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LIST OF ABBREVATIONS

1. M	Molar
2. mM	Milli molar
3. HCl	Hydrochloric acid
4. NaCl	Sodium chloride
5. SDS	Sodium Dodecyl Sulphate
6. SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
7. µl	Micro liter
8. ml	Milli liter
9. gm	Gram
10. mg	Milli gram
11. dcw	Dry cell weight
12. TEMED	N,N,N',N'-Tetramethylethylenediamine
13. DTT	Dithiothreitol
14. EDTA	Ethylene Diamine Tetra Acetic acid
15. DEAE-cellulose	Diethylaminoethyl cellulose
16. EC number	Enzyme commission number

Chapter: - 1

ABSTRACT

Amidase (EC 3.5.1.4) is a nitrile metabolizing enzyme, belongs to the family of hydrolases, which acts on carbon-nitrogen bonds other than peptide bonds. It usually occurs in prokaryotic and eukaryotic as an inducible intracellular enzyme, which is involved in nitrogen metabolism. 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). In current reasearch project the isolation, production and reaction conditions optimization of mesophilic amidase were undertaken. An aliphatic selective amide degrading gram positive *Bacillus* sp. MNB1 strain was isolated from thermal spring soil of Manikaran, Himachal Pradesh, India. Amidase production and reaction conditions were optimized. Amidase of *Bacillus* sp. MNB1 exhibited maximum biomass and activity in M2 medium at temperature 37ºC, pH 7.0 in the presence of acetamide as inducer. The enzyme showed maximum activity at pH 7.0, temperature 37 ºC and 40-50 mM of acetamide as substrate. The enzyme exhibited activity in 30-50% cut of ammonium sulphate precipitation and eluted fraction 0.1-0.5M exhibited amidase activity (secondary activity of amidase) confirmed its presence in the strain *Bacillus* sp. MNB1.

Key words: Amidase, hydroxamic acids, nitrile metabolizing enzyme.

Chapter 2: -

INTRODUCTION

Nitrile metabolizing enzymes are ubiquitous enzymes found in different organisms. Nitrilase enzyme directly converts organic nitrile into acids and nitrile hydratase-amidase system where nitrile is first converted to amides by NHase (nitrile hydratase) and then hydrolyzed to acids by amidase (Fig 1) Nitrile metabolizing enzymes hydrolyze organic nitriles (RC-N) which are common constituents of plants (Kobayashi *et al*, 1989) and also occur as intermediates of microbial metabolism (Kobayashi *et al*, 1993a).

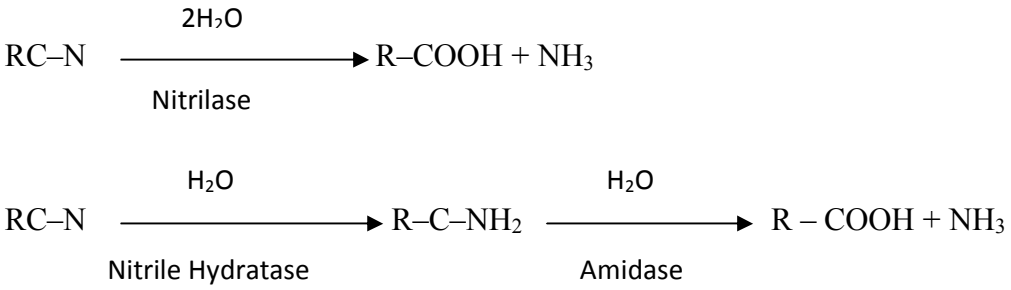


Figure 1: catalysis of nitrile hydrolysis via two enzymatic pathways (Joni Frederick, 2006)

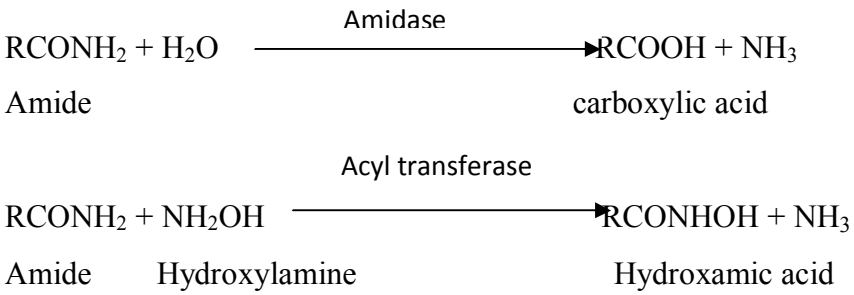
A number of Nitrile metabolizing enzymes have been reported in plants e.g. oranges (*Citrus sinesis*) barley, tobacco (Thiamann *et al*. 1977) and in bacteria e.g. *Brevibacterium* (Arnaud *et al*. 2001), *Arthrobacter* (Asano *et al*. 1982), *Nocardia*, *Klebsiella*, *Corynebacterium* and *Rhodococcus* (Langdahl *et al*. 1996) and in fungi e.g. *Fusarium lateritium*, *Aspergillus nidulans* (Hynes *et al.* 1970).

The biological functions of these enzymes in nature are carbon or nitrogen fixation in prokaryotes through hydrolysis of amides, the generation of properly charged tRNA in eubacteria through transfer of ammonia from glutamine (Curnow *et al.* 1997, Banerjee *et al.* 2002) and the degradation of neuromodulatory fatty acid amides in mammals (Cravatt *et al.* 1996).

Generally amidase (EC 3.5.1.4) is co-transcribed with nitrile hydratase. Amidase has dual activity as hydrolysis and acyl transfer.

Amidase (amidohydrolase) catalyzes the hydrolysis of amides to the corresponding acids and liberate ammonia. C-N bonds containing compounds are widely distributed in nature. Acyl transfer activity of amidase has a wide substrate affinity for a variety of aliphatic amides (formamide, acetamide, propanamide) and aromatic amides (benzamide, nicotinamide). Amidases enzymes have been found quite stable at 35-50ºC having half life 8 hours (Bhatia *et al*. 2013; Sharma. *et al*. 2012).

Amidases catalyze the transfer an acyl moiety to hydroxylamine to form hydroxamic acids (Fournand *et al.* 2001, Sharma *et al*. 2013). *Rhodococcus* sp. R312 possesses a wide spectrum aliphatic amidase which transfer acyl groups of amides, acids, hydrazine and esters to hydroxylamine to form the corresponding hydroxamic acid (Thiery *et al.* 1986).



Nitrile metabolizing enzymes have wide applications in commercial processes in paper manufacturing and waste treatment. Amidases have also been used in commercial production of acrylic acid (Kobayashi *et al*. 1993), antibiotics (Jallgeas *et al*. 1980), anti inflammatory agents (Gilligan *et al*. 1993) and herbicides (Bianchi *et al*. 1991). For efficient industrial applications, thermostability, enantioselectivity and substrate specificity are of prime importance. Presently directed evolution or by protein engineering were used for amidase activity improvement.

Amidases will also find application as industrial catalyst to their higher operational stability. This research has been undertaken to isolate and to optimize the production and reaction condition of *Bacillus* sp. MNB1 strain isolated from Manikaran thermal springs. This enzyme can be used for biotransformation reactions at mesophilic temperature.

Keeping above in view, the present research work entitled has been undertaken with the aim "Isolation, production and reaction conditions optimization of amidase of *Bacillus* sp. MNB-1". The objectives of this research work were:

1. Isolation of mesophilic amidase producing bacteria.
2. Optimization of culture and reaction conditions of bacterial isolate *Bacillus* sp. MNB1.
3. Protein profiling and zymography of amidase of *Bacillus* sp. MNB1.

Chapter 3: -

REVIEW OF LITERATURE

In nature many organic and inorganic nitriles (C-N bond containing compounds) are widely distributed and they are mainly hydrolyzed by nitrilase superfamily enzymes (Pace and Brenner 2001). Nitrile-metabolizing enzymes are relatively uncommon in eukaryotic organisms but they are largely distributed in bacteria (*Acinetobacter*, *Bacillus*, *Arthrobacter*, *Corynebacterium*, *Pseudomonas*, *Klebsiella*, *Norcadia*, *Rhocococcus*, *etc*), fungi (*Aspergillus nidulans*) and yeast (*Candida* sp, *Rhodotorula glutinis*, *Cryptococcus flavus*, *Geotrichum* sp. etc) which metabolize nitriles as a sole carbon and nitrogen source (Banerjee *et al.* 2002).

3.1 Amidase in bacteria: -

A thermophillic *Bacillus* strain was isolated by Thalenfeld and coworkers in 1975. it exhibited the maximal amidase activity at 55˚C and 30mM acetamide was used as inducer and substrate. *Klebsiella pneumoniae* was isolated from industrial sewage using acrylonitrile as sole source of nitrogen (Nawaz *et al.* 1991). Acetonitrile, acrylonitrile, benzonitrile and acrylonitrile plant waste water were used for enrichment, in order to isolate bacterial strains from soil. *Pseudomonas* species was isolated from soil samples by using succinate as carbon source and benzylcyanide, methyl, ethyl and methoxybenzyl cyanide as the different sole source of nitrogen.

Kobayashi *et al.* (1998) reported *Rhodococcus rhodochrous* J1 strain, which had one exceptional amidase enzyme having nitrile hydrolyzing activity. It surprisingly catalyzes C-N triple bonds containing nitrile to form an corresponding acid and ammonium stoichiometrically with amidase activity. Baek *et al.* (2002) purified a thermostable D-metheonine amidase isolated from *Brevibacillus bosrstelensis* BCS-1, it was found to be stable upto 65˚C and pH range from 6.5 to 10.0. Its maximal enzyme activity was measured at 65˚C and pH 9.5.

An acrylamide degrading *Burkholderia* sp. strain DR Y27 was isolated and aliphatic amides (acetamide and propionamide) were found to be best substrates. Maximal amidase

activity was observed at 40˚C and pH 8.0 (Syed *et al*. 2011). Sharma *et al.* (2012) isolated thermophilic aliphatic selective amidase from hot springs, which showed maximum activity at pH 7.0, temperature 55˚C. A novel thermosatable strain *Geobacillus subterraneus* RL-2a was isolated by Mehta and co- workers in 2013, had a wide spectrum for mid chain aliphatic and amino acid amides. It exhibited amidase activity in wide range of pH 4.5-11.5 and temperature stability from 40˚C- 90˚C. Abada *et al.* (2014) isolated an extracellular amidase enzyme producing *Pseudomonas putida* from agricultural soil. After isolating amidase optimum production was observed at 30˚C and pH 8.0 for 36 hours of incubation. Enzyme exhibited maximum activity for acetamide and acrylamide used as substrate. Gao et al. (2015) described N-acyl-D-amino acid amidohydrolase and D-amino acid amidase, which can be used in synthesis of D-amino acids.

3.2 Amidase in Yeast: -

Only few types of yeast have been reported to have nitrile hydrolyzing enzymes in comparison to bacteria. In 1933 *Candida utilis* was isolated had amide hydrolyzing activity by Gorr and Wagner. Brady *et al.* (1969) grown *Candida utilis*, which can catalyze the hydrolysis of wide spectrum amides. Prasad *et al.* (2005) isolated *Kluyveromyces thermotolerans* MGBY 37 from traditional fermented foods and beverages of Himachal Pradesh, India.

3.3 Amidase in soil: -

There are four amidases (amidohydrolases) which are used in nitrogen nutrition of plants; L-asparaginase (EC 3.5.1.1), L-glutaminase (EC 3.5.1.2), urease (EC 3.5.1.5), aliphatic amidase (EC 3.5.1.4) (Frankenberger, 1980). L- Asparagine amidohydrolase was first reported in soils by Drobni'k in 1956 which catalyzed the hydrolysis of L-asparagine to L-aspartate and ammonia. Higher activity of L-glutaminase is generally present in toluene treated soils compared to non treated soil. Its activity first detected in soil by Galstyan and Sakyan in 1973. This enzyme converts L-glutamine into L-glutamate and ammonia by hydrolysis.

Urea amidohydrolase is well characterized enzyme because of the use of urea as soil fertilizer. It causes the hydrolysis of urea into ammonia and carbon dioxide. It was first detected in soil by Rotini in 1935. Aliphatic amidases are also found in soils after degradation of plant leaves and in industrial waste disposal sites.

3.4 Amidase in fungi: -

Amongst all fungal strains *Aspergillus nidulans* is the most studied strain and it was reported to utilize formamide as nitrogen source and acetamide as sole source of carbon and nitrogen. Many amidases have been isolated as acetamidase which can hydrolyze aliphatic amides contain 1-6 carbon atoms; acetamide, butyramide, glycolamide, propionamide, acrylamide. Formamidase hydrolyses formamide and glycinamide. Acetamidase and formamidase are known to be inducible in nature. Another amidase type is known as general amidase, which have substrate specificity for both aromatic and alipahatic amides; benzamide, valeramide, phenylacetamide (Hynes, 1970)

3.5 Amidase in plants: -

Amidase enzymes are important enzymes found in great amount in the rhizosphere of plants. Apart from the amide hydrolysis activity these take part in the biosynthesis of IAA (Indole acetic acid) which is a plant hormone. A peptide amidase was partially purified from the oranges, which helps in hydrolysis of C-terminal amide groups into in peptides or N-protected amino acids and amino acids do not deaminate.

3.6 Amidase in animals: -

There are many reports on amidases in animals e.g. Bray *et al.* (1950) extracted aliphatic amidase from rat liver which was able to hydrolyze aliphatic acetamide and propioniamide but it shown higher activity for mid chain amides. Anandamide amidase or N-arachidonyl ethanolamine deacylase (EC 3.5.160) or fatty acid amidohydrolases (FAAH) was detected from porcine brain, was found to hydrolyze anandamide (arachidonyl ethanolamide) or fatty acid oleamide.

Nitrilase superfamily enzymes are also known as CN-hydrolases. The nitrilase enzyme superfamily is consisted of thiol enzymes. These enzymes are involved in the biosynthesis of natural products and post translational modifications in bacteria, fungi, plants and animals. Nitrilase superfamily can be classified in 13 branches on the basis of the presence of the additional domains, sequence identity and substrate specificity. Out of these 13 branches 9

branches are divided on the basis of nitrilase related sequences. Only one branch has nitrilase activity whereas other 8 branches are known to have amide hydrolysis or condensation activities. Nitrile hydratase are not members of nitrilase superfamily. All enzyme branches of nitrilase superfamily have a catalytic triad of glutamate, lysine and cysteine.

Nitrilase superfamily consists of 13 following branches (Branner *et al.* 2002): -

(i) Nitrilase
(ii) Aliphatic amidase
(iii) N-terminal amidase
(iv) Biotinidase
(v) Beta-Uridopropienase
(vi) Carbamylase
(vii) Prokaryotic NAD+ Synthetase
(viii) Eukaryotic NAD+ Synthetase
(ix) Apolipoprotein N – acyltransferase
(x) Nit and Nit Fhit
(xi) NB 11
(xii) NB12
(xiii) Non-fused outliers

3.7 Classification of amidase enzyme:

Amidases are classified in different types either on the basis of substrate specificity or amino acid sequences (Faurnand *et al*.2001, Sharma M. *et al.* 2009).

(a) Aliphatic amidases: - they catalyze the hydrolysis of aliphatic amides and produce corresponding acids and ammonia. These enzymes generally found in *Rhodococcus rhodochrous MB*, *Corynebacterium sp*, *Pseudomonas aeruginosa*, *Bacillus stearothermophillus* BR 388, *Geobacillus pallidus* BTP-5x and *Geobacillus pallidus* RAPc8.

(b) Aromatic amidases: - they utilize aromatic amides as substrate and convert into corresponding acids and ammonia. These are present in *Pseudomonas cepecia*, *Pseudomonas putida* and *Pseudomonas acidovorans*.

(c) Enantioselective amidases: - they catalyze hydrolysis of enantio-selectively hydrolyzed racemic amides to enantiopure product. Examples of the bacteria contain these amidases are *Rhodococcus sp*. R312, *Rhodococcus erythropolis* MP50.

(d) α-aminoamidases: - they hydrolyze only L-α-amino acids and are found in *Ochrabactrum anthropi* NCIMB 40321, *Mycobacterium neoaurum* ATCC 25795.

(e) Arylalkyl acylamidases: - they catalyze hydrolysis of N- acyl primary aromatic amides and amides to acids e.g. amidase of *Pseudomonas putida.*

(f) Wide spectrum amidases: - These amidases hydrolyze a variety of amides such as acetamide, propionamide, butyramide, benzamide and valeramide into corresponding acids. E.g. *Bacillus stearothernmophilus* BR 388 (Cheong *et al*. 2000) and *Brevibacterium* sp. R312.

Amidases are also classified into two categories on the basis of amino acid sequences.

I. Aliphatic amidases: - these belong to nitrilase superfamily which includes aliphatic amidases and hydrolyze only short chain aliphatic amides. These enzymes are homohexamers of approximately 230 kDa; each subunit contains a cysteine residue which acts as catalytic nucleophile. These enzymes are also known as aliphatic amidases but do not contain the central conserved GGSS-signature (Gly-Gly-Ser-Ser) in their amino acid sequence.

II. Signature amidases: - These enzymes have enantioselectivity and they belong to the GGSS-signature containing amidase family. It contains GGSS (Gly-Gly-Ser-Ser) signature motif in their primary sequence and also known as wide spectrum amidases.

3.8 Applications of amidase: -

Amidases exhibit two types of activity amidohydrolase and acyl transferase activity. Amidase of *Rhodococcus rhodochrous* is employed as biocatalyst in industrial production of acrylic acid. It was isolated from soil and was grown by utilizing nitrile via coupled action of nitrile hydratase that converts nitriles into corresponding amides and amidase hydrolyze amides to corresponding acids and ammonia (Kotlava *et al*, 1998).

3.8.1 Amidases in bioremediation: -

One of the amidases, such as arylamidase (α-amino acyl- peptide hydrolase; EC 3.4.11.2) catalyzes the hydrolysis of an N-terminal amino acid of peptides, amides or arylamides. It plays

an important role in nitrogen cycle process. As a variety of arylamides are present in soil and at least 14 amino acids have been studied and extracted. After hydrolysis of the amides, peptides or acrylamides, amino acids are released which are used as substrates for other enzymes as amidohydrolase involved in nitrogen mineralization.

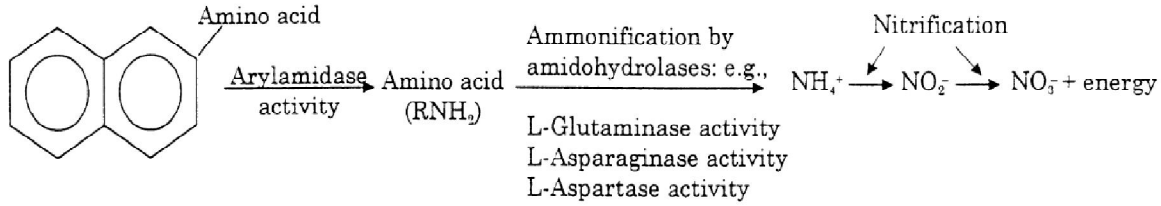


Figure 2: The reaction of hydrolysis of an N-terminal amino acid by arylamidase (Acosta-Martoanez V. *et al*. 2001).

Organic nitriles are used by nitrile metabolizing micro-organisms as source of carbon and nitrogen. Strain *Rhodococcus* M8 is widely used in the industrial production of acrylamide.

3.8.2 Amidase in acrylamide degradation: -

Amidase enzyme is also used in bioremediation field because of having ability to degrade acrylamide. Acrylamide is a building block which is used in the synthesis of polyacrylamide (PAM). Polyacrylamides are usually used as sewage-flocculating agents, coagulants in the crude oil recovery process and as adhesives also. And commercial polyacrylamide preparations may be contaminated with its toxic monomers i.e. acrylamide. Under environmental conditions polyacrylamide degrades to acrylamide which are known as neurotoxicant, carcinogen, teratogen. And now the presence of acrylamide in processed food products has become a very serious health issue. Species of genera *Pseudomonas*, *Bacillus*, *Rhodococcus* produce amidases which convert acrylamide to ammonia and acrylic acid (Syed *et al*. 2011).

3.8.3 Amidase in Pharmaceutical industry: -

a. For production of antibiotics: -

Peptides which contain D amino-acid exhibit greater antimicrobial properties than peptides containing corresponding L-isomers, because D-isomers would seem to be more stable than L-isomers against proteolytic digestion. D-amino acids are used as intermediate for the

production of pharmaceuticals, food additives and agrochemicals. In pharmaceutical industries D-amino acids are used in synthesis of semi synthetic antibiotics and have been reported to enhance the microbial activity against biofilms of wound microorganisms.

Basically D-phenylglycine, D-cystein and D-aspartic acid are used for the synthesis of Beta-lactum antibiotics, insecticides and synthetic sweeteners. D-amino acids as D-glumatic acid, D-aspartic acid, D-alanine usually found in raw milk of cows and sheep. D-amino acid synthesized sweeteners can be used in food products for diabetes patients (Gao *et al.* 2015). Thermostable D-amidases are used because of having high amidase activity and D-stereospecificity for D-amino acid amides. That produces optically pure D-amino acids from DL-racemic mixtures without any by-products formation. D-stereospecific amino acid amidase catalyze hydrolysis of D-amino acid amides to yield D-amino acid and ammonia, e.g. amidase from *Bacillus sp.* BCS-1, D-methionine amidase from *Brevibacillus borstelnsis* BCS-1 (Baek *et al*. 2002).

b. Amidase product (Hydroxamic acid) applications: -

Amidase enzymes have acyl transfer activity; amides transfer acyl groups of amides, acids, esters to hydroxylamine (cosubstrate of amides) to form hydroxamic acid and ammonia by using ping pong bi bi mechanism. Different amides are converted into their corresponding hydroxamic acids (Faurnand *et al.* 1998).

Many amidases producing bacteria have been isolated which known to have acyl transfer activity; *Rhodococcus rhodochrous* MP50 (Hirrlinger *et al*. 1996), *Rhodococcus sp.* R312 (Bigey *et al*. 1999), *Geobacillus pallidus* (Makhongela *et al.* 2007), Geobacillus pallidus BTP-5x (Sharma *et al.* 2009). Enzymatic biotransformation is eco-friendly that does not yield toxic by-products. As amidases are used to hydrolyze hydroxylamine into corresponding hydroxamic acids which have an important role in pharmaceuticals (Sharma *et al.* 2009) as well as in waste water treatment (Table 1). They are inhibitors of enzymes e.g. matrix metalloproteinases (MMP) (Leung *et al.* 2000, Hidalgo *et al.* (2001), angiotensin converting enzymes (ACE), ureases, peroxidases, prostaglandin H synthetase and are thus considered as ideal drug molecules. Fatty hydroxamic acids have anti inflammatory property and also used to treat chronic asthma. (Hamer *et al*.1998)**.**

Marimastat is a hydroxamic acid (Naglich *et al.* 2001) which is at an advanced clinical stage as an anticancer drug. Phase III clinical trials have shown that Marimastat may stop the growth of non-small cell lung cancer by stopping blood flow to the tumor (National Cancer Institute, 2008). In AIDS α-amino hydroxamic acids are used as anti-HIV agent (Gao *et al*. 1995). α-aminohydroxamic acid derivatives are used in tissue remodelling because of potential inhibition properties against metalloprotease. Acetohydroxamic acid has been investigated for having anti malarial properties and has been recommended for treatment of anemia and ureaplasma infections. Few fatty hydroxamic acids have also found a potent anti inflammatory agent (Fournand *et al*. 1998).

Some of hydroxamic acids (mid-chain or long-chain hydroxamic acids and polymerizable unsaturated hydroxamic acids) are also used in waste water treatments because of having metal chelating properties, which help to eliminate metal contaminating metal ions.

Product of Amide (Hydroxamic acids)	**Applications**	**References**
α-aminohydroxamic acid derivatives	Involves in tissue remodelling	Fournand *et al*. (1998)
N-formyl hydroxylamine BB-3497	Antibacterial Applications	Muri *et al.* (2002)
Idrapril	Render cardio protective effects	Muri *et al.* (2002)
Fatty hydroxamic acids	Anti inflammatory -To treat chronic Asthma	Hamer *et al.* (1996)
Acrylic acid	Commercial commodity Chemical	Nawaz *et al.* (1991)
2-arylpropionic acid (Profens)	Non-steroidal antiinflammatory drugs	Bianchi *et al.* (1991)

Table 1: Applications of hydroxamic acids in pharmaceutical industry (Sharma M. *et al.* 2009)

Chapter 4: -

MATERIALS AND METHODOLOGY

4.1. Sample Collection: -

The soil sample was collected into sterile vials from "Hot Springs of Manikaran, Himachal Pradesh; India". Sample was kept at 4ºC till further processing.

4.2 Isolation of thermophilic nitrile and amide hydrolyzing bacteria: -

To 50ml of minimal basal media (KH_2PO4 2.0gm, NaCl 1.0gm, $MgSO_4.7H_2O$ 0.2gm, $FeSO_4.7H_2O$ 30mg (per litre) and pH 7.0) 100 µl acetonitrile was added as a sole source of carbon and nitrogen. For inoculation of medium, 1gm of soil was dissolved in 50ml water. 5ml of suspension (after sedimentation) was added to above minimal basal media. Flask was incubated at 37ºC, 160 rpm for 48 hours. After 48 hours 5ml of the enriched medium was again inoculated in fresh minimal basal medium containing 100 µl acetonitrile and incubated again at 37ºC, 160 rpm for 48 hours (Cramp *et al*.1997).

Cell suspension from the broth medium was diluted in saline and 50µl of diluted samples were plated on nutrient agar plates [Peptone 1%, Yeast extract 1%, NaCl 0.5%, Glucose 2%, and Agar 3% (per litre)] and incubated at 37ºC for 24 hours. Plates were screened for bacterial growth and isolated colonies were again streaked on fresh prepared nutrient agar plates and incubated at 37ºC for 48 hours the isolated bacterial strains were characterized by Gram's staining method.

4.3 Screening of bacterial isolates for amidase activity

Bacterial isolates were screened for amidase activity as per procedure described in subsequent sections.

4.4 Production of amide hydrolyzing enzyme

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) (Langdahl *et al.* 1996) was prepared, 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.

4.5 Amidase assay: -

In 1ml of reaction mixture 100µl cell suspension, 200µl inducer and 700µ phosphate buffer 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 (Fawcett and Scott 1960). One unit of amidase activity was defined as amount of enzyme (cells/protein) that hydrolyzed the amide to release 1 µmole of NH_3 released min^{-1} under assay conditions. The standard curve was prepared using 0.02–0.2 µ mole of NH_4Cl/ml in buffer: 0.1M HCl (1:1) mixture.

4.6 Acyl transfer assay: -

Acyl transfer activity of amidase was determined by taking 1 ml of reaction mixture containing 50 µl cell suspension, 0.1M acetamide, 0.2 M hydroxylamine HCl and 0.1 M phosphate buffer pH 7.0. Reaction mixture was incubated at 37˚C for 5 minutes. In control cell suspension was obviated, which was added after stopping the reaction. Reaction was stopped by adding $FeCl_3$ solution (2.1 ml of $FeCl_3$ (27.5% w/v) and 0.53 ml of 12.5% HCl). Red/brown color complex formed due to reaction of iron with hydroxamic acid. Absorbance was taken at 540nm.

4.7 Characterization of selected strains: -

Since one isolate MNB1 was found to have amidase activity amongst all the isolates, for colony characterization Gram's staining was performed. Therefore, that strain was selected for further procedure.

4.8 Optimization of production of amidase producing *Bacillus* strain MNB1: -

To increase the biomass as well as the amidase enzyme activity of MNB1 different growth and activity affecting parameters were optimized.

4.8.1 Effect of inducer: -

Different nitriles and amides (acetonitrile, acrylonitrile, benzonitrile, acrylamide and acetamide) were used as inducer to find the maximal amidase induction. Medium with nitriles and amides inoculated with the inoculum. Inoculated media were incubated in incubator for 48 hours at 37˚C. Enzyme activity was determined for each inducer. The amidase activity was assayed by using five substrates acetonitrile, acrylamide, acetamide, acrylonitrile and benzonitrile.

4.8.2 Selection of suitable production medium for amidase of *Bacillus* sp. MNB1: -

To select the suitable media for high biomass production and high amidase activity in *Bacillus* sp. MNB1 six different media were freshly prepared (Table. 2). These media were inoculated with 10% v/v seed culture and induced with 50mM acetamide and incubated at 37˚C, 160 rpm for 48 hours incubation period.

Table 2: - Composition of production media for amidase production in *Bacillus* sp. MNB1: -

M1	KH_2PO4 0.5gm, K_2HPO_4 0.5gm, glycerol 10.0gm, yeast extract 1.0gm and peptone (per litre) (Kobayashi *et al.*1993)
M2	Malt extract 3.0 gm, yeast extract, peptone 5.0gm and glycerol 10.0gm (per litre) (Kobayashi *et al.*1989)
M3	Yeast extract 15.0 gm, tryptone 30.0gm and NaCl 5.0gm (per litre) (Piotraschke *et al.* 1994)
M4	Glucose 15.0gm, malt extract 3.0gm, yeast extract 3.0gm and peptone 5.0gm (per litre) (Watanabe *et al.* 1987)
M5	Glucose 2.0gm, NaCl 5.0gm, yeast extract 15.0gm and tryptone 30.0gm (per litre) (Piotraschke *et al.*1994)
M6	KH_2PO_4 3.4gm, K_2HPO_4 4.3gm, $CaCl_2$ 0.005gm, $MgSO_4.7H_2O$ 0.5gm, yeast extract 2.5gm, glucose 10gm, acetamide 10.0gm and 0.5 ml. of trace element solution containing $ZnSO_4.7H2O$ 0.5mg, $FeSO_4.7H_2O$ 0.8, $CuSO_4.5H_2O$ 0.8mg, $MnSO_4.7H_2O$ 0.6mg (per litre, pH 7.5) (Chacko *et al.* 2012)

4.8.3 Effect of pH on the production of amidase of *Bacillus* sp. MNB1: -

To determine the optimal pH for the growth of amiadse of MNB1, M2 media having different pH ranges (pH 3.0-10.0) were prepared and supplemented with 50mM acetamide. M2 media of different pH were inoculated with 5ml of 24 hours old inoculum. Inoculated flasks were incubated at 37˚C, 160 rpm for 48 hours. After 48 hours enzyme assay was performed.

4.8.4 Effect of incubation temperature on production of amidase *Bacillus* sp. MNB1: -

M2 medium (pH 7.0) having 50mM acetamide, was inoculated with 10% v/v of inoculum and incubated at different temperature range (30-60˚C), at 160 rpm for 48 hours. Enzyme activity was observed at temperature range (30-60˚C).

4.8.5 Effect of concentration of inducer (acetamide) for amidase production: -

Effect of inducer (acetamide) concentration was determined for maximal production and amidase activity. Different acetamide concentrations (10 - 100mM) were inoculated in previous optimized medium and incubated at 37˚C, 160 rpm for 48 hours.

4.8.6 Effect of incubation period (time course) on production of *Bacillus* sp. MNB1: -

To determine the time in which amidase of MNB1 was induced by the inducer in the medium, 5 ml of seed culture was transferred in M2 medium (pH 7.0) with 50 mM acetamide and incubated at 37˚C, 160 rpm. After every 4 hours of incubation, cells were taken and subjected for enzyme assay.

4.9 Optimization of reaction conditions for assay of amidase activity of *Bacillus* sp. MNB1 strain: -

4.9.1 Buffer pH: -

Amidase assay was performed in different buffer systems, having different pH ranges 3.0 to 11.0.

4.9.2 Incubation temperature: -

Enzyme assay was performed at different temperatures ranging from 10-60^{o}C to find out the optimum temperature of amidase activity.

4.9.3 Substrate Concentration: -

Different concentrations of acetamide were used ranging from 10mM to 100mM in potassium phosphate buffer (pH 7.0) to study inhibitory effect of substrate concentration on amidase activity.

4.9.4 Effect of incubation time on the amidase activity: -

Enzyme activity was taken after different incubation periods (5-60 minutes) to determine the most suitable incubation time for maximal amidase activity.

4.9.5 Stability of amidase of *Bacillus* sp. MNB1: -

Reactions were carried for different incubation period (30 minutes, 1 hour, 2 hours, 3 hours, 4 hours) at 37˚C to check the time at which enzyme loss its activity.

4.10 Protein profiling of amidase of *Bacillus* sp. MNB1: -

MNB1 was cultured in different media (table 2) induced with 50mM acetamide and incubated in orbital shaker at 160 rpm, 37˚C for 48 hours. Cells were harvested after 48 hours at 10,000 x g for 10 minutes and sonicated. Crude cell free extract was run on SDS-PAGE to get to see the expression of protein in different media (Table 2).

4.10.1 Homogenization of cells: -

Strain was grown in optimized production conditions and after centrifugation, cells were washed 2-3 times with the 0.1 M phosphate buffer (pH 7.0) and resuspended in the same buffer containing 1mM DTT and 1mM EDTA. Cells were disrupted by using 20 cycles of sonication (1 minute of beating and 1 minute of resting) in Hilscher UP200S ultrasonicator processor. After sonication homogenate was centrifuged at 10,000 x g for 15 minutes, to separate the protein extract from the cell pellets. Amidase presence in supernatant and cell pellet was determined by using enzyme assay. Lowry's method was used for estimation of protein concentration in supernatant.

4.10.2 Ammonium sulphate precipitation for purification of proteins: -

To precipitate out the proteins ammonium sulphate precipitation was done. Ammonium sulphate was dissolved in initial concentration of 10% of total volume of extract and kept overnight at 4˚C. 10% - 60% cuts of ammonium sulphate were given. After every cut of ammonium sulphate protein precipitate was washed with 0.1 M phosphate buffer (pH 7.0) and resuspended in same buffer.

4.10.3 Anion-Exchange (DEAE-Cellulose) Chromatography: -

DEAE-Cellulose chromatography was further used to purify the protein. DEAE-Cellulose resin was activated by treating it with 0.5 N HCl and 0.5 NaOH. Resin was packed into the column and washed with 0.1 M phosphate buffer (pH 7.0). Protein sample of 30% cut of ammonium

sulphate was loaded on to the column. After loading of protein sample column was washed with 0.1M phosphate buffer and elute was collected as wash out. Then the protein is eluted out by using NaCl solution ranges from 0.1 M-1.0 M. Fractions were collected and enzyme assay was performed.

4.10.4 SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis): -

Required stock solutions-

(a) 1 M Tris HCl (pH 6.8); 100 ml: -

12.1gm Tris

HCl to adjust the pH

Added distilled water to make up the volume.

(b) 2 M Tris HCl (pH 8.8); 100 ml: -

24.2gm Tris

HCl to adjust the pH 8.8

Distilled water was added to make up the volume.

(c) 10% (w/v) SDS; 100 ml

(d) 1% (w/v) glycerol; 100 ml

(e) 1% (w/v) bromophenol blue; 10 ml

(f) Electrophoretic buffer (1000 ml): -

3.0gm Tris

14.4gm Glycine

Volume was made up to 1000 ml with distilled water.

Working solutions: -

Solution A- 30% w/v Acrylamide stock solution (29.2gm acrylamide and 0.8gm bis-acrylamide; 100 ml)

Solution B- 4x separating gel buffer (2M Tris-HCl (pH 8.8) 75 ml, 10% SDS 4ml, distilled water 21ml; 100 ml)

Solution C- 4x stacking gel buffer (1M Tris HCl (pH 6.8) 50 ml, 10% SDS, distilled water 46 ml; 100 ml)

10% ammonium persulphate

12.5% separaritng gel: -

Solution A	7.5ml
Solution B	4.5ml
10% ammonium persulphate	0.07 ml
TEMED	0.01 ml
Distilled water	6.0 ml
Total volume	18.08 ml

8% stacking gel: -

Solution A	0.9 ml
Solution C	1.5 ml
10% ammonium persulphate	0.018 ml
TEMED	0.01 ml
Distilled water	3.6 ml
Total volume	6.028 ml

For SDS-PAGE first 12.5% separaritng gel was prepared and left for polymerization of the gel and 8% stacking gel was prepared (Bollag and Stuart, 1991). After polymerization of the gel 25 µl protein samples with 5µl of 5x sample buffer (1M Tris-HCl (pH 6.8) 0.6 ml, 50% glycerol 5ml, 2-mercaptoethanol 0.5 ml, 10% SDS 2ml, 1% bromophenol blue 1 ml, distilled water 0.9 ml) were loaded in the wells and allow to run the samples at 100 mV.

4.10.5 Native PAGE (Native Polyacrylamide gel electrophoresis): -

Stock solutions:-

Native PAGE of protein sample was done for the further process of zymography. 10% separating gel and 8% stacking gel was used. First stock solutions were made (Bollag and Stuart, 1991).

Solution A- 30% acrylamide stock solution (29.8gm acrylamide and 0.8gm bis-acrylamide)

Solution B- 4x separating buffer (1.5 M Tris HCl (pH 8.8) 18.2gm in 100 ml distilled water)

Solution C- 4x stacking buffer (0.5 M Tris HCl (pH 6.8) 6.0gm in 100 ml distilled water)

10% separating gel: -

Solution A	3.4 ml
Solution B	2.5ml
10% ammonium persulphate	50 µl
TEMED	10µl
Distilled water	4.17 ml
Total volume	10.130 ml

5% Stacking gel: -

Solution A	0.67 ml
Solution C	1.0 ml
10% ammonium per sulphate	30 µl
TEMED	5 µl
Distilled water	2.3 ml
Total volume	3.005 ml

10% Separating gel was allowed to set between the plates and then 5% stacking gel solution was poured on the separating gel and comb was fixed on the stacking gel. After polymerization of the gel comb was removed and 25 µl protein samples and 5x sample loading buffer (1M Tris-HCl (pH 6.8) 3.1 ml, glycerol 5ml, 1% bromophenol blue 0.5 ml, distilled water 0.9 ml) without heat treatment, were loaded in the wells. Electrophoresis was carried out at 4˚C and 100 mV.

4.10.6 Zymography: -

After native PAGE gel was washed 2-3 times with 0.1 M phosphate buffer pH 7.0, and incubated in phosphate buffer containing 0.07 M acetamide and 0.7 M hydroxylamine HCl, at 37˚C for 30 minutes. After incubation gel was washed with distilled water. Acyl transfer activity was measured by pouring $FeCl_3$ solution onto the gel. Iron reacts with hydroxamic acid and developed the red brown bands and gel was observed to see the presence of red/brown band.

Chapter 5: -

RESULTS AND DISCUSSION

5.1 Isolation and screening of mesophilic bacterial isolates for amide activity: -

Soil samples were taken from hot springs of Manikaran, Himachal Pradesh, India. Amidase producing microorganisms were isolated by enrichment in nitriles and amides. Acetonitrile was added in defined basal medium and incubated at 50^{o}C and 37^{o}C, 160 rpm. Streak plating was done to isolate single pure strain. Five microorganisms were isolated and MNB1 (gram positive *Bacillus* strain) showed highest activity. This strain develops greenish pigment in DE (defined minimal medium) medium after 48 hours of incubation (Fig 3). Kotlava *et al*. (1998), Sogani *et al* (2011), Bhatia *et al.* (2013) and Thalenfeld *et al* (1975) used acetamide for enrichment and isolated *Rhodococcus rhodochrous*, *Bacillus* sp. strain, *Alcaligenes* sp. MTCC 10674, thermophilic *Bacillus* strain.

Figure 3*: Bacillus* sp. MNB1 grown in different production media

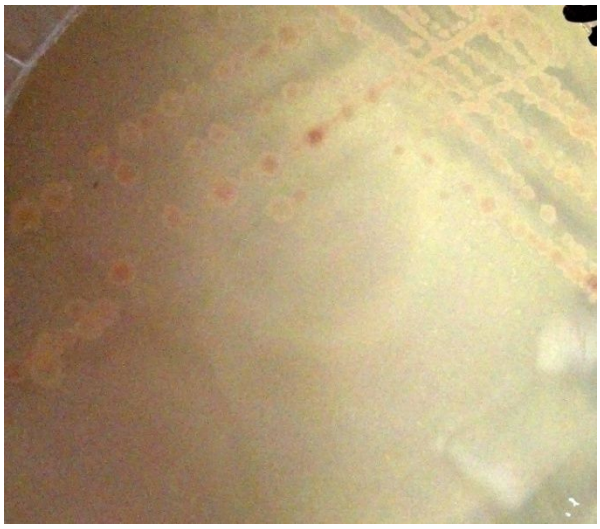


Figure 4: Colony morphology of *Bacillus* sp. MNB1 on nutrient agar

Strain was isolated for amidase activity by enrichment (using acetamide) and exhibited amidase activity. Isolate was named as MNB1 from Manikaran hot spring soil sample and found to have an inducible amidase (Fig. 4). Highest enzyme activity of amidase of MNB1 was observed in acetamide induced medium in comparision to acrylamide, acetonitrile, benzonitrile and acrylonitrile. Acetamide was found best substrate as well as inducer of amidase of MNB1. Isolate was detected as Gram positive bacteria (Fig. 5). Amidase of *Bacillus* sp. showed acyl transfer activity in acyl transferase assay.

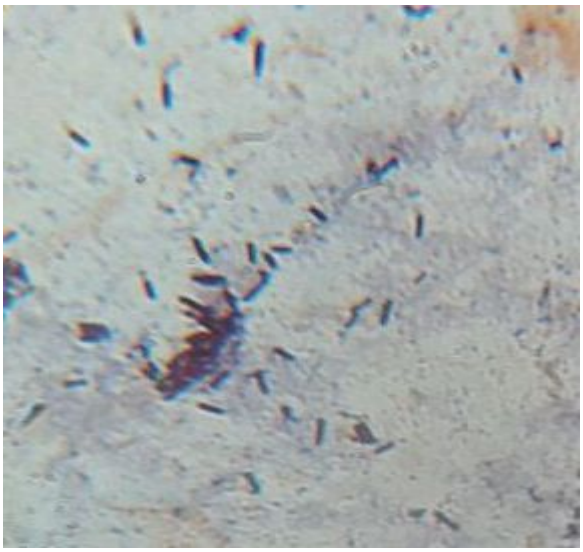


Figure 5: Gram staining of *Bacillus* sp.MNB1

5.2 Optimization of culture conditions of amidase of *Bacillus* sp. MNB1: -

5.2.1 Effect of inducer: -

Amidase of *Bacillus* sp. MNB1 showed maximum enzyme activity in acetamide induced medium and acetamide also found the best substrate amongst different substrates. Production of *Pseudomonas putida* MTCC 6809 was carried out in M6 (table 2) and acetamide was found to be suitable inducer (Chacko *et al.* 2012). *Bacillus* sp. (Thalenfield *et al.* 1976), *Pseudomonas aeruginosa* (Brammer *et al.* 1964), *Methylophilus methylotrophus* (Silman *et al*. 1989) also reported acetamide as inducer for amidase production.

5.2.2 Selection of production medium for amidase of *Bacillus* sp. MNB1: -

Bacillus sp. MNB1 was grown in six different media M1, M2, M3, M4, M5 and M6. Highest specific activity of amidase was observed in M2 medium 0.0095µmole/min/mg and total activity was 3.160 U/mg dcw (Fig. 6). Although higher biomass was achieved in M1 medium but total activity (0.470 U/mg dcw) was less in comparison to M2 medium. Hence it showed glycerol, malt extract, yeast extract and peptone enhances the amidase activity as well as biomass of MNB1. Kobayashi *et al.* (1989) reported nitrilase maximum production of *Rhodococcus rhodochrous* J1 in medium 2 (Table 2).

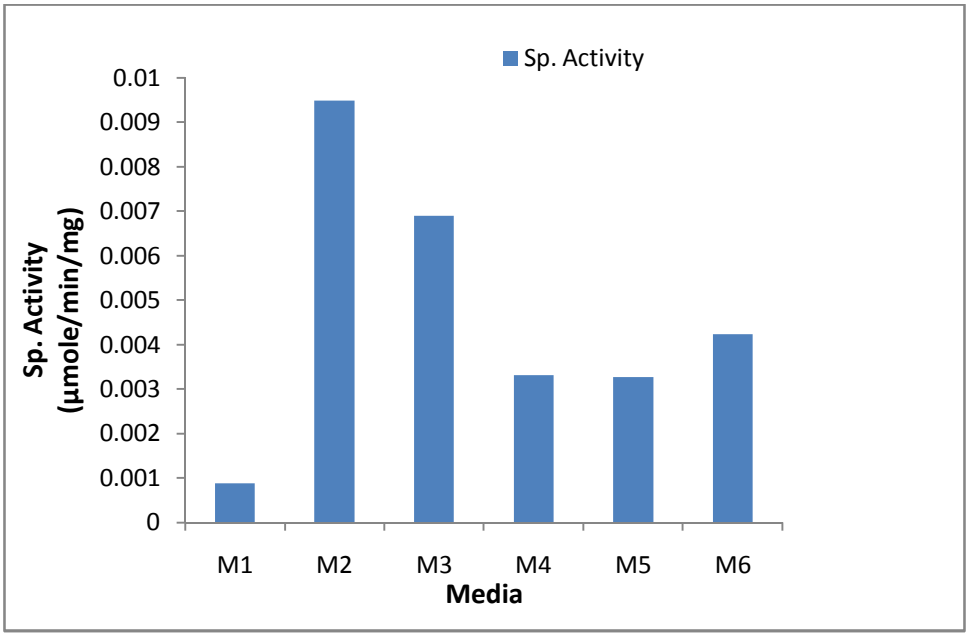


Fig 6: Production of *Bacllus* sp. MNB1 in different media (Whole cells) (Appendix)

5.2.3 Determination of optimum pH for production of amidase of *Bacillus* sp. MNB1: -

The pH of M2 medium was varied in range of pH 4.0-10.0 and used for culturing of *Bacillus* sp. MNB1 for high amidase production. Amidase was active within the range of pH 6.0-8.0. At pH 7.0 maximal amidase total activity (1.588 U/mg dcw) was observed. After pH 8.0 activity was decreased drastically (Fig. 7). 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).

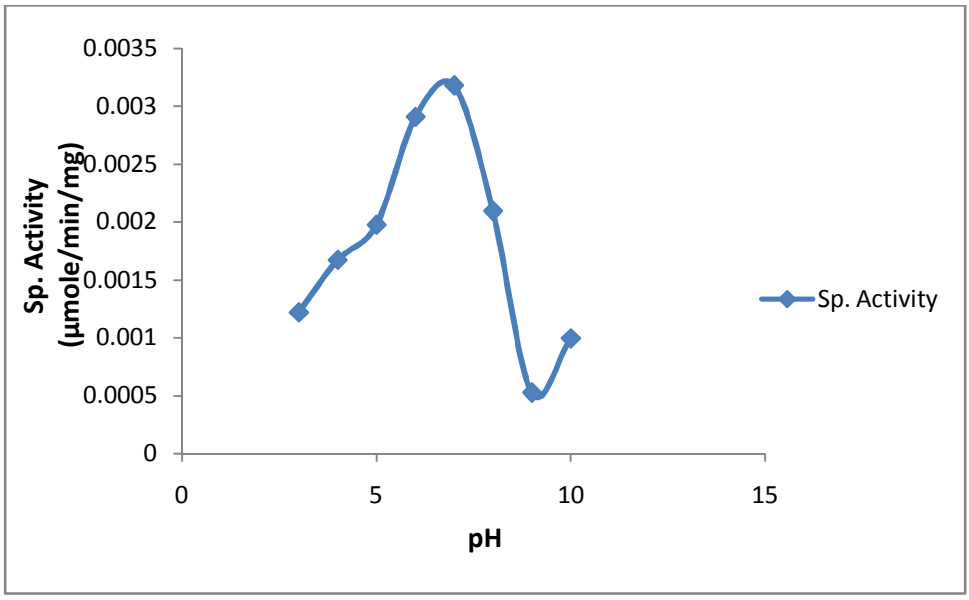


Fig 7: Effect of different pH on the growth *Bacillus* sp. of MNB1 (Whole cells) (Appendix)

5.2.4 Optimum incubation temperature for amidase production: -

Optimum temperature for amidase production in MNB1 was observed at 37^{o}C with maximal total activity of 0.0054 U/mg dcw (Fig. 8). *Mycobacterium neoaurum* ATCC 25795 (Hermes *et al.* 1994), *Pseudomonas aeruginosa* 86021A (Brammer e*t al.* 1964) also reported optimum incubation temperature to be 37^{o}C. Many organisms exhibited maximum amidase production at 30^{o}C (Sharma *et al.* 2009, Abada *et al.* 2014).

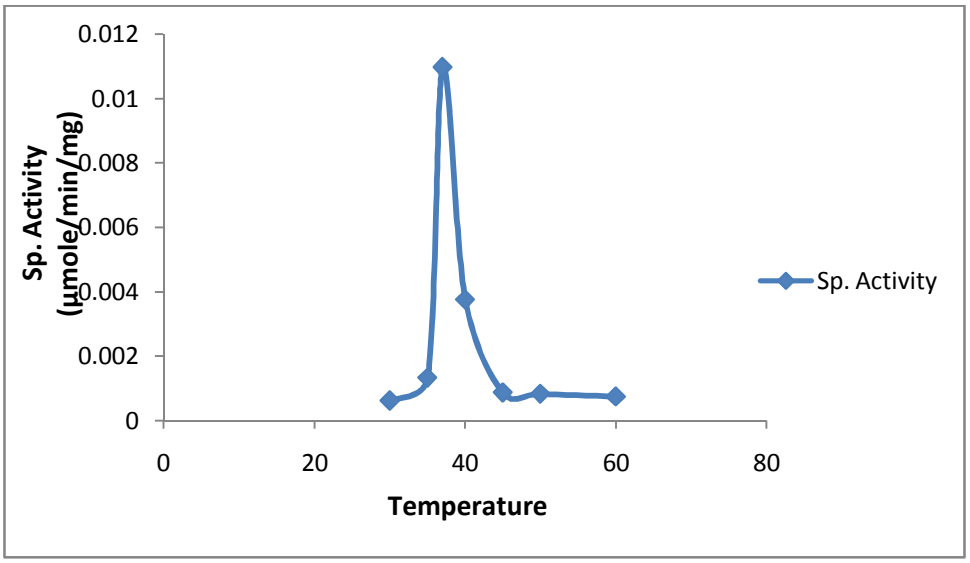


Fig 8: Effect of temperature on the production of amidase of *Bacillus* sp. MNB1 (whole cells) (Appendix)

5.2.5 Effect of inducer concentration: -

Highest total activity (4.6264 U/mg dcw) was observed in the medium containing 50mM acetamide (Fig.9). Although growth in 80mM acetamide containing medium was high in comparison to 50mM acetamide supplemented medium but total activity (4.1186 U/mg dcw) was low.

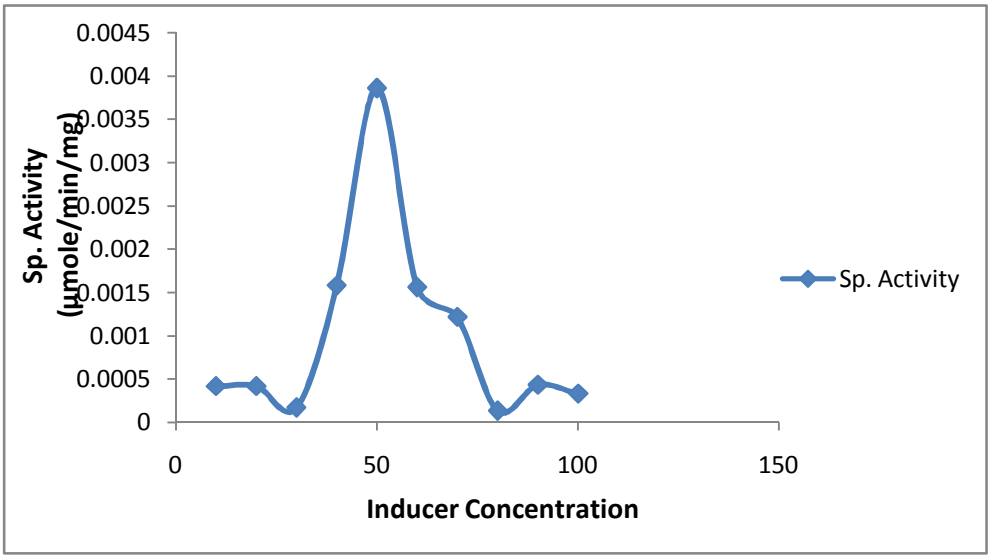


Fig 9: Effect of Inducer concentration on the production of amidase of *Bacillus* sp. MNB1 (whole cells) (Appendix)

5.2.6 Time course: -

MNB1 was incubated for varied time period. Samples were withdrawn after every 4 hours interval. Amidase highest total activity (0.0021 U/mg dcw) was observed in 48 hours old culture (Fig. 10). After 48 hours amidase activity was decreased drastically. *Xanthomonas agilis* NRRL21115 (Black *et al.* 1996), *Xanthomonas maltophila* ATCC 21764 (Bouvrette *et al.* 1991), *Klebsiella pneumoniae* NCTR1 (Nawaz *et al*. 1996), *Mycobacterium neoaurum* ATCC 25795 (Hernes *et al.* 1994), *Rhodococcus rhodochrous* J1 (Kobayashi *et al.* 1993a) and *Stenotrophomonas maltophilia* (Stelkes *et al.* 1995) also showed maximum amidase induction after 48 hours of incubation.

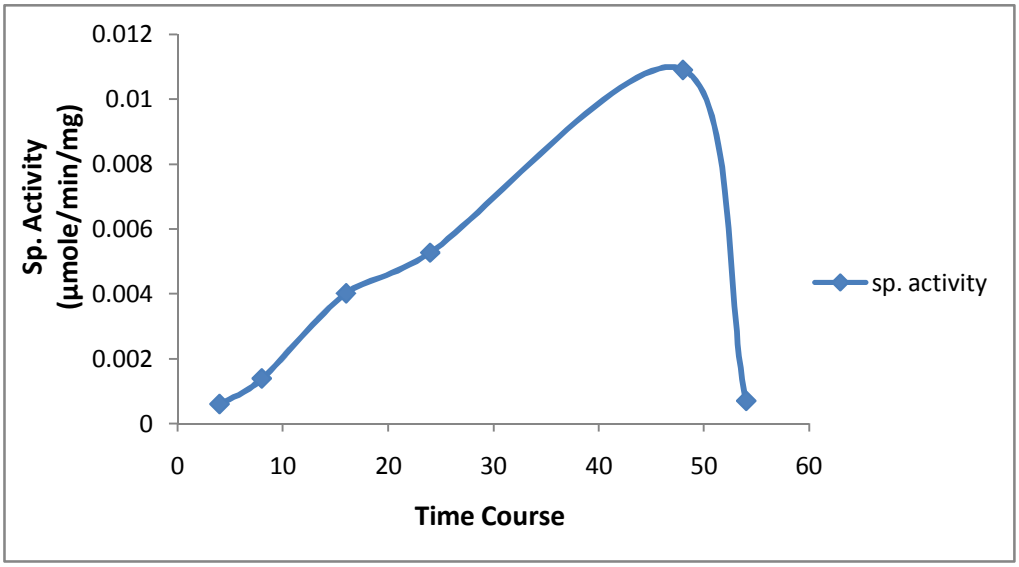


Fig 10: Effect of Incubation period on the production of amidase of *Bacillus* sp. MNB1 (Whole cells) (Appendix)

5.3 Optimization of reaction conditions for amidase activity of *Bacillus* sp. MNB1: -

5.3.1 Effect of Buffer pH: -

Amidase of MNB1 showed highest specific activity (0.090µmole/min/mg) at pH 7.0 (Fig.11). In majority of microorganisms amidase have optimum activity in the range of neutral pH, like *Geobacillus pallidus* RAPc8 (Makhongela *et al.* 2007), *Klebsiella pneumoniae* NCTR1 (Nawaz *et al.* 1996), *Geobacillus pallidus* BTP-5x (Sharma M. 2009), *MycobactKerium* ATCC 25795

activity in pH 7.0 to 9.5 (Hermes *et al.* 1994)**.** Beak *et al* (2003) reported two amidases from *Brevibacillus borstelensis* BCS-1, which exhibited maximum activity at pH 9.0 and 9.5 respectively.

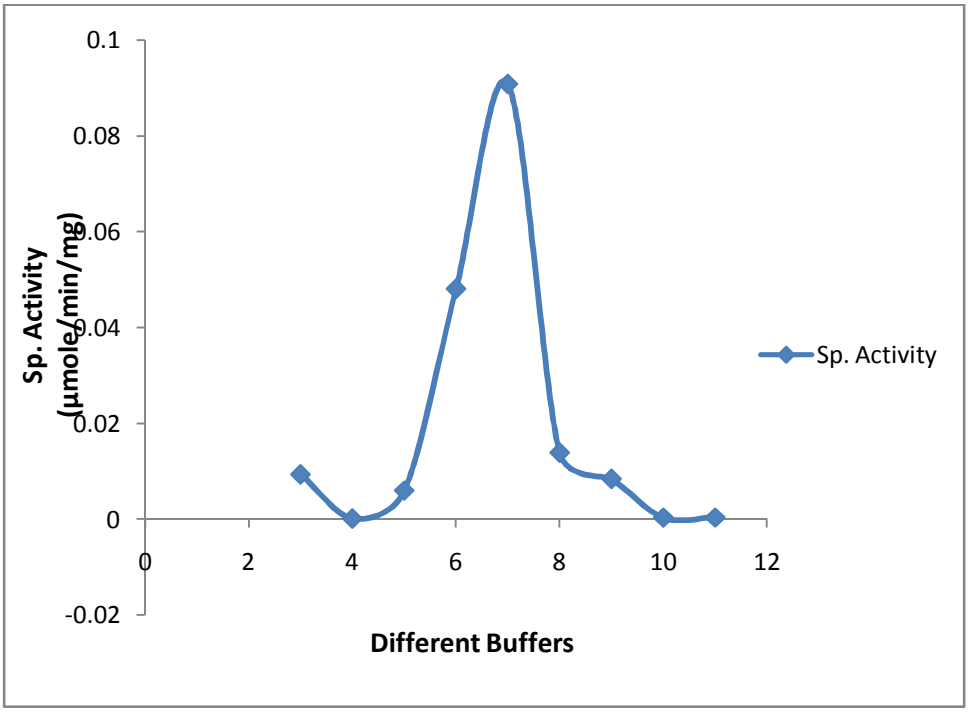


Fig 11: Effect of Buffer pH on amidase activity of *Bacillus* sp. MNB1 (whole cells) (Appendix)

5.3.2 Effect of Incubation temperature: -

Optimum reaction temperature for amidase specific acitivity of MNB1 was observed (0.0274µmole/min/mg) at 37^{o}C. Amidase activity gradually decreased above these optimum temperatures due to deanaturation of amidase enzyme (Fig. 12). Optimum reaction temperature for amidase activity of *Klebsiella pneumoniae* was reported at 30-40^{o}C. *Mycobcterium neoaurum* ATCC MP50 (Hirrlinger *et al*. 1996) and amidase from *Geobacillus pallidus* RAPc8 exhibited at 50^{o}C (Makhongela *et al.* 2007) and *G. Pallidus* BTP-5x exhibited maximum avtivity at 55^{o}C (Sharma M. *et al*. 2012).

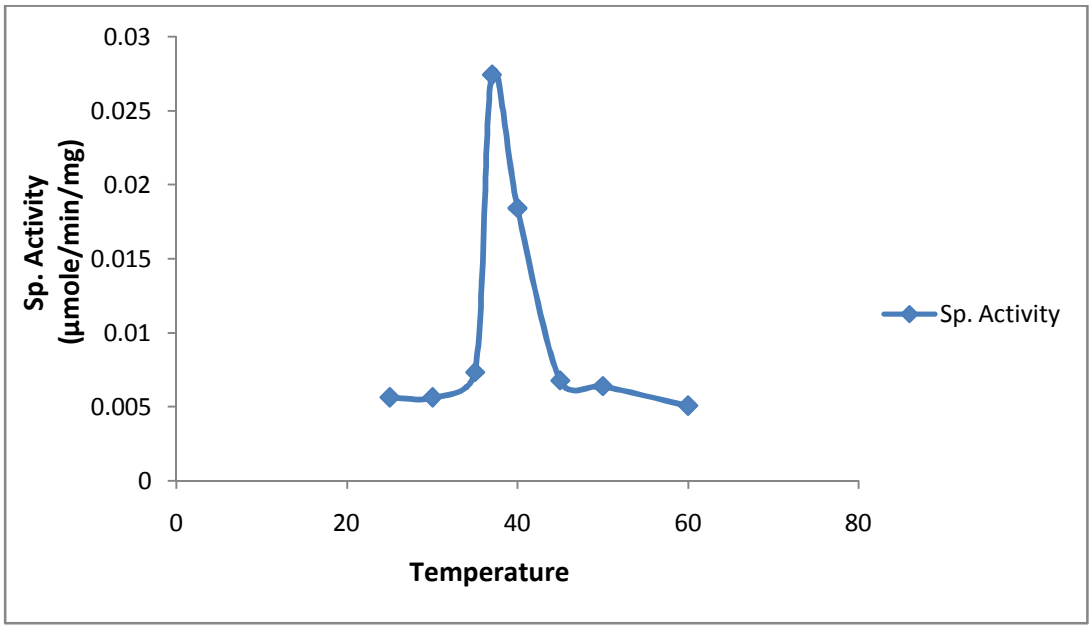


Fig 12: Effect of Reaction Temperature on amidase activity of *Bacillus* sp. MNB1 (whole cells) (Appendix)

5.3.3 Effect of Substrate concentration: -

Effect of substrate concentrations on amidase activity of MNB1 grown in M2 production medium was observed. Acetamide was used as a substrate in concentration ranging from 10mM to 100mM. Maximum amidase specific activity of MNB1 (0.0398µmole/min/mg) was observed at 50mM acetamide concentration (Fig. 13). Amidase of MNB1 exhibited substrate inhibition above 50mM acetamide concentration because of the saturation of enzyme active site. Recombinant amidase of *Bacillus stearothermophilus* displayed optimal activity with 120mM acrylamide and substrate inhibition was observed at higher concentration of acrylamide (Cheong *et al.* 2000).

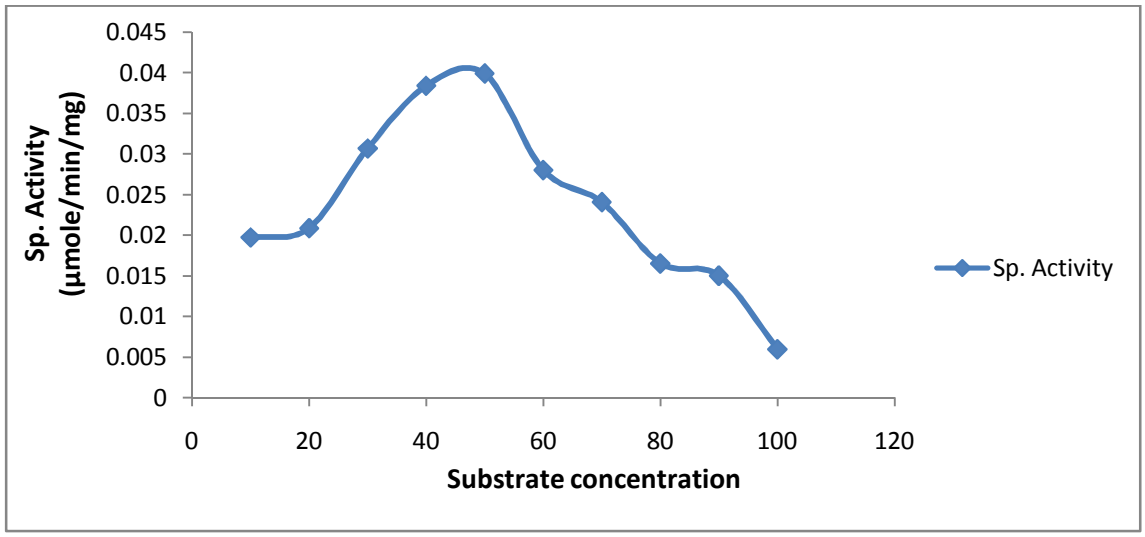


Fig 13: Effect of substrate concentration on amidase activity of *Bacillus* sp. MNB1 (whole cells) (Appendix)

5.3.4 Effect of Incubation period: -

Amidase of MNB1 showed the maximum specific activity 0.218µmole/min/mg for 15 minutes of incubation time (Fig. 14).

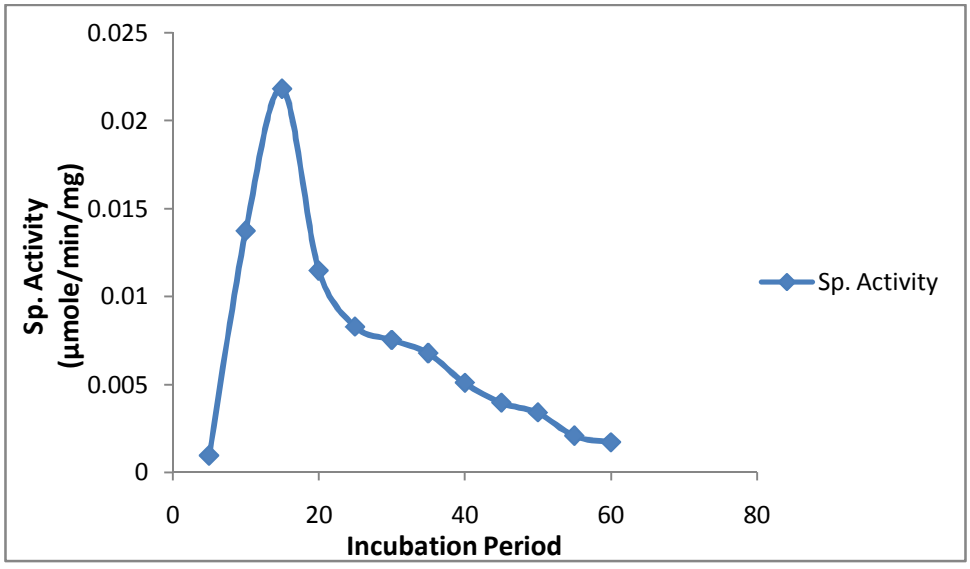


Fig 14: Effect of Incubation period on the amidase activity of *Bacillus* sp. MNB1 (whole cells) (Appendix)

5.3.5 Stability of amidase of *Bacillus* sp. MNB1: -

Incubation period for amidase activity reaction was increased from 30 minutes to 4 hours at 37°C. Specific activity (0.129µmole/min/mg) was observed to decrease after 30 minutes (Fig. 15). Amidase of MNB1 was found quite stable at 30 minutes but after that it becomes inactive for the substrate. The amidase of *Rhodococcus rhodochrous* J1 was reported to be quite stable when incubated at 30°C for 30 min but after that it lost amidase activity (Kobayashi *et al.* 1993a).

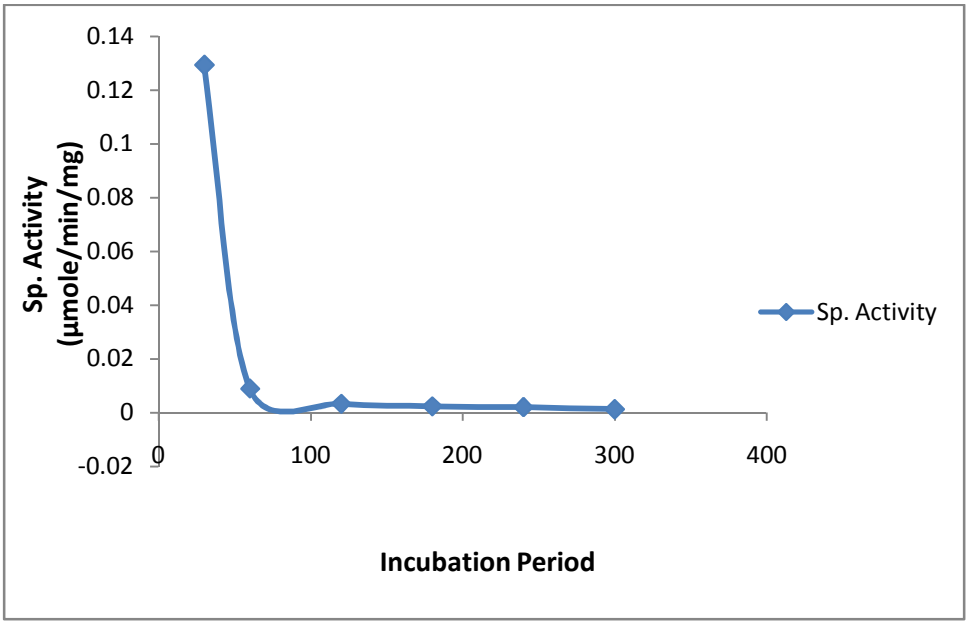


Fig 15: Effect of Incubation period on the stability of amidase of *Bacillus* sp. MNB1 (whole cells) (Appendix)

5.4 Protein profiling of *Bacillus* sp. MNB1: -

5.4.1 Sonication of cells: - Bacillus sp. MNB1 grown in different production media (M1-M6 table 2) at 37°C and further sonicated with 20 cycles of sonication (1 minute beating and 1 minute rest). The samples were further run on SDS-PAGE to see the protein expression profile. Medium 6 (M6) exhibited maximum expression of protein and maximum activity was also observed in M6 medium.

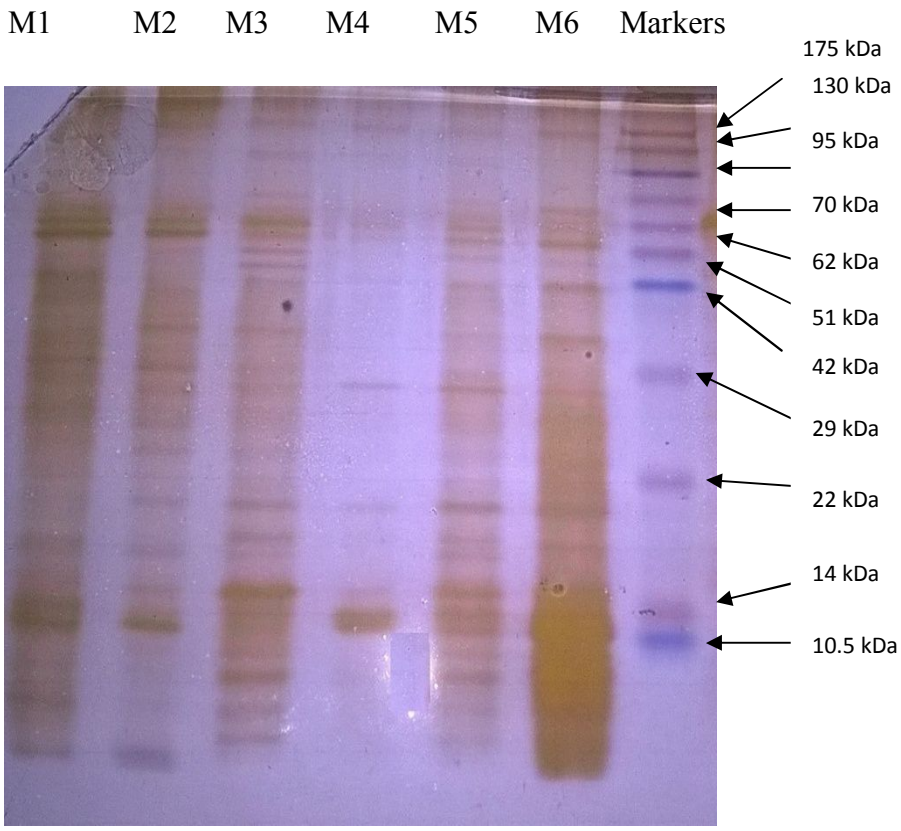


Fig 16: SDS-PAGE of crude extract of *Bacillus* sp. MNB1 grown in different production media (Table 2)

5.4.2 Ammonium salt precipitation of protein: -

Ammonium salt precipitation of cell free extract was done and highest protein concentration was observed in 10% cut of ammonium sulphate (0.36mg/ml) but high amidase activity was found in the cuts of 30-50% ammonium sulphate.

5.4.3 Anion exchange (DEAE-cellulose) chromatography: -

30% fraction of ammonium sulphate precipitation was loaded onto DEAE column and washed with two volumes of column and further eluted with NaCl gradient 0.1M-1.0M. The fractions of 0.1M-0.3M NaCl showed maximum activity. Hence, it is concluded that amidase of *Bacillus* sp. MNB1 is weak negatively charged protein.

5.4.4 Zymography: - Native gel was run and subjected for zymography to detect acyl transfer activity of amidase of MNB1. After iron chloride solution treatment, iron reacts with hydroxamic

acid and develops red/brown bands. The appearance of red/brown bands confirms acyl transfer activity in the amidase sample, which is the second signature property of amidase.

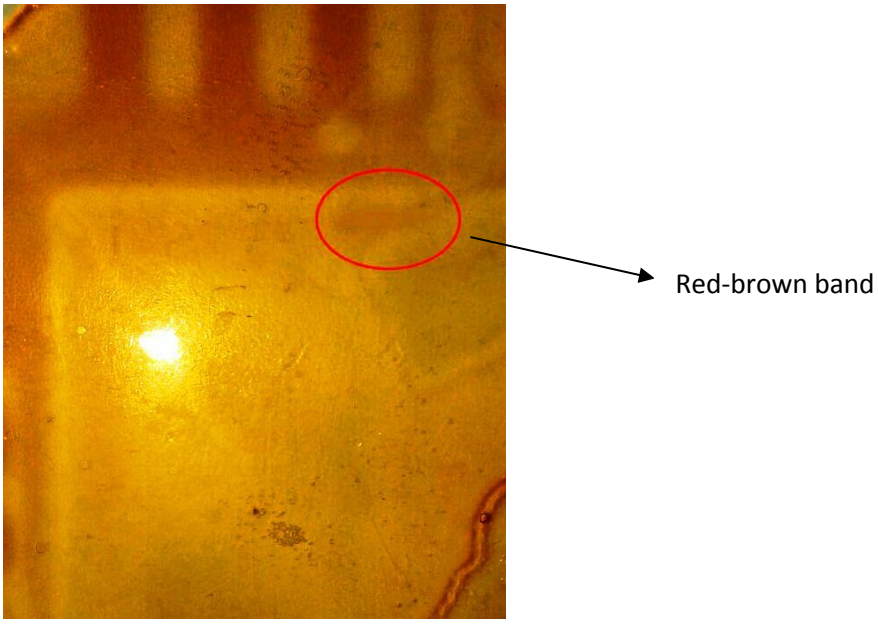


Fig 17: Zymography of Native PAGE gel

Chapter 6: -

CONCLUSION

Amidase is widely distributed in nature in plants, microorganisms, fungi, yeasts and animals and find wide applications in various industries and pharmaceutical companies. In the present report, an inducible amidase producing *Bacillus* sp. Strain was isolated from hot spring of Manikaran, Himachal Pradesh. *Bacillus* sp. MNB1 exhibited amide degrading activity and have acyl transfer activity for acetamide.

Optimization of culture conditions and enzyme assay condition led to increase the biomass and amidase activity. Bacillus sp. MNB1 showed maximum growth and activity in M2 medium, pH 7.0, temperature 37^{o}C in the presence of 50mM acetamide as inducer. MNB1 found active at pH 7.0, 37^{o}C and 50mM acetamide concentration. Amidase of *Bacillus* sp. MNB1 also has acyl transferring ability to produce hydroxamic acids. Amidase purification can be further subjected to different chromatographic separation to get purified protein.

Chapter 7: -

FUTURE PERSPECTIVE

In this research work acetamidase from gram positive *Bacillus* sp. MNB1 was isolated and culture and reaction conditions were optimized. Further work can be done:-

- Production optimization of *Bacillus* sp. MNB1 at bioreactor level and amidase purification by using different bioseparation techniques can be undertaken.
- Purified proteins can be characterized for its N-terminal sequence and this information will be further used for primer design for cloning experiments and over expression of gene in recombinant host.

Chapter 8: -

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Chapter 9: -

APPENDIX

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

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

9.3 LB (Luria-Bertani) Medium (1000ml), pH-7.0

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

9.4 Solution A (1000ml)

Phenol	1%
Sodium Nitroprusside	0.005%

9.5 Solution B (1000ml)

Sodium Hydroxide	0.5%
Sodium Hypochlorite	0.02 N

9.6 Optimization of culture conditions of *Bacillus* sp. MNB1

9.6.1 Selection of suitable production medium for amidase of *Bacillus* sp. MNB1: -

S.NO.	Different Media	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)* 2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50* mg added
1	M1	0.0141	0.0009	10.7	1.07	0.0008	0.470
2	M2	0.1083	0.0072	7.6	0.76	0.0095	3.610
3	M3	0.0682	0.0045	6.6	0.66	0.0068	2.275
4	M4	0.0287	0.0019	5.8	0.58	0.0033	0.959
5	M5	0.0225	0.0015	4.6	0.46	0.0032	0.752
6	M6	0.03554	0.0023	5.6	0.56	0.0042	1.184

9.6.2 Determination of optimum pH for production of amidase of *Bacillus* sp. MNB1: -

S.NO.	Different pH	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50* mg added
1	3	0.0462	0.0030	25.3	2.53	0.0012	0.6095
2	4	0.0225	0.0015	9	0.9	0.0016	0.8358
3	5	0.0829	0.0055	28.7	2.8	0.0019	0.9873
4	6	0.1263	0.0084	29	2.9	0.0029	1.4526
5	7	0.1997	0.0133	419	4.19	0.0031	1.5889
6	8	0.0942	0.0062	30.7	3	0.0020	1.0469
7	9	0.2369	0.0015	33	3	0.0005	0.7898
8	10	0.0897	0.0059	60.2	6.02	0.0009	0.4967

9.6.3 Effect of Inducer concentration on the production of amidase of *Bacillus* sp. MNB1: -

S.NO.	Different Concentration of inducer	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50* mg added
1	10	0.0564	0.0037	90	9	0.0004	1.8806
2	20	0.0507	0.0033	81	8.1	0.0004	1.6926
3	30	0.1111	0.0074	429	42.9	0.0001	3.7049
4	40	0.0236	0.0015	289	1	0.0015	0.7898
5	50	0.0823	0.0054	24	2.4	0.0022	4.6264
6	60	0.0631	0.0042	27	2.7	0.0015	2.1063
7	70	0.1094	0.0072	60	6	0.0012	3.6484
8	80	0.1235	0.0082	607	60.7	0.0001	4.1186
9	90	0.0507	0.0033	78	7.8	0.0004	1.6926
10	100	0.0214	0.0014	43	4.3	0.0003	0.7146

9.6.4 Optimum incubation temperature for amidase production: -

S.NO.	Temperature (˚C)	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50* mg added
1	30	0.0022	0.0001	2.4	0.24	0.0006	0.0001
2	35	0.0062	0.0004	3.1	0.31	0.0013	0.0004
3	37	0.0823	0.0054	0.5	0.5	0.0109	0.0054
4	40	0.0186	0.0012	3.3	0.33	0.0037	0.0012
5	45	0.0039	0.0002	3	0.3	0.0008	0.0002
6	50	0.0033	0.0002	2.7	0.27	0.0008	0.0002
7	60	0.0022	0.0001	2	0.2	0.0007	0.0001

9.6.5 Effect of time course on production of *Bacillus* sp. MNB1 amidase: -

S.NO.	Time Course	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50* mg added
1	4	0.0078	0.0005	8.9	0.89	0.0005	0.0005
2	8	0.0248	0.0016	12	1.2	0.0013	0.0016
3	16	0.0180	0.0012	3	0.3	0.0040	0.0012
4	24	0.0078	0.0005	1	0.1	0.0052	0.0005
5	48	0.0327	0.0021	2	0.2	0.0109	0.0021
6	64	0.0062	0.0004	6	0.6	0.0006	0.0004

9.7 Optimization of the assay conditions of *Bacillus* sp. MNB1: -

9.7.1 Effect of different buffers on amidase activity of *Bacillus* sp. MNB1: -

S.NO.	Different buffers	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50 *mg added
1	3	0.02821	0.0018	2	0.2	0.0094	0.9403
2	4	0.0005	3.7613	2	0.2	0.0001	0.0188
3	5	0.0180	0.0012	2	0.2	0.0060	0.6018
4	6	0.1443	0.0096	2	0.2	0.0481	4.8107
5	7	0.2725	0.0181	2	0.2	0.0908	9.0836
6	8	0.0417	0.0027	2	0.2	0.01391	1.3916
7	9	0.0253	0.0016	2	0.2	0.0084	0.8463
8	10	0.0011	7.5226	2	0.2	0.0003	0.0376
9	11	0.0011	7.5226	2	0.2	0.0003	0.0376

9.7.2 Effect of temperature on amidase of *Bacillus* sp. MNB1: -

S.NO.	Temperature (˚C)	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50 *mg added
1	25	0.0169	0.0011	2	0.2	0.0056	0.5642
2	30	0.0169	0.0011	2	0.2	0.0056	0.5642
3	35	0.0220	0.0014	2	0.2	0.0073	0.7334
4	37	0.0823	0.0054	2	0.2	0.0274	2.7457
5	40	0.0552	0.0036	2	0.2	0.0184	1.8430
6	45	0.0203	0.0013	2	0.2	0.006	0.6770
7	50	0.0191	0.0012	2	0.2	0.0063	0.6394
8	60	0.0152	0.0010	2	0.2	0.0050	0.5077

9.7.3 Effect of substrate concentration on amidase activity of *Bacillus* sp. MNB1: -

S.NO.	substrate concentration	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50 *mg added
1	10	0.0592	0.0039	2	0.2	0.0197	1.9747
2	20	0.0626	0.0041	2	0.2	0.0208	2.0875
3	30	0.0919	0.0061	2	0.2	0.0306	3.0654
4	40	0.1150	0.0076	2	0.2	0.0383	3.8365
5	50	0.1196	0.0079	2	0.2	0.0398	3.9870
6	60	0.0840	0.0056	2	0.2	0.0280	2.8021
7	70	0.0722	0.0048	2	0.2	0.0240	2.4072
8	80	0.0496	0.0033	2	0.2	0.0165	1.6549
9	90	0.0451	0.0030	2	0.2	0.0150	1.5045
10	100	0.0180	0.0012	2	0.2	0.0060	0.6018

9.7.4 Effect of reaction incubation period of *Bacillus* sp. MNB1: -

S.NO.	Incubation Period	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50*mg added
1	5	0.0028	0.0001	2	0.2	0.0009	0.0940
2	10	0.0411	0.0027	2	0.2	0.0137	1.3728
3	15	0.0654	0.0043	2	0.2	0.0218	2.1815
4	20	0.0344	0.0022	2	0.2	0.0114	1.1472
5	25	0.0248	0.0016	2	0.2	0.0082	0.8274
6	30	0.0225	0.0015	2	0.2	0.0075	0.7522
7	35	0.0203	0.0013	2	0.2	0.0067	0.6770
8	40	0.0152	0.0010	2	0.2	0.0050	0.5077
9	45	0.0118	0.0007	2	0.2	0.0039	0.3949
10	50	0.0101	0.0006	2	0.2	0.0033	0.3385
11	55	0.0062	0.0004	2	0.2	0.0020	0.2068
12	60	0.0050	0.0003	2	0.2	0.0016	0.1692

9.7.5 Stability of amidase of *Bacillus* sp.MNB1: -

S.NO.	Incubation Period	T. Amm	Rate	dcw/ml in mg	mg added	Sp. Act.	total activity
		(T-C)*2*0.2821	T. Amm/15		100 µl	Rate/mg cells added	sp. act.*10*50*mg added
1	30	0.3881	0.0258	2	0.2	0.1293	12.93
2	60	0.0265	0.0017	2	0.2	0.0088	0.8839
3	120	0.0095	0.0006	2	0.2	0.0031	0.3197
4	180	0.0067	0.0004	2	0.2	0.0022	0.2256
5	240	0.0056	0.0003	2	0.2	0.0018	0.1880
6	300	0.0033	0.0002	2	0.2	0.0011	0.1128