Enhancing production of organic acids by immobilized thermophilic nitrilase of *Pseudomonas* sp. KNB2

To be submitted as Major Project Report in partial fulfillment of the requirement for the degree of

Master of Technology

In

Industrial Biotechnology

Submitted by

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled "Enhancing production of organic acids by immobilized thermophilic nitrilase of *Pseudomonas* sp. KNB2", submitted by **Sidharth Sharma (DTU/14/M.TECH/098)**in the partial fulfillment of the requirements for the award of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance. The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honoring of any other degree.

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DECLARATION

I hereby declare that the M. Tech major project dissertation entitled, "'**Enhancing production of organic acids by immobilized thermophilic nitrilase of *Pseudomonas* sp. KNB2**" submitted by me to Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of M. Tech Industrial Biotechnology is a record of *bonafide* work carried out by me under the guidance of Dr. Pravir Kumar, Associate Professor, Department of Biotechnology, Delhi Technological University, New Delhi.

I, further declare that the work reported in this report has not been submitted, and will not be submitted, either in part or in full, for the award of any other degree or diploma of this University or of any other institute or university.

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Enhancing production of organic acids by immobilized thermophilic nitrilase of *Pseudomonas* sp. KNB2

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1. ABSTRACT

Nitrile compounds have a very diverse range of application in the production of organic acid and other chemicals. Nitrilase can hydrolyze nitriles into respective organic acid and ammonia.The wide distribution and potential of producing useful products with high commercial value, present it as a green catalyst from the view point of industrial biotechnological application.A previously isolated strain of bacteria with high thermophilic nitrilase activity was characterized using biochemical tests, which showed the present belonged to *Pseudomonas* genus and named *Pseudomonas* sp. KNB2. With the aim to increase the production of organic acids cells were immobilized using calcium alginate and polyacrylamide entrapment. Calcium alginate gives maximum activity with 0.6mg cell density per bead while polyacrylamide at 0.7 mg cells per bead.

The immobilized cells show optimum activity at a range of 45-60^{0}C, 6-8 pH using benzonitrile substrate at a concentration from 30mM to 50mM. The immobilized beads have stability up to 3 days and can be used up to 8-9 times. Use of metal ions such as Zn, Ag and Fe resulted in the decrease of activity of nitrilase.

2. INTRODUCTION

Nitriles are the chemical compounds, which are broadly used as a substrate in the production of carboxylic acids like acetic acid, mandelic acid and indole-3-acetic acid by the means of either chemical or enzymatic hydrolysis (Heinemann *et al.*, 2003). These compounds are abundantly formed in nature as well as produced xenobiotically. They have cyano groups in their structure as a functional group. Their most commonly occurring forms are cyanolipids, ricinine, cyanoglycosides, and phenyl acetonitrile i.e., mainly found in plants as well as microbial metabolism (Kobayashi *et al*., 1993).

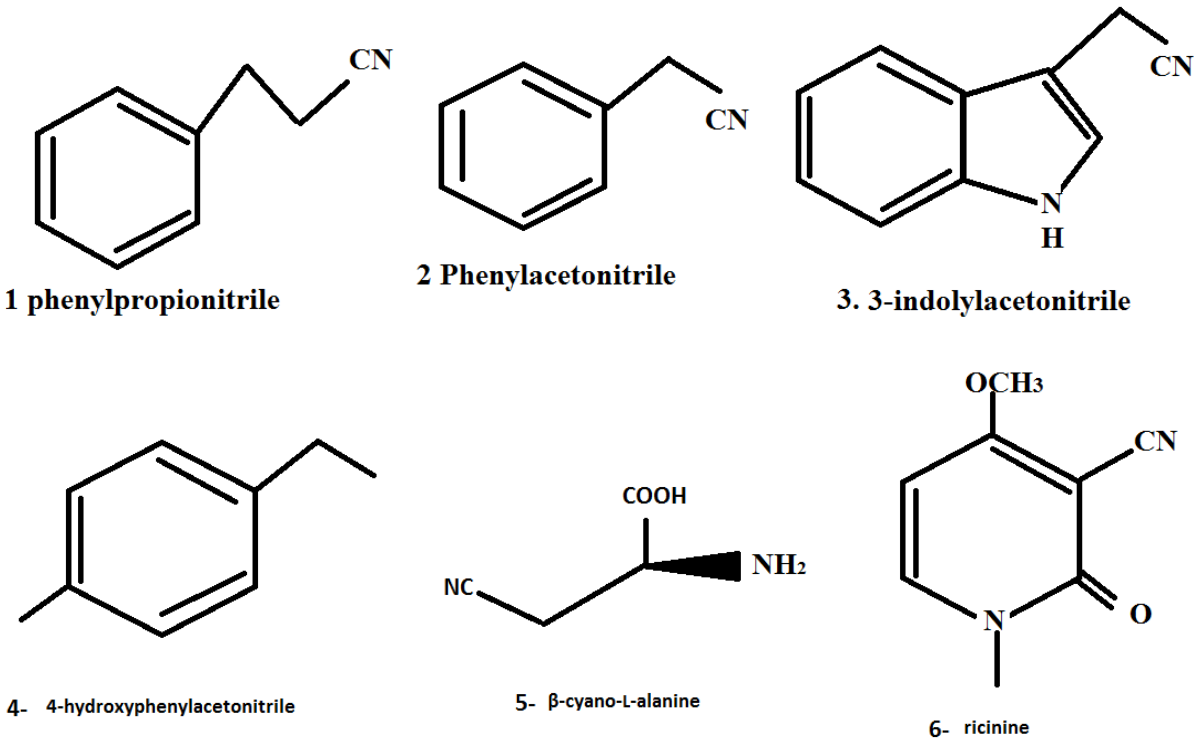


Figure 1: Examples of naturally occurring nitriles: (1)3- phenylpropionitrile, (2) phenylacetonitrile, (3) 3-indolylacetonitrile, (4) 4-hydroxyphenylacetonitrile, (5) β-cyano-L alanine, (6) ricinine (Banerjee *et al.*, 2002).

Nitrile compounds have a very diverse range of application in the manufacturing of plastics, cosmetics, organic chemicals and pharmaceutical product. But the presence of cyano functional group makes nitrile highly toxic in nature (Kobayashi *et al.*, 1990; Nagasawa *et al.*, 2000; Brady

et al., 2004). Cyanides have a property to inhibit the electron transport chain by binding the iron ion of cytochrome c oxidase leading to the inhibition of ATP synthesis (Gupta *et al*., 2010).

Nitrile hydrolysis can be performed by chemically or microbial systems. Chemical methods used for the hydrolysis of nitriles commonly produces undesirable byproducts and inorganic waste along with these reactions were carried out under harsh environmental conditions like strong acidic conditions with high temperature (Khandelwal *et al.*,2007). In contrast, biotransformation reactions which involve biocatalysts had shown greater specificity along with lesser production of unwanted byproducts (Rey *et al.*,2004).

Nitrile hydrolyzing biocatalysts have gained substantial importance in the field of industrial biotechnology due to their capability of manufacturing of organic acids, antibiotics and other chemicals and their role in treatment of toxic nitrile and cyanide wastes (Trott and Herald, 2001).

Carboxylic acid production from nitrile using nitrile hydrolyzing enzymes involves two different pathways: 1) Nitrilase (EC3.5.5.1) produce corresponding organic acid in a single step conversion of nitrile; and 2) It is a two steps reaction in which firstly, nitrile hydratases (EC 4.2.1.84) convert nitrile compound into amide and then in second step amidases (EC 3.5.1.4) subsequently transform amide into the corresponding organic acid (Zhou *et al.,* 2005)

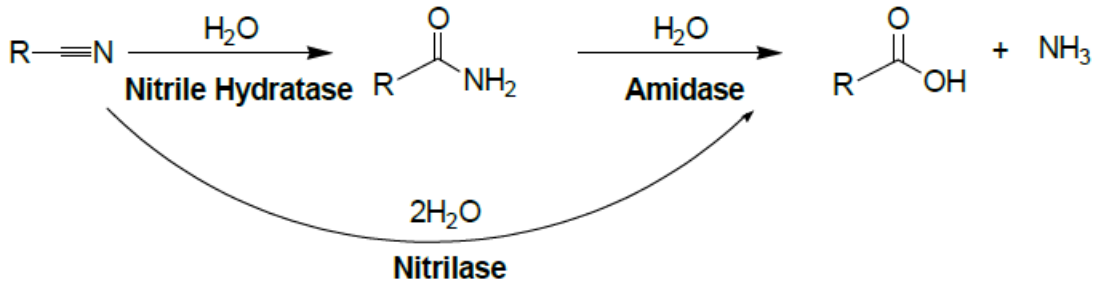


Figure 2: Nitrile hydrolysis by single and intermediate root (Kobayashi and Yamada, 1990)

Nitrilase enzyme is a part of nitrilase superfamily and found in both prokaryotes and eukaryotes. This nitrilase superfamily has been classified into 13 branches, among which nitrilase comes under the first branch along with cyanide hydratase. Members of the rest of the 12 branches have amidase activity and they were classified according to their amino acid sequences. Nitrile

hydratase, although have catalytic property to hydrolyze nitrile does not belong to this superfamily (Brenner, 2002).

Nitrilases is distributed extensively in nature, mainly in plants, filamentous fungi and bacteria. Nitrilases show regio- and enantio- selectivity and by these properties various nitrilases are classified (Singh *et al.,* 2006). Due to regio-selective properties production of chiral compounds is achieved. This wide distribution and potential of producing useful products with high commercial value, present it as a "green catalyst" from view point of industrial biotechnological application (Kobayashi *et al.,* 1994).

Recently, the bacterial strain *Pseudomonas sp.*KNB2 with relatively high thermophilic nitrilase activity was isolated and characterized in the departmental laboratory by using screening methods. Although the production of relative carboxylic acids is very prominent but free cell biocatalysis has been shown to be susceptible to reduction of activity to enzyme associated with the alteration in environmental factors like pH and temperature.

Industrial aspects of production of biotechnological products demands for an economical process including reusability of biocatalysts, better operational stability and high efficiency of catalysis. Considering these point in view immobilization came to be a very advantageous process to improve the biotransformation process. Immobilization is basically done to make the process of bioconversion more economical. By using immobilization biocatalysts can be recycled and the separation process of biocatalyst from the final product becomes very easy as compared to free cells (Sharma *et al*., 2011).

Based on the above points the current work entitled '**Enhancing production of organic acids by immobilized thermophilic nitrilase of *Pseudomonas* sp. KNB2'** has been undertaken with purpose to immobilize the *Pseudomonas sp.*KNB-2 for thermo stable nitrilase and optimization of the various parameter to enhance the productivity of the immobilized cells in the production of various organic acids.

3. REVIEW OF LITERATURE

3.1 NITRILASE SUPER FAMILY AND NITRILES

Organic compounds having R-C≡N bond are called as nitrile compounds. In the synthesis of many organic solvents they are formed as intermediate compounds. They also act as precursor or building block in the production of organic acids (Kobayashi *et al*., 1990). Many plant species known as cyanogenic plants produce natural nitriles as a part of their defense mechanism against herbivores. Some of these cyanogenic plant species are *Arabidopsis thaliana*, Barley, *Brassica rapa* (Bhalla *et al*., 2009).The naturally formed cyanogenic compounds like cyano lipids or cyanoglycosides are degraded by enzymes belonging to nitrilase super family and nitrile hydratase into organic acid and ammonia (Banerjee *et al*., 2002).

Nitrile compounds are also produced xenobiotically as they have an enormous range of application in manufacturing of organic chemicals, pharmaceutical products, cosmetics and plastics. But on the other side they are also toxic in nature due to the presence of cyanide functional group (Gupta *et al*., 2010). Degradation of nitriles can be achieved by both chemical methods and biocatalysis. Chemical methods have several drawbacks as they are carried out under harsh conditions like highly acidic or basic pH along with high temperature. In addition chemical methods of hydrolysis also lead into the formation of unnecessary byproducts and inorganic waste (Zhou *et al*., 2005).

On the other hand, biocatalysis came up as a promising tool using cell extracts, whole microbial cells, and immobilized cells or immobilized enzymes as catalysts in the production of various compound from their precursor (Zhou *et al*., 2005)

Catabolism of nitriles is carried out by nitrilases and nitrile hydratase-amidase in two distinct ways. Nitrilase transforms nitriles into respective acids and ammonia in a single step reaction while nitrile hydratase converts nitrile into amide and then amidase act on the amide to convert it into organic acid and ammonia in the presence of ammonia (Martinkova *et al*., 2009). Characterization of nitrilase is achieved in the terms of regio-selectivity and enantio-selectivity. Enantio-selectivity includes biotransformation of substrate with R and S enantiomers having difference in their respective Gibbs free energy. While the regio-selectivity used in the

production of the chiral compounds. These add up as a unique quality of nitrilase compared to the pathway carried out by nitrile hydratase and amidase (Singh *et al*., 2006). Here, figure 3 shows the reaction carried out by nitrilase and Nitrile hydratase-amidase.

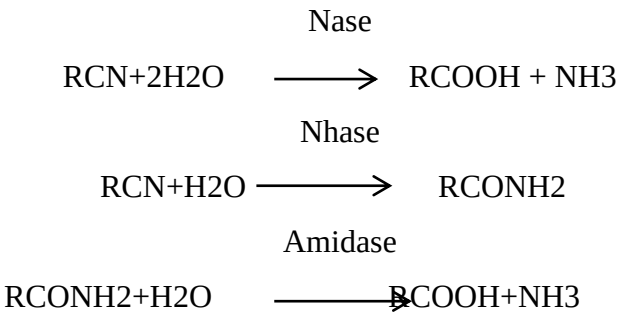


Figure 3- hydrolysis of nitriles by nitrilase and nitrile hydratase-amidase (Singh *et al*., 2006).

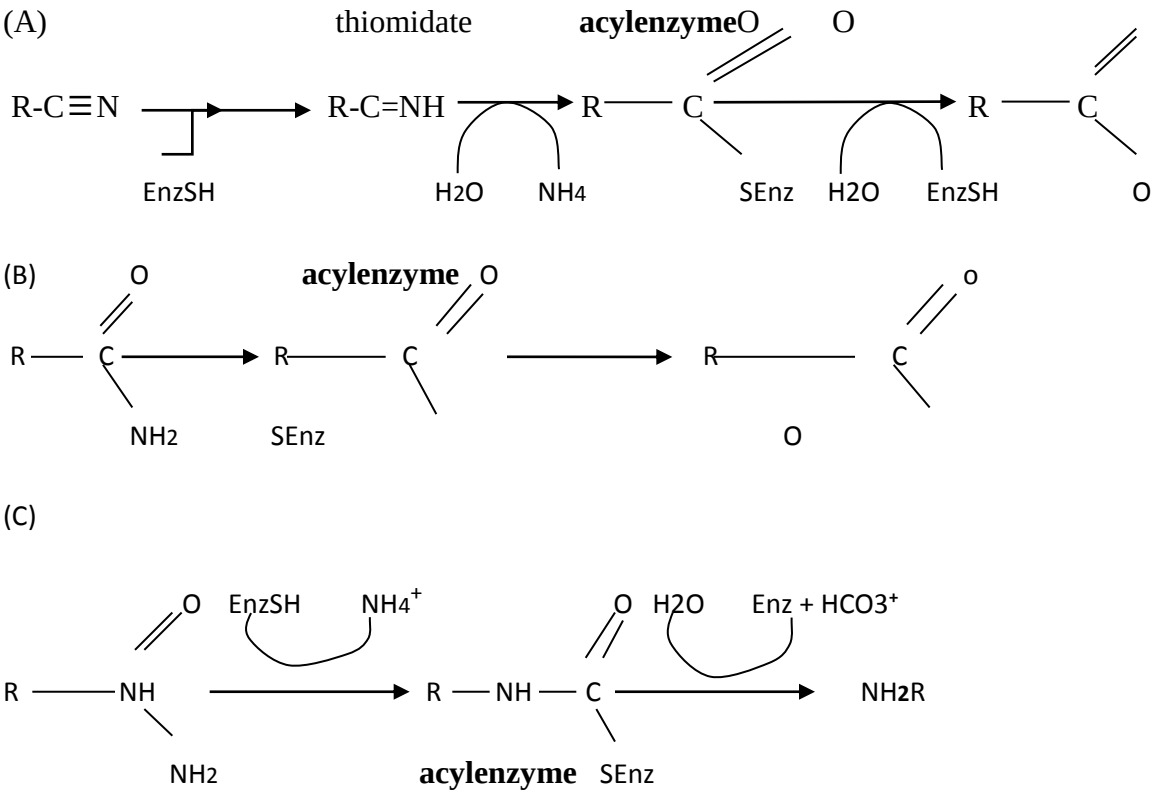
Nitrilase super family is categorized into 13 distinct families primarily on the basis of presence of additional domain and amino acid sequence similarity. The members of this enzyme superfamily have a thiol group (-SH) at their catalytic site. Nitrilase enzyme comes under the first branch of this super family. Other 12 branches constitutes of amidase of different type. All the 13 branches are "(1) aromatic nitrilase, (2) aliphatic amidase, (3) amino terminal amidae, (4) biotinidase (5) **β**-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).

Nitrile hydratase, which also hydrolyze nitrile into amide, is not a part of this enzyme super family (Reilly *et al*., 2003). Although, many of the branches of nitrilase superfamily shows amidase activity, but not all amidases comes under this family. That includes Ntn and triad hydrolase, amidase signature enzymes and thiol proteases, which are not the part of nitrilase superfamily. Amidase of several families having homologous Glu-Lys-Cys, are embraced as branches of nitrilase super family (Thuku *et al*., 2007).Nitrilases are related to aliphatic amidases in historical observations, that is the reason term nitrilase superfamily is used.

3.2 TYPES OF REACTION CATALYZED BY NITRILASE SUPERFAMILY

There are basically four types of reactions catalyzed by the enzymes of nitrilase super family which are shown in figure 3. These reactions are :- A) this type of reaction is performed by branch 1 enzymes mainly nitrilase in which nitrile is converted to carboxylic acid. Indole-3-acetonitrile is converted into indole-3-acetic acid, in plants, which is their growth hormone.

B) the most frequently observed reaction of amidase in the superfamily, of conversion of amide to acid. This type of reaction is observed in 2-4, 7 and 8 branch enzymes. C) It is a special case known as carbamylase reaction (Pace et al., 2000). It is also a type of amidase reaction which is carried out by enzymes belonging to branch 5 and 6. D) It is a reverse amidase reaction in which fatty acid is transferred to a polypeptide amino terminal. It is carried out by branch 9 N-acyl transferases (Pace et al., 2000).



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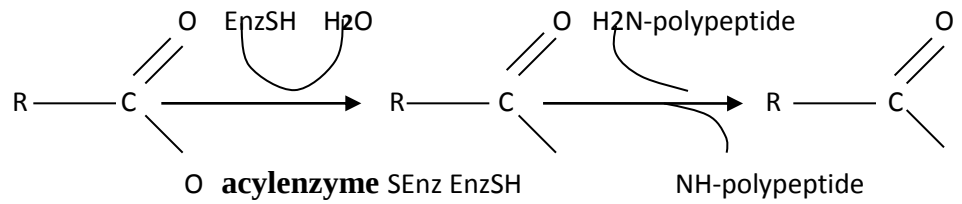


Figure 4: types of reactions catalyze by nitrilase super family (Pace et al., 2000)

It was observed that many microorganisms had shown the activity of enzymes of more than one branch of the nitrilase superfamily.

3.3 NITRILASES

First nitrilase was isolated and studied in the early 1960s in plants. There, it converts indole-3-acetonitrile into indole-3-acetic acid, which is a growth hormone (auxin) promotes the root production in plants (Thimann and Mahadevan *et al.,* 1964).Nitrilase had been in the focus of several studies from past few decades due to its wide application in chemical synthesis. In this period of time, nitrilase was isolated and characterized from various organisms including bacteria, filamentous fungi, yeasts and plants (Banerjee *et al.,* 2002). Many of the nitrilase producing microorganisms were used for the manufacturing of various carboxylic acids on large (industrial) scale. The economic potential of nitrilases came into light when nicotinic acid (Schmid *et al*., 2001) and (R) (-) mandelic acid (Shaw *et al.,* 2003) was successfully produced on industrial scale.

Enzymes belonging to nitrilase super family Aare found in both eukaryotes and prokaryotes. In microorganisms nitrilase activity has been found mainly in *Brevibacterium, Coryne bacterium, Nocaria, Arthrobacter, Rhodococcus* and *Pseudomonas* (Robinson *et al,* 1964). Nitrile hydratase-amidase activity is also found in those microbes; in some cases both type of nitrile hydrolyzing enzyme system is present in a single strain.

Most of the nitrilase reported are thermolabile. They are present in a vast range of mesophilic bacteria i.e. they cannot withstand high temperature of the reaction medium. This property limits the use of nitrilase in commercial applications. Though, this can be partially improved by either using immobilization methods or by using biphasic system (Rezende *et al*., 2000).

In the last few years some thermophilic nitrilases have been isolated and their detailed structural characterization has been carried out. The studies have shown that despite of having thermophilicity, they have structural and functional similarity to the mesophilic ones (Gong *et al.,* 2012).

3.3.1 STRUCTUAL PROPERTIES OF NITRILASE

Nitrilases (EC 3.5.5.1) constitute of thiol group at their catalytic site. Most of the nitrilases are homooligomers with a peptide protein of 40kDa (Harper, 1977). But in the case of *Nocardia*sp. (NCIB 11216) the individual subunits have a monomeric peptide of 47kDa. It was also observed that the subunits mentioned before self-associate to convert the enzyme into its active form. This is due to benzonitrile induced activation of nitrilase and the association of polypeptide lead into the formation of dodecamer complex of 560 kDa (Harper, 1977b).

Structural analysis revealed that nitrilase consists of monomeric subunits in a form of αβαβ-fold combined in a regular fashion of dimmers. In the other members of this super family these dimers are associated in oligomeric forms in different ways (Pace *et al.*, 2000).

Nitrilase also have the conserved amino acids Cys-Glu-Lys (catalytic triad), which leads to the substrate binding of the catalytic site of enzyme and leads to covalent catalysis (Brenner *et al.*, 2002). Nitrilase structure comprises of 30% of helical structure (O'reilly and Turner, 2003). In the recent research, it has been proposed that the catalytic triad includes an extra Glu residue which is structurally conserved and this makes it a catalytic tetrad (Thuku*et al*., 2009). Nitrilase obtained from *R .rhodochrous* J1 observed using electron microscopy, have an extended helix structure associated with a 480 kDa dimer complex, forming a homo oligomeric structure (Thuku and webber, 2007).

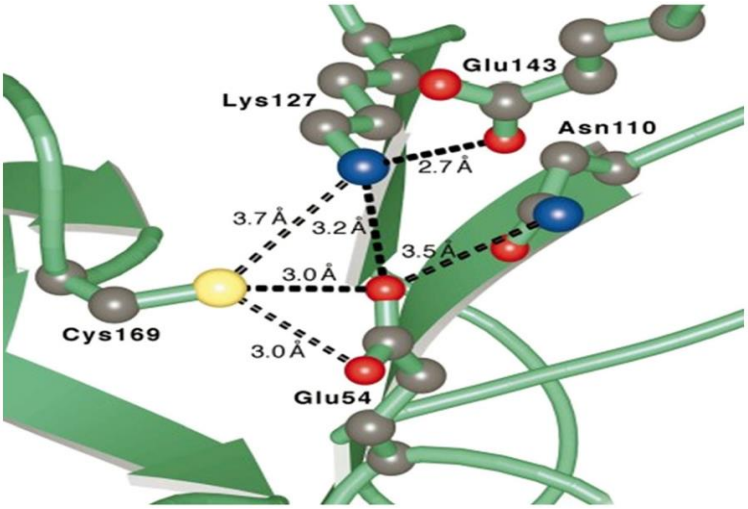


Figure-5 structure of nitrilase(Thuku and Webber, 2007)

Studies involving circular dichorism (C.D.) spectroscopy revealed secondary structure and that consists of 30% α-helix, 16% turn, 21% β-sheet and 33% other structure and the same is observed using electron microscope (Thuku *et al.,* 2009).

Many varieties of nitrilases consist of multimeric form, required to perform their function. Generally these varieties of nitrilases are homopolymers of 4 to 26 subunits (Goldlust and bohak, 1994). Molecular mass of single subunit of nitrilase vary from 32-47 kDa observed in a wide range of species (O'reilly and Turner, 2003).

3.3.2 SUBSTRATE SPECIFICITY OF NITRILASES

Nitrilase show a broad range of substrate specificity, so to simplify that they are categorized into 3 main categories: - Aliphatic, Aromatic, Aryl acetonitrilases (Gupta *et al.,* 2010). From the structural analysis, it is clear that nitrilase comprises of monomeric subunits which self associate to form a protein complex resulting into the activation of enzyme. As observed in *Nocardia* species monomeric subunit associate to form a dodecamer complex because of benzonitrile induction, it was also observed that this formation of complex can also be strengthened by increasing salt concentration and temperature (Harper *et al.,* 1977). This property of nitrilase is known as nature of enzyme expression or inducibility.

In the below table nitrilases of various bacterial species are shown with their substrate specificity and nature of enzyme expression.

BACTERIAL SP.	NATURE OF ENZYME EXPRESSION	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
Comamonastestosteronisp.	Inducible	Aliphatic nitriles
*R. rhodochrous*K22	Inducible	Aliphatic nitriles
*Pyrococcusabyssi*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
Halomonasnitrilicus sp. nov.	Inducible	Arylaliphatic nitriles

Table 1- List of nitrilase producing organisms and their corresponding substrate (Agarwal *et al.*, 2012).

By increasing the concentration of salts and in the presence of solvents the hydrophobic part of the monomers gets exposed and it leads into the association of them to form active unit of enzyme (Nagasawa *et al.,* 2000). It was reported that nitrilase have a cysteine residue at or near the catalytic site which is catalytically very important. Meanwhile, metal ion is required by nitrile hydratase which act as prosthetic group during catalysis. No such metal ion is required by nitrilase during catalysis (Kobayashi *et al.,* 1992).

3.3.3 CATALYTIC PROPERTY AND MECHANISM OF ACTION

The first ever nitrilase isolated by Mahadevan in 1964 was studied by various scholars. It was proposed that the thiol group present in the nitrilase attach to the nitrile carbon and perform nucleophilic attack on it. This results into the formation of a tetrahedral thiomidate (an enzyme linked intermediate), which is than hydrolyzed into ketone along with the liberation of NH_3 (Ammonia). With the addition of one water molecule Acyl-enzyme break down to form carboxylic acid. Later the enzyme gets regenerated. The same mechanism was also reported in hydrolysis of ricinine by the ricinine nitrilase (Robinson and Hook, 1964) (Stevenson *et al.,* 1992). In the later researches this proposed mechanism is widely accepted by many scholars (Banerjee *et al.,* 2009). Although, this was also reported in a research that the hydrolysis of nitrile mediated by nitrilase also produce amides as a byproduct (Pitrowski *et al.,* 2006).

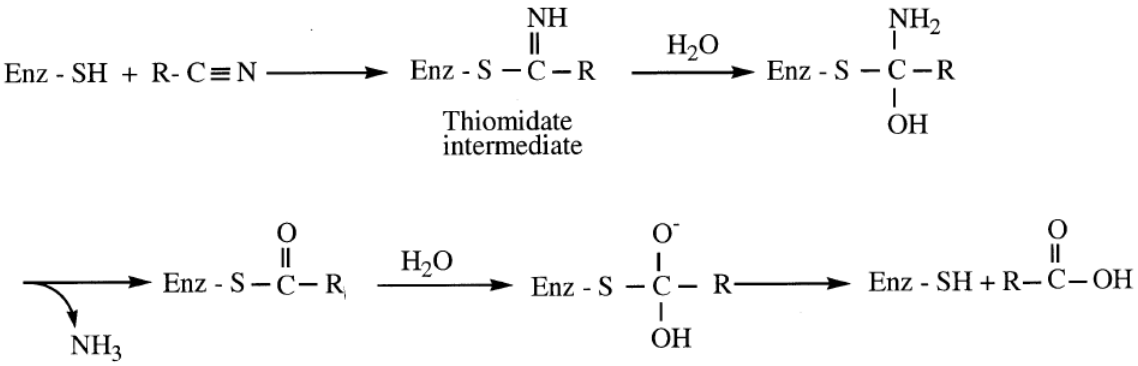


Figure 6- Catalytic mechanism of nitrilase (Kobayashi *et al.,* 1998)

Nitrilase activity in many bacteria got affected by many compounds either positively or negatively. Mercaptoethanol and DTT (dithiotreitol) boost the activity of nitrilase due to presence of thiol group (Layh*et al.,* 1998). On the other hand, metal ions like Ag, Zn and copper acts as inhibitor of catalytic property of enzyme. All these observation leads to a conclusion that cysteine present on the active site play a very significant role in the complete functioning of enzyme (Yamamoto *et al.,* 1991).

As mentioned before, there are some nitrilases which produce amides as a byproduct. In later studies, it was proposed that Amide production is due to the absence of positive charge from the reactant. Due to this the steric and electronic property of the interaction of reactant with enzyme, got changed (Chen *et al.,* 2009). It was also observed that due to the electron deficiency of α-substituent, the production of amide increases (Brady, 2010).

3.3.4 SOURCES OF NITRILASES

Nitrilase plays an extensive role in metabolism and defense of various organisms i.e. bacteria, plants, fungi and yeast. Nitrilase from microbial sources is important in the commercial production of many important organic compounds. This led the researchers to focus their studies on the microbial nitrilases.

3.3.4.1 Bacteria

The first nitrilase from a bacterial source was reported in 1964. It is a ricinine nitrilase obtained from a bacterial strain belonging to *Pseudomonas* genus, characterized on the basis of morphological properties (Robinson and Hook, 1964). Thereafter, many bacterial strains mainly belonging to the genera of *Rhodococcus, Nocardia, Acinetobacter, Alcaligenes, Pseudomonas* and *Corynebacterium* had been reported of showing nitrilase activity. Successful use of nitrilase obtained from *R. rhodochrous*J1, in industrial production of carboxylic acids, created a substantial hope on the enzymes of nitrilase superfamily. This lead the nitrilase in the lime light in the field of industrial biotechnology (Kobayashi *et al.,* 1989).

3.3.4.2 Filamentous fungi

In many fungal strains, nitrilase activity was reported which can convert indole-3-acetonitrile into indole-3-acetic acid. These strains belong to the fungal genera of *Penicillium, Fusarium, Aspergillus* and *Gibberell* (Thimann and Mahadevan, 1964). But the nitrilase from only *Fusarium solani* had shown significant activity in the degradation of herbicide bromoxynil (3, 5-dibromo-4-hydroxybenzontrile) and ioxynil (3, 5-diiodo-4-hydroxybenzonitrile). *F.solani* IMI 196840 was studied by a research group of Czech Republic and the result showed a promising biocatalyst for the utilization of fungi in nitrile transformation (Hsu and Camper, 1979). This new nitrilase from fungal source was further purified and immobilized and used in the production of organic acids.

3.3.4.3 Yeasts

There are over 60 strains of yeast which can metabolize nitriles. These include species of *Pichia, Candida, Hanseniaspora, Geotrichum, Candida, Debaromyces, and Aureobasidium.* They were isolated from soil, cyanide treatment bioreactor and food samples. But most of these species reported to have Nitrile-hydratase-amidase system of nitrile hydrolysis (Sharma *et al.,* 2011) (Brewis *et al.,* 1995). Only few were reported to have nitrilase system, showing nitrilase activity. Nitrilase mediated biotransformation from yeast source was reported to occur in the acidic conditions as well usually pH 4.0-7.0. This has advantage in the biotransformation of aminonitriles and hydroxy nitriles, which cannot withstand the neutral pH. The strain reported of showing this property is *Cryptococcus* sp. UFMG-Y28 (Rezende *et al.,* 2000).

3.3.4.4 Plants

Nitrilases have a crucial role in the defense mechanism and growth hormone production of plants. Nitrilases increase the production of auxin by converting indole-3-acetonitrile into indole-3-acetic acid. Auxin increases the induction of plant roots. The first nitrilase from a plant species was isolated from barley in 1964 (Thiman and Mahadevan, 1964). Other plants which are reported to show nitrilase activity are *Arabidopsis thaliana* (Plotrowski*et al*., 2001)and *Brassica rapa* (Rausch *et al.,* 1980). The *Brassica rapa* also known as Chinese cabbage have a magnificent activity of nitrilase for various aromatic and aliphatic nitrilases but it was reported that nitrilase from this plant shows poor activity for indole-3-acetonitrile (Rausch *et al.,* 1980).

Recent studies have demonstrated that plant nitrilase have cyanide detoxification function. And also many plant nitrilases are involved in the catabolism of cyanogenic glycosides (Piotrowski *et al*., 2001).

3.4 FACRORS INFLUENCING NITRILASE

3.4.1 NITROGENANDCARBON SOURCE

Carbon and nitrogen source have a significant impact on the cellular enzyme biosynthesis and cell proliferation, on the overall, affecting the enzyme yield. In some cases, there are physiological events like catabolic repression due to glycerol, glucose and fructose, which regulate the expression of enzyme in the cell (Mao *et al*., 2005). In *P.putida* MTCC5110 it was reported that in the presence of glycerol there is little nitrilase production but the biomass generated is very high in comparison to lack of glycerol in media (Shen *et al*., 2009). Glucose, starch, sodium acetate and mannitol are the optimum carbon sources while casamio acid and peptone are the optimum nitrogen sources for the nitrilase in bacteria (Mao *et al*., 2005).

3.4.2 METAL IONS

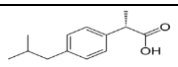
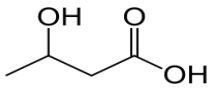
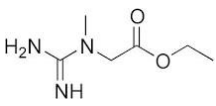
Nitrile hydratases requires Fe^{3+} and Co^{2+} as a cofactor for their activity and production also. While, in case of nitrilases no such requirement of cofactor was reported for its activity (Prasad *et al.*, 2010). But it was also observed by few research groups that some metal ions like Co^{2+}, Ag^{+} and Hg^{2+} inhibit nitrilase activity and Copper ion decreases the cell growth (Mao *et al*., 2005). These results were reported in *P.putida* MTCC5110 and *R.rhodochrous* J1. An opposite result was reported in *Alcaligenes* sp. ECU0401, where Cu^{2+} supported both high cell growth and enhanced specific activity of nitrilase (Brady, 2010). All these results give unclear results about the effect of metal ion on nitrilase activity. This demands the need to study the effect of metal ions more precisely on nitrilase.

3.4.3 IMMOBILIZATION

Immobilization of nitrilase can be achieved by both using whole cells and purified nitrilases. Most of the industrial protocols involving nitrile biocatalysis use immobilized biocatalysts. An immobilized lipase in sol-gel reported to have increased activity up to 100 fold as compared to the free cells (Reetz *et al.,* 1995). In case of nitrilase, immobilized cells were more stable and can hydrolyze more nitriles due to less substrate inhibition as compared to free cells. *A.faecalis* MTCC 126 had given activity at 65^0C after immobilization (Kaul *et al.,* 2006). The *Candida guilliermondii* CCT7207 cells were able to degrade some nitriles after immobilization, which it was not able to hydrolyze when present in free cell form (Rezende*et al*., 2000).

3.5 APPLICATION OF NITRILASE

Nitrilase catalyzed transformation of nitriles have enormous application in synthesis of organic acids mainly carboxylic acids like acetic acid, mandelic acid and indole-3-acetic acid by the means of either chemical or enzymatic hydrolysis (Heinemann *et al.,* 2003). Nitriles have a broad range of applications in industries as extractants, solvents, drug intermediates, pharmaceuticals, pesticides and herbicides intermediates in the synthesis of amines, amides, esters, heterocyclic compounds, etc. (Banerjee *et al.,* 2002).

PRODUCT	**APPLICATION**	**ENZYME SOURCE**	**STRUCTURE**	**REFERENCE**
Ibuprofen	Inflammatory drug	*Acinetobacter* sp		Yeom *et al.,* 2008
(R)-4-Cyano-3-hydroxybutyric acid	In the treatment of alcoholism and *source* of energy in the heart and brain	*Acinetobacter* sp		Yeom *et al.,*2008
Ethyl ester	Cholesterol lowering drug	*E. coli*		Stalker *et al.,* 1988

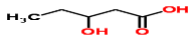
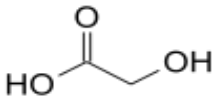
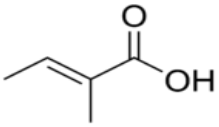
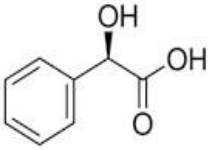
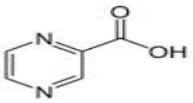
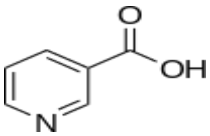
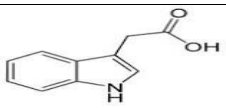
3-Hydroxyvaleric acid	Synthesis of polyester	*Acidovorax facilis*72W		Wu *et al.*,2008
Glycolic acid	In medical and industrial products	*Acidovoraxfacilis72W*		Wu *et al.*,2008
(E) -2- Methyl-2-butenoic acid	Used in making perfumes and flavoring agents.	*Acidovoraxfacilis72W*		Hann *et al.*, 2004.
R-(-) Mandelic acid	Semisynthetic cephalosporins pharmaceuticals	*Alcaligenes faecalisATCC 8750, Pseudomonas putida, Microbacterium paraoxydans and Microbacterium 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 properly)	Food source proteins		Mathew *et al.*,1998
Indole acetic acid (IAA)	Plant growth promotion	*Streptomycesgrise oviridisi*K61		Khamna *et al.*, 2010

Table 2- Application of nitrilase in production of commodity chemicals and fine chemicals.

4. METHODOLOGY

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.

4.1 ENZYME ASSAY

The Nitrilase activity was assayed in 1ml reaction mixture containing 0.1 M potassium phosphate buffer (pH-7.0) using 50 µl cell suspension and 50 mM acetonitrile as substrate at 50^{0}C for 15 min. The reaction was stopped by adding equal volume of 0.1M HCl. In the control, the nitrile was omitted during incubation and added after the reaction was stopped. It was centrifuged at 10,000 x *g*. The clear supernatant was collected and the pellet was discarded. The reaction mixture was centrifuged at 10,000 x g for 10 min, to 1 ml of the clear supernatant, 2.5 ml of solution A (containing 1% phenol, 0.005% sodium nitroprusside), was added, mixed well, kept at room temperature for 5 min and then 2.5 ml of solution B (containing 0.5% sodium hydroxide, 0.02N sodium hypochlorite) was added. The mixture was kept in boiling water bath for 5 min, cooled to room temperature and its absorbance was recorded at 640 nm (Fawcett and Scott 1960).

4.2 DRY CELL WEIGHT

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

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

4.3 IMMOBILIZATION PROCEDURE

4.3.1 CALCIUM ALGINATE IMMOBILIZATION:-

Three percent solution of sodium alginate was made by dissolving 3gm of it 100ml water. To 10ml of this solution, approximately 253 mg of cell dry weight was mixed. The concentration of sodium alginate can be varied between 6-12 % depending on the desired hardness. The beads are formed by dripping the polymer solution from a height of approximately 0.5cm into an excess (100 ml) of stirred 0.2M $CaCl_2$ solution with a syringe and a needle at room temperature.

4.3.2 IMMOBILIZATION USING POLYACRYLAMIDE:-

Buffered monomer solution was prepared by adding 1.1 g of bis-acrylamide and 20 g of acrylamide to a 100 ml of buffered solution (pH 7.0) of 0.1mM EDTA and 0.1M Tris-HCl in a beaker. To 10 ml of the buffered monomer solution of the above step, 320 mg cell and 0.1 ml of dimethylaminopropionitrile was added and mixed. To this solution 1.0 ml of freshly prepared ammonium persulphate solution (1%) was added to initiate polymerization. The solution was poured into a mold and left undisturbed, after approximately 10-30 minutes get hardened. The resulting gel was cut into small cubes of approximately 3mm per side. Alternatively, if smaller pieces are desired, the gel can be forced through a syringe fitted with a fine needle. Beads/discs were gently washed to the free enzyme off the gel surface in 10 ml of the washingsolution.

4.4 OPTIMIZATION OF REACTION CONDITION OF IMMOBILIZED CELLS FOR NITRILASE ACTIVITY

4.4.1 Effect of pH

Different buffers system with range of pH 3 to pH 9 were prepared and used for the identification of the optimum pH for the reaction condition. Nitrilase assay was carried out.

4.4.2 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.

4.4.3 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.

4.4.4 Optimum substrate Concentration

Maximum enzyme activity was obtained when benzonitrile was used as substrate. To determine the optimum substrate concentration, nitrilase enzyme assay of *Pseudomonas* sp. KNB2 i.e. immobilized by two different entrapment matrix, was performed using different concentration of benzonitrile (i.e. 20mM, 30mM, 40mM, 50mM, 60mM, and 70mM)

4.4.5 Effect of metal ions

Different metal ions with a final concentration of 1mM/L, to check there influence on enzyme activity. Nitrilase assay was carried out with immobilized cells of *Pseudomonas* sp. KNB2 under optimum conditions using benzonitrile as substrate.

4.4.6 Cell viability

Stability of immobilized cells was analyzed by placing cell beads into phosphate buffer at previously determined optimum reaction conditions. Nitrilase assay was performed after 24 hrs subsequently for 5 days.

4.4.7 Density of cells per bead

To obtain optimum density of cells (dry weight in mg) per bead, cells immobilized beads are made with dry weight ranging from 0.3 mg to 0.9 mg and specific activity was determined separately of each bead.

4.5 Characterization of bacteria

To identify the isolated bacterial strain i.e. showing thermophilic nitrilase activity, a series of biochemical tests were performed. The results of these biochemical tests were analyzed and linked to determine the genus and species of the present strain of bacteria. The detailed procedure of these tests is written blow:-

4.5.1 Gram staining:-

Prepared the smear and heat-fixed it. Stained the slide by flooding it with crystal violet for 1 min. Poured off excess dye and washed gently in tap water. Added Gram's iodine for 60 second by washing with iodine then added and left it on the smear. Washed with tap water and drained. Washed with 95% alcohol drop by drop until no more color flows from the smear. Washed the slides with distilled water and drained. Counter stained with safranin for 1 min. Washed, drained, blotted and examined under immersion oil at 100x objective.

4.5.2 Endospore staining

Prepared bacterial smears, air dried and heat fixed the smears. Dry and heat fixed the smears. Flooded the smear with Malachite green. Heated slides to steaming and steam for 5 minutes, adding more stain to the smear from time to time. Washed slide under slowly running tap water. Counter stained with safranin for 30 second. Washed smear with distilled water. Blot dried slides with absorbent/blotting paper. Endospore stained green and vegetative cells stained red.

4.5.3 Indole test

Bacterium to be tested was inoculated in tryptone water, containing amino acid tryptophan and incubated overnight at 37°C. 2. Followed by incubation, few drop of Kovac's reagent were added. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and conc.

HCl. Ehrlich's reagent is more sensitive in detecting indole production in anerobes and non-fermenters. Formation of a red or pink colored ring at the top is taken as positive.

4.5.4 Methyl Red (MR) Test

Bacterium to be tested was inoculated into glucose phosphate broth, which contains glucose and a phosphate buffer and incubated at 37°C for 48 hours. Over 48 hours the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acid. pH of the medium was tested by addition of 5 drops of MR.

4.5.5 Voges Proskauer (VP) Test

Bacterium to be tested was inoculated into glucose phosphate broth and incubated for at least 48 hours. 0.6 ml of alpha-naphthol will be added to the test broth and shaken. 0.2 ml of 40% KOH was added to the broth and shaken. Tube was allowed to stand for 15 minutes. Negative tube was held for one hour, since maximum color development occurs within one hour after addition of reagents.

4.5.6 Urease test

Inoculated a urea broth tubes with the microbe to be tested. Incubated at optimum temperature for 24 hours. If the organism has the ability to utilize urea, the medium would change its color from yellow to pink.

4.5.7 Sugar fermentation test

A single carbohydrate for each of batch medium was prepared. After inoculation a Durham tube was inserted. Kept one tube as un-inoculated or as a control. Incubated at optimum temperature for 24 hours. If the organism has the ability to ferment sugar the medium would change its color from yellow to pink. If the organism has the ability to produce gas, the inverted Durham tubes in the medium were detected the presence of a gas.

4.5.8 Catalase test

Inoculate Simmons Citrate Agar lightly on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old. Incubate at 35^{o}C to 37^{o}C for 18 to 24 hours. Some organisms may

require up to 7 days of incubation due to their limited rate of growth on citrate medium. Observe the development of blue color; denoting alkalinization.

4.5.9 Nitrate test

Inoculate nitrate broth with a heavy growth of test organism using aseptic technique. Incubate at an appropriate temperature for 24 to 48 hours Add one dropper full of sulfanilic acid and one dropper full of a α-naphthylamine to each broth. At this point, a color change to red indicates a positive nitrate reduction test. If you get a red color, then you can stop at this point. No color change indicates the absence of nitrite. If you do not get a red color, then you must proceed to the next step. Add a small amount of zinc (a toothpick full) to each broth. Zinc catalyzes the reduction of nitrate to nitrite. At this point, a color change to red indicates a negative nitrate reduction test because this means that nitrate must have been present and must have been reduced to form nitrite. No color change means that no nitrate was present. Thus no color change at this point is a positive result

4.5.10 Soft Agar Stabbing (Tube Method) for motility

Label the tubes of semisolid (or SIM) media with the names of the organisms. Flame and cool the inoculating needle, and insert it into the culture after flaming the neck of the tube. Remove the cap from the tube of medium, flame the neck, and stab it 2⁄3 of the way down to the bottom. Incubate the tubes at room temperature for 24 to 48 hours. Examine the SIM cultures for the presence or absence of a black precipitate along the line of the stab inoculation. A black precipitate of FeS indicates the presence of H2S. If desired, one can also test for indole production by adding 5 drops of Kovacs' reagent to the SIM cultures and looking for the development of a red color at the top of the deeps. In agar stab method a well dispersed growth from the line of inoculation is evidence of motility,

5. RESULTS:-

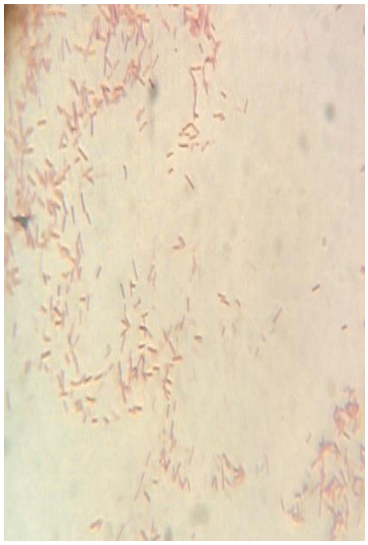
5.1 CHARACTERIZATION OF BACTERIAL CELLS:-

S.No	Biochemical Test	Observation	Result
1	Gram staining	Red color	Gram -ve
2	Morphology	Straight rod shaped	*Pseudomonas* sp.
3	Spore (sporing/non-sporing)	Unstained capsule observed	Non-sporing
BIOCHEMICAL TEST			
4	Sugar fermentation	Glucose	-ve
		Sucrose	-ve
		Lactose	-ve
5	Catalase test	Production of bubbles	+ve
6	MR/VP test	No ring appeared	MR -ve
		No ring appeared	VP -ve
7	Indole test	No ring appeared	-ve
8	Motility test	Bacterial growth observed along the stab	Motile (Unipolar)
9	Urease test	No color change observed	-ve
10	Citrate test	Intense blue color observed along the slant	+ve

11	Nitrate reduction test	red color observed	+ve

Table 3- Result of biochemical tests performed on *Pseudomonas* sp. KNB2

A



B

C

D

E

F

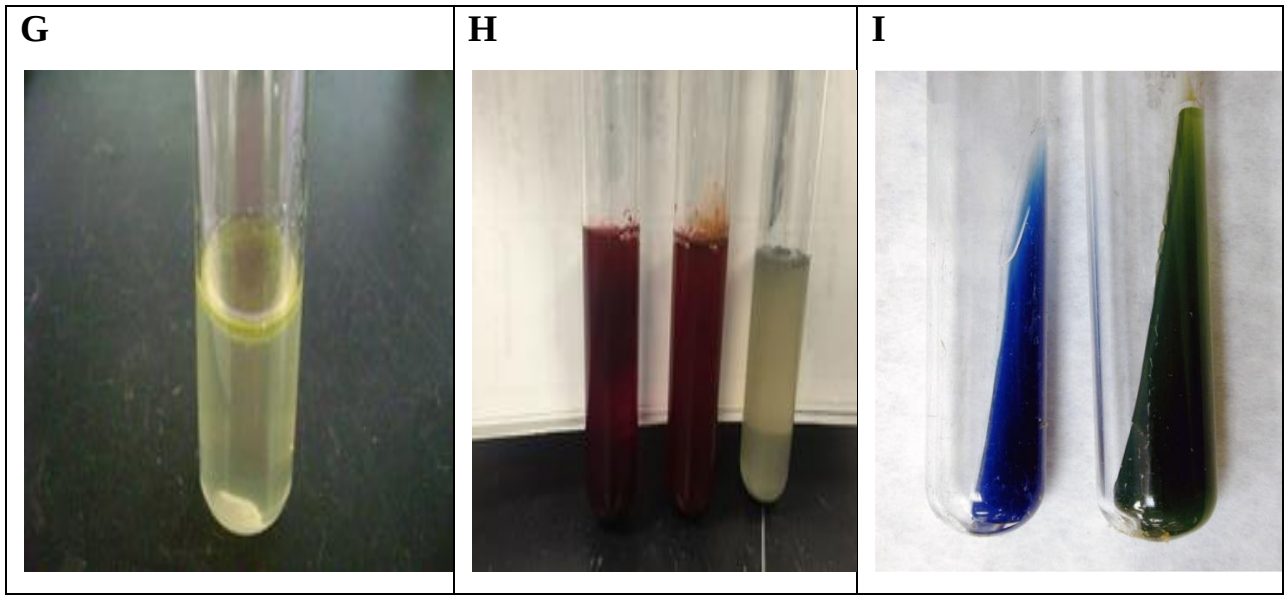


Figure 7:- A) Gram Staining (-ve), B) Sugar fermentation test (-ve), C) Catalase Test (+ve), D) MR/VP test (-ve), E) Indole test (-ve), F)Motility test, G) Urease Test (-ve), H) Citrate test (+ve), I) Nitrate reductase test (+ve)

The analysis of results showed that the present strain belongs to "*Pseudomonas"* genus and strain "*aeruginosa"*, which require more test for conformation. So, the strain was named *Pseudomonas* sp. KNB2.

5.2 OPTIMIZATION OF PRODUCTION CONDITIONS OF IMMOBILIZED *PSEUDOMONAS* SP. KNB2 NITRILASE

5.2.1 Effect of substrate

5.2.1.1 Using Calcium Alginate entrapment immobilization

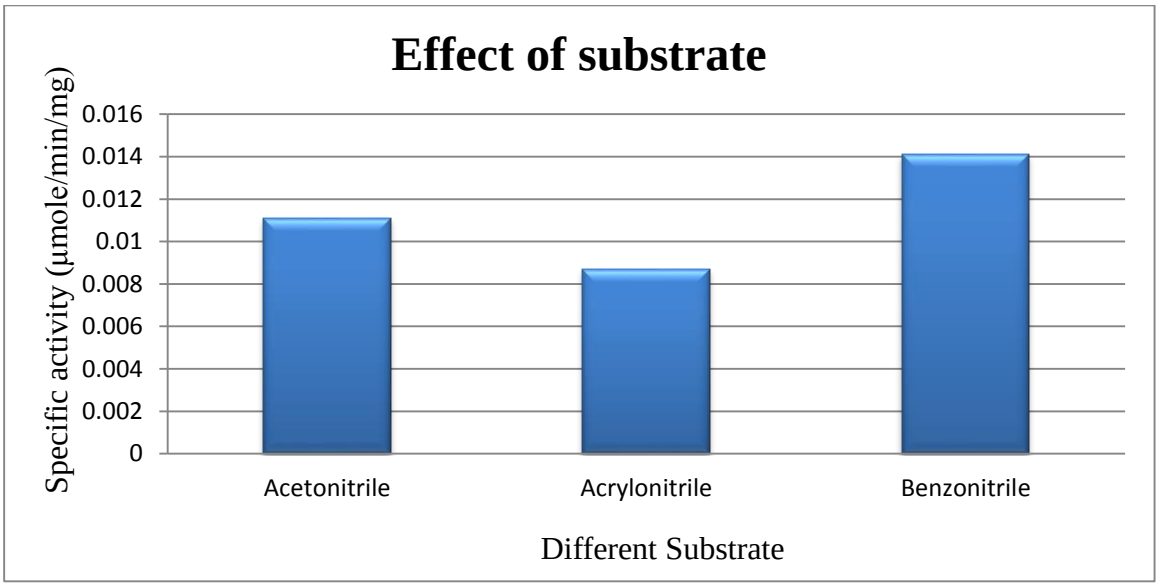


Figure 8- Specific activity of *Pseudomonas* sp. KNB2 with calcium alginate immobilization using different substrate.

To determine the substrate specificity of the nitrilase of immobilized cells of *Pseudomonas* Sp. KNB2, three different nitrile substrates i.e., benzonitrile, acetonitrile, acrylonitrile, were used in the enzyme assay. The cells immobilized in calcium alginate showed highest nitrilase activity with benzonitrile (0.0141µmole/min/mg) and polyacrylamide immobilized cells also used benzonitrile more efficiently (0.013 µmole/min/mg).

5.2.1.2 Using polyacrylamide entrapment immobilization

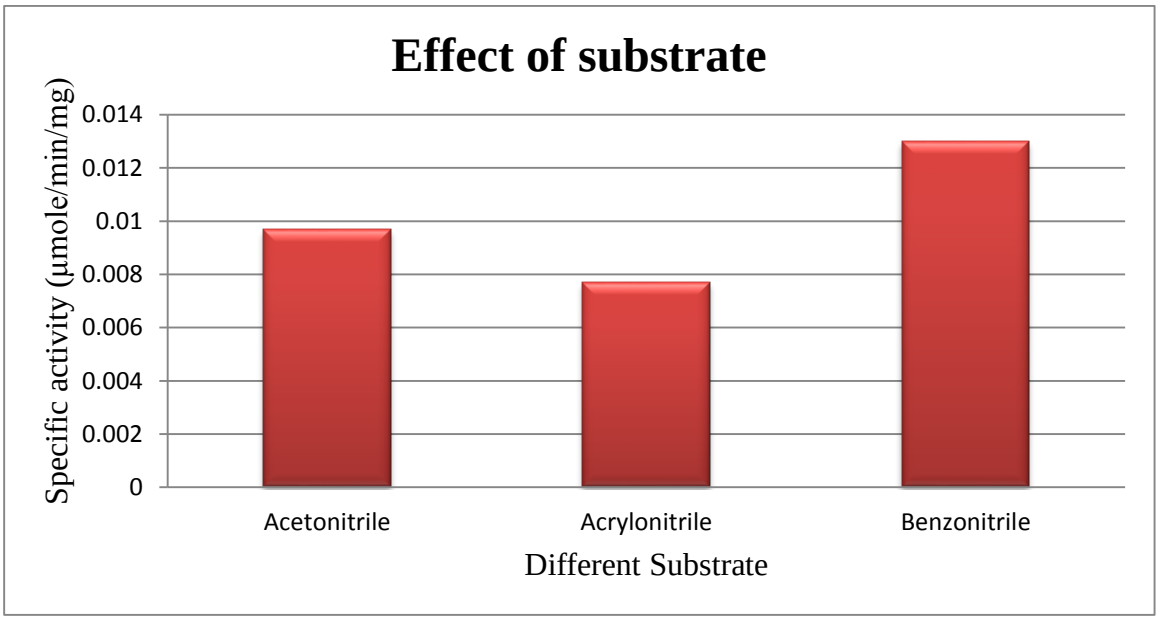


Figure 9- Specific activity of *Pseudomonas* sp. KNB2 with polyacrylamide immobilization using different substrate.

In the previous studies it was also reported that nitrilase showing more activity using benzonitrile as a substrate. Some examples are *Pseudomonas putida* (Nagasawa *et al*., 2000), *Bradyrhizobium japonicum* (Howden and Preston, 2009), *P. fluorescens* (Pace *et al*., 2001), Pseudomonas (Banerjee *et al*., 2006). These results depicts that the present nitrilase is specific to aromatic substrates.

5.2.2 Optimum density of cells (dry weight) per bead

5.2.2.1 Using Calcium Alginate entrapment immobilization

Cell beads were made using two different type of entrapment: - 1) calcium alginate and 2) polyacrylamide with a diameter of 3.0mm and 4.0 mm respectively. To optimize cell density per bead for obtaining maximum production, beads were prepares with varying cell density from 0.3 mg to 0.9 mg cells (dry weight) per bead

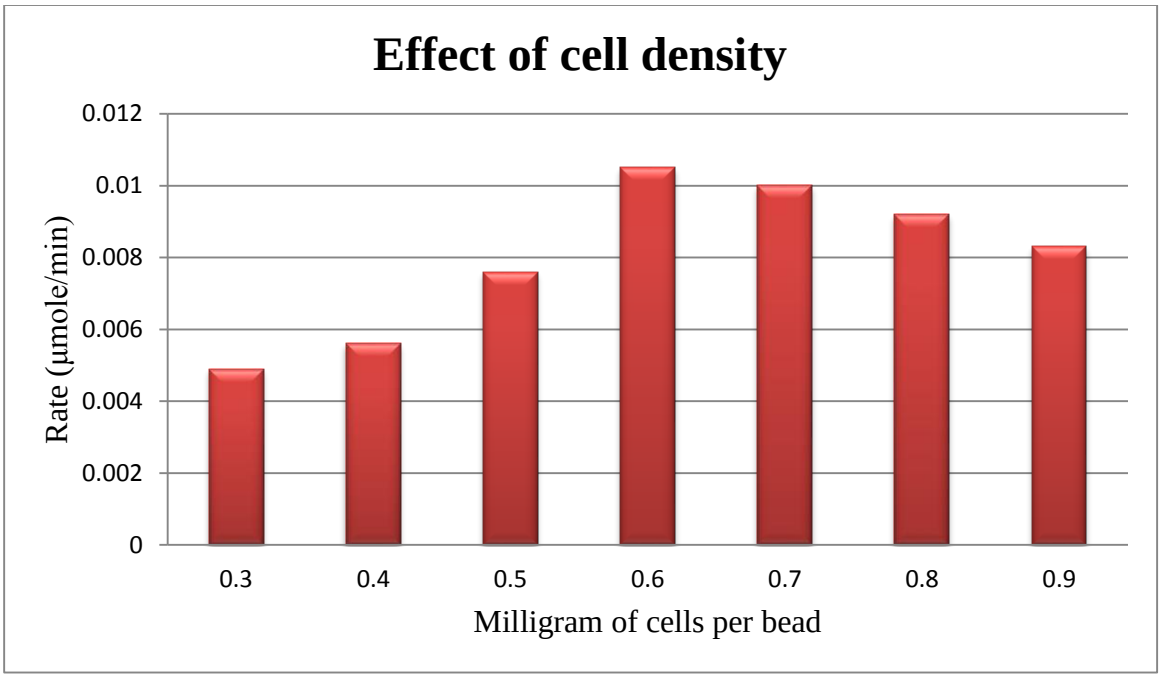


Figure 10- Conversion rate at different bead density (calcium alginate immobilization)

Conversion rate of cells within calcium alginate beads were highest at a cell density of 0.6 mg per bead.

5.2.2.2 Using polyacrylamide entrapment immobilization

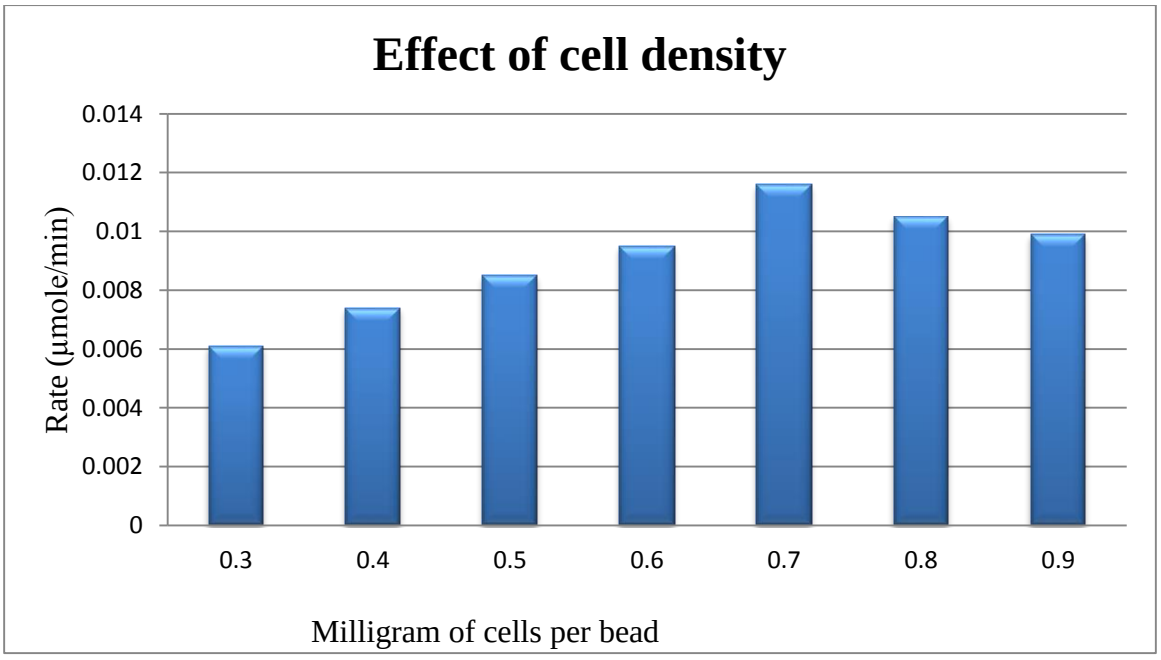


Figure 11- Conversion rate at different bead density (polyacrylamide immobilization)

Results showed that beads with calcium alginate entrapment had given maximum activity with 0.6 mg of cells while those with polyacrylamide entrapment had maximum activity with 0.7 mg of cells.

5.2.3 Effect of Substrate concentration

Many researchers previously reported that several nitrilase from different strains had shown substrate inhibition. After the optimum substrate concentration, Lowan *et al.*, (1989) had observed decline in the activity of nitrilase. When the present nitrilase activity was observed using different concentration of benzonitrile (20mM-70mM), the maximum activity was observed at 40mM (.0148µmole/min/mg

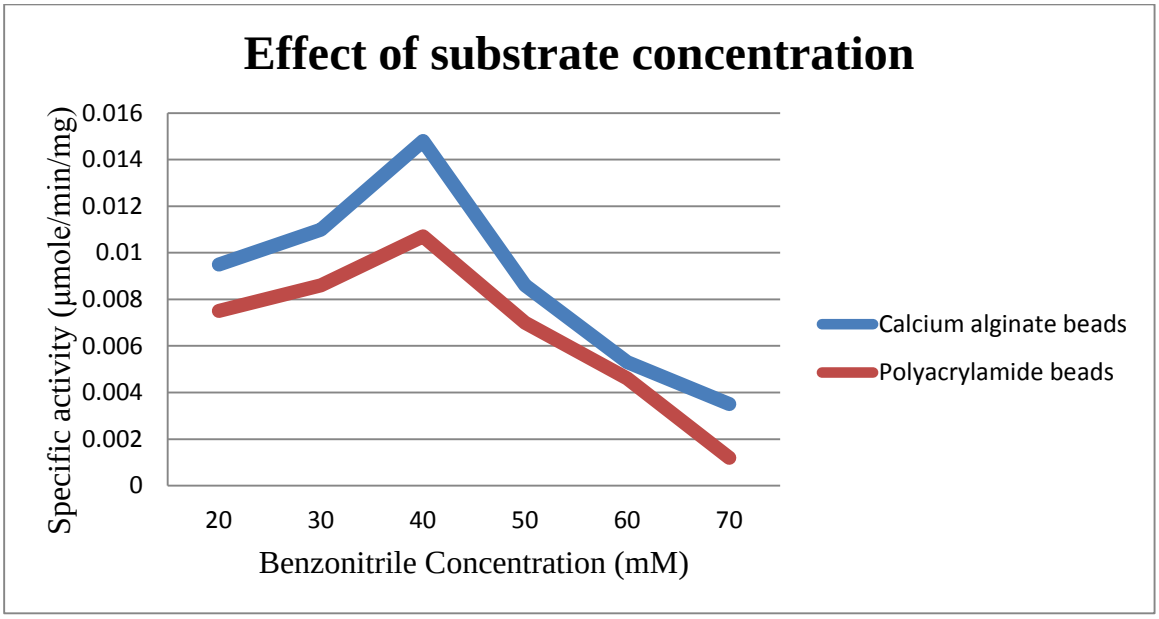


Figure 12- Specific activity of immobilized *Pseudomonas* sp. KNB2 using different substrate concentrations.

At 30mM and 50mM, enzyme had a significant activity. So, the immobilized cells have an optimum range of substrate concentration from 30-50mM. After 50 mM concentration of substrate there was rapid decrease in the enzyme activity, which was may be due to substrate inhibition.

5.2.4 Effect of pH

Nitrilase activity at different pH (4-9) was observed of immobilized cells. The results had shown that the present nitrilase had optimum pH from a range of 6 to 8 pH. At pH 7 maximum activity was observed, that is 0.0116 µmole/min/mg.

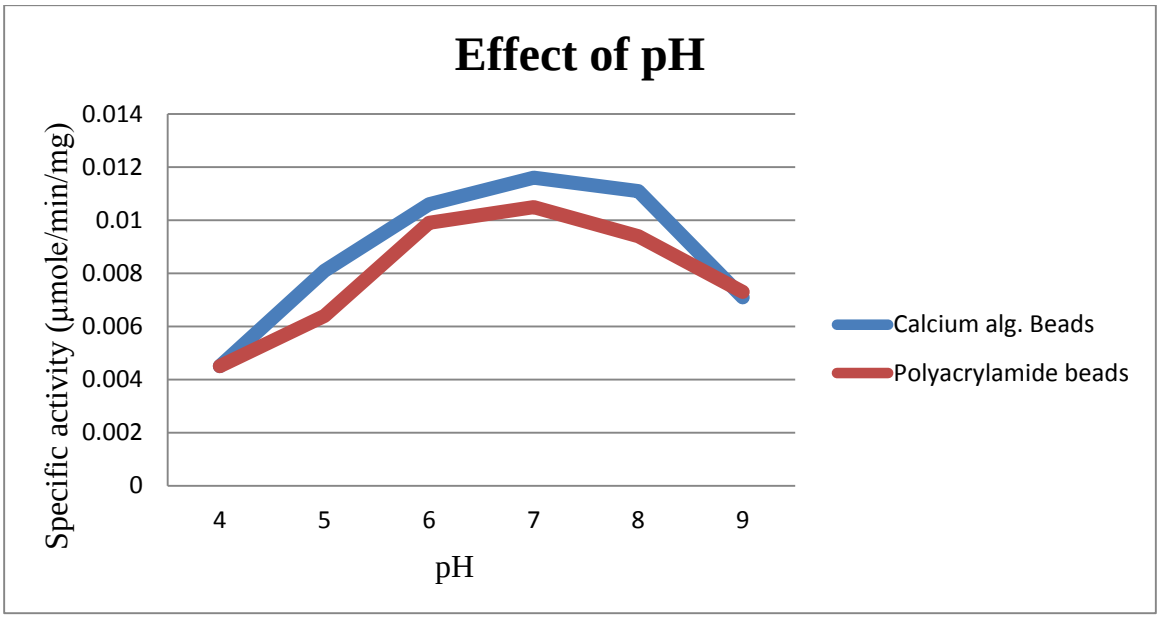


Figure 13- Specific activity of immobilized *Pseudomonas* sp. KNB2 at different pH

Previously, many nitrilase from different microbes had shown optimum pH activity at neutral pH for example *Arthrobacter* sp. Strain J1 pH -8.5/7.5, *Pseudomonas putida* pH-7, *Rhodococcus rhodochorus* ATCC 39484 pH-7.5. Nitrilase obtained from *Pseudomonas* sp. 13 characterized to have optimum pH value of 9.5 by using 10mM 2-mercaptoethanol in reaction medium (Rustler *et al*., 2008).

5.2.5 Effect of Temperature

For determining optimum temperature to obtain maximum production of immobilized nitrilase, specific activity was observed at different temperature ranging from 35^0C to 65^0C. From the results it was observed that optimum temperature of the present nitrilase was in the range of 45-60^0C.

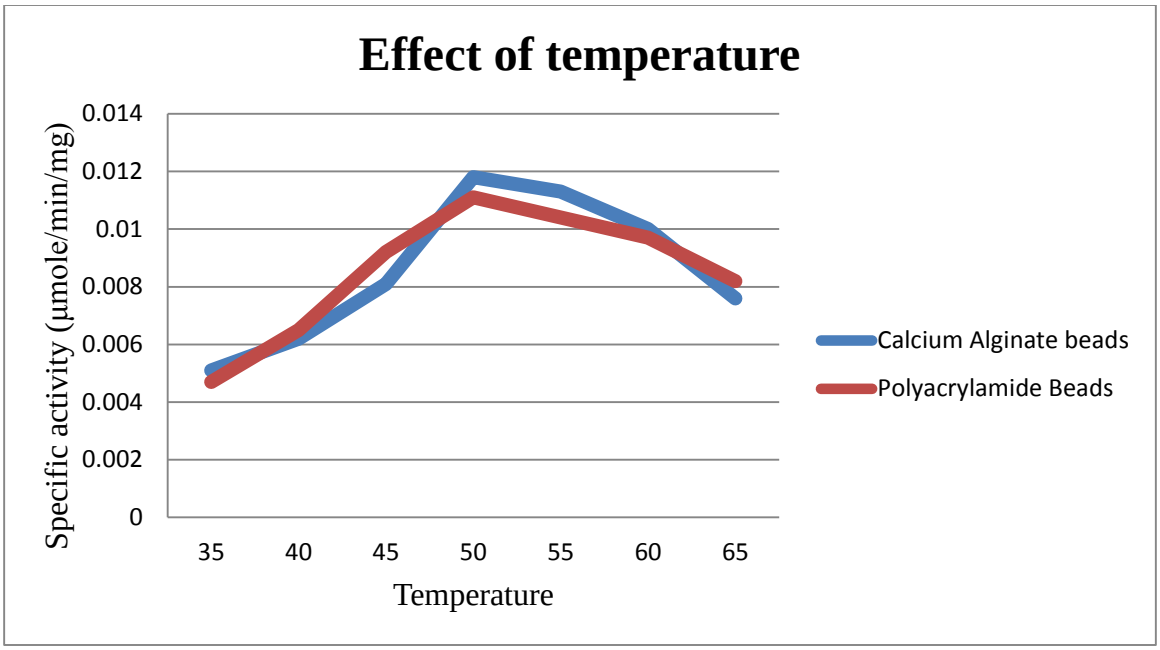


Figure 14- Specific activity of immobilized *Pseudomonas* sp. KNB2 at different temperature.

As the enzyme was a thermophile, it had maximum activity at 50^0C (0.0118µmol/min/mg). Some bacteria which had been reported with an optimum temperature of 40-45^0C were *P. fluorescens* EBC191 (45^0C) (Kiziak and Conradt, 2005), *P. putida* (40^0C) (Banerjee *et al*., 2006) and *Fusarium solani* O1 (40-45^0C) (Kaplan *et al*., 2006).

5.2.6 Effect of metal ions

To determine the effect of different metal ions on the activity of nitrilase, respective salts of the metal ions were added in the reaction system. In *R. rhodochrous* J1 it was reported that Cu^{2+} and Ag^{2+} inhibit the specific activity of enzyme. But in *Alcaligenes* sp. ECU0401, Cu^{2+} enhanced the specific activity and high biomass was also reported.

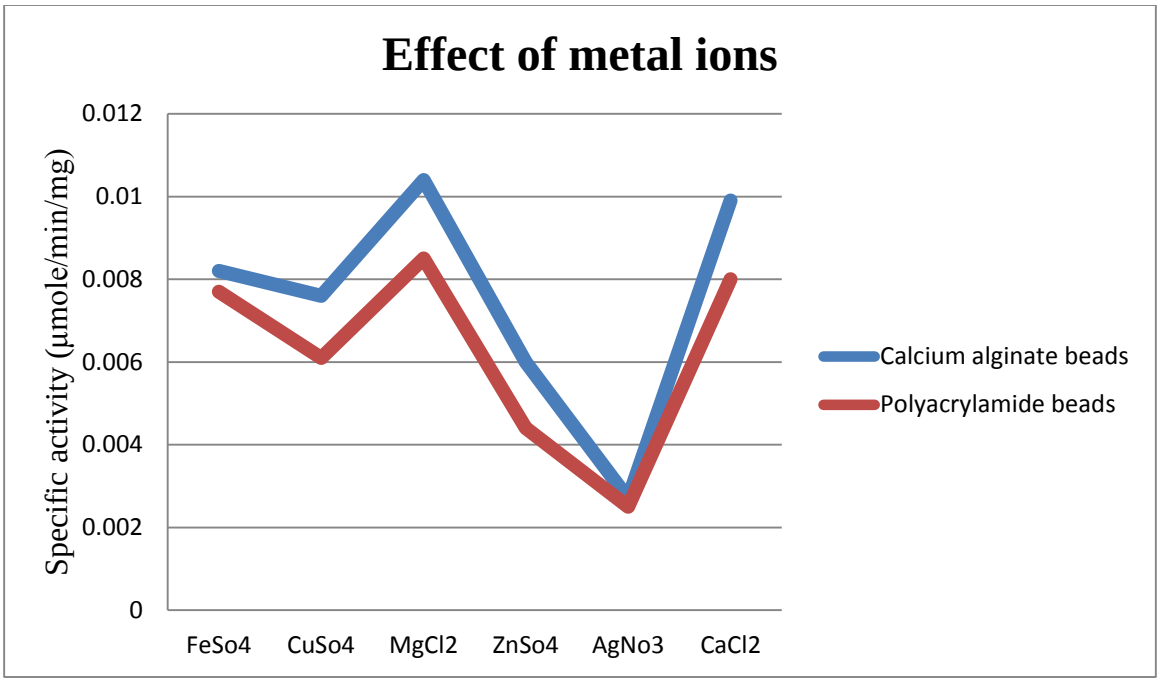


Figure 15- Specific activity of immobilized *Pseudomonas* sp. KNB2 using different metal ions.

From results, it was observed that Fe^{2+}; Mg^{2+} and Ca^{2+} do not have significant effect on the nitrilase activity, While, Zn, Ag^{2+} and Cu^{2+} inhibit the activity of immobilized nitrilase.

5.2.7 Reusability

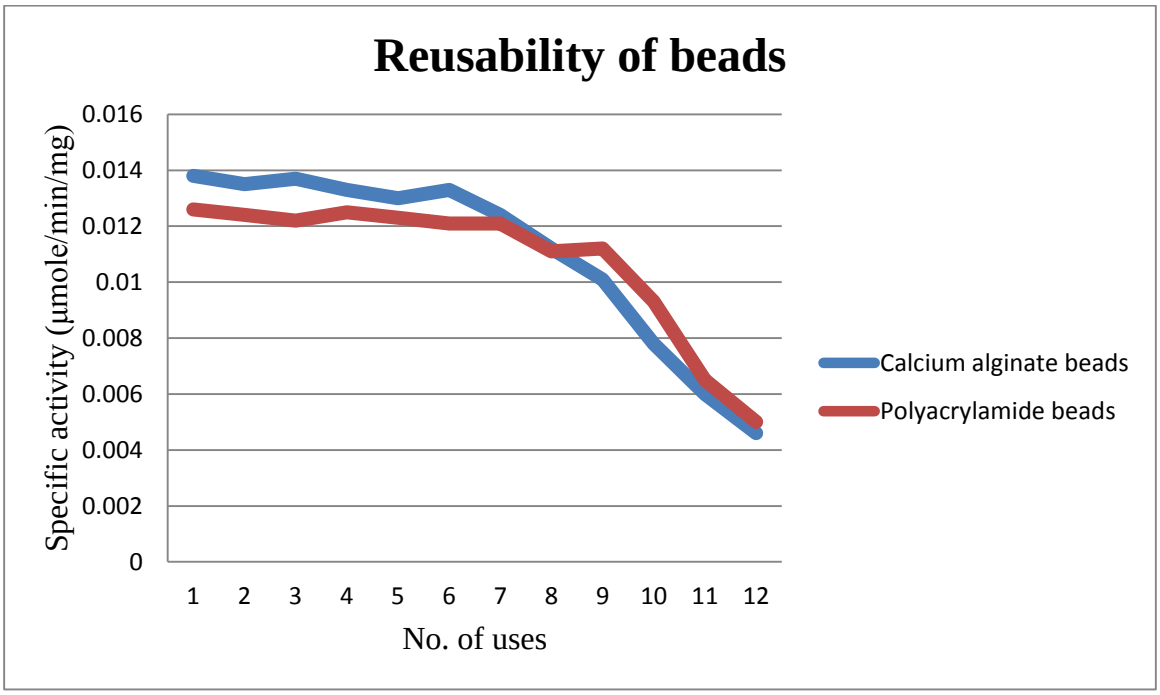


Figure16 - Specific activity of immobilized *Pseudomonas* sp. KNB2 after different no. of repeated uses.

Reusability of beads i.e. no. of times the immobilized cells beads can be reused was checked by performing enzyme assay repeatedly one by one. The specific activity was measured and it came out that beads retain their maximum activity when used 8 times repeatedly after that up to 11th time they showed more than half of their maximum specific activity. In the previous research it was reported that immobilized *Alcaligenes faecalis* ZJUTB10 can be reused 10 times and retain their full activity (Xue et al., 2013).

5.2.8 Cell viability

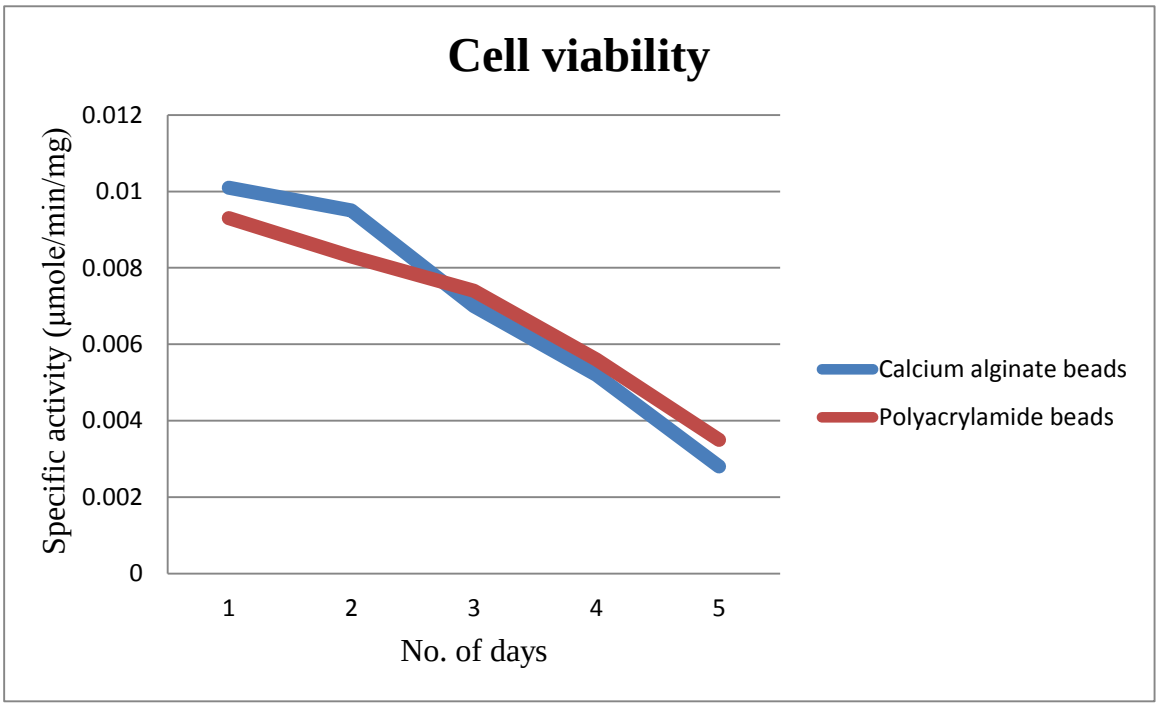


Figure 17 - Specific activity of immobilized *Pseudomonas* sp. KNB2 at specific intervals.

In the process of determining the stability of the immobilized cells, time period (day wise) up to which cell was viable for carrying out the nitrile hydrolysis and organic acid production, was determined by carrying out nitrilase enzyme assay after a period of 24 hours. It was observed that cells retain their activity till 3 days. Then it gets reduced to 40% of the first day on the 4th day.

6. CONCLUSION

A previously isolated bacterial strain having good thermophilic nitrilase activity was first characterized by using biochemical tests. The analysis of results showed that the present strain belongs to "*Pseudomonas"* genus and strain "*aeruginosa"*, which require more test for conformation. So, the strain was named *Pseudomonas* sp. KNB2.

Pseudomonas sp. KNB2 cells were immobilized using two different type of entrapment method i.e. calcium alginate and polyacrylamide. Immobilized cells with two different entrapments had shown substrate specificity towards benzonitrile. This indicates that the present nitrilase is specific towards aromatic nitriles. However, cells with calcium alginate entrapment showed more specific activity than those with polyacrylamide. The most suitable explanation of this result seems to be, due to mass transfer aspect.

The optimum density of cells per bead came out to be 0.6 mg for calcium alginate entrapment while 0.7 mg for polyacrylamide entrapment. Immobilized Pseudomonas sp.KNB2 showed a wide range of optimum production condition i.e., 45-60^{0}C temperature with 6-8 pH at benzonitrile (substrate) concentration ranging from 30mM to 50mM. The immobilized beads can be reused up to 8-9 times. The beads were stable for 3 days (72 hours). Metal ions like Zn and Ag adversely affects the activity of nitrilase enzyme due to presence of thiol group in cysteine residue of active site.

The results had shown the potential of the immobilized Pseudomonas sp. KNB2 for the bioconversion of nitriles and production of organic acids under harsh conditions. Now the immobilized cells of pseudomonas sp. KNB2 can be scaled up for the use in bioreactor, to determine their conversion rate.

7. FUTURE PROSPECTIVE

In the present day, there is high demand for clean production technologies globally. Biocatalyst mediated biotransformation fulfils this demand along with use of renewable raw material and energy. In this scenario, nitrilase can be a key player due to its catalytic property of one step hydrolysis of nitrile compound. By this two goals are achieved first is the production of various chemical compounds like organic acids, antibiotics and other pharmaceutical product and secondly, removal of hazardous nitrile compounds from the environment.

In the current work immobilized *Pseudomonas* sp. KNB2 nitrilase was optimized for enhancing the production of organic acids. By carrying forward this work, an integrated bioprocess system can be designed using packed bed reactor made of immobilized cells. And can be utilized for the large scale production of various carboxylic acids for commercial purpose.

In an another prospective, using CSTR (continuous stir tank reactor) system, a waste treatment unit can be designed so that the nitrile generated as waste from various chemical industries can be treated effectively and efficiently.

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9. APPENDIX

9.1 AMMONIA STANDARD

9.1.1 Reagents

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

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

9.1.2 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 PRODUCTION MEDIA

9.2.1 Minimal Basal Medium (1000ml), pH-7.0

KH_2PO_4	2gms
NaCl	1gm
$MgSO_4.7H_2O$	0.2gm
$FeSO_4.7H_2O$	30mg

9.2.3 Nutrient Agar Medium (1000ml), pH-7.0

Peptone	1%
Yeast Extract	1%
NaCl	0.5%
Glucose	2%
Agar	3%

9.3 Effect of pH on immobilized *Pseudomonas* Sp. KNB2 Nitrilase

9.3.1 Using Calcium Alginate entrapment immobilization

S.NO.	pH	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	4	0.0409	0.0027	5	0.6	0.0045
2	5	0.0735	0.0049	5	0.6	0.0081
3	6	0.0969	0.0064	5	0.6	0.0106
4	7	0.1051	0.0070	5	0.6	0.0116
5	8	0.1019	0.0067	5	0.6	0.0111
6	9	0.0646	0.0043	5	0.6	0.0071

9.3.2 Using polyacrylamide entrapment immobilization

S.NO.	pH	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	4	0.0477	0.0031	6.14	0.7	0.0045
2	5	0.0681	0.0045	6.14	0.7	0.0064
3	6	0.1040	0.0069	6.14	0.7	0.0099
4	7	0.1099	0.0073	6.14	0.7	0.0105
5	8	0.0995	0.0066	6.14	0.7	0.0094
6	9	0.0770	0.0051	6.14	0.7	0.0073

9.4 Effect of Temperature on immobilized *Pseudomonas* Sp. KNB2 Nitrilase

9.4.1 Using Calcium Alginate entrapment immobilization

S.NO.	Temp.	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	35	0.0478	0.0031	5	0.6	0.0051
2	40	0.0561	0.0037	5	0.6	0.0062
3	45	0.0734	0.0049	5	0.6	0.0081
4	50	0.1072	0.0071	5	0.6	0.0118
5	55	0.1028	0.0068	5	0.6	0.0113
6	60	0.0904	0.0060	5	0.6	0.0100
7	65	0.0684	0.0045	5	0.6	0.0076

9.4.2 Using polyacrylamide entrapment immobilization

S.NO.	Temp.	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	35	0.0497	0.0033	5	0.7	0.0047
2	40	0.0604	0.0046	5	0.7	0.0065
3	45	0.0888	0.0065	5	0.7	0.0092
4	50	0.1173	0.0078	5	0.7	0.0111
5	55	0.1102	0.0073	5	0.7	0.0104
6	60	0.1029	0.0068	5	0.7	0.0097
7	65	0.0870	0.0058	5	0.7	0.0082

9.5 Effect of substrate on immobilized *Pseudomonas* Sp. KNB2 Nitrilase

9.5.1 Using Calcium Alginate entrapment immobilization

S.NO.	Substrate	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	Acetonitril	0.1002	0.0066	6.4	0.6	0.0111
2	Acrylonitril	0.0789	0.0052	6.4	0.6	0.0087
3	Benzonitril	0.1271	0.0084	6.4	0.6	0.0141

9.5.2 Using polyacrylamide entrapment immobilization

S.NO.	Substrate	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	Acetonitril	0.1021	0.0068	6.4	0.7	0.0097
2	Acrylonitril	0.0814	0.0054	6.4	0.7	0.0077
3	Benzonitril	0.1369	0.0091	6.4	0.7	0.0131

9.6 Effect of Substrate concentration on immobilized *Pseudomonas* Sp. KNB2 Nitrilase:-

9.6.1 Using Calcium Alginate entrapment immobilization

S.NO.	Benzonitrile Conc. (mM)	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	20	0.0860	0.0057	6.2	0.6	0.0095
2	30	0.0992	0.0066	6.2	0.6	0.0110
3	40	0.1340	0.0089	6.2	0.6	0.0148
4	50	0.0779	0.0052	6.2	0.6	0.0086
5	60	0.0472	0.0032	6.2	0.6	0.0053
6	70	0.0318	0.0021	6.2	0.6	0.0035

9.6.2 Using polyacrylamide entrapment immobilization

S.NO.	Benzonitrile Conc. (mM)	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	20	0.0789	0.0052	7.1	0.7	0.0075
2	30	0.0911	0.0060	7.1	0.7	0.0086
3	40	0.1129	0.0075	7.1	0.7	0.0107
4	50	0.0740	0.0049	7.1	0.7	0.0070
5	60	0.0490	0.0032	7.1	0.7	0.0046
6	70	0.0132	0.0009	7.1	0.7	0.0012

9.7 Cell viability of immobilized *Pseudomonas* sp. KNB2 nitrilase: -

9.7.1 Using Calcium Alginate entrapment immobilization

S.NO.	Days	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
		(T-C)*2*0.2821	T. Amm/15		Per bead	Rate/mg cells added
1	1	0.0917	0.0061	5	0.6	0.0101
2	2	0.0857	0.0057	5	0.6	0.0095
3	3	0.0634	0.0042	5	0.6	0.0070
4	4	0.0471	0.0031	5	0.6	0.0052
5	5	0.0260	0.0017	5	0.6	0.0028

9.7.2 Using polyacrylamide entrapment immobilization

S.NO.	Days	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
		(T-C)*2*0.2821	T. Amm/15		Per bead	Rate/mg cells added
1	1	0.0977	0.0065	5	0.7	0.0093
2	2	0.0874	0.0058	5	0.7	0.0083
3	3	0.0785	0.0052	5	0.7	0.0074
4	4	0.0597	0.0039	5	0.7	0.0056
5	5	0.0373	0.0025	5	0.7	0.0035

9.8 Effect of metal ions on immobilized *Pseudomonas* sp. KNB2 nitrilase: -

9.8.1 Using Calcium Alginate entrapment immobilization

S.NO.	Metal ion	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	$FeSo_4$	0.0739	0.0049	5	0.6	0.0082
2	$CuSo_4$	0.0685	0.0046	5	0.6	0.0076
3	$MgCl_2$	0.0943	0.0062	5	0.6	0.0104
4	$ZnSo_4$	0.0541	0.0036	5	0.6	0.0060
5	$AgNo_3$	0.0248	0.0016	5	0.6	0.0027
6	$CaCl_2$	0.0893	0.0059	5	0.6	0.0099

9.8.2 Using polyacrylamide entrapment immobilization

S.NO.	Metal ion	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	$FeSo_4$	0.0822	0.0054	5	0.7	0.0077
2	$CuSo_4$	0.0750	0.0043	5	0.7	0.0061
3	$MgCl_2$	0.1001	0.0060	5	0.7	0.0085
4	$ZnSo_4$	0.0574	0.0031	5	0.7	0.0044
5	$AgNo_3$	0.0370	0.0018	5	0.7	0.0025
6	$CaCl_2$	0.0941	0.0056	5	0.7	0.0080

9.9 Optimum density of cells (dry weight) per bead of immobilized *Pseudomonas* sp. KNB2 nitrilase: -

9.9.1 Using Calcium Alginate entrapment immobilization

S.NO.	Density of cells(mg)		T. Amm	Rate
		T-C	(T-C)*2*0.2821	T. Amm/15
1	0.3	0.131	0.0739	0.0049
2	0.4	0.149	0.0840	0.0056
3	0.5	0.204	0.1150	0.0076
4	0.6	0.28	0.1579	0.0105
5	0.7	0.266	0.1500	0.0100
6	0.8	0.247	0.1393	0.0092
7	0.9	0.221	0.1246	0.0083

9.9.2 Using polyacrylamide entrapment immobilization

S.NO.	Density of cells(mg)		T. Amm	Rate
		T-C	(T-C)*2*0.2821	T. Amm/15
1	0.3	0.162	0.0914	0.0061
2	0.4	0.197	0.1112	0.0074
3	0.5	0.228	0.1286	0.0085
4	0.6	0.253	0.1427	0.0095
5	0.7	0.309	0.1743	0.0116
6	0.8	0.281	0.1585	0.0105
7	0.9	0.264	0.1489	0.0099

9.10 Reusability of immobilized *Pseudomonas* sp. KNB2 nitrilase: -

9.10.1 Using Calcium Alginate entrapment immobilization

S.NO.	No. times used (perbead)	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	1	0.1242	0.0082	5	0.6	0.0138
2	2	0.1218	0.0081	5	0.6	0.0135
3	3	0.1233	0.0082	5	0.6	0.0137
4	4	0.1201	0.0080	5	0.6	0.0133
5	5	0.1174	0.0078	5	0.6	0.0130
6	6	0.1198	0.0079	5	0.6	0.0133
7	7	0.1119	0.0074	5	0.6	0.0124
8	8	0.1013	0.0067	5	0.6	0.0112
9	9	0.0912	0.0060	5	0.6	0.0101
10	10	0.0706	0.0047	5	0.6	0.0078
11	11	0.0545	0.0036	5	0.6	0.0060
12	12	0.0430	0.0028	5	0.6	0.0046

9.10.2 Using polyacrylamide entrapment immobilization

S.NO.	No. times used (perbead)	T. Amm (T-C)*2*0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. Act. Rate/mg cells added
1	1	0.1328	0.0088	5	0.7	0.0126
2	2	0.1305	0.0087	5	0.7	0.0124
3	3	0.1282	0.0085	5	0.7	0.0122
4	4	0.1317	0.0087	5	0.7	0.0125
5	5	0.1299	0.0086	5	0.7	0.0123
6	6	0.1273	0.0084	5	0.7	0.0121
7	7	0.1276	0.0085	5	0.7	0.0121
8	8	0.1180	0.0078	5	0.7	0.0111
9	9	0.1183	0.0079	5	0.7	0.0112
10	10	0.0979	0.0065	5	0.7	0.0093
11	11	0.0685	0.0045	5	0.7	0.0065
12	12	0.0532	0.0035	5	0.7	0.0050