Production, purification, immobilization and application of penicillin acylase from *Acremonium sclerotigenum*

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JEEVAN LATA

DEPARTMENT OF BIOTECHNOLOGY
HIMACHAL PRADESH UNIVERSITY
SUMMER HILL, SHIMLA-5, INDIA

HIMACHAL PRADESH UNIVERSITY
NAAC ACCREDITED 'A' GRADE UNIVERSITY
DEPARTMENT OF BIOTECHNOLOGY
SUMMER HILL, SHIMLA, INDIA
Phone: +91-177-2831948, Fax: +91-177-2831948

Dr. Duni Chand
Chairman & Coordinator
(Ph. D. Appl. Microbiol) PDF (DAAD) Germany

Dated: .....

CERTIFICATE

This is to certify that **Ms. Jeevan Lata** has worked on the thesis entitled **"Production, purification, immobilization and application of penicillin acylase from *Acremonium sclerotigenum*"** under my supervision and guidance. The content of the thesis being submitted, to Faculty of Life Sciences, Department of Biotechnology, Himachal Pradesh University, Shimla for the award of degree of **Doctor of Philosophy in Biotechnology** contains original work done by her in the Department of Biotechnology, Himachal Pradesh University, Shimla. Further, no part of this work has been earlier submitted for any other degree of this or any other university.

(Duni Chand)

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Abbreviations

6-APA	6-aminopenicillanic acid
°C	Degree Celsius
dcw	Dry cell weight
DTT	Dithiothreitol
et al.	et alii
EDTA	Ethylene di amine tetra acetic acid
Fig.	Figure
g	Gram
g /l	Gram per litre
h	Hour
HIC	Hydrophobic interaction chromatography
HPLC	High performance liquid chromatography
kb	Kilo base
kDa	Kilo Dalton
L	Litre
LCB	Lactophenol cotton blue
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
MW	Molecular weight
N	Normal
NA	Not available
nm	Nanometer
OD	Optical density
NMR	Nuclear magnetic resonance
PA	Penicillin acylase
PAC	Penicillin G acylase
PEG	Polyethylene glycol
pH	Potential of hydrogen
*p*DAB	Paradimethylaminobenzeldehyde

PGA	Penicillin G acylase
PVA	Penicillin V acylase
rpm	Revolutions per minute
FTIR	Fourier Transform Infrared Spectroscopy
sp.	Species
SEM	Scanning Electron Microscope
SSP	Semisynthetic penicillins
$t_{1/2}$	Half life
TEMED	N, N, N', N', - Tetra methyl ethylene di amine
U	Units
UV	Ultraviolet
v/v	Volume by volume
w/v	Weight by volume
w/w	Weight by weight
µg	Microgram
µl	Microlitre
µM	Micromolar

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Chapter -1 Introduction

Enzymes are protein molecules and the most commonly used biocatalysts in various biotransformation processes, which are preferred over the conventional chemical conversion processes. The biotransformation processes offer multiple advantages, like less chemical load on the environment, higher efficiency and ability to dilute multiple downstream transformation attempts, while maintaining better product yield and recovery (Chandel *et al*., 2008). These are specific for a particular reaction and serves to accelerate the biochemical reactions by several orders of magnitude (Charles, 2003). Enzymes as drugs have two important features that distinguish them from all other types of drugs. First, enzymes often bind and act on their targets with great affinity and specificity. Second, enzymes convert multiple target molecules to the desired products. These two features make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that small molecules cannot. These characteristics have resulted in the development of many enzyme drugs for a wide range of disorders (Vellard, 2003).

During the last decade most of the biocatalytic processes for the synthesis of several antibiotics have been developed and penicillin acylases (Penicillin amidohydrolases, EC 3.5.1.11) which belong to the structural super family of the Ntn (N terminal nucleophile) hydrolases are the most widely used biocatalysts for the industrial production of semi synthetic β-lactam antibiotics (Srirangan *et al.,* 2013; Maresova *et al.,* 2014). Penicillin acylases are found in microorganisms such as bacteria, yeast and fungi are used to catalyze the hydrolysis of natural penicillin's. They are also used in the hydrolysis of cephalosporins, which yield another group of common antibiotics. Penicillin antibiotics were among the first drugs to be effective against many serious diseases, such as bacterial infections caused by syphilis, *Staphylococci* and *Streptococci* (Javadpour *et al.,* 2002). All penicillins are β-lactam antibiotics and used in the treatment of bacterial infections caused by susceptible, usually Gram-positive, organisms. Naturally occurring penicillins are increasingly becoming ineffective for treatment of various antibiotic-resistant pathogens, newer and effective antibiotics have to be discovered or developed. In this quest, semi-synthetic penicillins, with a natural β-lactam nucleus and a custom-designed side chain, look promising, although resistance to some semi-synthetic penicillin is also a

serious problem. Various semi-synthetic penicillins have greater selectivity against pathogens, increased resistance to β-lactamase and improved pharmacological properties compared to naturally occurring penicillins (Rathinaswamy *et al.*, 2012).

Based on their substrate specificity, penicillin acylases have been classified into three groups, penicillin G-acylases, penicillin-V-acylases and ampicillin acylases. They are both intracellular and extracellular enzymes, whose large scale production is achieved by nutrient controlled metabolism and by genotypic changes of the producing microorganisms (Arroyo *et al*., 2003). The first report on the enzyme penicillin acylase was in 1950 by Sakaguchi and Murao (1950) when they found the enzyme in mycelium of *Penicillium chrysogenum* capable of hydrolyzing penicillin G in to phenyl acetic acid and the unknown 6 APA (named penicin). In the course of more than 60-years history, penicillin G acylase (PGA) gained a unique position among enzymes used by pharmaceutical industry for production of β-lactam antibiotics (Maresova *et al*., 2014).

The discovery of 6-APA by scientists in the Beecham Research Laboratories was a major event in the fight against infection at a time when there was a real danger of the battle being lost owing to the emergence of bacteria resistant to available antibiotics and the recognition that some of the available agents had potentially serious adverse reactions. The introduction of immuno suppressive drugs, organ transplantation ,other major surgical procedures and hospital-acquired infections in general resulted in the emergence of 'difficult to treat' infections often caused by resistant bacteria, requiring the availability of new antimicrobial agents The discovery of 6-APA was followed by major advances in the development of the β-lactam antibiotics, with the saving of probably millions of lives and a massive reduction in morbidity from infection (Rolinson and Geddes, 2007).

The characteristics of penicillin G acylase isolated from different biological and environmental sources were found to be varied in different aspects including, substrate specificity, optimum pH, temperature tolerance etc necessitated extensive screening of microorganisms for isolation of novel penicillin acylases with higher compatibility with industrial deacylation requirements. Penicillin V acylase is produced mainly by moulds and actinomycetes. They are mainly intracellular enzymes but also genus *Fusarium*(Sudhakaran and Shewale, 1993) and actinomycetes *Streptomyces lavendulae* could produce extracellular acylases (Torres *et al*., 2000).

Penicillin acylases are involved in the industrial production of semi synthetic penicillins and cephalosporins, which remains the most widely used group of antibiotics (De Vroom, 1999). Beta-lactam antibiotics, in particular penicillins and cephalosporins, represent one of the world's major biotechnology markets. With annual sales of $15 billion, they make up ~ 65% of the total antibiotics market (Elander, 2003; Chandel *et al*., 2008; Pinotti *et al*., 2007) which explains such a tremendous and continuous interest in this enzyme (Maresova *et al.*, 2014). Semi synthetic pencillins exhibit better properties such as increased stability, easier absorption and fewer side effects. Penicillin G and V and represent a practical solution to the problem of adaptive microbial resistance to antibiotics (Bruggink *et al*., 1998; Hoople, 1998; Wegman et *al*., 2001). Both penicillin G acylase and penicillin V acylase used for industrial production of 6-APA. Penicillin V acylase has some advantages over penicillin G acylase like better stability and higher conversion rates. The industrial application of penicillin V acylase has so far been limited due to the non-availability of suitable bacterial strains and cost issues (Avinash *et al.*, 2016). Several bacteria and yeast have been used for the development of expression systems for the PGA production. Among them, periplasmic over production in *Escherichia coli* hosts is the state-of-art technology predominantly used on industrial scale (Maresova *et al*., 2017).

Most of the new semi synthetic penicillins are produced from 6-aminopenicillanic acid (6-APA), which in turn is produced mainly by enzymatic or chemical deacylation (Chisti and Moo-Young, 1991) of the natural benzyl penicillin. The chemical methods for producing 6-APA are environmentally burdensome and require the use of hazardous chemicals such as pyridine, phosphorous pentachloride, and nitrosylchloride (Matsumoto, 1993; Vandamme, 1988). In contrast, the enzymatic conversion is regio- and stereo-specific and the reaction conditions are mild (Parmar *et al.*, 2000).

The industrial production of 6-APA has undergone a remarkable transformation due to the reason that penicillin acylase catalyzed processes have replaced traditional chemical conversions. The traditional chemical synthesis of 6-APA began around 1970 at Gist-Brocades and consisted of a one pot deacylation of the fermentation product penicillin G using a procedure requiring hazardous chemicals and solvents. This approach remained in use for 15-20 years until it was largely

replaced by penicillin acylase catalyzed hydrolysis of penicillin G which affords 6-APA in good yield (Shewale and Sudhakaran, 1997).

High selectivity of penicillin acylase in a single step conversion reaction makes the enzyme a cost-effective and environmentally friendly alternative to conventional processes, which involve multiple chemical reactions (Chandel *et al*., 2008). The majority of deacylation in β-lactam production processes depend on penicillin G acylase. Developments indicated that the enzyme can also be successfully exploited in a synthetic direction (Giordano *et al.,* 2006). Subsequent production processes of valuable antibiotics such as amoxicillin and ampicillin are based on the condensation of an appropriate D-amino-acid derivative with 6-APA (Giordano *et al.,* 2006).

Penicillin acylases are useful as biocatalysts in many potentially valuable reactions such as protection and deprotection of amino groups of amino acids by direct enzymatic synthesis and acyl group transfer reactions. These strategies have been applied to the synthesis of peptides and their derivatives. As a classical example, penicillin G acylase from *E. coli* proved to be an efficient biocatalyst in the synthesis of the sweetener aspartame (Fuganti *et al*., 1986a).

Penicillin acylases have been shown to efficiently resolve racemic mixture of chiral compounds such as amino acids (Cardillo *et al.,* 1996; Fadnavis *et al.,*1997; Bossi *et al*., 1998; Ng *et al*., 2001) beta-amino esters, (Roche *et al*.,1999), amines (VanLangen *et al*., 2000 b; Guranda *et al.,* 2001;) and secondary alcohols (Fuganti *et al.,*1986 b; Svedasa *et al*.,1997) in aqueous media as well as in water co-solvent mixtures and anhydrous organic media. The resulting pure enantiomers can be used as intermediates in the synthesis of biologically active compounds. The PGAs of two bacterial species, i.e. *E. coli* and *A. faecalis* were used in these reactions. Only the PGAEc (PGA of *E.coli*) was used by as a free enzyme (Chilov *et al*., 2003; Deaguero *et al*., 2010, 2012; Topgi *et al.,* 1999). Kumaraguru and Fadnavis (2012) exploited PGAEc for resolution of a racemic mixture of 4- oxocyclopent- 2- en-1-yl 2- phenyl acetate in diisopropyl ether. Enantiomerically pure (R) and (S) 4-hydroxy-2-cyclopentenones are versatile intermediates for the synthesis of a large number of complex natural products such as prostaglandins, prostacyclins, thromboxane and nucleosides. PGA-catalyzed N-deacylation has been exploited in biosensors. Analytical devices called enzyme thermistors are used as sensors in flow meters for

gases and liquids.Yakovleva *et al.*(2013) developed sensitive enzyme thermistors based on immobilized PGA that were applied to determine penicillin G concentration ranging from 0.02 to 200 mM.

Keeping in view the potential of these hydrolases the present work entitled **"Production, purification, immobilization and application of penicillin acylase from *Acremonium sclerotigenum*"** has been undertaken with the following objectives.

I. Isolation of novel penicillin acylase producing microorganisms
II. Optimization of production condition and reaction parameters for penicillin acylase from *Acremonium sclerotigenum*
III. Purification of the penicillin acylase to homogeneity
IV. Molecular characterization of purified penicillin acylase from *Acremonium sclerotigenum*
V. Immobilization of penicillin acylase on different matrices
VI. Bench scale production of 6-amino penicillanic acid (6-APA)

Introduction

Penicillin acylases (penicillin amidohydrolases, EC 3.5.1.11 are a group of enzymes that catalyze the selective hydrolysis of the relatively stable side chain amide bond in penicillins and cephalosporins while leaving the labile β-lactam ring intact. For many years the enzymes capable of performing this unique transformation (mainly penicillin acylase from *E. coli)* have been widely studied as industrial biocatalysts for the modification of β-lactam antibiotics (Vandamme, 1980; Abbott, 1976; Bruggink *et al*., 1998). Nowadays penicillin acylases have become the focus of interest from the viewpoint of fundamental enzymology.

2.1 Historical background

Acylase activity was first described by workers in Japan (Sakaguchi and Murao, 1950) in *Penicillium chrysogenum* Q 176; they reported that the mycelia of this strain were capable of converting benzyl penicillin into phenyl acetic acid and a compound that they called "penicin". Further details of this reaction were subsequently given by Murao (1955), who showed that penicin was degraded by penicillinase to "penicic acid" which was strongly ninhydrin positive and reported that "penicin" had a melting point of 157 to 160°C. Kato (1953) suggested that penicin was in fact the penicillin nucleus 6-APA.The matter rested for some years until Batchelor and his colleagues (Batchelor *et al*., 1959) at the Beecham Research Laboratories reported that 6 APA had been detected in a fermentation brew of *P.chrysogenum* W5120 to which no side chain precursors had been added. The identity of this 6-APA was proved by these workers by virtue of the fact that, on phenylacetylation, benzylpenicillin was produced.

2.2 Occurrence of penicillin acylases in nature

Potential of enzymes extraction from different microbial sources, that are able to catalyze many industrial processes offer great biotechnological possibilities. This gives the possibility of choosing the most adequate industrial enzyme. An accurate selection of a given native enzyme may help to overcome a number of obstacles which hinder a massive implementation of enzyme derivatives as industrial catalysts (Bruggink *et al.,* 1998). Penicillin acylase occurs in many bacteria, yeasts and

filamentous fungi. More than a decade, this enzyme is commercially employed for the large-scale hydrolysis of penicillin G that is fermentatively produced by *Penicillium chrysogenum* (Shewale *et al*., 1990; Alvaro *et al*., 1992; Demain, 2000).

2.3 Sources of penicillin acylases

2.3.1 Bacterial sources

Penicillin G acylase: Penicillin G acylase is second most commonly used enzyme worldwide after glucose isomerase (Silva *et al*., 2006). Bacterial sources of penicillin acylase are *E.coli, Bacillus megaterium, Streptomyces lavendulae, Achromobacter* sp., *Proteus rettgeri, Actinoplanes* sp., *Bavista plumbe, Kluyvera atrophila, Pseudomonas melanogenum* (Parmar *et al*., 2000). *Shigella, Xenthomonas, Xylella, Arthrobacter viscous, Alcaligenes faecalis* and *Providencia rettgeri* (Grulich *et al.,* 2013). The enzyme is secreted either as intracellular or extracellular or inclusion bodies (Premalatha *et al*., 2013).

Penicillin V acylase: *Bacillus sphaericus* (Arroyo *et al*., 2002 a) and *S. lavendulae* produces an extracellular PVA. *Beijerinckia indica var. Bacillus subtilis* and *Pseudomonas acidovorans* produced PVA intracellularlly (Torres *et al*., 1999).

2.3.2 Fungal, actinomycetes or yeast sources

Pichia pastoris and *Rhodotorula glutinis* (yeast) have been used for cytoplasmic or extracellular expression (Grulich *et al*., 2013).Other fungal sources of penicillin acylase reported are *Penicillin chrysogenum, Fusarium* sp., *Alterneria, Cephalosporium, Cryptococcus, Emericellopsis, Mucor, Trichoderma, Trichophyton Trichosporon* and *Epidermophyton* (Moharram *et al*., 2013). Penicillin V has some advantage over penicillin G like better stability, higher conversion rates. But industrial application of PVA's has so far been limited due to the non-availability of suitable bacterial strain and cost issues (Avinash *et al*., 2016). This group of enzymes is classified into three subtypes based on substrate preferences: penicillin acylases (PVA) which preferentially hydrolyze penicillin V (phenoxymethyl penicillin, Type I), penicillin G acylases which prefer penicillin G (PG, benzylpenicillin; Type II) and ampicillin acylases which specifically hydrolyze ampicillin (Type III) (Hamilton-Miller, 1966; Vandamme and Voets, 1974).

Table 2.1 Main characteristics of some penicillin acylases from different sources

Class	Source	Preferable substrate	Induction of biosynthesis	Cell localization	References
Class I PA-V	*Streptomyces lavendulae*	Pen-V, Pen-K	No	Outside	Hamilton-Miller,1966 Torres-Bacete *et al*., 2015
	Dermatophytes	Pen-V	Yes	Inside	Hamilton-Miller, 1966
	Penicillium chrysogenum	Pen-V, Pen-K	Yes	Inside	Hamilton-Miller, 1966
	Bacillus sphaericus	Pen-V	No data	Outside	Pundle & Sivaraman, 1994
	Fusarium sp.	Pen-V	No data	Outside	Sudhakaran and Shewale, 1995
	Streptomyces mobaraensis	Pen-V	No data	Outside	Zhang *et al*., 2007
	Actinoplanes utahensis	Pen-K	No data	No data	Torres Bacete *et al.*, 2007
	Rhodotorula aurantiaca	Pen-V	No data	Inside	Kumar *et al.*, 2008
	Bacillus cereus ATUAVP1846	Pen-V, Ampicillin, Cephalexin	Constitutive	Inside	Sunder *et al.*, 2012
	Pectobacterium atrosepticum	Pen-V	No data	Outside	Avinash *et al*., 2014
	Acinetobacter sp.	Pen-V	Constitutive	Outside	Philem *et al.*, 2016
	Streptomyces sp. AAP1846	Pen-V	No data	Inside	Mukherji *et al*., 2014

Class II PA-G	*Bacillus megaterium*	Pen-G , Cephalexin, Cephaloglycine, Cephaloridine	No data	Outside	Savidge and Cole,1975
	Alcaligenes faecalis	Pen-G	No data	Inside	Verhaert *et al*., 1997
	E. coli	Pen-G, Pen-X	Yes	Inside	Hewitt *et al*., 2000
	Bacillus sp.	Pen-G	Yes	Inside	Rajendhran *et al.*, 2002
	Achromobacter sp.	Ampicillin	Inducible	Inside	Plhackovak *et al.*, 2003
	Providencia rettgeri	Pen-G	No data	Inside	Cheng *et al*., 2006
	Thermus thermophilus	Pen-K, Pen-G	No data	No data	Torres *et al.*, 2012
Class III Amp-PA	*Pseudomonas melanogenum*	Amp, Cephaloglycine, Cephadrine, Cephalexin	No data	Inside	Kim and Byun,1990

Amp - Ampicillin, Pen-G - Penicillin G, Pen-V - Penicillin V, Pen-K - Penicillin K, Pen-X - Penicillin X

PGA can be further sub-classified into Type II a, specific to an aromatic phenyl acetyl moiety and Type II b specific to an aliphatic moiety (Schmidt, 2010; Sudhakaran *et al.,* 1992). Type II a PGAs are the industrially most relevant enzymes and it was estimated that ~85% of enzymatically produced 6-APA (~7650 tons) originates from penicillin G, with the rest from penicillin V (Rajendran *et al.,* 2011; Sudhakaran *et al.,* 1992). While PGA activity has been detected in approximately 40 different microorganisms (including yeast, filamentous fungi, and bacteria), cell factories employed for large-scale production of PGA are limited to a few bacterial platforms with *Escherichia coli* as the major one (Rajendran *et al.,* 2011; Sudhakaran *et al.,* 1992).They belong to the Ntn hydrolases super family which is characterized by a catalytic serine or cysteine or threonine residue at the N-terminal end (Brannigan *et al.,* 1995). According to Valle *et al*. (1991) this enzyme may have a role in using aromatic compounds as alternative carbon source in free living state.

2.4 Structure and reaction mechanism

Crystal structures of PGA are known for the dimeric, precursor, and substrate-bound forms of the enzyme (Duggleby *et al.,* 1995; Done *et al.,* 1998; Alkema *et al.,* 2000; Hewitt *et al.,* 2000; McVey *et al.,* 2001). The crystal structure of the dimeric enzyme (Duggleby *et al.,* 1995) shows a catalytic center that includes the N-terminal serine residue of the α-subunit. This particularity made PGA the founding member of the Ntn (N-terminal nucleophile) hydrolase super family (Brannigan et *al.,* 1995). Structural analysis of different enzyme-substrate complexes (Done *et al.,* 1998; Alkema *et al*., 2000; McVey *et al.,* 2001), show ligand-induced conformational changes in the enzyme-binding pocketIn vitro refolding studies of PGA subunits have suggested a key role for α subunit during heterodimer folding: The β subunit is unable to fold properly in the absence of the subunit (Lindsay and Pain, 1991). The successful production of functional enzyme when the protein subunits are separately expressed inside the cytoplasm (Burtscher and Schumacher, 1992) indicates that the presence of the α-subunit in the same polypeptide precursor is not necessary for the successful assistance in the folding of the β-subunit. The structure of dimeric PGA (Fig. 2.1) showed that the C terminus of the β-subunit was nearby the N terminus of the α-subunit.

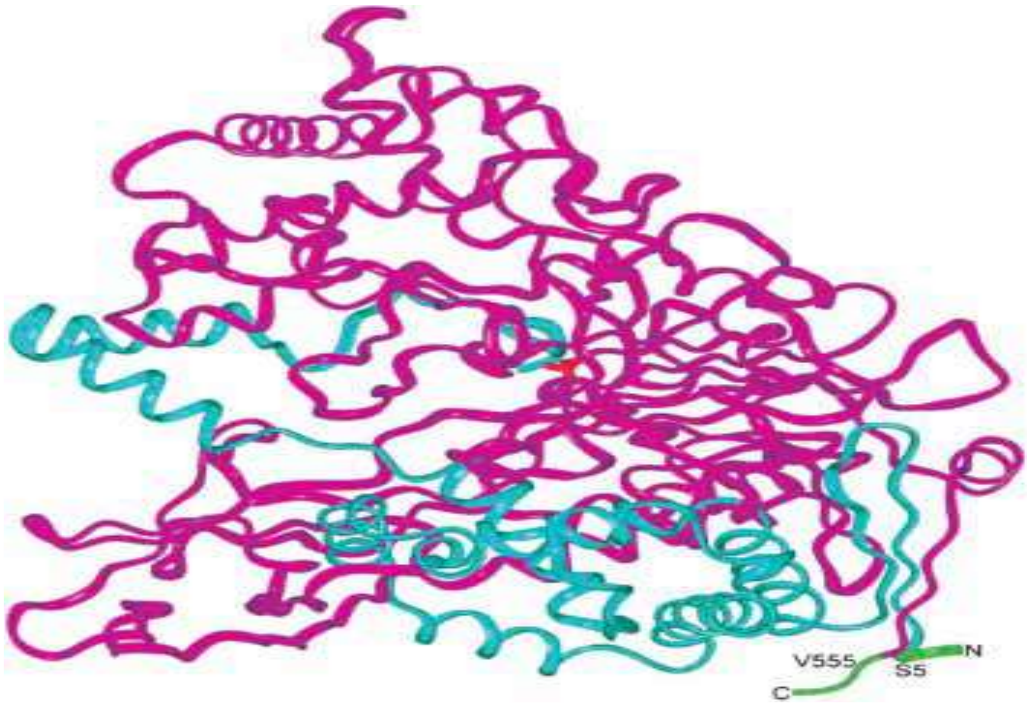


Fig.2.1 PGA dimeric structure (Flores *et al*., 2004)

The β-subunit shown in magenta and the α-subunit, in blue ribbons. The polypeptide regions trimmed from the N terminus of the α-subunit and from the C terminus of the β-subunit indicated in green. The amino acid residues to be connected with the four amino acids linker were labeled. In red, at the center of the molecule, the catalytic serine residue was indicated (Flores *et al*., 2004).

2.4.1 Mechanism of hydrolysis of penicillin G by penicillin acylase

PGA mediates the hydrolysis of penicillin G to yield 6-APA and PAA under slightly alkaline pH, resulting in the transfer of the phenyl acetyl moiety from 6-APA to water. Mechanistically, this deacylation process is similar to that of serine proteases (Fig. 2.2). A nucleophilic attack is brought upon the carbonyl carbon of the amide bond by the Oγ hydroxyl group of Ser (β1), resulting in the formation of a covalent intermediate of an acyl-enzyme complex via a tetrahedral transition state. When this transition state collapses through the involvement of another two AAs of Asn (β 241) and Ala (β 69), the first product, 6-APA, is released from the active site. The acyl-enzyme complex is deacylated via a nucleophilic attack by water (or another nucleophile), yielding the second product, phenyl acetic acid and the free enzyme (Arroyo *et al.*, 2003; Spence and Ramsden, 2007; Srirangan *et al.*, 2013). All steps are reversible; the condensation of acyl groups with a β-lactam nucleus becomes possible

under low water activities and acidic pH, yielding semisynthetic penicillins (Spence and Ramsden, 2007). Such catalytic reversibility solidifies the importance of PGA as a generic biocatalyst for producing a variety of β-lactam semi synthetic antibiotics.

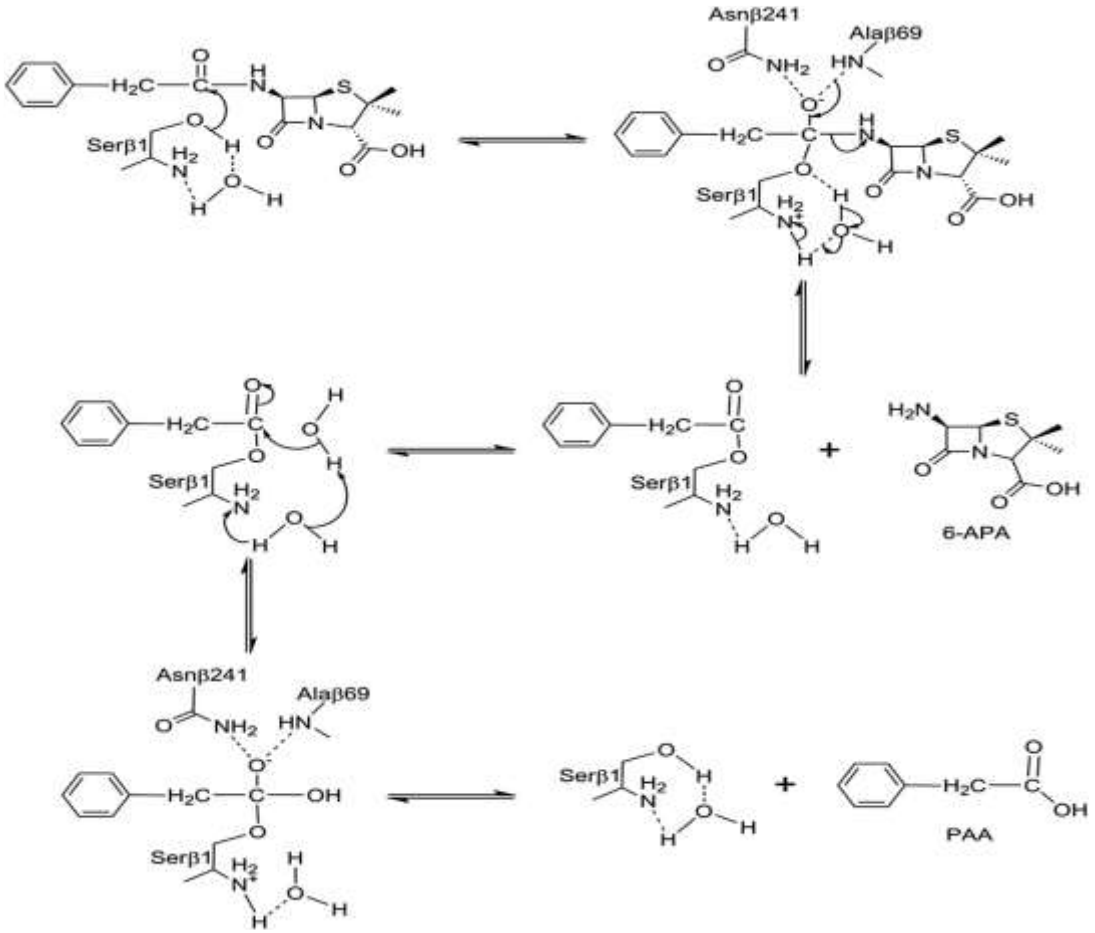


Fig.2.2 Proposed mechanism of hydrolysis of penicillin G mediated by PGA to yield PAA and 6-APA (Srirangan *et al.*, 2013)

2.5 Isolation and production of penicillin acylase

PGA is an enzyme produced by the bacteria as well as fungi in order to acylate or deacylate penicillin G into its constituents 6-APA and PAA or vice-versa (Bahman *et al.,* 2013). Production of active PGA from fungi, actinomyces or yeast has been achieved in different media (Savidge, 1984; Shewale and Sivaraman, 1989). The most widely used bacterial strains of *E. coli* are generally grown in a standard medium containing corn-steep liquor, peptone, and glucose and 0.1-0.2% phenylacetic acid (PAA) as inducer (Schomer *et al*., 1984; Shewale and Sivaraman, 1989). Higher concentrations of phenyl acetic acid suppress the formation of PGA. Salts of phenyl

acetic acid and its derivatives such as amides are also used to induce PGA production (Josef *et al.,* 1987; Vladimir *et al*., 1987).

Production of PGA was induced in recombinant *E.coli* JM101 transformed with Ppa102 plasmid (Ramirej *et al*.,1994 a,b;Ospina *et al*.,1995) and *E. coli* PPQEA11 (Sriubolmas *et al*., 1997) by various concentrations of isopropyl β-D thiogalactopyranoside (IPTG). PGA production had a saturation type of behavior with respect to the inducer concentration and IPTG had no negative effect even when used at a high concentration. This was believed to be an advantage over other recombinant systems in which the toxicity of PAA limits PGA production (Ohashi *et al.,* 1989). Exponentially fed batch cultures and controlled growth rates at reduced levels yielded an almost 10-fold increase in PGA specific activity. Some of the highest values reported were obtained this way.

Chemical mutagenesis of *E. coli* ATCC 11 105 with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) gave a mutant strain which was found to be four times more productive for PGA than the parent strain (Erarslan *et al.,*1991; Erarslan and Guray, 1991a; Kazan and Erarslan, 1996). Penicillin G acylase activity was induced in *Bacillus megaterium* in a medium consisting of yeast extract, sucrose and PAA (Illanes *et al*., 1994; Senthilvel and Pai, 1996). About 12.5% of the total 6-APA produced enzymatically involves the use of PVA (Vandamme, 1988).

Penicillin V acylase activity has been shown to occur mainly in fungi and actinomyces (Sudhakaran and Borkar,1984), but some strains of bacteria such as *Pseudomonas acidovorans* have shown good potential for industrial production of PVA (Lowe *et al.,*1981). Penicillin V acylase has also been isolated from *Beijerinckia indica* var. *Penicillium* SKB313 (Sudhakaran and Shewale, 1990) and mutant URES (Ambedkar *et al*., 1991), *Fusarium* sp. SKF235 (Sudhakaran and Shewale, 1993, 1995), *Penicillium chryogenum* (Whiteman and Abraham, 1996) and *Aeromonas* ACY95 (Ambedkar *et al.,* 1997; Deshpande *et al.,* 1996).

PenicillinV acylases are generally catabolically repressed by glucose (Shewale and Sivaraman, 1989). Penicillin V acylase production was enhanced in mutant strain UREM-5 of *Beijerinckia indica* by incorporating glucose, sodium glutamate, and vegetable oils in the medium (Ambedkar *et al.,* 1991). Highest specific production of PVA (244 IU/g dry cell weight) or 168% more than produced by the parent strain was

achieved by supplementing the medium with olive oil. It was claimed to be the highest specific PVA production (Ambedkar *et al.*, 1991; Stoppock and Wagner, 1993).

Bacillus WN - 14, a haloalkaliphilic bacterium, isolated from the Salt marshes of Wadi El - Natroun, Egypt, produced alkaline penicillin acylase enzyme in alkaline medium. *Bacillus* WN - 14 produced high yield of extracellular alkaline penicillin acylase enzyme at 40° C, pH 10.0 in 0.2 M Borax - NaOH buffer, in the presence of starch (1.0 %), potassium phenyl acetate (25.0 %), NaCl (12 %), yeast extract (0.3 %) and phenyl alanine (0.6 %). The crude enzyme extract showed maximum activity at pH 10.0 and the product (6 APA) was stable up to pH 11 (Ghanem et *al*., 1995). Javadpour *et al.* (2002) studied isolation of penicillin acylase producing *E. coli*. The optimal pH and temperature for penicillin acylase of the whole cells were determined to be 8.0 and 57°C, respectively. K_m value and activation energy of the enzymatic hydrolysis reaction of penicillin G by intracellular enzyme were estimated 0.004 mmol and 6.2 Kcal/mol, respectively.

A *Bacillus* sp. producing a high level of intracellular penicillin G acylase (PAC) was isolated. The PAC production in this strain was induced by phenylacetic acid. Various carbon and nitrogen sources were evaluated for their effect on growth and PAC production at 28°C and pH 7.0. Cells grown in medium supplemented with sucrose as carbon source and tryptone as nitrogen source, produced maximum activity of 6.45 and 8.92 U/mg) respectively. Maximum concentration of penicillin acylase (10.1 U/mg) was produced by the cells grown in the medium containing sucrose and tryptone, which was two-fold higher than the production in basal medium (Rajendhran *et al*., 2003). A fermentation process for the production of penicillin acylase (PA) by recombinant *Escherichia coli* using cheese whey as unique carbon source and inducer was developed. A design factorial 32 was used to evaluate the influence of independent variables (dissolved oxygen and cw concentration) on the ability of *E.coli* W3110/pPA102 to produce PA. Maximum specific PA activity of 781U g^{-1} was attained at 5g /L of CW and 3% dissolved oxygen. (Leon- Rodriguez *et al.*, 2006).

Table-2.2 Optimized growth parameters of some previously isolated penicillin acylase producing microorganisms

Organism	Optimized/optimum Parameters	PGA activity	References
Mutant strain of *E. coli* EP1 (pGL-5)	Carbon sources: glucose, sorbitol glycerol and PAA	Cell concentration-162 g wet weight/L (2.4 times higher compared to that of the original)	**Liu *et al.*, 2000**
***B. megaterium* ATCC 14945**	Carbon and nitrogen sources	Maximum PGA activity-138 IU/L using casein hydrolysate supplemented with 0.6 L of alcalase and cheese whey	**Pinotti *et al.*, 2000**
***B.megaterium* ATCC 14945**	Inoculum germination phase and inoculum size	Spore concentration 1.5×10^7 spores/ ml and germination during 24 and 72 h showed maximum PGA activity	**Pinotti *et al.*, 2002**
B*acillus* sp.	2-Level fractional design with seven components	2-step medium optimization resulted into 2-fold increase in PGA activity	**Rajendhran *et al* ., 2002**
***Bacillus* sp.**	Sucrose, PAA and tryptone	PGA activity was 2 fold higher than the production in basal medium	**Rajendhran *et al.*, 2003**
K. citrophila* penicillin G acylase expressed in *E. coli	TB medium (Tryptone, yeast extract, and glycerol), supplemented with IPTG (isopropylthio-beta-d-galactoside)	2.4 factor increased PGA activity	**Wen *et al.*, 2004**
***B. megaterium* ATCC 14945**	Vitamin solution	7-fold improvement in PGA activity. Ca^{2}+ (2.5mM) increased 2.6 fold specific activity of PGA. Exchange of natural signal peptide increased enzyme activity by 1.7 fold	**Yang *et al.*, 2006**

***B. megaterium* ATCC 14945**	Fed-batch cultivation using free amino acids and cheese whey supplemented media	Maximum PGA activity-220 IU/mL biomass, concentration 5.5 g/L using, during fed batch. In batch, PGA activity 160 IU/mL with biomass concentration (4.5 g/L)	**Silva *et al.*, 2006**
B. subtilis* WB 600 (pMA5*)	PGA production under batch and fed-batch culture using starch as carbon source.	5-fold increased PGA activity (1960 IU/mL) found upon feeding of hydrolyzed starch and tryptone in media with 19.6 U/l/h	**Zhang *et al.*, 2006**
Aspergillus niger	PGA production under batch culture using lactose as carbon source	Enzyme exhibited the highest enzyme activity (48 IU/ml) at pH 5.5	**Bashir *et al.*, 2008**
Bacillus cereus	Enzyme production was achieved using an optimal minimal medium containing glucose and $(NH_4)2HPO_4$.	Maximum PVA production at 30°C, pH 7 after 24 h	**Sunder *et al.*, 2012**
***Streptomyces* sp.**	Enzyme production was achieved using casein as carbon and nitrogen source	Enzyme activity was increased 3 fold	**Devi *et al.*, 2013**
Pichia pastoris	Production in rich YP medium containing yeast extract, peptone and biotin.	Highest enzyme activity was 1160U/L after 72 h of cultivation	**Maresova *et al.*, 2017**

Pinotti *et al.* (2007) reported production of penicillin G acylase (PGA) by *Bacillus megaterium*; using different medium composition. Using 51 g/L of casein hydrolyzed with alcalase1 and 2.7 g/L of phenyl acetic acid (PhAc), different carbon substrates tested, individually and combined: glucose, glycerol, and lactose (present in cheese whey). Glycerol and glucose showed to be effective nutrients for the microorganism growth but delayed the PGA production. Cheese whey increased enzyme production and cell mass. However, lactose (present in cheese whey) was not a significant carbon source for *B. megaterium*. PhAc, amino acids, and small peptides present in the hydrolyzed casein were the actual carbon sources for enzyme production. Replacement of hydrolyzed casein by free amino acids,10.0 g/L, led to a significant increase in enzyme production (app.150%), with a preferential consumption of alanine, aspartic acid, glycine, serine, arginine, threonine, lysine, and glutamic acid. A decrease of the enzyme production was observed when 20.0 g/L of amino acids were used. Ph Ac, up to 2.7 g/L, did not inhibit enzyme production, even if added at the beginning of the cultivation.

Supartono *et al*. (2008) investigated the production of penicillin G acylase from *Bacillus subtilis* BAC4. Enzyme production was carried out by batch fermentation, optimal activity for hydrolyzing penicillin G observed at 43^{o}C and pH 8.5. The highest production of extracellular PGA achieved was 2.05 U per litre of medium with a specific activity of 0.4 U/mg.

Bashir *et al*. (2008) reported production of penicillin acylase from *Aspergillus niger*. Lactose was used as a source of carbon. The enzyme activity was determined by quantification of 6-amino penicillanic acid in the reaction mixture. *Aspergillus niger* has shown the highest activity of enzyme (40 IU/ml). Out of seven different carbon sources studied, 0.4% lactose was found as the best carbon source, which exhibited the highest enzyme activity (48 IU/ml) at pH 5.5. The conversion rate of 3% and 5% potassium salt of benzyl penicillin by penicillin G acylase of *A. niger* was 35% and 26%, respectively. K_m value (0.82 mM) was promising in terms of better enzyme production**.** Two strains of *M. luteus* and a *Staphylococcus* spp capable of producing penicillin acylse were isolated from lonar lake water. Optimum penicillin acylse production time for *M. luteus* was 72 h and 48 h for *Staphyloccus* spp. Elevated temperature (40 to 50^{o}C) better suited penicillin acylse production. Glucose and sucrose stimulated penicillin acylse production by *M. luteus* and *Staphyloccus* spp

respectively. Penicillin acylse from *M. luteus* worked optimally at neutral to slightly alkaline pH and within 60 to 80^{o}C. *Staphylococcus* spp worked better at pH 6 (Tembhurkar *et al*., 2012).

Sunder *et al.* (2012) reported the isolation of a new PVA-producing strain ATUAVP1846 that was identified as *Bacillus cereus* using 16S rRNA and FAME analysis. *Bacillus cereus* ATUAVP1846 produced maximum PVA at 30°C, pH 7 after 24 h. Highest enzyme production was achieved using an optimal minimal medium containing 0.4% glucose and 0.3% $(NH_4)2HPO_4$. Moharram *et al*. (2013) studied penicillin V acylase production by *Aspergillus terreus* and *Penicillium chrysogenum*, producing 325 and 280 U/ml, respectively. Highest activity of penicillin V acylase produced by *A. terreus* and *P. chrysogenum* were obtained after 7 and 6 days of incubation, respectively at 35°C and initial pH 6. Dextrose or glucose was the best carbon source for penicillin V acylase produced by *A.terreus* and *P. chrysogenum*, respectively. Peptone was the best nitrogen source. Inoculation of cultures by 3 discs of fungi and incubation of cultures at 180 rpm shaking condition improved the enzyme Production.

Devi *et al*. (2013) reported isolation of novel intracellular penicillin V acylase producing actinomycetes, *Streptomyces* sp APT13 and the effect of media components on intracellular penicillin V acylase production. Enzyme activity was increased 3 fold in a medium with casein as carbon and nitrogen source. The enzyme production was found constitutive and enhanced by nutrient stress. Optimum activity was shown in pH 9.0 at 50°C. Magnesium was essential for enzyme production and deletion of calcium from the basal medium increased enzyme production which accompanied mycelial aggregation. Penicillin G acylase producing fungi were isolated and screened from lemon waste. The fungal microorganism was confirmed as *Penicillium chrysogenum* from colony morphology and microscopic characteristics (Lactophenol cotton blue staining). Media was optimized and yield of PGA from optimized media was found to be of higher concentration The enzyme was largely present on the supernatant at third day; similarly the production of PGA was also higher at optimized carbon source (glucose), pH (8) and temperature, 30°C (Mahendiran *et al*., 2013).

Velez *et al.* (2014) investigated the influence of medium composition, cultivation strategy, and temperature on PGA production by recombinant *E. coli* cells. Shake flask cultures carried out using incubation temperatures ranging from 18 to

28°C revealed that the specific enzyme activity achieved at 20°C (3000 IU g DCW^{-1}) was 6-fold higher than the value obtained at 28°C. Auto-induction and high cell density fed-batch bioreactor cultures were performed using the selected induction temperature, with both defined and complex media, and IPTG and lactose as inducers. Final biomass concentrations of 100 and 120 g DCW L^{-1}, and maximum enzyme productivities of 7800 and 5556 IU l^{-1} h^{-1}, were achieved for high cell density cultures using complex and defined media, respectively.

Arjun *et al.* (2016) reported isolation of penicillin G acylase producing bacterial strains, isolated from the forest soil in Western Ghats. The isolated bacterial strains were screened for PGA production and the strain (RGPGA 269), the bacterial strain showed 2.22 U/ml enzyme activity. The presence of PGA gene in the isolate was confirmed by PCR using the gene specific primers. Molecular identification of the strain was done by 16S r RNA gene amplification, sequencing and homology analysis of the sequence using NCBI BLAST. The phylogenetic analysis of the strain with selected sequences from database, showed that the isolate formed cluster with *Bacillus megaterium*.

Maresova *et al.* (2017) evaluated the potential of *Pichia pastoris* for the production of penicillin G acylase (PGAA) from *Achromobacter* sp. CCM 4824. Highest enzyme activity was 1160 U/L after 72 h of cultivation in rich YP medium containing 10 g/L yeast extract, 20 g/L peptone, 4 × 10−5% biotin.

2.5.1 Penicillin acylase production using response surface methodology

Application of the various statistical tools for the optimization of a simple and the most economical fermentation medium for penicillin acylase production have been reported by many researchers. In their study, Putri *et al*. (2015) optimized the Pac production medium by RSM using two variables (xylose as inducer and $CaCl_2$ as divalent cations) to obtain the optimum PAc specific activity from *Bacillus megaterium* btpac BD1. They have analyzed combinations of five different concentrations each of xylose (0.13 - 0.87 %) and $CaCl_2$ (0.64 - 4.36 mM) in a total of 22 experiments. CCD used for the analysis showed that in shake flask cultivations, xylose and $CaCl_2$ gave significant effects on volumetric activity and the quadratic model was in good agreement with the experimental results (R_2= 0.86 (p-value < 0.0001)). The maximum specific activity (130.669 ± 50.241) U/mg protein) was reached when xylose and $CaCl_2$ concentrations were 0.49% and 2.4 mM respectively,

and medium pH was around 7. Under such conditions, the activity of enzyme and protein concentration achieved was 1.318 ± 0.406 units' ml^{-1} and 0.01 ± 0.01 mg/ml. The shake flask validation experiments demonstrated that with such medium composition the volumetric activity, protein concentration and specific activity achieved were 1.294 ± 0.171 U ml^{-1}, 0.0102 ± 0.0003 mg ml^{-1} and 125.91 ± 13.31 units mg^{-1}, respectively. When the optimum medium composition was applied in 10 l bioreactor, the optimum volumetric activity (2.0687 ± 0.0820 U/ml) and protein concentration (0.0078 ± 0.0008 mg/ ml) was achieved 48 h after the start of the cultivation. However, the optimum PAc specific activity (1260.52 ± 27.57 U/mg protein) was achieved 18 h after the start of the cultivation.

Optimization of the medium for production of penicillin G acylase (PGA) from recombinant *E. coli* D H5α carrying a plasmid encoding pac from *Bacillus badius* was carried out based on response surface methodology (RSM). RSM is a useful method for studying the effect of several variables influencing the responses by varying them simultaneously and carrying out a limited number of experiments. The initial screening method of Plackett- Burman design gave rise to identification of fructose, yeast extract, and magnesium sulfate as significant medium components. A 23 full-factorial central composite design (CCD) was also applied to further optimize concentration of each significant variable. The optimal concentration for production of PGA consisted of 10 g/l fructose, 6 g/l yeast extract, 1.85 ml/l $MgSO_4.7H_2O$ (100 mM), 9 g/l disodium hydrogen phosphate, 4 g/l potassium dihydrogen phosphate, 0.75 g/l sodium chloride, 2ml/l thiamine HCl (100 mM), and 2ml/l calcium chloride (10 mM). The model prediction of PGA activity (14.11U/ml) at optimum conditions was verified experimentally (13.94U/ml). Through statistically designed optimization, the production of PGA was found to go up from 2.2U/ml to an average of 13.94±0.03U/ml. Thus the modified media has shown a 7.74-fold increase in PGA activity (Rajendhran *et al*., 2015).

2.5.2 Stability and activity of penicillin acylases

The following non genetic methods are commonly applied to obtain stable enzymes: (i) screening microorganisms for enzymes with enhanced intrinsic stability, (ii) use of various additives that can enhance the stability, (iii) immobilization of enzymes on a solid support and, (iv) chemical modification of enzymes. The catalytic performance of PAC increases at temperatures between 25-50°C. However, the

enzyme shows poor stability at temperatures above 35°C, and hence the stabilization of the enzyme against temperature is essential (Rajendhran and Gunasekaran, 2004). Thermostability can be important for enzymes intended for industrial use, as higher temperature operation becomes feasible for enhancing the reaction rate, shifting the thermodynamic equilibrium, increasing the solubility of the reactants, and decreasing the viscosity of the reaction medium (Kazan and Erarslan, 1997).

Thermal unfolding of penicillin acylases has been linked to their conformational mobility in water. The mobility can be reduced by reducing the amount of free water available. This may be achieved by adding stabilizing agent (Klibanov,1983,1989) such as polyolcompounds (Erarslan,1995), polyethylene glycols (Kazan and Erarslan,1997), bis-imidoesters (Erarslan and Ertan, 1995), neutral salts, albumins, other proteins, thiol reducing agents (Janecek, 1993) and sugar polyols (Larreta-Garde *et al.,* 1986,1988). The strategies for stabilizing enzymes are based on a two step model of irreversible inactivation, which involves a preliminary reversible unfolding followed by an irreversible inactivation (Mozhaev, 1993).

Here, N denotes native, R denotes reversibly denatured, and I denotes irreversibly inactivated forms of an enzyme. Stabilization methods generally aim at slowing down either of the two steps. Reversible unfolding is best prevented by immobilization, whereas additives such as sugar, surfactants, polyols and salts are effective against the irreversible step. The stabilization effects of salts are non-specific, and salting out ions decrease the availability of water molecules in solution, increase the surface tension and enhance hydrophobic Interaction. In the case of PAC, two subunits are held together by hydrophobic interactions. Thus, the presence of salts such as $(NH_4)_2SO_4$ and Na_2SO_4 favors the binding of the subunits in the native form, thereby decreasing dissociation. The addition of polyols often increases the stability of native proteins by a phenomenon attributed to the preferential exclusion of the additive from the vicinity of the protein, i.e., the preferential hydration of the protein surface (Cordt, 1994). The stabilization of penicillin acylase improved by up to 180% in the presence of trehalose after 30 min of incubation at 60°C (Azevedo *et al*., 1999). Glucose and sucrose gave two and four fold protection to PA respectively, and

the addition of various PEGs gave a 20-fold protection to PAC at 55°C. Dextran 11500 provided more than 100-fold protection to PAC against thermal inactivation at 55°C (Kazan and Erarslan, 1999). The addition of dextran polymers into the enzyme solutions may cause the exclusion of water molecules from the hydrophobic core of the enzyme globule, resulting in a more compact hydrophobic molecule with improved stability.

The formation of hydrogen bonds between dextran and water reduces the water activity of the enzyme solution; consequently, the enzyme molecule is more stable in the more structured dextran-water solvent than in water alone. The chemical modification of enzymes by cross-linking agents is a useful method to increase the stability of protein molecules against thermal unfolding. Chemical cross-linking provides a reinforcement of the native folded conformation of the enzyme molecule and reduces its thermal inactivation rate. Glutaraldehyde is the most commonly used agent for the artificial cross-linking of enzyme molecules. The cross-linking of PA with glutaraldehyde resulted in only a slight enhancement of the thermostability (Erarslan and Kocer, 1992). The half-life of the enzyme at 40°C increased from 30 min to 90 min, but at elevated temperatures, its stability remained unchanged. The effects of three different bisimidoesters as cross-linking agents on the thermostabilization of PA were investigated. A 15-fold higher thermostability of the enzyme was observed after cross-linking with dimethyladipimidate without much affecting the V_{max}, K_m and optimal temperature (Erarslan and Ertan, 1995). Dextrans with different molecular masses were converted into dialdehyde derivatives as a result of periodate oxidation and covalently linked to the ε-amino groups of lysine residues and the N-terminal ε-amino groups of the protein molecule by Schiffs base formation (Kazan *et al*., 1996).

Dextran dialdehyde- modified PA exhibited a nine fold increase in its inactivation half-life at 55°C without affecting its V_{max} value. Another method of stabilizing a prokaryotic enzyme is by expressing the gene heterologously in a eukaryotic host and thereby producing more stable glycosylated enzymes. The pac genes of *E. coli* and *P. rettgeri* were heterologously expressed in *Saccharomyces cerevisiae* and the enzymes were secreted from the yeast cell into the medium in contrast to bacterial hosts. Recombinant *E. coli* PAC was not glycosylated. However, the recombinant *P. rettgeri* PAC was partially and selectively *O*- glycosylated at the

α-subunit, while the β-subunit was not glycosylated and N-glycosylation was not detected (Ljubijankic *et al*., 2002).

The *P. rettgeri pac* gene cloned and expressed in the methylotrophic yeast *Pichia pastoris* has revealed that the recombinant active enzyme was secreted from the *P. pastoris* cells into the medium and the yield was higher (Sevo *et al*., 2002). The secreted recombinant enzyme was entirely N-glycosylated and exhibited a higher thermostability. However, other kinetic parameters were very similar to those of the corresponding non glycosylated enzymes produced in *P. rettgeri.* PAC catalyzes the hydrolytic reaction at slightly alkaline pH (7.5-8.0) and the synthetic reaction at lower pH (4.0-7.0). Therefore, in β-lactam synthesis, which is carried out at acidic pH, the stability of the enzyme at acidic pH is a vital factor. The chemical cross-linking of PAC by dimethyladipimidate resulted in enhancement of pH stability between 3.0 and 9.0 (Kazan *et al.,* 1996). Dextran-11500 dialdehyde crosslinked PAC exhibited 100% stability at pH values between 4.0-9.0 up to 120 minutes (Ertan *et al.,* 1997).

The stability of PA was enhanced by glycosylation with yeast mannan. A mannan dialdehyde derivative was used to cross-link the PA and resulted in a higher stability at an extreme pH condition and a 16-fold thermostabilization at 60°C. Chemical reactions of mannan dialdehyde groups with the lysine ε-amino groups of the PA molecules led to the formation of Schiffs base and highly stable glycozymes (Masarova *et al*., 2001a). Similarly, a four-fold increase in thermostability and pH stability was reported by cross-linking with carboxymethyl cellulose (Ozturk *et al*., 2002).

The use of organic solvents or water-cosolvent mixtures had increased the performance of enzymatic-β-lactam synthesis (Travascio *et al.,* 2002; Illanes *et al*., 2002). Thermodynamic equilibrium could be shifted towards the synthesis using hydrophobic solvents in the reaction medium (Illanes and Fajardo, 2001). Since PA loses activity in organic solvents or aqueous-organic mixtures, the stabilization of PA in organic solvents requires attention to accelerate synthetic reaction. The use of ethylene glycol or glycerol as an organic cosolvent enhanced the synthesis of ampicillin without inactivating the PA, as they are known enzyme stabilizers (Illanes and Fajardo, 2001). The chemical modification of PA by polyaldehyde polymer after co-immobilization with polyamine molecules generated nanoenvironments surrounding the immobilized PA resulted in 1000-fold stability in the presence of 90%

tetraglyme (Fernandez-Lafuente *et al*., 1999). The most significant improvements in catalytic activity obtained to date resulted from lyophilizing enzymes in the presence of excipients, which include polymers, sugars, and simple non buffer salts.

Penicillin acylase in the presence of a non buffer salt (KCl) resulted in a salt-enzyme composite which yielded a remarkably active biocatalyst in the organic solvent. PA was found to be 750-fold more reactive in hexane upon lyophilization in the presence of KCl. The preparation contained 98% (w/w) salt and 1% (w/w) each of enzyme and phosphate buffer (Lindsay *et al.*, 2002). The development of immobilized Penicillin acylase preparations has made the production process economically viable. PAC has been immobilized on various matrices, ranging from natural polymers to synthetic polymers (Kheirolomoom *et al.*, 2002). The practice of using non growing whole microbial cells rather than the purified enzyme is gaining importance due to the lower cost of production and high operational stability. The disadvantages of using whole cell systems are low specific activity and diffusional restrictions. The specific activity of PAC has been enhanced by genetic engineering, and diffusional restriction alleviated through cell permeabilization followed by cross-linking. *E. coli* cells with over expressed PAC have been permeabilized with N-cetyl-N,N, and trimethyl ammonium bromide (CTAB) followed by cross linking with glutaraldehyde. These cells were immobilized on various matrices, and proved to compete well with a biocatalyst prepared from isolated enzyme (Prabhune *et al*., 1992; Norouzian, *et al*., 2002).

Kazan and Erarslan (1997) studied the stabilization of a PGA from a mutant strain of *E. coli* ATCC 11 105 by polyethylene glycols (PEG) against thermal inactivation. The addition of PEGs to the PGA solution caused the exclusion of water from the hydrophobic core of the enzyme and resulted in a more compact hydrophobic molecule with improved thermal stability. The highest enhancement of thermostability, 20-fold above 50°C, was observed with PEG 4000 and PEG 6000. The effect of sugar polyols (sucrose, glucose, mannose, sorbitol, adonitol, xylitol) on stabilization factor values of PGA from mutant strain of *E. coli* ATCC 11105 was reported (Erarslan,1995).Sucrose was found to be the most effective thermostabilizing agent among all the polyol compounds studied. Immobilization of the enzyme also improved the thermal stability appreciably (Boccu *et al*., 1987; Erarslan and Guray, 1991b). Multipoint covalent immobilization of PGA from *Kluyvera citrophila*

improved thermostability up to 40-fold as the degree of activation of the support was increased. The thermal stability also increased (76-225- fold) as the contact time between the PGA and the activated support increased (Guisan *et al*., 1993).

A 24-fold additional stabilization of previously stabilized immobilized derivatives was achieved in terms of stabilization against irreversible thermal inactivation using chemical modifications with formaldehyde. The integrated effect of additional chemical modification and the previous multipoint covalent attachment gave PGA derivatives that were 50, 000 times more stable than the native PGA. These derivatives were also more stable than most of the commercial PGA preparations (Fernandez-Lafuente *et al*., 1999). The stabilization of the enzyme against pH by chemical cross-linking of the enzyme molecule has been reported (Erarslan, 1993; Kazan and Erarslan, 1996; Kazan *et al*., 1996; Rio *et al.*, 1995).

Kazan *et al.* (1996) reported that chemical cross-linking of the PGA enzyme from a mutant strain of *E. coli* ATCC 11105 resulted in the stabilization of the enzyme against pH. The cross-linking agent was dimethyladipimidate (DMA). Highest enhancement of pH stabilization (nearly 4-fold) was obtained at pH 7.0 and 8.0. In another study, the effect of polyhydric compounds (PHC), such as glucose, sucrose, and polyethylene glycols (PEG) was reported and the study concluded that sucrose, PEG 400 and PEG 4000 produced the greatest enhancements of pH stability of PGA (Kazan and Erarslan, 1996).

Thermal denaturation of penicillin acylase (PA) from *E. coli* has been studied by high-sensitivity differential scanning calorimetry as a function of heating rate, pH and urea concentration. It has been shown to be irreversible and kinetically controlled. Upon decrease in the heating rate from 2 to 0.1 $K\ min^{-1}$, the denaturation temperature of PA at pH 6.0 decreases by about 6 °C, while the denaturation enthalpy does not change notably giving an average value of 31.6 ±2.1 J g^{-1}. The denaturation temperature of PA reaches a maximum value of 64.5 °C at pH 6.0 and decreased about 15 °C at pH 3.0 and 9.5.

Grinberg *et al*. (2008) reported that the effect of pH induced changes in the denaturation enthalpy follow changes in the denaturation temperature. While studying penicillin acylase from *E. coli* they noticed that increasing the urea concentration caused a decrease in both denaturation temperature and enthalpy of PA, where

denaturation temperature obeyed a linear relation. The heat capacity increment of PA was not sensitive to the heating rate, nor to pH, and neither to urea. The denaturation transition of PA was approximated by the Lumry- Eyring model. The first stage of the process was assumed to be a reversible unfolding of the α-subunit. It activated the second stage involving dissociation of two subunits and subsequent denaturation of the β-subunit. Using this model the temperature, enthalpy and free energy of unfolding of the α-subunit, and a rate constant of the irreversible stage were determined as a function of pH and urea concentration

PGAs from *Bacillus badius* (Rajendhran and Gunasekaran 2007 b), *A. faecalis* (Verhaert *et al.,* 1997) and *A. viscosus* (Ohashi *et al*., 1988) exhibited the highest activity at 50°C. Zhang *et al.* (2014) reported characterization of a thermostable penicillin G acylase from an environmental metagenomic library. The optimum temperature for ACPG A001 PGA (*Achromobacter* PGA) activity was 60°C, which was higher than most of the reported PGAs. The purified enzyme retained approximately 55 and 80 % of activities at 30 and 40°C, respectively. ACPGA001 PGA retained approximately 15% of activity at 5°C, indicated that this enzyme could adapt to low temperature environments. The purified ACPGA001 PGA was stable at 40°C for 70 min and exhibited a half-life ($t_{1/2}$) of 60 min at 55°C. The thermostability of ACPGA001PGA was a little higher than that of *A. xylosoxidans*, which was the most thermostable PGA known. The effects of temperature on ACPGA001 PGA, including high thermostability and optimal temperature higher than that in its habitat, suggested that this enzyme is obtained from a non-indigenous microorganism of the Antarctic.

2.5.3 Effect of metal ions

Metal ions play important roles in the biological function of many penicillin acylases. The various modes of metal-protein interaction include metal-, ligand-, and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis acids or structural regulators (Riordan, 1977). The effect of various metal ions and chelators on the activity of penicillin acylase was evaluated. Irreversible inhibition of penicillin acylase was observed with PMSF by Kutzhbach and Rauenbusch (1974).

Rajendhran *et al*. (2003) reported the effect of the different metal ions Ag^{+}, Al^{3+}, Ca^{2+}, Co^{2+}, Cr^{3+}, Cs^{+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, K^{+}, Li^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} on *β*-lactam acylase, from *Bacillus badius.* None of the metal ions affected the

penicillin acylase activity significantly. Similarly, the addition of EDTA did not affect the PA activity, indicating that no metals are required for the enzymatic reaction. The PA activity was not inhibited by the addition of the histidine-specific reagent DEPC and tryptophan-specific NBS. However, a complete activity loss was observed with the addition of the serine-specific reagent PMSF, indicating the role of serine as an active site residue in the *B. badius* PAC. Active site titration revealed that the PAC of *B. badius* contains a single catalytic site, since PMSF inactivated the enzyme at an equimolar ratio. Sanjivkumar *et al*. (2012) have also found that metal ions, Ca^{2+}, Ba^{2+}, $Mg^{2+,}$ Hg^{2+} and Mn^{2+} had not shown any significant effect on penicillin acylase from *Bacillus subtilis.* Balci *et al*. (2014) has reported inhibition of penicillin acylase from *E. coli* ATCC11105 by $CuCl_2$, $FeCl_2$ and EDTA. The enzyme activity was strongly inhibited by PMSF. Zhang *et al*. (2014) reported effect of metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, K^{+}, Sr^{2+}, Mn^{2+} and Fe^{3+} on a thermostable penicillin G acylase ACPGA001 activity. EDTA also affected enzyme activity, indicating that metals are important for enzymatic reactions. Among the metal ions tested K^{+} and Mg^{2+} increased ACPGA001 PGA activity by 87.8 and 46 %, respectively.

2.5.4 Substrate specificity

One of the properties of enzymes that make them important as diagnostic and biocatalytic tools, is the substrate specificity they exhibit relative to the reactions they catalyze. A few enzyme exhibits absolute specificity that is they will catalyze one particular reaction. Penicillin acylases may accept different penicillins as substrates.

Chiang and Bennett (1967) described specificity of penicillin amidase from *Bacillus megaterium.* The enzyme was specific for benzyl penicillin and had a pH optimum between 8 and 9. Complete hydrolysis of benzyl penicillin was obtained at low substrate concentrations. At higher substrate concentrations, the hydrolysis of benzyl penicillin was incomplete, apparently due to enzyme inhibition by phenylacetic acid and 6-aminopenicillanic acid, which were formed during the hydrolysis. Under the assay conditions, phenylacetic acid was a competitive inhibitor of penicillin amidase with an inhibitor constant (K_i) of 0.45 M, whereas 6-aminopenicillanic acid was noncompetitive in nature with a K_i of 2.6×10^{-2} M. The Michaelis constant of this enzyme was found to be 4.5×10^{-3} M when benzyl penicillin was used as substrate.

Earlier studies on the specificity of penicillin G acylase were carried out using an unpurified, cell-bound enzyme preparation (Cole, 1969 a). The presence of cell

membranes impeded the diffusion of reactants so that the apparent K_m for penicillin G was greater than the values found for purified, soluble preparations. In order of rate of deacylation, the most susceptible penicillin substrates were p-hydroxybenzyl, DL-α-hydroxybenzyl, 2-furylmethyl, 2-thienylmethyl, DL-α-amino benzyl-propoxymethyl, isobutoxymethylpenicillin, Phenyl penicillin and DL-α-carboxybenzyl penicillin were not substrates and penicillin V was a poor substrate. Amides and esters of penicillins and cephalosporins with a thienylmethyl side-chain, phenylacetylglyeine and amides such as phenylacetamide were also reported as substrates (Cole, 1969 a,b). The same specificity was found in the direction of synthesis of penicillins and other acylamino compounds and the reaction was most effective when an amide or N-acylglycine was the acyl group donor in a transacylation reaction (Cole, 1969 c).

The kinetic parameters of several substrates of penicillin acylase from *Streptomyces lavendulae* have been determined. The enzyme hydrolyses phenoxymethyl penicillin (penicillin V) and other penicillins with aliphatic acyl-chains such as penicillin F, dihydro F, and K. The best substrate was penicillin K (octanoyl penicillin) with a kcat/K_m of 165.3 mM^{-1} s^{-1}. The enzyme also hydrolyze chromogenic substrates as NIPOAB (2-nitro-5-phenoxyacetamido benzoic acid), NIHAB (2-nitro-5-hexanoylamido benzoic acid) or NIOAB (2-nitro-5-octanoylamido benzoic acid), however it failed to hydrolyze phenylacetil penicillin (penicillin G) or NIPAB (2-nitro-5-phenylacetamido benzoic acid) and penicillins with polar substituents in the acyl moiety. These results suggested that the structure of the acyl moiety of the substrate is more determinant than the amino moiety for enzyme specificity (Torres-Guzman *et al.*, 2002).

Penicillin acylase from *Alcaligenes faecalis* has a very high affinity for both natural benzyl penicillin, (K_m= 0.0042mM) and colorimetric (6-nitro-3 phenyl acetamido benzoic acid, (K_m = 0.0045 mM) substrates as well as the product of their hydrolysis, phenylacetic acid (A\ = 0.016 mM). The enzyme was partially inhibited at high benzyl penicillin concentrations, but the triple SES complex formed still retained 43% of the maximal catalytic activity; the affinity of benzyl penicillin for the second substrate molecule binding site was much lower (As' = 54 mM) than for the first one. Phenylmethylsulfonyl fluoride was found to be a very effective irreversible inhibitor; completely inactivating the penicillin acylase from *A. faecalis* in a few minutes at micromolar concentrations, this compound was used for enzyme active site titration.

The absolute values of the determined kinetic parameters for enzymatic hydrolysis of 6-nitro-3-phenylacetamidobenzoic acid *(kcat* = 95 s^{-1} and *kcatIK*$_m$ = 2.1 ×$10^{-7}M^{-1}s^{-1}$) and benzyl penicillin were (K_{cat} = 54 s^{-1} and k_{cat}/K_m -1.3 ×$10^{-7}M^{-1}s^{-1}$) (Svedasa *et al*., 1997).

Sm-PVA (*Streptomyces mobaraensis* penicillin V acylase) was isolated as an acylase that catalyzes the hydrolysis of *N*-fatty-acyl-l-amino acids and *N*-fatty-acyl-peptides as well as capsaicin (Koreishi *et al.,* 2006). However, in this study, they concluded that *Sm*-PVA belongs to the-β-lactam acylase family judging from the polypeptide organization and the amino acid sequences. Hence, the hydrolytic activities toward various -β-lactam antibiotics such as Pen G, PenV, ampicillin, and Ceph C (cephalosporin) were measured in addition to capsaicin using *Sm*-PGA. They also used NIPOAB, which is efficiently hydrolyzed by PVA from *Fusarium oxysporum*, as a substrate (Kerr, 1993). *Sm*-PVA showed hydrolytic activity toward Pen V, NIPOAB, and Pen G in addition to capsaicin while it showed no activity toward ampicillin and Ceph C. The kinetic analysis showed that the hydrolytic reactions toward PenV, Pen G, NIPOAB, and capsaicin were consistent with the Michaelis-Menten reaction mechanism. The values of the kinetic constants for the hydrolysis of capsaicin, pen V, Pen G and NIPOAB were determined by Line weaver-Burk plots and Woolf-Augustinsoon-Hofstee plots, respectively. The determined values of the kinetic constants were Pen V *k*cat (s^{-1}) 270.8 ± 11, K_m (mM) 5.2±0.7, *k*cat/K_m (mM^{-1} s^{-1}) 51.9, POAA, K_i (mM) 11.0±0.7 (competitive), Pen G *k*cat (s^{-1}) 25.6±3.2, K_m (mM) 12.0±2.1, kcat/K_m^{-1} (mM^{-1} s^{-1}) 2.1. NIPOAB *k*cat (s^{-1}) 106.4±6.0, K_m (mM) 1.6±0.2, kcat/K_m^{-1} (mM^{-1} s^{-1}) 66.5, Capsaicin *k*cat (s^{-1}) 9.5±0.7, K_m (mM) 0.083±0.00, *k*cat/K_m (mM^{-1} s^{-1}) 114.0, pH 7.0 (-β-lactam antibiotics) or 7.5 (NIPOAB and capsaicin), temperature (37°C). *Sm*-PVA preferentially hydrolyzes PenV and NIPOAB with much higher *k*cat values than penicillin G and capsaicin. Hence, *Sm*-PVA was regarded to be PVA that belong to the -β-lactam acylase family, but with broader substrate specificity (Zhang *et al*., 2007).

The variation of kinetic parameters of penicillin V acylase from *Streptomyces lavendulae* with pH was used to gain information about the chemical mechanism of the hydrolysis of penicillin V catalyzed by this enzyme. The pH-dependence of *Vmax* showed that a group with a p*K* value of 6.45 (pK_1) must be unprotonated for activity. The pH-dependence of V_{max}/K_m showed that a group with a p*K* value of 7.1 (pK_1) must

be unprotonated and a group with a pK of 10.83 (pK_2) must be protonated for activity. The lower pK value corresponded to a group in the enzyme involved in catalysis and whose protonation state also affects binding. The higher pK value was only involved in binding. Results from chemical modification studies showed the importance of serine residues in the catalytic mechanism of the enzyme and pointed to the identity of the groups responsible for pK_1 and pK_2 as the α-amino nitrogen of the N-terminal residue and a lysine residue, respectively (Torres-Guzman *et al*., 2001).

Residue Phe 375 of cephalosporin acylase has been identified as one of the residues that is involved in substrate specificity. A complete mutational analysis was performed by substituting Phe375 with the 19 other amino acids and characterizing all purified mutant enzymes. Several mutations lead to a substrate specificity shift from the preferred substrate of the enzyme, glutaryl-7-ACA, towards the desired substrate, adipyl-7-ADCA. The catalytic efficiency ($k$cat/K_m) of mutant SY-77F375C towards adipyl-7-ADCA was increased 6-fold with respect to the wild-type enzyme, due to a strong decrease of K_m. The k_{cat} of mutant SY-77F375H towards adipyl-7-ADCA was increased 2.4-fold.The mutational effects point at two possible mechanisms by which residue 375 accommodated the long side chain of adipyl-7-ADCA, either by a widening of a hydrophobic ring-like structure that positions the aliphatic part of the side chain of the substrate, or by hydrogen bonding to the carboxylate head of the side chain. (Sio *et al*., 2004).

Kumar *et al.* (2008) reported substrate specificity of penicillin V acylase from yeast, *Rhodotorula aurantiaca* (NCIM 3425) on different synthetic and natural substrates. 5 mg/ml of various substrates such as penicillin G, amoxicillin trihydrate, ampicillin, cephalosporin C, cephalosporin G, cephalexin, cefaclor, cloxacillin, dicloxacillin, containing amide bonds were used as substrates instead of penicillin V for activity measurement by enzyme assay and reported that enzyme exhibited relatively high specificity for the side chain structure and penicillin V was the best substrate for the enzyme. Enzyme hydrolyzed synthetic substrates such as GCLH, GCLE and 3-VBA to more than 10% where as natural substrate (another analogue) such as penicillin G less than 10%. This contained benzyl side chain with amide bond at C 7 position and various ‘R’ groups attached at C 4 position of cepham (beta) - lactam ring. Enzyme had very less activity on clavulanate, ampicillin and amoxicillin and cephalosporin C. It hydrolyzed cephalexin and cefaclor more than 10% whereas

had no activity on cloxacillin and dicloxacillin. Relative activity exhibited by different substrates was, penicillin V-100 (%), penicillin G-9.3 (%), GCLH- 13.2 (%), GCLE-10 (%), 3-VBA 28.9 (%), cephalexin-10.3(%), cephaclore 13.6 (%), clavulanate K 3.5(%), amoxicillin trihydrate 5.7 (%), cephalosporin C 5.7(%), ampicillin Na 6(%).

ACPGA001 (*Achromobacter* penicillin G acylase) PGA was active against numerous β-lactams, ranging from natural to semi-synthetic penicillins and cephalosporins. When 500 mM substrates were used, the enzyme exhibited a maximum activity with penicillin G followed by cephalothin (65 %), cephaloridine (43.2 %), and cephalexin (28.4 %), penicillin V (28.2 %) and carbenicillin (22.6 %). No activity could be detected with ampicillin and amoxicillin. To compare the catalytic efficiencies of the ACPGA001 PGA with various substrates, kinetic parameters were determined. The obtained $k$cat/K_m values indicated that the enzyme had the strongest preference for penicillin G followed by cephalothin and cephaloridine. This characteristic indicated that ACPGA001PGA belongs to type II PGAs, which exhibited broad substrate specificity (Deshpande *et al*., 1994).

Ashar *et al.* (2015) studied substrate specificity of Fermenta Research Culture Collection (FRCC71) isolate with various betalactam antibiotic substrates. Cephalosporin-G (Ceph-G), penicillin-G (PenG), cephalexin (CPX), cefaclor (CCL), cefadroxil (CDL), cefprozil (CZL). Substrate specificity (%) of various substrates were as, Pen G - 131.9, Ceph G, 71.31,CPX -100, CDL - 83.48,CCL- 69.32, CZL-82.95. Each of 0.5% w/v substrate hydrolyzed with cell suspension in 50 mm sodium phosphate buffer for 15 minutes resulted in intermediate betalactam product which was assayed by reaction with chromogenic substrate *p*-dimethylaminobenzaldehyde and extent of yellow colouration was measured at 415 nm.

The hydrolase activity of penicillin acylase from *Bacillus subtilis* was checked with different-β-lactam antibiotics PenV, PenG, ampicillin, amoxicillin, cephalosporin C and cephalosporin G, along with appropriate controls. After 10 min at 40°C the solutions containing the potential alternate substrates were quenched and the presence of products was determined. The purified protein exhibited measurable enzymatic activity with PenV and the synthetic chromogenic substrate NIPOAB. There was negligible activity when PenG was used as substrate. The enzyme did not hydrolyze ampicillin or amoxicillin or cephalosporin. The enzyme was active in the pH range from pH 5.5 to 9.0 with maximal penicillin acylase activity at pH 6.6-7.4 with PenV.

The pKa values for the enzyme were calculated to be 6.5 and 8.5, which could be contributed by the ionization of histidine or by a carboxyl group in non-polar environment or by a cysteine and a basic amino acid, respectively. The apparent K_m of the enzyme using PenV as substrate was 40±1 mM and the V_{max} was 4.8± 0.2 µmol mg^{-1} min^{-1}. The K_m using NIPOAB was 0.63 mM and the V_{max} was 1.2 µmol mg^{-1} min^{-1} (Rathinaswamy *et al*., 2012).

2.6 Purification of penicillin acylase

Most of the industrial applications of enzymes do not always need homogenous preparations of the enzyme. However a certain degree of purity is required, depending upon the final application. Penicillin acylase has been utilized as whole cells but for the molecular characterization their purification has been described by various researchers. Several purification schemes have been reported specifically for penicillin acylases. Lagerlof *et al.* (1976) used a purification process in which the fermentation broth was first concentrated by centrifugation and filtration and then precipitated by 20% aqueous tannin and recovered by homogenization in cold acetone from which the enzyme was precipitated by a further acetone addition. The precipitates were dissolved in water and further purified by adsorption to carboxymethylcellulose (CMC), followed by elution with phosphate buffer (pH 8.0).

Erarslan *et al.* (1991) purified the PGA from to 350-fold and a high specific activity of 34.5 U/mg. Purification was achieved by disrupting the cells (Chisti and Moo-Young, 1991) removing the cell debris by centrifugation, and then the nucleic acids were removed by precipitation with streptomycin sulfate. The supernatant fluid from streptomycin sulfate precipitation was further precipitated by 40-60% ammonium sulfate. Precipitates were collected by centrifugation, dissolved in phosphate buffer (pH 8.0), dialyzed and applied to a DEAE-cellulose column. The pooled fractions were dialyzed and applied to a hydroxyapatite column. An enzyme with specific activity of 34.5 U/mg and yield of 39% was obtained. Senthilvel and Pai (1996) reported the purification of PGA from *B. megaterium* using ultra filtration followed by ammonium sulfate precipitation and gel filtration chromatography using Sephadex G-100. The enzyme was purified 6-fold with a highest specific activity of 2.62 U/mg in 15% yield.

Sudhakaran and Shewale (1995) described the purification of PVA from *Fusarium* sp. SKF 235 using a three-step procedure involving chromatography on CM-Sepharose, DEAE-Sephacel treatment followed by gel filtration on Bio-Gel P-100. A 182-fold purification with a yield of 9.3% was achieved by this method. A penicillin amidase, obtained from the exogenous medium of a *Bacillus megaterium* culture, was purified approximately 96-fold by means of two cycles of adsorption on, and elution from, Celite, followed by a further fractionation on carboxy methylcellulose. On the basis of sedimentation centrifugation analysis, the final preparation was homogeneous with an apparent molecular weight of approximately120000 Da. The enzyme is specific for benzyl penicillin and has a pH optimum between 8 and 9.The Michaelis constant of this enzyme was found to be 4.5 $\times 10^{-3}$ M when benzyl penicillin was used as substrate.

Two isoforms of the heterodimeric enzyme penicillin G acylase (EC 3.5.1.1 I) from *Providencia rettgeri* ATCC 31052 were purified to homogeneity. The isoforms exhibited comparable enzymatic activities but differed slightly in the molecular weight and PI of their respective α-subunit. The origin of this difference was traced to the partial conversion of the N-terminal Gln of the a-subunit to pyrrolidonecarboxylic acid (pyro-Glu). The boundaries of the mature enzyme within the translated DNA sequence of the wild-type propeptide (Gen Ban Km 86533) were determined. The results conclusively identified the length of the signal peptide and the position of the spacer cleaved from the propeptide to form the active heterodimer. The molecular weights of α and β subunits, based on these termini, were 23.7 and 62.2 kDa, respectively (Chiang and Bennett 1967; Klei *et al*., 1995).

Penicillin acylase of *Kluyvera citrophila* KY7844 was purified approximately 120-fold by DEAE-cellulose chromatography, hydroxyapatite chromatography and isoelectric focusing fractionation. Thus purified enzyme, with an approximate molecular weight of 63,000 Da appeared to be homogeneous in disc electrophoretic analysis, and showed isoelectric point (Ip) 8.12 and 13.0 units/mg of specific activity for cephalexin hydrolysis (Shimizu *et al.,* 1975). Penicillin acylase of *E. coli* NCIM 2400 has been purified to homogeneity using a combination of hydrophobic interaction chromatography and DEAE-cellulose treatment**.** Purification of this partially pure enzyme with DEAE-cellulose at pH 7.0-7.2 yielded an enzyme preparation of very high purity according to electrophoretic and ultracentrifugal

analyses, its specific activity being as high as 37 U/mg proteins. The purified enzyme has a molecular weight of 67,000 Da, sedimentation coefficient of 4.0S, and resolves into two forms upon isoelectric focusing. Overall recoveries ranged between 75 and 85 % (Mahajan and Borkar, 1984).

Erarslan *et al*. (1991) described purification of penicillin acylase from *E. coli* ATCC 11105 by DEAE-cellulose and hydroxyapatite column chromatography. The SDS-PAGE of the purified PA of the mutant strain showed two bands with different molecular weights. The corresponding molecular weights were about 23 000 and 59 000 Daltons for alpha and beta subunits, respectively. V_{max} and K_m values of the enzyme (specific activity: 24.81 U mg-^{1}, protein concentration: 0.56 mg cm^{-3}) were found to be 22.73 U cm^{-3} min^{-1}and 3.18 mmol dm^{-3} respectively. Verhaert *et al.* (1997) described purification of penicillin G acylase from *Alcaligenes faecalis.* Enzyme was purified to homogeneity by two chromatography steps. First the supernatant was loaded on a carboxymethyl cellulose column. Penicillin acylase active fractions were pooled and dialyzed. Subsequently, these fractions were loaded on hydroxyapatite gel. Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular mass determination of the penicillin G acylase showed that α and β subunits have molecular masses of 23.0 kDa and 62.7 kDa, respectively. Pseudo-affinity chromatographic supports for penicillin acylase purification were prepared and evaluated. Six supports were selected. These gels showed 70 to 100% of activity recovery. They were tested with an industrial clarified feedstock and with crude extract prepared in our laboratory. The six gels presented a high recovery of around 80% at 1.5- to 2-fold purification with industrial clarified and diafiltrated feedstock, and 70 to 100% of recovery with crude extract at two- to six-fold purification.

Vohra *et al.* (2001) have also described the purification of penicillin acylase. Penicillin G acylase (*pac)* gene was cloned into a stable *asd*C vector (pYA292) and expressed in *Escherichia coli*. Purified PAC protein near to homogeneity (92% elimination of soluble proteins) by hydrophobic interaction chromatography, attained high specific activity (16 units mg^{-1} protein) and 60% recovery of PAC protein The PAC protein showed two fragments of 23.9 kDa and 62.2 kDa on SDS-PAGE. Which corresponded to *α*-subunit and *β*-subunit of PAC protein respectively (Santarelli *et al.,* 2000).

Yang *et al*. (2001) reported purification of penicillin acylase of *B. subtilis* SIBAS205 by centrifugation, Al_2O_3 adsorption and phenyl-Sepharose CL-4B hydrophobic chromatography with a yield of 85% and a specific activity of 45 U/mg protein. Liu *et al*. (2003) studied purification of the penicillin G acylase using immobilized metal affinity membranes (IMAM) with modified regeneration cellulose with 9.11-fold. Zheng *et al.* (2003) have cloned and expressed *Alcaligenes faecalis* PGA gene encoding heterodimeric penicillin G acylase (PGA) in *Escherichia coli* and *Bacillus subtilis* respectively. The enzyme was fractionated with $(NH_4)_2SO_4$ and purified by DEAE-Sepharose CL-6B with a yield of 81%.The purified enzyme had a specific activity of 1.469 U/mg. The 23 kDa and 65 kDa bands corresponded with the subunit and β subunits. The gene encoding a novel penicillin G acylase (PGA), designated *pgaW*, was cloned from *Achromobacter xylosoxidans* and over expressed in *Escherichia coli*. The periplasmic extract fraction precipitated with ammonium sulfate at 30 to 60% saturation was loaded directly onto a hydrophobic interaction phenyl Sepharose CL-4B column (Amersham Pharmacia Biotech). Fractions containing PGA activity were pooled, concentrated by ultra filtration (cutoff of 30 kDa), and dialyzed in 20 mM Tris-HCl (pH 7.5). The resulting sample was applied to an ion-exchange Mono Q HR5/5 column (Amersham Pharmacia Biotech). The active fractions were pooled and desalted, two protein bands of 27.0 and 62.4 kDa were revealed by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis. All purification steps were carried out at room temperature in a fast protein liquid chromatography system (Cai *et al*., 2004).

Kim *et al*. (2004) reported one-step purification of Poly-His tagged penicillin acylase expressed in *E.coli* ATCC 11105. The maximum specific activity of the acylase purified by using one-step chromatography after osmotic shock was 38.5 U/mg and was recovered with the yield of 70%. 23 kDa (a) Histidine-tagged PGA proteins were identified to be 23 kDa (α subunit) and 62 kDa (β subunit). The purification of the periplasmic fraction by osmotic shock and that of purified acylase was increased by 2.6-fold and 19-fold, respectively, compared to the crude extract.

Wen *et al.* (2004) studied purification of His-tagged penicillin G acylase from *Kluyvera citrophila* in *Escherichia coli*. The DNA fragment encoding *Kluyvera citrophila* penicillin G acylase (KcPGA) was amplified and cloned into the vector pET 28b to obtain a C-terminus His-tagged fusion expression plasmid. The fusion protein

KcPGA was successfully over expressed in *Escherichia coli* BL21 (DE3). Fusion protein was purified in a single step by Ni-IDA affinity chromatograph to a specific activity of 35.3U/mg protein with a final yield of 89% representing a 23-fold purification. The SDS-PAGE showed that the enzyme consisted of two dissimilar α and β subunits, with approximate molecular mass of 23 and 62 kDa, respectively. The optimal pH and temperature of recombinant KcPGA were 8.5 and 55°C, respectively. The K_m and V_{max} were 17.6 µM and 23.8 U/mg, respectively.

Rathinaswamy *et al.* (2005) reported purification of penicillin acylase from *Bacillus subtilis*. The purified protein was a tetramer with an estimated molecular weight of 140 kDa on gel filtration, displayed PVA activity (5 mmol min^{-1} mg^{-1} specific activity). Several genes for the enzyme penicillin G acylase, isolated from four different micro-organisms (*Alcaligenes facaelis*, *Escherichia coli*, *Kluyvera cryocrescens* and *Providencia rettgeri*) were modified at their carboxy-termini to include His-tag fusions, then were expressed from the plasmid pET-24a (+) in *E.coli* JM109 (DE3) cells. All fusion proteins were purified to homogeneity in a single step by agar-based Co-IDA chromatography and purity of those enzymes was analyzed by SDS-PAGE. Each enzyme consisted of α and β heterologous subunits with similar molecular weights of approximate 23 and 62 kDa, respectively.

The enzymes (Af PGA, EcPGA, KcPGA, and PrPGA) from *Alcaligenes facaelis*, *Escherichia coli*, *Kluyvera cryocrescens* and *Providencia rettgeri* were purified by 40-183-, 107-and 17-fold, respectively, with yields of 27.6, 36.3, 33.6, and 19.7%, respectively. The specific activities of the purified enzymes were 18.2, 22.0, 12.8, and 2.0 U/mg, respectively (Cheng *et al*., 2006). Zhang *et al.* (2007) reported purification of penicillin V acylase from *Streptomyces mobaraensis*. The enzyme was purified from the culture broth of *S. mobaraensis* by column chromatographies on CM Sephadex C-50 gels and hydroxyapatite gels after precipitation with ammonium sulfate. An efficient downstream recovery of PGA from *E. coli* homogenate has been obtained using Rolquat (quaternary ammonium salt) and adsorption of the enzyme on Amp Seph (3.8 µmole ampicillin cm^{-3}) under pseudo-affinity conditions (Fonseca and Cabral, 2002). Cheng *et al*. (2006) synthesized 7.5 fold increased PGA from *Alcaligenes faecalis* when cells were permeabilized with 0.3% (w/v) cetyl-trimethylammonium bromide. The synthesized enzyme was found to be stable at 4°C for up to 30 days.

Kecilli *et al.* (2007) observed 10.3- and 35.5-fold purification with a recovery of 90% and 89% from *Penicillium chrysogenum* NRRL 1951 and *P. purpurogenum* crude extracts respectively, using a monolith column containing methacryloyl antipyrine. In another effort, Chen *et al*. (2007) purified and simultaneously immobilized PGA using a bifunctional membrane (epoxy and immobilized copper ion) and reported that 96.3% of the PGA activity could be retained through 26 reactions over more than 2 months.

Rajendhran and Gunasekaran (2007b) reported purification of penicillin -G acylase from cross-linked enzyme aggregates (CLEA) of *Bacillus badius* expressed in *E.coli*. The recombinant PAC was purified in a single step using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography with 77% yield (832 U) and 31-fold purification. Specific activity of the purified pac was 66.6 U mg^{-1}.

A mutant strain with high penicillin G acylase activity was derived from *Escherichia coli* ATCC 11105 by chemical mutagenesis, using N-methyl-N′-nitro-N-nitrosoguanidine. Penicillin acylase was synthesized from the mutant strain and purified by DEAE-cellulose and hydroxyapatite column chromatography followed by preliminary precipitation steps. V_{max} and K_m values of the enzyme (specific activity: 24·81 U mg^{-1}, protein concentration: 0.56 mg cm^{-3}) were found to be 22.73 U cm^{-3} mm^{-1} and 3.18 mM m^{-3} penicillin G, respectively. The enzyme was shown to be uncompetitively inhibited by excess of substrate. The inhibition by phenylacetic acid was found to be competitive, and 6-aminopenicillanic acid to be noncompetitive. Inhibition constants for excess of penicillin G, phenylacetic acid and 6-aminopenicillanic acid were estimated 74.20, 18.74 and 15.00 mmol dm^{-3} respectively, at pH 8.0 and 40°C. The activation energy of the enzymatic hydrolysis reaction of penicillin G was found to be 10.78 kcal mol^{-1}. Optimal pH and temperature values of the enzyme were determined as 8.0 and 60°C. Complete loss of the enzyme stability occurred when the enzyme was incubated at pH 10.0 for 2 days or at 50°C for 2 h (Erarslan *et al*., 2007).

Pinotti *et al*. (2007) studied the recovery of penicillin acylase produced by *E. coli* and *B. megaterium* through adsorption in Streamline SP XL, a cationic resin, using both packed and expanded beds. Stability assays showed that penicillin acylases from the two sources presented high irreversible deactivation at pH 4.0 and 4.5, but remained stable at pH 4.8. Adsorption experiments performed in a packed bed (PB), in

the pH range 4.8-5.8, showed highest adsorption yields at pH 4.8, for both enzymes. Using small expanded bed adsorption (EBA) columns, PGA was directly recovered and partially purified from *E.coli* crude extracts, *E.coli* homogenates and from *B. megaterium* centrifuged broth in a single unit operation. Recovery yields of 91.0, 55.0 and 7.4% and purification factors of 4.5-7.5 and 12.7-fold were achieved, respectively.

The elution yields of penicillin acylase obtained with these cationic EBA processes when working with *E.coli* homogenate and *B. megaterium* centrifuged medium were of 100 and 52%, respectively. An intracellular monomeric penicillin V acylase (PVA) of 36,000 Da exhibiting pI of 4.19, purified from newly identified yeast source, *Rhodotorula aurantiaca* (NCIM 3425). The enzyme was purified by hydrophobic interaction chromatography. The enzyme showed optimal activity at 45^0C and retained 80% activity after incubation at 45 8C and pH 5.5 for 1 h. The enzyme showed maximum activity at pH 5.5 and was very stable between pH 5.5-6.5 with optimum stability at pH 6.0. It exhibited 50% of its original activity after 30 min of incubation at 60°C. Enzyme hydrolyzed substrates with benzyl side chain but preferred penicillin V as primary substrate (Kumar *et al.,* 2008).

Supartono *et al*. (2008) reported purification of penicillin acylase from a new local strain of *Bacillus subtilis* BAC4 was purified by affinity chromatography column using sepharose 4B as a solid support and 1, 6-hexanediamine as a spacer arm with PAA as a ligand. The yield of the PGA was as 22.46% with purification of 2.13 times. The extracellular PGA of *B. subtilis* BAC4 consisted of two subunits, α and β. The smaller subunit (α) has a molecular weight of 57.8 kDa; and the bigger subunit (β) has a molecular weight of 62.1 kDa. Therefore, the relative molecular mass (Mr) of the extracellular PGA of *B. subtilis* BAC4 was estimated to be 120 kDa.

Rathinaswamy *et al.* (2012) reported the purification and characterization of the first penicillin acylase from *Bacillus subtilis*. YxeI, the protein annotated as hypothetical, coded by the gene *YxeI* in the open reading frame between *iol* and *hut* operons in *B. subtilis* was cloned and expressed in *Escherichia coli*, purified and characterized. The purified protein showed measurable penicillin acylase activity with penicillin V. The enzyme was a homotetramer of 148 kDa. The apparent K_m of the enzyme for penicillin V and the synthetic substrate 2-nitro-5-(phenoxy acetamido)-

benzoic acid was 40 mM and 0.63 mM, respectively, and the association constants were $8.93\times10^{2}M^{-1}$ and $2.51\times10^{5}M^{-1}$, respectively.

Orr *et al.* (2012) demonstrated purification of extracellularly produced penicillin G acylase using tangential flow filtration and anion-exchange membrane chromatography (TFF-AEMC) from *E.coli.* After cultivation in a bench-top bioreactor with 1L working volume using the developed host/vector system for high-level expression and effective secretion of recombinant penicillin G acylase (PAC), the whole culture broth was applied directly to the established system. One-step purification of recombinant PAC was achieved based on the dual nature of membrane chromatography (i.e. microfiltration-sized pores and anion-exchange chemistry) and cross-flow operations. Most contaminant proteins in the extracellular medium were captured by the anion-exchange membrane and cells remained in the retentate, whereas extracellular PAC was purified and collected in the filtrate. The batch time for both cultivation and purification was less than 24 h and recombinant PAC with high purity (19 U/mg), yield (72% recovery), and productivity (41 mg of purified PAC per liter of culture) was obtained.

Kumar *et al*. (2012) described partial purification of penicillin G acylase from *Bacillus subtilis.* The dialysate showed the activity was 1251.98 IU/ml. The maximum enzyme activity was observed at pH 7 and temperature 30°C. Philem *et al.* (2016) have also reported purification of penicillin V acylase from *Acinetobacter* sp. AP24. The enzyme was purified to homogeneity by cation-exchange chromatography using SP-sepharose resin. The PVA was a homotetramer with subunit molecular mass of 34 kDa. The enzyme was highly specific toward penicillin V with optimal hydrolytic activity at 40°C and pH 7.5.The enzyme was stable from pH 5.0 to 9.0 at 25 °C for 2 hr. The enzyme retained 75% activity after 1 hr of incubation at 40°C at pH 7.5. In their study Demircelik *et al.* (2017) reported the use of poly (2-hydroxyethyl methacrylate) based cryogel column containing hydrophobic N-methacryloyl-L-tryptophan (MATrp) functional monomer for the purification of penicillin acylase from *Penicillium chrysogenum*. Optimum pH was determined as 5.0 for PA adsorption and maximum adsorption capacity was obtained as 6.40 mg/g. PA adsorption increased up to 0.25 M salt concentration and decreased in higher salt concentrations. PA was purified with 76.3% yield and 332.3 purification factor.

2.7 Immobilization of penicillin acylase

In order to be useful as a biocatalyst the PGA enzyme preparation has to be active, robust and re-usable. One of the most effective ways to enhance stability for many enzymes is to immobilize them onto a solid support. In addition, immobilization may allow re-use of the catalyst and thus increase cost effectiveness. A number of immobilization methods have been used in this context (Hashem *et al.,* 2016). Each method shows superiority to the more traditional use of free cells, extracts or even immobilized whole cells.

Immobilized enzyme preparations attain higher activity and specificity and show better control of contamination (Ghada *et al*., 2016). The commercial viability of any enzyme depends on its operational stability and reusability. Enzymes in free form are thermo labile and cannot be reused, owing to their loss during downstream processing and purification of the product, (Bryjak and Trochimczuk, 2006; Heilmann *et al*., 2007). Immobilization is the most important technique for stabilizing enzyme activity and enhancing its operational life. Immobilization does not necessarily enhance the enzyme's stability, but this can be achieved by different modes of the immobilization matrix system (Heilmann *et al*., 2007). PGA is one of the most common commercially significant examples of enzyme reusability. In industry, the stability of immobilized protein during bioconversion is the most important parameter for obtaining the required yields and economizing the process. In the past, a number of immobilization systems have been patented and commercialized for PGA production (Bryjak and Trochimczuk, 2006; Giordano *et al*., 2006). Whole cells were being entrapped with a certain ratio of polyethylimine and glutaraldehyde and used as a catalyst for antibiotics' conversion into intermediates. In liquid, PGA has been immobilized on a number of novel carriers, such as ethylene glycol, dimethyl acrylate, Eupergit C, alumina beads, nylon fibers, silica support, zerogel, sepa beads, glycoxyl agarose, and a number of anionic exchangers (Giordano *et al.,* 2006; Wang *et al*., 2007).

The enzyme preparation with the highest specific activity (322 U/g dry matrix at 40°C and pH 8.0) was obtained by covalent binding of penicillin acylase to vinyl copolymers (Dhal *et al.,* 1985) because of large number of oxirane groups on oxirane-acrylic beads used for the covalent linkage. Loss of 16% of activity was reported after 20 cycles of operation at 40°C for the immobilized penicillin acylase. However,

treatment of penicillin acylase with DL-Dithiothreitol (DTT) as a stabilization agent, significantly reduced the deactivation of the enzyme and at 28°C, DTT-treated immobilized penicillin acylase was stable over 20 cycles. No activity loss occurred in repeated use within that range. DTT was said to cause additional bonds to form between the matrix and the enzyme molecules and hence the improved stability (Erarslan and Guray, 1991b). Singh *et al.* (1988) used double entrapment methodology for the immobilization of penicillin acylase from *E. coli* NCIM 2563 on agar-polyacrylamide resins. In spite of remarkable success of alginate and polyacrylamide supports in other applications (Illanes *et al*., 1996; Prabhune and Sivaraman, 1991) these were not suitable possibly because phosphate buffer was used and it is known to affect stability of such supports. Danzig *et al.* (1993) used penicillin acylase produced by genetically engineered *E. coli* and immobilized it on polyacrylamide carrier for improved 6-APA production. Many methods for the immobilization of penicillin acylase from *E. coli* ATCC 11105 have been reported (Erarslan *et al.*, 1989, 1990, 1991; Erarslan and Guray, 1991b).

Chemical modification of immobilized penicillin acylase from *E. coli* has been reported with formaldehyde followed by sodium borohydride reduction. The derivative modified with formaldehyde and further reduced with borohydride was much more stable than the original unmodified preparation (Blanco and Guisan, 1989). The multipoint covalent immobilization of penicillin acylase from *K. citrophila* (Alvaro *et al.*, 1992; Guisan *et al.*, 1993) stabilized the enzyme 10000-fold more relative to common soluble enzyme from *E.coli*. The use of copolymers of acrylonitrile, vinyl acetate, butylacrylate (Bryjak *et al.*, 1993), and ethyl acrylate (Bryjak *et al.*, 1989) with cross-linking agents such as divinylbenzene or ethylenedimethacrylate (Bryjak and Noworyta, 1993) has been reported. The stability of the immobilized preparation is mostly affected by multipoint attachment of the enzyme to the carrier surface (Bryjak *et al.*, 1993). The penicillin acylase immobilized on copolymers of butyl acrylates and ethyleneglycol dimethacrylate had a significantly enhanced reaction rate compared to penicillin hydrolysis by the native enzyme. (Bryjak and Noworyta, 1993).

Fonseca *et al*. (1993) used grace silica gel carrier activated by a silanization method for immobilization of penicillin acylase. The activity of the immobilized penicillin acylase on grace silica gel (Fonseca *et al.*, 1993) was almost the same as

that of the enzyme immobilized on the oxirane beads (Erarslan and Guray, 1991b) or on Eupergit C (Kraemer and Goekcek, 1987). Asahi Chemical Industry Co. of Japan had been using penicillin acylase from *B. megaterium* B-400, immobilized on partially aminated porous polyacrylonitrile (PAN) fibers. This preparation showed a sufficiently high specific activity of 2330 U/g dry carrier at 38°C in the broad pH range of 7.5-8.5.

Bianchi *et al.* (1996) described a new procedure for the immobilization of an industrial penicillin acylase by covalent coupling on the poly (methacrylic ester) resin, Amberlite XAD-7, modified by trans amidation with 1, 2-diethylenediamine and activated by glutaraldehyde followed by reduction with sodium borohydride resulting in a dramatic improvement of the operational stability of the immobilized enzyme without affecting the catalytic activity. Although most of the processes developed until the late 1980s were based on the PGA/ PVA production from purified microbial enzymes immobilized on solid supports, it soon became clear that PGA/PVA prepared from whole cell immobilization was more attractive from the economic view point (Savidge, 1984). However, the whole cell preparations had low specific activities compared with the immobilized enzymes. This disadvantage has been overcome to some extent by chemical mutations/genetic engineering.

Ramirej *et al.*(1994 a,b) have described the construction of a recombinant *E. coli* strain with PGA activity induced with IPTG and its use in exponentially fed batch-cultures to obtain high levels of expression. Ospina *et al.* (1995) used *E. coli* JM101ppA102 strain for whole cell immobilization by gel entrapment in agar. The highest activity obtained was 134 U/g biocatalyst, which is 10-13-fold higher as compared to the wild strain biocatalyst (Rio *et al.,* 1995). The stability of this PGA preparation at 37°C and pH 7.0 was 1035 h (half-life) in comparison with a half-life of 2400 h for a commercial enzyme immobilized by covalent linkage to an epoxy acrylic resin (Ospina *et al.,* 1992). The half-life of the free enzyme is only about 115 h (Greco *et al.,* 1983).

Chauhan *et al.* (1998) reported immobilization of *Chainia* penicillin V acylase. Partially purified acylase was adsorbed on kieselguhr and entrapped in polyacrylamide gel. The immobilized preparation proved effective with respect to retention of enzyme and enzyme activity even after 15 successful cycles. The pH optimum for crude enzyme was in the range of pH 7.5-8.0, and for the $(NH_4)_2\ SO_4$ fractions it was pH

8.5. The immobilized enzyme showed maximal activity at pH 9.5. The optimum temperature for acylase activity was at 55°C. The crude enzyme, ammonium sulfate fraction, and immobilized enzyme showed K_m value for penicillin V of 6.13 mM, 14.3 mM, and 17.1 mM, respectively.

Pereira *et al.* (1997) reported immobilization of penicillin G acylase on silica-glyoxyl. This technique in macro porous silica involves support activation with 3-glycidyloxy propyl tri methoxy silane, followed by acidic hydrolysis and oxidation with sodium periodate. The aldehyde-glyoxyl groups so formed react subsequently with the enzyme. The degree of activation affects the yield and stability of the enzyme immobilization. For 20 UI of enzyme, the results showed an immobilization yield equal to 100%. The immobilized enzyme has shown half-life of 23 minutes at 60ºC; under the same conditions, the soluble enzyme has no residual activity after a few minutes. A rigid support of sepa beads was able to stand in 90% dioxane without significant loss of PGA activity. The sepa beads were found to be an excellent carrier because of their robustness and the ease with which they separated from the substrate solution (Torres-Bacete *et al*., 2000).

Bahulekar *et al.* (1993) constructed macro porous beaded polymers of varying pore size coated with polyethylene mine and further derivatized with glutaraldehyde for use as immobilizers. Using this technique, they demonstrated efficient, highly stable, and specific binding on the supports. They also found that the optimum temperature shifted from 40 to 57 $^{\circ}$C and that the K_m increased from 31 to 128µmol. The carboxylic groups of PGA and glutaryl acylase were chemically aminated in a controlled way by reaction with ethylene diamine via 1-ethyl-3-dimethyl aminopropyl carbaimide coupling, which showed a noticeable stability, efficient recovery and reuse of the biocatalyst is a prerequisite for a commercially viable process in terms of obtaining a satisfactory yield of antibiotic intermediates 6-APA and 7-ADCA. Hydrophobic interaction chromatography that concentrates and purifies the enzyme in a single-step process was explored by Chen *et al.* (2007) and Wang *et al.* (2007) by fabricating macro porous weak cation-exchange methacrylate polymers to immobilize PGA. To obtain the desired yield of antibiotic intermediates, attention has been focused toward sturdy, robust supports like sepa beads, Eupergit C (Torres-Bacete *et al*., 2000), and nylon fibers (Eldin *et al*., 2000) for immobilization.

Cheng *et al*. (2006) successfully demonstrated the immobilization of permeabilized whole-cell PGA from *A. faecalis* using pore matrix cross-linking with glutaraldehyde that enhanced PGA activity7.5-fold. In this bioconversion, the yield of penicillin G to 6-APA was 75% in batch reactions up to 15 repeated cycles, and about 65% enzyme activity was retained at the end of the thirty first cycle.

Penicillin acylase from *Streptomyces lavendulae* had been covalently immobilized to epoxy-activated acrylic beads (Eupergit C). Consecutive modification of the matrix with bovine serum albumin lead to a new biocatalyst (ECPVA) with enhanced activity (1.5 fold) in the hydrolysis of penicillin V with respect to its soluble counterpart. This biocatalyst had a K_m value of 7.6 mM, slightly higher than K_m for native acylase (3 mM). ECPVA could be recycled for at least 50 consecutive batch reactions without loss of catalytic activity (Torres-Bacete *et al*., 2000).

The use of restrictive conditions during enzyme adsorption onto anionic exchangers allows the adsorption strength and enzyme stability to increase in the presence of organic solvents. This action suggests that the enzyme actively penetrates the polymeric beads, and becomes fully covered with the polymer. After the enzyme is inactivated, it can be desorbed to reuse the support. The possibility of improving the immobilization properties of an enzyme by site-directed mutagenesis of its surface opens a promising new scenario for enzyme engineering.

Abian *et al.* (2003) reported the immobilization of the wild-type and mutant PGA enzymes of *E.coli* on CNBr-Sepharose. Three mutations on the penicillin acylase surface (increasing the number of Lys in a defined area) were performed. They did not alter the enzyme's stability and kinetic properties; however, after immobilization on glyoxyl-agarose, the mutant enzyme showed improved stability under all tested conditions (e.g., pH 2.5 at 4°C, pH 5 at 60°C, pH 7 at 55°C, or 60% dimethylformamide) with stabilization factors ranging from 4 to 11 compared with the native enzyme immobilized on glyoxyl-agarose. Immobilization on glyoxyl-agarose occurred in less than 10 min and allowed enzyme activity to be preserved. The new mutant PGA derivative showed a half-life that was 100,000-fold higher than that of the soluble wild-type enzyme. Both immobilized enzymes presented similar activity and pH profiles.

Cross-linked enzyme crystals (CLECs) are highly stable novel biocatalysts. The CLEC approach is unique among stabilization technologies in that it results in

both stabilization and immobilization of the enzyme without the dilution of activity. the protein matrix is both the catalyst and the support. They are produced by stepwise crystallization of the enzyme and molecular cross-linking to preserve the crystalline structure. CLECs are extremely stable, not only with respect to temperature, but also to other inactivating agents such as organic solvents. In CLECs, the enzyme molecules are compacted almost to the theoretical limit, stabilization being a consequence of intense polar and hydrophobic interactions.

The CLEC of PAC from *E. coli* has been commercialized and has worked equally well in the hydrolysis and synthesis of β-lactam antibiotics. CLEC-PAC retained 70% of its original activity after 1000 batch reactions (Govardhan, 1999). Similarly, another novel preparation called cross-linked enzyme aggregates (CLEA) of PAC has been reported (Cao *et al.*, 2001). The CLEAs of PAC were prepared by slowly adding a precipitant ammonium sulfate, PEG 8000 or tert-butyl alcohol to a solution of the enzyme at 0°C and pH 8. In the next step, the physically aggregated PAC was subjected to chemical cross-linking using glutaraldehyde and then used as the biocatalyst. Compared with conventionally immobilized PAC and CLEC, these CLEAs possessed a high specific activity as well as high productivity and synthesis/hydrolysis (S/H) ratio in the synthesis of semi synthetic -β-lactam antibiotics in aqueous media. They were active and stable in a broad range of polar and non-polar organic solvents.

Penicillin G acylase from *E. coli* TA1 was immobilized by Cross-Linked Enzyme Aggregates (CLEA), immobilization. This biocatalyst and commercial immobilized penicillin G acylase (PGA-450) were used to study the effect of pH, temperature and substrate concentration on the synthesis of ampicillin from phenyl glycine methyl ester (PGME) and 6-aminopenicillanic acid (6-APA). Compared with PGA-450, this immobilized enzyme showed a high synthesis activity. The optimum conditions for synthetic activity was at pH 6, 25°C and 2:6 (6-APA: PGME) substrate ratio (Abedia *et al*., 2004).

Adriano *et al.* (2005) studied enzyme immobilization of penicillin acylase from *E.coli* on chitosan activated with glutaraldehyde, aiming to produce a cheap biocatalyst. Two different immobilization strategies were studied: one-point and multipoint covalent attachment to the solid matrix. The multipoint covalent attachment derivative had an 82% immobilization yield. It was 4.9-fold more stable

than the free enzyme at 50°C and 4.5-fold more stable than soluble enzyme at pH 10.0. The one-point derivative had an 85% immobilization yield. It was 2.7-fold more stable than the free enzyme at 50°C and 3.8-fold more stable than soluble PGA at pH 10.0. Chitosan can be loaded with PGA above 330 IU/g. Intraparticle diffusive effects, however, limited hydrolysis of penicillin G catalyzed by those derivatives at 37°C and 25°C. Operational stability assays were performed and the multipoint derivative exhibited a half-life of 40 hours.

Illanes *et al*. (2007) studied cephalexin production at high substrate concentration (750 mM) acyl donor with immobilized and cross-linked aggregates (CIEA) of PGA. To assess the operational stability and global productivity of the biocatalyst, 40.1 and 135.5 g of cephalexin/g of biocatalyst were obtained for PGA-450 and CLEA, respectively. Magnetic hydroxyl particles have been activated with epoxyl chloropropane by suspension polymerization and used for PGA immobilization. The activity of the immobilized enzyme remained constant at ~ 94% for up to 80 cycles of the bioconversion process (Wang *et al*., 2007).

Montes *et al.* (2007) used site-directed mutagenesis to add eight, new, homogeneously distributed glutamic residues throughout the enzyme's surface to study reverse immobilization using anionic exchanger (DEAE- or polyethylene mine-coated agarose). Zuza *et al.* (2007) investigated the effect of sepabeads EC-EP as a support matrix for covalent immobilization of penicillin G acylase from *E.coli* .The immobilization procedure involved the direct enzyme binding on polymers via epoxide groups. The highest activity coupling yield of 89.4% was achieved working at low enzyme loading (0.14 mg g^{-1}). The optimal pH for the immobilized enzyme activity was found to be 8.7. A slightly higher value for optimum temperature (31.5°C) was found for the immobilized enzyme in comparison with that displayed by the free one (27.5 ^{o}C).The K_m value for the free enzyme was determined to be 5.991.46 mmol dm^{-3}, whereas V_{max} value was 128.985.37 μmol min^{-1} mg^{-1}. The apparent value of K_m for the immobilized enzyme was 29.858.7 mmol dm^{-3}, approximately 5-fold higher than that of the free enzyme.

The immobilization of PGA from *E. coli* on a composite carrier consisting of an adsorbent resin and biocompatible chitosan were investigated. FT-IR and SEM analysis confirmed the structure of the composite carrier. The activity of the immobilized PGA on the chitosan-resin (IP-CsR) was about 1300 U (g dry carrier) $^{-1}$

with a protein loading of about 27mg (g dry carrier) $^{-1}$.Compared with the immobilized PGA on unmodified resin (IP-R), the specific activity of IP-CsR was enhanced about 2-fold. The operational, thermal and pH stability were investigated. IP-CsR maintained more than 75% initial activity after 35 cycles, while IP-R was active for only 10 cycles. The half-life at 50°C increased from 75 to 300 min and the most stable pH was changed from 8.0 to 5.5 (Jin *et al.*, 2008).

κ-Carrageenan hydrogel cross linked with protonated polyethylenemine (PEI1) and glutaraldehyde (GA) was prepared and evaluated as a novel biocatalytic support for covalent immobilization of penicillin G acylase (PGA).The method of modification of the carrageenan biopolymer was verified by FTIR, elemental analysis, Results showed that the gel's mechanical strength was greatly enhanced from 3.9 kg /cm^2 to 16.8 kg/cm^2 with an outstanding improvement in the gels thermal stability. The control gels were completely dissolved at 35°C, whereas the modified gels remained intact at 90°C. FTIR revealed the presence of the new functionality, aldehyde carbonyl group, at 1710 cm 21 for covalent PGA immobilization. PGA was successfully immobilized as a model industrial enzyme retaining 71% of its activity. The enzyme loading increased from 2.2 U/g (control gel) to 10 U/g using the covalent technique .The operational stability showed no loss of activity after 20 cycles (Elnashar *et al*., 2008). Sun *et al.* (2008) reported immobilization of penicillin acylase of *Alcaligenes faecalis* on epoxy type supports. Two novel supports ZH-EP (epoxy type) and ZH-HA (epoxy-amino type) were used to immobilize *Alcaligenes faecalis* penicillin acylase with Eupergit as a reference.

Zuza *et al.* (2009) presented an approach to stable covalent immobilization of chemically modified penicillin G acylase from *Escherichia coli* on Sepabeads carriers with high retention of hydrolytic activity and thermal stability. The two amino-activated polymethacrylate particulate polymers with different spacer lengths used in the study were Sepabeads EC EA and Sepabeads EC HA. The enzyme was first modified by cross-linking with polyaldehyde derivatives of starch in order to provide it with new useful functions. Such modified enzyme was then covalently immobilized on amino supports. The method provided a possibility to couple the enzyme without risking a reaction at the active site which might cause the loss of activity. Performances of these immobilized biocatalysts were compared with those obtained by the conventional method with respect to activity and thermal stability. The thermal

stability study shows that starch-PGA immobilized on Sepabeads EC-EA was almost 4.5-fold more stable than the conventionally immobilized one and 7-fold more stable than free non-modified PGA. Similarly, starch-PGA immobilized on Sepabeads EC-HA was around 1.5- fold more stable than the conventionally immobilized one and almost 9.5-fold more stable than free non-modified enzyme.

Wang *et al.* (2008) reported the successful improvement of covalent immobilization of PA in the MCFs (meso cellular silica foams) using p-benzoquinone as coupling reagent. The relative activity of PA in the MCFs was enhanced to 1.45 folds of that of free enzyme. The stability of PA was also ameliorated greatly by this practice. The activity of PA immobilized in MCFs was 1.23 folds of that of free enzyme in solution at the tenth cycle. The optimum pH of the immobilized PA shifted to pH 7.5 and the optimum reaction temperature rose from 45°C to 50°C. FT-IR spectroscopy showed no major secondary structural change for the PA confined in MCFs. Amino acids were used to quench excess activated groups in order to improve the thermo stability of the immobilized enzyme. PA co assembled with Dex 10 in mesopores retained 88% of its initial catalytic activity after heating at 50 °C for 6 h, as a result of glycine quenching the excess activated groups. This biomolecule enhanced the thermo stability of the enzyme preparation by 2-fold.

Wang *et al.* (2009) reported covalent immobilization of penicillin acylase using macromolecular crowding and glycine quenching. Macromolecular reagents were covalently assembled on the walls of meso cellular silica foams (MCFs) and paralleled enzyme molecules under microwave irradiation at low temperatures. The effects of kind and content of macromolecules on immobilization and the characteristics of the immobilized enzyme were investigated. The maximum specific activities of PA assembled with Dex 10 (Dextran, Mw 10000) (85.3 U/mg) and BSA (Bovine Serum Albumin) (112.7 U/mg) in MCFs under microwave irradiation were 1.73 and 1.31 times, respectively. The optimum reaction temperature rose from 45-55°C.

Bernardino *et al*. (2011) studied Immobilization of penicillin G acylase by entrapment in xerogel particles with magnetic properties, using 150 mM AOT/isooctane, which allowed for the formation of spherical micro and nano beads. The effects on PGA activity of different sol-gel precursors, additives, enzyme concentration, aging, drying conditions and mechanical stability were evaluated. A

mechanically stable carrier based on porous xerogels silica matrixes, starting from tetramethoxysilane (TMOS) with 65-67% PGA activity yield in these carriers allowed an immobilization yield of 74 mg protein /g dry sol-gel $^{-1}$ and 930 U/g dry sol-gel^{-1}. Eldin *et al.* (2012) studied covalent Immobilization of penicillin G acylase on to chemically activated surface of poly (vinyl chloride) membranes. PVC membranes were successfully modified with EDA, and as a result, their surface was functionalized with amine groups. The best results of catalytic activity of the immobilized enzyme were 0.020% EDA and 40°C, respectively. The reaction time with EDA for 15 min was optimum time. Aminated PVC membranes were activated with GA (G acylase). The optimum conditions of the activation process, including GA concentration, pH, reaction time, and reaction temperature, were GA-1.5%, pH- 8.0, 15 min, and 40°C, respectively. The modified PVC membranes were characterized with FTIR spectroscopy and TGA to prove the occurrence of fictionalization with amine groups. A characteristic band at 3357 cm^{-1} for amine groups was recognized.

Entrapment of permeabilized whole cells within a matrix is a common method for immobilization. Chitosan possesses distinct chemical and biological properties, which make it a suitable matrix for entrapment and immobilization of penicillin G acylase (PGA). *Escherichia coli* (ATCC 11105) cells were permeabilized using N-cetyl-N, N, N-trimethyl ammonium bromide (CTAB) (0.1% w/v, 45 min, 45 rpm) which then immobilized using glutaraldehyde (5% w/v) as cross-linker and chitosan (3% w/v) as the matrix. Permeabilization of cells caused 9% increase in penicillin G acylase (PGA) conversion after 15 min compared to the intact cells. Immobilization on chitosan decreased the conversion compared to un-immobilized treated cells (13%), the new biocatalyst showed maintained more than 90% of the initial activity after 20 cycles. Optimum conditions for immobilization of *E. coli* cells were: CTAB 0.1% w/v and glutaraldehyde 5% v/v (Bagherinejad *et al.*, 2012).

Bahman *et al.* (2013) studied immobilization of penicillin G acylase on Iron Oxide nanoparticles. Penicillin G acylase was immobilized onto iron oxide nanoparticles coated with polyethylenemine and then cross linked with glutaraldehyde solution. The FTIR spectrum of immobilized enzyme showed peak at 1648cm^{-1} which can be attributed to the C=N bonds of Schiff's base linkage formed between glutaraldehyde and amino group of penicillin G acylase. The optimal pH and temperature were determined to be 7.0 and 10.0 and 50 ºC and 75ºC for free and

immobilized penicillin G acylase, respectively. Thermal stabilities of both nano and free penicillin G acylase were studied. The K_m value of immobilized nanozyme was calculated from Line weaver Burck plot to be 0.23 μM while that of free penicillin G acylase was 0.28μM.

Avinash *et al.* (2017) in their study reported the whole cell immobilization of a recombinant PVA enzyme from *Pectobacterium atrosepticum* expressed in *E. coli*. Membrane permeabilization with detergent was used to enhance the cell bound PVA activity, and the cells were encapsulated in calcium alginate beads and cross linked with glutaraldehyde. Optimization of parameters for the biotransformation by immobilized cells showed, full conversion of Pen V to 6-APA achieved within 1h at pH 5.0 and 35˚C, till 4% (w/v) concentration of the substrate. The beads could be stored for 28 days at 4˚C with minimal loss in activity, and were reusable up to 10 cycles with 1h hardening in $CaCl_2$ between each cycle.

2.8 Applications of penicillin acylase

2.8.1 Synthesis of 6-APA by free enzyme

Production of semi-synthetic penicillins and cephalosporins, which remain the most widely used group of antibiotics (De Vroom, 1999). In addition, penicillin acylases are useful as biocatalysts in many potentially valuable reactions such as protection of amino and hydroxyl groups in peptide synthesis, as well as in the resolution of racemic mixtures of chiral compounds. The industrial production of 6-APA has undergone a remarkable transformation due to the fact that penicillin acylase-catalyzed processes have replaced traditional chemical conversions. The traditional chemical synthesis of 6-APA began around 1970 at Gist-Brocades and consisted of a one-pot deacylation of the fermentation product penicillin G using a procedure requiring hazardous chemicals and solvents. This approach remained in use for 15-20 years until it was largely replaced by the penicillin G catalyzed hydrolysis of penicillin G, which affords 6-APA in good yield.

According to some earlier studies alternative process combining penicillin V acylase and penicillin V could be more advantageous (Shewale and Sudhakaran 1997). Penicillin V shows higher stability in aqueous solutions at lower pH during extraction from the fermented broth, which could lead to a higher yield of 6-APA. Penicillin V acylases also achieve higher conversion at a higher substrate

concentration as compared with penicillin G acylases, and their broader optimal pH range reduces buffering requirements during hydrolysis. However, only 15% of all manufactured 6-APA worldwide is produced from penicillin V. Taking into account procedures involving both penicillin acylases, an annual worldwide production of 9,000 tons 6-APA is produced enzymatically from penicillin G and V (Demain, 2000).

Xue-jun *et al*. (2014) described the bioconversion of penicillin G in PEG 20000/dextran T 70 aqueous two-phase systems using the recombinant *Escherichia coli* A56 (ppA22) with an intracellular penicillin acylase as catalyst. The best conversion conditions were attained for: 7% (w/v) substrate (penicillin G), enzyme activity in bottom phase 52 U ml^{-1}, pH 7.8, temperature 37 °C, reaction time 40 min. Five repeated batches performed in these conditions. Conversions ratios between 0.9-0.99 mol of 6-aminopenicillanic acid (6-APA) per mol of penicillin G were obtained and volumetric productivity was 3.6-4.6 μmol min^{-1} ml^{-1}. In addition the product 6-APA directly crystallized from the top phase with a purity of 96%.

A dodecane/thermo sensitive polymer/water three-liquid-phase system was introduced for enzymatic hydrolysis of penicillin G (Pen G) for 6-aminopenicillanic acid (6-APA). The enzyme was covalently attached to the terminal of PEO-PPO-PEO polymer (L63), which would be transferred into a polymer coacervate phase at high temperature. 6-APA was primarily resided in the aqueous phase due to its zwitter ionic nature. More than 70% phenylacetic acid (PAA) was transferred into the organic phase using trioctylmethylammonium hydroxide and trihexyl-(tetradecyl) phosphonium bis 2, 4, 4-trimethylpentylphosphinate ionic liquids (Cyphos IL-104) mixture at pH 5.5, while most of Pen G resided in water. As a result, high operational pH was permitted in three-liquid-phase system, which lead to higher enzymatic activity (120 IU at 40°C) and stability (enzymatic half-time up to 55 h at 60°C) in comparison with the value in butyl acetate/water two-phase system. Two products in three-liquid-phase system might be automatically separated from the enzyme sphere into different phases at the same time, which facilitated the reaction equilibrium towards the product's side with 6-APA productivity of 80% at 42°C, pH 5.5 (Jiang *et al*., 2007).

2.8.2 Synthesis of 6-APA by immobilized enzyme

The external and internal mass transfers of penicillin G in the process of its enzymatic hydrolysis to 6-Aminopenicillanic acid under competitive and non-competitive inhibitions using a bioreactor with stationary basket bed of immobilized penicillin amidase have been analyzed. By means of the penicillin G mass balance for a single particle of biocatalysts, considering the specific kinetic model proposed and a mathematical expressions have been developed for describing the profiles of penicillin G concentrations and mass flows in the outer and inner regions of biocatalyst particles, as well as for estimating the influence of internal diffusion on its hydrolysis rate. Very low values of internal mass flow could be reached in the particles centre. Enzyme immobilization and using the basket bed, the rate of enzymatic reaction is reduced over 160 times compared to the process with free enzyme (Cascavala *et al.*, 2012).

Banerjee and Debnath (2007) described the continuous production of 6-APA in a packed column reactor by using agarose immobilized penicillin acylase as a block polymer. The strain *Escherichia coli* ATCC 11105 was used as enzyme source and penicillin G as substrate. Optimization of process parameters like temperature, pH and substrate mass fraction was carried out as an initial part of the work. The temperature, T = 37 °C, pH 8 and w = 2 % penicillin G substrate mass fraction were found optimal for 6-APA production.

The 6-APA produced at different time intervals was assayed by hydroxylamine method (Batchelor *et al.*, 1959). Substrate fractions varying from 1 % to 3 % were circulated through the column and 6-APA production was found in relation to the residence time in each case. Although w = 3 % substrate mass fraction gave a higher productivity of 18.79 mg L^{-1} h^{-1} of 6-APA at optimum residence time = 20-33 min as compared to 16.2 mg L^{-1} h^{-1} of 6-APA yield by 2 % substrate fraction but in terms of the yield (y p/s) the 2 % substrate showed y p/s = 0.81 h^{-1} and 3 % substrate showed y p/s = 0.62 h^{-1}. The 1 % substrate showed y p/s = 0.51 h^{-1}. The yield factor gives the ratio of the product formed to the initial substrate fraction. The importance of determining yield factor is to find out the optimum substrate concentration that is needed for product formation. Therefore, in terms of yield factor 2 % substrate was preferable as optimum substrate fraction. The stability of the packed column in course

of time varying from 3 days to 15 days was observed. The half life of immobilized enzyme was higher (5 days) as compared to the native enzyme (533 min).

Enzymatic hydrolysis of penicillin G for production of 6-amino-penicillanic-acid (6-APA) was achieved by using penicillin G acylase as catalyst in an aqueous-methyl isobutyl ketone (MIBK) system. The optimization was carried out and it was found that the best conversion was improved 10% more than the aqueous system, which was obtained at the conditions: initial pH 8.0, 5.0% (w/v) substrate (penicillin G) and temperature at 35°C, and the ratio of aqueous and organic phase was 3:1. The stability of the biocatalyst was studied at the operational conditions. After 5 cycles of semi-batch reactions, the residual activity of penicillin G acylase was 69.2% of the initial activity. There was no apparent loss of the yield of product. This process has a potential application in the industrial scale production of 6-APA because it simplifies the process effectively (Fang *et al*., 2010).

2.8.3 Enantioselectivity of PGA

According to the IUPAC recommendations, the preferential formation of one stereoisomer over another is an enzyme characteristic called stereoselectivity. When the stereoisomers are enantiomers, the phenomenon is called enantioselectivity (Moss, 1996). PGA was found to catalyze two principal reactions: enantioselective reverse hydrolysis and enantioselective hydrolysis

2.8.3.1 PGA enantioselectivity for production of pure chiral compounds

Many synthons or active pharmaceutical ingredients (APIs) are chiral compounds. Although enantiomers have identical chemical and physical properties in achiral environments (except for the rotation of polarized light plane) they behave differently in a chiral, biological environment. It was found that critical physiological processes show 100% stereoselectivity. In these terms, exogenously supplied enantiomerically-pure drugs may exhibit diverse interactions with chiral targets such as enzymes, receptors and ion channels (Aboul and Aboul, 1997). 76% of new active substances launched on the market in 2000 were enantiomerically pure substances and in 2001 enantiomerically pure substances represented 36% of the total amount of APIs available on the market (Massollini *et al*., 2006). Around 2007, the percentage of enantiomerically pure drugs introduced to the market represented 75% (Lavecchia *et al*., 2007). Enantioselective reverse hydrolysis and enantioselective hydrolysis may be

used for preparation of pure chiral compounds with industrial potential from racemic mixtures. Both reactions may be performed in water phase alone, supplemented with co-solvents and ionic liquids (ILs), or in organic solvents alone. The overwhelming majority of applications were described with immobilized PGA of *E. coli*, a robust catalyst convenient for biocatalyses under harsh conditions in organic solvents. In a few cases, immobilized PGAs from the bacteria *A. faecalis*, *A. viscosus*, and *K. citrophila* were also used in enantioselective resolutions.

2.8.3.2 Enantioselective reverse hydrolysis

Zmijewski *et al.* (1991) reported that PGA is a suitable catalyst for carrying out kinetically controlled acylation in resolution of racemates, the PGAs of two bacterial species, i.e. *E. coli* and *A. faecalis* were used in these reactions. Only the PGAEc was used by three research groups as a free enzyme (Chilov *et al*., 2003; Deaguero *et al*., 2010, 2012; Topgi *et al.,* 1999). PGA was applied in the resolution of a racemic mixture of (RS)-phenylglycinonitrile to(R)-phenylglycinonitrile, an important intermediate for the synthesis of antibiotics (Chilov *et al.,* 2003) and on racemic mixtures of α-amino acids, intermediates for the production of semi-synthetic β-lactam antibiotics, agricultural chemicals and other new drugs (Kim and Lee, 1996). Mixtures of glutamate and glutamine were also resolved by PGA with a high degree of enantiomeric excess (ee) while the racemic mixtures of aspartate, asparagine and serine were resolved with a lower degree of ee. However, these asymmetric syntheses of enantiomerically pure amino acids have never led to an economically viable process (Carboni *et al.,* 2006). Topgi *et al.* (1999) used PGAEc to synthesize (S)-enantiomer of ethyl 3-amino-4-pentynoate. The enantiomer was obtained after resolution of trimethyl silylated racemic mixture of ethyl 3-amino-4-pentynoate by immobilized PGAEc. (S)-enantiomer, an important chiral synthon for syntheses of bioactive pseudo peptides (Xemilofiban, an anti-platelet agent), was prepared with high reaction yield.

2.8.3.3 Enantioselective hydrolysis

Immobilized PGAEc was used to obtain enantiopure (2S, 3S) (+) -3-hydroxyleucine via enantioselective hydrolysis of N-phenylacetyl-3-hydroxyleucine racemic mixture. The product is an important substance for preparation of naturally occurring compounds cyclodepsipeptides, components of antibiotics containing

peptide lactones as APIs. Reaction mixture consisted of acetonitrile and water in a 4:6 ratio and ee (p) equaled 99% (Fadnavis *et al.*, 1997). Liu *et al.* (2006) used PGAKc for the preparation of L-tert-leucine by enantioselective hydrolysis of N-phenylacetyl-tert-leucine racemic mixture. D-tert-Leucine was prepared via acid-catalyzed hydrolysis of remaining D-enantiomer. Overall reaction yield of L-tert-leucine and D-tert-leucine was 80.6% and 83.1%, respectively, and ee of L-tert-leucine was 99%. Preparation of optically pure leucine derivatives is of great importance because these compounds can serve as chiral building blocks for pharmacologically active peptide components of anti-tumor or anti-AIDS drugs (Bomamrius *et al.*, 1995). Fadnavis *et al*. (2008) used PGAEc to resolve a racemic mixture of 2- chlorophenyl glycine and obtained (S)-enantiomer of 2-chlorophenyl glycine, an important intermediate in the synthesis of antiplatelet agent Clopidogrel, with high yield.

Kumaraguru and Fadnavis (2012) exploited PGAEc from *E. coli* for resolution of a racemic mixture of 4- oxocyclopent- 2- en-1-yl 2- phenyl acetate in diisopropyl ether. Enantiomerically pure (R) and (S) 4-hydroxy-2-cyclopentenones are versatile intermediates for the synthesis of a large number of complex natural products such as prostaglandins, prostacyclins, thromboxane and nucleosides. Chemo enzymatic synthesis of dideoxymannojirimycine involves enantioselective hydrolysis of a racemic mixture of N-phenylacetyl derivative of homoserine lactone performed by PGAEc. These compounds have potential use in a wide range of therapeutic strategies, including the treatment of viral infection (HIV), cancer, diabetes, tuberculosis and lysosomal storage diseases and as inhibitors of the growth of parasitic protozoa.

2.8.3.4 Protection and deprotection of reactive amino groups

PGAEc was used for the protection/deprotection of reactive groups of L-amino acid esters during the chemo enzymatic synthesis of biologically active dipeptides by Didziapetris *et al.* (1991) and Svedas and Beltser (1998). Biologically important peptides also contain D-amino acids. Carboni *et al.* (2004) performed for the first time PGAEc-catalyzed protection/deprotection of a series of esters of non-polar D-amino acids in an organic solvent. The ability of PGA to accept a D-enantiomer of a nucleophile was ascribed to modification of enantioselectivity of the PGA in the presence of non-aqueous medium. The use of biocatalysis in protection/deprotection represents a“green” alternative to commonly used chemical processes using toxic and expensive chemicals (Kadereit and Waldmann, 2001).

2.8.4 Synthesis of dipeptides

Conventional chemical synthesis of peptides requires donor and acceptor activation accompanied by protection of the reactive group under harsh conditions. Biocatalysis allows simple introduction of protecting, environment-friendly agents to reactive groups under mild conditions while the activation is still the matter of chemistry. Moreover, the number of protecting groups in biocatalysis can be significantly reduced and, in the case of very selective biocatalysts, no protecting groups are required. Proteases were reported as efficient catalysts for kinetically controlled synthesis of dipeptides with C^{α}-esterified amino acid as an activated acyl donor and N^{α},C^{α}-free amino acids as nucleophiles (Yokozeki and Hara, 2005). Khimiuk *et al*. (2003) presented a chemo enzymatic synthesis of various dipeptides containing D-(−) phenylglycine moiety from activated acyl donor D-(−) phenylglycine amide and specific L-amino acid in reactions catalyzed by PGA in aqueous solution. This is the first step towards the synthesis of enantiomerically pure diketopiperazine, which can have potent cytotoxic (Boger and Jiacheng, 1993) and antitumor (Gomez-Monterey *et al.,* 2008) properties. In this case, the application of PGA does not use the trait of enantioselectivity. Van Langen *et al*. (2000b) synthesized stereochemically pure phenylglycine dipeptides without any need for protecting groups using immobilized PGAEc. They used L-phenylglycine (nucleophile) and D-phenylglycine amide (acyl donor) or L-phenylglycine methyl ester as both nucleophile and acyl donor. In the former case, D-phenylglycyl-L-phenylglycine was prepared. When using the second pair of reactants-phenylglycyl-L-phenylglycine methyl ester was obtained. Both dipeptides in activated forms (as esters) undergo a ring closure to corresponding diketopiperazine (Purdie and Benoiton, 1973) that can be used as a synthon for fungicidal and antiviral agents (Svokos *et al.,*1971).

2.8.5 Exploitation of PGA-catalyzed N-deacylation in biosensors

Biosensor is defined as an analytical tool consisting of a biological component, a biosensing layer, and physical signal transducer. According to the mode of signal transduction, sensors are classified as electrochemical, optical, thermal and piezoelectric ones. Electrochemical sensors, in their turn, are divided into four types: potentiometric, amperometric, conductometric and ion-selective field-effect transistor sensors (Dzyadevich *et al*., 2006). Immobilized enzymes are especially used as biosensing components of electrochemical sensors that have a wide application in

clinical diagnostics, food quality control, monitoring of in-process variables of fermentations in bioreactors or environmental sensing. A new role of biosensors is envisaged in screening processes aimed at discovering new drugs (Keusgen, 2002).

More than half of the enzyme sensors reported are electrochemical biosensors (Hasanzadeh *et al*., 2012) that work on the following principle: a target analyte interacts with the biological part of the sensor (e.g. an enzyme immobilized on the surface of a pH-sensitive device, amperometric electrode or semiconductor structure) and the resulting biological signal (a charged product of enzymatic reaction) is converted into a physical signal by a transducer. The most frequently used immobilization techniques are enzyme entrapment and cross-linking methods (Poghossian *et al*., 2001). Potentiometric types of biosensors and those based on ion-selective field-effect transistors have attracted the attention of researches due to their small size, fast response time, the possibility of bulk production (Dzyadevich *et al.,* 2006; Keusgen, 2002), and their development went ahead very fast.

Liu *et al.* (1998) developed a biosensor for fermentation process control based on ion-selective transducer with field effect, the biological component of which was PGA.The response time of the sensor to different concentrations of penicillin G was 30s.The biosensor showed high sensitivity and long-term operational stability when employed for off-line monitoring penicillin G in culture broth during fermentation (Liu and Li, 2000). Analytical devices called enzyme thermistors (Mosbach *et al.*, 1975) are used as sensors in flow meters for gases and liquids. The evolution of heat is a general property accompanying biochemical transformations and immobilized enzymes (as a thin film) serve as biorecognition elements for thermometric measurements. Yakovleva *et al.*(2013) developed sensitive enzyme thermistors based on immobilized PGA that were applied to determine penicillin G concentration ranging from 0.02 to 200 mM.

2.9 PGA as a promiscuous enzyme

The enzyme capable of catalyzing several chemical reactions with completely different reaction mechanisms (Wu *et al*., 2005) is designated as a promiscuous enzyme. Research into the promiscuity of PGA of *E. coli* has been carried out in connection with the production of nitroalkanols, modified APIs and N-heterocycle derivatives.

2.9.1 Markovnikov addition reaction

The reaction mechanism of Markovnikov addition consists of electrophile addition onto a double bond of a nucleophile. Wu *et al.* (2006) studied the ability of PGA to catalyze Markovnikov addition with different vinyl esters as substrates. The enzyme activity decreased with increasing length of vinyl ester chain and lower reaction yields of products were achieved with more sterically hindered vinyl esters as reaction substrates**.** The effect of different substituents on imidazole ring in the reactions with vinyl esters was analogously studied. 4-Nitroimidazol used as the electrophile gave in these additions the highest yield. With pyrole, pyrazole and 1, 2, 4 triazole, the reaction yield was lower and the amount of the product formed in the reaction was decreasing in a descending order with 1, 2, 4 triazole, imidazole, pyrazole, and pyrole as electrophile. Markovnikov additions were also studied with the electrophile uracil; however, in this case the reaction gave a very low yield even after prolonged reaction time (8 days). PGA-catalyzed Markovnikov addition reaction with allopurinol (electrophile) was also tested (Wu *et al.*, 2005). The product of the reaction was N-heterocycle derivative, which indicated a potential synthetic route for bioactive N-derivatives of heterocyclic compounds.

2.9.2 Transesterification

Transesterification is a term used for reactions where an ester is transformed into another one through an interchange of the alkoxy moieties. Transesterification activity of penicillin G acylase of *E. coli* was described by Lindsay *et al.* (2002) who studied the activation effect of non buffer salts on PGA catalyzing trans-esterification reaction of phenoxyacetic acid methyl ester with 1-propanol in hexane. The enzyme was found to be over 750-fold more reactive upon lyophilizing in the presence of KCl. The same group (Lindsay *et al*., 2003) later focused its effort on identification of the salt mixture used at lyophilization of PGA and having the highest activation effect. The study revealed that the formulation consisting of 98% (w/w) of a salt mixture KAc: CsCl = 1:1, 1% (w/w) enzyme, and 1% (w/w) potassium phosphate buffer was 35 000-fold more active in hexane than the salt-free formulation. Thus, a remarkably active biocatalyst in hydrophobic media was obtained by lyophilizing an enzyme in the presence of non buffer salts.

Liu *et al*. (2011) reported the trans-esterification reaction of vinyl acetate with guaifenesin as a promiscous reaction of PGA. Guaifenesin, an API for the preparation

of anxiolytics and central myorelaxants, and its structurally similar derivatives were used to study substrate binding into the active site of the PGA during trans-esterification reactions. Although PGA has a broad substrate specificity (including substrates with phenyl acetyl or phenoxy acetyl moieties), it has no specificity for vinyl acetate. As 10% water solution, N-methylimidazole was found to modulate the enzyme activity and alter the catalytic ability of PGA from amidation to esterification. Liu *et al.* (2011) proposed that N-methylimidazole assisted the enzyme in ester bond formation. The presence of N-methylimidazole in active site of the enzyme may activate the substrate by enhancing the nucleophilicity of the hydroxyl group. Guaifenesin was found to be the best substrate for PGA-catalyzed trans-esterification among the group of related APIs.

2.9.3 Henry reaction

The reaction is known in organic chemistry and represents a formation of carbon-carbon bond in a reaction of nucleophilic nitroalkane with an electrophilic aldehyde or ketone. Products of this coupling are β-nitroalkanols that are useful for syntheses of intermediates for the preparation of many biologically active compounds, e.g. fungicides (Mikite *et al.*, 1982). Biocatalytic approaches to Henry (Nitroaldol) reaction were reviewed by Milner *et al.* (2012). There are two biocatalytic approaches to enantio-enriched products of the Henry reaction reported in the literature: direct enzyme-catalyzed Henry reaction or initial chemical formation of the β-nitroalkanols followed by enzymatic kinetic resolution of the stereoisomers. Lipases, transglutaminases, hydroxynitril lyases and proteases are known to catalyze Henry reaction.

Wang *et al.* (2010) used three acylases to catalyze the synthesis of β-nitroalkanols in the presence of DMSO: PGAEc, aminoacylase from *Aspergillus oryzae* and D-aminoacylase from *E. coli*. The yield obtained with immobilized PGA was very low but still detectable. Among these enzymes, D-aminoacylase was the best catalyst of the direct enzyme catalyzed Henry reaction. Since the reactions were carried out under harsh denaturing conditions (DMSO, 50°C), the role of DMSO itself in Henry reaction should be considered. In general, the effect of DMSO on the enzyme structure and its function differs according to physico-chemical traits of a protein (Johannesson *et al*., 1997) and co-solvent concentration (Roy *et al*., 2012). Wang *et al*. (2010) did not exclude that DMSO itself may affect a catalytic

promiscuity of enzymes. Busto *et al.* (2011) observed that the Henry reaction can also occur through non-specific catalysis since BSA itself efficiently catalyzes this reaction.

2.10 Decolourization of dyes

The water pollution caused by the textile dye effluent in hazardous for aquatic eco system .The high concentration of dyes causes many water borne diseases and increase BOD of the receiving water because of their complex structure and large molecular size. Dyes present in the water can cause health hazards like ulceration of skin, and mucous membrane, dermatitis, perforation of nasal septum and severer irritation of respiratory track. Its injection may cause vomiting, pain, hemorrhage and diarrhoea (Ponraj *et al.,* 2011). Dyes used in the textile industry are difficult to remove by conventional waste water treatment methods since they are stable to light, oxidizing agents and are resistant to aerobic digestion. The presence of carcinogens has also being reported in combined waste water of dyeing and printing units (Wong and Yuen, 1996).

Without adequate treatment these dyes will remain in the environment for an extended period of time (Olukanni *et al.,* 2006). Several methods were adapted for the reduction of dyes to achieve decolourization. These include physiochemical methods such as filtration, specific coagulation, use of activated carbon, chemical flocculation etc. Some of these methods (viz. reverse osmosis, nanofiltration, multiple effect evaporator (MEE) are found to be effective but quite expensive (Maier *et al.,* 2004). The chemical structures of the synthetic dye molecule are designed to resist fading on exposure to light or chemical attack, and this renders them recalcitrant. Among the biological treatment, however, the azo bond is reduced to amines, which are potentially carcinogenic. No single conventional technology can remove all types of dyes because their molecular structure and chemical properties vary widely and may be complex. Dyes adsorb to membrane of cells in conventional activated sludge and is poorly degraded. But these methods are not widely used because of high cost, low efficiency, (Hai *et al.,* 2007) and inapplicability to avoid variety of dyes as well as formation of toxic by-product and secondary pollution that can be generated by excessive use of chemicals. Therefore, a more efficient and cost effective treatment is needed.

Some natural microbial species, including bacteria, fungi, and algae, are capable of removing the colour of azo dye via biotransformation, biodegradation, or even mineralization. Decolourization of azo dyes by bacteria is typically initiated by azo reductases-catalyzed reduction or cleavage of azo bonds under anaerobic environment (Chang *et al.*, 2000). Azo dyes are not readily degraded by microbes, but microorganisms that are able to degrade azo dyes anaerobically have been isolated.The ability of fungus to degrade azo dyes was observed. It was observed that *Thelospora sp.* was able to decolourize methyl orange up to 97.1%. Cripps *et al.* (1990) reported that *P. chrysosporium* could remove 87-93% congo red dye. Jadhav (2007) reported decolourization of methyl red by using *Saccharomyces cerevisiae*. Methyl red (100 mg l^{-1}) was degraded completely within 16 min in plain distilled water under static anoxic condition, at the room temperature. Cells of *Saccharomyces cerevisiae* could degrade methyl red efficiently up to 10 cycles in plain distilled water. Analysis of samples synthesized with ethyl acetate from decolourized culture flasks in plain distilled water (pH 6.5) and at pH 9 using UV-VIS, TLC, HPLC and FTIR confirm biodegradation of methyl red into several different metabolites.

P. putida strain MR1 could decolorize methyl red and some other dyes like yellow FG (4.27%), red RH (16.69%), ponceau S (3.19%) and brown GR (7.28%). The variation in percent decolourization of different dyes by the isolate was attributed to the difference in structure and complexity of each (Sani and Banerjee, 1999). Different physicochemical parameters like temperature, pH, dye concentration and carbon source influence decolorization of textile dyes by the isolate. In this investigation, the optimum pH and temperature required for the efficient decolorization of dye methyl red by the *P. putida* strain MR1 in the liquid culture was 7.0 and 34°C, respectively. Dye decolorizing bacteria have a narrow pH range (Chen *et al.,* 2003). Maximum growth of isolate and maximum decolorization (94.63%) was achieved at pH 7.0. Increase in temperature proved to have a positive effect on the growth of the isolate and methyl red decolorization, which was maximum (0.828 and 91%, respectively) at 34°C. These observations could be attributed to the increase in enzyme activity and growth increase with the temperature (Asad *et al.,* 2006). (Kumar and Bhat, 2012) reported that maximum decolourization of azo dye- Red 3BN by *P .chrysogenum, A. niger,* and *Cladosporium sp.* was observed when maltose was used at a concentration of (1%). Whereas (Verma *et al.,* 2015) in their study found that

dextrose was most effective for RBBR dye decolorization by *Mucor hiemalis* MV04 (KR078215), among all carbon sources used. Decolorization ranged between 80 to 100% in 8 days. The primary mechanism of decolorization may involve dye adsorption/degradation by mycelium of fungi as well as the reduction of dye intensity in solution because of changes caused by them (Kumar *et al.,* 2012). The rate of dyes removal can be linked with the available co-substrates and with the exponential growth phase (Namdhari *et al.,* 2012). The fungi needs readily usable carbon source for their growth and production of secondary metabolites and extracellular enzymes (Verma *et al.,* 2015). The isolate decolorized methyl red maximally (99.65%) at dye concentration of 100 mg/l. Further increase in dye concentration resulted in decrease in the percentage of decolorization and cell growth. This might be due to toxicity of dye through the inhibition of metabolic activities (Asad *et al.,* 2006).

2.11 Conclusion

Penicillin acylases represent a diversified group of enzymes that are produced by various microorganisms, including Fungi, Eubacteria, Actinomycetes and Archaea. In order to be useful as a biocatalyst, penicillin acylases need to be stable and must show reusability. One of the most effective ways to enhance stability for many enzymes is to immobilize the enzyme onto a solid support. Immobilization may allow re-use of the catalyst and thus increase cost effectiveness. Even though these enzymes have been described under one bunch with respect to their industrial utility, there exists a large structural and functional melange between these homologues despite the presence of the common Ntn fold. Thus, in spite of their still demand in antibiotic production, there are convincing reasons that the role of penicillin acylases in natural physiology might not be too small to be discounted. It is thus imperative to adopt an integrated approach to study these enzymes in the metabolic network.

Chapter-3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

The penicillin G and penicillin V were purchased from Sigma (Germany). All the media components were purchased from HiMedia (Mumbai). Sodium alginate, polyvinyl alcohol, acrylamide, bis-acrylamide and all other organic and inorganic salts, various polar and nonpolar organic solvents and HPLC grade solvents were purchased from Merck, India. All chemicals were of analytical grades and were used as received.

3.1.2 Instruments

Encapsulator (B-390 Encapsulator, BUCHI Switzerland), HPLC system (Waters India Pvt Ltd), Reverse Phase Sun Fire column (Waters India Pvt Ltd), Fermenter (BioFlow C-32 New Brunswick Scientific, USA), Lyophilizer (Flexi-Dry MP ™, FTS USA), FTIR spectrometer (Shimadzu FTIR 8000 series, Japan),Varian Cary 50spectrophotometer,Cary Eclipse Fluorescent spectrophotometer etc were used in the present study.

3.1.3 Microorganism

A penicillin acylase producing fungal isolate used in the present study was isolated in the Department of Biotechnology, Himachal Pradesh University, Shimla from the soils of Shimla and Solan districts of (Himachal Pradesh, INDIA) and has been explored for its penicillin acylase activity. This fungus has been identified and deposited as *Acremonium sclerotigenum*, at National Fungal Culture Collection (NFCCI), Agharkar Institute, Pune, (INDIA).

3.2 Methods

3.2.1 Isolation of penicillin G resistant microorganisms

Soil samples were collected in sterile vials from different parts of Shimla and Solan districts of Himachal Pradesh and brought to the laboratory and stored at -20°C till further processing. For enrichment, 1g of soil sample was added to nutrient broth, pH-7.0 (for bacterial isolates) and potato dextrose media, pH-6.0 (for fungal isolates), both supplemented with 1% benzylpenicillin. For further isolation, loop full of

enriched samples were streaked on nutrient agar and potato dextrose agar supplemented with 1% benzylpenicillin.

3.2.2 Screening of penicillin acylase producers

All the penicillin G resistant bacterial and fungal isolates were screened for penicillin acylase using two methods:

3.2.2.1 Qualitative screening by bioassay methods

The *Serratia marcescens* ATCC 27117 overlay technique (Meevootisom *et al.,* 1983) was used for bioassay of penicillin acylase producers. Cultures grown at 28°C on nutrient agar plates for approximately 24 h were overlaid with agar containing penicillin G and *S. marcescens* ATCC 27117, a bacterial strain resistant to penicillin G but sensitive to 6-APA. The plate was incubated at 28°C for approximately 24 h and enzyme producers identified by the formation of a clear zone of inhibition on a red mat of growth.

3.2.2.2 Qualitative screening by measuring volumetric penicillin acylase activity

All the positive isolates were grown for 24 h (bacterial) and 72 h (fungal isolates), in nutrient broth (g/l) peptic digest of animal tissue-5, sodium chloride -5, beef extract -1.5, yeast extract- 1.5, pH- 7.0 ((bacterial) and potato dextrose broth (g/l) potatoes (infusion form) -20.00, dextrose-20.00, pH-6.0 (fungal). All the cultures were then screened for penicillin acylase activity by the method described by (Balasingham *et al.,* 1972). For the determination of nature of penicillin acylase whether it is extracellular or intracellular, the enzyme assays were performed both with fungal culture supernatant as well as with the free resting cells of bacterial and fungal pellet.

3.2.3 Production of penicillin acylase

Fungal spores (8.5×10^5 spores /ml) were seeded in production media containing dextrose-10, peptone-10.0, beef extract-10.0, yeast extact-5 all (g/l) of distilled water) pH-6.0 (Torres *et al.,* 2007), containing 50 ml of the medium supplemented with 1% penicillin G and incubated at 25ºC for 48 h on rotary shaker at 150 rpm. Penicillin acylase activity was assayed using the standard assay method as mentioned in the section (3.2.4)

3.2.4 Spectrophotometric assay of penicillin acylase

The penicillin acylase activity in the clear supernatant was assayed at 40°C using penicillin G (0.05M) as a substrate (Balasingham *et al.,* 1972) in 0.1M potassium phosphate buffer. After incubation, 0.5 ml of the reaction mixture was added to 3.5 ml of reagent solution (containing 0.5 ml of 0.5% *p*dimethylamino benzaldehyde in methanol, 2 ml of 20% glacial acetic acid and 1 ml of 0.05 M NaOH) and the absorbance measured at 415 nm. Each of the assays was performed in triplicate unless otherwise stated and only the mean values ± standard deviation was presented in all the experimental results.

One unit of penicillin acylase activity was defined as the amount of enzyme required to release 1 µmol of 6-APA per min under standard assay conditions. Specific activity of penicillin acylase was expressed as units per mg of protein.

3.2.5 Maintenance of the selected hyper producer of penicillin acylase

The selected fungal isolate was maintained on potato dextrose agar slants. Glycerol stocks of the spores were prepared in 10% glycerol and maintained at -20°C for uniform inoculum throughout the study.

3.2.6 Identification of the isolate

The taxonomic identification of the organism showing highest penicillin acylase activity was carried out by phylogenetic profiling based on 18S rRNA sequence at NFCCI, Agharkar Institute, Pune.

3.3 Optimization of production parameters for penicillin acylase from *Acremonium sclerotigenum*

Optimization of various physicochemical parameters using one factor at a time (OFAT) was done for the production of penicillin acylase from *Acremonium sclerotigenum.* To maximize the yield of enzyme various parameters viz. production media, carbon source, incubation temperature, undefined growth supplements, pH, inoculum size and production profile were standardized.

3.3.1 Screening of various media for penicillin acylase production from *Acremonium sclerotigenum*

For the experiment of media screening, 15 different media (M-1 to M-15) were tested, (components summarized in Table-3.1). All the media were seeded with an

inoculum density of 8.5 $\times 10^5$ spores/ml and incubated at 25°C for 48 h on rotary shaker at 150 rpm.

Table 3.1 Composition of different media used for production of extracellular penicillin acylase from *Acremonium sclerotigenum*

Sr. No	Media	Composition (g/l)	References
1	M-1	Yeast extract-25, Soya Peptone-5, Sodium Chloride-5, Calcium carbonate-2, pH-6.8	Rolinson *et al*., 1961
2	M-2	Yeast extract-2, Casein-2, pH-6.8	Rolinson *et al*., 1961
3	M-3	Yeast extract-10, Sodium Chloride-2, KH_2PO_4-1, $MgCl_2$-0.2, pH-7.2	Plhackova *et al*., 2003
4	M-4	Peptone-5, Beef extract-3, PAA-0.64	Meevotism *et al.*,1983
5	M-5	Tryptone-10**,** Yeast extract-5, Sodium Chloride-5	Enshasy *et al*., 2009
6	M-6	Dextrose-10, Peptone-10.0, Beef extract-10.0, Yeast extact-5.	Torres *et al.*, 2007
7	M-7	K_2HPO_4-1.0, $MgSO_4.7H_2O$-0.5, Calcium Chloride.H_2O-0.05, PAA-1.5, Yeast extract-3.0, pH-5.0	Rajendhran *et al*., 2003
8	M-8	Beef extract-10, Difco peptone-10, Sodium Chloride-5, PAA-2.0	Bodhe and Sivaraman, 1987
9	M-9	Peptone-5, Meat extract-3	Enshasy *et al*., 2009
10	M-10	Casein-4, Yeast extract-8, KH_2PO_4-3, K_2HPO_4- 4.2	Enshasy *et al*., 2009
11	M-11	Tryptone-10, Yeast extract-5, Sodium Chloride-10,Glucose-0.3	Enshasy *et al*., 2009
12	M-12	Yeast extract-5, PAA-2, NH_4Cl-1, KH_2PO_4-0.1, K_2HPO_4-1, $MgSO_4.7H_2O$-0.2	Enshasy *et al*., 2009
13	M-13	Yeast extract-4.0, Malt extract 10.0, Dextrose- 4.0	Kheirolomoom *et al.*, 2001
14	M-14	Glucose-4, $(NH_4)_2HPO_4$-3	Huang *et al*., 1960
15	M-15	Glucose-2, Polypeptone-5, Yeast extract-5, KH_2PO_4-1, $MgSO_4.7H_2O$-0.2, pH-5.5	Bashir *et al*., 2008

The production of penicillin acylase enzyme was optimized with the one-factor-at-a-time (OFAT) method of designing experiments to observe the possible optimum levels of the parameters by changing one value alone. To determine the best cultural conditions, stepwise modifications of the production medium was done that affect fungus biomass and penicillin acylase production. Penicillin acylase activity was assayed using the standard assay method as described earlier in section 3.2.4 and optimum conditions were selected for further studies.

3.3.2 Optimization of carbon source

Acremonium sclerotigenum was grown in media containing different carbon sources to evaluate their effect on penicillin acylase production. Twelve different carbon sources viz. glucose, starch, sucrose, fructose, glycerol, xylose, arabinose, maltose, galactose, sorbitol, melibiose and lactose were used individually at a concentration of 40 mM for penicillin acylase production from *Acremonium sclerotigenum.* In order to optimize concentration of carbon source, varied concentrations (10mM -100 mM) of selected carbon source (glucose) were used in the production medium (pH 6.0). Penicillin acylase activity was assayed using supernatant harvested after 48 h and selected glucose concentration was used in the medium for further studies.

3.3.3 Nitrogen source optimization

Different organic and inorganic nitrogen sources were evaluated for their effect on growth and penicillin acylase production by *Acremonium sclerotigenum.* Control was replaced with 1.0% (w/v) of soya peptone, tryptone, casamino acid, casamino acid hydrolysate, urea, acetamide, potassium nitrate, gelatine, ammonium chloride and ammonium sulphate, individually to study their effect on penicillin acylase production. Further, to find out the optimum concentration of the optimized nitrogen source (soya peptone), it was added at different concentrations ranging from (0.8- 2.6 %) (w/v) in the production medium (pH 6.0) containing glucose 40 mM. The enzyme activity was assayed using culture supernatant harvested after 48 h and optimized nitrogen source concentration was used in the medium for further studies.

3.3.4 Effect of undefined growth supplements

To achieve the maximum growth and extracellular release of penicillin acylase by *Acremonium sclerotigenum,* undefined growth supplements yeast extract and beef extract were individually seeded in production medium (pH 6.0). Further, in order to optimize concentration of yeast extract and beef extract, concentrations were varied from (yeast (0.2-1.2%) (w/v) and (1-4%) (beef extract) respectively to achieve maximum penicillin acylase production. The enzyme activity was assayed using culture supernatant harvested after 48 h and optimum growth supplement concentration was used in the medium for further studies.

3.3.5 Effect of temperature on enzyme production

An optimum cultivation temperature condition for maximum penicillin acylase production was determined by growing isolate with previously optimized carbon and nitrogen sources. The flasks in shaking conditions containing the optimized media were incubated at various temperatures (20, 25, 30, 35, 40 and 45°C) to see the effect of temperature on the production.

3.3.6 Effect of pH on enzyme production

The fungal isolate was cultivated in the pre optimized medium and pH of the medium was varied as 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Penicillin acylase activity was assayed using supernatant harvested after 48 h according to the method described by (Balasingham *et al.*, 1972) with suitable modifications.

3.3.7 Effect of inoculum size

The inoculum size must be large enough to reach threshold microbial population so that all the substrate particles could be metabolized with maximum release of enzyme. To study the effect of inoculum size on enzyme production different counts of spores (8.5×10^5 to $15.5\times10^{5)}$ were inoculated in the production medium. The enzyme activity was assayed using culture supernatant harvested after 48 h and optimized inoculum size was used in the production medium for further studies.

3.3.8 Growth and enzyme production profile of *Acremonium sclerotigenum*

In order to find the optimal time of incubation for the maximum penicillin acylase production, the sterile medium (pH 6.0) containing soya peptone (1.6%) glucose (40mM) yeast extract (1.6%) and beef extract (0.8%) was inoculated with 1.25×10^6 spores and incubated in orbital shaker for 7 days at 25°C. The pH profile and penicillin activity in culture filtrate was checked after an interval of 6 h. Fungal biomass yield was determined as dry cell weight (dcw) after filtration through Whatman filter paper No.1 of the culture medium (100 ml) and dried at 60°C until three concordant weights were recorded and dcw was calculated as follow:

$$\text{DCW (mg/ml)} = \frac{(Wt.of dried\ mycelium + filter\ paper) - Wt.of\ filter\ paper}{100}$$

3.4 Immobilization of penicillin acylase of *Acremonium sclerotigenum* on different matrices

In the present study, penicillin acylase from *Acremonium sclerotigenum* was immobilized using adsorption and gel entrapment methods. Crude Penicillin acylase was immobilized on different matrices, *viz.*, agar, alginate, silica and chitosan. The encapsulator (Buchi Encapsulator B-390) was used for formation of equal sized beads of alginate and chitosan. These matrices were compared for their penicillin acylase activity and immobilization efficiency.

3.4.1 Immobilization of penicillin acylase on agar

Agar (2%) was added to 20 ml of distilled water and mixture was autoclaved at 121°C and then cooled to 40°C in water bath. Penicillin acylase (1.22U/ml±0.4) was added to melted agar and thoroughly mixed (Kierstan and Coughlan, 1985; modified). Mixture was poured into petriplate and then cooled and kept for 30 minute at 4°C for solidification. After solidification gel discs were cut and stored in 125mM sodium citrate buffer for further use.

3.4.2 Immobilization of penicillin acylase on calcium alginate

Penicillin acylase (1.22U/ml±0.02) was added to 2% sodium alginate and mixed thoroughly. The viscous enzyme-alginate mixture was introduced drop wise into 100 mM calcium chloride ($CaCl_2$) solution with the encapsulator in stirring conditions to produce calcium alginate beads. The beads were kept for hardening at 4 ºC for 30 min. After 30 min, the beads were filtered, washed thoroughly 4 times and stored in sodium citrate (pH 5.0) at 4 ºC for further use. The hardening and washing solution were collected to calculate unbound and leached enzyme. The immobilization efficiency was determined as described in section 3.9.4 after calculating activity of total added penicillin acylase and residual activity of unbound enzyme in hardening and washing solution. Crucial parameters like concentration of sodium alginate (1.5-4 % w/v) and calcium chloride were optimized for penicillin acylase immobilization in alginate beads.

3.4.3 Immobilization of penicillin acylase on silica gel

Silica (3 g) was dissolved in minimum amount of sodium citrate buffer (50 mM, pH 5.0) and left overnight at room temperature for activation. Next day it was filtered with Whatman No. 1 filter paper. Swelling capacity was calculated as:

$$\textbf{Swelling capacity} = \frac{\textbf{Weight of Wet matrix} - \textbf{weight of Dry matrix}}{\textbf{Weight of Dry matrix}} \times 100$$

Free enzyme (2.5 ml, 1.22 U/ml) was added in 1g of wet silica (pellets). Binding time varied between 1 to 4 h at 4ºC with continuous stirring rate of 100 rpm, then it was filtered with Whatman No. 1 filter paper. Protein and enzyme activity was determined in both pellet and supernatant.

3.4.4 Immobilization of penicillin acylase on chitosan beads

Chitosan (2.0g) was dissolved in acetic acid solution (4.0%, 100 ml) and kept overnight. The mixture was introduced drop wise into sodium hydroxide solution (2.5 M, 250 ml) using the Encapsulator System (B-390) while stirring continuously and was kept for 30 min. The beads were filtered and washed twice with distilled water. The chitosan beads were cross-linked and activated by immersing into sodium hydroxide solution (pH -12.0,100 ml) containing 2.0 % epichlorohydrin at 50ºC for 2 h while stirring at 200 rpm. The resulting cross-linked chitosan beads were stirred in chilled ethanol (200 ml) for 30 min and then washed extensively with sodium citrate buffer (pH-5.0, 125m M). Now the beads were transferred into enzyme solution. Immobilization time was 2 h with continuous stirring rate of 100 rpm at 4ºC. Then enzyme bound chitosan beads were separated and washed with saline solution (40 ml, 0.5M NaCl) then with sodium citrate buffer (pH 5.0, 125m M) and stored at 4ºC in same buffer.

3.4.5 Comparison of the matrices for their activity and immobilization efficiency

These matrices, *viz.*, agar, calcium alginate, silica, and chitosan were compared for their immobilization efficiency and the matrix showing highest immobilization efficiency and penicillin acylase activity was selected for further studies. Penicillin acylase activity and immobilization efficiency were calculated as mentioned below.

3.4.6 Immobilized penicillin acylase assay

Penicillin acylase assay for immobilized penicillin acylase was performed in reaction mixture which contained 125mM sodium citrate (900µl) pH 5.0, immobilized penicillin acylase and penicillin G (50 mM), incubated at 40°C for 20 min. Reaction was stopped by removing bead/pellet from reaction mixture and absorbance was taken at 415 nm (One unit of immobilized penicillin acylase activity

corresponds to 1 µmol of 6 -APA formed per min by 1g of immobilized enzyme (weight of matrix included) under the standard assay conditions.

3.4.7 Immobilization efficiency

Immobilization efficiency (I.E.) of different matrices was calculated using following equation:

$$\textbf{Immobilization Efficiency } (\%) = \frac{A-B}{A} \times 100$$

Where A = Total Added enzyme (U) and B = Total unbound enzyme (U)

3.5 Optimization of reaction parameters for free and alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum* for 6-aminopenicillanic acid synthesis

Various reaction parameters such as buffer systems, pH, reaction time and temperature, substrate concentration and enzyme concentration has crucial effect on enzyme activity. Individual parameters were investigated at different level and optimized value was used in next reaction parameter for both free and immobilized penicillin acylase. All the experiments were performed in triplicate and the mean values applying standard deviations were reported.

3.5.1 Optimization of buffer system and pH for crude and immobilized penicillin acylase

In order to select suitable buffer system and its optimum pH for enzyme reaction, 0.1 M of different buffers, *viz.*, sodium-citrate buffer (pH 3.0-6.5), sodium-acetate buffer (3.5-6.0), potassium phosphate buffer (pH 7.0-9.5), tris-HCl (7.0-9.0) and glycine NaOH buffer (pH 8.0-10.5) were used. The penicillin acylase activity of crude and immobilized enzyme was determined with 50 mM penicillin G as substrate at 40^{o}C for 20 minutes. Enzyme activity was assayed as described earlier in the section 3.2.4 and 3.4.6 respectively.

3.5.2 Effect of buffer concentration

Enzyme reactions were carried out in different concentrations of selected sodium citrate buffer (25 mM-225mM) at optimum pH 5.0 (free) and 6.0 (alginate gel entrapped enzyme). Enzyme activity was assayed as described earlier in section 3.2.4 and 3.4.6 respectively.

3.5.3 Optimization of reaction temperature

The optimum temperature for the enzyme reaction was investigated by evaluating the free and immobilized penicillin acylase activity of *Acremonium sclerotigenum* at different temperatures (30°C-60°C) to determine the optimum reaction temperature for maximum penicillin acylase activity.

3.5.4 Effect of substrate and substrate concentration

To study the effect of substrate on penicillin acylase activity, two different substrates (50 mM) penicillin G and V were used in the reaction mixture. Concentration of the optimized substrate for both free and immobilized penicillin acylase was varied from 12.5 mM to 200 mM in the reaction mixture.

3.5.5 Effect of enzyme dose

Different amounts of enzyme, free enzyme (1.07-16.05 µg) and (6.75 -135 µg) immobilized enzyme were used in the reaction mixture to evaluate the optimum enzyme concentration. Enzyme activity was assayed as described earlier in section 3.2.4 and 3.4.6 respectively.

3.5.6 Effect of metal ions, inhibitors and additives

To study the effect of metal ions on the activity of free and immobilized penicillin acylase the reactions were performed at 1mM final concentration of metal ions ($MgCl_2.6H_2O$, $ZnSO_4.7H_2O$, $CoCl_2$, $CuSO_4.5H_2O$, $BaCl_2.2H_2O$, $HgCl_2$ and $CaCl_2.\ 2H_2O$) in reaction mixture. Similarly, effect of inhibitors and additives such as dithiothreitol (DTT), ethylene diaminetetra acetic acid (EDTA), phenyl methyl sulphonyl fluoride (PMSF), urea and 2-mercaptoethanol were also studied in 1mM final concentration in reaction mixture.

3.5.7 Effects of various organic solvents

The enzyme reaction for both free as well as alginate immobilized enzyme was performed in the presence of various organic solvents e.g. isoamylalchhol, butanol, ethanol, methanol, acetone, isopropanol, 1, 4-dioxan, phenol, hexane and benzene at final concentrations of 10% v/v. Enzyme activity was assayed at 40°C (free) and 50°C (immobilized) as described earlier in section 3.2.4 and 3.4.6 respectively.

3.5.8 Thermostability of crude and immobilized penicillin acylase of *Acremonium sclerotigenum*

The thermal stability of the penicillin acylase is one of the most important criteria when dealing with its applications. Thus, thermostability of enzyme was studied by pre-incubating the free and alginate gel entrapped enzyme at various temperatures i.e. 40°C, 45°C, 50°C, 55°C and 60°C for 120 minutes. The residual enzyme activity was assayed after an interval of 15 minutes as described earlier in section 3.2.4 and 3.4.6 respectively.

3.5.9 Reusability of alginate gel entrapped penicillin acylase

The most important and attractive advantage of immobilization is the reusability of penicillin acylase. The catalyst reusability was determined by measuring of the stability of the immobilized enzyme as a function of number of reusages. The recyclability of the immobilized penicillin acylase was assessed in the reaction mixture by repeated use of calcium alginate beads. Removal of beads from the reaction mixture stopped first enzyme reaction. Then the beads were thoroughly washed with sodium citrate buffer and then used for the second reaction and so on.

3.5.10 Shelf-life of penicillin acylase

Storage stability of free and alginate immobilized penicillin acylase from *Acremonium sclerotigenum* was investigated at 4°C and room temperature for 360 h (free) and 480 h (immobilized) respectively. The enzyme was stored at 4°C and room temperature and enzyme activity was checked after an interval of 24 h.

3.5.11 Optimization of time course of enzyme reaction at different temperatures

Time course of enzyme reaction at different temperatures (30°C-60°C.) was studied with 50 mM penicillin G (Free) and 75 mM penicillin G (immobilized) as substrate for 90 minutes. The enzyme reaction was evaluated by terminating the reaction at an interval of 10 minutes.

3.6 Purification of penicillin acylse from *Acremonium sclerotigenum*

The penicillin acylse from *Acremonium sclerotigenum* was purified to homogeneity to study its biochemical characteristics. The cell free supernatant obtained after separating fungal mycelium was used as crude enzyme. The amount of protein content and penicillin acylase activity was determined. This crude enzyme was

purified by applying different protein precipitation methods and chromatographic techniques. All the experiments pertaining to purification of enzyme were carried out at 4ºC. Hydrophobic interaction chromatography (HIC) was performed using manually packed column (Octyl-Sepharose 4 FF, Amersham Pharmacia Biotech (now GE Healthcare) and the protein eluted during chromatography was monitored by absorbance at 280 nm using UV-VIS spectrophotometer (Lab India). Penicillin acylase activity in the pooled fractions was assayed as per method described earlier in the section (3.2.4). Protein concentrations in the fractions were estimated by Bradford method (Bradford, 1976) using BSA as standard. SDS-PAGE (Laemmli, 1970) analysis of the purified enzyme fractions obtained from the chromatography was done to assess molecular weight of enzyme and purification status of enzyme. Native-PAGE was also performed to determine the native molecular weight of the enzyme (Bollag and Edelstein, 1991).

3.6.1 Preparation of crude enzyme extract

The penicillin acylase from *Acremonium sclerotigenum* was purified from the fermentation broth. The production medium pH-6.0 containing (w/v) soya peptone (1.6%) glucose (40 mM) yeast extract (1.6%) and beef extract (0.8%) was seeded with 1.25×10^5 spores/ml and the Erlenmeyer flasks were incubated at 25°C for 48 hrs in an incubator shaker (150 rpm). The cell free crude enzyme extract was obtained by filtration of fermented broth using Whatman filter paper No.1 at 4°C. The collected supernatant was designated as cell free crude enzyme and used further for salting out by ammonium sulphate precipitation.

3.6.2 Ammonium sulphate precipitation of extracellular penicillin acylase from *Acremonium sclerotigenum*

Ammonium sulphate was used for the concentration and partial purification of the crude penicillin acylase**.** The culture filtrate was subjected to various saturation of ammonium sulphate (10-90%). The ammonium sulphate precipitation table (Scopes, 1982) was followed to calculate required amount of ammonium sulphate to be added in crude enzyme. Ammonium sulphate was added in increasing concentration with continuous stirring and the contents were stored at 4°C for one hour. The precipitated proteins were recovered by centrifugation at 15,000 g at 4°C for 30 min and suspended in minimum amount of 125 mM Sodium citrate (pH 5.0). Protein concentration and enzyme activity were estimated in each sample. The enzyme assay

was carried out as described earlier in the section (3.2.4). The optimum penicillin acylase activity at a specific concentration of ammonium sulphate reflects the best concentration to attain maximum enzyme recovery. Based on result of above experiment, an initial cut of 10% was employed to crude enzyme (300ml) to precipitate the contaminating proteins and final concentration of ammonium sulphate (10-50%) was added to the supernatant to precipitate the protein of interest which was recovered by centrifugation and suspended in 6 ml of 125 mM sodium citrate (pH 5.0) and was taken for further purification of enzyme.

3.6.3 Protein estimation (Bradford, 1976)

The colorimetric Bradford assay was used for the estimation of protein. This assay is based on an absorbance shift in the dye coomassie when bound to arginine, hydrophobic amino acid residues present in the protein. The bound form of the dye is blue and has an absorption spectrum maximum at 595 nm. The increase of absorbance at 595 nm is proportional to the amount of bound dye and thus to the amount (concentration) of protein present in the sample.

Reagents

Bradford stock solution

Ethanol	100 ml (95%, v/v)
Phosphoric acid	200 ml (85%, v/v)
Coomassie Brilliant Blue	G 350 mg

Bradford working solution

Distilled water	425 ml
Ethanol15 ml	(95%, v/v)
Phosphoric acid 30 ml	(85%, v/v)
Bradford stock solution	30 ml

The reagents were mixed thoroughly and filtered through Whatman filter paper No. 1 and stored at room temperature in amber glass bottles.

Procedure

To 0.1 ml protein sample, 1ml of Bradford working reagent was added and mixed immediately by vortexing. The absorbance of the reaction mixture was read at

595 nm after 2-5 min. A standard curve was prepared by using 20 -200 µg/ml of bovine serum albumin.

3.6.4 Hydrophobic interaction (Octyl-Sepharose) chromatography

The ammonium sulfate precipitated crude enzyme was subjected to hydrophobic interaction chromatography (HIC) using 5 ml of manually packed Octyl-sepharose column at a flow rate of 0.5 ml/min. The Octyl group is covalently coupled to a cross-linked 4% agarose matrix by ether linkage, giving a hydrophobic medium with minimal leakage and no ionic properties. Protein samples are separated on the basis of their different hydrophobicity.

Reagents

Sodium citrate buffer

Binding buffer

Elution buffer

Octyl -sepharose column

Column description

Column Volume	5 ml
Matrix	Agarose
Hydrophobic ligand	Octyl

3.6.5 Preparation of the matrix

Octyl-sepharose was supplied pre-swollen in 20% ethanol. Since ethanol markedly reduces hydrophobic interaction it was essential to thoroughly wash the medium to remove all traces before applying the sample. The medium was washed with at least 10 volumes of distilled water and decanted. The slurry was prepared with binding buffer, in a ratio of 75% settled medium to 25% buffer.

3.6.6 Packing of Octyl-sepharose column

All solvents were equilibrated to the temperature at which the chromatography will be performed. The slurry was poured into the column in one continuous motion, minimizing the introduction of air bubbles. The vacant space of the column was filled with buffer.

3.6.7 Equilibration of column

Before applying the sample, the column was equilibrated with at least 2 column volumes of equilibration buffer. The column was pre-equilibrated with 1.5 M ammonium sulphate in 125 mM Sodium citrate (pH 5.0) for hydrophobic interaction.

Loading and binding of protein

Those salts which cause salting-out (ammonium sulphate) also promote binding to hydrophobic ligands. The crude enzyme (210.16 U) was precipitated by ammonium sulphate (10-50%, w/v saturation) and stored at 4^{o}C. The protein sample having 2.94 U/mg specific activity was applied in a solution of high salt concentration (1.5 M ammonium sulphate) on the Octyl-sepharose column and subjected to hydrophobic interaction chromatography.

3.6.8 Elution of the protein

After loading the protein sample, bound proteins were eluted with a decreasing gradient of ammonium sulphate from 1.5 M to 0.1M in 125 mM sodium citrate buffer (pH 5.0). Last fractions were taken in 125 mM sodium citrate buffer (pH 5.0). The flow rate of eluent was adjusted to 0.5 ml/min and 10 fractions of 2 ml each were collected at all concentrations of elution buffer. The protein eluted during chromatography was monitored by taking absorbance at 280 nm and protein estimation was done as described in section 3.6.3. Enzyme assay of all the collected fractions were also performed. The protein fraction rich in penicillin activity were pooled and processed further to establish its purity.

3.6.9 Determination of molecular mass [M_r]

Both the SDS and Native -PAGE were performed to determine the purity on native, molecular mass and its subunit molecular mass. MALDI-TOF analysis was done to determine exact molecular mass.

3.6.9.1 Polyacrylamide gel electrophoresis (PAGE)

The polyacrylamide gel electrophoresis was performed to analyze the molecular mass of the protein and purity of the fractions obtained from hydrophobic interaction chromatography (Laemmli 1970, Blackshear 1984, Bollag and Edelstein 1991).

Reagents

A. Stock solutions for SDS-PAGE and Native-PAGE

Tris-HCl	2 M (pH 6.8, 8.8)
Sodium dodecyl sulphate	10% (w/v)
Ammonium persulphate	10% (w/v)
Glycerol	50% (v/v)
Bromophenol blue	1% (w/v)
2-Mercaptoethanol	

B. Working solution for SDS-PAGE and Native-PAGE

I. Solution A (Acrylamide stock solution)

The acrylamide stock solution was prepared by dissolving 29.2 g acrylamide and 0.8 g bis-acrylamide in distilled water and stirred until completely dissolved. More distilled water was added to make final volume 100 ml.

II. Solution B (Separating gel buffer, 4 x)

The separating gel buffer for SDS-PAGE was prepared by mixing 75 ml of 2 M Tris-HCl (pH 8.8), 4 ml of SDS and 21 ml of distilled water for SDS-PAGE. However, for Native-PAGE preparation, SDS was replaced by distilled water.

III. Solution C (Stacking gel buffer, 4 x)

The stacking gel buffer (4 x) was prepared by mixing 50 ml 1 M Tris-HCl (pH 6.8), 4 ml of 10% SDS and 46 ml of distilled water. SDS was omitted in solution C for Native-PAGE.

IV. Electrophoresis buffer

One litre of electrophoresis buffer was prepared by dissolving 3 g Tris, 14.4 g glycine and 1 g SDS in distilled water. However, pH was adjusted to 8.3 before making the final volume 1 L. SDS was not added in case of electrophoresis buffer for Native-PAGE.

V. Sample buffer (5 x)

The sample loading buffer 5x (10 ml) was prepared by mixing 0.6 ml 1 M Tris-HCl (pH 6.8), 5 ml glycerol, 2 ml SDS, 0.5 ml 2-mercaptoethanol, 1ml bromophenol blue and 0.9 ml distilled water. However, SDS and 2-mercaptoethanol were replaced with distilled water for Native-PAGE

3.6.9.2 Preparation of gel for Native and SDS-PAGE

The gel electrophoresis system from Banglore Genei Pvt. Ltd. was used to perform the SDS-PAGE and Native-PAGE. To prepare one gel of 1.0 mm thickness for SDS and native-PAGE, 6 ml of freshly prepared separating gel (12 % for SDS-PAGE and Native -PAGE) mixture was poured between two glass plates sandwiched together with 1.0 mm spacers. The gel solution was layered with distilled water and allowed to polymerize for one hour. The water layer form top was removed using filter paper and 1 ml of freshly prepared stacking gel (4%) mixture was poured over it. The comb was inserted carefully to avoid air bubbles and kept for 1h for polymerization. The composition of separating and stacking gels are given below:

Table 3.2 Composition of separating and stacking gels

Composition	Stacking gel (4%)	Separating gel (12%)	Separating gel (10%)
Solution A	0.285 ml	2.80 ml	2.33 ml
Solution B	-	1.75 ml	1.75 ml
Solution C	0.500 ml	-	-
Distilled water	1.235ml	2.45 ml	2.92 ml
APS (10%)	30 µL	35 µL	35 µL
TEMED	5 µL	10 µL	10 µL

3.6.9.3 Protein molecular weight markers

I. SDS- protein molecular weight marker

The protein molecular weight markers for SDS-PAGE from Biochem life sciences (High Range, Molecular Weight 11-245 kDa) were used for molecular mass analysis of penicillin acylase of *Acremonium sclerotigenum*. The molecular weight of the marker proteins were as follows:

II. Native-protein molecular weight marker

The broad range molecular weight-native protein markers from Serva Electrophoresis, Heidelberg (Kit for Molecular weights 21-720 kDa Non-denaturing PAGE) were used for determination of native molecular weight of penicillin acylase

of *Acremonium sclerotigenum.* The molecular weights of marker proteins were as per given details:

Protein	**MW (kDa)**
Ferritin	20
Urease (hexamer)	545
Ferritin	450
Urease (trimer)	272
Lactate dehydrogenase	146
Bovine serum albumin	67
Ovalbumin	45
Trypsin inhibitor	21

C. Sample loading and electrophoresis

The protein samples for SDS-PAGE electrophoresis was prepared by mixing the sample with 5x sample loading buffer in the ratio of 4:1 (v/v) in an eppendorff tube and heated to 100^{o}C in a boiling water bath for 5 min, however for Native-PAGE, the samples were mixed with 5x sample loading buffer (without SDS). The prepared samples were loaded onto the gel and electrophoresis was performed at constant voltage of 35 V at 4^{o}C until the dye front reached 0.5 cm above the bottom of the gel.

D. Staining and destaining of gel

After completion of electrophoresis, the gel was carefully transferred to a clean gel staining box containing 50 ml staining solution and kept over the rocker for gentle shaking (10-15 min) at room temperature. The staining solution was poured off and gel was washed twice with distilled water. The gel destaining solution (50 ml) was added and gently shook over rocker for 1 h. The gel was transferred in to destain under continous shaking and was extensively destained overnight to obtain distinctively resolved protein bands. The composition of staining and destaining solution are given below:

Staining solution (1 L)	
Methanol	450 ml
Glacial acetic acid	100 ml
Coomassie blue R-250	1.0 g
Distilled water	450 ml

Destaining solution (1 L)	
Ethanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

E. Analysis of the gel

The images of gel were recorded by Gel documentation System (Alpha Innotech Corporation, USA). The molecular weight analysis was done using Alpha Digi Doc RT and Alpha Ease FC software.The molecular weight of the purified penicillin acylase of *Acremonium sclerotigenum* was determined by SDS/Native-PAGE and MALDI-TOF analysis.

3.6.10 Molecular characterization of purified penicillin acylase from *Acremonium sclerotigenum*

The biochemical characterization of the purified penicillin acylase of *Acremonium sclerotigenum* was performed to compare its properties with that of the other reported penicillin acylase. The biochemical properties (buffer system and pH, buffer molarity, thermal stability, K_m and $V_{max,}$ effect of metal ions and inhibitors, effect of organic solvents and storage stability) were investigated for the purified enzyme.

3.6.10.1 Optimization of buffer system and buffer pH

The buffer system and the pH optima of the penicillin acylase from *Acremonium sclerotigenum* was evaluated by measuring the enzyme activity at various pH values of different buffers (0.1 M) *viz.*, sodium-citrate buffer (pH 3.0-6.5), sodium-acetate buffer (3.5-6.0), potassium phosphate buffer (pH 7.0-9.5), tris HCl (7.0-9.0) and glycine NaOH buffer (pH 8.0-10.5). The purified penicillin acylase activity was determined in each buffer with 50 mM penicillin G at 40^{o}C for 20 minutes.

3.6.10.2 Effect of buffer molarity

To study the effect of buffer concentration, enzyme reactions were carried out at different molarity of selected sodium citrate buffer (pH 5.0) varied from 25 mM to 225 mM. The reaction was performed with penicillin G at 40^{o}C for 20 min.

3.6.10.3 Effect of reaction temperature

The optimum temperature for the enzyme reaction was investigated by measuring the activity at different temperature (30°C, 35°C, 40°C, 45°C, 50°C, 55°C and 60°C) in 125 mM sodium citrate (pH-5.0) with 50mM penicillin G for 20 minutes.

3.6.10.4 Effect of substrate concentration

To study the effect of substrate concentration on penicillin acylase activity, different concentrations of penicillin G, (12.5-200 mM) were used in reaction mixture containing 125 mM sodium citrate (pH 5.0) at 40°C.

3.6.10.5 K_m and V_{max} of purified penicillin acylase from *Acremonium sclerotigenum*

To determine the K_m and V_{max} of purified penicillin acylase of *Acremonium sclerotigenum* the concentration of penicillin G was varied from 12.5-200 mM. The initial velocity (V) of the reaction at various substrate concentrations was calculated and the K_m and V_{max} of purified penicillin acylase from *Acremonium sclerotigenum* were determined by Line Weaver Burk plot.

3.6.10.6 Effect of enzyme dose

Different concentrations of enzyme (0.073- 9.44µg) were used in reaction mixture to investigate the optimum concentration of penicillin acylase from *Acremonium sclerotigenum* while keeping concentration of substrate (50mM) fixed.

3.6.10.7 Effect of metal ions, additives and inhibitors

To study the effect of metal ions ($MgCl_2.6H_2O$, $ZnSO_4.7H_2O$, $CoCl_2$, $CuSO_4.5H_2O$, $BaCl_2.2H_2O$, $HgCl_2$ and $CaCl_2.2H_2O$) on the enzyme activity of purified penicillin acylase from *Acremonium sclerotigenum*, the reaction was performed at 1mM final concentration of metal ions in reaction mixture. Similarly effect of additives and inhibitors DTT, EDTA, PMSF, urea and 2-mercaptoethanol were also studied in 1mM final concentration in reaction mixture. Penicillin acylase activity was assayed in 125 mM sodium citrate at 40°C for 20 min using penicillin G (50 mM) as substrate.

3.6.10.8 Effects of various organic solvents

Various organic solvents i.e. isoamyl alcohol, butanol, ethanol, methanol, acetone, isopropanol, 1-4-dioxan, phenol, hexane and benzene were added in reaction mixture at final concentration of 10% (v/v). Penicillin acylase activity was assayed in 125 mM Sodium citrate at 40°C for 20 min using penicillin G (50mM) as substrate.

3.6.10.9 Thermostability of purified penicillin acylase

To evaluate the thermostability of enzyme, the enzyme was pre incubated at various temperatures i.e. 40°C, 45°C, 50°C, 55°C and 60°C for 120 minutes. The enzyme activity was assayed after an interval of 15 min at 40°C for 20 minutes using penicillin G (50 mM) as substrate.

3.6.10.10 Shelf life of purified penicillin acylase

The penicillin acylase from *Acremonium sclerotigenum* was stored at 4°C and room temperature for 240 h. To investigate its storage stability enzyme activity was determined after an interval of 24 h.

3.7 Bioprocess development for the bench scale production of 6 aminopenicillanic acid

Based on the results of optimization of reaction parameters for the free and immobilized enzyme, the penicillin acylase from *Acremo*nium *sclerotigenum* was used for development of bioprocess for the synthesis of 6-aminopenicillanic acid.

3.7.1 Batch & fed batch reaction at 50 ml

Under optimized reaction parameters, reaction volume was scale up to 50ml. Both batch and fed batch synthesis was performed with free and alginate entrapped penicillin acylase from *Acremo*nium *sclerotigenum* in 250 ml capped Erlenmeyer flask at 40°C and at 50°C for 1.5 and 2.5 h respectively under continues shaking. The 6-APA synthesis was quantified by HPLC.

The reaction was carried out in 125 mM sodium citrate buffer (pH-5.0) at 40°C for free enzyme and with 150 mM sodium citrate buffer (pH-6) for alginate gel entrapped penicillin acylase. The substrate was supplied both in single batch and step feeding in reaction up to 90 and 150 minutes respectively. 6-aminopenicillanic acid formed was estimated and quantified by HPLC.

3.7.2 Bioconversion of penicillin G to 6 aminopenicillanic acid at bench scale (1L)

Bioconversion of penicillin G to 6-APA was carried out at 1L scale using 125mM sodium citrate buffer (pH-5.0) at 40°C (free enzyme) and 150mM sodium citrate buffer (pH-6), 50 mM penicillin G and 27µg of immobilized enzyme in 1.5 L fermenter (BioFlow C-32 New Brunswick Scientific, USA).The temperature of the

fermenter vessel was maintained at 40°C (free) and at 50°C (immobilized) for 90 and 150 minutes respectively with impeller speed of 200 rpm.

Fig.3.1 BioFlow C-32 New Brunswick Scientific, USA, fermenter used for the bioconversion of penicillin G to 6-APA using alginate entrapped penicillin acylase from *Acremonium sclerotigenum*

3.7.3 Down stream processing of reaction mixture and characterization of 6-APA

3.7.3.1 Recovery of 6-APA from reaction mixture

After completion of the bioprocess, the beads were separated from the reaction mixture through centrifugation at 10,000 *g* for 15 min. The supernatant obtained was was freeze-dried (Flexi-Dry MP ™, FTS USA) to recover the powder of 6-APA.This powder was compared with the commercial 6-APA (by HPLC analysis), to assess the purity of the product.

3.7.3.2 Characterization of 6-APA

The product 6-APA was characterized and authenticated with the following methods.

3.7.3.3 UV visible spectroscopy

The UV-visible spectra were recorded by using a Varian Cary 50 spectrophotometer with quartz cuvette of 1 cm path length at room temperature. All the samples for UV-visible analysis were prepared by dissolving in distilled water and subjected to the UV-visible spectroscopy analysis at Sardar Vallabhbhai National Institute of Technology Surat, Gujarat, India.

3.7.3.4 Fluorescence spectroscopy

The fluorescence spectra were recorded by using a Cary Eclipse Fluorescent spectrophotometer with a quartz cuvette of 1 cm path length at room temperature. All the samples for fluorescence analysis were prepared by dissolving in distilled water and subjected to the fluorescence spectroscopy analysis at Sardar Vallabhbhai National Institute of Technology Surat, Gujarat, India.

3.7.3.5 Infrared spectroscopic analysis (FTIR)

Test sample (6- aminopenicillanic acid) was ground with IR grade potassium bromide (KBr) (1:10) pressed in to discs under vacuum using spectra lab pelletiser and compared with commercial authentic 6- aminopenicillanic acid. The IR spectrum was recorded in the region 4000-500/cm in Shimadzu FTIR 8000 series instrument at SAIF/CIL, Punjab University Chandigarh, India.

3.8 Application of penicillin acylase

3.8.1 Decolourization of dye by penicillin acylase and *Acremonium sclerotigenum*

Five different dyes (5mM) Reactive Blue 4, Remazol Brilliant Violet 5R, Methyl red, Indigo and Bismarck Y were initially screened to analyse the decolourization efficiency of penicillin acylase and *Acremonium sclerotigenum*. Dye which showed highest decolourization (%) was selected for further studies. After screening of dyes, various growth parameters of fungus *viz.* carbon source, nitrogen source, time of incubation, medium pH, inoculum density, decolourization profile and concentration of dye were standardized for maximum decolourization. The decolourization % was calculated as:

$$\frac{\textbf{Transmittance of test - Transmittance of control}}{\textbf{Transmittance of test}} \times \textbf{100}$$

3.8.2 Effect of carbon source

To determine the effect of different carbon sources on dye decolorization potential of *Acremonium sclerotigenum,* six different carbon sources (40mM) *viz*. starch, sucrose, fructose, galactose maltose, and dextrose, were added individually in the medium and the decolourization % was recorded. The carbon source was omitted from medium control.

3.8.3 Effect of nitrogen source

Nitrogen source supplementation in medium plays a critical role in dye decolorization activity. Seven different nitrogen sources (1%) (tryptone, peptone, urea, acetamide, ammonium sulphate, gelatin, soya peptone) were added in the medium for the decolourization (%) of methyl red .

3.8.4 Effect of temperature

To study the effect of different temperatures on dye decolorization efficiency of the fungus, the medium pH was adjusted to initial pH -6 and incubated at 25, 30, 35 and 45 °C 50 for 2 days. The decolourization % was calculated as per section 3.8.1.

3.8.5 Effect of medium pH

The pH of the optimized medium was varied from pH 3.0 to 9.0 and decolourization % for methyl red was recorded.

3.8.6 Effect of Inoculum density

Inoculum density was varied from 1.85×10^5 spores/ml to 1.65×10^5 spores/ml, to evaluate decolourization potential of *Acremonium sclerotigenum.*

3.8.7 Decolourization profile of methyl red using *Acremonium sclerotigenum*

The dye decolourization profile of methyl red was studied for 120 h. Samples were taken at the interval of 24 h to find the optimum time period for decolourization of methyl red.

3.8.8 Effect of dye concentration

Concentration of dye influences the decolourization rate. Dye concentration was varied from 1.25mM -25 mM. The decolourization % was calculated as per section 3.8.1.

Chapter - 4 Results and Discussion

Biotechnological applications of penicillin acylases (PA) have emerged as a serious alternative to traditional chemical procedures for the manufacture of β-lactam antibiotics, small peptides and pure isomers from racemic mixtures. However, penicillin acylases are involved mainly in the industrial production of semi synthetic penicillins (Calleri *et al.,* 2004).

The objectives of present study were to isolate a potential organism, to optimize various fermentation parameters, purification and characterization of purified penicillin acylase, immobilization of penicillin acylase on suitable matrix and also to investigate its potential in the synthesis of 6-Aminopenicillanic acid. Results of the experiments performed in this study are presented and discussed in the following sections:

4.1 Isolation of penicillin acylase producing microorganisms

Soil samples collected from different parts of Solan and Shimla district of Himachal Pradesh were cultured on nutrient agar (NA) and potato dextrose agar (PDA). NA plates were used to isolate bacterial isolates and PDA plates for fungal isolates. About 108 isolates were obtained from soil samples.

4.1.1 Screening of penicillin acylase producing microorganisms

The *Serratia marcescens* ATCC 27117 overlay technique (Meevootisom *et al.,* 1983) was used for bioassay of penicillin acylase producers. Cultures grown at 28°C on nutrient agar plates for approximately 24 h were overlaid with agar containing penicillin and *S. marcescens* ATCC 27117, a bacterial strain resistant to penicillin but sensitive to 6-APA. The plate was incubated at 28°C for approximately 24 h and enzyme producers identified by the formation of a clear zone on a red mat of growth. Out of 108 isolates, 11 bacterial and 4 fungal isolates showed positive reaction (Table 4.1).

Table 4.1 Morphological characteristics and penicillin acylase activity of selected microbial isolates

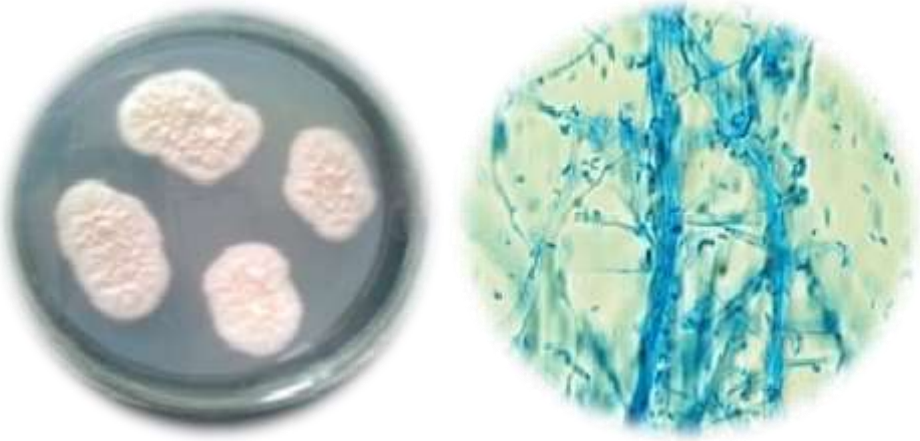
Isolate	Colony colour on PDA/NA	Growth pattern / Colony shape / Gram staining	Enzyme activity(U/ml)
Fungal Isolates			
PAF-1	White	Slow	0.05
PAF-2	Black	Rapid	0.01
PAF-3	Yellowish green	Rapid	0.13
PAF-4	**Peach**	**Slow/Velvety**	**0.34**
Bacterial Isolates			
PAB-1	Creamish	Circular , Gram -ve	0.02
PAB-2	Creamish	Circular, Gram -ve	0.01
PAB-3	Creamish	Circular, Gram +ve	0.03
PAB-4	Creamish	Irregular, Gram-ve	0.10
PAB-5	Yellow	Circular, Gram+ ve	0.05
PAB-6	Creamish	Circular, Gram -ve	0.03
PAB-7	Creamish	Wavy, Gram +ve	0.23
PAB-8	Light orange	Circular, Gram-ve	0.11
PAB-9	Creamish	Circular, Gram-ve	0.01
PAB-10	Creamish	Wavy, Gram-ve	0.09
PAB-11	Yellow	Irregular, Gram-ve	0.15

After primary screening the selected isolates were grown in potato dextrose broth and nutrient broth, incubated at 30°C in orbital shaker at 150 rpm. Penicillin acylase production was observed in 4 fungal isolates and 11 bacterial isolates. However, penicillin acylase production was recorded low in case of bacterial isolates as compared to fungal isolates (Table 4.1). Among four fungal isolates PAF-4 showed highest enzyme activity (0.34U/ml) and selected for further studies.

4.1.2 Identification and morphological characteristics of selected isolate PAF-4

Fungal isolate PAF-4 was identified as *Acremonium sclerotigenum* at National Fungal Culture Collection, Agharkar Institute, Pune (INDIA) .The production and characterization of penicillin acylase was further carried out with *Acremonium sclerotigenum* as the isolate showed highest penicillin acylase activity. It is a spore forming fungus which shows velvety growth on PDA, with peach coloured pigmentation after 48-96 hrs and creamish white coloured mycelium in submerged fermentation. Microscopic examination of lactophenol cotton blue stained fungus

revealed somewhat curved conidia, with large, sclerotia. The phialides were simple, awl-shaped, and erect from the substratum.



(A) **(B)**

Fig.4.1 Morphological characteristics of *Acremonium sclerotigenum* [Growth pattern on PDA plate (A) and LCB staining (B)]

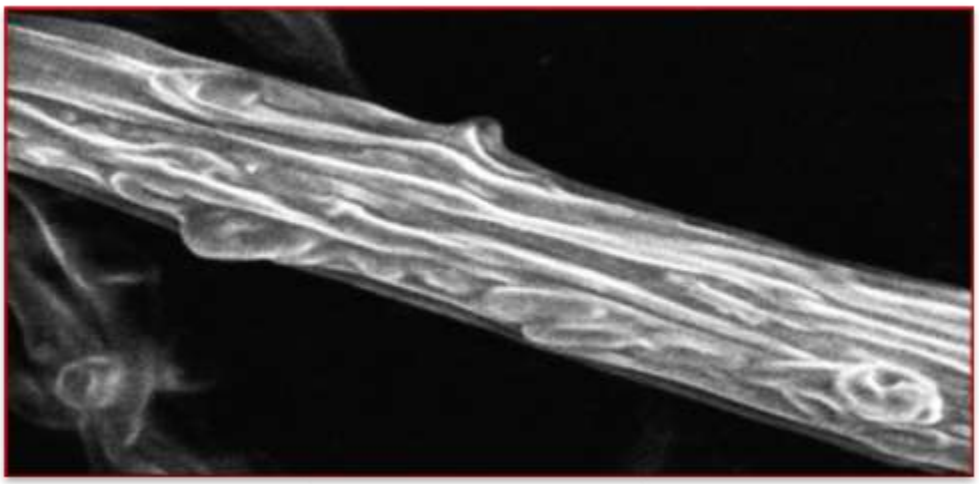


Fig.4.2 SEM image of *Acremonium sclerotigenum*

4.2 Optimization of fermentation parameters for penicillin acylase production from *Acremonium sclerotigenum*

4.2.1 Effect of medium on the production of penicillin acylase

To investigate the effect of media on the production of Penicillin acylase by *Acremonium sclerotigenum* it was grown in fifteen different media (M1-M15), (Compositions given earlier) and results have been summarized in Fig.4.3. The flask

cultivation was performed in Erlenmeyer flasks (250 ml) with working volume of 50ml. The inoculated flasks were incubated at 150 rpm and 25°C temperature. Among the different media used, Medium No. 6 (M6) containing (g/l) glucose-10, peptone-10.0, beef extract-10.0 and yeast extact-5, proved to be the best medium for the production of penicillin acylase. Highest enzyme activity (0.57±0.01U/ml) was recorded in this medium; hence it was selected for further studies. Similarly inducible penicillin G acylase was produced in *Bacillus megaterium* (Illanes *et al*., 1994) in a medium consisting of yeast extract, sucrose and PAA (Senthilvel and Pai, 1996). Enshasy *et al.* (2009) have used media containing tryptone (10), yeast extract (5), sodium chloride (10), and glucose (0.3) g/l for the production of penicillin acylase from *E.coli* ATCC11105. Devi *et al*. (2013) have found maximum production of penicillin acylase from *Streptomyces* sp. APT13 in starch casein broth containing (starch (10), KNO_3 (2), K_2HPO_4 (2), NaCl (2), casein (0.3) $MgSO_4$ (0.05), $FeSO_4$ (0.01), $CaCO_3$ (0.02) (g/l). Whereas Moharram *et al.* (2013) reported highest production of penicillin V acylase in a medium (g/l) containing glucose, 20; peptone 5; yeast extract, 5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.2 and 0.30 penicillin V as inducer

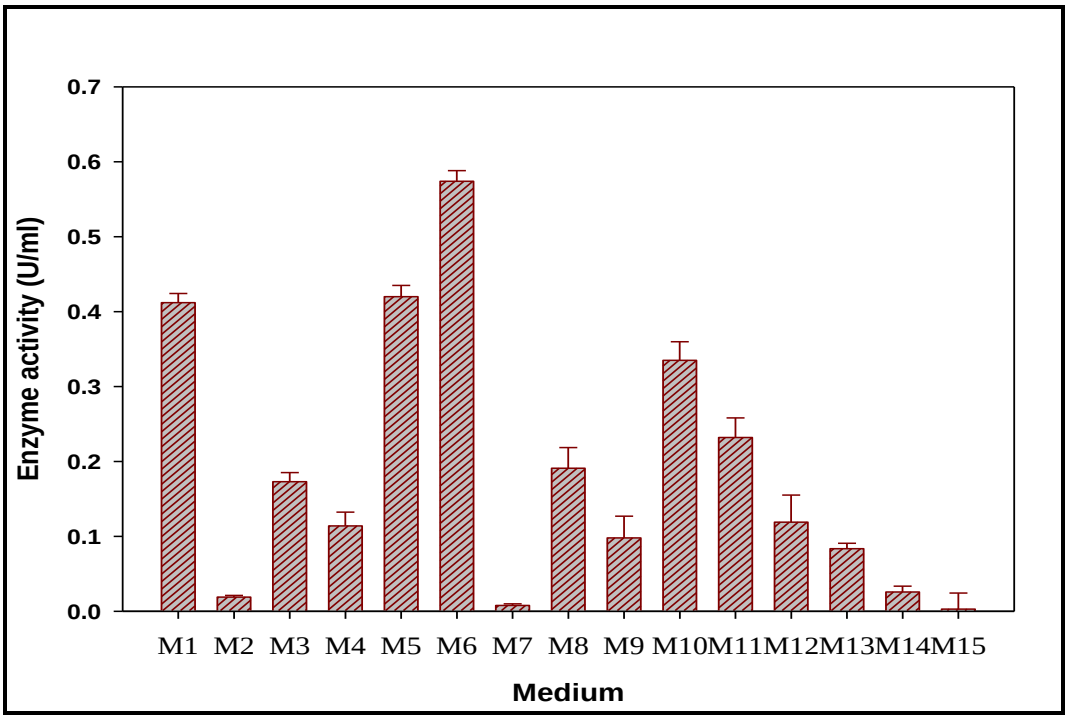
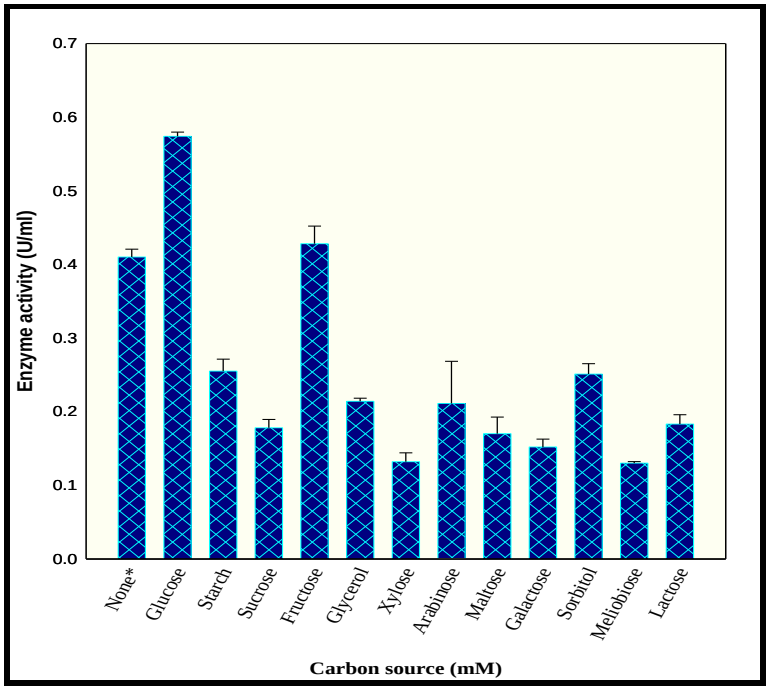


Fig.4.3 Effect of different media on the production of penicillin acylase from *Acremonium sclerotigenum*

4.2.2 Selection of suitable carbon source

Acremonium sclerotigenum was grown in the medium containing different carbon sources (40mM). Although the fungus utilized all the sugars supplemented in the production medium for its growth but highest enzyme production was observed with glucose (0.57±0.02 U/ml) as shown in Fig.4.4. Comparable enzyme activity was also observed with fructose (0.43±0.04U/ml) followed by sorbitol (0.25± 0.04U/ml). Glucose was selected and its varied concentration was also checked for maximum enzyme production. Highest enzyme activity was observed with 40mM glucose as shown in the Fig.4.5. Further increase in the glucose concentration lead to decrease in the enzyme production due to metabolic inhibition as glucose repression was reported in many fungal strains. Devi *et al.* (2013) reported threefold increase in enzyme production when 0.03% casein was used as carbon source for the production of penicillin acylase of *Streptomyces* sp. Whereas Moharram *et al.* (2013) reported that glucose and dextrin were the most favorable carbon sources for penicillin V acylase production by *P. chrysogenum* and *A.terreus.* Highest PVA production from *B. cereus* ATUAVP1846 was achieved (4.89 IU/g) using an optimal minimal medium containing 0.4% glucose as carbon source (Sunder *et al.*, 2012).



*Corresponds to (0.41±0.01U/ml)

Fig.4.4 Effect of different carbon sources on production of penicillin acylase from *Acremonium sclerotigenum*

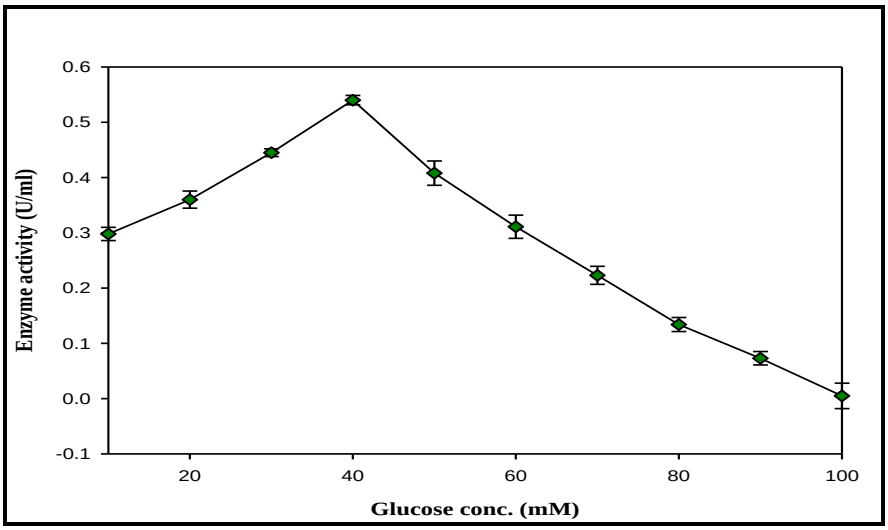
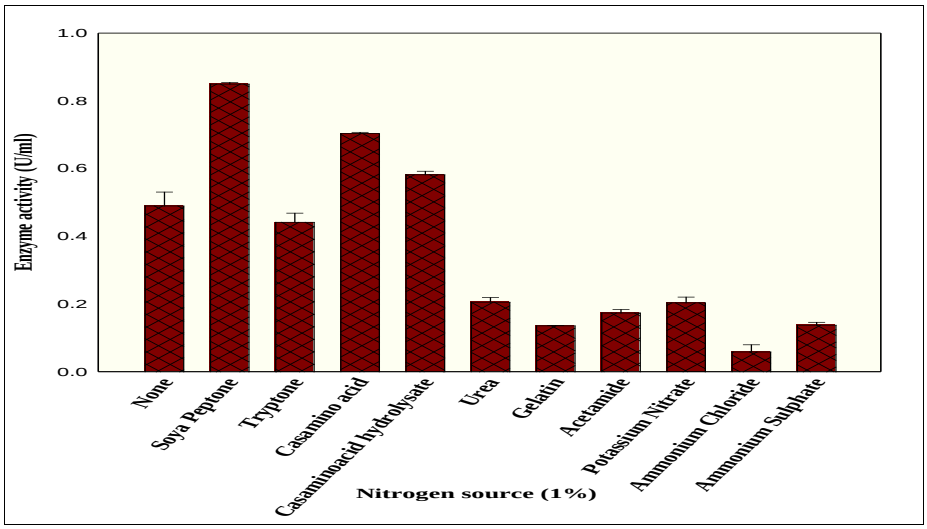


Fig.4.5 Effect of glucose concentration on the production of penicillin acylase from *Acremonium sclerotigenum*

4.2.3 Selection of suitable nitrogen source

Various organic and inorganic nitrogen sources were used at 1.0% (w/v) concentration in production medium (pH 6.0) and highest production (0.85±0.01 U/ml) was observed with soya peptone (Fig.4.6) followed by casamino acid (0.71±0.01U/ml) and casamino acid hydrolysate (0.58±0.02U/ml). Soya peptone was selected and its varied concentrations were also checked for maximum enzyme production. Highest enzyme activity was observed with 1.4 % soya peptone as shown in the Fig. 4.7.



***** Corresponds to (0.49±0.01U/ml)

Fig.4.6 Effect of nitrogen source on the production of penicillin acylase from *Acremonium sclerotigenum*

Further increase in the soya peptone concentration lead to decrease in the enzyme production. Similarly Rolinson *et al.* (1961) reported maximum production of

penicillin V acylase in a medium supplemented with (0.5%) soya peptone as nitrogen source. In contrast to present study highest enzyme production (8.92 IU/g) was achieved using an optimal minimal medium containing 0.3% $(NH_4)_2HPO_4$ as nitrogen source (Sunder *et al*., 2012). Devi *et al.* (2013) found maximum enzyme production when 0.03% casein was used as nitrogen source. Peptone was the most suitable nitrogen source for penicillin V acylase production from *A. terreus* and *P.chrysogenum*, respectively (Moharram *et al*., 2013). Kumar *et al.* (2008) reported enhanced production of penicillin V acylase from *R. aurantiaca* when tryptone was used in the minimal medium as nitrogen source.

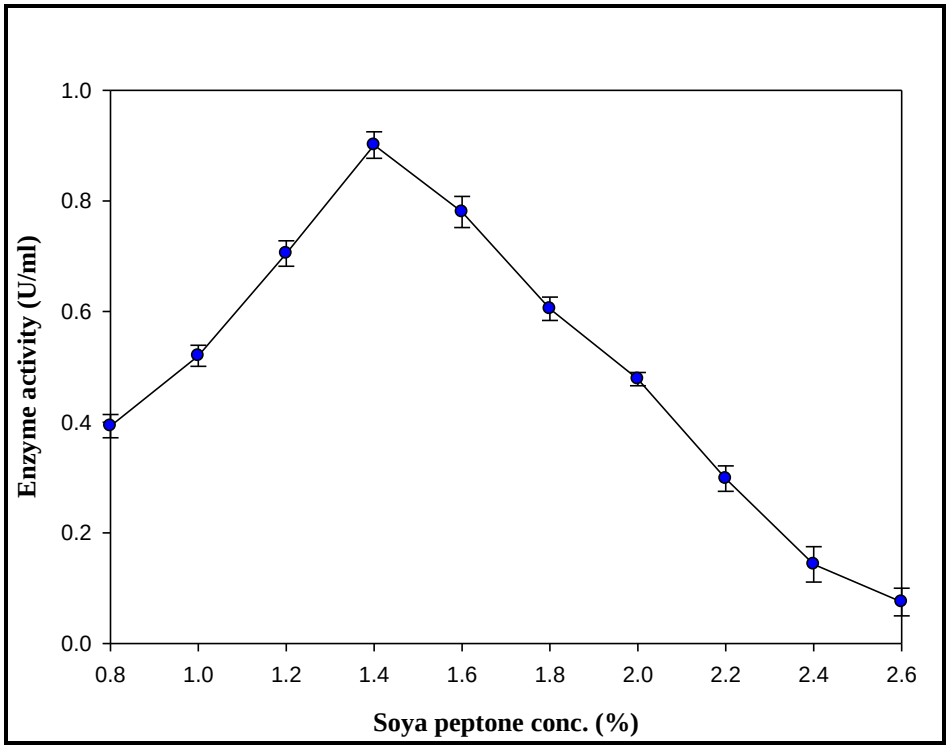


Fig.4.7 Effect of soya peptone concentration on the production of penicillin acylase from *Acremonium sclerotigenum*

4.2.4 Optimization of concentration of undefined growth supplements

The addition of 0.8.% (w/v) beef extract and 1.6% yeast extract as growth supplement to the production medium gave maximum penicillin acylase production of (0.89±0.02U/ml) (Fig.4.8) and (0.90±0.03U/ml) (Fig.4.9) respectively. Yeast extract is an excellent source of many nutrients. It is rich in amino acids, vitamins and many low molecular weight growth factors. Due to this property it is widely used in medium formulation for the induction of different primary and secondary metabolites. Decrease in the production of penicillin acylase after addition of increasing

concentration of yeast extract may be due to the effect of some accumulated low molecular weight enzyme inhibitors in the yeast extract, when used in higher concentration (Enshasy *et al*., 2009). In the earlier studies, Rolinson *et al.* (1961) observed an appreciable increase in enzyme production when yeast extract was used at a concentration of 2.5%. Whereas Bodhe and Sivaraman (1987) have used 10g/l beef extract for the production of penicillin acylase from *Kluyvera citrophila*. Tembhurkar *et al.* (2012 found maximum enzyme production in a medium composed of beef extract (3g/l). Torres *et al.* (2000) reported maximum production of penicillin V acylase (3.67 IU/l) when 0.2% yeast extract was used in the minimal medium.

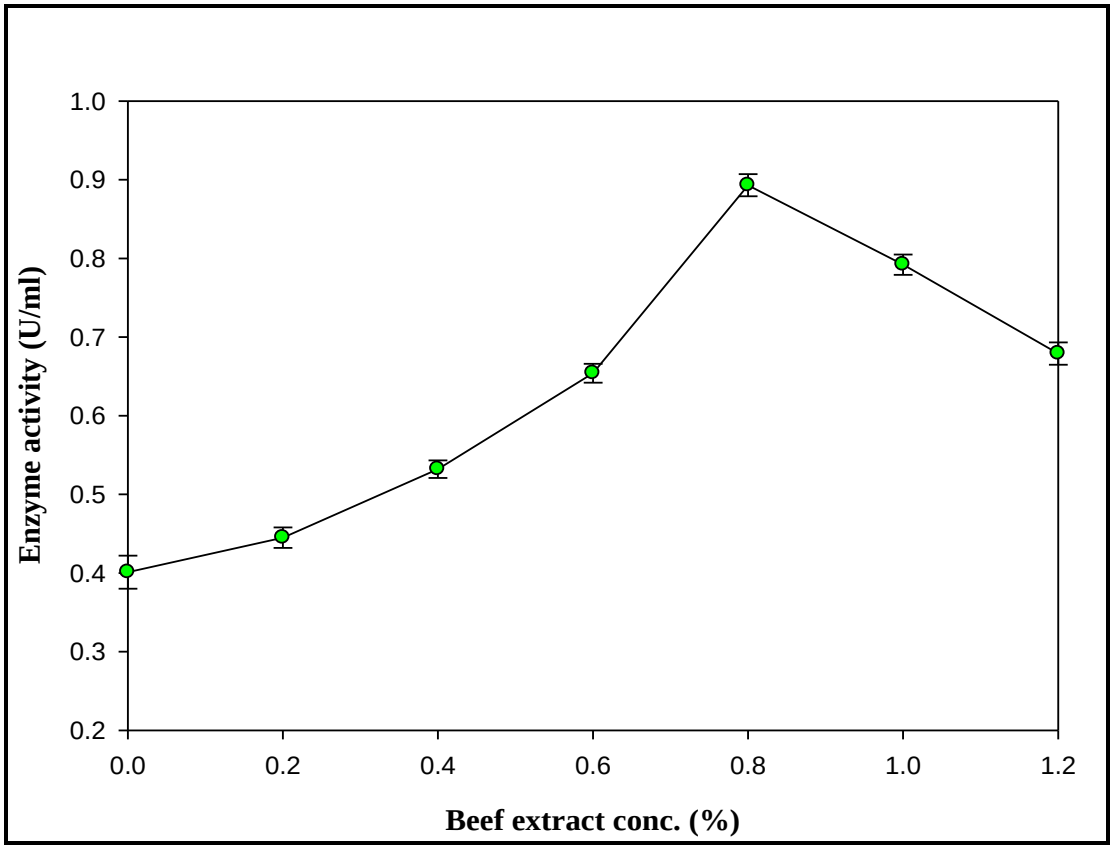


Fig.4.8 Optimization of concentration of beef extract for the production of penicillin acylase from *Acremonium sclerotigenum*

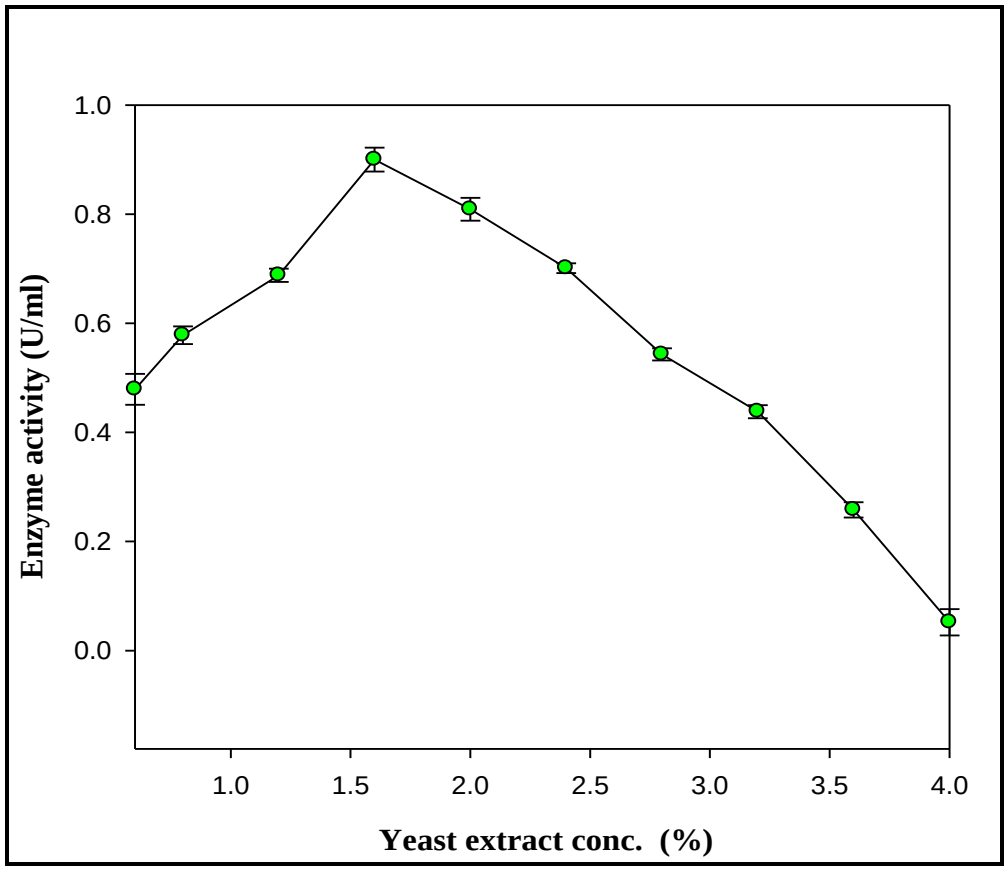


Fig.4.9 Optimization of concentration of yeast extract for the production of penicillin acylase from *Acremonium sclerotigenum*

4.2.5 Optimization of medium pH

The variation in pH alters acid-base equilibrium, which greatly affects the uptake of nutrients in the medium. Therefore, pH before inoculation, during the growth and harvesting needs to be evaluated. The organism was grown in the selected medium in the pH range of 4-9. The highest enzyme production was recorded at pH-6.0 (0.89±0.04U/ml) as shown in (Fig.4.10). The reduction in the production of penicillin acylase may be due to the reason that fungi are known to grow and secrete the desired proteins in acidic pH. Initial pH facilitates transport of several enzymes across the cell membrane thus unfavorable pH of production medium will limit the growth rate and enzyme production by reducing the accessibility of nutrients (Bajpai *et al*,.1997). Similarly Devi *et al.* (2013) have also reported a maximum enzyme production (3.2 µM /g/min) at pH-6.0. In contrast to standardization Tahir *et al*. (2009) reported maximum penicillin amidase production (18.3 U/ml/min) at pH 2.5 from thermo tolerant *Bacillus* sp. MARC-0103. Torres *et al*. (2000) reported an

optimum pH value of 6.8 for maximum penicillin acylase production. Enshasy *et al*. (2009) obtained maximum enzyme production (27.5 U/ml) at medium pH 7.5.

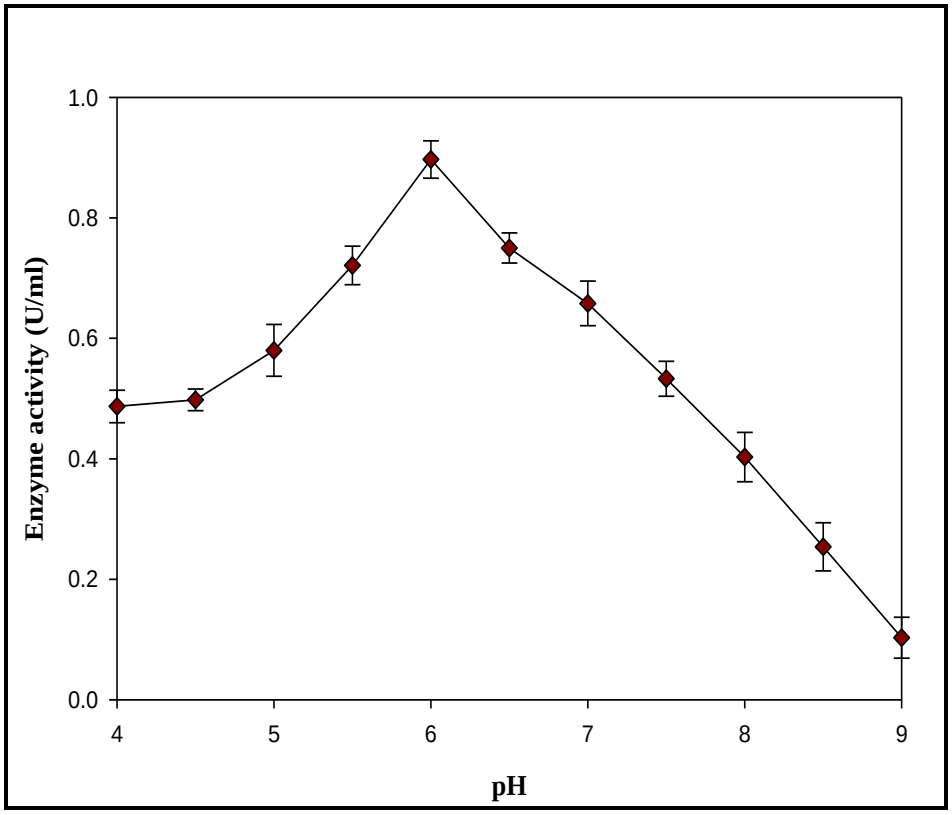


Fig.4.10 Effect of medium pH on the production of penicillin acylase from *Acremonium sclerotigenum*

4.2.6 Optimization of production temperature

The influence of temperature on the production of penicillin acylase was studied by growing the fungus at temperatures ranging from 20°C to 45°C. The enzyme produced in appreciably higher amount at 25°C (0.90±0.02U/ml) as compared to other temperatures (Fig.4.11). Whereas gradual decrease in enzyme production was observed at temperature above 30°C. Similarly Pundle & SivaRaman (1994) have also reported the production of PVA from *B. sphaericus* at 25°C. Kumar *et al.* (2008) reported maximum production of penicillin V acylase from *Rhodotorula aurantiaca* NCIM 3425 (12 IU/g DW) at 27°C. PVA was optimally produced by *S. lavendulae* an actinomyces, at 28°C (Torres *et al.*, 2000). *Bacillus cereus* ATUAVP1846 produced maximum PVA at 30°C (8.93IU/g DW). PVA production decreased drastically at higher temperatures (Sunder *et al.*, 2012). Tahir *et al*. (2009) reported an appreciable increase in the production of penicillin amidase (17.3 U/ml/min) when incubation temperature was raised from 30°C to 37°C and marked decrease in the production of

enzyme above 37°C. The difference in optimum temperature may be due to the microorganisms which may require a different temperature for their growth from the other.

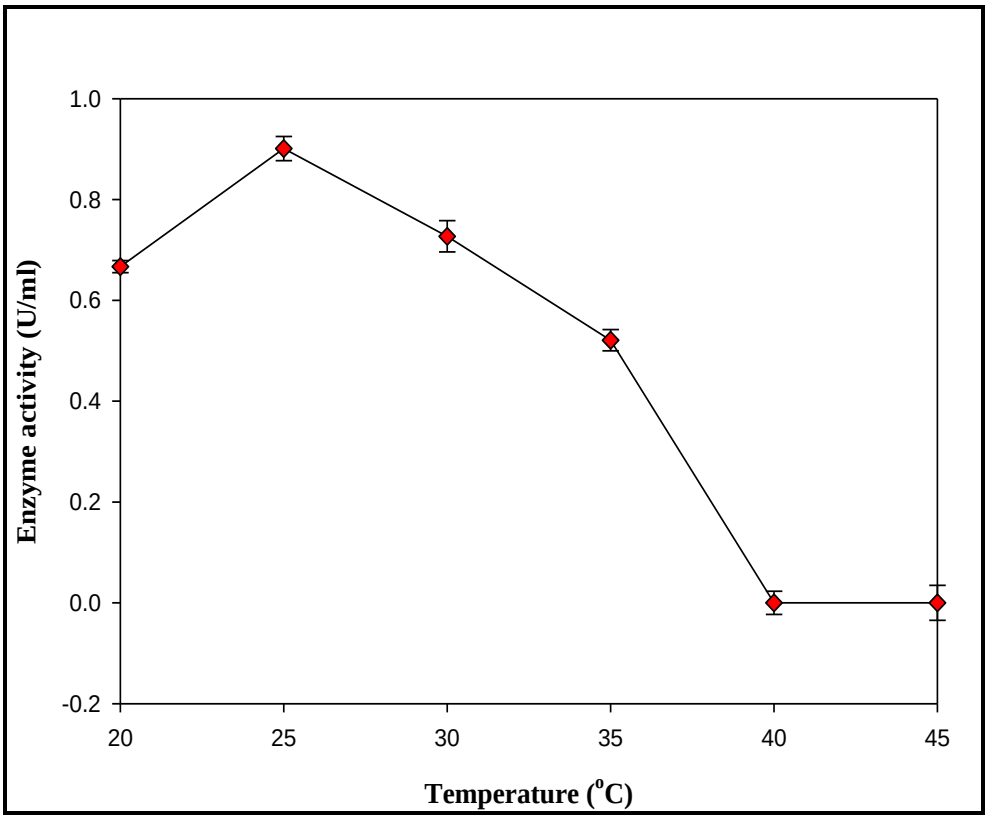


Fig.4.11 Effect of temperature on the production of penicillin acylase from *Acremonium sclerotigenum*

4.2.7 Effect of inoculum size on the production of penicillin acylase

To study the effect of inoculum size on the production of penicillin acylase the medium was seeded with inoculum in the range 8.4×10^5 to 15.5×10^5 spores/ml and the highest enzyme production was recorded at inoculum size of 12.5×10^5 spores/ml (0.95U/ml ± 0.05) (Fig. 4.12). With the further increase in spore count the production of the enzyme reduced which may be attributed to the depletion of nutrients in the production medium. In contrast to our study Pinotti *et al.* (2002) reported a spore suspension of 8.5×10^7 spores/ml optimal for the production (115 IU/l) of penicillin acylase from *Bacillus megaterium.* Sunder *et al*. (2012) used an inoculum size of 10% (v/v) for the maximum production of penicillin acylase from *Bacillus cereus* ATUAVP1846. Similarly Kumar *et al*. (2008) reported an inoculum size of 10% (v/v) for the maximum production of penicillin acylase from *Rhodotorula aurantiaca*. Tahir

et al. (2009) standardized an inoculum size of 6% (v/v) for the production of penicillin acylase from *Bacillus* sp. MARC-0103.

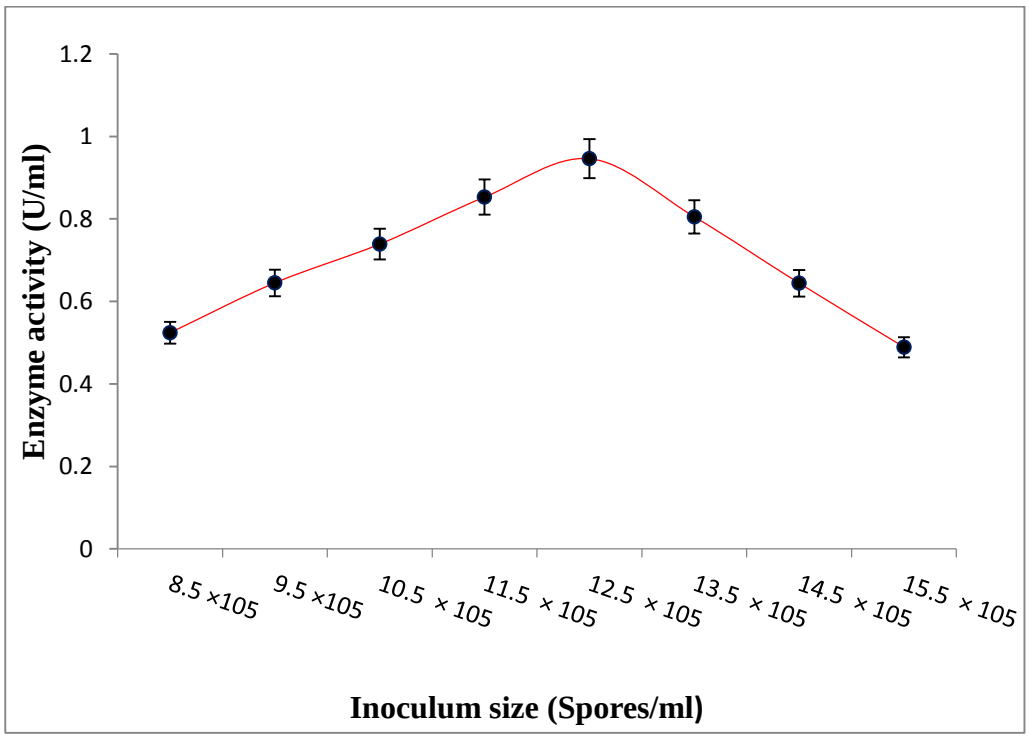


Fig. 4.12 Effect of inoculum size on the production of penicillin acylase from *Acremonium sclerotigenum*

4.2.8 Time course of penicillin acylase production from *Acremonium sclerotigenum*

Time course of penicillin acylase production was studied in the optimized production medium containing glucose (40mM), soya peptone (1.4%), yeast extract (1.6%) and beef extract (0.8%), inoculum size of 12.5×10^5 spores/ml, pH-6.0. The active cell growth continued up to 78 hrs and then gradually declined. Up to 48 h of growth, the pH of the fermentation broth decreased and then increased slightly with increase in time. The maximum penicillin acylase activity (1.23U/ml) was observed after 48 h of cultivation with 3.58 mg/ml dcw fungal biomass (Fig.4.13). Three fold increase in penicillin acylase production was recorded when *Acremonium sclerotigenum* was cultivated in optimized culture conditions. An incubation period of 24 h was found to be optimum for production of PVA from *B. cereus* afterwards gradual decrease in production was reported. Cell mass increased up to 18 h, then remained almost constant until 48 h, and decreased on further incubation (Sunder *et al.*, 2012). Tahir *et al.* (2009) reported an incubation time of 72 hrs for the production of penicillin acylase from *Bacillus* sp. Enshasy *et al.* (2009) found maximum

production of penicillin acylase (40U/L) from *E.coli* after 19 h of incubation with a cell mass of 4.25g/l. Whereas an incubation period of 15-18 h was found to be optimum for the production of PVA from *R. aurantiaca*, further incubation led to drastically decreased PVA production. *R. aurantiaca* cell mass increased constantly up to 36 h and slightly decreased thereafter, however, PVA activity reached maximum at 18 h and drastically decreased on further incubation (Kumar *et al* ., 2008).

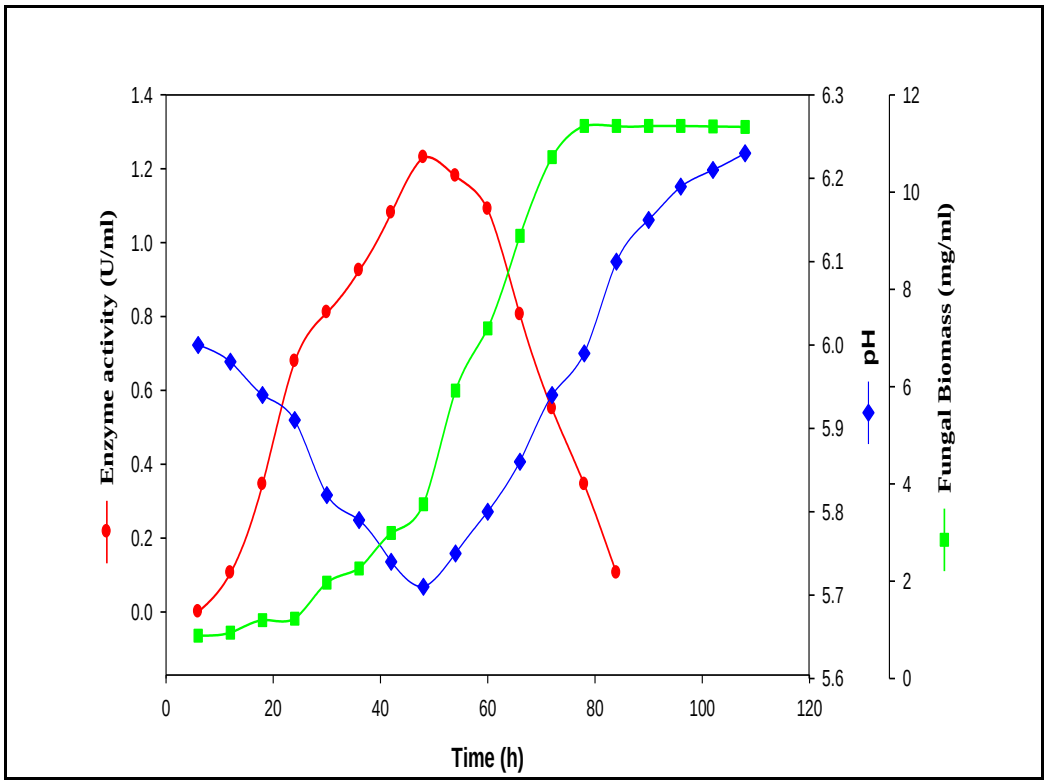


Fig. 4.13 Time course of penicillin acylase production from *Acremonium sclerotigenum*

4.3 Immobilization of penicillin acylase from *Acremonium sclerotigenum* on different matrices

In the present study, penicillin acylase from *Acremonium sclerotigenum* (1.22 U/ml) was immobilized by three methods- entrapment in agar and calcium alginate, adsorption on silica gel and covalent bonding with chitosan beads. Different immobilization parameters were studied to find out optimum immobilization conditions.

4.3.1 Comparison of the matrices for their penicillin acylase immobilization efficiency and penicillin acylase activity

Various matrices used for penicillin acylase immobilization has been shown in Fig. (4.14). Buchi Encapsulator B-390 used for synthesis of alginate beads. Among these matrices, sodium alginate beads showed highest enzyme activity (0.84U/g± 0.04) and immobilization efficiency (68.85%) and silica showed least enzyme activity and immobilization efficiency (Table 4.)

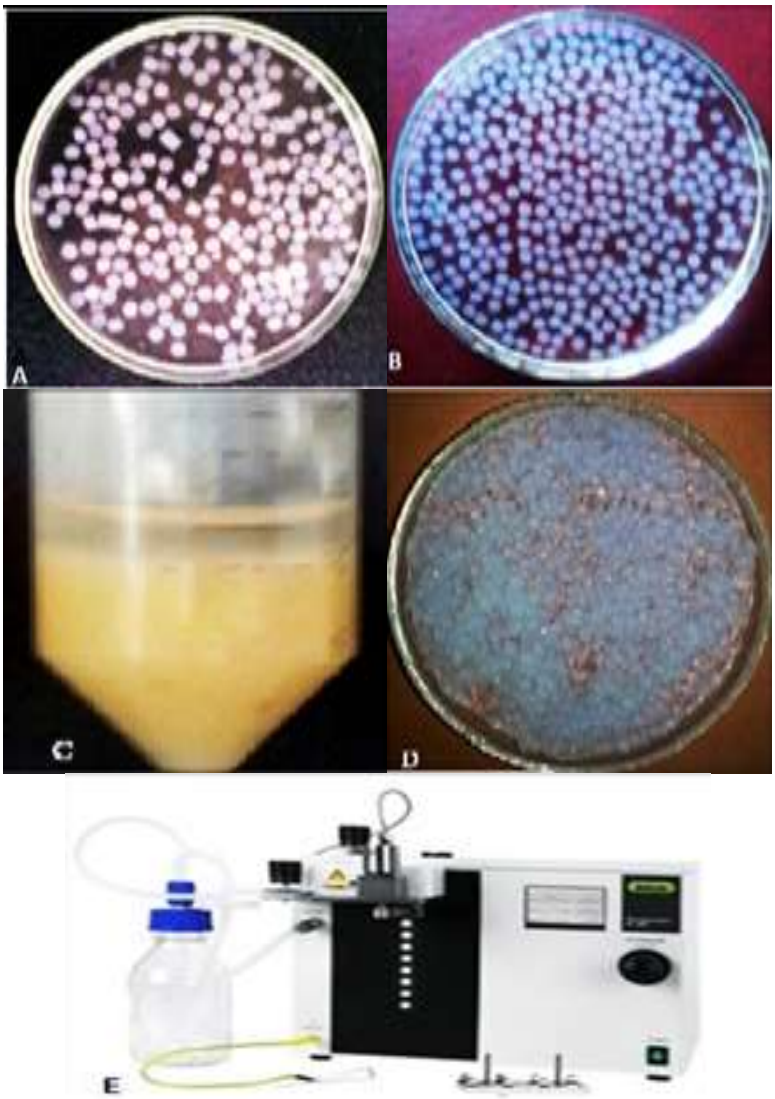


Fig.4.14 Different matrices used for immobilization: (A) Agar discs (B) Sodium alginate (C) Silica (D) Chitosan (E) Buchi encapsulator B-390.

Table 4.2 Different matrices with their penicillin acylase immobilization efficiency and penicillin acylase activity

S.No.	Name of Matrix	Penicillin acylase activity(IU/g matrix)	Immobilization Efficiency (%)
1	Agar	0.78 ±0.02	63.83
2	Chitosan	0.65±0.03	53.24
3	Sodium alginate	0.84±0.01	68.85
4	Silica	0.48±0.01	39.28

4.3.2 Optimization of immobilization time of penicillin acylase on sodium alginate

Binding time varied between 1 h to 5 h at 4ºC with continuous stirring rate of 100 rpm. Highest enzyme activity (0.84±0.05 U/g matrix) was recorded after a binding time of one hour for sodium alginate beads. After one hour there was a decline in enzyme activity (Fig.4.15). Decrease in penicillin acylase activity with increased time might be due to hardening of gel which heads to a stage of diffusion barrier of both the substrate and the product released. Similarly Avinash *et al* .(2016) also reported a hardening time of 1 hour for the beads containing entrapped whole cells of *E. coli* expressing a highly active penicillin V acylase.

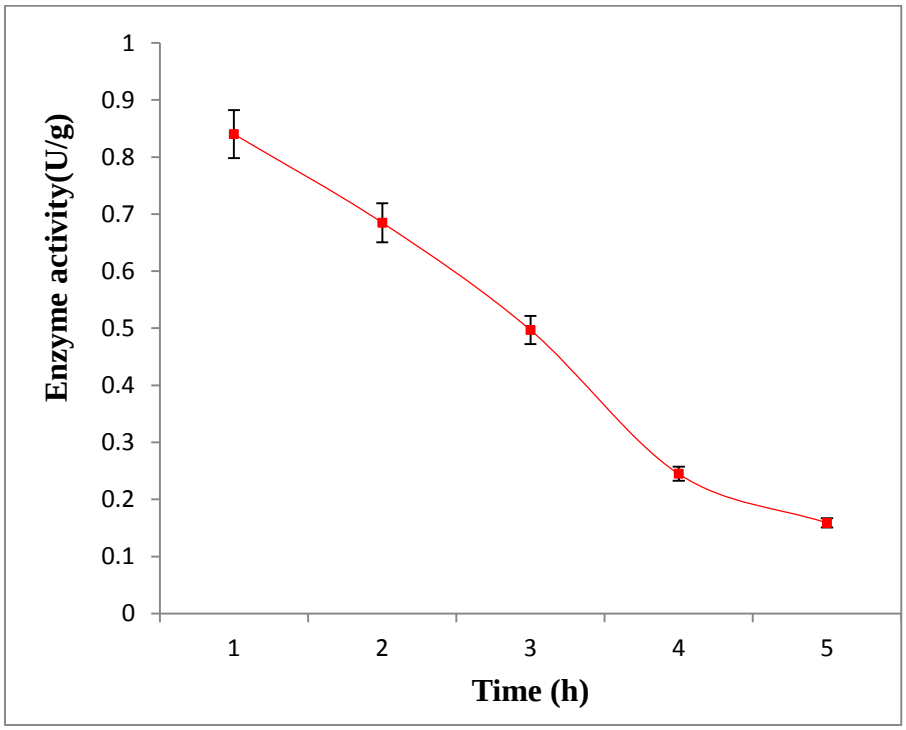


Fig 4.15 Effect of immobilization time on alginate entrapped penicillin acylase from *Acremonium sclerotigenum*

4.3.3 Effect of sodium alginate concentration

The optimum gel strength is the key factor of the beads as it provides better operational durability of beads as well as diffusion of both substrate and products. Sodium alginate is the most frequently used immobilization matrix, the formation and properties of alginate beads depend upon different parameters such as nature and concentration of the cations and alginate concentration Sodium alginate concentration was varied from 1.5-4 % (w/v) Maximum enzyme activity was recorded when sodium alginate was used at a concentration of 2% (0.84±0.04) (Fig.4.16). When the sodium alginate concentration is increased beyond 2% the thickness of the membrane capsule decreases (Blandino *et al.*, 1999), and this is presumably due to the fact that increasing the number of biopolymer molecules per unit solution, the binding sites for Ca^{2+} ions also increases. As a result a more densely cross-linked gel structure is probably formed, and consequently results in the formation of thinner walls. The dense membrane is expected to create diffusion resistance through the beads. Similarly Avinash *et al.* (2016) also used 2% alginate for the immobilization of whole cells of *E. coli* expressing a highly active penicillin V acylase.

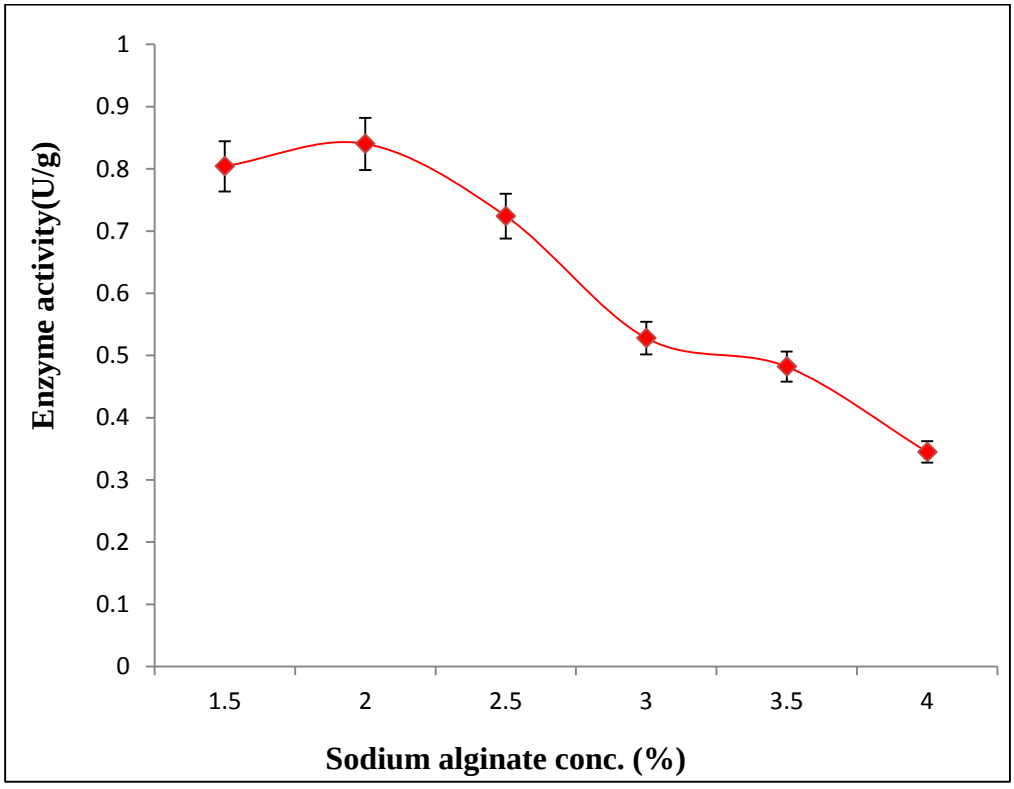


Fig.4.16 Effect of sodium alginate concentration on alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.3.4 Effect of divalent ions concentration on immobilized penicillin acylase

To study the effect of divalent ions (Ca^{2+}) on immobilized penicillin acylase concentration of $CaCl_2$ was varied from 50-350 mM. Maximum activity was observed at 100 mM concentration (0.847±0.04) (Fig.4.17). High concentration of calcium chloride causes change in pH which might be responsible to decrease the catalytic activity of enzyme in microenvironment of beads. In contrast to present study Avinash *et al.* (2016) used 0.2 M $CaCl_2$ for the immobilization of whole cells of *E.coli* in alginate beads.

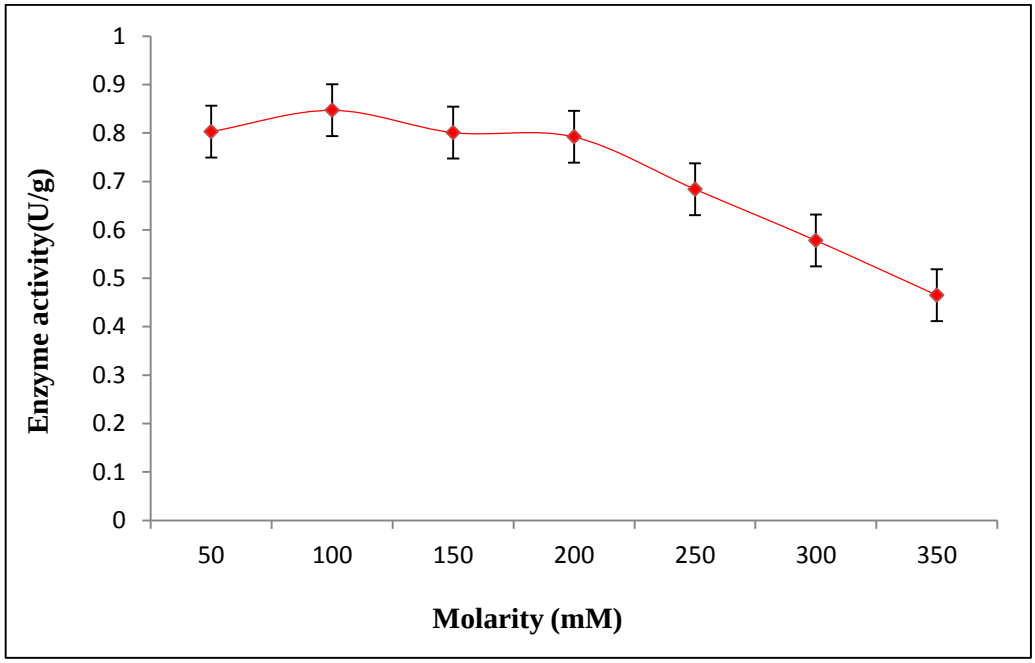


Fig.4.17 Effect of divalent ion concentration on immobilized penicillin acylase from *Acremonium sclerotigenum*

4.3.5 Bead size optimization

In the immobilized enzyme system as the substrate has to diffuse for the enzymatic reaction to take place, the size of the bead has significant effect on the rate of hydrolysis. The bead size also determines the suitability for reactor configuration. To determine the optimum size of beads for the enzyme reaction, different sized beads (dia. 200 µm, 300 µm, 450 µm, 750 µm 1000 µm and 2000 µm) prepared using airflow-assisted nozzle in Encapsulator system (Fig. 4.18). The highest activity (0.85±0.02 U/g) (Fig.4.18) was observed with 1000µm bead size. With increase in bead size a decrease in enzyme activity was observed. Therefore, bead size 1000 µm was selected for further studies. It may be due to that 1000 µm offered lesser diffusion resistance.

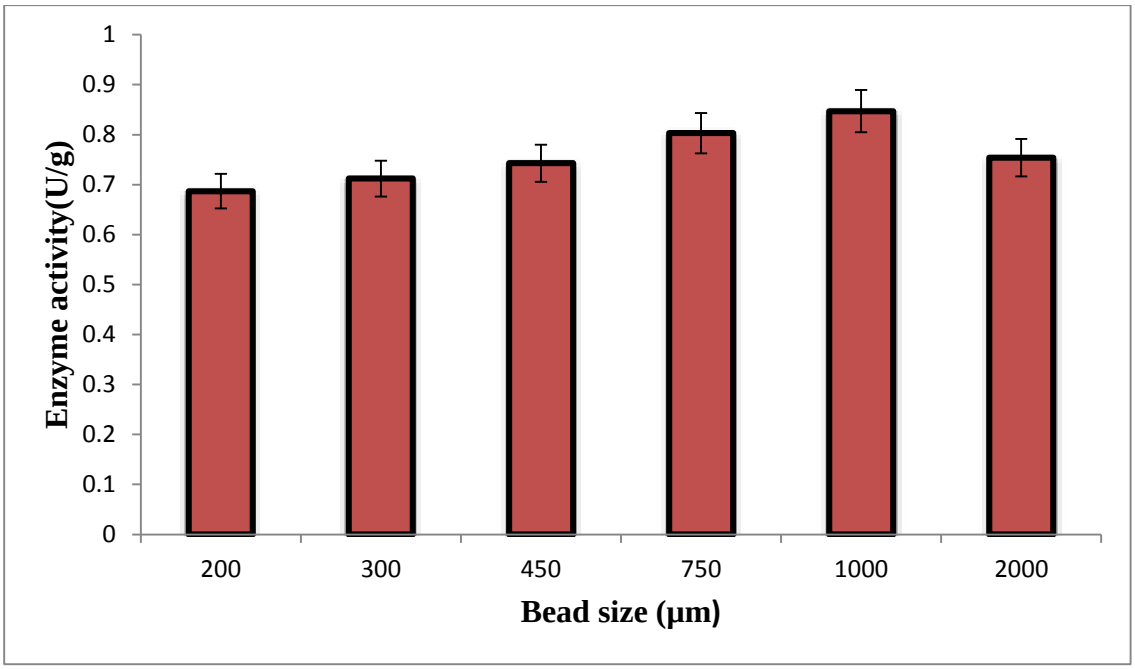


Fig.4.18 Optimization of bead size of alginate gel for penicillin acylase from *Acremonium sclerotigenum*

4.4 Optimization of reaction parameters for free and alginate entrapped penicillin acylase from *Acremonium sclerotigenum* for 6-aminopenicillanic acid synthesis

It is very important to optimize various process parameters for the biocatalysts before it is used at pilot or industrial scale for any bioprocess. Hence following reaction parameters were optimized for both free and alginate gel entrapped penicillin acylase before they could be used for 6- APA synthesis at pilot scale:

4.4.1 Effect of Buffer pH

Substrate binding and catalysis are often dependent on charge distribution on substrate and in particular enzyme molecules. Hence enzyme activity is very much dependent on pH of buffer system. Penicillin acylase exhibited activity in broad pH range in both free and immobilized form. The enzyme activity decreased gradually at higher pH and completely inactivated at alkaline pH. Highest enzyme activity was observed at pH 5.0 (1.03±0.04 U/ml) (Fig.4.19) for free and at pH-6.0 (0.90U/g±0.04) (Fig.4.20) for immobilized penicillin acylase with sodium citrate buffer.

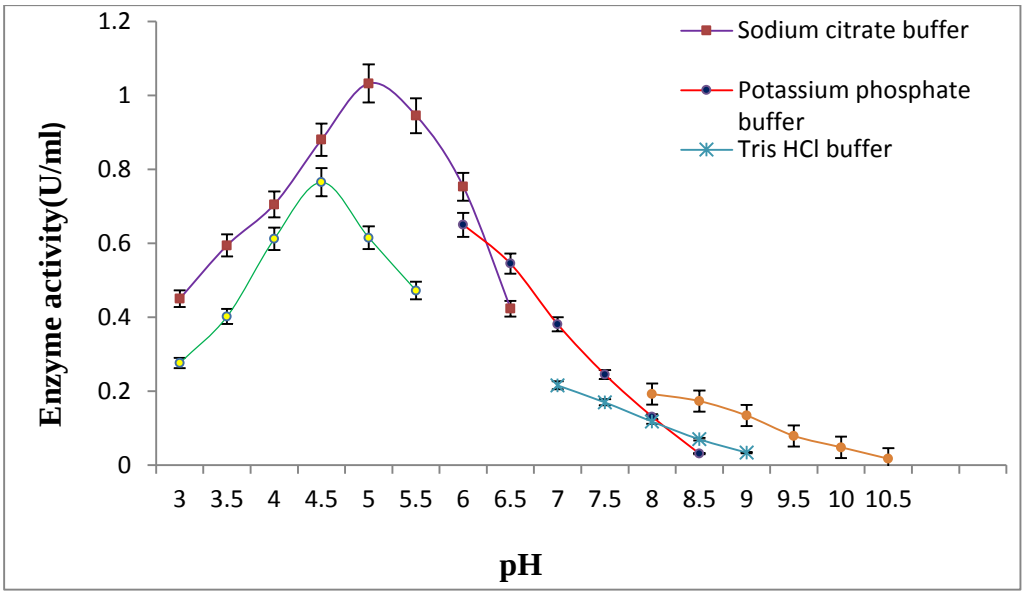


Fig.4.19 Effect of buffer pH on free penicillin acylase from *Acremonium sclerotigenum*

Kumar *et al*. (2008) reported sodium citrate buffer pH -5.5 for the assay of penicillin acylase activity of *R. aurantiaca*. Potassium phosphate buffer, pH- 8 was used by Torres *et al*. (1999) for the activity of penicillin acylase from *Streptomyces lavendulae.* Similarly Jose *et al*. (2003) also used potassium phosphate buffer, pH -8 for the determination of penicillin acylase activity from *Aspergillus fumigatus* and *Mucor gryseocianum.*

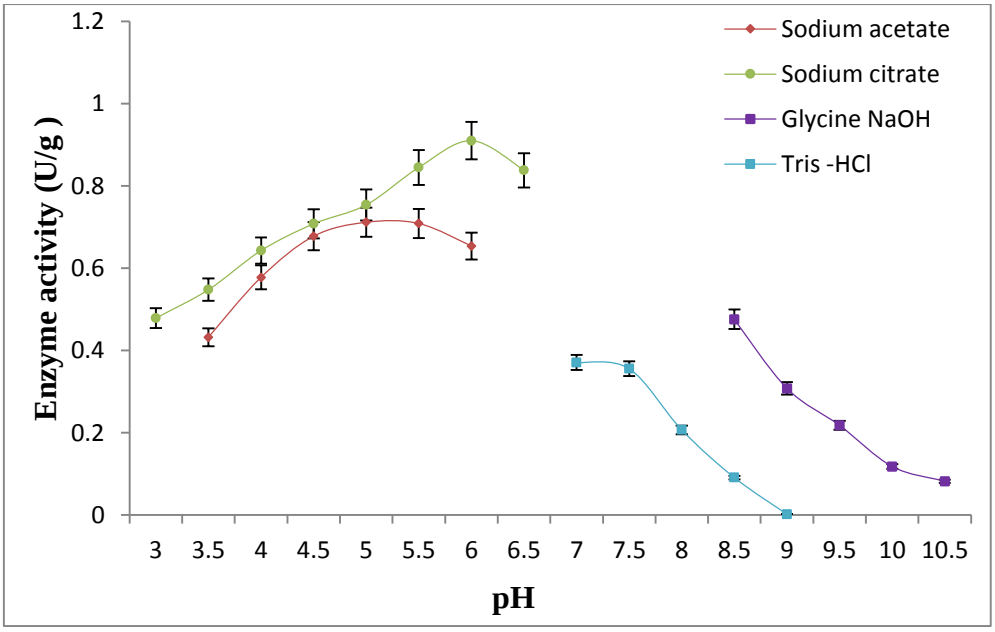


Fig.4.20 Effect of buffer pH on alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

The immobilization of enzymes to charged supports often leads to displacement of optimum pH for an immobilized enzyme higher or lower pH (Leontievsky *et al.*, 2001; Lu *et al.*, 2007). Avinash *et al.* (2016) reported maximum enzyme activity of calcium alginate immobilized penicillin acylase from *E.coli* with acetate buffer at pH-4.0. Bahman *et al.* (2013) reported maximum activity of immobilized penicillin acylase with phosphate buffer pH-7.5.

4.4.2 Buffer concentration

The effect of varied concentrations (25-250 mM) of sodium citrate buffer on penicillin acylase was investigated and highest enzyme activity (1.20±0.04U/ml) was recorded in 125mM sodium citrate buffer for free penicillin acylase and (0.92±0.04 U/g) in 150mM sodium citrate buffer for alginate immobilized penicillin acylase (Fig.4.21). As the molarity of buffer increased, enzyme activity decrease which might be due the polar surface groups of enzyme and buffer interact electrostatically. Penicillin acylase from fungi *Aspergillus fumigatus* and *Mucor gryseocianum* showed maximum enzyme activity at 0.05 M phosphate buffer (Joes *et al*., 2003). Kumar *et al*. (2008) reported optimum penicillin acylase activity with 0.1M sodium citrate buffer from *Rhodotorula aurantiaca* NCIM 3425. Avinash *et al.* (2016) reported maximum enzyme activity of penicillin acylase from *E.coli* immobilized on calcium alginate in 0.1 M acetate buffer. Bahman *et al.* (2013) reported maximum activity of penicillin G acylase immobilized onto iron oxide nanoparticles with 0.1 M phosphate buffer.

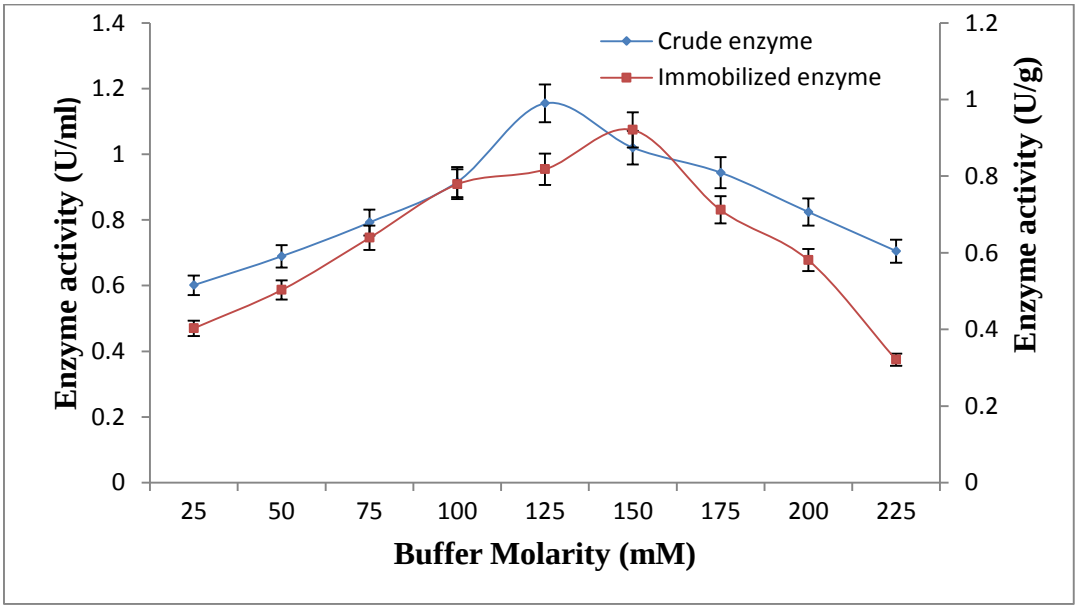


Fig.4.21 Optimization of buffer concentration for free and alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*.

4.4.3 Reaction temperature

The penicillin acylase activity was assayed in sodium citrate buffer, at different reaction temperatures (30-60ºC). The maximum enzyme activity (1.35±0.03 U/ml) for free penicillin acylase was observed at 40ºC whereas maximum enzyme activity (0.95±0.02 U/g) for immobilized penicillin acylase was recorded at 50°C (Fig.4.22). This effect could be related to a conformational restriction of the enzyme structure bound to the support, which enhances its thermal stability. Kumar *et al.* (2008) reported an optimum reaction temperature of 40°C for penicillin acylase activity from *Rhodotorula aurantiaca.* Similarly Joes *et al.* (2003) also reported 40°C optimum temperature for penicillin acylase activity determination from *Aspergillus fumigatus* and *Mucor gryseocianum.* Torres-Bacete *et al.* (2000) reported an optimum temperature 60°C for the immobilized penicillin acylase from *Steptomyces lavendulae.* Whereas Moharram *et al.* (2013) found 37°C as optimum reaction temperature for the activity of penicillin acylase from *Aspergillus terreus* and *Penicillium chrysogenum*. Whereas Liu *et al*. (2016) reported 45°C as optimum temperature for penicillin acylase immobilized on magnetic $NiFe_2O_4$ nanorods. Similarly Avinash *et al.* (2016) reported maximum enzyme activity of calcium alginate immobilized penicillin acylase from *E.coli* at 45°C.

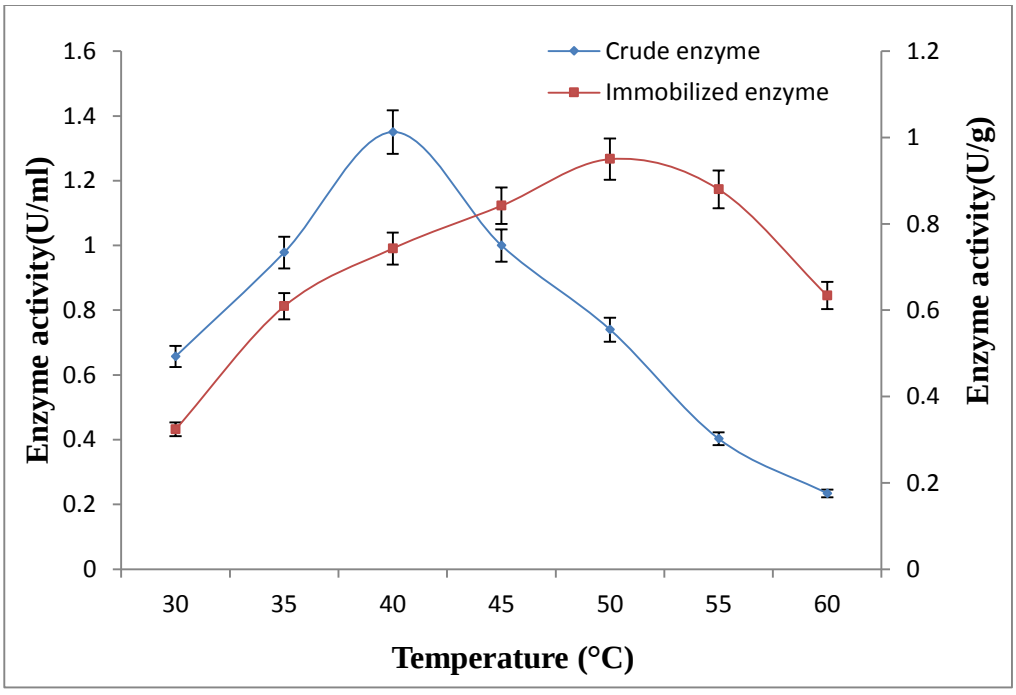


Fig.4.22 Effect of reaction temperature on free and alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.4.4 Optimization of substrate affinity and concentration

To study the effect of different substrates on enzyme activity, penicillin G and penicillin V (sodium salt) were used at a concentration of 50mM. The results have been depicted in Fig.4.23 and Fig. 4.24 respectively. Maximum enzyme activity (1.62 ±0.04 U/ml) (Fig.4.24) was recorded with 50 mM penicillin G for free penicillin acylase. Though alginate gel entrapped penicillin acylase exhibited optimum activity (1.40±0.04U/g) with 75mM penicillin G (Fig.4.24). Above this concentration there was no increase in activity probably due to limitation of enzyme amount in comparison to substrate. The substrate concentration was higher in immobilized enzyme as compare to free enzyme (50 mM). Lineweaver-Burk plot between 1/V and 1/[S] was used for the calculation of K_m and V_{max} values (Fig. 4.25). The kinetic parameters (K_m and V_{max}) of an enzyme undergo variations after immobilization, which indicates change in affinity for the substrate. These variations may occur due to several reasons like protein conformational changes induced by the attachment to the support, steric hindrances and diffusional effects. Effect of these factors results in decrease or increase of the value of apparent K_m. The decrease in the K_m indicates faster reaction rate, whereas an increase of the K_m suggest the requirement of higher substrate concentration to achieve the same reaction rate.The K_m and V_{max} values calculated from Lineweaver-Burk plot for alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum* were found to be 18.21mM and 1.56 U/g respectively. Increase in K_m and decrease in V_{max} values might be due to the hindrance of substrate to penetrate in matrix for enzyme-substrate reaction.

In earlier studies Braun *et al.* (1989) reported a K_m value of 2.22 x $10^{-3}M^{-1}$ for penicillin G acylase immobilized on chitosan. Jose *et al*. (2003) used 20 mM pencillin G as substrate for maximum penicillin acylase activity from *Aspergillus fumigatus* and *Mucor gryseocianum.* While Montes *et al.* (2007) reported maximum activity of anionic exchanger immobilized penicillin acylase from *Escherichia coli* when penicillin G was used at 10 mM concentration Luo and Zhang (2010) reported K_m and V_{max} values of 8.92 $\times 10^{-5}$ mol/L and 28.64 μmol/min, respectively for penicillin acylase immobilized in epoxy-activated magnetic cellulose microsphere.Whereas Tembhurkar *et al*. (2012) reported 0.5% pencillin G as optimum for maximum penicillin acylase activity from *Micrococcus luteus* and *Staphylococcus* sp. The V_{max} value of the magnetic $NiFe_2O_4$ nanorods immobilized PGA (2.783 mmol mg^{-1} min^{-1}) was about 3.3 times higher than that of the free PGA (0.837 mmol mg^{-1}min-1); The K_m for the immobilized PGA (275.5 mmol L^{-1}) was around 19.4 times higher than that

of the free PGA (14.2 mmol L^{-1}). The great increase in K_m indicated a lower substrate affinity of the immobilized PGA and revealed that it was necessary to use higher substrate concentration to achieve the same velocity (Liu *et al*., 2016).

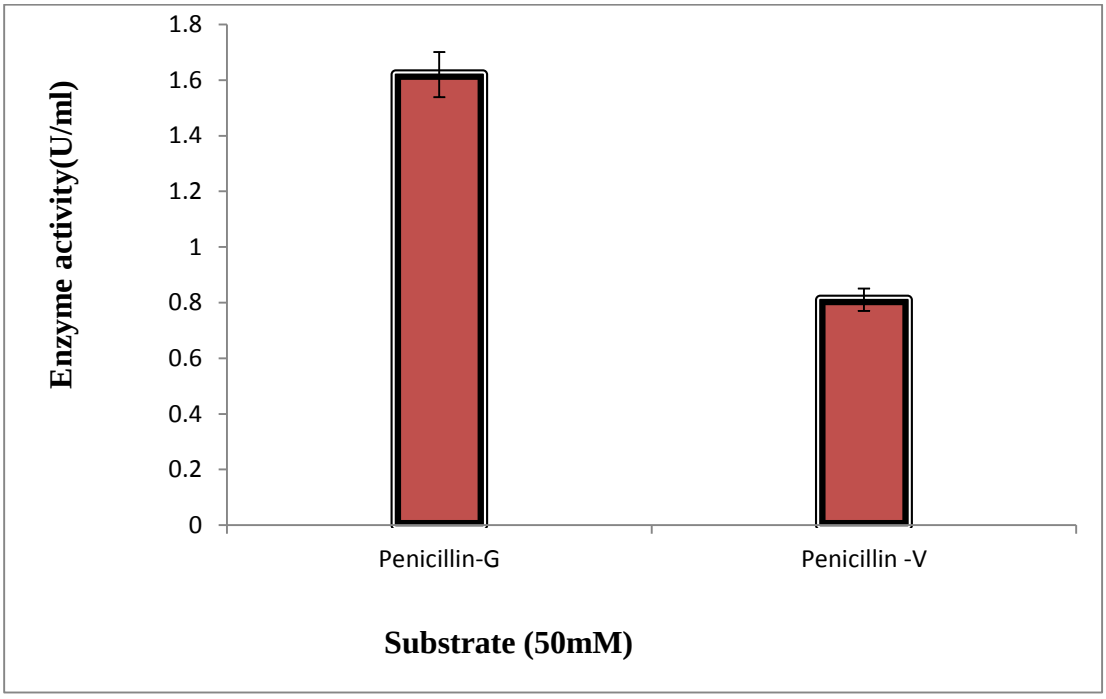


Fig.4.23 Substrate affinity of penicillin acylase from *Acremonium sclerotigenum*

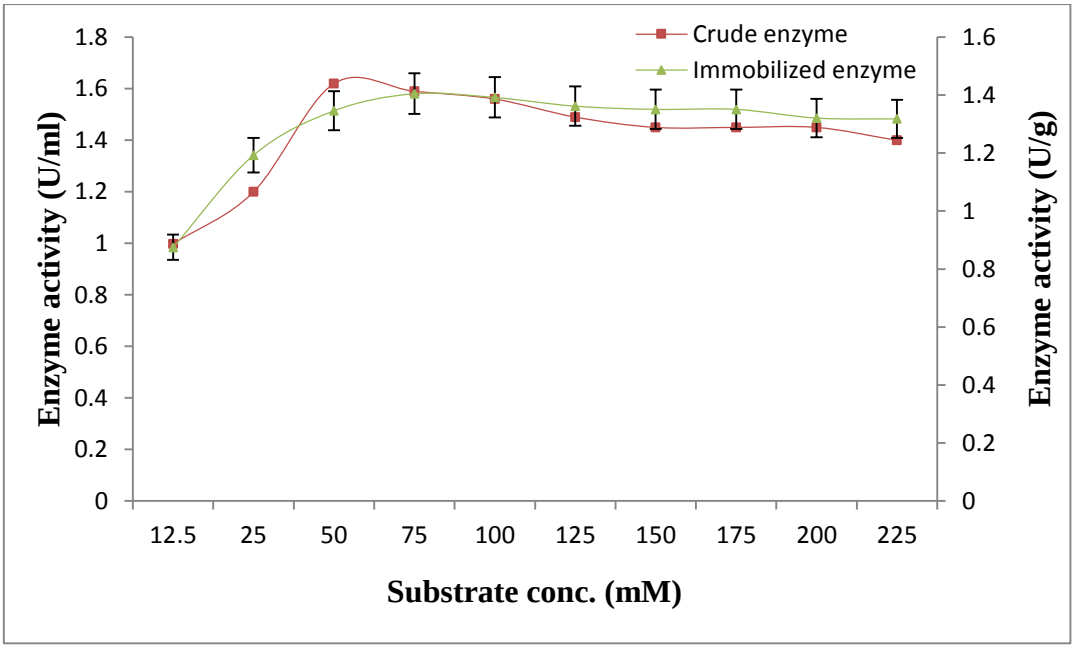


Fig.4.24 Effect of substrate (penicillin G) concentration on free and alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

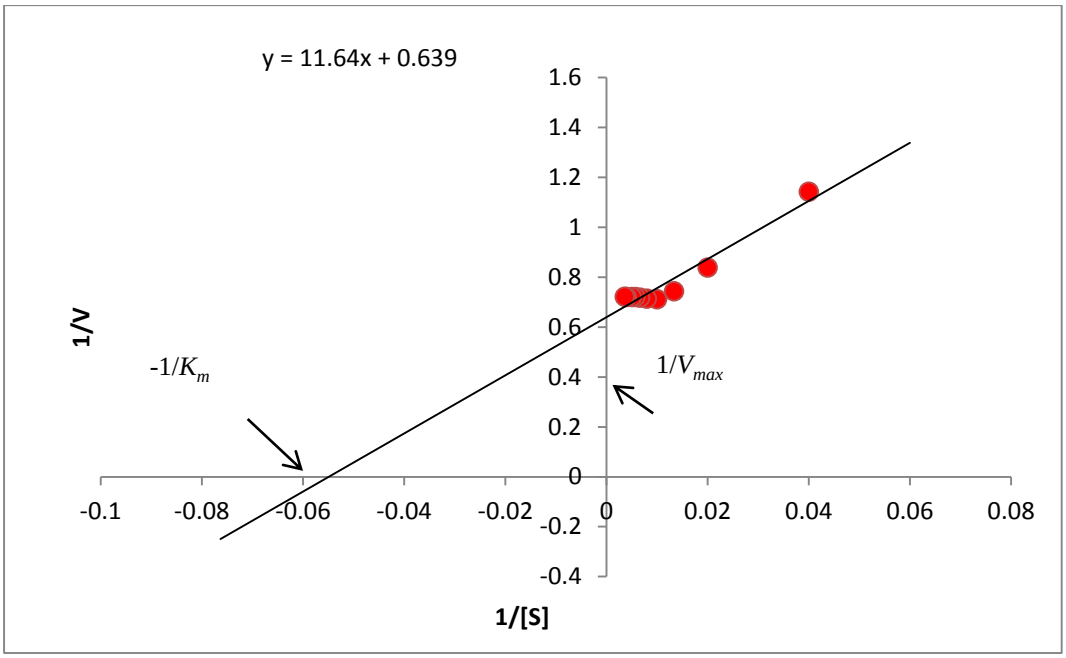


Fig.4.25 Determination of K_m and V_{max} of alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.4.5 Optimization of enzyme dose

To workout the zone of equivalence between substrate and catalyst, varied enzyme concentration 1.07-16.05µg (free) and 6.75-135µg (immobilized) were used. Maximum enzyme activity (16.07±0.04 U/mg) was found with 7.49 µg (Fig.4.26) of crude and (7.48±0.01U/mg) with 27 µg of immobilized penicillin acylase (Fig.4.27).

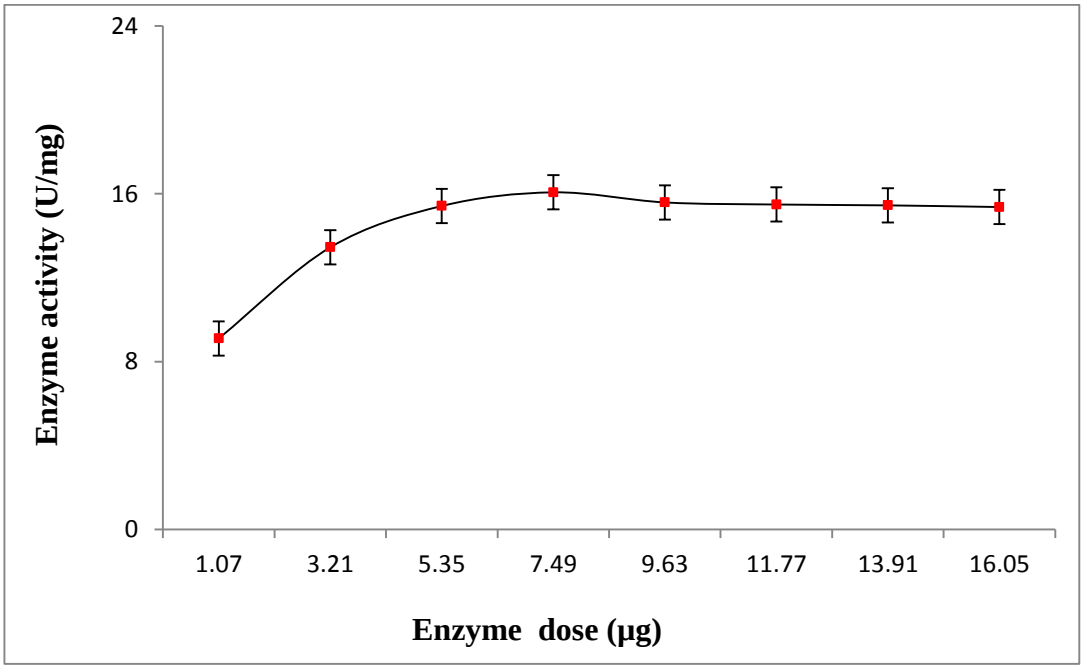


Fig.4.26 Optimization of enzyme dose of free penicillin acylase from *Acremonium sclerotigenum*

Further increase in the enzyme concentration resulted in no appreciable increase in enzyme activity probably due to decrease in substrate enzyme ratio with increasing concentration of enzyme. In contrast to our results Torres -Bacete *et al.* (2000) used 40 µg of penicillin acylase for enzyme activity from *Streptomyces lavendulae.* Whereas Rathinaswamy *et al.* (2012) has reported optimum activity with 5µM penicillin acylase from *Bacillus subtilis.*

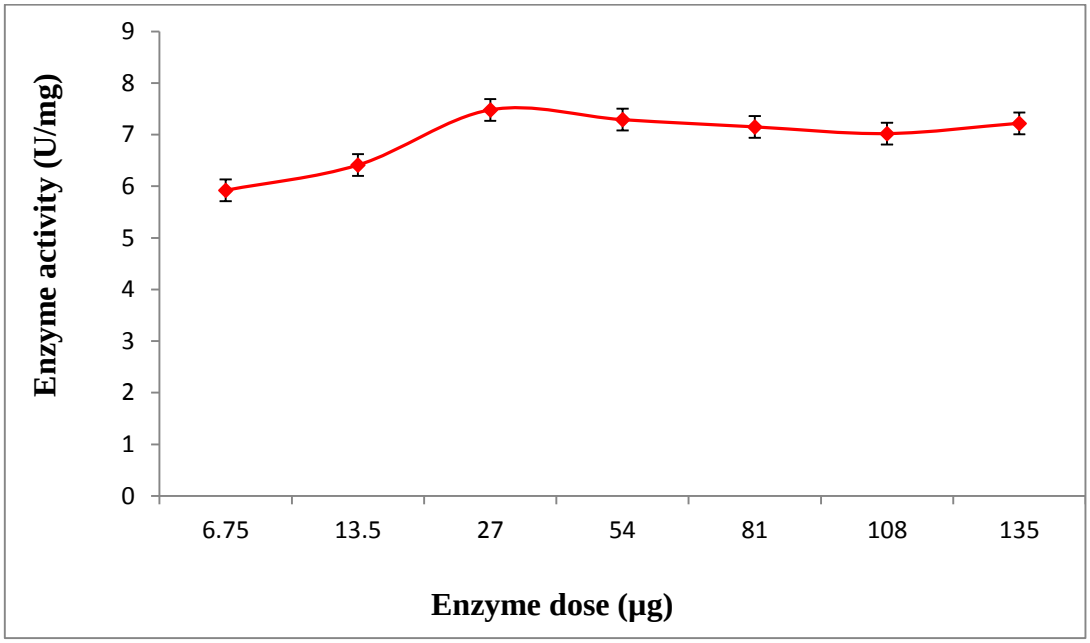


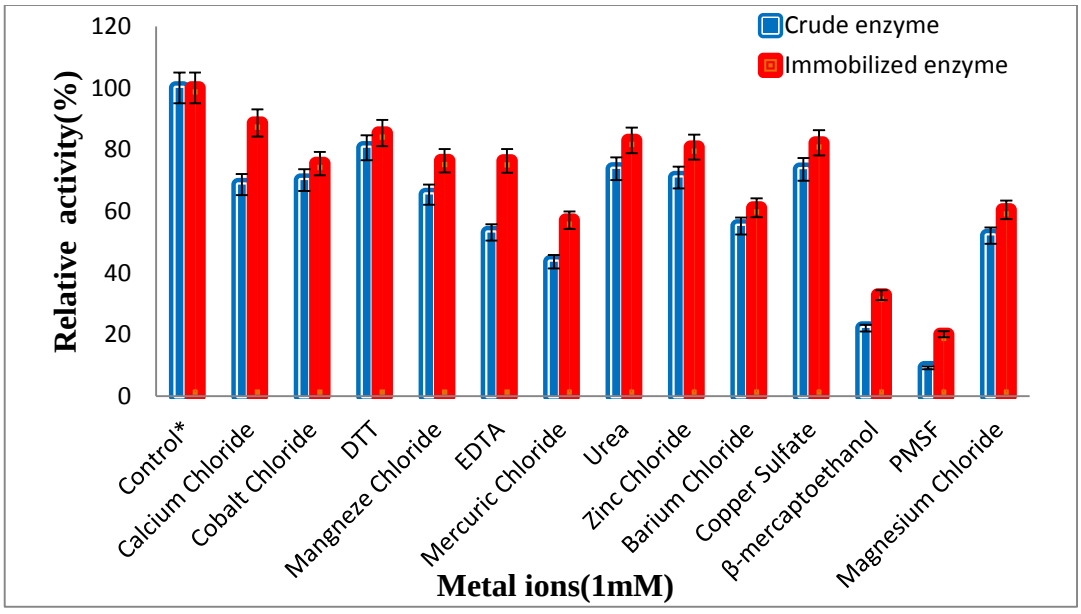
Fig.4.27 Optimization of concentration of alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

Prabhune and Sivaraman (1990) reported maximum enzyme activity of penicillin acylase of *E.coli* when enzyme was used at a concentration of 1.67mg. Zuza *et al.* (2007) used 0.1g immobilized penicillin acylase from *E.coli* for optimum enzyme activity. Whereas Hormigo *et al*. (2009) used 2µg of penicillin acylase from *Streptomyces lavendulae* for optimization studies. Whereas Liu *et al.* (2016) reported that magnetic $NiFe_2O_4$ nanorods immobilized penicillin acylase activity was optimum at 1.0 mg/ml PGA.

4.4.6 Effect of metal ions, inhibitors and additives

Metal ions play important roles in the biological function of many enzymes. The various modes of metal-protein interaction include metal-, ligand-, and enzyme-bridge complexes. Metals can serve as electron donors or acceptors, Lewis acids or structural regulators (Riordan, 1977). The effect of various metal ions and chelators on the activity of penicillin acylase was evaluated (Fig.4.28). The activity of penicillin

acylase was strongly inhibited by PMSF, $HgCl_2$, and β-mercaptoethanol both in free and immobilized penicillin acylase. In gel entrapped enzyme Inhibition was less as compared to free enzyme. None of the metal ions enhanced penicillin acylase activity as compared to control (Fig.4.28). Irreversible inhibition of penicillin acylase was observed with PMSF by Kutzhbach and Rauenbusch (1974). Similar to our results Sanjivkumar *et al*. (2012) have also found that metal ions, $Ca^{2+,}$ $Ba^{2+,}$ $Mg^{2+,}$ Hg^{2+} and Mn^{2+} had not shown any significant effect on penicillin acylase from *Bacillus subtilis.* Balci *et al*. (2014) has reported inhibition of penicillin acylase from *E.coli* ATCC11105 by $CuCl_2$, $FeCl_2$ and EDTA. The enzyme activity was strongly inhibited by PMSF. Zhang *et al*.(2014) reported effect of metal ions such as Ca^{2+},Mg^{2+}, $Zn^{2+,}$ $Cu^{2+,}$ K^{+}, $Sr^{2+,}$ Mn^{2+} and Fe^{3+} on a thermostable penicillin G acylase ACPGA001 activity. EDTA also affected enzyme activity, indicating that metals are important for enzymatic reactions. Among the metal ions tested K^{+} and Mg^{2+}increased ACPGA001 PGA activity by 87.8 and 46 %, respectively. Organic reagent such as N, Dimethylformamide (DMF) is usually involved in the synthesis of semi-synthetic beta-lactam antibiotics (Yang *et al.*, 2002). This enzyme was resistant to high concentration of organic reagents including 4 M urea and 40 % DMF, which was an advantage for ACPGA001 PGA in industrial use.



*****Corresponds to 1.62±0.04 U/ml for free enzyme 1.40U/g±0.04 for immobilized enzyme

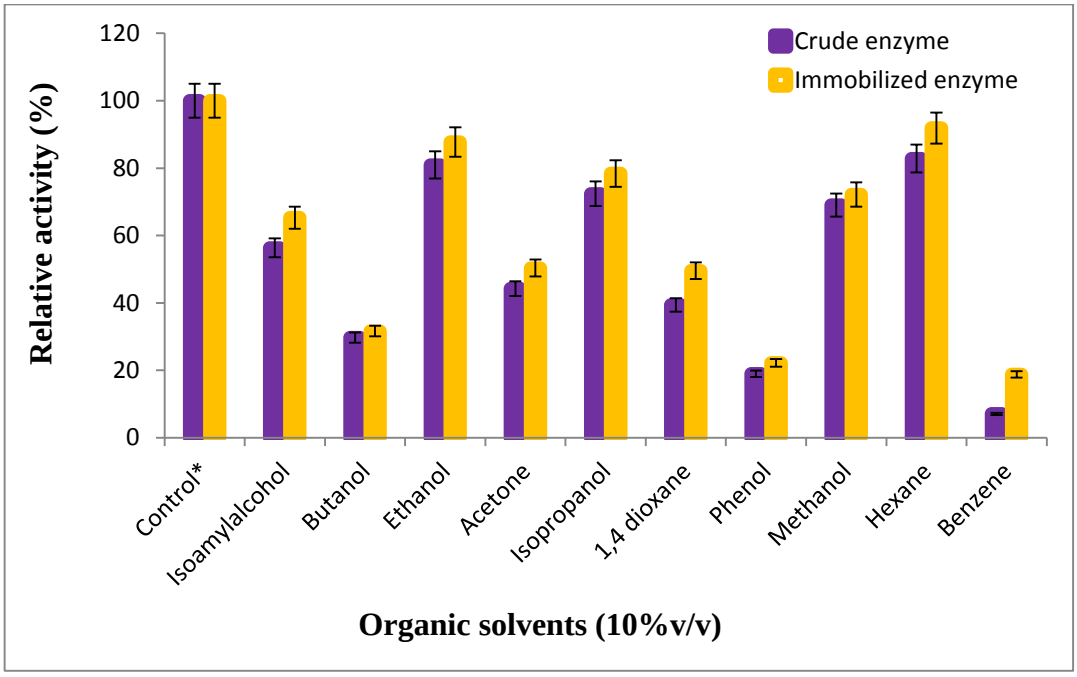
Fig.4.28 Effect of metal ions and inhibitors on free and immobilized penicillin acylase from *Acremonium sclerotigenum*

Rajendhran *et al*. (2003) reported the effect of the metal ions Ag^{+}, $Al^{3+,}$ $Ca^{2+,}$ $Co^{2+,}$ $Cr^{3+,}$ Cs^{+}, $Cu^{2+,}$ $Fe^{2+,}$ $Hg^{2+,}$ K^{+}, Li^{+}, $Mg^{2+,}$ $Mn^{2+,}$ $Ni^{2+,}$ Pb^{2+} and Zn^{2+} on *β*-lactam acylase, from *Bacillus badius.* None of the metal ions affected the PAC activity significantly. Similarly, the addition of EDTA did not affect the PAC activity, indicating that no metals are required for the enzymatic reaction. The PAC activity was not inhibited by the addition of the histidine-specific reagent DEPC and tryptophan-specific NBS. A complete activity loss was observed with the addition of the serine-specific reagent PMSF, indicating the role of serine as an active site residue in the *B. badius* PAC.

4.4.7 Effect of various organic solvents

The use of organic solvents as reaction media for biocatalytic reactions has proven to be an extremely useful approach to expand the range and efficiency of the practical applications of biocatalysis (Khmelnitsky and Rich, 1999). Penicillin acylase catalyzed reactions were also performed in water restricted systems. Various polar and non-polar organic solvents were added in reaction mixture in concentration of 10% (v/v) and results are illustrated in Fig.4.29. After performing the reaction in various organic solvents, the residual activity recorded was 91.89 % in hexane, 87.75% in ethanol and 78.41% in isopropanol in case of immobilized enzyme, while free penicillin acylase exhibited residual activity of (82.83%) with hexane followed by ethanol (80.94%). The enzyme activity was inhibited to a greater extent by phenol and benzene. Immobilized penicillin acylase showed less inhibition in phenol, benzene and butanol as compared to free penicillin acylase. The effects of various organic solvents on penicillin acylase-catalyzed synthesis of β-lactam antibiotics (pivampicillin and ampicillin) have been investigated in water-solvent mixtures. The rates of penicillin acylase-catalyzed reactions were found to be significantly reduced by the presence of even small amount of organic solvent. The rate of enzyme catalysis was extremely low in the presence of ring-structured solvents which include cycloaliphatics (cyclohexanol, cyclohexanone), aliphatics (dioxane, tetrahydrofuran), and aromatics (pyridine, phenol) and acids while enzyme activities were fully restored after removing the solvents (Kim and Lee, 1996). Arroyo *et al*. (2000) observed deactivation of penicillin V acylase in the presence of 1, 4 dioxane, tetrahydrofuran and pyridine. Balci *et al*. (2014) reported inhibitory effect of isoamyl alcohol and acetonitrile at (10-30%) and tolerance to ethanol and DMSO at 10% concentration A significant improvement in the stability of the crosslinked enzyme aggregates (CLEA) of penicillin G acylase in the presence of organic solvents was also reported. Dioxane was selected as a suitable cosolvent to study biocatalyst stability because it is highly

polar and deleterious for penicillin acylase (Abian *et al*., 2001). The half-life of CLEA was 8.9 h in 75% dioxane (Wilson *et al*., 2004).

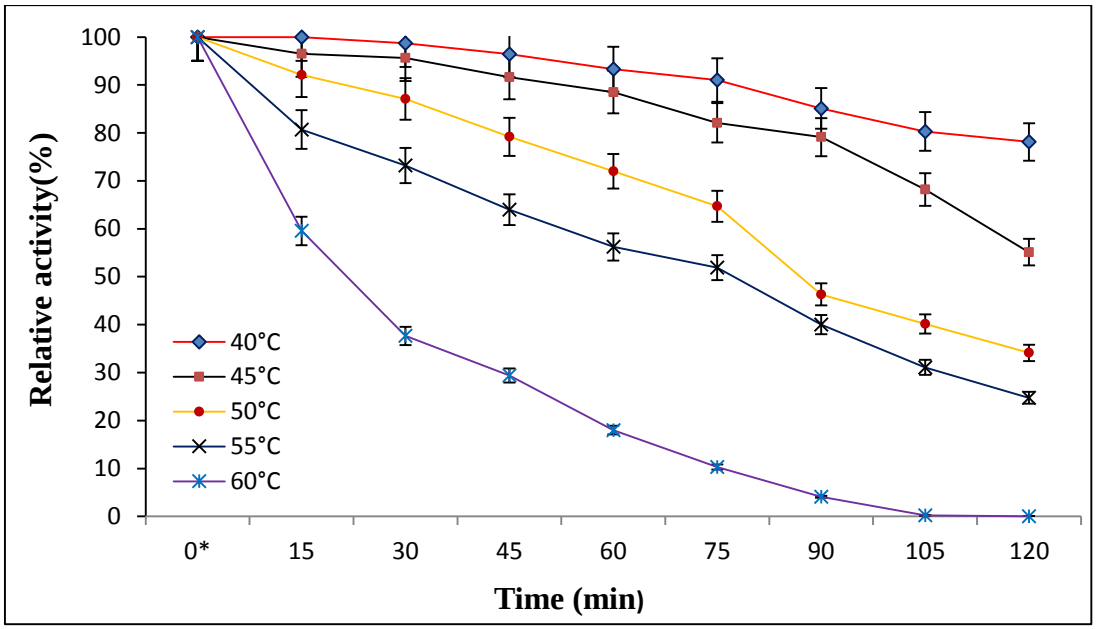


*Corresponds to 1.62±0.04 U/ml for free enzyme 1.40U/g±0.04 for immobilized enzyme

Fig.4.29 Effect of organic solvents on free and alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.4.8 Thermostability of enzyme

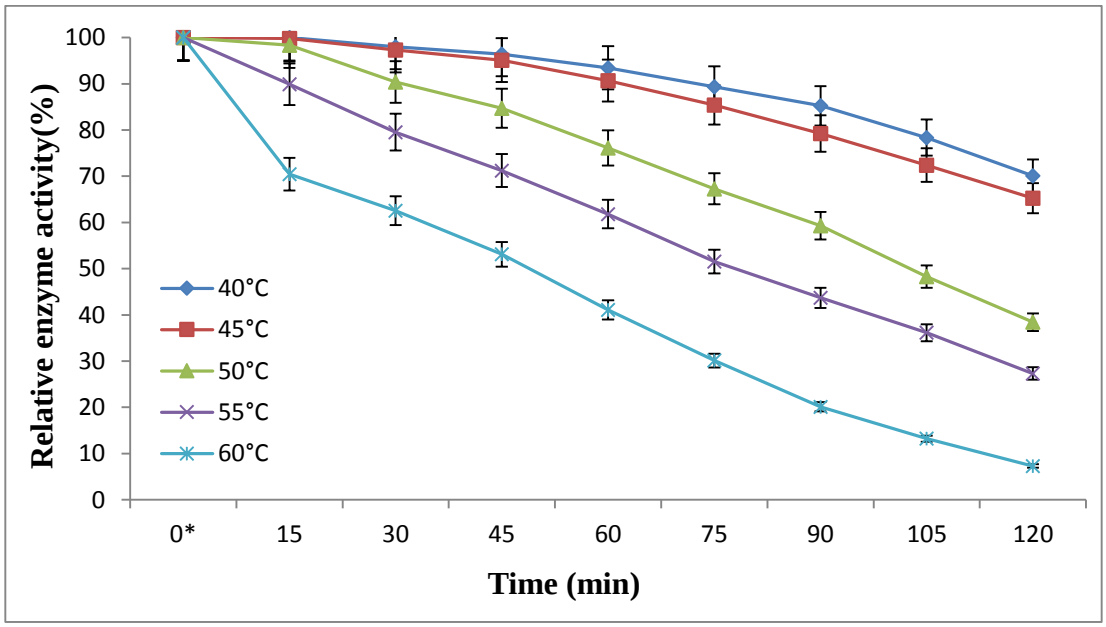
Thermal stability of the enzyme is one of the most important parameter when dealing with its industrial applications. Enzymes are in fact very sensitive to high temperature, because of their proteinacious nature. Increase in temperature results in denaturation of the enzyme which leads to a decrease in effective concentration and hence a decrease in reaction rate.The crude penicillin acylase from *Acremonium sclerotigenum* was found to be appreciably stable at 40°C to 45°C. The half life of the enzyme at 55°C and 60°C was 75 minutes and 15 minutes respectively (Fig.4.30). The immobilized penicillin acylase was more stable than the free one at relatively high temperature, indicating a better thermal stability. The increase in the thermal stability of immobilized PGA was caused by the stabilization through the weak intermolecular forces and the prevention of the autolysis of the PGA (Lei *et al*., 2009). The immobilized penicillin acylase exhibited a half life of 90 minutes and 45 minutes at 55°C and 60°C respectively (Fig.4.31).



*Corresponds to 1.61±0.04 U/ml

Fig. 4.30 Thermostability profile of free penicillin acylase from *Acremonium sclerotigenum*

In earlier studies Harmigo *et al.* (2009) have reported half life of penicillin acylase from *Streptomyces lavendulae* 96 h at 40°C. The enzyme was quite unstable at 60°C and half life of enzyme was 15minutes .The catalytic performance of PGA increases at temperatures between 25ºC and 50ºC. However, the enzyme shows poor stability at temperatures above 35ºC (Hassan, 2016).



*Corresponds to 1.39 U/g ± 0.04

Fig.4.31Thermostability profile of alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

In previous studies, Pereira *et al.* (1997) have reported a half life of 23 minutes at 60ºC for silica-glyoxyl immobilized penicillin acylase from *E.coli*. Composite carrier consisting of an adsorbent resin and biocompatible chitosan immobilized penicillin acylase has shown half-life of 300 minutes at 50°C (Jin *et al.,* 2008). Harmigo *et al.* (2009) have reported that half life of penicillin acylase from *Streptomyces lavendulae* was 96 hrs at 40°C. Ling *et al.* (2016) reported that PGA immobilized on magneticFe_3O_4@chitosan nanoparticles retained 80% and 45% activity after incubation for 180 min at 50°C and 55°C, respectively.

4.4.9 Reusability of alginate gel entrapped penicillin acylase

The most important and attractive advantage of immobilization is the reusability of enzyme. The catalyst reusability was investigated by measuring the stability of the immobilized enzyme as a function of number of reusages. The recyclability of the immobilized penicillin acylase was assessed in the reaction mixture by repeated use of calcium alginate beads (Fig.4.32). Results revealed that the immobilized enzyme beads could be used for up to 5 cycles only. The activity loss could be attributed to enzyme deactivation and protein leakage during washing and in the repeated uses (Chao *et al*., 2013).

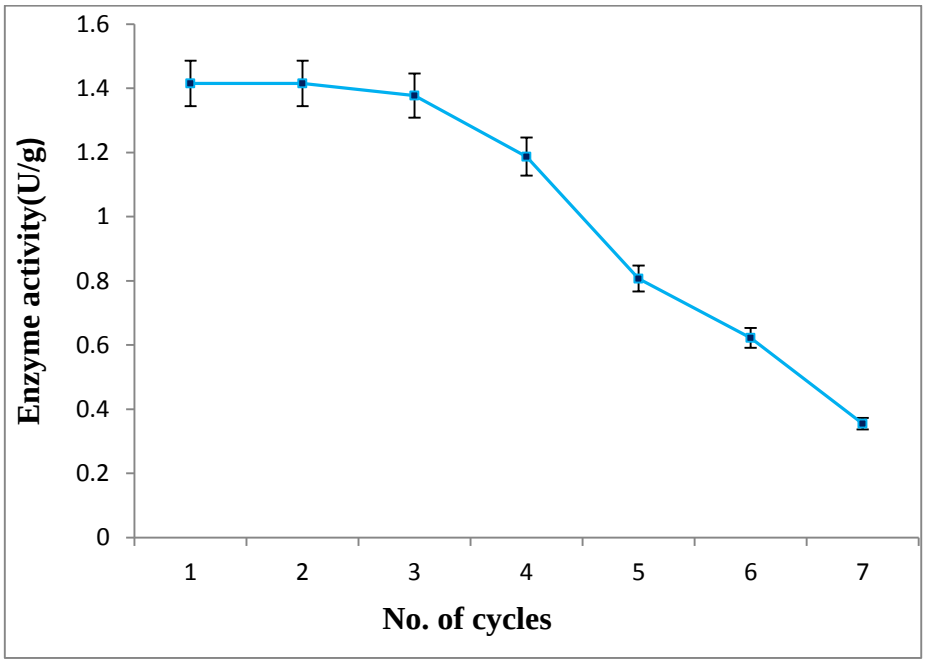


Fig.4.32 Reusability of alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

The operational stability of the immobilized PGA in repeated cycles in reaction use of revealed that immobilized PGA retained its activity even after ten

cycles of use (Shah *et al.,* 2008). The recyclability of the immobilized *E. coli* cell system was low, as a set of beads could only be used for three cycles (1h each) (Avinash *et al.,* 2016). Whereas Luo and Zhang (2010) reported that PGA immobilized in magnetic cellulose microsphere exhibited a better reusability, retaining 95.9% residual activity after being used 10 times. The immobilized PGA retained over 70 % residual activity after being used 10 times (Liu *et al*., 2016).

4.4.10 Shelf life

Shelf life of any enzyme of industrial bioprocess is very significant parameter. Therefore shelf life of penicillin acylase from *Acremonium sclerotigenum* was studied at both 4°C and at room temperature by pre incubating free enzyme for 360 h and immobilized penicillin acylase for 480 h. The results obtained have been depicted in Fig. 4.33 and 4.34 respectively. The free enzyme has lost 37.79% of its initial activity on storage at 4°C for 360 h, whereas at room temperature the enzyme activity declined gradually and enzyme had lost 50% of its activity after 288 h. The immobilized penicillin acylase showed better shelf-life as compared to crude enzyme at 4^0C and at room temperature. The half life of the immobilized enzyme at room temperature was 384 hrs (Fig.4.34). In earlier study Wang *et al.* (2007) reported that penicillin acylase retained 50% of their initial activity after 9 days.

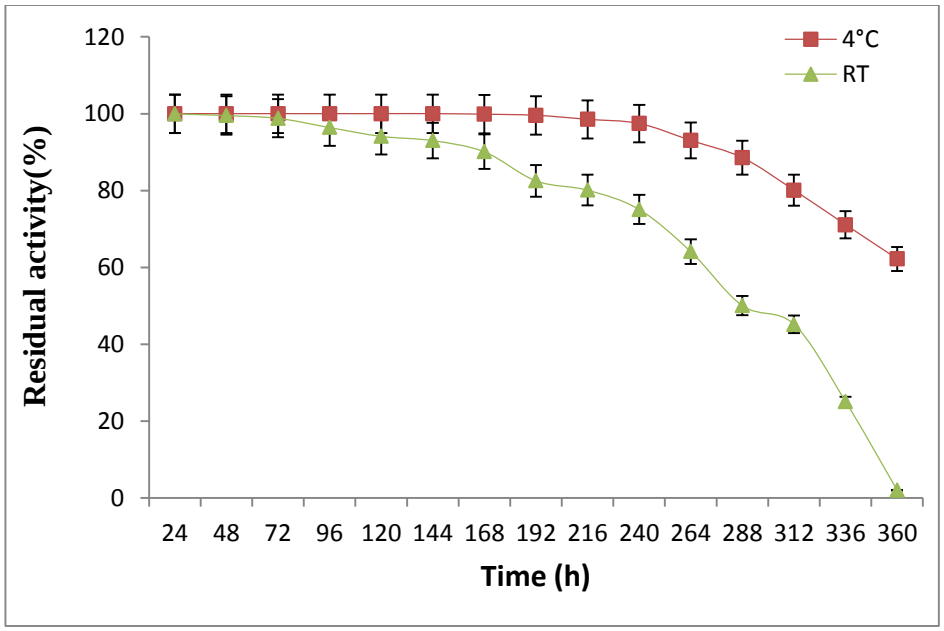


Fig. 4.33 Shelf life of free penicillin acylase from *Acremonium sclerotigenum*

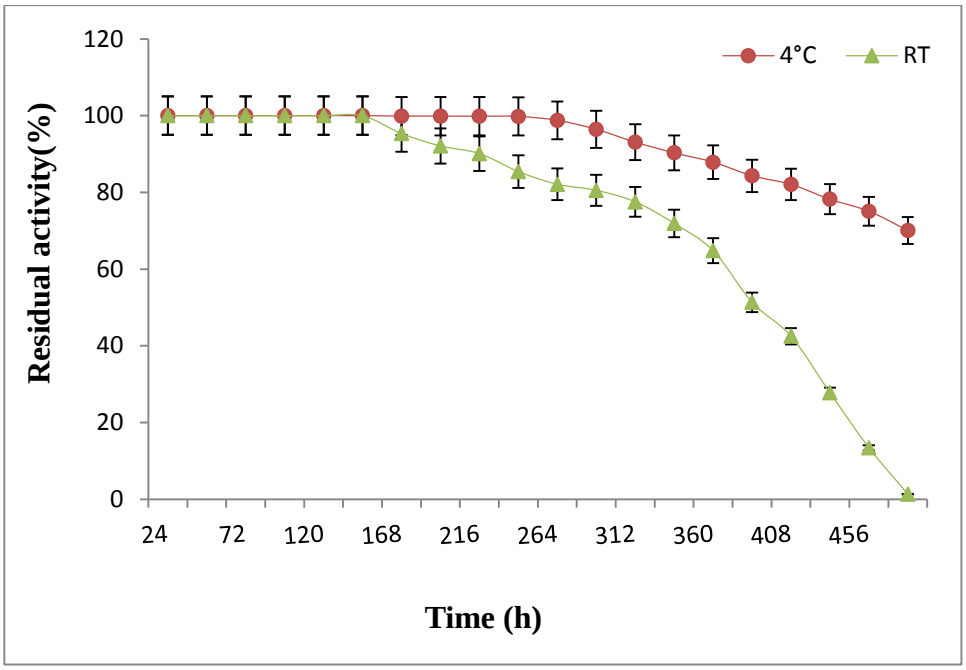


Fig.4.34 Shelf life of alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

In contrast to our study, Shah *et al*. (2008) evaluated the stability of the PGA-Am-SBA-15 enzyme at 4°C for 6 months and did not observe any significant loss in activity of immobilized enzyme over a period of 6 months and retained the initial activity when stored for 30 days at RT. The immobilized (cross-linked) whole cells of *E. coli* showed good storage stability when stored in 50 mM $CaCl_2$ at 4°C, effecting 80% conversion to 6-APA in 60 min even after 28 days (Avinash *et al.*,2017).

4.5 Purification of penicillin acylase

4.5.1 Ammonium sulphate fractionation

Crude enzyme was subjected to ammonium sulphate saturations from 10 to 90 %. Proteins started precipitating from 10% saturation. Protein of interest started precipitating after 10% saturation with ammonium sulphate and highest specific activity of penicillin acylase (49.66 U/mg protein) was obtained with 50% saturation (Table 4.2). Erarslan *et al.* (1991) has used 40-60% ammonium sulphate for precipitation of penicillin acylase. Whereas Esther *et al.* (2005) has used 1.5 M ammonium sulphate for precipitation of penicillin acylase from *E. coli*. Philem *et al.* (2016) reported a concentration of 30-50% for precipitation of penicillin V acylase from *Acinetobacter* sp. AP24.

Table 4.3 Ammonium sulphate fractionation profile of penicillin acylase from *Acremonium sclerotigenum*

S.No.	Saturation (%)	Specific activity (U/mg)	Protein (mg/ml)
1	10	5.4	0.045
2	20	7.75	0.073
3	30	10.93	0.092
4	40	13.82	0.105
5	50	49.66	0.418
6	60	1.854	0.523
7	70	0.679	0.602
8	80	0.100	0.00
9	90	0.032	0.00

4.5.2 Hydrophobic interaction chromatography

The crude enzyme having (516 U) total activity and (16.07 U/mg) specific activity was subjected to 50% saturation of ammonium sulphate and then subjected to hydrophobic interaction chromatography. HIC makes use of surface hydrophobicity interaction of protein and column packing material in the presence of high salt concentrations. Since, different amino acids have different hydrophobicity; HIC can be used to separate proteins and enzymes with different compositions. The results of protein elution and penicillin acylase activity profile of Octyl-sepharose chromatography shown in Fig. (4.35). Purification profile of penicillin acylase from *Acremonium sclerotigenum* summarized in Table-4.3. The enzyme was purified up to (11.24)-fold with a yield of (7.56 %). Kumar *et al.* (2008) have also reported purification of penicillin V acylase from yeast, *Rhodotorula aurantiaca* NCIM 3425 by hydrophobic interaction chromatography using Octyl-sepharose column. Yang *et al*. (2001) reported purification of penicillin acylase from *B.subtilis* SIBAS205 by centrifugation, Al_2O_3 adsorption and phenyl-Sepharose CL-4B hydrophobic chromatography with a yield of 85%. The purified enzyme had a specific activity of 45 U/mg protein. Lagerlof *et al.* (1976) used a purification process in which enzyme was purified by adsorption to carboxymethylcellulose (CMC). Erarslan *et al.* (1991) purified the PGA enzyme to 350-fold with high specific activity of 34.5 U/mg and yield of 39% using DEAE-cellulose ad hydroxyapatite column. Senthilvel and Pai (1996) reported the purification of PGA from *B. megaterium* using ultra filtration followed by ammonium sulfate precipitation and gel filtration chromatography using Sephadex G-100. The enzyme was purified 6-fold with a highest specific activity of

2.62 U/mg in 15% yield. Sudhakaran and Shewale (1995) described purification of PVA from *Fusarium* sp. SKF 235 using a three-step procedure involving chromatography on CM-Sepharose, DEAE-Sephacel treatment followed by gel filtration on Bio-Gel P-100. A 182-fold purification with a yield of 9.3% was achieved by this method.

Vohra *et al.* (2001) described the purification of penicillin acylase. Penicillin G acylase (*pac)* gene from *E.coli* ATCC 11105 was cloned into a stable *asd* C vector (pYA292) and expressed in *Escherichia coli*. Purified PAC protein near to homogeneity (92% elimination of soluble proteins) by hydrophobic interaction chromatography, attained high specific activity (16 U mg^{-1} protein) and 60% recovery of PAC protein. Zhang *et al.* (2007) reported purification of penicillin V acylase from *Streptomyces mobaraensis.* The enzyme was purified from the culture broth of *S. mobaraensis* by column chromatographies on CM Sephadex C-50 gels and hydroxyapatite gels. Kecilli *et al.* (2007) observed 10.3 and 35.5 fold purification with a recovery of 90% and 89% from *Penicillium chrysogenum* NRRL 1951 and *P. purpurogenum* crude extracts respectively, using a monolith column containing methacryloyl antipyrine. Demircelik *et al*. (2017) reported penicillin acylase purification from *Pe*nicillium *chrysogenum* using supermacroporous hydrophobic affinity sorbents with 76.3% purification yield and 332.3 purification factor.

Table 4.4 Purification summary of penicillin acylase from *Acremonium sclerotigenum*

Purification Stage	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (U/mg)	Total Activity (U)	Yield (%)	Fold Purification
CFE	300	0.107	32.1	16.07	516.00	100	1
ASP	6ml	0.418	2.50	49.66	124.15	24.06	3.09
HIC	12ml	0.018	0.216	180.77	39.04	7.56	11.24

CFE: Cell-free Extract; ASP: Ammonium Sulphate Precipitation; Hydrophobic Iinteraction chromatography

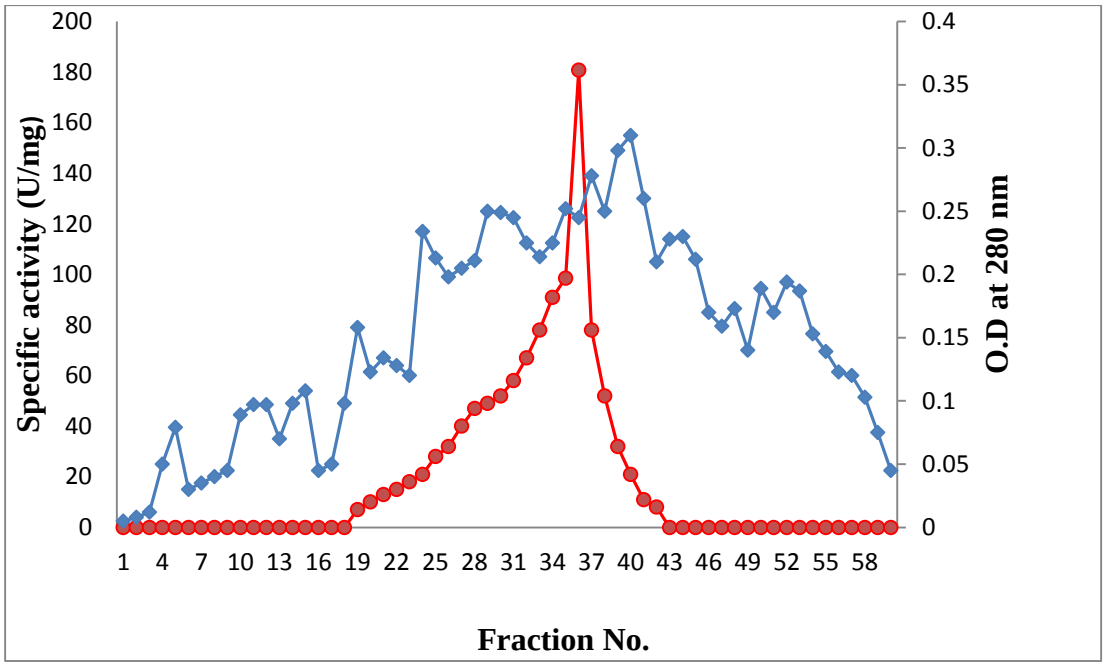


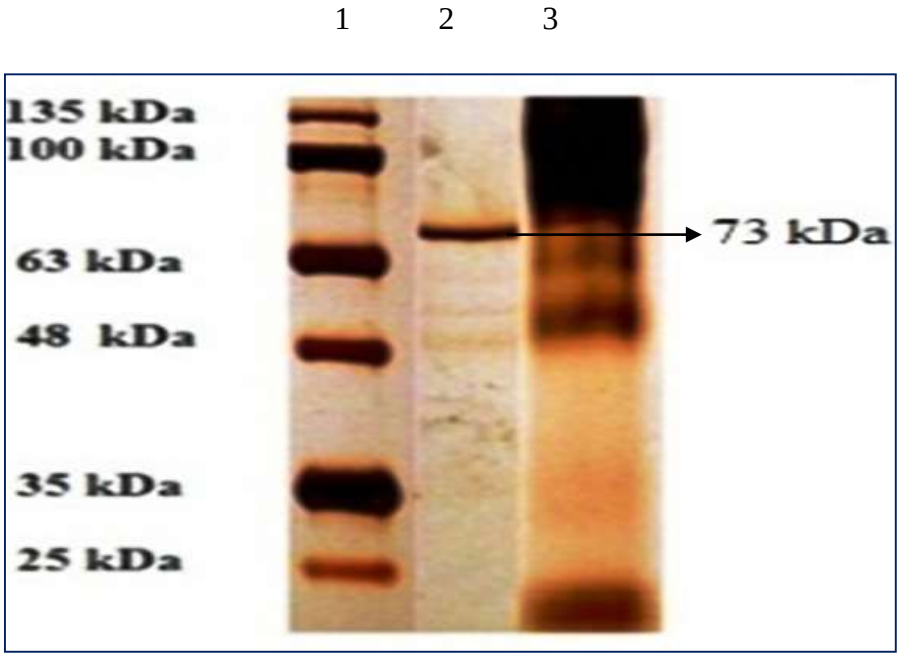
Fig.4.35 Protein elution and enzyme activity profile of hydrophobic interaction chromatography of penicillin acylase from *Acremonium sclerotigenum*

4.5.3 Electrophoretic characterization of penicillin acylase from *Acremonium sclerotigenum*

4.5.3.1 SDS- PAGE analysis of purity and molecular mass determination

The analysis of fungal penicillin acylase under reducing and denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the purified penicillin acylase possesses a single band of molecular weight 73 kDa as visualized with silver staining. The characteristics of Electrophoretic pattern revealed that *Acremonium sclerotigenum* penicillin acylase was a homogeneous protein (Fig.4.36). In earlier studies Shimizu *et al.* (1975) reported purification of penicillin acylase (EC 3.5.1.11) of *Kluyvera citrophila* with an approximate molecular weight of 63,000 Da. The SDS-PAGE of the purified PA of the mutant strain *E.coli* ATCC 11105 showed two bands with different molecular weights. The corresponding molecular weights were about 23000 and 59000 Daltons for alpha and beta subunits, respectively (Erarslan, *et al.*, 1991).Verhaert *et al*. (1997) described purification of penicillin G acylase from *Alcaligenes faecalis*. Molecular mass determination of the penicillin G acylase showed that α and β subunits have molecular masses of 23.0 and 62.7kDa, respectively. Pinotti *et al*. (2008) determined molecular weight of an intracellular monomeric penicillin V acylase (PVA) 36,000 Da. The extracellular PGA of *B. subtilis* BAC 4 consisted of two subunits, α and β. The smaller subunit (α) has a

molecular weight of 57.8 kDa, and the bigger subunit (β) has a molecular weight of 62.1 kDa. Therefore, the relative molecular mass (Mr) of the extracellular PGA of *B. subtilis* BAC4 was estimated to be 120 kDa (Supartono *et al*., 2008). Rathinaswamy *et al.* (2012) reported the purification and characterization of the penicillin acylase from *Bacillus subtilis*. The enzyme was a homotetramer of 148 kDa. Demircelik *et al*. (2017) reported purification of penicillin acylase from *Pe*nicillium *chrysogenum*, was a heterodimer with subunit molecular weight of 23kDa and 63 kDa.



Lane 1: Molecular Weight Marker (kDa)

Lane 2: HIC Fractions

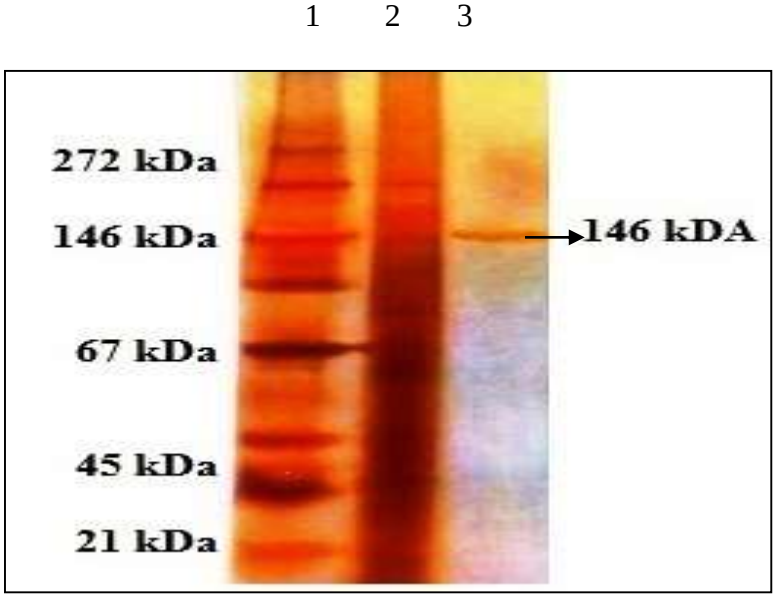
Lane 3: Crude extract

Fig.4.36 SDS PAGE of purified penicillin acylase from *Acremonium sclerotigenum*

4.5.3.2 Native polyacrylamide gel electrophoresis

A single band of 146 kDa was observed in Native-PAGE analysis. This protein band was carefully cut and assayed colorimetrically for penicillin acylase activity (207.24 U/mg±3.1) (Fig.4.37). It is confirmed that the native penicillin acylase of *Acremonium sclerotigenum* was biologically active and appeared to be a homozyme that consists of two identical subunits of approximately 73 kDa (homodimer) and 146 kDa in native form (Fig. 4.37). In previous studies Rathinaswamy *et al.* (2012) reported the purification and characterization of the penicillin acylase from *Bacillus*

subtilis .The enzyme was a homotetramer of 148 kDa. Demircelik *et al*. (2017) reported purification of penicillin acylase from *Pe*nicillium *chrysogenum* enzyme was a heterodimer with molecular weight of 86 kDa in native form.



Lane 1: Molecular Weight Marker (kDa)

Lane 2: Cell free Extract

Lane 3: HIC Fraction

Fig.4.37 Native PAGE of purified penicillin acylase from *Acremonium sclerotigenum*

4.5.3.3 MALDI -TOF analysis

4.5.3.3.1 Sinapic acid (SA) or 3, 5-Dimethoxy-4-hydroxycinnamic acid matrix preparation

Matrix solution was prepared according the recommended guidelines from Bruker Guide to MALDI sample preparation as described. Matrix solution A: A saturated solution of sinapic acid in ethanol. Matrix solution B: A saturated solution of sinapic in TA 30 solvent (30:70 [v/v] acetonitrile: 0.1%TFA in water).

4.5.3.3.2 Sample preparation and analysis

0.5 µl matrix solution A was deposited on the MALDI target plate and allowed to dry. Then 1 part of matrix solution B was mixed with 1 part analyte solution. 0.5 µl of the matrix/analyte mixture was deposited on the matrix spot and allowed to dry. Penicillin acylase mass was confirmed by MALDI-TOF mass spectrometry using a Model Autoflex Speed (Bruker, USA). Purified fractions were mixed (1:1, v/v) with 3,

5-Dimethoxy-4-hydroxycinnamic acid matrix (Sigma) and MALDI-TOF spectra were acquired in positive mode. MALDI showed a prominent peak of 144268.666Da, corresponding to the molecular weight of 146 kDa of penicillin acylase from *Acremonium sclerotigenum* (Fig, 4.38). In previous studies Avinash *et al.* (2015) reported purification of penicillin V acylase from *Pectobacterium atrosepticum*. The molecular weight was ascertained using Matrix-associated laser desorption ionization-mass spectrometry (MALDI, Perkin-Elmer) using a sinapinic acid matrix. MALDI showed a single peak of 39, 191 Da, corresponding to the subunit molecular weight of PaPVA enzyme. The native molecular weight was estimated to be 154 kDa. Penicillin V acylase was purified from *Acinetobacter* sp. AP24. Purity of the enzyme was confirmed by SDS PAGE and MALDI-TOF MS analysis. In MALDI-TOF, a prominent peak of 34 kDa molecular mass was observed, which was in agreement with the result of in SDS-PAGE (Philem *et al.*, 2016).

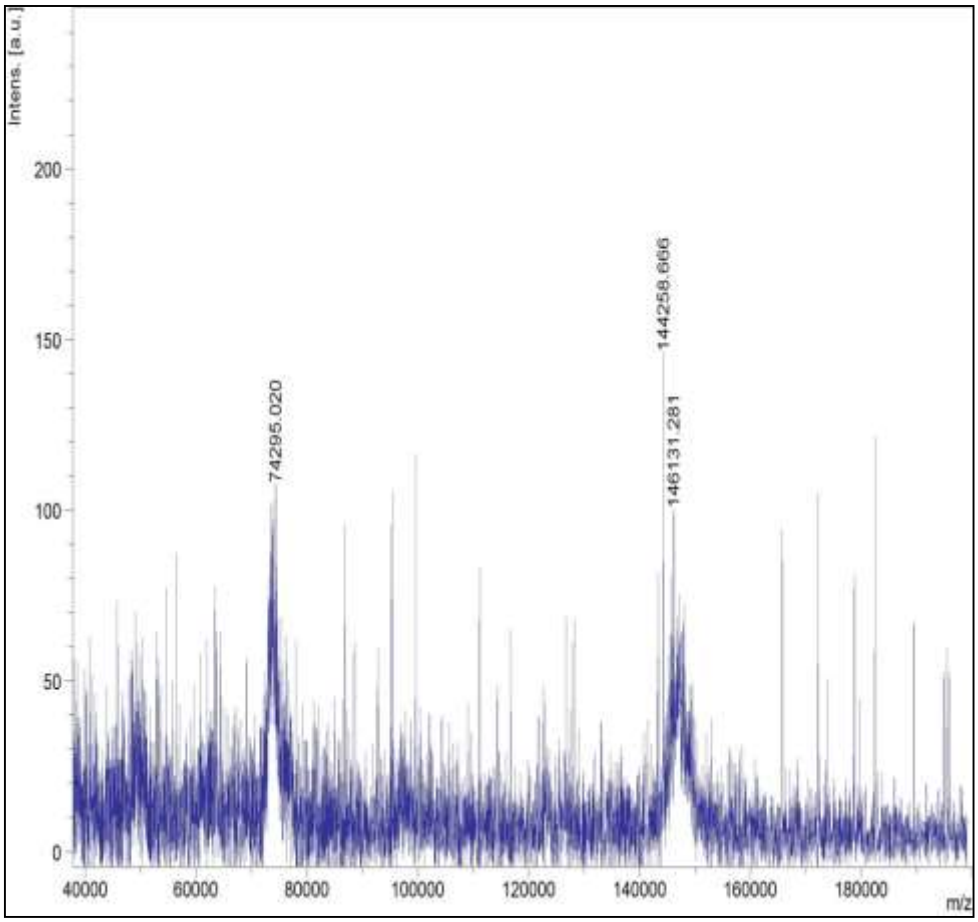


Fig.4.38 MALDI TOF of purified penicillin acylase from *Acremonium sclerotigenum*

4.6 Characterization of purified penicillin acylase from *Acremonium sclerotigenum*

Purified penicillin acylase of *Acremonium sclerotigenum* was characterized for various physicochemical parameters with appropriate / optimal enzyme content.

4.6.1 pH optimum

Substrate binding and catalysis are often dependent on charge distribution on substrate and in particular enzyme molecules. Hence enzyme activity is very much dependent on pH of buffer system.The purified penicillin acylase from *Acremonium sclerotigenum* showed similar pattern of activity as observed with the crude enzyme i.e. maximum activity (207.22U/mg±0.04) was observed at pH-5 in sodium citrate buffer (Fig.4.39), whereas activity decreased sharply in alkaline pH. The enzyme was stable at lower pH which is favorable for the stability of penicillins and 6-APA. Similarly Kumar *et al*. (2008) reported an optimum pH-5.5 for purified penicillin V acylase from yeast, *Rhodotorula aurantiaca* NCIM 3425, with citrate buffer.

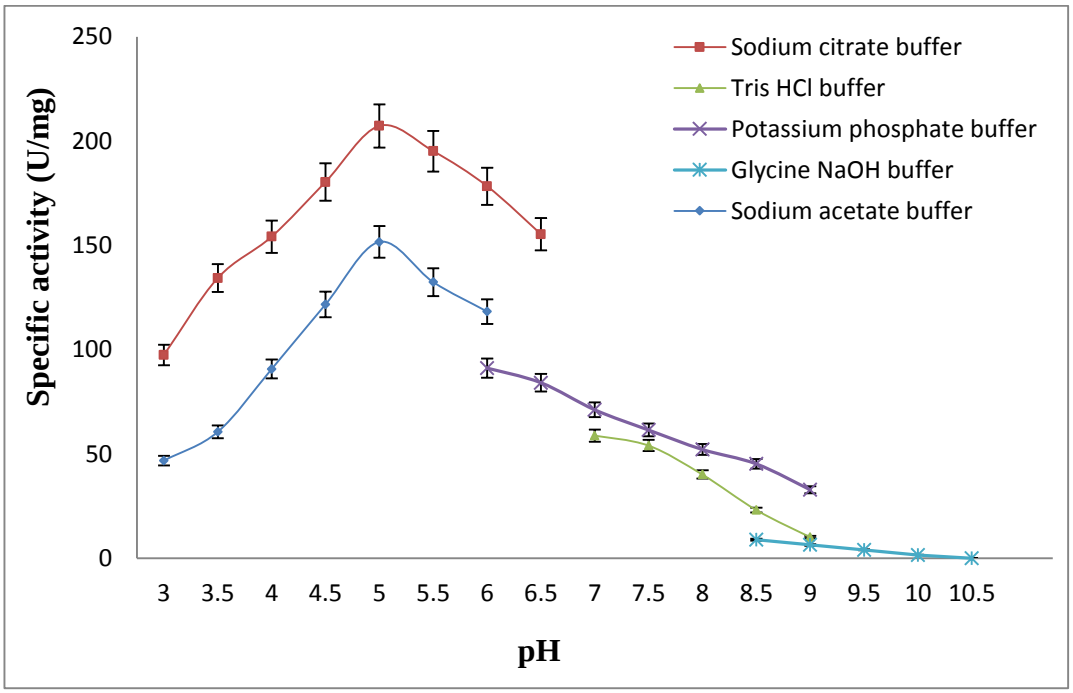


Fig. 4.39 Effect of buffer pH on purified penicillin acylase from *Acremonium sclerotigenum*

In contrast to our study, Cai *et al*. (2004) reported maximum activity of thermostable penicillin G acylase from *Achromobacter xylosoxidans* at pH-9 in Na_2HPO_4-NaOH buffer. Whereas Yang *et al*. (2001) used sodium phosphate buffer, pH 7.5 for maximum activity of penicillin acylase from *Bacillus subtilis.* Tris-HCl

buffer with pH 7.8 was reported by Zhang *et al*. (2007) for the optimum activity of purified penicillin V acylase from *Streptomyces mobaraensis.*

4.6.2 Buffer molarity

The effect of buffer molarity on the activity of purified penicillin acylase illustrated in Fig. 4.40. Highest enzyme activity (220.33±0.08U/mg) was recorded in 125 mM sodium citrate buffer at pH-5.0. In contrast to our study, Kumar *et al*. (2008) reported maximum activity of purified penicillin V acylase from yeast, *Rhodotorula aurantiaca* NCIM 3425, with 0.1M citrate buffer. Rathinaswamy *et al.* (2012) reported maximum activity of purified penicillin acylase from *Bacillus subtilis* with 50mM potassium phosphate buffer.

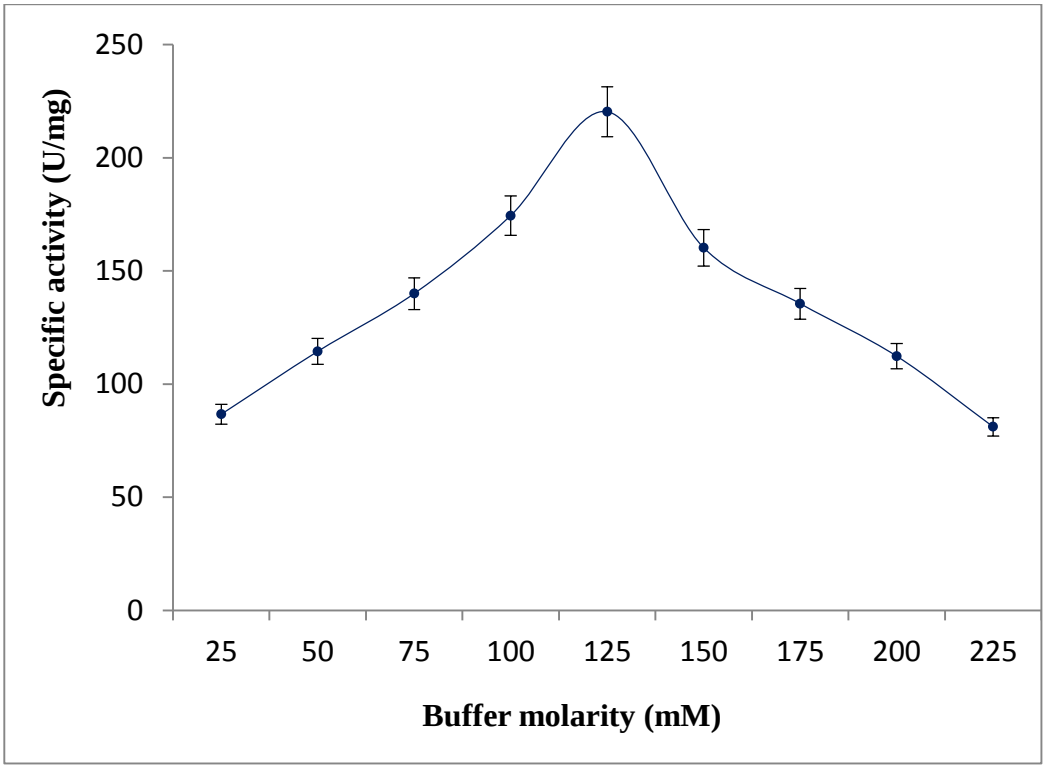


Fig.4.40 Effect of sodium citrate buffer molarity on purified penicillin acylase from *Acremonium sclerotigenum*

4.6.3 Temperature optimum

The effect of reaction temperature on purified penicillin acylase illustrated in Fig.4.41. There was gradual increase in the enzyme activity reported from 30-40°C there after the activity declined sharply. Maximum penicillin acylase activity was recorded at 40°C (225.4±0.08U/mg). Similarly Rathinaswamy *et al.* (2012) have also observed maximum activity of purified penicillin acylase from *Bacillus subtilis* at

40°C. Kumar *et al*. (2008) reported maximum activity for purified penicillin V acylase from yeast, *Rhodotorula aurantiaca* (NCIM 3425) at 45°C. Whereas Zhang *et al*. (2007) reported optimal activity of purified penicillin V acylase at 37°C from *Streptomyces mobaraensis.*

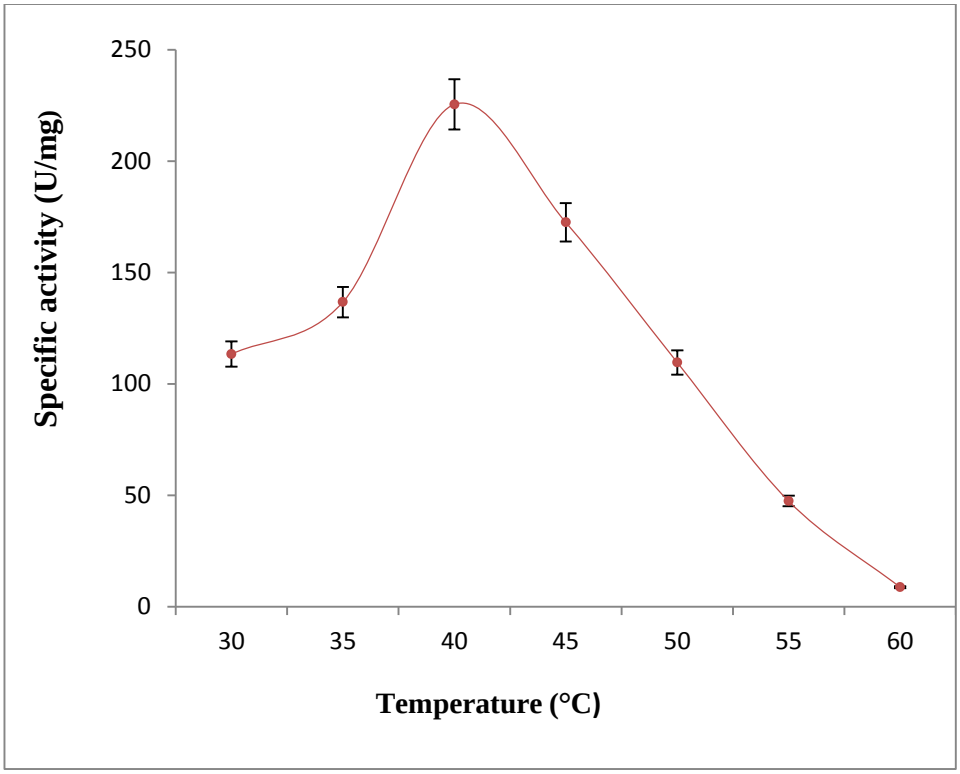
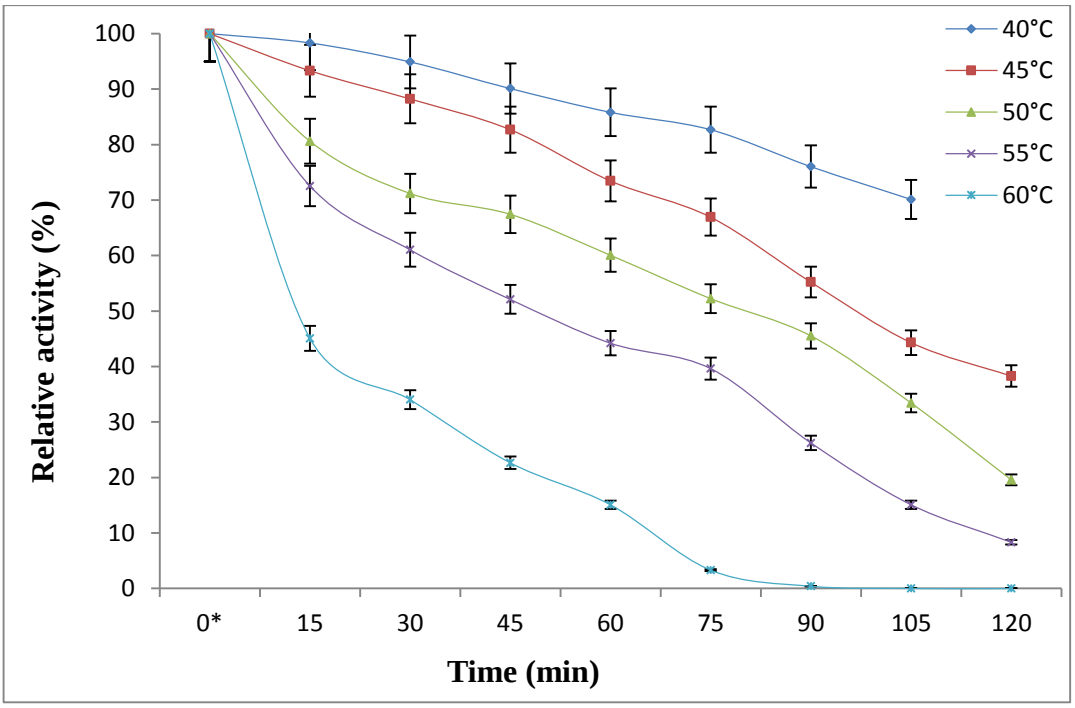


Fig. 4.41 Effect of temperature on purified penicillin acylase from *Acremonium sclerotigenum*

4.6.4 Thermal stability of purified penicillin acylase

The purified penicillin acylase was fairly stable at 40°C for 120 minutes where as at 45°C and 50°C the purified penicillin acylase has retained 38% and 20% of the residual activity respectively after 120 minutes. Activity declined at faster rate at 55°C and 60°C with a $t_{1/2}$ of 45 min and <20 min respectively (Fig.4.42). In contrast to present study Kumar *et al*. (2008) reported that penicillin V acylase from yeast; *Rhodotorula aurantiaca* NCIM 3425 retained 100% activity at 40°C for 30 minutes. The enzyme lost its 50% activity at 60°C after 30 min and only 14% and 6% of the activity was left when it was incubated for 120 minutes at 50°C and 60°C, respectively. His-tagged penicillin G acylase enzyme activity degraded above 40 °C and was lost completely above 60 °C after 30 min exposure (Wen *et al*., 2004).



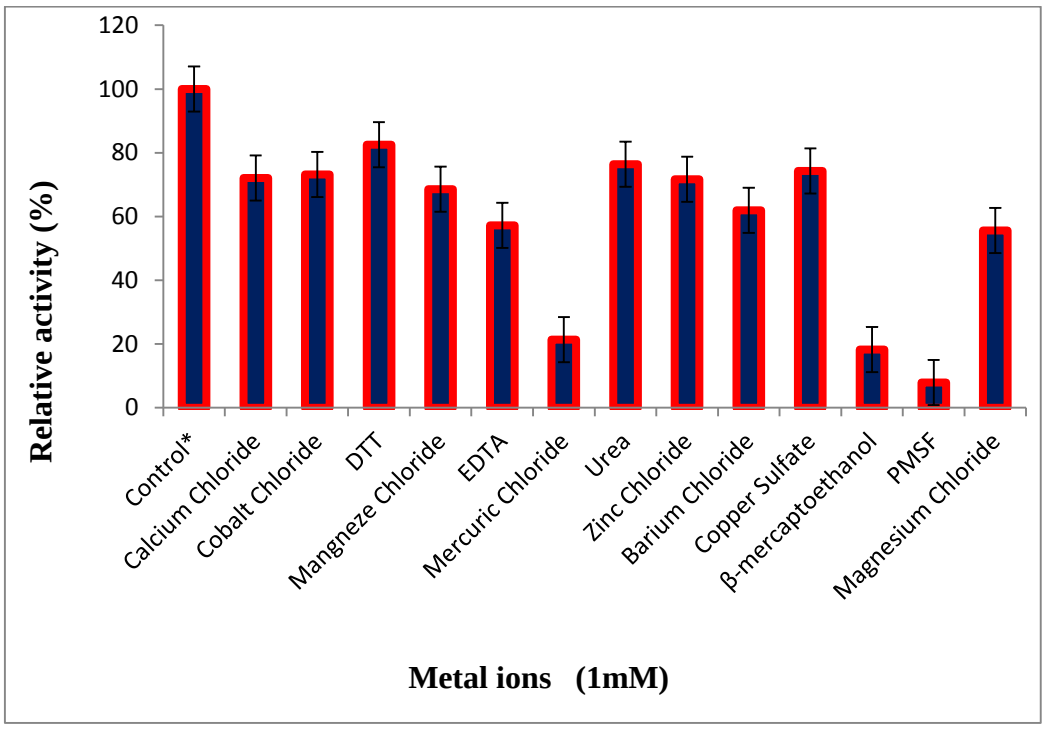
*Corresponds to 225.4 ± 0.08U/mg.

Fig. 4.42 Thermal stability profile of purified penicillin acylase from *Acremonium sclerotigenum*

4.6.5 Effect of metal ions inhibitors and additives

The effect of metal ions and inhibitors on purified penicillin acylase from *Acremonium sclerotigenum* was studied and the results (Fig. 4.43) were similar to those obtained with crude enzyme. The activity of purified penicillin acylase was also strongly inhibited by PMSF, $HgCl_2$, and β- mercaptoethanol. None of the metal ions enhanced penicillin acylase activity as compared to control. None of the metal ions Ag+, Al $^{3+,}$ $Ca^{2+,}$ $Co^{2+,}$ $Cr^{3+,}$ Cs^{+}, $Cu^{2+,}Fe^{2+,}$ $Hg^{2+,}$ K^{+}, Li^{+}, $Mg^{2+,}$ $Mn^{2+,}$ $Ni^{2+,}$ Pb^{2+} and Zn^{2+} affected the PAC activity significantly. Similarly, the addition of EDTA did not affect the PAC activity, indicating that no metals are required for the enzymatic reaction. A complete activity loss was observed with the addition of the serine-specific reagent PMSF, indicating the role of serine as an active site residue in the *B. badius* PAC (Rajendhran and Gunasekaran, 2007a). Metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, K^{+}, Sr^{2+}, Mn^{2+} and Fe^{3+} affected ACPGA001 PGA activity. EDTA also affected enzyme activity, indicating that metals are important for enzymatic reactions. Among the metal ions tested, K^{+} and Mg^{2+} increased ACPGA001 PGA activity by 87.8 and 46 %, respectively (Zhang *et al.*, 2014). Kumar *et al*. (2008) have reported the effect of reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol

(BME) enhanced the enzyme activity at 1 and 5 mM. The enzyme activity was severely inhibited by (PMSF) at very low concentration 0.5 mM .

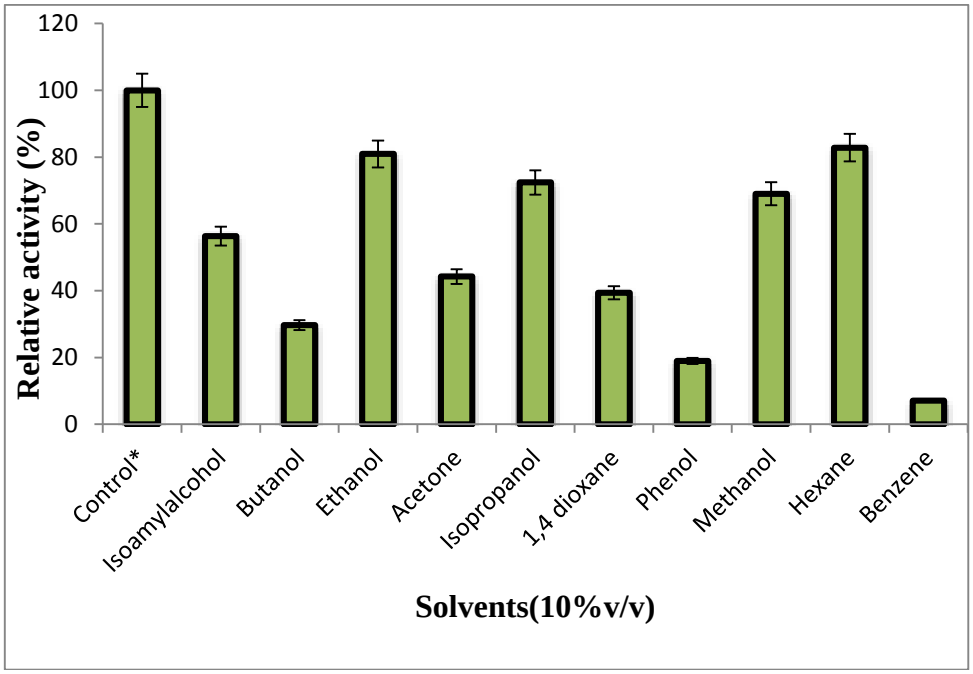


*Corresponds to C (225.4±0.08U/mg).

Fig.4.43 Effect of metal ions and inhibitors on purified penicillin acylase from *Acremonium sclerotigenum*

4.6.6 Effect of various organic solvents

Penicillin acylase activity was also performed in water restricted system and the results obtained were very much similar to those with crude enzyme (Fig. 4.44). Among the various organic solvents, the relative activity with respect to control (without solvent) was 82.83% in hexane, 80.94% in ethanol, 72.41% in isopropanol. The enzyme activity was inhibited to a greater extent by phenol and benzene. Direct binding of solvents to the specific binding sites of enzyme could be the contributing factor that determines modulation in the activity of protein/enzyme (Liepinsh and Otting, 1997). The water miscible solvents methanol, 2- propanol, n-butanol inhibited the enzyme activity where as ethanol stimulated it up to 10% at 3% (v/v) concentration Aprotic polar solvent acetone inhibited the enzyme activity to some extent, where as 1,4-dioxane severely inhibited it when enzyme was incubated for 15 min with 3 and 7% (v/v) of solvents. Water immiscible solvents, such as phenol severely inhibited the PVA activity. Hexane stimulated PVA activity at 3 and 7% (v/v) (Kumar *et al.*, 2008).



*Corresponds to (224.8±0.08U/mg)

Fig.4.44 Effect of various organic solvents on purified penicillin acylase from *Acremonium sclerotigenum*

4.6.7 Optimization of substrate concentration and determination of K_m and V_{max} of purified penicillin acylase from *Acremonium sclerotigenum*

The optimum substrate concentration for purified penicillin acylase was recorded at 50mM (Fig. 4.45) and the highest activity observed was 290.55U/mg ± 0.06, with further increase in substrate concentration the enzyme activity became almost constant because of saturation of enzyme active site with the substrate. Lineweaver-Burk plot between 1/V and 1/[S] was used for the calculation of K_m and V_{max} values which were found to be 13.32 mM and 333.33 µmol mg^{-1} min^{-1} respectively (Fig 4.46). Wen *et al*. (2004) reported the K_m and V_{max}, 17.6 µM and 23.8 U/mg penicillin G, respectively from His-tagged penicillin G acylase from *Kluyvera citrophila* in *Escherichia coli*. V_{max} and K_m values of the penicillin G acylase of *Escherichia coli* ATCC 11105 enzyme (specific activity: 24·81 Umg^{-1}, protein concentration0.56 mg cm^{-3}) were found to be 22.73 U cm^{-3} mm^{-1} and 3.18 mM m^{-3} penicillin G, respectively (Erarslan *et al*., 2007). Penicillin acylase (the purified protein) from *Bacillus subtilis* exhibited measurable enzymatic activity with 5µM

PenV (Rathinaswamy *et al.*, 2012). Kumar *et al*. (2008) described penicillin V as best substrate (75 mM) for purified penicillin V acylase from yeast, *Rhodotorula aurantiaca.*

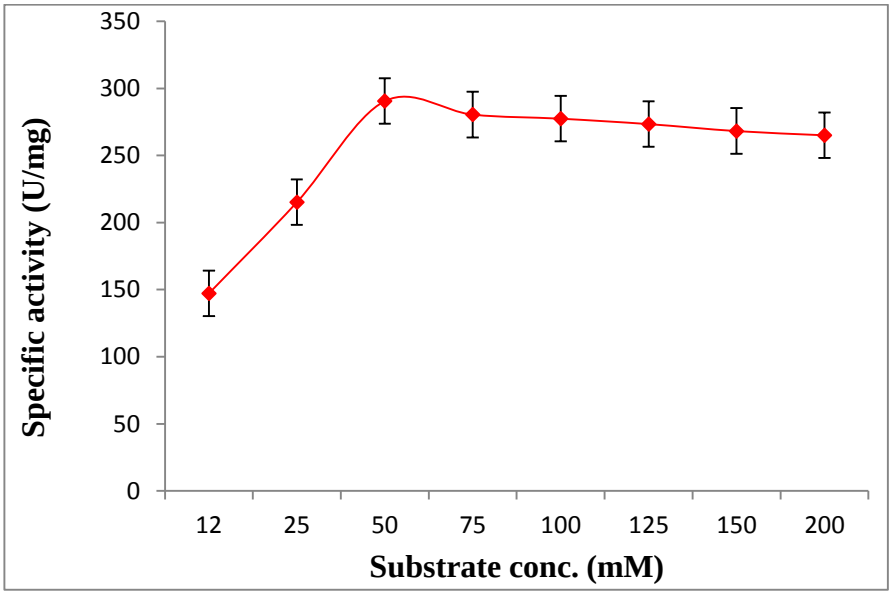


Fig.4.45 Effect of substrate concentration on purified penicillin acylase of *Acremonium sclerotigenum*

Kim *et al.* (2004) reported optimum activity of purified poly-his tagged penicillin G acylase expressed in *E. coli* with 15mM penicillin G as substrate. Rathinaswamy *et al.* (2012) reported K_m of the enzyme using PenV as substrate was 40±1 mM and the V_{max} was 4.8 ±0.2 µmol mg^{-1} min^{-1}.Michaelis Menten constant for purified penicillin V acylase was calculated from Lineweaver- Burk plot and found to be 20 mM (Kumar *et al.*, 2008).

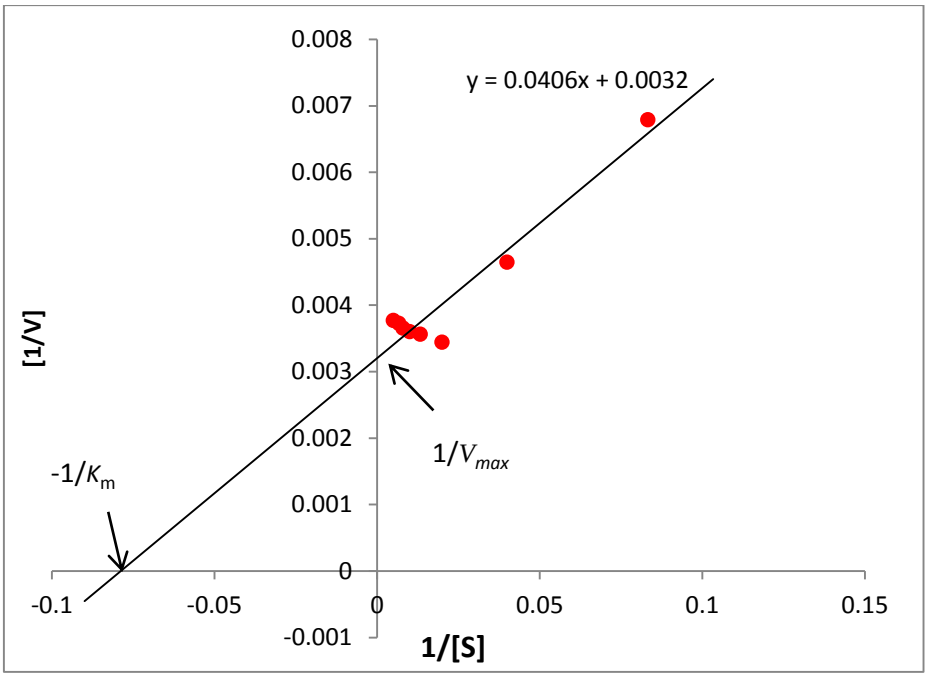


Fig.4.46 Determination of K_m and V_{max} of purified penicillin acylase from *Acremonium sclerotigenum*

4.6.8 Effect of enzyme dose

The enzyme reaction was carried out at different concentrations of purified penicillin acylase in the reaction mixture ranging from 0.073 µg to 9.44 µg and maximum activity (395.40 U/mg ±0.06) was recorded in the presence of 0.6 µg of enzyme (Fig. 4.47). Koreishi *et al*. (2007) used 1.5 µg of purified aminoacylase from *Streptomyces mobaraensis.* Kumar *et al*. (2008) reported 50 µg enzyme concentration for the assay of purified penicillin V acylase from yeast, *Rhodotorula aurantiaca.*

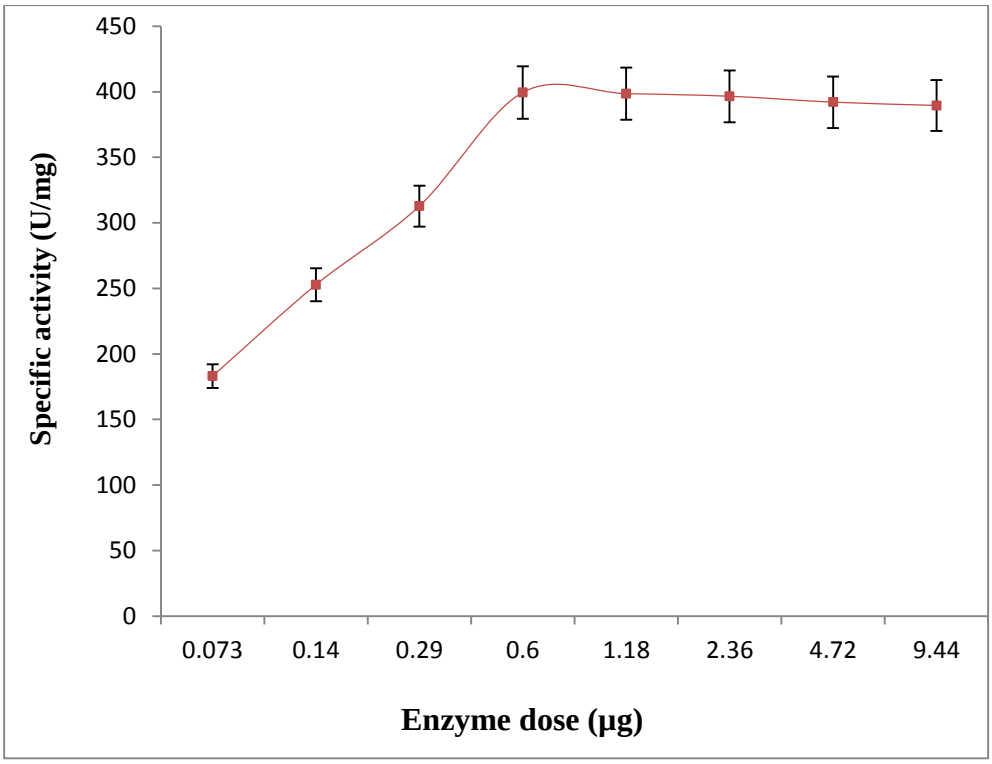


Fig.4.47 Effect of enzyme dose on purified penicillin acylase from *Acremonium sclerotigenum*

4.6.9 Storage stability of purified penicillin acylase

Shelf-life of purified penicillin acylase from *Acremonium sclerotigenum* was studied at 4°C and room temperature (Fig. 4.48). The enzyme was fairly stable at 4°C and retained 65.8% of residual activity even after 240 hrs. Whereas at room temperature, enzyme activity of purified penicillin acylase gradually started decreasing and half-life of enzyme was approximately 192 hrs at room temperature (RT). The His-tagged penicillin G acylase from *Kluyvera citrophila* in *Escherichia coli* can be stored at 4°C for 10 days without significant change of activity. The enzyme retains 76% of its activity after storage at room temperature for 10 days (Wen *et al*., 2004).

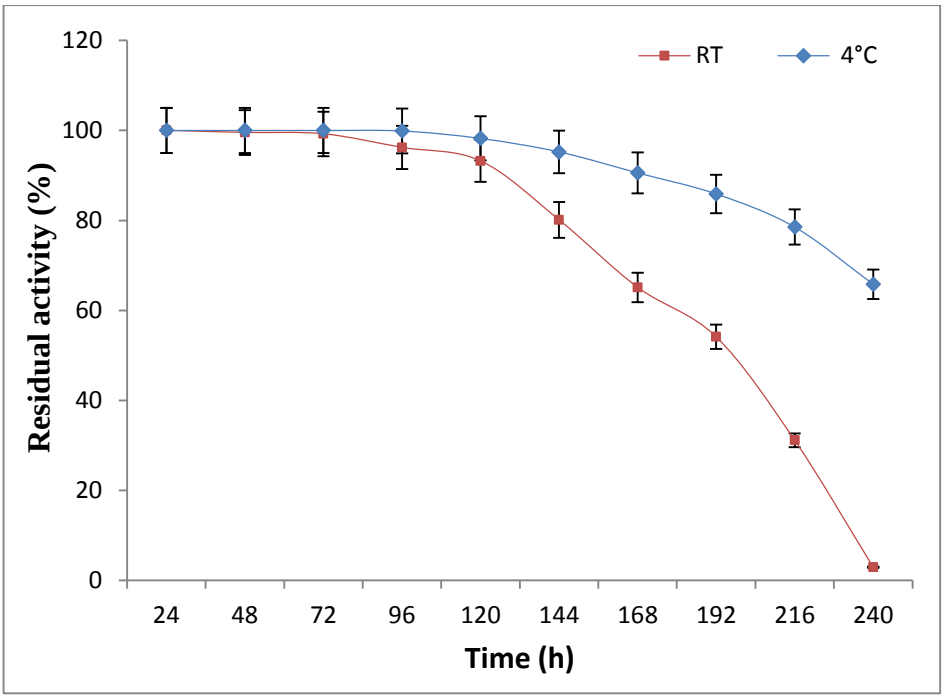


Fig.4.48 Storage stability of purified penicillin acylase from *Acremonium sclerotigenum*

4.7 Bioprocess development for conversion of penicillin-G to 6-APA using penicillin acylase from *Acremonium sclerotigenum*

4.7.1 Effect of substrate concentration using free and immobilized enzyme for conversion of penicillin G to 6-APA

Various concentrations of substrate (penicillin G) were tested in different concentration to evaluate the effects of substrate concentration by keeping the enzyme concentration (7.49 µg) for free and (27µg) for immobilized enzyme constant on conversion rate. In all combinations the conversion rate was slow but as the concentration increased up to 50 mM, the amount of product concentration also increased with all concentrations of penicillin G up to 50mM, after that there was no significant increase observed as shown in Fig. 4.49. Maximum amount of 6-APA (40.75 mM) was formed after 30 min. of reaction at 40°C using free penicillin acylase. In case of immobilized enzyme the reaction rate was initially slow might be due to limited diffusion in matrix, The amount of product concentration was higher in alginate entrapped enzyme as compared to when reaction was performed for 30 minutes in 1.5 ml of reaction mixture with free enzyme. Maximum amount of 6-APA (42.45mM) (Fig.4.50) was formed after 50 minutes of reaction at 50°C.

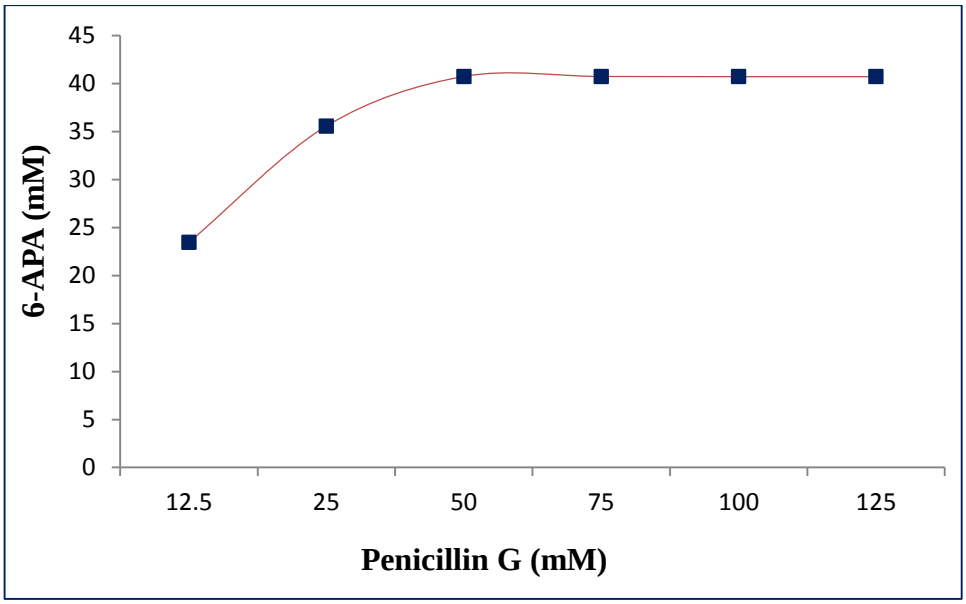


Fig.4.49 Effect of substrate concentration on conversion rate using free penicillin acylase from *Acremonium sclerotigenum*

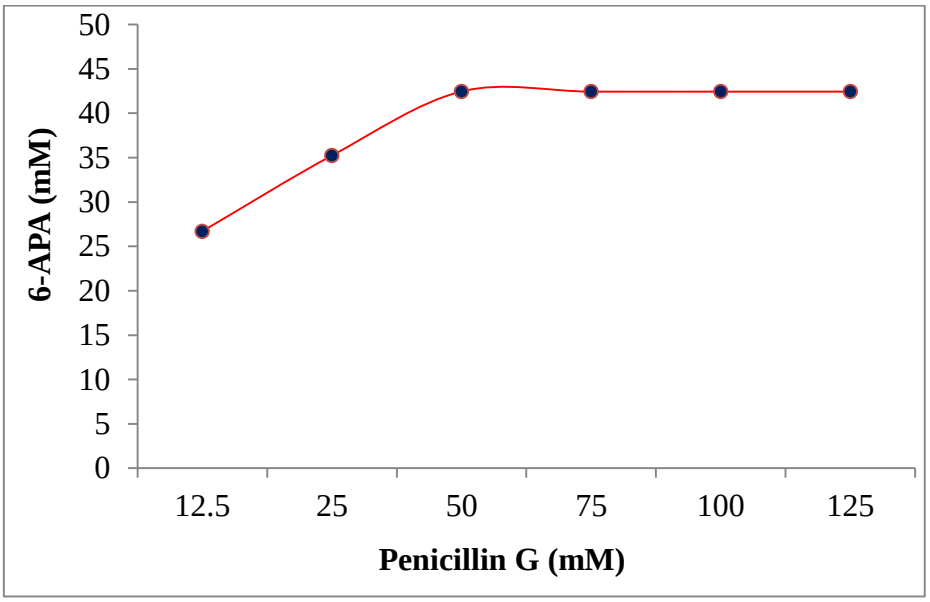


Fig.4.50 Effect of substrate concentration on conversion rate using alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.7.2 Effect of enzyme dose on conversion rate using free and immobilized penicillin acylase

To check the effect of enzyme concentration for maximum conversion of substrate to product, assay was performed by varying the concentration of biocatalyst amount while keeping the concentration of substrate constant for both free and alginate immobilized enzyme (Fig.4.51). Free enzyme at a concentration of 7.49 µg

showed highest conversion with 50mM penicillin acylase in 30 minutes. Whereas calcium alginate beads entrapped 27 µg of enzyme concentration showed highest conversion (Fig.4.52) with 50mM penicillin acylase in 50 min. No significant increase was observed with additional enzyme concentration may be due to mass transfer limitations or due to lower substrate-to-biocatalyst ratio in immobilized enzyme.

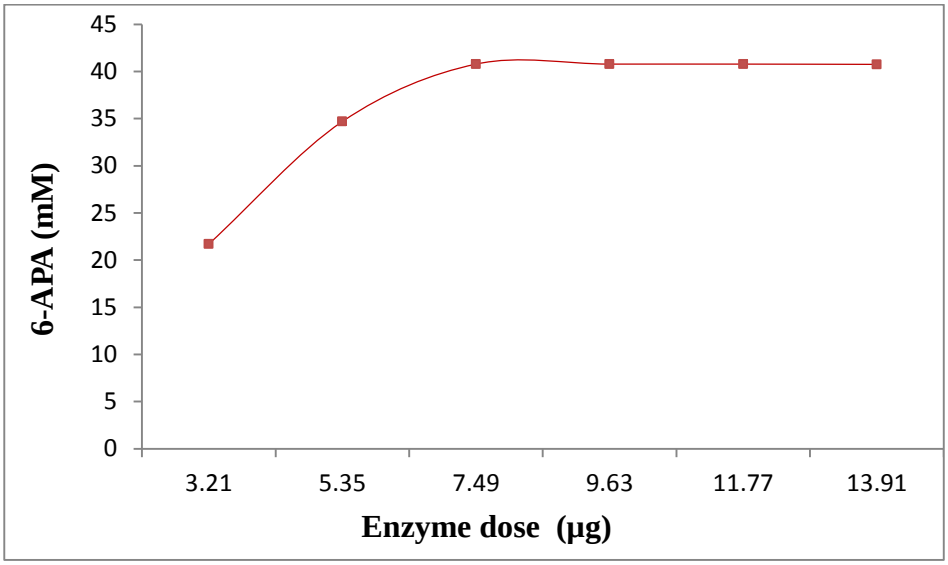


Fig.4.51 Effect of enzyme dose on conversion rate using free penicillin acylase from *Acremonium sclerotigenum*

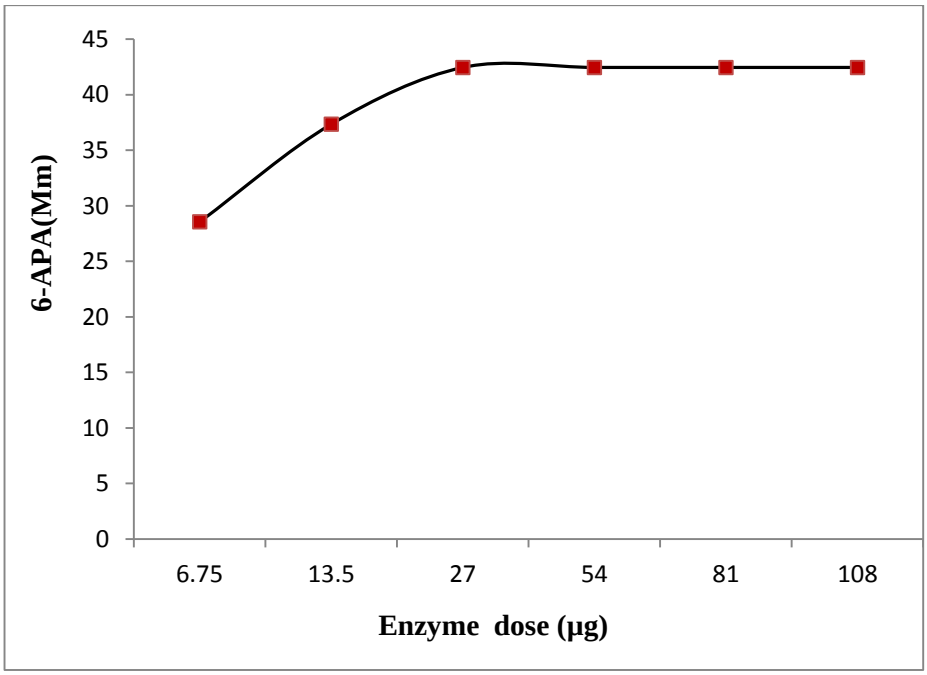


Fig.4.52 Effect of enzyme dose on conversion rate using alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.7.3 Time course of enzyme reaction for conversion of penicillin-G to 6-APA using free and immobilized penicillin acylase

To find out the best time and temperature combination for maximum conversion of substrates into product, time course of enzyme reaction at different temperatures (30˚C, 35˚C, 40˚C, 45˚C ,50˚C 55˚C and 60˚C) was investigated by terminating the reaction at different intervals of time. Maximum amount of 6-APA (40.75 mM) was formed after 30 minutes of reaction at 40˚C (Fig. 4.53). Whereas in case of immobilized enzyme maximum amount of 6-APA (42.45 mM) was formed after 50 minutes of reaction at 50˚C (Fig.4.55). In previous studies Bhattacharjee *et al.* (1996) observed 40-45 mole percent conversion to 6-APA after 60 minutes. Whereas Cao *et al.* (2004) reported that 0.9-0.99 mol of 6-aminopenicillanic acid (6-APA) per mol of penicillin G was produced by penicillin acylase from *E.coli.*

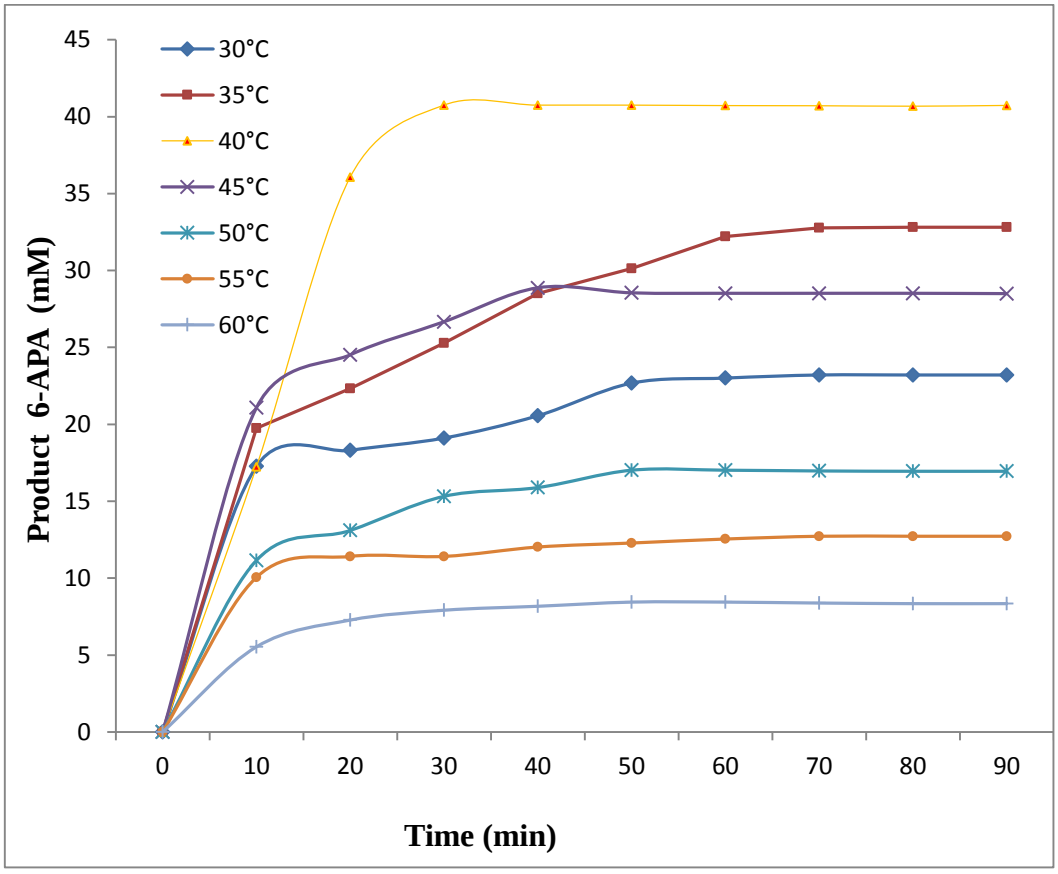


Fig.4.53 Time course of enzyme reaction at different temperatures using free penicillin acylase from *Acremonium sclerotigenum*

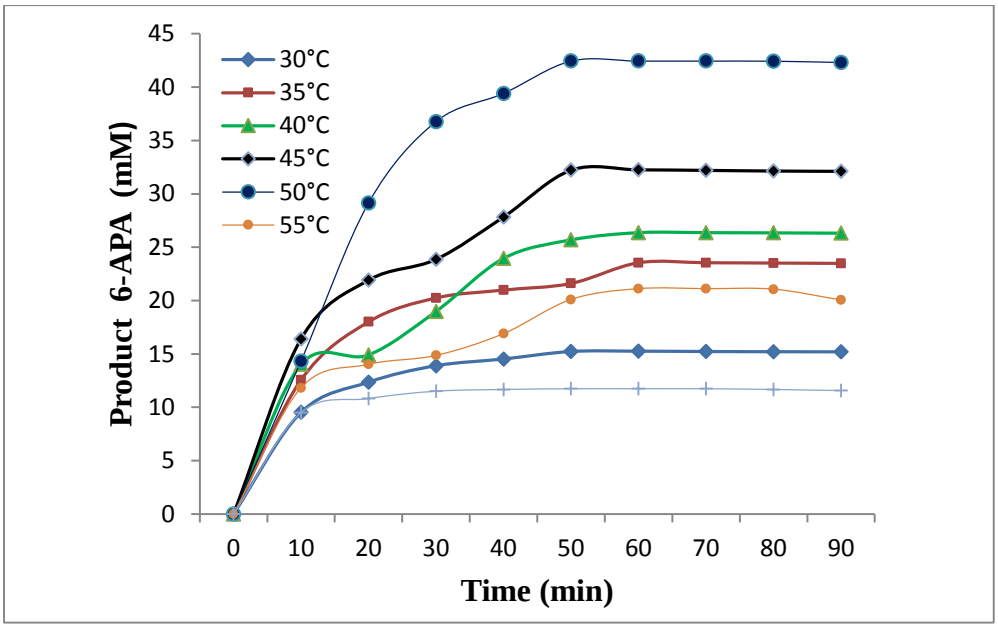


Fig.4.54 Time course of enzyme reaction at different temperatures using alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.7.4 Batch reaction at 50 ml scale using free and alginate gel entrapped penicillin acylase

The HPLC study of batch reaction showed that total amount of 6-aminopenicillanic acid produced was 42.10 mM using free penicillin acylase and 44.50 mM using alginate immobilized penicillin acylase in batch reaction at 50 ml scale. HPLC chromatogram showed peak of penicillin G at retention time (3.922) (Fig.4.55) along with the peak of 6- aminopenicillanic acid standard and synthesized sample was (Fig. 4.56) & (Fig. 4.57) at retention time 3.287 and 3.232 min respectively.

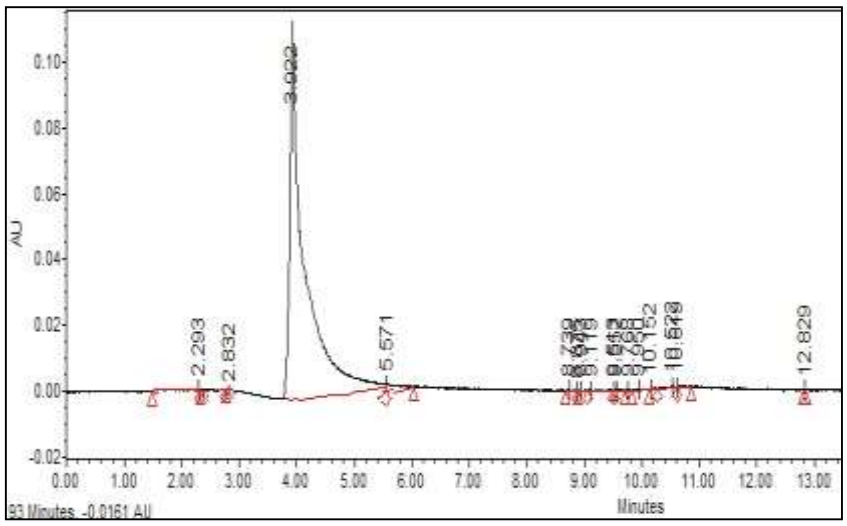


Fig.4.55 HPLC chromatogram of standard penicillin G (RT-3.922 min.)

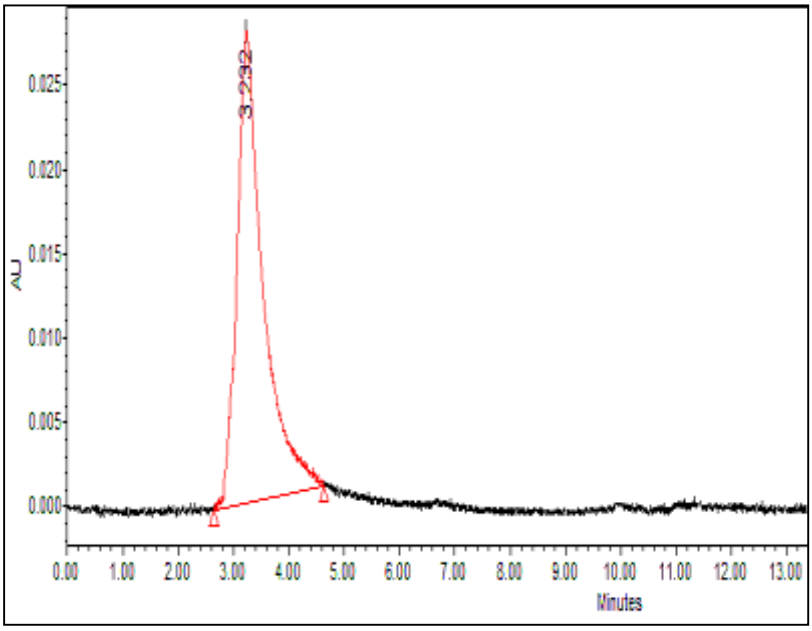


Fig. 4.56 HPLC chromatogram of synthesized 6 -APA (RT-3.232 min.)

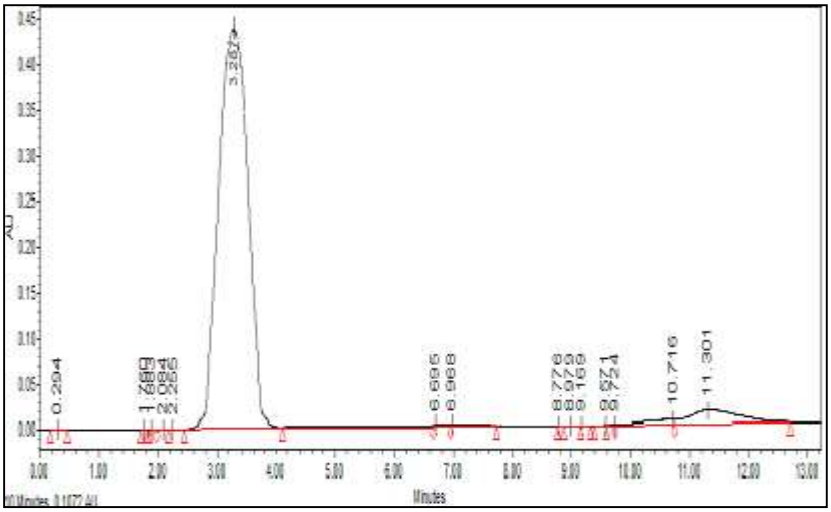


Fig.4.57 HPLC chromatogram of standard 6-APA (RT-3.287 min)

4.7.5 Fed batch reaction at 50 ml scale using free and alginate immobilized penicillin acylase

In order to increase the molar conversion yield, a study was also conducted by step feeding of substrate even after 30 minutes in case of free enzyme and after 50 minutes for immobilized enzyme. In order to keep the reaction moving in the forward direction. After 30 min, (i.e. before feeding of substrates) 42.35 mM 6-APA was formed from 50 mM penicillin acylase using free enzyme (84.70% conversion) (Fig.4.58) Whereas in case of immobilized enzyme 44.49 mM 6-APA was formed

from 50 mM penicillin acylase (88.98 % conversion) (Fig.4.59). The molar conversion yield did not increase after fed batch. So for bench scale (1L) synthesis of 6-APA batch reaction was undertaken.

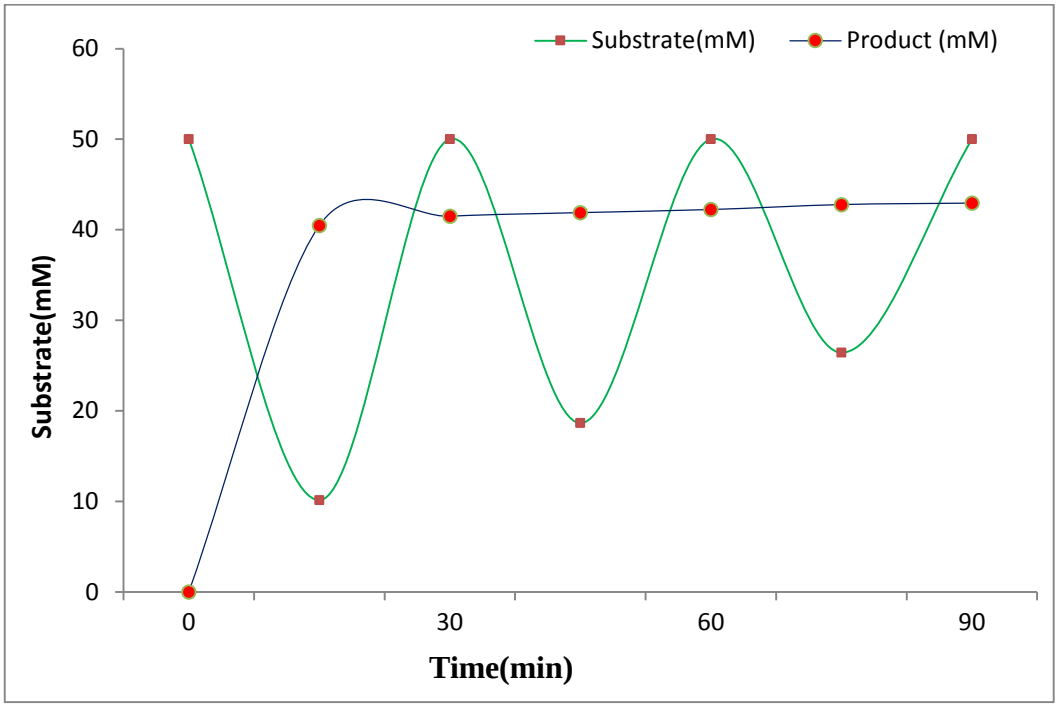


Fig.4.58 Fed batch reaction at 50 ml scale using free penicillin acylase from *Acremonium sclerotigenum*

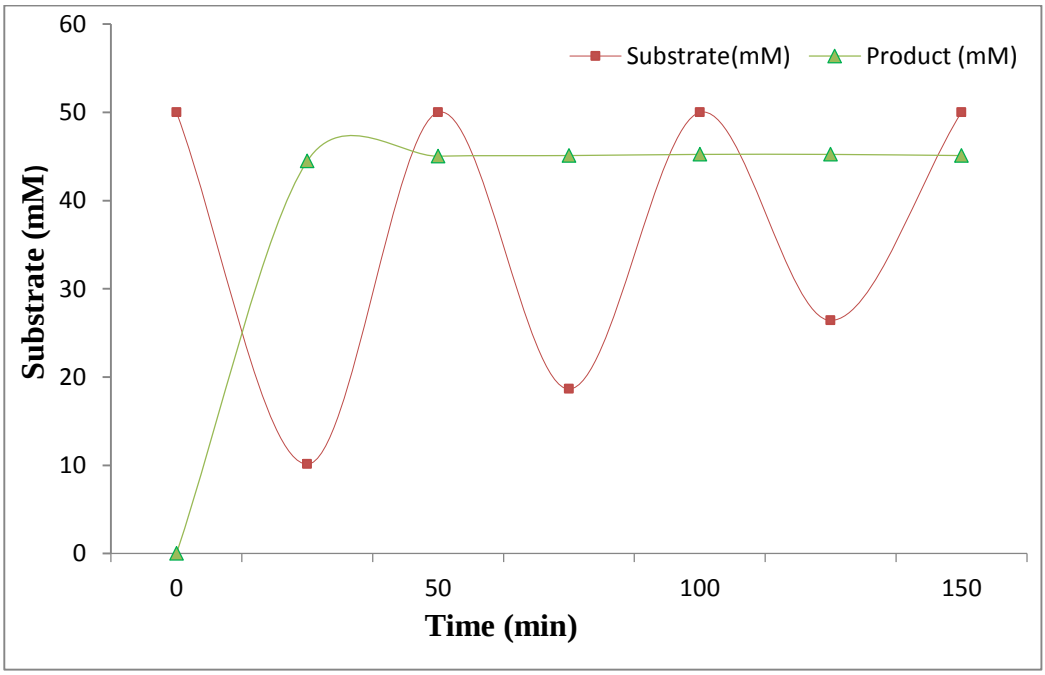


Fig.4.59 Fed batch reaction at 50 ml scale using alginate gel entrapped penicillin acylase from *Acremonium sclerotigenum*

4.7.6 Bench scale production of 6-APA

Batch reaction was carried out at 1L scale using free and alginate entrapped penicillin acylase. The temperature of the fermenter vessel was maintained at 30°C for 90 minutes for free enzyme and at 50°C for 150 minutes for immobilized enzyme with impeller speed of 200 rpm. At the end of the reaction 43.92 mM 6-APA (87.84% molar conversion yield) was obtained at 150 minutes (immobilized enzyme). The percentage purity of the sample was 54.33% and the productivity was 17.41mg/L/h Samples were withdrawn at regular interval of time. Banerjee and Debnath (2007) reported productivity of 18.79 mg L^{-1} h^{-1} of 6-APA from agrarose immobilized penicillin acylase of *E.coli*. Jiang *et al*. (2007) reported 6-APA productivity of 80% at 42°C, pH 5.5.Whereas Hernandez and Fernandez (2011) recorded the highest quantity of 6-APA obtained was 226.16 mg/l after 40-minutes reaction. In contrast to present study Avinash *et al.* (2017) reported 100% conversion of 4% penicillin V to 6-APA by penicillin V acylase of *Pectobacterium atrosepticum* expressed in *E. coli*. Xue-jun *et al*. (2014) described the bioconversion of penicillin G in PEG 20000/dextran T 70 aqueous two-phase systems using the recombinant *Escherichia coli* A56 (ppA22) with an intracellular penicillin acylase as catalyst. The best conversion conditions were attained for: 7% (w/v) substrate (penicillin G), enzyme activity in bottom phase 52 U ml^{-1}, pH 7.8, temperature 37 °C, reaction time 40 min. Conversions ratios between 0.9-0.99 mol of 6-aminopenicillanic acid (6-APA) per mol of penicillin G were obtained and volumetric productivity was 3.6-4.6 μmol min^{-1} ml^{-1}. In addition the product 6-APA directly crystallized from the top phase with a purity of 96%.

4.7.7 Recovery of 6 APA

After completion of the reaction the beads were separated from the reaction mixture .The supernatant obtained was collected and was freeze-dried (Flexi-Dry MP TM, FTS USA) to recover the powder of 6-APA (Fig.4.60). Straw yellow coloured powder of 6-APA was recovered.

(Lyophilizer)

Fig.4.60 6-APA obtained after lyophilization

4.7.8 Characterization of 6 -APA

6-APA formed was lyophilized and characterized by UV -Vis, Fluorescence and FTIR spectroscopy.

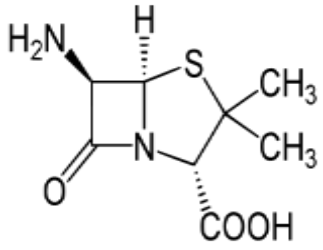


Fig. 4.61 6-Aminopenicillanic acid (Torres *et al*., 2000)

4. 7.9 UV-Visible and Fluorescence spectroscopy

The fluorescence and UV-Vis spectra were recorded respectively by using a Cary Eclipse Fluorescent spectrophotometer and Varian Cary 50 spectrophotometer with a quartz cuvette of 1 cm path length at room temperature. All the samples for UV and Fluorescence analysis were prepared by dissolving in distilled water and subjected to the UV-Visible spectroscopy analysis. The UV-Vis spectrum revealed a broad peak with λ_{max} 238nm for standard (Fig.4.62) and also for the synthesized product (Fig.4.63).The UV-Vis absorbance spectra of the standard and the synthesized product matches and thus confirms that the product is 6-APA.

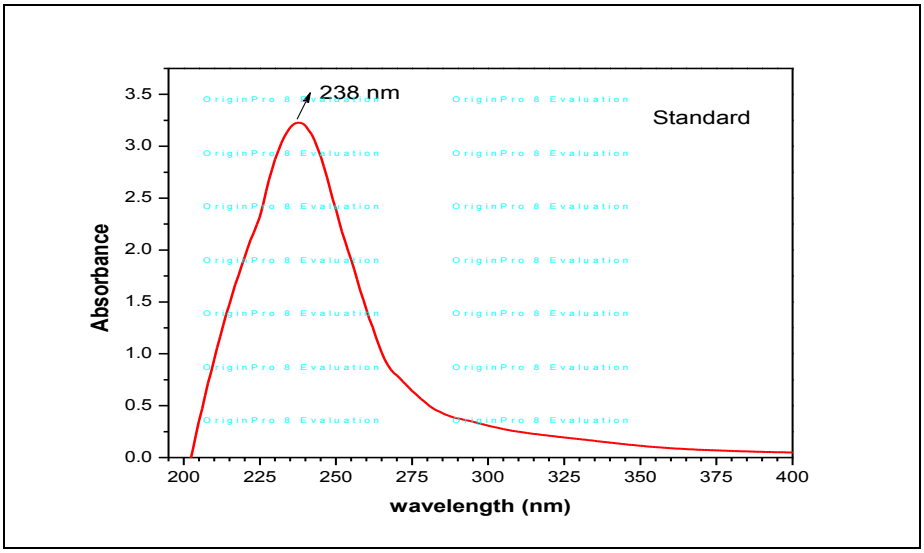


Fig.4.62 The UV-Vis spectrum of Standard 6-APA

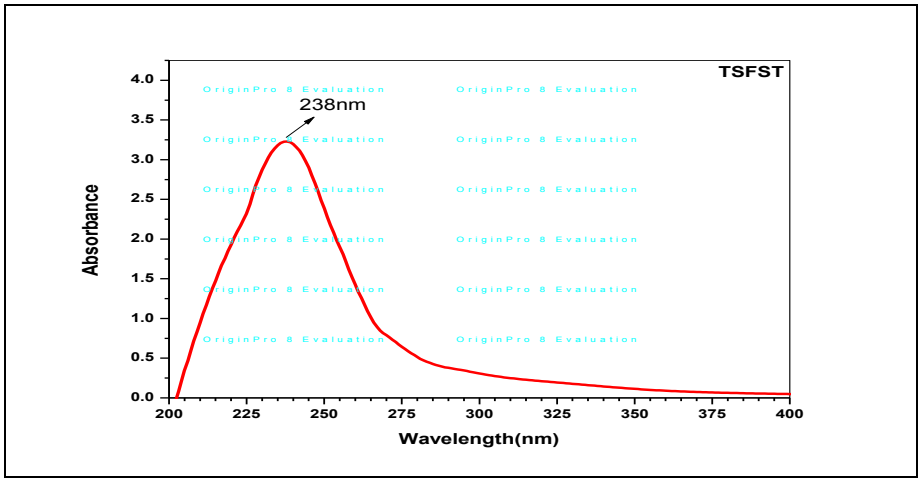


Fig.4.63 UV-Vis spectrum of synthesized 6-APA

4.7.10 The Fluorescence spectra

The fluorescence was recorded at the excitation wavelength of 390 nm and emission was observed at 425 nm for standard (Fig.4.64) and at 450 nm for the synthesized product (Fig.4.65). The fluorescence peak for standard was slightly broad however for the synthesized product the peak was almost sharp.

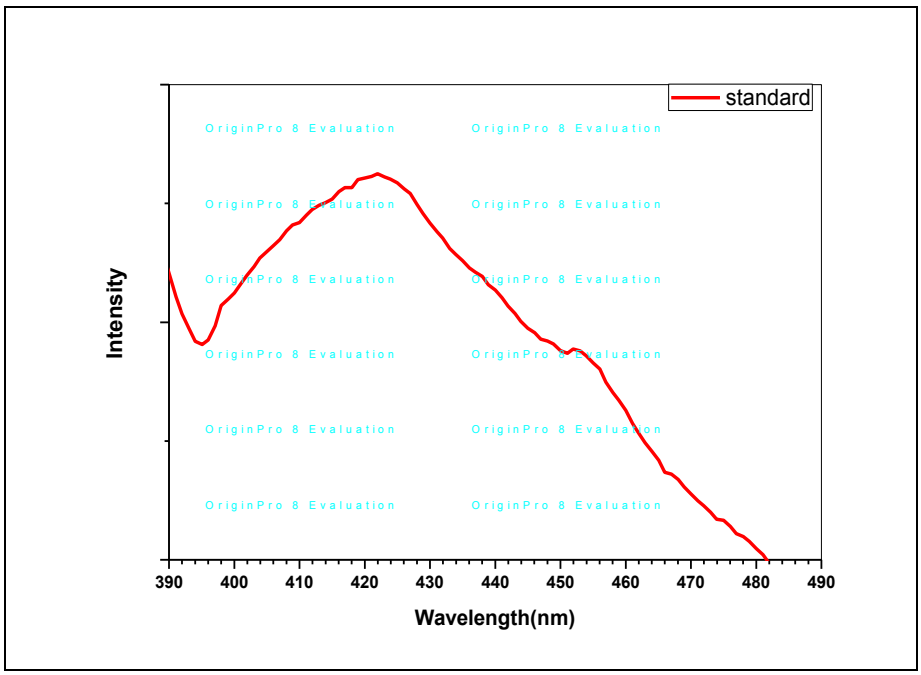


Fig.4.64 Fluorescence spectrum of the standard 6- APA

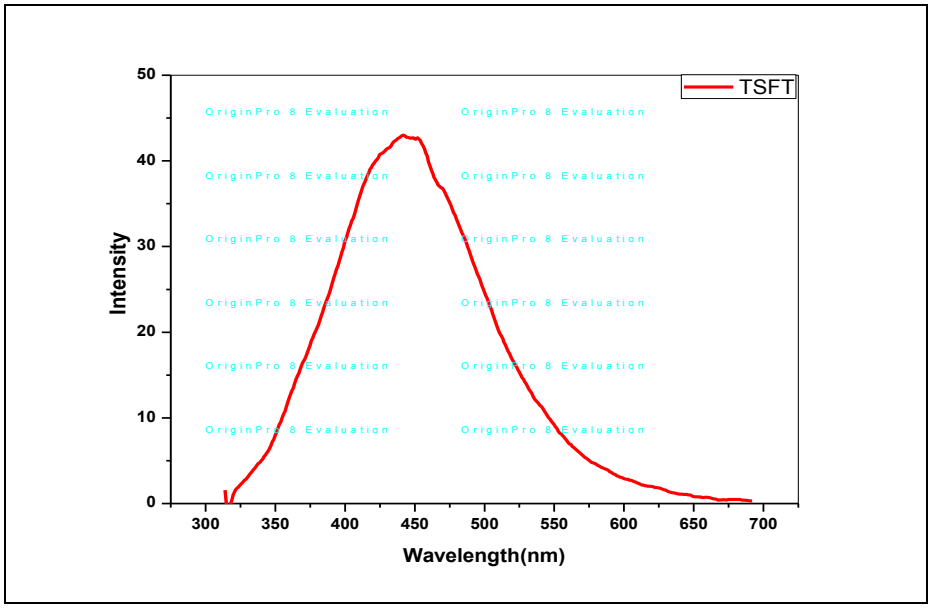


Fig.4.65 Fluorescence spectrum of synthesized 6- APA

4.7.11 Fourier transform infrared spectroscopy (FTIR)

The spectrum shows the peak for primary amine group at the 3470 cm-1 which is due to the presence of -NH2 group in the 6-APA. The medium intensity peak at 2942 was attributed to the asymmetric Sp3 -C-H stretch. The peak at 1706 cm-1 was because of -C=O (carbonyl) stretching) due to presence of C=O group in lactam ring. The peak at 1227 cm^{-1} was because of the C-O stretch and one at 1028 cm^{-1} was because of C-N stretching. The peak at 884 cm^{-1} was attributed to the $-NH_2$ wagging and the peak at 686 cm^{-1} was attributed to the C-S stretching vibrations (Fig.4.66). Thus these observations successfully confirms the functional groups present in the compound and which may be attributed to the 6-APA.

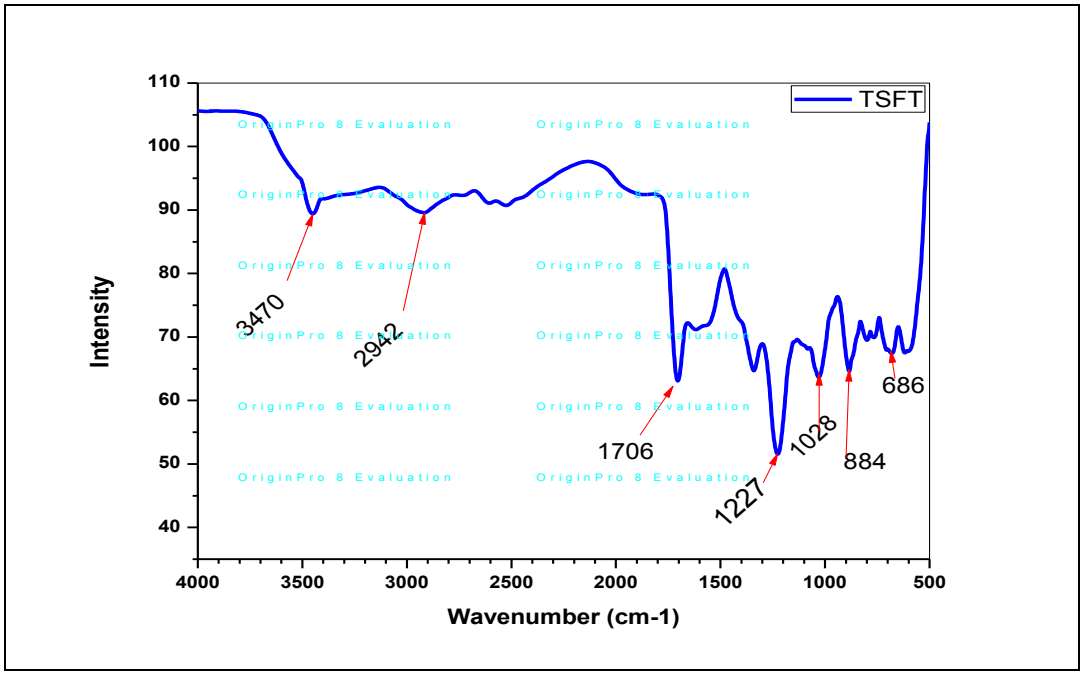


Fig. 4.66 FTIR spectrum of synthesized 6-APA

4.8 Dye decolourization by penicillin acylase and *Acremonium sclerotigenum*

Major classes of synthetic dyes include azo, anthroquinone and triaryl methane dyes, and many of them are toxic or contain carcinogenic compounds with long turnover times (Hartman *et al.*, 1978). Azo dyes are major synthetic dyes extensively used in various industries. A bulk of azo dye production is lost to domestic and industrial wastewater (Zollingre, 1987; Olliggard *et al.*, 1999). They become an

integral part of industrial wastewater due to their extensive use (Alhassani *et al.*, 2007). Inefficient dyeing processes, poor handling of dye effluent and insufficient treatment of dye wastes of industries lead to contamination of the soil and water bodies (Nigam *et al.*, 1996a). A conventional biological wastewater treatment process is not very efficient in treating a dye wastewater due to the low biodegradability of dyes. Physical or chemical-treatment processes, used for treatment of dyes do not offer a solution as they generate a significant amount of the sludge and cause secondary pollution due to the formation of toxic by products (Khehra *et al.*, 2005). The ability of microorganisms to carry out dye decolorization has received much attention as it is a cost effective method for removing dyes from the wastewater (Verma and Dutta, 2003; Moosvi *et al.*, 2005).

4.8.1 Screening of Dyes for decolourization by penicillin acylase and *Acremonium sclerotigenum*

Five different dyes (5mM) were initially screened to analyse the decolourization efficiency of penicillin acylase and *Acremonium sclerotigenum* (Table-4.5). Dye which showed highest decolourization (%) was selected for further studies. Out of these 5 different dyes methyl red showed decolourization (%) of 2.61% and 29.5% with penicillin acylase and *Acremonium sclerotigenum* respectively. Methyl red was selected as the dye of choice (Fig.4.67). Since the fungus proved more efficient in decolourization study when compared to penicillin acylase. *Acremonium sclerotigenum* was selected for decolourization study.

Table 4.5 Decolourization of various dyes with *Acremonium sclerotigenum* and penicillin acylase

Dye	**Decolourization by *Acremonium sclerotigenum (%)***	**Decolourization by Penicillin acylase (%)**
Reactive Blue 4	0	0
Remazol Brilliant Violet 5R	11.75±0.03	4.5±0.03
Methyl red	29.5±0.04	2.61±0.04
Indigo	8.25±0.03	5.05±0.02
Bismarck Y	4.2±0.01	2.05±0.03

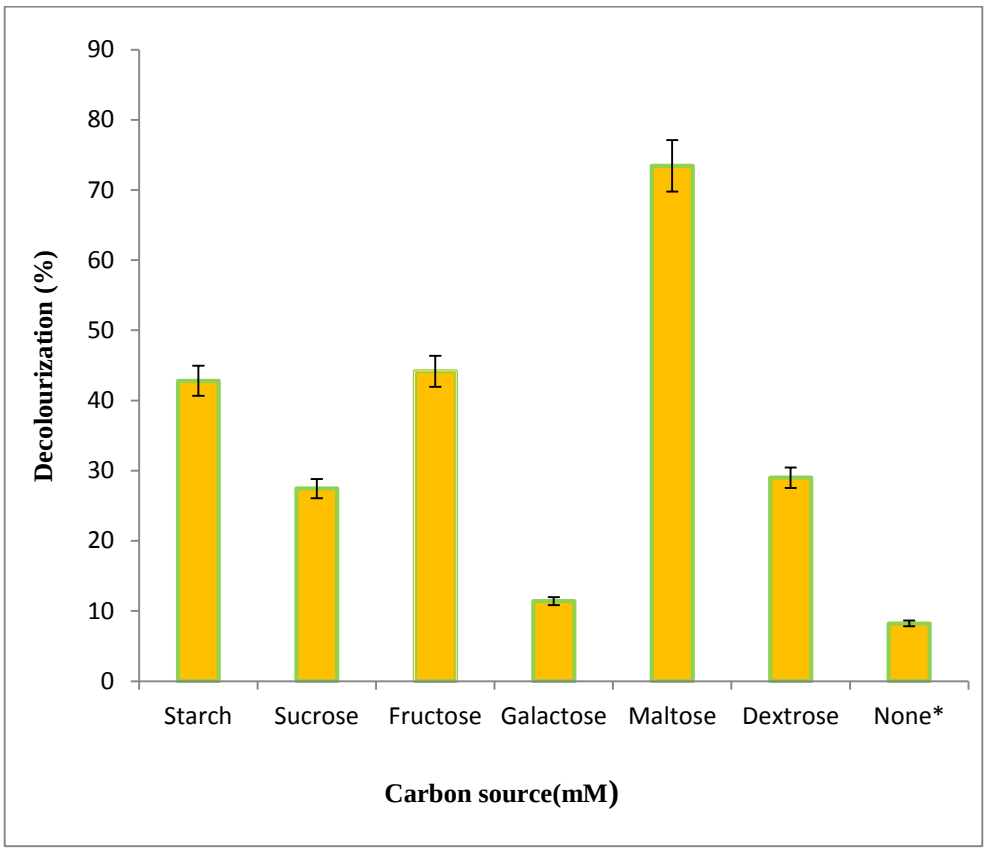
Fig.4.67 Dye decolourization of methyl red by *Acremonium sclerotigenum* (A) Control (B) Test

Effect of physicochemical parameters viz. composition of medium, pH of medium, temperature, concentration of spores, and concentration of dye, on methyl red decolourization focused the optimal conditions required for decolourization.

4. 8.2 Effect of carbon source

To determine the effect of different carbon sources on dye decolorization by *Acremonium sclerotigenum* six carbon sources (40mM) starch, sucrose, fructose, galactose maltose, and dextrose, were used and the highest decolourization was recorded when maltose was used as carbon source and decolourization recorded was 73.45±0.08 % (Fig.4.68). Similarly (Kumar and Bhat, 2012) reported that maximum decolourization of azo dye- Red 3BN by *P .chrysogenum, A. niger*, and *Cladosporium sp.* was observed when maltose was used at a concentration of (1%). Whereas (Verma *et al.*, 2015) in his study found that dextrose was most effective for RBBR dye decolorization by *Mucor hiemalis* MV04 (KR078215). Decolorization ranged between 80 to 100% in 8 days. The primary mechanism of decolorization may involve dye adsorption/degradation by mycelium of fungi as well as the reduction of dye intensity in solution because of changes caused by them (Kumar *et al.*, 2012). The rate of dyes removal can be linked with the available co-substrates and with the exponential growth phase (Namdhari *et al.*, 2012). However at higher concentrations the same carbon sources on metabolism produced organic acids, which in turn decreased the pH

of media. The fungi needs readily usable carbon source for their growth and production of secondary metabolites and extracellular enzymes (Verma *et al.*, 2015).



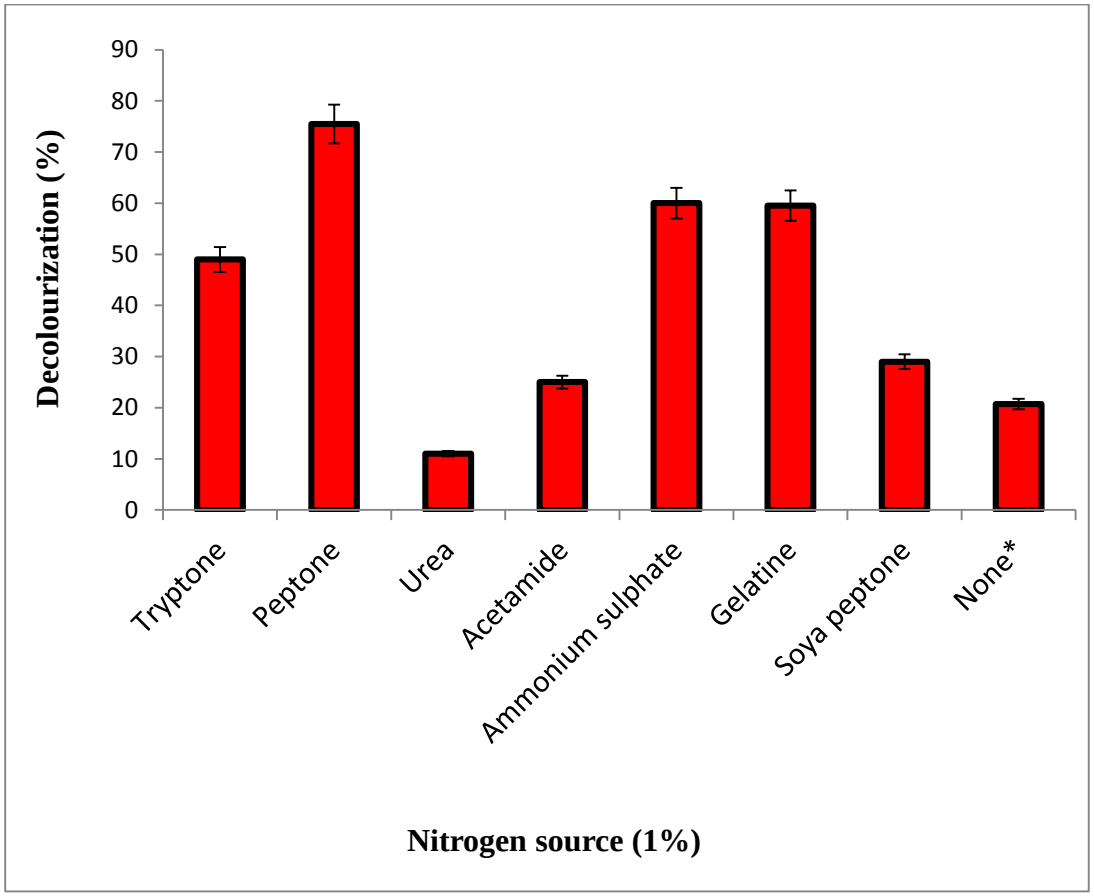
*Corresponds to 8.23 ± 0.01%

Fig. 4.68 Effect of different carbon sources on dye decolorization potential of *Acremonium sclerotigenum*

4.8.3 Effect of nitrogen source

Nitrogen source supplementation in medium plays a critical role in dye decolorization activity. The amount of nitrogen present in the media effects dye decolorization by altering the enzyme production by fungi. Nitrogen in the form of ammonium ions acts as a nutrient for the growth of fungal mycelium. The rate of decolourization of dye molecules depends on the rate of breaking of azo (-N,N-) bonds in the dye molecule (Senthilkumar *et al*., 2011). Seven different nitrogen sources (1%) were used and highest decolourization was recorded when peptone was used as nitrogen source and decolourization was 75.30±0.04% (Fig.4.69). This indicates that the rate of decolourization increased up to (Kumar *et al*., 2012) reported that peptone supported the decolourization of Red 3BN by *A. niger,* and *Cladosporium* sp. (90%). In contrast Singh *et al.* (2014) found the range of

decolorization of Brown GR with ammonium chloride, ammonium sulphate, ammonium nitrate, yeast extract and peptone, 72%, 64%, 68%, 68 % and 76% respectively with *Aspergillus* sp. For several fungal species the ligninolytic enzyme activity is suppressed rather than stimulated by high nitrogen concentrations (20-100mM) (Verma *et al.*, 2015).



*Corresponds to 20.70 ±0.04 %

Fig. 4.69 Effect of nitrogen source on dye decolourization potential of *Acremonium sclerotigenum*

4. 8.4 Effect of temperature

The incubation temperature affected the growth and decolourization potential of the *Acremonium sclerotigenum.* To study the effect of varying temperature on dye decolorization efficiency of the fungus, the medium pH was adjusted to initial optimum pH -6 and incubated at 25, 30, 35, 45 and 50°C for 2 days. It was found that with an increase in temperature from 25 to 30°C decolorization rate increased and

then a further increase in temperature to 45°C drastically affected dye decolorization potential of fungal isolate. Maximum decolorization of methyl red (76.45 ± 0.06%) was observed at 30°C (Fig.4.70). Suppressed decolourization at 45°C might be due to the loss of viability or deactivation of the enzymes responsible for decolourization at higher temperature (Saratale *et al*., 2011).

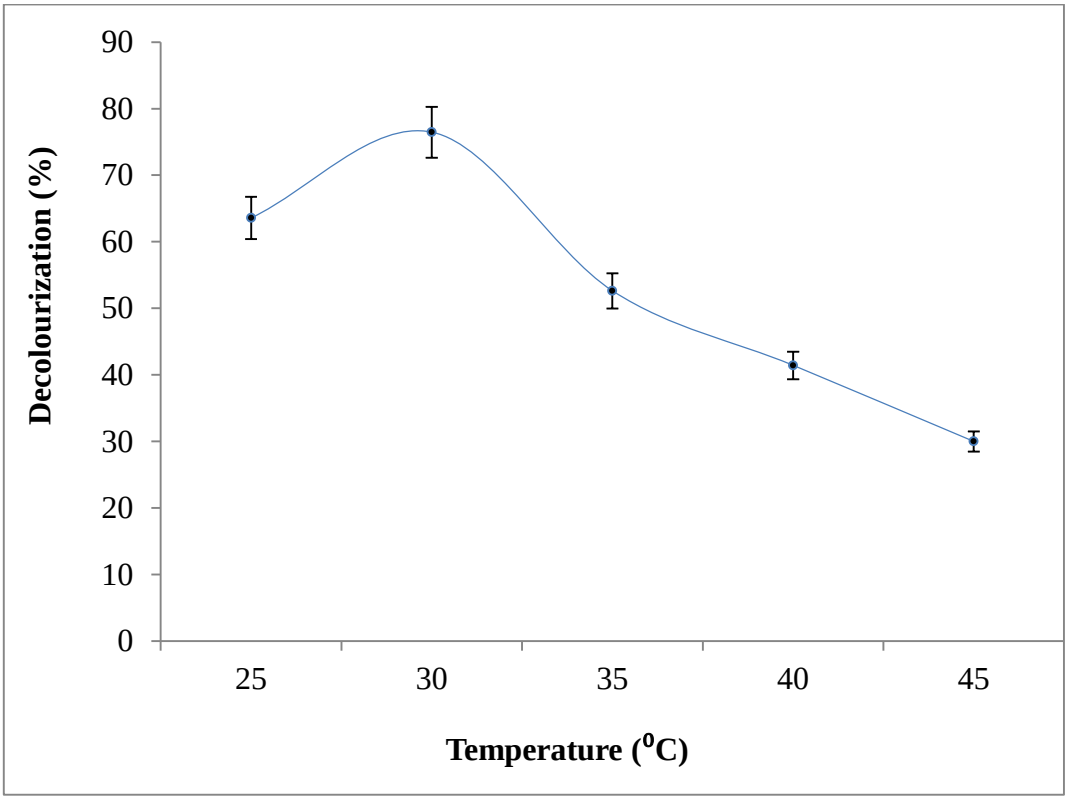


Fig.4.70 Effect of incubation temperature on dye decolourization potential of *Acremonium sclerotigenum*

4. 8.5 Effect of medium pH

Fungus has a strong capability to grow on wide range of pH. It affects not only the biosorption capacity but also the colour of the dye solution and the solubility of some dyes. Therefore pH is an important factor in colour removal (Fu and Viraraghavan, 2000). The pH range of the medium varied from 3.0 to 9.0 and highest dye decolourization was observed with pH 6.0.The decolourization (%) was 76.58±0.06 % (Fig 4.71), the percentage of decolourization declined as pH was altered. Fu and Viraraghavan (1999) and Fu and Viraraghavan (2000) reported that initial pH of the dye solution significantly influenced the chemistry of both dye

molecules and fungal biomass. They found the effective initial pH for the decolourization of Basic blue 9 and Acid blue 29 was 6.0 and 4.0 respectively.

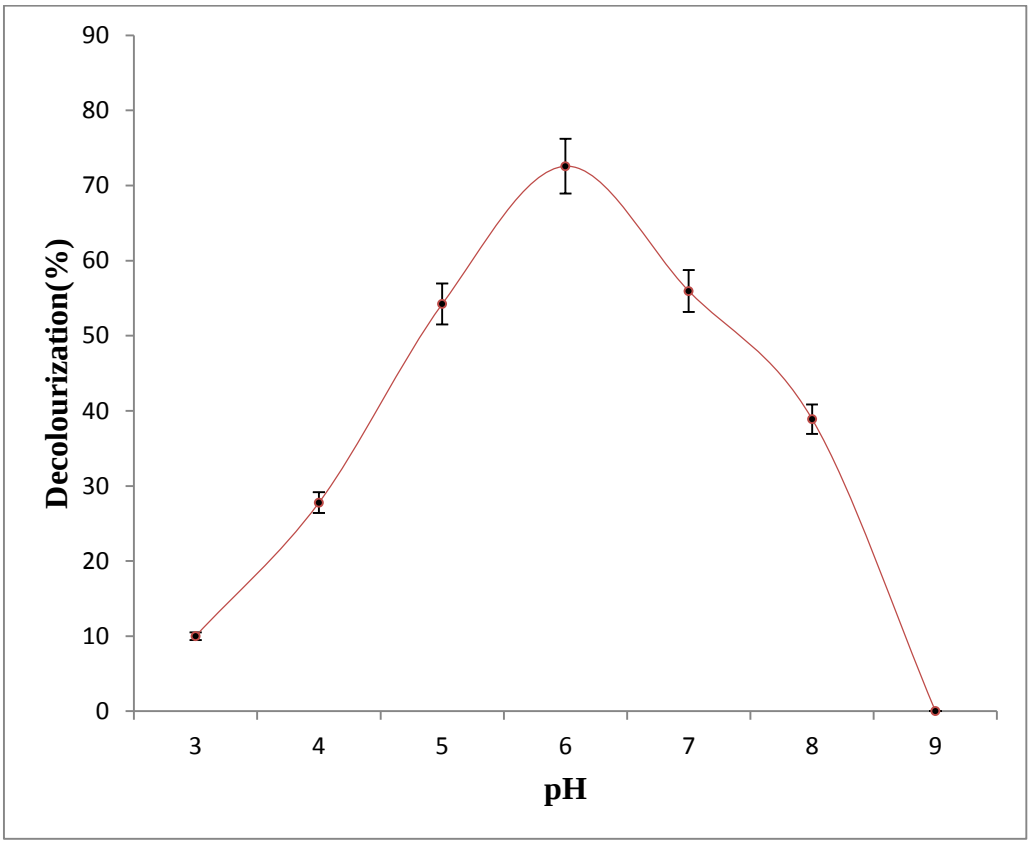


Fig 4.71 Effect of medium pH on dye decolourization potential of *Acremonium sclerotigenum*

4.9.6 Effect of inoculum density

Inoculum density was varied in the medium and highest decolourization of the dye was recorded with an inoculum density of 9.5×10^5 spores/ml and decolourization % was 79.66 ± 0.03% (Fig.4.72). Influence of the inoculum size on decolorization of the Red 3BN by *P.chrysogenum*, *A. niger* and *Cladosporium sp.* was studied by Kumar *et al.* (2012). *A.niger and P.chrysogenum* were found equally effective in the decolorizing Red 3BN and showed more than 95% decolorization of the dye. The ideal volume of inoculum was found to be 2% for *P.chrysogenum* and 10% for *A.niger.*

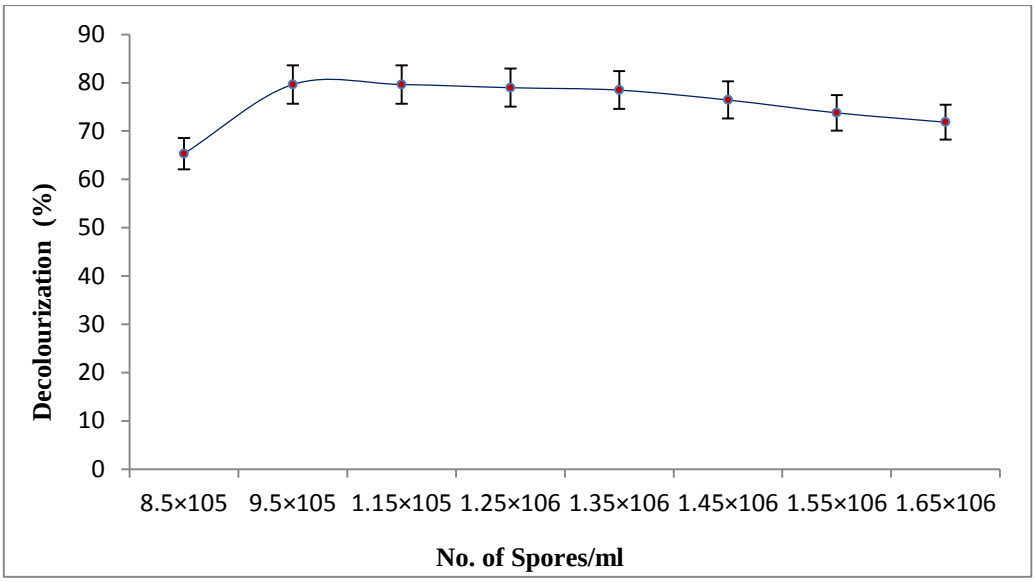


Fig.4.72 Effect of Inoculum density on dye decolourization potential of *Acremonium sclerotigenum*

4.8.7 Decolourization profile of methyl red using *Acremonium sclerotigenum*

The dye decolourization profile was studied for 120 h. Samples were taken at the interval of 24 h and the highest dye decolourization was observed after 72 h (83.65±0.02%) (Fig.4.73). In contrast to our results Kumar *et al.* (2012) reported the time course of decolorization of red 3BN under optimum conditions by *P.chrysogenum*, *A.niger* and *Cladosporium* sp. and observed that both *P.chrysogenum* and *A.niger* were capable of executing nearly 100% decolorization of red 3BN under their respective optimal conditions while *Cladosporium sp.* exhibited slightly lower level of decolorization activity after 6 days. The difference may be due to the structure of the dye and growth characteristics of the organism.

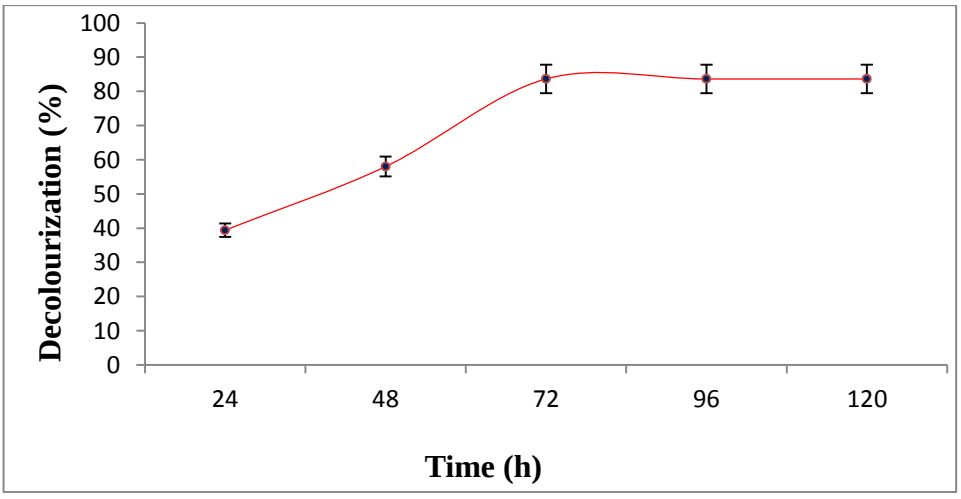


Fig 4.73 Decolourization profile of methyl red using *Acremonium sclerotigenum*

4.8.8 Effect of dye concentrations

Concentration of dye influences the decolourization rate. Dye Concentration was varied from 1.25mM-25 mM and highest decolourization (89.85 ± 0.06 %) (Fig. 4.74) was observed when methyl red was used at a concentration of 10 mM. The rate of decolourization declined with increased concentration of methyl red. The Increased dye concentration had an adverse effect on dye decolorizing efficiency of the fungi. Also, the class of the dye which defines its structure also influences the dye degradation (Banat *et al*., 1996; Robinson *et al.*, 2001; Kadapn *et al*., 2000). This might be attributed to the toxicity of dye to bacterial cells through the inhibition of metabolic activity, saturation of the cells with dye products, inactivation of transport system by the dye or the blockage of active site of azo reductase enzymes by the dye molecules (Mabrouk and Yusuf, 2008). For industrial applications, the microorganisms should be able to bear high concentrations of the dye since the dye concentration in a typical industrial effluent can vary between 10 and 50 mg (Chitra *et al.*, 2013; Basak *et al.*, 2013).

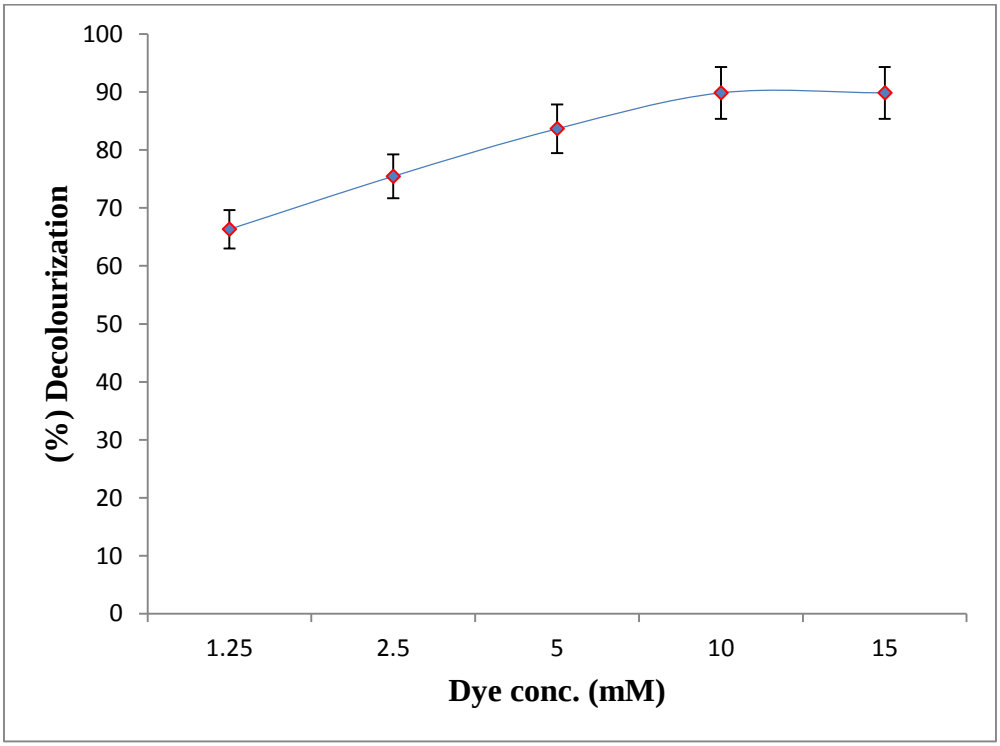


Fig.4.74 Effect of dye concentration on dye decolourization potential of *Acremonium sclerotigenum*

4.8.9 UV- visible spectrophotometric analysis

After the optimization of various parameters, degradation of dye by fungus *Acremonium sclerotigenum* was further confirmed by UV-visible spectroscopy (Fig.4.75).The spectrum of the dye degradation by fungus was plotted in shimadzu UV- Spectrophotometer (λ_{max} 472nm). Decrease in absorbance of test in comparison to control confirms the decolourization of methyl red.

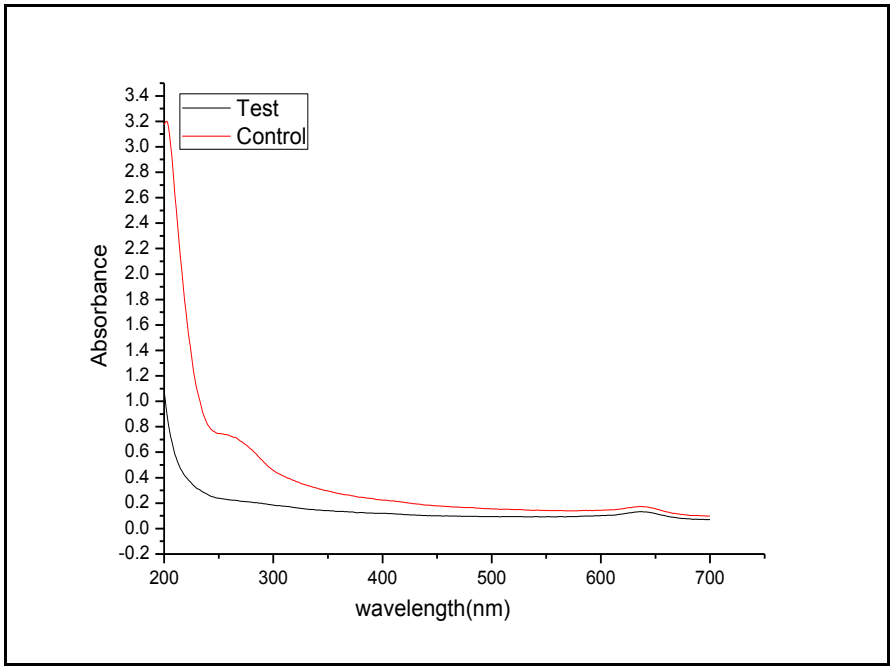


Fig. 4.75 UV-visible spectrum of methyl red after decolourization

Fungal isolate PAF-4 has been isolated from the soil of Himachal Pradesh and was identified at National Fungal Culture Collection, Agharkar Institute Pune, India as *Acremonium sclerotigenum*- 3406. Since the large amount of this enzyme has been needed for industrial applications, microbial sources are found to be best for the bulk production of penicillin acylase in economic way. The present work explores the potential of penicillin acylase from *Acremonium sclerotigenum* producing extracellular penicillin acylase. Various physicochemical parameters were optimized to maximize the production of penicillin acylase the enzyme has been purified characterized and immobilized on various matrices. The application of immobilized enzyme has been evaluated for bench scale production of 6-APA. The major findings of the research work has been summarized below:

5.1 Isolation and screening of penicillin acylase producing microorganisms

The soil samples from different areas of Himachal Pradesh were screened for the penicillin acylase producing microorganisms. Out of total 15 positive microbial isolates, fungal isolate PAF-4 showed highest enzyme activity and was used for further studies. The isolate was identified as *Acremonium sclerotigenum* at National Fungal Culture Collection, Agharkar Institute Pune, India.

5.2 Optimization of culture conditions for penicillin acylase production from *Acremonium sclerotigenum*

Various physico-chemical parameters *viz.* selection and optimization of production medium, pH, incubation temperature, inoculum size, and incubation time were optimized for the optimum growth and production of penicillin acylase from *Acremonium sclerotigenum.* The fungal isolate was grown on fifteen different media. The results suggested that the production medium containing (g/l) glucose (40mM), soya peptone (1.4%), yeast extract (1.6%) and beef extract (0.8%), inoculum size of 1.25×10^6 spores/ml, pH-6.0 was most suitable for the production of penicillin acylase. The maximum penicillin acylase production was observed at pH 6.0 and the optimum temperature for the growth of *Acremonium sclerotigenum* was found to be 25°C. The variation in inoculum size (8.5×10^5-15.5×10^5 spores/ ml) showed increase in penicillin acylase activity with increase in number of spores up to 12.5×10^5 spores/ml. The maximum penicillin acylase activity (1.23 U/ml) was observed after 48 h of

cultivation with 3.58 mg/mL dcw fungal biomass. Threefold increase in penicillin acylase production was recorded when *Acremonium sclerotigenum* was cultivated under optimized culture conditions.

5.3 Immobilization of penicillin acylase from *Acremonium sclerotigenum*

Penicillin acylase from *Acremonium sclerotigenum* (1.22U/ml) was immobilized broadly by three methods - entrapment in agar and calcium alginate, adsorption on silica gel and covalent bonding with simple chitosan beads. Among these matrices, calcium alginate beads have shown maximum penicillin acylase activity (0.840 U/g) and immobilization efficiency (68.85 %). Silica has shown least penicillin acylase activity and immobilization efficiency.

5.4 Optimization of reaction parameters for crude and immobilized penicillin acylase from *Acremonium sclerotigenum*

The crude and immobilized enzyme from *Acremonium sclerotigenum* was characterized for various reaction parameters like buffer and pH, buffer molarity, reaction temperature, substrate concentration, effect of metals ions and solvents, thermostability, time course and self-life. Sodium citrate buffer (100mM, pH 5.0) and (150mM, pH 6.0) was found to be the most suitable for the assay of crude and immobilized penicillin acylase from *Acremonium sclerotigenum*. The optimum reaction temperature for crude and immobilized penicillin acylase was 40°C and 50°C respectively. Free penicillin acylase has shown maximum enzyme activity (1.62U/ml) with 50 mM penicillin G and 7.49µg of free enzyme whereas immobilized penicillin acylase showed the maximum activity (1.42 U/g matrix) with 150 mM sodium -citrate buffer, pH 6.0, when incubated at 50°C for 20 min. with 75 mM penicillin-G. The K_m and V_{max} values calculated from Lineweaver-Burk plot for immobilized penicillin acylase from *Acremonium sclerotigenum* was found to be 18.21mM and 1.56 U/g respectively. None of the metal ions have any significant positive effect on penicillin acylase activity as compared to control. The activity of penicillin acylase was strongly inhibited by PMSF, $HgCl_2$ and β-mercaptoethanol but less inhibition was observed in case of alginate entrapped enzyme. Among the various organic solvents, the residual activity recorded was 91.89 % in hexane, 87.75% in ethanol and 78.41% in isopropanol in case of immobilized enzyme, while free penicillin acylase exhibited residual activity of (82.83%) with hexane followed by ethanol (80.94%). The enzyme activity was inhibited to a greater extent by phenol and benzene. While performing

reaction in biphasic solvent system. Immobilized penicillin acylase showed less inhibition in phenol, benzene and butanol as compared to free enzyme. Immobilized enzyme was fairly stable at 40°C and 45°C, at 50°C the enzyme has retained 40% of residual activity after 120 min. The half life of the enzyme at 55°C and 60°C was 90 and 45 minutes respectively. Whereas the half life of the free enzyme at 55°C and 60°C was 75 minutes and 15 minutes respectively. Shelf life of penicillin acylase from *Acremonium sclerotigenum* was studied at both 4°C and at room temperature by pre incubating free enzyme for 360 h and alginate entrapped penicillin acylase for 480 h. The free enzyme has lost 37.79% of its initial activity on storage at 4°C after 360 h, whereas at room temperature the enzyme activity declined sharply and enzyme has lost 50% of its initial activity after 288 h. The immobilized penicillin acylase showed better shelf-life as compared to crude enzyme at 4°C and at room temperature. The half life of the alginate entrapped enzyme at room temperature was 384 h.

5.5 Purification of penicillin acylase from *Acremonium sclerotigenum*

Purification of penicillin acylase was done by hydrophobic interaction chromatography. The crude enzyme was obtained after centrifugation of 48 h old fermentation broth at 15,000 g for 15 min at 4ºC. The supernatant was subjected for different methods of protein precipitation. Maximum penicillin acylase activity was obtained after 10-50% saturation of ammonium sulphate and the ammonium sulphate precipitated protein (3 mg) was subjected to hydrophobic interaction chromatography (Octyl sepharose). All the positive fractions were analyzed for protein contents and enzyme activity. The active fractions were assessed for purity by SDS-PAGE and Native-PAGE. The protein analyzed by the SDS-PAGE and the Native-PAGE resulted a single band of approximately 146 kDa with subunit molecular mass73 kDa. Which showed that penicillin acylase was a dimer. The enzyme was purified up to 11.24 fold with a yield of 7.56% and 180.77U/mg specific activity.

5.6 Characterization of purified penicillin acylase from *Acremonium sclerotigenum*

Various parameters were evaluated to find the optimum reaction conditions for maximum activity of purified penicillin acylase. The enzyme showed the maximum activity (399.40 U/mg) with 125 mM sodium citrate buffer, pH 5.0, when incubated at 40°C for 20 minutes with 50 mM penicillin G. Lineweaver-Burk plot between 1/V and 1/[S] showed K_m *and* V_{max} values 13.32 mM and 333.33 μmol mg^{-1}

min^{-1} respectively. The effect of metal ions and inhibitors on purified penicillin acylase from *Acremonium sclerotigenum* was studied and the purified enzyme showed similar pattern as crude enzyme. The activity of purified penicillin acylase was also strongly inhibited by PMSF, $HgCl_2$, β-mercaptoethanol, phenol and benzene. The purified penicillin acylase was fairly stable at 40°C for 120 minutes, whereas 38% of residual activity was retained at 45°C.While at 50°C the purified penicillin acylase has retained 20% of the residual activity after 120 minutes. Enzyme activity declined at faster rate with a $t_{1/2}$ of 45 min and <20 min at 55°C and 60°C respectively. The purified penicillin acylase found to be less thermostable than crude penicillin acylase, moreover storage stability was also lesser than the crude enzyme. The enzyme was fairly stable at 4°C and retained 65.8% of residual activity even after 240 h. Whereas at room temperature, enzyme activity of purified penicillin acylase gradually started decreasing and half-life of enzyme was approximately 192 h at room temperature (RT).

5.7 Bench scale production of 6-APA

Batch reaction was carried out at 1L scale using free and alginate entrapped penicillin acylase, at the end of the reaction 43.52 mM 6-APA (87.84% molar conversion yield) was obtained after 150 minutes. The percentage purity of the sample was 54.33% and the productivity was 17.41mg/L/h. Samples were withdrawn at regular interval of time. 6-APA produced was lyophilized and characterized by UV Visible, Fluorescence and FTIR spectroscopy.

5.8 Dye decolourization by penicillin acylase and *Acremonium sclerotigenum*

Five different dyes (5mM) were initially screened to analyse the decolourization efficiency of penicillin acylase and *Acremonium sclerotigenum*. Dye which showed highest decolourization (%) was selected for further study. Out of these 5 different dyes methyl red showed decolourization (%) of 2.61% and 29.5% with penicillin acylase and *Acremonium sclerotigenum* respectively. Methyl red was selected as the dye of choice. Since the fungus proved more efficient in decolourization study than penicillin acylase therefore *Acremonium sclerotigenum* was used for further decolourization study. Highest decolourization was recorded in the culture medium containing maltose (40mM), peptone (1%) (pH-6.0) when incubated at 30°C with 10 mM methyl red.

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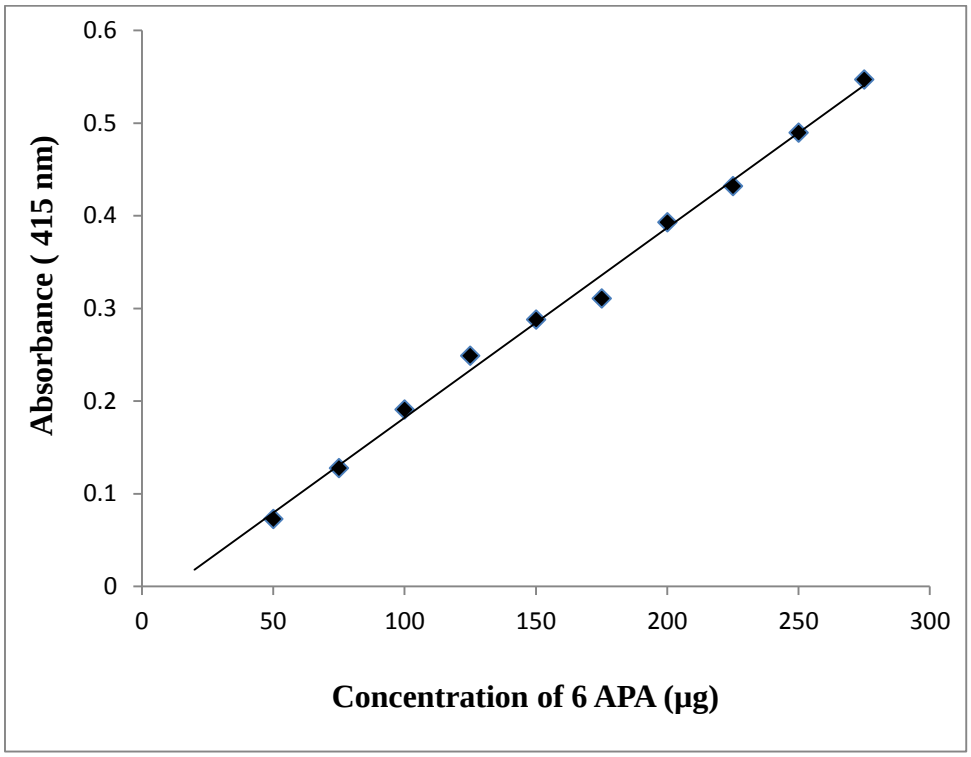
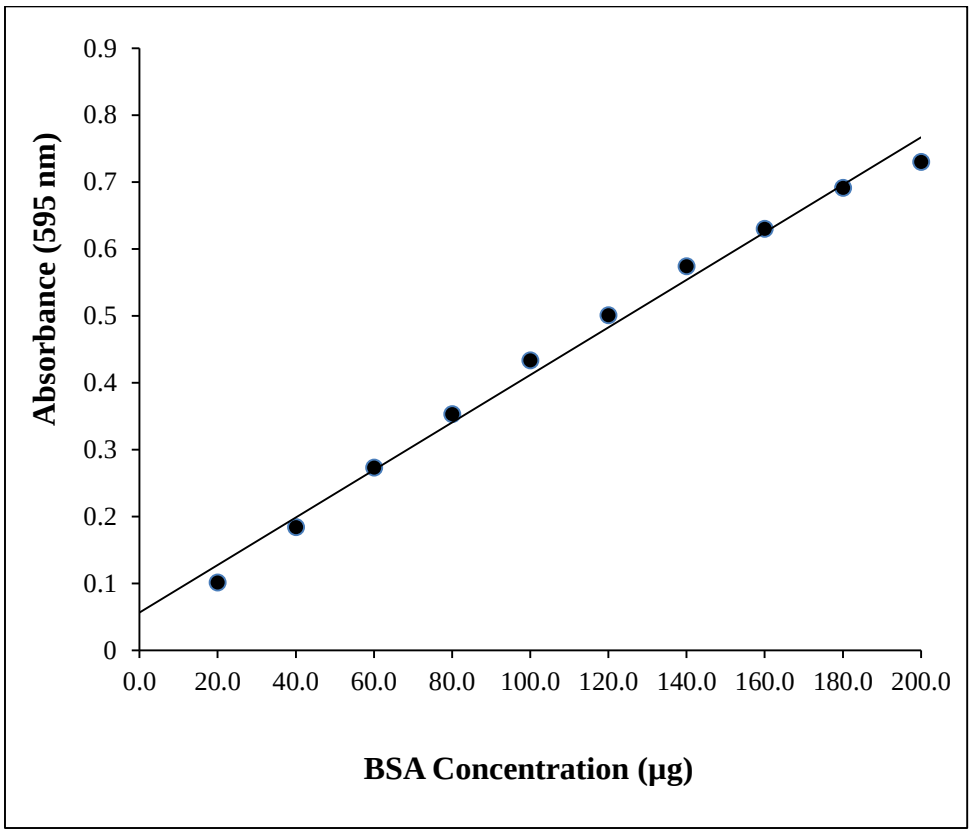


Standard curve of 6-APA



Bradford standard for protein estimation

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A Study on the Decolourization of Methyl Red by *Acremonium sclerotigenum*

Jeevan Lata[1], Charu Mehta[2], Neena Devi[3] and Duni Chand[4]*
Department of Biotechnology[1, 2, 3, 4]
Himachal Pradesh University, Summer Hill, Shimla 171005, India[1, 2, 3, 4]
Email: jeevanshimlabiotech@gmail.com,[1] *mehtacharu818@gmail.com*[2],
nthakur24@gmail.com[3],*dunichand_2000@yahoo.com*[4]
*Corresponding author: Dr. Duni Chand, Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla, HP-173212. Phone: 9418276448; Fax: 01772832153; Email: dunichand_2000@yahoo.com

Abstract

Dyes are extensively used in textile, leather and paper industries. But unsafe disposal of these dyes into water bodies and other places causes serious damage to the environment and negative impact on the human, plant and animal life. Textile effluent treatment is one of the greatest challenge. Use of microbes is a frequently applied process for the decolorization of textile dyes. Microbial decolorization offers an eco- friendly and cost competitive alternative to chemical decomposition process. Methyl red is a carcinogenic textile dye which is used for the colouration of various products. The aim of this research was to explore the ability of the fungal isolate *Acremonium sclerotigenum* to decolorize textile dyes. The fungus was isolated from soil of industrially contaminated areas and identified as *Acremonium sclerotigenum* at NFCCI, Pune. The isolate had shown maximum dye decolourization after 72 hrs (89.85%) in a medium containing dextrose (40mM), soya peptone (1.4%), yeast extract (1.6%) and beef extract (0.8%),inoculum size of 9.5×10^5 spores/ml, pH-6.0. with 10mM methyl red. Present study showed the potential of *Acremonium sclerotigenum* as microbial dye decolourization agent.

Keywords: Dye, Decolourization, *Acremonium sclerotigenum*, Methyl red

1. INTRODUCTION

Dyes are an important class of chemicals widely used in many industrial processes, like in leather, textile and printing, food, and cosmetics industries. They become an integral part of industrial wastewater due to their extensive use [1]. Inefficient dyeing processes, poor handling of dye effluent and insufficient treatment of dye wastes of industries lead to contamination of the soil and water bodies [2].A conventional biological wastewater treatment process is not very efficient in treating a dye wastewater due to the low biodegradability of dyes. Physical or chemical-treatment processes, used for treatment of dyes do not offer a solution as they generate a significant amount of the sludge and cause secondary pollution due to the formation of by products [3].

Azo dyes are major synthetic dyes extensively used in various industries. The process of applying dye on fabric is inefficient and approximately 10-15% of the dyes are released in to environment .The first contaminant observed in waste water is color and are visible in water even at concentrations as low as 1 ppm [4]. Dyes escape the conventional wastewater treatment process as they are recalcitrant's due to their synthetic origin and complex stable structure [5, 6]. Synthetic dyes are extensively used in textile dyeing paper printing, colour photography, pharmaceutical, and food, cosmetic and other industries [7].

Approximately 10,000 different dyes and pigments are used industrially, and over 0.7 million tonnes of synthetic dyes are produced annually worldwide. In 1991, the world production of dyes was estimated at 668,000 t [8] of which an estimated 70 percent were azo dyes [9]. A bulk of azo dye production is lost to domestic and industrial wastewater r[8 ,10]. Major classes of synthetic dyes include azo, anthroquinone and triaryl methane dyes, and many of them are toxic or contain carcinogenic compounds with long turnover times [11].The ability of microorganisms to carry out dye decolorization has received much

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POSTER ABSTRACT
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Studies on penicillin acylase production from *Acremonium sclerotigenum*

JeevanLata, Neena Devi and Dunichand*

Department of Biotechnology, HP University Campus, Shimla 171 005, India

* e mail: dunichand_2000@yahoo.com, www.hpuniversity.nic.in

Penicillin acylase or amidohydrolase (EC 3.5.1.1) has been still a target of research because of its demand in the enzyme market as it plays a very crucial role inthe deacylation of the penicillin into the 6-aminopenicilanic acid (6-APA)which is the key intermediate in the commercial manufacture of semi-synthetic penicillins and cephalosporins which remains the most widely used group of antibiotics. In the present studies screening, isolation and optimization of parameters for extracellular production of penicillin acylase from *Acremonium sclerotigenum* was carried out. Out of 120 isolates from soil samples collected from industrially contaminated areas, *Acremonium sclerotigenum* maximum penicillin acylase production (0.946 U/ml) was recorded when grown in medium supplemented with yeast extract1.2% (w/v), beaf extract 0.5%(w/v), soya peptone1.2% (w/v), glucose70 mM, 1% penicillin G as substrate inducer, inoculum size of 1.25×10^6/ml spores.Maximum PA production was observed after 48 h at pH 6.0 at 25°C. Optimization studies resulted in about 3-fold enhancement (0.946 U/ml) in overall enzyme activity.

Batch and Fed-batch Decolourization of Congo Red By Penicillin Acylase from *Acremonium Sclerotigenum* KJ194116.1

Jeevan lata[1] , Swati Sharma[1] , Neena Devi[1] and Duni Chand[1]

[1]*Himachal Pradesh University, Shimla, India.*

Synthetic organic colourants (i.e. azo dyes) are used commonly in different industries ranging from food, textile production, printing and pharmaceuticals. The release of dyes into the environment causes water pollution, and the coloured waste water represents a serious environmental problem and a major public health concern. Immobilized penicillin acylase from *Acremonium sclerotigenum* was investigated for its dye decolorization potential. Immobilization of penicillin acylase was done by entrapment method in 3% sodium alginate. Immobilized enzyme showed highest decolorization at 350C with Tris- HCl buffer at pH 9.0 and incubation time of 6 hrs. The reusability of the immobilized biocatalyst was also evaluated with repeated-decolorization experiments and found to retain above 50% dye decolourization efficiency up to 2nd cycle. Bench scale decolorization experiments were performed with immobilized enzyme using batch and fed-batch supply of dye in the simulated waste water. Fed-batch showed better results as compared to batch and decolourization of dye was about 92.54%.The IR spectra of the congo red dye after batch and fed batch showed absence of peak for aromatic N=N stretching vibrations in the FTIR spectrum. That showed the complete decolorization of Congo red by fungal penicillin acylase. Key words: Congo red; *Acremonium sclerotigenum*; Penicillin acylase; Decolourization; Azo dyes; Immobilized.

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Application of statistical experimental design for enhanced production of acyltransferase from *Bacillus* sp. APB-6

Neena Devi, Pankaj Kumari, Jeevan Lata and Duni chand*

Department of Biotechnology, Gyan Path, HP University Campus, Summer Hill, Shimla 171 005, India

* e mail: dunichand_2000@yahoo.com

Optimization of physico-chemical parameters for enhanced production of acyltransferase by *Bacillus* sp.APB-6 were carried out using one-variable-at a-time strategy(OVAT) and statistical designs of response surface methodology(RSM) to describe the relationship between tested variables.Optimization resulted into 3.8 folds increase in the acyltransferase production (448.44 Umg^{-1} dcw).Based on results of Plackett-Burman design, combined effect of variables viz. pH, temperature, inoculum size and non-substrate inducer acetonitrile on production of acyltransferase were investigated at four levels, through the statistical analysis of central composite design (CCD).The statistical optimization showed further increase in acyltransferase activity of 1.06 folds.The generated model was also validated by repeating the experiments under optimized condition which resulted acyltransferase production of 474.8 U mg^{-1} dcw (Predicted response 464.71 U mg^{-1} dcw).After performing optimization of all production parameters using statistical design, 4.2 fold increase in acyltransferase production (519.03 U mg^{-1} dcw) was recorded with 3.0%(w/v) acetamide, 1.5%(w/v) yeast extract, 0.5%(w/v) NaCl, 0.2%(w/v) glucose, pH 7.0 at 30°C , 8%(v/v) inoculum size and acetonitrile as non substrate inducer (70 mM) for 18h

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Enhanced Production of Acyltransferase from *Rhodococcus pyridinivorans* using Statistical Experimental Design

Neena Devi, Pankaj Kumari, Jeevan Lata and Duni Chand *
Department of Biotechnology, Himachal Pradesh University, Shimla-171005, India

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Abstract: Optimization of growth parameters for enhanced production of acyltransferase by *Rhodococcus pyridinivorans* were carried out using one-variable-at-a-time strategy (OVAT) and statistical designs of response surface methodology (RSM) to describe the relationship between tested variables. Optimization resulted into 3.8 folds increase in the acyltransferase production (448.44 ± 7.00 Umg^{-1} dcw). Based on results of Plackett-Burman design, combined effect of variables viz. pH, temperature, inoculum size and non-substrate inducer acetonitrile on production of acyltransferase were investigated at four levels, through the statistical analysis of central composite design (CCD). The statistical optimization showed further increase in acyltransferase activity of 1.06 folds. The validation of the experiments was also done by point determination in generated model with actual and predicted response level under optimized condition which resulted acyltransferase production of 480.8 ± 2.01 U mg^{-1} dcw (Predicted response 474.71 U mg^{-1} dcw). After performing optimization of all production parameters, 4.2 fold increase in acyltransferase production (519.03 ± 1.093 U mg^{-1} dcw) was recorded with 3.0 % (w/v) acetamide, 1.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 0.2 % (w/v) glucose, pH 7.0 at 30°C, 8 % (v/v) inoculum size and acetonitrile in multistep feeding as non substrate inducer (70 mM) for 18 h.

Keywords: *Rhodococcus pyridinivorans*, one variable at a time (OVAT), central composite design (CCD), acyltransferase production.

Introduction

Amidases or amidohydrolase is the nitrilase super family enzymes which are prevalent in prokaryotes and eukaryotes. The biological functions of amidases vary widely, but they are typically involved in carbon and nitrogen metabolism in prokaryotes. In nitrile metabolism, amidases possess duel catalytic activity viz. hydrolysis of amides to corresponding acids and acyltransferase activity in presence of hydroxylamine to form hydroxamic acids [22].The use of acyl transfer activity of amidase may be used to convert amides to hydroxamic acids. In acyltransferase catalyzed biotransformation, amides acts as acyl-group donors and hyroxylamine as acyl-group acceptors. Acyltransferase activity of amidase is exploited mainly for the synthesis of pharmaceutically active hydroxamic acids [29, 14 ,11]. Hydroxamic acids are known to possess high chelating properties. Some of hydroxamic acids such as a-aminohydroxamic acids, synthetic siderophores, and acetohydroxamic acid have also been investigated as anti-human immunodeficiency virus agents or anti-malarial agents or have been recommended for treatment of urea plasma infections and anaemia [9, 14]. Moreover, some fatty hydroxamic acids have been studied as inhibitors of cyclooxygenase and 5-lipoxygenase with potent anti-inflammatory activity [12].

Amidases from microorganisms have gained im-

*Corresponding author (Duni Chand)
E-mail: < dunichand_2000@yahoo.com >