"Immobilization and reaction condition optimization of amidase of *Bacillus* sp. MNB-1."

to be submitted as Major Project Report in partial fulfilment of the requirement for the degree of

M. Tech.

Submitted by

SHASHANK KUMAR SINGH

(2K14/IBT/14)

Delhi Technological University, Delhi

Under the supervision of

Dr. Pravir Kumar

Associate Professor

Department of Biotechnology

Delhi Technological University, Delhi

CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"Immobilization and reaction condition optimization of amidase of *Bacillus* sp. MNB-1."**, submitted by **Shashank Kumar Singh (DTU/14/M.TECH/96)** in the partial fulfilment 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 honouring of any other degree.

Date:

Dr. PRAVIR KUMAR
Associate Professor
(Project Mentor)
Department of Bio-Technology
Delhi Technological University

Dr. D. Kumar
Professor and Head
Department of Bio-Technology
Delhi Technological University

DECLARATION

I hereby declare that the M. Tech major project dissertation entitled, "**Immobilization and reaction condition optimization of amidase of *Bacillus* sp. MNB-1.**" 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 *bona fide* 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.

Date-

Shashank Kumar Singh
Roll No. 2K14/IBT/14

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Research is a higher concept. It brings to test our patience, vigour and dedication. Every result arrived is a beginning for a higher achievement. My project is a small drop in an ocean. It needs the help of friends and guidance of experts in the field, to achieve something new.

I found my pen incompetent to express my thanks to my supervisor **Dr. Pravir Kumar, Associate Professor, Department of Biotechnology DTU** under whose kind and worthy guidance and supervision I had the opportunity to carry out this work. It was only due to her advice, thoughtful comments, constructive criticism, and continuous vigil over the progress of my work with personal interest that it has taken this shape. He has been a great source of encouragement. To get an opportunity to carry out the project work in the well-equipped, ever developing laboratories in our institution.

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With a silent prayer to the almighty I take this opportunity to express my gratitude to all those who have supported me in completing my dissertation work as a part of my degree program.

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"Immobilization and reaction condition optimization of amidase of *Bacillus* sp. MNB-1."

Shashank Kumar Singh

Email: shashankgr11@gmail.com

ABSTRACT

Amidase is a nitrile metabolizing enzyme, belongs to the family of hydrolases, which acts on carbon-nitrogen bonds other than peptide bonds. It catalyzes hydrolysis of amide to carboxylic acid and ammonium. Amidases find wide applications in various industries as commodity chemicals (e.g. acrylic acid, nicotinic acid *etc* (.or pharmaceutically important acids (organic acids and hydroxamic acids). Characterization of bacterial strain was carried out and expected to be *Bacillus subtilis.* The bacterial cells, which produce amidase enzyme, were immobilized in sodium aliginate and agarose with agar polymer gels. The optimum pH, temperature and substrate specificity, substrate concentration, metal ion effect, reusability and cell viability for the activity of amidase in the immobilized cells were analyzed, respectively. The enzyme activity of the immobilized cells shows optimum activity with in a wide range of pH (6-9) temperature (20-40^{o}C) and substrate concentration (30mM-70mM). The reusability and cell viability also increased considerably. The amidase of the immobilized cells was reported slightly more stable and active in calcium alginate matrix than agarose+agar matrix however, both the candidate are efficient to be used in production of important products at bioreactor level.

INTRODUCTION

Nitrile metabolizing enzymes occupy a niche of potential biocatalysts in the chemical industries for conversion of nitriles to a wide range of products and intermediates too. Nitrilases and amidases are quite attractive as mild and selective catalysts in synthesis of fine chemicals due to their inherent stereo and enantio-selectivity. Nitrile hydratases (NHase) are the metalloenzymes, that catalyze hydration of nitriles to amides and are also used since past in industrial production of acrylamide and nicotinamide. Naturally, these enzymes are involved in biosynthesis of natural product and post-translational modification in fungi, plants, animals, and few prokaryotes. Amidases (E.C. 3.5.1.4-amidohydrolases) comprises of an interesting group of ubiquitous C-N bond cleaving enzymes in biological systems that possesses dual activity, which made them and model for advanced research due to their industrial and pharmaceutical applications (Fournand *et.al.* 1998, Kobayashi *et al.* 1993, Nawaz *et al.* 1994). The dual catalytic activities of amidases *i.e.* i) hydrolysis of amides to corresponding acids and ii) acyl transfer activity in presence of hydroxylamine to form hydroxamic acids. Amidases are usually co-transcribed with nitrile hydratase and stereo and enantio-selectivities that have been reported to be generally associated with amidases in the bi-enzymatic pathway (Mayaux *et al.* 1990, Kotlova *et al.* 1998). In nitrilase superfamily except nitrilase enzyme, all others (*i.e.* aliphatic amidase, amino-terminal amidase, biotinidase, apolipoprotein N-acyltransferase *etc*) catalyse amide hydrolysis or amide condensation (Pace and Brenner 2001). Naturally these enzymes functions as carbon or nitrogen fixtures in prokaryotes by hydrolysis of amides, the production of charged tRNAGln in eubacteria through transfer of ammonia from glutamine (Curnow *et al.* 1997, Banerjee *et al.* 2002) and in mammals the indignity of neuromodulatory fatty acid amides (Cravatt *et al.* 1996).

Substrate specificity is the basis of different types of amidases that have been reported *e.g.* _-amino amidase (Jallageas *et al.* 1980, Van del Tweel *et al.* 1993), enantioselective amidase (Mayaux *et al.*1990, Hirrlinger *et al.* 1996) and adipamidase. Majorly amidases are categorized into two groups: i) aliphatic amidases (Asano *et al.* 1982) and ii) aromatic amidases (Hirrlinger *et al.* 1996). *Corynebacterium* sp. (Gilligan *et al.* 1993), *Rhodococcus rhodochrous* M8, *Pseudomonas aeruginosa* (Kelly and Clarke 1962), *Bacillus stearothermophilus* BR388 (Cheong and Oriel 2000)

and *Geobacillus pallidus* RAPc8 (Makhongela *et al.* 2007) were reported to have aliphatic amidases while *Rhodococcus* sp. R312 (Fournand *et al.*1998), *R. erythropolis* MP50 (Hirrlinger *et al.* 1996), *Pseudomonas chlororaphis* (Ciskanik *et al.* 1995), *Mycobacterium neoaurum* ATCC 25795 (Hermes *et al.*1994) possessed amidases that actively hydrolyse a range of aliphatic, aromatic and heterocyclic amides.

Widely used in industry in combination with nitrile hydratase for the generation of significant organic acids *e.g.* acrylic acid, *p*aminobenzoic acid, nicotinic acid, pyrazinoic acid and many more (Banerjee *et al.* 2002), amidases are also utilized as industrial catalyst in treatment of effluents (Nawaz *et al.* 1996, Madhavan *et al.* 2005). Acyl transferase activity of it has been explored for synthesis of pharmaceutically active hydroxamic acids (Fournand *et al.*1998, Holmes 1996).
Efficient industrial applications involve vital properties of amidases such as catalytic activity, enantio-selectivity, substrate specificity and thermostability that must be observed for further improvements. Presently majority of microbial amidases characterized, cloned and engineered are from mesophilic microorganisms and attempts have been made to enhance either of the above mentioned properties by directed evolution or by protein engineering (Ryabchenko *et al.* 2006, Krieg *et al.* 2005, Trott *et al.* 2002). Inspite of many advantages, these enzymes find restricted application in industries because of their limited stability at elevated temperature, pH, ionic strength, *etc*. Thermophilic microorganisms are adapted to high temperatures and their enzymes under extremes of environmental conditions mediate biological reactions. Such conditions are often met in industrial operations. Thermostable enzymes have been extensively explored as they are excellent biocatalyst in industrial processes (Fujiwara 2002, Niehaus *et al*, 1999). Amidases from thermophiles will be more suitable biocatalyst for industry due to their higher operational stability. Among the hydroxamic acids, acetohydroxamic acid finds applications as anti-human immunodeficiency virus agent or antimalarial agent and recommended for treatment of ureaplasma infections and anaemia (Holmes 1996, Fournand *et al.* 1998).

World's biotechnological industries higher demands are the basis for improvement in enzyme productivity. The progression of extraordinary techniques for enhancing the enzyme's shelf life is also its major consequence. These necessities are expected to smoothen the progress of large-scale and monetary formulation. Immobilization of whole cells offer a great basis for growing

availability of enzyme to substrate along with high turnover number over a significant period of time. Various natural and synthetic ways have been assessed for their efficiency for whole cell immobilization. At present free enzymes are losing their role in the industrial applications because immobilized cells offer extended availability that shorten excessive purification and downstream processes. Further studies must make an effort at adoption of rational and logistic techniques along with resourcefully customized supports to revive up the state of enzyme immobilization and supply new vistas to the industrial sector.

Keeping in view the advantages offered by immobilization, the present research work entitled has been undertaken with the aim **"Immobilization and reaction condition optimization of amidase of *Bacillus* sp. MNB-1".** The objectives of this research work were:

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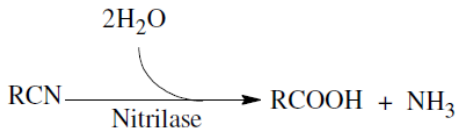
1. Characterization of bacterial strains
2. Immobilization of cells in different entrapment matrices.
3. Optimization of reaction conditions of immobilized cells of *Bacillus* sp. MNB1.

REVIEW OF LITERATURE

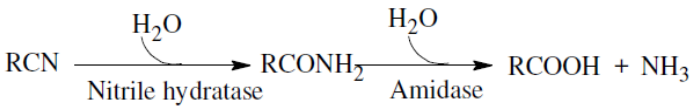
Compounds containing C-N bonds are broadly distributed in nature and these include organic cyanides or nitriles [R-C_N], inorganic cyanides [H-C_N], acid amides [RC(=O)-NH2], secondary amides [R-C(=O)NH-R´] and N-carbamyl amides [R-NH-C(=O)-NH2]. Nitrilase superfamily enzymes are mainly responsible for hydrolyzing these compounds (Pace and Brenner 2001) because that attack either the planar carbon of an amide or cyano carbon of a linear nitrile, using a residue of cysteine that is conserved (Stevenson *et al.* 1990, Bork and Koonin 1994). Enzymes like signature amidases (Patricelli and Cravatt 2000), amidotransferases (Zalkin and Smith, 1998) and N-terminal nucleophile hydrolases also acts on C-N bond-containing substrates, though they are unrelated to nitrilase superfamily (pace and Brenner 2002).

Nitrile-metabolizing enzymes are comparatively rare in eukaryotic organisms but they are mainly distributed in bacteria (, *Pseudomonas*, *Klebsiella*, *Norcadia*, *Bacillus*, *Acinetobacter*, *Corynebacterium*, *Arthrobacter*, *Rhocococcus*, *etc*), fungi (*Aspergillus nidulans*) and yeast (*Candida* sp, *Geotrichum* sp., *Rhodotorula glutinis*, *Cryptococcus flavus*, *etc*) that metabolize nitriles as only source of nitrogen and carbon. 2 types of reaction pathways are usually followed by microbes for the nitrile hydrolysis:

(1) A one-step pathway in which nitrilase enzyme converts nitriles to their consequent acids and ammonia.



(2) A bi-enzymatic pathway in which nitriles are first hydrated to corresponding amides by nitrile hydratases (NHases) and subsequently amides are hydrolyzed to acids and ammonia by amidases:



Cyanide hydratase and cyanide dihydratase enzymes are closely related to nitrilases in terms of protein structure and amino acid sequence similarities, but these enzymes only hydrolyze inorganic cyanide (H-C_N) substrates efficiently to acid and amide (O'Reilly and Turner 2003).

Naturally, carbon-nitrogen (C-N) bonds formation and cleavage are central processes in both eukaryotic and prokaryotic organisms. Hydrolysis of C-N bond occur in plants, animals and fungi where they play an essential role in production of natural substances like biotin and auxins, etc. that are usually needed for the amino acid substrates and proteins deamination purpose. (Pace and Brenner 2001). Particularly in the plants, activities like this are implicated in metabolism of nutrients, as well as also in deterioration of the compounds that are toxic as well as cyanogenic too (Piotrowski *et al.* 2001). Whereas, post translational modification and also the C-N bonds condensation is quite important in the biochemical processes. Although nitrile-metabolism activities are comparatively uncommon in plants and animals, they are normally observed in bacteria that utilize nitriles as a sole source of carbon and nitrogen. However, the physiological role of nitrile-degrading enzymes in these microbes is not fully understood (Banerjee *et al.* 2002).

OCCURRENCE AND BIOLOGICAL FUNCTIONS OF AMIDASES IN NATURE

Nitrile metabolising enzymes play a very significant role in C–N bonds formation and cleavage reactions in both prokaryotic as well as eukaryotic organisms for the production of natural substances such as auxin and biotin, etc. They also play important role in the modification of amino acids, nutrient metabolism, in the post-translational of proteins (Piotrowski et al. 2001) and degradation of toxic cyanogenic compounds. C–N bond reactions are also observed in bacteria and archea specifically those that have an ecological relationship with plants and animals (Pace and Brenner 2001). Even though activities of nitrile metabolism are relatively infrequent in plants and animals yet they are commonly seen in bacteria that metabolize nitriles as a sole source of carbon and nitrogen (Banerjee et al. 2002).

Naturally carbon or nitrogen fixation in prokaryotes is the main biological function of this enzyme. This is done either by hydrolysis of amides or by the generation of properly charged tRNAGln in

eubacteria through transamidation of misacylated Glu- tRNAGln by amidolysis of Glutamine in bacteria (Curnow et al. 1997). Indole-3-acetic acid (IAA) formation in Agrobacterium and catabolism of neuromodulatory fatty acid amides in mammals are its property (Cravatt et al. 1996; Patricelli and Cravatt 2000). With the help of amidase acyltransferase activity microbes synthesize hydroxamic acids and exploit their chelating properties to obtain iron from iron scarce environments.

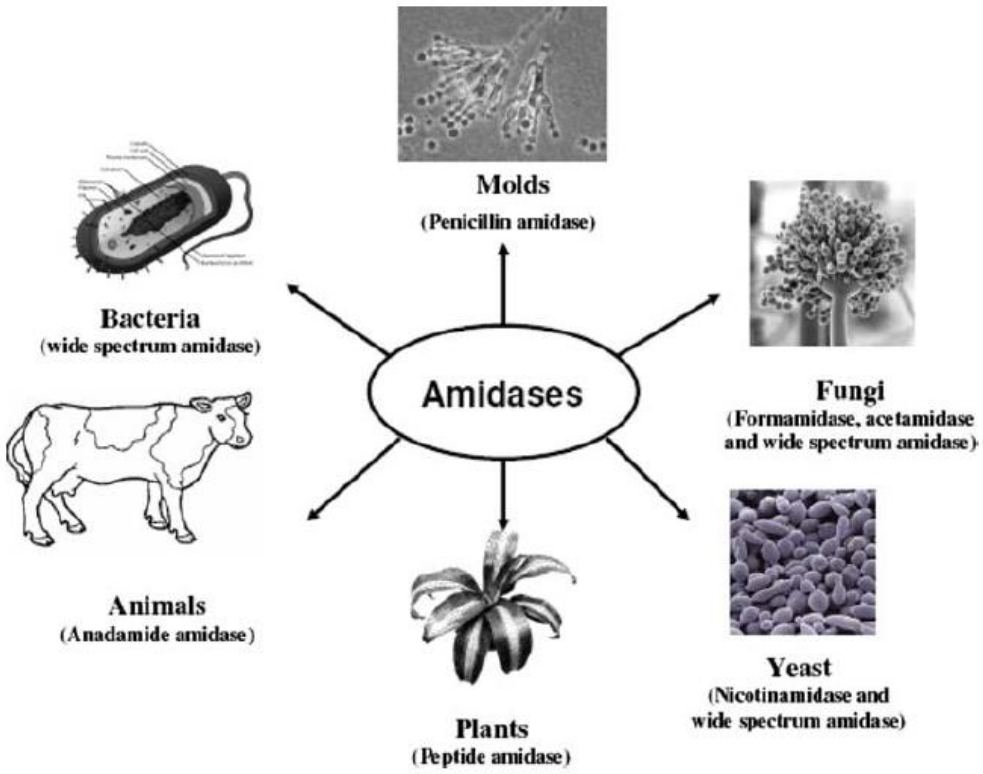


Fig.2.1- Occurrence of amidase in nature (Sharma et al. 2009)

Amidases from animals

The animal tissue enzymes were first studied by Bray and co-workers (Bray *et al.*1950) because of their capability to hydrolyze amides. The porcine brain enzyme was purified in 1995 (Ueda *et al.*1995). It had the ability to hydrolyzed anadamide (arachidonyl ethanolamide) to arachidonate (Arreaza *et al.* 1997) and the enzyme was named as anadamide amidase or fatty acid amidohydrolyse (FAAH) or Narachidonyl ethanolamine deacylase (E.C. 3.5.1.4 or 3.5.160). Being an endogenous ligand for cannabinoid receptors and a putative neurotransmitter, it also catalyzed the hydrolysis of fatty acid oleamide and ethanolamides (Arreaza *et al.* 1997).

Amidases from plants

Peptide amidases are found in plants, since they catalyze hydrolysis of C-terminal amide groups of peptides or N-protected amino acids. A highly stereo and regio-selective enzyme i.e. peptide amidase is obtained from oranges *Citrus sinesis* L**.** It hydrolyse all proteinogenic except L-proline as substrate and requires L-configured amino acid at the C-terminus (Kammermeier-Steinke *et al.* 1993).

Amidases from fungi

Various kinds of amidases are found like formamidase that acts on formamide and glycinamide, an acetamidase that acts on C 1-6 aliphatic amides and also wide spectrum amidase that acts on aliphatic and arylamide (Hynes and Pateman 1970). They were all found in *Aspergillus nidulans.* Expression of these amidases have also been studied at gene level (Hynes and Pateman 1970).

Amidases from yeasts and molds

The degradation of asparagine, urea and acetamide along with *Candida utilis* cells was studied by Gorr and Wagner in 1933. A purified amidase was obtained by Brady from *C.utilis* that proved to be efficiently hydrolyse several other aliphatic amides (Brady 1969). Nicotinamide specific amidase was purified by Joshi and Handler (1962) from *Torula cremoris.* Around fifty four yeast strains were isolated and purified by Brewis and co-workers from cyanide treatment bioreactor and they showed that *Candida fabianii* possess amidase too (Brewis *et al.* 1995). *Candida famata* (Linardi *et al.* 1996), *Candida guilliermondii*, *Rhodotorula glutinis*, *Cryptococcus flavus*, *Cryptococcus* sp. and *Geotrichum* sp. (Rezende *et al.* 2004) also harbour different amidases. Molds like *Fusarium oxysporum* and *Penicillium chrysogenum* (Alvarez *et al.* 1993) exhibits very good amidase activity.

Amidases from bacteria

Several amidases have been obtained from bacteria that were further purified and then characterized. Certain bacteria holds more than one amidases like *Rhodococcus* sp. R312 that possesses an enantioselective amidase, an aliphatic amidase and several specific amidases too (Arnaud *et al.* 1976).

Amidase classification

Various people have been working over amidases classification but they haven't been successful in giving a proper classification of this enzyme. (Pace and Brenner 2001, Fournand and Arnaud 2001). The major categorization of amidases on the basis of amino acid sequence, phylogenetic relationship and also catalytic activity seems to have developed more logical sense and hence it is described below.

Based on catalytic activity

On the basis of catalytic activity (*i.e.* substrate specificity), amidases can be further classified into six classes:

a) Aliphatic amidases-These amidases catalyze the hydrolysis of aliphatic amides to corresponding acids and ammonia. Amidases of this category are from *Rhodococcus* sp. (Bhalla *et al.* 1997), *Pseudomonas aeruginosa*, *Bacillus* sp. (Thalenfield and Grossowizc 1976).

b) Aromatic amidases- These amidases prefer aromatic substrates over aliphatic amides *e.g. Pseudomonas putida*, *P. cepacia* and *P. acidovorans* which hydrolyse phenylacetamide (Betz and Clarke 1973).

c) Enantioselective amidases-These amidases enantio-selectively hydrolyze racemic amides to enantiopure product. The aliphatic amidase from *Rhodococcus* sp. R 312 (Mayaux *et al.* 1990), *R. erythropolis* JCM 6823, *Rhodococcus* sp. N-774 (Hashimoto *et al.* 1991), *Rhodococcus* sp. (Mayaux *et al.* 1991), *R. rhodochrous* J1 (Kobayashi *et al.* 1993a), *R. erythropolis* MP 50 and *Bacillus* sp. BR 449 (Hirrlinger *et al.* 1996) belong to this group.

d) _-amino amidases-They hydrolyze only L-_- amino amides and have been reported from *Ochrobactrum anthropi* NCIMB 40321 (van den Tweel *et al.* 1993)/ *Mycobacterium neoaurum* ATCC 25795 (Hermes *et al.* 1994).

e) Arylalkyl acylamidases- Such amidases catalyze the hydrolysis of N-acyl primary aromatic amines and aromatic amines to acids *e.g.* amidase of *Pseudomonas putida* (Shimizu *et al.* 1992).

f) Wide spectrum amidases-These amidases hydrolyze a variety of amides such as acetamide, propionamide, butyramide, benzamide and valeramide into corresponding acids. Such amidases were reported from *Bacillus stearothernmophilus* BR 388 (Cheong and Oriel 2000) and *Brevibacterium* sp. R312 (Soubrier *et al.* 1992).

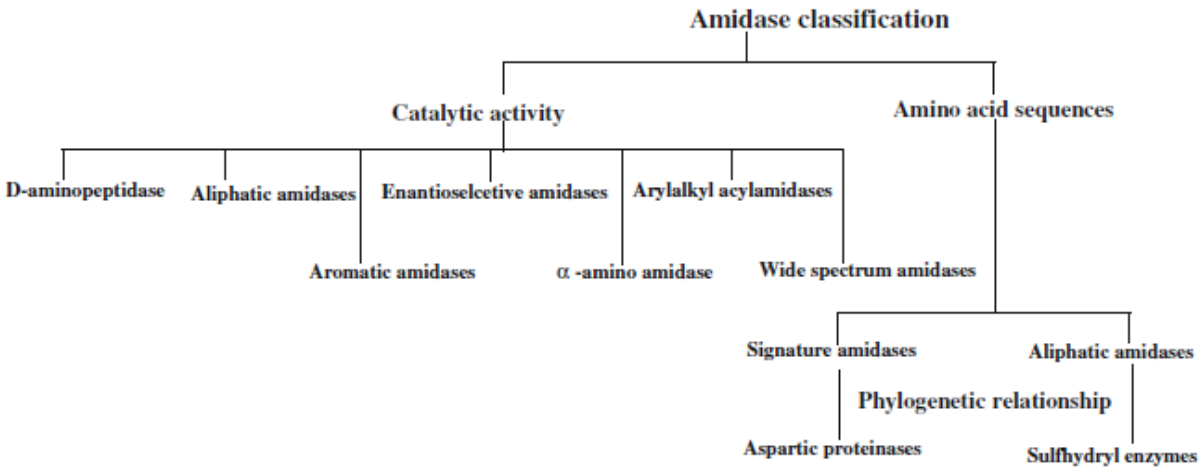


Fig.2.2-classification of amidase

Evolutionary aspects

Phylogenetic analysis of nucleotide sequences or amino acid sequences of aliphatic amidase, enantioselective amidases and nitrilase showed that all of them were descended from a common ancestor and members of sulfhydryl enzyme family. The comparison of signature sequence of enantioselective amidases with active site sequences of various types of proteases revealed that Asp 191 and Ser 195 of *Rhodococcus* amidase are also present within the active site sequence of aspartic proteinases (Kobayashi *et al.* 1997).

Types of reactions catalyze by amidases

Generally amidases possess four kinds of activities that includes ester transferase activity, acyltransferase activity, amidotransferase activity and acid transferase activity. The mechanism of

hydrolysis of amides that needs two substrates and products is quite difficult to study (amide and water) and two products (carboxylate and ammonia). In the case of amide hydrolysis, the initial concentrations of the substrates in the mixture cannot be defined because one of the reactants being water. However, amidase are often able to transfer the acyl moiety of amide to hydroxylamine to form hydroxamates. (Maestracci *et al.* 1984).

In 1970, the activity of amide transferase was showed by Clarke in *Pseudomonas aeruginosa*. It was reported that ester transferase activity was limited in comparison to amide transferase activity. Amide transferase activity was much more extended to corresponding acids and esters. It has been shown that acetamide and propionamide proved to be better substrates for the hydrolysis and acyl transfer reactions. But it was seen that propionamide hydrolysed quickly as a substrate while acetamide got transferred quickly to hydroxylamine.

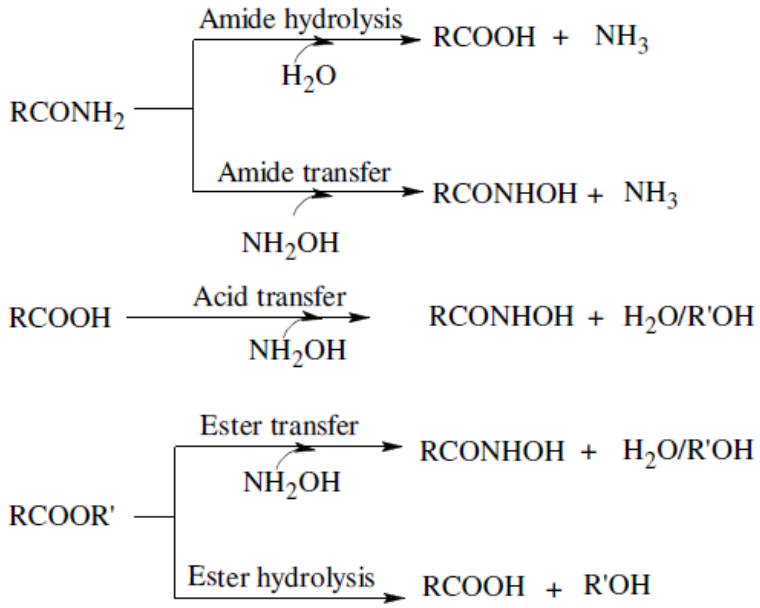


Fig.2.3- various types of reactions catalysed by amidase

Mechanism of amidase action

Amidase is involves in the transfer of acyl group of acetamide onto hydroxylamine that pursue Bi-bi Ping-Pong enzymatic reaction mechanism. The enzyme fastens itself to acetamide and becomes an E-S-acyl intermediate along with the synthesis of ammonia. Just like the acyl transfer reaction

a nucleophilic agent like hydroxylamine reacts with the intermediate to yield acetohydroxamic acid and then lastly after the synthesis of complete product, the enzyme attains its original state. In 1997, Kobayashi and colleagues planned an amidase hydrolytic mechanism that involved the carbonyl group of the amide which undergoes nucleophilic attack and then finally resulting in the formation a tetrahedral intermediate. This intermediate is further converted to an acyl-enzyme complex with immediate removal of ammonia. The complex is then hydrolyzed to an acid upon the addition of a water molecule.

Induction and repression of amidase

Generally amidases are co-transcribed and translated along with nitrile hydratases. Most of the amidases reported are inducible in nature. Kelly and Clarke (1962) used different amides and concluded that few of them acted both substrate inducers while others were found to be non-substrate inducers. In presence of succinate and ammonium chloride containing medium, when *Pseudomonas aeruginosa* was grown, acetamide and propionamide acted as substrate inducers while *N*methylformamide, *N* methylacetamide, *N*-ethylpropionamide, lactamide and methyl carbonate were utilized as non-substrate inducers.

Brammer and Clarke (1964) reported that induction caused by acetamide and *N*-acetylacetamide can be reversed by addition of other amides like cyanoacetamide and thioacetamide as these acted as repressors. This repression was because of structural similarity that led to mutual competition for an amide binding site.

Microorganism	**Inducer**	**Nature**	**Reference**
Bacillus pallidus Dac521	Benzonitrile or acetonitrile or benzamide	Inducible	Cramp *et al.* (1997)
Bacillus sp.	Acetamide and cyano-acetamide		Thalenfield and Grossowicz (1976)
Brevibacterium sp. R 312	Acetamide		Maestracci *et al.* (1984)
Variovorax paradoxus	DL- tert-leucine amide		Krieg *et al.* (2002)
Delftia acidovorans strain 16			Hongpattarakere *et al.* (2005)
Kluyveromyces			Prasad *et al.* (2005)

thermotolerans MGBY 37			
Pseudomonas putida PPW-3		Constitutive	Wyndham and Slater (1986)
Pseudonocardia Thermophile			Egorova *et al.* (2004)

Table 2.1-Inducers of amidase

Effect of metal ions and inhibitors

Investigation of effects of metal ions and inhibitors was done by several investigators and researchers over the amidase activity. In the year 1979 it was reported by Gregoriou and Brown that binding of hydroxyurea and urea at the active site of enzyme caused the inhibition of aliphatic amides in *P.aeruginosa*. It was seen that some amidases from microbes were quite tender towards chemicals like HgCl2 and AgNO3 (Hirrlinger *et al.* 1996, Kobayashi *et al.* 1993,). Low inhibition was observed in *Corynebacterium* sp. amidase in presence of Hg2+ and *p*chloromercuribenzoate. While no such effects was observed under the presence of iodoacetate and *N*ethylmaleimide. In presence of Cu2+, Hg2+, Ni2+ and Co2+, complete inhibition of amidase from *Rhodococcus* sp. was reported Nawaz *et al.* (1994). While partial inhibition of the amidase activity was seen in presence of Mg2+.

A considerably improved amidase activity was seen in presence of Fe2+, Cr2+ and Ba2+.seen that serine protease inhibited the L-carnitine amidase from DSM 6320. Amidase of *R. rhodochrous* M8 was highly effected by the presence of heavy metals ions (Kotlova *et al.* 1999) and the presence of serine protease inhibitor (PMSF) and chelators (EDTA and *o*phenanthroline). While on the other hand DTT addition in the reaction improved the amidase activity by 1.5 fold. Inhibition from the thermophile *Brevibacillus borstelensis* BCS-1 was observed by DTT, 2-mercaptoethanol, and EDTA. It was also observed that Co2+and Mn2+ strongly activated the enzyme (Baek *et al.* 2003).

Thermostable amidases such as the *Pseudonocardia thermophila* amidase showed the enzyme activity inhibition when visualized in the presence of metal ions in the medium. (Egorova *et al.* 2004). Total inhibition of the *Pseudomonas* sp. MCI3434 strain amidase was observed (R-stereoselective) in presence of HgCl2, AgNO3, PbCl2, ZnSO4, CuCl2, etc. It was shown to be 67% in presence of Fe(NH4)2(SO4)2 while inhibition was recorded to be 78% in presence of FeCl3. Increased enzyme activity was observed by 138% in presence of DTT. Various carbonyl reagents, protease inhibitor and chelating agents were not found to affect the activity of amidase (Komeda *et al.* 2004).

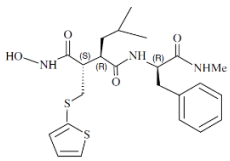
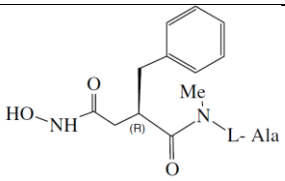
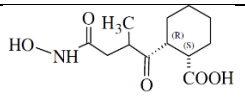
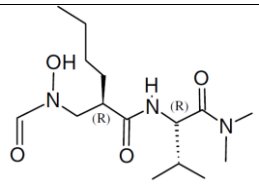
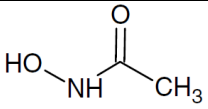
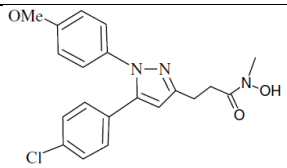
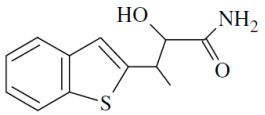
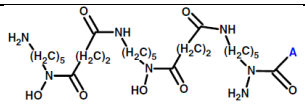
Immobilization

Immobilization is a process to reuse the enzyme utilized in distinct reactions. Amidase can be immobilized using either the whole cells or the purified form of amidase enzymes. Industrial usage of enzymatic reactions use immobilized biocatalysts. For instance, application of 2-3% (w/v) of alginate beads were appropriate for the production of amidase at very high activity after 36 h of incubation. Among the previously used substrates, acetamide (2% w/v) has been found to be a good inducer of amidase. Recently, it was identified that immobilized catalysts have capacity to be recycled up to five runs (Chacko. Et al 2012). Moreover, for the immobilization of whole cells, the optimal conditions such as initial biomass, gel concentration, curing period of beads, and calcium ion concentration in the production medium were elucidated. Further, *E. coli* cells entrapped in alginate were consumed and the maximum enzymatic activity of 6.2 U/mL was obtained after 12 h of incubation under optimized culture conditions (Madhavan et al 2005).

Applications

Due to the operation of biocatalysts under the mild conditions of salt concentration, pH, temperature and lower energy consumption, enzymes are being widely used in green chemistry. Since, they are also highly specific and free from the undesired products their application is found widely in the synthesis of several organic molecules that are gaining lots of importance in the research areas as well as industries. Application of enzymes in biotransformation is found to be very effective as well as eco-friendly too. Lots of products are the results of several

biotransformation taking place today. Bacterial amidases have also been proved to be very effective tool for transformation of amides in the past.

Product	Structures	Uses	references
Hydroxamic acid Marimastat		To treat small cell lung cancers	Muri *et al.* (2002)
Inhibitor of LTA		Anti-inflammatory	
Idrapril		Render cardio protective effects	
N-formyl hydroxylamine BB-3497		Antibacterial agent	Muri *et al.* (2002)
Acetohydroxamic acid (Lithostat)		-To treat ureaplasma -Anaemia -Anti-HIV agent	Holmes (1996);Gao *et al.* (1995)
Fatty hydroxamic acids	 **Tepoxalin**  **Zileuton**	Anti-inflammatory -To treat chronic asthma	Hamer *et al.* (1996)
Desferrioxamine B (Desferal)		Antimalarial	Tsafack *et al.* (1995)

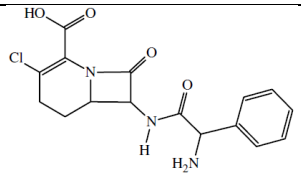
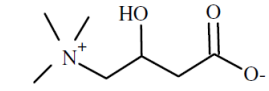
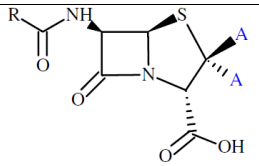
Acrylic acid	$H_2C{=}CH{-}COOH$	Commercial commodity chemical	Nawaz *et al.* (1991)
Loracarbef (Lorabid)		β lactum antibiotics	Black *et al.* (1996)
L-carnitine	 R-3-hydroxyl-4-trimethylamino butanoic acid	-to treat disorders of heart and skeletal muscle due to accumulation of long chain fatty acids	Joeres and Kula (1994)
Penicillin G derivatives	 Penicillin core structure	Semi synthetic antibiotics	Bovenberg *et al.* (1996);
Wastewater treatment	Amidase	Biological tool for detoxification of nitriles/amide containing industrial effluent	Bigey *et al.* (1999), Thompson *et al.* (1988)
Peptide synthesis	Peptide amidase	modify side chains of amino acids	Stelkes-Ritter *et al.* (1995)
Biosensor	Amidase	waste water treatment of industrial effluents	Silva *et al.* (2009)

Table2.2- Application of amidases in production of various fine chemicals

MATERIAL AND METHODS

A measophilic amidase containing strain was isolated from the samples collected from Manikaran HP and was assigned name as *Bacillus sp.* MNB1. All the production conditions are optimized like, different medium composition, pH, temperature, inducer concentration and time course of incubation on production of amidase etc. Isolated bacterial strain was found out to be producer of amidase having optimal activity at 25-35^{o}C and pH 7.

3.1 Production of amide hydrolysing bacteria: -

Selected isolates were streaked on the nutrient agar plates. Single bacterial colony from each plates taken and were inoculated in 50 ml of LB medium [LB (Luria-Bertani) Medium (1% Peptone, 1%Yeast Extract, 0.5%NaCl, 2% Glucose (per litre, pH 7.0)] and incubated for 24 hours. 5 ml inoculum was transferred into the 50 ml minimal basal medium containing 20mM glucose, with acetonitrile and without acetonitrile.

Fresh DE medium (defined basal medium) (NaCl 20gm, $MgCl_2.6H_2O$ 3.0gm, K_2HPO_4 1.03gm, $MgSO_4.7H_2O$ 0.3gm, KH_2PO_4 0.75gm, $CaCl_2.2H_2O$ 0.15gm and KCl 0.5gm (per litre) was prepared (Langdahl *et al.* 1996), containing 1ml of the trace element solutions $FeCl_2.4H_2O$ 2000mg, $MnCl_2.4H_2O$ 100 mg, $CoCl_2.6H_2O$ 250 mg. (per 100 ml.) supplemented with 50mM inducer and 10% v/v seed culture and incubated at 37ºC for 48 hours in orbital shaker at 160 rpm.

After 48 hours cells were centrifuged at 10,000 x g for 15 minutes at 4ºC. Cell pellets was washed with 0.1M potassium phosphate buffer of pH 7.0. After washing cell pellet was suspended in the 0.1 M potassium phosphate buffer (pH 7.0) and OD was taken at 660nm it was 25 OD for MNB1, then the cell suspension was kept at 4ºC for enzyme assay procedure.

3.2 Amidase assay: -

In 1ml of reaction mixture 100µl cell suspension, 200µl inducer and 700µ phosphate buffer (pH 7.0) was taken. Test tubes were incubated at 37ºC for 15 minutes. Reaction was stopped by adding of 0.1M HCl in the test tubes. In the control, the cell suspension was omitted during incubation and added after the reaction was stopped. These test tubes were centrifuged at 10000 x g. Pellet was discarded and supernatant was collected. 1 ml of supernatant was taken and 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. Cooled to room temperature and colour was observed and absorbance was recorded at 640nm. One unit of amidase activity was defined as amount of enzyme (cells/protein) that hydrolysed the amide to release 1 µmole of NH3 released min^{-1} under assay conditions (Fawcett and Scott 1960). The standard curve was prepared using 0.02–0.2 µ mole of NH4Cl/ml in buffer: 0.1M HCl (1:1) mixture.

3.3 Dry cell weight: -

1.5 ml microfuge tubes were placed in hot air oven at 80 °C for overnight meanwhile the lid of microfuge tubes was open during whole process. These tubes then cooled at the room temperature. Weight of each microfuge tubes was measured. 1 ml of culture from different cultivation media were taken in to the microfuge tubes 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 tubes were placed in to the hot-air oven for overnight at 80 °C and cooled. There after Weight of each labelled tubes were taken and dry cell weight were calculated

3.4 Characterization of bacterial strain

3.4.1 Gram's staining

A smear of bacterial colony was prepared and heat-fixed. Stained the slide by flooding it with crystal violet for 1 min and poured off excess dye and washed gently in running water**.** Now Gram's iodine added over smear for 60 second and washed with water and drained. Then Grams iodine was washed with 95% alcohol drop by drop until no more colour flows from the smear.

Washed the slides with distilled water and drained. A counter stain safranine was applied over the smear and left for 1 min. Finally washed, drained, blotted and examined under immersion oil at 100x objective of a microscope (Libenson and Mcilory 1955).

3.4.2 Endospore staining

Prepare the bacterial smears and heat fixed it. Then dry and heat fix the smears properly. Now place the side on staining rack Flood the smear with Malachite green. Heat the slides by placing it over boiling water bath for 5 minutes, and keep on adding more stain to the smear from time to time. Wash the slide under slowly running water and counter stain with safranin for one minute. Wash smear with distilled water, blot dry slides with absorbent/blotting paper. Observe the slide under microscope, endospore stain green and vegetative cells stain red.

3.4.3 Biochemical test

3.4.3.1 Indole test

Bacterium to be tested was inoculated in tryptone water, which contains amino acid tryptophan and incubated overnight at 37°C. After incubation time a few drops 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 anaerobes and non-fermenters. Formation of a red or pink coloured ring at the top is taken as positive (MacFaddin and Jean, 1980).

3.4.3.2 Methyl Red (MR) Test

The 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. After 48 hours of incubation the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acidic. The pH of the medium was tested by the addition of 5 drops of methyl red (MR) (Cowan, 1953).

3.4.3.3 Voges - Proskauer (VP) Test

Bacterium to be tested was inoculated into glucose phosphate broth and incubated for at least 48 hours. After incubation 0.6 ml of alpha-naphthol will be added to the test broth and shaken. Then 0.2 ml of 40% KOH was added to the broth and shaken. The tube was allowed to stand for 15 minutes. The negative tube was held for one hour, since maximum color development occurs within one hour after addition of reagents (Barry and Feeney, 1967).

3.4.3.4 Citrate utilization test or Simmon′s citrate agar 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 35ºC to 37ºC 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 alkalinisation.

3.4.3.5 Urease test

Inoculate a urea broth tubes with the microbe to be tested. Incubate at 37ºC temperature for 24 hours. If the organism has the ability to utilize urea, the medium changes its color from yellow to pink.

3.4.3.6 Sugar fermentation test

Use a single carbohydrate for each of medium batch medium prepared. After inoculation insert a Durham tube and keep one tube as un-inoculated to be used as control. Incubate all at 37ºC temperature for 24 hours. Observations are recorded after incubation, if the organism has the ability to ferment sugar the medium changes its colour from yellow to pink. If the organism has the ability to produce gas. The inverted Durham tubes in the medium detect the presence of gas.

3.4.3.7 Starch hydrolysis test

Prepare a starch agar plate and allow it to solidify. Label each of the starch agar plates with the name of the organisms to be incubated. Using sterile technique, make a single streak inoculation of each organism into the center of its appropriately labeled plate. Incubate the bacterial inoculated plates for 48 hours at 37ºc in an inverted position. Flood the surface of the plates with iodine solution with a dropper for 30 second. Pour off the excess iodine solution and observe the plate.

3.4.3.8 Catalase test

Bacterial strain of interest was inoculated in a slant or plate and incubated at optimal temperature for 24 hours. After incubation a 3-4% solution of H_2O_2 in need to be added in the slant or can be performed by picking a bacterial colony and placing it in H_2O_2 solution. A positive test give bubbles formation.

3.5 Immobilization procedure

3.5.1 Sodium 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.

3.5.2 Immobilization using Agarose + Agar:-

A 3% solution of Agarose+Agar was prepared and boiled. Buffer solution having cells of bacillus spp MNB1 was mixed with the solution of Agarose+Agar. The solution was poured into a pittre plate and left undisturbed, after approximately 30 minutes A+A get gelatinized. The resulting gel was cut into small discs of approximately 1cm. Prepared discs were stored at 4^0C in buffer;

3.6 Optimization of reaction condition of immobilized cells for amidase activity

3.6.1 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. Amidase assay was carried out.

3.6.2 Substrate specificity

Specificity towards different substrates for nitrilase of *Bacillus sp. MNB1* was screened for aromatic nitriles like (benzonitriles) aliphatic nitriles like (acetonitrile and acrylonitrile), aliphatic amides like (acetamide and acrylamide). Amidase activities were measured by performing assay.

3.6.3 Incubation temperature

To find out the optimum temperature of amidase of *Bacillus sp. MNB1* in reaction condition, nitrilase assay was done in different incubation temperature ranges from 30ºC to 65ºC.Effect on the nitrilase activity was analyzed by measuring Amidase activity.

3.6.4 Optimum substrate Concentration

Maximum enzyme activity was obtained when Acetamide was used as substrate. To determine the optimum substrate concentration, enzyme assay of *Bacillus* sp. MNB1 was performed using different concentration of Acetamide (i.e.10mM, 20mM, 30mM, 40mM, 50mM, 60mM, and 70mM)

3.6.5 Cell viability

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

3.6.6 Effect of metal ions

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

RESULTS AND DISCUSSIONS

4.1 Characterization of bacterial strain

S.No	Tests	Observation	Result
1	Gram staining	Purple colour	Gram +ve
2	Morphology	Straight rod shaped	*Bacillus sp.*
3	Endospore staining	Green colored endospores	Endospore formation
Biochemical Test			
3	Sugar fermentation	Glucose	+ve
		Sucrose	-ve
		Lactose	-ve
4	Catalase test	Production of bubbles	+ve
5	Indole test	Formation of red or pink colour ring at the top	+ve
6	MR/VP test	No color formation	MR -ve
		Pink/ red color formation	VP +ve
7	Citrate Test	Bubble formation	+ve
8	Starch hydrolysis test	A clear zone around bacterial growth	+ve
9	Urease test	For ammonia production due to colour change yellow to pink	+ve

Table 4.1-biochemical test results

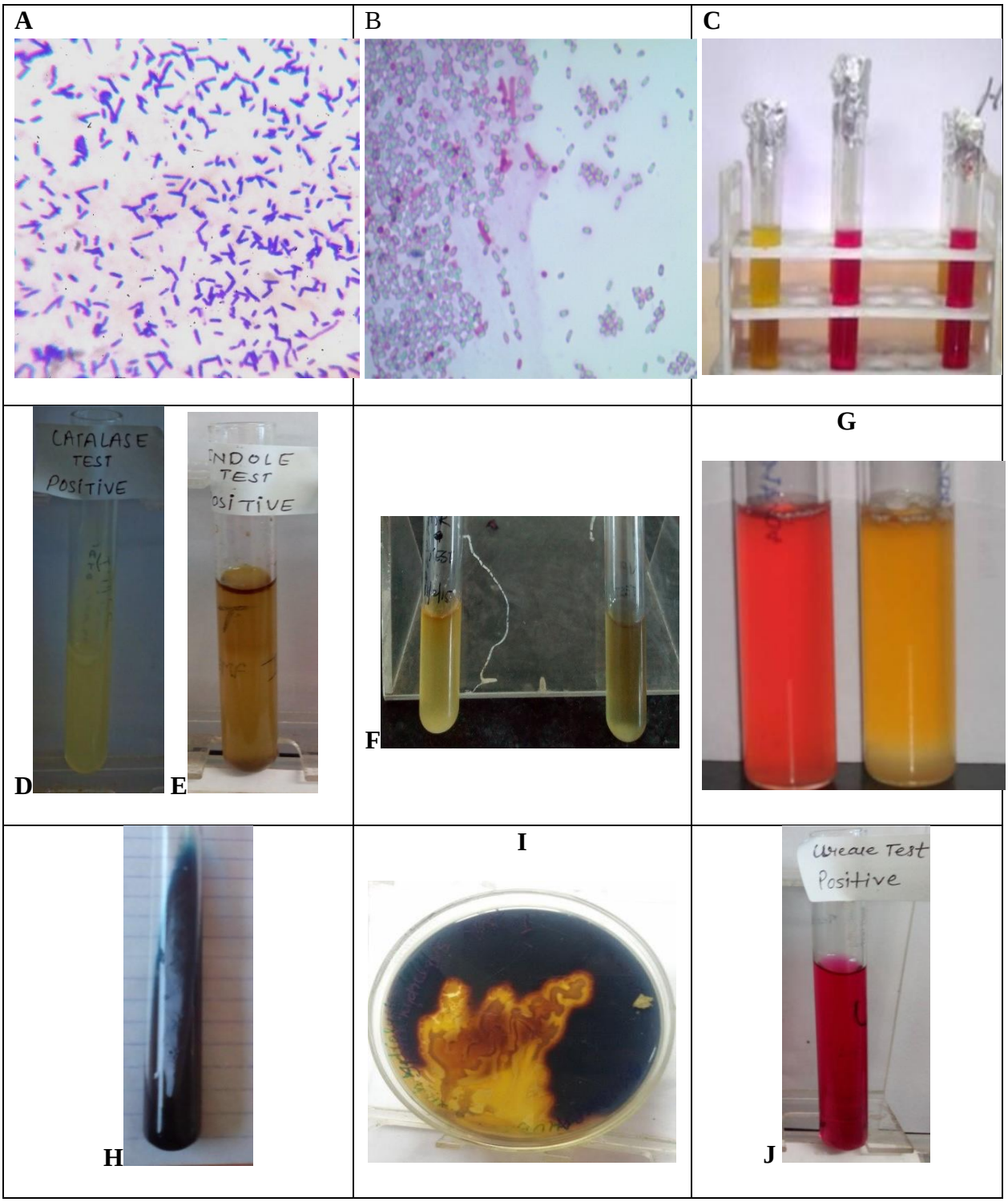


Fig.4.1: A-Gram's staining (+ ve) B-Endospore staining C- Sugar fermentation (+ ve for glucose-first one from left) D-Catalase test (+ ve) E-Indole test (+ ve) F-MR test (- ve) G- VP test (+ ve) H-Citrate test (+ ve) I- Starch hydrolysis J- Urease test (+ ve)

Several biochemical test are carried out to characterize the bacterial strain. From observed results it was concluded that the given bacterial strain was of *Bacillus subtilis* , but furthermore detailed analysis is required for clear identification of given bacterial strain. For the time being bacterial strain was named '*Bacillus sp.* MNB1'

4.2 Reaction Condition Optimization for immobilized *Bacillus sp.* MNB1

4.2.1 Substrate specificity-

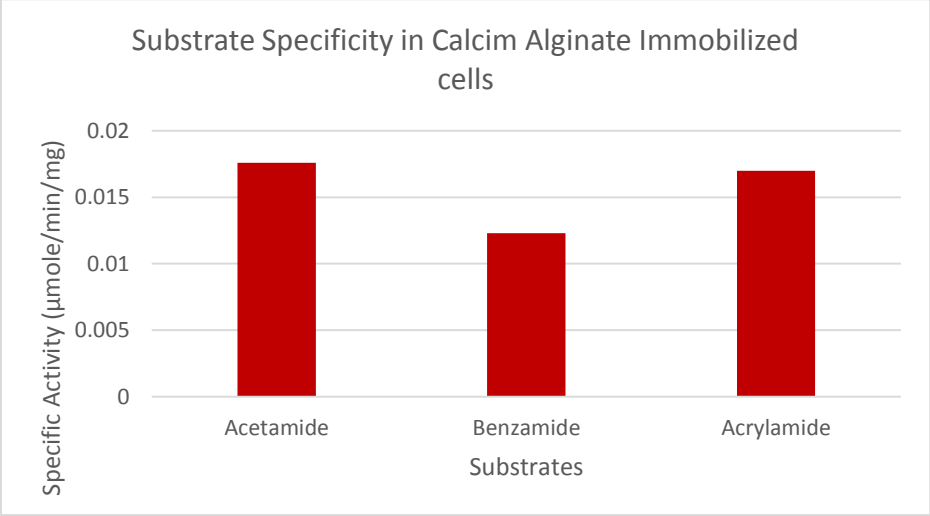


Fig.4.2- Specific activity of amidase in cells immobilized in Ca-alginate using different substrate.

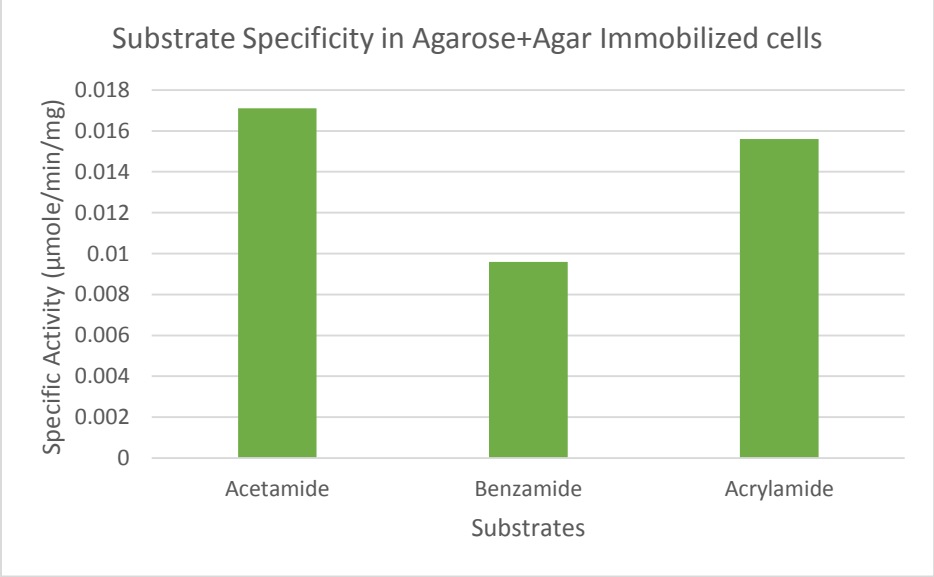


Fig. 4.3- Specific activity of amidase in cells immobilized in agarose+agar using different substrate.

Categorization of an amidase on the basis of its substrate specificities is difficult to because of its versatile behavior. Some amidases are specific for aliphatic amides (Asano *et al.* 1982, Makhongela *et al.* 2007), some hydrolyze amide of amino acids (Stelkes-Ritter *et al.* 1995) and others attack aromatic amides (Hirrlinger *et al.* 1996), aryl or aryloxypropionamide (Mayaux *et al.* 1990, 1991). To identify the substrate specificity of amidase of immobilized cells, different aliphatic and aromatic and substrate are used (Acetamide, benzamide and acrylamide). It was found that amidase of *bacillus sp.* MNB1 has higher specificity towards aliphatic amides-acetamide and acrylamide. It indicated that this amidase is highly selective for short chain amides. Cells immobilized in calcium alginate showed higher activity of amidase than immobilized in agarose+agar.

4.2.2 Bead density –

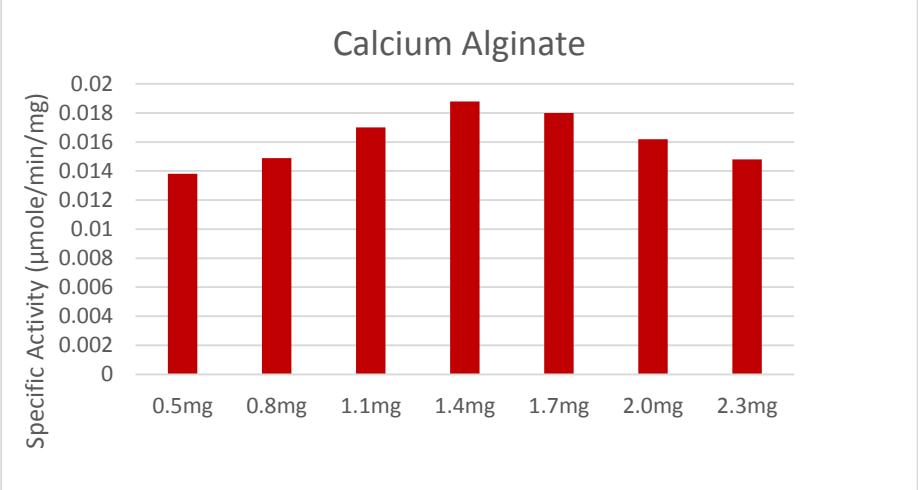


Fig. 4.4- Specific activity at different bead density (Ca-alginate immobiliztion)

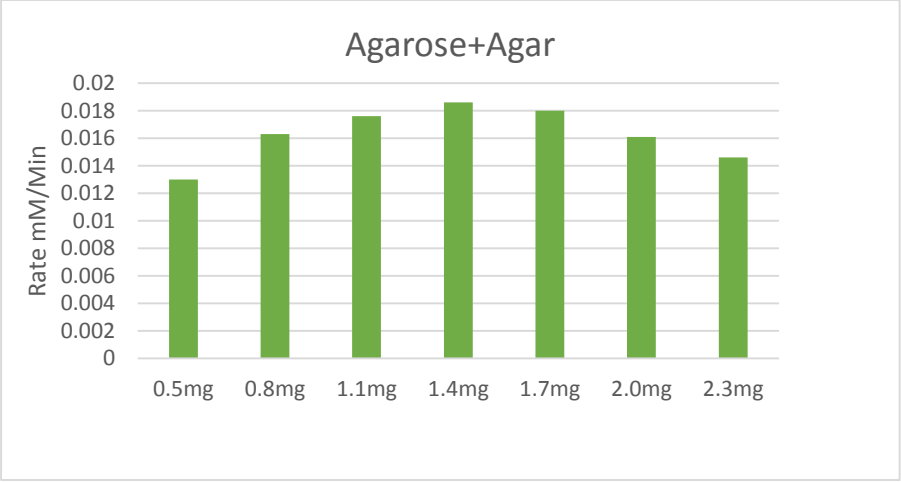


Fig. 4.5- Conversion rate at different bead density (agarose+agar immobiliztion)

Beads of calcium alginate and agrose+agar are prepared having cell density ranging from 0.5mg to 2.3mg/beads (DCW). To obtain optmum bead density of cells in immobilization matrix for production of amidase. Results showed that both cells entrapped in Ca-alginate and agrose+agar give their maxmum activity ay a cell density of 1.4mg (DCW)/bead.

4.2.3 Reusability:

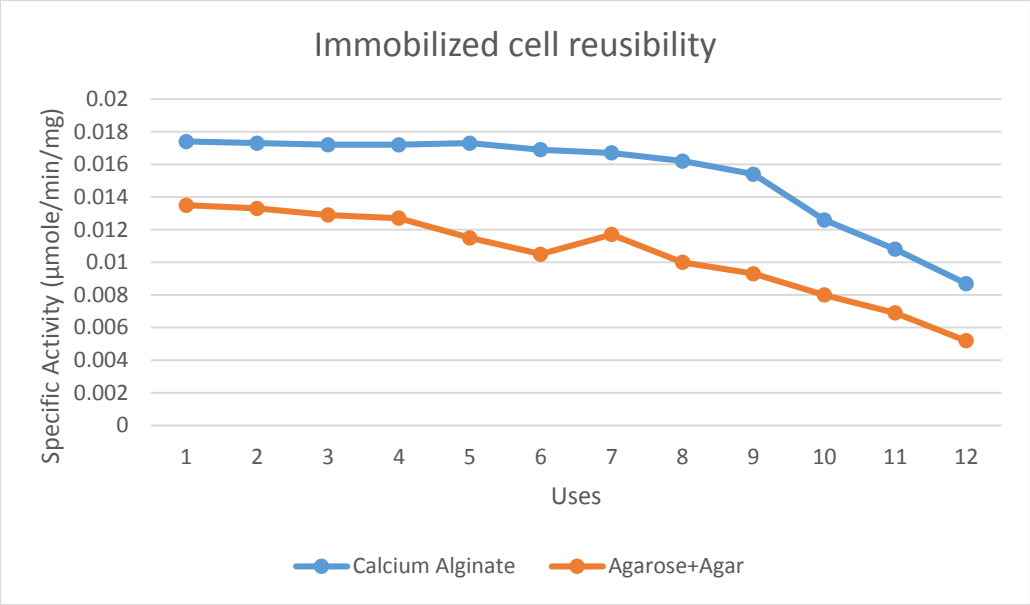


Fig. 4.6- Specific activity of immobilized *Bacillus sp.* MNB1 after different no. of repeated uses.

To estimate reusability of immobilized cells of *bacillus Sp*. MNB1, beads were used consecutively and continuously. It was observed that immobilized catalysts could be recycled up to 8 to 9 times with efficient activity as shown in Fig.4.6. Although amidase activity decreased after the 9^{th} time, almost 50% of the enzyme activity was retained in 11 and 12^{th} time. To obtain consistent amidase production fresh production medium need to be supplied after each run. The surface of the beads was suitable for the growth of cells because the supply with the nutrients and oxygen was better thus cells gradually grew on the surface of the gel with increasing cycles.

4.2.4 Optimum Substrate Concentration-

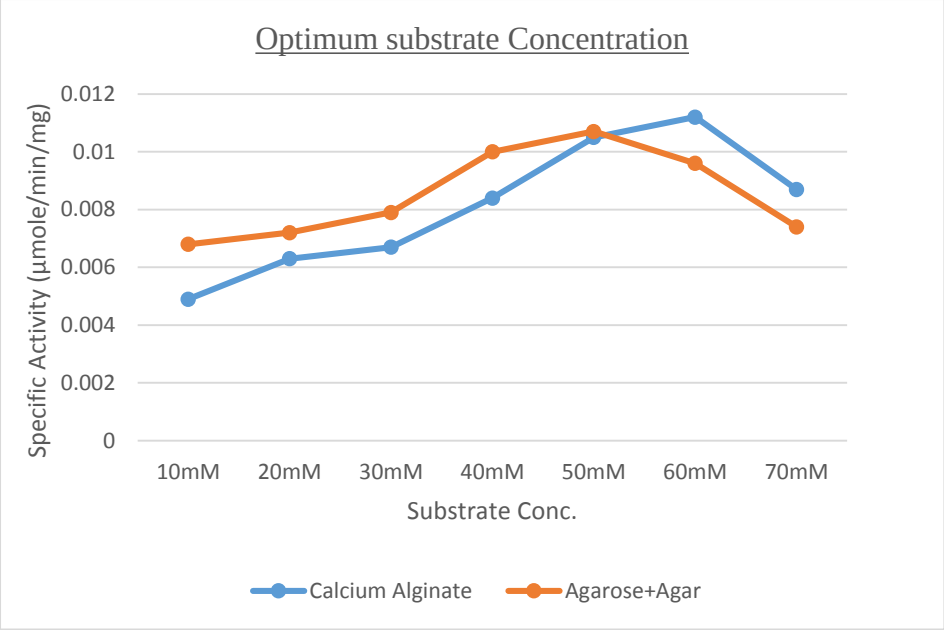


Fig. 4.7- Specific activity of immobilized *Bacillus sp.*MNB1 using different substrate concentrations.

Effect of substrate concentrations on amidase activity of immobilized MNB1 was observed. Acetamide was used as a substrate in concentration ranging from 10mM to 70mM. Maximum amidase specific activity of MNB1 was observed at 50mM in Agarose+agar immobilized cells and 60mM in calcium alginate immobilized cells (Fig.4.7). Amidase of MNB1 exhibited substrate inhibition above 50mM and 60mM acetamide concentration in agarose+agar and calcium alginate respectively, because of the saturation of enzyme active site. Recombinant amidase of *Bacillus stearothermophil* demonstrated higher activity with substrate conc. of 120mM (acrylamide) and inhibition by higher substrate was observed at higher concentrations of acrylamide (Cheong *et al.* 2000).

4.2.5 Effect of pH-

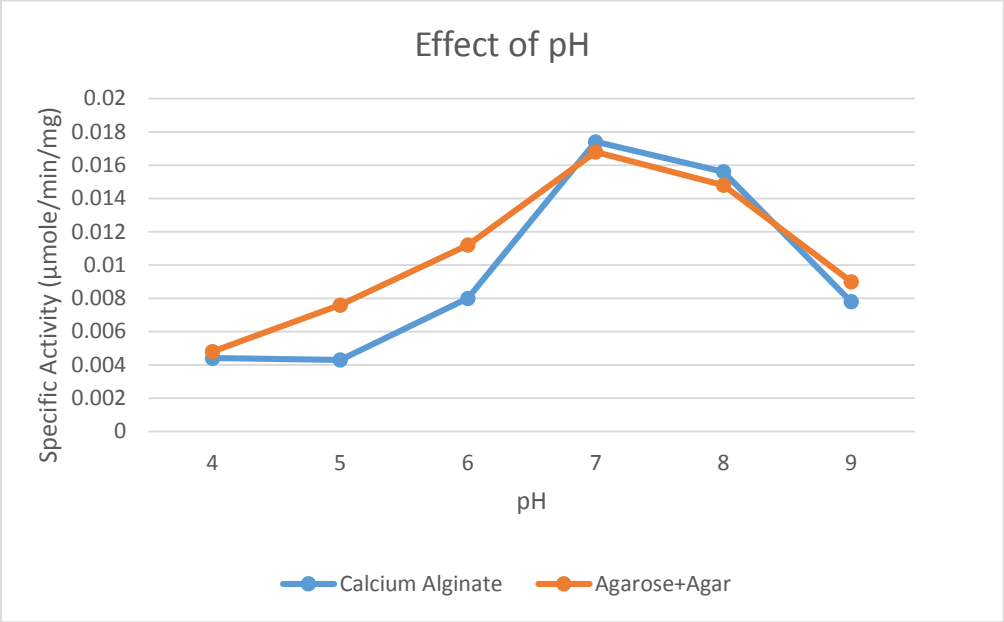


Fig. 4.8- Specific activity of immobilized *Bacillus sp.* MNB1 at different pH

The pH of reaction buffer was varied in range of pH 4.0-9.0 and used to optimize pH conditions for *Bacillus* sp. MNB1. Amidase of immobilized cells was found active within the range of pH 6.0-8.0. At pH 7.0 maximal amidase activity was observed. After pH 8.0 activity was decreased drastically (Fig.4.8). Optimum pH of *Brevibacillus borstelensis* BCS-1 was observed in the range of pH 6.5-10.0 (Baek *et al*. 2002). Shukor isolated an acrylamide hydrolyzing *Bacillus cereus* strain DRY 135, have optimum pH of 6.8-7.0. Many researchers have observed that amidase production is found to be maximum at pH 7.0-7.5 in different microorganisms like *Alcaligenes*, *Corynebacterium*, *Klebsiella etc.* (Sharma *et al.* 2009).

4.2.6 Effect of Temperature-

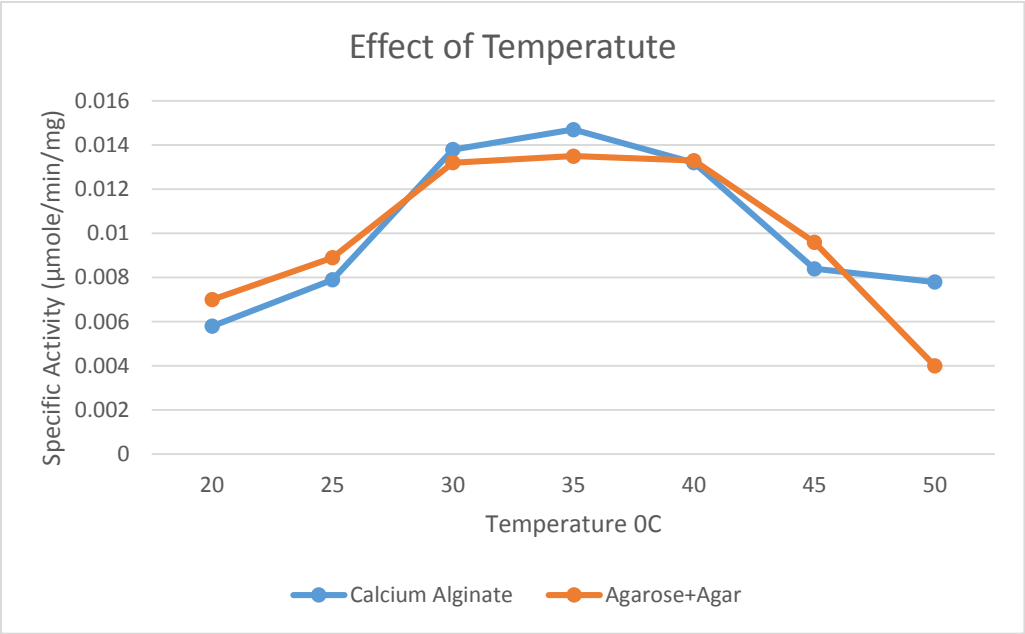


Fig. 4.9- Specific activity of immobilized *Bacillus sp.* MNB1 at different temperature.

The stability of the enzyme is strongly linked to temperature. Optimum reaction temperature for amidase of immobilized cells of Bacillus Sp. MNB1 was observed within range of 20^{o}C to 50^{o}C. Amidase of immobilized cell showed an optimal activity between 25^{o}C -40^{o}C with maximum activity at 35^{o}C. Amidase activity gradually decreased above these optimum temperatures due to deanaturation of amidase enzyme (Fig.4.9). Previous studies on amidase have observed optimal reaction temperature for amidase activity of *Mycobcterium neoaurum* ATCC MP50 (Hirrlinger *et al*. 1996), *Klebsiella pneumoniae* was recorded at 30-40^{o}C. And amidase from *Geobacillus pallidus* RAPc8 showed an optimal activity at 50^{o}C (Makhongela *et al.* 2007) and *G. Pallidus* BTP-5x demonstrated highest activity at 55^{o}C (Sharma M. *et al*. 2012).

4.2.7 Effect of Metal ions

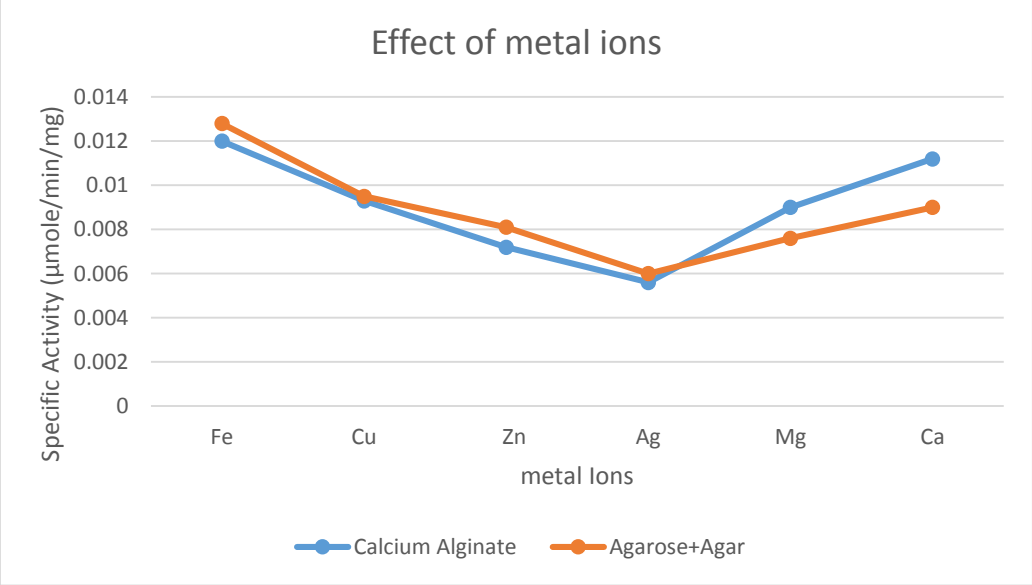


Fig. 4.10- Specific activity of immobilized *Bacillus sp.* MNB1 using different metal ions

Effect of various metal ions on the activity of amidase of immobilized bacillus Sp. MNB1 was studied at 1mM concentration and the results have been summarized in (fig.4.10). Amidase of MNB1 was strongly inhibited by AgNO3, which indicated the presence of free thiol residue at the active site of the enzyme. In related researches conducted by other researchers, metal chelating agent like EDTA had slightly inhibited the activity of this enzyme which showed that either enzyme in non-metallic or metal ions is tightly bound to the active site (Sharma *et al*. 2012). Calcium ions showed a favorable effect over amidase of cells immobilized in calcium alginate and in the presence of $FeCl_3$ maximum residual activity was recorded.

4.2.8 Viability of Immobilized cell

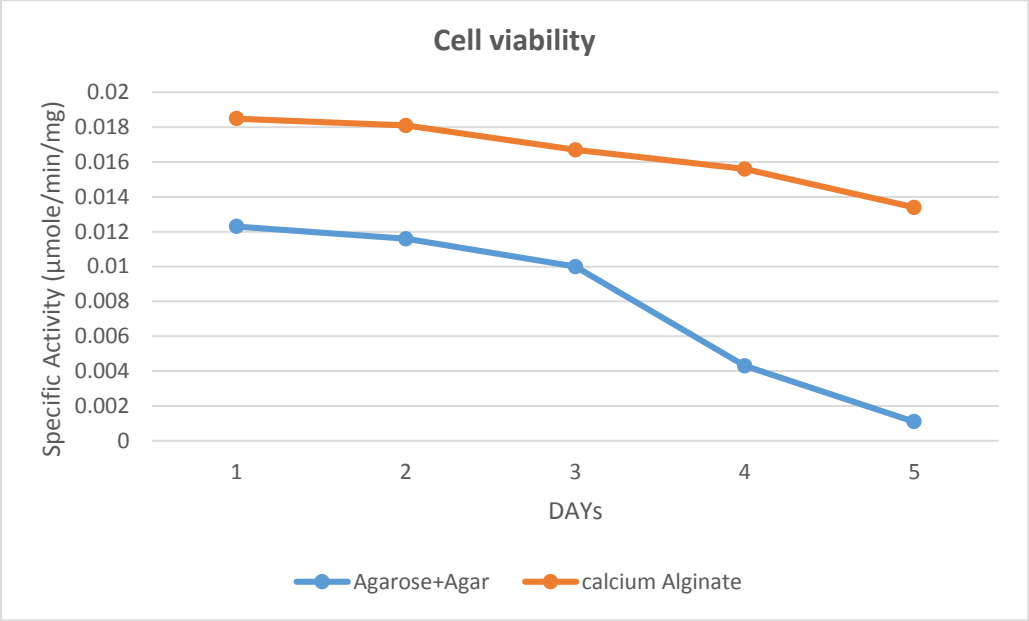


Fig. 4.11- Specific activity of immobilized *Bacillus sp.* MNB1 at specific intervals.

To determine the viability of cells for optimal production of amidase. Activity of amidase was analyzed on every 24hrs cycle for five days. Amidase of cells immobilized in Ca-alginate gave a better stability for about 5 days with only reduction of 20-30% reduction in amidase activity. On the other hand activity of amidase of cells immobilized in agrose+agar reduced drastically more than 50% after 3rd day of the process (Fig.4.11).

CONCLUSION

The potential for improvement in amidase production may offer significant advantages in some bioconversion processes. Before proceeding with immobilization procedures several biochemical test are carried out to characterize the bacterial strain. From observed results it was concluded that the given bacterial strain was of *Bacillus subtilis* , but furthermore detailed analysis is required for clear identification of given bacterial strain. For the time being bacterial strain was named '*Bacillus sp.* MNB1'.

The entrapment of cells of *Bacillus sp* MNB1 in Calcium-alginate gel beads and agrose+agar beads take into account a utilitarian method because of the simplicity of the method, inexpensive, inertness and nontoxicity of the gel. A slight decrease in enzyme activity in agrose+agar beads when compared to calcium alginate beads, was observed in the current study. This may be characteristic to relative high substrate mass transfer which may be due to difference in pore size. After immobilization amidase of *Bacillus sp.* MNB1 responded to a wider range of temperature (20^{o}C to 40 ^{o}C), pH (6 to 9), and substrate concentration (30mM to 70mM) relatively higher than amidase of free cells. Cell viability (4-5 days) and reusability (up to 9 to 10 times) also increased considerably when entrapped in immobilization matrix. These all advantages are attributed to the stable environment and protection provided by the immobilization matrix to the cells.

An enhanced enzyme production is observed when calcium ions are added to the media containing cells entrapped in Ca-alginate matrix. Previous researches also reported the same which could be due to Ca ions stabilize the calcium alginate matrix and this is why growth medium could be eked out with Ca ions to increase the structural stability of gel beads. The unstability of Calcium alginate matrix in the presence of some cations (Mg++ or K+), which are the major nutrients of living cells can be overcome by adding Ca ions in the medium.

It is therefore suggested that both the candidate are efficient to be used in production of important products at bioreactor level. Further a detailed study needs to be undertaken at bioreactor level to conclude whether amidase of immobilized *Bacillus sp.* MNB1 offer significant commercial advantages in a bioconversion process or not.

FUTURE PERSPECTIVE

Commercially important carboxylic acids like Acrylic acid and pharmacologically active hydroxamic acids (Marimastat, Inhibitor of LTA, etc.,) are produced on a large scale using amidase-producing organisms as cell factories. Amidase also have applications in bioremediations like waste treatment and industry effluent treatment due to its surface modification functions. A large number of researchers examines the microbiological, enzymological, and molecular biological expressions of amidase and have made its industrial application a desirable choice.

Immobilization provide greater stability, reusability, cost efficiency etc advantages over free cell applications in industries. Mesophilic amidase is easily available and can be isolated from several natural resources, but due to its fragile nature its industrial application is limited. Immobilization could become a light of new hope for the industrial application of mesophilic amidases. Current studied work on the immobilization of an amidase producing bacteria which could be a blessing for the industrial applications. Further study is required to make this process commercially viable like-

- Production optimization of immobilized *Bacillus* sp. MNB1 at bioreactor level.
- Production of commercially important carboxylic acids using immobilized *Bacillus* sp. MNB1 and purification by using different bioseparation techniques.
- To analyze application of immobilized *Bacillus* sp. MNB1 for waste water treatment.

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APPENDIX

Minimal Basal Medium (1000ml), pH-7.0

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

Solution A (1000ml)

Phenol	1%
Sodium Nitroprusside	0.005%

Solution B (1000ml)

Sodium Hydroxide	0.5%
Sodium Hypochlorite	0.02 N

Reaction condition optimization

Substrate specificity-

Immobilization in Calcium alginate

Substrate	**T. Amm** **(T-C)*2*0.2821**	**Rate** **T. Amm/15**	**Sp. Act.** **Rate/mg cells added**
Acetamide	0.397	0.0264	0.0176
Benzamide	0.278	0.0185	0.0123
Acrylamide	0.383	0.0255	0.0170

Immobilization in Agarose+Agar

Substrate	**T. Amm** **(T-C)*2*0.2821**	**Rate** **T. Amm/15**	**Sp. Act.** **Rate/mg cells added**

Acetamide	0.386	0.0257	0.0171
Benzamide	0.217	0.0144	0.0096
Acrylamide	0.350	0.0233	0.0156

Bead density-

Immobilization in Calcium alginate

S.NO.	mg	(T-C)	T. Amm	rate
1	0.5	0.369	0.208	0.0138
2	0.8	0.398	0.224	0.0149
3	1.1	0.453	0.255	0.0170
4	1.4	0.501	0.282	0.0188
5	1.7	0.482	0.271	0.0180
6	2.0	0.433	0.244	0.0162
7	2.3	0.394	0.222	0.0148

Immobilization in Agarose+Agar

S.NO.	mg	(T-C)	T. Amm	rate
1	0.5	0.347	0.195	0.0130
2	0.8	0.436	0.245	0.0163
3	1.1	0.471	0.265	0.0176
4	1.4	0.497	0.280	0.0186
5	1.7	0.479	0.270	0.0180
6	2.0	0.430	0.242	0.0161
7	2.3	0.390	0.220	0.0146

Reusability

Immobilization in Calcium alginate

S.NO.	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
	(T-C)*2*0.2821	T. Amm/15			Rate/mg cells added
1	0.393	0.0262	3	1.5	0.0174
2	0.390	0.0260	3	1.5	0.0173
3	0.387	0.0258	3	1.5	0.0172
4	0.388	0.0258	3	1.5	0.0172

5	0.391	0.0260	3	1.5	0.0173
6	0.382	0.0254	3	1.5	0.0169
7	0.377	0.0251	3	1.5	0.0167
8	0.365	0.0243	3	1.5	0.0162
9	0.347	0.0231	3	1.5	0.0154
10	0.284	0.0189	3	1.5	0.0126
11	0.244	0.0162	3	1.5	0.0108
12	0.197	0.0137	3	1.5	0.0087

Immobilization in Agarose+Agar

S.NO.	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
	(T-C)*2*0.2821	T. Amm/15			Rate/mg cells added
1	0.305	0.0203	3	1.5	0.0135
2	0.300	0.0200	3	1.5	0.0133
3	0.291	0.0194	3	1.5	0.0129
4	0.286	0.0190	3	1.5	0.0127
5	0.259	0.0172	3	1.5	0.0115
6	0.238	0.0158	3	1.5	0.0105
7	0.264	0.0176	3	1.5	0.0117
8	0.227	0.0151	3	1.5	0.0100
9	0.211	0.0140	3	1.5	0.0093
10	0.181	0.0120	3	1.5	0.0080
11	0.157	0.0104	3	1.5	0.0069
12	0.119	0.0079	3	1.5	0.0052

Optimum substrate Concentration

Immobilization in Calcium alginate

S.NO.	Substrate conc.	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
	(mM)	(T-C)*2*0.2821	T. Amm/15			Rate/mg cells added
1	10	0.148	0.0098	4	2	0.0049
2	20	0.190	0.0126	4	2	0.0063
3	30	0.203	0.0135	4	2	0.0067
4	40	0.254	0.0169	4	2	00084
5	50	0.317	0.0211	4	2	0.0105
6	60	0.336	0.0224	4	2	0.0112

7	70	0.263	0.0175	4	2	0.0087

Immobilization in Agarose+Agar

S.NO.	Substrate conc.	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
	(mM)	(T-C)*2*0.2821	T. Amm/15			Rate/mg cells added
1	10	0.205	0.0136	4	2	0.0068
2	20	0.217	0.0144	4	2	0.0072
3	30	0.238	0.0158	4	2	0.0079
4	40	0.300	0.0200	4	2	0.0100
5	50	0.321	0.0214	4	2	0.0107
6	60	0.289	0.0192	4	2	0.0096
7	70	0.223	0.0148	4	2	0.0074

Cell viability-

Immobilization in Calcium alginate

S.NO.	DAY	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
		(T-C)*2*0.2821	T. Amm/15			Rate/mg cells added
1	1	0.417	0.0278	3	1.5	0.0185
2	2	0.409	0.0272	3	1.5	0.0181
3	3	0.377	0.0251	3	1.5	0.0167
4	4	0.353	0.0235	3	1.5	0.0156
5	5	0.303	0.0202	3	1.5	0.0134

Immobilization in Agarose+Agar

Effect of pH-

Immobilization in Calcium alginate

S.NO.	pH	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	4	0.108	0.0072	1.5	1.5	0.0048
2	5	0.171	0.0114	1.5	1.5	0.0076
3	6	0.253	0.0168	1.5	1.5	0.0112

4	7	0.379	0.0258	1.5	1.5	0.0168
5	8	0.333	0.0222	1.5	1.5	0.0148
6	9	0.203	0.0135	1.5	1.5	0.0090
7	10	0.168	0.0112	1.5	1.5	0.0074

Immobilization in Agarose+Agar

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	0.099	0.0066	1.5	1.5	0.0044
2	5	0.098	0.0065	1.5	1.5	0.0043
3	6	0.181	0.0120	1.5	1.5	0.0080
4	7	0.392	0.0261	1.5	1.5	0.0174
5	8	0.350	0.0233	1.5	1.5	0.0156
6	9	0.177	0.0118	1.5	1.5	0.0078
7	10	0.154	0.0102	1.5	1.5	0.0068

Temperature-

Immobilization in Calcium alginate

S.NO.	Temp.	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. A
1	20	0.131	0.0087	1.5	1.5	0.0058
2	25	0.179	0.0119	1.5	1.5	0.0079
3	30	0.311	0.0207	1.5	1.5	0.0138
4	35	0.332	0.0221	1.5	1.5	0.0147
5	40	0.299	0.0199	1.5	1.5	0.0132
6	45	0.189	0.0126	1.5	1.5	0.0084
7	50	0.176	0.0117	1.5	1.5	0.0078

Immobilization in Agarose+Agar

S.NO.	Temp.	T. Amm (T-C)x2x0.2821	Rate T. Amm/15	dcw/ml in mg	mg added Per bead	Sp. A
1	20	0.156	0.0106	1.5	1.5	0.0070
2	25	0.201	0.0134	1.5	1.5	0.0089
3	30	0.298	0.0198	1.5	1.5	0.0132
4	35	0.304	0.0202	1.5	1.5	0.0135

5	40	0.300	0.0200	1.5	1.5	0.0133
6	45	0.217	0.0144	1.5	1.5	0.0096
7	50	0.091	0.0060	1.5	1.5	0.0040

Metal ions-

Immobilization in Calcium alginate

S.NO.	Metal ion	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
		(T-C)*2*0.2821	T. Amm/15			Rate/mg cells added
1	FeSO4	0.217	0.0144	2.4	1.2	0.0120
2	CuSO4	0.166	0.0110	2.4	1.2	0.0093
3	ZnSO4	0.131	0.0087	2.4	1.2	0.0072
4	AgNO3	0.102	0.0068	2.4	1.2	0.0056
5	MgCl2	0.162	0.0108	2.4	1.2	0.0090
6	CaCl2	0.203	0.0135	2.4	1.2	0.0112

Immobilization in Agarose+Agar

S.NO.	Metal ion	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.
		(T-C)*2*0.2821	T. Amm/15			Rate/mg cells added
1	FeSO4	0.231	0.0154	2.4	1.2	0.0128
2	CuSO4	0.171	0.0114	2.4	1.2	0.0095
3	ZnSO4	0.147	0.0098	2.4	1.2	0.0081
4	AgNO3	0.109	0.0072	2.4	1.2	0.0060
5	MgCl2	0.137	0.0091	2.4	1.2	0.0076
6	CaCl2	0.163	0.0108	2.4	1.2	0.0090