of polyacrylonitrile and maximum ammonia released at this polymer concentration (Fig.4.35). Further increase in polymer concentration didn't yield any further increase in ammonia release. Increase in polymer concentration beyond 15 g/l resulted in marginal improvement of protein adsorption. This study further confirms that adsorption of protein is not the sole criteria for efficient surface modification of polyacrylonitrile with enzyme. Enzymatically treated samples of polymer concentrations of 5

and 15 g/l were chosen for FTIR analysis. A sharp peak was detected at 1541 cm^{-1} at 15 g/l while no distinct peak was detected at 5 g/l of polyacrylonitrile concentration (Fig.4.36).

If we compare the ammonia release from 10g/l PAN during enzymatic treatment at pH 5.8 in three different batches (Fig.4.30, Fig. 4.34 and Fig. 4.35) it was found that there was significant difference in ammonia released in supernatant under similar experimental conditions. So to understand the differences in ammonia release, initial specific activity of amidase in three batches were tried to correlate with final ammonia release data. From the result (Fig.4.37) it can be concluded that lower the initial amidase activity higher the ammonia release during enzymatic treatment of PAN. However, activity has to be higher than zero or intermittent addition of amidase may improve the conversion of cyano group to carboxylic acid functional group in PAN. This may be explained interms of competition for adsorption sites between nitrile hydratase and amidase during enzymatic treatment of polymer. Ideally, nitrile hydratase has to adsorb first followed by adsorption of amidase for efficient conversion of cyano group of PAN to carboxylic acid group.

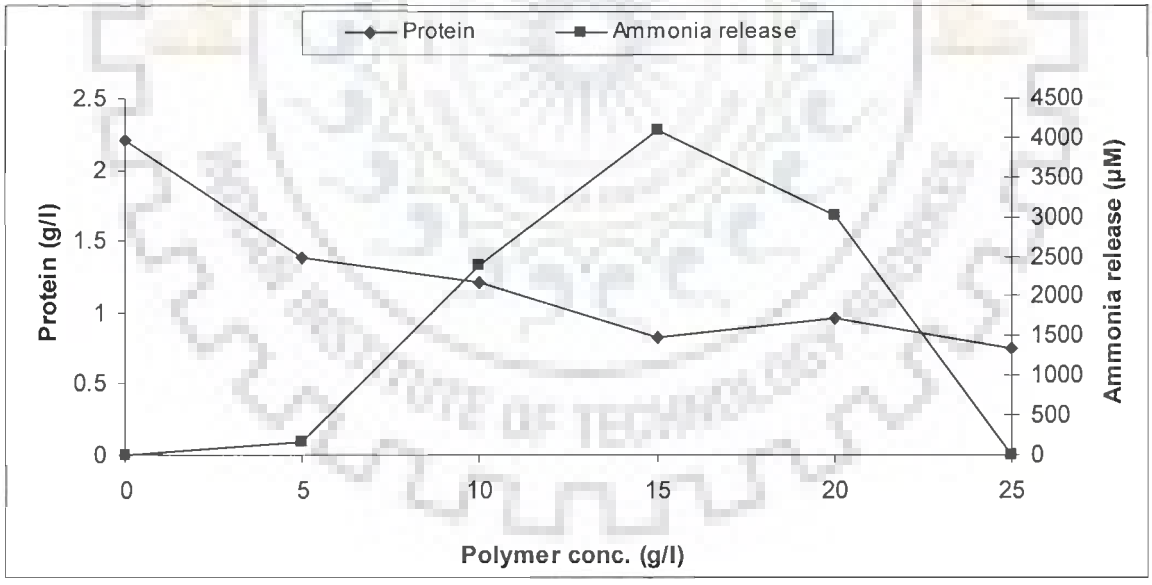


Fig.4.35. Effects of PAN concentration on protein adsorption and ammonia release during enzymatic treatment of PAN at 37°C, pH 5.8.

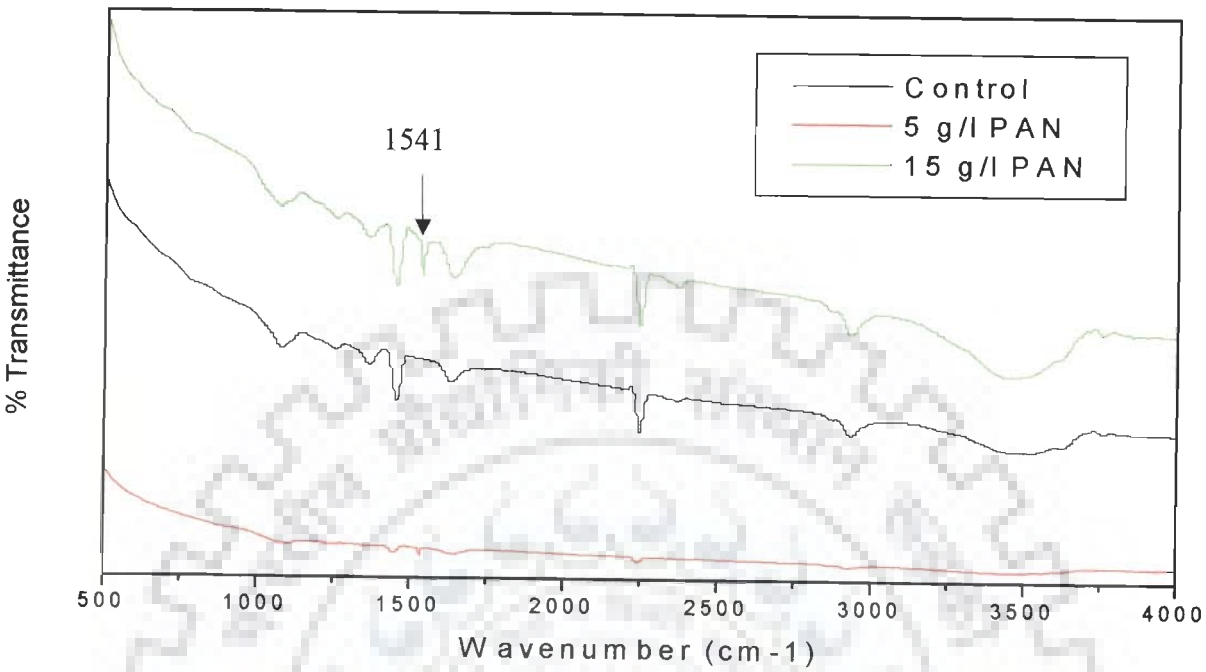


Fig.4.36. FTIR spectra of 5 g/l and 15 g/l enzyme treated PAN and control PAN.

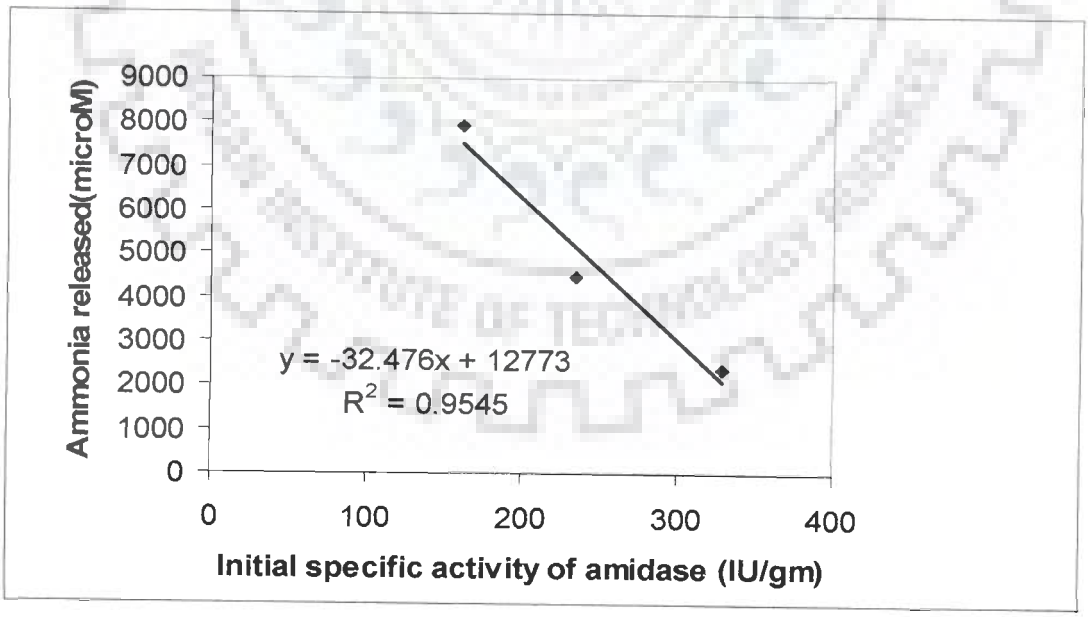


Fig.4.37: Effect of initial specific amidase activity on enzymatic treatment of polyacrylonitrile at pH 5.8.

4.3.9 Effect of enzymatic treatment on other PAN co-polymers

Enzymatic treatment of other PAN co-polymers was also studied at pH 7. From, the unbound protein profile in reaction mixture with three co-polymers (PMA, PAN-co-butadine-co-styrene and PAN-co-methacrylate), it can be concluded that minimum protein adsorption occurred with PAN-co-butadiene-co-styrene and maximum adsorption achieved with PMA. Nearly 40 % protein remained as unbound protein with PMA while protein adsorption was minimum (68% protein remained as unbound protein) on PAN-co-butadine-co-styrene and 54% protein remained in solution with PAN-co-methacrylate (Fig.4.38). However, no ammonia was released till 36 hours of treatment with any of these co-polymers.

In FTIR spectra, no distinct peak around 1540 cm^{-1} was detected on these PAN co-polymers but peak at 1638 cm^{-1} get broader only with PAN-co-butadine-co-styrene which shows that there was a formation of amide groups on PAN-co-butadine-co-styrene (Fig.4.39). This indicates that adsorption of nitrile hydratase was probably higher in this co-polymer.

In other enzyme treated PAN co-polymers, no peak was detected at 1547 cm^{-1} and peak around 1638 cm^{-1} (Fig.4.40 and 4.41) was marginally got broader. In the dye binding assay with these enzyme treated co-polymers, decrease in absorbance at 486 nm was also not observed. Therefore, it can be concluded that carboxyl groups were not formed on these polymers. From these results, it seems that nitrile metabolizing enzymes system of *Amycolatopsis* sp.IITR215 is more specific for PAN than other co-polymers. There are no reports on enzymatic treatment of other copolymer with nitrile hydratase/amidase system. However, Fischer-Colbrie et al. (2007) reported that *Micrococcus luteus* was not able to hydrolyze highly crystalline polymer and minimum concentration of co-monomer required was reported to be 5%. So from their study they concluded that highly crystalline polymer doesn't support surface hydrolysis (Fischer-Colbrie *et al.,* 2007). In the present study no such influence was studied but it was observed that specific protein adsorption probably play a role in enzymatic treatment of polymer.

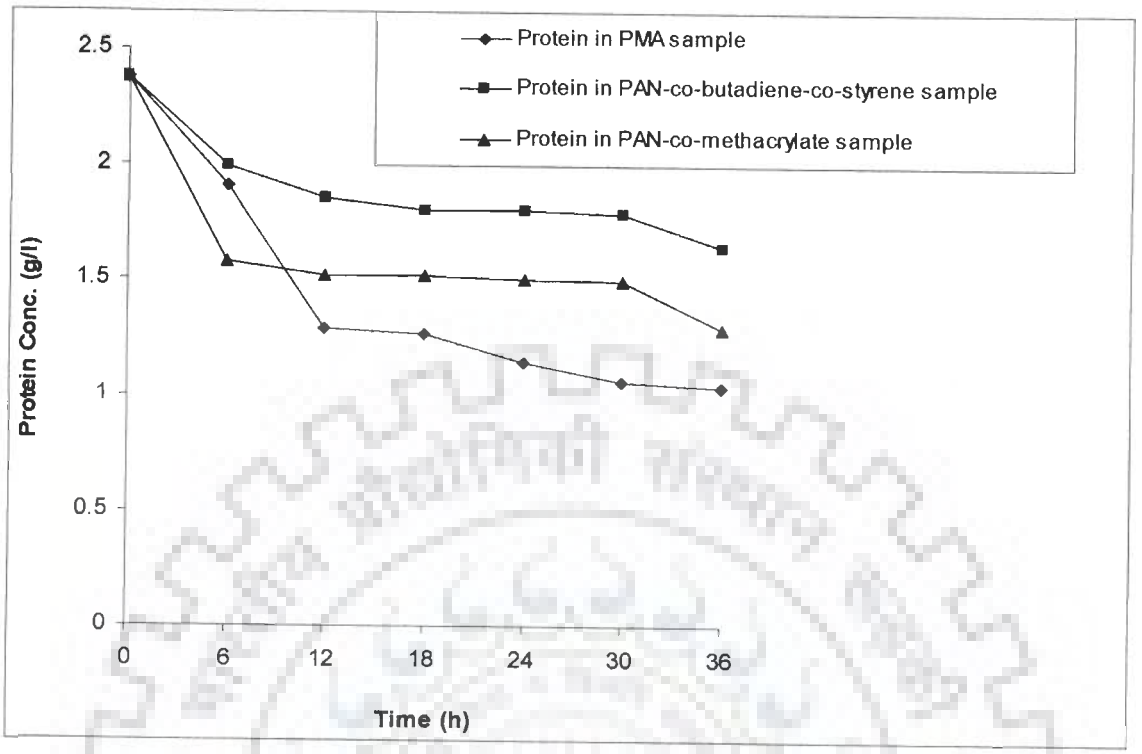


Fig.4.38. Protein adsorption profile on other PAN co-polymers at 37°C, pH 7.0.

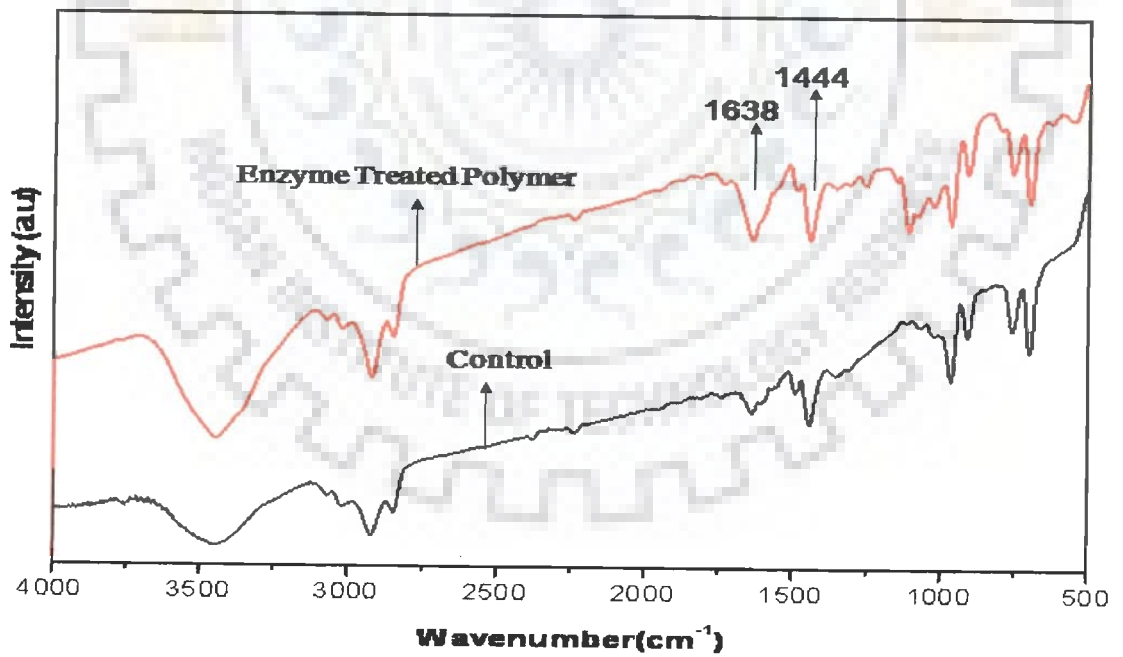


Fig.4.39. FTIR-spectra of PAN-co-butadiene-co-styrene (from 37°C, pH 7.0).

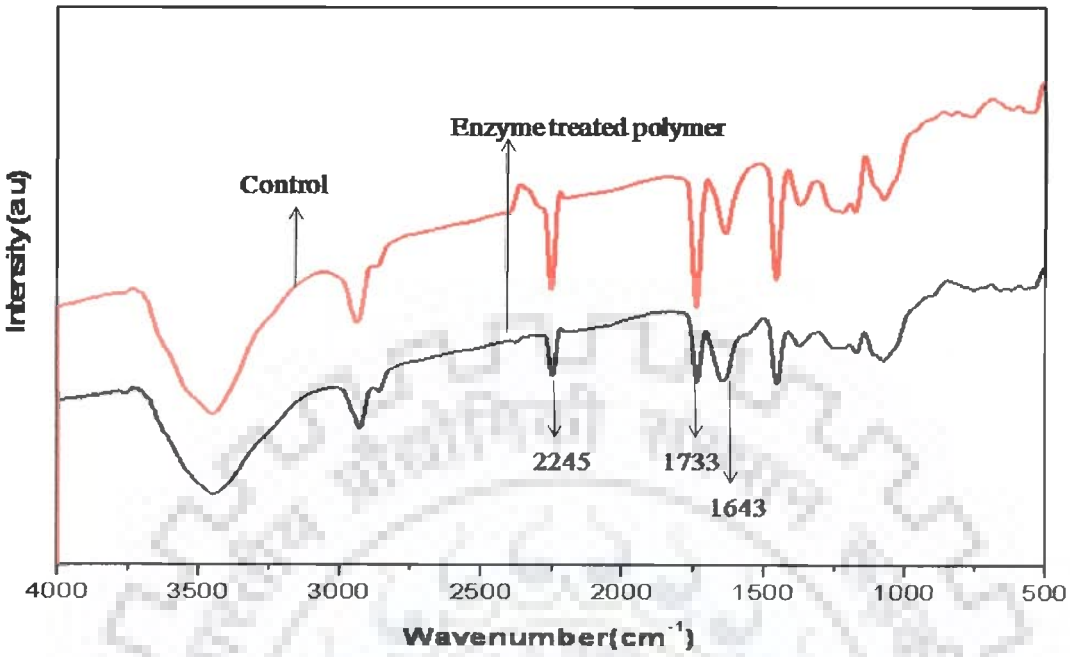
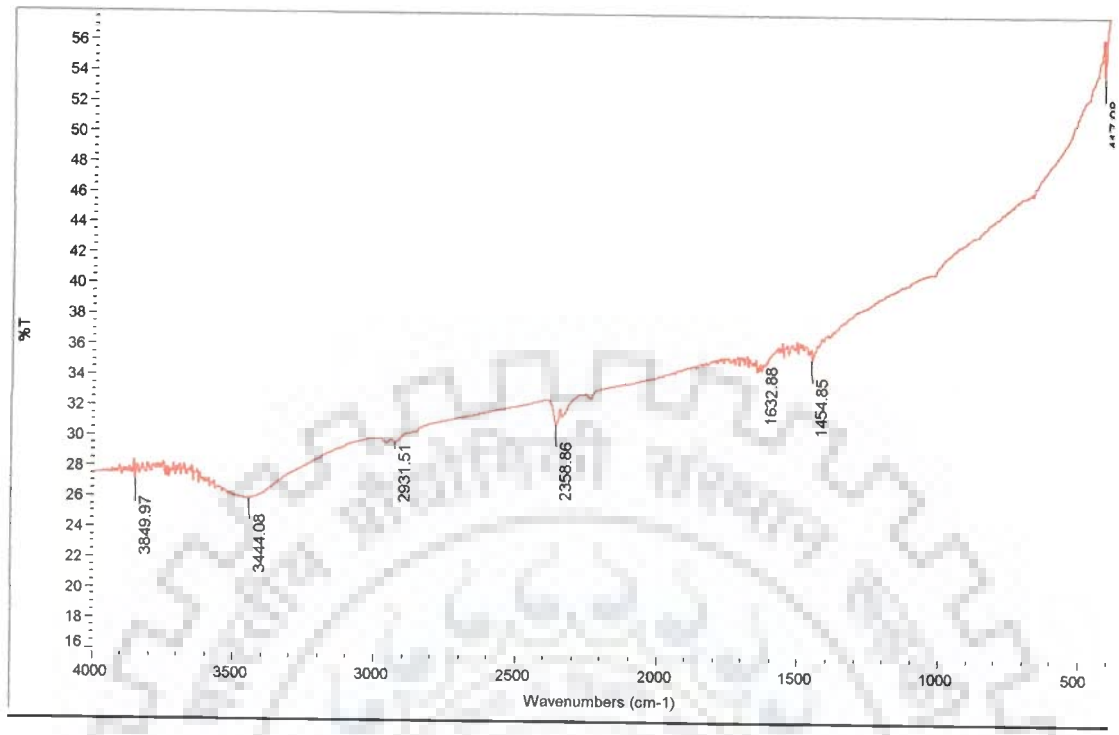


Fig.4.40. FTIR-spectra of PAN-co-methacrylate (from 37°C, pH 7.0).

(a)



(b)

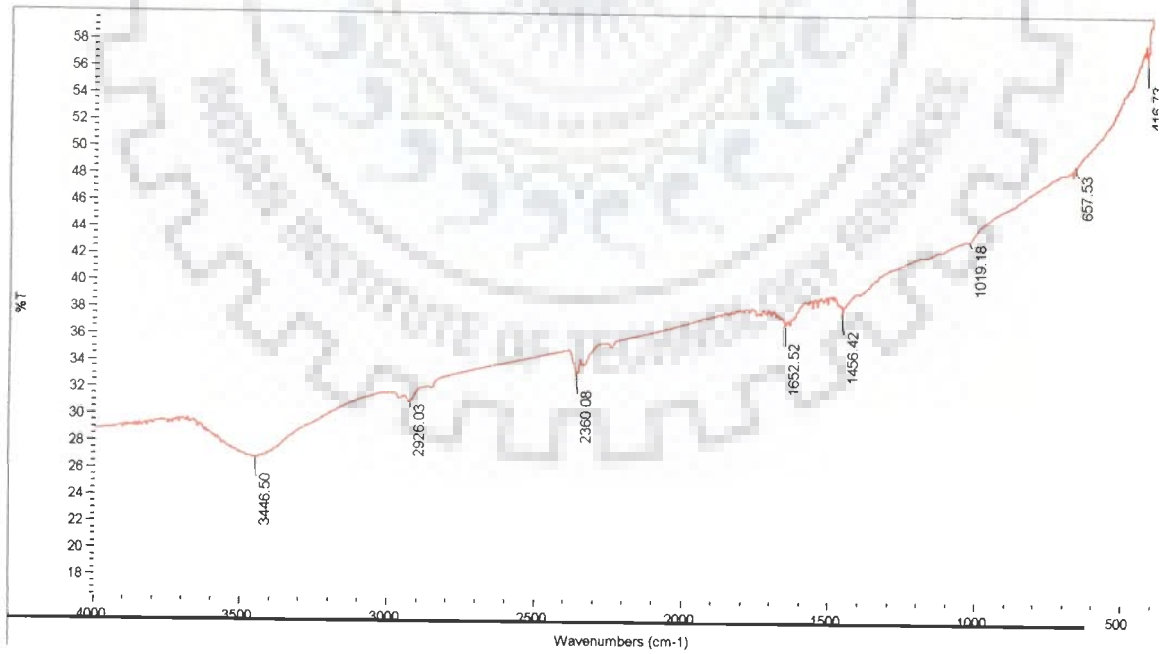


Fig.4.41. FTIR spectra of enzyme treated PMA (a) PMA-Control (b) Enzyme treated PMA.

In this section, enzymatic treatment of polyacrylonitrile was studied with respect to pH, temperature, and polymer concentration. It was observed that ammonia release and conversion of –CN group to –COOH group was marginally better at 37°C as compared to 45°C. The effect of pH was more significant and it was observed that at pH 5.8, the conversion of –CN group to –COOH group was nearly two times higher than at pH 7.0. The unbound protein concentration profile in the reaction mixture indicates that specific binding enzyme plays an important role in the enzymatic treatment efficiency. The specific activities of bound nitrile hydratase and amidase at two pH values indicates that at pH 5.8 more specific binding of nitrile hydratase was observed as compared to pH 7.0. Specific activity of bound amidase on PAN didn't differ much at these pH values but stability of bound amidase on PAN at pH 5.8 was higher than at pH 7.0. The polymer concentration also found to have an impact on enzymatic treatment efficiency and in this study it was observed that 15 g/l was the optimum concentration under experimental conditions.

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RESEARCH PUBLICATIONS

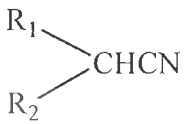
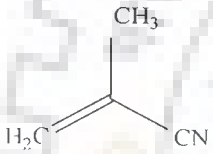
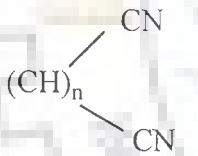
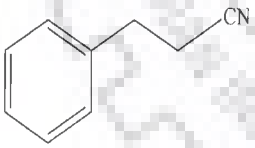
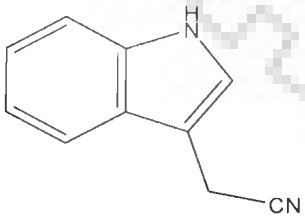
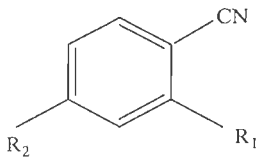
- **Vikash Babu,** Shilpi and Bijan Choudhury (2010). Nitrile metabolizing potential of *Amycolatopsis* sp.*IITR215*. Process Biochemistry.45, 866–873

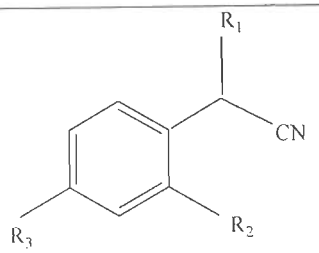
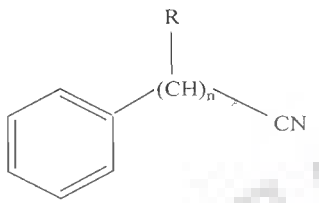
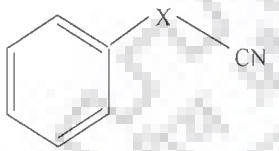
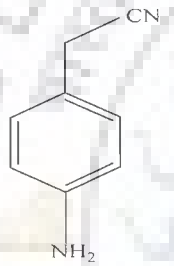
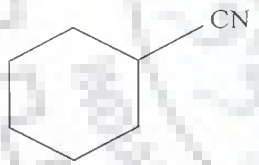
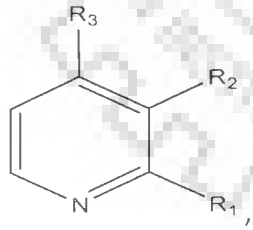
- **Vikash Babu** and Bijan Choudhury. Nitrile metabolizing enzyme from *Amycolatopsis* sp.IITR215 for surface modification of polyacrylonitrile. **Communicated in journal of applied polymer science. Manuscript ID-APP-2010-12-4035**

- **Vikash Babu,** Shilpi, Meenu Gupta and Bijan Choudhury. 2010. Opportunities and Challenges for Enzymatic Surface Modification of Synthetic Polymers. In: Green Chemistry for Environmental Sustainability (Eds. Sharma, S.K. and Mudhoo, A.), Taylor & Francis Group, LLC, Florida, Boca Raton, USA, pp. 93-104.

APPENDIX I

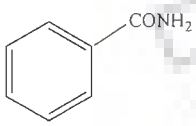
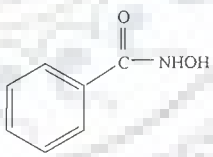
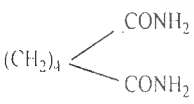
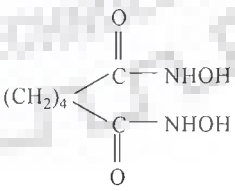
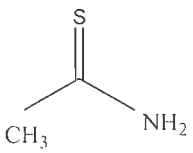
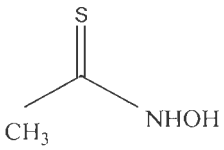
Chemical structure of used nitriles

Structure	Substitution	Substrate
	R_1=H, R_2= H	Acetonitrile
	R_1=CH_3, R_2= H	Propionitrile
	R_1= CH_3CH_2, R_2= H	Butyronitrile
	R_1= $CH_3(CH_2)_2$, R_2= H	Valeronitrile
	R_1= $CH_3(CH_2)_3$, R_2= H	Hexanenitrile
	R_1= CH_3, R_2= CH_3	Isobutyronitrile
	R_1= $(CH_3)_2CH$, R_2= H	Isovaleronitrile
	.....	Acrylonitrile
	-----	3-hydroxypropionitrile
	-----	Methacrylonitrile
	n =1	Malononitrile
	n = 3	Glutaronitrile
	n = 4	Adiponitrile
	-----	Hydrocinnamonitrile
	-----	Idole-3-acetonitrile
	R_1=H, R_2= H	Benzonitrile
	R_1=H, R_2= OH	4- Hydroxybenzonitrile

	R_1= H, R_2= H, R_3= H R_1= OH, R_2= H, R_3= H	Phenyl acetonitrile Mandelonitrile
	R= H, n=3	4- Phenyl butyronitrile
	X= O X= S	Phenoxy acetonitrile Phenyl thioacetonitrile
	-----	4- Aminobenzyl cyanide
	-----	Cyclohexanecarbonitrile
	R_1= CN R_2= H R_3= H	2-Cyano pyridine
	R_1= H R_2= CN R_3= H	3-Cyano pyridine
	R_1= H R_2= H R_3= CN	4- Cyanopyridine

APPENDIX II

Chemical structure of used amides and their corresponding hydroxamic acids

Substrate Structure	Substitution	Name	Corresponding Hydroxamic acid	Substitution	Name
$CH_2=CH-CONH_2$	-------	Acrylamide	$CH_2=CH-C(=O)-NHOH$	-------	Acrylo-hydroxamic acid
$R_1CH(R_2)-CONH_2$	R_1- H R_2- H	Acetamide	$R_1CH(R_2)-C(=O)-NHOH$	R_1- H R_2- H	Aceto-hydroxamic acid
	R_1- CH_3 R_2- H	Propionamide		R_1- CH_3 R_2- H	Propiono-hydroxamic acid
	R_1- $CH_3(CH_2)_3$ R_2- H	Hexanamide		R_1- $CH_3(CH_2)_3$ R_2- H	Hexano-hydroxamic acid
	R_1- CH_3 R_2- CH_3	Isobutyramide		R_1- CH_3 R_2- CH_3	Isobutyro-hydroxamic acid
		Benzamide			Benzo-hydroxamic acid
		Adipamide			Adipo-hydroxamic acid
		Thio-acetamide			Thio-aceto-hydroxamic acid