1. ABSTRACT

Microbial nitrilases are biocatalysts of industrial interest and being used for the production of commercial and pharmaceutically important products. In the present research work, an inducible thermophilic nitrilase containing strain was isolated by enrichment method from the dump site of Kirti Nagar, Delhi and was designated as *Pseudomonas* sp. KNB-2.

The effect of different nitriles and amides, different medium composition, pH, temperature, inducer concentration and time course of incubation on production of nitrilase was optimized. It was found that benzonitrile (inducer) in the concentration of 0.3% (v/v), pH 7 and 55^{o}C in M1 medium were most suitable conditions for maximum synthesis of nitrilase and produced dry cell biomass of 16.24 g/L. Nitrilase exhibited temperature stability in wide range of 45 ^{o}C to 65^{o}C and had optimum temperature of 45^{o}C, pH7 and exhibited maximum activity in benzonitrile, though it also catalyzed the conversion of acrylonitrile and acetonitrile.

Nitrilase activity was observed in 30-40% cut of ammonium sulphate precipitation and two bands of molecular weight 43 kDa and 47 kDa were observed in SDS-PAGE analysis.

Keywords: Thermophilic nitrilase, *Pseudomonas* sp. KNB-2, Benzonitrile

2. INTRODUCTION

Nitriles are organic compounds that have cyanide as a functional group (RC≡N),and are abundantly present in the nature. They occur in the form of cyanolipids, ricinine, cyanoglycosides, and phenylacetonitrile (figure -1) in plant and found as intermediates in microbial metabolism (Kobayashi *et al.*, 1993).

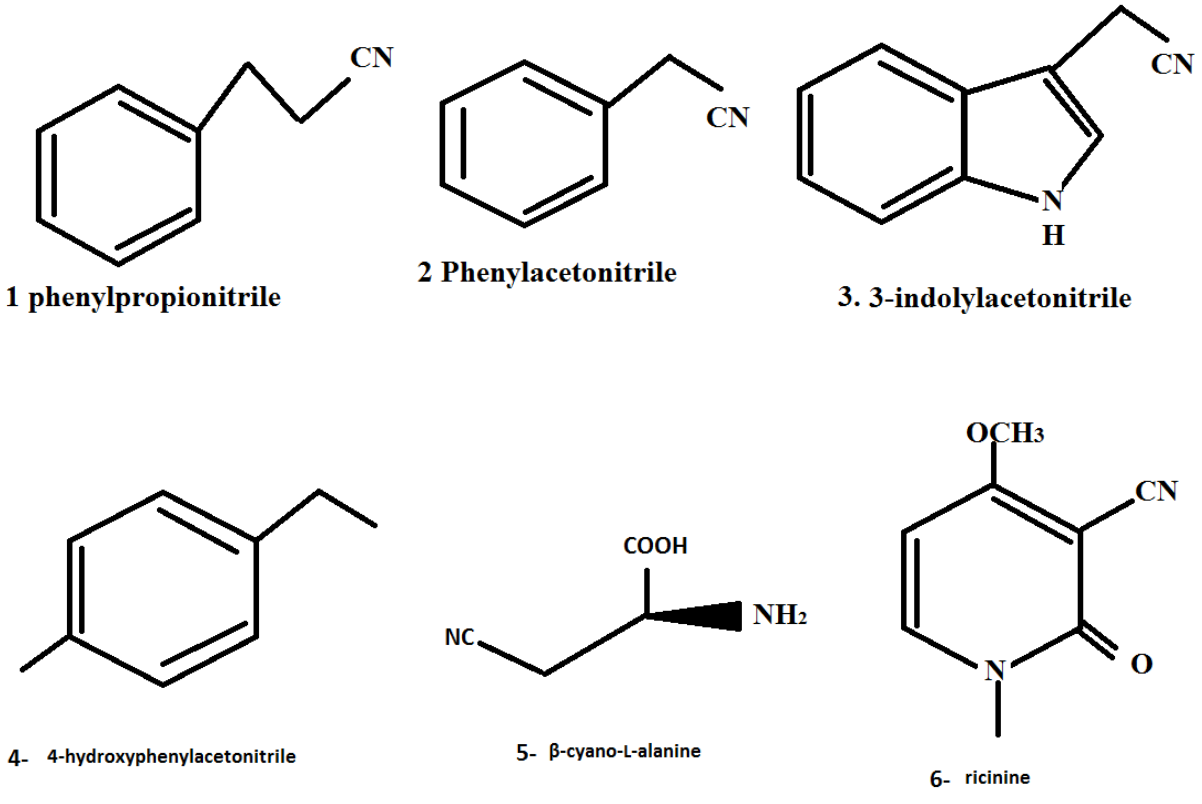


Figure 1:Examples of nitriles occurring in nature: (1)3- phenylpropionitrile, (2) phenylacetonitrile, (3) 3-indolylacetonitrile, (4) 4-hydroxyphenylacetonitrile, (5) β-cyano-L alanine, (6) ricinine (Banerjee *et al.*, 2002)

Nitriles are the most hazardous compounds due to the presence of cyano group in their structure and they are very toxic in nature (Kobayashi *et al.*,1998; Nagasawa *et al.*,2000; Brandao and Bull, 2003). In addition to the toxic nature, some of them are also carcinogenic and mutagenic. Cyanides are fast poisons which inhibit the electron transport chain by blocking of

transport of electron in respiratory cycle by binding the iron ion of cytochrome c oxidase and cause inhibition of ATP synthesis (Gupta *et al.,* 2010).

Nitrile hydrolyzing biocatalysts have important pharmaceutical and industrial Interest as they can be utilized for the treatment of nitrile and poisonous cyanide containing wastes and as agents for the manufacturing of many chemicals, antibiotics and vaccines. (Trott and Heald, 2001)

Nitrile hydrolysis is biochemically catalysed by nitrile-transforming enzymes that are present in wide range of plants (Thimann & Mahadevan, 1964), fungi [e.g. *Myrothecium verrucaria, Fusarium solani,* (Cluness and Herper1993)] and mesophilic bacteria [e.g. *Corynebacterium, Arthrobacte, Klebsiella, Nocardia, Brevibacterium and Rhodococcus* (Langdahl *et al.,* 1996)] and in thermophilic bacteria like *Pseudomonas fluorescens, Alcaligenes faecalis,* etc.

Three different types of enzymatic pathways were found in the bacteria that may hydrolyze the nitriles into the corresponding carboxylic acids and ammonia. Nitrilase (EC 3.5.5.1) or Nase that directly convert nitriles to the corresponding acid and NH3 while other two enzyme are nitrile hydratase (EC 4.2.1.84) or Nhase that convert nitriles to the corresponding amides that finally transform in to the respective acid by the action of amidases (EC 3.5.1.4).

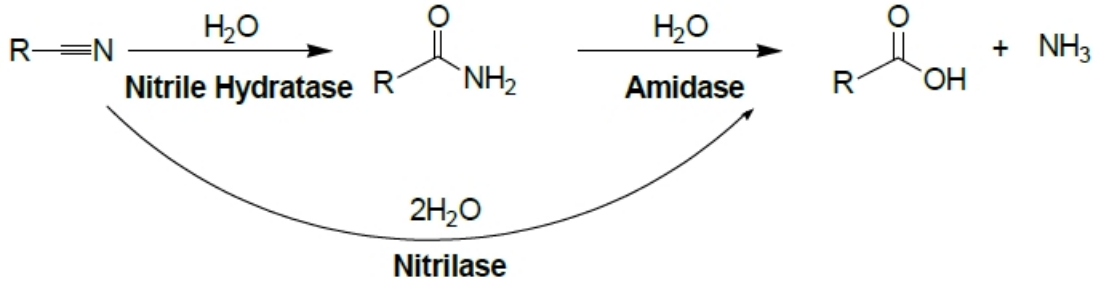


Figure 2: nitrile hydrolysis by single and intermediate root (Kobayash and Yamada, 1990)

Due to its regio and enatio- selectivity these enzymes have potential existence in biosynthesis of organic compounds by the conversion of readily available nitriles in to the valuable amides and acids without changing the other group (Banerjee *et al.,* 2006). They also use in the synthesis of commercially viable acrylamide (Kobayashi *et al.,* 1993b), antibiotics (Jallageas et al., 1980), herbicides, anti-inflammatory agents (Gilligan *et al.,* 1993) and nitriles

transformation by chemical means often include harsh condition which are detrimental to the environments. Biotransformation of nitriles, using nitrile metabolising enzymes overcome these problems andmay offer the additional advantage of production of valuable organic compound (Bianchi *et al.,* 1991).

Till now, many mesophilic nitrilases have been reported but due to having low stability at higher temperature they find few commercial applications. Due to resistant to chemical and physical denaturation and also to proteolysis, research group started exploring thermal niches to isolate thermophilic nitrilase producing bacterial strains. (Almatawa and Cowan, 1999)

Keeping above in view, the present work entitled **'Isolation and Characterization of Nitrilase of thermophilic *Pseudomonas* sp.KNB-2***'* has been undertaken with the aim to characterize production and reaction condition of "*Pseudomonas* Sp.KNB-2*"* for thermostable nitrilase. The objectives of this research work were:

1. To isolate and screen thermophilic nitrilase producing microbes.

2. To optimize culture condition for nitrilase production

3. Characterization of reaction condition of thermophilic nitrilase of *Pseudomonas* Sp.KNB-2

3. REVIEW OF LITERATURE

3.1. NITRILES AND NITRILASE SUPER FAMILY ENZYMES

Nitriles are a type of organic compounds that contains bond as R-C≡N. Most of them are produced as in intermediate act as precursor or building blocks of many organic solvents synthesis. Some of the identified plant species *Arabidopsis thaliana*, Barley, Chinese cabbage (*Brassica rapa)* known as cynogenic plants which produce natural nitriles for their defensive purpose against herbivores. In the nature, these cyanogenic substances (cyano lipid, cyano glycoside *etc*.) are hydrolyzed to nontoxic acid and ammonia by nitrilase super family enzymes.

Nitrilase and Nhase-amidase are most common forms, which is most prevalent in microorganisms, fungi and plants. Nitrilase is one of member of the nitrilase super family and it exists in the first class of nitrilase metabolizing enzymes. These families contains thiol group (SH) at the catalytic site for perform the action these enzyme utilize by the plants microbes fungi for their posttranslational modification. Nitrilase family classified in to 13 distinct families on the basis of on the basis of additional domains presence and sequence similarity. Branches of nitrilase super family enzymes based on sequences and substrate of enzyme are "(1) aromatic nitrilase, (2) aliphatic amidase, (3) amino terminal amidae, (4) biotinidase (5) beta-ureidopropionase (6) carbamylase, (7) prokaryotic NAD synthase, (8) eukaryote NAD synthase, (9) apolipoprotein N-acyltransferase (10) Nit and Nit fit, (11) NB11, (12) NB12 and (13) non-fused outliers. (Pace and Brenner 2001)

Hydrolysis of nitrile is catalyzed by two distinct ways; a nitrilase system "Nase" that converts nitriles directly into corresponding acids and ammonia, or by a nitrile hydratase "Nhase" which first convert it to amide which is further hydrolyzed into the acid in the presence of water by the action of amidase enzyme.

$$RCN + 2H_2O \xrightarrow{\text{Nase}} RCOOH + NH_3$$

$$RCN + H_2O \xrightarrow{\text{Nhase}} RCONH_2$$

$$RCONH_2 + H_2O \xrightarrow{\text{Amidase}} RCOOH + NH_3$$

A wide range of microorganisms including Brevibacterium, Corynebacterium, Nocardia, *Arthrobacter,Rhodococcus* and *Pseudomonas* has been shown to contain a nitrilase or nitrile hydratase–amidase, or both. Nitrilase have the broad range of application in the synthesis chemical pharmaceutical or therapeutic (as for the antibiotic production), paints industries and effluents treatments plants

Nitrilase have been reported in wide range of mesophilic bacteria species,these enzymes of the mesophilles are thermolabile and have inability to withstand high temperature, hence find limited commercial applications. Though operational stability of these enzymes can be increase either by immobilization or using biphasic system. However in last some decades these enzymes have been successfully used for the production of acrylamide at large scale and recently nicotinic acids in small scale production. (Rezende and Dias, 2001)

Recently, some of the examples of nitrile transforming enzymes from thermophilic micro-organisms had been reported. However, in the course of the past 5 years scientist have isolated and carried detailed structural and functional characterization of both nitrile hydratase/amidase and nitrilase enzymes from moderately thermophilic bacteria. While showing the higher degree of thermostability expected of thermophile-derived enzymes, they share functional and structural similarity to their mesophilic counter parts. The typical pattern of specificity is retained, where the nitrile hydratases are apparently restricted to aliphatic substrates while the nitrilases show broad specificity with a preference for aromatic or heterocyclic nitriles. (Harper *et al.*, 1985)

3.1.2. TYPES OF REACTIONS CATALYZE BY NITRILASE SUPER FAMILLY

Nitrilase super family of the enzymes carries out four type of the reaction which are Shown in figure 3. It was found that some of the microorganisms might be having more than one branch of the thirteen super families. (Pace *et al.*, 2001)

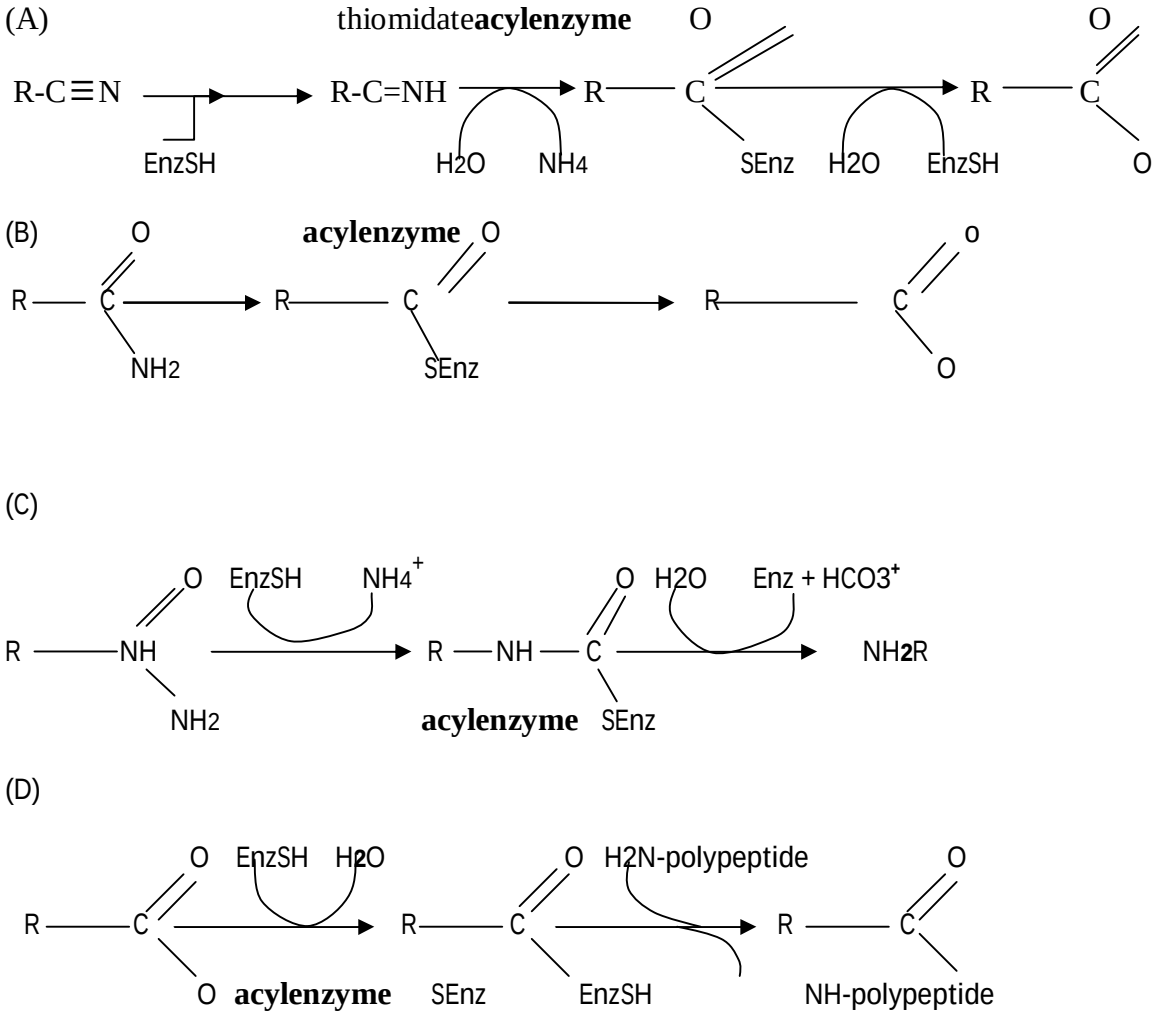


Figure 3: Types of reactions catalyze by nitrilase super family (Pace *et al.*, 2001)

3.2. BIOSYNTHESIS OF NITRILE COMPOUNDS

In the nature the cynogenic glycosides, a most common type of nitriles are widely dispersed. Two multifunctional UDPG-glucosyltransferase and cytochromes P450 enzymes catalysed the biosynthesis of cyano glycoside and in this pathway two intermediate (agylocones and α-hydroxynitriles) are deriverd from the amino nitriles. (Moller and Bjarnholdt 2008) (Figure – 4)

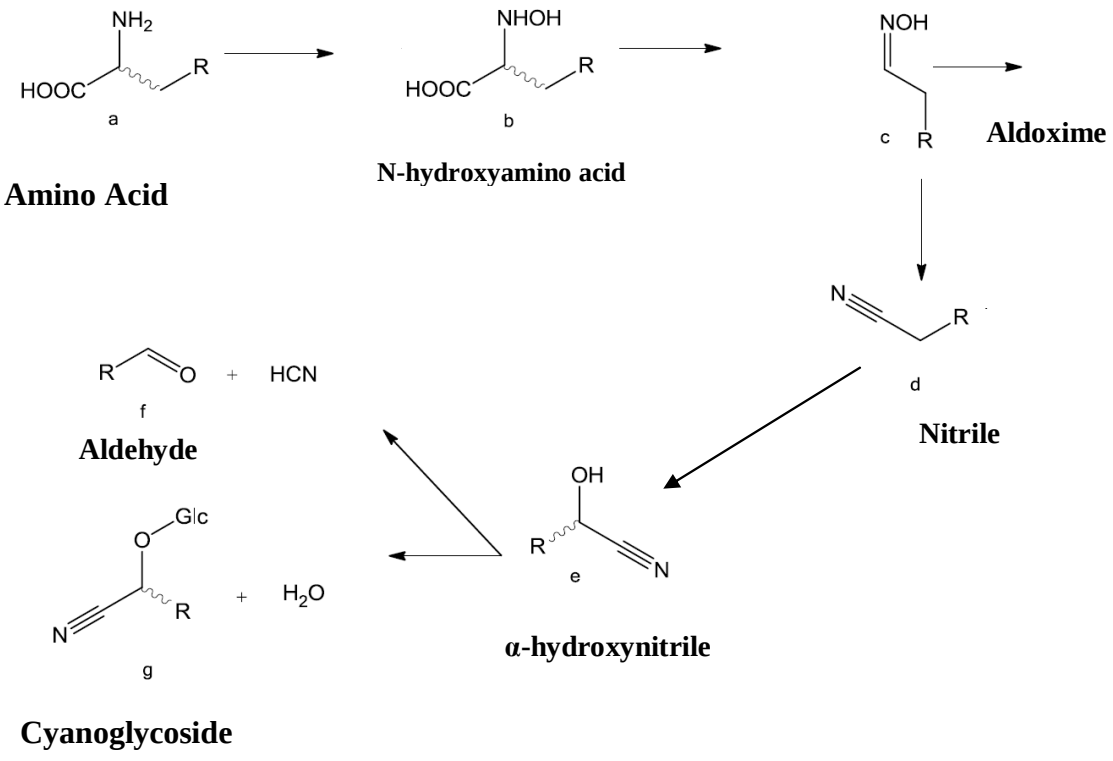


Figure 4: biosynthesis of nitrile compounds. (Moller and Bjarnholdt, 2008)

The biosynthesis of glucosinolates which are mostly decomposed into nitriles or isothiocyanates is comprised of three parts,

1) Elongation of the amino acid
2) Conversion into a parent glucosinolate, including conjugation with cysteine as the sulfur Donor
3) Secondary modification of the parent glucosinolate (Wittstock and Halkier, 2002).

3.3. NITRILASES

First nitrile-metabolizing enzyme (nitrilase) was discovered nearly 55 years ago in 1964, and it converted indole-3-acetonitrile into indole-3-acetic acid which is auxin a growth hormones in plants for root production (Thimann and Mahadevan *et al.*, 1964). Nitrilases (EC 3.5.5.1) are thiol group containg enzymes that hydrolyze nitriles into the corresponding carboxylic acids and ammonia and after release of free ammonia liberate free active enzyme. Now a day's

severalbacterial nitrilases (Turner and O'Reilly 2003) and plants nitrilase (Piotrowski *et al.,* 2001; Fernandes *et al.,* 2006) have been identified. The presence of the nitriles hydrolyzing enzymatic activity occurs in 3 out of 21 plant families (*Musaceae, Cruciferae, and Gramineae*) (Mahadevan and Thimann 1964) and also in a limited number of fungal genera (*Fusarium, , Penicillium Aspergillus*) (Harper 1977) that shows the relative rarity of this activity. Nitrile-hydrolyzing activity is more common in bacteria; still without broad screening it is almost difficult to judge the actual distribution frequency.

*Arthrobacter, Klebsiella, Acinetobacter, Corynebacterium, Pseudomonas, Nocardia, Rhodococcus,etc,*are identified bacteria which are capable of transforming nitrile to the acid and ammonia and they utilize nitriles as nitrogen and carbon source however their role in bacteria is unclear. While in plants, these activities are associated with metabolism of the nutrients, especially in the degradation of glucosinolates (Bestwick *et al.,* 1993) and in the formation of indole acetic acid as growth factor (Bartel and Fink 1994). Cyanide detoxification in some higher plants, also essentially utilize nitriles hydrolyzing pathway (Piotrowski *et al.,* 2001).

Almost all nitrilase enzymes are exists as homooligomers peptide protein and having monomer size of 40kDa. However also it has also been observed in *Nocardia* sp. (of NCIB 11216) the monomers individual subunits have 47 kDa and these different subunits of nitrilase self-associate to convert the enzyme to the active form. After association, nitrilase produce a 560 kDa dodecamer complex enzyme due to benzonitrile-induced activation (Harper 1977b).The enzymatic activity is depends on assembly of these subunits, pH, temperature, inducer concentration and in some instance substrates concentration (O'Reilly and Turner 2003).

Members of the nitrilase superfamily occur in both prokaryotic and eukaryotic species. Nitrilases are characterized by monomers subunits structurally having a conserved αββα-fold which combine in a regular fashion to form dimers. In different members of the super family, these dimers associate in different ways to form oligomeric complexes. In nitrilase these form of association of subunits often having spirals or helical structure to make complex. (Pace and breneer.,.2001)

An additional feature of the nitrilase are the presence of conserved amino acids Cys, Glu, Lys (catalytic triad) which involve in covalent catalysis, in which the substrate binds to the cysteine residues present at the catalytic site of the enzyme (Brenner *et al.,* 2002). Recently it has been suggested that this triad includes an extra, structurally conserved Glu residue which is not instantly apparent from sequential conservation, in this manner forming a catalytic tetrad (Kimani *et al.,* 2007; Thuku *et al.,* 2009).

It was observed that the releases of ammonia and amides after the formation of tetrahedral intermediate depends on the hydrolysis of thioimidate as if the bond between the carbon and nitrogen atom hydrolyze then the formation of ammonia takes place but if the bonds between the carbon and the sulphur atom hydrolyze then the amide will form and it was also concluded that these small differences dependent on rather small differences in the local electronic environment (Jandhyala *et al.,* 2005).

The structural analysis of nitrilase revealed that helices form 30% of the nitrilase structure. All the branches show some sequence similarity and also different classes show specificity to the different substrate. (Turner and O'reilly 2003)

3.3.1. STRUCTURAL PROPERTY OF NITRILASE

Brenner and his colleagues, found the structure of nitrilase enzyme which contains a sandwich folds α-β-β-α, (abba-abba) and conserved catalytic triad (Glu-Lys-Cys) which are essential for the catalytic function of its active site. This result was achieved by Sewell's group to confirm that the nitrilase from the *R. rhodochrous* J1 a 480 kDa complex, a dimer, and an extended helix associate to form homo oligomeric structural (Thuku and Weber 2007). The J1 nitrilase model have showed an extended C shaped-terminus and these enzyme also have two significant insertions that intersperse in the spiral oligomer and lead to spiral extention, at same time 3Dstructures of these helical homo-oligomers of the nitrilase were determined by electron microscopy.

G. pallidus RAPc8 Nitrilase showed different structural forms, as crescent-like, circular, c-shaped through electron microscopy and image classification (Williamson *et al.,* 2010) for determining secondary structure of protein nitrilases from *F. solani* IMI, and *F.solani* O1196840. Circular dichroism (C.D) spectroscopy was used to determine the secondary structure and both

nitrilases structures exhibited almost identical contents in their secondary structure, consisting of 30% of α-helix, 21% of β-sheet, 16% turn, and 33% of other structures (Thuku *et al.*, 2009).

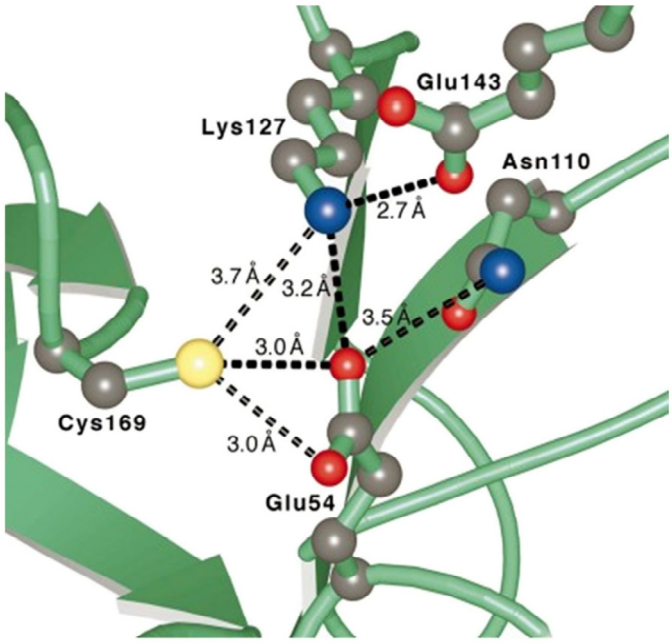


Figure-5structure of nitrilase (Thuku and Weber, 2007)

Most variety of nitrilase found to have multimer structure to perform the function. Usually they are consisting of 4 to 26 subunits and are homopolymers. This association implies the strong hydrophicity of conserved regions. (Goldlust and Bohak, 1994) but in case of nitrilase from *Pyrococcus abyssi* and also from *Klebsiella ozenae* that are specific for bromoxylin functionally active as in dimer conformation dimer (Bhalla *et al.*, 1992; Stalker *et al.*, 1988; Mueller *et al.*, 2006). There are wide ranges of nitrilase reported to have distinct molecular mass of individual subunits in the range of 32-47 (O'Reilly and Turner, 2003). And they are oligomerize to form dimer or multimer complex. (Thuku *et.,al* 2001)

3.3.2. CATALYTIC PROPERTY OF NITRILASE

Now it has been well known that in most cases for the activation of nitrilase enzyme, all subunits of the protein complex self-associate to form an active enzyme. As the monomers a 47 kDa subunit of *Nocardia* species associates to produce a 560 kDa dodecamer active complex due

to benzonitrile -induced activations. Also this association could be enhance by increasing temperature and salt concentration. (Harper *et al.*, 1977)

Nitrilase of *R.rhodochrous* sp. when it incubated in substrate get converted into activated forms by association of nitrilase subunits to form a catalytically active complex hence substrate presence can also influence the activation of nitrilase enzyme (Stevenson *et al.*, 1992). The presence of high enzyme concentration, salt and organic solvents can cause activation of nitrilases. (Nagasawa *et al.*, 2000)

In the presence of salt and solvents the hydrophobic patches get exposed and cause association of monomers to form active enzyme. Nitrile hydratase requires metal ions as prosthetic group to perform the action however nitrilases do not requires any cofactor like metal ions. Meanwhile nitrilases were reported to have catalytically important cysteine residues at catalytic site, or near the catalytic site (Kobayashi et al., 1992). In *R. rhodococcus* K22 nitrilase (figure-5) one conserved cys residue at 170th position was found in the catalytic site (Kobayashi *et al.*, 1992).

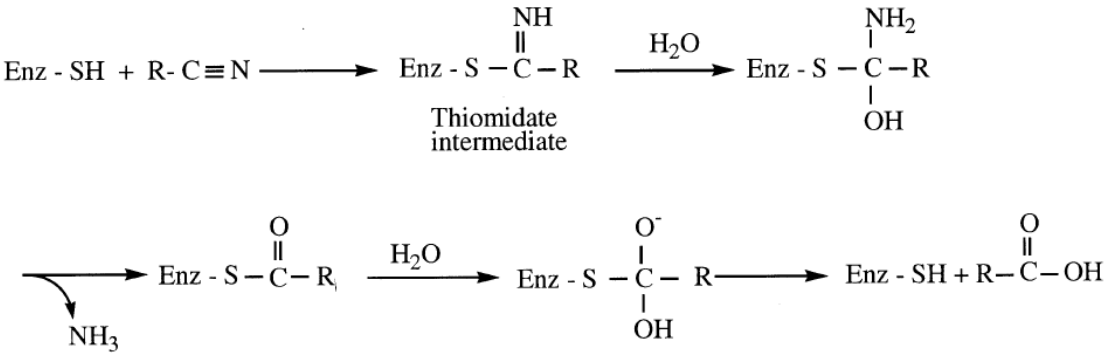


Figure 6 - catalytic mechanism of nitrilase (kobayashi *et al.*, 1998)

3.3.3. MECHANISM OF CATALYTIC REACTION

In the year when the first nitrilase was identified Robinson and Hook proposed that in the hydrolysis of ricinine follow same mechanism given by Stevenson *et al.*, (1992) and was catalyzed by the ricinine nitrilase (Hook and Robinson, 1964) This proposed mechanisms have been extensively accepted by many researchers till date (Banerjee and Sharma 2004), however, in recently reported study amide compound also formed when the nitrilase catalyzed the

hydrolysis of nitriles (Piotrowski *et al.*, 2006). Acids and ammonia is a usual end product of the nitrile hydrolysis by the nitrilase enzyme but in some cases amides can also forms as byproducts.

Hydrolysis of the nitriles as a substrate start with nucleophilic attack of thiol group of the cysteine residue on the carbon atom of a nitrile [a]; a tetrahedral thioimidate, enzyme-linked intermediate is formed (by the action of water molecule to the thiomidate intermediate results in release of free form of NH3 [b & d]. For elimination of the free ammonia nitrogen atom should be positively charge that occurs by stabilizing the Glu residue. Further addition of water molecules to the reaction ensures the release of free ammonia with regeneration of active enzyme [e & f] (Fernandez *et al.*, 2006).

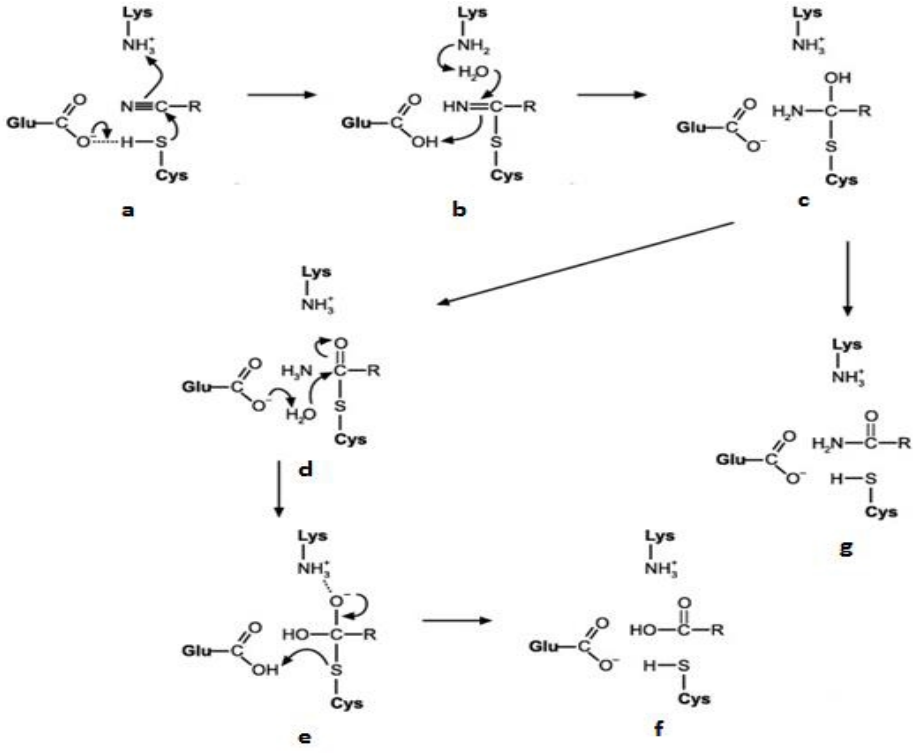


Figure 7- Mechanism of nitrile hydrolysis (Fernandez *et al.*, 2006).

Any reagents containing metal ions like silver or copper strongly inhibit the catalytic activity of nitrilase enzymes. This also means that presence of cysteine at active site is very important to make nitrilase enzyme functional (Layh *et al.*, 1998; Yamamoto *et al.*, 1991). Instead of corresponding acid formation nitrilase can also form amides (**g**). this happens when small positive charge is not present on the reactants such as lysine residue have positively charge

side chain and it plays important role by providing the positive charge to the reactants. The electronic and steric property or interaction of the reactants to the catalytic site of the enzyme decides the production of amide (amount). The amount of amide is higher when the α-substituent is electron-deficient. Also the amount of amide from the (S)-enantiomer of (R,S)-mandelonitrile is higher than that from the (R)-enantiomer (Fernandes *et al.*, 2006).

A high amount of amide is produced by plant nitrilases. A mixture of asparagine and aspartic acid is usually formed from β-cyano-L-alanine (a common substrate of plant nitrilases) (Piotrowski, and Osswald, 2012).

3.3.4. SOURCE OF THERMOPHILIC NITRILASE

Nitrilase enzymes extensively founds in the nature. Bacteria, plants, and fungi have been reported to have nitrilase which serves various roles in nutritional metabolisms and defense. Microbial nitrilase can be utilized for production of the compound of commercial value. Based upon the substrate specificity nitrilase from the wide variety of source can be categorized into three major classes, namely aliphatic nitrilases, aromatic nitrilases and aryl-aceto nitrilases.

3.3.4.1. Bacteria

The first nitrilase was isolated in 1964 from *Corynebacterium* and had ability to hydrolyze ricinine nitrile.Afterward many microorganisms eg.genera of *Corynebacteriums Nocardia sp., Acinetobacter, Alcaligenes sp., Pseudomonas, Rhodococcus sp* etc were isolated and characterized for the nitrilase activity. (Table-1)

Several nitrilase enzymes have been purified, characterized, immobilized and cloned and expressed in recombinant hosts and utilized in industrial production of commercially important products.

Bacteria –Formation type	**Nature of enzyme**	**Optimum pH /temperature (°C)**	**Stability pH /temperature (°C)**	**Substrate specificity**	**Bacteria –Formation type**
Pseudomonas sp	Inducible	-	-	7.4-8.8 /	NMethyl/ethyl3cyano4 methoxy-2-pyridone
Nocardia sp. NCIB 11216	Inducible	5/60	8 / -	-	Aromatic nitrile
Brevibacterium	Constituti	-	7 / 35	/ below	-

strainR312	ve			30	
Pseudomonas sp. 13	Inducible	4/70	9 / 55	7-11 / below 60	B-Cyano-L-alanine
Acinetobaeter sp. APN	Inducible	-	-	-	αAminonitriles
R. rhodochrous J1	Inducible	8/78	7.6 / 45	-/ 2050	Aliphatic, aromatic nitriles
Comamonastestosteron sp.	Inducible	-	-	-	Aliphatic nitriles
Alcaligenes faecalis JM3	Inducible	8/75	7.5 /45	78 / 2050	Arylacetonitriles
*Acinetobacter sp.*AK226	Inducible	5/58	8 / 50	5.88 / below 60	Aliphatic, heterocyclic nitriles
A. faecalis ATCC 8750	Inducible	7.5 /45	6.5	8 / below 50	Arylacetonitriles
R. rhodochrous PA34	Inducible	7.4/45	7.5 / 35	/ below 35	Aromatic, aliphatic nitriles
R. rhodochrous K22	Inducible	6/65	5.5 / 50	/ below 55	Aliphatic nitriles
Klebsiell aozaenae	Constitutive	7/37	9.2 / 35	-	Bromoxynil
Bacillus pallidus Dac521	Inducible	6/70	7.6 / 65 6	9 / below 65	Aromatic nitriles
R. rhodochrous NCIMB 11216	Inducible	4/50	-	Aromatic nitriles	R. rhodochrousNCIMB 11216
P. fluorescens DSM 7155	Inducible	130	9 / 55	-	Arylacetonitriles
N. globerula NHB2	Inducible	-	-	-	Aromatic, unsaturated aliphatic nitriles
P. putida	Inducible	4/12	7 / 40	6.58 - 8/ below 50	Arylacetonitriles
Pyrococcus abyssi GE5	Inducible	7.4 / 80 4.5	8.5 / 6090	Aliphatic dinitriles	PyrococcusabyssiGE5 –Inducible
Pyrococcus abyssi GE5	Inducible	/60	7.4 / 80 4.5	8.5 / 6090	Aliphatic dinitriles
Acidovorax facilis 72W	Inducible	/570	/ 65	510 / below 60	Aliphatic dinitriles

Rhodococcussp. NDB1165	Inducible		8 / 45	/ below 50	Aromatic and unsaturated aliphatic nitriles
Bradyrhizobium japonicum USDA110	Inducible	4/55	-	-	Mandelonitrile,phenylacetonitrile
P. aeruginosa 10145	Inducible	-	-	-	Aromatic nitriles
Halomonas nitrilicus sp.	Inducible	-	-	-	Arylaliphatic nitriles
Bacillus subtilis ZJB 063	Constitutive	-	-	-	Arylacetonitriles
Alcaligenessp. ECU0401	Constitutive	3/76	8 / 40	/ below 50	Aliphatic and aromatic nitriles
P. fluorescens Pf 5	Inducible	1/38	7 / 45	/ below 65	Dinitriles
Streptomyces sp. MTCC 7546	Inducible	-	-	-	Aliphatic nitriles
Arthrobacternitroguajacolicus ZJUTB06 99	Inducible	-	6.5 / 40	/ below 50	Aliphatic and aromaticnitriles

Table -1 List of nitrilase possessing bacteria (Gong *et al*., 2012; Microbial Cell Factories,)

3.3.4.2. Filamentous fungi

In previous research several fungal strains has been identified to have nitrilase activity. These fungal belong to genera *Gibberella, Penicillium multicolor, Aspergillus niger* and *Fusarium solani,* that found to have transformation activity to convert indole-3-acetonitrile (IAN) into indole-3-acetic acid (IAA). Till now only nitrilase activity of *Fusarium solani* (fungal nitrilase 47 kda) have been explained more accurately to have the ability to degrade 3,5-dibromo-4 hydroxybenzonitrile (bromoxynil) and 3,5-diiodo-4-hydroxybenzonitrile (ioxynil) herbicides (Camper and Hsu, 2004).

3.3.4.3. Yeasts

In yeast, till date no nitrilase activity have been reported however the earlier reported nitrile transforming enzymes from different species of yeast confirmed presence of only nitrile hydrates and amidase. Reported nitrile metabolizing yeast were isolated from the soil, bioreactor that are treated with cyanide and also from the fermented food products. These species includes

*Saccharomyces, Pichia, Candida, Debaryomyces, Williopsis, Geotrichum, Hanseniaspora, Exophiala, Aureobasidium Kluyveromyces, Torulopsis, Rhodotorula*and *Cryptococcus*.

3.3.4.4. Plants

Plant nitrilase have important existence in nitrilase super family. Earlier studies postulated the importance only based upon the transformation of IAN to IAA (auxin) plant hormones required for the root induction results in growth in the plants.However the mechanisms of nitrilase action in the biosynthesis of plant auxin is still unclear. First nitrilase was isolated from the plant (barley) by (Thiman and Mahadevan, 1964). Plants are known for synthesizing nitriles for metabolizing the nutrients for their growth and also are capable to hydrolyze the cyanide or toxic compounds as dense mechanism against herbivores. Nitrilase from the *Brassica rapa* exhibited both aliphatic and aromatic hydrolyzing activity and recent study on nitrilase from chinese cabbage seedlings explains that it can significantly transform IAN.

BACTERIA	
Acido vorax facilis 72W	Gavagan et al. 1999
Acinetobacter sp. (strain AK226)	Yamamoto and Komatsu 1991
Alcaligenes faecalis ATCC 8750	Yamamoto et al. 1992
Alcaligenes faecalis JM3	Nagasawa et al. 1990
Bacillus sp. strain OxB-1	Kato et al. 2000
Bacillus pallidus Dac521	Almatawah et al. 1999
Comamonas testosteroni	Levy-Schil et al. 1995
Klebsiella pneumoniae ssp	Kobayashi et al. 1989
Pseudomonas	Hook and Robinson 1964
Pseudomonas sp. (SI)	Dhillon et al. 1999
Pseudomonas DSM 7155	Layh et al. 1998
Nocardia (Rhodococcus) NCIB11216	Harper 1977
b Nocardia (Rhodococcus) NCIB11215	Harper 1985
Rhodococcus rhodococcus K22	Kobayashi et al. 1990
Rhodococcus ATCC39484	Stevenson et al. 1992
Rhodococcus rhodochrous PA-34	Bhalla et al. 1992
Rhodococcus rhodochrous J1	ozaenae Stalker et al. 1988
FUNGI	
Fusarium oxysporum f.sp.	melonis Goldlust and Bohak 1989
Fusarium solani IMI196840	Harper 1977a
Fusarium oxysporum	

Cryptococcus sp. UFMG-Y28	
Aspergillus niger	
Penicillium multicolor	
Exophiala oligosperma R1	
PLANT	
Arabidopsis thaliana	Piotrowski et al. 2001 Osswald et al. 2002
Barley	Mahadevan and Thimann 1964
Chinese cabbage (Brassica rapa)	Rausch and Hilgenberg 1980

Table 2- Distribution of nitrilase producing organism in nature

3.3.5. CLASSIFICATION OF NITRILASE

Based upon the substrate specificity, nitrilase can be categorized into three major classes, namely aliphatic nitrilases, aromatic nitrilases and arylaceto nitrilases.

BACTERIAL SP.	**NATURE OF ENZYME**	**NITRILES NATURE CATAGORIES**
*R. rhodochrous*J1	Inducible	Aliphatic, aromatic nitriles
R. rhodochrous PA34	Inducible	Aromatic, aliphatic nitriles
N. globerula NHB2	Inducible	Aromatic, unsaturated aliphatic nitriles
Rhodococcus sp. NDB1165	Inducible	Aromatic and unsaturated aliphatic nitriles
Alcaligenes sp. ECU0401	Constitutive	Aliphatic and aromatic nitriles
*Arthrobacternitrogua jacolicus*ZJUTB06 99	Inducible	Aliphatic and aromaticnitriles
Nocardia sp. NCIB 11216	Inducible	Aromatic nitrile
Bacillus pallidus Dac521	Inducible	Aromatic nitriles
P. aeruginosa 10145	Inducible	Aromatic nitriles
Comamonas testosteronisp.	Inducible	Aliphatic nitriles

R. rhodochrous K22	Inducible	Aliphatic nitriles
Pyrococcus abyssi GE5	Inducible	Aliphatic dinitriles
*Acidovorax facilis*72W	Inducible	Aliphatic dinitriles
*Bradyrhizobium japonicum*USDA110	Inducible	Mandelonitrile, Phenylacetonitrile
Halomonasnitrilicus sp. nov.	Inducible	Arylaliphatic nitriles
A. faecalis JM3	Inducible	Arylacetonitriles
A. faecalis ATCC 8750 –	Inducible	Arylacetonitriles
P. fluorescens DSM 7155	Inducible	Arylacetonitriles
P. putida	Inducible	Arylacetonitriles
Bacillus subtilis ZJB 063	Constitutive	Arylacetonitriles
Halomonas nitrilicus sp. nov.	Inducible	Arylaliphatic nitriles

Table 3- List of nitrilase producing organisms and their corresponding substrate.

3.3.6. CHARACTERIZATION OF NITRILASE

Many nitrilase have been reported that can be characterized on the basis their substrate specificity, their optimum and maximum temperatures and pH, molecular weight number of subunits etc. (Kobayashi *et al.,* 1998).

3.3.6.1. SUBSTRATE SPECIFICITY

Nitrilase from various sources show varied substrate specificity some are specific for aliphatic substrate, while other shows aromatic or aryl acetonitrile, enantioselectivity. The first nitrile-converting enzyme was found in barley leaves in the 1960s (Thimann and Mahadevan 1964). This nitrilase catalyzed the conversion of 3-indolylacetonitrile to 3-indolylacetic acid – a plant auxin. It was later found that 3-phenylpropionitrile is a preferred substrate for this enzyme, where the product of this reaction, 3-phenylpropionic acid, also functions as a plant auxin (Vorwerk *et al.,* 2001).

Based on the substrate specificities nitrilase classified in to different subclasses eg. aromatic nitrilase occur in nitrilase *rhodococcus* J1 and in the fungi, *Aspergillus, Penicillium* etc. (Kobayashi *et al.,* 1989; Kaplan *et al.,* 2011). Similarly aliphatic nitrilase found in *Acinetobacter,*

Comamonas, Acidovorax, Pseudomonas, (Martínková and Křen, 2010) Arylacetonitrilases were commonly found in *Alcaligenes, Pseudomonas, Halomonas, Arthroderma, Nectria,* etc. These enzymes usually act enantiospecifically (O'Reilly and Chmura *et al.*, 2008; Veselá *et al.*, 2013)

A bromoxynil (3, 5-dibromo-4-hydroxybenzonitrile) specific nitrilase was found in *Klebsiella* and *Pneumoniae*sp. ozaenae (Stalker *et al.,* 1988). In terms of amino acid sequence similarity, nitrilases are closely related to fungal cyanide hydratases and bacterial cyanide dihydratases. However, in contrast to nitrilases which have broad substrate specificities, cyanide hydratases and dihydratases exhibit a strong preference for hydrogen cyanide. (O'Reilly and Turner, 2003)

Table 4:Optimum pH and maximum pH stability of nitrilase

Optimum pH	**pH ranges**	**ORGANISM**	**COMMENTARY**	**REFERENCES**
7.4	5-10	*Fusarium solani*	pH 5.0: about 40% of maximal activity, pH 10.0: about 75% of maximal activity	Yamamoto *et al.,* 1990
6	5-10	*Fusarium solani*	-	Christian H *et al.*,2001
6.8	4-9	*Pseudomonas fluorescens*	over 50% of maximal activity within this range	Dufour and Storer, 1995
5.5	5-8	*Rhodococcus sp.*	stable between	Nagasawa and Shimizu 1993
6	5-11.5	*Fusarium solani*	pH 6.0: about 55% of maximal activity, immobilized enzyme, pH 11.5: about 50% of maximal activity	Prepechalova and Martinkova 2001
6.5	9.5	*Fusarium solani*		Kruse. and Mellon1953
6.7	6.5-8.8	*Brassica rapa subsp. pekinensis*	half-maximal activity	Fawcet *et al.*,1960

7	6-8.6	*Alcaligenes sp.*	the nitrilase activity was low at pH less 7.0 or pH above 8.6	Kaul and Banerjee 2004
7	5-9	*Fusarium solani*	-	Mana and Spohn. 2000
7.2	8.5	*Aspergillus niger*	-	Kaul and Banerjee 2004
7.8	9.1	*Fusarium solani*	optimal activity within	Reymon *et al.*, 2004

3.3.6.2. Substrate Specificity

Substrate	**Products**	**Microorganism**	**References**
Mandelonitrile + 2 h2o	Mandelic acid + NH3	*Bradyrhizobium japonicum* Sulfolobus solfataricus *Pseudomonas fluorescens* *Rhodococcus rhodochrous* *Pseudomonas putida*	Fernandes and mateo, 2006 Hardy *et .,al*1986 Kaplan e*t.,al* bezouška, raczynska *et.,al* 2001 Bork and koonin, (1994)
(Methylthio) acetonitrile + h2o	(Methylthio) indole-3-acetic acid + NH3	*Arabidopsis thaliana* *Synechocystis sp.*	kobayashi *et.,al* 2006
(Phenylthio) acetonitrile + h2o	(Phenylthio) indole-3-acetic acid + NH3	*Arabidopsis thaliana* *.Acinetobacter sp.* *Pseudomonas sp.* *Rhodococcus sp.*	Wyatt. And linton (1988) Yanaka et.,al 2001 Nagasawa et.,al 1992 Kaul and banerjee 2006

Acrylonitrile + h2o	Acrylic acid + NH3	*Fusarium solani* *Fusarium oxysporum* *Halomonas sp. Alphach3* *Pseudomonas sp.* *Pseudomonas sp.* *Rhodococcus rhodochrous* *Rhodococcus rhodochrous*	Stelkes. And kula, (1995) Patricelli and cravatt (2000) Yamada and beppu (1991) Conn et.,al (1981) Tany and yamada, (1982) Zalkin and smith, (1998)
Benzonitrile + h2o	Benzoic acid + NH3	*Bradyrhizobium japonicum* *Hordeum vulgare* *Pseudomonas fluorescens* *Rhodococcus rhodochrous* *Pseudomonas sp.* *Pseudomonas putida* *Bradyrhizobium japonicum* *Hordeum vulgare* *Pseudomonas fluorescens*	Faber *et.,al* (1995) Banerjee *et al.,* 2006 Kobayashi *et al.,* 1998 Lévy-schil *et al.,* 1989) Soubriere *et al.,*1995 Nagasawa *et al.,* 2004 Nakamura *et al.,* 2001 Yamada *et al.,*1990 Pace *et al.,* 1992
Phenylglycinenitrile + 2 h2o	Phenylglycine + NH3	*Sulfolobus solfataricus* *Pseudomonasfluorescens*	Janowitz, and trompetter 2009 Bhalla *et.,al* 2009
Ricinine + h2o		*Pseudomonas sp.*	Pace and brenner,

			(2001)
Succinonitrile + 2 h2o	Succinic acid + NH3	*Alcaligenes faecalis*	Nakai and hasegawa, 2000
Succinonitrile + h2o	Succinate + NH3	*Rhodococcus rhodochrous*	O'reilly *et.,al* 2003

Table 5- substrate specificity of nitrilase

3.3.6.3. Nitrilase inducers

Nitrilases are in general inducible enzymes, which can be induced by substrates or their analogues (apart from toxic nitriles such as mandelonitrile). For instance, acetonitrile induced nitrilase expression in *Fusarium oxysporum* (Goldlust and Bohak 1989); isobutyronitrile or isovaleronitrile in *Rhodococcus rhodochrous* J1 (Nagasawa *et al.*, 1988); propionitrile in *Rhodococcus* sp. NDB 1165 (Prasad *et al.*, 2007) or benzonitrile in *Pseudomonas aeruginosa* 10145 (Alonso *et al.*, 2008) have been reported.

Some constitutive nitrilases have also been found such as those from *Klebsiella ozaenae, Bacillus subtillis* ZJB-063 or *Rhodococcus rhodochrous* J1 (Stalker *et al.*, 1988; Zheng *et al.*, 2008; Kobayashi *et al.*, 1989). 2-Cyanopyridine seems to be a good nitrilase inducer for filamentous fungi, such as those from *Aspergillum, Fusarium* or *Penicillium* (Martínková *et al.*, 2009). In contrast, isovaleronitrile and ε-caprolactam act as powerful nitrilase inducers in rhodococci strains (Nagasawa *et al.*, 1990).

Table 6: Nitrilase optimum temperature and temperature stability

Optimum temperature	Temperature stability	ORGANISM	COMMENTARY	References
40	30-45	*Fusarium oxysporum*	-	Garnerand Ramakanth, (1986)
40	25-50	*Rhodococcus fascians, Rhodococcus rhodochrous*	-	Davies and Ichihara 1994
40	30-45	*Arthrobacter sp., Fusarium oxysporum f. sp. melonis, Rhodococcus rhodochrous*	-	Theil, F. and Ballschuh, S. (1996)
40	35-50	*Acidovorax facilis*	in vivo assay, recombinant cells	Ojika and Yamada (1993)
44	30-55	*Alcaligenes faecalis*	strain JM3	J and Willetts (1998)
45	40-65	*Rhodococcus rhodochrous*	recombinant nitrilase	Stolz and Knackmuss, (1997)
45	35-58	*Aspergillus niger, Bradyrhizobium japonicum*	-	Bisp and Ingvorsen (1996)
45	40-60	*Fusarium solani*	-	J. P. and Bull, A. T. (2002)
45	40-55	*Alcaligenes sp.*	-	A. M. and Leisinger, T. (1987)
50	40-60	*Acinetobacter sp.*	-	R and Cowan (1999)

55	45-65	*Rhodococcus rhodochrous*		

3.3.6.4. MOLECULAR WEIGHT

Almost all nitrilase enzymes are exists as homooligomers peptide protein and having monomer size of 40kDa. However also it has also been observed in *Nocardia* sp. NCIB 11216 the monomers individual subunits have 47 kDa. All known fungal nitrilase are high molecular weight protein typically more than 400 kDa (O reilly and turner, 2003) and therefore the purification method of choice is gel filtration (Thuku *et al.,* 2009). There are wide ranges of nitrilase reported to have distinct molecular mass of individual subunits in the range of 32-47 (O'Reilly and Turner, 2003)

MOLECULAR WEIGHT OF SUBUNITS	**SUBUNITS**	**ORGANISM**	**COMMENTARY**	**REFERENCE**
43000	Homodecamer	*Pseudomonas putida*		Banerjee and kaul, 2006
34000	Homodecamer	*Bradyrhizobium japonicum*		Rantwijk and Sheldon, (2004)
37600	Decamer	*Alcaligenes sp.*	recombinant enzyme, gel filtration	Dube and Peterson, (2006)
41000	Dimer	*Rhodococcus rhodochrous*	strain J1	Effenberger and Bohme, (1994)
41200	Dimer	*Rhodococcus rhodochrous*	sedimentation equilibrium analysis, after recovery conditions for hydrolysis of acrylonitrile	Beard, and Page, (1998)

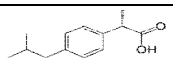
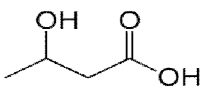
42000	Homodecamer or homoundecamer	*Brassica napus*	-	Horinouchi, and Beppu, (1998)
44000	Homododecamer	*Arabidopsis thaliana*	gel filtration	Sharma, and Banerjee, (2003)
45000	Homododecamer	*Arabidopsis thaliana*	-	Harper et.,al 1977
46000	Decamer	*Alcaligenes faecalis*	strain ATCC 8750	Theil, and Ballschuh, (1996)
48000	Dimer	*Rhodococcus rhodochrous*	gel filtration	Parratt, and Willetts, (1998)
55000	Homotetradecamer	*Fusarium oxysporum*	-	Bisp and Ingvorsen (1996)
55000	Homotetradecamer	*Fusarium oxysporum f. sp. melonis*	-	Hardicre, and Bull, (2001)
55000	Homooligomer	*Fusarium solani*	gel filtration	
56000	Dodecamer	*Nocardia sp.*	gel filtration, substrate catalyzedassociation of inactive monomers	Goldlust and Bohak, (1989)
56000	Dodecamer	*Nocardia sp.*	strain NCIB11216 and strain NCIB11215	Harper *et.,al* (1976)
56000	Monomer	*Rhodococcus sp.*	-	Ryuno, and Yamada, (1986)
56000	Homododecamer	*Rhodococcus rhodochrous, Rhodococcus sp.*	-	Takeuchi, and Yamada, (1988)
58000		*Acinetobacter sp.*	-	Horinouchi, and Beppu, (1993)
58000	Homooctamer	*Fusarium solani*	gel filtration	Willson, and

				Benedik, (2003)
58000	Homooctamer	*Fusarium solani*	-	
58000	Homooctamer	*Fusarium solani*	gel filtration	Effenberger, F. and Bohme, J. (1994)
60000	Homotetradecamer	*Aeribacillus pallidus*	-	
60000	Homotetradecame	*Aeribacillus pallidus*	native enzyme, gel filtration	
60000	Homotetradecame	*Aeribacillus pallidus*	-	Takeuchi, K. and Yamada, H. (1991)
62000	Homooctamer	*Fusarium solani*	gel filtration	Horinouchi, and Beppu (1998)
62000	Homooctamer	*Fusarium solani*	-	Turner, and Nelson, (1997)
65000	Decamer	*Rhodococcus rhodochrous*	-	Sharma, and Banerjee, (2003)
65000	Multimer	*Aspergillus niger*	above 650000 Da	Appel, and Bairoch, (2005)

Table 7- Molecular characterization of purified nitrilase enzyme

3.3.7. APPLICATION OF NITRILASE

Nitriles are finding a broad range of uses in industries as solvents, extractants, pharmaceuticals, drug intermediates (chiral synthons), pesticides and herbicides (bromoxynil, ioxynil, etc.), intermediates in the synthesis of amines, amides, esters, heterocyclic compounds, etc. (Banerjee et al., 2002)

PRODUCT	**APPLICATION**	**ENZYME SOURCE**	**STRUCTURE**	**REFERENCE**
Ibuprofen	Inflametory drug	*Acinetobactersp*		Worg *et al.,* 2003
(R)-4-Cyano-3-hydroxybutyric acid	In the treatment of alcoholism and *source* of energy in the heart and brain	*Acinetobactersp*		Worg. *et al.*,2003

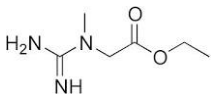
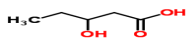
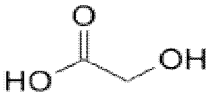
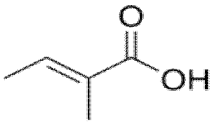
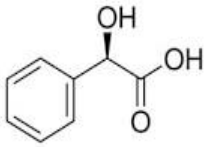
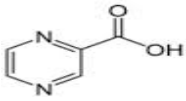
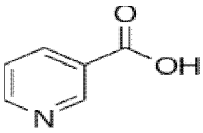
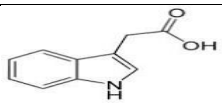
Ethyl ester	Cholesterol lowering drug	*E. coli*		Sosedov and Stalker (1988)
3-Hydroxyvaleric acid	Synthesis of polyester	*Acidovoraxfacilis7* 2W		Wu.s *et al.*,2008
Glycolic acid	In medical andindustrial products	*Acidovoraxfacilis7 2W*		Wu.S *et al.*,2008
(E) -2- Methyl-2-butenoic acid	Used in making perfumes andflavoring agents.	*Acidovoraxfacilis7 2W*		Hann *et al.*, 2004.
R-(-) Mandelic acid	Semisynthetic cephalosporinspharmaceuticals	*Alcaligenesfaecalis ATCC 8750, Pseudomonas putida,Microbacteriumparaoxydansa ndMicrobacterium liquefacians*		Detzel *et al.*,2011
Pyrazinoic acid	Antimicrobial action against Mycobacterium	*R.rhodochorusJ1*		Kobayashi et.,al 1990
Nicotinic acid	Cholesterol-lowering drugs (also help the nervous system function poperly)	Food source protiens		Mathew *et al.*,1998
Indole acetic acid (IAA	Plant growth promotion	*Streptomycesgrise oviridisi*K61		Khamna *et al.*, 2010

Table 8- Application of nitrilase in production of commodity chemicals and fine chemicals.

4. METHODOLOGY: -

4.1. Sample Collection: -

The soil samples were collected into sterile vials from dumping regions of "Kirti Nagar Industrial area, New Delhi; India". Samples were kept at 4ºC till further processing.

4.2. Isolation of thermophilic nitrilase bacteria: -

To 50ml of minimal basal media (KH2PO4 1.0gm, NaCl 1.0gm, MgSO4.7H2O 0.2gm, FeSO4.7H2O 30 mg (per liter) and pH 7.0). 100µlwas added as a sole source of nitrogen and carbon. For inoculation of medium, 1gm of soil was dissolved in 50ml water and after followed by serial dilution 5ml of suspension (after sedimentation) was added to above minimal basal media. Medium was incubated at temperature of 50ºC for 48 hours. After 48 hours the 5 ml of enriched culture mediums was again inoculated in to freshminimal basal medium for 48 hours supplemented with benzonitriles. (Cramp *et al.,* 1997)

Cell suspension from broth medium was diluted in saline and 50 µl of diluted samples were plated on nutrients agar plates [Peptone 1%, Yeast extract 1%, NaCl 0.5%, Glucose 2%, and Agar 3% (per liter)] and incubated at 50ºC for 24 hours. Plates were screened for bacterial growth and isolated colonies were again streaked on fresh prepared nutrient agar plates and incubated at 50ºC for 48 hours the isolated bacterial strains were characterized by Gram's staining method.

4.3. CELL AND COLONY CHARACTERISATION

For characterization of strain of bacterial colonies from streaked nutrient agar plates Gram staining method was performed. Cells were observed under the microscope

Screening of bacterial isolates for nitrilase activity: -

Bacterial colonies from streaked plates were characterized by Gram staining methods and also screened for nitrilase activity through enzyme assay.

4.4 PRODUCTION OF NITRILASE PRODUCING BACTERIA

Selected isolates were streaked on the nutrients agar plates. Single bacteria colony from each plates taken and were incubate in 50ml of LB medium (1% Peptone, 1%Yeast Extract, 0.5%NaCl, 2% Glucose (per litre, pH- 7.0) and incubate for 24 hours.

Single bacterial colony from each plates taken and were inoculated in freshly prepared Seed medium (LB) and grown for overnight after wash 10%v/v of inoculum is added to freshly prepared production medium (KH_2PO4 2.0gm, NaCl 1.0gm, $MgSO_4.7H2O$ 0.01gm, $(NH4)_2SO_4$ 8.6gm $FeSO_4.7H_2O$ 0.01gm and 20mM glucose (per liter) and pH7.0) and LB (1% Peptone, 1%Yeast Extract, 0.5%NaCl, 2% Glucose (per litre) pH 7) 20mM/µl of inducer and incubated at 50ºC for 48 hours. After 48 hours LB and production media containing cells were centrifuged at 1000xg for 10 minutes at 4 ºC. Cell pellets was washed two times with 0.1M potassium phosphate buffer of pH 7.0. After washing cell pellet was suspended in the potassium phosphate buffer of pH 7.0 and OD was taken at 660nm. It was 3.6OD and then these are kept at 4 ºC for the enzyme assay procedure.

4.5. ENZYME ACTIVITY DETERMINATION ASSAY

4.5.1. NITRILASE ACTIVITY AND AMIDASE ACTIVITY ASSAY

Nitrilase activity was determined using a modified phenol-hypochlorite ammonia detection method (Weather *et al.*, 1967). This method is based on the conversion of nitriles to their corresponding acids and ammonia with subsequent quantification of the ammonia. The ammonia reacts with hypochlorite and phenol, catalyzed by sodium nitroprusside, to form indophenol which yields an intense blue compound.

Available substrate acetonitrile, benzonitrile, acrylonitrile, acetamide and acrylamide were chosen for the enzyme essay. In each reaction of 1ml reaction mixture containing 0.1M potassium phosphate buffer (pH-7.0), 100µl cell suspension and 50µl substrate concentration were added and incubated at 50^oC for 15 min incubated. To stop the reaction equal volume of 0.1M HCl was added. In the control, the cell suspension was omitted during incubation and added after the reaction was stopped. These reaction mixtures were centrifuged at 10000 xg. Pellet was discarded and supernatant was collected. To 1 ml of supernatant, 2.5 ml of solution A(1% phenol, 0.005% sodium nitroprusside) was added and kept at room temperature for 5

minutes. Then 2.5 ml of solution B (0.5% sodium hydroxide, 0.02N Sodium hypochlorite) was added. These test tubes were incubated in boiling water bath for 5 minutes. Cooled to room temperature and colour was observed and absorbance was recorded at 640nm (Fawcett and Scott 1960). One unit of enzyme activity was defined as amount of enzyme (cells/protein) that hydrolysed the nitriles to release 1 µmole of NH3 released min^{-1} under assay conditions. The standard curve was prepared using 0.02–0.2 µ mole of NH4Cl/ml in buffer: 0.1M HCl (1:1) mixture.

4.5.2. NITRILE HYDRATASE ACTIVITY ASSAY

The nitrile hydratase activity measured for benzonitrile in the reaction mixture of 1ml containing 0.1 M potassium phosphate buffer (pH-7.0), 100µl cell suspension and 50µl substrate concentration were added and incubated at 10^{o}C for 1 hour. To stop the reaction equal volume of 0.1M HCl was added absorbance was taken at 240 nm.

4.6. DRY CELL WEIGHT

1.5 ml eppendorf tube were placed in hot air oven at 80 °C for overnight meanwhile the lid of eppendorf was open during whole process. These tubes then cooled at the room temperature. Weight of each eppendorf was measured. 1 ml of culture from different cultivation media were taken in to the eppendorf tube. Tubes were centrifuged at 10000 xg for 10 min. supernatant was discarded and then pellet was washed two times with 0.1M phosphate buffer.

Again these eppendorf tubes were placed in to the hot-air oven for overnight at 80 °C and cooled. There after Weight of each labeled tubes were takenand dry cell weight were calculated.

4.7. OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION OF NITRILASE BY *Pseudomonas* sp. KNB-2

To maximize the production of the nitrilases of *Pseudomonas*spKNB-2, several different production conditions were used to screen optimum yield of the nitrilase. Strain was grown on different media of composition, different pH, different inducer, incubation temperature and different inducer concentration. Optimization of the production condition was analyzed through enzyme assay and by measuring biomass concentration.

First step in production optimization was to prepared fresh inoculum by inoculating the primary *Pseudomonas*sp.KNB-2 colonies grown on agar nutrients plate (mother culture) in to LB medium. Inoculum was incubated for 24 hours at 50°C and used as seed culture.

4.7.1. Selection of suitable production medium for nitrilase of *Pseudomonas* KNB-2

To select the suitable media for high biomass and high nitrilase activity in KNB-2, eight different media were freshly prepared, incubated with 10%v/v seed culture of *Pseudomonas*sp.KNB-2 and induced with 0.20% (v/v) benzonitrile. These cultures were incubated at 50°C for 24 hours. After 24 hours Dry cell weight was measured and nitrilase activity was checked for each mediums

MEDIA	COMPOSITION	REMARKS
1. Luria-Bertani (LB)	0.5% (w/v) yeast extract	Autoclave at 121°c. For 20 mints then stored at 4 °c. or directly put in laminar for further use
	1.0% (w/v) peptone	
	1.0% (w/v) nacl	
	2.0% (w/v) glucose	
2. Minimal Basal Media (MAB)	(NH4)SO4 0.16% (w/v)	for growth of the bacteria contain nitriles as sole source of carbon and nitrogen
	$MgSo_4$ 0.025% (w/v)	
	$CaCl_2$ 0.005% (w/v)	
	KH_2PO_4 0.05% (w/v)	
3. M1	KH_2PO_4 0.2% (w/v)	
	NaCl, 0.1% (w/v)	
	$MgSO_4 \cdot 7H_2O$, 0.001% (w/v)	
	$(NH4)_2SO_4$, 0.86% (w/v)	
	$FeSO_4 \cdot 7H_2O$ 0.01% (w/v)	
	Sodium succinate, 20mM or	

	Glucose 0.18% (w/v)	
4. M2	Glycerol 1.0% (w/v)	
	KH_2PO_4 0.05% (w/v)	
	K_2HPO_4, 0.05% (w/v)	
	$MgSO_4 \cdot 7H_2O$, 0.01% (w/v)	
	Yeast extract, 0.1% (w/v)	
	Peptone 5% (w/v)	
5. M3	Glycerol, 1.0% (w/v)	
	Peptone,0.5% (w/v)	
	Yeast extract, 0.3% (w/v)	
	Malt extract, 0.3% (w/v)	
6. M4	Tryptone 3.0% (w/v)	
	Yeast extract 1.5% (w/v)	
	NaCl 0.5% (w/v)	
7. M5	Glucose, 1.5% (w/v)	
	Peptone, 0.5% (w/v)	
	Yeast extract, 0.3% (w/v)	
	Malt extract, 0.3% (w/v)	
8. M6	Yeast extract, 1.5% (w/v)	
	NaCl, 0.5% (w/v)	
	Tryptone, 3.0% (w/v)	
	Glucose, 0.2% (w/v)	

Table 9: Compositions of media screened for nitrilase production by *Pseudomonas Sp.* KNB-2

4.7.2. EFFECT OF NITRILES AND AMIDES ON NITRILASE PRODUCTION

To identify the hyper inducer for nitrilase vis-à-vis growth of *Pseudomonas* sp. KNB-2, various nitriles (Acetonitrile, benzonitrile, and acrylonitrile) and available amides (acetamide and acrylamide) at a concentration of 0.10% (v/v) and0.20% (v/v) respectively were used as inducer in 50 ml of M1 production media (pH-7). Different induced medium were inoculated with 24 hours old precultured seed medium (10% v/v). Culture medium was incubated at 50°C for 24 hours. After 24 hours DCW was measured and harvested cells were screened by nitrilase assay.

4.7.3. EFFECT OF pH ON THE NITRILASE PRODUCTION

To study the effect on the growth of *Pseudomonas* sp.KNB-2 different alkaline and acidic media was prepared by adjusting their pH ranges from pH 3 to pH 11. These medium was inoculated with 10% (v/v) seed culture (24 hours). All these cultureswere supplemented with

0.10% v/v benzonitrile as an inducer and incubated at 50°C for 24 h and the nitrilase activity was assayed.

4.7.4. EFFECT OF INCUBATION TEMPERATURE ON NITRILASE PRODUCTION

Selected media with pre optimize media in previous steps was used to for the cultivation of the *Pseudomonas* sp.KNB-2 at varied incubation temperature ranges of 30-60ºC. After 24 hours the cells were harvested and enzyme assay was performed.

4.7.5. EFFECT OF INDUCER CONCENTRATION ON AMIDASE PRODUCTION

To study the effects of inducer concentration on the nitrilase activity inducer used in the range of 0.1% (v/v), 0.2% (v/v), 0.3% (v/v) 0.4% (v/v) 0.6% (v/v) and 0.8% (v/v) inoculated into the M1 medium. These cultures were incubated at 50 ºC for 24 hours. Cells were harvested and enzyme assay was performed.

4.8. OPTIMIZATION OF REACTION CONDITIONS FOR NITRILASE ACTIVITY (WHOLE CELLS)

4.8.1. EFFECT OF BUFFERS SYSTEM AND pH

Different buffers system with range ofpH 3 to pH 11 were prepared and used for the identification of the optimum pH for the reaction condition. Nitrilase assay was carried out in such various buffer systems which are as sodium acetate buffer (pH3 to pH6), phosphate buffer (pH7 to pH 9), and sodium hydroxide buffer (pH10 and pH11).

4.8.2. SUBSTRATE SPECIFICITY

Specificity towards different substrates for nitrilase of *Pseudomonas* sp. KNB-2 was screened for aromatic nitriles like (benzonitriles) aliphatic nitriles like (acetonitrile and acrylonitrile), aliphatic amides like (acetamide and acrylamide). Nitrilase activities were measured by performing assay.

4.8.3. EFFECT OF SUBSTRATE CONCENTRATION ON NITRILASE ACTIVITY

Different concentrations of benzonitrile were used in the range of 15µl to 50 µl in 0.1M Potassium phosphate buffer (pH 7.0) reaction mixture.

Effect of substrate concentration was analyzed by measuring nitrilase activity.

4.8.4. INCUBATION TEMPERATURE

To find out the optimum temperature of nitrilase of *Pseudomonas* sp. KNB-2in reaction condition, nitrilase assay was done in different incubation temperature ranges from 30ºC to 65ºC.Effect on the nitrilase activity was analyzed by measuring nitrilase activity.

4.9. ISOLATION AND CHARACTERIZATION OF NITRILASE OF KNB-2 STAIN.

4.9.1. SONICATION OF *KNB-2* STRAIN

Cells were disrupted through mechanical shearing. Whole cell suspension dissolved in 0.1M K_2HPO_4 buffer solution;containing 0.1 mM of DTT and 0.1mM EDTA were sonicated. Sonication was done for 20 minutes (one cycle, 30 seconds beating and 30 seconds rest)20 of running cycle. Beaker containing cells suspended in buffer solution were kept in ice to provide sufficient cooling.

4.9.2. AMMONIUM SULPHATE PRECIPITATION METHODS

At high concentration of salts proteins aggregate and precipitate from the solution. This technique is known as salting out as at different salt concentration different proteins precipitate hence used for the protein purification.

To express the percent saturation for ammonium sulphate a simple equation is used, grams of ammonium sulphate needed for making x% of solution from X_0%

$$G= 515 (x-x_0)/100-0.27X \{\text{for 1 liter solution at temperature of } 0ºC\}$$

For precipitating the protein, the cell free extract from sonication was taken as sample. 10% ammonium salt was gently added to the sample protein and allowed it for stirring on magnetic

stirrer for 2 hours at 0ºC. Sample was centrifuge at 10,000gx for 10 minutes pellet was collected and supernatant was taken for further stepwise consistent increase salt concentration of 20%, 30%, 40%, 50% and 60% were done.

4.9.3. DIALYSIS

Dialysis is a typical method which use for changing the buffering solution of the protein as well as for concentrating protein solution by dialyzing in hygroscopic environment (Sephadex and PEG). For dialyses, the dialysis begs were activated by boiling in 1mM EDTA solution for removing the manufacturing contaminants. Samples were taken in to the dialysis begs and sealed and further stirred in dialysis buffer (20% PEG solution in 0.1 phosphate buffer) at 0ºC.

4.9.4. PROTEIN CONTRATION DETERMINATION METHODS

Determination of protein concentration was done by Lowry methods (Lowry *et al.*,1951). In this method 0.2 ml protein solution was taken and then 2ml of alkaline $CuSO_4$was added and mixed and kept at room temperature for 10 minutes. After that 0.2 ml of Folin Ciocalteau reagent (1:1 water and reagents) was added and again incubates for 30 minute at room temperature for 30 minutes in dark. Absorbance was taken at 680nm against control that has only 2ml of $CuSO_4$ reagent and 0.2ml of Folin Ciocalteau reagent. (BSA standard was prepared to calculate the unknown protein concentration).

4.9.5. SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE ELECTROPHORESIS (LAEMMLI'S METHOD, 1970) BUFFERS

Reagents

- Acrylamide
- N,N'- Methylene bis acrylamide
- Ammonium persulfate
- N,N,N',N'- Tetramethylethylenediamine (TEMED)
- Tris- hydroxymethylaminomethane
- SDS

- HCl
- Glycine
- Glycerine
- Acetic acid
- Methanol
- Commassie Brilliant Blue G
- Bromophenol Blue
- Protein Marker (Low molecular weight marker)

STOCK SOLUTIONS

Solution A- 30% Acrylamide solution

29.2gm acrylamide
0.8gm bis-acrylamide
Added water to made volume 100ml

Solution B- 1.5 M Tris buffer (pH-8)

Dissolved 18.17g of Tris and 0.4g of SDS in water, adjusted for pH with HCl and madeup to 100 ml

Solution C- 0.5 M Tris buffer (pH-6.8)

Dissolved 0.4gm of SDS and 6.06 gm of Tris in DH_2O, adjusted for the pH 6.8 with HCl and made upto 100 ml.

Solution D- 10% APS

Added 1ml of water to 0.1g of APS (PREPARE JUST PRIOR TO USE)

	Separating gel (12.5%) in ml	Separating gel (10%) in ml	Stacking gel(8%)in ml
Solution A	7.5		0.9
Solution B	4.5		-
Solution C	-		1.5
Solution D	0.07		0.018
TEMED	0.01		0.01
Water	6		3.6

Table 10:Composition of gel solution for a 1mm thick gel

Stock solution-10% SDS solution

10g of SDS in 100 ml water

Sample solubilizing buffer (SSB) (1X)

SDS	10%
Tris-HCl buffer(pH6.8)	10mM
Glycerine	20%
Bromophenol blue	0.05%

Table 11: Composition of sample solubilising buffer

For 10 ml

Tris-HCl (0.1M)	1ml
Glycerol	2ml
SDS	1g
BPB	0.05g
Water	Volume makeup to 10ml

Table 12: composition for 10ml solution

Tris – HCl (pH6.8)

Stock (0.1M) = 100ml

Weighed 1.21g and dissolved it in water, volume made-up to 100ml

Electrophoretic buffer

Tris	0.025M
Glycine	0.192M
SDS	0.1%

Table-13: composition of electrophoretic buffer

Added 10ml of 10% SDS solution to 3g of Tris and 14.4g of glycine and made upto 1000ml with water

Staining solution

CBB	2.5g
Methanol	500ml
Acetic acid	100ml

Table-14: Composition of staining solution

Madevolume upto 1000 ml

Destaining solution

To 250ml methanol, 70ml of acetic acid, and added water to madeup volume to 1000ml.

Gel preparation

1. Glass plates and spacers were cleaned with mild detergent, wiped with 70% ethanol and dried up with tissue.
2. Clamped two plates on casting stand with the help of spacers.
3. Examined the setup for any leakage.
4. Separating gel was poured with the help of micropipette.
5. Water or methanol was immediately poured over it to level the gel.
6. Left for polymerization

7. Drained water.
8. Then after polymerization of separating gel,separating gel was immediately poured
9. Comb was insertedto make wells and allowed it to polymerize.

Sample preparation

1. Samples were prepared in the appropriate volume of 1x sample solubilizing buffer (laemmli buffer) and lysis buffer
2. Heat the sample in boiling water bath for 15minutes (keep the sample at $4^{O}C$ for 1to 2 min and centrifuge at 10,000 rpm for 30sec, take the supernatant in sample).

Sample loading and Electrophoresis

1. Load the protein samples into the corresponding well along with molecular weight marker protein in respective wells.
2. Electrophoretic unit were run at 30mA until the tracking dye were about to leave the separating gel. (Run the gel at 80V, when dye front enters the separating gel, increase the voltage to 100-120V).

1. After that, gel were removed and placed in the staining tray and flooded with staining solution, incubated for 20 minutes.
2. After that staining solution was discarded and gel were kept in destaining solution until bands were clearly visible.(for destaining, after adding solution, keep the tray in incubator ($37^{O}C$).

5. RESULTS AND DISCUSSION.

5.1. Isolation and screening of thermophilic bacterial isolates for nitrilase activity

Samples collected from dumping site of Kirti Nagar New Delhi and were stored at 4°C. Primary isolations were performed by success enrichment method. The soil sample was inoculated in to basal medium at 50°C for 24 day supplemented with different inducer as acetonitrile, benzonitrile, acrylonitrile, acetamide and acylamide. Enrichment in high temperature and nitrile or amides as sole carbon and nitrile (benzonitrile, acetonitrile) was also used for isolation of many nitrile/amide metabolizing strains. *Pseudomonas* sp.KNB-2 was also isolated using enrichment in benzonitrile supplemented mineral salt medium at 50°C. Cells were observed under the microscope that showed the presence of Gram negative bacteria because of appearance of pink and rod shaped cells (fig-9). Many gram negative strains (P*seudomonas, Klebsiella* producing nitrilase were reported in literature. (Perriva *et al.,* 1998; Watanabe *et al.,* 1987; Nawaz *et al.,* 1998; Kaul *et al.,* 2007)

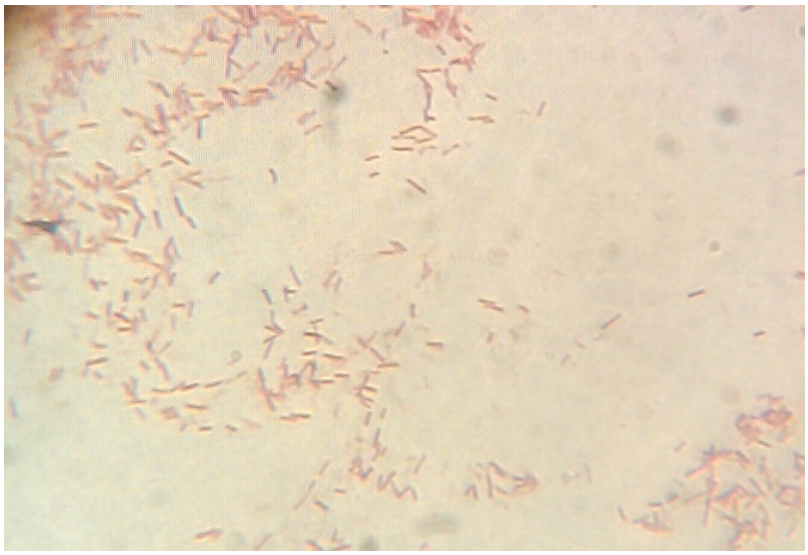


Figure 8: Gram staining of strain showing pink colour and rod shape.

5.2. EFFECT OF NITRILES AND AMIDES ON NITRILASE PRODUCTION

Five different available nitriles and amides were used as the inducer to enhance the production of nitrilase in *Pseudomonas* sp. KNB-2 culture. Highest nitrilase activity was found in the benzonitrile 0.0045µmole/min/mg (Fig-9).

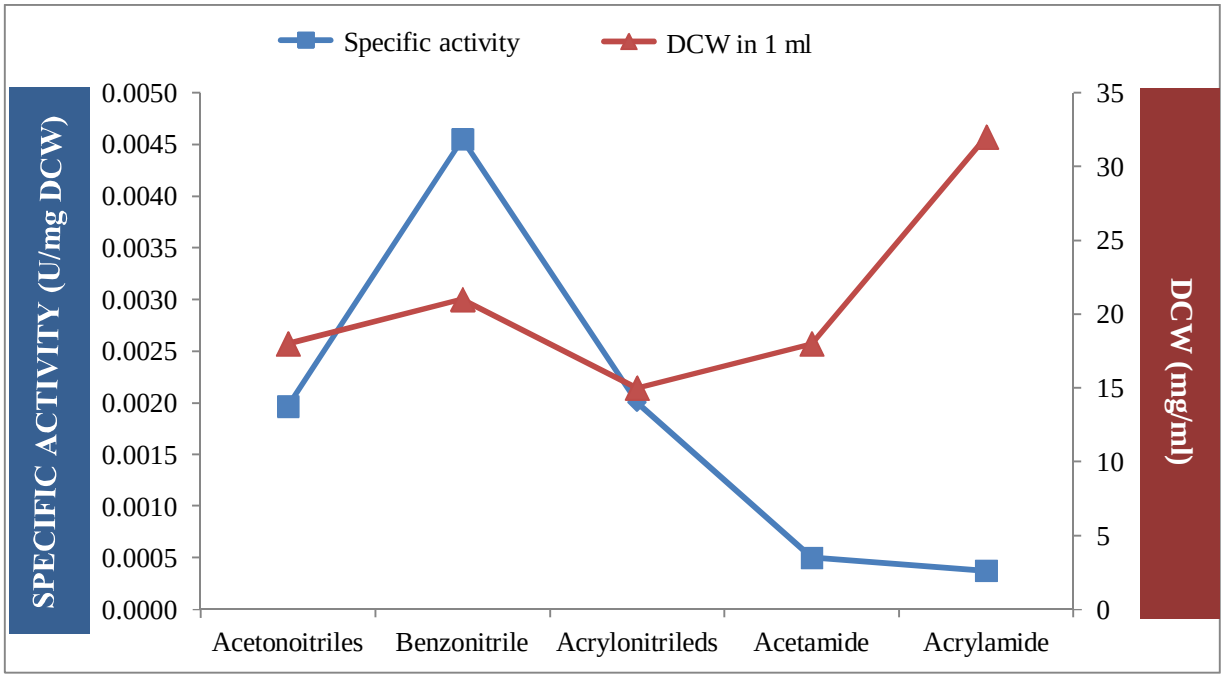


Figure 9: Graph of nitrilase activity in different inducers

Many researchers have reported the isolation of nitrilase producing *Pseudomonas*straines by enrichment in benzonitrile. *Bradyrhizobiumjaponicum*, (Howden and Preston, 2009), *Pseudomonas* (Banerjee *et al.*, 2006),*Hordeum vulgare*(Faber *et.,al*1995), *Pseudomonas putida* (Nagasawa *et al.*, 2004),Pseudomonas fluorescens (Pace *et al.*, 1992)

5.3. OPTIMIZATION OF PRODUCTION CONDITION FOR NITRILASE PRODUCING BACTERIAL STRAIN

5.3.1. Production Medium

Eight different types of media including LB, MINIMAL BASAL MEDIA, M1, M2, M3, M4, M5, and M6 were screen to achieve highest growth of the nitrilase producing bacteria simultaneously to yield highest nitrilase activity. M1 medium found to have maximum nitrilase

activity 0.0071µmole/min/mg. However highest cell growth was observed in LB medium (92 mg/ml DCW) (Fig-10). In M1 medium dry cell weight was 36 mg of DCW/ml of culture medium. Yusuf and Chaubey have reported nitrilase activity in M1 medium in *Fusarium proliferatum* using benzonitrile as inducer (Farnaz Yusuf and Asha Chaubey, 2013).

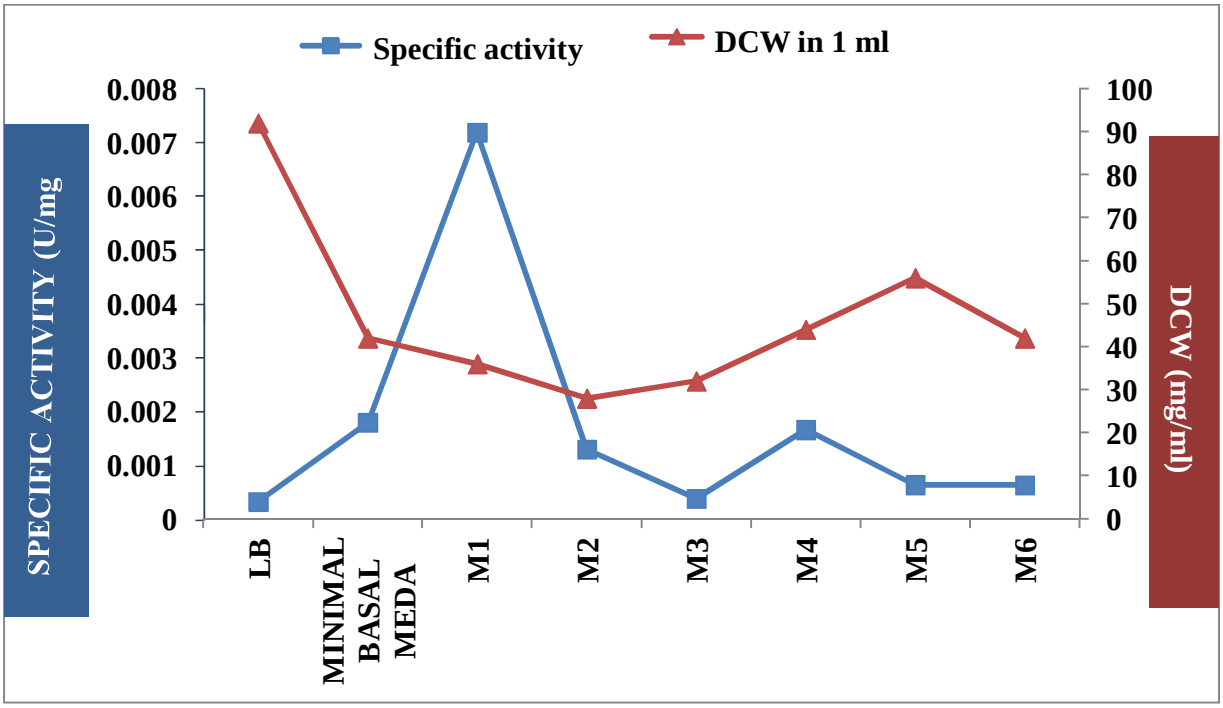


Figure 10: Graph of Specific activity curve in different medium

5.3.2 OPTIMIZATION AT DIFFERENT pH MEDIUM

M1 Medium having different pH is used for cultivation of nitrilase producing strains. It was found that at neutral pH 7 and pH 8 strains have highest enzymatic activity that is 0.0163 µmole/min/mg and 0.014 µmole/min/mg. At pH7 and pH 8 the DCW was measured as 14 and 18mg/ml (Fig-11). While in alkaline pH-10 condition growth was high but it didn't exhibit comparable activity.

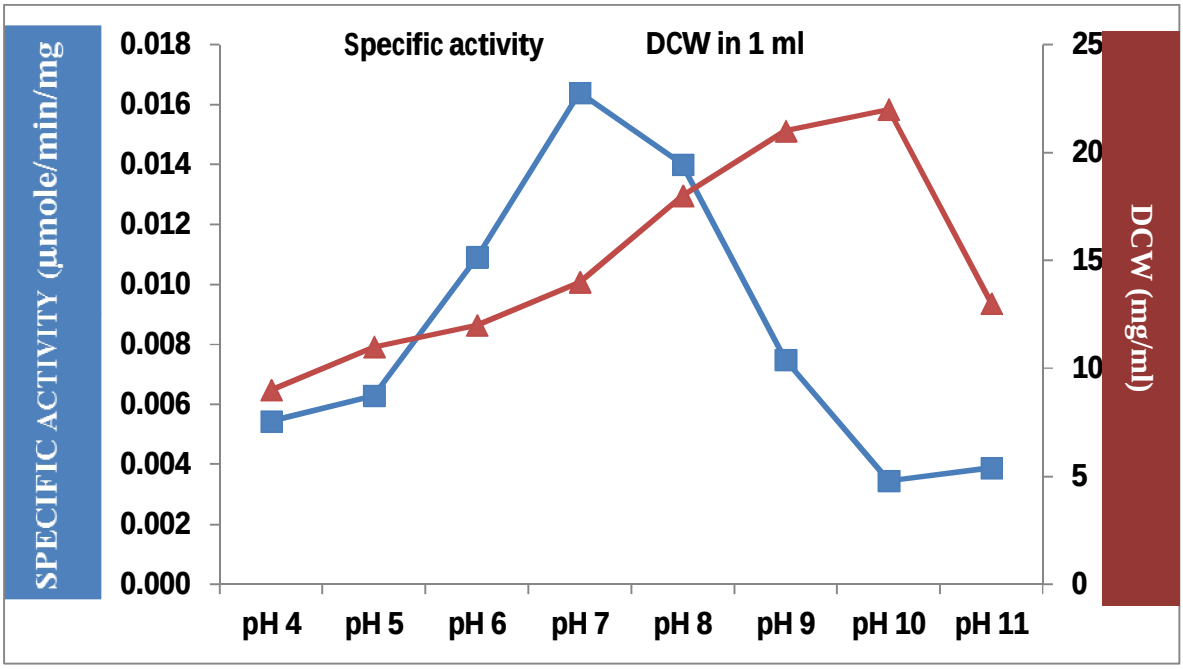


Figure 11: Graph of Specific activity and growth curve at different pH

some of the nitrilase producing microorganism that have optimum pH among 7 to 8 e.g.*Fusarium solani* optimum pH7.8-9.1 (Harper *et.,al* 1977), *Arthrobacter sp.* strain J1 pH 8.5/7.5 (Bandyopadhyay and Nagasawa, 1986), *Rhodococcus rhodochorus* ATCC 39484, pH (7.5) (Stevenson and Feng 1992), *Pseudomonas putida* pH (7) (Banerjee and Kaul 2006).

5.3.3. OPTIMIZATION IN DIFFERENT INCUBATION TEMPERATURE

Pseudomonas sp.KNB-2 was grown in M1 medium (pH 7) and incubate at temperature ranges from 30ºC, 35 ºC, 40 ºC, 45 ºC, 50 ºC, 55 ºC and 60 ºC. Maximum activity was found at 55 ºC that is 0.033 µmole/min/mg (Fig-12). Enzyme activity was found to increase consistently upto 55ºC and thereafter decrease sharply at 60ºC. While the growth of the bacteria strains was continuously decreased as increase with the temperature the maximum growth was observed at 30 ºC but at this temperature the nitrilase activity was found to be very low.

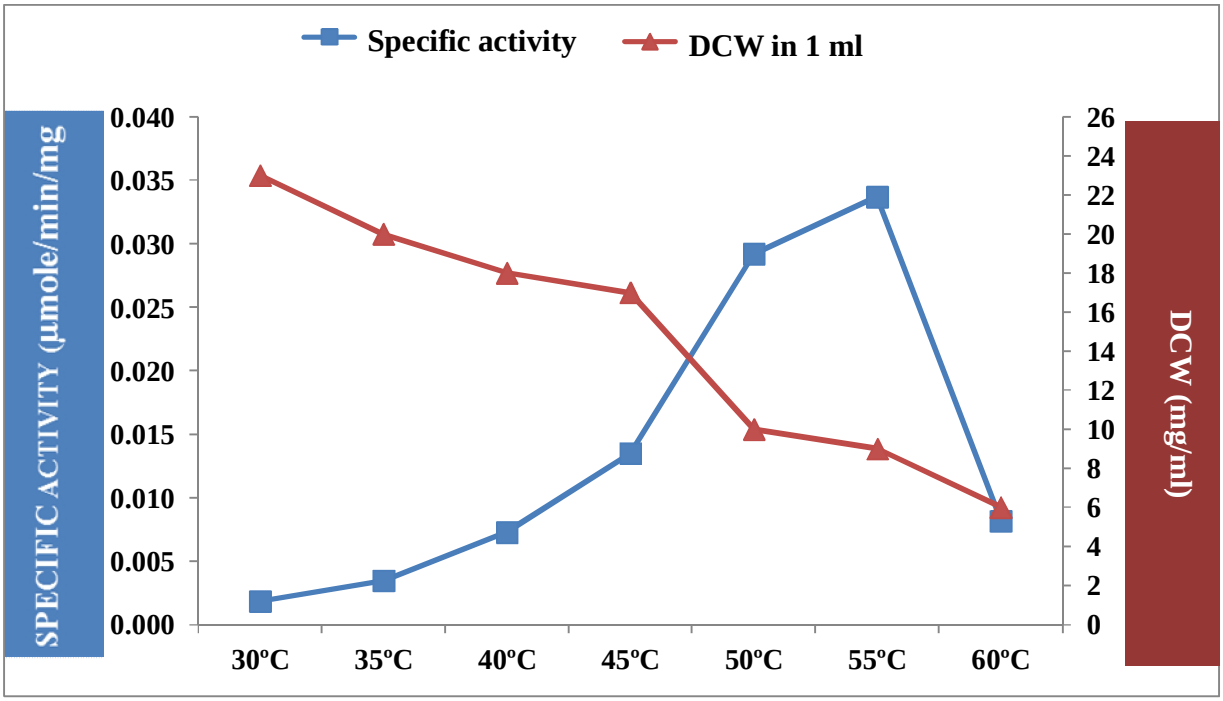


Figure 12: Graph of specific activity and growth curve at different temperature.

Some thermophilic gram negative bacteria like *Alcaligene sp.* (Mollar and Leisinger, 1987), *Fusarium solani* (J. P. and Bull, 2002), *Rhodococcus rhodochrous* (Stolz and Knackmuss, 1997), *Pseudomonas pudida* (Banerjee and Kaul, 2006). Reported to have Optimum production temperature at 55ºC

5.3.4. OPTIMIZATION IN DIFFERENT INDUCER CONCENTRATION

Benzonitrile used as inducer in range of 0.1% (v/v), 0.2% (v/v), 0.3% (v/v) 0.4% (v/v) and 0.8% (v/v) was inoculated into the M1 medium. These cultures were incubated at 55 ºC culture for 24 hours. Essay was performed to identify the activity of the culture sample and dry weight was calculated. At 0.3% (v/v) benzonitrile concentration, nitrilase activity was found highest (0.015 µmole/min/mg) (Fig-13). However *Pseudomonas* sp. KNB-2growth was observed highest at 0.2% (v/v) benzonitrile concentration that is 17.56 mg DCW/ml of culture medium.

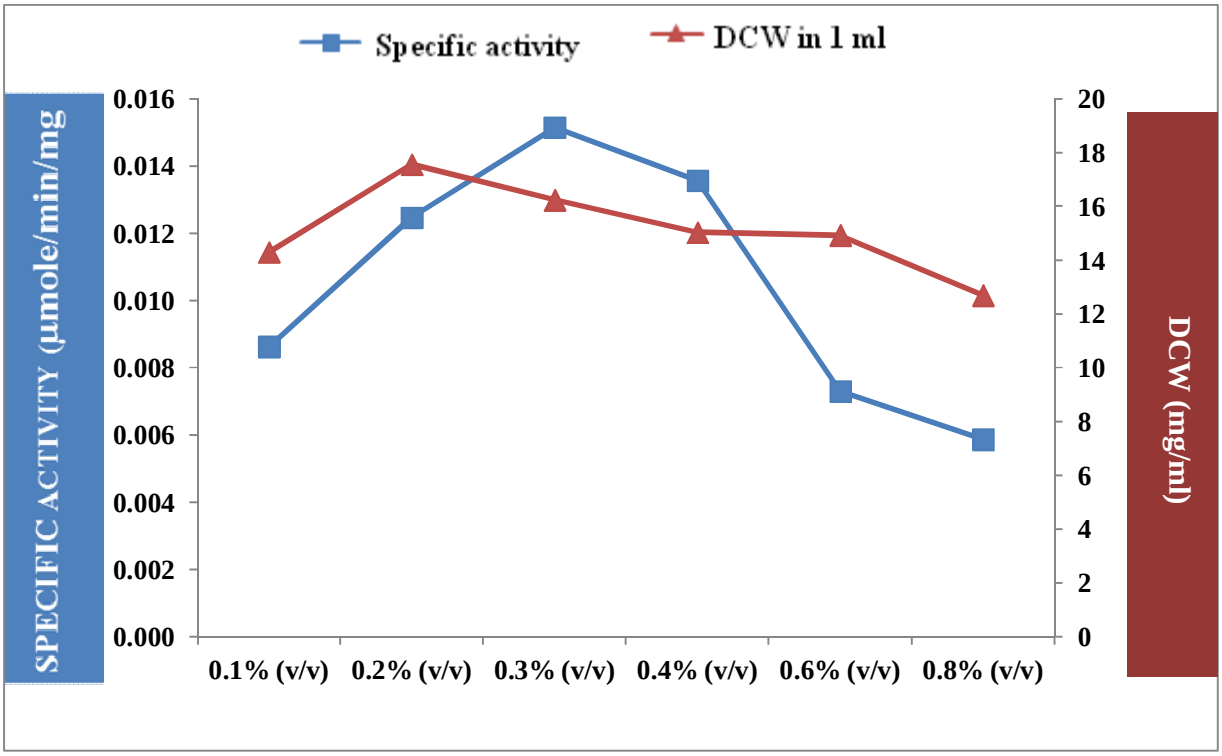


Figure 13: Graph of specific activity and growth curve at different benzonitrile concentration.

5.4. OPTIMIZATION OF REACTION CONDITIONS FOR NITRILASE ACTIVITY (WHOLE CELLS)

Biocatalytic activity was measured at different buffer pH, benzonitrile concentration, incubation temperature and different substrate.

5.4.1. BUFFERS SYSTEM AND pH

Different buffer systems with range of pH3 to pH11 were used to get the optimum pH for reaction condition. Highest activity was found to be at pH-7 in phosphate buffer (0.0100 µmole/min/mg) (Fig-14).

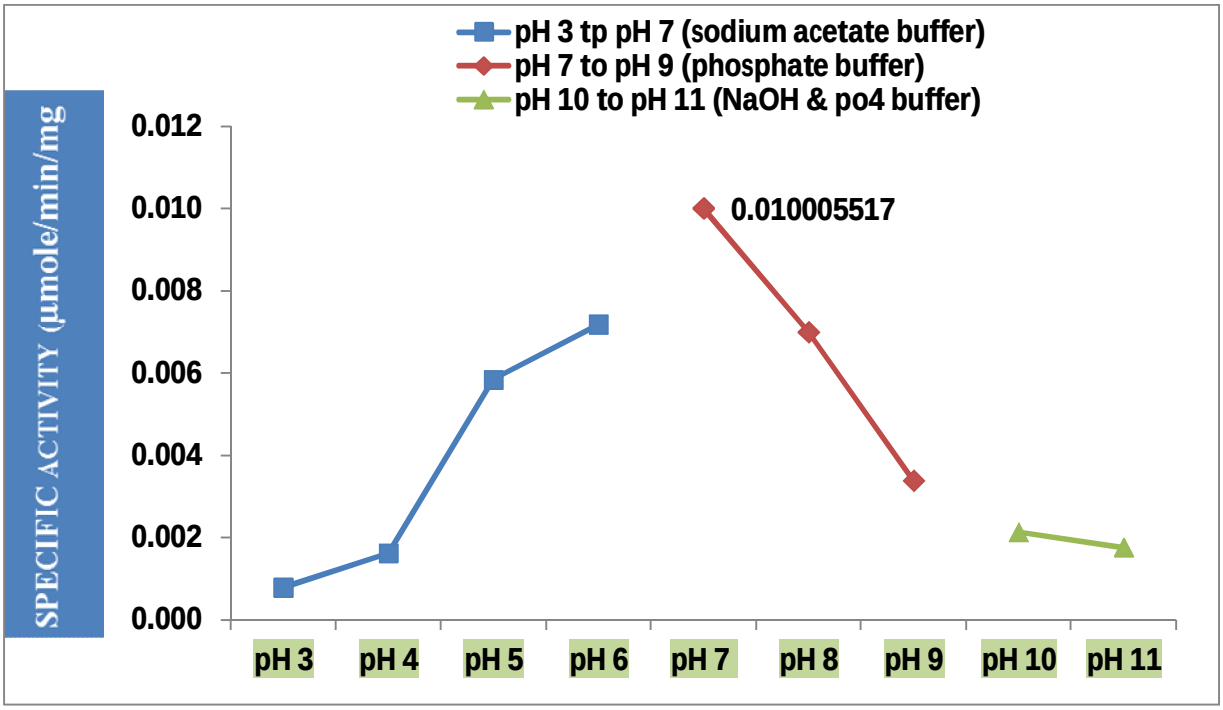


Figure 14: Graph ofspecific activity at different buffer system and pH

Some reported bacterial species that shows optimum nitrilase activity at pH 7 is *Fusarium solani* (Harper *et.,al* 1977), *Arthrobacter sp.* strain J1 (Bandyopadhyay and Nagasawa, 1986), *Rhodococcus rhodochorus* ATCC 39484 (Stevenson and Feng, 1992), *Pseudomonas putida*. (Banerjee and Kaul 2006)

5.4.2. SUBSTRATE SPECIFICITY.

To identify the substrate specificity different substrate were used as acetonitrile, benzonitrile, acrylonitrile, acetamide and acrylamide in the nitrilase assay. It was found that nitrilase of *Pseudomonas* sp. KNB-2 has highest specificity towards benzonitrile (0.017 µmole/min/mg) (Fig-15).

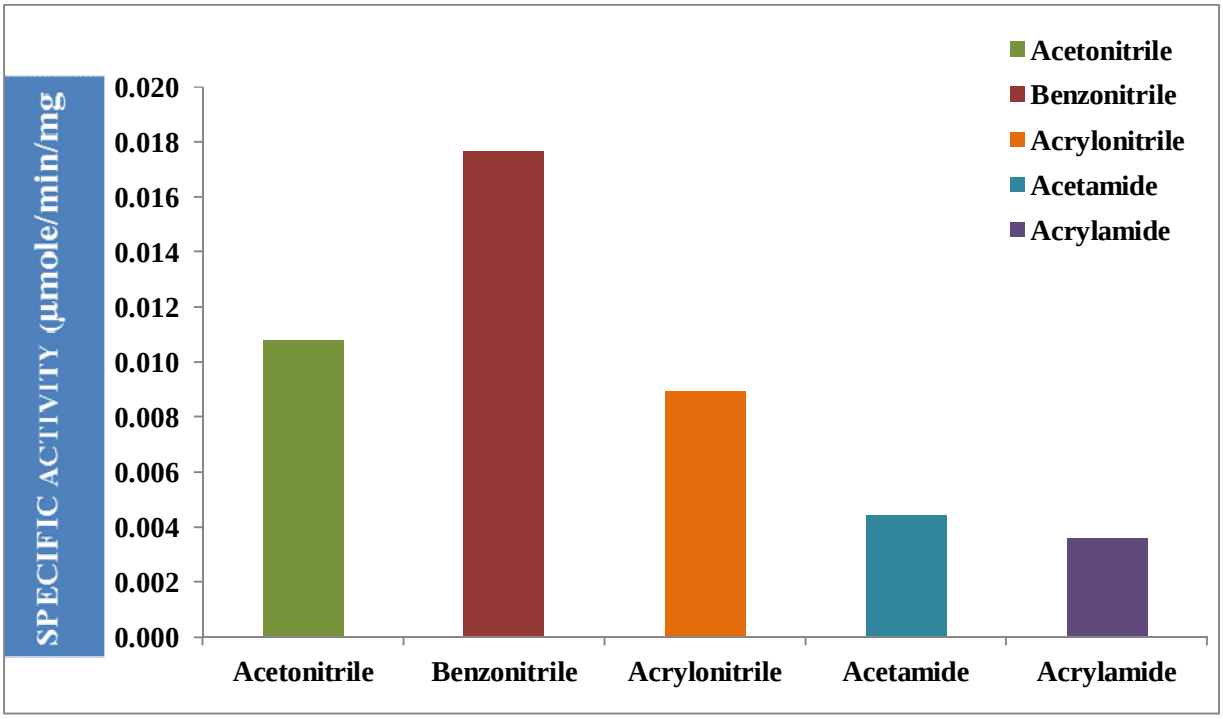


Figure 15: Graph of specific activity in different substrate

Nitrilase from bacterial species have reported to shown specificity to benzonitrile. *Bradyrhizobium japonicum*, (Howden and Preston, 2009), *Pseudomonas* (Banerjee *et al.,* 2006), *Hordeum vulgare* (Faber *et.,al*1995),*Pseudomonas putida* (Nagasawa *et al.*, 2004),*Pseudomonas fluorescens* (Pace *et al.*,1992).

5.4.3. EFFECT OF BENZONITRILE CONCENTRATION ON NITRILASE ACTIVITY

Different concentration of substrate (benzonitrile) is used in range of 15µl to 50 µl. highest nitrilase activity (0.0128 µmole/min/mg) was found at 30 µl benzonitrile concentration (Fig-16). After 30 µl of benzonitrile concentration nitrilase activity was decreased that might be due to substrate inhibition. Lowan *et al*., (1989) and kaul *et al.,* (2007) reported many nitrilase from different strain which reported to be substrate inhibited.

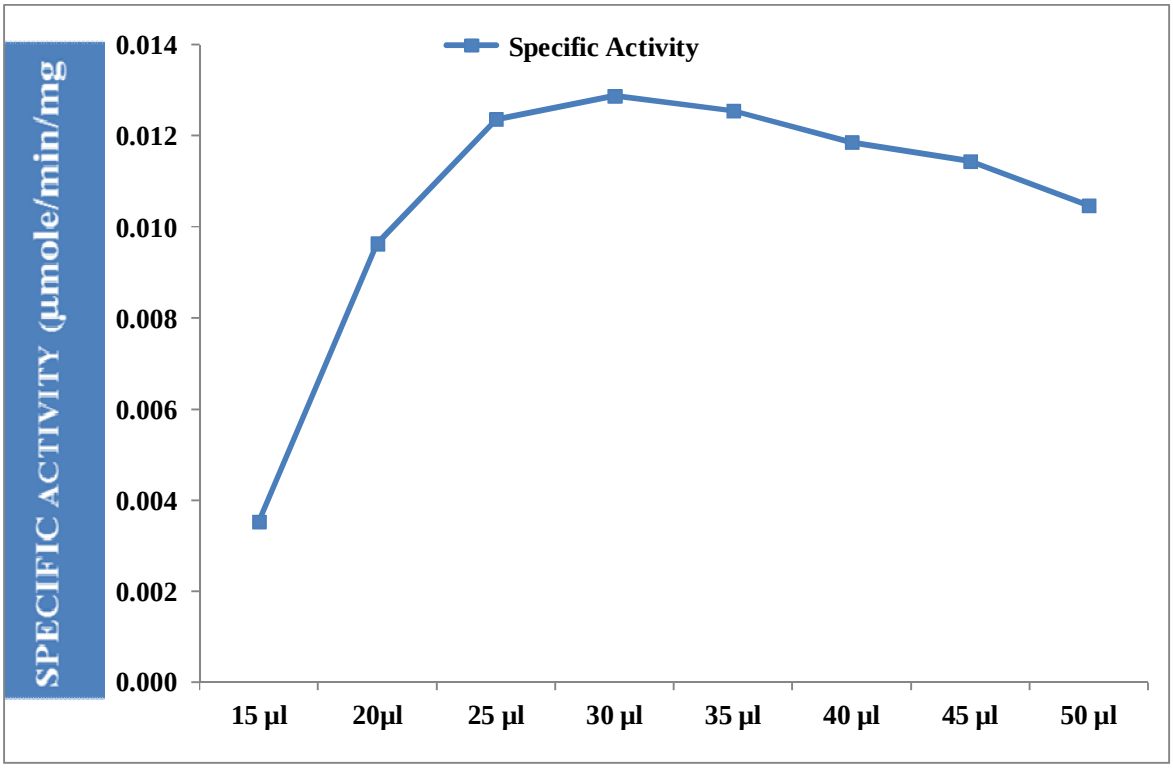


Figure 16: Graph of specific activity graph at different Benzonitrile (substrate) concentration

5.4.4. INCUBATION TEMPERATURE

For determining optimum incubation temperature to achieve highest nitrilase activity assay was carried out at different temperature ranges from 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C and 65°C. It was observed that the nitrilase enzyme of *Pseudomonas* KNB-2 has highest Nitrilase activity at 45°C. But the activity is stable in wide temperature range from 45°C to 65°C.

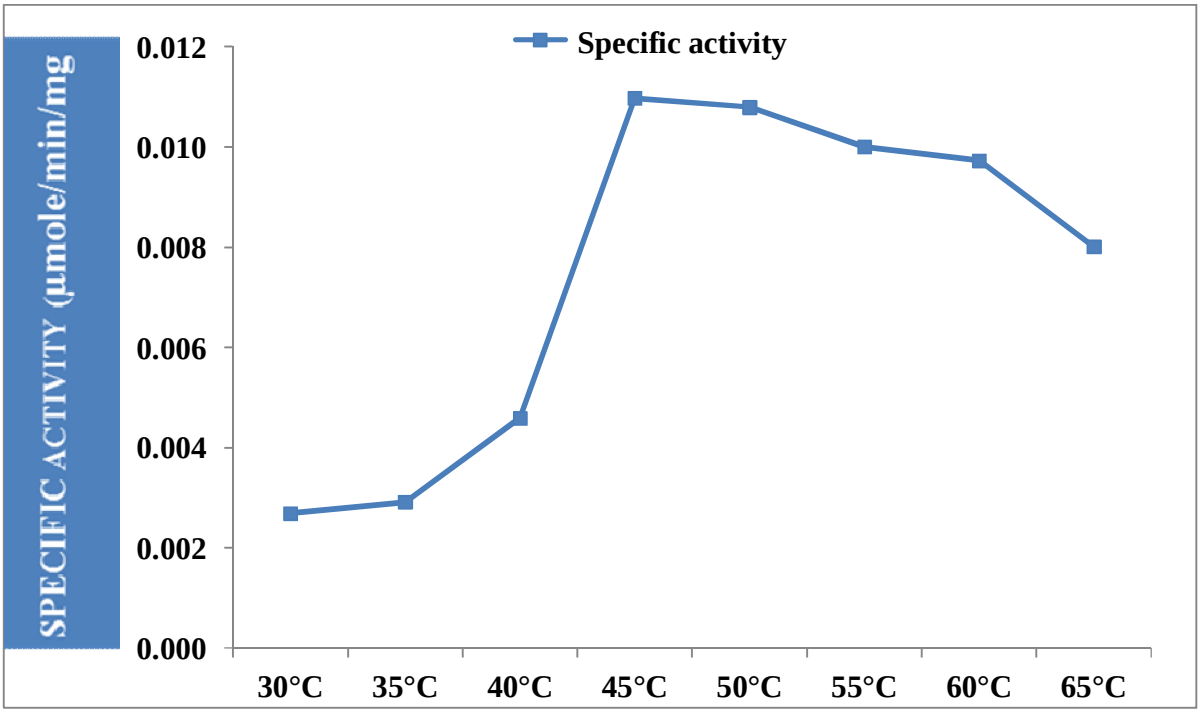


Figure 17: Graph of specific activity at different incubation temperature

Highest nitrilase activity of pseudomonas sp. KNB-2 at 45°C 0.01098 µmole/min/mg(Fig-17). Also some nitrilase have been reported to have optimum incubation temperature at 45°C eg. As *Arthrobacter sp.* strain J1 (40/30°C) (Bandyopadhyay and Nagasawa, 1986), *Rhodococcus rhodochorus* J1 (45°C) (Kobayashi and Nagasawa, 1989), *Aspergillus niger* K10 (Kaplan and Nikolau, 2006), *Fusarium solani* O1 (40-45°C) (Vejvoda and Kaplan, 2008), *Pseudomonas fluorescens* EBC191 (45°C) (Kiziak and Conradt, 2005), *Pseudomonas putida* (40°C) (Banerjee and Kaul, 2006).

5.5. ISOLATION AND CHARACTERIZATION OF NITRILASE OF *Pseudomonas* sp.*KNB-2*

After optimization of the production and reaction conditions, *Pseudomonas sp*. KNB-2 was grown in the M1 production media supplemented with 0.30% (v/v) of benzonitrile, acetonitrile, and acrylonitrile as inducer. Cell cultures were harvested by centrifugation. Supernatant was discarded and pellet was dissolved minimal volume of 0.1M pH-7 phosphate buffer.

5.5.1 SONICATION OF *Pseudomonas* KNB-2 *cells.*

To the 9 ml of phosphate buffer suspended with harvested cells, 1 mM DTT and 1mM EDTA were added. Sonication was carried out for 20 minutes of 20 cycles in cold condition. Protein concentration was measured by the Lowry method.

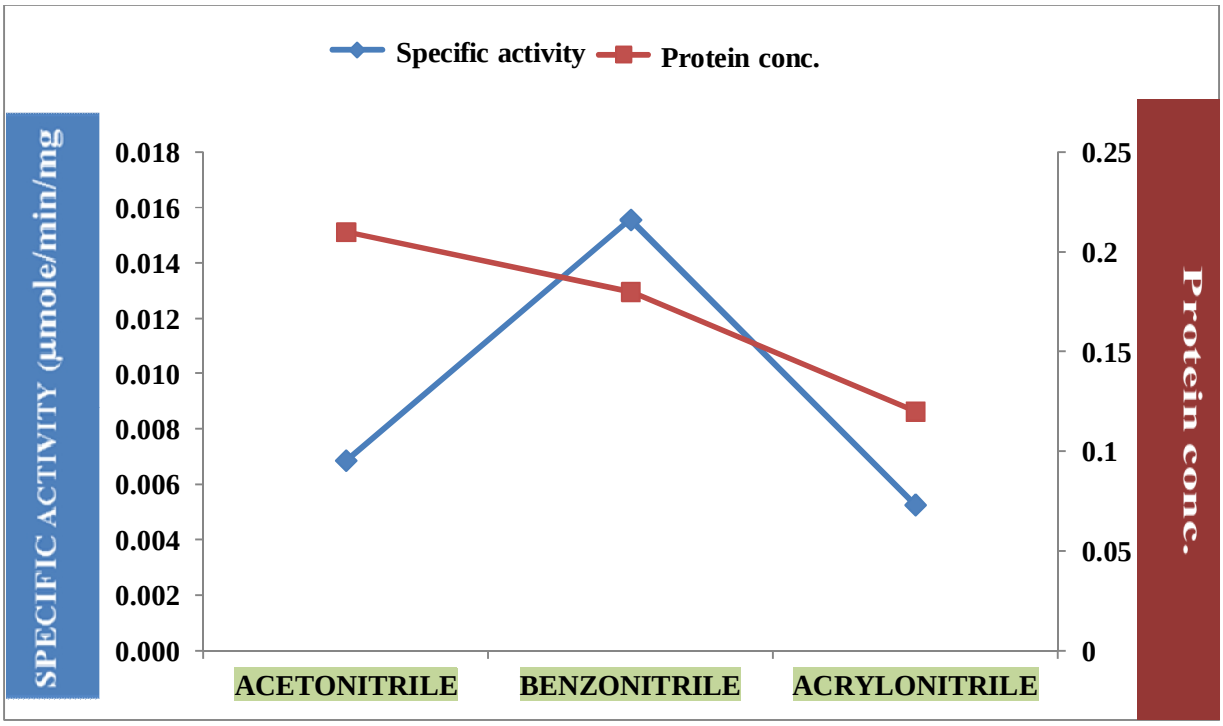


Figure 18: Graph of Specific activity of sonicated cells induced by different substrate

Highest nitrilase activity of 0.015µmole/min/mg was found in benzonitrile induced sample (Fig-18).

5.3. AMMONIUM SALT PRECIPITATION

Sonicated sample suspended in 0.1M pH-7 phosphate buffer was subjected to salt precipitation. Initially all samples were dissolved with 10% w/v of ammonium sulphate. Samples were incubated for 2 hours at 0°C. These were centrifuged at 10,000 xg for 10 minutes. Pellets were collected (labelled with 10% AC/BZ/ACR) and supernatant were taken into other tubes same process repeated up to 60% of ammonium salts. Each fractions of ammonium salt later were to dialysis. Each samples were taken in to dialysis begs and begs was immersed in to the dialysis buffer (PEG).

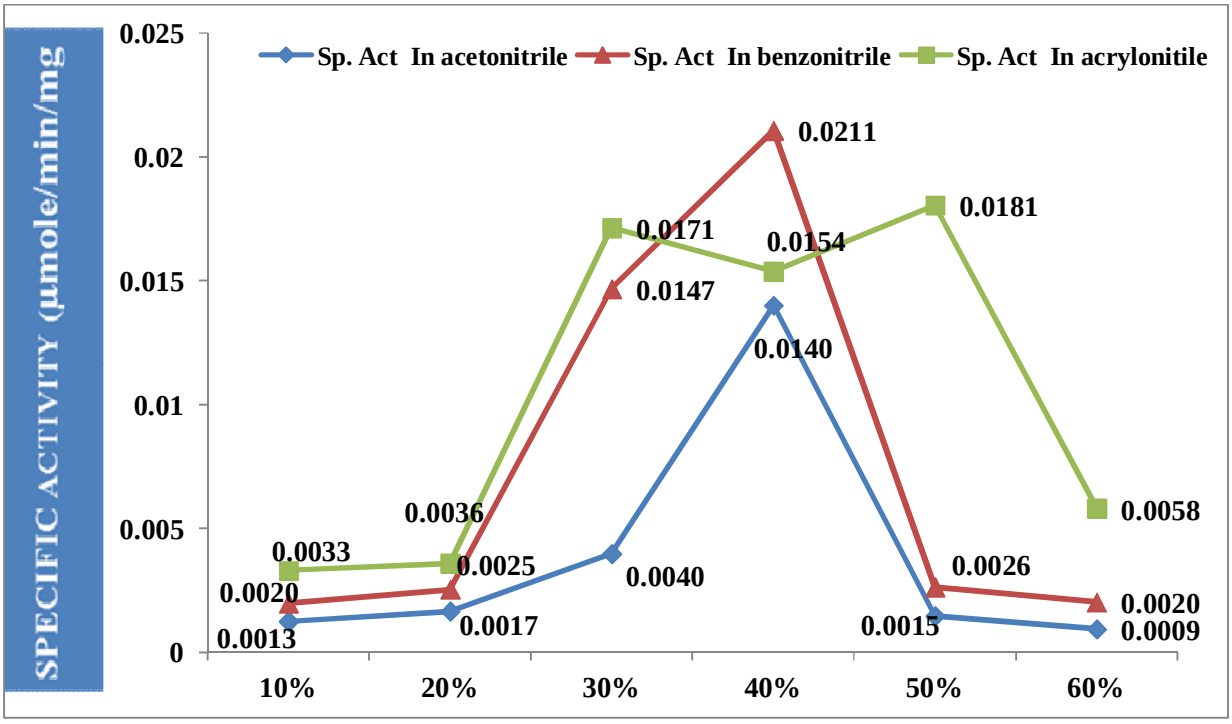


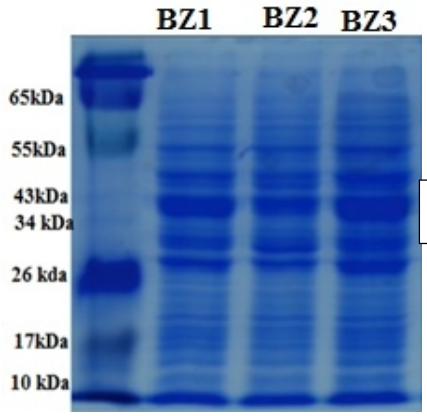
Figure 19: Graph of Specific activity of different fraction

Highest activity of nitrilase was found at 40% of ammonium salt precipitation i.e. 0.02106 µmole/min/mg the protein concentration of the sample (40%) was 0.13mg/ml (Fig-19). Though acrylonitrile induced sample showed nitrilase activity in 30-50% cut and acetonitrile induced cells exhibited maximum nitrilase activity in 40% ammonium sulphate fraction.

5.4. MOLECULAR WEIGHT ANALYSIS

After dialysis, the concentrated samples were run 12.5% of acrylamide gel and two clear bands were visualized and two bands of molecular weight 43 and 47 kDa were observed (Fig-21).
Many nitrilase of different microorganisms reported to have subunit weight in the range of 35-47. Few examples are*Pseudomonas putida*(43 kDa) (Banerjee and kaul, 2006), *Aspergillus niger*(43kDa) (recombinant enzyme) malandra and falesburge (2011),*Nocardia sp*. (47 kDa) (O'Reilly and Turner, 2003))

The sample will be further purified to get the purified protein and holozyme composition will confirmed by native PAGE.

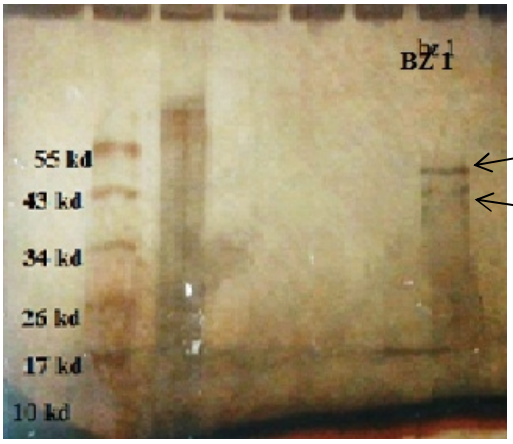


Sodium deodecyl sulphate-polyacrylamide gel electrophoresis (12%)

43 kDa bands

Figure 22: SDS PAGE analysis of crude sample showing nitrilase activity.

Two clear bands at 43kDa and 47kDa are showing



47 kDa band

43 kDa band

Figure 21: SDS PAGE of 47 kDa and 43 kDa protein bands

6. CONCLUSION

The results presented in this study emphasize the importance of growth medium in achieving high biomass concentration and the subsequent impact on achieving higher activity. Different production media including LB, basal media, M1, M3, M4, M6, M10 AND M14 had screened for achieving high biomass concentration and the subsequent impact on achieving higher activity. Although LB media was supported for the higher biomass generation but M1 production media was found to have highest nitrilase activity,

Further, to find out the appropriate condition to achieve highest nitrilase activity in the productions and reaction condition optimization of production condition and reaction condition have been done.

For highest nitrilase production the appropriate parameters are identified as benzonitrile used as inducer, M1 production Media, pH-7, incubation temperature 50°C, and 0.3% (v/v) inducer concentration. Similarly to achieve highest nitrilase activity the appropriate parameter of reaction condition (were screened and it was observed that it shows higher nitrilase activity in phosphate buffer system). pH 7, benzonitrile as inducer (0.3%), and incubation temperature 45°C,

Hence it is concluded the *Pseudomonas*sp. KNB-2 is having thermophilic inducible nitrilase, which is specific to benzonitrile and have optimum reaction temperature is 45°C, pH-7 and may have monomeric subunit, molecular weight of 43kDa/47kDa.

7. FUTURE PROSPECTIVE

Developing biocatalyst with appropriate activities and stability are a “top priority” of many industries. Therefore present and future research efforts would be focused on improving enzyme properties, such as substrate spectrum, stability and function at extreme environments. The screening and discovery of novel nitrilase producing strain or new nitrile converting enzymes with greater potential is still currently going on.

Secondly the substrate spectrum of nitrilase needed to be widened. In the long term, narrow substrate spectrum is limiting factor in the development ofgeneral purpose catalysts.

So protein engineering of nitrilase for improved operational stability is current research interest of chemist and biotechnologist.

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9. APPENDIX

9.1. AMMONIA STANDARD

Reagents

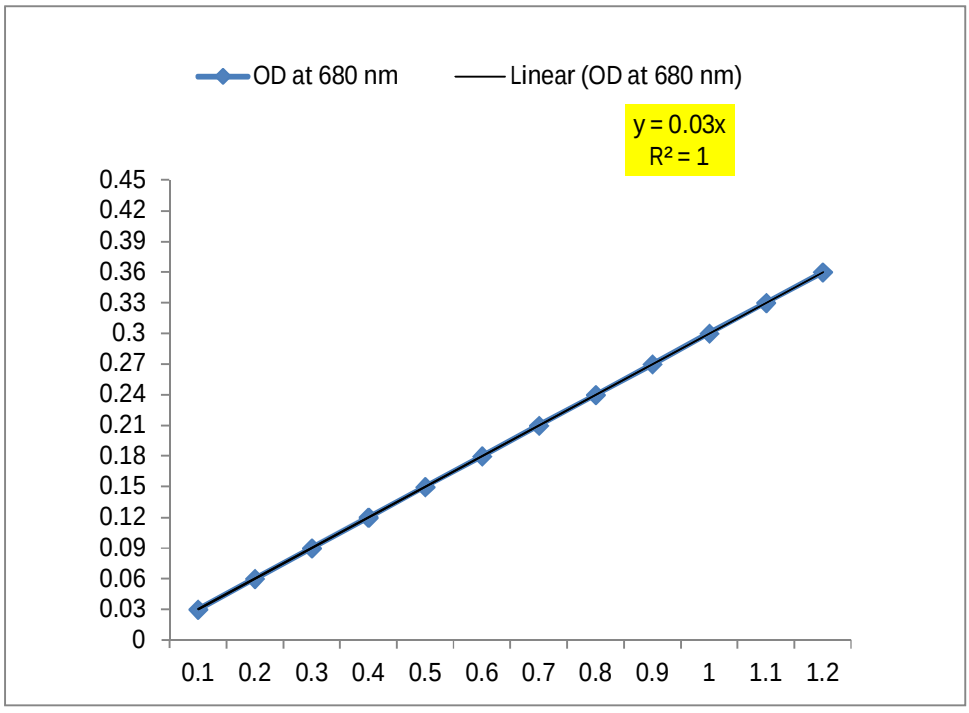
Reagent A: 1% of phenol and 0.005% sodium nitroprusside

Reagent B: 0.5N sodium hydroxide and 0.02N sodium hypochlorite

Method

1. Add 100 µl of the standard ammonium solution to 2.5 ml reagent A present in the 1.5 ml of Eppendorf tube.
2. Add 2.5ml of reagent B to the reaction mixture above and vortex.
3. Leave to incubate at room temperature for 15 minutes.
4. Transfer contents of Eppendorf tube to 2 ml cuvette and read absorbance at 640nm

9.2. STANDARD GRAPH TO ESTIMATE THE PROTEIN CONCENTRATIONS.



BSA standards in the range 0.2 – 0.9 mg/ml were prepared in duplicate, and 100 ml of each added to 5 ml protein concentration determination dye reagent. Standards were incubated at room temperature for a minimum of 5 minutes. The absorbance at 595 nm was read using a Beckman Coultier DU 800 Spectrophotometer. Unknown samples were prepared and the concentration determined from the standard curve.

1. Table a: Effects of nitriles and amides on nitrilase production

indcer	**T 1**	**C**	**T-C**	**T. Amm (T-C)x2x0.2821**	**Rate T. Amm/15**	**mg cells in 50 MICROLITRE**	**Sp. Act.**	**total activity U/mg DCW**	**DCW in 1 ml**
Acetonoitriles	0.249	0.202	0.047	0.0265	**0.0018**	0.9	**0.0020**	**1.9643**	18
Benzonitrile	0.296	0.169	0.127	0.0717	**0.0048**	1.05	**0.0045**	**4.5494**	21
Acrylonitrileds	0.28	0.24	0.04	0.0226	**0.0015**	0.75	**0.0020**	**2.0060**	15
Acetamide	0.461	0.449	0.012	0.0068	**0.0005**	0.9	**0.0005**	**0.5015**	18
Acrylamide	0.197	0.181	0.016	0.0090	**0.0006**	1.6	**0.0004**	**0.3761**	32

2. Table b: Effects of different production media

media	**T 1**	**C**	**T-C**	**T. Amm (T-C)x2x0.2821**	**Rate T. Amm/15**	**mg cells in 50 MICROLITRE**	**Sp. Act. rate/ mg of cells**	**total activity U/mg DCW**	**DCW/ML**
LB	0.193	0.154	0.039	0.0220	0.0015	4.6	**0.0003**	**0.3189**	92
MINIMAL BASAL MEDA	0.267	0.167	0.1	0.0564	0.0038	2.1	**0.0018**	**1.7911**	42
M1	0.967	0.623	0.344	0.1941	0.0129	1.8	**0.0072**	**7.1883**	36
M2	0.51	0.462	0.048	0.0271	0.0018	1.4	**0.0013**	**1.2896**	28
M3	0.197	0.181	0.016	0.0090	0.0006	1.6	**0.0004**	**0.3761**	32
M4	0.269	0.172	0.097	0.0547	0.0036	2.2	**0.0017**	**1.6584**	44
M5	0.43	0.383	0.047	0.0265	0.0018	2.8	**0.0006**	**0.6314**	56
M6	0.346	0.311	0.035	0.0197	0.0013	2.1	**0.0006**	**0.6269**	42

3. Table c: optimization in diff. pH of medium

pH	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg cells in 50 µl	Sp. Act. rate/ mg of cells	total activity U/mg DCW	DCW/ML
pH 4	0.321	0.256	0.065	0.0367	0.0024	0.45	**0.0054**	**5.4330**	9
pH 5	0.375	0.283	0.092	0.0519	0.0035	0.55	**0.0063**	**6.2917**	11
pH 6	0.903	0.729	0.174	0.0982	0.0065	0.60	**0.0109**	**10.9079**	12
pH 7	0.975	0.67	0.305	0.1721	0.0115	0.70	**0.0164**	**16.3887**	14
pH 8	0.858	0.523	0.335	0.1890	0.0126	0.90	**0.0140**	**14.0005**	18
pH 9	0.733	0.524	0.209	0.1179	0.0079	1.05	**0.0075**	**7.4868**	21
pH 10	0.599	0.498	0.101	0.0570	0.0038	1.10	**0.0035**	**3.4536**	22
pH 11	0.435	0.368	0.067	0.0378	0.0025	0.65	**0.0039**	**3.8771**	13

4. Table d: Production optimization at diff incubation temperature

temperature	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg cells in 50 µl	Sp. Act. rate/ mg of cells	total activity U/mg DCW	DCW/ML
30ºC	0.177	0.121	0.056	0.0316	0.0021	1.15	**0.0018**	**1.8316**	23
35ºC	0.375	0.283	0.092	0.0519	0.0035	1	**0.0035**	**3.4604**	20
40ºC	0.903	0.729	0.174	0.0982	0.0065	0.9	**0.0073**	**7.2719**	18
45ºC	0.975	0.67	0.305	0.1721	0.0115	0.85	**0.0135**	**13.4965**	17
50ºC	0.856	0.468	0.388	0.2189	0.0146	0.5	**0.0292**	**29.1879**	10
55ºC	0.927	0.524	0.403	0.2274	0.0152	0.45	**0.0337**	**33.6848**	9
60ºC	0.647	0.582	0.065	0.0367	0.0024	0.3	**0.0081**	**8.1496**	6

5. Table e:Production optimization at diff. inducer concentrations

inducer concentration	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg of cells in 50 µl	Sp. Act. rate/ mg of cells	total activity U/mg DCW	DCW/ML
0.1% (v/v)	0.646	0.482	0.164	0.0925	0.0062	0.715	**0.0086**	**8.6274**	14.3
0.2% (v/v)	0.723	0.432	0.291	0.1642	0.0109	0.878	**0.0125**	**12.4664**	17.56
0.3% (v/v)	0.958	0.631	0.327	0.1845	0.0123	0.812	**0.0151**	**15.1472**	16.24
0.4% (v/v)	0.853	0.582	0.271	0.1529	0.0102	0.7515	**0.0136**	**13.5638**	15.03
0.6% (v/v)	0.856	0.711	0.145	0.0818	0.0055	0.7465	**0.0073**	**7.3060**	14.93
0.8% (v/v)	0.623	0.524	0.099	0.0559	0.0037	0.6345	**0.0059**	**5.8687**	12.69

6. Table f:reaction condition opt. in diff buffer system of diff pH

buffer pH	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	pH 3 tp pH 7 (sodium acetate buffer)	pH 7 to pH 9 (phosphate buffer)	pH 10 to pH 11 (NaOH & po4 buffer)	pH 3 tp pH 7 (sodium acetate buffer)	pH 7 to pH 9 (phosphate buffer)	pH 10 to pH 11 (NaOH & po4 buffer)	DCW/ML
pH 3	0.231	0.214	0.017	0.0096	0.0006	**0.0008**			**0.7875**			
pH 4	0.328	0.293	0.035	0.0197	0.0013	**0.0016**			**1.6213**			
pH 5	0.624	0.498	0.126	0.0711	0.0047	**0.0058**			**5.8366**			
pH 6	0.737	0.582	0.155	0.0875	0.0058	**0.0072**			**7.1799**			
pH 7	0.689	0.473	0.216	0.1219	0.0081		**0.0100**			**10.0055**		
pH 8	0.623	0.472	0.151	0.0852	0.0057		**0.0070**			**6.9946**		16.24
pH 9	0.659	0.586	0.073	0.0412	0.0027		**0.0034**			**3.3815**		
pH 10	0.57	0.524	0.046	0.0260	0.0017			**0.0021**			**2.1308**	
pH 11	0.434	0.396	0.038	0.0214	0.0014			**0.0018**			**1.7602**	

7. Table g: substrate specificity

substrate	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg of cells in 50 µl	Sp. Act. rate/ mg of cells	total activity U/mg DCW	DCW/ML
Acetonitrile	0.786	0.528	0.258	0.1456	0.0097		**0.0108**	**10.7825**	
Benzonitrile	0.935	0.512	0.423	0.2387	0.0159		**0.0177**	**17.6783**	
Acrylonitrile	0.678	0.464	0.214	0.1207	0.0080	0.9	**0.0089**	**8.9436**	18
Acetamide	0.389	0.283	0.106	0.0598	0.0040		**0.0044**	**4.4300**	
Acrylamide	0.284	0.198	0.086	0.0485	0.0032		**0.0036**	**3.5942**	

8. Table h: effect of benzonitrile concentration on nitrilase activity

SUBSTRATE CONCENTRATION	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg of cells in 50 µl	Sp. Act. rate/ mg of cells	total activity U/mg DCW	DCW/ML
15 µl	0.475	0.399	0.076	0.0429	0.0029		**0.0035**	**3.5205**	
20µl	0.641	0.433	0.208	0.1174	0.0078		**0.0096**	**9.6349**	
25 µl	0.743	0.476	0.267	0.1506	0.0100	0.812	**0.0124**	**12.3679**	16.24
30 µl	0.86	0.582	0.278	0.1568	0.0105		**0.0129**	**12.8775**	
35 µl	0.682	0.411	0.271	0.1529	0.0102		**0.0126**	**12.5532**	

40 µl	0.726	0.47	0.256	0.1444	0.0096		**0.0119**	**11.8584**
45 µl	0.649	0.402	0.247	0.1394	0.0093		**0.0114**	**11.4415**
50 µl	0.564	0.338	0.226	0.1275	0.0085		**0.0105**	**10.4687**

9. Table i: effect of incubation temperature in reaction condition

incubation temperature	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg of cells in 50 µl	Sp. Act. rate/ mg of cells	total activity U/mg DCW	DCW/ML
30℃	0.269	0.211	0.058	0.0327	0.0022	0.812	**0.0027**	**2.6867**	16.24
35℃	0.286	0.223	0.063	0.0355	0.0024		**0.0029**	**2.9183**	
40℃	0.342	0.243	0.099	0.0559	0.0037		**0.0046**	**4.5859**	
45℃	0.468	0.231	0.237	0.1337	0.0089		**0.0110**	**10.9783**	
50℃	0.537	0.304	0.233	0.1315	0.0088		**0.0108**	**10.7930**	
55℃	0.549	0.333	0.216	0.1219	0.0081		**0.0100**	**10.0055**	
60℃	0.582	0.372	0.21	0.1185	0.0079		**0.0097**	**9.7276**	
65℃	0.578	0.405	0.173	0.0976	0.0065		**0.0080**	**8.0137**	

10. Table j: nitrilase activity in diff induced sonicated samples

inducer	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg of cells in 50 µl	Sp. Act. rate/ mg of cells	Total activity U/mg DCW	DCW/ML	protein conc. Mg/ml
ACETONITRILE	0.816	0.597	0.219	0.1236	0.0082	1.2	**0.0069**	**6.8644**	24	0.21
BENZONITRILE	0.948	0.617	0.331	0.1868	0.0125	0.8	**0.0156**	**15.5625**	16	0.18
ACRYLONITRILE	0.714	0.588	0.126	0.0711	0.0047	0.9	**0.0053**	**5.2659**	18	0.12

11. Table k: nitrilase activity in diff cut of diff induced sample

inducer	salt concentration	T 1	C	T-C	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	mg of cells in 50 µl	Sp. Act In acetonitrile	Total activity U/mg DCW	DCW/ML	Protein conc. Mg/ml
ACETONITRILE	10%	0.324	0.284	0.04	0.0226	0.0015	1.2	0.0013	1.2538	24	0.23
	20%	0.341	0.288	0.053	0.0299	0.0020	1.2	0.0017	1.6613	24	0.2
	30%	0.723	0.596	0.127	0.071	0.0048	1.2	0.0040	3.9807	24	0.16

					7						
	40%	0.863	0.416	0.447	0.2522	0.0168	1.2	0.0140	14.0110	24	0.133
	50%	0.234	0.187	0.047	0.0265	0.0018	1.2	0.0015	1.4732	24	0.23
	60%	0.222	0.192	0.03	0.0169	0.0011	1.2	0.0009	0.9403	24	0.066
BENZONITRILE	10%	0.236	0.194	0.042	0.0237	0.0016	0.8	0.0020	1.9747	16	0.2
	20%	0.254	0.2	0.054	0.0305	0.0020	0.8	0.0025	2.5389	16	0.17
	30%	0.898	0.586	0.312	0.1760	0.0117	0.8	0.0147	14.6692	16	0.13
	40%	0.921	0.473	0.448	0.2528	0.0169	0.8	0.0211	21.0635	16	0.1
	50%	0.412	0.356	0.056	0.0316	0.0021	0.8	0.0026	2.6329	16	0.1
	60%	0.342	0.299	0.043	0.0243	0.0016	0.8	0.0020	2.0217	16	0.067
ACRYLONITRILE	10%	0.243	0.164	0.079	0.0446	0.0030	0.9	0.0033	3.3016	18	0.24
	20%	0.267	0.181	0.086	0.0485	0.0032	0.9	0.0036	3.5942	18	0.2
	30%	0.782	0.372	0.41	0.2313	0.0154	0.9	0.0171	17.1350	18	0.17
	40%	0.721	0.353	0.368	0.2076	0.0138	0.9	0.0154	15.3797	18	0.14
	50%	0.763	0.331	0.432	0.2437	0.0162	0.9	0.0181	18.0544	18	0.13
	60%	0.485	0.346	0.139	0.0784	0.0052	0.9	0.0058	5.8092	18	0.1