DEVELOPMENT OF BIOCATALYTIC PROCESSES FOR HYDROXAMIC ACID AND ACID HYDRAZIDE SYNTHESIS

Ph.D. THESIS

by

SHILPI

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)
DECEMBER, 2013

DEVELOPMENT OF BIOCATALYTIC PROCESSES FOR HYDROXAMIC ACID AND ACID HYDRAZIDE SYNTHESIS

A THESIS

Submitted in partial fulfilment of the

requirements for the award of the degree

of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

by

SHILPI

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE - 247 667 (INDIA)
OCTOBER, 2013

INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled, "**DEVELOPMENT OF BIOCATALYTIC PROCESSES FOR HYDROXAMIC ACID AND ACID HYDRAZIDE SYNTHESIS"** in partial fulfilment of the requirements for the award of the Degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during the period from January, 2008 to December, 2013 under the supervision of Dr. Bijan Choudhury, Associate Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee, India.

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

(SHILPI)

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

(Bijan Choudhury)
Date: Supervisor

The Ph.D. Viva-Voce Examination of **Ms. SHILPI,** Research Scholar has been held on…….

Supervisor Chairman, SRC External Examiner

Head of the Department/Chairman, ODC

ABSTRACT

Hydroxamic acids and acid hydrazides are important chemical compounds with considerable applications in pharmaceutical, medicinal, polymer and agrochemical industries. Nicotinic acid hydroxamate, a heterocyclic class of hydroxamic acid has roles as bioligand, urease inhibitor, antityrosinase, antioxidant, antimetastatic and vasodilating agents due to strong metal chelating ability and possibly their NO releasing property. Isoniazid and its derivatives are potent antituberculosis agents and isoniazid has been employed in the treatment of tuberculosis for over half a century. Chemical methods for syntheses of hydroxamic acids and acid hydrazides present several disadvantages and thus there is a need to explore efficient enzymatic routes for syntheses of these compounds. Amidases have been studied as biocatalyst for syntheses of hydroxamic acids and acid hydrazides, however amidase catalysed bioprocesses for productions of nicotinic acid hydroxamate and isoniazid are still not developed. The major objective of the present thesis was to develop biocatalytic processes for syntheses of nicotinic acid hydroxamate and isoniazid using acyltransferase activity of whole cell amidase.

At the beginning of this thesis, **Chapter 1** introduces briefly about roles of hydroxamic acids and acid hydrazides in various industrial applications with special emphasis on nicotinic acid hydroxamate and isoniazid. The present scenario of different existing chemical and enzymatic methods for syntheses of these compounds was discussed. Further, it also dealt with the limitations and disadvantages associated with the chemical methods and suitability of amidase catalyzed biocatalytic methods to overcome these drawbacks was highlighted. Finally the objectives to be attained in this study were specified in brief.

Followed by this, **Chapter 2** presents (1) the detail review on hydroxamic acids and acid hydrazides, their structures, functions and industrial applications of nicotinic acid hydroxamate and isoniazid; (2) a detailed description of various chemical methods of syntheses of these compounds; (3) information on enzymatic routes for syntheses of hydroxamic acids and acid hydrazides reported in various literatures, factors affecting enzyme catalyzed processes, advantages of enzymatic methods over chemical methods; (4)

and finally classification, mechanism of action, occurrence and properties of reported amidases with their industrial applications in various fields.

Further**, Chapter 3** deals with materials and methods used in present work. Detailed descriptions of methods, different experimental techniques utilized in this study were mentioned.

Detailed results, obtained during this work along with discussion are presented in **Chapter 4**. This chapter is divided into four sections. The work presented in **Section 1** aimed at screening of suitable bacterial isolate producing amidase enzyme having acyltransferase activity. Among ten available nitrile metabolizing bacterial isolates, 6b2 was selected as biocatalyst as it possessed acyltransferase activity for a broad range of amides, higher acyltransferase to amide hydrolase activity ratio for heterocyclic amides and the highest mole ratio of product (nicotinic acid hydroxamate) to by-product (nicotinic acid). Isolate 6b2 was identified as *Bacillus smithii* strain IITR6b2, based on biochemical characteristics and 16S rDNA sequence. The influences of various physiochemical parameters on amidase production were studied using one variable at a time approach. Amidase was found to be inducible in nature. Under optimized conditions, maximum amidase production was obtained in mid exponential growth phase at 48 h with glycerol (10 g/l) as carbon source, phenylacetonitrile (10 mM) as sole source of nitrogen and inducer at pH 7.0 and temperature 45 °C. Whole cell amidase had pH and temperature optima of 7.0 and 55 °C respectively. Acyltransferase activity of amidase was thermally stable with half lives of 29, 14 and 10 h at 30, 45 and 55 °C respectively. Amidase showed acyltransferase activity for a broad range of amides like aliphatic, aromatic and heterocyclic amides with highest activity for nicotinamide. Whole cell amidase was compatible in presence of both water miscible and water immiscible solvents at 15% (v/v) concentration. This solvent compatible property is highly desirable for syntheses of hydroxamic acids and acid hydrazides from hydrophobic amides that are poorly soluble in aqueous medium.

Although amidases from different microbial sources having acyltransferase activity for a broad range of amides have been studied, only a few have been reported to develop bioprocesses for syntheses of hydroxamic acids. Bioprocess development for synthesis of nicotinic acid hydroxamate using acyltransferase activity of *B. smithii* strain IITR6b2 is described in **Section 2**. Amidase with acyltransferase activity for nicotinamide is suitable

for nicotinic acid hydroxamate production. However amidase can also simultaneously hydrolyzes nicotinamide to nicotinic acid. Nicotinic acid is an undesirable by-product and thus any biocatalytic process involving amidase for nicotinic acid hydroxamate production needs to have high ratio of acyltransferase to amide hydrolase activity. Isolate *B. smithii* strain IITR6b2 was found to have 28 fold higher acyltransferase to amide hydrolase activity. This higher ratio resulted in limited undesirable by-product, nicotinic acid synthesis. Further the effects of various parameters on bioconversion yield were investigated in detail. The optimal substrate/co-substrate ratio, pH, temperature, incubation time and resting cells concentration were 200/250 mM, 7.0, 30 °C, 40 min and 0.7 mg_{dcw}/ml respectively. Under these optimized reaction conditions, 94.5% molar conversion of nicotinamide to nicotinic acid hydroxamate was achieved. To avoid substrate inhibition effect, a fed batch process based on the optimized parameters was developed and a molar conversion yield of 89.4% with the productivity of 52.9 $g/h/g_{dcw}$ was achieved in laboratory scale. Finally, 6.4 g of powder containing 58.5% (w/w) nicotinic acid hydroxamate was recovered after lyophilisation and further purification resulted in 95% pure product.

In **Section 3**, a biocatalytic route for the synthesis of isoniazid, in aqueous system is presented. Acyltransferase activity of *B. smithii* strain IITR6b2 was utilized for its ability to transfer acyl group of isonicotinamide to hydrazine-2HCl in aqueous medium. Whole cell amidase possessed 3 folds higher acyltransferase activity as compared to amide hydrolase activity for isonicotinamide and this ratio was further improved to 4.5 by optimizing concentration of co-substrate hydrazine–2HCl. Various key parameters were optimized and under the optimum reaction conditions of pH (7.0, phosphate buffer 100 mM), temperature (30 °C), substrate/co-substrate concentration (100/1000 mM) and resting cells concentration (2.0 mg_{dcw}/ml), 90.4% conversion of isonicotinamide to isoniazid was achieved in 60 min. Under these conditions, a fed batch process for production of isoniazid was developed and resulted in the accumulation of 439 mM of isoniazid from 500 mM isonicotinamide with 87.8% molar conversion yield and productivity of 6.0 $g/h/g_{dcw}$. Finally, 9.1 g of powder containing 33.3% (w/w) isoniazid was recovered after lyophilisation and further purification resulted in 94% pure product.

In addition, amidase from *B. smithii* strain IITR6b2 was purified and characterized as reported in **Section 4**. Purified amidase was characterized for temperature optima, pH optima, substrate specificity, organic solvent compatibility, effects of chemical reagents

including metals, inhibitors and surfactants and was compared with amidases reported in literature from other microbial sources. Intracellular amidase was purified to 12.11 fold with yield of 37.5% by a procedure involving ammonium sulphate precipitation (30-60%), ultrafiltration by amicon (10 kD), column chromatographies with Q sepharose and phenyl sepharose resins. The purified amidase was a monomer with molecular mass of 63 kDa as determined by gel filtration chromatography and SDS-PAGE. Purified amidase had pH and temperature optima of 7.0 and 45 °C respectively. The substrate specificity of purified amidase was determined for both acyltransferase and amide hydrolase activities. It had highest acyltransferase activity with nicotinamide followed by benzamide and hexanamide. This amidase also hydrolyzed different amides but acyltransferase activity was considerably higher as compared to amide hydrolase activity. Purified amidase was not a metalloenzyme as metal chelating reagent EDTA did not affect the enzyme activity. The amidase activity was highly inhibited by heavy metal ions and –SH modifying reagents (DTNB and NEM), while reducing agent DTT improved enzyme activity to 2.2 fold suggesting thiol group involvement at amidase active site. Purified amidase was compatible with organic solvents and maintained more than its 90% activity in 10% (v/v) of methanol, ethanol, isopropanol, acetonitrile, 1-Decanol, DMF, Xylene, hexane, heptane and n-hexadecane. In presence of methanol, ethanol and isopropanol at 20% (v/v) concentration activity reduced to 76, 56 and 53% respectively. Amidase activity was enhanced to 285, 302, 365 and 157% in the presence 0.05% (v/v) of non ionic surfactants such as Tween 20, Tween 60, Tween 80 and Triton X-100 respectively. On the other hand SDS and CTAB caused complete inhibition of activity. The substrate specificity profiles of purified amidase and whole cell amidase were found considerably different and it led to predict the presence of one more amidase having affinity for short chain aliphatic amides. This second amidase was partially purified. The two amidases purified from *B. smithii* strain IITR6b2 totally differed in substrate specificity and temperature optima, while pH optima were nearly same.

Finally, in **Chapter 5**, summary of the complete work and the conclusions drawn are presented. In conclusion, the present work demonstrates the development of efficient bioprocesses for nicotinic acid hydroxamate and isoniazid syntheses using acyltransferase activity of *B. smithii* strain IITR6b2. These results also presents that this bacterial isolate can be a potential biocatalyst for syntheses of other hydroxamic acids and acid hydrazides.

RESEARCH PUBLICATIONS

Agarwal S, Gupta M, Choudhury B (2013) Bioprocess development for nicotinic acid hydroxamate synthesis by acyltransferase activity of *Bacillus smithii* strain IITR6b2. **Journal of Industrial Microbiology and Biotechnology** 40, 937–946.

Agarwal S, Gupta M, Choudhury B (2013) Solvent free biocatalytic synthesis of isoniazid from isonicotinamide using whole cell of *Bacillus smithii* strain IITR6b2. **Journal of Molecular Catalysis B: Enzymatic** 97, 67–73.

ACKNOWLEDGEMENTS

During past few years stay in **IITR** I have been accompanied and supported by many people. With immense pleasure, I take this opportunity to express my gratitude for all those persons who contributed directly or indirectly during this work of thesis.

First and foremost, I would like to express my sincere gratitude and reverence to my supervisor, Dr. Bijan Choudhury for his expert guidance, invaluable suggestions, scientific expertise and encouragement throughout the study. I thank him for his patience, many fruitful discussions and also for rectifying my mistakes. His interest for the subject and his perseverance as a researcher, have always motivated me to be persistent and to work harder.

I would also like to acknowledge DRC chairman Dr. Partha Roy, thesis advisory committee members Dr. Naveen K. Navani, assistant professor, Department of Biotechnology and Dr. C. B. Majumder associate professor, Department of Chemical engineering for their valuable comments, suggestions and timely help in various issues of the research work.

I am extremely grateful to Dr. R. Prasad, Head of Department of Biotechnology for providing the necessary research facilities and an excellent working environment in the department. I am thankful to all the faculty members of my department for their continuous inspiration and co-operation whenever required. I am thankful to University Grants Commission, New Delhi for all the financial support in the form of Research Fellowship and facilities availed to me during the course of this work.

I extend a note of thanks to the staff members of Department of Biotechnology especially Mr. Jain, Mr. V.P Saini, Shashi madam, Surita madam, Mr. Lokesh and Anil ji for all the assistance provided by them during the entire research work.

I would like to thank my research colleagues at Bioprocess engineering laboratory Dr. Vikash, Dr. Rohit, Gaurav, Meenu, Pawan, Swapan ji, Sidharth, Rukmini, Nupur, Archana, Shailender for their kind cooperation during the work.

I would like to thank my colleagues and friends Abhijeet, Rekha, Rajnikant, Parmesh, Peeyush, Jitendra, Geetu, Supriya, Silva, Tapas, Rajbala, Bivekanand, Prabhat, Shivendra, Tamogna, Atin, Shailendra, Anamika, Nidhi, Shweta, Pradeep, Manju, Megha and Sonali for their loving support and assistance in numerous ways.

Naturally, it was impossible to enjoy these years of research without the occasional distraction of friends and family. A great deal of appreciation goes to my dear friends Saurabh, Swati, Santosh, Mansi, Chandrakant, Rashmi, Gaurav, Yogesh, Sandeep and Prabhat sir who have been with me during my varying moods and temperament. The selfless support and the timely attention rendered by all these individuals have contributed in my overall development and also to achieve all this. I deeply appreciate their friendship, encouragement and company during the stay in IITR.

Most importantly, I would like to express my deep gratitude to my beloved parents for their boundless love, guidance and support which encouraged me throughout and helped me endure periods of difficulties with cheer. They have been my greatest source of energy and inspiration. I would like to extend my sincere thanks to my sisters, for their love and supports. My special thanks to my uncle Dr. S. Pandey for his constant encouragement and fruitful guidance in all aspects of my life.

SHILPI

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
AU	Arbitrary unit
µM	Micromolar
BLAST	Basic local alignment search tool
BOP	(Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
BSA	Bovine serum albumin
CDMT	2-chloro-4,6-dimethoxy-1,3,5-triazine
CTAB	Cetyl trimethylammonium bromide
CFE	Cell free extract
DCC	*N,N'*-Dicyclohexylcarbodiimide
DEAE	Diethylaminoethyl
DEPA	Di-ethylphosphoramidate
DMCPCA	2,2-dimethylcyclopropanecarboxamide
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMTMM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
dNTP	Deoxyribonucleotide triphophate
E.C.	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
e.e.	Enantiomeric excess
et al	And others

etc	Et cetera
Fig	Figure
g	Gram
h	Hour
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
INH	Isoniazid
INA	Isonicotinic acid
i.e.	That is
kb	Kilobase
KDa	Kilo Dalton
kHz	Kilo Hertz
l	Litre
MB	Mineral base
M	Molar
mM	Millimolar
mg	Milligram
min	Minute
ml	Millilitre
N	Normal
NCBI	National Center for Biotechnology Information
NAH	Nicotinic acid hydroxamate
NA	Nicotinic acid
NEM	N-Ethylmaleimide
nm	Nanometer
NMM	N-methylmorpholine
NMR	Nuclear magnetic resonance
NO	Nitrogen oxide
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
pH	Potential of hydrogen
PAN	Polyacrylonitrile
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
PVC	Polyvinyl chloride
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
SEM	Scanning Electron Microscopy
sp.	Species
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	N,N,N’,N’-tetramethylenediamine
THF	Tetrahydrofuran
UV	Ultraviolet
vol	Volume
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Hydroxamic acids, an important class of organic compounds containing –CONROH group are key constituents in several biological processes, organic, coordination and medicinal chemistry (Fazary *et al*., 2001, Griffith *et al*., 2004, Mulchay *et al*., 2005, Alagta *et al*., 2008, Codd 2008). Considerable biochemical interests have been shown in these organic compounds due to their versatile biological properties and low toxicities. Hydroxamic acids form very stable metal chelates, thus are effective inhibitors of several metalloenzymes such as histone deacetylase, peroxidase, matrix metalloproteinase and urease (Farkas *et al*., 2000, Hockly *et al*., 2003, Ouellet *et al*., 2004, Kontogiorgis *et al*., 2005, Ul-Haq *et al*., 2009). Hydrazides (R-CO-$NHNH_2$), acylated derivatives of hydrazine, are of great significance in the syntheses of various organic compounds, pharmaceuticals, polymers and agrochemicals (Mohamed 1994, Sinha *et al*., 2005, Woo *et al*., 2007, Kumar *et al*., 2010, Kudelko *et al*., 2012). Like hydroxamic acids, hydrazides are also effective chelating agents widely used in analytical chemistry (Nehru *et al*., 2001, Mohamed *et* al., 2006). Several chemical methods for syntheses of hydroxamic acids and acid hydrazides from carboxylic acids, esters, acid chlorides and aldehydes have been recommended in various literatures (Nikam *et al*., 1995, Reddy *et al*., 2000, Luca *et al*., 2001, Zhang *et al*., 2002, Porcheddu and Giacomelli, 2006, Saha *et al*., 2010). These reactions often require expensive O/N- protected hydroxylamine, toxic coupling reagents, reflux conditions and usually carried out in highly alkaline reaction medium with different organic solvents. The requirement of strong alkaline reaction condition of these methods, make recovery of product cumbersome due to additional requirement of neutralization by concentrated HCl. Further, the chemical routes for syntheses of these compounds are multistep processes; result in several impurities due to side reactions and in many instances incomplete reactions due to less reactive substrates. Alternatively development of single step biotransformation processes for hydroxamic acids and acid hydrazides syntheses will provide a simple, economic, convenient and environmental friendly route.

Biocatalysis has been reported as a valuable tool for syntheses of numerous pharmaceuticals and fine chemicals. Unique characteristics of acyltransferase activity of several amidases and their ability to synthesize hydroxamic acids and acid hydrazides have

been studied in detail; however only few reports are available on amidase catalyzed bioprocesses for syntheses of these compounds (Fournand *et al*., 1997, 1998a, Kobayashi *et al*., 1999, Makhongela *et al*., 2007, Pandey *et al*., 2011, Bhatia *et al*., 2013). Hence development of biocatalytic route for productions of pharmaceutically important hydroxamic acids and acid hydrazides are of great interest. In present study, efficient biocatalytic processes for hydroxamic acid and acid hydrazide syntheses were developed from corresponding amides using the acyltransferase activity of amidase.

Hydroxamic acids have been investigated as antibacterial, antimetastatic, antimalarial, antituberculosis and anti human-immunodeficiency virus agents (Miller 1989, Malley *et al*., 1994, Pepeljnjak *et al*., 2005). Nicotinic acid hydroxamate (NAH), a heterocyclic class of hydroxamic acid is a nicotinic acid derivative with several significant pharmaceutical and analytical applications. The NAH can serve as whitening agent and antioxidant in cosmetics, medicines and food processing industries (Bissett *et al*., 1999, Oblong *et al*., 2003, Chen *et al*., 2011). NAH is a potent inhibitor of monophenolase and diphenolase activities of mushroom tyrosinase in vitro (Lin *et al*., 2012). NAH found its role as a bioligand in development of a heterobimetallic novel wave like co-ordination polymer and Ruthenium (III) dimethyl sulfoxide pyridine hydroxamic acid complex, a potential antimetastatic agent (Mulchay *et al*., 2005, Griffith *et al*., 2008). A new class of bifunctional metallodrug was developed exploiting the well established anticancer properties of Pt and the NO releasing ability of 3- pyridine hydroxamic acid (Griffith *et al*., 2007). NAH is also reported to be effective for treatment of hyperammonemia (Konishi *et al*., 1974), as urease inhibitor (Takebe *et al*., 1982), vasodilating agent (Bailey *et al*., 1972, Harrison and David, 1979). Several metal complexes (Ni, Co and Vn) of NAH have also been synthesized (Aliyu and Nwabueze, 2005, 2008, Aliyu *et al*., 2010).

Hydrazides and their derivatives are reported to possess antitumor, antiviral, antimalarial, antibacterial, antifungal, anticancer, anticorrosive and anti-inflammatory activities (Mohamed *et al*., 1997, Quraishi *et al*., 2001, Narasimhan *et al*., 2010, Kumar *et al*., 2010). Isoniazid (INH), the hydrazide of isonicotinic acid is an important first-line antitubercular drug and is given in combination with rifampicin and ethambutol (Ziskind *et al*., 1953, Toit *et al*., 2006, Imramovsky *et al*., 2007). This drug has been used in the treatment of tuberculosis for over half a century. In recent years several isoniazid derivatives have been developed with antimycobacterial, antimicrobial and antiviral activities (Sinha *et al*., 2005, Sriram *et al*., 2005, Judge *et al*., 2013).

The common method for the production of hydroxamic acids is via the reaction of activated carboxylic acids with O/N-protected hydroxylamine, followed by an additional step of catalytic hydrogenation with palladium to synthesize the free hydroxamic acids (Nikam *et al*., 1995, Luca *et al*., 2001, Devocelle *et al*., 2003, Kokare *et al*., 2007). These methods suffer from disadvantages of requiring expensive hydroxylamine derivatives, toxic coupling reagents along with acid activation step that sometimes results in low yield. Another economical way to synthesize hydroxamic acids is the reaction of hydroxylamine or O-benzyl hydroxylamine with esters (Pinung *et al*., 1995, Ho *et al*., 2005, Gissot *et al*., 2005). The route of hydroxamic acid synthesis from corresponding ester involves use of organic solvents such as THF, methanol or ethanol and reaction is favoured under strong alkaline condition (pH>10) and sometimes nitrogen atmosphere. Due to strong alkaline conditions, this method is not recommended for ester derivatives that contain base sensitive groups (Reddy *et al*. 2000).

The common method reported for hydrazide synthesis is reaction of corresponding ester with excess of hydrazine monohydrate in alcoholic solution under strong basic and refluxing conditions (Yale *et al*., 1953, Zhang *et al*., 2002). During the process highly reactive esters yield considerable amount of diacylhydrazine, a by-product. While less reactive esters require harsh reaction conditions and longer reaction time to complete the process. Another method for hydrazide synthesis is reaction of carboxylic acid with hydrazine in presence of coupling agents (Zhang *et al*. 2002) or under microwave irradiation condition (Saha *et al*., 2010). This method results in low yield due to an extra carboxylic acid activation step and synthesis of different by-products leads to complicated recovery of desired product.

In view of these disadvantages and limitations associated with chemical methods, it may be worthwhile to develop enzymatic processes for syntheses of these compounds. Amidase (amidohydrolase, EC 3.5.1.4) enzyme has attracted significant attentions in various pharmaceutical, chemical industries and bioremediation. In past, acyltransferase activity of amidases has been studied in detail for hydroxamic acids and acid hydrazides syntheses (Fournand *et al*., 1997, 1998a, 1998b, Kobayashi *et al*., 1999, Andrade *et al*., 2007, Vejvoda *et al*., 2011, Bhatia *et al*., 2013). Acyltransferase activity of amidases involves the transfer of acyl group of amide to co-substrate hydroxylamine or hydrazide which leads to syntheses of hydroxamic acid and acid hydrazide respectively. The study on acyltransferase activity of amidases showed that these compounds can be produced in

aqueous medium at neutral pH with higher product yield using a few milligrams of biocatalyst (Fournand *et al*., 1998a, 1998b, Pandey *et al*., 2011, Bhatia *et al*., 2013). Moreover, the selection of biocatalysts with higher acyltransferase to amide hydrolase activity leads to high molar conversion of product with minimal by-product.

With obvious industrial and medical applications of nicotinic acid hydroxamate and isoniazid, there is an increasing demand for improved syntheses methods of these compounds with high yield and productivity. Hence nicotinic acid hydroxamate and isoniazid were selected for the present study, dealing with amidase catalyzed bioprocesses development. In earlier reported literature, bioprocesses for syntheses of few hydroxamic acids have been developed. First reported bioprocess for acetohydroxamic acid was developed by Fournand et al. (1997) and 54-61% (mol/mol) conversion was achieved. Later Pandey et al. (2011) reported an improved method with 93% molar conversion of 300 mM acetamide to acetohydroxamic acid in 30 min using *Bacillus sp.* ABP-6. Bioprocess for benzohydroxamic acid synthesis was reported recently by Bhatia et al. (2013) with *Alcaligenes sp.* MTCC 10674. They developed a fed-batch method to avoid substrate inhibition effect of benzamide and recovered 33 g of benzohydroxamic acid with 24.6 g/l/h productivity. Although amidase catalyzed biocatalytic processes for syntheses of aceto and benzohydroxamic acids have been reported, there is no report available on biotransformation process for synthesis of nicotinic acid hydroxamate.

On the other hand lipase catalyzed enzymatic methods for isoniazid synthesis have already been reported in literature. Yadav et al. (2005) explored the enzymatic route of isoniazid synthesis by working in non-aqueous media under milder reaction conditions using lipase as a catalyst. This process resulted in 52% conversion in 24 h. They further improved the method by using microwave irradiation with immobilized lipase and process led to a conversion of 54% in 4 h (Yadav and Saiure, 2007). 1, 4-Dioxane, was used in lipase catalysed synthesis as solvent is highly flammable and combines with atmospheric oxygen to form explosive peroxide on prolonged exposure to air. It is a known carcinogen to animals and is classified as a possible carcinogen to human (Leung and Paustebach, 1990, Kano *et al*., 2009, Kasai *et al*., 2009). Hence an improved enzymatic synthesis method with high conversion rate in aqueous medium will offer an economic and greener approach for isoniazid synthesis. Surprisingly, hydrazinolysis of isonicotinamide by acyltransferase activity of amidase has not been reported yet for the preparative scale synthesis of isoniazid.

Amidase mediated conversions of amides to hydroxamic acids and acid hydrazides have received interest in recent years due to mild reaction conditions, reduced by-products, less waste, good bioconversion yields, ease of enzyme immobilization and choice of different biocatalysts based on its substrate affinity. The starting material of these enzymatic reactions are amides that are cheaper than corresponding acids and esters which are used in chemical methods of synthesis and also reactions take place in aqueous medium leading to a greener approach with reduced cost of bioprocess.

Based on these observations and unavailability of efficient bioprocesses for nicotinic acid hydroxamate and isoniazid production, the current investigation was performed. During this work, the biocatalytic processes for nicotinic acid hydroxamate and isoniazid syntheses were developed with whole cell amidase of *Bacillus smithii* strain IITR6b2. Further amidase was purified to characterize its biochemical properties including optimum temperature, pH, substrate specificity, organic solvent compatibility etc.

The present research work entitled "Development of biocatalytic processes for hydroxamic acid and acid hydrazide synthesis" was undertaken with the following objectives.

1. Screening and selection of microorganism for amidase enzyme having acyltransferase activity.
2. Optimization of amidase enzyme production using shake flask.
3. Optimization of reaction conditions for acyltransferase activity of whole cell enzyme and its characterization.
4. Bioprocess developments for hydroxamic acid/ acid hydrazide synthesis.
5. Purification of enzyme and its characterization.

CHAPTER 2

LITERATURE REVIEW

2.1 Hydroxamic acids

Hydroxamic acids show a broad range of biological activities and are of biochemical interest to a great extent for chemical biology and medicinal chemistry (Fazary *et al*., 2001, Griffith *et al*., 2004, Codd 2008, Lin *et al*., 2012). The biological properties of hydroxamic acids are due to their affinity to chelate various transition metals (O'Brien *et al*., 2000, Farkas *et al*., 2000, Mulchay *et al*., 2005, Codd 2008, Shotor *et al*., 2010) and possibly NO releasing property under certain conditions (Shirota *et al*., 1991, Devocelle *et al*., 2003, Griffith *et al*., 2004). These compounds constitute a remarkable class of bioligands that forms very stable chelates with a number of metal ions and hence are potent inhibitors of several metalloenzymes (Grant *et al*., 2001, Cali *et al*., 2004, Griffith *et al*., 2011). In microorganism, natural occurring hydroxamic acids are involved in the transport of extracellular iron by chelation through the microbial cell wall (Miller 1889).

2.1.1 General structure of hydroxamic acids

Hydroxamic acids, important ligands in coordination chemistry are of general formula, RxC(O)N(Ry)OH (Rx= alkyl/aryl; Ry= H/alkyl/aryl) (Fig 2.1). These compounds are weak organic acids with pKa value of N-OH proton in range of 8.5-9.4 in aqueous medium (Fazary 2005). The chelation of metals by hydroxamic acids takes place through oxygen of the -NHOH and carbonyl groups. Hydroxamic acids undergo two consecutive deprotonation events depending on the pH of aqueous solution and two (*O, O*) bidentate chelation modes of ligands (singly deprotonated or doubly deprotonated) are available to metal ions (Türkel 2011). The release of first proton of -OH group results in hydroxamato form of ligand with coordination moiety $(CO)NHO^-$ (first deprotonation step). Further metal induced release of second proton from NHO^- results in the hydroximato type of ligand (second deprotonation step). These hydroxamate and hydroximate ions contribute to several resonance structures (Codd 2008) (Fig 2.2).

Hydroxamic acids exhibit cis/trans (Z/E) isomerism as a result of free rotation around the C-N bond and also keto (RxC(O)N(Ry)OH)/ iminol (RxC(OH)NOH) isomerism (Codd 2008, Saldyka and Mielke 2007) (Fig 2.3). The X-ray crystal structures of simple hydroxamic acids, such as formo and acetohydroxamic acids reveal that these compounds adopt the cis (Z) configuration in solid state while C and N substituted hydroxamic acids, such as N-methyl-4-methyl benzohydroxamic acid and N-(3-cyanophenyl) acetohydroxamic acid are present in trans (E) configuration due to steric effects. It was observed that the type of C and N substituents affect the stability of metal-hydroxamic acid complex. Presence of an electron donating group as C and N substituent results in stable metal-hydroxamate complex due to increased negative charge on the coordinating oxygen atom.

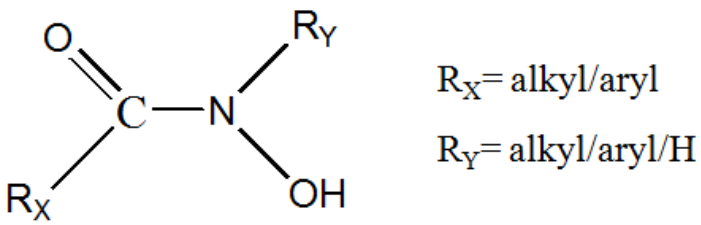


Fig 2.1 General structure of hydroxamic acids

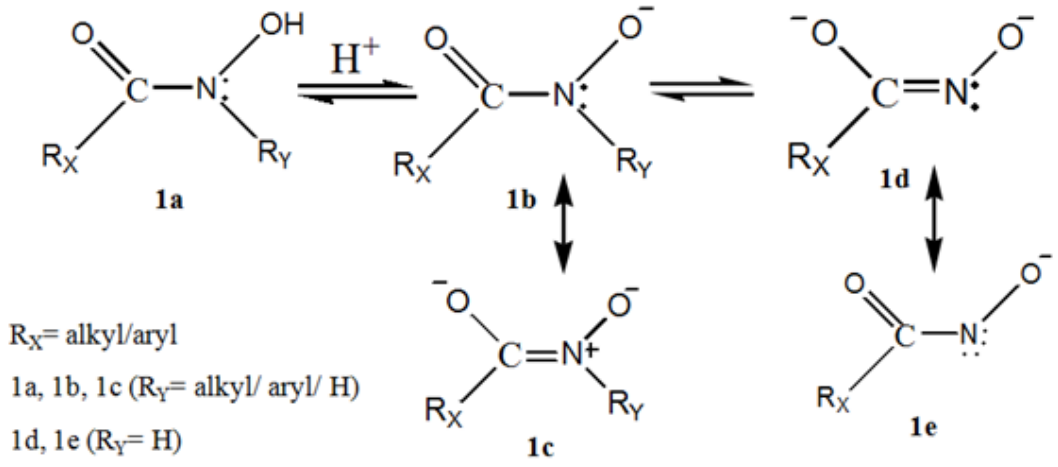


Fig 2.2 Hydroxamic acid (1a), hydroxamate anion (1b, 1c), hydroximate dianion (1d, 1e) (adapted from Codd 2008)

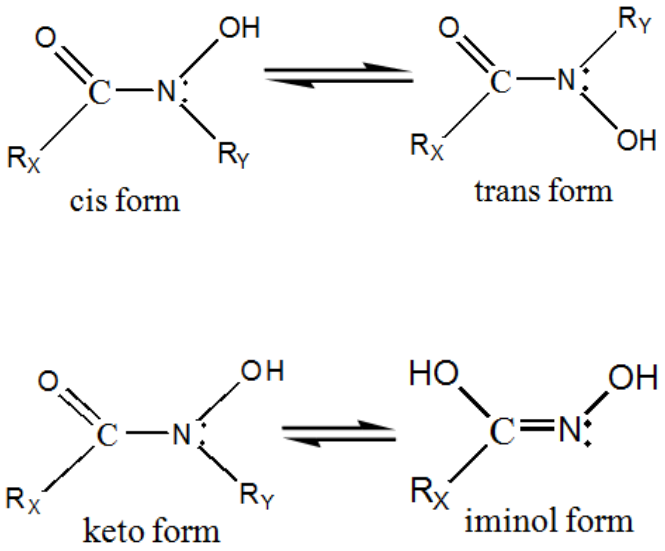


Fig 2.3 Representation of cis/trans and keto/iminol isomerism of hydroxamic acids

2.1.2 Functions of hydroxamic acids

Due to their ability to coordinate to metal ions (Buglyó and Pótári, 2005, Bátka and Farkas, 2006, Seda *et al*., 2006, Farkas *et al*., 2009), these are effective inhibitors of metalloenzymes such as matrix metalloproteinases, histone deacetylase, tyrosinase, peroxidase, urease and carbonic anhydrase (Kontogiorgis *et al*., 2005, Verma 2012, Codd *et al*., 2009, Kwak *et al*., 2013, O'Brien *et al*., 2000, Hassan *et al*., 1997, Scozzafava and Supuran, 2000). It was reported that the hydroxamic acid moiety (R-CO-NHOH), is the key pharmacophore in hydroxyurea, an anticancer drug (Fazary *et al*., 2001). Desferrioxamine, a trihydroxamate siderophore chelates iron and is used for treatment of acute iron poisoning, hemochromatosis, aceruloplasminemia and aluminium toxicity. It removes excess iron from blood of iron overloaded patients (Miller 1989, Fazary *et al*., 2001, Miyajima *et al*., 1997). Hydroxamic acids have metal binding ability and therefore can be used for removal of contaminating metal ions from industrial waste (Ahuja *et al*., 1996, Pandey *et al*., 2011). A chelating polymeric ligand, poly (hydroxamic acid)-poly (amidoxime) has been reported to remove several contaminating heavy metal ions from waste water (Lutfor and Mashitah, 2011). Formo and acetohydroxamic acids offer considerable advantages in nuclear fuel reprocessing by allowing the separation of neptunium from uranium (Taylor *et al*., 1998). Hydroxamic acids as self-assembled monolayers (SAMs) have been studied showing corrosion inhibitory properties for carbon steel electrode surface (Alagta *et al*., 2008). Acetohydroxamic acid or Lithostat is an effective urease inhibitor and has been reported to inhibit ureolytic activity in mice, dogs

and men (Hase and Kobashi, 1967, Fishbein 1967, Summerskill *et al*., 1968, Pandey *et al*., 2011). Gao et al. (1995) reported that hydroxyurea enhanced the anti HIV-1 (human immunodeficiency virus type-1) potency of nucleoside. Azaindole N-methyl hydroxamic acids have been investigated as potent HIV-1 integrase inhibitors (Tanis *et al*., 2010). Salicylhydroxamic acid has significant applications in treatment of thalassemia major, urinary tract infections, as trypanocidal agent and viral growth inhibitor (Fazary *et al*., 2001). Derivatives of acetohydroxamic acid, suberoylanilide hydroxamic acid, 3-and 4-pyridine hydroxamic acid and benzohydroxamic acid have been shown to possess antitumor activity (Li *et al*., 2004, Griffith *et al*., 2008, Pal and Saha, 2012). 3-pyrdine hydroxamic acid has been used as building block in rational design of a heterobimetallic novel wave like coordination polymer (Mulchay *et al*. 2005).

2.1.3 Nicotinic acid hydroxamate (NAH)

Nicotinic acid hydroxamate (NAH), also known as 3-pyridine hydroxamic acid, is one of the nicotinic acid derivative in which the hydroxamic acid moiety (-CONHOH) is present at third carbon of the pyridine ring (Bailey *et al*., 1972). Nicotinic acid derivatives are attractive compounds due to their pharmaceutical and analytical applications (Lukevits 1995). Lin et al. (2012a) reported NAH as a more potent inhibitor of monophenolase and diphenolase activities of mushroom tyrosinase in vitro than the other structural analogues of nicotinic acid derivatives. Simultaneously NAH also exhibited dose dependent diphenylpicrylhydrazyl (DPPH), anti-low density lipoprotein peroxidation and hydroxyl radical scavenging activities. NAH dose dependently inhibited tyrosinase activity and reduced the melanin contents in murine melanoma B16F10 cells under non cytotoxic concentrations (below 50 μm) (Lin *et al*., 2012b). These results indicate that NAH has potential medicinal value for treatment of hyper pigmentation of skin. On the other hand nicotinic acid hydroxamate is a ligand with two coordination sites i.e. the pyridine nitrogen and the hydroxamic acid moiety and these sites are juxtaposed in such a manner that they both cannot coordinate to the same metal ion. This property was utilized in the design of a heterobimetallic novel wave like coordination polymer using NAH as bridging scaffolds (Mulchay *et al*., 2005). Development of a new class of bifunctional metallodrug was reported based on the anticancer properties of Pt and the nitric oxide releasing ability of hydroxamic acids. 3- and 4-pyridine hydroxamic acids were selected for this as they could coordinate Pt^{II} or Pt^{IV} via the pyridine nitrogen thus leaving hydroxamic acid moiety free to release cytotoxic nitric oxide (Griffith *et al*., 2007). Ruthenium (III) dimethyl sulfoxide

pyridine hydroxamic acid complexes were developed as potential antimetastatic agents in which pyridine nitrogen coordinate Ru^{III} leaving the hydroxamic acid moiety free to bind Zn at the active site of matrix metalloproteinases (Griffith *et al*., 2008).

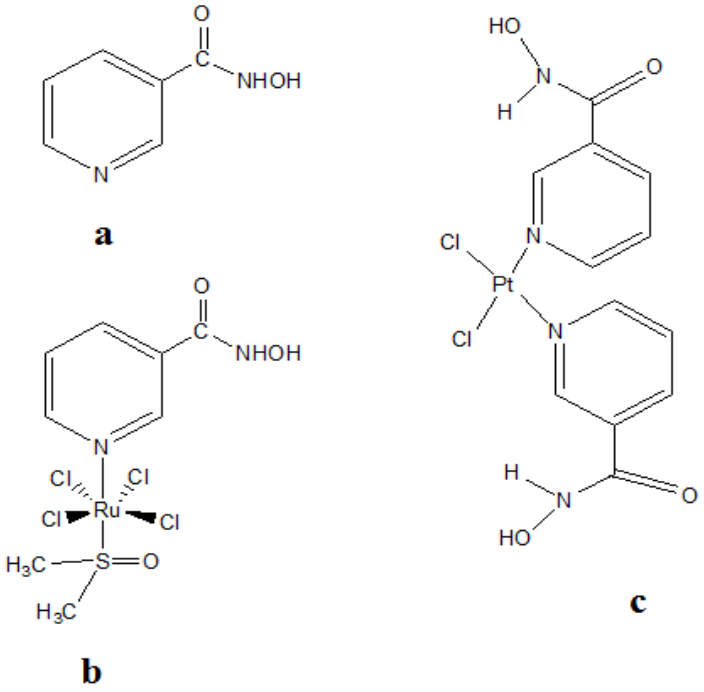


Fig 2.4 Nicotinic acid hydroxamate **(a)**, Ruthenium (III) dimethyl sulfoxide pyridine hydroxamic acid complex (Griffith *et al*., 2008) **(b)**, bifunctional metallodrug based on 3-pyridine hydroxamic acid complex with Pt (Griffith *et al*., 2007**) (c)**

2.2 Acid hydrazides

Acid hydrazides comprise a group of acylated derivatives of hydrazine with general formula $RCONHNH_2$ (Fig 2.5). Hydrazides are important precursor and intermediates in the syntheses of certain organic compounds, amides, heterocyclic compounds, aldehydes and hydrazones (Feuer and Brown 1970, Sinha *et al*., 2005, Roveda *et al*., 2009, Kumar *et al*., 2010, Kudelko an Zieliński, 2012, Refat and Fadda, 2013). In analytical chemistry, these are used as metal chelating agents. Hydrazides may coordinate with metal ions and two atoms (N, O) binding sites of these ligands are accessible to metal ions and hence like hydroxamic acids these are also potent chelating agents (Nehru *et al*., 2001, Odunola *et al*., 2002, Galal *et al*., 2009, Ain *et al*., 2013, Sathayadevi *et al*., 2014). These are reported to possess antitumor, antibacterial, anticancer and anti-inflammatory activities (Narasimhan *et al*., 2010).

2.2.1 General structure of acid hydrazides

Acid hydrazides and their analogues are of significant interest due to their ability to coordinate to a range of transition metals. Coordination mode of hydrazides to metals has been determined by analysis of infrared and NMR spectra of several hydrazide metal complexes and corresponding free ligands (David *et al*., 1999, Odunola *et al*., 2002, 2003, Bouslimani *et al*., 2010, Salawu *et al*., 2011, Ain *et al*., 2013). Hydrazides may coordinate to metals through amino nitrogen and carbonyl oxygen atoms and result in a five-membered metallocycle (Fig 2.6a). The monohydrazides function as bidentate ligands (Fig 2.6a) while dihydrazides function as asymmetric or symmetric bis-bidentate ligands (Fig 2.6b) (Miminoshvili 2009). These compounds exist in either amide or imide form depending on pH of medium (Fig 2.7).

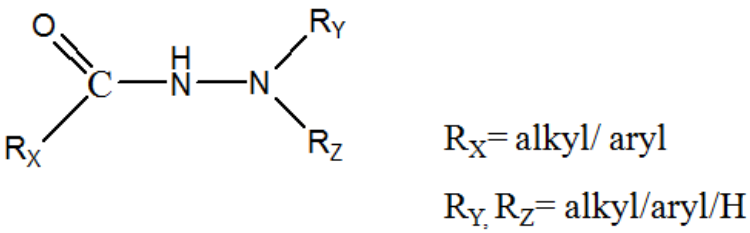


Fig 2.5 General structure of acid hydrazides

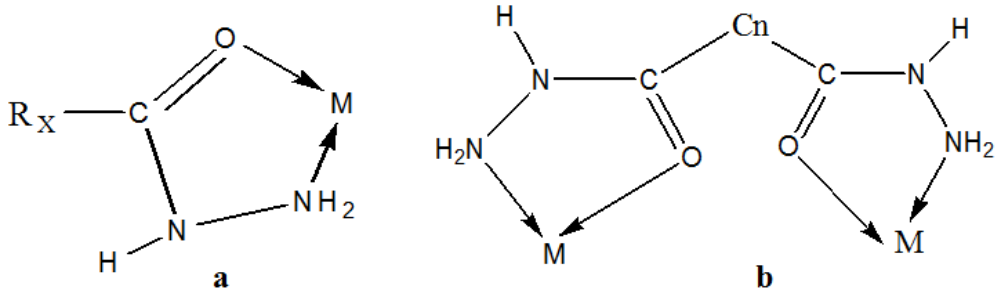


Fig 2.6 Monohydrazide as bidentate ligand **(a),** dihydrazide as bis-bidentate legend **(b)**

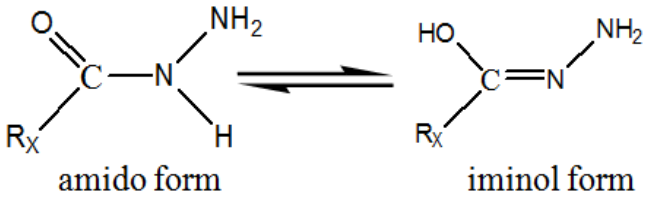


Fig 2.7 Representation of amido/iminol isomerism of acid hydrazides

2.2.2 Applications of acid hydrazides

Acid hydrazides, their derivatives and metal complexes exhibit antitubercular, antimalarial, anticonvulsant, anti-inflammatory, anti HIV, antitumor, antibacterial and antifungal properties (Zemlyakow *et al*., 1997, Semina *et al*., 1998, Kulakov *et al*., 2007, Zemtosova *et al*., 2008, Joshi *et al*., 2008, Galal *et al*., 2009, Wardakhan *et al*., 2013, Kumar *et al*., 2013). Aromatic polyamide hydrazides possess properties such as easy processability into fibres and films, high tensile strength and high electrical conductivity of their complexes with transition metals (Mohamed 1994). Palladium (II) complexes with benzo and pyridine carbohydrazides possessed antioxidant properties. These complexes were reported to be more effective inhibitors of superoxide and DPPH free radical than free hydrazides (Ain *et al*., 2013). Aryl hydrazides are the versatile resin-linkers for the rapid and efficient solid phase syntheses of a variety of chemically modified peptides (Woo *et al*., 2007). Aromatic hydrazides have been investigated as stabilizers for rigid poly vinyl chloride (PVC) against oxidative thermal degradation (Mohamed 1997). Hydrazides of amino acids are used as carboxyl protecting agents in peptide industry. Some organic acid hydrazides; such as salicylic acid hydrazide, benzoic acid hydrazide and cinnamic acid hydrazide are good corrosion inhibitors of mild steel in hydrochloric acid media (Quraishi *et al*., 2001). Thiosalicylhydrazide-modified magnetic nanoparticles have been reported for removing heavy metal ions from industrial waste water (Zargoosh *et al*., 2013). Derivatives of hydrazides are shown to exhibit strong gelation ability in benzene, chloroform and 1,2-dichloroethane (Zhang and Li., 2008).

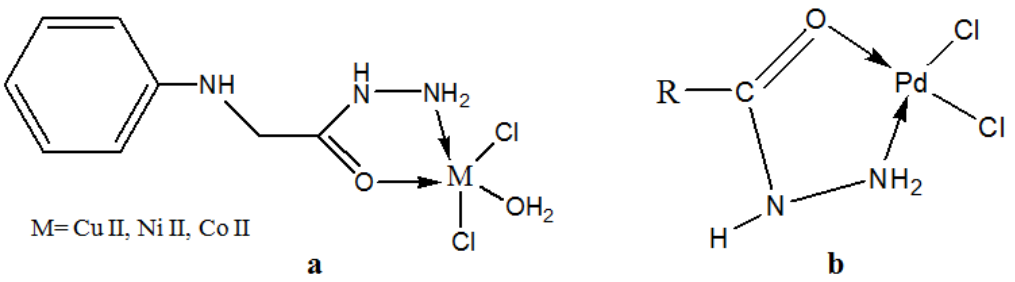


Fig 2.8 Some hydrazide metal complexes

2.2.3 Isoniazid

Isonicotinic acid hydrazide, also known as isoniazid (INH) is an important antituberculosis agent and is employed in treatment of tuberculosis in combination of other drugs such as rifampicin, ethambutol (Ziskind *et al*., 1953, Toit *et al*., 2006). This combination therapy approach considerably reduces the period required for treatment. It is a prodrug and must be activated by KatG enzyme of *M. tuberculosis*. Isoniazid inhibits the synthesis of mycolic acid, which is an essential component of the mycobacterial cell wall (Winder *et al*., 1970).

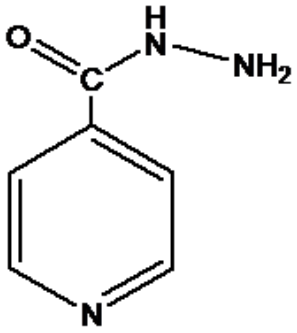


Fig 2.9 Isoniazid

2.3 Methods of hydroxamic acids and acid hydrazides syntheses

Biological significances of hydroxamic acids and acid hydrazides can be gauged from the number of developed chemical and enzymatic methods of synthesis. Chemical methods of synthesis from esters, acids and aldehydes require various organic solvents,

highly alkaline reaction medium, sometimes nitrogen atmosphere, coupling reagents for acid activation, O/N- protected hydroxylamine, an additional step of catalytic hydrogenation with palladium to release the free hydroxamic acid and/or refluxing of few hours under basic conditions. Enzymatic synthesis of hydroxamic acid/acid hydrazide is an interesting alternative to chemical synthesis method. Biocatalytic methods for hydroxamic acids/acid hydrazides syntheses utilize acyltransferase activity of amidase and lipase enzymes (Fournand and Arnaud 2001, Hacking *et al*., 2001). Microbial enzymes catalyse reactions under mild physiochemical conditions.

2.3.1 Chemical methods for hydroxamic acids syntheses

Chemical methods for hydroxamic acids syntheses involve reaction of carboxylic acids, esters, acid anhydride, acid chloride and aldehydes with either hydroxylamine or derivative of hydroxylamine in strong basic medium. If derivative of hydroxylamine is used, protected hydroxamic acid is formed and one extra step of catalytic hydrogenation with palladium is required to synthesize free hydroxamic acid.

2.3.1.1 From activated carboxylic acids

The common method for the preparation of hydroxamic acids is via the reaction of O/N-protected hydroxylamine such as NH_2-O-Bn, N-t-BOC-O-THP, O-bis (phenoxycarbonyl) hydroxylamine and N-t-BOC-O-TBDMS, with activated carboxylic acids (Devocelle *et al*., 2003, Kokare *et al*., 2007). These derivatives of hydroxylamine are expensive and some are not available commercially. The synthesis of hydroxamic acid derivative is followed by catalytic hydrogenation with palladium to get the desired product, hydroxamic acid (Nikam *et al*., 1995). Each method differs by the involvement of activating group. Different methods of synthesis are shown to use peptide coupling reagents such as chloroformates, DCC, BOP or phosphonic derivatives (Luca *et al*., 2001). Coupling reagents are toxic and expensive in various cases, and their removal after reaction completion may be difficult. To obtain main reaction product, extra purification steps are often required due to various side reactions. Luca et al. (2001), used different [1,3,5] triazine derivatives as the coupling agents. They treated carboxylic acid with 2-chloro-4, 6-dimethoxy-[1,3,5] triazine (CDMT) and *N*-methylmorpholine (NMM) in THF to form activated ester. CDMT is an irritating agent to respiratory system, skin and eyes hence other coupling agents; 4-(4, 6-dimethoxy [1,3,5] triazin-2-yl)-4-

methylmorpholinium chloride (DMTMM) and cyanuric chloride were also used. The reaction was carried out under the same conditions employed before and lower conversions were obtained than those when reaction was performed with CDMT.

2.3.1.2 From esters

The economical way of making hydroxamic acids and its derivatives is the reaction of hydroxylamine or o-benzylhydroxylamine with esters in presence of a strong base (Pinung *et al*., 1995, Ho *et al*., 2005). It eliminates the activation step as required in case of carboxylic acids, but to overcome poor electrophilicity of unactivated esters, use of tri methyl aluminium ($AlMe_3$) is required (Pinung *et al*., 1995, Gissot *et al*., 2005). The method is limited in scope as $AlMe_3$ is hazardous and very reactive. Reaction of hydroxylamine with esters involves use of organic solvents such as THF, methanol or ethanol and sometimes nitrogen atmosphere. Reddy et al. (2000), reported that these reactions do not proceed under neutral conditions (pH=7) and always require an alkaline medium (pH>10) for reaction progress. Gissot et al. (2005), tested different organic bases in the reaction of ethyl benzoate with o-benzylhydroxylamine in THF at -78 °C. They reported that at least 3 equivalent of base was needed for this reaction to neutralise hydrochloric acid and to form nucleophilic N-centred anion. Sodium and lithium hexamethyldisilazide were found to be efficient bases. The synthesis from esters generally takes few hours to 3 days and after completion, reaction media needs to be neutralized by concentrated HCl for product recovery. Hydroxamic acid synthesis from esters under basic conditions is not appropriate with ester derivatives that contain base sensitive groups (Reddy *et al*., 2000).

2.3.1.3 Form aldehydes

Syntheses of hydroxamic acids from aldehydes follows Angeli and Rimni reaction in which N-hydroxybenzenesulphonamide is reacted with aldehyde in presence of strong basic conditions and methanol. The reaction results in required hydroxamic acid along with the benzensulfinic acid as a by-product which is difficult to separate from the main product (Porcheddu and Giacomelli, 2006).

2.3.2 Chemical methods of acid hydrazides syntheses

2.3.2.1 From esters

The most common method employed in acid hydrazide synthesis is hydrazinolysis of corresponding ester with hydrazine monohydrate in alcoholic solution under basic conditions. The reaction involves refluxing methyl or ethyl ester with an excess of hydrazine monohydrate, with or without addition of a solvent such as ethanol (Yale *et al*., 1953). During hydrazides syntheses from esters, some highly reactive esters yield considerable amount of diacylhydrazine, a by-product. While less reactive esters may need harsh reaction conditions and longer reaction time (from few hours to 2-3 days) to complete the process. Generally these reactions are spontaneous, exothermic and complete in 5-9 hr. It is hazardous and energy intensive reaction and can lead to decomposition or degradation of desired products. This method is not suitable for α, β- unsaturated esters, as the main product of reaction is pyrazolidinone resulting from an undesired intramolecular Michael type addition (Cacic *et al*., 2006).

2.3.2.2 From carboxylic acids

Acid hydrazide is also synthesized by reaction of carboxylic acid with hydrazine in presence of coupling reagents. This method usually results in low yield due to an extra acid activation step and complex product recovery. Different coupling agents such as 1-hydroxybenzotriazole (HoBt), Bis (trimethylsilyl) acetamide and 1-(3-dimethylaminopropyl)-3-(ethylcarbodimide) hydrochloride (EDC) have been used for hydrazides syntheses. During the reaction; acids, coupling reagent, hydrazine and hydrazide react with each other to form different by-products. It was observed that the sequence of reagents addition affected the reaction products and yield. These impurities were very difficult to remove from the desired product. Modification of the addition sequence of reagents gave better results in terms of higher yield and product purity (Zhang *et al*., 2002). Another method for hydrazide synthesis from carboxylic acid involves use of microwave irradiation. The reaction mixture containing carboxylic acid and hydrazine was irradiated under microwaves for 60-200 seconds at 900 Watt at 2.45 GHz in absence of any solvent (Saha *et al*., 2010).

2.3.2.3 From acid chloride and acid anhydride

Acid chloride and anhydride approach to synthesize hydrazides results in several impurities due to side reactions, low yields and incomplete reaction. The reaction also shows diacylation because of highly reactive nature of these substrates.

2.3.3 Enzymatic methods of hydroxamic acids and acid hydrazides syntheses

Enzymatic methods for hydroxamic acids and acid hydrazides syntheses utilize acyltransferase activity of amidase and lipase enzymes (Fournand *et al*., 1998a, 1998b, Dadd *et al*., 2001, Pandey *et al*., 2011, Vaysse *et al*., 1996, Yadav and Borkar, 2010). Lipases are acyltransferase enzymes like amidases. Enzymatic syntheses of hydroxamic acids and acid hydrazides depend on the co-substrate used in the reaction mixture. Both hydroxylamine and hydrazine are efficient nucleophiles and show nucleophilic attack on acyl enzyme complex leading to formation of hydroxamic acid and acid hydrazide respectively due to transfer of acyl group of substrate to co-substrate. Lipases are used for hydroxylaminolysis and hydrazinolysis of fatty acids or their esters leading to formation of corresponding fatty hydroxamic acids and fatty hydrazides in non aqueous media. However amidases are used for syntheses of short and medium chain length aliphatic, aromatic, heterocyclic hydroxamic acids and acid hydrazides from corresponding amides in aqueous media. For a biocatalytic reaction to take place, different parameters are needed to be optimized because enzymes are biological catalysts and reaction conditions can affect their catalytic activity that will lead to low bioconversion with poor product yield.

2.4 Advantages of enzymatic methods over chemical synthesis methods

As an alternative to chemical synthesis, environmental friendly biocatalytic route for hydroxamic acids and acid hydrazides syntheses have been suggested in various literatures (Fournand *et al*., 1998a, 1998b, 2001, Kobayashi *et al*., 1999, Dadd *et al*., 2001, Sharma *et al*., 2011, Bhatia *et al*., 2013). Major advantages of the biocatalytic route are mild reaction conditions, reaction media devoid of organic solvents, cheaper substrates, good bioconversion yield in shorter time period with minimum by-products formation and ease of enzyme immobilization. Amidase and lipase catalysed enzymatic methods are simple, economical and convenient as it does not require any crucial work-up after reaction completion that involves acidification, doesn't require toxic coupling reagents and

expensive O/N-protected hydroxylamine, omit an extra step of catalytic hydrogenation of hydroxamic acid derivatives with palladium to release free hydroxamic acid.

2.5 Detailed study of biocatalytic route to hydroxamic acids and acid hydrazides syntheses

2.5.1 Lipase

Lipases (EC 3.1.1.3) are ubiquitous enzyme with considerable physiological potential and industrial applications. These are widely distributed in microbes, plants and animals. Most of the industrial microbial lipases are of fungal origin as fungal enzymes are generally extracellular that facilitates their easy extraction from media. Lipases show broad substrate specificities and various natural oils, esters of fatty acids, fatty acids and synthetic triglycerides are choice of substrates (Aravindan *et al*., 2006, Kaushik *et al*., 2010, Kapoor and Gupta, 2012). They catalyse the hydrolysis of fats into fatty acids and glycerol in aqueous media at water-lipid interface while catalyse the reverse reaction in organic solvents (Reetz 2002). Lipases show specificity towards fatty acid (position of fatty acid in glycerol backbone, its chain length and degree of unsaturation) and also the alcohol part of substrates (Gaur *et al*., 2008, Kapoor and Gupta, 2012). Many lipases show the phenomenon of interfacial activation in which a helical oligopeptide unit shields the lipase active site and upon interaction with a hydrophobic surface it undergoes movement in a way to expose the active site. This feature distinguishes lipase from esterase (Reetz 2002, Kapoor and Gupta, 2012). Lipases are choice of enzymes in various industrial processes such as detergents, baking, oils and fat, leather and paper processing, biopolymer synthesis, biodiesel production, treatment of fat containing waste effluent and enantiopure syntheses of pharmaceuticals (Jaeger and Eggert, 2002, Hasan *et al*., 2006, Patil *et al*., 2011).

2.5.1.1 Mechanism of action of lipase

Lipases catalyze a wide range of bioconversion reactions such as hydrolysis, esterification, transesterification (alcoholysis and acidolysis) and aminolysis (Reetz 2002) (Fig 2.10). The active site of lipase is characterized by a catalytic triad composed of serine, histidine and aspartate amino acids. The mechanism of lipase catalysed hydroxamic acids and acid hydrazides syntheses follow a ping pong mechanism and two tetrahedral intermediates are formed during the reaction. Carbon from the ester bond of substrate

undergoes nucleophilic attack by hydroxyl group of serine residue present at active site of lipase resulting in formation of a tetrahedral intermediate. This intermediate gives an acyl-enzyme complex which leads to release of an alcohol molecule. A water or hydroxylamine/ hydrazine molecule then attacks the acyl enzyme complex to give another tetrahedral intermediate. This intermediate releases the final product that may be fatty acid, fatty hydroxamic acid or fatty acid hydrazide depending on the co-substrate that attack at acyl enzyme complex as a nucleophile (Kapoor and Gupta, 2012).

2.5.1.2 Lipase catalysed hydroxamic acids and acid hydrazides syntheses

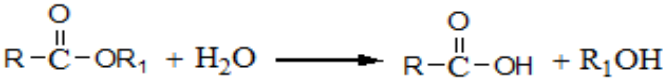
Lipases have been used for syntheses of a broad range of fatty hydroxamic acids and fatty hydrazides using different substrates. Developments in lipase catalysed synthesis methods were reported from time to time with use of different substrates, solvents and a range of biocatalysts. Servat et al. (1990) reported the enzymatic syntheses of fatty hydroxamic acids using lipase of *Mucor miehei* by reaction of fatty acids or methyl esters of fatty acid with hydroxylamine. Different reaction parameters such as temperature, biocatalyst quantity, mole ratio of substrates and reaction time were studied to obtain the optimum reaction conditions. Lipase from *Candida parapsilopsis* was used as biocatalyst for fatty hydroxamic acid production in a biphasic medium. Both fatty acids and esters of fatty acid were used to study their effects on enzyme catalysed acyl group transfer on hydroxylamine. Esters of fatty acid were more efficient substrates than free fatty acids. It was found that this lipase catalysed both aminolysis and synthesis simultaneously but aminolysis was the preferred reaction (Vaysee *et al*., 1997). Yadav et al. (2005) reported the synthesis of isoniazid, an antitubercular drug from the reaction of ethyl isonicotinate with hydrazine hydrate in 1, 4-dioxane solvent with different immobilized lipases and studied the kinetics of reaction. Reaction resulted in 52% conversion in 24h. Synthesis of fatty hydroxamic acid from palm oil and hydroxylamine was reported in a biphasic medium in presence of immobilized lipase (Suhendra *et al*., 2005). The use of immobilized lipase provided a simple separation of enzyme from reaction mixture. Fatty acid hydrazides were synthesized effectively from palm oils using immobilized lipozyme enzyme with hydrazine hydrate at neutral pH (Mohamad *et al*., 2008). Benzoic acid hydrazide synthesis has been done from phenyl benzoate and hydrazine using an immobilized lipase. Three different immobilized lipases were screened to select the best lipase for hydrazinolysis (Yadav and Borkar, 2010). Al-Mulla et al. (2010) reported lipase catalysed syntheses of fatty thiohydroxamic acids from palm oil and thiohydroxamic acid

in presence of hexane. Phenyl hydroxylaminolysis of canola and palm kernel oil was studied in biphasic organic/aqueous medium by lipozyme catalysed reaction and the procedure was found applicable for other low cost and easily available vegetable oils (Jahangirian *et al*., 2011a). Jahangirian et al. (2011b) synthesized fatty hydroxamic acid derivatives using Lipozyme TL IM biocatalyst in a biphasic medium by reaction of palm kernel oil and N-substituted hydroxylamine.

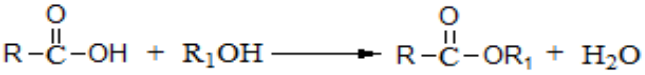
Table 2.1 Lipase catalysed hydroxamic acids and acid hydrazides syntheses

Enzyme	Substrate	Co-substrate	Product	References
Lipase from *Mucor miehei*	Fatty acids, fatty acid esters	Hydroxylamine	Fatty hydroxamic acid	Servat et al. (1990)
Lipase from *Candida parapsilosis*	Fatty acids, methyl esters of fatty acids	Hydroxylamine	Fatty hydroxamic acid	Vaysse et al. (1996)
Novozyme 435 and Lipozyme IM20	Ethyl isonicotinate	Hydrazine hydrate	4-pyridine carboxylic acid hydrazide	Yadav et al. (2005)
Lipozyme	Palm oil, palm olein, palm kernel, palm stearin	Hydrazine hydrate	Fatty acid hydrazide	Mohamad et al. (2008)
Candida antarctica Lipase B	Phenyl benzoate	Hydrazine hydrate	Benzoic acid hydrazide	Yadav and Borkar (2010)
Lipase from *Mucor miehei* and *candida antarctica*	Fatty acid esters, olive oil, sesame oil, corn oil	Hydroxylamine	Fatty acid hydrazide	Carpenter et al. (2010)
Lipozyme TL IM and Lipozyme RM IM	Canola oil, palm stearin oil, palm kernel oil	Phenyl hydroxylamine	Phenyl fatty hydroxamic acid	Jahangirian et al. (2011a)
Lipozyme TL IM	Palm kernel oil	(N-methyl, N-propyl, N-benzyl) hydroxylamines	(N-methyl, N-propyl, N-benzyl) hydroxamic acids	Jahangirian et al. (2011b)

1. Hydrolysis

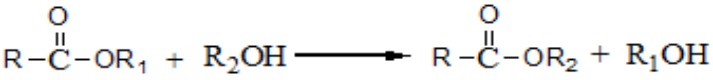


2. Esterification

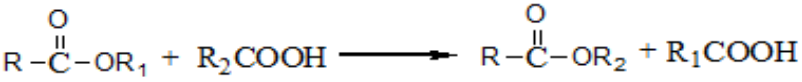


3. Transesterification

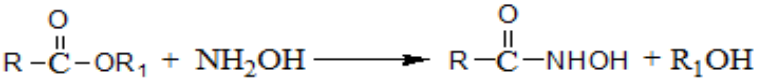
a. Alcoholysis



b. Acidolysis



4. Aminolysis



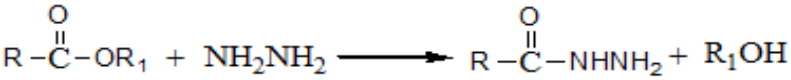
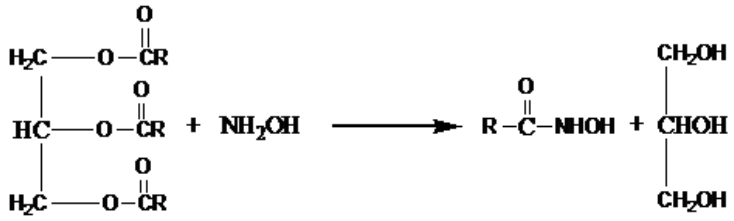


Fig 2.10 Lipase catalyzed reactions (adapted from Kapoor and Gupta, 2012)



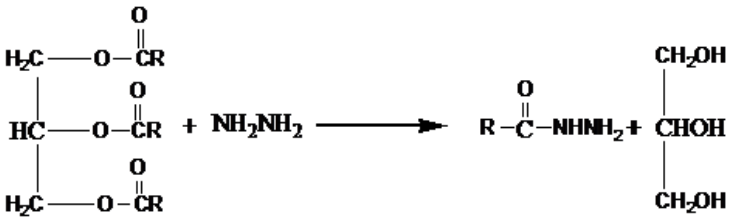


Fig 2.11 Reaction schemes of lipase catalyzed hydroxylaminolysis and hydrazinolysis of triacylglycerides

2.5.2 Amidase

Amidases [EC 3.5.1.4] are widely distributed enzymes in nature and have diverse applications (Ramakrishna *et al*., 1999, Fournand and Arnaud, 2001, Banerjee *et al*., 2002, Sharma *et al*., 2009). Amidases are involved in carbon and nitrogen metabolism in both prokaryotic and eukaryotic cells (Banerjee *et al*., 2002). Amidases catalyse the hydrolysis of amide to carboxylic acid and ammonia. In a bi-enzymatic pathway, amidase in combination with nitrile hydratase is involved in bioconversion of nitriles to acids in two steps with amide as an intermediate. Therefore these enzymes are widely employed for the syntheses of important organic acids through biotransformation of nitriles and amides. Most of the amidases are also reported to exhibit acyltransferase activity besides amide hydrolysis (Fournand and Arnaud, 2001, Egorova *et al*., 2004, Pandey *et al*., 2011). Due to acyltransferase activity microorganisms synthesize hydroxamic acids to intake environmental iron, exploiting their chelating properties. Acyltransferase activity of amidases is used mainly for syntheses of pharmaceutically important hydroxamic acids and acid hydrazides.

Purification of amidases from various microorganisms utilizing different steps has been reported by many researchers (Table 2.2). These purified amidases were characterized in term of molecular weights, number of subunits, optimum pH, optimum temperature and results are shown in table 2.3. Amidases are found to have broad range of substrate specificity. Some amidases have affinity only for aliphatic amides, while others catalyze biotransformation of broad range of amides including aliphatic, aromatic and heterocyclic amides. Some amidases are also reported to have affinity for amides of amino acids. Substrate specificity profiles of various amidases are summarized in table 2.4.

Table 2.2 Different purification steps, yield (%) and purification fold of amidases from various microorganisms

Microorganism	Purification steps	Yield (%)	Purification fold	References
Arthrobacter sp. J-1	Seven: Protamine sulphate, DEAE-cellulose, ASF, Hydroxyapatite, 1st Sephadex G-200, 2nd Sephadex G-200, 3rd Sephadex G-200	3.3	47	Asano *et al*., 1982
Rhodococcus sp. strain NCTR 4	Four: Acetone fractionation, ASF, DEAE-Sepharose, Mono Q, Superose 12	20	138	Nawaz *et al*., 1994
Mycobacterium neoaurum ATCC 25795	Three: ASF, HiLoad Superdex, Mono-Q	12	280	Hermes *et al*., 1994
Klebsiella pneumoniae NCTR 1	Four: Acetone, DEAE, ASF, Gel filtration	10	168	Nawaz *et al*., 1996
Rhodococcus erythropolis MP50	Three: Ion exchange chromatography, Phenyl sepharose, Desalting step	25	6.18	Hirrlinger *et al*., 1996
Rhodococcus rhodochrous M8	Two: Fractionation with Propanol, Mono Q	57	88	Kotlova *et al*., 1999
Ochrobactrum anthropi SV 3	Four: ASF, DEAE Toyopearl, Butyl Toyopearl, FPLC Superdex 200	7.81	10.9	Komeda and Asano 2000
Ochrobactrum anthropi NCIMB 40321	Four: ASF, Mono Q, Superdex 200, Alkyl superose	35	98	Sonke *et al*., 2005
Geobacillus pallidus RAPc8	Two: Heat treatment, S-300 HR	247	6	Makhongela *et al*., 2007
Brevibacterium iodinum TPU 5850	Four: ASF, DEAE-Toyopearl, Butyl-Toyopearl, MonoQ HR10/10	52	17.4	Komeda and Asano 2008
Rhodococcus erythropolis AJ270	Two: Ni-NTA, Superose S10-300	33.8	6.9	Xue *et al*., 2011
Geobacillus pallidus BTP-5x MTCC 9225	Three: DEAE-I, Sephacryl S-200, DEAE-II	6.7	6.2	Sharma *et al*., 2013
Alcaligenes sp. MTCC 10674	Three: ASF, DEAE-Sepharose, Sephacryl-100	6	6.3	Bhatia *et al*., 2013
Geobacillus subterraneus RL-2a	Four: Heat treatment, ASF, DEAE, Sephadex-200	9.54	52.04	Mehta *et al*., 2013

Table 2.3 Biochemical properties of amidases purified and characterized from various microorganisms

Microorganism	Nature	Substrate specificity	Molecular weight (MW)		Optimum temperature (°C)	Optimum pH	References
			Native (kDa)	No. Of subunits and MW (kDa)			
Arthrobacter sp. J1	Inducible	Aliphatic amides	320	8 (39)	35	7.0	Asano *et al*., 1982
Brevibacterium sp. R312	Inducible	Aliphatic amides (wide spectrum)	180	4 (43)	-	-	Thiery *et al*., 1986
Brevibacterium sp. R312	Inducible	Aryloxypropionamide	120	2 (46)	-	-	Mayaux *et al*., 1990
Mycobacterium neoaurum ATCC 25795	-	α-H- and α-alkyl-substituted amino acid amides	136	(40)	50	8.0-9.5	Hermes *et al.* 1994
Rhodococcus sp. strain NCTR 4	Constitutive	Aliphatic amides	360	- (44.5)	40	8.5	Nawaz *et al*., 1994
Pseudomanas chlororaphis B23	Inducible	Aliphatic and aromatic amides	105	2 (54)	50	7.0-8.6	Ciskanik *et al.* 1995
Klebsiella pneumonia NCTR 1	Inducible	Aliphatic amides	62	Monomer	65	7.0	Nawaz *et al*., 1996
Rhodococcus erythropolis MP50	Inducible	Aromatic amide	480	- (61)	55	7.5	Hirrlinger *et al*., 1996
Rhodococcus rhodochrous M8	Constitutive	Aliphatic amide	150	4 (43)	55-60	7.0	Kotlova *et al*., 1999
Ochrobactrum anthropi SV3	Inducible	Amino acid amides	40	-	45	8.5-9.5	Komeda and Asano 2000
B. stearothermophilus BR388	Inducible	Wide spectrum amidase	-	-	55	7.0	Cheong and Oriel 2000

Table 2.3 Continued

Microorganism	Nature	Substrate specificity	Molecular mass		Optimum temperature (°C)	Optimum pH	References
			Native (kDa)	No. Of subunits and MW (kDa)			
Sulfolobus solfataricus	-	Aliphatic and aromatic amides	55	Monomer	7.5	95	d'Abusco *et al*., 2001
Agrobacterium tumefaciens d3	Inducible	Aromatic amides	490	8 (63)	-	-	Trott *et al*., 2001
*Brevibacillus borstelensis*BCS-1	-	D-amino acid amides	199	6 (30)	70	9.5	Baek *et al.* 2003
Pseudonocardia thermophila	Constitutive	Broad substrate spectrum	108	2 (52)	7.0	70	Egorova *et al*., 2004
Sulfolobus todaii strain 7	-	S-2-phenyl-propionamide	47	Monomer	7.0-8.0	75	Suzuki and Ohta 2006
Brevundimonas diminuta TPU 5720	-	L-amino acid amides	-	-	7.5	50	Komeda *et al*., 2006
Geobacillus pallidus RAPc8	-	Aliphatic and unsaturated amides	218	6 (38)	7.0	50	Makhongela *et al*., 2007
Brevibacterium iodinum TPU 5850	-	D-amino acid amides	290	10 (30)	7.2	35	Komeda and Asano 2008
Rhodococcus sp. N-771	-	Aliphatic and aromatic amides	107	2 (54.7)	5.9	55	Ohtaki *et al.*,2010
Nesterenkonia strain AN1		Small aliphatic amides	-	2 (30.1)	6.5-7.5	30	Nel *et al.*, 2011
Geobacillus pallidus BTP-5x MTCC 9225	Inducible	Small aliphatic amides	158	4 (38.5)	8.0	60	Sharma *et al*., 2012
Geobacillus subterraneus RL-2a	constitutive	broad spectrum of amides	111	3 (37)	6.5	70	Mehta *et al*., 2013
Alcaligenes sp. MTCC 10674	-	-	114	2 (52) (49)	8.0	45	Bhatia *et al*., 2013

Table 2.4 Substrate specificity of amidases from various microorganisms

Microorganism	Substrate specificity	Selectivity	References
Brevibacterium strain R312 DSM 6230	2-(4)-hydroxyphenoxylpropionamides, 2-aryl and 2-aryloxylpropionamides L-carnitine amide	(R)-enantioselective (L)-stereoselective	Mayaux *et al*., 1990 Joeres and Kula 1993
Rhodococcus sp. Strain NCTR 4	Aliphatic amides: acetamide, propionamide, butyramide, isobutyramide, acrylamide	-	Nawaz *et al*., 1994
Mycobacterium neoaurum ATCC 25795	Broad range of α-H and α-alkyl substituted amino acid amides, aliphatic amides: acetamide and propionamide	(L)- stereoselective	Hermes *et al*., 1994
Klebsiella pneumoniae NCTR 1	Aliphatic amides: acetamide, butyramide, acrylamide, propionamide	-	Nawaz *et al*., 1996
Rhodococcus erythropolis MP50	Aliphatic amides: acetamide, propionamide, butyramide, hexanamide, isobutyramide, acrylamide etc., aromatic amides: benzamide, phenylacetamide, aromatic α-substituted amides: 2-phenylpropionamide, naproxen amide, ketoprofen amide	(S)-enantioselective	Hirrlinger *et al*., 1996
Rhodococcus rhodochrous M8	Aliphatic amides: acrylamide, acetamide, propionamide	-	Kotlove *et al*., 1999
Ochrobactrum anthropi SV3	D-amino acid amides	(D)-stereoselective	Komeda and Asano 2000
Sulfolobus solfataricus	Very broad substrate spectrum	(S)-enantioselective	D'Abusco *et al*. 2001
Brevibacillus borstelensis BCS-1	D-amino acid amides: D-methioninamide, D-phenyl-alaninamide, D-leucine-p-nitroanilide etc., esters: D-alanine benzyl ester, arylamides	(D)-stereoselective	Baek *et al*., 2003

Table 2.4 Continued

Microorganism	Substrate specificity	Selectivity	References
Pseudonocardia thermophila	Aliphatic amides, aromatic amides, heterocycles, amino acid amides	(S)-enantioselective	Egorova *et al*., 2004
Ochrobactrum anthropi NCIMB 40321	Aliphatic and aromatic α-hydrogen-α-amino acid amides, α,α-disubstituted α-amino acid amides, α-hydroxy acid amide, DL-mandelic acid amide, α-*N*-hydroxyamino acid amide, DL-*N*-hydroxyphenylalanine amide	(L)-stereoselective	Sonke *et al*., 2005
Geobacillus pallidus RAPc8	low molecular weight aliphatic amides: acetamide, acrylamide, propionamide, flouroacetamide, formamide, isobutyramide, lactamide	(D)- stereoselective towards lactamide	Makhongela *et al*., 2007
Brevibacterium iodinum TPU 5850	D-methioninamide, D-glutaminamide, D-phenylalaninamide, D-lysinamide	(D)-stereoselective	Komeda and Asano 2008
Nesterenkonia strain AN1	Only aliphatic amides: propionamide, fluoroacetamide, butyramide, acetamide	-	Nel *et al*., 2011
Rhodococcus erythropolis AJ270	Aliphatic amides: acetamide, propionamide, acrylamide, aromatic amide: benzamide; α-substituted amides, O-benzylated β-hydroxy amides, N-benzylated β-amino amides	(S)-enantioselective	Xue *et al*., 2011
Geobacillus pallidus BTP-5x MTCC 9225	Aliphatic amides- acrylamide, acetamide, propionamide, lactamide	-	Sharma *et al*., 2012
Geobacillus subterraneus RL-2a	Aliphatic amides- formamide, lactamide, propionamide, butyramide, isobutyramide, methacrylamide, valeramide, aromatic amides: benzamide, sulfanilamide, heterocycles: nicotinamide, isonicotinamide, amino acid amides: L-leucinamide, L-prolinamide	(L)- stereoselective	Mehta *et al*., 2013

2.5.2.1 Amidase producing organism

Most of the reported amidases are of bacterial origin from different genera: *Rhodococcus, Pseudomonas, Bacillus, Brevibacterium, Alcaligenes, Arthrobacter, Nocardia, Mycobactrium, Micrococcus, Helicobacter and Streptomyces*. Amidases from different bacterial sources possess diverse range of substrate specificity. Ergova et al. (2004) purified an amidase from *Pseudomonas aeroginosa* having substrate affinity for a wide range of amides. On the other hand amidase from DSM 6230 appeared to have specificity only for L-carnithine amide (Joeres and Kula, 1993). More than one amidases have been reported in strain *Rhodococcus sp.* R312; an aliphatic amidase, an enantioselective amidase and an L-amino amidase (Arnaud *et al*., 1976, Fournand and Arnaud, 2001). Three amidases have also been reported in a fungal strain *Aspergillus nidulans*; a formamidase, an acetamidase and a wide spectrum amidase (Hynes and Pateman, 1970). Another report of amidase from fungal source includes an acetamidase from *Aspergillus candidus* (Rahim *et al*., 2003). There are some reports in literature regarding the enzymes from yeasts that can catalyse the formation of ammonia and carboxylic acid from amide. A purified protein from *Torula cremoris* was reported to hydrolyse only nicotinamide (Joshi and Handler, 1962). On the other hand Brady (1969) purified a protein from cells of *Candida utilis* which efficiently catalysed acrylamide, acetamide, propionamide, butyramide, hexanamide and valeramide hydrolysis. Kammermeier-Steinke et al. (1993) partially purified a regio- and stereoselective peptide amidase from *Citrus sinensis* L. Arreaza et al. (1997) described the presence of an anandamide amidase or N-arachidonyl ethanolamide deacylase in rat brain. Anandamide along with fatty acid ethanolamides and oleamide was substrate for this amidase.

2.5.2.2 Classification of amidase

The amidase enzyme has been classified on the basis of its catalytic activity, amino acid sequence and phylogenetic relationship (Fournand and Arnaud, 2001, Sharma *et al*., 2009).

Substrate specificity profile of an amidase is the basis for the catalytic activity based classification. Two types of amidases are illustrious. The first type of amidase is aliphatic amidases that have affinity for only short chain aliphatic amides like acetamide, acrylamide and propionamide. The aliphatic amidases from *Arthrobacter sp*. J1 (Asano *et al*., 1982), *Rhodococcus sp*. R312 (Maestracci *et al*., 1984), *Pseudomonas aeruginosa*

(Clarke 1970), *Methylophilus methylotrophus* (Silman *et al*., 1991), *Rhodococcus sp*. Strain NCTR 4 (Nawaz *et al*., 1994), *Klebsiella pneumonia* NCTR 1 (Nawaz *et al*., 1996), *Helicobacter pylori* (Skouloubris *et al*. 1997), *Rhodococcus rhodochrous* M8 (Kotlove *et al*., 1999), *Geobacillus pallidus* RAPc8 (Makhongela *et al*., 2007) and *Nesterenkonia* strain AN1 (Nel *et al*., 2011) belong to this category. The second type of amidases are amidases which have affinity for mid chain length aliphatic amides, aromatic amides and heterocyclic amides (Sharma *et al*., 2009). Generally second type amidases are associated with nitrile hydratase and are responsible for bacterial nitrile metabolism and also exhibit enantioselectivity. The aliphatic enantioselective amidases from *R. rhodochrous* J1 (Kobayashi *et al*., 1993), *Bacillus sp*. BR449 (Kim and Oriel, 2000), *Rhodococcus sp.* N-774 (Hashimoto *et al*., 1991), *R. Erythropolis* AJ270 (Xue *et al*.,2011), *Rhodococcus sp.* R312 (Fournand *et al*., 1998b), *Pseudonocardia thermophila* (Egorova *et al*., 2004), *R. erythropolis* MP50 (Hirrlinger *et al*., 1996), *Sulfolobus solfataricus* (D'Abusco *et al*., 2001), *Rhodococcus sp.* N-771 (Ohtaki *et al*., 2010) and *Geobacillus subterraneus* RL-2a (Mehta *et al*., 2013) belong to this group.

Considerable similarities in amino acid sequences of various nitrilase, b-alanine synthase, cyanide hydratase and aliphatic amidase (first group amidase having affinity for short chain aliphatic amides) have been observed after multiple alignment. Presence of numerous conserved motifs was observed in these proteins (Bork and Koonin, 1994). One of these conserved motifs possesses a nucleophilic cysteine, involved in catalysis. A different conserved motif contains an invariant glutamic acid that possibly also takes part in catalysis. Based on sequence similarities and resemblance in catalysed reactions, it can be predicted that these enzymes comprise a group having similar catalytic mechanism (sulfhydryl enzymes). On the other hand significant homology was observed in sequences of the second type of aliphatic amidase (amidase having affinity for wide substrate range), various indole acetamide hydrolase and 6-amino hexanoatecyclic dimer hydrolase. These enzymes contained a highly conserved motif (GGSS) and conserved different amino acids like glycine, aspartic acid and serine placed at 17, 19 and 23 amino acids downstream of GGSS sequence. Sequence similarity between these enzymes demonstrates that these enzymes represent a common amidase family and are distinct to nitrilase (Chebrou *et al*., 1996).

Evolutionary relationship of amidases

Aliphatic amidases appear to be associated evolutionary with nitrilases as they possess amino acid sequence similarity with them. These amidases and nitrilases are believed to be from a common ancestry and part of sulfhydryl enzyme group (Fournand and Arnaud, 2001). In this type of amidases an invariant cysteine residue at active site was reported which act as the nucleophile during catalytic mechanism. The second type of amidases (enatioselective amidases with wide substrate range) possesses a highly conserved signature sequence (GGSS). Kobayashi et al. (1997) determined that the real active site residues residing in this conserved signature sequence are Asp191 and Ser195. It was reported that the sequence containing Asp191 and Ser195 residues in amidase of *R. Rhodochrous* J1was also present within the active site sequences of aspartic proteinases. Consequently aspartic proteases and second type of amidases are probably evolutionary related.

2.5.2.3 Mechanism of action of amidases

The reaction mechanism of amidase catalysed acyltransferase reactions with hydroxylamine and hydrazine is shown in figure 2.12. Maestracci et al. (1986) studied the reaction kinetics of aliphatic amidase of *Rhodococcus sp*. 312. They used acetamide as substrate and hydroxylamine as co-substrate and shown the mechanism was of "Ping Pong Bi-Bi type" and both the hydrolysis and acyltransferase reactions involve the same mechanism. Amidase reacts with amide to give an intermediate acyl enzyme complex with the release of first product ammonia. The nucleophilic agent (co-substrate) water or hydroxylamine then reacts with acyl enzyme complex which leads to the formation of final product carboxylic acid or hydroxamic acid respectively with the release of enzyme. Later Kobayashi et al. (1998b) proposed the reaction mechanism of amidase catalysed hydrolysis. During the reaction, amino acid of amidase (nucleophile) attacks on the carbonyl group of substrate (amide) and results in the formation of a tetrahedral intermediate. The intermediate was transformed into an acyl enzyme complex with the release of ammonia. This acyl enzyme complex was subjected to nucleophilic attack by water leading to hydrolysis of amide to acid. Fournand et al. (1998a) determined that the wide spectrum amidase from *Rhodococcus sp.* R312 catalysed the acyltransferase reaction leading to transfer of acyl group of amide to another co-substrate hydrazine. Only few amidases have been reported to catalyse the acyl group transfer from amide to hydrazine

and the same reaction mechanism is involved here (Fournand *et al*., 1998a, Kobayashi *et al*., 1999). Hydrazine ($RCONHNH_2$), a weak base but due to α-effect it is a strong nucleophile and readily acts as efficient acyl group acceptor, leading to syntheses of acid hydrazides.

2.5.2.4 Amidase catalysed reactions

Amidases catalyse different types of reactions (Fig 2.13), such as amide hydrolase, amide acyltransferase, acid acyltransferase and ester acyltransferase reactions (Clark *et al*., 1970, Thiery *et al*., 1986, Fournand *et al*, 1998a, 1998b). Clark et al. (1970) reported amide hydrolysis and amide acyltransferase reactions for aliphatic amidase from *P. aerogenosa* in presence of water and hydroxylamine respectively. He also showed that corresponding esters and acids of acetamide and propionamide also acted as acyl group donor for acyltransferase reactions. However the ester acyltransferase activity was only 1% of amide acyltransferase activity. Later Fournand et al. (1998a, 1998b) also observed acyltransferase reactions with acids or esters as substrate but the reaction rates were much lesser than those reported with amide substrates. Hence only amide hydrolase and amide acyltransferase activities of amidase represent a significant route for acids, hydroxamic acids and acid hydrazides syntheses.

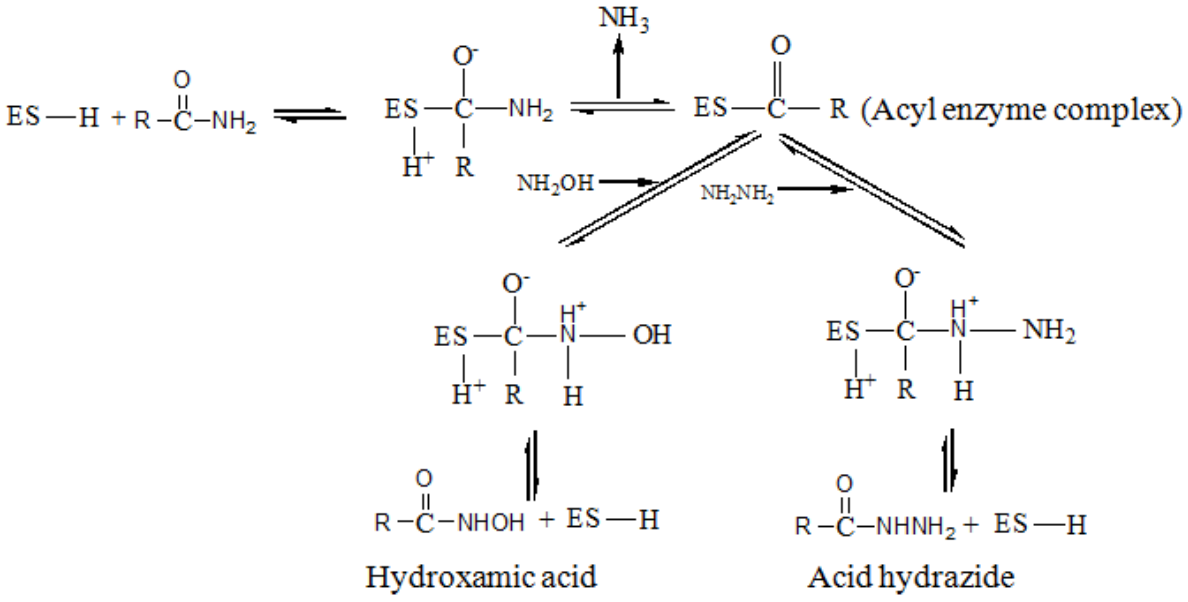
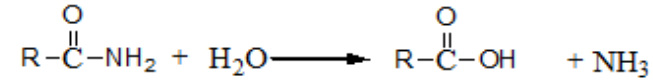
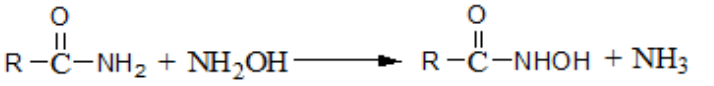


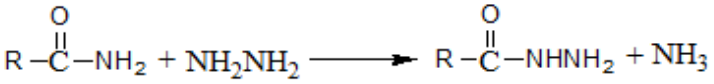
Fig 2.12 Mechanism of amidase catalyzed acyltransferase reaction for hydroxamic acids and acid hydrazides syntheses from amides (adapted from Sharma *et al*., 2009)

1. Amide hydrolysis

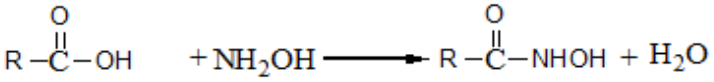


2. Amide transfer





3.Acid transfer



4. Ester transfer

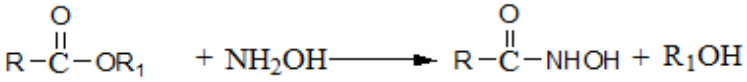


Fig 2.13 Amidase catalyzed reactions (Maestracci *et al*., 1986)

2.5.2.5 Amidase catalyzed amide hydrolase reactions

Nitriles (RCN) are important organic compounds in chemical industry that lead to formation of a variety of amides and carboxylic acids by hydrolysis. Chemical methods for nitrile hydrolysis require heating under strongly basic or acidic conditions resulting in low yield and undesirable side reactions. Such harsh conditions do not favour the hydrolysis of nitriles containing acid or alkaline labile functional groups. Enzyme catalysed nitrile hydrolysis is an efficient eco-friendly alternative to chemical processes because of milder reaction conditions, chemo-, stereo- and regioselectivity and high yield with less by-products. Nitrile metabolizing enzymes comprise of three classes of enzymes, nitrilase [EC 3.5.5.1], nitrile hydratase [EC 4.2.1.84] and amidase (Gong *et al*., 2012, Prasad and Bhalla, 2010, Sharma *et al*., 2009). Nitrilase catalyzes the direct conversion of nitriles to carboxylic acids and ammonia. In the second route, nitriles are metabolized in two steps by nitrile hydratase and amidase enzymes. In first step nitriles are converted to corresponding amides by nitrile hydratase and then amide is transformed to carboxylic acid and ammonia by amidase. Amide hydrolase activity of amidases has been utilized in many industrial applications (Section 2.6).

2.5.2.6 Amidase catalysed acyltransferase reactions for hydroxamic acids and acid hydrazides syntheses

Lots of reports are available on amidase catalysed acyltransferase reactions describing substrate specificity, reaction mechanism involved, enzyme kinetics, different acyl group donors and acceptors, affinity of acyl enzyme complex towards co-substrate (hydroxylamine and hydrazine) and reaction conditions. Earlier work on enzyme catalysed hydroxamic acid synthesis involves substitution of amide groups of asparagine and glutamine with hydroxylamine resulting in formation of aspartohydroxamic and glutamohydroxamic acids (Grossowicz *et al*., 1950). Later a purified enzyme from *M. avium* catalysed hydroxamic acid synthesis from butyric and valeric acid using hydroxylamine as co-substrate (Kimura 1959). Thiery et al. (1986) reported that aliphatic amidase from *Rhodococcus sp.* R312 catalysed the transfer of acyl groups of amides, esters and acids to hydroxylamine resulting in formation of hydroxamic acids. Hirrlinger and Stolz (1997) studied the amidase from *R. erthrypolis* MP50 for the production of optically active (S) 2-phenylpropionohydroxamate which was chemically transformed by lossen rearrangement to chiral amines. Simultaneously they observed that acyltransferase activities with acetamide, 2-phenylpropionamide and phenylacetamide substrates were higher as compared to amide hydrolase activities with same substrates.

First initiative towards bioprocess development for hydroxamic acid synthesis was taken by Fournand et al. (1997). A bioprocess was developed for bench scale production of acetohydroxamic acid using amidase from *Rhodococcus sp.* R312, immobilized on Duolite A-378 resin. After 90 min of incubation, 54-61% (mol/mol) conversion was achieved with 40-21% (w/w) purity. Further Fournand et al. (1998a) used the wide spectrum amidase from *Rhodococcus sp.* R312 overproduced in *E.coli.* to identify several potential acyl group donors and acyl group acceptors for acyltransferase reaction. Aliphatic carboxylic acids were found to be 10-1000 times less efficient as substrate than their corresponding short chain amides. It was also reported that acyl enzyme complex exhibited highest affinity for hydrazine and hydroxylamine because of their polarity. This recombinant amidase was shown to catalyse the syntheses of hydroxamic acids and acid hydrazides from corresponding amides and high bioconversion yield was obtained in short duration of time (10-60 min) with small amount of enzyme. Later the detailed kinetic study on acyltransferase reaction was done with the enantioselective amidase of the same strain and the reaction found to follow a “Ping Pong bi-bi” mechanism. The amidase possessed

acyltransferase activity for a wide spectrum of amides and was also enantioselective towards α-substituted amides. Only water and hydroxylamine have been found to be efficient acyl acceptors for this amidase and no acid hydrazide synthesis was reported when hydrazine was used as co-substrate (Fournand *et al*., 1998b). Kobayashi et al. (1999) reported that the amidase from *R. rhodochrous* J1 catalysed the acyl group transfer of a range of amides to hydrazine leading to acid hydrazides syntheses.

Biotransformation of benzonitrile instead of benzamide to benzohydroxamic acid by *R. rhodochrous* in presence of hydroxylamine was investigated by Dadd et al. (2001). Egorova et al. (2005) reported a thermoactive amidase from thermophilic actinomycete *P. thermophila* showing acyltransferase activity for a broad spectrum of amides. On the other hand amidase from *G. pallidus* was found to be active only on low molecular weight aliphatic amides (Makhongela *et al*., 2007). Sharma et al. (2011) worked on bioprocess development for acetohydroxamic acid production employing acyltransferase activity of amidase of *G. pallidus* BTP-5x MTCC and achieved 90-95% molar conversion (0.28 M) with 40% product purity after 20 min reaction time under optimized conditions. Later Pandey et al. (2011) reported an improved bioprocess for acetohydroxamic acid synthesis using DTT treated resting cell of *Bacillus sp.* ABP-6 and reported a higher molar conversion of 93% after 30 min at 45 °C in a one litre reaction mixture. After lyophilisation, 62 g powder containing 34% (w/w) acetohydroxamic acid was recovered. Recently Bhatia et al. (2013) reported a bioprocess for benzohydroxamic acid synthesis using amidase of *Alcaligenes sp*. MTCC 10674. They developed a fed-batch method to avoid substrate inhibition effect of benzamide and recovered 33 g of benzohydroxamic acid with 24.6 g/l/h productivity from 1 l of reaction mixture. Cells of *P. aeruginosa* strain L10 containing amidase were immobilized in a reverse micellar system. Both free and immobilized cells were used to determine apparent kinetic constants for acyltransferase reaction using aliphatic, aromatic, and amino acid amides. The decrease in Km values by 2-7 folds were obtained in reverse micelles system as compared to free biocatalyst (Bernardo *et al*., 2013).

Table 2.5 Summary of amidase catalysed bioprocesses developed for acetohydroxamic acid synthesis from acetamide

Enzyme source	Reaction time (min)	Bioconversion (mol/mol)	Product purity (w/w)	Reference
Rhodococcus sp. R312	90	54-61%	40-21%	Fournand *et al*. 1997
Geobacillus pallidus BTP-5x MTCC 9225	20	90-95%	40%	Sharma *et al*., 2011
Bacillus sp. AB-6	30	93%	34%	Pandey *et al*., 2011

2.5.3 Factors affecting enzyme catalysed acyltransferase reactions

2.5.3.1 Choice of substrates as acyl group donor

Several acyl group donors, such as acids, esters and amides have been studied for amidase catalyzed acyltransferase reactions. Few reported amidases showed ester and acid acyltransferase activities along with the amide acyltransferase activity. However ester acyltransferase activity of *P. aeroginosa* amidase was found to be very less, about 1% as compared to amide acyltransferase activity (Clark 1970). Only amide was found to be potent acyl group donor in acyltransferase reaction, catalysed by amidase from *R. rhodochrodus* J1 (Kobayashi *et al*., 1993). Enantioselective amidase from *Rhodococcus sp.* 312 was shown to transfer acyl groups of amides, acids and esters to the hydroxylamine, thus leading to formation of corresponding hydroxamic acids but the reaction rate with amide was much faster than those with corresponding ester and acid as acyl group donors (Fournand *et al*., 1998b). These results suggested that amide was better acyl group donor, hence it was preferred by most of researchers (Pacheco *et al*., 2005, Vejvoda *et al*., 2011, Pandey *et al*., 2011, Bhatia *et al*., 2013).

Lipase catalysed fatty hydroxamic acids and hydrazides syntheses involved use of different oils such as canola oil, palm stearin oil and palm kernel oil. These oils comprised different composition of saturated and unsaturated fatty acids with 12 to 22 carbon atoms in aliphatic chain (Mohamad *et al*., 2008, Carpenter *et al*., 2010, Jahangirian *et al*., 2011a, 2011b). Fatty hydroxamic acids, fatty acid hydrazides and their derivatives can be synthesized by utilizing cheaper and easily available oils. Vaysee et al. (1997) used both

fatty acid and fatty acid methyl ester as acyl group donor and found that the transfer of acyl group from ester to hydroxylamine was preferentially catalysed. Suhendra et al. (2005) studied the lipase catalysed synthesis of fatty hydroxamic acid directly from commercial palm oil.

2.5.3.2 Selection of suitable biocatalyst for development of a bioprocess

To perform an enzymatic reaction, the selection of biocatalyst is an essential requirement. Selection of enzyme should be based on its catalytic activity toward different substrates, thermo stability at reaction temperature, solvent tolerance, rate of reaction, substrate and product inhibitions or activations and higher conversion. Amidases show different affinity towards different amides for formation of acyl enzyme complex. Only very short chain aliphatic amides were reported to be efficient substrates for aliphatic amidase of *Rhodococcus sp.* 312 while the enantioselective amidase of the same strain exhibited the highest affinity for the amides containing a hydrophobic moiety and lacking a polar group (Fournand *et al*., 1998a, 1998b). On the other hand, the different acyl enzyme complexes exhibit different affinity for hydroxylamine or hydrazine. As in some cases if acyl group of intermediate complex is short, polar or unsaturated it may show high affinity for hydroxylamine or hydrazine than acyl group with long acyl side chain (Fournand *et al*., 1998b). The possible reason might be that a long acyl side chain hindered nucleophilic attack by co-substrate to acyl enzyme complex. Similarly, sometimes lipase preferentially catalysed transfer of acyl group to hydroxylamine from esters of fatty acid than free fatty acids (Vaysee *et al*., 1997). The specificity of lipase also depends upon position of fatty acid in glycerol backbone, its chain length and degree of unsaturation. Hence before start of process development, biocatalyst screening is must for selection of most efficient enzyme for that substrate and process. Immobilized enzymes are also good alternative than free enzymes and make product recovery easy.

2.5.3.3 Undesirable side reactions

Amidase and lipase catalyzed acyltransferase reactions are performed in aqueous or biphasic lipid/aqueous medium. During enzyme catalysed hydroxamic acids and acid hydrazides syntheses, few undesirable side reactions can also take place due to presence of co-substrates hydroxylamine/hydrazine and water in reaction mixture. Water and hydroxylamine/hydrazine are in competition with each other for nucleophilic attack at

intermediate acyl enzyme complex and at the same time hydroxamic acid/acid hydrazide and carboxylic acid can be produced (Fournand *et al*., 1998a). However rate of acyl group transfer to hydroxylamine/hydrazine is much faster to transfer to water. It was reported that the carboxylic acids are not efficient substrate for acyltransferase reaction of amidase at pH 7.0 or 8.0 and hence synthesis of carboxylic acid is irreversible reaction. On the other hand few amidases also exhibit affinity for hydroxamic acids possibly due to presence of amide bond and due to this, hydroxamic acid produced during reaction can also be hydrolysed to corresponding carboxylic acid (by-product) (Fournand *et al*., 1998b).

Lipase catalysed syntheses of hydroxamic acids can take place by aminolysis of either a fatty acid esters or free fatty acids. When esters are choice of substrate, both hydrolysis and aminolysis reactions take place simultaneously due to competition between both acyl acceptors (hydroxylamine and water) for acyl donor but aminolysis is the preferred reaction. Higher concentration of hydroxylamine can be an alternative to reduce side reaction i.e. hydrolysis of esters. Vaysee et al. (1997) reported that at 1M hydroxylamine concentration, fatty hydroxamic acid synthesis was maximum while the hydrolysis was reduced to 60% of its highest value.

2.5.3.4 Substrate/co-substrate ratio

The mole ratio of reactants is one of the most effective parameter for desired higher molar conversion and better yield. The higher acyl acceptor concentration (co-substrate) can be used to reduce both undesirable hydrolysis reactions i.e. amide hydrolysis and hydroxamic acid hydrolysis. The substrate/co-substrate ratio has influence on amidase catalysed hydroxamic acid/acid hydrazide synthesis and is also related to type of substrate used. A relative amide and hydroxylamine/hydrazine excess can result in inhibition of amidase. The inhibition of enzyme catalysed reaction due to excess of amide concentration may be due to formation of an inactive complex in which two amide substrates are bound on enzyme (Fournand *et al*., 1998a). To get a suitable substrate/co-substrate mole ratio, reactions are needed to be carried with several possible combinations of amide and hydroxylamine/hydrazine.

A higher concentration of hydroxylamine also shifted the equilibrium favourably in lipase catalysed hydroxylaminolysis reactions. In lipase catalysed synthesis, mole ratio of hydroxylamine to palm kernel oil or other substrate increased the reaction yield to its

maximum up to a certain point and further increase in hydroxylamine concentration resulted in the decrease of yield. This reduction in yield beyond a point could be due to saturation of hydroxylamine in aqueous phase (Servat *et al*., 1990, Al-mulla *et al*., 2010).

2.5.3.5 Effect of solvents

When the reactants or the products are poorly soluble in aqueous media or rate of reaction is slow, non aqueous enzymatic catalysis is an efficient approach (He *et al*., 2006, Zhang *et al*., 2011, Li *et al*., 2012). The conversion of oils to fatty hydroxamic acids is found to be affected by the organic solvents used in the processes. In lipase catalysed reaction hydrolysis is likely to be significant in aqueous media, whereas non aqueous reaction media can be used to suppress this side reaction. Organic solvents differ in their ability to solubilise the substrates and products of the reaction and its effect on enzyme stability and activity (Patil *et al*., 2011). The log P value of solvents is a widely used parameter to describe solvent polarity and its effects on enzyme activity. Mohamad et al. (2008) reported that the solvents with log P value about 3.0 resulted in very good conversion. It might be due to the fact that hydrophobic solvents maintain the catalytic activity of enzyme without disturbing its micro aqueous layer. Investigation on the effects of different organic solvents on the hydroxylaminolysis of palm oil was done by Suhendra et al. (2005), and hexane was found the best solvent for fatty hydroxamic acid synthesis with higher yield.

2.5.3.6 Effect of reaction time

Time course of enzymatic reactions is a good indicator of performance of biocatalyst used. Reactions are needed to be optimized with respect to reaction time to achieve high yield and reduce process expenses. In case of acyltransferase reactions, a shorter reaction time also reduces the extent of side reactions (amide hydrolysis and product hydrolysis).

2.5.4 Properties of amidase enzyme

Unlike chemical catalysts, enzymes are highly selective for substrates in terms of regio- and enantiomers. These properties make enzymes more useful in reactions where this regio- and enantioselectivities are highly essential such as for the production of optically active compounds of industrial interest and surface modification of synthetic

polymers (Ewert *et al*., 2008, Takabe *et al*., 2003, Guebitz and Cavaco-Paulo, 2008, Saxena *et al*., 2011).

2.5.4.1 Enantioselectivity

Enzymes are chiral catalysts and have been used widely for kinetic resolution of a racemic mixture for syntheses of optically active enantiopure compounds (Wandel *et al*., 2001, Kaul *et al*., 2004, Mukherjee *et al*., 2010, Siódmiak *et al*., 2013, Richard *et al*., 2013). Several enantioselective amidases have been reported in literature (Yamamoto *et al*., 1992, Fournand *et al*., 1998b, Hayashi *et al*., 1997, Kotlova *et al*., 1999, Snell and Colby, 1999, Krieg *et al*., 2002, Baek *et al*., 2003, Komeda and Asano, 2008). A D-stereospecific amino acid amidase from *O. anthropi* SV3 catalyzed the synthesis of D-amino acids from D-amino acid amides (Komeda *et al*., 2003). Hirrlinger and Stolz (1997) studied the amidase from *R. erthrypolis* MP50 for the production of optically active (S) 2-phenylpropionohydroxamate. An enantioselective amidase from *Ps. Chlororaphis* B23 was purified and characterized which hydrolysed aromatic amides (2-phenylpropionamide, 2-(4-chlorophenyl)-3-methylbutyramide and phenyalaninamide) to corresponding S-isomers (Ciskanik *et al*., 1995). A purified amidase from *R. erythropolis* MP50 catalysed an almost 50% conversion of racemic 2-phenylpropionamide, [2-(6-methoxy-2-naphthyl) propionamide] and [2-(3'-benzoylphenyl) propionamide] to S-acids (e.e.> 99%) (Hirrlinger *et al*., 1996). Doran et al. (2004) demonstrated that an amidase gene from *Microbacterium sp*. AJ115 was expressed in *E. coli*. This expressed and purified amidase catalysed the S-selective hydrolysis of racemic (R, S)-2-phenylpropionamide to S-2-phenylpropionic acid (e.e.> 82%, 50% conversion). Suzuki and Ohta (2006) reported a thermostable archaeal amidase from *S. tokodaii* strain 7 that enantioselectively hydrolyse (R, S)-2-phenylpropionamide to S-acid. (S)-4-chloro-3-hydroxybutyric acid, a precursor of Atorvastatin was synthesized from 4-chloro-3-hydroxybutyramide using the enantioselective purified recombinant amidase of *R. erythropolis* (Park *et al*., 2008).

2.5.4.2 Regioselectivity

Regioselectivity is a property of enzymes to distinguish among similar functional groups located in different regions of substrate molecule (Danieli *et al*., 1994, Park *et al*., 2003, Hann *et al*., 2004, Wuensch *et al*., 2012). Amidases also exhibit regioselectivity, hydrolysing only one amide group in a compound with two or more amide functional

groups. Diamides are selectively monohydrolysed, allowing prochiral compounds to be desymmetrized, thus generating a chiral centre. Yokoyama et al. (2004) studied an amidase from *R. rhodochrous* IFO 15564 that catalysed the enzymatic desymmetrization of prochiral diamides (α,α- disubstituted malonamides) to (R)-carbomyl acetic acids in high enantiomeric excess. Recently a regioselective amidase from *R. erythropolis* AJ270 has been reported to catalyze the hydrolysis of different prochiral α,α- disubstituted malonamides to corresponding carbomylacetic acids under mild reaction conditions (Zhang *et al*., 2011).

2.6 Other applications of amidases

2.6.1 Synthetic applications

Biocatalysis has been evolved as a valuable tool for industrial production of fine chemicals. It involves the use of enzymes and microbes to carry out chemical reactions under mild reaction conditions. Amidase enzyme has a considerable role in syntheses of hydroxamic acids, acid hydrazides, organic acids, optically active and enantiopure intermediates and surface modifications of polymers. Some of the reported applications of amidases in organic syntheses are discussed below.

2.6.1.1 Production of important organic acids

Amidases are generally co-expressed with nitrile hydratases (Tani *et al*., 1989, Kobayashi *et al*., 1993, Yeom *et al*., 2007, Ewert *et al*., 2008, Cantarella *et al*., 2010**,** Bhatia *et al*., 2013). The combined action of these enzymes in a two step pathway has been used for the production of important organic acids such as indole-3-acedic acid (Kobayashi *et al*., 1995), *trans*-4-cyanocyclohexane-1-carboxylic acid (Yamamoto *et al*., 1992), 3-hydroxyalkalonic acid (Hann *et al*., 2003), acrylic acid (Cantarella *et al*., 2013), benzoic acid (Cantarella *et al*., 2006, Kubáč *et al*., 2006) through the biotransformation of nitriles. Amidase has also been used for the production of organic acid from amide directly (Martínková *et al*., 2000). Adipic acid, an important precursor of nylon 66 was synthesized from adiponitrile by nitrile hydratase/amidase system of *Rhodococcus* sp. R312 (Moreau *et al*., 1993). Biotransformation of a broad range of nitriles (aliphatic, aromatic and heterocyclic with different substituent) to corresponding acids was performed using nitrile hydratase and amidase of *R. rhodochrous* AJ270 (Meth-Cohn and Wang, 1997). The combined action of these enzymes has also been reported for the enzymatic synthesis of

nicotinic acid (vitamin B_3) (Kubáč *et al*., 2006, Cantarella *et al*., 2012). Developments in enzymatic synthesis of nicotinic acid from 3-cyanopyridine were made by use of a continuous stirred membrane reactor utilizing nitrile hydratase and amidase of *M. imperiale* CBS 498–74 (Canatarella *et al*., 2010, 2011). Recently a recombinant amidase was used for the production of 2-chloronicotinic acid, an intermediate for syntheses of pesticides and medicines (Jin *et al*., 2013).

2.6.1.2 Production of optically active compounds

Numerous therapeutic drugs, plant protecting agents and natural products are chiral compounds and generally exhibit specific biological effect only in one enantiomeric form. The enantioselective biotransformation of nitriles and amides has attracted increasing interest in recent years due to the versatile utility of the optically active organic acids. Amidases can be enantioselective, producing single stereoisomer via selective enzyme catalysed hydrolysis of racemic amides (Layh *et al*., 1994, Eichhorn *et al*., 1997, Wang and Feng, 2000, Yeom *et al*., 2007, Wang *et al*., 2010). In addition it can display regioselectivity, producing optically active compound by desymmetrization of prochiral diamides (Yokoyama *et al*., 2004, Zhang *et al*., 2011). In a nitrile hydratase/amidase system, it has been observed that enantioselectivity is generally associated with amidase enzyme (Yeom *et al*., 2007, Gilligan *et al.*, 1993). Moreover, many amidases have been used widely for the syntheses of optically active compounds such as S-naproxen (Layh *et al*., 1994), (***R***)-piperazine-2-carboxylic acid, (***S***)-piperazine-2-carboxylic acid, (***S***)-piperidine-2-carboxylic acid (Eichhorn *et al*., 1997), S-(+)-ibuprofen (Snell and Colby, 1999), (S)-3-substituted 4-cyanobutanoic acids (Wang *et al*., 2002), (R)-2-chlormandelic acid, (R)-2-hydroxy-4-phenylbutyric acid (Osprian *et al*., 2003), (S)-2,2-dimethylcyclopropane carboxylic acid (Yeom *et al*., 2007), (R)-2,2-dimethylcyclopropane carboxylic acid (Wang *et al*., 2010), (S)-mandelic acid (Chmura *et al*., 2013). A production process of S-(+)-2, 2-dimethylcyclopropanecarboxamide (S-DMCPCA), an intermediate of cilastatin was developed by bio-resolution of R, S-DMCPCA using a R-stereoselective recombinant amidase from *D. Tsuruhatensis* (Yang *et al*., 2011).

2.6.1.3 Surface modification of polymers

Utilization of enzymes for modifications of surface properties of natural and synthetic polymers is an eco-friendly alternative to classical chemical or physical methods

(Fischer-Colbrie *et al*., 2004, De Geyter *et al*., 2007, Guebitz and Cavaco-Paulo, 2008). Enzymatic routes include milder reaction conditions and offer chemo- and regioselectivities. On the other hand chemical methods require harsh reaction conditions (strong acid or alkaline agents and high energy), coupling reagents and chemical binders leading to deterioration of other properties of polymers (Silva and Cavaco-Paulo, 2008). In earlier reported literature nitrile metabolizing enzymes (nitrilase, nitrile hydratase and amidase) have been used for surface modification of synthetic polymers such as polyacrylonitrile (PAN), polyamide 6, polyamide 6.6 with improved properties. Tauber et al. (2000) reported the partial conversion of –CN groups of granular PAN40 and PAN190 to the -COOH group while -CN groups of acrylic fibre were hydrolysed to $–CONH_2$ only by nitrile hydratase and amidase of *R. rhodochrous* NCIMB 11216. Nitrile hydratase and amidase from *A. tumefaciens* have been found to convert polyacrylonitrile to polyacrylic acid (Fischer-Colbrie *et al*., 2006). Nitrile hydratase and amidase from *Amycolatopsis sp*. IITR215 catalysed the conversion of cyano groups of polyacrylonitrile to carboxylic acid groups (Babu and Choudhury, 2012). An aryl acylamidase from *Nocardia farcinica* was used to hydrolyze polyamide 6 and resulted in increased hydrophilicity (Sonja Heumann *et al*., 2008).

2.6.2 Bioremediation

Nitriles and amides are widely used in many industrial applications in the form of drug intermediates, plastics, extractants, solvents, pesticides etc. As a result these are major pollutants in the industrial effluents and waste water. These are highly mutagenic, toxic and carcinogenic compounds and their degradation to less toxic carboxylic acids is required (Ahmed and Farooqui, 1982, Johammsen *et al*., 1986). Nitrile and amide biodegradation by nitrile and amide metabolizing enzymes are a promising way for detoxification of these compounds (Banerjee *et al*., 2002, Bhalla *et al*., 2005, Sorokin *et al*., 2005, Feng *et al*., 2009, Yusuf *et al*., 2013, Rahim *et al*., 2012). A number of microorganisms have been reported to degrade nitriles and amides through these enzymes (Nawaz *et al*., 1994, Shanker *et al*., 1990, Acharya and Desai, 1997, Cowan *et al*., 1997, Wang *et al*., 2001, Wang *et al*., 2004, Shukor *et al*., 2009, Sathesh Prabu and Thatheyus, 2006, Martínková *et al*., 2009, Chen *et al*., 2010, Bao *et al*., 2010, Santosh kumar *et al*., 2011, Jebasingh *et al*., 2013). *Arthrobacter sp*., isolated from waste water of Indian Petrochemical Corporation Limited (IPCL) could catalyzed the conversion of acetonitrile to acetamide and then to acid (Narayanasamy *et al*., 1990). *Pseudomonas sp*., isolated from

tropical garden soil by enrichment was found to degrade high concentrations of acrylamide by amidase (Shanker *et al*., 1990). Acrylamide was degraded to acrylic acid by immobilized cells of *Pseudomonas sp*. and *X. maltophilia* (Nawaz *et al*., 1993). A stable mixed microbial consortium containing nitrilase, nitrile hydratase and amidase was developed to treat highly toxic waste water containing acrylonitrile, succinonitrile, cyanopyridine, acrylic acid and acrylamide (Wyatt and Knowles, 1995). A Mixed culture capable of degrading different nitriles (acrylonitrile, acetonitrile and benzonitrile) was obtained by enrichment method from activated sludge of waste water treatment plant. This mixed culture possessed different degradation pathways, as acrylonitrile and acetamide were degraded by a two step pathway catalyzed by nitrile hydratase plus amidase. However benzonitrile was degraded by nitrilase leading to direct formation of benzoic acid and ammonia (Li *et al*., 2007).

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Nicotinamide and hydroxylamine-HCl were obtained from Himedia laboratories (India). Nicotinic acid hydroxamate, isonicotinamide and hydrazine-2HCl were purchased from Sigma-Aldrich (USA). Isoniazid was obtained from Loba Chemie (India). Among amides and nitriles; acetamide, acrylamide, benzamide, acetonitrile, propionitrile and phenylacetonitrile were purchased from S.D. Fine Chem. (India). While butyramide, isobutyramide, adipamide, methacrylamide, DL-lactamide, malonamide, glutaronitrile and benzamide were obtained from Sigma-Aldrich (USA). Different culture media components and solvents were obtained from S.D. Fine Chem. (India). Prepacked gel filtration column (Hiload Superdex 75 HR) was purchased from GE Healthcare. Q sepharose, phenyl sepharose resins and empty jacketed glass column were procured from Sigma-Aldrich (USA). Reagents for gel electrophoresis (PAGE) were purchased from Himedia laboratories (India) while, SDS-PAGE protein molecular size markers were purchased from Genei (USA). Amicon Ultra (10 kD) was procured from Millipore (USA). All other chemicals and reagents used were of analytical or HPLC grade as per requirement, procured from various commercial sources.

3.2 Bacterial isolates and media composition

3.2.1 Bacterial isolates

In the present study, the objective was to develop biocatalytic processes for hydroxamic acid and acid hydrazide syntheses using acyltransferase activity of amidase enzyme. Different bacterial isolates for nitrile metabolizing enzymes were isolated previously in lab from various soil and water samples collected by Babu V. (2010). These nitrile metabolizing bacterial isolates were used in the present study for screening of suitable isolate containing amidase enzyme with acyltransferase activity for heterocyclic amides. These bacterial isolates were maintained in mineral base medium. The sources of these bacterial isolates are listed in table 3.1.

Table 3.1 Sources of bacterial isolates

S. No.	Bacterial isolates	Sources	
		Description of source	Sample type
1.	1a	Activated sludge of Ranbaxy, Chandigarh	Water
2.	1b	Activated sludge of Ranbaxy, Chandigarh	Water
3.	3a	Soil near the ETP of Ranbaxy, Chandigarh	Soil
4.	3d	Soil near the ETP of Ranbaxy, Chandigarh	Soil
5.	4a	Soil near the ETP of Nectar life science, Chandigarh	Soil
6.	6b	Sewage, Mohali	Soil
7.	6b2	Sewage, Mohali	Soil
8.	6bD	Sewage, Mohali	Soil
9.	9a	Hot spring (temp. 45 °C), Vashishtha (Manali, H.P.)	Water
10.	9b	Hot spring (temp. 45 °C), Vashishtha (Manali, H.P.)	Water

3.2.2 Composition of mineral base media (MBM)

The amidase production was studied in mineral base medium and its composition is listed in table 3.2, 3.3 and 3.4.

Table 3.2 Composition of mineral base media (pH 7.0)

Components	Composition
Glycerol	10 g/l
Tri sodium citrate	0.2 g/l
Potassium dihydrogen phosphate	1.35 g/l
Di potassium phosphate	0.87 g/l
Trace element solution	1 ml/l
Mineral base solution	100 ml/l

Trace element and mineral base solutions were added into the medium after sterilization (at 121 °C, 15 psi for 20 min). Further 10 mM of phenylacetonitrile (nitrogen source) was added into it.

Table 3.3 Composition of trace element solution (pH 7.0)

Components	Composition
H_3BO_3	0.3 g/l
$CoCl_2.6H_2O$	0.2 g/l
$ZnSO_4.7H_20$	0.2 g/l
$MnCl_2.4H_2O$	0.03 g/l
$Na_2MoO_4.H_2O$	0.03 g/l
$NiCl_4.6H_2O$	0.03 g/l
$CuCl_2.2H_2O$	0.01 g/l

Table 3.4 Composition of mineral base solution (pH 7.0)

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

3.3 Culture growth conditions

Preculture was prepared by inoculation of bacterial colony grown over nutrient agar for 3 days at 45 °C to 50 ml of the sterile mineral base medium in a 250 ml Erlenmeyer flask. The organism was grown aerobically for 36 h at 45 °C and 200 rpm in an incubator shaker. The inoculums (500 µl) was added into 100 ml of same sterile medium in a 500 ml Erlenmeyer flask and incubated under similar conditions. The bacterial cells were harvested at around mid exponential phase of growth (OD_{600}= 0.6-0.9) by centrifugation at 10,000 x g for 10 min at 4 °C and washed twice with 100 mM potassium phosphate buffer (pH 7.0). Bacterial cells were suspended in same buffer and here after it were referred as resting cells. Bacterial cell growth was monitored by measuring optical density spectrophotometrically. For optical density measurement, 2 ml of culture broth was

centrifuged at 10,000 x g for 10 min at 4 °C and washed cell pellet was re-suspended in 2 ml of isotonic solution (0.85% NaCl) and optical density was determined at 600 nm.

3.4 Enzyme assays

3.4.1 Acyltransferase activity assay for hydroxamic acid synthesis

The acyltransferase activity assay for hydroxamic acid synthesis was performed in a reaction mixture (1 ml) having following composition- 400 µl of amide solution (250 mM in 100 mM phosphate buffer, pH 7.0), 500 µl of hydroxylamine-HCl solution (1000 mM in distilled water, neutralized with 10.0 N NaOH) and 100 µl of resting cells in phosphate buffer (100 mM, pH 7). After incubation of reaction mixture at 45 °C for 10 min, the reaction was stopped by adding 1 ml of acidic $FeCl_3$ solution (356 mM $FeCl_3$ in 0.65 N HCl) in 500 µl of reaction mixture. The reaction mixture was centrifuged at 10,000 x g for 8 min, and the clear supernatant was collected for spectroscopic estimation of hydroxamic acid at λ=500 nm. Reaction mixture without resting cells was also tested for any possible spontaneous chemical synthesis of hydroxamic acid. One unit of acyltransferase activity was defined as that amount of resting cells (mg dry cell weight= mg_{dcw}) which catalysed the formation of one µmol of hydroxamic acid in 1 min under the assay conditions. Dry cell weight was determined as discussed in section 3.6.

3.4.2 Amide hydrolase activity assay

The amide hydrolase activity assay was performed in a reaction mixture (1 ml) having following composition- 800 µl of amide solution (125 mM in 50 mM phosphate buffer, pH 7.0), 100 µl of 50 mM phosphate buffer and 100 µl of resting cells in phosphate buffer (50 mM, pH 7). Reaction mixture without resting cells was tested for any possible spontaneous reaction. A control reaction was also conducted having only resting cells in the absence of substrate. After incubation of reaction mixture at 45 °C for 10 min, the reaction was terminated by the addition of 10 µl of 1N HCl and cells were removed by centrifugation at 10,000 x g for 8 min. Supernatant was collected for ammonia estimation using Berthelot method (Weatherburn 1967). One unit of amide hydrolase activity was defined as that amount of resting cells (mg_{dcw}) which catalysed the formation of one µmol of ammonia in 1 min under the assay conditions.

3.4.3 Hydroxamic acid hydrolase activity assay

The hydroxamic acid hydrolase activity assay was performed in a reaction mixture (1 ml) of the following composition- 800 µl of nicotinic acid hydroxamate solution (125 mM in 50 mM phosphate buffer, pH 7), 100 µl of 50 mM phosphate buffer and 100 µl of resting cell in phosphate buffer (50 mM, pH 7.0). Blank reaction was conducted in the absence of enzyme. After incubation of reaction mixture at 45 °C for 10 min, the reaction was terminated by the addition of 10 µl of 1N HCl and cells were removed by centrifugation at 10,000 x g for 8 min. Supernatant was collected for HPLC analysis. One unit of hydroxamic acid hydrolase activity was defined as that amount of resting cells (mg_{dcw}) which catalysed the formation of one µmol of nicotinic acid in 1 min under the assay conditions.

3.4.4 Acyltransferase activity assay for hydrazide synthesis

The acyltransferase activity assay for hydrazide synthesis was performed in a reaction mixture (1 ml) having following composition- 400 µl of amide solution (250 mM in 100 mM phosphate buffer, pH 7.0), 500 µl of hydrazine-2HCl solution (1000 mM in distilled water, neutralized with 10.0 N NaOH) and 100 µl of resting cells in phosphate buffer (100 mM, pH 7.0). After incubation of reaction mixture at 45 °C for 20 min, the reaction was stopped by adding 10 µl of 2 N HCl and cells were removed by centrifugation at 10,000 x g for 8 min. Clear supernatant was collected for HPLC analysis of isoniazid. Reaction mixture without resting cells was also tested for any possible spontaneous chemical synthesis of isoniazid. One unit of acyltransferase activity was defined as that amount of resting cells (mg_{dcw}) which catalysed the formation of one µmol of isoniazid in 1 min under the assay conditions.

3.4.5 Hydrazide hydrolase activity assay

The hydrazide hydrolase activity assay was performed in a reaction mixture (1 ml) of the following composition- 800 µl of isoniazid solution (125 mM in 50 mM phosphate buffer, pH 7.0), 100 µl of 50 mM phosphate buffer and 100 µl of resting cells in phosphate buffer (50 mM, pH 7.0). Blank reaction was conducted in the absence of enzyme. After incubation of reaction mixture at 45 °C for 20 min, the reaction was terminated by the addition of 10 µl of 2 N HCl and cells were removed by centrifugation at 10,000 x g for 8 min. Supernatant was collected for HPLC analysis of isonicotinic acid. One unit of

hydrazide hydrolase activity was defined as that amount of resting cells (mg_{dcw}) which catalysed the formation of one µmol of isonicotinic acid in 1 min under the assay conditions.

3.5 Analytical methods

3.5.1 Determination of molar extinction coefficient for nicotinic acid hydroxamate/ Fe(III) complex

Nicotinic acid hydroxamate (NAH) formed was quantified spectrophotometrically by determining the absorbance of red brown complexes of hydroxamic acid with Fe(III) (Fournand *et al*., 1997a). Molar extinction coefficient ($Ɛ_M$) of the NAH/Fe(III) complex was determined at λ=500 nm. Solutions of NAH were prepared in the concentration range of 0.5-4.0 mM in phosphate buffer (50 mM, pH 7.0) and one ml of freshly prepared $FeCl_3$ solution (356 mM $FeCl_3$ in 0.65 N HCl) was added in 500 µl of each sample. Absorbance was read at λ=500 nm and a standard curve was plotted to obtain mean extinction coefficient value (Ɛm) (Fig 3.1)**.** $Ɛ_M$ value of $4.14X10^2$ l mol^{-1} cm^{-1} was obtained from the slope of standard plot.

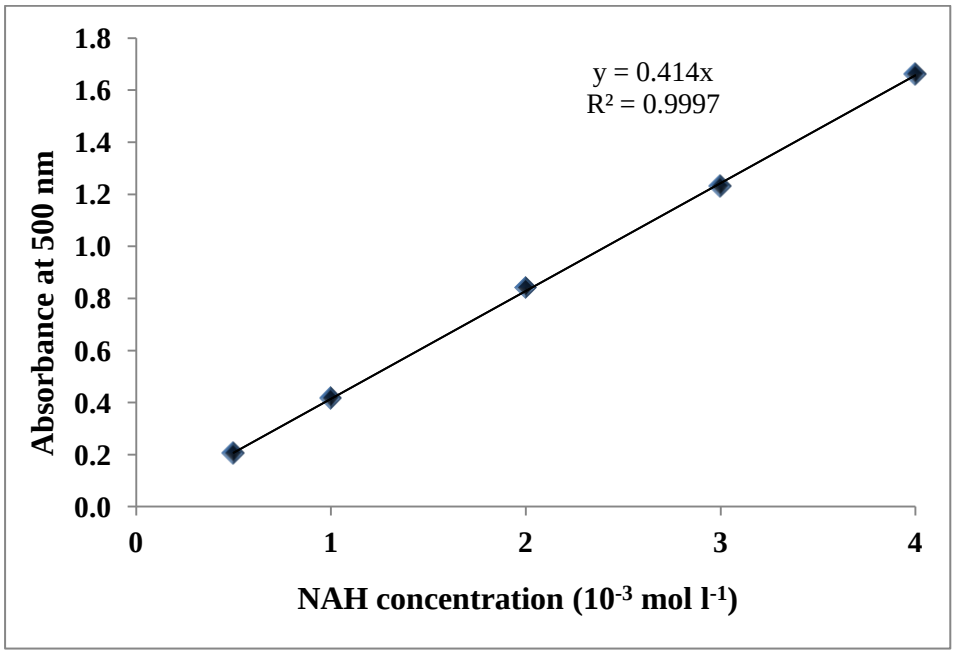


Fig 3.1 Standard plot by spectrophotometric method for nicotinic acid hydroxamate

3.5.2 Validation of Spectrophotometer method with HPLC for nicotinic acid hydroxamate determination

To validate the spectrophotometer method of nicotinic acid hydroxamate determination, HPLC analysis of NAH was also carried out with three samples. These samples were prepared by incubating resting cells with nicotinamide/hydroxylamine-HCl (100/500 mM) under assay conditions (50 mM phosphate buffer pH 7.0 and 45 °C) and samples were taken at an interval of 5, 10 and 20 min. It was observed that both methods are comparable for determination of nicotinic acid hydroxamate concentration in reaction mixture (Table 3.5), hence analysis of NAH was done using spectrophotometric method instead of HPLC method.

Table 3.5 Comparison of spectrophotometric and HPLC methods for nicotinic acid hydroxamate (NAH) analysis under assay conditions

Sample	HPLC NAH (mM)	Spectrophotometer NAH (mM)
S1	31.5±0.15	30.7±0.28
S2	68.3±0.38	68.9±0.47
S3	86.2±0.60	84±0.62

*Difference in NAH amount estimated by spectrophotometer and HPLC methods was not statistically significant (analysed by one way ANNOVA, p value >0.01)

3.5.3 Standard plot for ammonia estimation

Ammonia generated in the reaction mixture by the action of amidase on amide was assayed by Berthelot method and ammonia released data was used for the measurement of amide hydrolase activity of whole cells. This method converts ammonium ions to a coloured complex. Absorbance at 640 nm gives the measure of ammonia released. Solution (0.2 ml) of ammonium chloride was prepared in the concentration range of 0.01-2.0 mM in phosphate buffer (50 mM, pH 7.0) and 0.2 ml each of 0.33 M sodium phenolate, 0.01% sodium nitroprusside and 0.02 M sodium hypochlorite solutions were added into it. The mixture was heated in a boiling water bath for two minutes and then cooled in an ice bath for two minutes. 1.2 ml of distilled water was then added to each sample and a blue coloured solution was obtained. Absorbance was read at λ=640 nm and a standard plot of

ammonia concentration vs. O.D was plotted to calculate ammonia concentration in the enzyme assay (Fig 3.2). The reagents used were prepared as given in the table 3.6.

Table 3.6 Composition of reagent used for Berthelot method

Reagents	Concentration	Components
Sodium phenolate	0.33 M	Phenol, Sodium hydroxide in distilled water
Sodium hypochlorite	0.02 M	Sodium hypochlorite solution in distilled water
Sodium nitroprusside	0.01%	Sodium nitroprusside in distilled water

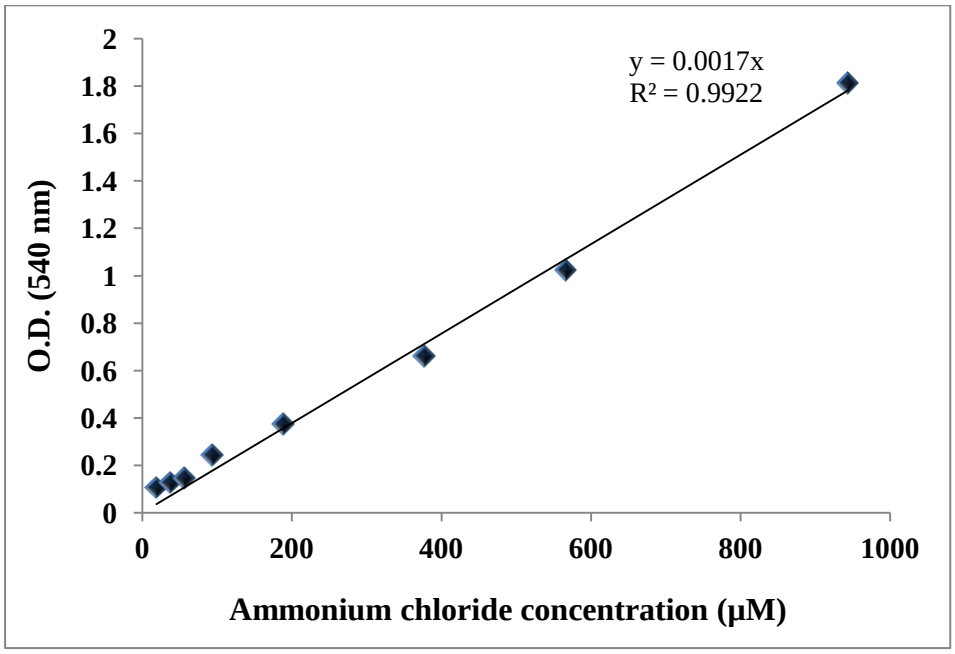


Fig 3.2 Standard plot of ammonium chloride using Berthelot method

3.5.4 HPLC analysis

HPLC analysis was carried out with Varian Prostar HPLC with Waters spherisorb® 10 µm ODS2, 4.6 X 250 mm analytical column. The analysis of nicotinic acid hydroxamate, nicotinic acid and nicotinamide were carried out at a mobile phase flow rate of 1.0 ml/min at 210 nm and mobile phase used was 10% (v/v) acetonitrile in milliQ water

adjusted to pH 2.8 by H_2SO_4. The analysis of isonicotinamide, isoniazid and isonicotinic acid were carried out at a mobile phase (20 mM KH_2PO_4 with 5% (v/v) acetonitrile) flow rate of 1.0 ml/min at 210 nm. 20 µl of the sample was injected. HPLC profiles of nicotinic acid hydroxamate, nicotinic acid, nicotinamide, isoniazid, isonicotinic acid and isonicotinamide are presented as fig 3.3a- 3.3h. The standards plots for quantitative analysis of these compounds were prepared from commercially available pure compounds and are shown in fig 3.4a-3.4f.

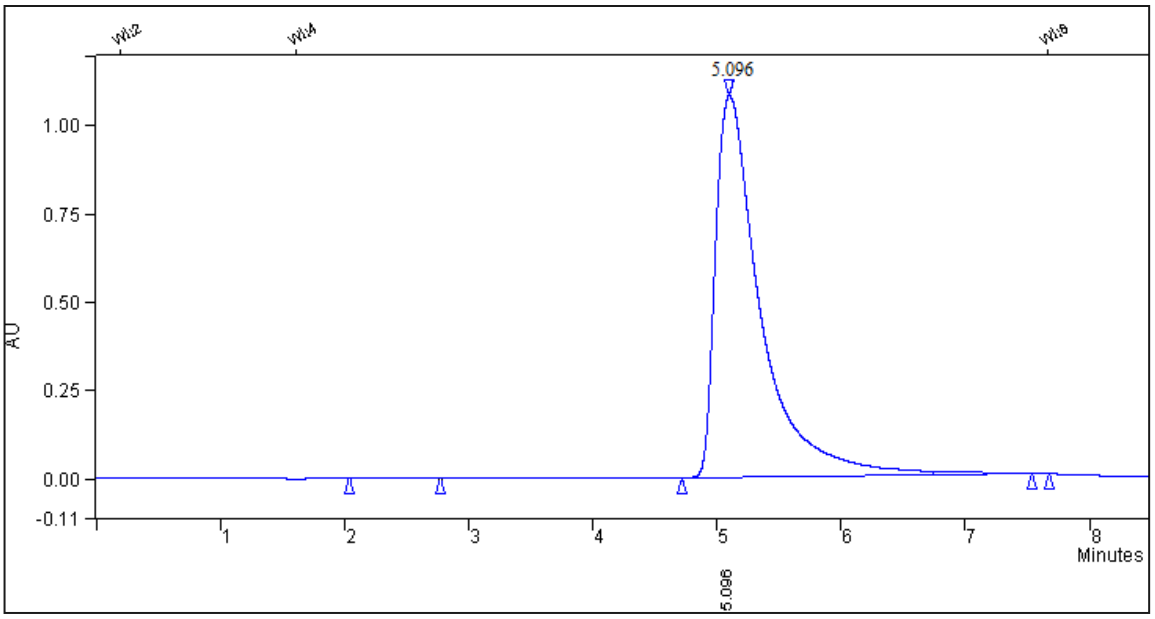


Fig 3.3a HPLC chromatogram of standard nicotinic acid hydroxamate

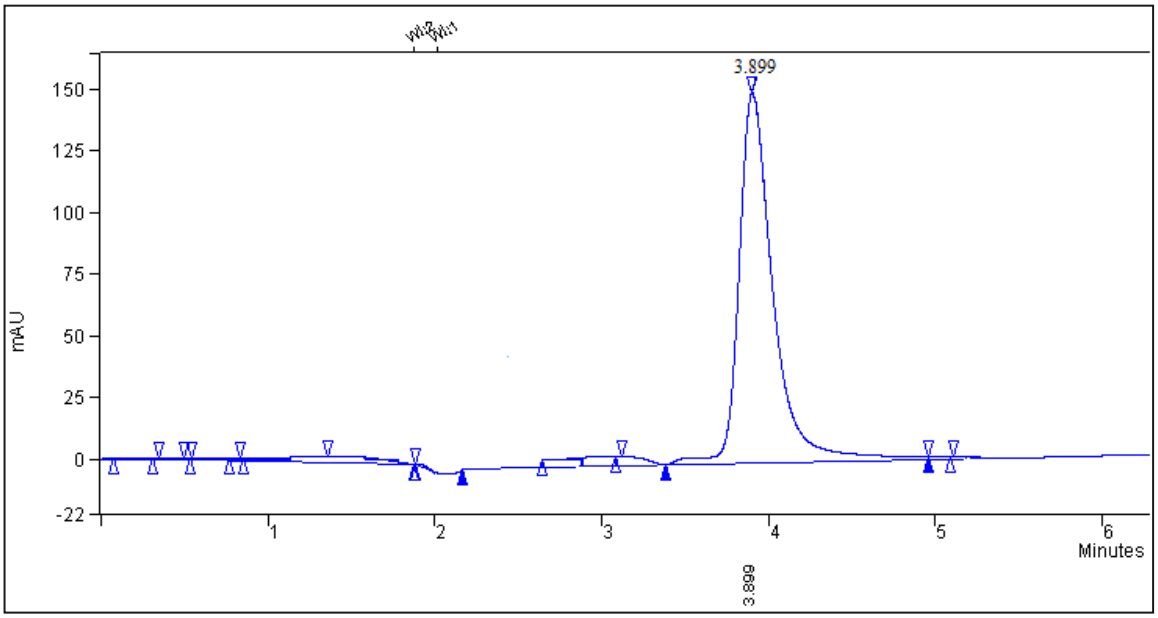


Fig 3.3b HPLC chromatogram of standard nicotinic acid

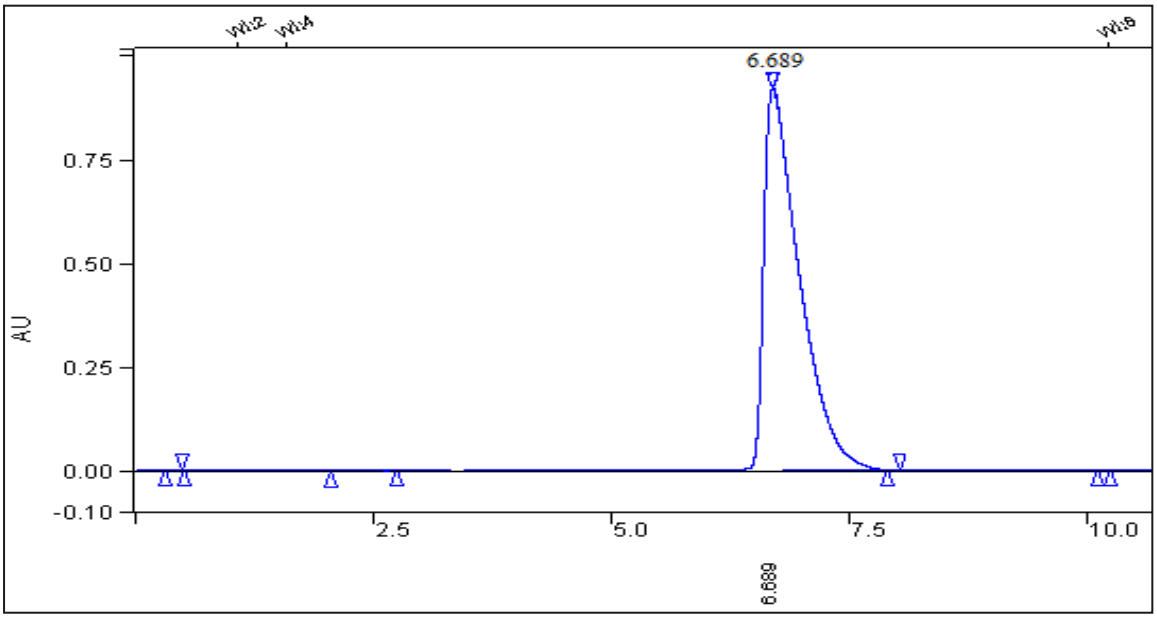


Fig 3.3c HPLC chromatogram of standard nicotinamide

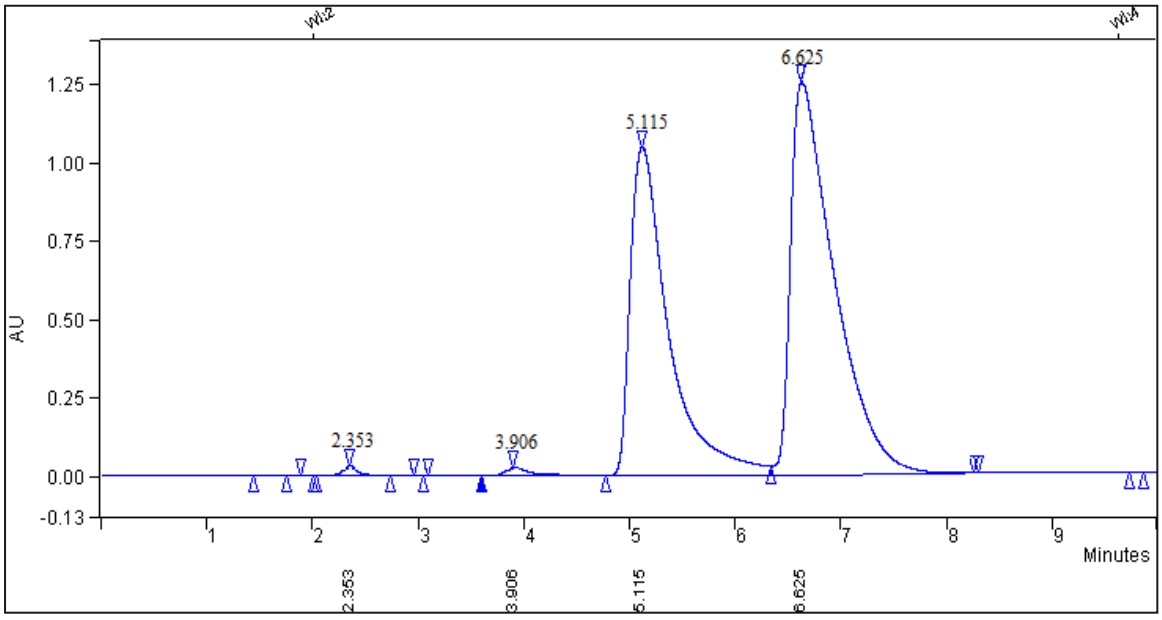


Fig 3.3d HPLC chromatograms of nicotinic acid hydroxamate, nicotinic acid and nicotinamide in reaction mixture

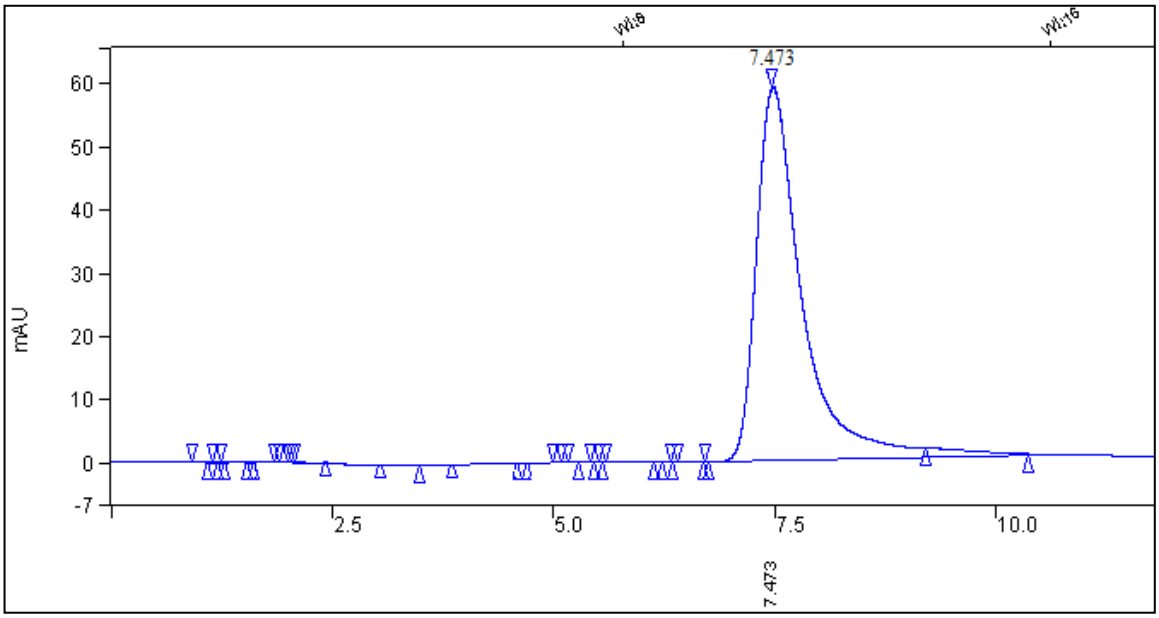


Fig 3.3e HPLC chromatogram of standard isoniazid

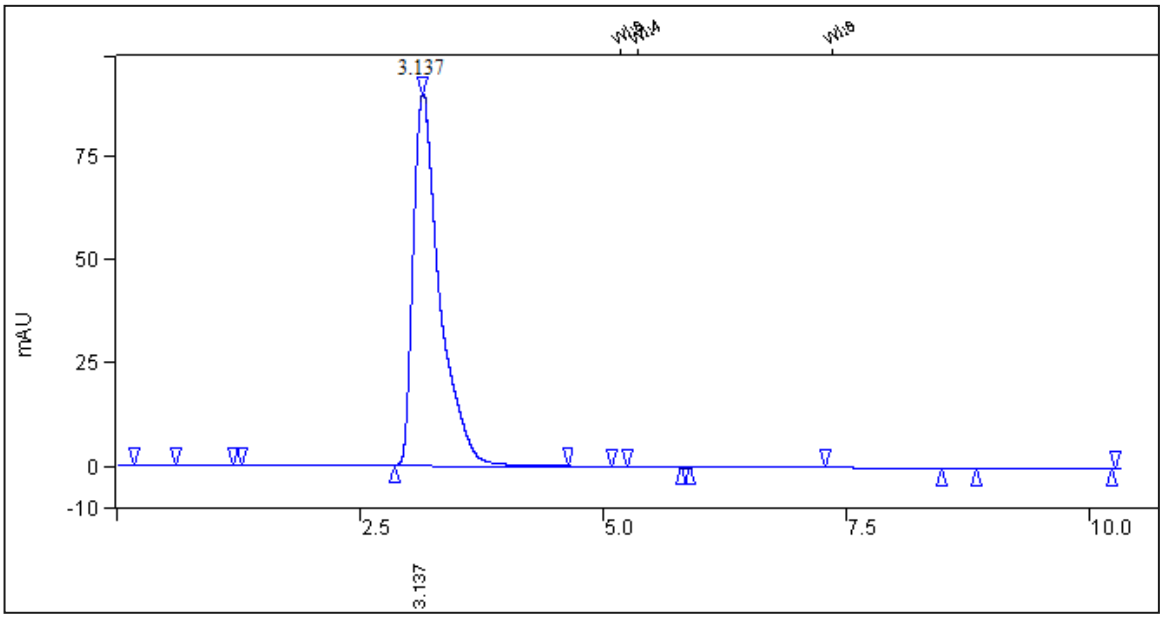


Fig 3.3f HPLC chromatogram of standard isonicotinic acid

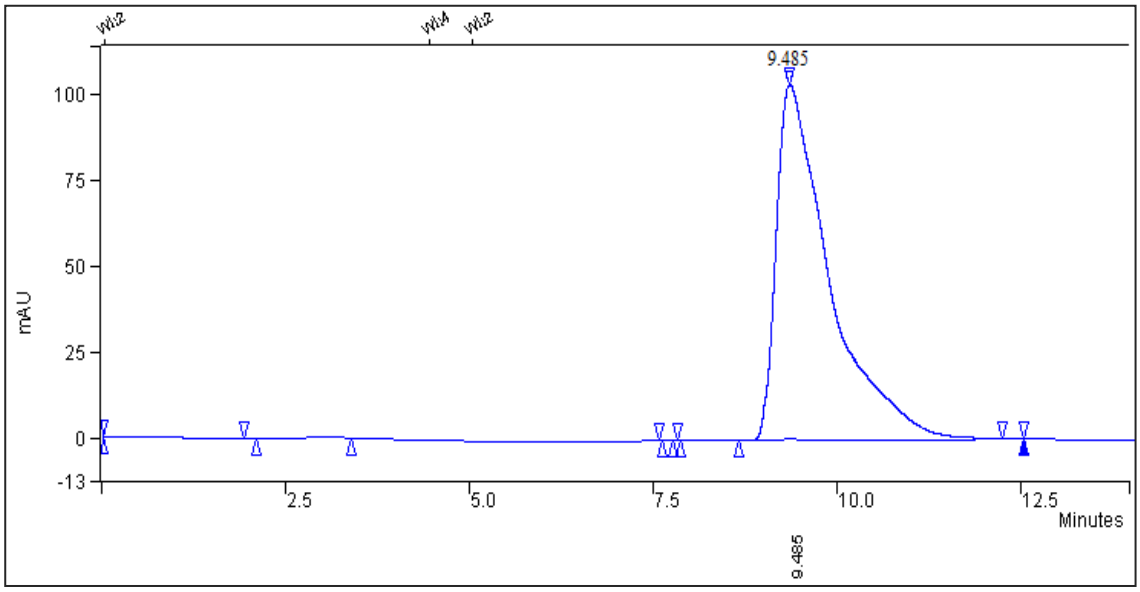


Fig 3.3g HPLC chromatogram of standard isonicotinamide

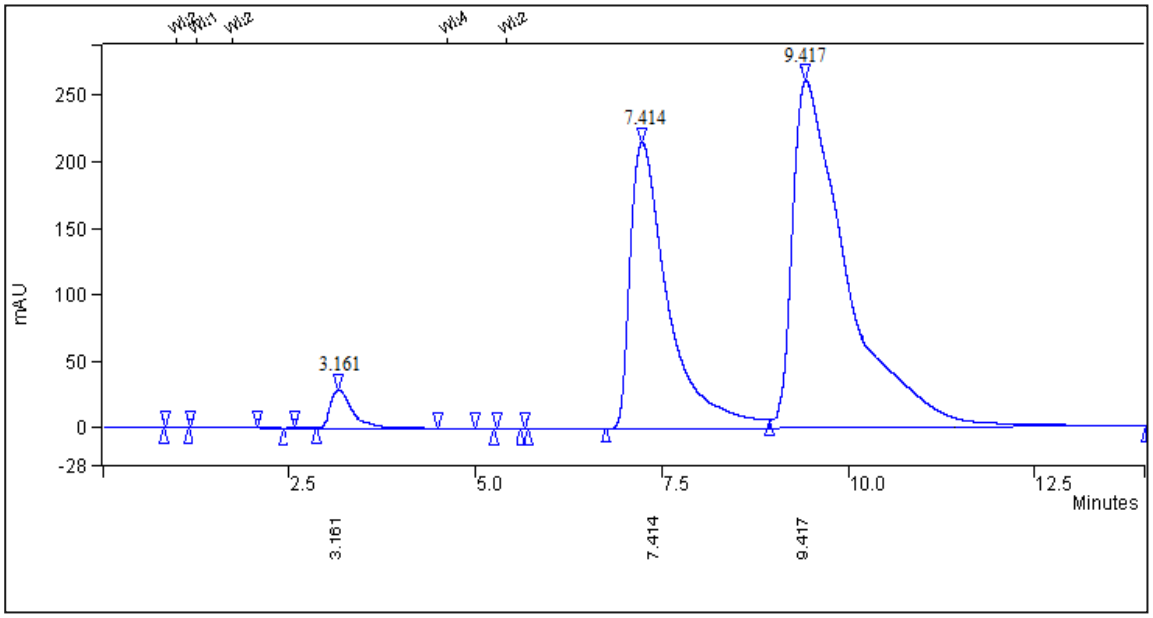


Fig 3.3h HPLC chromatograms of isoniazid, isonicotinic acid and isonicotinamide in reaction mixture

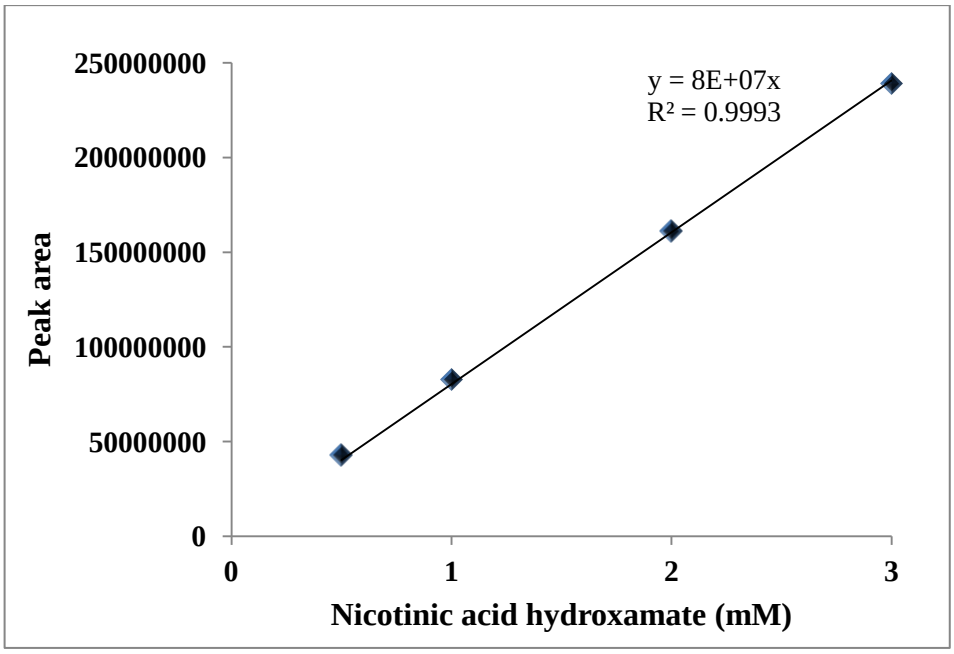


Fig 3.4a Standard plot of nicotinic acid hydroxamate by HPLC

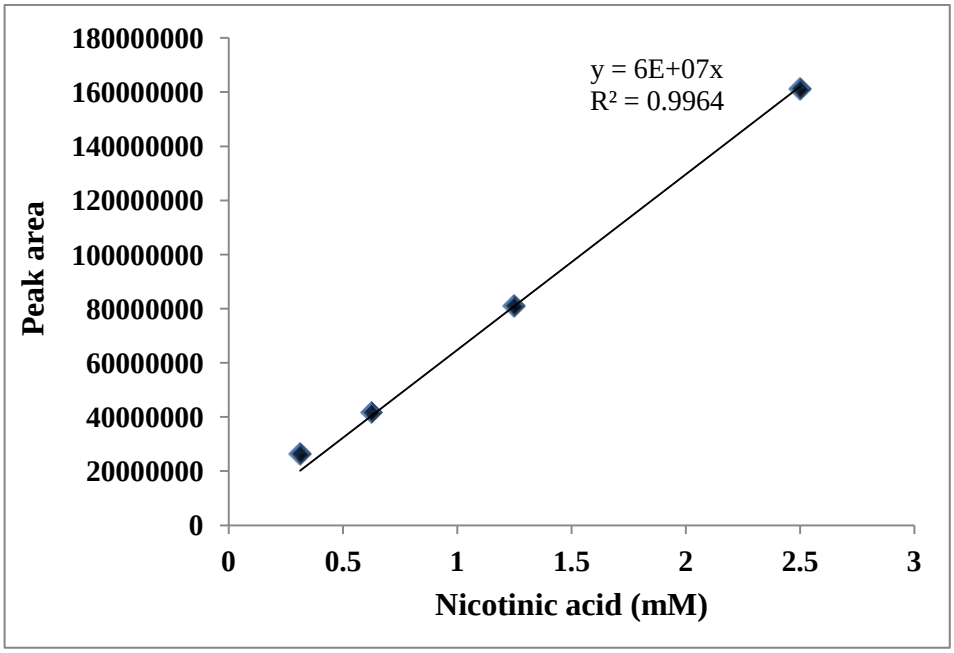


Fig 3.4b Standard plot of nicotinic acid by HPLC

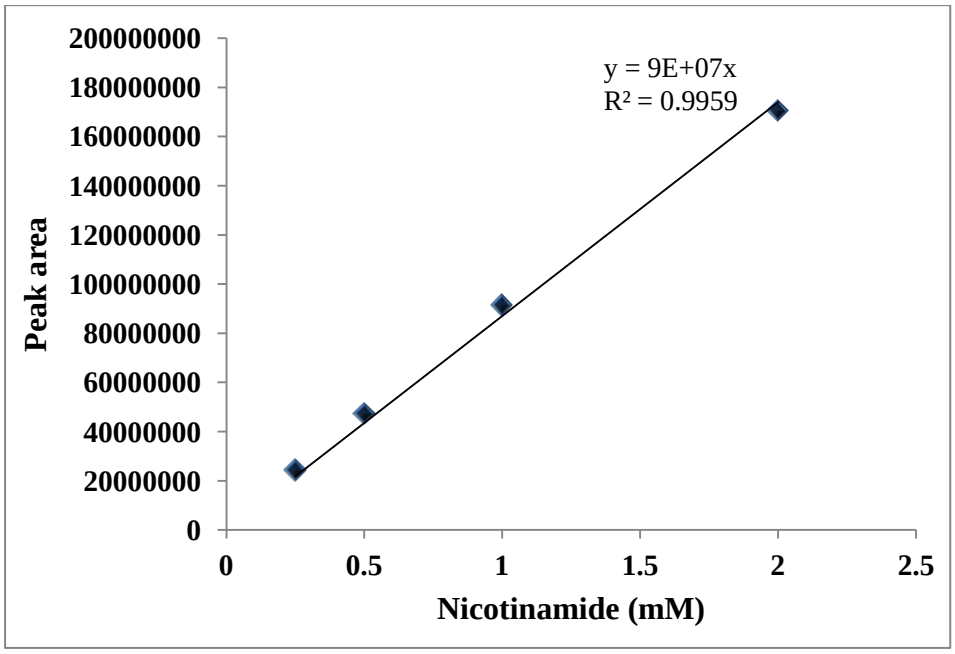


Fig 3.4c Standard plot of nicotinamide by HPLC

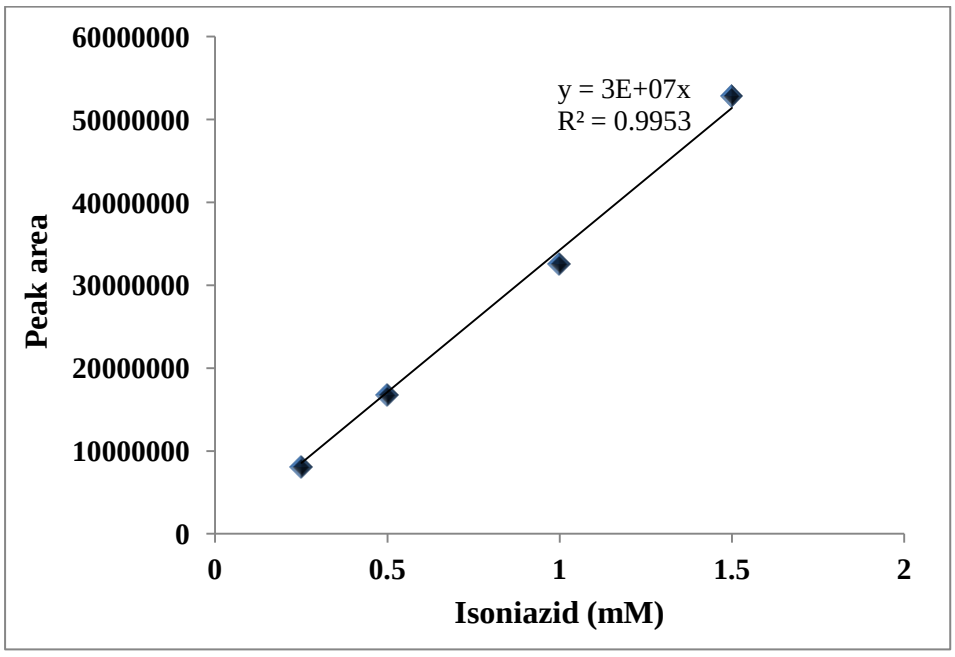


Fig 3.4d Standard plot of isoniazid by HPLC

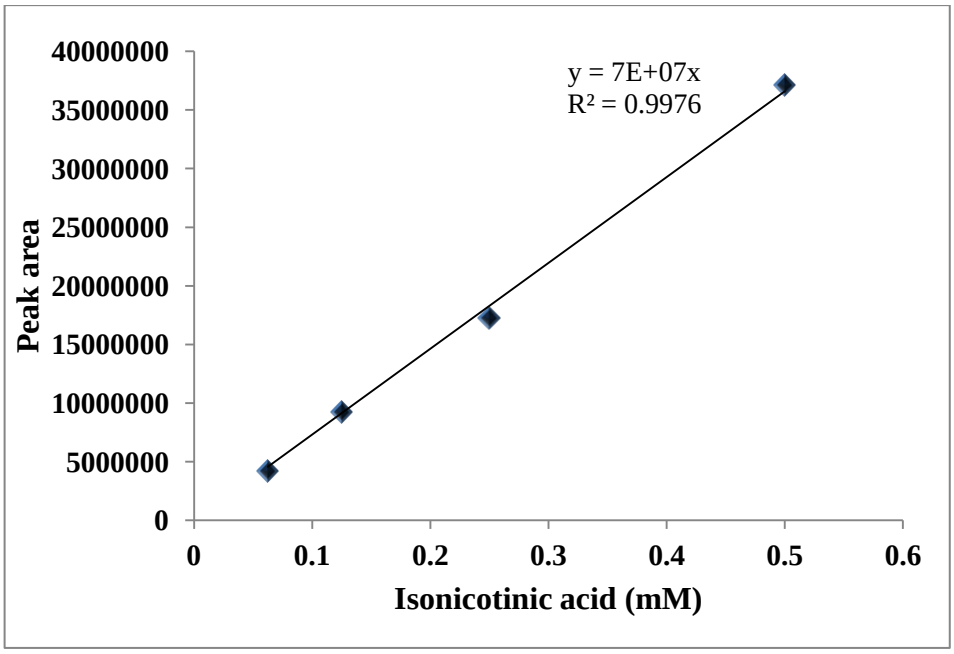


Fig 3.4e Standard plot of isonicotinic acid by HPLC

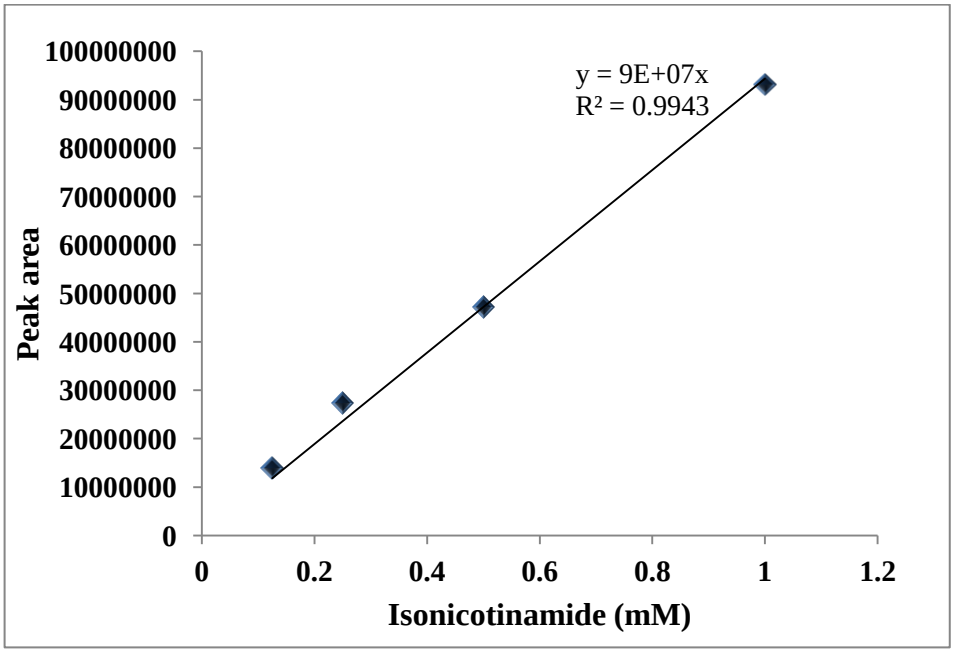


Fig 3.4f Standard plot of isonicotinamide by HPLC

3.6 Biomass quantification

Samples collected at different intervals were centrifuged at 10,000 x g for 10 min at 4 °C and washed pellet was suspended in saline solution (0.85% NaCl) followed by taking optical density of the suspension at 600 nm. Dry cell weight (cell biomass) was calculated from a standard plot, between cell concentration (g_{dcw}/l) and optical density (O.D.). A standard plot was prepared using the following method. In brief, culture broth was centrifuged at 10,000 x g for 10 min at 4 °C and the resulting pellet was suspended in 4 ml of saline solution. 2 ml of this suspension was kept for drying (at 55 °C) till a constant weight was achieved and dry weight was determined while remaining 2 ml was used to make different dilutions of cells suspension and O.D. were taken at 600 nm. Finally a standard plot was prepared by plotting optical density against cell concentration as shown in fig 3.5.

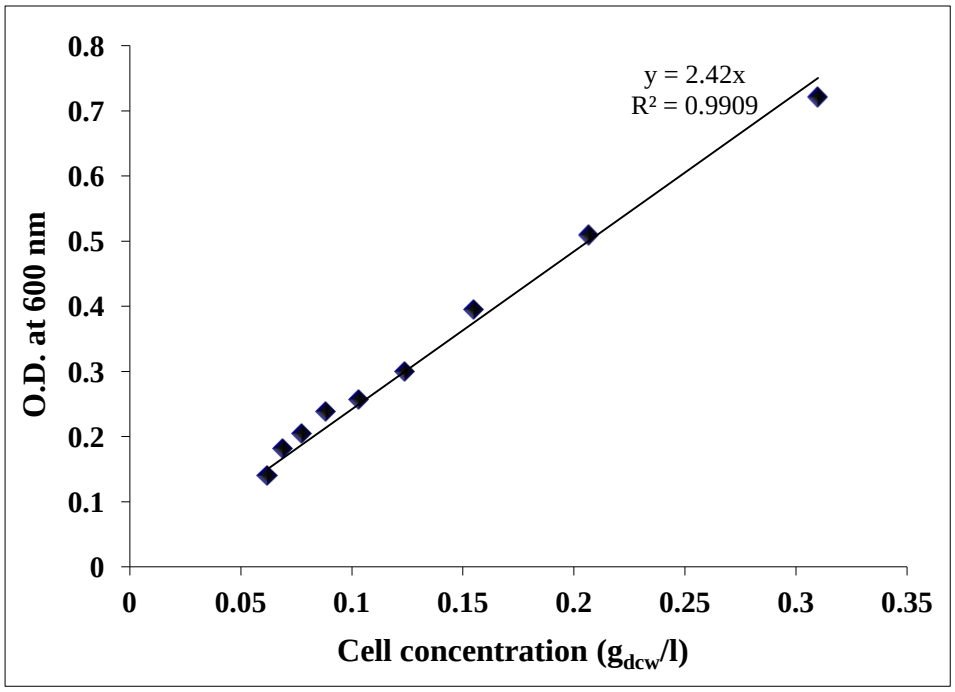


Fig: 3.5 Standard plot for cell concentration

3.7 Identification and characterization of 6b2 isolate

The identification of the isolate was carried out on the basis of the biochemical characteristics and nucleotide sequence analysis of amplified 16S rDNA.

3.7.1 Biochemical characterization

Carbon substrate utilization by isolate was determined using the standardized Biolog Phenotype GEN III plates. After incubation at 37 °C for 24 h, the microplate was read on a Biolog system (MicroStation™ System/ MicroLog Hayward, USA).

3.7.2 16S rRNA gene amplification and DNA sequencing

For the 16S rDNA sequence analysis, extraction of the chromosomal DNA was done by DNA extraction kit (Himedia). The 16S rDNA was amplified by polymerase chain reaction with the universal primers pair p16S-8 (5'-AGAGTTTGATCCTGGCTCAG-3') and p16S-1541 (5'-AAGGAGGTGATCCAGCCGCA-3') (Zheng *et al*., 2007) in a thermal cycler under the following conditions: 5 min at 95 °C, 30 cycles of 40 s at 95 °C, 1 min at 55 °C, 2 min at 72 °C and a final extension was performed for 10 min at 72 °C. A volume of 50 µl of PCR reaction mixture was consisting of following components:

PCR assay buffer (10X)	5.0 µl
dNTP mix (10 mM)	4 µl
Forward primer (20 pmol/µl)	2 µl
Reverse primer (20 pmol/µl)	2 µl
Template DNA (10 ng/µl)	1 µl
Taq polymerase (5 unit/µl)	2 µl
Milli 'Q' water	34 µl

3.7.3 Agarose gel electrophoresis for genomic DNA and amplified product

Isolated DNA and amplified PCR product was analyzed on 0.8% (w/v) agarose gel containing EtBr using gel documentation system.

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

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

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

Procedure: 0.8% (w/v) agarose gel was prepared by dissolving appropriate amount of agarose in 50 ml of 1.0x TBE buffer and heated in microwave oven until the solution became transparent. The solution was cooled to about 50 °C and 2.5 µl of EtBr stock solution was added to get a final concentration of 5µg/ml. The warm agarose was mixed and poured into the horizontal gel casting tray containing a gel comb at the proper place. The gel was allowed to solidify at room temperature. The gel comb was removed carefully and the gel tray along with gel was submerged in tank buffer (TBE). The DNA samples were prepared by adding 1 µl of gel loading dye to 5 µl of DNA solution and were loaded into the well along with the marker DNA to determine the size of amplified fragment. The electrodes present at two sides of the tank were connected to power supply and 50 V direct current was applied. When the dye migrated to 2/3rd of the gel, the electric current was turned off and DNA was visualized under gel documentation system (BIORAD).

3.7.4 Sequencing and molecular phylogenetic analysis

The amplified PCR product was sequenced by Ocimum biosolutions, Hyderabad (India). The sequence obtained was compared with sequences in NCBI (http://www.ncbi.nlm.gov/Blast.cgi) GenBank database using the nucleotide BLAST program to determine phylogenetic position. Multiple alignments of most similar sequences and query sequence were done by CLUSTAL W software. The construction of phylogenetic tree by the neighbor-joining method and a bootstrap analysis to evaluate the tree topology were performed by Phylip version 3.69.

3.7.5 Nucleotide sequence accession number

The partial 16S rRNA gene sequence of isolate *Bacillus smithii* strain IITR6B2 determined in this study has been deposited in GenBank database under the accession number JX157878.

3.8 Characterization of acyltransferase activity of whole cell amidase

3.8.1 Effect of temperature

To study the effect of temperature on acyltransferase activity of whole cell amidase, the enzyme assay was performed with 100 µl of resting cells (0.14 mg_{dcw}/ml) at different temperatures (25 to 70 °C) keeping all other standard assay conditions (100 mM

nicotinamide, 500 mM hydroxylamine-HCl, 50 mM phosphate buffer, pH 7.0, reaction volume- 1ml) constant for 10 min. The maximum activity of enzyme was considered as 100%.

3.8.2 Effect of pH

The optimum pH for the acyltransferase activity was determined for a pH range of (4.0-10) in following buffers (50 mM): sodium acetate buffer (pH 4.0-5.8), potassium phosphate buffer (pH 5.8-8.0), borate buffer (pH 8.0-9.2), and sodium carbonate buffer (pH 9.2-10). Acyltransferase activity in each buffer was measured with 100 µl of resting cells (0.13 mg_{dcw}/ml) keeping all other standard assay conditions (55 °C, 100 mM nicotinamide, 500 mM hydroxylamine-HCl, reaction volume- 1ml) constant for 10 min. The maximum activity of enzyme was considered as 100%.

3.8.3 Thermal stability

Thermal stability of whole cell amidase was determined at 30, 45 and 55 °C. Whole cell suspensions (0.16 mg_{dcw}/ml) in phosphate buffer (100 mM, pH 7) were incubated at 30, 45 and 55 °C for 12 h and samples (100 µl) were withdrawn at interval of 2 h to determine the residual acyltransferase activity. Acyltransferase activity was determined under standard assay conditions (100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer 50 mM, pH 7, 55 °C, 10 min, reaction volume- 1ml). The natural logarithm of residual acyltransferase (lnRA) activity was plotted against time.

3.8.4 Substrate specificity

The following amides were tested for substrate specificity determination of amidase enzymes for both acyltransferase and amide hydrolase activity: aliphatic saturated amides acetamide, propionamide, butyramide, isobutyramide, hexanamide; aliphatic saturated diamides- malonamide, adipamide; aliphatic unsaturated amides- acrylamide, methacrylamide; aromatic amides- benzamide, 4-Hydroxybenzamide, salicylamide; heterocyclic amides- nicotinamide, isonicotinamide. Enzymatic reaction (reaction volume- 1ml) for acyltransferase activity assay were performed with 100 µl of resting cells (0.36 mg_{dcw}/ml), 10 mM amide substrate, 500 mM hydroxylamine-HCl at 55 °C and phosphate buffer 50 mM, pH 7.0 for 10 min. Amide hydrolase activity assay was performed under similar assay conditions in absence of co-substrate hydroxylamine-HCl.

3.8.5 Organic solvent compatibility of whole cell amidase

The compatibility of the whole cell acyltransferase activity with several solvents (methanol, ethanol, isopropanol, acetone, Di-methyl formamide, Di-methyl sulfoxide, hexane and heptane) was determined. Resting cells were incubated with these solvents at their final concentration of 15 and 30% (v/v) (0.19 mg_{dcw}/ml resting cells, final volume 100 µl) at 30 °C for 30 min and the residual acyltransferase activity was determined under standard assay conditions (100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer 50 mM, pH 7, 55 °C, 10 min, reaction volume- 1ml). The acyltransferase activity was assumed as 100% in the absence of any organic solvent.

3.9 Bioprocess development for nicotinic acid hydroxamate synthesis

3.9.1 Determination of different activities of whole cell amidase

To determine the various enzymatic reactions involved during nicotinic acid hydroxamate synthesis; amide acyltransferase, amide hydrolase and hydroxamic acid hydrolase activities of whole cell amidase were determined using methods described in section 3.4.1, 3.4.2 and 3.4.3 respectively. Further, to confirm that the acyltransferase activity belong to amidase, following experiments (3.9.2, 3.9.3) were carried out with whole cells.

3.9.1.1 Effect of amidase inhibitor on acyltransferase, amide hydrolase and hydroxamic acid hydrolase activities of whole cell

Amidase inhibitor Di-ethyl phosphoramidate (DEPA) at three different final concentrations (1, 5, 10 mM) was pre-incubated with resting cells (0.5 mg_{dcw}/ml) at 30 °C for 30 min. After pre-incubation; resting cells were tested for residual acyltransferase, amide hydrolase and hydroxamic acid hydrolase activities. Activities obtained after incubating resting cells without inhibitor were taken as 100%.

3.9.1.2 Thermal stability profile of acyltransferase, amide hydrolase and hydroxamic acid hydrolase activities of whole cells

Thermal stability profiles of acyltransferase, amide hydrolase and hydroxamic acid hydrolase activities of whole cell enzyme were determined at 45 °C. Whole cell suspension

(0.5 mg_{dcw}/ml) in phosphate buffer (100 mM, pH 7) was incubated at 45 °C and samples (100 µl) were withdrawn at regular interval to determine the residual activities.

3.9.2 Effects of substrate and co-substrate concentrations on acyltransferase activity

To study the effect of nicotinamide concentration on amide acyltransferase activity, varied concentrations of nicotinamide (10-500 mM) at constant hydroxylamine-HCl concentration (500 mM) were used. Acyltransferase activity assay was performed with 100 µl of resting cells (0.16 mg_{dcw}/ml) under optimized assay conditions (55 °C, phosphate buffer 50 mM, pH 7, 10 min, reaction volume- 1ml). The Effect of hydroxylamine-HCl concentration on acyltransferase activity and by-product formation was studied in the range of 0-1500 mM keeping nicotinamide concentration constant (100 mM). Acyltransferase activity assay was performed with 100 µl of resting cells (0.14 mg_{dcw}/ml) under optimized assay conditions (55 °C, phosphate buffer 50 mM, pH 7, 10 min, reaction volume- 1ml).

3.9.3 Optimization of substrate and co-substrate concentration combination

This experiment was performed in such a manner that concentration of nicotinamide was varied from 50-1000 mM at different hydroxylamine-HCl concentrations ranging from 100-1000 mM in the reaction mixture. Total 55 combinations were used to find out the optimum concentration combination for highest acyltransferase activity with 100 µl of resting cells (0.12 mg_{dcw}/ml) under optimized assay conditions (55 °C, 50 mM phosphate buffer pH 7, 10 min, reaction volume- 1ml).

3.9.4 Effect of reaction temperature on molar conversion and by-product formation

To evaluate the effect of temperature on molar conversion of nicotinamide to NAH and by-product formation, enzymatic reactions with optimized substrate/co-substrate concentrations combination (200/250 mM) were performed at various temperatures (30, 35, 45 and 55 °C) in 25 ml of 50 mM phosphate buffer (pH 7) using 0.5 mg_{dcw}/ml resting cells. Samples were taken at interval of 10 min for 90 min for analysis of NAH and nicotinic acid concentration.

3.9.5 Effect of resting cells on conversion and time course of reaction

Cell concentration varying from 0.3-0.9 mg_{dcw}/ml were used to find out the optimum concentration of resting cells and time course of bioconversion of nicotinamide to nicotinic acid hydroxamate under optimized conditions of temperature (30 °C), substrate/co-substrate concentration combination (200/250 mM) and 25 ml of 50 mM phosphate buffer (pH 7).

3.9.6 Fed batch biotransformation at 50 ml scale

Fed-batch biotransformation was carried out in 250 ml Erlenmeyer flask containing 50 ml of reaction mixture with initial nicotinamide and hydroxylamine-HCl concentration of 200 and 250 mM respectively and 0.7 mg_{dcw}/ml cells in phosphate buffer (50 mM, pH 7) at 30 °C. Powdered nicotinamide (1.22 g) and highly concentrated solution (2 ml, 5 M, pH 7) of hydroxylamine-HCl were fed in subsequent two feeds after 40 and 80 min to maintain the residual nicotinamide and hydroxylamine concentration around 200 and 250 mM respectively. 500 µl of sample was withdrawn at every 10 min during the reaction and monitored for nicotinic acid hydroxamate, nicotinic acid and nicotinamide concentration. Effort was made to maintain the reaction volume constant around 50 ml. A control experiment was also conducted with same parameters without enzyme for any spontaneous chemical reaction.

3.9.7 Effect of nicotinic acid hydroxamate concentration on acyltransferase activity

The effect of NAH on acyltransferase activity was determined for a NAH concentration range of 0-500 mM under assay conditions (200 mM nicotinamide, 250 mM hydroxylamine-HCl, 50 mM phosphate buffer, pH 7, 45 °C, cell concentration- 0.7 mg_{dcw}/ml).

3.9.8 Recovery of nicotinic acid hydroxamate

After completion of fed batch process, the reaction mixture was centrifuged (10,000 x g for 20 min) to separate the cells. The supernatant obtained was freeze-dried to recover pinkish white powder. HPLC analysis of this powder was carried out to determine nicotinic acid hydroxamate and nicotinic acid concentration (w/w). The product was further purified by the extraction of 200 mg of this powder with 5 ml of acidified acetone

(with 11.5 N HCl). The extract was filtered and evaporated to yield a liquid. The HPLC analysis of this liquid was done to confirm the presence of nicotinic acid hydroxamate and its purity.

3.10 Bioprocess development for isoniazid synthesis

3.10.1 Effect of temperature

To study the effect of temperature on acyltransferase activity of whole cell amidase for isoniazid synthesis, enzyme assay was performed with 100 µl of resting cells (1.0 mg_{dcw}/ml) at different temperatures (25 to 70 °C) keeping other standard assay parameters (100 mM isonicotinamide, 500 mM hydrazine-2HCl, 50 mM phosphate buffer, pH 7.0, reaction volume- 1ml) constant for 20 min. The maximum activity of enzyme was considered as 100%.

3.10.2 Effect of pH

The optimum pH for the acyltransferase activity for isoniazid synthesis was determined for a pH range of (4.0-10) in following buffers (50 mM): sodium acetate buffer (pH 4.0-5.8), potassium phosphate buffer (pH 5.8-8.0), borate buffer (pH 8.0-9.2), and sodium carbonate buffer (pH 9.2-10). Acyltransferase activity in each buffer was measured with 100 µl of resting cells (1.0 mg_{dcw}/ml) keeping other standard assay parameters (55 °C, 100 mM isonicotinamide, 500 mM hydrazine-2HCl, reaction volume- 1ml) constant for 20 min. The maximum activity of enzyme was considered as 100%. The effect of molarity of potassium phosphate buffer at optimum pH 7 was studied for a molarity range of 25 mM to 500 mM under assay conditions.

3.10.3 Effect of substrate and co-substrate concentration on acyltransferase activity

To study the effect of isonicotinamide concentration on amide acyltransferase activity, varied concentrations of isonicotinamide (10-500 mM) at constant hydrazine-2HCl concentration (500 mM) were used. Acyltransferase activity assay was performed with 100 µl of resting cells (1.0 mg_{dcw}/ml) under optimized assay conditions (55 °C, phosphate buffer 100 mM, pH 7, 20 min, reaction volume- 1ml). Effect of hydrazine-2HCl concentration on acyltransferase activity and by-product formation was studied in the range of 0-1500 mM keeping isonicotinamide concentration constant (100 mM).

Acyltransferase activity assay was performed with 100 µl of resting cells (1.0 mg_{dcw}/ml) under optimized assay conditions (55 °C, phosphate buffer 100 mM, pH 7, 20 min, reaction volume- 1ml).

3.10.4 Optimization of substrate and co-substrate concentrations combination

This experiment was performed in such a manner that concentration of isonicotinamide was varied from 25-500 mM at different hydrazine-2HCl concentrations ranging from 250-1500 mM in the reaction mixture. Total 42 combinations were used to find out the optimum concentrations combination. Acyltransferase activity assay was performed with 100 µl of resting cells (1.0 mg_{dcw}/ml) under optimized assay conditions (55 °C, 100 mM phosphate buffer pH 7, 20 min, reaction volume- 1ml).

3.10.5 Effect of reaction temperature on molar conversion and by-product formation

To evaluate the effect of temperature on molar conversion of isonicotinamide to isoniazid and by-product formation, enzymatic reactions with optimized substrate/co-substrate concentrations combination (100/1000 mM) were performed at various temperatures (30, 35, 45 and 55 °C) in 25 ml of 100 mM phosphate buffer (pH 7) using 1.0 mg_{dcw}/ml resting cells. Samples were taken at interval of 15 min for 120 min for analysis of isoniazid and isonicotinic acid.

3.10.6 Effect of resting cells on conversion and time course of reaction

Cell concentration varying from 1.0-2.5 mg_{dcw}/ml were used to find out the optimum concentration of resting cells and time course of bioconversion of isonicotinamide to isoniazid under optimized temperature (30 °C) and substrate/co-substrate (100/1000 mM) concentration combination in 25 ml of 100 mM phosphate buffer (pH 7).

3.10.7 Fed batch biotransformation at 50 ml scale

Fed-batch biotransformation was carried out in 250 ml Erlenmeyer flask containing 50 ml of reaction mixture with initial isonicotinamide and hydrazine-2HCl concentration of 100 and 1000 mM respectively and 2.0 mg_{dcw}/ml resting cells in phosphate buffer (100 mM, pH 7) at 30 °C. Powdered isonicotinamide (0.61 g) and highly concentrated solution (1 ml, 5 M, pH 7) of hydrazine-2HCl were fed in subsequent seven feeds at an interval of

60 min to maintain the residual isonicotinamide and hydrazine-2HCl concentration around 100 and 1000 mM respectively. 500 µl of sample was withdrawn at every 30 min during the reaction and monitored for isoniazid, isonicotinic acid and isonicotinamide concentrations. Effort was made to maintain the reaction volume constant at around 50 ml. A control experiment was also conducted with same parameters without enzyme for any spontaneous chemical reaction.

3.10.8 Recovery of isoniazid

A fed batch process was performed at 50 ml scale and after 5 h; reaction was stopped by centrifugation at 10,000 x g for 20 min at 4 °C. The supernatant obtained was freeze-dried to recover white powder. Isoniazid was further purified by extraction of this powder with 120 ml of methanol for 1 h. The methanolic solution was filtered, evaporated and obtained powder was analysed using HPLC for its purity.

3.11 Cell lysis for the recovery of amidase enzyme

3.11.1 Enzymatic method

Lyophilized bacterial cells (5.0 g/l) in phosphate buffer (25 mM, pH 7) were incubated with different concentrations of lysozyme (0.5, 1.0, 2.0 and 5.0 g/l) at 30 °C for 1 hour in incubated shaker at 200 rpm for lysis. Cell debris was removed by centrifugation at 10,000 x g for 10 min at 4 °C. Supernatant was analysed for the protein concentration and enzyme assay. Cell debris was again suspended in the same amount of phosphate buffer for determining the unrecovered amidase activity.

3.11.2 Mechanical methods

Bacterial cell suspensions (5.0 g/l) were sonicated on icebath in 5 ml glass tubes using a Hielscher ultrasonic processor UP100H (100W, 30 kHz) for a total sonication time of 2, 4, 6, 8, 10 and 12 min (30s pulse-on and 30s pulse-off). Cell debris was removed by centrifugation at 10,000 x g for 10 min at 4 °C. Supernatant was analysed for the protein concentration and enzyme assay. Cell debris was again suspended in the same amount of phosphate buffer for determining the unrecovered amidase activity.

3.12 Protein precipitation

3.12.1 Precipitation by ammonium sulphate

Bacterial cell suspension (5 g/l) was lysed by incubating with 1 g/l lysozyme at pH 7, 30 °C for 1h. Cell debris was removed by centrifugation at 10,000 x g for 10 min at 4 °C. Proteins in the supernatant were serially precipitated by different concentration of ammonium sulphate which was calculated using the following formula:

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

S_2 = Final % saturation

S_1= Initial % saturation

Initially ammonium sulphate was added to cell free extract up to 30% saturation. This 30% saturated suspension was kept at 4 °C for 15 min, and then gently stirred for 5 min. The precipitate formed was collected by centrifugation. Again ammonium sulphate was added to the supernatant obtained from previous step to 60% saturation, kept at 4 °C for 15 min and then gently stirred for 5 min. The precipitate was collected by centrifugation. Now ammonium sulphate was added to supernatant obtained from previous step to 90% saturation, kept at 4 °C for 15 min and then gently stirred for 5 min. The precipitate was collected by centrifugation. The precipitated proteins obtained from each step of ammonium sulphate precipitation were dissolved in 5 ml of potassium phosphate buffer (25 mM, pH 7). These precipitated proteins and supernatant obtained after 90% saturation were assayed for amidase acyltransferase activity and protein concentration.

3.12.2 Sodium sulphate precipitation

Proteins in cell free extract were serially precipitated by 10, 20, 30 and 40% of sodium sulphate. Precipitated proteins were separated by centrifugation at 10,000 x g for 15 min at 4 °C. These precipitated proteins and supernatant were assayed for amidase acyltransferase activity and protein concentration. Amidase acyltransferase activity was determined under standard assay conditions of 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer 50 mM, pH 7, 45 °C, 10 min.

3.13 Steps of amidase purification

All the amidase purification steps were carried out at a temperature below 10 °C using 25 mM potassium phosphate buffer (pH 7). The protein preparations at different

stages of amidase purification were analyzed for amidase acyltransferase activity and protein concentration. The following steps were performed during amidase purification.

3.13.1 Cell growth and preparation of cell-free extract

Cells of *B. smithii* strain IIR6b2 were grown in mineral base medium (section 3.2.2) containing 10 mM phenylacetonitrile as mentioned in section 3.3. Cells were harvested after 48 hours of growth by centrifugation at 10,000 x g for 10 min at 4 °C. Biomass (100 mg_{dcw}) was suspended in 10 ml of 25 mM potassium phosphate buffer (pH 7) and the resulting mixture was vortexed until a homogenous suspension was obtained. Cells were lysed by adding 10 mg lysozyme in 10 ml of cell suspension and incubated at 30 °C for 1 h. Cell debris was removed by centrifugation at 10,000 x g for 10 min and the clear supernatant (crude cell free extract) was used for further amidase purification steps.

3.13.2 Ammonium sulphate precipitation

The crude cell free extract (10 ml) was brought to 30% (w/v) saturation with addition of solid ammonium sulphate under stirring. The 30% saturated suspension was kept at 4 °C for 15 min and then gently stirred for 5 min, and the resulting precipitant formed was removed by centrifugation at 10,000 x g for 15 min at 4 °C. Again ammonium sulphate was added to supernatant to 60% saturation, kept at 4 °C for 15 min and then gently stirred for 5 min. The precipitate was collected by centrifugation at 10,000 x g for 15 min at 4 °C. This protein pellet precipitated at 30-60% saturation containing amidase enzyme was re-suspended in 2 ml of potassium phosphate buffer (25 mM, pH 7).

3.13.3 Ultrafiltration

The protein fraction (2 ml) precipitated between 30-60% ammonium sulphate saturation was desalted and then concentrated to 1 ml with centrifugal concentrators (Amicon ultra, Millipore) with molecular weight cut off of 10 kDa. Ultrafiltration was carried out in centrifuge at 5000 rpm and at a temperature of 4 °C. Initially protein sample was concentrated to 1.0 ml and then 25 mM buffer was added to reservoir of centrifugal concentrator to bring the sample volume to 5 ml. This process was repeated to three times to desalt the protein sample and finally protein sample after desalting was concentrated to 1.0 ml.

3.13.4 Anion exchanger chromatography

This step of amidase purification was performed with an ÄKTA prime plus, GE Healthcare Life sciences. The clarified supernatant (1 ml) after ultrafiltration was loaded on an anion exchanger Q sepharose column (resin packed in a PTFE, jacketed column, 1.0 cm x 30 cm, bed volume 24 ml) at a flow rate of 1.3 ml/min. The column was pre-equilibrated with buffer A (25 mM phosphate buffer, pH 7) and the same buffer was used to wash the column until no unbound protein was detected in the flow through. Bound proteins were eluted from the column by a step gradient (40 ml for each 20, 25, 30, 35, 40, 45 and 50 %) of 1 M NaCl in buffer A at a flow rate of 1.3 ml/min and fractions of 2.5 ml were collected. Amidase acyltransferase activity in pooled fractions of each peak was monitored under assay conditions. The active fractions showing amidase activity, eluted at 35% of 1M NaCl were pooled (30 ml) desalted and concentrated (1 ml) by centrifugal concentrators (Amicon ultra, Millipore) with molecular weight cut off of 10 kDa.

3.13.5 Hydrophobic interaction chromatography

The desalted and concentrated protein sample (1 ml) after ion exchange chromatography was mixed with 1 ml of 2 M NH_4SO_4 (in 25 mM phosphate buffer, pH 7) to equilibrated it. This protein fraction was loaded on a phenyl sepharose column (1.0 cm x 12 cm, bed volume 10 ml), after pre-equilibration with 1 M NH_4SO_4. The column was washed by gravity flow with 2.5 column volumes of 1M NH_4SO_4 to remove the unbound proteins. The elution of bound protein was done by a step gradient (25 ml for each 75, 62.5, 50, 37.5, 25, 12.5 and 0%) of 1 M NH_4SO_4 and fractions of 2 ml were collected. Protein in these fractions was detected using a Varian UV-VIS Spectrophotometer at 280 nm. Amidase acyltransferase activity in pooled fractions of each peak was monitored under assay conditions. The active fractions showing amidase activity, eluted at 0.375 M of NH_4SO_4 were pooled (24 ml), desalted and concentrated by centrifugal concentrators (Amicon ultra, Millipore) with molecular weight cut off of 10 kDa for PAGE analysis.

3.14 Polyacrylamide gel electrophoresis (PAGE)

3.14.1 Native-PAGE analysis

Purity of pooled, concentrated and desalted active fraction of HIC was determined by native-PAGE and further purification was found to be unnecessary. Non-denaturing

polyacrylamide gel electrophoresis was performed in a 10% native resolving gel and 5% staking gel in duplicates, one for purity analysis and another for zymogram.

3.14.2 Zymogram

To confirm that the purified protein band in the native-PAGE gel corresponds to amidase enzyme, gel was stained using acyltransferase activity assay according to modified method of Egorova et al. (2004). Native-PAGE gel was washed with distilled water and incubated in 25 ml of phosphate buffer (50 mM, pH 7) containing nicotinamide (100 mM) and hydroxylamine-HCl (500 mM) at 45 °C for 10 min. After incubation, the gel was washed with buffer and then acidic ferric chloride solution was used to cover the gel for visualization of a dark red brown band corresponding to the amidase acyltransferase activity in gel.

3.14.3 SDS-PAGE analysis and molecular mass determination

To evaluate the molecular weight of the purified enzyme, denaturing SDS-PAGE was conducted. Purified amidase was analysed under both reductive and non-reductive conditions on a 5% polyacrylamide-stacking gel and 10% polyacrylamide-resolving gel according to the method of Laemmli (1970). The molecular weight standard used was a mixture of reference proteins: 97.4 kDa, Phosphorylase B; 66 kDa, Bovine serum albumin; 43 kDa, Ovalbumin; 29 kDa, carbonic anhydrase; 20.1 kDa, soybean trypsin inhibitor and 14.3 kDa, lysozyme.

3.14.4 Reagents of PAGE

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

Solution B: Resolving gel buffer, 1.5 M Tris-Cl, pH 8.8

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

Solution D: 10% (w/v) SDS

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

Solution F: TEMED (N, N, N‟, N‟- tetramethylenediamine)

Table 3.7 Solutions for preparing resolving and stacking gels

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

3.14.5 Casting of gel

Gel electrophoresis was performed with a Mini-PROTEAN Tetra Cell system (Bio-Rad) according to manufacturer's instructions. Resolving gel was prepared by mixing all the components followed by addition of TEMED before pouring. The mixture was gently mixed and poured between the plates by 1 ml micro-pipette and leaving the adequate space at the top for pouring the stacking gel after polymerization. Resolving gel was overlaid by distilled water to prevent oxygen from diffusing into the gel and inhibiting polymerization. After completion of polymerization, overlay was poured-off and washed the top of gel several times with de-ionized water to remove any un-polymerized acrylamide. Mixture of stacking gel was prepared followed by addition of TEMED and it was poured similarly as the resolving gel and comb was immediately inserted into the stacking gel mixture to form the wells. After polymerization of stacking gel, comb was carefully removed and formed wells were rinsed with de-ionized water and polymerized gel was used for electrophoresis.

3.14.6 Sample preparation

Gel-loading buffer for NATIVE-PAGE (5X): 0.313 M Tris-Cl (pH 6.8), 0.1% bromophenol blue, 50% (v/v) glycerol.

Gel-loading Buffer for non-reductive SDS-PAGE (5X): 0.313 M Tris-Cl (pH 6.8), 0.1% bromophenol blue, 50% (v/v) glycerol, 10% (w/v) SDS.

Gel-loading Buffer for reductive SDS-PAGE (5X): 0.313 M Tris-Cl (pH 6.8), 2% (v/v) β-mercaptoethanol, 0.1% bromophenol blue, 50% (v/v) glycerol, 10% (w/v) SDS.

Protein samples and gel loading buffer in the ratio of 20:5 was mixed and loaded on the gel for electrophoresis. For SDS-PAGE (reducing conditions) the samples were mixed with gel loading buffer and boiled for 5 min.

3.14.7 Electrophoresis

Tris-glycine buffer (For Native–PAGE, 1X): 25 mM Tris, 250 mM glycine (pH 8.3)

Tris-glycine buffer (For SDS-PAGE, 1X): 25 mM Tris, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS

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

3.14.8 Determination of molecular weight of purified amidase by size exclusion chromatography

The purity and molecular mass of the purified native amidase was also evaluated by size exclusion (gel-filtration) chromatography using ÄKTA prime plus, GE Healthcare Life Sciences. Concentrated active fraction of HIC (1 ml) at flow rate of 0.5 ml/min was loaded on a Hiload Superdex 75 column (1.6 cm x 60 cm, GE Healthcare, Life sciences) equilibrated with 25 mM phosphate buffer pH 7. The protein was eluted with 120 ml of the same buffer at a flow rate of 0.5 ml/min. Only a single major peak was observed, and the corresponding fractions contained the amidase acyltransferase activity.

3.14.9 Protein estimation by Bradford method

Preparation of Bradford Reagent

Bradford reagent was prepared by dissolving 20 mg of the dye Coomassie Brilliant Blue G250 in 10 ml ethanol and 20 ml of 85% phosphoric acid was added in it. The final

volume was adjusted to 200 ml with distilled water and solution was filtered through Whatman filter paper no. 2. The reagent was stored in an amber bottle and kept at 4 °C.

Procedure

Initially a series of various concentrations of the standard protein solution (bovine serum albumin) were prepared in the range 0-100 µg/ml in 25 mM phosphate buffer of pH 7.0. Further 1 ml of the Bradford reagent was added in 200 µl of each protein sample and mixed well. A reagent blank was also set up by adding 1 ml of the reagent to 200 µl 25 mM phosphate buffer of pH 7.0. Now absorbance was measured at 595 nm after 5 min using a spectrophotometer. A standard plot for standard concentration of protein BSA (µg/ml) against O.D. was plotted (Fig 3.6) and the amount of protein in the unknown sample was determined by referring to standard plot.

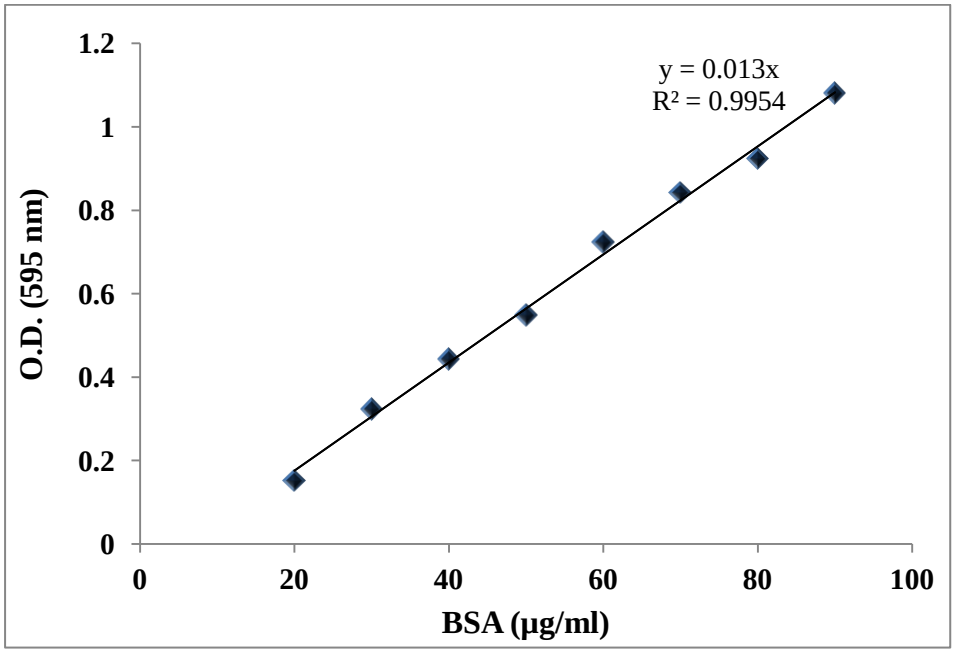


Fig 3.6 Standard plot of BSA by Bradford method

3.15 Characterization of purified amidase

3.15.1 Effect of temperature

To study the effect of temperature on acyltransferase activity of purified amidase, enzyme assay was performed at different temperatures (25 to 70 °C) with 100 µl of

purified amidase (16 μg/ml) keeping all other standard assay parameters (100 mM nicotinamide, 500 mM hydroxylamine-HCl, 50 mM phosphate buffer, pH 7.0, reaction volume- 1ml) constant for 10 min. The maximum activity of enzyme was considered as 100%.

3.15.2 Effect of pH

The optimum pH for the enzymatic reaction was determined for a pH range of (4.0-10) in following buffers (50 mM): sodium acetate buffer (pH 4.0-5.8), potassium phosphate buffer (pH 5.8-8), borate buffer (pH 8.0-9.2), and sodium carbonate buffer (pH 9.2-10). Acyltransferase activity in each buffer was measured with 100 μl of purified amidase (16 μg/ml) keeping all other standard assay parameters (45 °C, 100 mM nicotinamide, 500 mM hydroxylamine-HCl, reaction volume- 1ml) constant for 10 min. The maximum activity of enzyme was considered as 100%.

3.15.3 Substrate specificity

Different amides (section 3.8.4) were tested for substrate specificity of purified amidase enzymes for both acyltransferase and amide hydrolase activities. Enzymatic reaction (reaction volume- 1ml) for acyltransferase activity assay was performed with 100 μl of purified amidase (35 μg/ml), 10 mM amide substrate and 500 mM hydroxylamine-HCl at 45 °C, pH 7 for 10 min. Amide hydrolase activity assay was performed under similar assay conditions in absence of co-substrate hydroxylamine-HCl.

3.15.4 Effects of chemical reagents, inhibitors and metal ions on enzyme activity

The effect of different metal ions on the enzyme activity was investigated by pre-incubation of purified amidase with metal ions at three different final concentrations (1, 5, 10 mM) at 30 °C for 30 min (16 μg/ml amidase, final volume 100 μl). The enzyme activity after incubation was assayed under standard assay conditions (100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer 50 mM, pH 7 and 10 min, reaction volume-1ml). The activity of the enzyme incubated in the absence of any metal ion was taken as 100%. The effects of various chemical reagents such as disulphide bond (-SH) reducing agents (dithiothreitol), metal chelators (1, 10-phenanthroline, EDTA); -SH group inhibitors (n-ethylmaleimide and DTNB), oxidizing agents (H_2O_2, ammonium persulphate) and Di-ethyl phosphoramidate on the acyltransferase activity were examined. These reagents were

incubated at 30 °C for 30 min with the purified amidase at their final concentrations ranging from 1–10 mM (16 μg/ml amidase, final volume 100 μl). The enzyme activity after incubation was assayed under standard assay conditions. Enzyme activity determined without these reagents, under similar conditions was assumed as 100%. The reaction mixture with respective metal/ chemical reagent but without enzyme served as blank.

3.15.5 Organic solvent compatibility of purified amidase

The compatibility of the acyltransferase activity of purified amidase with several water miscible and immiscible solvents was determined. Purified amidase was incubated with these solvents at their final concentration of 10, 20 and 30% (v/v) at 30 °C for 30 min (16 μg/ml amidase, final volume 100 μl) and the residual acyltransferase activity was determined under standard assay conditions (100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer 50 mM, pH 7, 45 °C, 10 min, reaction volume- 1ml). The acyltransferase activity was assumed as 100% in the absence of any organic solvent.

3.15.6 Effect of surfactants and detergents

The effects of different non ionic, ionic and cationic (Triton X-100, Tween 20, Tween 40, Tween 80, sodium deoxycholate, sodium cholate, SDS and CTAB) surfactants on acyltransferase activity were studied. These surfactants at their final concentration of 0.05, 0.1 and 0.2% were incubated with purified amidase at 30 °C for 30 min (7 μg/ml amidase, final volume 100 μl) and the residual activity was assayed under standard assay conditions (100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer 50 mM, pH 7, 45 °C, 10 min, reaction volume- 1ml). The enzyme activity was assumed as 100% in the absence of any surfactant. The reaction mixture with respective surfactant but without enzyme served as blank.

CHAPTER 4

RESULTS AND DISCUSSION

Results and discussion of this study are presented in four sections:

Section 4.1 Screening, selection, characterization of microbial isolate producing amidase enzyme with acyltransferase activity and whole cell enzyme characterization

Section 4.2 Bioprocess development for nicotinic acid hydroxamate synthesis

Section 4.3 Bioprocess development for isoniazid synthesis

Section 4.4 Purification of amidase enzyme and its characterization

SECTION 4.1

4.1.1 Screening of biocatalyst for bioprocess development

4.1.1.1 Screening of bacterial isolates for amidase producing isolate

Various nitrile metabolizing bacterial isolates, previously isolated from different sources (soil and water samples of different parts of India) were screened for amidase activity. Among these bacterial isolates three isolates (6bD, 6b2 and 3a) were found to produce amidase enzyme (Table 4.1). Most of the studied amidases are also reported to exhibit acyltransferase activity besides amide hydrolysis in presence of co-substrate hydroxylamine or hydrazine (Fournand *et al*., 1998a, 1998b, Kobayashi *et al*., 1999, Makhongela *et al*., 2007, Bhatia *et al*., 2013). Therefore acyltransferase activity profiles of these amidase producing isolates were studied to confirm their utility for hydroxamic acids syntheses. Further, criteria of biocatalyst selection were based on acyltransferase activity towards heterocyclic amides, high acyltransferase to amide hydrolase activity ratio and molar conversion efficiency with minimum by-product formation.

Table 4.1 Screening of bacterial isolates producing amidase enzyme

Nitrile metabolizing isolates	Amidase positive isolates
1a	
1b	
3a	Amidase positive
3d	
4a	
6b	
6b2	Amidase positive
6bD	Amidase positive
9a	
9b	

4.1.1.2 Study of acyltransferase activity profile with different substrates

Whole cell amidases of three isolates exhibited acyltransferase activity for a wide range of amides and thus it seems that these amidases are suitable for hydroxamic acids syntheses with broad choice of amides (Table 4.2). In present study, aim was to develop bioprocesses for nicotinic acid hydroxamate and isoniazid syntheses. So a biocatalyst with high affinity towards heterocyclic amides was required. From substrate specificity profiles of three whole cell amidases it was observed that all three had affinity for nicotinamide and isonicotinamide, however maximum acyltransferase activity was obtained with amidase of 6b2 isolate for both substrates.

Table 4.2 Acyltransferase activity profile of whole cell amidases of bacterial isolates

Substrate	Acyltransferase activity (U/mg)		
(10 mM)	6bD	6b2	3a
Acetamide	4.81±0.18	6.75±0.24	0.36±0.03
Propionamide	3.58±0.23	2.32±0.13	0.74±0.05
Butyramide	1.63±0.13	3.20±0.11	0.84±0.13
Isobutyramide	0.77±0.07	0.87±0.10	1.13±0.09
Adipamide	0.10±0.08	0.42±0.15	0.32±0.04
Acrylamide	2.70±0.17	1.71±0.24	0.18±0.08
Methacrylamide	0.11±0.05	0.40±0.14	0.10±0.02
Nicotinamide	**4.24±0.33**	**6.80±0.37**	**1.32±0.08**
Isonicotinamide	**1.16±0.10**	**2.86±0.21**	**0.41±0.05**
Benzamide	3.63±0.12	6.32±0.29	0.10±0.04

Enzyme assay conditions: 10 mM amide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

4.1.1.3 Study of acyltransferase to amide hydrolase activity ratio

To achieve higher yield of hydroxamic acid and acid hydrazide syntheses from amide with minimum by-product (acid) formation it is desirable to have a biocatalyst with high acyltransferase and minimum amide hydrolase activities. Therefore high ratio of acyltransferase to amide hydrolase activity was chosen as criteria for selection of suitable bacterial isolate for further work. Higher the ratio, lower the formation of by-product. 6bD and 6b2 isolates exhibited higher acyltransferase activity than amide hydrolase activity for

most of the amides while 3a isolate showed higher amide hydrolase activity than acyltransferase activity for all amides (Table 4.3). This difference in acyltransferase and amide hydrolase activities of whole cell amidases is due to different affinity of acyl enzyme complex towards water and hydroxylamine. It is notable that the acyltransferase activity was significantly higher for 6b2 and 6bD isolates than 3a isolate towards heterocyclic amides (Table 4.2). On the other hand, ratios of acyltransferase to amide hydrolase activity for nicotinamide and isonicotinamide were also highest for 6b2 isolate followed by 6bD while for 3a isolate this ratio was less than 1.

Table 4.3 Acyltransferase to amide hydrolase activity ratio of bacterial isolates

Substrate	Ratio (acyltransferase/amide hydrolase activity)		
(10 mM)	6bD	6b2	3a
Acetamide	1.28	1.31	0.72
Propionamide	1.36	0.74	0.32
Butyramide	6.08	5.05	0.56
Isobutyramide	0.96	7.77	0.54
Adipamide	0.72	9.44	0.32
Acrylamide	1.34	0.45	0.29
Methacrylamide	1.46	3.39	0.48
Nicotinamide	**4.10**	**7.58**	**0.73**
Isonicotinamide	**0.95**	**2.45**	**0.64**
Benzamide	6.26	6.75	0.06

Enzyme assay conditions: For acyltransferase activity assay- 10 mM amide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0) and 45 °C. For amide hydrolase activity assay- 10 mM amide, phosphate buffer (50 mM, pH 7.0) and 45 °C.

4.1.1.4 Study of molar conversion efficiency and by-product formation

A further criterion of biocatalyst selection was molar conversion efficiency achievable by these isolates for nicotinic acid hydroxamate synthesis. Enzymatic reactions were performed with 100 mM of nicotinamide and 500 mM hydroxylamine–HCl for 45 minutes with whole cell amidases of all three isolates and product (nicotinic acid hydroxamate) along with by-product (nicotinic acid) formed were analysed (Table 4.4). Maximum 74.6% molar conversion of nicotinamide to NAH was achieved by 6b2 isolate while by-product formed was only 2.6%. This bioconversion yield obtained with 6b2

isolate was significantly higher to those obtained with 6bD (30.1%) and 3a (18.1%) isolates.

Among these three isolates, 6b2 isolate was selected for further studies as it possess notable acyltransferase activity for heterocyclic amides, high acyltransferase to amide hydrolase activity ratios for nicotinamide and isonicotinamide and the highest molar conversion efficiency with minimum by-product formation.

Table 4.4 Biocatalytic performances of three bacterial isolates for nicotinic acid hydroxamate synthesis

Isolates	Nicotinic acid hydroxamate (NAH) (mM)	Nicotinic acid (NA) (mM)	Product (NAH)/by-product (NA) ratio
6bD	30.15±1.72	3.66±0.77	8.23
6b2	74.64±2.52	2.63±0.39	28.38
3a	18.09±1.06	8.73±0.50	2.07

Reaction conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C, incubation time 45 min.

4.1.2 Identification of selected 6b2 isolate

4.1.2.1 Morphological characterization of 6b2 isolate

Morphological characterizations of isolate 6b2 were carried out by plating of isolate 6b2 in agar plate, Gram's staining and scanning electron microscopy. Colony of isolate 6b2 on agar plate had rounded configuration, convex elevation, smooth surface and white colour (Fig. 4.1). Isolate 6b2 was Gram positive rods as determined by Gram's staining (Fig. 4.2a) and scanning electron microscopy also confirmed rod shape of 6b2 isolate (Fig. 4.2b). Further, isolate 6b2 was characterized with respect to growth temperatures and initial growth pH (Table 4.5).

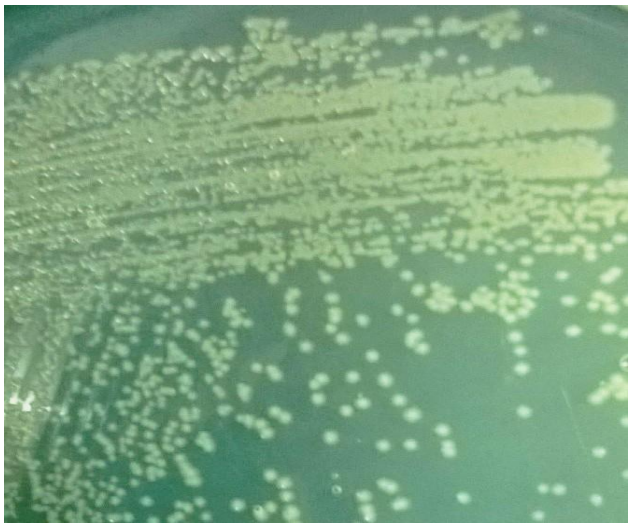


Fig.4.1 Growth of 6b2 isolate on agar plate

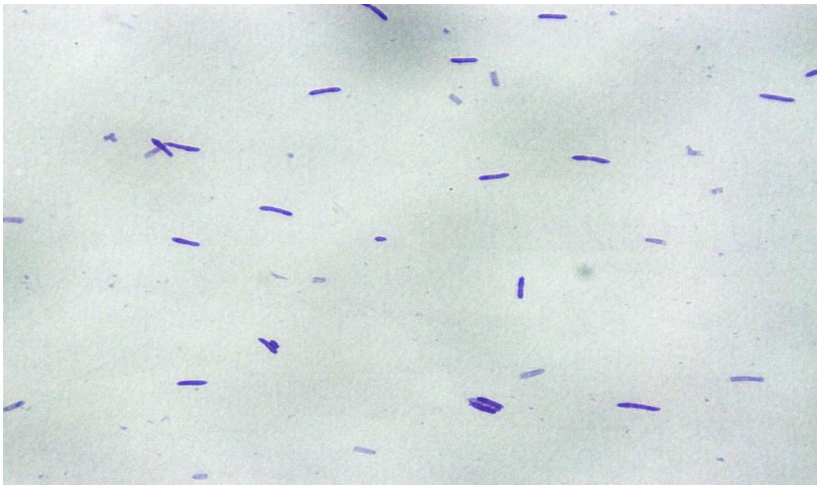


Fig.4.2a Gram's staining of 6b2 isolate

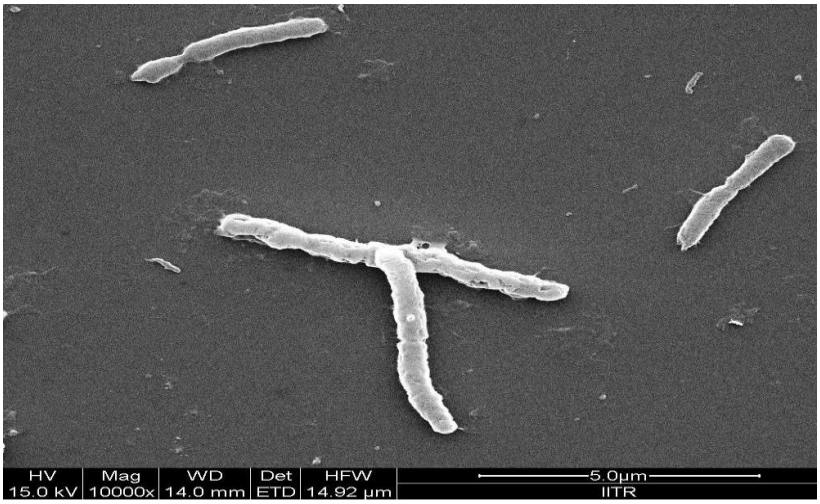


Fig.4.2b Scanning electron microscopy of 6b2 isolate

Table 4.5 Morphological and growth characterization of 6b2 isolate

Tests	Results
Colony morphology	
Configuration	Round
Margin	Entire
Elevation	Convex
Surface	Smooth
Density	Opaque
Colour	White
Gram's reaction	+
Motility	-
Fluorescence	-
Growth at temperature	
25 °C	-
30 °C	+
35 °C	+
40 °C	+
45 °C	+
50 °C	+
55 °C	+
60 °C	+
65 °C	-
Growth pH	
4.5	-
5.0	+
5.5	+
6.0	+
6.5	+
7.0	+
7.5	+
8.0	-

4.1.2.2 Biochemical characterization of 6b2

A biochemical analysis of isolate 6b2 was carried out using Biolog system to determine its carbon substrate utilization profile. Substrate utilization profile and cellular growth were measured spectrophotometrically. According to the data from the Biolog system (Table 4.6), the isolate was identified as *Bacillus* genus.

Table 4.6 Data from BioLog system

D-Raffinose	+	Glucoronamide	Bl
D-Glucose	+	α-Ketoglutaric acid	+
D-Sorbitol	+	Acetoacetic acid	-
Gelatin	+	Sucrose	+
Pectin	Bl	N- Acetyl D-mannosamine	+
4-Hydroxyphenylacetic acid	Bl	L- Fucose	+
Tween 40	+	Fructose -6 phosphate	-
Dextrin	+	L- Histidine	-
D-Lactose	+	Mucic acid	+
D-Mannose	+	D-Malic acid	+
D-Mannitol	+	Propionic acid	-
Glycyl-L-proline	+	D-Turanose	+
D-Galactouronic acid	Bl	N-Acetyl D-galactosamine	+
Pyruvic acid methyl ester	+	L-Rhamnose	+
		D-Aspartic acid	Bl
Amino butyric acid	+	L- pyroglutamic acid	+
D-Maltose	+	Quinic acid	+
D-Melibiose	+	L- Malic acid	+
D-Fructose	+	Acetic acid	-
D-Arabitol	+	Stachyose	+
L-Alanine	+	N-Acetylneuraminic acid	+
L-Galactonic acid-γ lactone	Bl	Inosine	+
D-Lactic acid methyl ester	+	D-Serine	-
β-Hydroxyl butyric acid	+	L-Serine	Bl
D-Trehalose	+	D-Saccharic acid	+
β-methyl d-glucoside	+	Bromo succinic acid	+
D-Galactose	+	Formic acid	-
Myo-inositol	+	Sodium lactate	+
L-arginine	+	Troleandomycin	-
D-Gluconic acid	+	Lincomycin	-
Lactic acid	+	Vancomycin	-
α-Hydroxy D-L butyric acid	+	Nalidixic acid	+
Cellobiose	+	Aztreonam	-
D- Salicin	+	Fusidic acid	-
3-Methyl glucose	+	Rifamycin SV	-
Glycerol	+	Guanidine hydrochloride	-
L-aspartic acid	+	Tetrazolium violet	Bl
Glucuronic acid	Bl	Lithium chloride	-
Citric acid	Bl	Sodium butyrate	Bl
α-ketobutyric acid	+	D-Serine	-
Gentiobiose	+	Minocycline	Bl
N-Acetyl-D-glucosamine	+	Niaproof-4	-
D-Fucose	+	Tetrazolium blue	Bl
D-Glucose -6 phosphate	-	Potassium tellurite	+
Glutamic acid	+	Sodium bromate	-

(+)- Positive, (-)- Negative, (Bl)- Borderline

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

Isolated DNA was visualized on agarose gel (Fig. 4.3a) and 16s rDNA was amplified by using universal primers. A PCR product of about 1.5 kb was obtained as visualized on agarose gel (Fig. 4.3b). The amplified PCR product was sequenced by Ocimum biosolutions, Hyderabad (India).

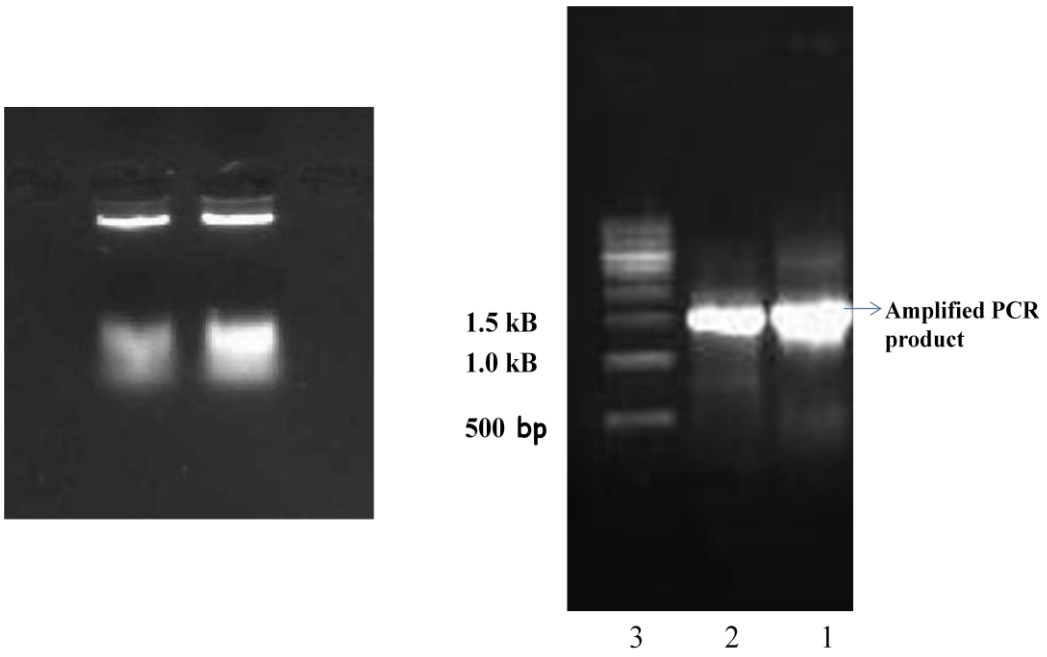


Fig 4.3 (a) Genomic DNA from 6b2 isolate **(b)** amplified 16S rDNA (lane1 and 2), Marker DNA (range 500-1500 bp, lane 3)

4.1.2.4 Phylogenetic analysis

Partial 16S rDNA sequence 6b2 isolate was compared with sequences in GenBank database by searching with the BLAST program provided by the National Center for Biotechnology Information. Comparative 16S rDNA sequence analysis suggested a strong similarity between isolate 6b2 and *Bacillus smithii* strains. Isolate 6b2 showed 99% similarity with *Bacillus smithii* strain TBMI12 (GeneBank accession no. EF010852), *Bacillus smithii* strain R-7170 (GeneBank accession no. AY373319), *Bacillus smithii* strain NRS-173 (GeneBank accession no. NR_036987) and *Bacillus smithii* strain T7 (GeneBank accession no. EU628681). Based on the partial 16S rDNA sequence of 6b2 isolate and 20 similar strains, a phylogenetic tree was constructed by neighbor-joining method (Fig. 4.4b). The sequence (Fig. 4.4a) was deposited in the Genbank database with an accession

no. **JX157878**. Based on biochemical characteristics and 16S rDNA sequence analysis, the isolate was identified as *Bacillus smithii* and referred as *Bacillus smithii* strain IITR6b2.

GCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGCAAGACGGGGATAACTCCGGGAAACCGGGGCT
AATACCGGATAATATCTTCCTTCGCATGAAGGAAGGTTGAAAGGCGGCGCAAGCTGCCGCTTGCAGATGG
GCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG
GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGCGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTG
TCAGGGAAGAACAAGTACCGTTCGAACAGGGCGGTACCTTGACGGTACCTGACCAGAAAGCCACGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC
GCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGA
CTTGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACC
AGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGCTTCCACCCTTTAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC
TTCGCTACCTCTAGAGATAGGAGGGTTCCCCTTCGGGGGACGGAGTGACAGTGGTGCATGGTTGTCGTCA
GCTCGTGTCGTGAGATTGTTGGGTTAAGTCCCGCAACGAGCGCACCCTGACCTTAGTTGCCAGCATTCAG
TTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGTCGCGAAACCGCGAGGTGGAGCCA
ATCCCAAAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTA
ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTT
GCAACACCCGAAGTCGGTGAGGTAACCCTTACGGGAGC

Fig 4.4a Partial 16S rRNA gene sequence of *Bacillus smithii* strain IITR6b2 submitted to GenBank database under accession no. JX157878

Table 4.7 Homology of 16S rDNA of isolated bacteria from other strains (data obtained from http:/blast.ncbi.nlm.nih.gov/Blast.cgi)

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
EU652724.1	*Bacillus smithii* strain T4 16S ribosomal RNA gene, partial sequence	2505	2505	100%	0.0	99%
EU628681.1	*Bacillus smithii* strain T7 16S ribosomal RNA gene, partial sequence	2505	2505	100%	0.0	99%
FJ490609.1	*Bacillus sp.* 2n 16S ribosomal RNA gene, partial sequence	2501	2501	100%	0.0	99%
AB271749.1	*Bacillus smithii* gene for 16S rRNA, partial sequence	2499	2499	100%	0.0	99%
AY373319.1	*Bacillus smithii* strain R-7170 16S ribosomal RNA gene, partial sequence	2497	2497	100%	0.0	99%
JF749811.1	*Bacillus sp*. BPTK2 16S ribosomal RNA gene, partial sequence	2492	2492	99%	0.0	99%
NR_036987.1	*Bacillus smithii* strain NRS-173 16S ribosomal RNA, partial sequence	2484	2484	100%	0.0	99%
JQ435663.1	*Bacillus smithii* strain PR3 16S ribosomal RNA gene, partial sequence	2483	2483	100%	0.0	99%
FJ430068.1	*Bacillus sp*. F92 16S ribosomal RNA gene, partial sequence	2483	2483	99%	0.0	99%
EF592490.1	*Bacillus sp*. MS-1 16S ribosomal RNA gene, partial sequence	2479	2479	100%	0.0	99%
EF010852.1	*Bacillus smithii* strain TBMI12 16S ribosomal RNA gene, partial sequence	2447	2447	100%	0.0	99%
FJ572204.1	*Bacillus smithii* strain GBPI2 16S ribosomal RNA gene, partial sequence	2379	2379	95%	0.0	99%
GQ293457.1	*Bacillus smithii* strain CMB-B1 16S ribosomal RNA gene, partial sequence	2292	2292	91%	0.0	99%
X60643.1	*B.smithii* 16S ribosomal RNA	2287	2287	97%	0.0	97%
AY603079.1	*Bacillus sp.* STB9 16S ribosomal RNA gene, partial sequence	2285	2285	100%	0.0	97%
D78316.1	*Bacillus smithii* strain JCM9076 DNA for 16S ribosomal RNA, partial sequence	2279	2279	97%	0.0	97%
AB362281.1	*Bacillus aeolius* gene for 16S rRNA,	2254	2254	97%	0.0	97%

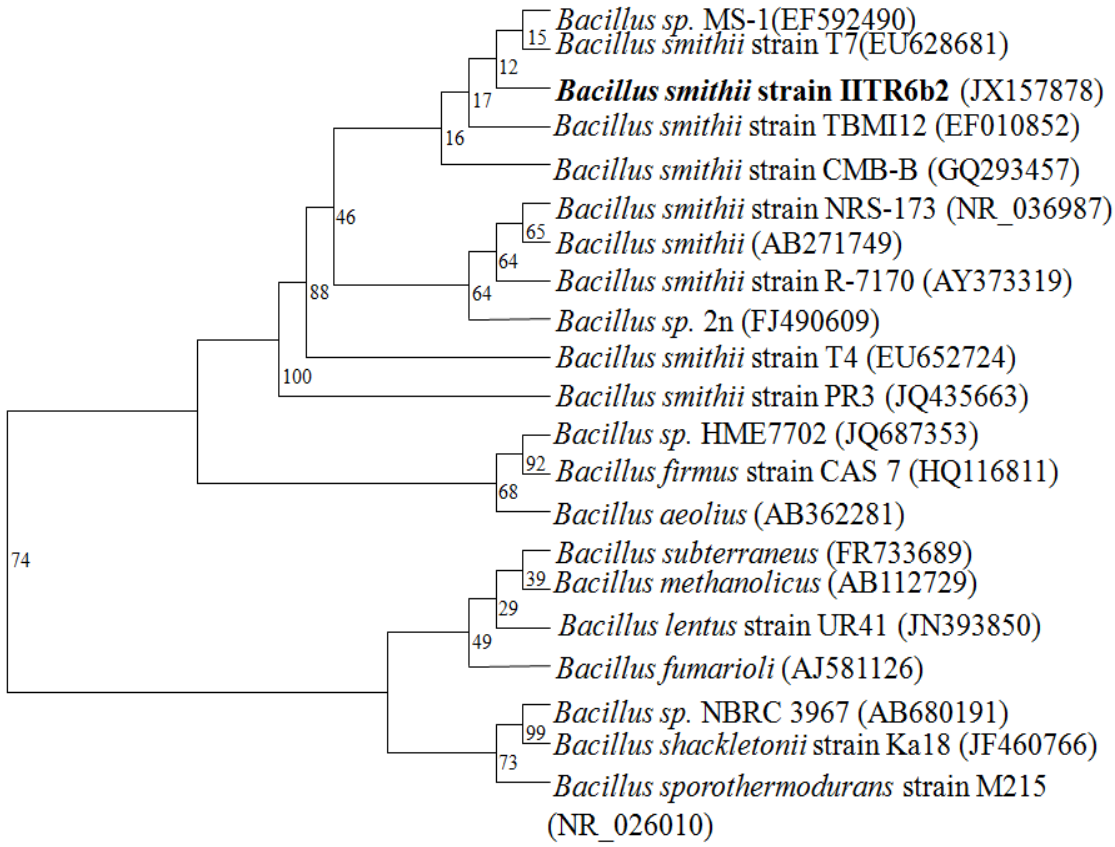


Fig 4.4b Phylogenetic tree of *Bacillus smithii* strain IITR6b2 and related strains based on the 16S rDNA sequence. Numbers after the name of organisms are accession numbers of published sequences

4.1.3 Production medium optimization

Enzymes production by microorganism is greatly influenced by different physiochemical and environmental factors. Therefore, various environmental parameters are needed to be optimized to enhance the enzyme production. In the present study, optimization of different parameters for *B. smithii* strain IITR6b2 was carried out with respect to acyltransferase activity and bacterial cell growth. The experiments were designed for maximum production of amidase (checked by acyltransferase activity) by optimizing culture conditions by one variable at a time (OVAT) approach. Effects of various parameters such as incubation temperature (25-65 °C), initial medium pH (4.5-8.0), type of carbon source, concentration of carbon source (2.5-25 g/l), type of nitrogen source and concentration of nitrogen source (2.5-30 mM) on amidase production and cell growth were studied. Samples were removed from production medium at different time intervals for analysis of acyltransferase activity and bacterial cell growth. Optimum

acyltransferase activity with corresponding cell biomass was selected for comparative analysis during optimization of any single parameter.

4.1.3.1 Effect of temperature on amidase production

The effect of temperature on cell growth and amidase production was investigated by varying incubation temperature from 25 to 65 °C, and the results are presented in Fig. 4.5. Isolate was capable of growth and amidase production in temperature range of 30–60 °C and failed to grow at 25 and 65 °C. Increase in amidase production as well as cell growth was observed with rise in incubation temperature and maximum amidase production (13.92±1.02 U/mg) was obtained at 45 °C with moderate biomass (0.36±0.04 mg/ml). Incubation temperature above 45 °C, supported higher cell growth with maximum growth at 55 °C, while amidase production at 55 °C was 74% of the optimum value and it further decreased to 60% at 60 °C. Decrease in amidase production at higher temperature may be due to thermal denaturation of amidase enzyme leading to reduced enzyme activity. At lower temperature (30 and 35 °C) slow growth was observed while amidase productions were 51 and 68% of optimum value at 30 and 35 °C respectively. In contrast to these results, 30 °C was found to be optimum temperature for amidase production by *Alcaligenes sp.* MTCC 10674 (Bhatia *et al*., 2013).

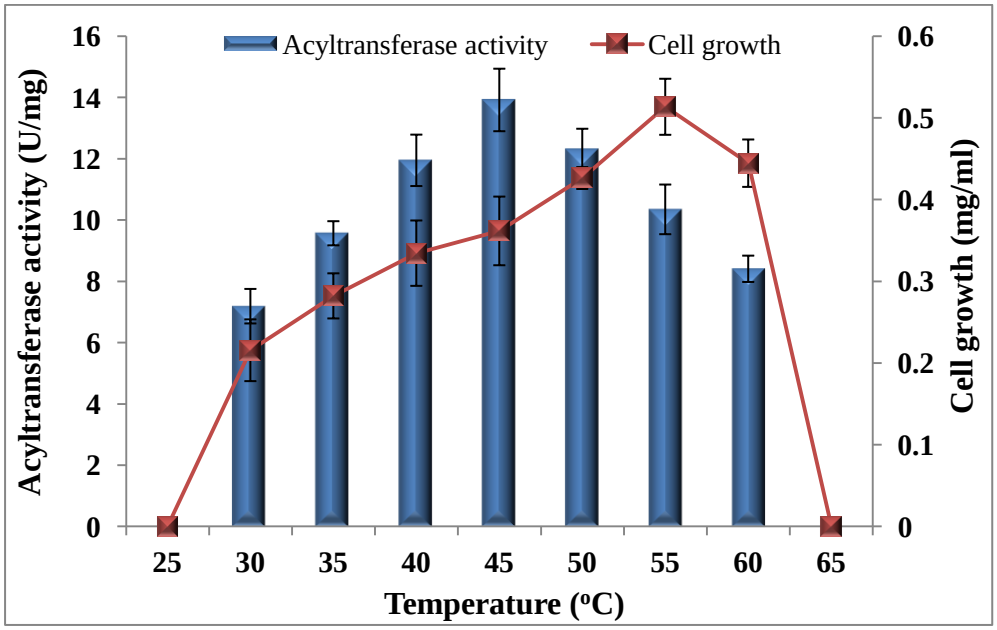


Fig. 4.5 Effect of temperature on amidase production
Other environmental parameters: carbon source- glycerol (5 g/l), nitrogen source- glutaronitrile (10 mM), pH 7.0
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

4.1.3.2 Effect of initial medium pH on amidase production

The effect of initial pH of medium on cell growth and amidase production by isolate is presented in Fig. 4.6. The growth and amidase production varied with change in pH in a range of 5.0-7.5 while no growth was observed when pH of the medium was below 5.0 and above 7.5. In spite of the continued decrease in cell growth with increase in pH, amidase production attained a maximum level at pH 7.0 (14.8±0.58 U/mg) and declined above it. At pH 6.5 and 7.5, 88% and 79% amidase productions were obtained respectively. However at pH value below 6.0, amidase production was very low but cell growth steadily increased with decrease in pH. Maximum microbial growth was achieved at pH 5.0 although amidase production was only 52% of the optimum value. Therefore, optimum pH for amidase production was neutral pH and in subsequent studies, the initial pH of medium was adjusted to 7.0. Amidase production from *Alcaligenes sp.* MTCC 10674 has been reported to be highest at pH 7.5 while bacterial growth was highest at pH 7.0 (Bhatia *et al*., 2013).

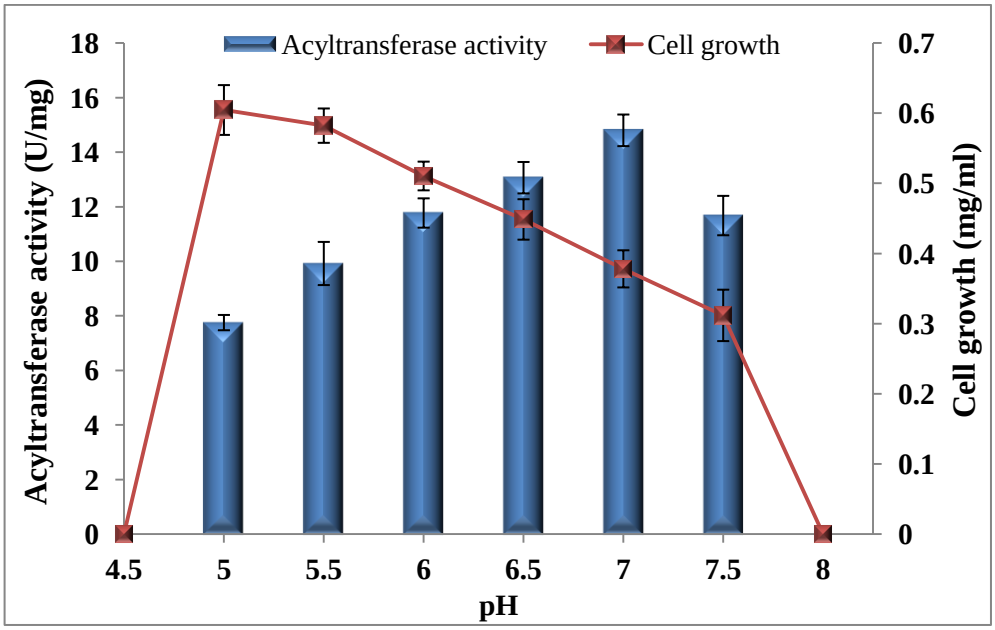


Fig 4.6 Effect of initial medium pH on amidase production
Other environmental parameters: carbon source- glycerol (5 g/l), nitrogen source- glutaronitrile (10 mM), temperature 45 °C
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

4.1.3.3 Effects of different carbon sources on amidase production

The effects of various organic carbon sources on the biomass and amidase production by the isolate were investigated at 5 g/l concentration and the results are summarized in Fig. 4.7. The isolate was able to grow in various carbon sources, however significant variations in both cell growth and amidase production were observed with carbon sources. Among these carbon sources, although glucose and fructose supported more growth, they could not result in higher amidase production. The highest amidase (15.25±0.65 U/mg) was produced by *B. smithii* strain IITR6b2 in the presence of glycerol, which also produced higher biomass (0.34±0.03 mg/ml) than sucrose, maltose and lactose. Minimum cell growth was observed with lactose followed by maltose while amidase productions were only 41 and 58% of the maximum value respectively. Therefore, in this study, glycerol was selected as the most suitable carbon source in terms of amidase production and used in the later experiments. Maximum production of amidase from *Delfia tsuruhatensis* ZJB-05174 (82.06 U/l) and *Rhodococcus erythropolis* MTCC 1526 (489.14 U/g) was observed when glucose and sorbitol were used as carbon source respectively (Yuan-Shan *et al*., 2008, Vaidya *et al.*, 2009).

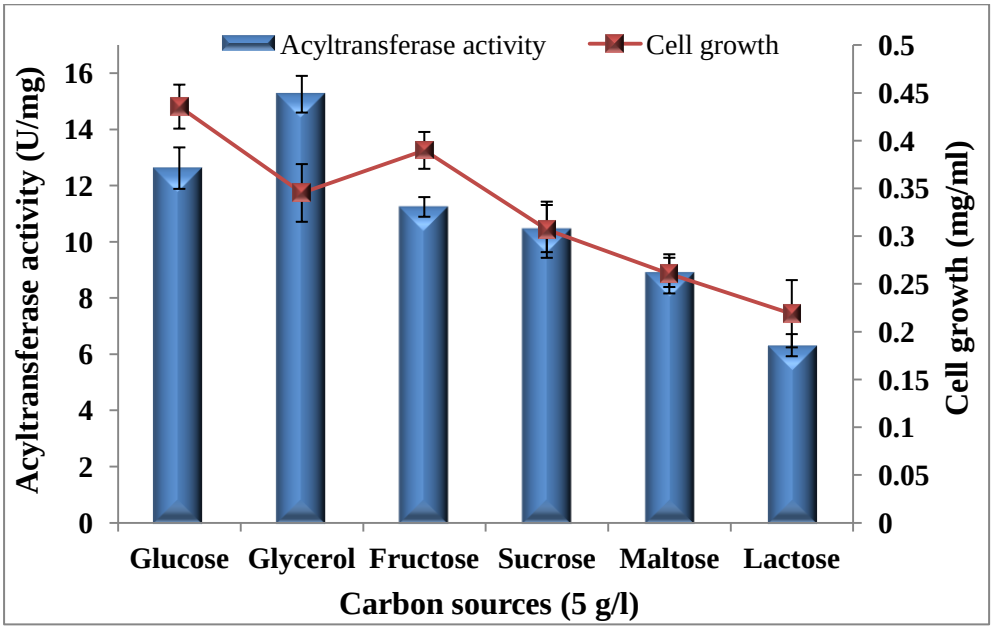


Fig 4.7 Effects of different carbon sources on amidase production
Other environmental parameters: nitrogen source- glutaronitrile (10 mM), pH-7.0, temperature -45 °C
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C

4.1.3.4 Effect of glycerol concentration on amidase production

Glycerol concentrations of 2.5 to 25 g/l were used in the production medium to determine the optimum concentration for amidase production. As shown in Fig. 4.8, both cell growth and amidase production progressively increased with the increase of glycerol concentration up to 10 g/l. Further increase beyond this concentration resulted in decline in amidase production while cell growth was highest at 15 g/l concentration and above this concentration (15 g/l) cell growth also reduced notably. The maximum amidase production in medium containing 10 g/l of glycerol was about 17.86±0.42 U/mg and 10 g/l of glycerol was selected as the optimum concentration for further work.

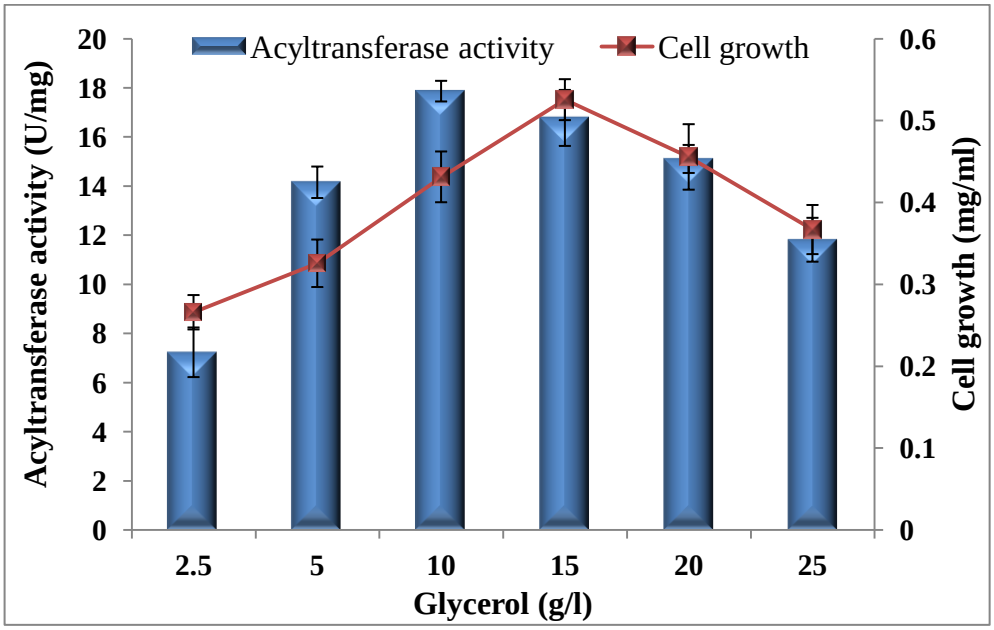


Fig 4.8 Effect of glycerol concentration on amidase production
Other environmental parameters: carbon source- glycerol (2.5-25 g/l), nitrogen source- glutaronitrile (10 mM), pH-7.0, temperature- 45 °C
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

4.1.3.5 Effect of different nitrogen sources on amidase production

The effects of various nitrogen sources (nitriles and amides) on cell growth and amidase production were investigated (Fig. 4.9). To differentiate the inducible and constitutive nature of amidase production, ammonium chloride was also used as sole nitrogen source. Very low amidase production (3.75±0.52 U/mg) was observed when ammonium chloride was used as sole nitrogen source while the cell growth was highest (0.57±0.02 mg/ml). The additions of nitriles and amides (10 mM) resulted in induction of

amidase production to a remarkable level, confirming inducible nature of amidase enzyme of *B. smithii* strain IIT6b2. In literature, most of the amidase are reported to be inducible (Maestracci *et al*., 1984, Hirrlinger *et al*., 1996, Nawaz *et al*., 1996, Rahim *et al*., 2003, Nampoothiri *et al*., 2005, Yuan-Shan *et al*., 2008, Pandey *et al*., 2011, Bhatia *et al*., 2013) while few are reported to be constitutive (Nawaz *et al*., 1994, Egorova *et al*., 2004, Hongpattarakere *et al*., 2005, Prasad *et al*., 2005). Out of the eight inducers tested in the present study acetonitrile, acetamide and nicotinamide favoured cell growth while few nitriles such as propionitrile, butyronitrile, phenylacetonitrile and benzonitrile reduced the bacterial cell growth. Yeast (1 g/l) was also used as nitrogen source and resulted in moderate amidase production (13.13±1.0 U/mg) and cell growth (0.48±0.04 mg/ml).

The effects of acetonitrile and butyronitrile on amidase production were almost the same as that of benzonitrile. The additions of inducers such as glutaronitrile and phenylacetonitrile enhanced the amidase production significantly to its maximum level. On the other hand uses of acetamide (5.05±0.37 U/mg) and nicotinamide (5.17±0.61 U/mg) seem to result in poor induction of enzyme production. Among eight inducers studied, phenylacetonitrile resulted in maximum amidase production (20.74±0.69 U/mg) and was chosen as nitrogen source for further studies.

4.1.3.6 Effect of phenylacetonitrile concentration on amidase production

Maximum induction of amidase production was obtained by the addition of phenylacetonitrile in the culture medium. In order to determine the optimum concentration of phenylacetonitrile for amidase production, phenylacetonitrile concentration was varied from 2.5 to 30 mM in the production medium (Fig. 4.10). The amidase production gradually increased with the increase in the phenylacetonitrile concentration and maximum amidase production (21.6±0.98 U/mg) was achieved at 10 mM. Further increase in phenylacetonitrile concentration above 10 mM resulted in 19 and 34% declines in amidase productions at 20 and 25 mM respectively. On the other hand, cell growth was also increased by increasing the phenylacetonitrile concentration up to 15 mM (0.40±0.03 mg/ml) and considerable reduction in cell growth was observed beyond 15 mM. Higher concentration of phenylacetonitrile (30 mM) inhibited both the cell growth and amidase production. Therefore, for amidase production 10 mM of phenylacetonitrile was found to be the optimum value.

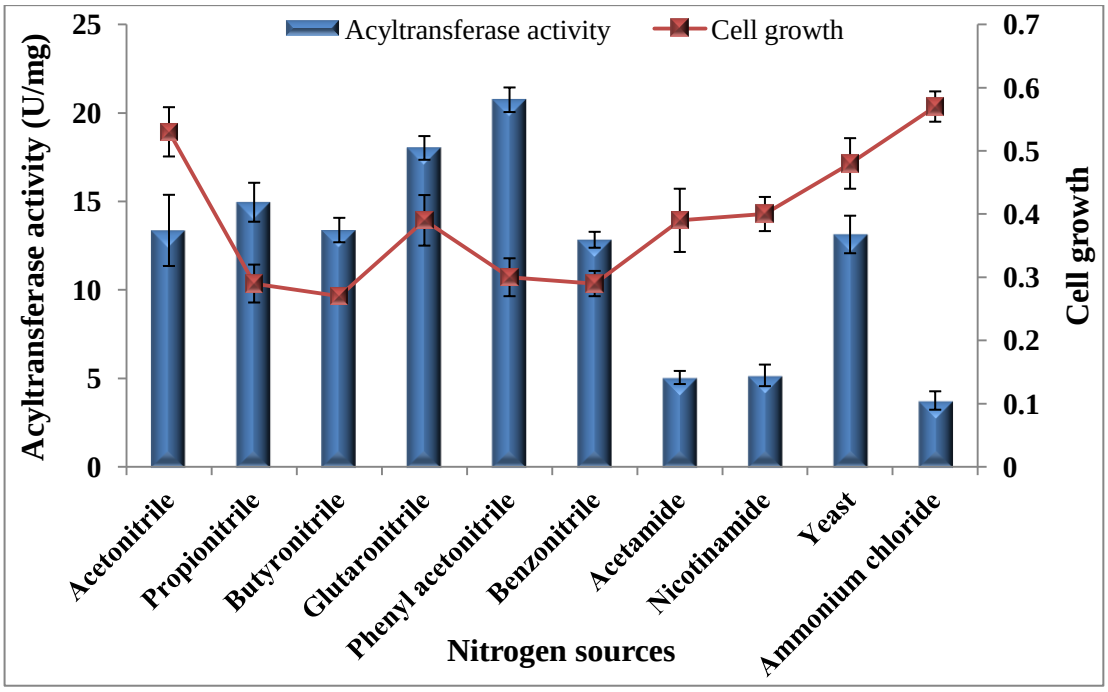


Fig 4.9 Effect of different nitrogen sources on amidase production

Other environmental parameters: carbon source- glycerol (10 g/l), pH-7.0, temperature 45 °C Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

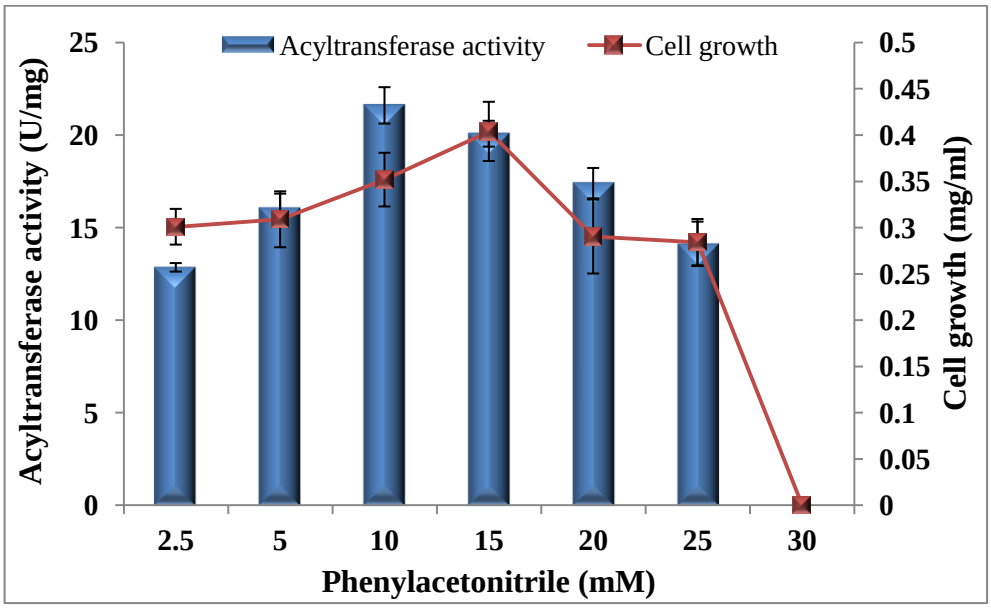


Fig. 4.10 Effect of phenylacetonitrile concentration on amidase production

Other environmental parameters: carbon source- glycerol (10 g/l), nitrogen source- phenylacetonitrile (2.5-25 mM), pH-7.0, temperature 45 °C

Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

In can be concluded that amidase production from *B. smithii* strain IITR6b2 was affected by incubation temperature, media pH, type of carbon and nitrogen sources and their concentrations. Optimization results revealed that at a temperature of 45 °C, initial medium pH 7.0, 10 g/l glycerol and 10 mM of phenylacetonitrile would result in maximum amidase production. In presence of ammonium chloride as a sole nitrogen source, highest biomass was achieved while amide acyltransferase activity was very low confirming the inducible nature of amidase.

4.1.4 Growth curve of *Bacillus smithii* strain IITR6b2

For *B. smithii* strain IITR6b2, cell growth and amidase production profiles were simultaneously studied with respect to incubation time under optimized conditions. Broth was withdrawn for amidase activity assay at an interval of 6 h till 84 h and the results are displayed in Fig. 4.11. It was observed that the production of amidase was related with the cell growth of the organism. Initially biomass increased slowly with significant lag phase and amidase production started during this phase after 18 h, however it was very less. After the lag phase, bacterial biomass increased exponentially and entered the log phase. During this phase the amidase production was also increased with time and maximum activity obtained at around mid exponential phase (culture time of 48 h). Subsequently the culture entered the stationary phase after 60 h and a rapid fall in amidase activity was observed with the prolonged incubation time. The possibility of reducing the lag phase can be achieved by adopting a suitable inoculum development method.

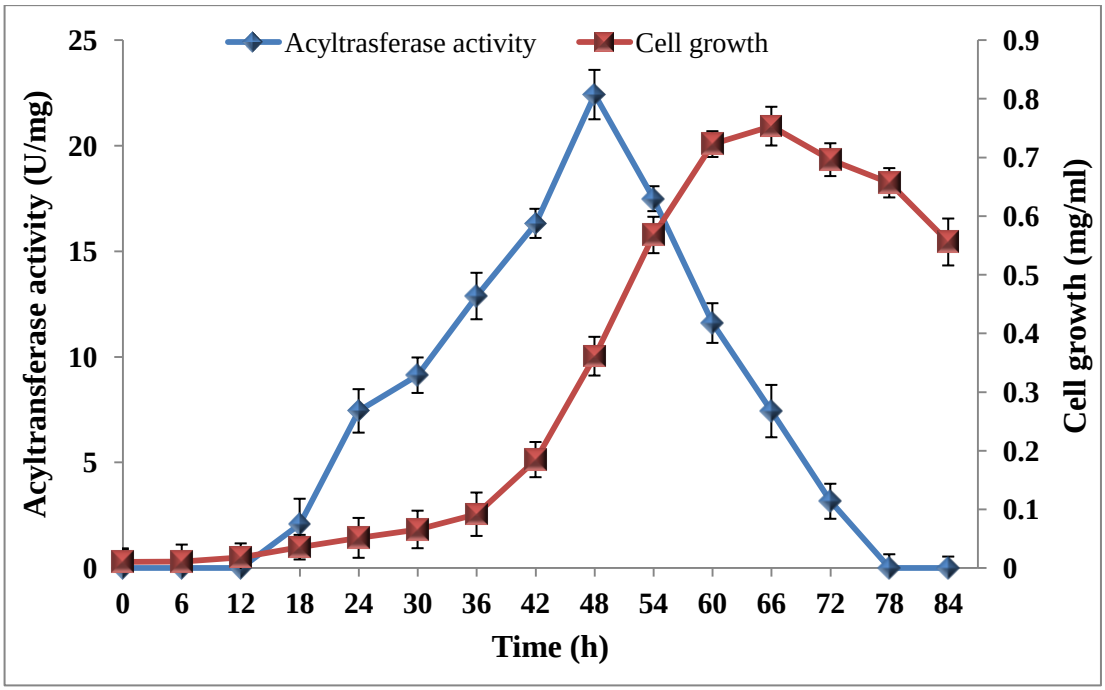


Fig. 4.11 Growth curve of *Bacillus smithii* strain IITR 6b2
Optimized environmental parameters: carbon source- glycerol (10 g/l), nitrogen source-phenylacetonitrile (10 mM), pH-7.0, temperature 45 °C
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C

4.1.5 Characterization of acyltransferase activity of whole cell amidase

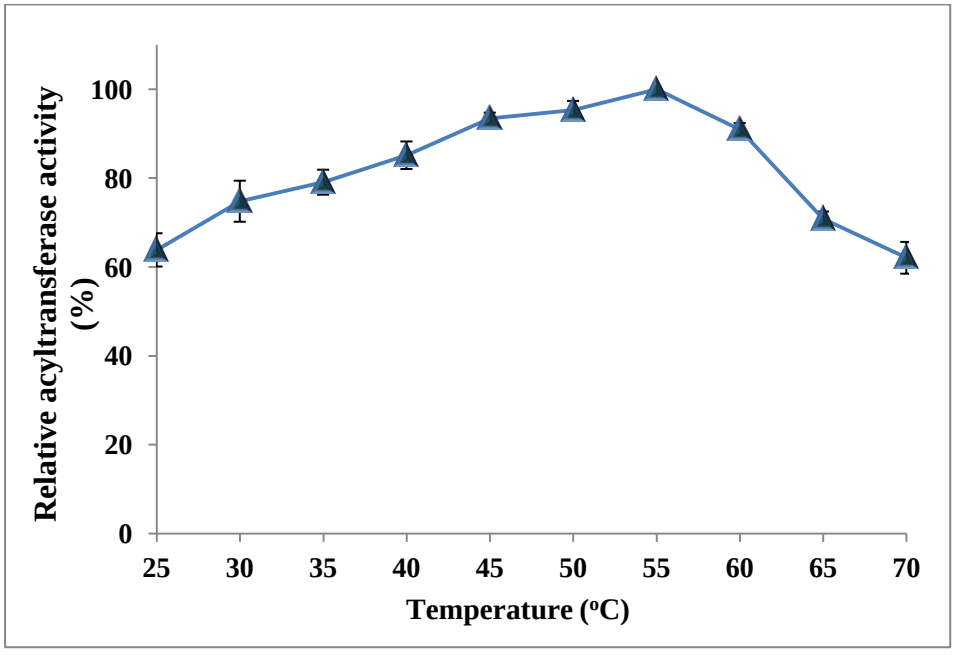
4.1.5.1 Effect of temperature on acyltransferase activity

The effect of temperature on acyltransferase activity of whole cell amidase was investigated at different temperatures (25-70 °C). Whole cell amidase was found to be active in a broad temperature range of 30-60 °C. As depicted in Fig 4.12, acyltransferase activity increased with increase in reaction temperature from 25-55 °C and showed maximum activity at 55 °C with marginally lower activities at 45 and 50 °C. Nearly 85% and 91% relative activities were found at 40 and 60 °C respectively whereas 95% activity was found at 50 °C. *Rhodococcus sp.* N-771 amidase also showed an optimum temperature of 55 °C but above 60 °C its activity decreased sharply (Ohtaki *et al*., 2010).

4.1.5.2 Effect of pH on acyltransferase activity

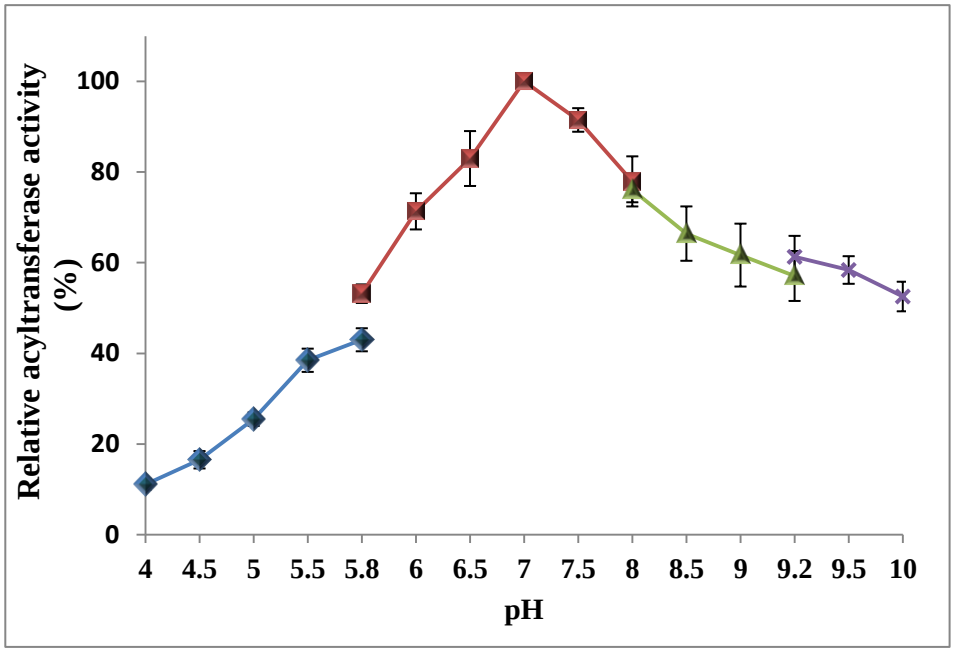
The effect of initial reaction pH on acyltransferase activity was studied over a pH range of 4 to 10 and results are shown in Fig. 4.13a. These results indicate that pH considerably affected the acyltransferase activity with optimum value obtained at pH 7.0.

Acidic conditions were found to be adverse for whole cell amidase activity and 71% and 11% of optimum activity retained at pH 6 and 4 respectively. Above optimum pH value (7.0) notable reduction in activity was observed with 52% activity at pH 10. The molarity of potassium phosphate buffer at optimum pH 7.0 was varied from 25 mM to 500 mM to determine its optimum value. Potassium phosphate buffer of 50 mM (pH 7.0) was found to show maximum acyltransferase activity (Fig. 4.13b). Amidases of *Rhodococcus sp.* R312 (Fournand *et al*., 1998b) and *Geobacillus pallidus* (Makhongela *et al*., 2007) are reported to have optimum activity at pH 7.0. On the other hand amidases from *Mycobacterium neoaurum* ATCC 25795 and *Brevibacillus borstelensis* BCS-1 showed maximum activity at pH 8.0 (with no amidase activity below pH 6.0) and 9.5 respectively (Hermes *et al*., 1994, Baek *et al*., 2003).



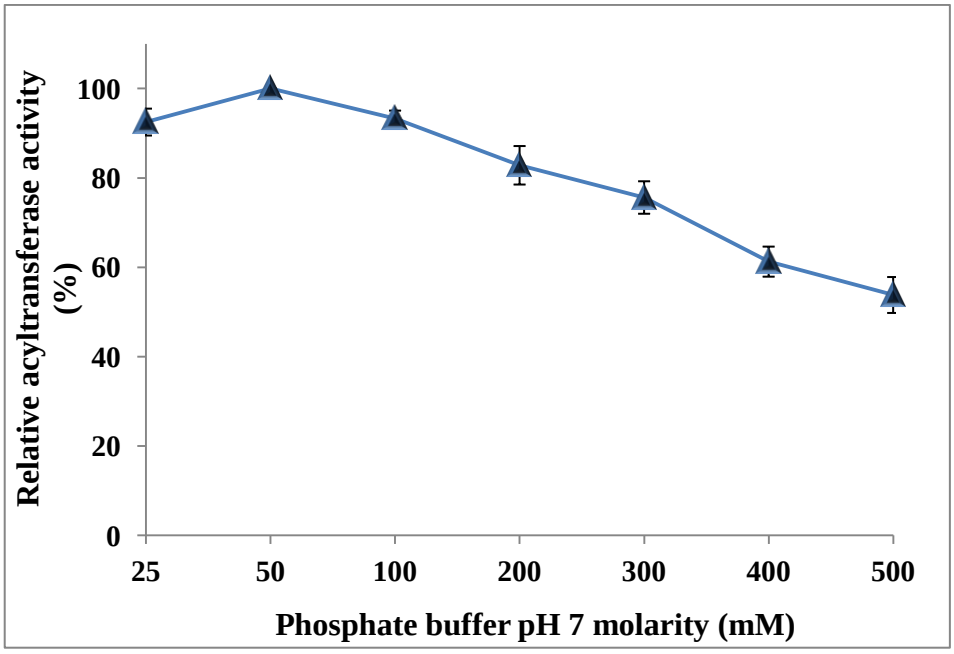
* 100% activity corresponds to 22.39 U/mg

Fig.4.12 Effect of temperature on acyltransferase activity
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0).



* 100% activity corresponds to 20.28 U/mg

Fig. 4.13a Effect of pH on acyltransferase activity
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, 55 °C.



* 100% activity corresponds to 21.12 U/mg

Fig 4.13b Effect of molarity of potassium phosphate buffer (pH 7.0) on acyltransferase activity
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (pH 7.0), 55 °C.

4.1.5.3 Thermal stability of acyltransferase activity

For successful commercialization of a bioprocess, an enzyme needs to be stable at operational conditions. So a study was carried out to find out the thermal stability of the whole cell enzyme with acyltransferase activity at 30, 45, and 55 °C. The whole cell amidase retained 86%, 74% and 61% of its initial activity after 8 h of incubation at 30, 45 and 55 °C. The natural logarithm of residual acyltransferase (lnRA) activity was plotted against time (Fig. 4.14) and the half-lives of whole cell enzyme at specific temperatures were determined. The results indicated that the whole cell enzyme was relatively stable at 30 °C with a half-life of 29 h and at 45 and 55 °C half-lives were 14 and 10 h, respectively. Thus this enzyme is found to be reasonably stable at experimental conditions.

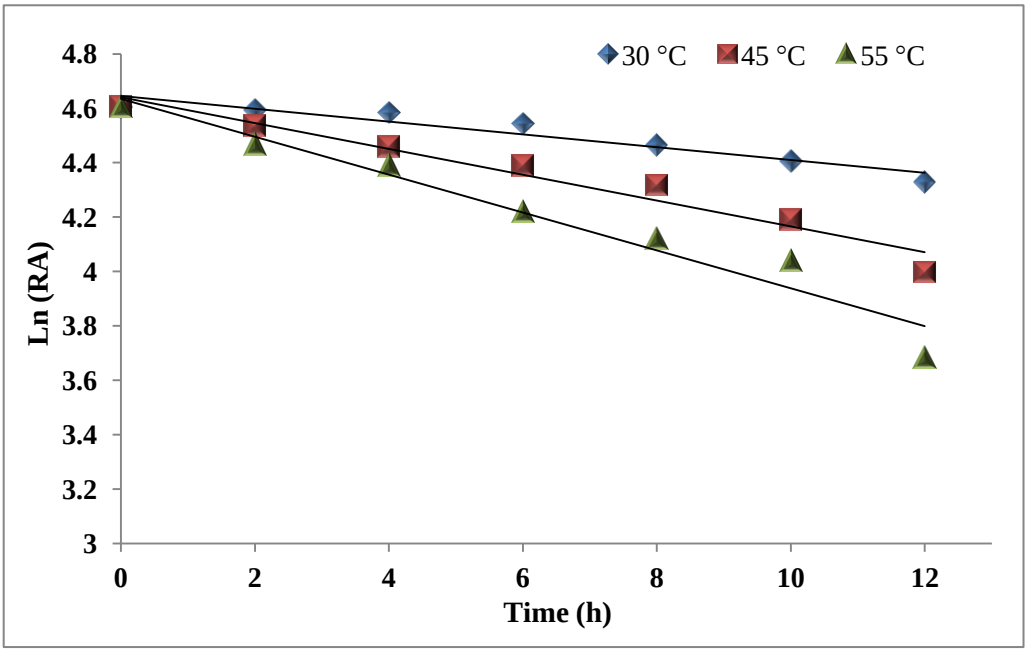


Fig. 4.14 Thermal stability of acyltransferase activity
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 55 °C.

4.1.5.4 Substrate Specificity of whole cell amidase

Whole cell amidase showed acyltransferase and amide hydrolase activities for a broad range of amides. This whole cell amidase was highly active on aliphatic, heterocyclic and aromatic amides however no acyltransferase and amide hydrolase activities were detected for amino acid amides. Maximum acyltransferase activity was

obtained for nicotinamide (100%) followed by acetamide (98.3%), benzamide (94.6%) and hexanamide (86.8%). The acyltransferase activity profile for aliphatic amides was contradictory as higher activity was obtained with either very short carbon chain amides (acetamide) or for long carbon chain amides (hexanamide) while moderate activity was obtained for mid carbon chain amides (butyramide). These results indicate the probable presence of more than one amidase in *B. smithii* strain IITR6b2, one amidase having activity for short chain aliphatic amides and another one having affinity for long and mid chain aliphatic amides along with aromatic and heterocyclic amides. The presence of more than one amidases can be further confirmed by purifying enzymes.

Substrate specificity profile for amide hydrolase activity was different as compared to acyltransferase activity profile (Table 4.8). Highest amide hydrolase activity was obtained for short chain aliphatic amides, acetamide (100%) and propionamide (71.4%). On the other hand only 19%, 19.9% and 20.4% of optimum activities were reported for nicotinamide, benzamide and hexanamide respectively. Thus for efficient hydroxamic acids syntheses nicotinamide, hexanamide and benzamide can be chosen as substrate.

Table 4.8 Substrate specificity profile of acyltransferase and amide hydrolase activities of whole cell amidase

Substrate (10 mM)	Relative acyltransferase activity (%)	Relative amide hydrolase activity (%)
Acetamide	98.3±3.1	100
Propionamide	39.84±2.7	71.48±4.7
Butyramide	44.49±3.3	12.64±2.5
Isobutyramide	14.71±1.2	2.84±0.17
Hexanamide	86.85±3.9	20.4±2.7
Acrylamide	19.82±1.7	75.8±4.1
Methacrylamide	3.04±0.95	1.45±0.28
Malonamide	2.11±0.61	1.47±0.82
Adipamide	9.5±1.1	1.63±0.94
Nicotinamide	100	19.01±1.7
Isonicotinamide	45.22±4.3	21.07±4.2
Benzamide	94.65±4.9	19.91±3.4
4-Hydroxybenzamide	1.71±0.62	0.58±0.31
Salicylamide	0	0
DL-lactamide	6.73±1.3	7.38±2.0

Enzyme assay conditions: For acyltransferase activity assay- 10 mM amide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 55 °C. For amide hydrolase activity assay- 10 mM amide, phosphate buffer (50 mM, pH 7.0) and 55 °C.

4.1.5.5 Organic solvent compatibility of whole cell amidase

Some hydrophobic amides like benzamide, 4-Hydroxybenzamide, salicylamide and hexanamide are poorly soluble in aqueous medium and solubility of these amides can be increased by addition of water miscible solvents to aqueous phase. Hence compatibility of amidase in presence of organic solvents is required to work with higher concentration of these amides for hydroxamic acids or acid hydrazides syntheses. Generally, efficiency of enzymatic reactions in presence of organic solvents is low due to denaturation of enzymes caused by solvents. In literature, very few reports are available on amidase activity in presence of organic solvents (Makhongela *et al*., 2007, Mehta *et al*., 2013). Therefore the compatibility of whole cell amidase in the presence of solvents was determined by incubating resting cells at 30 °C for 30 min at concentration of 15 and 30% (v/v) and

results are summarised in Fig 4.15. The whole cell amidase was compatible in presence of both water miscible (methanol, ethanol, isopropanol, DMF and DMSO) and water immiscible solvents (hexane and heptane) at 15% (v/v) concentration. Highest acyltransferase activity was obtained with hexane (105%) followed by DMSO (103%) and heptane (98%). More than 80% activity was retained with methanol (95%), ethanol (91%) and isopropanol (84%) while 58% activity was retained in presence of acetone. Whole cell amidase was stable even at 30% (v/v) of DMSO, hexane and heptane. However at higher concentration (30%) of water miscible solvents, loss in enzyme activity was observed and 75%, 52% and 47% activities were maintained in presence of methanol, ethanol and isopropanol respectively. Whole cell amidase showed higher organic solvent compatibility than other amidase reported in literature. The activity of purified amidase from *Geobacillus subterraneus* RL-2a reduced to 33%, 18% 13% and 6% in presence of DMSO, propanol, ethanol and acetone respectively (Mehta *et al*., 2013). Hence these findings signify the suitability of whole cell amidase for biotransformation of hydrophobic amides to hydroxamic acids or acid hydrazides at higher molar concentration.

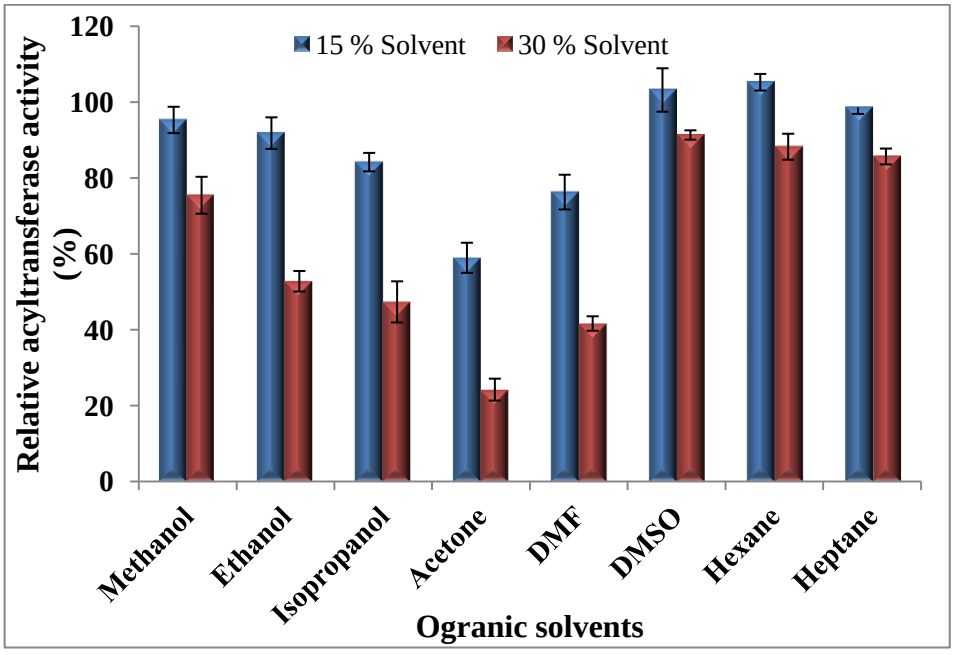


Fig. 4.15 Organic solvent compatibility of whole cell amidase
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 55 °C.

Section 4.2

Bioprocess development for nicotinic acid hydroxamate synthesis

Nicotinic acid hydroxamate (NAH), also known as 3-pyridine hydroxamic acid, is one of the nicotinic acid derivatives in which the hydroxamic acid moiety (-CONHOH) is present at third carbon of the pyridine ring (Bailey *et al.,* 1972). In recent years, increased interest in nicotinic acid hydroxamate resulted in higher attention on development of improved production methods with high yield and productivity. Chemical methods of nicotinic acid hydroxamate synthesis involve reaction of carboxylic acid or ester with O/N-protected hydroxylamine such as NH_2-O-Bn under strong basic conditions in solvents like methanol, ethanol, tetrahydrofuran and dimethylformamide (Kokare *et al.,* 2007, Gissot *et al.,* 2005). Sometimes carboxylic acids are found to be reluctant in the activation step that results in poor yield of product in the coupling reaction. After reaction, O-alkylated derivative of NAH is obtained and this needs further catalytic hydrogenation with palladium to release the free hydroxamic acid. Development of a single step biotransformation process for NAH synthesis will provide a simple, convenient and environmental friendly economic route. In recent past, acyltransferase activity of amidases has been utilized for hydroxamic acids and acid hydrazides syntheses (Fournand *et al.,* 1998a, 1998b, Pandey *et al.,* 2011, Bhatia *et al.,* 2013). To achieve higher yield of hydroxamic acid by minimizing the undesirable by-product (acid) formation from amide it is desirable to have a biocatalyst with high acyltransferase to amide hydrolase activity ratio. Hence whole cell amidase of *B. smithii* strain IITR6b2 having acyltransferase activity can be used as a potential biocatalyst for NAH synthesis.

In this section, a bioprocess for synthesis of nicotinic acid hydroxamate from nicotinamide using acyltransferase activity of *B. smithii* strain IITR6b2 was described. During the study, special emphasis was placed on process development for NAH production with minimum formation of by-product, nicotinic acid. Different process parameters were optimized for efficient production of NAH by fed batch biotransformation in order to achieve high product concentration.

4.2.1 Enzymatic reactions during nicotinic acid hydroxamate synthesis

The amidase from *Rhodococcus sp.* strain R312 catalysed amide hydrolysis, amide acyltransferase, acid acyltransferase and hydroxamic acid hydrolase reactions (Fournand *et al.,* 1998b). It was suggested that during amidase catalysed hydroxamic acid synthesis, hydroxamic acid can be formed by transfer of acyl groups of both amide and acid to hydroxylamine. Simultaneously by-product (acid) can also be synthesized by hydrolysis of both amide and hydroxamic acid. Whole cell biocatalyst of *B. smithii* strain IITR6b2 was found to possess amide acyltransferase, amide hydrolase and hydroxamic acid hydrolase activities but not acid acyltransferase activity (Table 4.9). Amide acyltransferase activity was 28 and 12.3 folds higher as compared to amide hydrolase and hydroxamic acid hydrolase activities respectively (at 100 mM substrate concentration). Although whole cell exhibited all these activities, to confirm that these reactions are mediated by amidase enzyme system of the isolate, a known amidase inhibitor (Di-ethyl phosphoramidate) was used at 1, 5 and 10 mM concentrations. The results (Table 4.9) indicate that the extent of inhibitions of whole cell acyltransferase and hydrolase activities were nearly similar at different concentrations of inhibitor. Thus the inhibition profile of these activities suggested that amidase enzyme of *B. smithii* strain IITR6b2 possess amide acyltransferase, amide hydrolase and hydroxamic acid hydrolase activities. Further confirmation was carried out by performing the thermal stability studies of these three activities at identical conditions (pH 7.0 and 45 °C). From the results (Fig. 4.16) it can be observed that all three activities followed similar deactivation profile. Thus it was confirmed that these three activities belong to same protein. The advantage of using this whole cell of *B. smithii* strain IITR6b2 is that hydrolase activities were significantly lower than acyltransferase activity hence synthesis of by-product, nicotinic acid is limited. Based on these results the possible enzymatic route for NAH synthesis by whole cell amidase of *B. smithii* strain IITR6b2 is shown in Fig. 4.17.

Table 4.9 Comparison of *B. smithii* strain IITR6b2 catalysed reactions and effect of amidase inhibitor (Di-ethyl phosphoramidate) on them

Reactions catalysed	Enzyme activity (U/mg_{dcw})	Inhibitor[a] (Residual enzyme activity %)		
		Di-ethyl phosphoramidate		
		1 mM	5 mM	10 mM
Amide acyltransferase	16.1±0.32	79.5±2.7	46.6±2.4	41.3±4.9
Amide hydrolase	0.57±0.02	72±2.1	41±2.3	36.1±1.1
Hydroxamic acid hydrolase	1.3±0.03	66.2±3.2	38.6±1.9	27.7±2.8
Acid acyltransferase	ND			

a. Reaction conditions: Inhibitor was preincubated with whole cell suspensions at 30 °C for 30 min and then whole cells were tested for residual enzyme activities.
Enzyme assay conditions: phosphate buffer (50 mM, pH 7.0), 55 °C; for amide acyltransferase activity- 100 mM nicotinamide and 500 mM hydroxylamine-HCl, for amide hydrolase activity- 100 mM nicotinamide, for hydroxamic acid hydrolase activity- 100 mM NAH, for acid acyltransferase activity- 100 mM nicotinic acid 500 mM hydroxylamine-HCl. ND- not detected

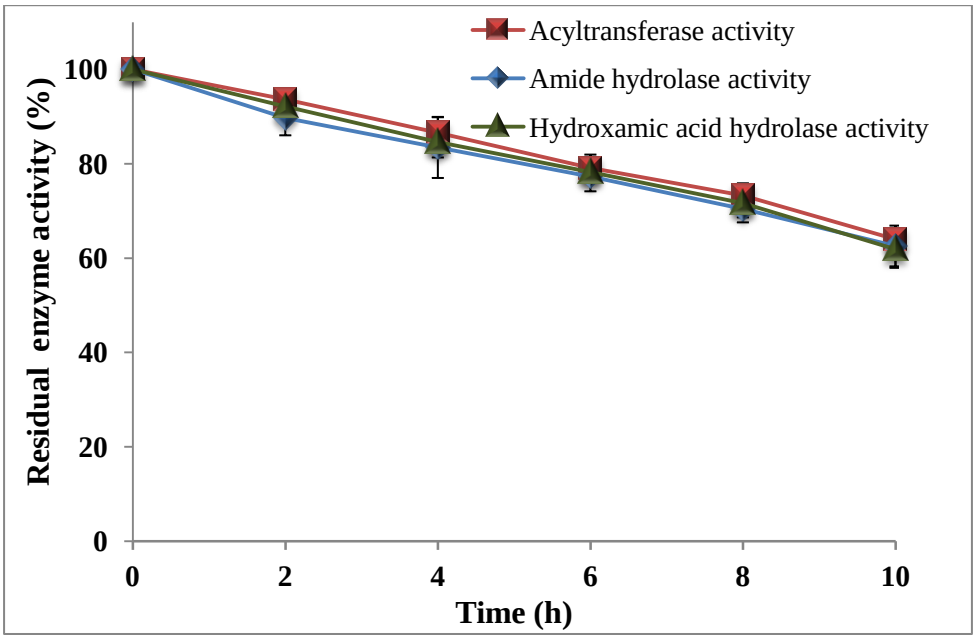


Fig 4.16 Thermal stability profile of whole cell acyltransferase activity, amide hydrolase activity and hydroxamic acid hydrolase activity at 45 °C

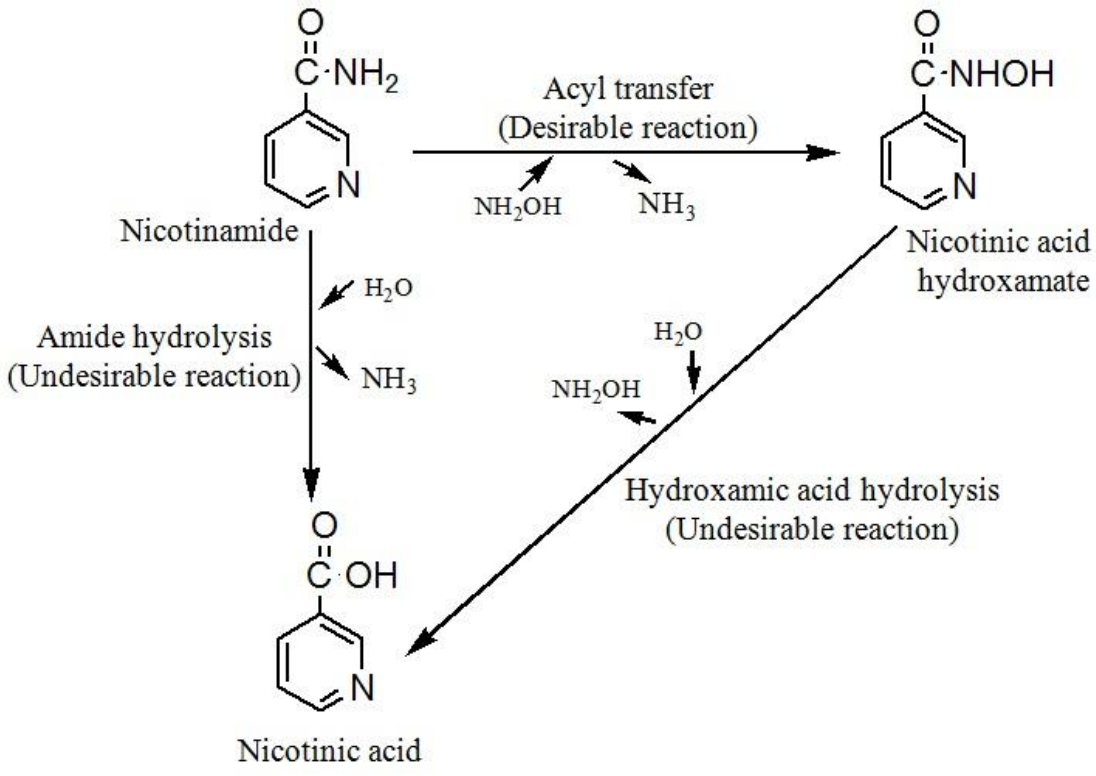


Fig 4.17 Enzymatic reactions during nicotinic acid hydroxamate synthesis catalysed by *B. smithii* strain IITR6b2

4.2.2 Effects of temperature and pH on acyltransferase activity for NAH synthesis

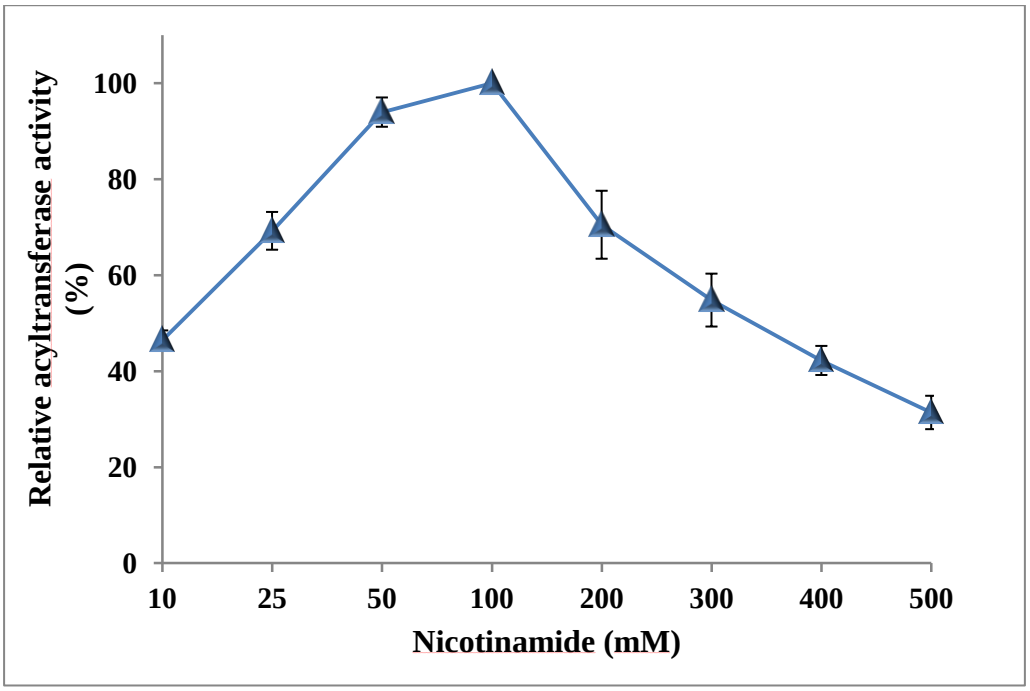
Whole cell amidase was found to be active in a broad temperature range of 30-60 °C with maximum activity at 55 °C (section 4.1.5.1). The effect of pH on the acyltransferase activity was also studied and it was found to be highest at pH 7.0, 50 mM potassium phosphate buffer (section 4.1.5.2).

4.2.3 Study of effects of substrate and co-substrate concentrations on acytransferase activity and mole ratio optimization

B. smithii strain IITR6b2 catalysed NAH synthesis is a bi-substrate reaction involving an acyl group donor (nicotinamide) and acyl group acceptor (hydroxylamine-HCl). It was expected that concentrations of both substrates may influence the acyltransferase activity and side reaction (amide hydrolysis). Thus there was a need to study the effect of substrate and co-substrate concentrations on acyltransferase activity and optimize both substrates concentrations.

4.2.3.1 Effect of substrate (nicotinamide) concentration

Enzymatic processes are usually inhibited by high substrate concentration; hence performance of acyltransferase activity of whole cell amidase at different nicotinamide concentrations was evaluated at constant hydroxylamine-HCl (500 mM) concentration. Rise in acyltransferase activity was observed as the nicotinamide concentration increased from 10-100 mM with maximum acyltransferase activity at 100 mM (Fig. 4.18). Increasing the amide concentration increased acyltransferase activity because of more acyl enzyme complex formation due to increase in availability of substrate for enzyme. A further increase in the amide concentration led to decrease in the enzyme activity and 68.6% reduction in acyltransferase activity was observed at 500 mM suggesting the inhibitory effect of nicotinamide.



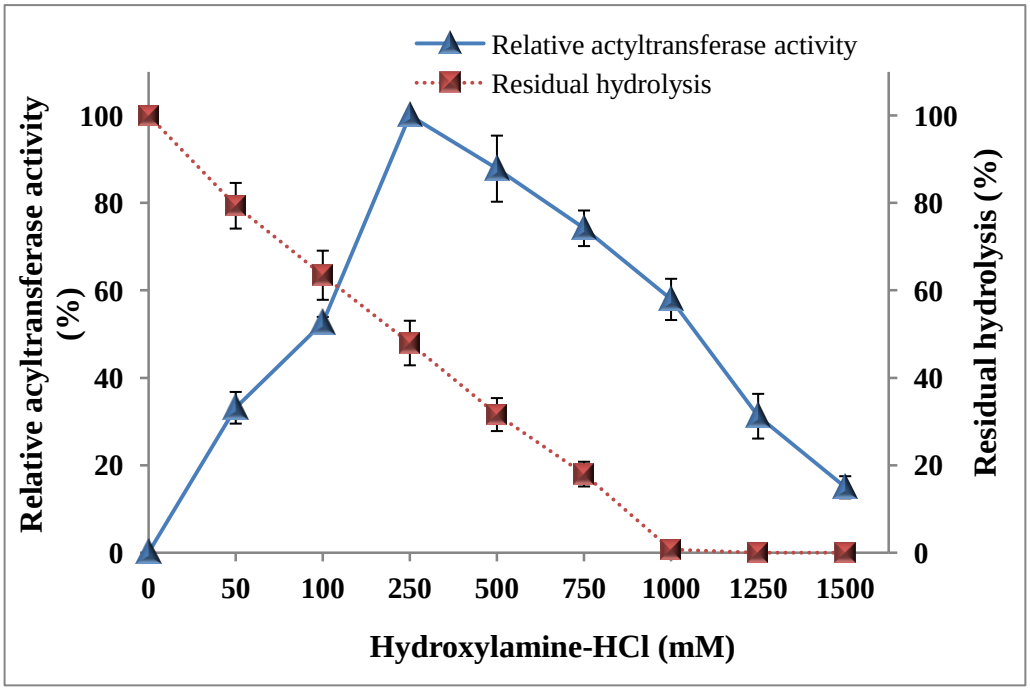
* 100% activity corresponds to 17.24 U/mg

Fig 4.18 Effect of substrate (nicotinamide) concentration on acyltransferase activity
Enzyme assay conditions: nicotinamide (10-500 mM), hydroxylamine-HCl (500 mM), phosphate buffer (50 mM, pH 7.0), 55 °C.

4.2.3.2 Effect of hydroxylamine-HCl (co-substrate) concentration

The effect of hydroxylamine-HCl concentration on acyltransferase activity was studied at various hydroxylamine-HCl concentrations (0-1500 mM) keeping nicotinamide concentration (100 mM) constant. Fournand et al. (1998a) reported that an increase in

concentration of hydroxylamine-HCl reduced the undesirable hydrolysis of amide. Hence effect of hydroxylamine-HCl concentration on residual hydrolysis reaction was also studied. It was observed that acyltransferase activity of amidase of *B. smithii* strain IITR6b2 increased with increase in concentration of hydroxylamine up to 250 mM but above this concentration decline in enzyme activity was observed probably due to inhibition by higher concentration of hydroxylamine-HCl (Fig. 4.19). Hydroxylamine being a more potent nucleophile than water has higher affinity for acyl enzyme complex and considerably reduces undesirable residual amide hydrolysis, if present in reaction mixture. In this study, decline in residual hydrolysis was also observed with increase in co-substrate concentration and residual hydrolysis reduced to 79% if only 50 mM hydroxylamine is present in reaction mixture and it declined to 48% at optimum hydroxylamine-HCl concentration (250 mM).



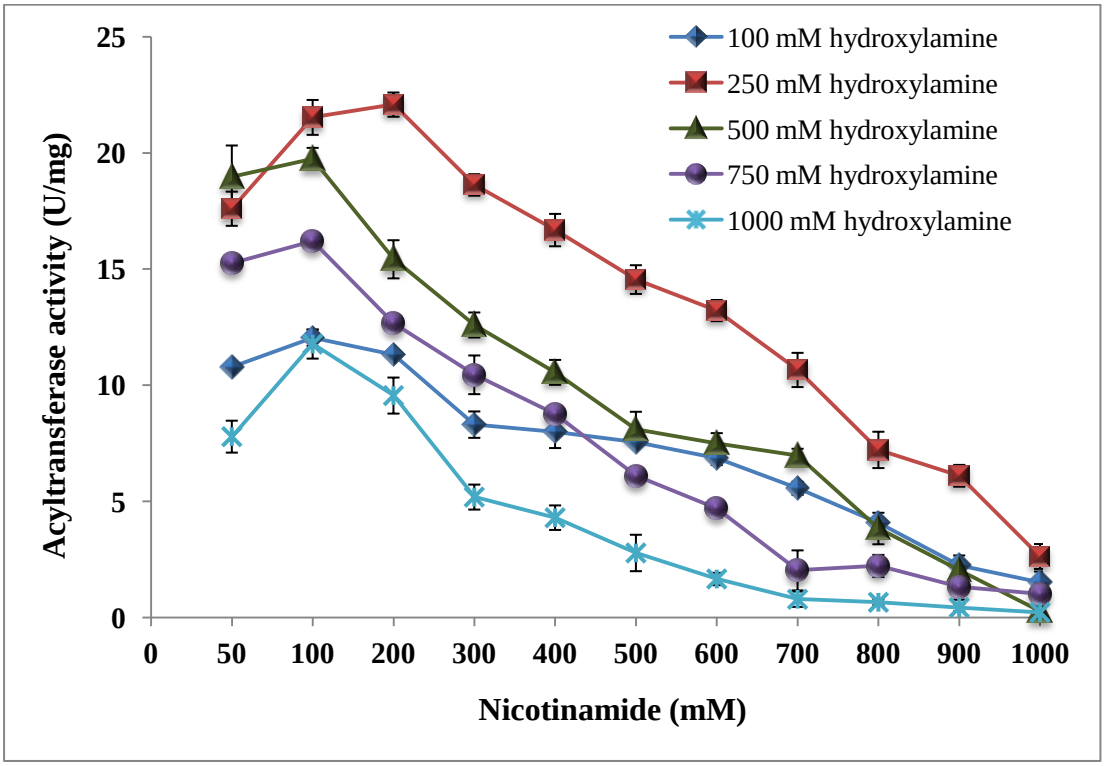
* 100% activity corresponds to 19.45 U/mg

Fig 4.19 Effect of co-substrate (hydroxylamine-HCl) concentration on acyltransferase activity
Enzyme assay conditions: nicotinamide (100 mM), hydroxylamine-HCl (0-1500 mM), phosphate buffer (50 mM, pH 7.0), 55 °C.

4.2.3.3 Optimization of substrate and co-substrate concentration combination

During the determination of the optimum concentrations of nicotinamide and hydroxylamine–HCl, concentration of only one substrate was varied whereas the other

substrate was kept constant. The possibility of determined concentrations of nicotinamide and hydroxylamine-HCl being suboptimal cannot be ruled out. Thus to find the true optimum mole ratio of substrates (nicotinamide and hydroxylamine-HCl); reactions were performed with several possible ratios of amide and hydroxylamine-HCl. Initially, acyltransferase activity of whole cell enzyme increased with an increase in nicotinamide concentration and thereafter it continuously decreased with amide concentration for all hydroxylamine-HCl concentrations studied. The highest acyltransferase activity was obtained with 250 mM hydroxylamine-HCl and 200 mM nicotinamide followed by 250 mM hydroxylamine-HCl and 100 mM nicotinamide. It was found that increased concentration of hydroxylamine-HCl was also inhibitory to the enzyme activity at all amide concentrations (Fig. 4.20). From these results, 200 mM of nicotinamide and 250 mM of hydroxylamine-HCl were chosen for further work.



* 100% activity corresponds to 22.09 U/mg

Fig 4.20 Optimization of substrate and co-substrate concentrations
Enzyme assay conditions: nicotinamide (50-1000 mM), hydroxylamine-HCl (100-1000 mM), phosphate buffer (50 mM, pH 7.0), 55 °C.

4.2.4 Effect of reaction temperature on molar conversion and by-product formation

It was important to decide the operating temperature for bioconversion by considering the optimum temperature (55 °C) and stability of enzyme. Time course of bioconversions at 30, 35, 45 and 55 °C were investigated for 90 min to choose the optimum temperature of bioprocess for NAH synthesis. From the results (Fig. 4.21) it can be observed that initial conversion of substrate to product was higher at 55 °C. However after 30 min, the concentration of product was higher at 30 °C. At 45 and 55 °C, highest molar conversions achieved were 84.8 and 81.6% respectively in 30 min and maximum molar conversion (90.4%) was obtained at 30 °C in 40 min. It was also observed that at 45 and 55 °C, the concentration of product decreased after 30 minutes whereas at 30 °C, the product concentration decreased after 40 minutes. The rate of decrease of product concentration with time was higher at 55 °C as compared to 30 °C. To explain this discrepancy in conversion at later stage, the by-product (nicotinic acid) concentration was also determined at these temperatures. It was observed that the by-product (nicotinic acid) formation was 3.4 times higher at 55 °C as compared to 30 °C. Thus this lower conversion of substrate to NAH at 55 °C was due to the higher by-product formation. Similar trend of higher by-product formation was also observed at 45 °C. Pandey *et al*. (2011) also reported decrease in concentration of acetohydroxamic acid with time at all temperatures after achieving maximum conversion. This study suggest that better thermal stability and higher conversion (90.4%) of nicotinamide to nicotinic acid hydroxamate along with only 1.4% conversion to by-product at 30 °C , made it more suitable to choose 30 °C as operating temperature.

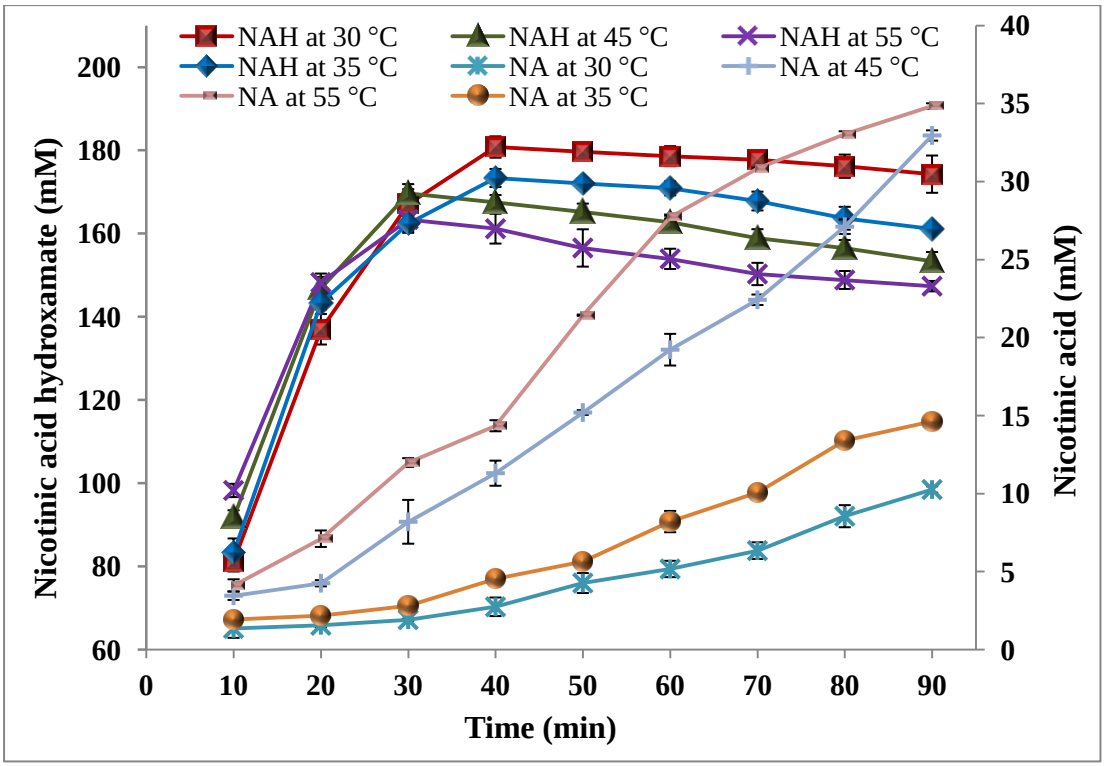


Fig 4.21 Effect of reaction temperature on molar conversion and by-product formation Reaction conditions: nicotinamide (200 mM), hydroxylamine-HCl (250 mM), 25 ml phosphate buffer (50 mM, pH 7.0), 0.5 mg_{dcw}/ml resting cells.

4.2.5 Effect of resting cells on conversion and time course of reaction

The amount of biocatalyst used and process time is crucial for development of an economic bioprocess. The nicotinamide bioconversion to NAH in the reaction mixture containing 200/250 mM substrate/co-substrate at 30 °C with different cell concentrations are shown in Fig. 4.22. Molar conversion of nicotinamide to NAH increased with increase in resting cells concentration from 0.3-0.7 mg_{dcw}/ml due to availability of more acyl enzyme complex. However at 0.9 mg_{dcw}/ml concentration, maximum conversion was 96.3% which is marginally higher than the 94.5% conversion observed at 0.7 mg_{dcw}/ml in 40 min. It illustrates that the additional cells did not improve the conversion significantly. This may be due to mass transfer limitations or lower substrate to biocatalyst ratio. To reduce both cost and possibility of hydroxamic acid hydrolysis (due to higher enzyme availability) during biotransformation process 0.7 mg_{dcw}/ml resting cells with 200/250 mM initial substrates (nicotinamide/ hydroxylamine-HCl) concentrations at 30 °C were used for fed batch reaction.

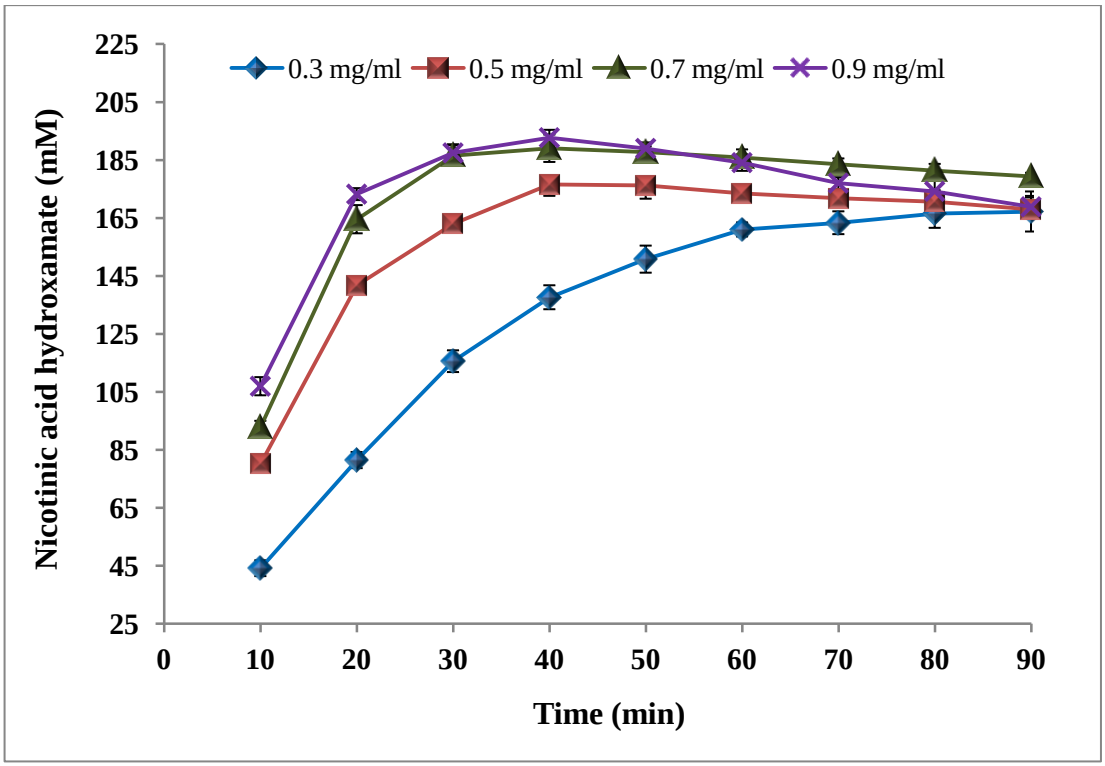


Fig 4.22 Effect of resting cells concentration (DCW) on biocatalytic synthesis of nicotinic acid hydroxamate and time course of reaction
Reaction conditions: nicotinamide (200 mM), hydroxylamine-HCl (250 mM), 25 ml phosphate buffer (50 mM, pH 7.0), 30 °C.

4.2.6 Fed batch process development at 50 ml scale

Substrate inhibition is a common problem in enzyme mediated processes which can be partially overcome by fed batch method using low initial substrate concentration followed by subsequent feeds of substrate during process (Sharma *et al*., 2011, Zhang *et al*., 2011a). As it was observed that higher initial concentration of the substrate/co-substrate (nicotinamide and hydroxylamine-HCl) had inhibitory effects on molar conversion, a fed batch process was developed under optimized reaction conditions. The investigation of biotransformation performance of whole cell amidase of *B. smithii* strain IITR6b2 showed that when the reaction pH, concentration of substrate/co-substrate, temperature and resting cells concentration were 7, 200/250 mM, 30 °C and 0.7 mg_{dcw}/ml respectively, excellent molar bioconversion yield (94.5%) was achieved with in 40 min. Hence the optimum feeding strategy for production of NAH was pulse feedings of nicotinamide and hydroxylamine-HCl (200/200 mM) at 40 min interval. It was predicted (from nicotinamide consumption profile) that in first 40 min, nearly 50 mM of hydroxylamine-HCl remain unutilized hence only 200 mM of it was fed during the

subsequent addition of substrates. Periodic analysis of reaction mixture revealed that after two feedings of substrates (200/200 mM), an accumulation of 536 mM of nicotinic acid hydroxamate was obtained in 120 min (Fig. 4.23). Further addition of third feed resulted in no significant enhancement of NAH production.

This bioprocess resulted in 89.4% molar conversion of 600 mM nicotinamide to NAH in 120 min at a production rate of 52.9 g/h/g_{dcw} with 4.5% undesirable by product, nicotinic acid. During this whole process 36.5% decrease in resting cells concentration (measured by optical density at 600 nm) due to lysis and 40.6% loss in acyltransferase activity was observed. The effect of NAH concentration on acyltransferase activity was also studied (Fig. 4.24) and 76.4% decrease in acyltransferase activity was observed at 500 mM concentration of NAH. Thus this confirms that product inhibition along with cell lysis and low enzyme stability are responsible for no further conversion of substrate beyond second feeding of substrates.

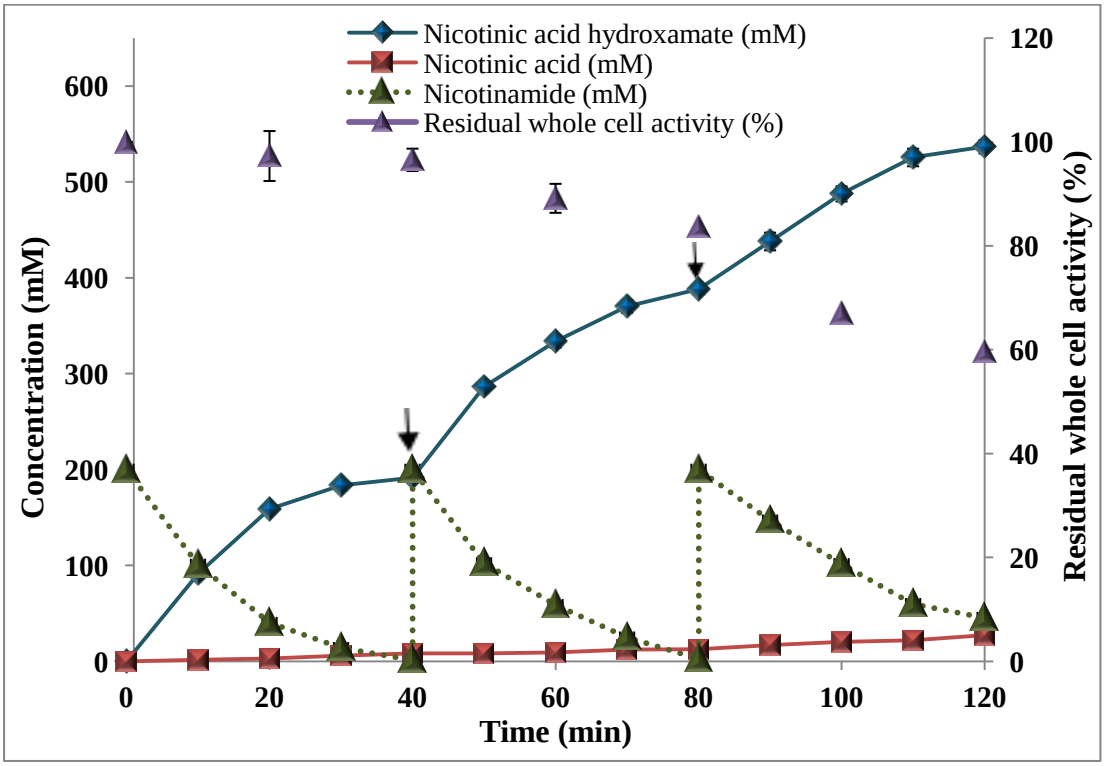


Fig 4.23 Time course of production of nicotinic acid hydroxamate during fed batch biotransformation process (↓ feed of nicotinamide and hydroxylamine–HCl)
Reaction conditions: 50 ml phosphate buffer (50 mM, pH 7.0), 30 °C, resting cells concentration (0.7 mg_{dcw}/ml).

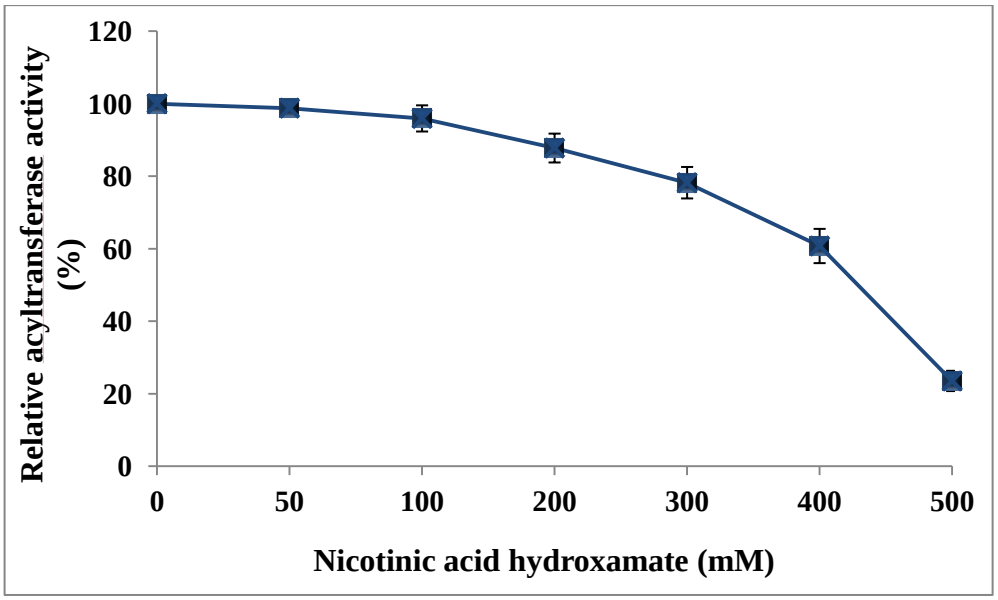


Fig 4.24 Effect of nicotinic acid hydroxamate concentration on acyltransferase activity
Reaction conditions: 200 mM nicotinamide, 250 hydroxylamine-HCl, cell concentration- 0.7 mg_{dcw}/ml, phosphate buffer (50 mM, pH 7.0), 55 °C.

4.2.7 Recovery of nicotinic acid hydroxamate

The supernatant obtained was freeze-dried to recover 6.4 g of pinkish white powder. HPLC analysis of this powder was carried out and it was found that powder contains 58.5% (w/w) NAH and 2.6% (w/w) nicotinic acid. Further purification of NAH was done by solvent extraction, filtration and evaporation, yielding 121 mg of liquid from 200 mg of powder. The HPLC analysis of this liquid confirmed the presence of nicotinic acid hydroxamate and purity level of 95% (w/w) was obtained.

An efficient biotransformation process for NAH production has been developed with high molar conversion and limited side reaction in shorter time. The starting material of this bioprocess is nicotinamide, cheaper than acid and ester of nicotinic acid which are commonly used in chemical methods for NAH synthesis and also this reaction takes place in aqueous medium leading to reduced cost of bioprocess. This is the first report on biocatalytic synthesis of any hydroxamic acid at this molar concentration with high conversion rate. Previously Fournand et al. (1997), used amidase from *Rhodococcus sp.* R312, immobilized on Duolite A-378 resin for bench scale production of acetohydroxamic acid and achieved only 54-61% (mol/mol) conversion. While Pandey et al. (2011) reported an improved bioprocess for acetohydroxamic acid synthesis using DTT treated resting cell

of *Bacillus sp.* ABP-6 and observed molar conversion of 93% of acetamide (300 mM) to acetohydroxamic acid. The productivity obtained in the present study was significantly higher than the previously reported productivity for acetohydroxamic acid and benzohydroxamic acid syntheses (Fournand *et al.*, 1997, Pandey *et al.*, 2011, Bhatia *et al.*, 2013).

Section 4.3

Bioprocess development for isoniazid synthesis

Isonicotinic acid hydrazide, also known as isoniazid (INH) is the first-line antitubercular drug and is given in combination with rifampicin and ethambutol (Ziskind *et al*., 1953, Toit *et al*., 2006). Chemical methods reported for isoniazid synthesis involve hydrazinolysis of 4-cyanopyridine, isonicotinamide or ethyl isonicotinate with hydrazine hydrate in aqueous or alcoholic solution in presence of sodium hydroxide at 100 °C under reflux conditions. These reactions are spontaneous, exothermic and complete in 4-9 h (Sycheva *et al*., 1972, Ray *et al*., 2004, Baizer *et al*., 1956). 4-cyanopyridine is prepared from expensive γ-picoline whereas less reactive ester requires longer reaction time (from few hours to 2-3 days). Generally these reactions are multistep, expensive, hazardous, energy intensive and can lead to decomposition of desired product. Hence an enzymatic synthesis method with high conversion rate in aqueous medium will provide an economic route and greener approach for isoniazid synthesis. The acyltransferase activity of amidase results in transfer of acyl group of amide to co-substrate hydroxylamine or hydrazine in aqueous medium (Thiery *et al*., 1986, Fournand *et al*., 1998a, Kobayashi *et al*., 1999) and it has been applied for synthesis of few hydroxamic acids (Fournand *et al*., 1997, Hirrlinger and Stolz 1997, Sharma *et al*., 2011, Pandey *et al*., 2011, Vejvoda *et al*., 2011, Bhatia *et al*., 2013). Although the ability of amidase to transfer acyl group of amide to hydrazine has been reported (Fournand *et al*., 1998a, Kobayashi *et al*., 1999), surprisingly bioprocess development for hydrazides syntheses using amidase has not been explored till now. In this work the hydrazinolysis of isonicotinamide by whole cell amidase of *B. smithii* IITR6b2 was applied for the preparative scale synthesis of isoniazid.

The current work focused on the synthesis of isoniazid from isonicotinamide and hydrazine-2HCl in aqueous medium using acyltransferase activity of whole cell amidase of *B. smithii* strain IITR6b2. Whole cells possessed higher acyltransferase to amide hydrolase activity and this ratio was further improved by optimizing hydrazine-2HCl concentration. Various reaction parameters such as temperature, pH, substrate/co-substrate concentration, resting cells concentration and time course of reaction were optimized and a fed batch process was developed.

4.3.1 Enzymatic reactions during isoniazid synthesis

Amidase of *B. smithii* strain IITR6b2 was capable of catalysing transfer of acyl group of amide to hydroxylamine and water (section 4.2.1) to synthesize hydroxamic acid and acid respectively. Few amidases have also been reported to catalyse the acyl group transfer from amides to hydrazine, leading to synthesis of acid hydrazides (Fournand *et al*., 1998a, Kobayashi *et al*., 1999). During amidase catalysed hydrazide synthesis an acyl enzyme complex is formed which is subjected to nucleophilic attack by either co-substrate hydrazine or water leading to hydrazide or acid synthesis respectively (Fournand *et al*., 1998a). Whole cell of *B. smithii* strain IITR6b2 possessed acyltransferase (2.68±0.24 U/mg_{dcw}) activity with isonicotinamide as substrate and hydrazine-HCl as co-substrate and hence was suitable for isoniazid synthesis. Whole cell enzyme also possessed amide hydrolase (0.87±0.05 U/mg_{dcw}) activity but not acid acyltransferase activity. Difference in acyltransferase and hydrolase activities might be due to the different affinity of hydrazine and water for acyl enzyme complex and hydrazine being a more potent nucleophile (due to α effect) attacks acyl enzyme complex faster than water. Rate of transfer of acyl group of isonicotinamide to hydrazine (500 mM) was 3 folds higher as compared to amide hydrolase reaction rate. Higher the ratio of acyltransferase to hydrolase activity less the by-product (isonicotinic acid) will be formed. One of the objective of this work was to explore the synthesis of isoniazid by whole cell amidase with minimum formation of by-product, isonicotinic acid. Whole cells of *B. smithii* strain IITR6b2 also showed hydrazide hydrolase activity (0.15±0.01 U/mg_{dcw}) when isoniazid was used as substrate in absence of hydrazine-2HCl but it was only 5.6% of amide acyltransferase activity. Kobayashi et al. (1999) also reported benzoic acid hydrazide hydrolase activity catalysed by amidase of *Rhodococcus rhodochrous* J1. Based on these activities, the possible reaction mechanism during whole cells catalysed isoniazid synthesis from isonicotinamide is shown in Fig. 4.25.

Table 4.10 Comparison of *B. smithii* strain IITR6b2 catalysed reactions

Reactions catalysed	Enzyme activity (U/mg_{dcw})	Acyltransferase to amide hydrolase activity ratio	Acyltransferase to hydrazide hydrolase activity ratio
Amide acyltransferase	2.68±0.24		
Amide hydrolase	0.87±0.05		
Hydrazide hydrolase	0.15±0.012	3.0	17.8
Acid acyltransferase	ND		

Enzyme assay conditions: phosphate buffer (50 mM, pH 7.0), 55 °C; for amide acyltransferase activity- 100 mM isonicotinamide and 500 mM hydrazine-2HCl, for amide hydrolase activity- 100 mM isonicotinamide, for hydrazide hydrolase activity- 100 mM isoniazid, for acid acyltransferase activity- 100 mM isonicotinic acid 500 mM hydrazine-2HCl. ND- not detected

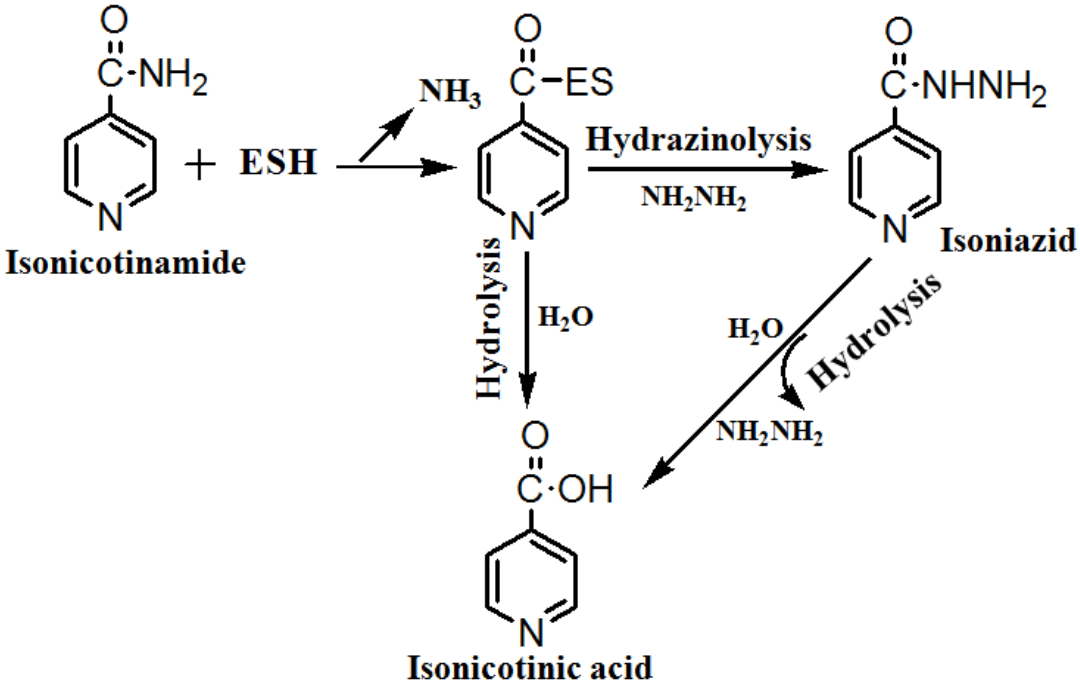


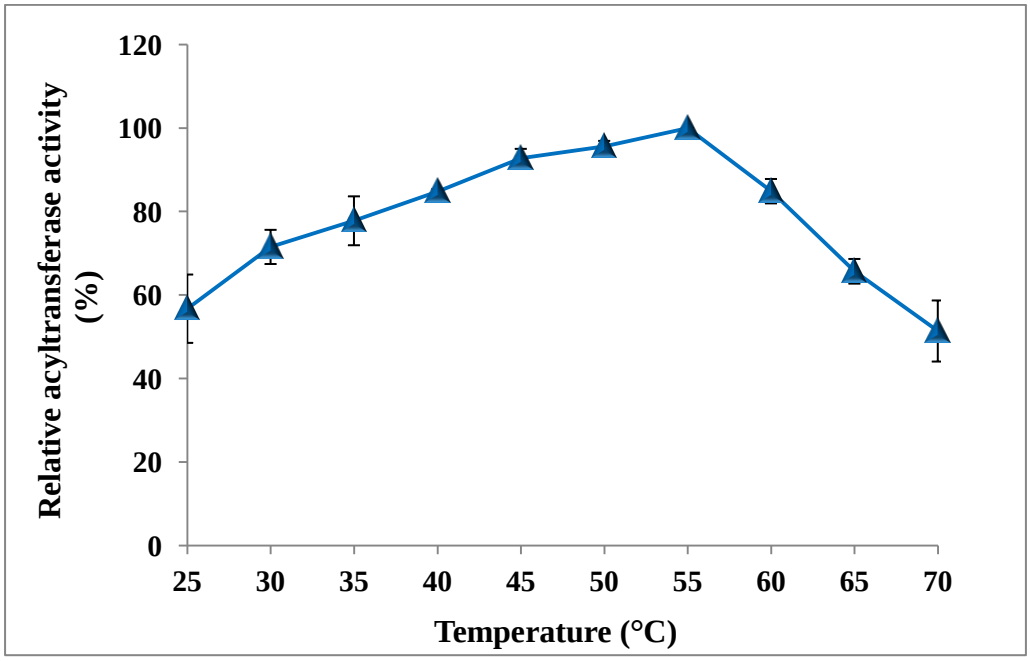
Fig 4.25 Enzymatic reactions during isoniazid synthesis catalysed by *B. smithii* strain IITR6b2

4.3.2 Effect of temperature and pH on acyltransferase activity for isoniazid synthesis

To study the influence of reaction temperature on acyltransferase activity for hydrazide synthesis, the reactions were carried out at temperature range of (25-70 °C). As depicted in Fig. 4.26, the maximum acyltransferase activity was obtained at 55 °C,

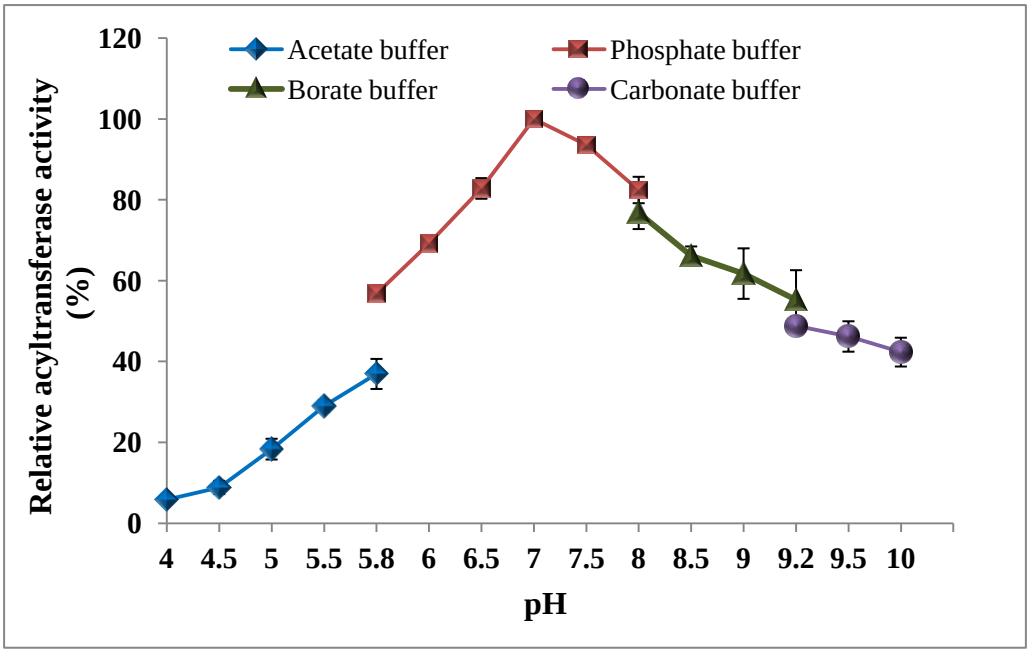
however no significant increase in activity with temperature was observed with increase in reaction temperature from 45 to 55 °C. Above 55 °C, a sharp decrease in activity was observed due to probable thermal inactivation of whole cell enzyme. At 25 and 70 °C relative acyltransferase activities were 56 and 51% of optimum respectively.

The effect of reaction pH on acyltransferase activity was investigated in a pH range of 4-10 using various buffers (50 mM). Results suggested that the whole cell enzyme was active at narrow range and activity increased gradually with increasing pH reaching the maximum at pH 7 (Fig. 4.27). It was found that it retain 69% and 42% of its optimum activity at pH 6 and 10 respectively. The molarity of potassium phosphate buffer at optimum pH 7 was varied from 25 mM to 500 mM to find out its optimum value. Potassium phosphate buffer of 100 mM (pH 7.0) was found to show maximum acyltransferase activity (Fig. 4.28). In earlier reported studies, the highest acyl transfer activity of amidase of *Rhodococcus rhodochrous* J1 for benzoic acid hydrazide synthesis was reported at pH 8.5 (Kobayashi *et al*., 1999).



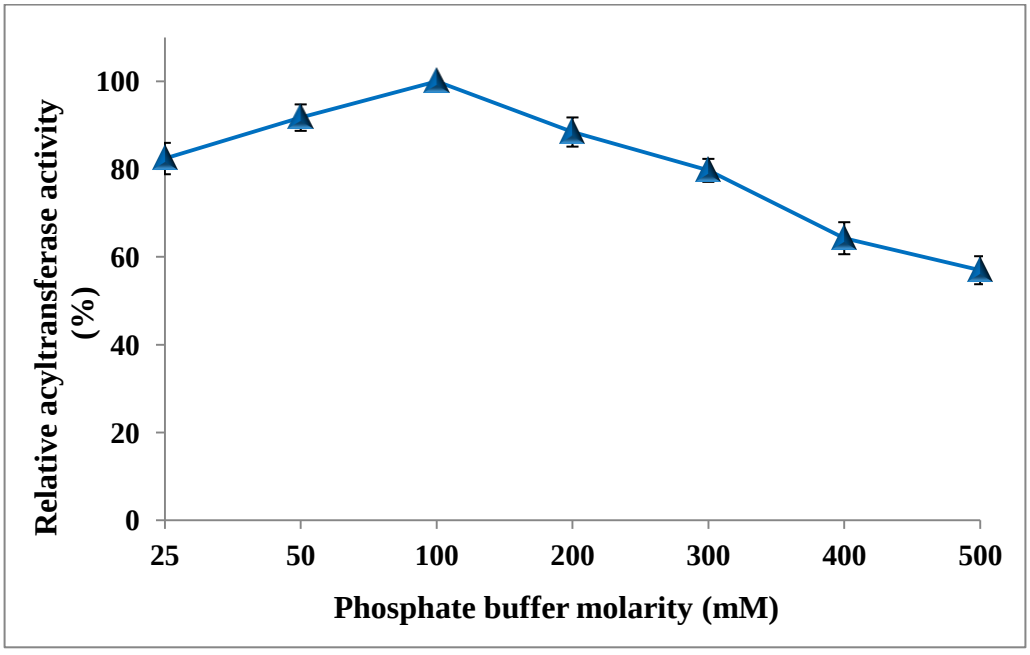
* 100% activity corresponds to 2.51 U/mg

Fig 4.26 Effect of temperature on acyltransferase activity for isoniazid synthesis
Enzyme assay conditions: isonicotinamide (100 mM), hydrazine–2HCl (500 mM), phosphate buffer (50 mM, pH 7.0).



* 100% activity corresponds to 2.63 U/mg

Fig 4.27 Effect of pH on acyltransferase activity for isoniazid synthesis
Enzyme assay conditions: isonicotinamide (100 mM), hydrazine-2HCl (500 mM), 55 °C.



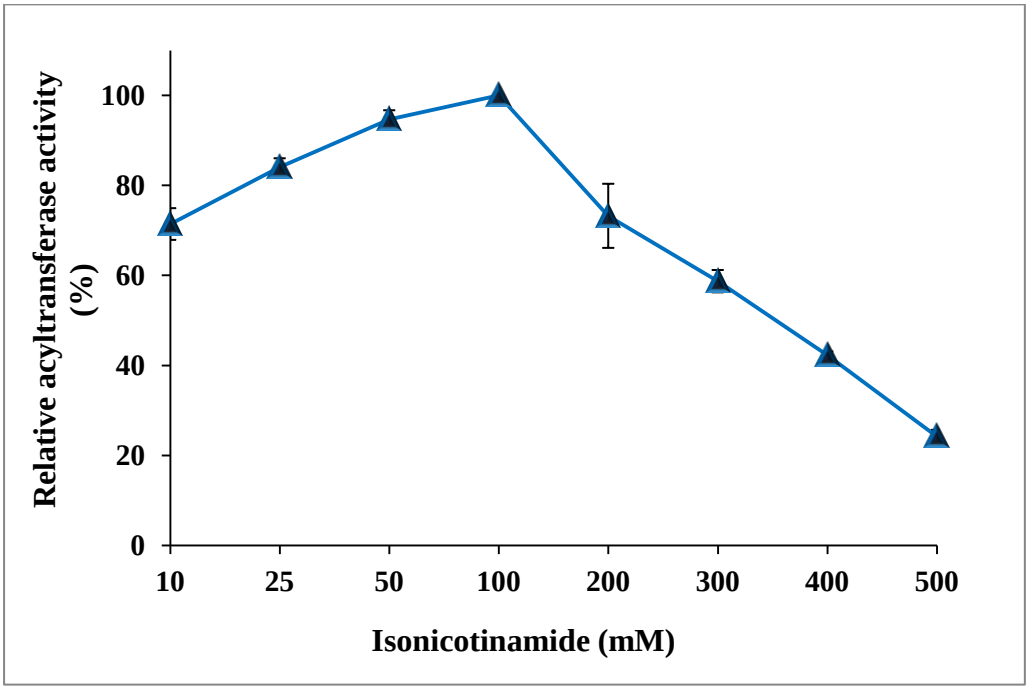
* 100% activity corresponds to 2.84 U/mg

Fig 4.28 Effect of molarity of potassium phosphate buffer on acyltransferase activity
Enzyme assay conditions: isonicotinamide (100 mM), hydrazine-2HCl (500 mM), phosphate buffer (pH 7.0), 55 °C.

4.3.3 Study of effects of substrate, co-substrate concentrations on acytransferase activity and mole ratio optimization

4.3.3.1 Effect of isonicotinamide (substrate) concentration

The concentration of isonicotinamide was varied from 10-500 mM keeping the concentration of hydrazine–2HCl constant (500 mM). Rise in acyltransferase activity was observed as the concentration of isonicotinamide increased from 10-100 mM with maximum acyltransferase activity achieved at 100 mM of isonicotinamide. Whole cell enzyme was found to tolerate higher concentration of isonicotinamide but above 100 mM decrease in enzyme activity was observed (Fig. 4.29). At 500 mM, it reduced to 24% of maximum enzyme activity suggesting the substrate inhibition.



* 100% activity corresponds to 2.71 U/mg

Fig 4.29 Effect of substrate (isonicotinamide) concentration on acyltransferase activity

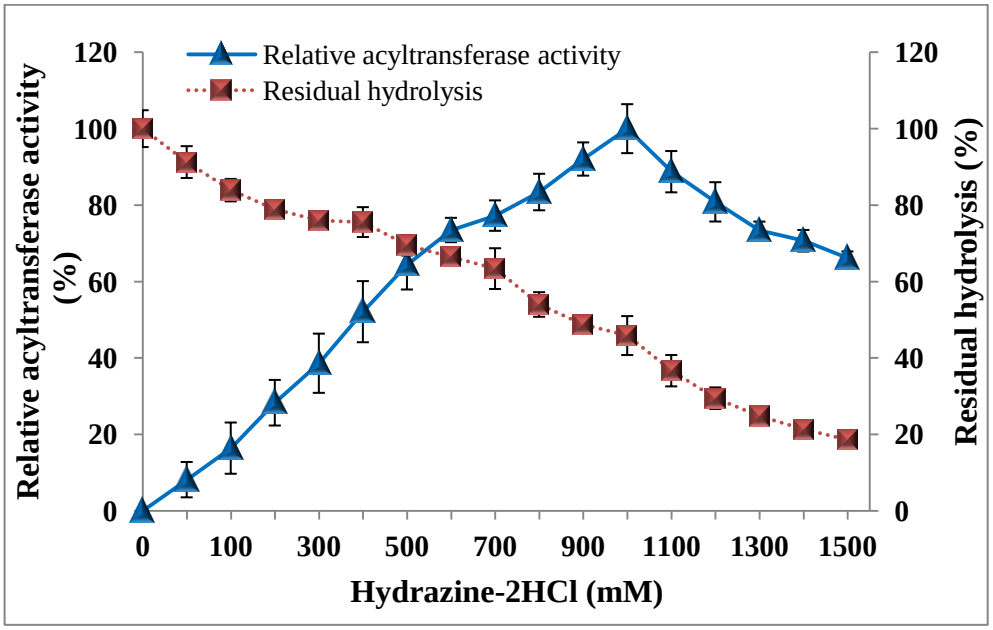
Enzyme assay conditions: isonicotinamide (10-500 mM), hydrazine-2HCl (500 mM), phosphate buffer (100 mM, pH 7.0), 55 °C.

4.3.3.2 Effect of hydrazine-2HCl (co-substrate) concentration

The effect of hydrazine-2HCl on acyltransferase activity and residual hydrolysis during acyltransferase reaction was studied at different hydrazine concentrations (100-1500 mM) keeping isonicotinamide concentration (100 mM) constant. Higher hydrazine-

2HCl concentration resulted in increased acyltransferase activity up to 1000 mM and above this concentration decline in activity was observed due to inhibition by hydrazine-2HCl (Fig. 4.30). The acyltransferase activity was highest at 1000 mM (4.39±0.33 U/mg_{dcw}). Higher concentration of co-substrate was found to be inhibitory to hydrolysis of amide and thus by-product formation decreased with increasing hydrazine-2HCl concentration. Amide hydrolysis reduced to 45% at 1000 mM and 18% at 1500 mM hydrazine concentration-2HCl. Fournand et al. (1998a) reported 26% of residual hydrolysis with propionamide substrate at 800 mM hydrazine concentration. This reduction in hydrolysis in presence of co-substrate confirmed the fact that hydrazine had higher affinity for acyl enzyme complex than water thus it reduced the undesirable amide hydrolysis.

These results showed that higher concentration of co-substrate was not inhibitory to acyltransferase reaction up to 1000 mM concentration. This was contrary to results obtained with hydroxylamine-HCl (Section 4.2.3.2) where concentration of co-substrate higher than 250 mM was inhibitory to acyltransferase reaction. At optimum concentrations of isonicotinamide (100 mM) and hydrazine-2HCl (1000 mM), acyltransferase to hydrolase activity ratio further improved from 3 to 4.5 (Fig. 4.31). In summary, a high ratio of acyl acceptor (hydrazine-2HCl) to acyl donor (isonicotinamide) improved acyltransferase (hydrazinolysis) reaction, and suppressed hydrolysis.



* 100% activity corresponds to 4.39 U/mg

Fig 4.30 Effect of co-substrate (hydrazine-2HCl) concentration on acyltransferase activity. Enzyme assay conditions: isonicotinamide (100 mM), hydrazine-2HCl (0-1500 mM), phosphate buffer (100 mM, pH 7.0), 55 °C.

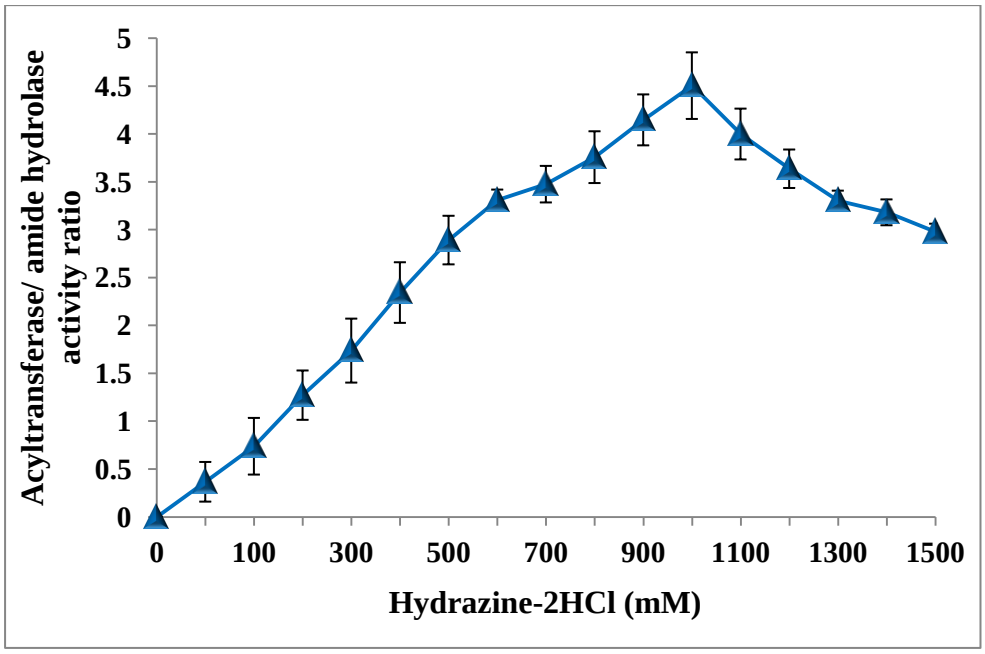


Fig 4.31 Effect of co-substrate concentration on acyltransferase to amide hydrolase activity ratio

4.3.3.3 Optimization of substrate and co-substrate concentrations combination

To select the optimum mole ratio of substrates (isonicotinamide and hydrazine-2HCl); reactions were performed with several possible ratios of amide and hydrazine-2HCl. Highest acyltransferase activity was obtained with 100 mM of isonicotinamide and 1000 mM of hydrazine-2HCl (Fig. 4.32). Therefore all the subsequent experiments were performed with 100 and 1000 mM of isonicotinamide and hydrazine-2HCl respectively.

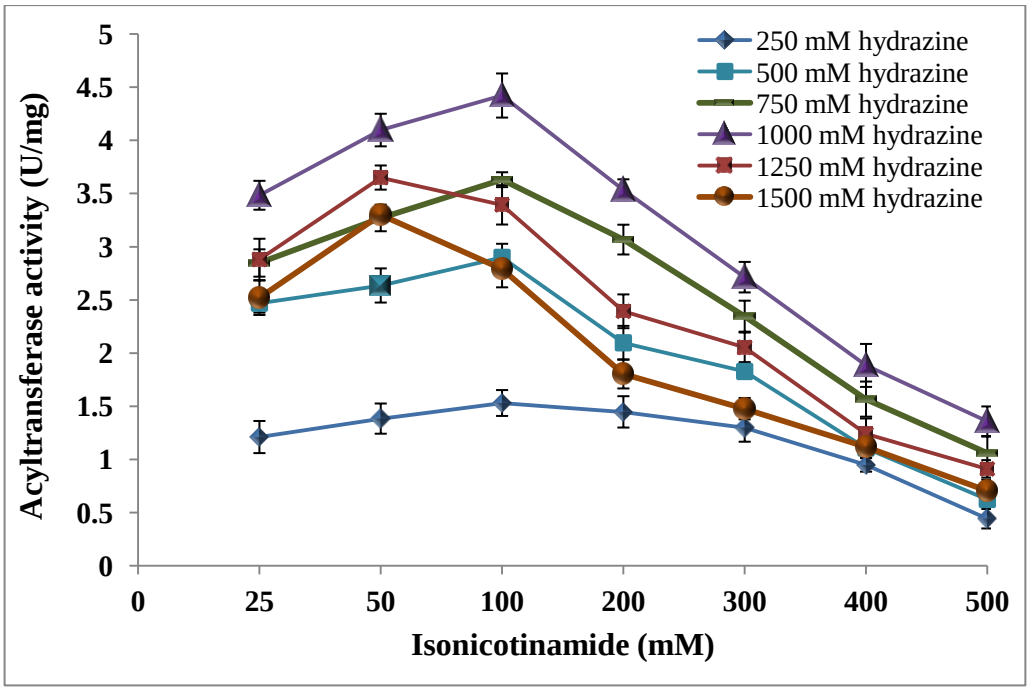


Fig 4.32 Optimization of substrate and co-substrate concentrations

Enzyme assay conditions: isonicotinamide (25-500 mM), hydrazine-2HCl (250-1500 mM), phosphate buffer (100 mM, pH 7.0), 55 °C.

4.3.4 Effect of reaction temperature on molar conversion and by-product formation

It was already found that 55 °C is the optimum temperature for whole cell acyltransferase activity. The effects of temperature on molar conversion of isonicotinamide to isoniazid and isonicotinic acid were studied for 120 min at various temperatures (30, 35, 45 and 55 °C). Initial conversion rates were found to increase with an increase in temperature from 30 to 55 °C for first 45 min (Fig. 4.33). However maximum conversion of 89.6% was achieved at 30 °C in 90 min. There was a marginal difference in the rate of conversion at 45 and 55 °C and maximum conversions achieved were 81.9% and 78.7% respectively in 75 min and thereafter the concentration of isoniazid decreased with time probably due to product hydrolysis. This lower conversion rate at higher temperature can be explained by isonicotinic acid (by-product) analysis. By-product formation rates were 1.8 and 1.6 folds higher at 55 and 45 °C as compared to 30 °C respectively. It may be due to higher rate of side reactions (amide and hydrazide hydrolysis) at higher temperatures. This study suggested that the final conversion of isonicotinamide to isoniazid was higher at 30 °C temperature with less by-product (7.6%) formation. As a result the optimal temperature was chosen to be 30 °C for bioprocess development. However possibility of

lower by-product formation at temperatures lower than 30 °C cannot be ruled out. Lower temperatures also decreased acyltransferase activity simultaneously (Section 4.3.2).

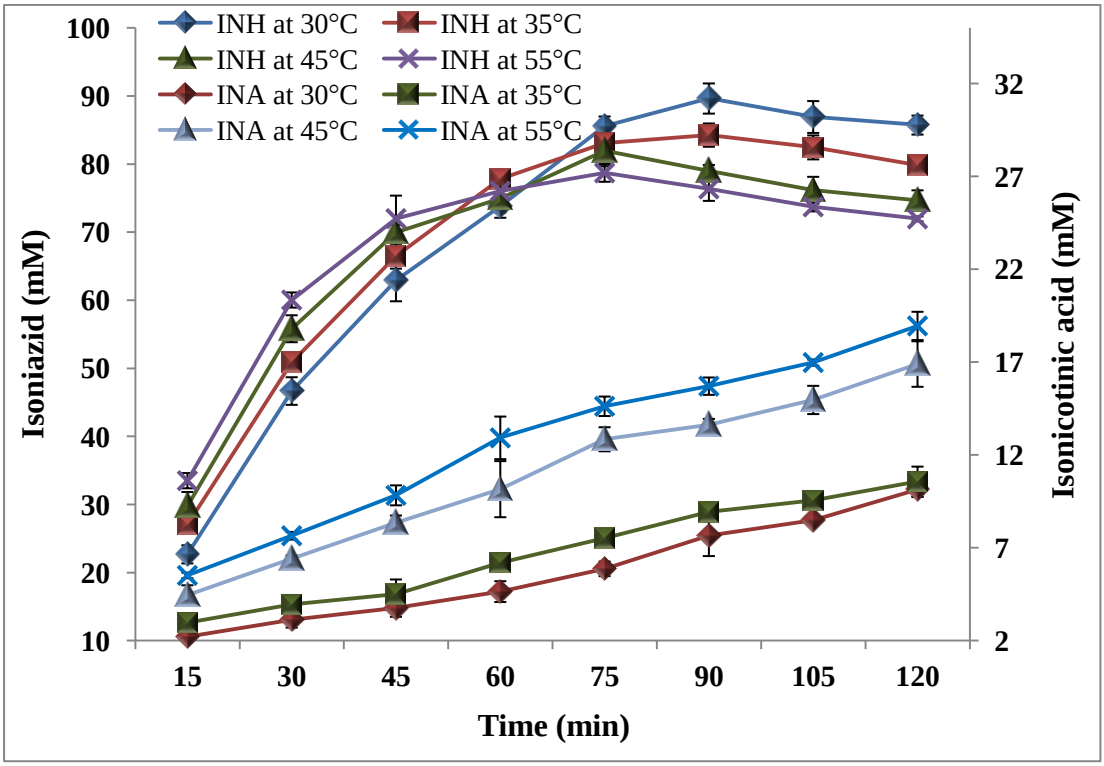


Fig 4.33 Effect of reaction temperature on molar conversion and by-product formation Reaction conditions: isonicotinamide (100 mM), hydrazine-2HCl (1000 mM), 25 ml phosphate buffer (100 mM, pH 7.0), 1.0 mg_{dcw}/ml resting cells.

4.3.5 Effect of resting cells concentrations on conversion and time course of reaction

The **e**ffects of whole cell concentrations on the molar conversion and time course of reaction were studied in the range of 1.0 to 2.5 mg_{dcw}/ml under optimized substrate/co-substrate concentrations of 100/1000 mM at 30 °C. The conversion rate increased with increase in cell concentration and attained a conversion of 90.4% at 2.0 mg_{dcw}/ml while the overall conversion time reduced to 60 min from 90 min (Fig. 4.34). With increase in cell concentration to 2.5 mg_{dcw}/ml, the initial conversion rate was marginally higher but maximum conversion achieved was nearly same and no reduction in reaction time was observed. It indicated that an excess amount of cells would not increase the molar conversion of isonicotinamide, which might be due to increase in concentration of catalyst above the substrate concentration or mass transfer limitations. A lower amount of the biocatalyst and shorter reaction time will reduce the bioprocess development cost. Hence

whole cell concentration of 2.0 mg_{dcw}/ml was used for fed batch process development with intermittent addition of substrates (100/100 mM) at 60 min interval.

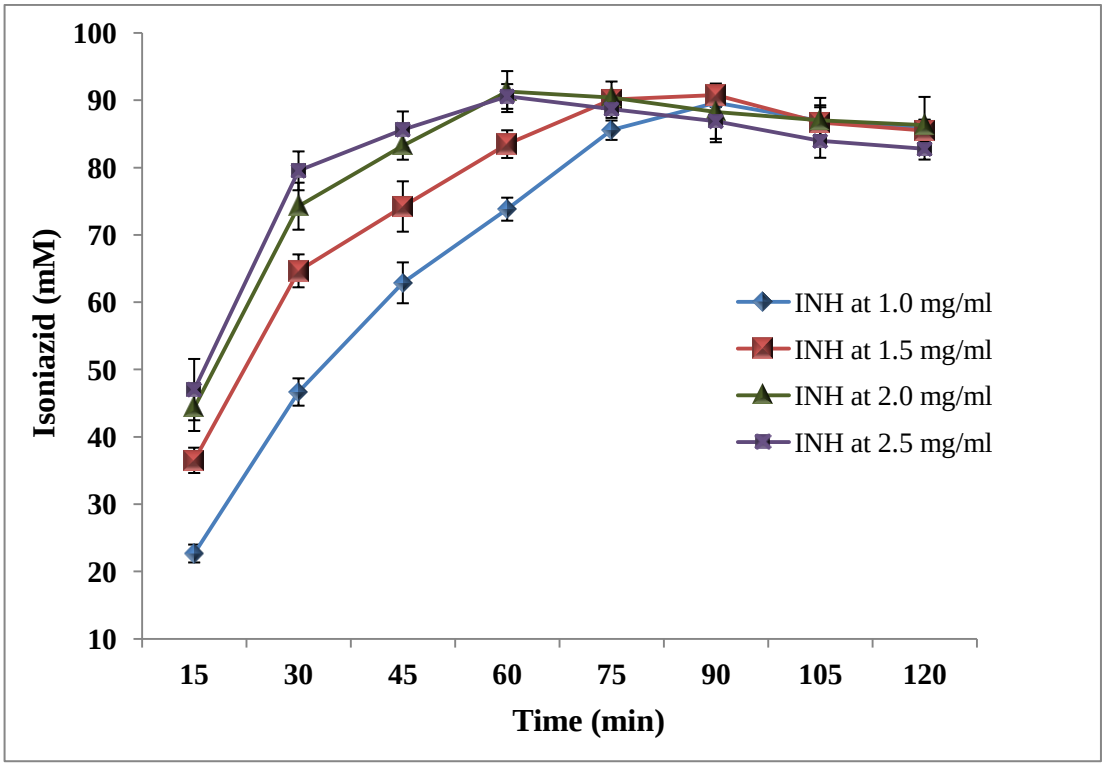


Fig 4.34 Effect of resting cells concentration (DCW) on biocatalytic synthesis of isoniazid and time course of reaction
Reaction conditions: isonicotinamide (100 mM), hydrazine-2HCl (1000 mM), 25 ml phosphate buffer (100 mM, pH 7.0), 30 °C.

4.3.6 Fed batch bioprocess development at 50 ml scale

Acyltransferase activity of whole cell enzyme was inhibited by isonicotinamide concentration above 100 mM, hence fed batch biotransformation can be a useful strategy in order to obtain high yield of isoniazid with high productivity. Previous study suggested that under optimized conditions of pH (7.0, phosphate buffer 100 mM), temperature (30 °C), substrate/co-substrate concentration (100/1000 mM) and cell concentration (2.0 mg_{dcw}/ml), high molar bioconversion yield (90.4%) was achieved with in 60 min. Feedings of substrates (100 mM each) were made at an interval of 60 min. The accumulations of isoniazid and isonicotinic acid during biotransformation process with seven feedings of substrates is shown in Fig. 4.35. There was no inhibitory effect on rate of conversion of isonicotinamide to isoniazid up to third feeding and during 4^{th} feed 79% conversion was observed with respect to conversion achieved in first feed. After 4^{th} feed, sharp decline in

conversion rate of isonicotinamide was observed and it reduced to 35%, 22.6 %, and 8.6% during 5th, 6th and 7th feed. Hence for maximum conversion of substrate to product with minimum by-product formation fed batch process should be continued till 4th feed only. A total of 439 mM of isoniazid was accumulated by 87.8% molar conversion of isonicotinamide (500 mM) with only 7.8% by product in 5 h at a production rate of 6.0 g/h/g_{dcw}. During this whole process 32.7% loss in acyltransferase activity and 48.6% decrease in resting cells concentration (measured by optical density at 600 nm) due to lysis was observed after 5 h. Therefore sharp decrease in conversion rate after 4th feed was probably due to combined effects of product inhibition, decrease in acyltransferase activity and cell lysis.

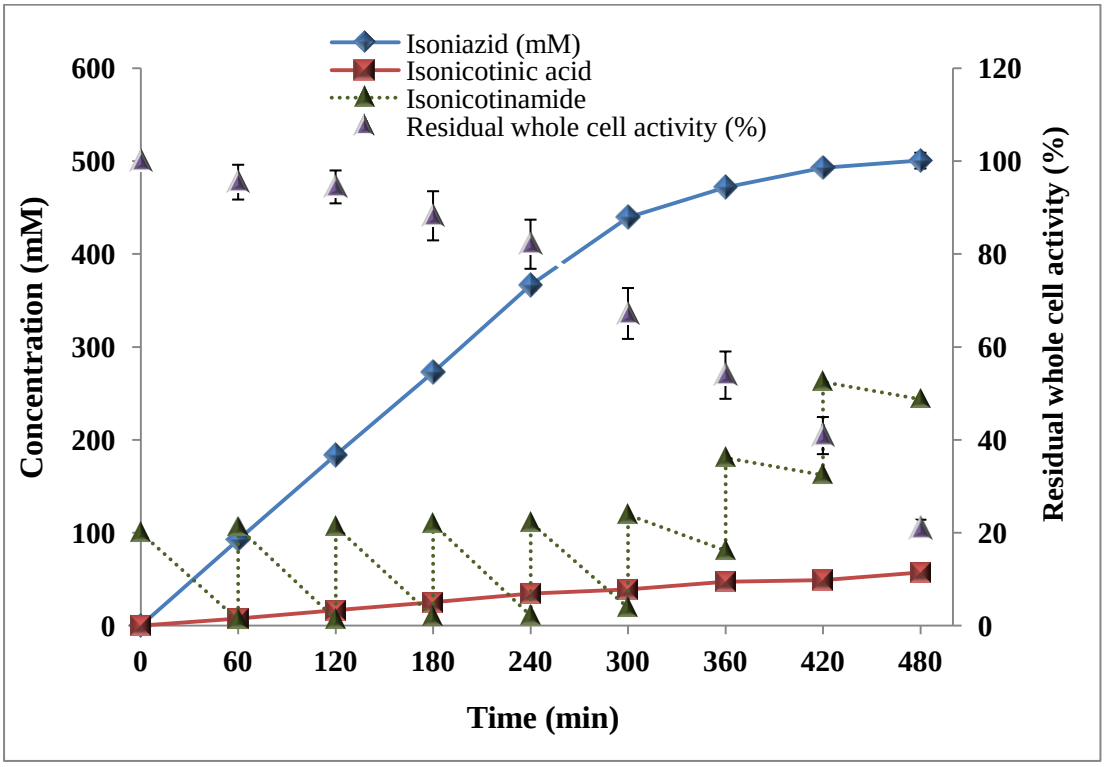


Fig 4.35 Time course of production of isoniazid during fed batch biotransformation process
Reaction conditions: 50 ml phosphate buffer (100 mM, pH 7.0), 30 °C, resting cells concentration (2.0 mg_{dcw}/ml).

4.3.7 Recovery of isoniazid

The supernatant obtained was freeze-dried and a white powder (9.1 g) was recovered. On HPLC analysis of this powder it was found that powder contains 33.3%

(w/w) isoniazid and 2.5% (w/w) isonicotinic acid. Further purification was done by solvent extraction to recover isoniazid as white powdery solid (3.1 g) with 94% purity.

Fed batch bioprocess for isoniazid synthesis has been successfully developed for high molar conversion with limited side reactions using acyltransferase activity of *B. smithii* strain IITR6b2. Yadav et al. (2005) has developed a lipase catalyzed enzymatic route for isoniazid synthesis by working in non-aqueous media and under milder reaction conditions. This process resulted in 52% conversion in 24 h. Further improvement in method was done by using microwave irradiation with immobilized lipase and process led to a conversion of 54% in 4 h (Yadav and Sajgure 2007). 1, 4-Dioxane, used in lipase catalysed synthesis is a known carcinogen to animals and is classified as a possible carcinogen to human (Kano *et al*., 2009, Kasai *et al*., 2009, Leung and Paustenbach, 1990). The advantages of present method include aqueous medium, higher conversion in shorter reaction time and mild reaction conditions. These findings can contribute to industrial production of isoniazid via a biocatalytic route in future. However further development in conversion can be achieved by adopting immobilized enzyme which may improve the stability of the enzyme under process conditions.

Section 4.4

Purification of amidase enzyme and its characterization

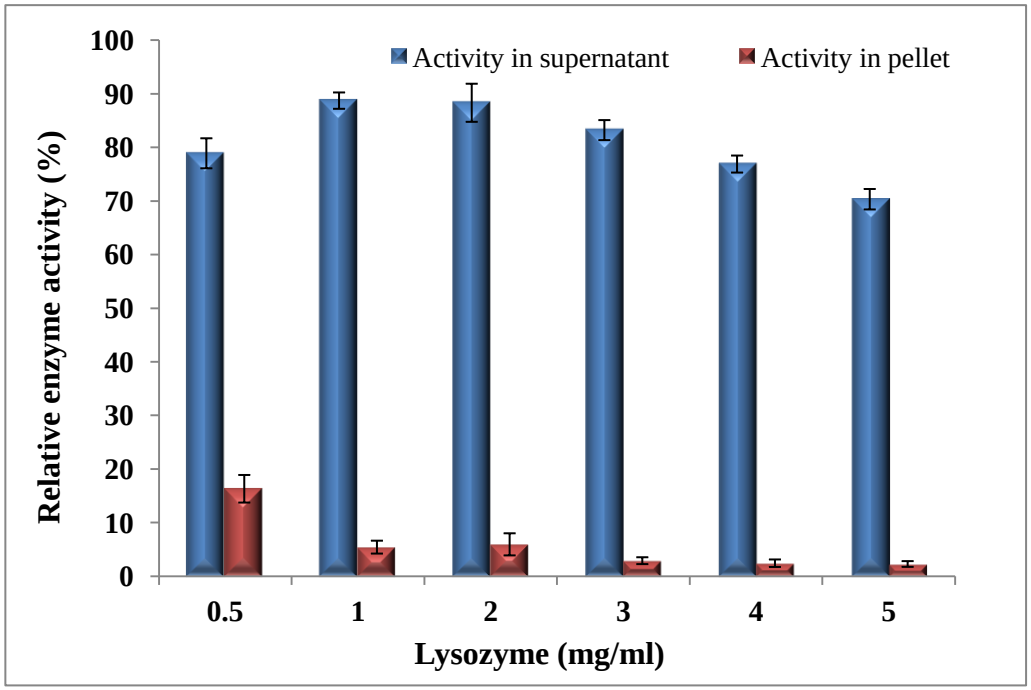
4.4.1 Methods of cell lysis for recovery of amidase enzyme

Amidase is an intracellular enzyme in nature and to purify it, disruption of bacterial cells is required to release the proteins. Lysis of whole cells of *B. smithii* strain IITR6b2 for recovery of cell free extract containing amidase enzyme is an important step of purification process. Thus adopted methods need to be cheap and should be able to recover maximum amidase. Bacterial cell disruptions for recovery of intracellular proteins are often performed by lysozyme, sonication and French press methods (Kotlova *et al*., 1998, d'Abusco *et al*., 2001, Vejvoda *et al*., 2010, Skouloubris *et al*., 2001). In the present study, bacterial cell lysis was performed by lysozyme treatment and sonication and comparison of efficiency of these methods was carried out based on maximum amidase recovery in cell free extract. During enzymatic lysis of cells, recovery of amidase in supernatant increased as the lysozyme concentration increased up to 1 mg/ml and about 88.7% of whole cell activity was recovered in the supernatant (Fig. 4.36). The activity remained in corresponding lysed cells was only 5.4%. Further increase in lysozyme concentration did not improve amidase recovery in the supernatant.

Cell lysis with sonication resulted in about 69.5% recovery of whole cell amidase activity in supernatant after 8 min of sonication (Fig. 4.37). Further increase in sonication time reduced the enzyme activity both in supernatant and lysed cells. This decrease in recovery with time might be due to thermal denaturation of amidase caused by heat generation during sonication. Maximum 69.5% recovery of whole cell amidase in supernatant indicates that cells were not lysed completely. Hence use of lysozyme (1 mg/ml) was selected to lyse bacterial cells.

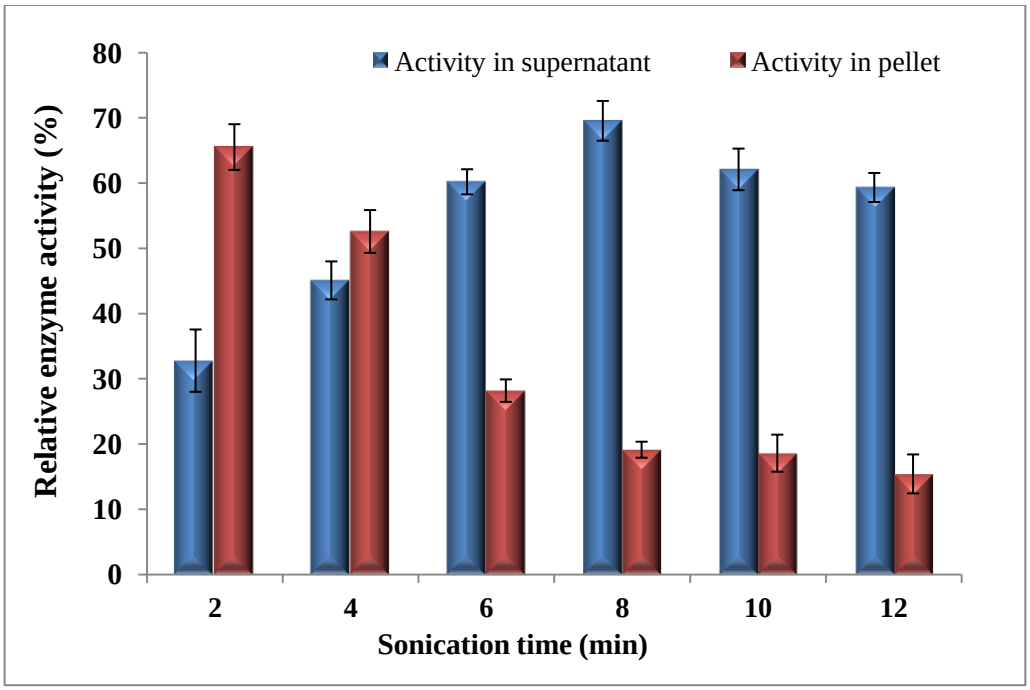
In the earlier reports of bacterial cell lysis, sonication and French press methods were found to be efficient for recovery of amidase enzyme from *Ochrobactrum anthropi* NCIMB 40321 (Sonke *et al*., 2005), *Burkholderia sp.* strain DR.Y27 (Syed *et al*., 2012), *Brevibacterium iodinum* (Komeda and Asano, 2008) and *Mycobacterium neoaurum* ATCC 25795 (Hermes *et al*., 1994) respectively.

.



* 100% activity corresponds to 81.42 U/ml

Fig 4.36 Bacterial cell lysis by lysozyme for recovery of amidase enzyme
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.



* 100% activity corresponds to 66.04 U/ml

Fig 4.37 Cell lysis by sonication for recovery of amidase enzyme
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

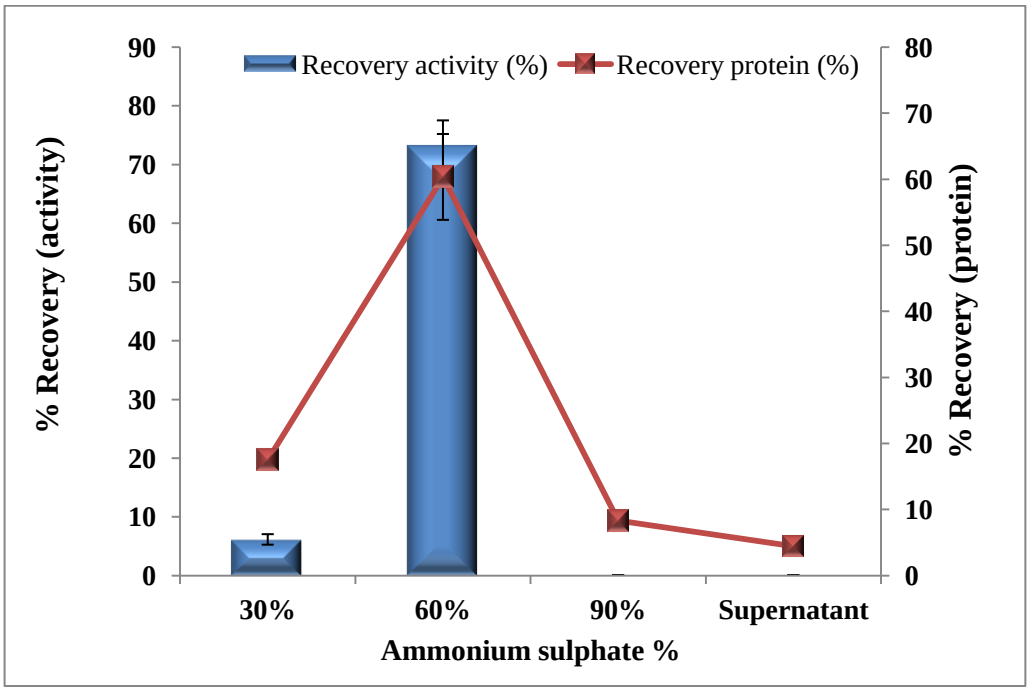
4.4.2 Protein precipitation

Cell free extract obtained after cell lysis was needed to be concentrated before loading on chromatography column. In literature most of the schemes used for amidase purification involved a precipitation step, using either ammonium sulphate or acetone or isopropanol fractionation followed by different chromatographic methods (Mehta *et al*., 2013, Nawaz *et al.*, 1996, Kotlova *et al*., 1999, Baek *et al*., 2003). Precipitation is generally used as concentration step during early stages of purification process and it also removes contaminating proteins to some extent. In this study two salts, ammonium sulphate and sodium sulphate were used as precipitating agents to study their efficiency for protein precipitation and recovery of amidase enzyme.

Precipitation by ammonium sulphate was done by step wise addition at different concentrations (30%, 60% and 90% saturation) to the cell free extract (Fig. 4.38). Amidase activity and protein concentration of each precipitated protein fraction were analysed to determine the optimum ammonium sulphate concentration for maximum amidase precipitation. Precipitation at 30% saturation was less effective resulting in 17.4% protein precipitation and only 6.1% recovery of amidase. At 30-60% of ammonium sulphate saturation, amidase enzyme was preferentially precipitated resulting in 73.1% recovery of amidase activity. However, for concentration higher than 60%, a decrease in both protein precipitation and amidase recovery were observed, as most of the amidase was precipitated during 30-60% saturation level. Cell free extract obtained after ammonium sulphate precipitation at 60-90% saturation level contained only 4.4% protein and no amidase activity indicating the effectiveness of ammonium sulphate for amidase precipitation from cell free extract.

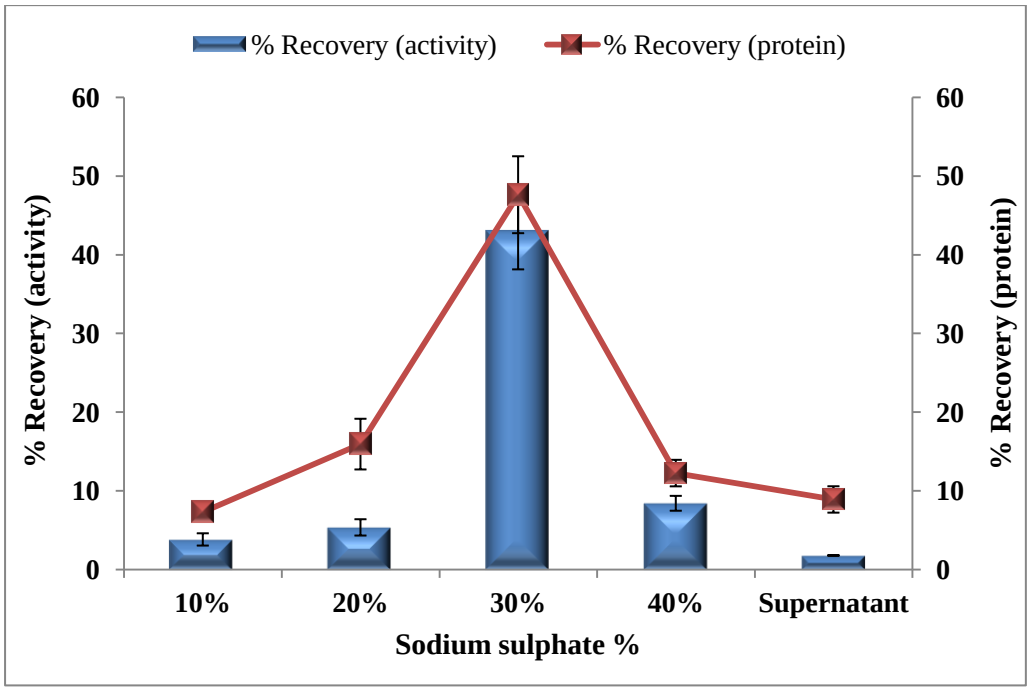
To study the efficiency of sodium sulphate as precipitating agent, different concentrations (10, 20, 30 and 40%) were used to precipitate proteins from cell free extract (Fig. 4.39). At 10% sodium sulphate concentration, 7.3% protein was precipitated and only 3.8% amidase activity was recovered. Sodium sulphate concentration was further increased to 20% to recover 15.9% protein and 5.3% amidase enzyme. Maximum precipitation of protein (47.6%) was obtained at 30% sodium sulphate with 42.9% amidase recovery. After precipitation with 40%, amidase activity of supernatant was also analysed and it was only 1.7% of total activity. From these results it is obvious that sodium sulphate salt was inhibitory to amidase activity as only 60.4% overall recovery of amidase was obtained while 1.7% remained in cell free extract.

Thus it was concluded that sodium sulphate was not effective as precipitating agent of amidase enzyme of *B. smithii* strain IITR6b2. With ammonium sulphate salt, overall amidase recovery higher than 79% was obtained with maximum 73.1% recovery at 30-60% saturation. Therefore, to minimize the effect of salts and achieve optimal amidase recovery, 30-60% saturated ammonium sulphate fractionation was used to precipitate amidase for further purification steps. Similar to the present study there are other reports regarding the use of ammonium sulphate fractionation in the purification of amidase from *Geobacillus subterraneus* RL-2a (Mehta *et al*., 2013), *Ochrobactrum anthropi* NCIMB 40321 (Sonke *et al*., 2005), *Brevibacillus borstelensis* BCS-1 (Baek *et al*., 2003), *Klebsiella pneumoniae* NCTR 1 (Nawaz *et al*., 1996) and *Rhodococcus sp.* (Nawaz *et al*., 1994).



* 100% activity corresponds to 88.28 U/ml

Fig 4.38 Protein precipitation by ammonium sulphate fractionation
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.



* 100% activity corresponds to 74.17 U/ml

Fig 4.39 Protein precipitation by sodium sulphate fractionation
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0), 45 °C.

4.4.3 Purification of amidase from *Bacillus smithii* strain IITR6b2

Protein purification is generally a multistep process involving the combinations of different steps like precipitation, ultrafiltration, heat treatment, ion exchange, hydrophobic interaction, gel permeation and affinity column chromatographies (Yao *et al*., 2013, Dheeman *et al*., 2011, Gaur *et al*., 2008, Sinsuwan *et al*., 2010, Haddar *et al*., 2009). Steps followed for purification of amidase from different microorganisms varied from simple to complicate. Makhongela et al. (2007) purified amidase from *Geobacillus pallidus* RAPc8 in just two steps: heat treatment and gel permeation chromatography. However, amidase from *Rhodococcus sp.* was purified by acetone precipitation, ammonium sulphate precipitation, DEAE sepharose, Mono Q and superpose 12 chromatographies (Nawaz *et al*., 1994).

Amidase from *B. smithii* strain IITR6b2 was also purified in four consecutive steps of ammonium sulphate precipitation (30-60%), ultrafiltration by amicon (10 kD), anion exchange (with Q sepharose resin) chromatography and hydrophobic interaction (with phenyl sepharose resin) chromatography to an electrophoretic homogeneity. In the existing literature, Q sepharose has been used for amidase purification from recombinant *E. coli* (Fournand *et al*., 1997), *Rhodococcus sp.* Strain R312 (Fournand *et al*., 1998b),

Xanthobacter agilis (Briggs *et al*., 1996) and *Variovorax paradoxus* (Krieg *et al*., 2002). There are reports regarding the use of phenyl sepharose matrix for purification of amidase of *Rhodococcus erythropolis* MP50 (Hirrlinger *et al*., 1996) and *Pseudonocardia thermophila* (Egorova *et al*., 2004).

The cell free extract (CFE) obtained after treatment with 1 mg/ml lysozyme was subjected to ammonium sulphate fractional precipitation. The protein fraction precipitated between 30-60% salt saturation was collected and this step resulted in precipitation of approximately 51% of total protein and a 1.46 fold increase in amidase specific activity (35.3 U/mg) with 75.4% recovery. The protein sample obtained after the ammonium sulphate fractionation step was concentrated, desalted (by amicon 10 kD) and loaded on a Q sepharose column. This concentration and desalting step resulted in 83.6% recovery which is higher to recovery obtained in previous precipitation step (75.4%). This unusual increase in % recovery in later step is possibly due to removal of NH_4SO_4 salt from protein sample that was probably inhibitory to acyltransferase activity resulting in less apparent % recovery.

In the anion exchange chromatography step, elution of bound proteins was done by step wise gradient of 1 M NaCl (20-50%) and the elution profile of the proteins obtained from the Q sepharose column are presented in Fig. 4.40. Total eight peaks were obtained. Fractions corresponding to each peak were clubbed and used for amidase activity determination (Table 4.11). Amidase activity was detected in fifth peak corresponding to proteins eluted at 35% of 1 M NaCl. The specific activity of amidase fraction purified using Q sepharose was 168.2 U/mg and purification fold was 6.97 while the enzyme yield was 74.5%.

For further purification, the pooled active fractions of this step were concentrated, desalted and loaded onto a phenyl sepharose column. The bound proteins were eluted by step wise gradient of 1 M NH_4SO_4 (75-0%) and the elution profile of proteins is shown in Fig 4.41. Fractions corresponding to each peak were clubbed and used for amidase activity assay (Table 4.12). Amidase activity was detected in fourth peak corresponding to proteins eluted at 0.375 M NH_4SO_4 and this step resulted in overall 12.11 fold purification. Purity of concentrated active fractions of hydrophobic interaction chromatography was confirmed by native-PAGE (Section 4.4.4). After all these steps the amidase of *B. smithii* strain IITR6b2 was purified to 12.11 fold with specific activity of 292.2 U/mg of protein and amidase yield of 37.5% was obtained with respect to the cell-free extract. Results of

different steps during amidase purification are summarized in table 4.13. This procedure of purification of amidase from *B. smithii* strain IITR6b2 resulted in high homogeneity (Fig 4.42) and obtained amidase was used for further characterisation.

The obtained amidase yield (37.5%) for *B. smithii* strain IITR6b2 was significantly higher as compared to reported yields obtained after amidase purification from *Geobacillus subterraneus* RL-2a (9.54%) (Mehta *et al*., 2013), *Geobacillus pallidus* BTP-5x MTCC 9225 (6.7%) (Sharma *et al*., 2013), *Brevundimonas diminuta* TPU 5720 (1.1%) (Komeda *et al*., 2006), and *Klebsiella pneumonia* (10%) (Nawaz *et al*., 1996). It was comparable to amidase purified from *Ochrobactrum anthropi* NCIMB 40321 (35%) (Sonke *et al*., 2005), *Rhodococcus erythropolis* AJ270 (33.8%) (Xue *et al*., 2011). However obtained yield was less than those obtained from *Rhodococcus rhodochrous* (57%) (Kotlova *et al*., 1999) and *Brevibacterium iodinum* TPU 5850 (52%) (Komeda and Asano, 2008). The fold purification of this amidase was lesser than the 168 fold purification of *Klebsiella pneumonia* (Nawaz *et al*., 1996), 138 fold of *Rhodococcus sp.* (Nawaz *et al*., 1994), 98 fold of *Ochrobactrum anthropi* NCIMB 40321 (Sonke *et al*., 2005), 52 fold of *Geobacillus subterraneus* RL-2a (Mehta *et al*., 2013). In contrast it was higher as compared to 6.2 fold purification obtained from *Geobacillus pallidus* BTP-5x MTCC 9225 (Sharma *et al*., 2013), 6.9 fold from *Rhodococcus erythropolis* AJ270 (Xue *et al*., 2011) and 6 fold from *Geobacillus pallidus* RAPc8 (Makhongela *et al*., 2007).

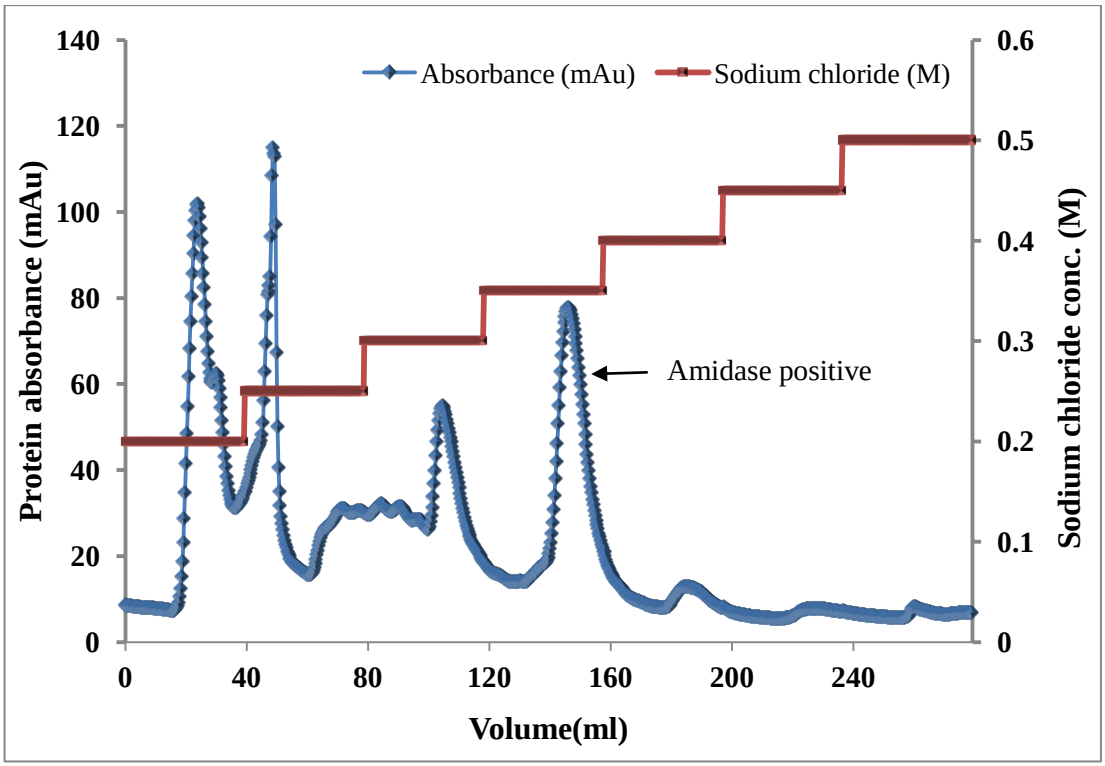


Fig 4.40 Elution profile of proteins on ion exchange chromatography column (Q **sepharose)**

Table 4.11 Acyltransferase activity of clubbed fractions of ion exchange chromatography

Peak no.	NaCl concentration (mM)	Enzyme activity
1	200	ND
2	200-250	ND
3	250	ND
4	300	ND
5	350	Amidase*
6	400	ND
7	450	ND
8	500	ND

*Amidase acyltransferase activity with nicotinamide substrate

ND- No amidase acyltransferase activity detected with nicotinamide

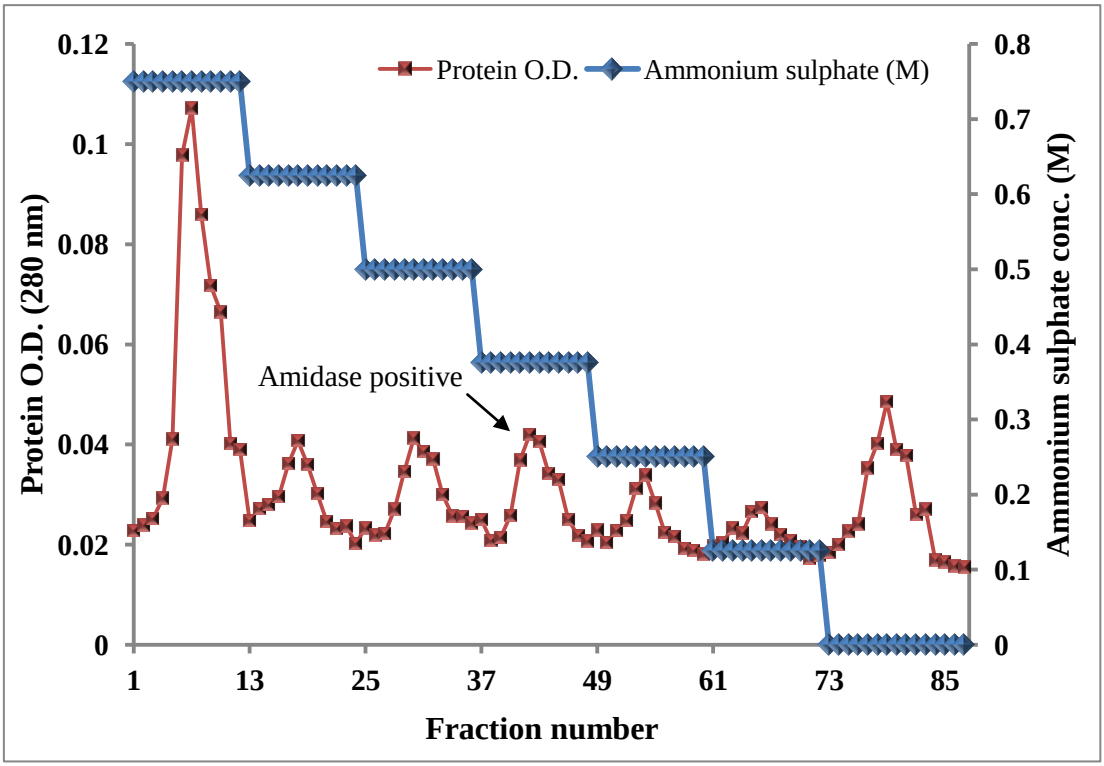


Fig 4.41 Elution profile of proteins on hydrophobic interaction chromatography column (**phenyl sepharose**)

Table 4.12 Acyltransferase activity of clubbed fractions of hydrophobic interaction chromatography

Peak no.	NH_4SO_4 concentration (mM)	Enzyme activity
1	750	ND
2	625	ND
3	500	ND
4	375	Amidase*
5	250	ND
6	125	ND
7	0	ND

*Amidase acyltransferase activity with nicotinamide substrate
ND- No amidase acyltransferase activity detected with nicotinamide

Table 4.13 Different purification steps of amidase from *B. smithii* strain IITR6b2

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	52.74	1272.94	24.13	1	100
Ammonium sulphate precipitation (30-60 %)	27.2	960.17	35.29	1.46	75.4
Concentration/ desalting (by amicon 10 kD)	23.94	1065.21	44.48	1.84	83.6
Ion exchange chromatography	5.63	947.67	168.20	6.97	74.5
Hydrophobic interaction chromatography	1.63	477.79	292.19	12.11	37.5

4.4.4 Native poly acrylamide gel electrophoresis (Native-PAGE)

After hydrophobic interaction chromatography, all the active fractions having amidase activity were pooled, concentrated and desalted. To confirm the homogeneity of purified amidase this sample was subjected to native-PAGE. Protein obtained as a result of four purification steps gave a single band in Native-PAGE gel (Fig. 4.42a).

4.4.5 Zymogram

Acyltransferase activity of the cell free extract and purified protein was further confirmed by zymogram analysis using the modified method of Egorova et al. (2004). Zymogram analysis resulted in one red-brown band at the same position as was in the native-PAGE gel (Fig. 4.42b). Zymogram confirmed the amidase activity and purity of purified amidase obtained from *B. smithii* strain IITR6b2.

4.4.6 Molecular mass determination by SDS-PAGE and gel filtration chromatography

The molecular mass of purified amidase was determined with 10% SDS-PAGE under reducing and non-reducing conditions (Fig. 4.43). Samples of different purification steps were analysed on SDS-PAGE gel. Purified protein was also subjected to gel filtration

column to determine both molecular weight and purity. Gel filtration chromatography resulted in a single major peak confirming purity of purified amidase (Fig. 4.44). SDS-PAGE gel analysis revealed that the number of bands significantly reduced from Q sepharose step onwards demonstrating the efficiency of purification steps. The purified protein showed a single band at same position under both reducing and non-reducing conditions indicating the monomeric nature of purified amidase. The molecular weight of the amidase protein was calculated from electrophoretic mobility and was found to be 63 kDa. While gel filtration chromatography on a Superdex 75 column also revealed that the molecular mass of the native protein is approximately 63 kDa. The results of SDS-PAGE along with gel filtration chromatography confirmed that purified amidase from *B. smithii* strain IITR6b2 is a monomer with molecular mass (M_r) of 63 kDa.

Amidases from *Ochrobactrum anthropi* SV3 (Okazaki *et al*., 2007), *Sulfolobus solfataricus* (d'Abusco *et al*., 2001), *Klebsiella pneumoniae* NCTR 1 (Nawaz *et al*., 1996) and *Mycobacterium smegmatis* (Mahenthiralingam *et al*., 1993) are monomer like purified amidase of *B. smithii* strain IITR6b2 with molecular weights of 40, 56, 62 and 47 kDa respectively. Many of the amidase are reported to have quaternary structure and consist of identical subunits. Amidases from *P. thermophila* (Egorova *et al*., 2004), *Ochrobactrum anthropi* NCIMB 40321(Sonke *et al*., 2005), *M. Methylotrophus* (Wyborn *et al*., 1994), *P. chlororaphis* B23 (Ciskanik *et al*., 1995), *Rhodococcus erythropolis* AJ270 (Xue *et al*., 2011) and *Rhodococcus sp.* N-771 (Ohtaki *et al*., 2010) are homodimers with molecular weights of 108, 66, 123, 105, 110 and107 kDa respectively. The amidase from *Geobacillus pallidus* BTP-5x MTCC 9225 (Sharma *et al*., 2013) and *R. Rhodochrous* M8 (Kotlova *et al*., 1999) are homotetramer with molecular weight of 158 and 150±20 kDa respectively. While the amidase from *Geobacillus pallidus* RAPc8 (Makhongela *et al*., 2007) has homohexameric structure with molecular weight of 218 kDa and amidase from *Rhodococcus erythropolis* MP50 (Hirrlinger *et al*., 1996) is an octamer with an apparent molecular mass of 480 kDa.

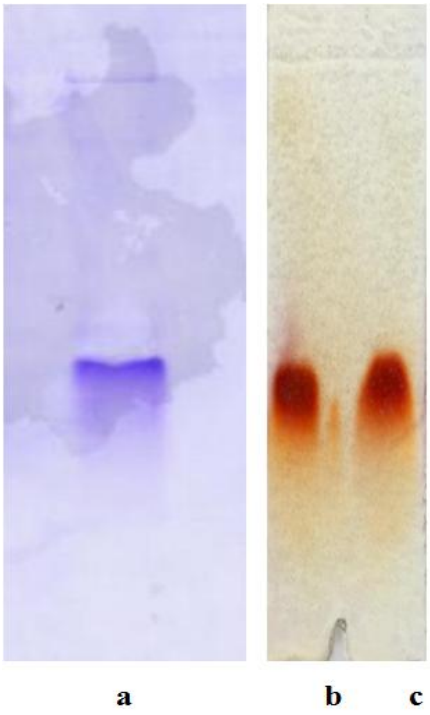


Fig 4.42 a) native-PAGE of active fractions of HIC, **b)** Zymogram of active fraction of HIC, **c)** Zymogram of cell free extract

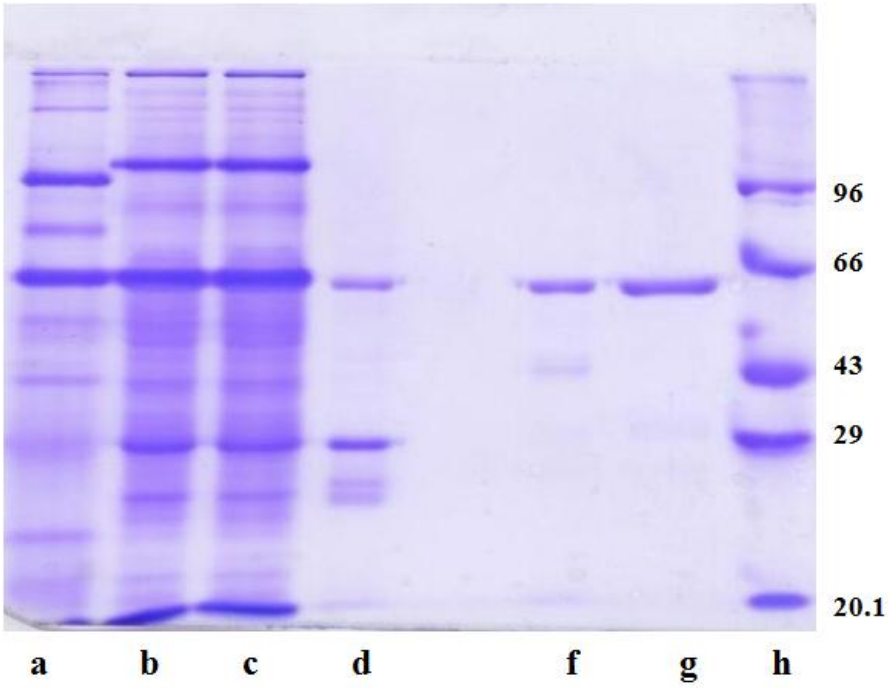


Fig 4.43 SDS-PAGE (10% w/v) analysis of amidase at different purification steps, crude extract **a**, ammonium sulphate precipitation **b**, 10 kD amicon concentration **c**, 350 mM NaCl fractions from Q sepharose column **d**, 375 mM NH_4SO_4 fractions from phenyl sepharose non reducing **f**, HIC reducing **g**, molecular weight markers **h**

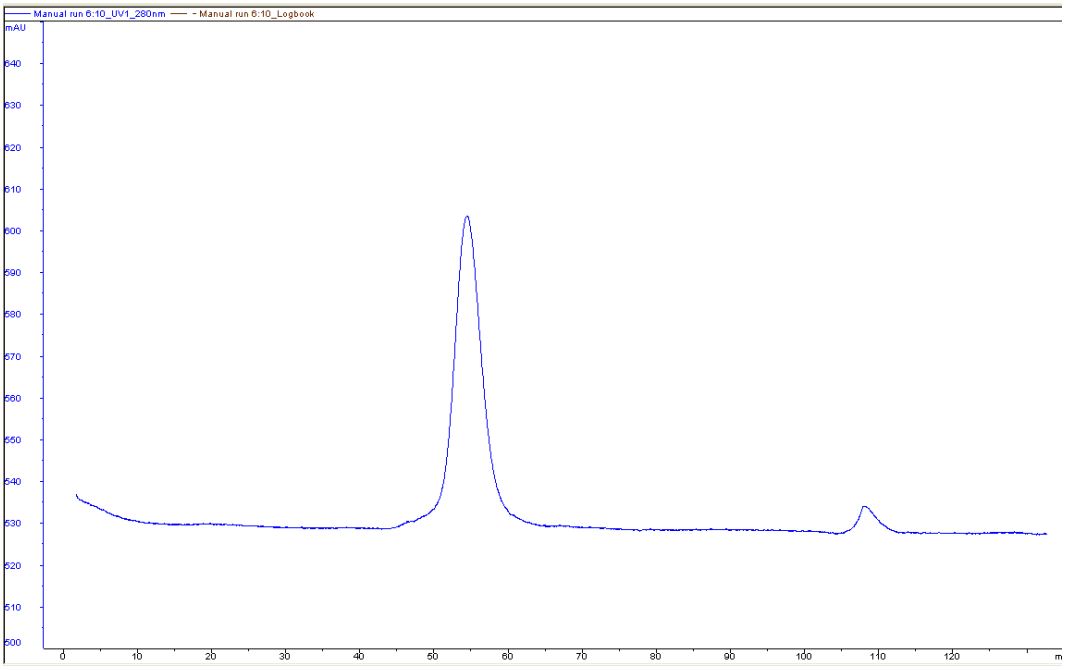
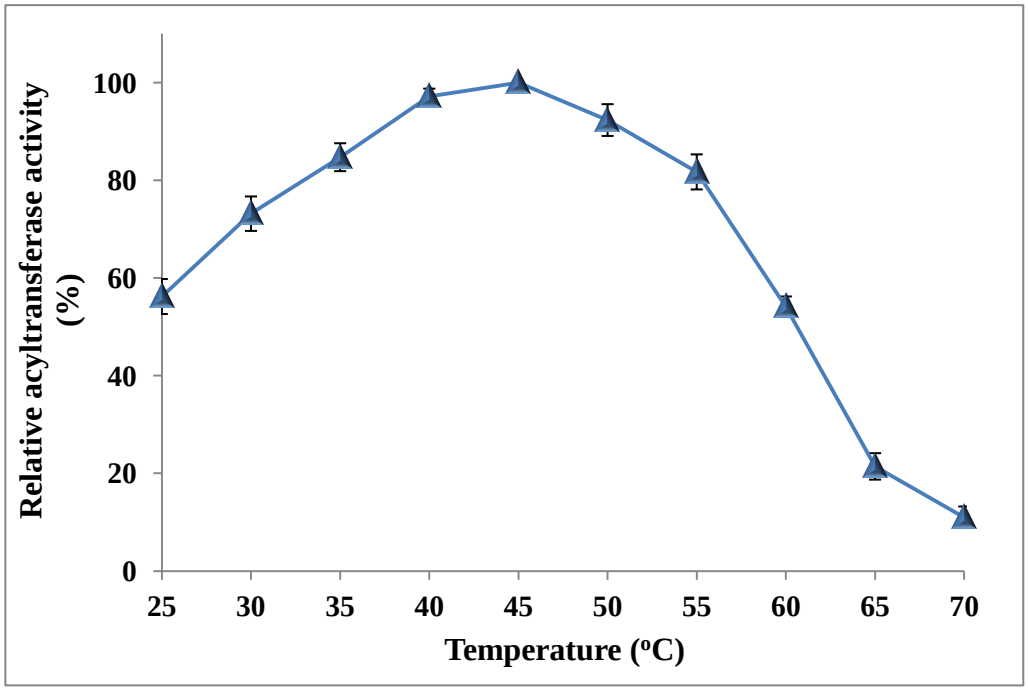


Fig 4.44 Elution profile of amidase active fraction of hydrophobic interaction chromatography column on gel filtration column (HiLoad 16/60 Superdex 75)

4.4.7 Effect of temperature on acyltransferase activity

The effect of reaction temperature on amidase activity is shown in Fig. 4.45. Purified amidase was active in temperature range of 25-60 °C. Increase in enzyme activity was observed with increase in temperature and optimum temperature for amidase activity was 45 °C. Amidase activity declined significantly above 55 °C with 78 and 89% reductions in activity at 65 and 70 °C respectively. The optimum temperature of purified amidase of this study is higher than the optimum value of amidases reported from *Nesterenkonia* Isolate (30 °C) (Nel *et al*., 2011), *Brevibacterium iodinum* (35 °C) (Komeda and Asano, 2008), *Rhodococcus sp*. (40 °C) (Nawaz *et al*., 1994) and *Rhodococcus erythropolis* AJ270 (40 °C) (Xue *et al*., 2011).

However, the results are significantly different from that of the amidase of *Sulfolobus solfataricus* MT4 (D'Abusco *et al*., 2001), *Brevibacillus borstelensis* BCS-1 (Baek *et al*., 2003), *Sulfolobus tokodaii* strain 7 (Suzuki and Ohta, 2006), *Geobacillus subterraneus* RL-2a (Mehta *et al*., 2013) and *Pseudonocardia thermophila* (Egorova *et al*., 2004). The optimal temperatures of amidases from these microorganisms were 95, 90, 75, 70 and 70 °C respectively.

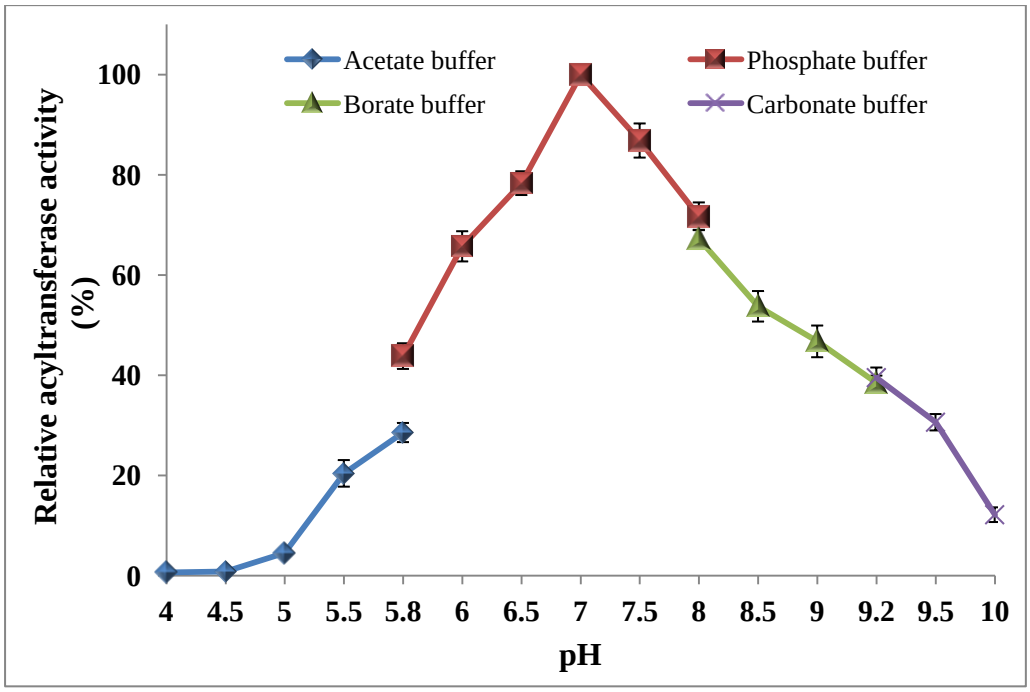


* 100% activity corresponds to 243.75 U/mg

Fig. 4.45 Effect of temperature on acyltransferase activity of purified amidase
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0).

4.4.8 Effect of pH on acyltransferase activity

The effect of pH on amidase activity was determined for pH range of 4-10. Maximum amidase activity was obtained at pH 7 in the potassium phosphate buffer (Fig 4.46). The relative activities at pH 6 and 8 were 65.7% and 71.6% of maximum respectively and decreased significantly above pH 9 and below pH 5.8. The pH optima of amidase of *B. smithii* strain IITR6b2 is similar to the optimum pH value of amidases from *Pseudonocardia thermophila* (Egorova *et al*., 2004), *Brevibacterium iodinum* (Komeda and Asano, 2008), *Geobacillus pallidus* RAPc8 (Makhongela *et al*., 2007), *Klebsiella pneumoniae* NCTR 1 (Nawaz *et al*., 1996) and *R. erythropolis* AJ270 (Xue *et al*., 2011). However few amidases showed their optimum activity either at basic pH (Baek *et al*., 2003, Hermes *et al*., 1993, Komeda and Asano, 2000) or acidic pH (Stelkes-Ritter *et al*., 1995). The optimum pH values for amidase from *Rhodococcus rhodochrous* M8 (Kotlova *et al*., 1999) and *Ochrobactrum anthropi* NCIMB 40321 (Sonke *et al*., 2005) were in a wide range of pH 5-8 and 6-8.5 respectively.



* 100% activity corresponds to 231.24 U/mg

Fig. 4.46 Effect of pH on acyltransferase activity of purified amidase
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, 45 °C.

4.4.9 Substrate specificity

Amidases reported from various sources are found to have different substrate specificities. Some of them catalyze acyl group transfer and amide hydrolysis of small aliphatic amides (Kotlova *et al*., 1999, Nel *et al*., 2011, Makhongela *et al*., 2007), while some have affinity for broad range of amides including aliphatic, aromatic and heterocyclic amides (D'Abusco *et al*., 2001, Fournand *et al*., 1998b, Egorova *et al*., 2004). Few of these amidases have affinity for amides of amino acids (Sonke *et al*., 2005). The substrate specificity of purified amidase of *B. smithii* strain IITR6b2 was determined by checking both acyltransferase and amide hydrolase activities with various amides and ratio of these two activities for all substrates was also determined.

Purified amidase exhibited acyltransferase activity with wide spectrum of amides; however a large difference in activity with different amides was observed under similar experimental conditions (Table 4.14). The highest acyltransferase activity was obtained with nicotinamide followed by benzamide and hexanamide. Very low activity was observed with substituted benzamide. This difference in activity with benzamide and

substituted benzamide (4-Hydroxybenzamide and salicylamide) was probably due to presence of –OH group at para and ortho position of benzene ring causing steric hindrance at enzyme active site. Significant variation in acyltransferase activity towards aliphatic amides was found with highest activity obtained with hexanamide followed by butyramide. Acyltransferase activity of *B. smithii* strain increased with increase in carbon chain length of aliphatic monoamides. Short chain aliphatic amides like acetamide, acrylamide, propionamide and isobutyramide were poor substrates for acyltransferase activity. It was also noted that branching in aliphatic amides also caused significant reduction in activity possibly due to steric hindrance. This purified amidase from *B. Smithii* strain IITR6b2 was shown to catalyze acyl group transfer to co-substrate hydroxylamine from aliphatic, aromatic and heterocyclic amides similar to amidases from *Pseudonocardia thermophila* (Egorova *et al*., 2004), *Sulfolobus solfataricus* (D'Abusco *et al*., 2001)., *Rhodococcus sp.* Strain R312 (Fournand *et al*., 1998b) and *G. subterraneus* RL-2a (Mehta *et al*., 2013). In contrast, the amidase from *Geobacillus pallidus* RAPc8 (Makhongela *et al*., 2007) and *Rhodococcus sp.* R312 (Fournand *et al*., 1997) showed highest acyltransferase activity towards short chain aliphatic amides.

The substrate specificity for amide hydrolase activity is also reported in table 4.14. Like acyltransferase activity, amide hydrolase activity of purified amidase was also highest for heterocyclic and aromatic amides. However in contrast to acyltransferase activity maximum amide hydrolase activity was found with isonicotinamide followed by benzamide and nicotinamide. These results suggest that acyl enzyme complex of nicotinamide had relatively higher affinity for hydroxylamine than water resulting in higher acyltransferase activity for nicotinamide substrate. These results are similar to amidase of *G. Pallidus* RAPc8 that showed maximum acyltransferase activity with isobutyramide, although amide hydrolase activity was not highest with same amide (Makhongela *et al*, 2007). Among aliphatic amides, short chain amides were hydrolysed at low rate and hydrolase activity increased with increase in carbon chain length. Maximum activity was obtained with hexanamide followed by butyramide, propionamide, acrylamide, isobutyramide and acetamide. Amidases with broad range of amide hydrolase activity were isolated from *Pseudonocardia thermophila* (Egorova *et al*., 2004), *Sulfolobus solfataricus* (D'Abusco *et al*., 2001), *Rhodococcus erythropolis* MP50 (Hirrlinger *et al*., 1996) and *G. subterraneus* RL-2a (Mehta *et al*., 2013). In contrast to these results, the amidase from *Geobacillus pallidus* RAPc8 (Makhongela *et al*, 2007), *Geobacillus pallidus*

BTP-5x MTCC 9225 (Sharma *et al*., 2013), *R. rhodochrous* M8 (Kotlova *et al*., 1998) and *Nesterenkonia* Isolate (Nel *et al*., 2011) showed higher amide hydrolase activity with aliphatic amides such as acetamide and propionamide. On the other hand the presence of L-selective stereospecific amidase and d-stereoselective amidase has been reported in *Ochrobactrum anthropi* NCIMB 40321 (Sonke *et al*., 2005) and *Brevibacterium iodinum* (Komeda and Asano, 2008) with specificity towards only amino acid amides.

Table 4.14 Substrate specificity profile of purified amidase

Substrate (10 mM)	Relative acyltransferase activity (%)	Relative amide hydrolase activity (%)	Ratio (acyltransferase/ amide hydrolase activity)
Acetamide	4.62±1.4	10.4±1.6	3.2
Propionamide	11.29±2.1	25.91±2.3	3.0
Butyramide	43.71±2.8	55.94±1.0	5.5
Isobutyramide	10.28±2.7	10.76±1.9	6.9
Hexanamide	81.13±3.1	82.72±2.6	6.0
Acrylamide	3.56±1.3	11.2±1.3	2.2
Adipamide	4.76±1.7	4.37±0.34	7.8
Nicotinamide	100	90.31±2.2	7.8
Isonicotinamide	39.89±3.3	100	2.8
Benzamide	86.91±2.6	94.53±2.1	6.5
4-Hydroxybenzamide	1.13±0.12	6.46±0.73	1.2
Salicylamide	0	0	0
DL-lactamide	0	0	0

* 100% acyltransferase activity corresponds to 132.85 U/mg
* 100% amide hydrolase activity corresponds to 18.78 U/mg
Enzyme assay conditions: phosphate buffer (50 mM, pH 7.0), 45 °C, for acyltransferase activity assay- 10 mM amide, 500 mM hydroxylamine-HCl, for amide hydrolase activity assay- 10mM amide

4.4.10 Effects of chemical reagents and inhibitors

The effects of different chemical reagents and inhibitors such as tetra-sodium ethylenediamine tetra acetate (EDTA), 1, 10-phenanthroline, dithiobis-nitrobenzoic acid

(DTNB), N-ethylmaleimide (NEM), dithiothreitol (DTT), ammonium persulphate (APS), H_2O_2 and diethyl phosphoramidate (DEPA) on acyltransferase activity of purified amidase were determined (Table 4.15).

Metal chelating reagents EDTA and 1, 10-phenanthroline had no considerable effects on activity indicating that the purified amidase was not a metal dependent enzyme. The enzyme activity was almost stable even at higher concentration of EDTA (10 mM). This was in agreement with previous reports on amidases from *Geobacillus pallidus* RAPc8 (Makhongela *et al*., 2007), *Pseudonocardia thermophila* (Egorova *et al*., 2004), *Brevibacterium iodinum* TPU 5850 (Komeda and Asano, 2008), *Geobacillus pallidus* BTP-5x MTCC 9225 (Sharma *et al*., 2013) which showed no inhibition of activity in presence of metal chelating agents. In contrast to these data, amidase from *Klebsiella pneumoniae* NCTR 1 (Nawaz *et al*., 1996), *Rhodococcus sp*. (Nawaz *et al*., 1994) and *Brevundimonas diminuta* TPU 5720 (Komeda *et al*., 2006) were reported to be metal associated enzymes containing Fe, Co or both metals.

The acyltransferase activity was completely inhibited with mercury and silver metal ions (Fig. 4.47). The inhibition of amidase by these heavy metal ions (thiol reactive metals) indicated the importance of thiol (-SH) containing amino acid residues in the enzyme function and it was further confirmed by the inhibitory effects of other –SH modifying reagents (DTNB and NEM) on activity. Complete inhibition of enzyme activity was observed by DTNB (99% at 1 mM) and NEM (98% at 1 mM). Inhibitory effects of –SH modifying reagents were also reported with amidases of *Geobacillus pallidus* BTP-5x MTCC 9225 (Sharma *et al*., 2013), *Klebsiella pneumoniae* NCTR 1 (Nawaz *et al*., 1996), *Rhodococcus sp*. (Nawaz *et al*., 1994) and *Rhodococcus erythropolis* MP50 (Hirrlinger *et al*., 1996). The presence of DTT (reducing agent) caused a 1.6 fold (at 1 mM) increase in activity and at higher concentration of DTT (10 mM) this increase was 2.2 fold. Similar 1.5 fold increase in activity was observed for amidase of *Geobacillus pallidus* (Makhongela *et al*., 2007) and *Rhodococcus rhodochrous* M8 (Kotlova *et al*., 1999). In contrast to activation by S-S reducing reagents, inhibition of acyltransferase activity to significant level was observed by oxidizing agents (APS and H_2O_2). These data indicate that sulfhydryl group is involved in the mechanism of action of the purified amidase of *B. smithii* strain IITR6b2. In previous studies, the occurrence of sulfhydryl group at amidase active site was reported in amidases from *Geobacillus pallidus* BTP-5x MTCC 9225 (Sharma *et al*., 2013), *Klebsiella pneumoniae* NCTR 1 (Nawaz *et al*., 1996), *Arthrobacter*

sp. J-l (Asano *et al*., 1982), *Pseudonocardia thermophila* (Egorova *et al*., 2004) and *Geobacillus pallidus* RAPc8 (Makhongela *et al*., 2004). The effect of a known amidase inhibitor on purified amidase activity was also studied. Thus 59% and 63% inhibitions were observed at 5 and 10 mM concentrations respectively.

4.4.11 Effects of metal ions

The effects of metals ions on acyltransferase activity were determined at three different concentrations (1, 5, 10 mM) of various metal salts shown in Fig. 4.47. The activity in the absence of any metal was taken as 100%. Strong inhibition of amidase activity occurred with heavy metal ions (characteristic thiol reactive reagents) such as Hg^{++} (97% inhibition at 1mM), Ag^{++} (95% inhibition at 1 mM) and Cu^{++} (97% inhibition at 1 mM) while Zn^{++} inhibited activity up to 92% at 10 mM concentration. Heavy metal ions also inhibited the enzyme activity of *Pseudonocardia thermophila* (Egorava *et al*., 2004), *Geobacillus subterraneus* RL-2a (Mehta *et al*., 2013), *Geobacillus pallidus* BTP-5x MTCC 9225 (Sharma *et al*., 2013), *Rhodococcus erythropolis* MP50 (Hirrlinger *et al*., 1996) and *Rhodococcus sp*. (Nawaz *et al*., 1994). As depicted in Fig. 4.47, the metal ions such as Mn^{++}, Mg^{++}, Ca^{++} and Co^{++} were slight activators of enzyme activity, increasing the activity by about 7-30%. In addition, these results also showed that Ba^{++} and Na^{+} had very little effect on the acyltransferase activity even at higher concentration (10 mM).

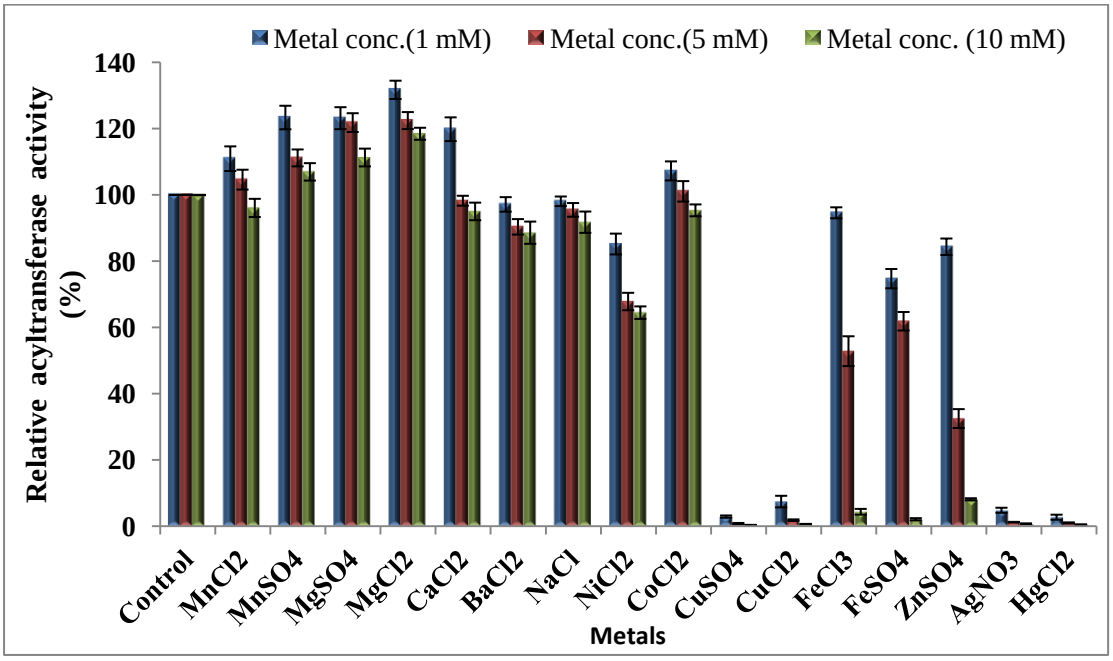


Fig 4.47 Effect of metal ions on acyltransferase activity
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7), 45 °C.

Table 4.15 Effect of chemical reagents and inhibitors on acyltransferase activity

Chemical reagents/ inhibitors		Relative enzyme activity (%)
Control		100
Metal cheaters		
EDTA	1 mM	104.35±4.4
	5 mM	102.35±3.1
	10 mM	100.71±2.8
1,10-Phenanthroline	1 mM	103.66±3.0
	5 mM	98.20±4.5
	10 mM	97.84±3.6
Thiol modifying reagents		
Dithiobis-nitrobenzoic acid (DTNB)	0.1 mM	38.68±4.8
	0.5 mM	21.53±3.8
	1 mM	1.04±0.14
N-ethylmaleimide (NEM)	0.1 mM	34.48±3.1
	0.5 mM	17.25±3.6
	1 mM	2.12±1.1
Reducing agent		
DTT	1 mM	165.84±3.3
	5 mM	198.13±4.1
	10 mM	223.86±4.5
Oxidizing agent		
Ammonium persulphate	1 mM	81.74±3.3
	5 mM	26.5±4.0
	10 mM	ND
H_2O_2	0.1 %	12.02±3.1
	0.2 %	5.4±1.6
	0.3 %	2.57±0.73
Amidase inhibitor		
Di-ethyl phosphoramidate	1 mM	73.13±3.2
	5 mM	41.84±4.3
	10 mM	37.26±3.6

Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7), 45 °C.

4.4.12 Organic solvent compatibility

Acyltransferase activity of purified amidase was studied in various organic solvents at a concentration of 10, 20 and 30% (v/v) by incubating at 30 °C for 30 min (Fig 4.48). In the presence of solvents (10%), highest acyltransferase activity was obtained with DMSO (110%). While purified amidase maintained more than its 90% activity in following solvents: methanol, ethanol, isopropanol, acetonitrile, 1-Decanol, DMF, Xylene, hexane, heptane and n-hexadecane at 10% concentration. Amidase was able to maintain its 76, 56 and 53% activity in methanol, ethanol and isopropanol at 20% (v/v) concentration. Even in presence of 30% (v/v) of solvent significant residual amidase activity was obtained in following solvents: methanol (65%), DMSO (88%), xylene (70%), hexane (73%), heptanes (71%), n-octane (81%), isooctane (79%) and n-hexadecane (91%). In contrast to these results, purified amidase activity of *G. Pallidus* was decreased to 50, 58, 60 and 48 % by addition of only 5% of following solvents: methanol, ethanol, isopropanol and n-butanol respectively while DMF and DMSO completely inhibited the enzyme (Makhongela *et al*., 2007).

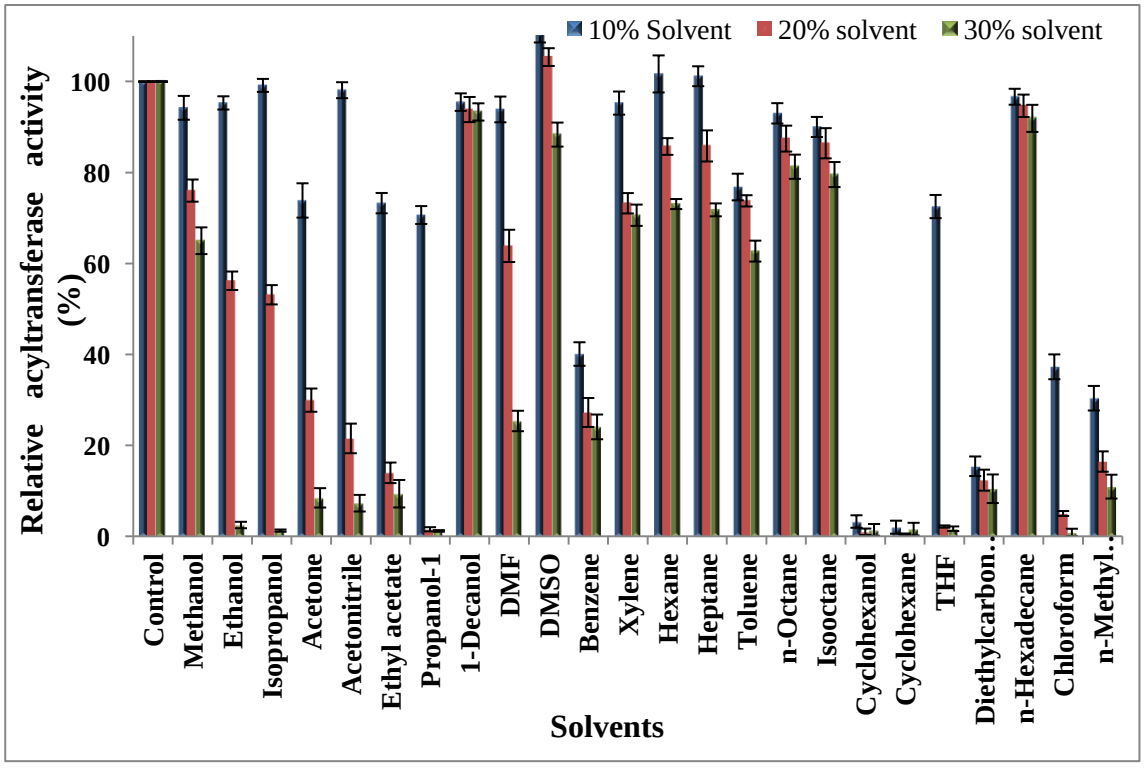


Fig 4.48 Organic solvents compatibility for acyltransferase activity
Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7), 45 °C.

4.4.13 Effects of surfactants and polyethylene glycols

Different types of surfactants: anionic surfactants (SDS, sodium cholate and sodium deoxycholate), cationic surfactants (CTAB) and non-ionic surfactants (Tween 20, Tween 60, Tween 80 and Triton X-100) were selected to study their effects on amidase activity.

The results in table 4.16 show that all detergents had significant effects on amidase activity. Possibly these surfactants caused changes in the conformation of amidase protein resulting in activation, stabilization or deactivation. Non-ionic Surfactants such as Tween 20 (2.8 fold), Tween 60 (3 fold), Tween 80 (3.6 fold) and Triton X-100 (1.5 fold) increased the enzyme activity when present at 0.05% concentration. This activation of enzyme activity may be due to the conformational changes in amidase protein thus modifying the accessibility of substrate to the active site. Further, amidase activity was also enhanced by 1.9 and 2 folds in the presence of sodium cholate and deoxycholate (anionic surfactants) respectively while another anionic surfactant SDS along with CTAB at 0.05 % concentration inhibited amidase activity completely. Effects of polyethylene glycols (synthetic polymers) on amidase activity were also studied. With PEG 4000 and PEG 6000, 2.9 and 3 fold enhancements in amidase activity were observed. Activation of enzyme activity by surfactants and polyethylene glycols has been reported in various literatures (Liu *et al*., 2000, Jung *et al*., 2003, Yoon and Robyt, 2005, Guncheva *et al*., 2007).

Table 4.16 Effects of surfactants and PEG on acyltransferase activity

Surfactants/ PEG	Relative enzyme activity (%)		
	0.05% (v/v)	**0.1% (v/v)**	**0.2% (v/v)**
Control	100	100	100
Anionic surfactants			
SDS	ND	ND	ND
Sodium cholate	196.31±3.4	147.33±3.5	2.73±1.3
Sodium deoxycholate	209.84±3.1	198.20±3.0	191.91±4.4
Cationic surfactant			
CTAB	ND	ND	ND
Non-ionic surfactants			
Tween 20	285.86±4.2	257.58±5.5	213.01±3.2
Tween 60	302.25±5.0	268.13±3.2	227.25±4.9
Tween 80	365.06±3.9	335.01±4.1	317.74±4.1
Triton X100	157.27±3.2	115.26±3.0	16.89±2.6
PEG 4000	294.31±2.5	275.38±4.6	247.36±3.1
PEG 6000	302.69±3.7	286.94±4.3	259.89±5.6

Enzyme assay conditions: 100 mM nicotinamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7), 45 °C.

4.4.14 Presence of second amidase in *B. smithii* strain IITR6b2

The results obtained from substrate specificity data of whole cell amidase (Section 4.1.5.4) and purified amidase (Section 4.4.9) were contradictory. The whole cell amidase showed highest acyltransferase activity with nicotinamide followed by acetamide, benzamide and hexanamide respectively. On the other hand, purified amidase showed maximum acyltransferase activity for nicotinamide followed by benzamide and hexanamide; however this time activity for acetamide substrate was only 4.6% of the maximum activity (with nicotinamide). These results suggested the presence of more than one amidase in *B. smithii* strain IITR6b2. The presence of more than one amidase was confirmed by zymogram analysis of cell free extract of bacterial isolate. Nicotinamide and acetamide along with co-substrate hydroxylamine-HCl were used to develop red brown band on native-PAGE gels to locate the amidase band position (Fig 4.49). With nicotinamide substrate, only one band on native-PAGE gel was obtained while with

acetamide substrate, two bands were observed at totally different positions. One band out of two bands obtained with acetamide was at the same position as obtained with nicotinamide. These results confirmed the presence of two amidases in *B. smithii* strain IITR6b2 strain. One amidase (I) purified in section 4.4.3 had affinity for both nicotinamide and acetamide; however affinity for acetamide was very less as compared to nicotinamide. The second amidase (II) had highest affinity for acetamide while it was not active for nicotinamide. Further confirmation needed to be carried out by separating the second amidase from cell free extract and study its substrate specificity profile.

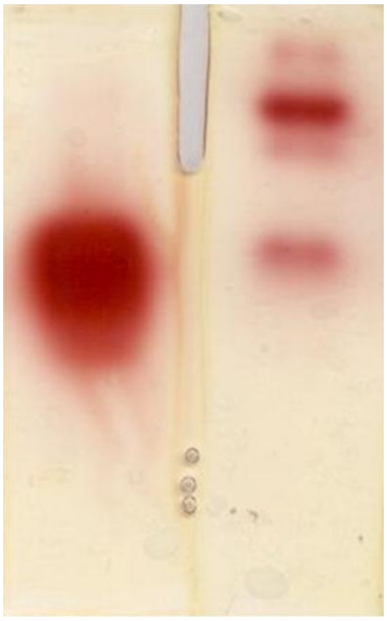


Fig 4.49 Zymogram of cell free extract for confirmation of presence of two amidases in *B. smithii* strain IITR6b2 with substrates, **a)** nicotinamide **b)** acetamide and hydroxylamine-HCl

4.4.15 Partial purification of amidase II from cell free extract

The crude extract containing high acyltransferase activity for acetamide and nicotinamide was prepared as discussed previously (Section 4.4.3) and loaded on Q sepharose column. In this anion exchange chromatography step, elution of bound proteins was done by step wise gradient of 1 M NaCl (20-50%) and the elution profile of the proteins obtained from the Q sepharose column are presented in Fig. 4.50. Total eight peaks were obtained. Fractions corresponding to each peak were clubbed and used for

amidase activity determination using acetamide as substrate (Table 4.17). Amidase activity was detected in fourth and fifth peak corresponding to proteins eluted at 30% and 35% of 1 M NaCl. Acyltransferase activity for acetamide substrate in fifth peak was due to presence of amidase I (purified in Section 4.4.3) while activity in fourth peak was due to amidase II.

For further purification, the pooled active fractions (only of 30% NaCl) of this step were concentrated, desalted and loaded onto a phenyl sepharose column. The bound proteins were eluted by step wise gradient of 1 M NH_4SO_4 (75-0%) and the elution profile of proteins is shown in Fig 4.51. Fractions corresponding to each peak were clubbed and used for amidase activity assay (Table 4.18). Amidase activity was detected in third peak corresponding to proteins eluted at 0.5 M NH_4SO_4. The elution profile of amidase II on phenyl sepharose column was different to the amidase I which was eluted at 0.375 M of NH_4SO_4.

The molecular mass of purified amidase II was determined by 10% SDS-PAGE under both reducing and non-reducing conditions (Fig. 4.52). SDS-PAGE gel analysis revealed that amidase II was partially purified as some contaminating proteins were present in lane b and c. Major protein band under non reducing condition (lane b) was not present under reducing condition (lane c) and a new band was observed. These results suggest multimeric nature of amidase II. However amidase II was not completely purified, hence it can’t be possible to predict about its molecular weight and subunit composition.

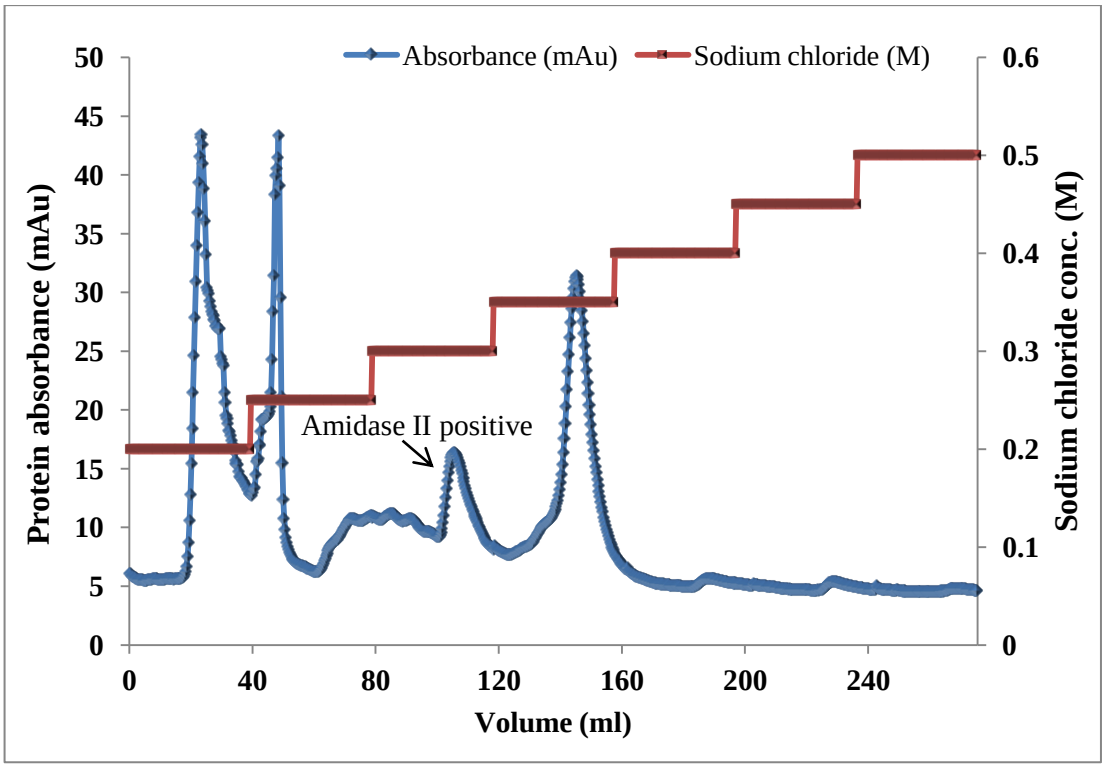


Fig 4.50 Elution profile of proteins on ion exchange chromatography column (Q **sepharose)**

Table 4.17 Acyltransferase activity of clubbed fractions of ion exchange chromatography

Peak no.	NaCl concentration (mM)	Enzyme activity
1	200	ND
2	200-250	ND
3	250	ND
4	300	Amidase II*
5	350	Amidase I*
6	400	ND
7	450	ND
8	500	ND

*Amidase acyltransferase activity with acetamide substrate
ND- No amidase acyltransferase activity detected with acetamide

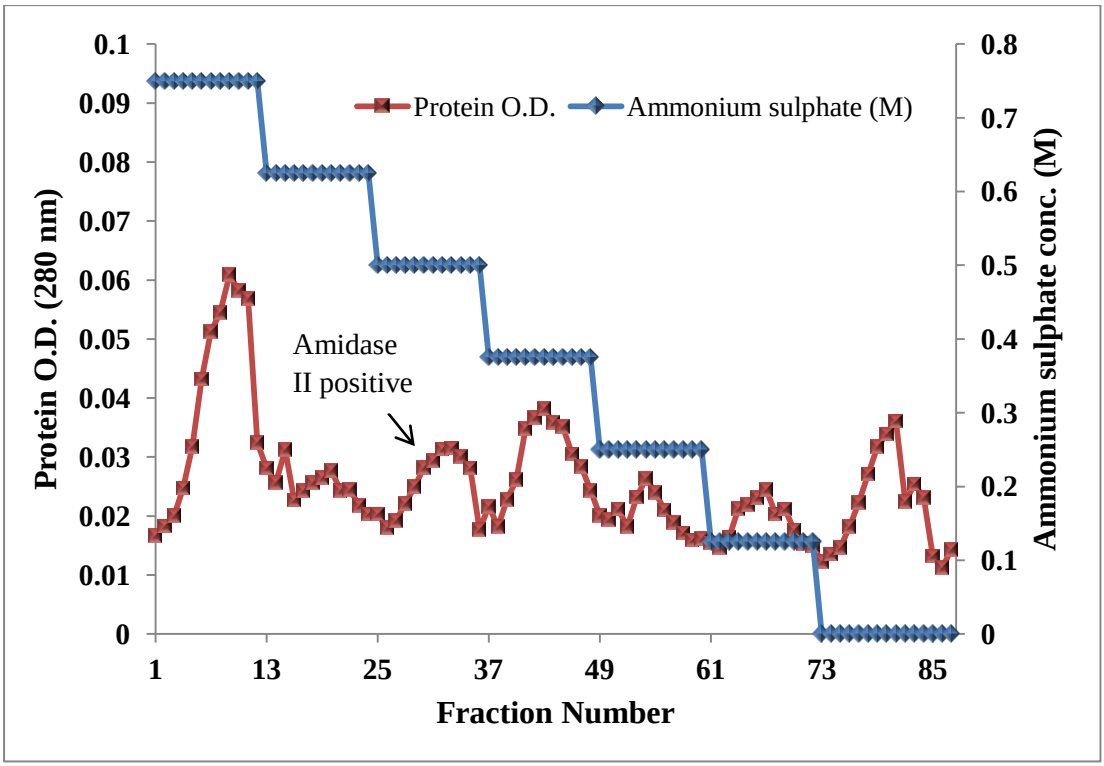


Fig.4.51 Elution profile of proteins on hydrophobic interaction chromatography column (**phenyl sepharose**)

Table 4.18 Acyltransferase activity of clubbed fractions of hydrophobic interaction chromatography

Peak no.	NH_4SO_4 concentration (mM)	Enzyme activity
1	750	ND
2	625	ND
3	500	Amidase II*
4	375	ND
5	250	ND
6	125	ND
7	0	ND

*Amidase acyltransferase activity with acetamide substrate

ND- No amidase acyltransferase activity detected with acetamide

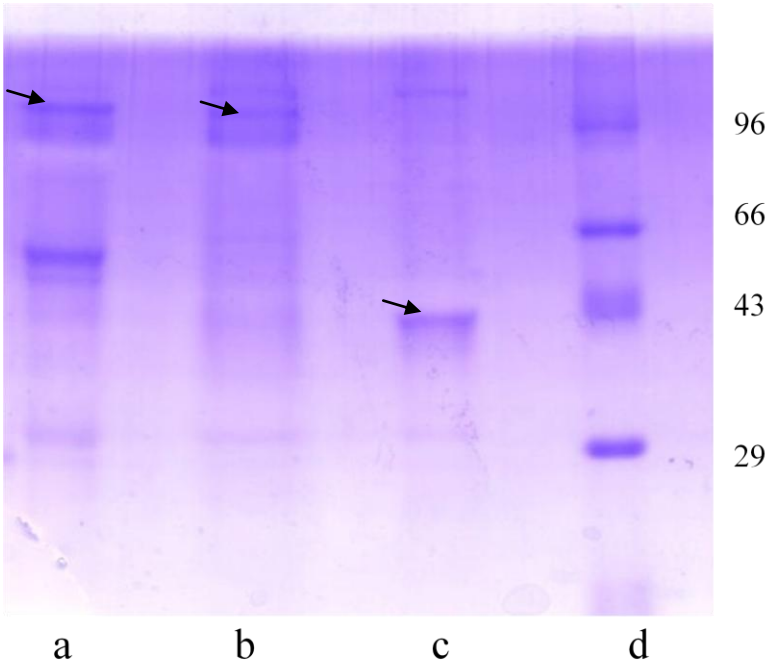


Fig. 4.52 SDS-PAGE (10% w/v) analysis of second amidase, 300 mM NaCl fractions from Q sepharose column **a**, 500 mM NH_4SO_4 fractions from phenyl sepharose non reducing **b**, HIC reducing **c**, molecular weight markers **d**.

4.4.16 Substrate specificity

Partial purified amidase II exhibited acyltransferase and amide hydrolase activities for only small chain aliphatic amides like acetamide, propionamide and acrylamide (Table 4.19). Maximum acyltransferase and amide hydrolase activities were observed with acetamide followed by propionamide, acrylamide and butyramide. These results indicated that with increase in carbon chain length of aliphatic amides amidase activity decreased probably due to steric hindrance created by carbon chain. These results justified the presence of two amidases in *B. smithii* strain IITR6b2. One amidase showed high affinity for heterocyclic amides, aromatic amides, long chain aliphatic amides and considerably low activity towards short chain aliphatic amides (Section 4.4.9). On the other hand amidase II showed affinity only for short chain aliphatic amides. The whole cell amidase substrate specificity profile (Section 4.1.5.4) was the result of combine action of both the amidases.

Table 4.19 Substrate specificity profile of partial purified amidase II

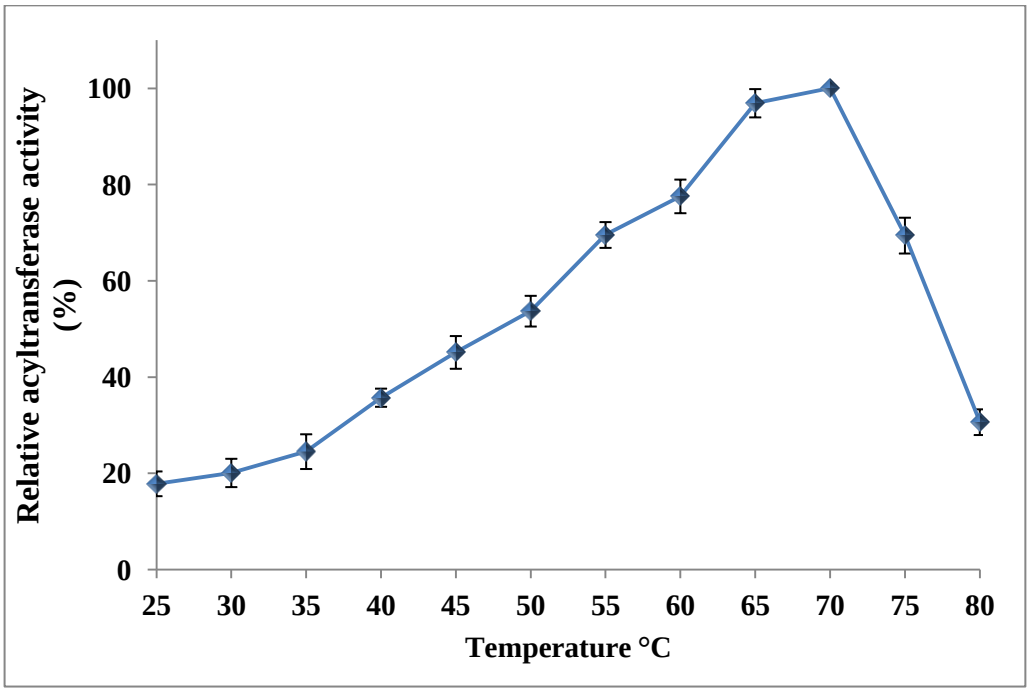
Substrate (10 mM)	Relative acyltransferase activity (%)	Relative amide hydrolase activity (%)	Ratio (acyltransferase/ amide hydrolase activity)
Acetamide	100	100	1.43
Propionamide	28.61±2.9	64.35±4.3	0.62
Butyramide	3.47±1.1	8.62±2.0	0.55
Isobutyramide	0	0	0
Hexanamide	0	0	0
Acrylamide	17.83±3.4	68.27±4.8	0.36
Adipamide	0	0	0
Nicotinamide	0	0	0
Isonicotinamide	0	0	0
Benzamide	0	0	0
4-Hydroxybenzamide	0	0	0
Salicylamide	0	0	0
DL-lactamide	5.72±1.3	7.24±1.9	1.13

* 100% acyltransferase activity corresponds to 69.72 U/mg
* 100% amide hydrolase activity corresponds to 48.65 U/mg
Enzyme assay conditions: phosphate buffer (50 mM, pH 7.0), 45 °C, for acyltransferase activity assay- 10 mM amide, 500 mM hydroxylamine-HCl, for amide hydrolase activity assay- 10mM amide

4.4.17 Effect of temperature on acyltransferase activity

To differentiate amidase I and amidase II, the optimum temperature and pH of amidase II were also studied. The effect of reaction temperature on amidase II activity is shown in Fig. 4.53. Partial purified amidase was active in a broad temperature range of 25-80 °C. Increase in enzyme activity was observed with increase in temperature and optimum temperature for amidase activity was 70 °C. Amidase activity declined notably above 70 °C with 30 and 70% reductions in activity at 75 and 80 °C respectively. The optimum temperature of partial purified amidase II (70 °C) was significantly different from the purified amidase I (45 °C, Section 4.4.7). For amidase I, drastic decline in amidase activity was observed above 55 °C, on the other hand partial purified amidase II was highly

thermoactive. In previous reports, amidase from *Geobacillus subterraneus* RL-2a (Mehta *et al*., 2013) and *Pseudonocardia thermophila* (Egorova *et al*., 2004) showed optimum activity at 70 °C.

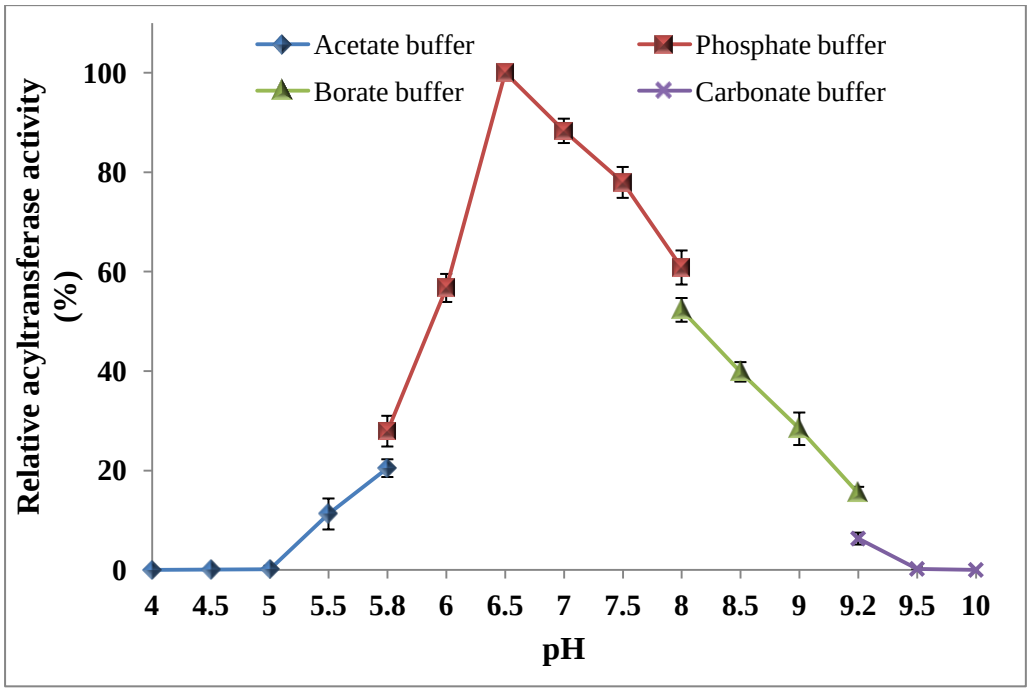


* 100% activity corresponds to 157.21 U/mg

Fig 4.53 Effect of temperature on acyltransferase activity of purified amidase II
Enzyme assay conditions: 100 mM acetamide, 500 mM hydroxylamine-HCl, phosphate buffer (50 mM, pH 7.0).

4.4.18 Effect of pH on acyltransferase activity

The Effect of pH on amidase activity was determined for pH range of 4-10. Maximum amidase activity was obtained at pH 6.5 in the potassium phosphate buffer (Fig. 4.54). The relative activity at pH 6 and 7 were 56.7 and 88.3% respectively and decreased significantly above pH 8 and below pH 6.0. It was observed that partial purified amidase II was active at a narrow pH range as compare to amidase I (Section 4.4.8); however optimum pH values were nearly similar for both amidases.



* 100% activity corresponds to 145.78 U/mg

Fig 4.54 Effect of pH on acyltransferase activity of purified amidase II

Enzyme assay conditions: 100 mM acetamide, 500 mM hydroxylamine-HCl, 70 °C.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Summary

In the present study, suitable bacterial isolate having amidase enzyme with acyltransferase activity for heterocyclic amides was selected for bioprocesses development. Production medium optimization for selected isolate was carried out with respect to acyltransferase activity and further acyltransferase activity of whole cell amidase was characterized. Various reaction parameters such as temperature, pH, substrate/co-substrate concentration, resting cells concentration and time course of reaction were optimized and efficient bioprocesses for NAH and isoniazid syntheses were developed using the acyltransferase activity of whole cell amidase. Further, amidase of *B. smithii* strain IITR6b2 was purified and characterized. The results of present investigations are summarized briefly as follows.

Screening, selection, characterization of bacterial isolate producing amidase enzyme with acyltransferase activity and whole cell enzyme characterization

- Various available nitrile metabolizing bacterial isolates previously isolated from soil and water samples were screened for amidase enzyme with acyltransferase activity. Among these isolates, 6b2 was selected for further studies as it possess acyltransferase activity for a broad range of amides, higher acyltransferase to amide hydrolase activity ratio for heterocyclic amides and the highest mole ratio of product (NAH) to by-product (nicotinic acid).
- Based on biochemical characteristics and 16S rDNA sequence analysis, the strain was identified as *Bacillus smithii* and referred as *Bacillus smithii* strain IITR6b2. The sequence was deposited in the GenBank data base with an accession no. JX157878.
- Production medium optimization results revealed that temperature of 45 °C, initial medium pH 7.0, 10 g/l glycerol and 10 mM of phenylacetonitrile would result in maximum amidase production. In presence of ammonium chloride as sole nitrogen source, highest biomass was achieved while acyltransferase activity was very low confirming the inducible

nature of amidase. Maximum amidase production was obtained at mid exponential phase (culture time of 48 h).

- Whole cell amidase showed maximum acyltransferase activity at 55 °C with marginally lower activities at 45 and 50 °C. Reaction pH considerably affected the acyltransferase activity with optimum activity obtained at pH 7.0.
- Acyltransferase activity of amidase was relatively stable at 30 °C with half life of 29 h and at 45 °C and 55 °C half lives were 14 and 10 h respectively.
- Amidase showed activity with broad range of amides like aliphatic, aromatic and heterocyclic amides. The highest acyltransferase activity was obtained with nicotinamide followed by acetamide, benzamide and hexanamide. However amide hydrolase activity was highest with aliphatic amides like acetamide, acrylamide and propionamide.
- Amidase was compatible in presence of both water miscible and water immiscible solvents at 15% (v/v) concentration. Highest acyltransferase activity was obtained with hexane (105%) followed by DMSO (103%) and heptane (98%). Amidase maintained more than 80% activity in water miscible solvents such as methanol (95%), ethanol (91%) and isopropanol (84%). Loss in enzyme activity was observed in presence of 30% (v/v) of methanol, ethanol and isopropanol and 75%, 52% and 47% activities were observed respectively.

Bioprocess development for nicotinic acid hydroxamate synthesis

- Whole cell biocatalyst of *B. smithii* strain IITR6b2 was found to possess amide acyltransferase, amide hydrolase (nicotinamide substrate) and hydroxamic acid hydrolase activities. Amide acyltransferase activity was 28 and 12.3 folds higher as compared to amide hydrolase and hydroxamic acid hydrolase activities respectively.
- Thermal stability studies at 45 °C and the inhibition profile (in presence of amidase inhibitor DEPA) of these activities suggested that these three activities belong to same protein.
- Maximum acyltransferase activity was obtained at 100 mM of nicotinamide (hydroxylamine-HCl, 500 mM) while for hydroxylamine-HCl, the optimum value was 250 mM (nicotinamide, 100 mM).
- To find the true optimum mole ratio of substrates (nicotinamide and hydroxylamine-HCl); reactions were performed with several possible ratios of amide and hydroxylamine-HCl. The highest acyltransferase activity was obtained with 250 mM hydroxylamine-HCl and 200 mM nicotinamide.

- At 45 and 55 °C, highest molar conversions of nicotinamide to NAH achieved were 84.8 and 81.6% respectively in 30 min whereas maximum 90.4% molar conversion was obtained at 30 °C in 40 min along with only 1.4% conversion to by-product.
- Molar conversion of nicotinamide to NAH increased with increase in resting cells concentration from 0.3-0.7 mg_{dcw}/ml. However at 0.9 mg_{dcw}/ml concentration, maximum conversion was 96.3% which is marginally higher than the 94.5% conversion observed at 0.7 mg_{dcw}/ml in 40 min.
- A fed batch process based on the optimized parameters with two feeding of substrates (200/200 mM) at 40 min interval was developed. This bioprocess resulted in 89.4% molar conversion of 600 mM nicotinamide to NAH in 120 min at a production rate of 52.9 g/h/m_{gdcw} with 4.5% undesirable by-product, nicotinic acid.
- Finally, 6.4 g of powder containing 58.5% (w/w) NAH was recovered after lyophilization and further purification resulted in 95% pure product.

Bioprocess development for isoniazid synthesis

- Whole cell biocatalyst of *B. smithii* strain IITR6b2 was found to possess amide acyltransferase, amide hydrolase (isonicotinamide substrate) and hydrazide hydrolase activities. Amide acyltransferase activity was 3 and 17.8 folds higher as compared to amide hydrolase and hydrazide hydrolase activities respectively.
- Maximum acyltransferase activity for isoniazid synthesis was obtained at 55 °C and above 55 °C, a sharp decrease in activity was observed. Whole cell enzyme was active at narrow range of pH and activity increased gradually with increasing pH with maximum value at pH 7.0.
- Maximum acyltransferase activity was obtained at 100 mM of isonicotinamide (hydrazine-2HCl, 500 mM) while for hydrazine-2HCl, the optimum value was 1000 mM (at constant isonicotinamide, 100 mM).
- At optimum concentrations of isonicotinamide (100 mM) and hydrazine-2HCl (1000 mM), acyltransferase to hydrolase activity ratio further improved from 3 to 4.5 and corresponding reduction in by-product formation was 54%.
- To select the optimum mole ratio of substrates (isonicotinamide and hydrazine-2HCl); reactions were performed with several possible ratios of amide and hydrazine-2HCl. Highest acyltransferase activity was obtained with 100 mM of isonicotinamide and 1000 mM of hydrazine-2HCl.

- Highest 89.6% molar conversion of isonicotinamide to isoniazid was achieved at 30 °C in 90 min with less by-product (7.6%) formation. By-product formation rates were 1.8 and 1.6 folds higher at 55 and 45 °C as compared to 30 °C respectively.
- Molar conversion of isonicotinamide to isoniazid increased with increase in resting cell concentration and attained a conversion of 90.4% at 2.0 mg_{dcw}/ml while the overall conversion time reduced to 60 min from 90 min.
- Based on optimized parameters, a fed batch process for production of isoniazid was developed. A total of 439 mM of isoniazid was accumulated with 87.8% molar conversion of isonicotinamide (500 mM) with only 7.8% by-product in 5 h at a production rate of 6.0 $g/h/g_{dcw}$.
- Finally, 9.1 g of powder containing 33.3% (w/w) isoniazid was recovered after lyophilization and further purification resulted in 94% pure product.

Purification of amidase enzyme and its characterization

- Among cell lysis methods, maximum 88.7% amidase activity was recovered in supernatant when cells were treated with lysozyme concentration of 1mg/ml. Cell lysis with sonication resulted in about 60% recovery of amidase activity in supernatant after 8 min of sonication.
- Ammonium sulphate was proved to be effective for precipitation of proteins fraction having amidase enzyme rather than sodium sulphate. Nearly 60% proteins were precipitated and 73.1% amidase activity was recovered by ammonium sulphate fractionation at a of 30-60% saturation.
- Amidase was purified by four consecutive purification steps and 12.11 fold purification with a specific activity of 292.2 U/mg of protein and amidase yield of 37.5% was obtained.
- The results of SDS-PAGE along with gel filtration chromatography confirmed that purified amidase from *B. smithii* strain IITR6b2 was a monomer with molecular mass (M_r) of approximately 63 kDa.
- The purified amidase was active in the temperature range of 25-60 °C and optimum temperature for the amidase activity was 45 °C. Maximum acyltransferase activity was obtained at pH 7.0 in the potassium phosphate buffer.
- The purified amidase exhibited acyltransferase activity for a wide spectrum of amides; however large variations in amidase activities for different amides were observed. The highest acyltransferase activity was obtained with nicotinamide while amide hydrolase

activity showed maximum activity towards isonicotinamide. Short chain aliphatic amides were poor substrates for both activities of amidase.

- Strong inhibition of amidase activity occurred with heavy metal ions such as Hg^{++} (97% inhibition at 1mM), Ag^{++} (95% inhibition at 1 mM) and Cu^{++} (97% inhibition at 1 mM). Metal ions such as Mn^{++}, Mg^{++}, Ca^{++} and Co^{++} were activators of enzyme activity, increasing the activity by about 7 to 30%.
- Metal chelating reagents EDTA and 1, 10-phenanthroline had no significant effects on the amidase activity. The inhibition of amidase by heavy metal ions and –SH modifying reagents (DTNB and NEM) indicated the importance of thiol (-SH) containing amino acid residues at active site of the amidase. The presence of DTT (reducing agent) caused a 2.2 fold (at 10 mM) increase in the acyltransferase activity. Oxidizing agents (APS and H_2O_2) were found to have inhibitory effect on amidase activity.
- Purified amidase maintained its 76, 56 and 53 % (v/v) activity in methanol, ethanol and isopropanol at 20% concentration. Even in presence of 30% (v/v) of solvent significant amidase activity was retained with following solvents: methanol (65%), DMSO (88%), xylene (70%), hexane (73%), heptanes (71%), n-octane (81%), isooctane (79%) and n-hexadecane (91%).
- Non-ionic Surfactants such as Tween 20 (2.8 fold), Tween 60 (3 fold), Tween 80 (3.6 fold) and Triton X-100 (1.5 fold) increased the enzyme activity when present at 0.05% concentration. SDS and CTAB inhibited amidase activity completely (at 0.05%).
- Presence of another amidase (II) in *B. smithii* strain IITR6b2 was confirmed by zymogram analysis of native gel.
- This amidase II showed maximum acyltransferase and amide hydrolase activity for only short chain aliphatic amides**.**
- The optimum temperature and pH for the amidase II activity was 70 °C and 6.5 respectively.

Conclusions

The results obtained from the present work demonstrate the potential of *B. smithii* strain IITR6b2 as a biocatalyst for syntheses of NAH and isoniazid. The whole cell amidase showed considerable thermal stability at different temperatures and showed acyltransferase activity for a broad range of amides therefore has potential for productions of industrially important hydroxamic acids and acid hydrazides. The amidase was

compatible in presence of both water miscible and water immiscible solvents at 15% (v/v). This feature renders this amidase suitable for biotransformation of hydrophobic amides to hydroxamic acids or acid hydrazides at higher molar concentration.

Efficient biotransformation processes for NAH and isoniazid productions have been developed utilizing acyltransferase activity of this isolate for high molar conversion with limited side reaction in shorter time. This is the first study which reports amidase catalyzed bioprocesses for the syntheses of NAH and isoniazid. The starting material of these bioprocesses are corresponding amides, cheaper than acids and esters which are commonly used in chemical methods for NAH and isoniazid syntheses and also these reactions take place in aqueous medium leading to reduced cost of bioprocess. This process resulted in 89.4% molar conversion of nicotinamide to nicotinic acid hydroxamate in 120 min with only 4.5% by-product, nicotinic acid. Formation of by-product was limited due to high ratio of acyltransferase to hydrolase activities of whole cells. During the fed batch process after two subsequent feedings further conversion to product was almost stopped probably due to cell lysis and product inhibition of acyltransferase activity. This process can be extended to higher molar concentration by the use of a bioreactor with continuous removal of NAH formed during reaction and immobilized bacterial cells, which will provide stability to biocatalyst resulting in its repeated use and reduction in process cost.

During bioprocess development for isoniazid synthesis, by optimizing various parameters as hydrazine-2HCl concentration, temperature and resting cells concentration, reaction was directed to the preferred hydrazinolysis hence synthesis of by-product (isonicotinic acid) was limited. The fed batch process for isoniazid synthesis resulted in 87.8% molar conversion of isonicotinamide to isoniazid after four subsequent feedings in 5 h with only 7.8% by-product, isonicotinic acid. Till now the biocatalytic route to isoniazid synthesis using lipase enzyme has been reported, however this process occurs at a slow rate in 1, 4-Dioxane and resulted in lower conversion (54%). The reported bioprocess is a greener method, does not involve any hazardous reagent and takes place under mild reaction conditions. These results demonstrated that enzymatic synthesis of isoniazid using whole cells of *B. smithii* strain IITR6b2 might present an efficient alternative route to the chemical synthesis procedures. Further improvements in process can be expected by immobilization of biocatalyst. In conclusion, *B. smithii* strain IITR6b2 has potential for the commercial productions of NAH and isoniazid in future.

REFERENCES

1. Acharya A, Desai AJ (1997) Studies on utilization of acetonitrile by *Rhodococcus erythropolis* A10. World Journal of Microbiology and Biotechnology 13(2), 175-178.
2. Ahuja M, Rai AK, Mathur PN (1996) Adsorption behaviour of metal ions on hydroximate resins. Talanta 43(11), 1955-1963.
3. Ahmed AE, Farooqui MY (1982) Comparative toxicities of aliphatic nitriles. Toxicology letters 12(2), 157-163.
4. Ain QU, Ashiq U, Jamal RA, Mahrooof–Tahir M (2013) Synthesis, spectroscopic and radical scavenging studies of Palladium (II)-hydrazide complexes. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 115, 683-689.
5. Aliyu AO, Nwabueze JN (2005) Complexation of nicotinohydroxamic acid with nickel (II). European Journal of Scientific Research, 18(3), 354-359.
6. Aliyu AO, Nwabueze JN (2008) Complexation of cobalt with nicotinohydroxamic acid and its microbial sensitivity. International Journal of Physical Sciences 2(7), 167-172.
7. Alagta A, Felhösi I, Bertoti I, Kálmán E (2008) Corrosion protection properties of hydroxamic acid self-assembled monolayer on carbon steel. Corrosion Science 50, 1644-1649.
8. Al-mulla EAJ, Yunus WMZW, Ibrahim NAB, Rahaman MZA (2010) Enzymatic synthesis of palm olein-based fatty thiohydroxamic acids. Journal of Oleo Science 59, 569-573.
9. Aliyu AO, Egwaikhide PA, Okeniyi SO, Akporhonor CEE (2010) Complexation of vanadium (IV) with hydroxamate chelators and their microbial sensitivity. Electronic Journal of Environmental, Agricultural and Food Chemistry 9(6), 1019-1024.
10. Andrade J, Karmali A, Carrondo MA, Frazão C (2007).Structure of amidase from *Pseudomonas aeruginosa* showing a trapped acyl transfer reaction intermediate state. Journal of Biological Chemistry 282(27), 19598-19605.
11. Aravindan R, Anbumathi P, Viruthagiri T (2007) Lipase applications in food industry. Indian Journal of Biotechnology 6(2), 141.
12. Arreaza G, Devane WA, Omeir RL, Sajnani G, Kunz J, Cravatt BF, Deutsch DG (1997) The cloned rat hydrolytic enzyme responsible for the breakdown of anandamide

also catalyzes its formation via the condensation of arachidonic acid and ethanolamine. Neuroscience letters 234(1), 59-62.

13. Asano Y, Tachibana M, Yamada YTH (1982) Purification and characterization of amidase which participates in nitrile degradation. Agricultural and Biological Chemistry 46(5), 1175-1181.

14. Babu V, Shilpi, Choudhury B (2010) Nitrile metabolizing potential of *Amycolatopsis sp.* IITR215. Process Biochemistry 45, 866–873.

15. Babu V (2010) Development of nitrile metabolizing enzyme for surface modification of polyacrylonitrile. Ph.D. Thesis. IIT Roorkee, INDIA

16. Babu V, Choudhury B (2012) Competitive adsorptions of nitrile hydratase and amidase on polyacrylonitrile and its effect on surface modification. Colloids and Surfaces B: Biointerfaces 89, 277-282.

17. Baek DH, Song JJ, Lee SG, Kwon SJ, Asano Y, Sung MH (2003) New thermostable d-methionine amidase from *Brevibacillus borstelensis* BCS-1 and its application for d-phenylalanine production. Enzyme and Microbial Technology 32, 131–139.

18. Baizer MM, Dub M, Gister S, Steinberg NG (1956) Synthesis of isoniazid from citric acid. Journal of the American Pharmaceutical Association 45(7), 478-480.

19. Banerjee A, Sharma R, Banerjee U (2002) The nitrile-degrading enzymes: current status and future prospects. Applied Microbiology and Biotechnology 60(1-2), 33-44

20. Bátka D, Farkas E (2006) Pb(II)-binding capability of aminohydroxamic acids: Primary hydroxamic acid derivatives of α-amino acids as possible sequestering agents for Pb (II). Journal of inorganic biochemistry 100(1), 27-35.

21. Bao M, Chen Q, Li Y, Jiang G (2010) Biodegradation of partially hydrolyzed polyacrylamide by bacteria isolated from production water after polymer flooding in an oil field. Journal of hazardous materials 184(1), 105-110.

22. Bauer R, Hirrlinger B, Layh N, Stolz A, Knackmuss H-J (1994) Enantioselective hydrolysis of racemic 2-phenylpropionitrile and other (R,S)-2-arylpropionitrile by a new bacterial isolate, *Agrobacterium tumefaciens* strain d3. Applied Microbiology and Biotechnology 42, 1-7.

23. Bauer R, Knackmuss H-J, Stolz A (1998) Enantioselective hydration of 2-arylpropionitriles by a nitrile hydratase from *Agrobacterium tumefaciens* strain d3. Applied Microbiology and Biotechnology 49, 89-95.

24. Bailey DM, Wood D, Johnson RE (1972) Lowering of serum lipid levels by "masked" nicotinic acid derivatives. Journal of Medicinal Chemistry 15, 344-348.

25. Bernardo M, Pacheco R, Serralheiro MLM, Karmali A (2013) Production of hydroxamic acids by immobilized *Pseudomonas aeruginosa* cells: kinetic analysis in reverse micelles. Journal of Molecular Catalysis B: Enzymatic 93, 28-33.

26. Bhatia RK, Bhatia SK, Mehta PK, Bhalla TC (2013) Bench scale production of benzohydroxamic acid using acyltransfer activity of amidase from *Alcaligenes sp.* MTCC 10674. Journal of Industrial Microbiology and Biotechnology 40, 21-27.

27. Bhatia RK, Bhatia SK, Mehta PK, Bhalla TC (2013) Production and characterization of acyl transfer activity of amidase from *Alcaligenes sp.* MTCC 10674 for synthesis of hydroxamic acids. Journal of Microbial and Biochemical Technology 5(1), 001-005.

28. Bhatia SK, Mehta PK, Bhatia RK, Bhalla TC (2013) Simultaneous purification of nitrile hydratase and amidase of *Alcaligenes sp.* MTCC 10674. 3 Biotech DOI 10.1007/s13205-013-0163-z

29. Bissett DL, Hamilton OH, Adams JA (1999) Method of treating skin irritation WO 99/47141

30. Bork P, Koonin EV (1994) A new family of carbon-nitrogen hydrolases. Protein Science 3(8), 1344-1346.

31. Bouslimani N, Clément N, Rogez G, Turek P, Choua S, Dagorne, S, Welter R (2010) Stability, molecular structures and magnetic properties of dinuclear iron complexes supported by benzoic hydrazide derivative ligands. Inorganica Chimica Acta 363(1), 213-220.

32. Brady BL (1969) The acylamide amidohydrolase of *Candida utilis*: purification and properties. Journal of General Microbiology 59(1), 47-55.

33. Briggs BS, Kreuzman AJ, Whitesitt C, Yeh WK, Zmijewski M (1996) Discovery, purification, and properties of o-phthalyl amidase from *Xanthobacter agilis*. Journal of Molecular Catalysis B: Enzymatic 2(1), 53-69.

34. Buglyó P, Pótári N (2005) Study of the interaction between oxovanadium (IV) and hydroxamic acids. Polyhedron 24(7), 837-845.

35. Buranasilp K, Charoenpanich J (2011) Biodegradation of acrylamide by *Enterobacter aerogenes* isolated from wastewater in Thailand. Journal of Environmental Sciences 23(3), 396–403.

36. Cacic M, Trkovnik M, Cacic F, Has-Schon E (2006) Synthesis and Antimicrobial Activity of Some Derivatives on the Basis (7-hydroxy-2-oxo-2H-chromen-4-yl)-acetic Acid Hydrazide. Molecules 11(2), 134-147.

37. Cali P, Naerum L, Mukhija S, Hjelmencrantz A (2004) Isoxazole-3-hydroxamic acid derivatives as peptide deformylase inhibitors and potential antibacterial agents. Bioorganic & medicinal chemistry letters 14(24), 5997-6000.

38. Carpenter CA, Kenar JA, Price NP (2010) Preparation of saturated and unsaturated fatty acid hydrazides and long chain C-glycoside ketohydrazones. Green Chemistry 12(11), 2012-2018.

39. Cantarella M, Cantarella L, Gallifuoco A, Spera A (2006) Use of a UF-membrane reactor for controlling selectively the nitrile hydratase–amidase system in *Microbacterium imperial* CBS 498-74 resting cells case study: Benzonitrile conversion. Enzyme and Microbial Technology 38, 126–134.

40. Cantarella M, Cantarella L, Gallifuoco A, Intellini R, Kaplan O, Spera A, Martínkov´a L (2008) Amidase-catalyzed production of nicotinic acid in batch and continuous stirred membrane reactors. Enzyme and Microbial Technology 42, 222–229.

41. Cantarella L, Gallifuoco A, Malandra A, Martínková L, Pasquarelli F, Spera A, Cantarella M (2010) Application of continuous stirred membrane reactor to 3-cyanopyridine bioconversion using the nitrile hydratase–amidase cascade system of *Microbacterium imperial* CBS 498-74. Enzyme and Microbial Technology 47(3), 64-70.

42. Cantarella M , Cantarella L, Gallifuoco A, Spera A, Martinkova L (2012) Nicotinic acid bio-production by *Microbacterium imperiale* CBS 489-74: Effect of 3-cyanopyridine and temperature on amidase activity. Process Biochemistry 47, 1192–1196.

43. Cantarella L, Gallifuoco A, Martinkova M L, Spera A and Cantarella M (2011) High-yield continuous production of nicotinic acid via nitrile hydratase-amidase cascade reactions using cascade CSMRs. Enzyme and Microbial Technology 48, 345-350.

44. Cantarella L, Gallifuoco A, Spera A, Cantarella M (2013) Nitrile, amide and temperature effects on amidase-kinetics during acrylonitrile bioconversion by nitrile-hydratase/amidase in-situ cascade system. Bioresource technology 142, 320-328.

45. Chebrou H, Bigey F, Arnaud A, Galzy P (1996) Study of the amidase signature group. Biochimica et Biophysics Acta 1298, 285-293.

46. Chacko S, Ramteke PW, Joseph B (2012) A comparative study on the production of amidase using immobilized and dehydrated immobilized cells of *Pseudomonas putida* MTCC 6809. Journal of Genetic Engineering and Biotechnology 10, 121–127.

47. Chebrou H, Bigey F, Arnaud A, Galzy P (1996) Study of the amidase signature group. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology 1298(2), 285-293.

48. Chen CH, County T, Chien MY, County TY, Hou WC, City T, Lin YH, County T (2011) Method for scavenging free radicals and inhibiting tyrosinase and melanin. US 20122 00339898A1.

49. Chen CY, Chen SC, Fingas M, Kao CM (2010) Biodegradation of propionitrile by *Klebsiella oxytoca* immobilized in alginate and cellulose triacetate gel. Journal of hazardous materials 177(1), 856-863.

50. Cheong TK, Oriel PJ (2000) Cloning of a wide-spectrum amidase from *Bacillus stearothermophilus* BR388 in *Escherichia coli* and marked enhancement of amidase expression using directed evolution. Enzyme and microbial technology 26(2), 152-158.

51. Chmura A, Rustler S, Paravidino M, van Rantwijk F, Stolz A, Sheldon RA (2013) The combi-CLEA approach: enzymatic cascade synthesis of enantiomerically pure (S)-mandelic acid. Tetrahedron: Asymmetry 24(19), 1225-1232.

52. Ciskanik LM, Wilczek JM, Fallon RD (1995) Purification and Characterization of an Enantioselective Amidase from *Pseudomonas chlororaphis* B23. Applied and environmental microbiology 61(3), 998-1003.

53. Clark PH (1970) The aliphatic amidases of *Pseudomonas aeruginosa.* Advances in Microbial Physiology 4, 179-222.

54. Codd R (2008) Transversing the coordination chemistry and chemical biology of hydroxamic acids. Coordination Chemistry reviews 252, 1387-1408.

55. Codd R, Braich N, Liu J, Soe CZ, Pakchung AA (2009) Zn (II)-dependent histone deacetylase inhibitors: suberoylanilide hydroxamic acid and trichostatin A. The International Journal of Biochemistry & Cell Biology 41(4), 736-739.

56. Cramp R, Gilmour M, Cowan DA (1997) Novel thermophillic bacteria producing nitrile-degrading enzymes. Microbiology 143, 2313-2320.

57. Crosby J, Moilliet J, Parratt JS, Turner NJ (1994) Regioselective hydrolysis of aromatic dinitriles using a whole cell catalyst. Journal of the Chemical Society, Perkin Transactions 1 13, 1679-1687.

58. D'Abusco AS, Ammendola S, Scandurra R, Politi L (2001) Molecular and biochemical characterization of the recombinant amidase from hyperthermophilic archaeon *Sulfolobus solfataricus*. Extremophiles 5, 183-192.

59. D'Abusco AS, Casadio R, Tasco G, Giangiacomo L, Giartosio A, Calamia V, Marco SD, Chiaraluce R, Consalvi V, Scandurra R, Politi L (2005) Oligomerization of *Sulfolobus solfataricus* signature amidase is promoted by acidic pH and high temperature. Archea 1, 411-423.

60. Dadd MR, Claridge TDW, Pettman AJ, Knowles CJ (2001) Biotransformation of benzonitrile to benzohydroxamic acid by *Rhodococcus rhodochrous* in the presence of hydroxylamine. Biotechnology Letters 23, 221-225.

61. Danieli B, Riva S (1994) Enzyme-mediated regioselective acylation of polyhydroxylated natural products. Pure and applied chemistry 66(10), 2215-2218.

62. David L, Rusu M, Cozar O, Rusu D, Todica M, Balan C (1999) Spectroscopic and magnetic investigations of some transition metal complexes with N-4-methoxyphenyl-N-4-chlorobenzoyl hydrazide as ligand. Journal of molecular structure 482, 149-152.

63. Devocelle M, McLoughlin BM, Sharkey CT, Fitzgerald DJ, Nolan KB (2003) A convenient parallel synthesis of low molecular weight hydroxamic acids using polymer-supported 1-hydroxybenzotriazole. Organic and Biomolecular Chemistry 1, 850-853.

64. De Geyter N, Morent R, Leys C, Gengembre L, Payen E (2007) Treatment of polymer films with a dielectric barrier discharge in air, helium and argon at medium pressure. Surface and Coatings Technology 201(16), 7066-7075.

65. Dheeman DS, Antony-Babu S, Frías JM, Henehan G (2011) Purification and characterization of an extracellular lipase from a novel strain *Penicillium sp*. DS-39 (DSM 23773). Journal of Molecular Catalysis B: Enzymatic 72(3), 256-262.

66. Doran JP, Duggan P, Masterson M, Turner PD, O'Reilly C (2005) Expression and purification of a recombinant enantioselective amidase. Protein Expression and Purification 40, 190–196.

67. Draper P (1967) The aliphatic acylamide amidohydrolase of *Mycobacterium smegmatis*: Its inducible nature and relation to acyl-transfer to hydroxylamine. Journal of General Microbiology 46, 111-123.

68. Egorova K, Trauthwein H, Verseck S, Antranikian G (2004) Purification and properties of an enantioselective and thermoactive amdase from the thermophilic actinomycete *Pseudonocardia thermiophila.* Applied Microbiology and Biotechnology 65, 38-45.

69. Eichhorn E, Roduit JP, Shaw N, Heinzmann K, Kiener A (1997) Preparation of (S)-piperazine-2-carboxylic acid,(R)-piperazine-2-carboxylic acid, and (S)-piperidine-2-carboxylic acid by kinetic resolution of the corresponding racemic carboxamides with stereoselective amidases in whole bacterial cells. Tetrahedron: asymmetry 8(15), 2533-2536.

70. Emmanuel Joshua Jebasingh S, Lakshmikandan M, Rajesh RP, Raja P (2013) Biodegradation of acrylamide and purification of acrylamidase from newly isolated bacterium *Moraxella osloensis* MSU11. International Biodeterioration & Biodegradation 85, 120-125.

71. Ewert C, Lutz-Wahl S, Fischer L (2008) Enantioselective conversion of a-arylnitriles by *Klebsiella oxytoca*. Tetrahedron: Asymmetry 19, 2573–2578.

72. Fazary AE (2005) Thermodynamic studies on the protonation equilibria of some hydroxamic acids in $NaNO_3$ solutions in water and in mixtures of water and dioxane. Journal of Chemical & Engineering Data 50(3), 888-895.

73. Fazary AE, Khalil MM, Fahmy ALY, Tantawy TA (2001) The role of hydroxamic acids in biochemical processes. Medical Journal of Islamic Academy 14(3), 109-116.

74. Farkas E, Enyedy EA, Micera G, Garribba E (2000) Coordination modes of hydroxamic acids in copper (II), nickel (II) and zinc (II) mixed-ligand complexes in aqueous solution. Polyhedron 19, 1727-1736.

75. Farkas E, Csapó E, Buglyó P, Damante CA, Natale GD (2009) Metal-binding ability of histidine-containing peptidehydroxamic acids: Imidazole versus hydroxamate coordination. Inorganica Chimica Acta 362(3), 753-762.

76. Feuer H, Brown Jr F (1970) Chemistry of hydrazides. X. The reduction of cyclic and acyclic hydrazides with diborane. The Journal of Organic Chemistry 35(5), 1468-1471.

77. Fischer-Colbrie G, Matama T, Heumann S, Martinkova L, Cavaco Paulo A, Guebitz, G. (2007) Surface hydrolysis of polyacrylonitrile with nitrile hydrolysing enzymes from *Micrococcus luteus* BST20. Journal of biotechnology 129(1), 62-68.

78. Fishbein WN (1967) Urease inhibitors for hepatic coma: Inhibition of ^{14}C-urea hydrolysis in mice by alkyl hydroxamates: I. Methodology; Acetohydroxamic acid. Biochemical Medicine 1(2), 111-128.

79. Fournand D, Arnaud A, Galzy P, Bigey F, Ratomahenina R (1997) Biocatalyst improvement for the production of short-chain hydroxamic acids. Enzyme and Microbial Technology 20, 424-431.

80. Fournand D, Pirat JL, Bigey F, Arnaud A, Galzy P (1997a) Spectrophotometric assay of aliphatic monohydroxamic acids and α-, β-, and γ-aminohydroxamic acid in aqueous medium. Analytica Chimica Acta 353, 359-366.

81. Fournand D, Arnaund A and Galzy P (1998a) Study of the acyl transfer activity of a recombinant amidase overproduced in an *Escherichia coli* Strain. Application for short-chain hydroxamic acid and acid hydrazide synthesis. Journal of Molecular Catalysis B: Enzymatic 4, 77-90.

82. Fournand D, Bigey F and Arnaud A (1998b) Acyl transfer activity of an amidase from *Rhodococcus sp.* Strain R312: Formation of a wide range of hydroxamic acids. Applied and Environmental Microbiology 64(8), 2844.

83. Fournand D, Arnaud A (2001) Aliphatic and enantioselective amidases: from hydrolysis to acyl transfer activity. Journal of Applied Microbiology 91, 381-393.

84. Fragoso A, Pacheco R, Karmali A (2012) Investigation of structural effects and behaviour of *Pseudomonas aeruginosa* amidase encapsulated in reversed micelles. Process Biochemistry 47, 264–272.

85. Gao WY, Mitsuya H, Driscoll JS, Johns DG (1995) Enhancement by hydroxyurea of the anti-human immunodeficiency virus type 1 potency of 2′-β-fluoro-2′, 3′-dideoxyadenosine in peripheral blood mononuclear cells. Biochemical pharmacology 50(2), 274-276.

86. Galal SA, Hegab KH, Kassab AS, Rodriguez ML, Kerwin SM, El-Khamry AMMA, El Diwani HI (2009) New transition metal ion complexes with benzimidazole-5-carboxylic acid hydrazides with antitumor activity. European journal of medicinal chemistry 44(4), 1500-1508.

87. Gaur R, Gupta A, Khare SK (2008) Purification and characterization of lipase from solvent tolerant *Pseudomonas aeruginosa* PseA. Process Biochemistry 43, 1040–1046.

88. Gilligan T, Yamada H, Nagasawa T (1993) Production of S-(+)-2-phenylpropionic acid from (R,S)-2-phenylpropionitrile by the combination of nitrile hydratase and stereoselective amidase in *Rhodococcus equi* TG328. Applied microbiology and biotechnology 39(6), 720-725.

89. Gissot A, Volonterio A, Zanda M (2005) One-step synthesis of *O*-Benzyl hydroxamates from unactivated aliphatic and aromatic esters. Journal of Organc Chemistry 70, 6925-6928.

90. Gong JS, Lu ZM, Li H, Shi JS, Zhou ZM, Xu ZH (2012) Nitrilases in nitrile biocatalysis: recent progress and forthcoming research. Microbial cell factories 11(1), 1-18.

91. Grant SK, Green BG, Kozarich JW (2001) Inhibition and structure-activity studies of methionine hydroxamic acid derivatives with bacterial peptide deformylase. Bioorganic Chemistry 29(4), 211-222

92. Griffith DM, Szőcs B, Keogh T, Suponitsky KY, Farkas E, Buglyó P, Marmion CJ (2011) Suberoylanilide hydroxamic acid, a potent histone deacetylase inhibitor; its X-ray crystal structure and solid state and solution studies of its Zn (II), Ni (II), Cu (II) and Fe (III) complexes. Journal of Inorganic Biochemistry 105(6), 763-769.

93. Griffith D, Zangrando E, Alessio E, Marmon C (2004) A novel ruthenium nitrosyl complex which also contains a free NO-donor moiety. Inorganica Chimica Acta 357, 3770-3774.

94. Griffith D, Bergamo A, Pin S, Vadori M, Mőller-Bunz H, Sava G, Marmion CJ (2007) Novel platinum pyridine hydroxamic acid complexes: Synthesis, characterisation, X-ray crystallographic study and nitric oxide related properties. Polyhedron 26, 4697-4706.

95. Griffith D, Cecco S, Zangrando E, Bergamo A, Sava G, Marmion CJ (2008) Ruthenium(III) dimethyl sulfoxide pyridinehydroxamic acid complexes as potential antimetastatic agents: synthesis, characterization and in vitro pharmacological evaluation. Journal of Biological Inorganic Chemistry 13, 511-520.

96. Grossowicz N, Wainfan E, Borek E, Waelech H (1950) The enzymatic formation of hydroxamic acids from glutamine and asparagines. Journal of Biological chemistry. 187, 111-125.

97. Guebitz GM, Cavaco-Paulo A (2008) Enzymes go big: surface hydrolysis and functionalisation of synthetic polymers. Trends in biotechnology 26(1), 32-38.

98. Guncheva M, Zhiryakova D, Radchenkova N, Kambourova M (2007).Effect of nonionic detergents on the activity of a thermostable lipase from *Bacillus stearothermophilus* MC7. Journal of Molecular Catalysis B: Enzymatic 49(1), 88-91.

99. Hacking MAPJ, Rantwijk FV, Sheldon RA (2001) Lipase catalysed acylation of hydroxylamine and hydrazine derivatives. Journal of Molecular catalysis B: Enzymatic 11, 315-321.

100. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M (2009). Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21:

Purification, characterization and potential application as a laundry detergent additive. Bioresource Technology 100(13), 3366-3373.

101. Hann EC, Eisenberg A, Fager SK, Perkins NE, Gallagher FG, Cooper S M, Gavagan JE, Stieglitz B, Hennessey SM, DiCosimo, R (1999) 5-Cyanovaleramide production using immobilized *Pseudomonas chlororaphis* B23. Bioorganic & medicinal chemistry 7(10), 2239-2245.

102. Hann EC, Sigmund AE, Fager SK, Cooling FB, Gavagan JE, Ben-Bassat A, Chauhan S, Payne MS, Hennessey SM, DiCosimo R (2003) Biocatalytic hydrolysis of 3-hydroxyalkanenitriles to 3-hydroxyalkanoic acids. Advanced Synthesis & Catalysis 345(6-7), 775-782.

103. Hann EC, Sigmund AE, Fager SK, Cooling FB, Gavagan JE, Bramucci MG, Chauhan S, Payne MS, DiCosimo R (2004) Regioselective biocatalytic hydrolysis of (E, Z)-2-methyl-2-butenenitrile for production of (E)-2-methyl-2-butenoic acid. Tetrahedron, 60(3), 577-581.

104. Hasan F, Shah AA, Hameed A (2006) Industrial applications of microbial lipases. Enzyme and Microbial Technology 39, 235-251.

105. Hase JI, Kobashi K (1967) Inhibition of *Proteus vulgaris* urease by hydroxamic acids. Journal of biochemistry 62(3), 293-299.

106. Harrison JD, David AD (1979) Experimental studies of the use of DTPA and other agents to limit the systemic burden of plutonium after wound contamination. Radiation research 77, 534-546.

107. Hashimoto Y, Nishiyama M, Ikehata O, Horinouchi S, Beppu T (1991) Cloning and characterization of an amidase gene from *Rhodococcus species* N-774 and its expression in *Escherichia coli*. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1088(2), 225-233.

108. Hassan SS, El-Bahnasawy RM, Rizk NM (1997) Potentiometric determination of salicylhydroxamic acid (urinary struvite stone inhibitor) based on the inhibition of urease activity. Analytica chimica acta 351(1), 91-96.

109. Hayashi T, Yamamoto K, Matsuo A, Otsubo K, Muramatsu S, Matsuda A, Komatsu KI (1997) Characterization and cloning of an enantioselective amidase from *Comamonas acidovorans* KPO-2771-4. Journal Of Fermentation and Bioengineering 83(2), 139-145.

110. He JY, Sun ZH, Ruan WQ, Xu Y (2006) Biocatalytic synthesis of ethyl (S)-4-chloro-3-hydroxy-butanoate in an aqueous-organic solvent biphasic system using *Aureobasidium pullulans* CGMCC 1244. Process Biochemistry 41(1), 244-249.

111. Heumann S, Eberl A, Fischer-Colbrie G, Pobeheim H, Kaufmann F, Ribitsch D, Cavaco-Paulo A, Guebitz GM (2009) A novel aryl acylamidase from *Nocardia farcinica* hydrolyses polyamide. Biotechnology and Bioengineering 102(4), 1003-1011.

112. Hermes HFM, Tandler RF, Sonke T, Dijkhuizen L, Meijer EM (1994) Purification and characterization of an L-Amino amidase from *Mycobacterium neoaurum* ATCC 25759. Applied and Environmental Microbiology 60(1), 153-159.

113. Hirrlinger B, Stolz A, Knackmuss HJ (1996) Purification and properties of an amidase from *Rhodococcus erythropolis* MP 50 which enantioselectively hydrolyzes 2-arylpropionamides. Journal of Bacteriology 178(12), 3501-3507.

114. Hirrlinger B, Stolz A (1997) Formation of a chiral hydroxamic acid with an amidase from *Rhodococcus erythropolis* MP 50 and subsequent chemical lossen rearrangement to a chiral amine. Applied and Environmental Microbiology 63(9), 3390-3393.

115. Hockly E, Richon VM, Woodman B, Smith DL, Zhou X, Rosa E, Sathasivam K, Ghazi-Noori S, Mahal A, Lowden PA, Steffan JS, Marsh JL, Thompson LM, Lewis CM, Marks PA, Bates GP. (2003) Suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, ameliorates motor deficits in a mouse model of Huntington's disease. Proceedings of the National Academy of Sciences, 100(4), 2041-2046.

116. Ho CY, Strobel E, Ralbovsky J, Galemmo RA (2005) Improved solution-and solid-phase preparation of hydroxamic acids from esters. Journal of Organic Chemistry 70, 4873-4875.

117. Hongpattarakere T, Komeda H, Asano Y (2005) Purification, characterization, gene cloning and nucleotide sequencing of D-stereospecific amino acid amidase from soil bacterium: *Delftia acidovorans*. Journal of Industrial Microbiology and Biotechnology 32(11-12), 567-576.

118. Hynes MJ, Pateman JA (1970) The use of amides as nitrogen sources by *Aspergillus nidulans*. Journal of general microbiology, 63(3), 317-324.

119. Imramovský A, Polanc S, Vinšová J, Kočevar M, Jampílek J, Rečková Z, Kaustová J (2007) A new modification of anti-tubercular active molecules. Bioorganic & medicinal chemistry 15(7), 2551-2559.

120. Jahangirian H, Haron MJ, Silong S, Yusof NA (2011a) Enzymatic synthesis of phenyl fatty hydroxamic acids from canola and palm oils. Journal of Oleo Science 60, 281-286.

121. Jahangirian H, Haron MJ, Yusof NA, Silong S, Kassim A, Rafiee-Moghaddam R, Peyda M, Gharayebi Y (2011b) Enzymatic synthesis of fatty hydroxamic acid derivatives based on Palm kernel oil. Molecules 16, 6634-6644.

122. Jaeger K, Eggert T (2002) Lipases for biotechnology. Current Opinion in Biotechnology 13, 390-397.

123. Jin L-Q, Liu Z-Q, Xu J-M, Zheng R-C, Zheng Y-C, Shen Y-C (2013) Efficient biocatalytic hydrolysis of 2-chloronicotinamide for production of 2-chloronicotinic acid by recombinant amidase. Catalysis Communications 38, 6-9.

124. Joeres U, Kula MR (1994) Purification and characterization of amicrobial L-carnitine amidase. Applied Microbiology and Biotechnology 40, 606-610.

125. Johannsen FR, Levinskas GJ, Berteau PE, Rodwell DE (1986) Evaluation of the teratogenic potential of three aliphatic nitriles in the rat. Fundamental and Applied Toxicology 7(1), 33-40.

126. Joshi JG, Handler P (1962). Purification and properties of nicotinamidase from *Torula cremoris*. Journal of Biological Chemistry 237(3), 929-935.

127. Joshi SD, Vagdevi HM, Vaidya VP, Gadaginamath GS (2008) Synthesis of new 4-pyrrol-1-yl benzoic acid hydrazide analogs and some derived oxadiazole, triazole and pyrrole ring systems: A novel class of potential antibacterial and antitubercular agents. European journal of medicinal chemistry 43(9), 1989-1996.

128. Judge V, Narasimhan B, Ahuja M, Sriram D, Yogeeswari P, De Clercq E, Pannecouque C, Balzarini J (2013) Synthesis, antimycobacterial, antiviral, antimicrobial activity and QSAR studies of N2-acyl isonicotinic acid hydrazide derivatives. Medicinal Chemistry 9(1), 53-76.

129. Jung YJ, Lee JK, Sung CG, Oh TK, Kim HK (2003) Nonionic detergent-induced activation of an esterase from *Bacillus megaterium* 20-1. Journal of Molecular Catalysis B: Enzymatic 26(3), 223-229.

130. Kammermeier-Steinke D, Schwarz A, Wandrey C, Kula MR (1993) Studies on the substrate specificity of a peptide amidase partially purified from orange flavedo. Enzyme and microbial technology 15(9), 764-769.

131. Kay-Shoemake JL, Watwood ME, Sojka RE, Lentz RD (1998) Polyacrylamide as a substrate for microbial amidase in culture and soil. Soil Biology and Biochemistry 30 (13), 1647-1654.

132. Kapoor M, Gupta MN (2012) Lipase promiscuity and its biochemical applications. Process Biochemistry 47, 555-569.

133. Kano H, Umeda Y, Kasai T, Sasaki T, Matsumoto M, Yamazaki K, Nagano K, Arito H, Fukushima S (2009) Carcinogenicity studies of 1,4-dioxane administered in drinking-water to rats and mice for 2 years. Food and Chemical Toxicology 47(11), 2776-2784.

134. Kasai T, Kano H, Umeda Y, Sasaki T, Ikawa N, Nishizawa T. 2009. Two-year inhalation study of carcinogenicity and chronic toxicity of 1,4-dioxane in male rats. Inhalation Toxicology 21(11), 889-897.

135. Kaul P, Banerjee A, Mayilraj S, Banerjee UC (2004) Screening for enantioselective nitrilases: kinetic resolution of racemic mandelonitrile to (R)-(−)-mandelic acid by new bacterial isolates. Tetrahedron: Asymmetry 15(2), 207-211.

136. Kaushik R, Marwah RG, Gupta P, Saran S, Saso L, Parmar VS, Saxena RK (2010) Optimization of lipase production from *Aspergillus terreus* by response surface methodology and its potential for synthesis of partial glycerides under solvent free conditions. Indian Journal of Microbiology 50(4), 456–462.

137. Kim S-H, Oriel P (2000) Cloning and expression of the nitrile hydratase and amidase genes from *Bacillus* sp. BR449 into *Escherichia coli.* Enzyme and Microbial Technology 27, 492–501.

138. Kimura T (1959) Studies on metabolism of amides in *Mycobacteriaceae*: IV. Formation and hydrolysis of hydroxamate. Journal of Biochemistry 46(11), 1399-1410.

139. Kobayashi M, Komeda H, Nagasawa T, Nishiyama M, Horinouch S, Beppu T, Yamada H and Shimizu S (1993) Amidase coupled with low molecular-mass nitrile hydratase from molecular-mass nitrile hydratase from *Rhodococcus rhodochrous* J1. European Journal of Biochemistry 217, 327-336.

140. Kobayashi M, Suzuki T, Fujita T, Masuda M, Shimizu S (1995) Occurrence of enzymes involved in biosynthesis of indole-3-acetic acid from indole-3-acetonitrile in plant-associated bacteria, *Agrobacterium* and *Rhizobium*. Proceedings of the National Academy of Sciences 92(3), 714-718.

141. Kobayashi M, Fujiwara Y, Goda M, Komeda H, Shimizu S (1997) Identification of active sites in amidase: evolutionary relationship between amide bond-and peptide bond-

cleaving enzymes. Proceedings of the National Academy of Sciences 94(22), 11986-11991.

142. Kobayashi M, Goda M, Shimizu S (1998a) Nitrilase catalyzes amide hydrolysis as well as nitrile hydrolysis. Biochemical and Biophysical Research Communications 253, 662–666.

143. Kobayashi M, Goda M, Shimizu S (1998b) The catalytic mechanism of amidase also involves nitrile hydrolysis. FEBS Letters 439, 325-328.

144. Kobayashi M, Goda M, Shimizu S (1999) Hydrazide synthesis: novel substrate specificity of amidase. Biochemical and Biophysical Research Communications 256, 415–418.

145. Kotlova EK, Chestukhina GG, Astaurova OB, Leonova TE, Yanenko AS, Debabov VG (1999) Isolation and primary characterization of an amidase from *Rhodococcus rhodochrous*. Biochemistry (Moscow) 64(4), 384-389.

146. Kokare ND, Nagawade RR, Rane VP, Sinde DB (2007) Design, synthesis and utilization of a novel coupling reagent for the preparation of O-alkyl hydroxamic acids. Tetrahedron Letters 48, 4437-4440.

147. Komeda, H., & Asano, Y. (2000). Gene cloning, nucleotide sequencing, and purification and characterization of the D-stereospecific amino-acid amidase from *Ochrobactrum anthropi* SV3. European Journal of Biochemistry 267(7), 2028-2035.

148. Komeda H, Ishikawa N, Asano Y (2003) Enhancement of the thermostability and catalytic activity of d-stereospecific amino-acid amidase from *Ochrobactrum anthropi* SV3 by directed evolution. Journal of Molecular Catalysis B: Enzymatic 21, 283–290.

149. Komeda H, Hariyama N, Asano Y (2006) L-Stereoselective amino acid amidase with broad substrate specificity from *Brevundimonas diminuta*: characterization of a new member of the leucine aminopeptidase family. Applied microbiology and biotechnology 70(4), 412-421.

150. Komeda H, Asano Y (2008) A novel d-stereoselective amino acid amidase from *Brevibacterium iodinum*: Gene cloning, expression and characterization Enzyme and Microbial Technology 43, 276–283.

151. Konishi T, Kayano M, Tanabe F (1974) Pharmaceutical composition for remedy of hyperammoniemia. U.S. patent 3961064.

152. Kontogiorgis CA, Papaioannou P, Hadjipavlou-Litina DJ (2005) Matrix metalloproteinase inhibitors: a review on pharmacophore mapping and (Q) SARs results. Current medicinal chemistry 12(3), 339-355.

153. Krieg L, Ansorge-Schumacher MB, Kula MR (2002) Screening for amidases: isolation and characterization of a novel D-Amidase from *Variovorax paradoxus*. Advanced Synthesis & Catalysis 344(9), 965-973.

154. Kubáč D, Cejkova A, Masak J, Jirku V, Lemaire M, Gallienne E, Bolte J, Stloukal R, Martinkova, L (2006) Biotransformation of nitriles by *Rhodococcus equi* A4 immobilized in Lentikats. Journal of Molecular Catalysis B: Enzymatic 39, 59-61.

155. Kudelko A, Zieliński W (2012) Microwave-assisted synthesis of 2-styryl-1, 3, 4-oxadiazoles from cinnamic acid hydrazide and triethyl orthoesters. Tetrahedron Letters 53(1), 76-77.

156. Kulakov IV, Nurkenov OA, Gazalieva MA, Zhaugasheva SK, Isakova ZO (2007) Synthesis and tuberculostatic activity of N-aminoacetic acid hydrazides and acylhydrazides based on ephedrine alkaloids. Pharmaceutical Chemistry Journal 41(11), 620-624.

157. Kumar D, Maruthi Kumar N, Ghosh S, Shah K (2012) Novel bis (indolyl) hydrazide–hydrazones as potent cytotoxic agents. Bioorganic & medicinal chemistry letters 22(1), 212-215.

158. Kumar D, Chadda S, Sharma J, Surain P (2013) Syntheses, spectral characterization, and antimicrobial studies on the coordination compounds of metal ions with schiff base containing both aliphatic and aromatic hydrazide moieties. Bioinorganic chemistry and applications, http://dx.doi.org/10.1155/2013/981764.

159. Kumar P, Narasimhan B, Yogeeswari P, Sriram D (2010) Synthesis and antitubercular activities of substituted benzoic acid N′-(substituted benzylidene/furan-2-ylmethylene)-N-(pyridine-3-carbonyl)-hydrazides. European journal of medicinal chemistry 45(12), 6085-6089.

160. Kwak SY, Yang JK, Choi HR, Park KC, Kim YB, Lee YS (2012) Synthesis and dual biological effects of hydroxycinnamoyl phenylalanyl/prolyl hydroxamic acid derivatives as tyrosinase inhibitor and antioxidant. Bioorganic & medicinal chemistry letters 23(4), 1136-1142.

161. Layh N, Stolz A, Böhme J, Effenberger F, Knackmuss HJ (1994) Enantioselective hydrolysis of racemic naproxen nitrile and naproxen amide to S-naproxen by new bacterial isolates. Journal of biotechnology 33(2), 175-182.

162. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259), 680-685.

163. Leung HW, Paustenbach DJ (1990) Cancer risk assessment for dioxane based upon a physiologically based pharmacokinetic approach. Toxicology Letters 51(2), 147-162.

164. Li XF, Lu ZH, Zhao GL, Wu H, Yu YG (2012) A facile whole-cell biocatalytic approach to regioselective synthesis of monoacylated 1- β-d-arabinofuranosylcytosine: Influence of organic solvents. Bioresource Technology 114, 6-11.

165. Li Q, Guedes da Silva MFC, Pombeiro AJ (2004) Diorganotin (IV) derivatives of substituted benzohydroxamic acids with high antitumor activity. Chemistry-A European Journal 10(6), 1456-1462.

166. Li T, Liu J, Bai R, Ohandja DG, Wong FS (2007) Biodegradation of organonitriles by adapted activated sludge consortium with acetonitrile-degrading microorganisms. Water research 41(15), 3465-3473.

167. Lin Z-J, Zheng R-C, Lei L-H, ZhengY-G, ShenY-C (2011) Ferrous and ferric ions-based high-throughput screening strategy for nitrile hydratase and amidase. Journal of Microbiological Methods 85, 214–220.

168. Lin YS, Chen SH, Haung WJ, Chen CH, Chien MY, Lin SY, Hou WC (2012a) Effects of nicotinic acid derivatives on tyrosinase inhibitory and antioxidant activities. Food Chemistry 132, 2074-2080.

169. Lin YS, Chuang MT, Chen CH, Chien MY, Hou WC (2012b) Nicotinic acid hydroxamate downregulated the melanin synthesis and tyrosinase activity through activating the MEK/ERK and AKTGSK3*β* signalling pathways. Journal of Agricultural and Food Chemistry 60, 4859-4864.

170. Liu YY, Xu JH, Hu Y (2000) Enhancing effect of Tween-80 on lipase performance in enantioselective hydrolysis of ketoprofen ester. Journal of Molecular Catalysis B: Enzymatic 10(5), 523-529.

171. Lutfor MR, Mashitah MY. (2011) Synthesis of Poly (hydroxamic Acid)-Poly (amidoxime) Chelating Ligands for Removal of Metals from Industrial Wastewater. Journal of Chemistry 8(3), 1038-1043.

172. Lukevits È (1995) Pyridine derivatives in the drug arsenal (150 years of pyridine chemistry). Chemistry of Heterocyclic Compounds 31, 639-650.

173. Luca LD, Giacomelli G, Taddei M (2001) An easy and convenient synthesis of Weinreb amides and hydroxamates. Journal of Organic Chemistry 66, 2534-2537.

174. Mahenthiralingam E, Draper P, Davis EO, Colston MJ (1993) Cloning and sequencing of the gene which encodes the highly inducible acetamidase of *Mycobacterium smegmatis*. Journal of general microbiology 139(3), 575-583.

175. Makhongela HS, Glowacka AE, Agarkar VB, Sewell BT, Weber B, Cameron RA, Cowan DA, Burton SG (2007) A novel thermostable nitrilase superfamily amidase from *Geobacillus pallidus* showing acyltransfer activity. Applied Microbiology and Biotechnology 75, 801-811.

176. Maestracci M, Thiery A, Arnaund A, Galzy P (1986) A study of the mechanism of the reaction catalysed by the amidase from *Brevibacterium sp.* R312. Agricultural and Biological Chemistry 50, 2237-2241.

177. Maestracci M, Bui K, Thièry, Arnaud A, Galzy P (1984) A study of the inhibition of an amidase with a wide substrate spectrum and its consequences for the bioconversion of nitriles. Biotechnology Letters 6(3), 149-154.

178. Maestracci M, Thiery A, Bui K, Arnaud A, Galzy P (1984) Activity and regulation of an amidase (acrylamide amidohydrolase EC 3.5.1.4) with a wide substrate spectrum from a *Brevibacterium Sp.* Archives of Microbiology 138, 315-320.

179. Malley SD, Grange JM, Hamedi-Sangsari F, Vila JR (1994) Synergistic anti-human immunodeficiency virus type 1 effect of hydroxamate compounds with 2', 3'-dideoxyinosine in infected resting human lymphocytes. Proceedings of the National Academy of Sciences 91(23), 11017-11021.

180. Martínkov´a L, Vejvoda V, K˘ren V (2008) Selection and screening for enzymes of nitrile metabolism. Journal of Biotechnology 133, 318–326.

181. Martínková L, Křen V, Cvak L, Ovesná M, Přepechalová I (2000) Hydrolysis of lysergamide to lysergic acid by *Rhodococcus equi* A4. Journal of biotechnology 84(1), 63-66.

182. Martínková L, Klempier N, Bardakji J, Kandelbauer A, Ovesná M, Poda˘rilová T, Kuzmaa, M, P˘repechalová I, Griengl H, Kren V (2001) Biotransformation of 3-substituted methyl (*R,S*)-4-cyanobutanoates with nitrile- and amide-converting biocatalysts. Journal of Molecular Catalysis B: Enzymatic 14, 95–99.

183. Martínková L, Uhnáková B, Pátek M, Nešvera J, Křen V (2009) Biodegradation potential of the genus *Rhodococcus*. Environment International 35(1), 162-177.

184. Mayaux JF, Cerebelaud E, Soubrier F, Faucher D, Petre D (1990) Purification, cloning, and primary structure of an enantiomer-selective amidase from *Brevibacterium sp.* strain R312: structural evidence for genetic coupling with nitrile hydratase. Journal of bacteriology 172(12), 6764-6773.

185. Mehta PK, Bhatia SK, Bhatia RK, Bhalla TC (2013) Purification and characterization of a novel thermo-active amidase from *Geobacillus subterraneus* RL-2a. Extremophiles 17(4), 637-48.

186. Meth-Cohn O, Wang MX (1997) An in-depth study of the biotransformation of nitriles into amides and/or acids using *Rhodococcus rhodochrous* AJ2701. Journal of the Chemical Society Perkin Transactions 1, (8), 1099-1104.

187. Miller MJ (1989) Syntheses and therapeutic potential of hydroxamic acid based siderophores and analogues. Chemical Reviews 89, 1563-1579.

188. Miminoshvili ÈB (2009) Metal hydrazide complexes. Journal of Structural Chemistry 50(1), 168-175.

189. Miyajima H, Takahashi Y, Kamata T, Shimizu H, Sakai N, Gitlin JD (1997) Use of desferrioxamine in the treatment of aceruloplasminemia. Annals of neurology 41(3), 404-407.

190. Mohamed NA (1994) Novel wholly aromatic polyamide-hydrazides. Part V: Structure-molecular-weight-thermal-stability relationships. Polymer degradation and stability 44(1), 33-42.

191. Mohamed NA (1997) Aromatic hydrazides as stabilizers for rigid PVC against thermo-oxidative degradation. Polymer Degradation and Stability 56, 317-329**.**

192. Mohamed HA, Ali SA, Ramadan RM (2006) Spectroscopic and thermal studies of chromium (III), molybdenum (VI) and ruthenium (0) complexes of maleic hydrazide. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 64(4), 913-917.

193. Mohamad S, Yunus WMZW, Haron MJ, Rahman MZA (2008) Enzymatic synthesis of fatty hydrazides from Palm oils. Journal of Oleo Science 57, 263-267.

194. Mönster A, Villain L, Scheper T, Beutel S (2013) One-step-purification of penicillinG amidase from cell lysate using ion-exchange membrane adsorbers. Journal of Membrane Science 444, 359–364.

195. Moreau JL, Bernet N, Arnaud A, Galzy P (1993) Isolation of *Brevibacterium sp.* R312 mutants potentially useful for the enzymatic production of adipic acid. Canadian journal of microbiology 39(5), 524-528.

196. Mukherjee C, MacLean ED, Cameron TS, Jha A (2010) Enzyme-assisted kinetic resolution of novel 2-naphthol Mannich bases. Journal of Molecular Catalysis B: Enzymatic 62(1), 46-53.

197. Mulchay C, Dolgushin F, Krot KA, Griffth D, Marmion CJ (2005) Synthesis, characterisation and speciation studies of heterobimetallic pyridinehydroxamate-bridged Pt(II)/M(II) complexes (M = Cu, Ni, Zn). Crystal structure of a novel heterobimetallic 3-pyridinehydroxamate-bridged Pt(II)/Cu(II) wave-like coordination polymer. Dalton Transactions 1993-1998.

198. Nampoothiri KM, Roopesh K, Chacko S, Pandey A (2005) Comparative study of amidase production by free and immobilized *Escherichia coli* cells. Applied Biochemistry and Biotechnology 120, 97-108.

199. Nawaz, MS, Franklin W, Cerniglia CE (1993) Degradation of acrylamide by immobilized cells of a Pseudomonas sp. and *Xanthomonas maltophilia*. Canadian journal of microbiology 39(2), 207-212.

200. Nawaz MS, Khan AA, Bhattacharayya D, Siitonen PH, Cerniglia CE (1996) Physical, biochemical, and immunological characterization of a thermostable amidase from *Klebsiella pneumoniae* NCTR 1. Journal of Bacteriology 178(8), 2397-2401.

201. Nawaz MS, Khan AA, Seng JE, Leakey JE, Siitonen PH, Cerniglial CE (1994) Purification and characterization of an amidase from an acrylamide-degrading *Rhodococcus sp*. Applied and Environmental Microbiology 60(9), 3343-3348.

202. Narasimhan B, Kumar P, Sharma D (2010) Biological activities of hydrazide derivatives in the new millennium. Acta Pharmaceutica Sciencia 52, 169-180.

203. Narayanasamy K, Shukla S, Parekh L J (1990) Utilization of acrylonitrile by bacteria isolated from petrochemical waste waters. Indian journal of experimental biology 28(10), 968.

204. Nehru K, Athappan P, Rajagopal G (2001) Ruthenium (II)/(III) complexes of bidentate acetyl hydrazide Schiff bases. Transition Metal Chemistry 26(6), 652-656.

205. Nel AJM, Tuffin IM, Sewell BT, Cowan DA (2011) Unique aliphatic amidase from a psychrotrophic and haloalkaliphilic *Nesterenkonia* isolate. Applied and Environmental Microbiology 77(11), 3696-3702.

206. Nikam SS, Korenberg BE, Johnson DR, Doherty AM (1995) Synthesis of hydroxamic acids: $Pd/BaSo_4$ as a new catalyst for the deprotection of O-benzyl hydroxamates. Tetrahedron Letters 36, 197-200.

207. O'Brien EC, Farkas E, Gil MJ, Fitzgerald D, Castineras A, Nolan KB (2000) Metal complexes of salicylhydroxamic acid (H_2Sha), anthranilic hydroxamic acid and benzohydroxamic acid. Crystal and molecular structure of $[Cu(phen)_2(Cl)]Cl{\cdot}H_2Sha$, a

model for a peroxidase-inhibitor complex. Journal of inorganic biochemistry 79(1), 47-51.

208. Oblong JE, Bissett DL, Biederman KA (2003) Method of regulating skin appearance with Vitamin B3 compound. European patent 0 896 522 B1.

209. Odunola OA, Adeoye IO, Woods JAO, Gelebe AC (2003) Synthesis and characterization of nickel (II) complexes of benzoic acid and methyl substituted benzoic acid hydrazides and X-Ray structure of $Ni[C_6H_5CONHNH_2]3Cl_2 \cdot 3CH_3OH$. Synthesis and reactivity in inorganic and metal-organic chemistry 33(2), 205-221.

210. Odunola OA. Adeoye IO, Woods JA (2002) Synthesis and structural features of copper (II) complexes of benzoic acid and methyl substituted benzoic acid hydrazides and X-ray structure of $Cu[C_6H_5CONHNH_2]_2(NO_3)_2$. Synthesis and reactivity in inorganic and metal-organic chemistry, 32(4), 801-817.

211. Ohtaki A, Murata K, Sato Y, Noguchi K, Miyatake H, Dohmae N, Yamada K, Yohda M and Odaka M (2010) Structure and characterization of amidase from *Rhodococcus sp.* N-771: Insite into the molecular mechanism of substrate recognition. Biochimica et Biophysica Acta 1804, 184-192.

212. Olaofe OA, Burton SG, Cowan DA, Harrison STL (2010) Improving the production of a thermostable amidase through optimising IPTG induction in a highly dense culture of recombinant *Escherichia coli*. Biochemical Engineering Journal 52, 19–24.

213. Okazaki S, Suzuki A, Komeda H, Yamaguchi S, Asano Y, Yamane T (2007) Crystal structure and functional characterization of a D-stereospecific amino acid amidase from *Ochrobactrum anthropi* SV3, a new member of the penicillin-recognizing proteins. Journal of molecular biology 368(1), 79-91.

214. Osprian I, Fechter MH, Griengl H (2003) Biocatalytic hydrolysis of cyanohydrins: an efficient approach to enantiopure α-hydroxy carboxylic acids. Journal of Molecular Catalysis B: Enzymatic 24, 89-98.

215. Ouellet M, Aitken SM, English AM, Percival MD (2004) Aromatic hydroxamic acids and hydrazides as inhibitors of the peroxidase activity of prostaglandin H_2 synthase-2. Archives of biochemistry and biophysics 431(1), 107-118.

216. Pal D, Saha S (2012) Hydroxamic acid–A novel molecule for anticancer therapy. Journal of advanced pharmaceutical technology & research 3(2), 92.

217. Pandey D, Singh R, Chand D (2011) An improved bioprocess for synthesis of acetohydroxamic acis using DTT (dithiothreitol) treated resting cells of *Bacillus sp.* APB-6. Bioresource Technology 102(11), 6579-86.

218. Pacheco R, Karmali A, Serralheiro MLM, Haris PI (2005) Application of Fourier transform infrared spectroscopy for monitoring hydrolysis and synthesis reactions catalyzed by a recombinant amidase. Analytical Biochemistry 346, 49–58.

219. Park HG, Do JH, Chang HN (2003) Regioselective enzymatic acylation of multi-hydroxyl compounds in organic synthesis. Biotechnology and Bioprocess Engineering 8(1), 1-8.

220. Park HJ, Uhm KN, Kim HK (2008) R-stereoselective amidase from *Rhodococcus erythropolis* No. 7 acting on 4-chloro-3-hydroxybutyramide. Journal of microbiology and biotechnology, 18(3), 552-559.

221. Patil D, Dev Bappaditya, Nag A (2011) Lipase catalyzed synthesis of 4-methoxy cinnamoyl glycerol. Journal of molecular catalysis B: Enzymatic 73, 5-8.

222. Pepeljnjak S, Zorc B, Butula I (2005) Antimicrobial activity of some hydroxamic acids. Acta Pharmaceutica-Zagreb 55(4), 401-408.

223. Pinung MC, Chau JH-L (1995) A convenient procedure for the preparation of amino acid hydroxamates. Journal of Organic Chemistry 60, 8084-8085.

224. Porcheddu A, Giacomelli G (2006) Angeli-Rimini reaction on solid support: A new approach to hydroxamic acids. Journal of Organic Chemistry 71, 7057-7059.

225. Prasad S, Sharma DR, Bhalla TC (2005) Nitrile- and amide-hydrolysing activity in *Kluyveromyces thermotolerans* MGBY 37. World Journal of Microbiology & Biotechnology 21, 1447–1450.

226. Prasad S, Bhalla TC (2010) Nitrile hydratases (NHases): At the interface of academia and industry. Biotechnology advances 28(6), 725-741.

227. Quraishi MA, Sardar R, Jamal D (2001) Corrosion inhibition of mild steel in hydrochloric acid by some aeromatic hydrazides. Materials Chemistry and Physics 71, 309-313.

228. Raadt A, Klempier N, Faber K, Griengl H (1992) Chemoselective enzymatic hydrolysis of aliphatic and alicyclic nitriles. Journal of the Chemical Society, Perkin Transactions 1, (1), 137-140.

229. Rahim MA, Saxena RK, Gupta R, Sheoran A, Giri B (2003) A novel and quick plate assay for acetamidase producers and process optimization for its production by *Aspergillus candidus*. Process Biochemistry 38, 861-866.

230. Rahim MBH, Syed MA, Shukor MY (2012) Isolation and characterization of an acrylamide-degrading yeast *Rhodotorula sp.* strain MBH23 KCTC 11960BP. Journal of Basic Microbiology 52(5), 573-581.

231. Ramakrishna C, Dave H, Ravindranathan M (1999) Microbial metabolism of nitriles and its biotechnological potential. Journal of Scientific and Industrial Research, 58(12), 925-947.

232. Ray SC, Nandi LN, Sing B, Prasad H, Maharaj S, Sarkar PK, Dutta P, Roy SK, Yadav SN, Bandyopadhyay (2004) Process for the synthesis of isonicotinic acid hydrazide. U.S. patent 6734309 B1.

233. Reddy AS, Kumar MS, Ravindra G (2000) A convenient method for the preparation of hydroxamic acids. Tetrahedron Letters 42, 6285-6288.

234. Refat HM, Fadda AA (2013) Synthesis and antimicrobial activity of some novel hydrazide, benzochromenone, dihydropyridine, pyrrole, thiazole and thiophene derivatives. European journal of medicinal chemistry 70, 419-426.

235. Reetz MT (2002) Lipases as practical biocatalysts. Current Opinion in Chemical Biology 6, 145-150.

236. Richard G, Nott K, Nicks F, Paquot M, Blecker C, Fauconnier ML (2013) Use of lipases for the kinetic resolution of lactic acid esters in heptane or in a solvent free system. Journal of Molecular Catalysis B: Enzymatic 97, 289-296.

237. Roveda JG, Clavette C, Hunt AD, Gorelsky SI, Whipp CJ, Beauchemin AM (2009) Hydrazides as tunable reagents for alkene hydroamination and aminocarbonylation. Journal of the American Chemical Society 131(25), 8740-8741.

238. Rustler S, Stolz A (2007) Isolation and characterization of a nitrile hydrolysing acidotolerant black yeast—*Exophiala oligosperma* R1. Applied Microbiology and Biotechnology 75, 899–908.

239. Santoshkumar M , Veeranagouda Y, Lee K , Karegoudar TB (2011) Utilization of aliphatic nitrile by *Paracoccus sp.* SKG isolated from chemical waste samples. International Biodeterioration & Biodegradation 65,153-159.

240. Saha A, Kumar R, Kumar R, Devakumar C (2010) Development and assessment of green synthesis of hydrazides. Indian Journal of Chemistry 49, 526-531.

241. Salawu OW, Abdulsalam AO (2011) Synthesis, characterization and biological activities of Cd (II) complexes with hydrazide ligands. Der Pharma Chemica 3(4), 298-304.

242. Sałdyka M, Mielke Z (2007) Keto–iminol tautomerism in acetohydroxamic and formohydroxamic acids: Experimental and theoretical study. Vibrational Spectroscopy 45(1), 46-54.

243. Sathyadevi P, Krishnamoorthy P, Butorac RR, Cowley AH, Dharmaraj N (2014) Novel ONN pincer type copper (II) hydrazide complexes: An investigation on the effect of electronegativity and ring size of heterocyclic hydrazides towards nucleic acid/protein binding, free radical scavenging and cytotoxicity. Inorganica Chimica Acta 409, 185-194

244. Saxena RK, Misra S, Rawat I, Gupta P, Dutt K, Parmar VS (2011) Production of 1, 3 regiospecific lipase from *Bacillus sp*. RK-3: Its potential to synthesize Cocoa Butter Substitute. Malaysian Journal of Microbiology 7(1) 41-48

245. Sathesh Prabu C, Thatheyus AJ (2007) Biodegradation of acrylamide employing free and immobilized cells of *Pseudomonas aeruginosa*. International biodeterioration & biodegradation 60(2), 69-73.

246. Scozzafava A, Supuran CT (2000) Carbonic anhydrase and matrix metalloproteinase inhibitors: sulfonylated amino acid hydroxamates with MMP inhibitory properties act as efficient inhibitors of CA isozymes I, II, and IV, and N-Hydroxysulfonamides inhibit both these Zinc enzymes 1. Journal of medicinal Chemistry 43(20), 3677-3687.

247. Seda SH, Janczak J, Lisowski J (2006) Synthesis and reactivity of copper (II) metallacrowns with S-phenylalanine and 2-picolinehydroxamic acids. Inorganica chimica acta 359(4), 1055-1063.

248. Semina II, Shilovskaya EV, Tarasova RI, Baichurina AZ, Pavlov VA, Tikhonova N A, Valeeva IKh, Garaev RS (2002) Mechanisms of the psychotropic action of phosphorylated carboxylic acid hydrazides. Pharmaceutical Chemistry Journal 36(4), 165-169.

249. Servat F, Montet D, Pina M, Galzy P, Arnaud A, Leodon H, Marcou L, Graille J (1990) Synthesis of fatty hydroxamic acids catalysed by the lipase of the *Mucor Miehei.* Journal of American Oil Chemist's Society 67(10), 646-649.

250. Shanker R, Ramakrishna C, Seth PK (1990) Microbial degradation of acrylamide monomer. Archives of microbiology 154(2), 192-198.

251. Sharma M, Sharma NN, Bhalla TC (2013) Purification studies on a thermo-active amidase of *Geobacillus pallidus* BTP-5x MTCC 9225 isolated from thermal springs of Tatapani (Himachal Pradesh). Applied Biochemistry and Biotechnology 169, 1–14.

252. Sharma NN, Sharma M, Bhalla TC (2011) An improved nitrilase–mediated bioprocess for synthesis of nicotinic acid from 3-cyanopyridine with hyperinduced *Nocardia globerula* NHB-2. Journal of Industrial Microbiology and Biotechnology 38, 1235-1243.

253. Sharma M, Sharma NN, Bhalla TC (2011) Biotransformation of acetamide to acetohydroxamic acid at bench scale using acyl tranferase activity of amidase of *Geobacillus pallidus* BTP-5x MTCC 9225. Indian Journal of Microbiology 52(1), 76-82.

254. Sharma M, Sharma NN, Bhalla TC (2009) Amidases: versatile enzymes in nature. Reviews in Environmental Science and Biotechnology 8, 343-366.

255. Shirota FN, DeMaster EG, Lee MJC and Nagasawa HT (1999) Generation of nitric oxide and possibly nitroxyl by nitrosation of sulfohydroxamic acids and hydroxamic acids. NITRIC OXIDE: Biology and Chemistry 6, 445–453.

256. Shotor SNM, Kassim K, Bahron H, Tajuddin AM, Yusof FZ, Marmion CJ, Rajab NF (2010, December). In-vitro cytotoxicity studies of vanadyl complexes with hydroxamic acid series. In Science and Social Research (CSSR), 2010 International Conference on (pp. 1283-1286). IEEE.

257. Shukor MY, Gusmanizar N, Ramli J, Shamaan NA, MacCormack WP, Syed MA (2009) Isolation and characterization of an acrylamide-degrading Antarctic bacterium. Journal of Environmental Biology 30(1), 107-112.

258. Shukor MY, Gusmanizar N, Azmi NA, Hamid M, Ramli J, Shamaan NA, Syed MA (2009) Isolation and characterization of an acrylamide-degrading *Bacillus cereus*. Journal of Environmental Biology 30(1), 57-64.

259. Siódmiak T, Ziegler-Borowska M, Marszałł MP (2013) Lipase-immobilized magnetic chitosan nanoparticles for kinetic resolution of (R,S)-ibuprofen. Journal of Molecular Catalysis B: Enzymatic 94, 7-14.

260. Silman NJ, Carver MA, Jones CW (1991) Directed evolution of amidase in *Methylophilus methylotrophus*; purification and properties of amidases from wild-type and mutant strains. Journal of general microbiology 137(1), 169-178.

261. Silva C, Cavaco-Paulo A (2008) Biotransformations in synthetic fibres. Biocatalysis and Biotransformation 26(5), 350-356.

262. Sinha N, Jain S, Tilekar A, Upadhayaya RS, Kishore N, Jana GH, Arora SK (2005) Synthesis of isonicotinic acid N′-arylidene-N-[2-oxo-2-(4-aryl-piperazin-1-yl)-ethyl]-hydrazides as antituberculosis agents. Bioorganic & medicinal chemistry letters 15(6), 1573-1576.

263. Sinsuwan S, Rodtong S, Yongsawatdigul J (2010) A NaCl-stable serine proteinase from *Virgibacillus sp.* SK33 isolated from Thai fish sauce. Food Chemistry 119(2), 573-579.

264. Skouloubris S, Labigne A, De Reuse H (1997) Identification and characterization of an aliphatic amidase in *Helicobacter pylori*. Molecular microbiology 25(5), 989-998.

265. Skouloubris S, Labigne A, De Reuse H (2001) The AmiE aliphatic amidase and AmiF formamidase of *Helicobacter pylori*: natural evolution of two enzyme paralogues. Molecular microbiology 40(3), 596-609.

266. Šnajdrová R, Kristová-Mylerová v, Crestia d, Nikolaou K, Kuzmaa M, Lemaire M, Gallienne E, Bolte J, Bezouška K, K˘ren V, Mart´ınková L (2004) Nitrile biotransformation by *Aspergillus niger*. Journal of Molecular Catalysis B: Enzymatic 29, 227–232.

267. Snell D, Colby J (1999) Enantioselective hydrolysis of racemic ibuprofen amide to s-(+)-ibuprofen by *Rhodococcus* AJ270. Enzyme and microbial technology 24(3), 160-163.

268. Sonke T, Ernste S, Tandler RF, Kaptein B, Peeters WPH, Friso B. J. van Assema FBJ, Wubbolts MG, Schoemaker HE (2005) L-Selective amidase with extremely broad substrate specificity from *Ochrobactrum anthropi* NCIMB 40321. Applied and Environmental Microbiology 71(12), 7961-7973.

269. Sorokin DY, Van Pelt S, Tourova TP, Takaichi S, Muyzer G (2007) Acetonitrile degradation under haloalkaline conditions by *Natronocella acetinitrilica* gen. nov., sp. nov. Microbiology 153(4), 1157-1164.

270. Sriram D, Yogeeswari P, Madhu K (2005) Synthesis and in vitro and in vivo antimycobacterial activity of isonicotinoyl hydrazones. Bioorganic & medicinal chemistry letters 15(20), 4502-4505.

271. Stelkes-Ritter U, Wyzgol K, Kula MR (1995) Purification and characterization of a newly screened microbial peptide amidase. Applied microbiology and biotechnology 44(3-4), 393-398.

272. Suzuki Y, Ohta Y (2006) Identification of a thermostable and enantioselective amidase from the thermoacidophilic archaeon *Sulfolobus tokodaii* strain 7. Protein Expression and Purification 45, 368–373.

273. Suhendra D, Yunus WMZW, Haron M J, Basri M, Silong S (2005) Enzymatic synthesis of fatty hydroxamic acids from palm oil. Journal of Oleo Science 54, (1), 33-38.

274. Summerskill WH, Thorsell F, Feinberg JH, Aldrete JS (1968) Effects of urease inhibition in hyperammonemia: clinical and experimental studies with acetohydroxamic acid. Gastroenterology 54(1), 20.

275. Sycheva TP, Pavlova TN Shchukina MN (1972) Synthesis of isoniazid from 4-cyanopyridine. Pharmaceutical Chemistry Journal 6(11), 696-698.

276. Syed MA, Ahmad SA, Kusnin N, Shukor MYA (2012) Purification and characterization of amidase from acrylamide-degrading bacterium *Burkholderia sp.* strain DR. Y27. African Journal of Biotechnology 11(2), 329-336.

277. Takabe K, Mase N, Hisano T, Yoda H (2003) Highly regioselective lipase-catalyzed acetylation and hydrolysis of acyclic α, ω-terpenediols and their diacetates. Tetrahedron letters 44(16), 3267-3269.

278. Takebe S, Kobashi K, Hase J, Koizumi T (1982) Kinetic study on ureolysis in rats. Chemical and Pharmaceutical .Bulletin 31(2), 600-604.

279. Tani Y, Kurihara M, Nishise H (1989) Characterization of nitrile hydratase and amidase, which are responsible for the conversion of dinitriles to mononitriles, from *Corynebacterium sp*. Agricultural and biological chemistry 53(12), 3151-3158.

280. Tanis SP, Plewe MB, Johnson TW, Butler SL, Dalvie D, DeLisle D, Dress KR, Hu Q, Huang B, Kuehler JE, Kuki A, Lu W, Peng Q, Smith GL, Solowiej J,, Tran KT, Wang H, Yang A, Yin C, Yu X, Zhu H (2010) Azaindole *N*-methyl hydroxamic acids as HIV-1 integrase inhibitors-II. The impact of physicochemical properties on ADME and PK. Bioorganic & medicinal chemistry letters 20(24), 7429-7434.

281. Tauber MM, Cavaco-Paulo A, Robra KH, Gübitz GM (2000) Nitrile hydratase and amidase from *Rhodococcus rhodochrous* hydrolyze acrylic fibers and granular polyacrylonitriles. Applied and environmental microbiology 66(4), 1634-1638.

282. Taylor RJ, May I, Wallwork AL, DDenniss IS, Hill NJ, Galkin BY, Zilberman BY, Fedorov YS (1998) The applications of formo- and aceto-hyroxamic acids in nuclear fuel reprocessing. Journal of Alloys and Compounds 271-273, 534-537.

283. Thiery A, Maestracci M, Arnaund A, Galzy P (1986) Acyltransferase activity of the wide spectrum amidase of *Brevibacterium sp.* R312. Journal of General Microbiology 132, 2205-2208.

284. Toit LCD, Pillay V, Danckwerts MP (2006) Tuberculosis chemotherapy: current drug delivery approaches. Respiratory Research 7, 118.

285. Toogood HS, Brown RC, Line K, Keene PA, Taylor SJC, McCagueb R, Littlechild JA (2004) The use of a thermostable signature amidase in the resolution of the bicyclic synthon (rac)-g-lactam. Tetrahedron 60, 711–716.

286. Trott S, Bauer R, Knackmuss HJ, Stolz A (2001) Genetic and biochemical characterization of an enantioselective amidase from *Agrobacterium tumefaciens* strain d3. Microbiology 147(7), 1815-1824

287. Türkel N (2011) Stability of metal chelates of some hydroxamic acid ligands. Journal of Chemical & Engineering Data 56(5), 2337-2342.

288. Ul-Haq Z, Wadood A, Uddin R (2009) CoMFA and CoMSIA 3D-QSAR analysis on hydroxamic acid derivatives as urease inhibitors. Journal of enzyme inhibition and medicinal chemistry 24(1), 272-278.

289. Vaidya BK, Mutalik SR, Joshi RM, Nene SN, Kulkarni BD (2009) Enhanced production of amidase from *Rhodococcus erythropolis* MTCC 1526 by medium optimisation using a statistical experimental design. Journal of Industrial Microbiology and Biotechnology 36, 671–678.

290. Vaysse L, Dubreucg E, Pirat J-L, Galzy P (1997) Fatty hydroxamic acid biosynthesis in aqueous medium in the presence of the lipase-acyltransferase from *Candida parapsilosis*. Journal of Biotechnology 53(6), 41-46.

291. Vejvoda V, Martínková L, Veselá AB, Kaplan O, Wahl SL, Fischer L, Uhnáková B (2011) Biotransformation of nitriles to hydroxamic acids via a nitrile hydratase-amidase cascade reaction. Journal of Molecular Catalysis B: Enzymatic 71, 51-55.

292. Vejvoda V, Kaplan O, Kubac D, Kren V, Martínková L (2006) Immobilization of fungal nitrilase and bacterial amidase-two enzymes working in accord. Biocatalysis and Biotransformation 24(6), 414-418.

293. Vejvoda V, Kubáč, D, Davidová, A, Kaplan O, Šulc M, Šveda O, Chaloupková, Martínková L (2010) Purification and characterization of nitrilase from *Fusarium solani* IMI196840. Process Biochemistry 45(7), 1115-1120.

294. Verma RP (2012) Hydroxamic Acids as Matrix Metalloproteinase Inhibitors, In Matrix Metalloproteinase Inhibitors (pp. 137-176), Springer Basel.

295. Wandel U, Machado SS, Jongejan JA, Duine JA (2001) Enzymes involved in the glycidaldehyde (2, 3-epoxy-propanal) oxidation step in the kinetic resolution of racemic glycidol (2, 3-epoxy-1-propanol) by *Acetobacter pasteurianus*. Enzyme and microbial technology 28(2), 233-239.

296. Wang MX, Feng GQ (2000) Enantioselective synthesis of chiral cyclopropane compounds through microbial transformations of trans-2-arylcyclopropanecarbonitriles. Tetrahedron Letters 41(33), 6501-6505.

297. Wang C, Lee C, Cheng P (2001) Acrylonitrile removal from synthetic wastewater and actual industrial wastewater with high strength nitrogen using a pure bacteria culture. Water Science & Technology 43(2), 349-354.

298. Wang MX, Liu CS, Li JS (2002) Enzymatic desymmetrization of 3-alkyl-and 3-arylglutaronitriles, a simple and convenient approach to optically active 4-amino-3-phenylbutanoic acids. Tetrahedron: Asymmetry 12(24), 3367-3373.

299. Wang CC, Lee CM, Chen LJ (2004) Removal of nitriles from synthetic wastewater by acrylonitrile utilizing bacteria. Journal of Environmental Science and Health, Part A 39(7), 1767-1779

300. Wang, Yuan-Shan, Xu JM, Zheng RC, Zheng YG, Shen YC (2008) Improvement of amidase production by a newly isolated *Delftia tsuruhatensis* ZJB-05174 through optimization of culture medium. Journal of Microbiology and Biotechnology 18(12), 1932–1937.

301. Wang YS, Zheng RC, Xu JM, Liu ZQ, Cheng F, Feng ZH, Liu LL, Zheng YG, Shen YC (2010) Enantioselective hydrolysis of (R)-2, 2-dimethylcyclopropane carboxamide by immobilized cells of an R-amidase-producing bacterium, *Delftia tsuruhatensis* CCTCC M 205114, on an alginate capsule carrier. Journal of industrial microbiology & biotechnology 37(5), 503-510.

302. Wardakhan WW, EL-Sayed NN, Mohareb RM (2013) Synthesis and anti-tumor evaluation of novel hydrazide-hydrazone derivatives. Acta pharmaceutica 63(1), 45-57.

303. Weatherburn MW (1967) Phenol-hypochlorite reaction for determination of ammonia. Analytical chemistry 39(8), 971-974.

304. Winder FG, Collins PB (1970) Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis.* Journal of General Microbiology 63, 41-48.

305. Woo Y, Mitchell AR, Camarero JA (2007) The use of aryl hydrazide linkers for the solid phase synthesis of chemically modified peptides. International Journal of Peptide Research and Therapeutics 13, 181-190.

306. Wuensch C, Glueck SM, Gross J, Koszelewski D, Schober M, Faber K (2012) Regioselective enzymatic carboxylation of phenols and hydroxystyrene derivatives. Organic Letters 14(8), 1974-1977.

307. Wyatt JM, Knowles CJ (1995) The development of a novel strategy for the microbial treatment of acrylonitrile effluents. Biodegradation 6(2), 93-107.

308. Wyborn NR, Mills J, Williams SG, Jones CW (1996) Molecular characterisation of formamidase from *Methylophilus methylotrophus*. European Journal of Biochemistry 240(2), 314-322.

309. Xue Z, Chao Y, Wang D, Wang M, Qian S (2011) Overexpression of a recombinant amidase in a complex auto-inducing culture: purification, biochemical characterization, and regio- and stereoselectivity. Journal of Industrial Microbiology and Biotechnology 38, 1931-1938.

310. Yale HL, Losee K, Martins J, Holsing M, Perry FM, Bernstein (1953) Chemotherapy of experimental tuberculosis. VIII. The synthesis of acid hydrazides, their derivatives and related compounds.75, 1933-1942.

311. Yadav GD, Sajgure A D (2007) Synergism of microwave irradiation and enzyme catalysis in synthesis of isoniazid. Journal of Chemical Technology and Biotechnology 82, 964–970.

312. Yadav GD, Joshi SS, Lathi PS (2005) Enzymatic synthesis of isoniazid in non-aqueous medium. Enzyme and Microbial Technology 36, 217-222.

313. Yadav GD, Borkar IV (2010) Lipase-catalysed hydrazinolysis of phenyl benzoate: kinetic modelling approach. Process Biochemistry 45, 586-592.

314. Yang Z-Y, Ni Y, Lub Z-Y, Liao X-R, Zheng Y-G, Suna Z-H (2011) Industrial production of S-2,2-dimethylcyclopropanecarboxamide with a novel recombinant R-amidase from *Delftia tsuruhatensis*. Process Biochemistry 46, 182–187.

315. Yamamoto K, Ueno Y, Otsubo K, Yamane H, Komatsu KI, Tani Y (1992) Efficient conversion of dinitrile to mononitrile-monocarboxylic acid by *Corynebacterium sp.* C5 cells during tranexamic acid synthesis. Journal of fermentation and bioengineering 73(2), 125-129.

316. Yao C, Cao Y, Wu S, Li S, He B (2012) An organic solvent and thermally stable lipase from *Burkholderia ambifaria* YCJ01: purification, characteristics and application for chiral resolution of mandelic acid. Journal of Molecular Catalysis B: Enzymatic (85-86), 105-110.

317. Yeom S-J, Kim H-J, Oh D-K (2007) Enantioselective production of 2,2-dimethylcyclopropane carboxylic acid from 2,2-dimethylcyclopropane carbonitrile using the nitrile hydratase and amidase of *Rhodococcus erythropolis* ATCC 25544. Enzyme and Microbial Technology 41, 842–848.

318. Yokoyama M, Kashiwagi M, Iwasaki M, Fuhshuku KI, Ohta H, Sugai T (2004) Realization of the synthesis of α, α-disubstituted carbamylacetates and cyanoacetates by either enzymatic or chemical functional group transformation, depending upon the substrate specificity of *Rhodococcus* amidase. Tetrahedron: Asymmetry 15(18), 2817-2820.

319. Yoon SH, Robyt JF (2005) Activation and stabilization of 10 starch-degrading enzymes by Triton X-100, polyethylene glycols, and polyvinyl alcohols. Enzyme and microbial technology 37(5), 556-562.

320. Yusuf F, Chaubey A, Jamwal U, Parshad R (2013) A new isolate from *Fusarium proliferatum* (AUF-2) for efficient nitrilase production. Applied Biochemistry and Biotechnology 171, 1022–1031.

321. Zhang XB, Li M (2008) Synthesis and self-assembly of novel hydrazide derivatives containing multi-alkoxy chains with different lengths. Journal of Molecular Structure 892(1), 490-494.

322. Zhang LB, Wang DX, Wang MX (2011) Microbial whole cell-catalyzed desymmetrization of prochiral malonamides: practical synthesis of enantioenriched functionalized carbamoylacetates and their application in the preparation of unusual α-amino acids. Tetrahedron 67(31), 5604-5609.

323. Zhang Z-J, Pan J, Liu J-F, Xu J-H, He Y-C, Liu YY (2011a) Significant enhancement of (R)-mandelic acid production by relieving substrate inhibition of recombinant nitrilase in toluene-water biphasic system. Journal of Biotechnology 152, 24-29.

324. Zhang X, Breslav M, Grimm J, Guan K, Huang A, Liu F, Maryanoff CA, Palmer D, Patel M, Qian Y, Shaw C, Sorgi K, Stefanick S, Xu D (2002) A new procedure for preparation of Carboxylic acid hydrazides. Journal of Organic Chemistry 67, 9471-9474.

325. Zargoosh K, Abedini H, Abdolmaleki A, Molavian MR (2013) Effective removal of heavy metal ions from industrial wastes using thiosalicylhydrazide-modified magnetic nanoparticles. Industrial & Engineering Chemistry Research 52(42), 14944-14954.

326. Zemtsova MN, Zimichev AV, Trakhtenberg PL, Belen'kaya RS, Boreko EI (2008) Synthesis and antiviral activity of 4-quinolinecarboxylic acid hydrazides. Pharmaceutical Chemistry Journal 42(10), 571-573.

327. Zemlyakov AE, Kur'yanov VO, Tsikalova VN, Chirva VY (1997) Synthesis of conjugates of glyco amino acids and glycopeptides with carboxylic acid hydrazides. Chemistry of natural compounds 33(5), 568-570.

328. Ziskind MM, Calovich E, Joffko J, Jacobs S (1953) The use of the iso-nicotinic acid hydrazides in the treatment of tuberculosis. CHEST Journal 24(5), 535-544.

329. Zheng R-C, Wang Y-S, Liu Z-Q, Xing L-Y, Zheng Y-G, Shen Y-C (2007) Isolation and characterization of *Delftia tsuruhatensis* ZJB-05174, capable of R-enantioselective degradation of 2,2-dimethylcyclopropanecarboxamide. Research in Microbiology 158, 258-264.

330. Zheng YG, Chen J, Liu ZQ, Wu MH, Xing LY, Shen YC (2008) Isolation, identification and characterization of *Bacillus subtilis* ZJB-063, a versatile nitrile-converting bacterium. Applied microbiology and biotechnology 77(5), 985-993.

331. Zubareva GI, Adeev SM, Radushev AV, Gomzikov AI, Zubarev MP (1998) Treatment of wastewaters to remove metal ions by flotation with hydrazides of aliphatic carboxylic acids. Russian journal of applied chemistry 71(2), 283-287.