Penicillin G Acylase from *Arthrobacter viscosus* (ATCC 15294): Production, Biochemical Aspects and Structural Studies

A THESIS SUBMITTED BY

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UNDER THE GUIDANCE OF

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DIVISION OF BIOCHEMICAL SCIENCES
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.....Dedicated to my beloved parents

and to my mentor

CERTIFICATE

Certified that the work incorporated in the thesis entitled: "**Penicillin G acylase from *Arthrobacter viscosus* (ATCC 15294): Production, Biochemical aspects and Structural Studies**", submitted by Mr. Ambrish Rathore, for the Degree of ***Doctor of Philosophy***, was carried out by the candidate under my supervision at Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India. Material that has been obtained from other sources is duly acknowledged in the thesis.

Asmita Prabhune

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DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Penicillin G acylase from *Arthrobacter viscosus* (ATCC 15294): Production, Biochemical aspects and Structural Studies**", submitted by me for the Degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at Division of Biochemical Sciences, National Chemical Laboratory, Pune, India, under the guidance of Dr. Asmita Prabhune. The work is original and has not formed the basis for the award of any other degree, diploma, associateship, fellowship and titles, in this or any other University or other institution of higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis

Ambrish Rathore

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C O N T E N T S

	Page No.
Acknowledgement	ii
List of abbreviations	iv
Abstract	v
Chapter 1: General Introduction	1
Chapter 2: Screening and Optimization of Fermentation Parameters	33
Chapter 3: Production, Purification and Biochemical Characterization of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294	47
Chapter 4: Identification and Characterization of Active site residues of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294	62
Chapter 5: CTAB capped gold nanoparticles for colorimetric detection of Hydrolysis of antibiotics by penicillin G acylase	74
Chapter 6: Immobilization studies of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294	87
Summary and Conclusions	98
Appendix: Preliminary X-Ray crystallography study of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294	100
References	107
Publications	130

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Ambrish Rathore

LIST OF ABBREVIATIONS

6-APA	:	6- amino penicillanic acid
7-ADCA	:	7-amino 3-deacetoxy cephalosporanic acid
7-ACA	:	7-amino cephalosporanic acid
ATCC	:	American Type Culture Collection
AU	:	Absorption Unit
CAs	:	Cephalosporin acylases
CPB	:	Citrate Phosphate buffer
h	:	hour
HNBBr	:	2-hydroxy 5-nitrobenzylbromide
IU	:	International Unit
LB	:	Luria-Bertani
L	:	litre
m	:	metre
mol	:	mole
M	:	molar
mM	:	milli molar
mm	:	milli meter
min	:	Minute
M.W. /Mr	:	molecular weight
NBS	:	N-bromosuccinimide
NCIM	:	National Collection of Industrial Microorganisms.
nm	:	Nano metre
Ntn	:	N-terminal nucleophile
OD	:	Optical density/Absorbance
PAA	:	Phenyl Acetic Acid
PAs	:	Penicillin acylases
PAGE	:	poly acrylamide gel electrophoresis
PB	:	Phosphate Buffer
PDAB	:	p-dimethylaminobenzaldehyde.
PGA	:	Penicillin G acylase
pen G	:	Penicillin G (Benzyl penicillin)
pen V	:	Penicillin V (phenoxymethyl penicillin)
rpm	:	revolution per minute
SDS	:	sodium dodecyl sulphate
Tris-Cl	:	tris-(hydroxymethyl) amino methane chloride
U	:	Unit
Å	:	Angstrom
kDa	:	kilo Dalton
°C	:	degree centigrade
µg	:	microgram
µl/µL	:	microlitre
µM	:	micromolar

Abstract

Abstract

Penicillin acylases (penicillin amidohydrolase, EC 3.5.1.11) are used in the commercial production of 6-amino penicillanic acid (6-APA), the starting compound for the synthesis of semi-synthetic penicillins. Penicillin acylases, a subclass of β-lactam antibiotic acylase family, catalyze the selective hydrolysis of relatively stable amide bond in penicillins and some cephalosporins while leaving the labile β-lactam ring intact. The enzyme mediated 6-APA production is the preferred route over chemical route as they lead to almost completion under appropriate conditions of pH, temperature and substrate concentration. Enzymatic routes are environ-friendly, as they require lesser number of steps with hazardous chemicals and most of the times these enzymatic reactions are in aquous conditions.

The enzymes are classified in three types based on their substrate specificities: the penicillin V acylases (PVAs) (type I) which are usually of fungal origin, specifically act on phenoxymethyl penicillin/penicillin V (pen V); penicillin G acylases (PGAs), which are usually of bacterial origin, catalyse the hydrolysis of benzyl penicillin/penicillin G (pen G) preferably and bacterial ampicillin acylases which specifically act on ampicillins (D-aminobenzyl penicillins).

Penicillin acylases (PAs) could be placed in N-terminal nuleophile (Ntn) hydrolase superfamily. Ntn hydrolases are a class of enzymes that share a common fold of αββα core structure possessing an N-terminal catalytic nucleophile residue. In PGA, N-terminal nucleophile is serine. The Ntn hydrolases invariably produce their active form from the corresponding precursor by an intra-molecular autocatalytic cleavage to create a free amino group at the nucleophile residue.

The capacity of penicillin G acylases (PGAs) to catalyze the acylation of amino group of key intermediates can be used in the environment-friendly synthesis of semi-synthetic β-lactam antibiotics. PGAs are also useful for other applications such as peptide synthesis, removal of protecting groups and separation of racemic mixtures of certain compounds.

The present study “Penicillin G Acylase from *Arthrobacter viscosus* (ATCC 15294): Production, Biochemical Aspects and Structural Studies” was

taken up with the objectives of screening PGA producing culture and optimization of fermentation parameters for PGA production from *Arthrobacter viscosus* and purification of the enzyme. Substrate specificity study was performed to probe the binding behaviour of the enzyme to various semi synthetic antibiotics which is useful in protein engineering. Biochemical characterization of PGA such as pH and temperature optimization, pH and temperature stability are pre-requisite for any industrially important enzyme. Active site studies were performed to evaluate the kinetics of catalysis and study the role of various amino acids taking part in the catalysis. CTAB capped gold nano particles were explored for deriving a novel and simple methodology for rapid screening of PGA producers. Industrial applicability of *Arthrobacter viscosus* PGA (*Av*PGA) was performed by immobilization study of PGA on iron oxide nano particles. Use of nano particles has advantage of very high surface area and less diffusional barriers compared to other support matrices and iron oxide nano particles have advantage of easy separation by magnetic precipitation. Initial crystallization trials were performed to screen the crystallization conditions for *Av*PGA.

Arthrobacter viscosus, being a gram positive bacterium, secretes PGA in the culture broth, as it does not have any periplasmic space. PGA produced by gram negative bacteria such as *E. coli*, which is most studied, is accumulated in periplasmic space. *Av*PGA has advantage over other PGAs as it will require fewer steps in downstream processes. Many steps such as disruption of cells and centrifugation steps can be avoided when using *Arthrobacter viscosus* which produce enzyme of interest in the culture broth.

The thesis is divided in to the following chapters:

Chapters 1: General Introduction

Detailed literature survey, significance and the objectives of the study are included in this chapter.

Chapters 2: Screening and Optimization of Fermentation Parameters

This chapter includes the study related to methodology for screening PGA producing cultures using *Serratia marcescens* culture which is resistant to penicillin G but sensitive to 6-aminopenicillanic acid. PGA producing cultures show a clear zone of inhibition around it. Then various fermentation parameters were tried for optimum PGA production. These parameters

included testing different medium, various carbon and nitrogen sources, inducer concentration, medium pH and temperature, inoculum size and fermentation time for maximum enzyme production.

Chapters 3: Production, Purification and Biochemical Characterization of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

The enzyme PGA from *Arthrobacter viscosus* is secreted in culture broth. The enzyme was produced using optimum fermentation condition in shake flask condition and then purification of enzyme from culture broth was performed using various chromatographic techniques. The enzyme was purified using alumina matrix packed in column, followed by hydrophobic interaction (octyl sepharose) and ion exchange (Q-sepharose) chromatography. Enzyme characterization was performed after checking its purity by gel electrophoresis. Molecular weight determination by SDS-PAGE and MALDI-TOF were performed. Various biochemical parameters like optimum pH, pH stability, optimum temperature, temperature stability, kinetic parameters were determined by using purified enzyme.

Chapters 4: Identification and Characterization of Active site residues of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

This chapter includes identification and characterization of active site residues of the enzyme. Enzyme was treated with different modifying reagent. Incubation of PGA with arginine modifying reagent, phenyl glyoxal, inactivated the enzyme. The enzyme is also inactivated by tryptophan modifying reagent, N-bromosuccinimide (NBS). The time dependent kinetics was performed to identify the role of active site amino acid residue. Penicillin G, phenylacetic acid and 6-aminopenicillaninc acid significantly lower the extent of inactivation by arginine and tryptophan modifying reagent.

Chapters 5: CTAB capped gold nanoparticles for colorimetric detection of Hydrolysis of antibiotics by penicillin G acylase

CTAB capped gold nanoparticles were used for colorimetric detection of hydrolysis reaction of penicillin G by PGA. The pink coloured nano particles turned purple when both, substrate, pen G, and enzyme, PGA were present. Colour was not changed by the presence of substrate alone. When the nano particles were mixed with 6-APA, the colour change was observed, proving that nano particles are sensing the hydrolysis reaction by PGA. This property

of these nano particles is very beneficial in designing biosensors to screen the PGA producing culture.

Chapter 6: Immobilization studies of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

Penicillin G acylase is an industrially important enzyme and it is commercially used in the form of immobilized system. PGA from *Arthrobacter viscosus* was purified and immobilized on iron oxide nano particles to test its compatibility with the matrix. Enzyme had good activity when immobilized on iron oxide nano particles. The effect of enzyme loading on nano particles was also performed for its industrial

Summary and Conclusions

This section of the thesis gives summary of the findings and conclusion derived from the work. Future aspects of the work are also discussed in this section.

Appendix: Preliminary X-Ray crystallography study of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

X-ray crystallography is a strong tool to decipher the 3D structure of protein molecules. Some preliminary screening was performed using standard screens from Hampton Research to obtain protein crystal. Protein crystals were obtained in some of the conditions. Further crystallization trials are to be performed to get good quality crystals for data collection.

CHAPTER 1

General Introduction

1.1 Enzymatic catalysis

Enzymes are biocatalysts which find applications in numerous industrial processes. They are employed in the form of intact microbial cells or pure enzyme. More than 2500 enzymes have been identified to date of which around 250 are used as biocatalysts in various commercial processes (Woodley, 2000). Enzyme catalyzed reactions are cheaper than their chemical counter parts, as they require mild conditions of pH and temperature with fewer byproducts, thus making these processes environment-friendly. Enzymes have found applications in the manufacturing of food, animal feed, textiles, petrochemicals, detergents, pulp and paper, fine chemicals etc. in the form of immobilized biocatalyst. One such example in the fine chemical industry is the use of penicillin G acylase. Penicillin G acylases (PGAs), a member of β-lactam acylase family acting on a broad range of natural and semi-synthetic β-lactam antibiotics, are industrially important enzymes which are being used to hydrolyze natural penicillins to produce 6-amino penicillanic acid (6-APA) (Figure 1.1). They catalyze the hydrolysis of the relatively stable linear amide bond of penicillins to produce the β-lactam nucleus, 6-APA, which is the core compound in the production of various types of semi-synthetic antibiotics. They are also used in industrial production of semi-synthetic penicillins and cephalosporins via, 6-APA and 7-amino-3-deacetoxy cephalosporanic acid (7-ADCA) at acidic or neutral pH values (4.0-7.0).

Despite the availability of alternative antimicrobials, penicillins (along with semi-synthetic penicillins) are one of most important antibiotics in terms of annual production and prescription volume (Rajendhran and Gunasekaran, 2004), amounting about 19% of the estimated world wide antibiotic market (Parmar et al, 2000). Excessive use of these antibiotics has led to the development of resistance in pathogens and newer semi-synthetic antibiotics are required to overcome resistance problem. Penicillin G and Penicillin V are natural penicillins, produced in large quantity by fermentation. About 16,000 tons of fermentative penicillin G and V are produced annually for therapeutic purposes (Bruggink and Roy, 2001). Only a small amount of these penicillins is used directly as therapeutic agents while majority of amount is used for production of 6-APA (Rajendhran and Gunasekaran, 2004).

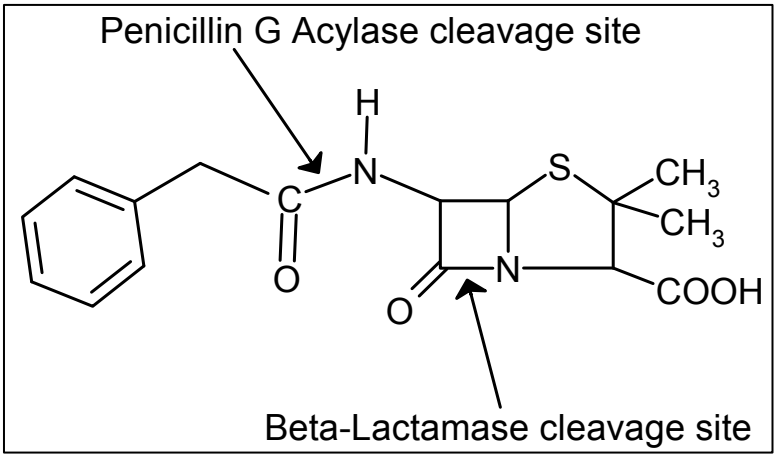


Figure 1.1: Penicillin G molecule showing the respective hydrolytic bonds, the site of action of PGA and beta-lactamase

1.2 β-Lactam Antibiotics

The era of antibiotic started with the discovery of penicillin by Alexander Fleming in 1928. At that time, nobody took notice of Fleming's discovery. Use of penicillin did not begin until 1939 when Howard Florey and Ernst Chain isolated the active ingredient and developed a powdery form of the penicillin (Abraham, 1981; Bennet and Chung, 2001). The effectiveness of penicillin against bacterial infection had been first demonstrated in mice. Later sufficient material was prepared with great difficulty to treat a few human patients (Nayler, 1991). Industrial scale penicillin production was realized as a direct result of World War II.

A new penicillin-like antibiotic was discovered in 1945, when Giuseppe Brotzu isolated the mould *Cephalosporium acremonium* from a sewer pipe in Sardinia. Crude filtrates of this organism displayed the same activity against *S. aureus* as shown for the *Penicillium* strains and the antibacterial substance was named cephalosporin C (Nicholas et al, 1995).

Antibiotics are substances or compounds that kill bacteria (bactericidal) or inhibit the growth of microorganisms (bacteriostatic), even at very low concentrations. Majority of the clinically used antibiotics have been obtained from actinomycetes, especially *Streptomyces* species. *Bacillus* species and fungi also have yielded few useful antibiotics. They can be classified based on their chemical structure, microbial origin, and spectrum of activity or mode of

action. One class of antibiotics that work by inhibiting the synthesis of peptidoglycan in bacterial cell walls is β-lactam antibiotics. They have superior inhibitory action on the bacterial cell wall synthesis, broad spectrum of antibacterial activity, low toxicity and outstanding efficacy against various bacterial strains. They are responsible for inhibition of cross-linking in peptidoglycan layer by binding to and inactivating transpeptidase enzyme. Transpeptidase or any other protein that binds to penicillin is known as penicillin binding protein (PBP). PBPs vary in their binding affinity to any penicillin or β-lactam antibiotics. Structural resemblance of penicillin to D-alanyl-D-alanine portion of cell wall glycopeptide makes it potent antibiotic as it specifically inhibits linking of neighboring subunits via transpeptidation. Transpeptidation reaction gives structural support to cell wall. Once the cell walls are faulty, it allows water to flow into the cells causing it to burst. β-lactam antibiotics can be classified based on their core structure (nucleus) as:

1. **Penicillins:** They are also called penams. They can be classified into two types according to the source. Natural penicillins are penicillin G (Pen G) and penicillin V (Pen V) and the Semi-synthetic penicillins e.g. amoxicillin, penicillinase-resistant cloxacillin, methicillin.
2. **Cephalosporins:** These are classified in generations I, II, III, IV and V. Along with cephamycin, they form a sub-group called **cephems**.
3. **Carbapenems:** eg imipenem, meropenem, entrapenem
4. **Monobactams:** eg aztreonam

Some other less common β-lactam nuclei (core structures) include penems, oxacephem and clavam.

Figure 1.2 illustrates the structural features of some of these β-lactam nuclei. Some natural and semi-synthetic beta lactam antibiotics with their structural drawings are shown in figure 1.3 and figure 1.4. A list of various beta lactam antibiotics and their class are listed in table 1.1. Various new beta lactam antibiotics have been produced after the resistance developed among microorganisms against natural penicillins. A list of some of earlier antibiotics with their year of discovery and administration is tabulated in table 1.2.

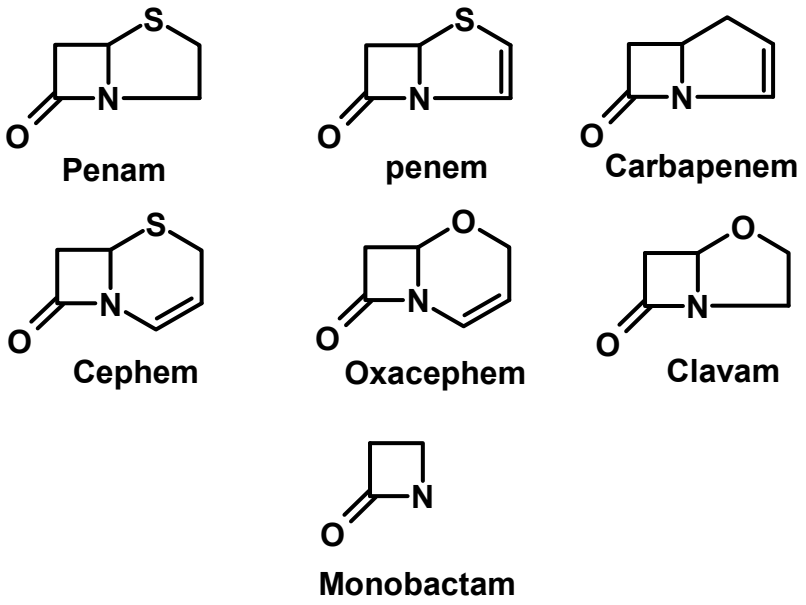


Figure1.2: Structural drawings of β-lactam antibiotic nucleus

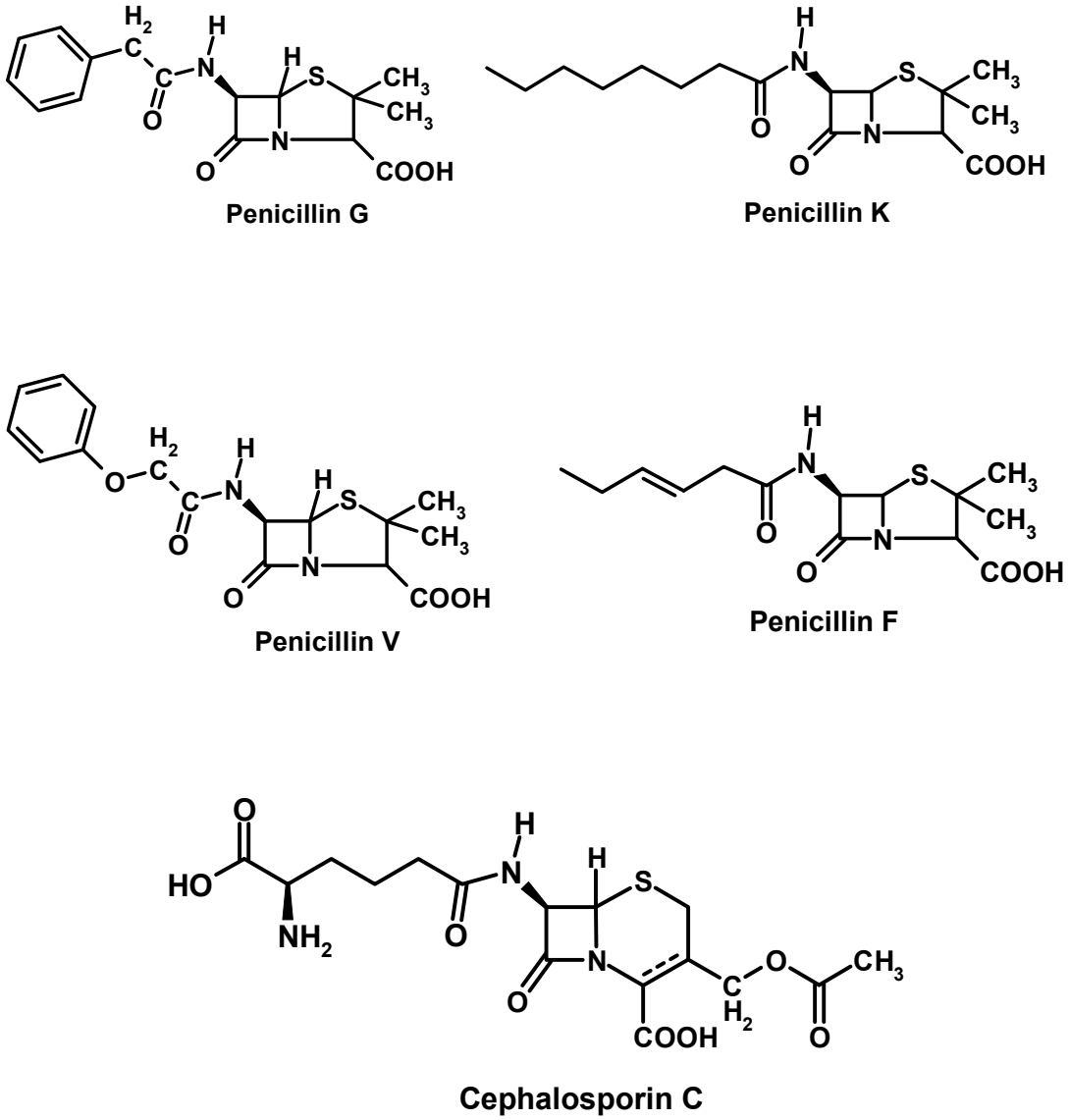
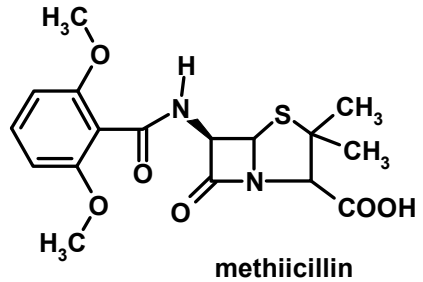
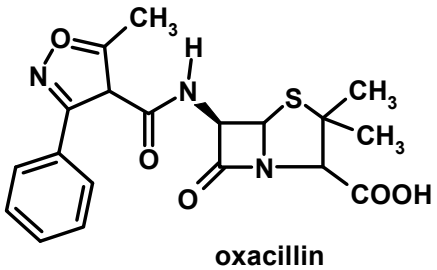


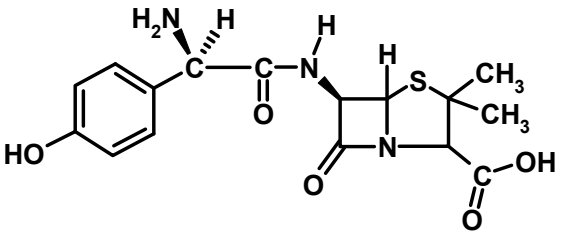
Figure 1.3: Structural drawings of some natural β-lactam antibiotics



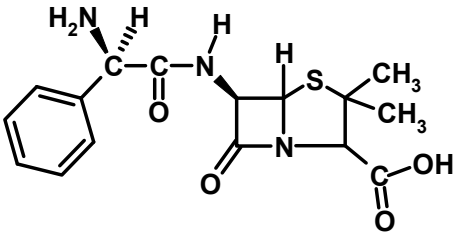
methiicillin



oxacillin



Amoxicillin



Ampicillin

Figure 1.4: Structural drawings of some semi-synthetic β-lactam antibiotics

Table 1.1: List of Natural and Semi-symthetic β-lactam antibiotics

β-lactams	**Penicillins (penams)**	**Extended spectrum**		***Aminopenicillins***: Amoxicillin[#], Ampicillin[#] (pivampicillin, hetacillin, Bacampicillin, Metampicillin,
				Carboxypenicillins: Carbenicillin (carindacillin), Ticarcillin, Temocillin
				Ureidopenicillins: Azlocillin, Pipeacillin, Mezlocillin
				Others: Mecillinam (Pivmecillinam), Sulbenicillin
		Narrow Spectrum	**Beta lactamase sensitive**	**Benzylpenicillin (G)[#]:** clometocillin, Benzathine benzylpenicillin[#], Procaine benzapenicillin[#], Azidocillin, Penamecillin
				Phenoxymethylpenicillin (V)[#]: Propicillin, Benzathine phenoxymethylpenicillin, Pheneticillin
			Beta lactamase resistent	Cloxacillin[#] (Dicloxacillin, Flucloxacillin), Oxacillin, Methicillin, Nafcillin
	Penems	Faropenem		
	Carbapenems	Biapenem, Ertapenem, *antipseudomonal* (Doripenem, Imipenem, Meropenem), Panipenem		
	Cephalosporins/ (cephems)	**1st (PEcK)***		Cefazolin[#], Cefacetrile, Cefadroxil, Cefalexin, Cefaloglycin, cefalonium, Cefaloridine, Cefalothin, Cefapirin, Cefatrizine, Cefazedone, Cefazaflur, Cefradine, Cefroxadine, Ceftezole
		2nd (HEN)$		Cefaclor, Cefamandole, Cefminox, Cefonicid, Ceforanide, Cefotiam, Cefprozil, Cefbuperazone, Cefuroxime, Cefuzonam, *cephamycin* (Cefoxitin, Cefotetan, Cefmetazole), *carbacephem* (Loracarbef)
		3rd		Cefixime[#], Ceftriaxone[#], *antipseudomonal* (Ceftazidime[#], Cefoperazone), Cefcapene, Cefdaloxime, Cefdinir, Cefditoren, Cefetamet, Cefmenoxime, Cefodizime, Cefotoxime, Cefpimizole, Cefpiramide, Cefpodoxime, Cefsulodin, Cefteram, Ceftibuten, Ceftiolene, Ceftizoxime, *oxacephem* (Flomoxef, Latamoxef[‡])
		4th (Antips-)&		Cefepime, Cefozopran, Cefpirome, Cefquinome
		5th		Ceftobiprole, Ceftaroline fosamil
	Monobactams	Aztreonam, Tigemonam, Carumonam, Tabtoxin		
	β-lactamse Inhibitor	Sulbactam, Tazobactam, *clavam* (Clavulanic acid)		
	Combinations	Co-amoxiclav (Amoxicillin/clavulanic acid)[#], Imipenem/cilastatin[#], Ampicillin/sulbactam (Sultamicillin), Piperacillin/Tazobactam		

#, World Health Oraganization Essential medicines; ‡, Withdrawn from market; *, (P- *Proteus mirabilis,* Ec- *Escherichia coli,* K- *Klebsiella pneumoniae*); $, (H-*Haemophilus influenzae*, E-*Enterobacter aerogenes*, N-*Neisseria*); &, Antipseudomonal

Table 1.2: Some of the antibiotics with their year of discovery or introduction

Year	Event	Country
1929	penicillin discovered	England
1932	sulfonamides (Prontosil) discovered	Germany
1939	gramicidin discovered	United States
1942	penicillin introduced	England and United States
1943	streptomycin discovered	United States
1943	bacitracin discovered	United States
1945	cephalosporins discovered	Italy
1947	chloramphenicol discovered	United States
1947	chlortetracycline discovered	United States
1949	neomycin discovered	United States
1950	oxytetracycline discovered	United States
1952	erythromycin discovered	United States
1956	vancomycin discovered	United States
1957	kanamycin discovered	Japan
1960	methicillin introduced	England and United States
1961	ampicillin introduced	England
1961	spectinomycin reported	United States
1963	gentamicin discovered	United States
1964	cephalosporins introduced	England
1966	doxycycline introduced	United States
1967	clindamycin reported	United States
1971	tobramycin discovered	United States
1972	cephamycins (cefoxitin) discovered	United States
1972	minocycline introduced	United States

1.3 Semi-synthetic penicillins to overcome bacterial resistance

Despite the effectiveness of penicillin in curing a wide range of diseases, infections caused by certain strains of staphylococci cannot be cured because the latter produces an enzyme, penicillinase (β-lactamase), capable of destroying the antibiotic (Figure 1.1). Moreover, the over prescription of the penicillin drugs led to the development of strains resistant to current antibiotics. Enterococci and other bacteria known to cause respiratory and urinary tract infections were found intrinsically resistant to the action of penicillins.

In order to produce novel penicillins effective against resistant bacteria, a combined fermentation and chemical approach is used which leads to the production of semi-synthetic penicillins. Appropriate chemical treatment of a

biological precursor to penicillin, isolated from bacterial cultures, resulted in a number of so-called semi-synthetic penicillins. After the mid 1950s a wide range of new penicillins has been developed this way. Semi-synthetic penicillins exhibit enhanced properties - such as increased stability, easier absorption and fewer side effects - than pen G and pen V, and represent a practical solution to the problem of adaptive microbial resistance to antibiotics.

Ampicillin and amoxicillin are both active against most aerobic gram-positive cocci. *S. aureus* is usually resistant to ampicillin and amoxicillin. The anaerobic gram positive cocci and rods are generally susceptible to ampicillin, so are some aerobic gram negative bacilli such as *E. coli*, *Proteus mirabilis*, and *Haemophilus influenzae.* Ampicillin and penicillin are the drugs of choice for the treatment of infections caused by Group B streptococci. Ampicillin, when combined with an aminoglycoside, is effective in treating intra-amniotic infections. Oxacillin or Nafcillin are used in the treatment of complicated skin infections.

Carbenicillin has a similar spectrum of activity as that of ampicillin but is also active against *Pseudomonas aeruginosa*, whereas *Klebsiella pneumoniae* is resistant to carbenicillin. Ticarcillin is two to four times more potent than carbenicillin against *P. aeruginosa*. Piperacillin and mezlocillin have greater activity against gram-negative enteric organisms and also provide coverage against most anaerobes and enterococcus. Dicloxacillin is an orally-active semi-synthetic penicillin used to treat infections by bacteria such as *Staphylococcus aureus*.

Table 1.3: Some semi-synthetic β-lactam antibiotics in commercial use

Amoxicillin	Dicloxacillin	Oxacillin
Ampicillin	Flucloxacillin	Piperacillin
Bacampicillin	Methicillin	Pivampicillin
Carbenicillin	Mezlocillin	Pivmecillinam
Cloxacillin	Nafcillin	Ticarcillin

The most important of these are methicillin and ampicillin, the former is remarkably effective against penicillinase-producing *staphylococci* and the

latter is not only active against all organisms normally killed by penicillins, but also inhibits growth of *enterococci* and many other bacteria. In fact, methicillin was the first successful semisynthetic penicillin introduced in 1959 to overcome the problems that arose from the increasing prevalence of penicillinase-producing *S. aureus* (Livermore, 2000).

In 1980s, CI-867, a semi-synthetic penicillin exhibited broad-spectrum activity *in vitro* against gram-positive cocci. It was active against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Weaver and Bodey, 1980). All of these newly prepared or identified antibiotics (Table 1.3) have varying effect on different types of microbial pathogens. This has provided evidence for the direct influence of the side chain on spectrum of penicillins and researchers started concentrating on the chemical structure of the side chain.

1.4 β-lactam acylases

Enzymes which act on a variety of β-lactam antibiotics are known as β-lactam acylases and are classified based on their substrate specificities. They are broadly grouped into two classes:

(A) Penicillin acylases and

(B) Cephalosporin acylases

(A) Penicillin acylases (EC 3.5.1.11) specifically hydrolyze the amide bond connecting the β-lactam nucleus to the side chain of penicillins (Figure 1.1). The EC number denotes that they are hydrolases acting on carbon-nitrogen bonds other than peptide bonds in linear amides. They are also termed as penicillin amidase (Sakaguchi and Murao, 1950; Murao, 1955; Claridge et al., 1960, Murao and Kashida, 1961; Claridge et al., 1963), penicillin deacylase (Cole, 1964), benzylpenicillin acylase (Huang et al., 1963) penicillin splitting and synthesizing enzyme (Rolinson et al., 1960; Kaufman and Bauer, 1960), acyl transferase (Kaufmann and Bauer, 1964). Enzymatic hydrolysis of penicillins to 6-APA was first reported by Sakaguchi and Murao (1950) with the enzymes obtained from *Penicillium chrysogenum* Q176 and *Aspergillus oryzae*. According to the type of substrate preferably hydrolyzed, penicillin acylases are classified into Penicillin V acylase (PVA), Penicillin G

acylase (PGA) and ampicillin acylase which preferably cleave pen V, pen G and ampicillin, respectively. Figure 1.5 illustrates the reaction catalyzed by these enzymes leading to the production of 6-APA, a key intermediate in the manufacture of semi-synthetic penicillins. Besides penicillins, penicillin acylases catalyze the hydrolysis of amides, acylamino acids, and ester derivatives of the carboxylic acid side chains of penicillins. Substrate susceptibility is determined by the acyl moiety and some of the non-penicillin compounds being better substrates than the corresponding penicillins (Cole, 1964; Kaufmann and Bauer, 1964; Kutzbach and Rauenbusch, 1974). However, some recent reports on PAs have shown that they have broad substrate specificity, hydrolyzing more than one type of penicillin. PVA from *Streptomyces lavendulae* also acts on aliphatic penicillins like penicillin F, dihydroF and K (Torres et al., 2002). A newly discovered penicillin acylase from *Actinoplanes utahensis* is described (Torres-Bacete et al., 2007), which can hydrolyze various natural aliphatic penicillins and penicillin K was found to be the best substrate.

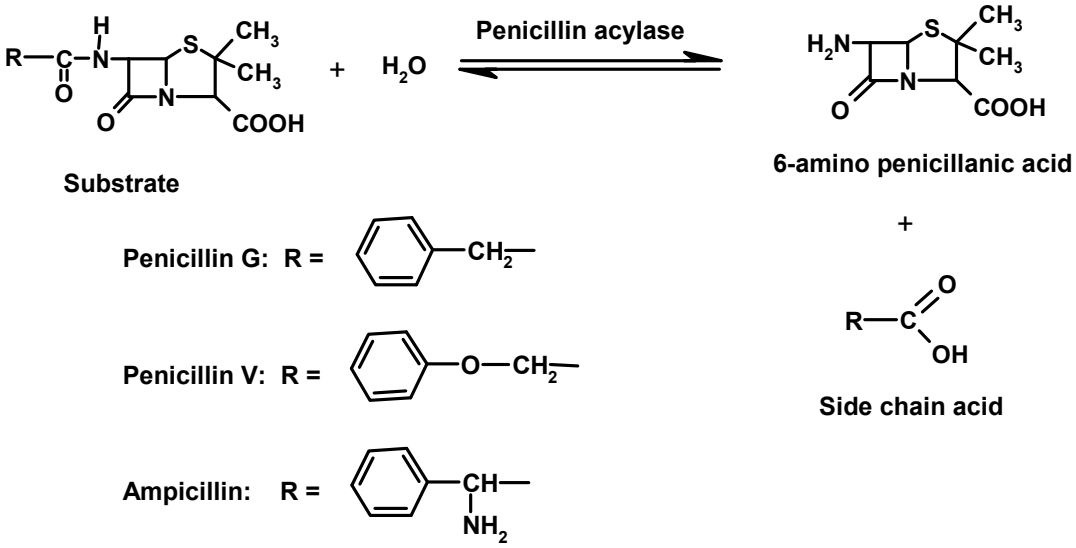


Figure 1.5: Penicillin acylase catalyzed reaction leading to the production of 6-amino penicillanic acid and corresponding side chain acid

(1) Penicillin V acylase (type I): Type I, the phenoxymethyl penicillin acylase (PVA) has little activity towards penicillin G (pen G) or other derivatives. PVA reversibly cleave the amide bond between the side chain phenoxymethyl group and the β-lactam nucleus of pen V. Earlier belief was

that PGA is produced mainly by bacterial cultures and PVA by molds (Claridge et al., 1963), but later it had been established that both PGAs and PVAs are distributed in bacteria, actinomycetes, yeasts, and fungi (Vandamme and Voets, 1974; Sudhakaran and Borkar, 1985a, 1985b; Shewale and Sudhakaran, 1997). Sudhakaran and Borkar (1985b) have presented a list of microorganisms producing penicillin V acylase. PVA occur mainly in moulds and actinomycetes, although bacterial PVA has been isolated from *Erwinia aroideae* (Vandamme and Voets, 1975), *Rhodotorula glutinis* var. *glutinis* (Vandamme and Voets, 1973), *Bacillus sphaericus* (Olsson and Uhlen, 1986) and *Pseudomonas acidovorans* (Lowe et al., 1981). Most of the PVAs are mainly intracellular enzymes. PVA from *Fusarium* sp. SKF 235 (Sudhakaran and Shewale, 1995) and actinomycete *Streptomyces lavendulae* ATCC 13664 (Torres-Bacete et *al*., 2001) are extracellular acylases. PVA from *Rhodotorula aurantiaca* is produced intracellularly (Kumar et al, 2009).

(2) Penicillin G acylase (type II): Type II, the benzylpenicillin acylase or PGA has broader substrate specificity. In addition to pen G they act on a range of N-phenyl acetyl compounds. PGA hydrolyses pen G to produce 6-aminopenicillanic acid (6-APA) and phenylacetic acids. PGA has been studied from *E. coli* (Cole, 1969; Schumacher et al., 1986), (*Kluyvera citrophila* (Barbero et al., 1986; Martin et al., 1991) *Arthrobacter viscosus* (Ohashi et al*.,* 1989), *Alcaligenes faecalis* (Verhaert et al., 1997), *Bacillus megaterium* (Chiang and Bennet, 1967; Martin et al., 1995), *Proteus rettgeri* (McDonough et al., 1999), *Achromobacter xylosoxidans* (Cai et al., 2004). Most PGAs in gram negative organisms are usually periplasmic. Gram positive bacteria do not have rigid cell wall and lack periplasmic space. PGA produced by them is secreted in the culture broth. PGAs produced by *Arthrobacter viscosus and Bacillus megaterium* are extracellular (Ohashi et al*.,* 1989; Martin et al., 1995). A thermo stable PGA from *Achromobacter xylosoxidans* has been cloned and characterized (Cai et al, 2004).

Bacterial PGAs also catalyze the reverse reaction i.e. the synthesis of penicillins from 6-APA and phenyl acetic acid and its derivatives. The study of PGA remained the basis for all research on penicillin acylases. A list of penicillin G acylase producing microorganisms is summarized in table 1.4.

Table 1.4: List of Microorganisms that produce penicillin G acylase

Organism	Reference
Escherichia sp.	Rolinson et al. (1960)
Pseudomonas sp.	Huang et al. (1960)
E. coli ATCC 9637	Kaufman and Bauer (1960)
Aerobacter cloacae	Claridge et al. (1960)
Bacillus subtilis var *niger*	Claridge et al. (1960)
Mycobacterium phlei	Claridge et al. (1960)
Nocardia F D 46973, ATCC 13635	Huang et al. (1960)
Xanthomonas sp.	Huang et al. (1963)
Flavobacterium	Huang et al. (1963)
Proteus rettgeri F D 13424	Huang et al. (1963)
E. coli NCIB 9465	Holt and Stewart (1964)
Alcaligenes faecalis BRL 1237, 1238	Cole and Sutherland (1966)
Proteus rettgeri ATCC 9919, 9250	Cole et al. (1967)
Bacillus megaterium ATCC 14945	Chiang and Bennett (1967)
E. coli NCIB 8134, 8879, 8949	Cole (1967)
Rhodopseudomonas Spheroids	Nara et al. (1971)
Streptomyces ambofaciens SPSL 15	Nara et al. (1971)
E. coli ATCC 11105	Bauer et al. (1971)
Kluyvera citrophila KY 3641	Okachi et al. (1972)
Pseudomonas aeruginosa KY 3591, KY 8501	Okachi et al. (1972)
E. coli BMN, KY 8219, KY 8268, KY 8275, KY 8289	Okachi et al. (1973)
Proteus morganii KY 4035, KY 405	Okachi et al. (1973)
Arthrobacter viscosus 8895GU	Ohashi et al (1989)
Achromobacter sp. CCM 4824	Skrob et al. (2003)
Bacillus badius	Rajendhran and Gunasekaran (2007)

(3) Ampicillin acylase (type III): Type III, ampicillin acylases act on the antibiotics with phenyglycine derived side chain, such as ampicillin and cephalexin. Ampicillin acylase from *Pseudomonas melanogenum* was first described in 1973 (Okachi et al., 1973). This enzyme has a completely different substrate range as it catalyzes both the synthesis and hydrolysis of ampicillin but shows no activity with penicillin G or V. Ampicillin acylase from *Pseudomonas melanogenum* has been purified and its properties has been studied (Kim and Byun, 1990)

(B) Cephalosporin acylases (CAs) are a group of enzymes that hydrolyze cephalosporin C (CPC) and/or glutaryl 7-aminocephalosporanic acid (GL-7ACA) to produce 7-amino cephalosporanic acid (7-ACA). Two types of cephalosporin acylases have been found: glutaryl 7-ACA acylase (GL-7ACA acylase) and cephalosporin C acylase (CPC acylase). GL-7ACA acylase has high activity on GL-7ACA but much lower activity on CPC. CPC acylase is active on both CPC and GL-7ACA. Advantage of CPC acylase over GL-7ACA acylase is that the former can be employed directly to convert CPC to 7-ACA while GL-7ACA is used in the production of 7-ACA from GL-7ACA which is derived from CPC through oxidation by D-amino acid oxidase (Isogai et al., 1990). Like penicillin acylases are useful in the production of 6-APA, cephalosporin acylases are valuable in the production of 7-ACA, which can be used to synthesize semisynthetic cephalosporins. Irrespective of the low sequence similarity between PAs and CAs, the structural homology at their active sites is impressive. However, despite this structural conservation, they catalyse very different substrates. A detailed account on various aspects of cephalosporin acylases has been reported (Kumar et al., 1993; Sonawane et al., 1993). Several genes encoding cephalosporin acylases from different sources have been isolated and cloned (Matsuda and Komatsu, 1985; Matsuda et al., 1987; Aramori et al., 1991a, 1991b; Yang et al., 1991; Ishiye and Niwa, 1992; Ishii et al., 1994). The gene encoding a cephalosporin acylase from *Pseudomonas sp*. 130 (CA-130) has been expressed in *Escherichia coli* and properties of the enzyme have been studied (Zhou et al., 1997; Li et al., 1998; Chen et al., 1998).

The semi-synthetic cephalosporins represent one of the commonly prescribed antibiotics classes. Consequently, there is huge demand for the

intermediates of semisynthetic cephalosporins, 7-ACA and 7-ADCA (Oh et al., 2003). In order to meet this need, alternative routes for the production of the intermediates are investigated to replace the currently used processes (Bruggink et al., 1998). One of these alternative routes comprises a one-step enzymatic deacylation of either the fermentation product adipyl-7-ADCA (Crawford et al., 1995) to 7-ADCA or the fermentation product Cephalosporin C (CPC) to 7-ACA by cephalosporin acylases. It is difficult to classify cephalosporin acylases based on their substrates as their physiological role and true substrates *in vivo* have not yet been clarified (Li et al, 1999). They have been divided into five types (CAI-CAV) based on their gene structure, molecular masses and enzyme properties (Li et al., 1999) and are summarized in Table 1.5.

Table 1.5: Summary of cephalosporin acylases

Type	Precursor	Signal	α-subunit	Spacer	β-subunit	Source
I	70	29aa	16	ND	54	*Pseudomonas* sp. GK16
	70	ND	16	10aa	54	*Pseudomonas* sp 130
	70	27aa	16	8aa	54	*Pseudomonas* sp C427
II	89	29aa	28	ND	61	*Pseudomonas* sp A14
	80	-	22	ND	58	*Pseudomonas* sp SE83 [acy II]
III	80	-	22	-	58	*Pseudomonas diminuta* N176
	80	-	22		58	*Pseudomonas diminuta* V22
IV	64	-	40	ND	22	*Pseudomonas* sp SE83 [acy II]
	64	-	40	ND	22	*Pseudomonas* sp V22
V	70	27aa		70		*Bacillus laterospous* J1

ND, not determined; signal, signal peptide; spacer, spacer peptide; -, no signal or spacer peptide

1.5 Ntn hydrolase superfamily

The members of the Ntn hydrolase superfamily have an N-terminal nuclephilic residue, Ser, Thr or Cys whose side chain O or S atom acts as nucleophile and its own free α-amino group acts as base in catalysis (Brannigan et al., 1995). Residue at the N-terminus serves as the nucleophile and proton donor in the catalytic process. The representative fold of this superfamily is composed of a four-layered catalytically active αββα core structure. This core structure consists of two antiparallel β-sheets packed against each other, and they are sandwiched by a layer each of α-helices on either side (Figure 1.6) (Brannigan et al., 1995). The members of the Ntn-hydrolase family consist of aspartylglucosaminidase (AGA) (Oinonen et al., 1995), glutamine phosphoribosyl-pyrophosphate (PRPP) amidotransferase (Smith et al., 1994), PGA (Duggleby et al., 1995), the 20S subunit of the proteasome (Löwe et al., 1995), glucosamine-6-phosphate synthase (Isupov et al., 1996), penicillin V acylase (Suresh et al., 1999), glutaryl 7-aminocephalosporanic acid acylase (Lee et al., 2000) and cephalosporin acylase (Kim et al., 2000). The comparison of structures between members of the family shows great variability in the number of secondary structural elements and in details of their arrangement (Oinonen and Rouvinen, 2000). Another reported characteristic of these enzymes is that the respective precursor undergoes an intramolecular autocatalytic cleavage to remove a peptide to generate the active enzyme (table 1.6 gives an over view of Ntn hydrolase family members).

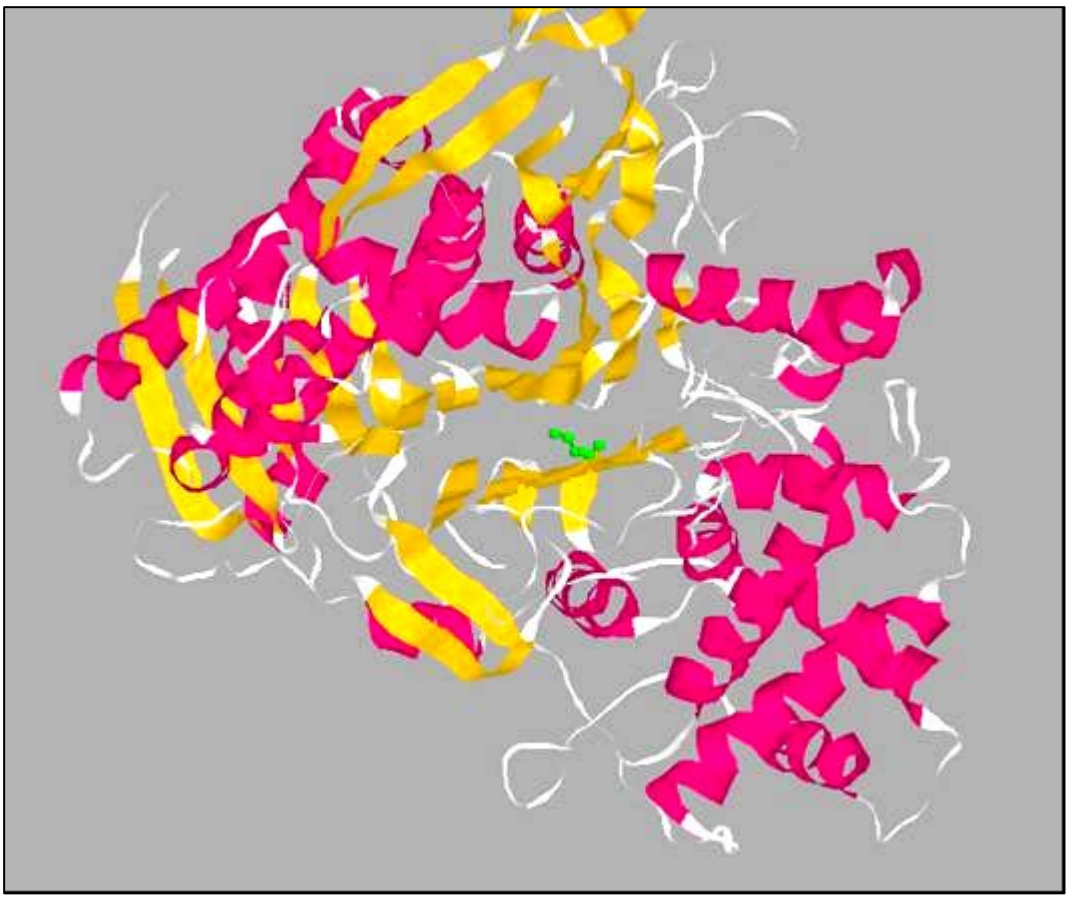


Figure 1.6 PGA molecule (PDB ID, 1PNK) showing four layered αββα core structure with the active site serine (green) in ball and stick model.

Table 1.6: Ntn-hydrolase family members (PVA: Penicillin V Acylase; PGA: Penicillin G Acylase; CA: Cephalosporin Acylase; AGA: Aspartylglucosaminidase; GAT: Glutamine amido transferase; GCA: Glutaryl 7-aminocephalosporanic acid acylase).

	PVA	**PGA**	**CA**	**Proteasomes**	**Human AGA**	**Bacterial AGA**	**GAT**	**GCA**
Structure	Homo-tetramer	αβ-dimer	αβ-dimer	7-member ring?	(αβ)2 hetero-tetramer	(αβ)2 hetero-tetramer	Homotetramer	(αβ)2 hetero-tetramer
Source	Bacterial, fungal,	Bacterial	Bacterial	Archaea yeast	Human	Bacterial	Bacterial	Bacterial
Catalytic function	Penicillin V Acylase	Penicillin G Acylase	Deacylation of Cephalosporin	Protease	Glycosyl-asparaginase	Glycosyl-asparaginase	Amidotransferase	Deacylation of Cephalosporin
Activation	Propeptide removal	Cleaveage in to subunits	Cleaveage in to subunits	Propeptide removal	Cleaveage in to subunits	Cleaveage in to subunits	Propeptide removal	Cleaveage in to subunits
WT structure PDB code	2PVA, 3PVA	1PNK, 1GK9	1FM2, 1GK0, 1GK1	1PMA	1APY, 1APZ	1AYY, 2GAW	1GPH, 1ECF	1OR0
Nucleophile	C1	S264	S1β	T1	T206	T152	C1	S170
Activation site	L2 – G1 – C1	T262-G263-S264	Q168α-G169α-S1β	L2 – G1 – T1	H204-D205-T206	H150-D151-T152	E2 – E1 – C1	Q168-G169-S170
Oxyanion hole in Catalysis	Asn175, Tyr82	A331, N504	N244β, V70β	-	T257, G258	T203, G204	N101, G102	H192, water
Precursor structure PDB code	-	1E3A	1KEH	1RYP	-	9GAA, 9GAC, 9GAW, 1P4K. 1P4V	-	1OQZ
Oxyanion Hole in Autocatalysis	Asn175, Tyr82	A332, N504	Water, H23β	-	T224	T170, water	N101, G102	N413, H192
Proposed general base in autocatalysis	-	Water	Water	Water	Water/D205	D151	Water	Water
Conformational Strain	Yes	Probable	Probable	γ-turn	Probable	Yes	Probable	Yes
Extra cleavage	-	Y260-P261	-	-	Q189-D191, E197-T198	-	-	G160-D161

1.6 Physiological role of PGA

Despite the ability of PGA to hydrolyze penicillin G efficiently, it does not have a function in bacterial antibiotic resistance, and its physiological role still remains unclear. Though, it has been hypothesized that the PGA gene is related to pathways involved in the assimilation of aromatic compounds as carbon sources. It was suggested that penicillin acylase is involved in the degradation of phenoxyacetylated compounds for the generation of phenoxyacetic acid, which may be used as a carbon source and could act as an inducer of the degradative pathway (Merino et al., 1992; Valle et al, 1991). Penicillin acylases are able to hydrolyze phenylacetylated compounds, the products that may then be fed into 4-hydroxyphenylacetic acid degradative pathways, thus enhancing the catabolic versatility of *E. coli*. Although evidence explains the role for penicillin acylase, this pathway is of little use when *E. coli* lives as a parasite, since usually richer carbon sources will be then readily available. However, when *E. coli* moves into its free living state, for example in soil, the utilization of alternative carbon sources becomes necessary. Phenyl acetic acid derivatives would be available abundantly in this kind of non-parasitic environment (Burlingame and Chapman, 1983). Broad substrate range of PGA together with the fact that its synthesis is activated by PAA has favored that PGA is a scavenger enzyme for natural compounds containing a phenylacetate or hydroxyphenylacetate residue in ester or amide linkage (Diaz et al, 2001). This hypothesis is further supported by the fact that *pac* gene encoding the PGA is located in the vicinity of the *hpa* cluster responsible for the degradation of 3- and 4-hydroxiphenylacetate in the chromosome of *E. coli* W (Galan et al, 2004)

1.7 Industrial Application of penicillin acylase

PGAs are extensively used in the production of 6-APA, the precursor for synthesis of various semi-synthetic penicillins. The natural penicillins such as benzylpenicillin (penicillin G) and phenoxymethylpenicillin (penicillin V) are used as substrates for penicillin acylases to produce 6-APA (Shewale and Shivaraman, 1989; Rajendhran et al, 2003). More than 60% of commercial 6-APA is produced enzymatically and It is estimated that more than 85% of enzymatically obtained 6-APA is from PGA activity alone and the remaining from penicillin V acylase (Shewale et al, 1989; Rajendhran et al, 2003).

Annual consumption of PGA is estimated to be in the range of 10–30 million tons (de Souza et al., 2005). Because of high industrial importance of penicillin acylase, much effort has been taken to obtain penicillin acylase from different sources with altered specificities and to increase its industrial applications (Rajendhran et al, 2003). Table 1.7 summarizes the profound applications of penicillin G acylase.

The penicillin acylase also has ability to catalyze the condensation of acyl group and 6-APA molecule for production of semi-synthetic β-lactam antibiotics. Use of penicillin acylase as catalyst in the synthetic direction was first demonstrated in 1960 by Kaufman and Bauer, who reported the *E. coli* penicillin acylase catalyzed formation of penicillin G from 6-APA and phenylacetic acid. Since then it has been used for synthesis of a variety of semi-synthetic β-lactam antibiotics. Semi-synthetic penicillin derivatives exhibit better properties such as more stability against β-lactamase, easier absorption, and fewer side effects than penicillin G or penicillin V and solve problems of microbial resistance to antibiotics [Valle et al, 1991]. Penicillin G acylase from *E. coli*, *Kluyvera citrophila, Bacillus megaterium* have been extensively used in the industrial production of 6-APA and semi-synthetic antibiotics. PGA from *Arthrobacter viscosus* been recently used for enzymatic synthesis of some cephalosporins (Terreni et al., 2007). Besides from this important application, penicillin acylase is also capable of hydrolyzing phenyl acetyl derivatives of a number of peptides, resolution of racemic mixture and enantioselective acylation (Chandel et al., 2008; Rajendhran et al., 2004). Apart from penicillin production, PAs are also used in other industries, in peptide synthesis or acyl group transfer reactions (van Langen et al., 2000b). Amidase from *E. coli* is used in the synthesis of artificial sweetener aspartame (Fuganti and Grasselli, 1986) and diphenyl dipeptides, whose derivatives are used as food additives, fungicidal, antiviral and anti-allergic compounds (van Langen et al., 2000a). PAs can be used to resolve racemic mixtures of chiral compounds such as amino acids (Bossi et al., 1998), β-amino esters (Roche et al., 1999), amines and secondary alcohols (Svedas et al., 1996).

Table 1.7: Industrial applications of penicillin G acylase

Mechanism	Industrial application	References
Peptide-synthesis (fortification of amino groups)	Production of d-phenyl dipeptides, esters applied in food additives, chitinase inhibitors, production of antifungal, anti allergic and antiviral compounds	van Langen et al, (2000b)
Resolution of racemic mixture	Chiral compounds (amino acids) removal	Fadnavis et al, (1997)
	Loracarbef, Carba cephalosporin antibiotic synthesis	Cainelli et al, (1997)
	Xemilofiban, anti-platlet synthesis	Topgi et al, (1999)
	β-Amino acids synthesis	Roche et al, (999)
Cephalothion synthesis	Cephalothin production from 7-ADCA and amide derivatives of 2-thienylacetic acid (2-TA) using PGA	Shaw et al, (2000)
Enantioselectivity (modulation of enantioselectivity of immobilized PGA)	Hydrolysis of racemic iso-propylamide of mandelic acid. Derivatives were used for the preparation of cefamandole and cefonicid	Rocchietti et al, (2002)
Enantioselective acylation	Synthesis of a series of structurally related compounds-2-aryloxy-2-aryl acetic acids together with a thioisostere derivatives	Massolini et al, (2006)
Chiral hydrolysis (hydrolysis of prochiral diethyl and dimethyl phenylmalonate)	Synthesis of +(−) methyl penylmalonate	Cabrera et al, (2007)

1.8 Screening Methods

Since the importance of penicillin acylase in hydrolyzing natural penicillin to 6-APA by Sakaguchi and Murao (1950), search for newer sources was realized. Earlier approach for screening microorganism included selective growth on mineral medium containing benzylpenicillin as sole carbon source in screening medium and testing for hydrolysis by measuring 6-APA by bioassay after treatment with phenylacetyl chloride (Kameda et al., 1961). Selective growth on corn steep liquor medium and other penicillin fermentation media have also been employed for screening penicillin acylase producers (Batchelor et al., 1961; Huang et al., 1963). Walton (1964) used chromogenic substrate, N-phenylacetyl-4-nitroanilide; a colorless compound in screening medium which produces yellow 4-nitroaniline upon hydrolysis, but this method was not very sensitive due to low solubility of the substrate. Szewczuk et al. (1980) has used paper discs saturated with phenylacetyl-4-aminobenzoic acid as a substrate for such screens. After contact with bacterial colonies on agar plates for about 30 seconds, the paper disc is treated with H-acid. Red spots are developed if enzyme activity is present. The intensity of the red spots, measured densitometrically, was reported to correlate quantitatively with the acylase activity in the bacterial colonies. The disadvantage of this method was that it involves several steps and that some solutions for color development have to be freshly prepared. Moreover, it generally worked well with bacteria capable of producing only cell-bound but not extracellular acylases.

A microbiological method for screening penicillin acylase positive cultures has been developed by adopting the method of 6-APA detection (Oostendorp, 1972) based on *Serratia marscecens* ATCC 27117 which is sensitive to 6-APA but resistant to penicillins (Meevootisom et al., 1983). A clear zone is developed around the test culture when the enzyme activity is present.

A simple and rapid assay for screening PGA producing bacteria is developed (Zhang et al., 1983). The method is based on using the NIPAB (2-nitro-5-phenylacetamidobenzoic acid) test paper, which are applied on bacterial colonies on agar plates. A bright yellow color indicates the presence of PGA activity.

1.9 Regulation of PGA production

Penicillin G acylase is regulated at both transcriptional and post-transcriptional level. There are three major regulatory mechanisms involved in penicillin acylase synthesis: phenyl acetic acid (PAA) induction, catabolite repression and thermoregulation. Benzylpenicillin acylase production by *E. coli* is stimulated in the presence of phenyl acetic acid (Kaufman and Bauer, 1960) or ammonium phenylacetate (Sikyta and Slezak, 1964). The highest productivity by cells has been reported when phenylacetate was the sole source of carbon and energy (Vojtisek and Slezak, 1975). The penicillin G acylase production is increased in presence of PAA and this induction results from relief of penicillin G acylase gene from PaaX repressor (Kim et al, 2004). Enzyme synthesis in *E. coli* is repressed at high levels of dissolved oxygen (Vojtisek and Slezak, 1975). Penicillin G acylase production is not induced by phenylacetic acid in *Proteus rettgeri* (Daumy et al, 1982). Suggested mechanism for PAA induction is that a *pac* specific repressor protein encoded by *pacR* is located inside the *pac* structural gene and transcribed in the direction opposite to that of *pac* (Merino et al., 1992)

The pac gene expression is subjected to catabolite repression by glucose. Complete catabolite repression by glucose and partial repression by acetate have been reported (Vojtisek and Slezak, 1975). cAMP stimulates enzyme production and overcomes completely repression by glucose in *E. coli* (Gang and Shaikh, 1976). The enzyme production is not subject to catabolite repression by glucose but repression of pac gene occurs due to C_4-dicarboxylic acids of Krebs' cycle such as succinate, fumarate and malate (Daumy et al., 1982). Temperature-dependent synthesis of penicillin acylase occurs in all organisms. This enzyme is produced properly when the cells are grown at suboptimal temperatures (28 °C or below) (Vandamme and Voets, 1974). Optimal temperature for enzyme production in *E. coli* was found to be between 24 °C and 28 °C, no activity was produced when grown at 37 °C (Vojtisek and Slezak, 1975). Proteolytic processing of Penicillin acylase is a temperature sensitive process. At higher temperature improper folding of enzyme protein occur which results into inactive enzyme production (Lindsay et al., 1991).

1.10 Enzyme assay procedures

Enzyme assay can be carried out based on either the substrate concentration or estimation of product. The penicillin acylase assays are generally based on determination of penicillin degradation products, 6-APA or side chain carboxylate. In recent years, chromogenic and fluorogenic substrates for penicillin acylase assays have been employed. Assay methods for the determination of penicillin acylase activity fall into two main groups.

The first group of methods is based on the quantitative determination of products formed during enzymatic hydrolysis of penicillins. The earliest procedures to assay penicillin acylase required the collection of aliquots and are multistage, laborious, and not very sensitive. Alicino described iodometric method of estimating 6-APA after removal of penicillin G by extraction (Alicino et al, 1961), requiring eight equivalents of iodine per mole of 6-APA. Method described by Bachelor et al. involved the use of penicillin substrates and paper chromatography to separate 6-APA from the substrate, treatment of 6-APA with phenylacetylchloride followed by bioassay of the benzylpenicillin (Bachelor et al, 1961). Pruess and Johnson (1965) used S^{35} labeled benzylpenicillin as substrate and the 6-APA separated by chromatography was assayed by autoradiography. Solvent extraction had been used for the separation of penicillin substrate, followed by the colorimetric assay of 6-APA with hydroxylamine (Cole et al, 1975).

Some more rapid and direct procedures include colorimetric assay of 6-APA without separation of the substrate, using p-dimethylaminobenzaldehyde (Bomstein and Evans, 1965) and titrimetric determination of side chain carboxylic acid released using a pH stat (Sjoberg et al, 1967). Assay of 6-APA with D-glucosamine (Shaikh et al, 1973), ninhydrin (Baker, 1979) and fluorescamine (Baker, 1985) have also been reported as well as the assay of phenylacetic acid by gas chromatography (Chiang and Bennet, 1967). Assay based on detection of the substrate and products by HPLC has also been reported (Daumy et al, 1982; Tewari and Goldberg, 1988)

Another colorimetric assay for penicillin acylase activity has been carried out using phenylacetyl-4-aminobenzoate as substrate, the amino benzoate released being assayed by diazotization and coupling with H-acid

(Szewczuk et al, 1980). 6-APA produced in the reaction has been assayed microbiologically with *Serratia marscescens* ATCC 27117 which is sensitive to 6-APA and insensitive to penicillins (Oostendorp, 1972)

The second group of methods assaying penicillin acylase activity includes substrates other than β-lactam antibiotics such as phenylacetyl derivatives yielding coloured products during enzymatic cleavage. These methods permit continuous monitoring of the penicillin acylase hydrolysis. Among these, the most commonly used for PGA activity is 6-nitro-3-phenylacetamido benzoate (NIPAB) (Kutzbach and Rauenbusch, 1974). An assay for penicillin G acylase based on fluorogenic substrate has been described (Ninkovic et al, 2001). The method is based on the release of fluorescent 7-amino-4-methyl-coumarin through cleavage of phenylacetyl-4-methyl-coumaryl-7-amide by penicillin G acylase.

1.11 Catalytic mechanism of PGA action

PGA from *Escherichia coli* is the most studied and used enzyme for commercial purposes (Demain, 2000; Gabor et al., 2005). The mechanism of penicillin acylase involves the formation of an acyl-enzyme intermediate as described for the serine protease chymotrypsin. The crystal structure of PGA was solved in 1995 and revealed a single amino acid catalytic centre (Duggleby *et al*., 1995). The hydroxyl group of the serine that is located at the N-terminal end of the β-subunit (Serβ1) is activated, via a bridging water molecule, by its own α-amino group and the serine oxygen attacks the carbonyl carbon atom of the substrate, forming an oxyanion tetrahedral intermediate that is stabilized via hydrogen bonds by the main chain amide of Alaβ69 and the side chain nitrogen of Asnβ241. Rearrangement of electrons leads to the collapse of the intermediate resulting in release of the leaving group and a covalent acyl-enzyme intermediate. The enzyme is subsequently deacylated by a nucleophile, which leads via a similar tetrahedral intermediate to the free enzyme and the acylation product of the nucleophile. In this way, β-lactam antibiotics can be cleaved to yield the free β -lactam nucleus and the side-chain carboxylic acid when the nucleophilic attack is performed by water (hydrolysis). However, when the acyl donor is an activated synthetic side chain (either an amide or an ester), a nucleophilic attack by a β-lactam nucleus such as 6-APA will yield a semi-synthetic β-lactam antibiotic through

a process called aminolysis. Since the discovery of the catalytic mechanism for penicillin acylase, in which an N-terminally located nucleophile is the key residue (Duggleby *et al*., 1995), several other enzymes have been found to posses a similar mechanism. These enzymes are referred to as the N-terminal nucleophile (Ntn) hydrolases and contain a typical four-layered αββα-core structure (Brannigan *et al*., 1995).

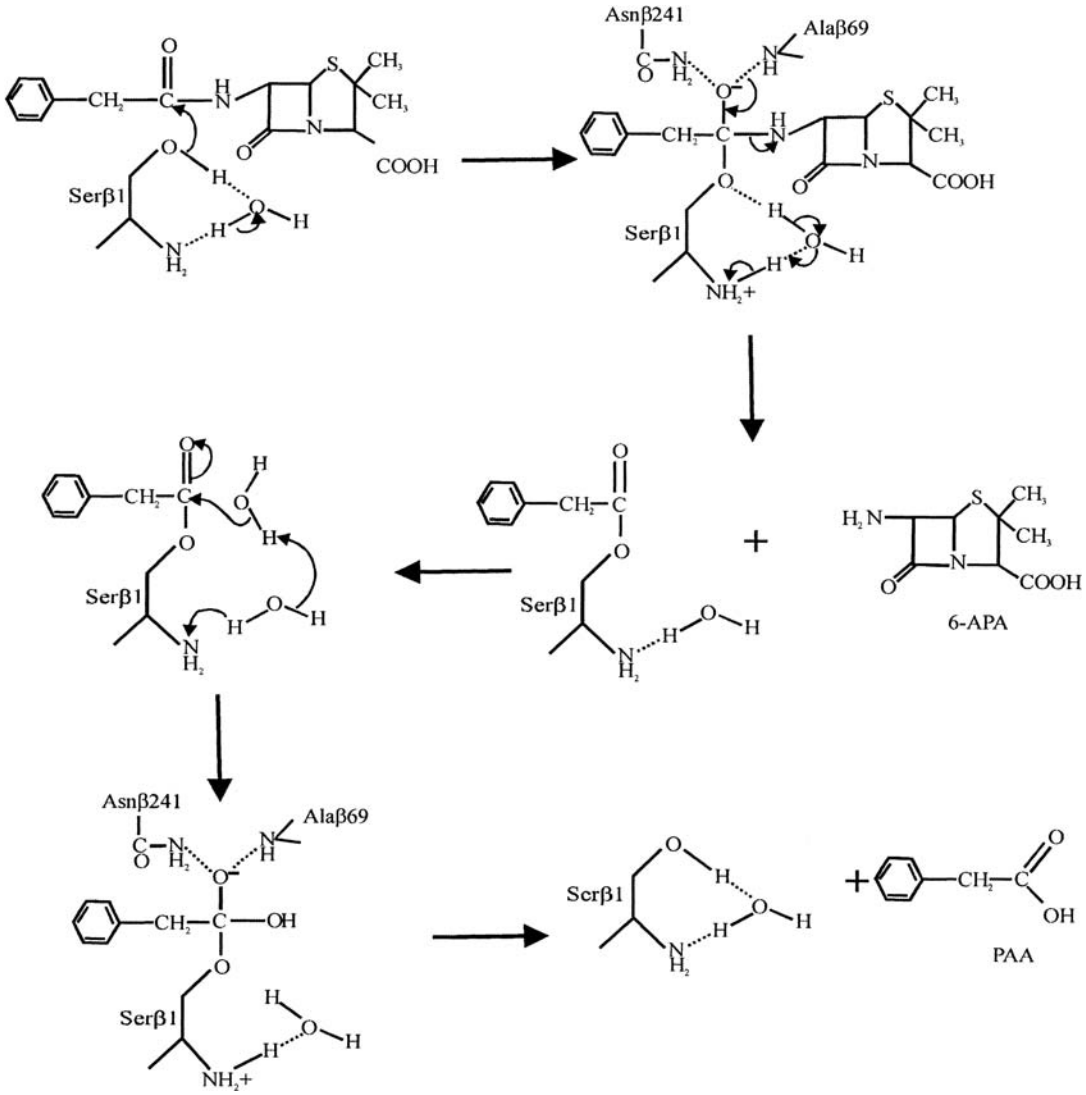


Figure 1.7: Proposed mechanism for action of hydrolysis by PGA. The carbonyl carbon atom of the amide bond of pen G is attacked by the Serβ1 and a covalent acyl-enzyme is formed through a tetrahedral transition state, which is further stabilized by the H-bonds to Asnβ241 and Alaβ69. The tetrahedral intermediate then forms a seryl-acyl enzyme and releases the 6-APA. The acyl enzyme is attacked by water to form a second tetrahedral intermediate by the same mechanism with the interaction of Asnβ241 and Alaβ69. Finally, this intermediate collapses to release the PAA. Dotted lines represent possible hydrogen bonds (Duggleby et al, 1995)

1.12 Molecular property of Penicillin G Acylase

The molecular weight and subunit structure data of the well characterized PGA are summarized in table 1.8. Penicillin G acylase is a heterodimer consisting of small α subunit and large β subunit, which are formed by processing of precursor protein (Brannigan et al, 1995; McVey et al, 2001). PGA from *E. coli* has been thoroughly investigated. In 1974, Kutzbach and Rauenbusch studied its general properties. The mature enzyme is a 80 kDa heterodimer of 24 kDa α-subunit and 64 kDa β-subunit, comprising of 209 and 566 aminoacids, respectively (Duggleby, 1995; McVey, 1997). It is produced as a 96 kDa cytoplasmic precursor pre-pro-protein. Post-translational processing requires translocation through the cytoplasmic membrane (Schumacher et al., 1986; Burtscher and Schumacher, 1992; Hewitt et al., 2000) to the periplasm using the 26-amino acid signal peptide that is subsequently cleaved off. The 54-amino-acid spacer peptide that connects the α and β chains which may influence the final folding of the chains (Oliver et al., 1985), is cleaved on the carboxyl side first between Thr263 and Ser264 (Choi et al., 1992), giving rise to the N-terminal of the β-subunit, serine, which is the active catalytic residue. However, the β-chain alone is not catalytic (Daumy, 1985). Kinetic studies showed that the autoproteolysis in PGA is intramolecular (Kasche et al., 1999). These endopeptidase cleavages require an intact carboxyl terminus. This type of processing is found in the synthesis and processing of preproinsulin and other eukaryotic hormones and is unique for a prokaryotic enzyme.

PGA from *Kluyvera citrophila* ATCC 21285 is reported to be composed of two nonidentical subunits of 23 and 62 kDa (Barbero et al., 1986), in contrast to the previous findings (Shimizu et al., 1975). Its nucleotide sequence is 80% similar to *E. coli* ATCC11105 PGA (Schumacher et al., 1986), indicating a common ancestor. *Proteus rettgeri* is an 86 kDa enzyme composed of two essential non-identical subunits. Like *E. coli* PGA, the beta subunit contained a serine residue required for enzymatic activity, and the alpha subunit contained the domain that imparts specificity for the penicillin side chain (Daumy et al., 1985; Klei et al., 1985). The enzymes from different sources had similar substrate specificity but differed in molecular weight,

isoelectric point, and electrophoretic mobility in polyacrylamide gels and did not antigenically cross-react. However, most PGAs have a similar subunit configuration, structure and substrate range as *E. coli* PGA. This indicates divergent evolution although they have evolved beyond any obvious sequence homology. *A. viscosus* penicillin G acylase has molecular weight of 81,000 and this enzyme is made up of two distinct α and β subunits with molecular weights of 24,000 and 60,000 respectively. *A. viscosus* penicillin G acylase is translated as a single precursor polypeptide consisting of signal sequence, alpha peptide, spacer and beta peptide (Ohashi et al, 1989).

Table 1.8: Molecular weight and subunit structure of some PGAs

Microorganism	Holoenzyme	Subunit		Reference
	M_r	α	β	
E. coli	85,500	20,500	65,000	Schumacher et al, (1986
K. citrophila	85,200	23,600	61,600	Barbero et al, 1986
P. rettgeri	90,000	24.500	66,000	Daumy et al, 1985
A. viscosus	81,000	24,000	60,000	Ohashi et al, 1989
B. megaterium	86,020	26,950	59,070	de Souza et al, 2005
A. xylosoxidans	NA	27,000	62,400	Cai et al, 2004
B. badius	85,000	25,000	62,000	Rajendran and Gunasekaran, 2007

NA, not available

1.13 Post-translational processing of PGA

The important rate-limiting step in the production of active enzyme is the efficiency of proteolytic processing of the precursor. The temperature dependent proteolytic processing has been already reported (Oh et al, 1987; Lindsay and Pain, 1991). Surprisingly, this processing has a lot of resemblance with the processing of some higher eukaryotic peptide preprohormones. This processing phenomenon, very rare in prokaryotic systems, is also observed in *Bradyrhzobium japonicum* cytochrome *bc,* (Trumpower, 1990) *Bacillus subtilis* spore coat proteins (Aronson et al., 1989) and *Bacillus polynyxa* amylase (Uozumi et al., 1989).

Considerable progress has been made in the understanding of post-translational modification process in prokaryotic systems (Thony-Meyer et al., 1992). This process constitutes two essential steps, the translocation of precursor to the periplasmic membrane and the processing of precursor by proteolytic cleavage. The most studied maturation pathways of PGA have been that of *E. coli* ATCC 11105. The maturation pathway of the precursor protein comprises the following steps:

i. The synthesis of precursor polypeptide (preproPGA) containing a signal peptide, α-subunit, spacer peptide and β-subunit from the pac gene.
ii. The precursor protein is transported across the cytoplasmic membrane into the periplasmic space followed by removal of signal peptide (proPGA);
iii. Proteolytic cleavage at the N-terminus of the β-subunit.
iv. Removal of spacer peptide from the α-subunit by sequential C-terminal proteolytic processing in two or three steps.

A schematic representation of the maturation pathway of *E.* coli PGA is set out in Figure 1.8. The post-translational modifications apparently are not brought about by specific processing enzymes as the *pac* structural gene yields active enzyme heterologously (Sizmann et al., 1990). For translocation, especially in *E. coli,* there is Sec translocase that selectively export the protein which contains some signature in the signal sequence (Economou, 2000). Sec system required the sec chaperone for the translocation process (Chou et al., 1999). Another major pathway identified in the protein translocation is the twin arginine translocation (Tat) pathway, completely Sec-independent and is involved in the translocation of proteins that are incompatible with the Sec translocase (Ignatova et al., 2003). For the proteolytic cleavage an enzyme DegP was identified. It is found in *E. coli* periplasm and function simultaneously as the protease and chaperone (Lin et al., 2001).

The processing of *E. coli* PGA gene has been shown to be temperature sensitive, the precursor polypeptide accumulating without production of active enzyme at 42 °C (Oliver et al., 1985). Western blot analysis with anti-β subunit serum confirmed that, at 37 °C, the PGA gene is transcribed and translated, but the precursor polypeptide becomes incorrectly folded, which could not be further processed to form active enzyme (Dai et al., 2001).

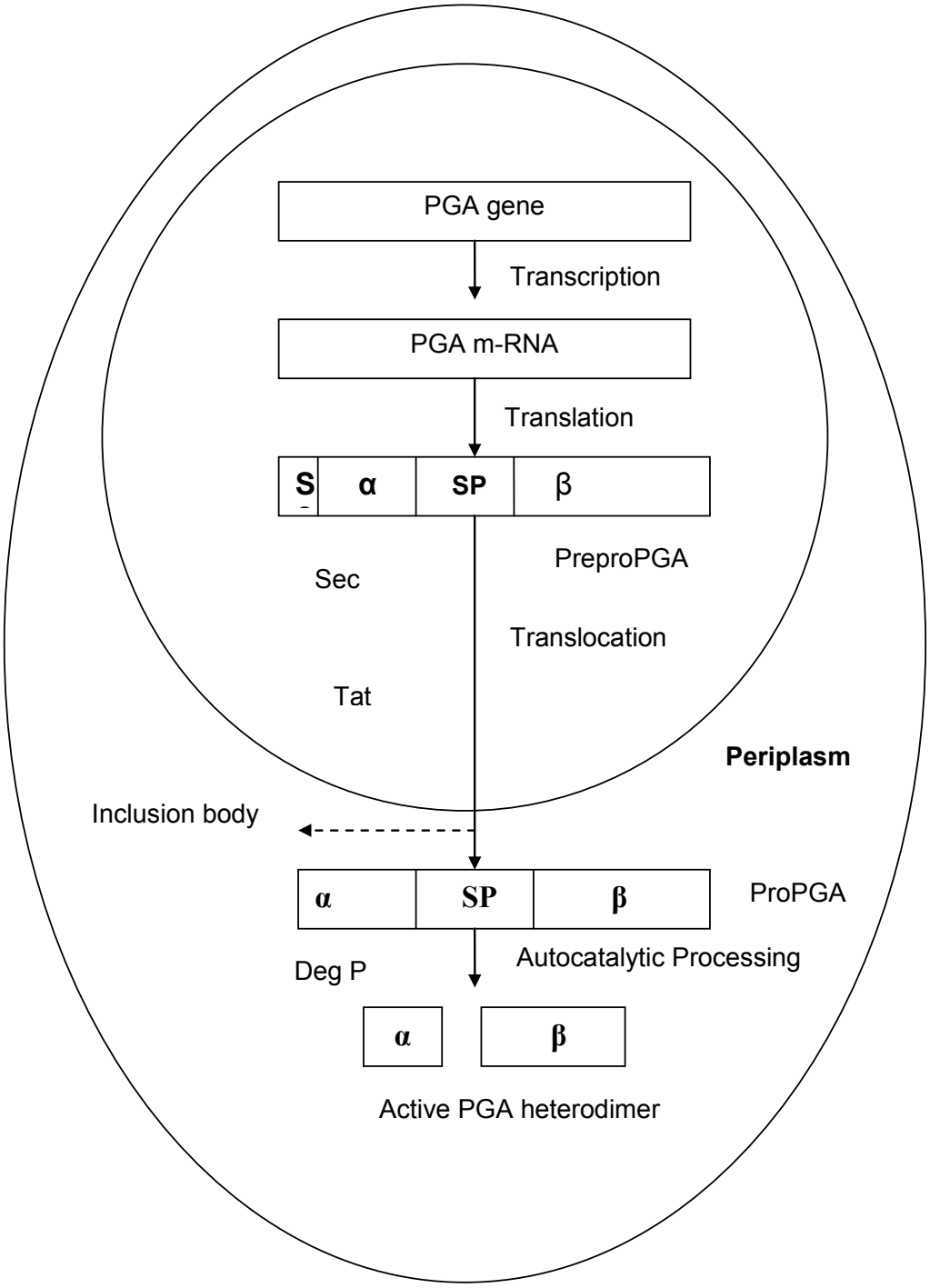


Figure 1.8: Steps involved in the Post Translational processing of penicillin G acylase precursor of *E. coli*. (S, Signal Peptide; SP, spacer peptide)

1.14 Applied aspects of Penicillin G acylases

6-APA is the precursor for all types of semi-synthetic penicillins. It can be produced either by chemical or enzymatic hydrolysis. Enzymatic route of 6-APA production has almost replaced the chemical route due to low cost and high efficiency of enzymatic conversions (~80% - 90%) (Elander, 2003) and it can be achieved by using immobilized enzymes. High cost, thermal and pH degradation, nonreusability and difficulty in recovery of the solution form of PGA have prompted immobilization of the enzyme on solid supports. All the strategies of immobilized biocatalyst technology have been applied for immobilization of penicillin G acylase. These include:

a) immobilization of microbial whole cells in or on support by entrapment, crosslinking and covalent binding,

b) enzyme immobilization by adsorption on carriers, covalent binding to supports, intramolecular crosslinking, entrapment in gels and encapsulation within semi permeable membranes.

Enzymes can be fixed to a carrier by physical forces, such as hydrophobic interaction, van der Waals binding or ionic interactions. These are generally too weak to keep the enzyme fixed to the carrier under industrial conditions of high reactant and product concentrations and high ionic strength. Covalent binding of the enzyme to a support is stable and has generally been favoured in the case of penicillin G acylase. It has been immobilized onto various matrices ranging from natural polymers to synthetic polymers and efforts are being undertaken to improve the kinetics of immobilized PGA (Kheirolomoom et al, 2002). Some examples of matrices include DEAE-cellulose beads, carboxymethyl cellulose, XAD-7, MCM-41 (Self et al, 1969; Warburton et al, 1972; Warburton et al, 1973; Carleysmith et al, 1980; Prabhune et al, 1992; Guisan et al, 1993; Fernandez-Lafuente et al,1999; He et al, 2000). Fatty lipid biocomposite films have been used for immobilization of PGA. Lipid bilayers offer biofriendly environment, flexibility and inertness and are versatile matrices (Phadtare et al, 2002). A detailed study related to various industrial scale support matrices and technologies have been reviewed by Kallenberg et al. (2005).

Basically, three methods of enzyme immobilization can be distinguished:

(i) Support binding, usually by covalent attachment, to a prefabricated carrier. The carrier can be a synthetic resin, a biopolymer or an inorganic material;
(ii) Entrapment via inclusion of an enzyme in a polymer network (gel lattice) or a membrane device such as a hollow fibre or a microcapsule. The physical restraints generally are too weak, however, to prevent enzyme leakage entirely. Hence, additional covalent attachment is generally required;
(iii) Cross-linking of enzyme aggregates or crystals, using a bifunctional reactant, to prepare carrierless macroparticles.

Cross-linked enzyme crystals (CLECs) are the latest development in the immobilization techniques which have been used for PGAs. CLECs are highly stable novel biocatalysts. It results in both stabilization and immobilization of enzyme without any dilution in activity. The protein matrix is both the catalyst and support. The technology involves the crosslinking of enzyme microcrystals (l-100 pm) with bifunctional reagents, such as glutaraldehyde. The CLEC of PGA from *E. coli* has been commercialized and has proven to work equally well in hydrolysis and synthesis of β-lactam antibiotics. Similarly another novel preparation called cross-linked enzyme aggregates (CLEAs) of PGA has been reported (Cao et al, 2001). The CLEAs of PGA are prepared by slowly adding a precipitant ammonium sulphate, 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 PGA is subjected to chemical cross-linking using glutaraldehyde and then used as the biocatalyst. Compared with conventionally immobilized PGA and CLEC, these CLEAs possess a high specific activity as well as high productivity and synthesis/hydrolysis (S/H) ratio in the synthesis of semisynthetic β-lactam antibiotics in aqueous media. Furthermore, they are active and stable in a broad range of polar and nonpolar organic solvents.

Penicillin G acylase also finds application in designing biosensors for measurement of the penicillin G content in penicillin fermentation broth. A biosensor based on an H+ ion sensitive field effect transistor (H+ (-) ISFET) and penicillin G acylase has been reported to e (Liu et al, 1998). The importance of PGA in biosensor is obvious as methods are being patented for such biosensors (Chou et al, 2009)

CHAPTER 2

Screening and Optimization of Fermentation Parameters

2.1 SUMMARY

This chapter includes the study related to methodology for screening PGA producing cultures using *Serratia marcescens* culture which is resistant to penicillin G but sensitive to 6-aminopenicillanic acid. PGA producing cultures show a clear zone of inhibition around it. Then various fermentation parameters were tried for optimum PGA production by *Arthrobacter viscosus* ATCC 15294. Nutrient broth (NB) was found optimum medium among other tested media for the PGA production. NB was used as basal medium for further parameter optimization. These parameters included testing various carbon and nitrogen sources, inducer concentration, medium pH, temperature, and fermentation time for maximum enzyme production.

2.2 INTRODUCTION

Due to excessive use of antibiotics, resistance towards these antibiotics has occurred among micro organisms. So there is need for novel penicillin G acylase producers with enhanced and altered activities to produce more potent semisynthetic antibiotics. In past few years there has been a major shift from chemical route to enzymatic route for production of the 6-APA and 7-ACA, which are major building blocks of many semi-synthetic antibiotics. They are of great importance to the pharmaceutical industry for the production of semi-synthetic β-lactam antibiotics via the key intermediates 6-APA and 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) (Parmar et al. 2000; Sudhakaran et al. 1992). Penicillin acylase with broad substrate specificity is exploited for the commercial production of semi synthetic penicillins as well as cephalosporins (Shewale and Sivaraman 1989). Apart from mainly being used for the hydrolysis of natural β-lactams, penicillin acylases are also used for catalyzing the acylation of the amino group of key intermediates by appropriate esters or amides leading to the formation of semi-synthetic β-lactam antibiotics (Hernandez *et al*. 1999). Due to the high industrial importance of PGA, efforts have been made to isolate new sources of PGA with broad substrate specificity to extend its industrial application (Shewale et al. 1990). PGA is widespread among microorganisms including bacteria, fungi, and yeasts. Most of them are bacteria, but some fungi and yeasts also produce it (Vandamme and Voets 1974). The PGAs of Gram-

negative bacteria are accumulated in the periplasmic space, whereas the PGAs from Gram-positive bacteria like *Bacillus megaterium* (Chiang et al. 1967) and *Arthrobacter viscosus* (Ohashi et al. 1989) are secreted in the culture broth.

Many methods have been developed to screen microorganisms for PGA production. A detailed survey of these methods has been mentioned in General Introduction. A widely used method for screening PGA producers is adopted by Meevootisom et al. (1983). They developed a simple and rapid method for screening bacteria producing either cell-bound or extracellular penicillin acylase. They modified a microbiological method for detection of 6-APA (Oostendorp, 1972). The microbiological method was quite sensitive and specific, and is also suitable for the detection of acylase-producing colonies in specific cases, such as a search for the transfer of the penicillin acylase gene to recipient bacteria.

For its use in industrial purposes, any fermentation needs to be optimized for its growth conditions, so that the culture produces maximum amount of the product. Growth conditions are usually optimized for carbon and nitrogen sources, pH of the medium, temperature, inoculum size to be used, time for product isolation and many more. Production of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294 was standardized by media manipulation and optimizing the fermentation parameters.

2.3 MATERIALS AND METHODS

Materials

All media components were obtained from HiMedia, India. Potassium salt of Penicillin G, different semi-synthetic penicillins and cephalosporins were gift from Hindustan Antibiotics, Pune. p-dimethylaminobenzaldehyde (PDAB) was obtained from Qualigens. All other chemicals used were of analytical grade or high purity chemicals available comercially. All media were prepared in distilled water and all buffers were prepared in glass distilled water. PDAB reagent was prepared by dissolving 0.1 gm of PDAB in 17 ml of methanol.

Bacterial strains

The following standard type cultures were obtained from National Collection of Industrial Microorganisms (NCIM): *Arthrobacter viscosus* 2451, *Proteus vulgaris* 2857, *Proteus vulgaris* 2813, *Proteus vulgaris* 2027, *Serratia marcescens* 5061. *Arthrobacter viscosus* ATCC 15294 was a kind gift from Prof. Zoya Ignataova, Germany. All these cultures were routinely maintained on Nutrient Agar slants containing 0.1% phenylacetic acid.

Methods for screening of PGA producers

The screening of the microbial cultures for PGA production was carried out in two phases.

Phase 1: Bioassay for Detection of PGA activity

The initial screening was done by the *S. marcescens* overlay technique according to the modification of the plate assay method of Oonstendrop (1972) as described by Meevotism et al. (1983). The test microorganism was point inoculated on a nutrient agar plate (2%) and incubated for about 24 to 48 hours at 28 °C. After the incubation, the plate was overlaid with 10 ml of soft nutrient agar (1%) containing 10 mg/ml pen G (w/v) and 1 ml of *S. marcescens* 5061, grown overnight in nutrient broth. The plate was then incubated at 28 °C for colour to develop. The occurrence of a zone of clearance around the test microorganism on the lawn of *S. marcescens* was used as an approximate measure of PGA production by the test culture.

Phase 2: Quantitative screening for extracellular PGA producers

Screening was done to screen the potential PGA producers and to quantitate the extracellular and intracellular production. The test cultures were grown in 10 ml of Nutrient Broth for 24-28 hours at 28 °C. An aliquot of 2 ml of the culture was centrifuged at 5000 rpm for 5 minutes. The cell bound and extracellular activity was determined by the method of Balasingham et al (1972). An aliquot of 100 µl of clear supernatant was used for the extracellular PGA activity and the pellet obtained from 2 ml culture was used for any intracellular activity. The reaction was done at pH 7.0 in 1 ml of 0.1 M phosphate buffer containing either cell or supernatant and pen G at a concentration of 10 mg/ml. 1ml buffer containing only pen G (10 mg/ml)

served as substrate blank. Suitable cell or supernatant blanks were also prepared in the same buffer for any background reading by cells or media components in the supernatant. The reaction mixtures and blanks were incubated at 40 °C in water bath. After 30 min, the reaction was quenched by addition of 1 ml of citrate phosphate buffer (CPB, pH ~2.5). An aliquot of 0.5 ml was mixed with 1.5 ml of CPB. 2 ml of PDAB reagent was added and after 2 min of incubation, the absorbance was taken at 415 nm.

PGA Enzyme assay

The enzyme activity of penicillin G acylase was determined by measuring the amount of 6-APA produced in a reaction mixture containing 10 mg/ml penicillin G in 0.1 M phosphate buffer, pH 7.0, when incubated at 40 °C (Balasingham et al. 1972). The 6-APA produced was estimated spectrophotometrically at 415 nm, after reaction with p-dimethylaminobenzaldehyde (PDAB), according to Bomstein and Evans (Bomstein and Evans, 1965), modified by Shewale (Shewale et al., 1987). One unit of PGA activity is defined as the amount of enzyme required for producing 1 µmole of 6-APA in one minute under the assay conditions (40 °C and pH 7.0)

Batch Fermentation

Inoculum was prepared by growing a single colony into 10 ml of NB medium and incubating at 28 °C for overnight at 150 rpm. Batch fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of fermentation medium inoculated with 10% v/v of seed culture at 30 °C at 150 rpm. Nutrient broth contained (gm/L): peptone, 10; beef extract, 10; NaCl, 5; pH adjusted to 7.0. In successive experiments either the nitrogen (0.3%, w/v) or the carbon (2.0%, w/v) was used. Nutrient broth was used as the basal media for various optimization parameters.

Aeration study was performed at shake flask level in 500 ml Erlenmeyer flask containing various volumes of NB medium. Fermentation was carried out by inoculating the medium with 10% of seed culture. For optimal fermentation time, samples were removed at various time intervals for PGA activity measurements. An aliquot was centrifuged and activity was measured in the clear supernatant. All other experiments were performed in

250 ml Erlenmeyer flask containing 50 ml of medium. Various concentrations of phenylacetic acid were tested for the extent of induction of enzyme. The effect of initial pH of the medium was performed by assessing the fermentation in the range of pH from 5.0 - 11.0. Time kinetics of enzyme production was performed to check the production time.

2.4 RESULTS AND DICUSSION

2.4.1 Screening of PGA producers

The results of screening for PGA producing microorganisms are summarized under the two phases as described before.

Phase 1: **Bioassay for PGA production**

The test microorganisms were screened for activity of penicillin G acylase and some are presented here. Some of them showed significant activity in phase 1 of screening. Zone of clearance produced by the bacterial culture *Arthrobacter viscosus* ATCC 15294 against lawn of *S. marcescens* indicated that test microorganism is a potential PGA producer as seen in figure 2.1.

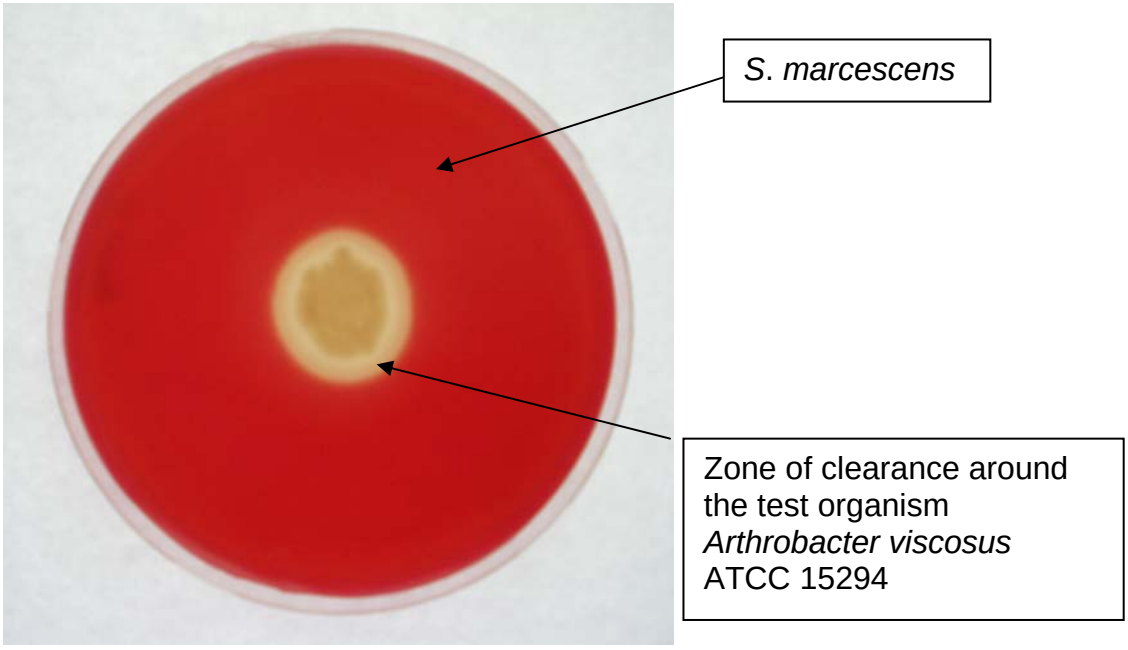


Figure 2.1: Plate assay: Zone of clearance around the colony of test organism against the red lawn of *Serratia marcescens* indicated positive penicillin acylase producer

Phase 2: Quantitative screening for extracellular PGA producers

The results obtained are shown in figure 2.2. Here, the test organisms were compared for production of PGA either intracellularly or extracellularly. The screening was done by estimating the PGA production by the PDAB assay as described before. Of all the test organisms, *Arthrobacter viscosus* ATCC 15294 showed maximum extracellular production of PGA, while *Proteus vulgaris* NCIM 2027 showed maximum intracellular PGA production. *Arthrobacter viscosus* ATCC 15294 was selected for production and further optimization of various production conditions.

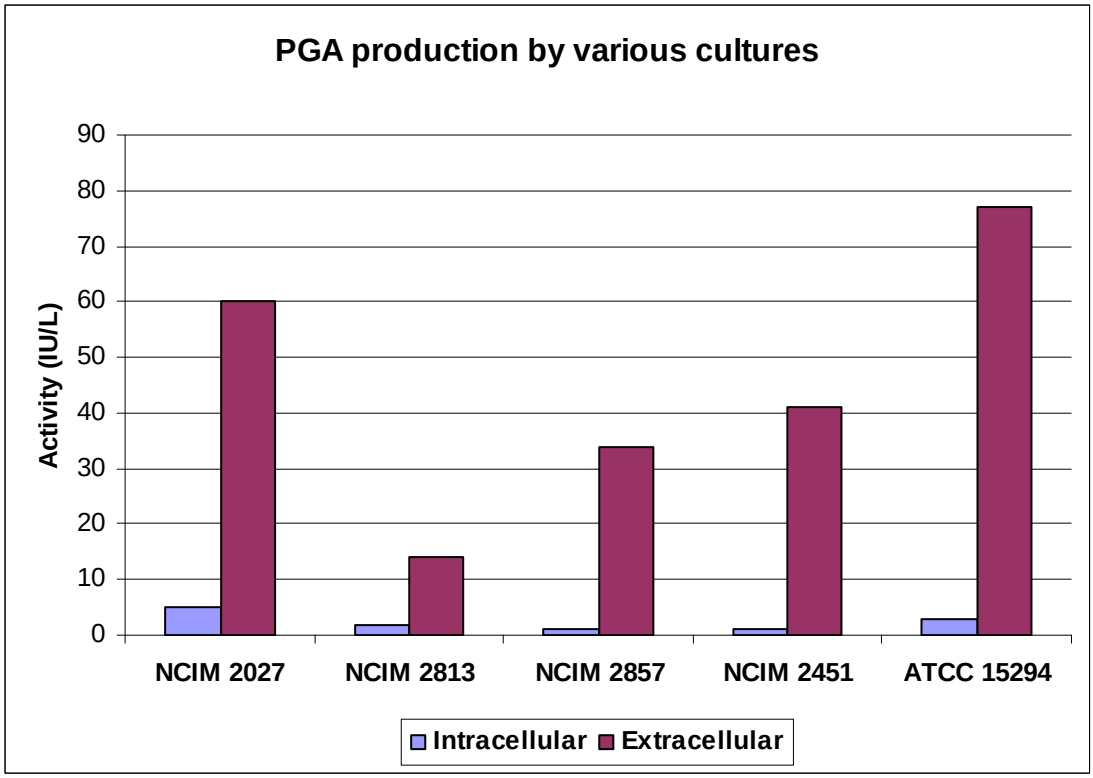


Figure 2.2: comparison of intracellular and extra cellular PGA production by various test cultures. It is clear that *Arthrobacter viscosus* ATCC 15294 produces highest level of PGA among the tested microorganisms.

2.4.2 Optimization of fermentation parameters

The effect of medium components on the growth of *Arthrobacter viscosus* was studied to improve enzyme production. Different sugars, sugar alcohols, organic and inorganic nitrogen were studied for their effect on growth and enzyme production (Tables 2.1 and 2.2). Also different concentrations of the inducer, phenylacetic acid were studied.

Effect of Carbon source

As seen in Table 2.1, best results were obtained with galactose and maltose. It was observed that galactose has the highest impact on the specific PGA production. There is 27% increase in the specific PGA production as compared to the control when no sugar added. Maltose has highest effect on increasing the cell mass but overall PGA production is low when it is added to the medium. Addition of sucrose in the medium showed an increase in the PGA activity and cell mass. Addition of mannose gave a slight increase in the activity whereas lactose showed no difference. Rajendran et al. (2003) has demonstrated that sugar alcohols show an increase in specific PGA activity in *Bacillus* sp. In *Arthrobacter viscosus*, these sugar alcohols inhibit the activity when added to the minimal medium.

Senthilvel and Pai (1996) have reported that the production of PGA by *Bacillus megaterium* was increased by sucrose and repressed by glucose. These results are in agreement with these results. Glucose and fructose showed increase in cell mass but repressed the PGA activity. These effects were also reported by Shewale and Sudhakaran (1989) where PGA production was repressed by glucose and fructose in *E. coli* ATCC 11105.

Table 2.1: Effect of various carbon sources on PGA production from *Arthrobacter viscosus* ATCC 15294

Carbon Source (20 g l^{-1})	PGA activity (u l^{-1})	Cell Dry Weight (g l^{-1})	Specific Activity (u g^{-1})
None	4543.2	5.2	874
Glucose	1185.2	7.0	169
Mannitol	493.8	4.1	120
Sorbitol	3160.5	5.6	564
Glycerol	1382.7	7.4	187
Fructose	1185.2	7.0	169
Galactose	6814.8	6.1	1117
Maltose	6814.8	8.3	821
Sucrose	5629.6	6.6	853
α-Lactose	4444.4	6.3	706
D (+) Mannose	5530.9	17.3	320
Specific activity: units per gram cell dry weight (CDW)			

Effect of Nitrogen source

The influence of organic and inorganic sources on PGA production was tested. Although the presence of nitrogen is essential for proper cell growth, supplementation of the minimal medium with various organic and inorganic nitrogen sources does not have much effect on enzyme production (Table 2.2).

Table 2.2: Effect of Various Inorganic and Organic Nitrogen source on PGA production by *Arthrobacter viscosus*

Nitrogen Source (3 g l^{-1})	PGA activity (u l^{-1})	CDW (g l^{-1})	Specific activity (u g^{-1})
None	10469.1	4.6	2276
Potassium nitrate	6814.8	5.1	1336
Ammonium sulphate	5432.1	5.2	1045
Ammonium chloride	4148.2	5.3	783
Glutamate	6024.7	6.7	899
Urea	2963.0	4.0	741
Yeast extract	6716.1	5.8	1158
Casein	9777.8	6.1	1603
Casein Acid hydrolysate	4543.2	6.1	745
Casein enzyme hydrolysate (Tryptone)	8691.4	4.8	1811
Soybean Casein Digest Medium	10567.9	4.7	2249

As shown in Table 2.2, a slight increase in PGA production was observed when soybean casein digest medium was used as a supplement to the minimal medium. On the other hand, supplementation with organic nitrogen source like tryptone, yeast extract, casein acid hydrolysate decreased the PGA production levels. This decrease was also observed when inorganic salts such as ammonium sulphate, ammonium chloride were added. Presence of urea in the medium has adverse effect on enzyme production. Rajendran et al (2003) have also reported reduction in PGA production when urea was added as a nitrogen supplement to the medium. Casein enzyme hydrolysate has been widely used for the extracellular PGA production by *Bacillus megaterium* (Savidge & Cole, 1975; Gentina et al. 1997). However it does not increase specific PGA production in *Arthrobacter viscosus*.

Effect of Phenylacetic acid

As shown in Figure 2.3, the PGA production was induced by addition of PAA to the medium. Maximum PGA production is obtained at a concentration of 0.2% (w/v). The stimulatory effect of phenylacetic acid has been shown by Kaufmann and Bauer (1960) in *E. coli*. Higher concentrations have an inhibitory effect on PGA production (Fig. 2.3).

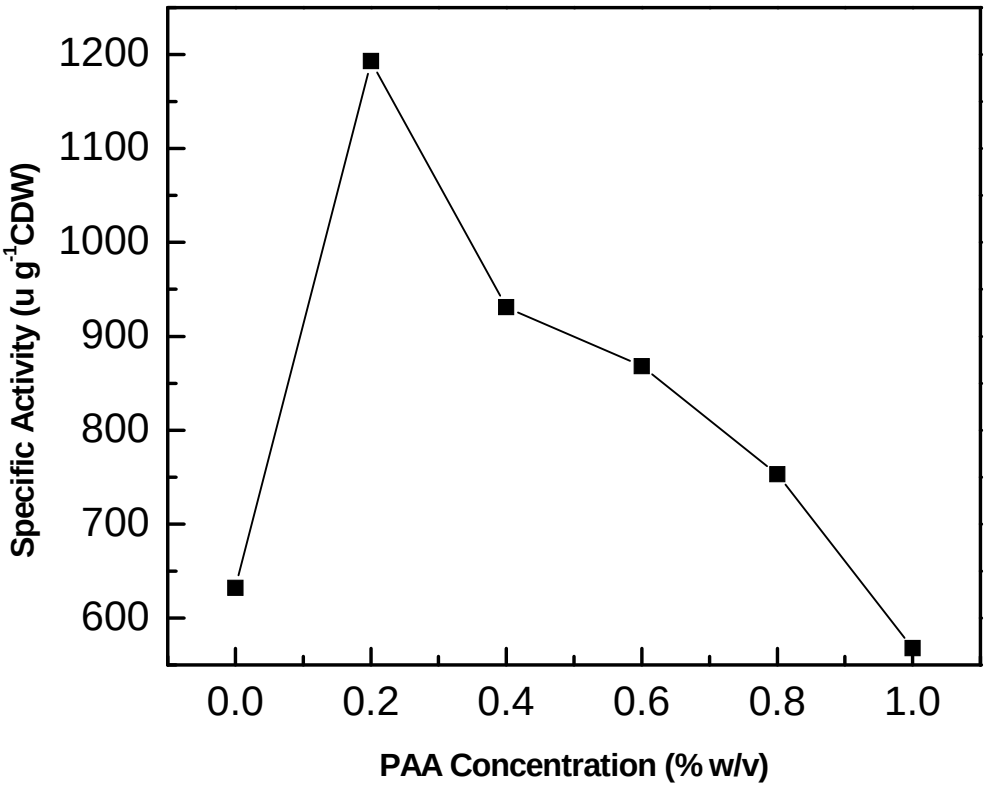


Figure 2.3: Effect of phenyl acetic acid on PGA production by *Arthrobacter viscosus*

Effect of Initial pH

As shown in the Fig. 2.4, PGA production is severely affected when the initial pH of the medium is too acidic or basic. Enzyme production was almost constant in the range pH 6.0 and 9.0 (including). Maximum enzyme production was observed when the initial pH of the medium was 7.0.

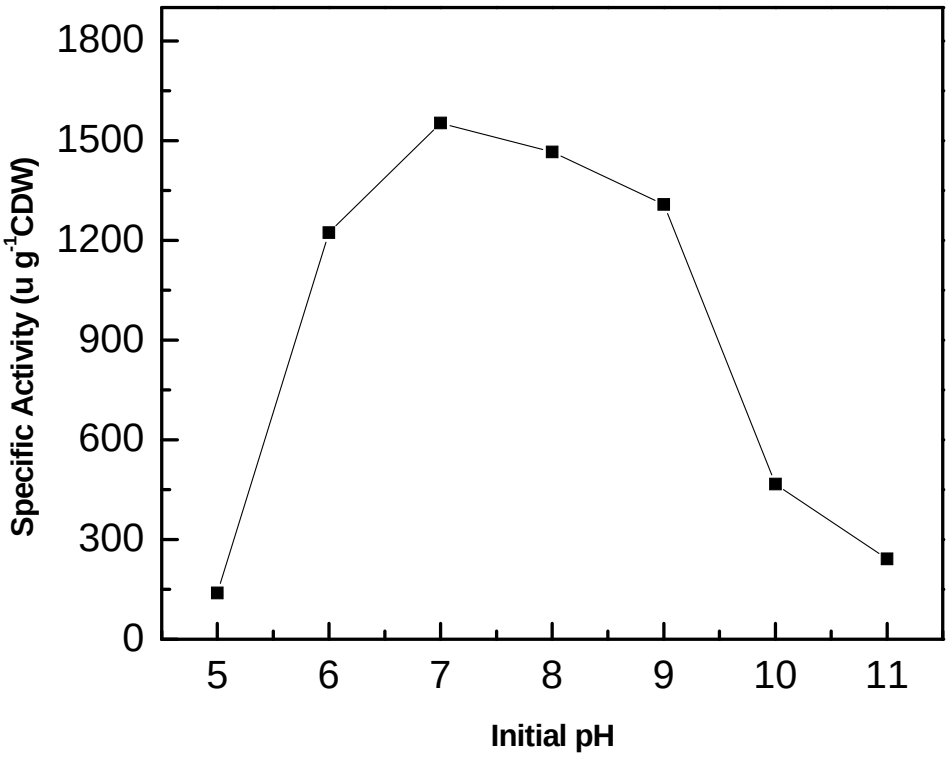


Figure 2.4: Effect of Initial pH on Penicillin G Acylase production by *Arthrobacter viscosus*

Effect of Aeration

The effect of aeration was assessed by varying the volume of culture medium in 500 ml Erlenmeyer flasks. As shown in Fig 2.5, maximum PGA production was achieved when 75 ml medium was used for enzyme production. Increasing the medium content in flasks above 75 ml decreased PGA yield.

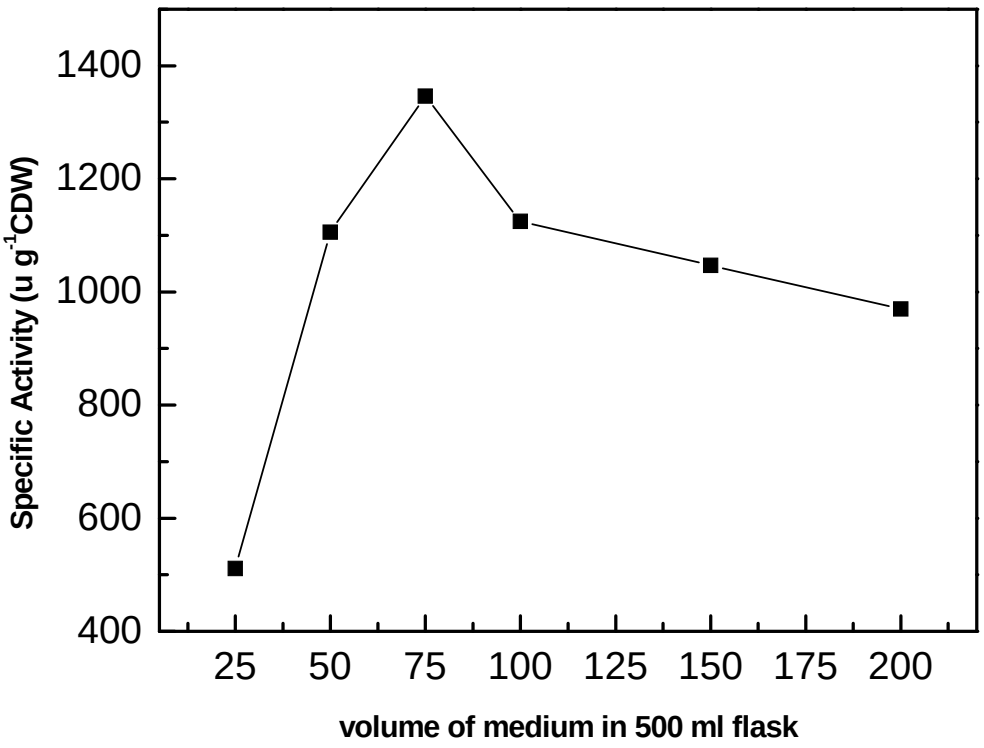


Figure 2.5: Effect of Aeration on specific activity of Penicillin G Acylase by *Arthrobacter viscosus*

Effect of Fermentation time

The culture time for maximum PGA production was assessed by fermentation till 72 hours and activity was checked at various time intervals. As seen from Figure 2.6, specific PGA activity was maximal after 48 hours till 60 hours. The activity and cell mass dropped after 60 hours.

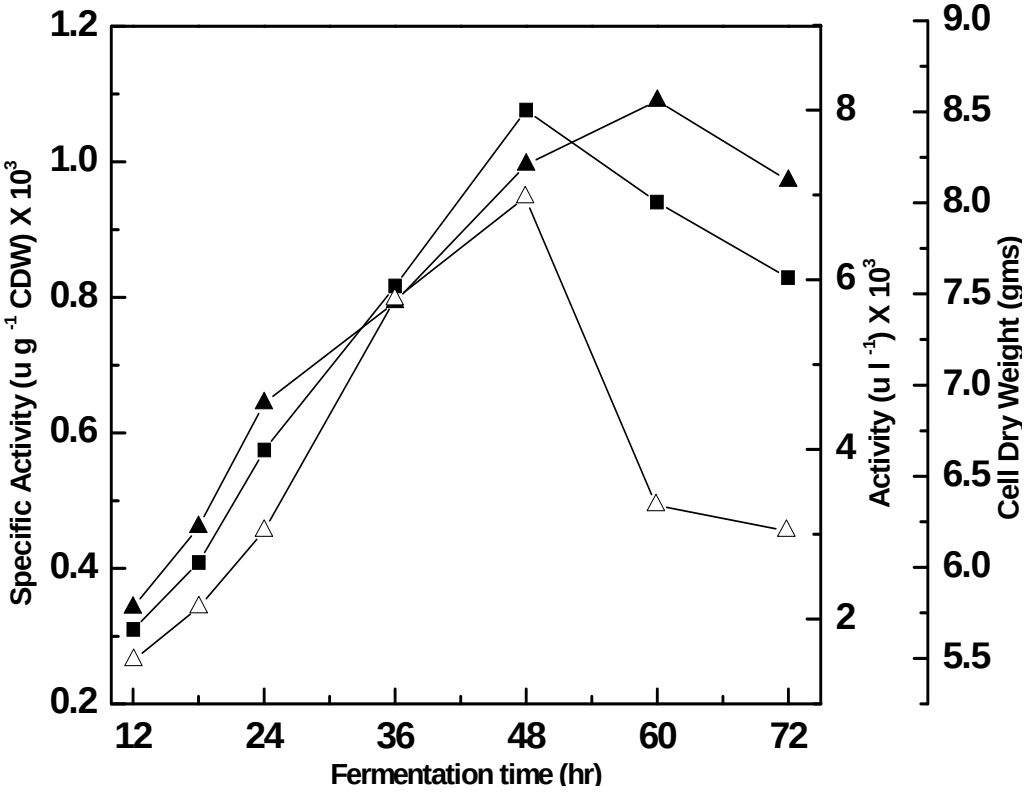


Figure 2.6: Effect of fermentation time of *Arthrobacter viscosus* on Penicillin G Acylase production (—■—), specific activity (—▲—) and cell dry weight (—△—),

Conclusion

Among the cultures tested for potent PGA producer, *Arthrobacter viscosus* ATCC 15294 shows maximus extracellular PGA production. Cell bound activity was found less in cultures. There is an advantage of extracellular enzyme production over intracellular, as it requires less operational steps (down stream processing) in enzyme purification. *Arthrobacter viscosus* ATCC 15294 was chosen for further studies. Some of

fermentation conditions were optimized for enhancing the PGA production. Phenylacetic acid was an inducer essentially required for the PGA production. Senthilvel and Pai (1996) reported that the production of PGA in *Bacillus megaterium* was increased by sucrose and repressed by glucose and fructose. In this study it was found that galactose, sucrose and maltose increased the PGA production whereas glucose and fructose repressed the PGA production.

Savidge and Cole (1975) reported the use of casein enzyme hydrolysate as complex nitrogen source for PGA production by *Bacillus megaterium*. In this study 0.3% casein enzyme hydrolysate showed slight increase in the PGA production. In sugars, galactose showed best results with 1.3 fold increase in enzyme production. Next to galactose, maltose and sucrose showed increase in enzyme units, which is in agreement with Rajendran et al (2003). Similarly phenylacetic acid, which is an inducer, showed high impact on the PGA production. 0.2% of PAA was found optimum for PGA production. Supplementation of the minimal medium with various inorganic nitrogen salts such as potassium nitrate, ammonium sulphate and ammonium chloride increased cell growth, but not PGA production. Rajendran *et al*, (2003) reported that the addition of tryptone and peptone increased the PGA activity in *Bacillus* sp. without increasing the cell growth and also increase in the specific activity. In this study both tryptone and peptone showed increase in cell mass but not in PGA activity and specific activity.

Thus optimum PGA production can be achieved by fermentation for 48-60 hours at a pH of 7.0 with an inducer concentration of 0.2%. Based on above results, *Arthrobacter viscosus* produced maximum concentration of PGA (8 U ml^{-1}, 1346 u g^{-1} of Cell Dry Weight) when the fermentation was carried out for 48 hours at pH 7.0 with 0.2% (w/v) PAA in 75 ml of medium in 500 ml Erlenmeyer flask at 150 rpm.

CHAPTER 3

Production, Purification and Biochemical Characterization of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

3.1 SUMMARY

Penicillin G acylase produced by *Arthrobacter viscosus* ATCC 15294 was produced by using optimized fermentation conditions. The enzyme is secreted in culture broth. The purification of PGA was done using the clear supernatant after the removal of cells by centrifugation. The enzyme was purified to homogeneity by using various chromatographic techniques. First clear supernatant was passed through alumina column, then elution of PGA with 24% ammonium sulphate solution at pH 8.0. Active fractions were pooled and loaded on pre-equilibrated octyl sepharose column and elution with decreasing concentration of ammonium sulphate at pH 8.0. The active fractions were pooled, dialyzed against 10 mM Tris-Cl buffer (pH 8.0) and loaded on Q-sepharose column at pH 8.0. The active fractions were re-chromatographed on the same column. Purity of the enzyme was performed by Non-denaturing PAGE and subunit molecular weight was calculated by SDS-PAGE with standard molecular weight markers. Subunit molecular weights were confirmed by MALTI-TOF mass spectroscopy. The purified enzyme was characterized for its optimum pH and temperature. pH and temperature stability of the enzyme was also performed. Substrate specificity of the enzyme was also performed to check the binding behaviour.

3.2 INTRODUCTION

Penicillin acylase (EC 3.5.1.11) is an enzyme that catalyzes the deacylation of β-lactam antibiotics; the resulting product, 6-aminopenicillanic acid (6-APA), is widely used in the production of a variety of semisynthetic penicillins. Penicillin acylase genes have been cloned from *Escherichia coli, Bacillus sphaericus, Proteuis rettgeri, Pseudomonas* sp. strain GK16, and *Kluyvera citrophila* to characterize the gene and enhance the enzyme productivity. Generally, enzymes once secreted can be easily isolated, but the above organisms do not secrete penicillin acylase. *Arthrobacter viscosus* was found to secrete this enzyme into the cultured medium. *A. viscosus*, like *E. coli*, needs phenylacetic acid (PAA) to induce penicillin G acylase (Ohashi *et al*, 1989). As described in the General Introduction, PGA is produced by a variety of microorganisms. Most of them produce PGA intracellularly. PGA from *B. megaterium* and *A. viscosus* are secreted in the culture broth and thus making the purification of enzyme easier and cost effective.

For production of PGA from *Arthrobacter viscosus* ATCC 15294, various known media were studied and nutrient broth (NB) was found to be optimum. The enzyme was then purified using chromatography.

3.3 MATERIALS AND METHODS

Materials

All media components were obtained from Hi-Media, India. Alumina matrix was obtained from SRL. p-dimethylaminobenzaldehyde was obtained from Qualigens. Octyl sepharose and Q-sepharose were from Sigma, USA. SDS-PAGE molecular weight markers were obtained from Bangalore Genei, INDIA. 30 kDa centricons were obtained from Millipore Corporation, UK. All buffers were prepared in glass distilled water. All other chemicals used were commercially available high purity or analytical grade compounds.

Enzyme assay

PGA activity was measured by the method of Bomstein and Evans (1965) as modified by Shewale et al. (1987). Potassium salt of pen G was used as a substrate. 1 ml reaction mixture contained 10 µl of enzyme and substrate at a concentration of 10 mg/ml in 0.1 M phosphate pH 7.0. 1 ml of substrate without enzyme served as a blank. The reaction mixture was incubated for 10 min at 40 °C. The reaction was stopped by the addition of 1 ml citrate phosphate buffer (CPB), pH 2.5. An aliquot of 0.1 ml was mixed with 1.9 ml of CPB and 2 ml of 0.6% (w/v) PDAB prepared in methanol was added. 6-APA was estimated at 415 nm. One unit of PGA activity is defined as the amount of enzyme required for producing 1 µmole of 6-APA in one minute under the assay conditions (40 °C and pH 7.0).

Microorganism

Arthrobacter viscosus ATCC 15294 was maintained on a nutrient agar slant with phenyl acetic acid (0.1%) added as inducer. Loop full of this culture was inoculated in 10 ml of nutrient broth and incubated at 28 °C for 24 h. This culture was used as seed culture.

Culture medium

Nutrient broth (composition: peptone (1%), beef extract (1%), NaCl (0.5%). Phenyl acetic acid at a concentration of 0.2% was added as inducer. The pH

was adjusted between 7.0 and 7.5. The media were sterilized at 15 lb pressure for 20 min.

Enzyme production under shake flask condition

Enzyme production was performed under shake flask condition in 1 L Erlenmeyer flask. Seed culture was prepared by inoculating a single colony in 10 ml Nutrient broth and incubating at 28 °C under shaking condition. Batch fermentation was carried out under optimal conditions for production of penicillin G acylase as described earlier, in 1 L flasks containing 150 ml of fermentation medium inoculated with 10% (v/v) seed culture at 28 °C with shaking for 60 hrs. After the fermentation was complete, the cells were removed by centrifugation at 5000 rpm at 10 °C. The clear supernatant obtained was used for further purification of enzyme.

Purification of PGA

Enzyme present in clear supernatant was purified by using different chromatography techniques: ion exchange on alumina column, followed by hydrophobic interaction chromatography on octyl-sepharose column, followed by ion exchange chromatography on Q-sepharose column. Purity of the enzyme was checked on non denaturing poly acrylamide gel electrophoresis (Native-PAGE). *A. viscosus* produces PGA extracellularly along with polysaccharides. The polysaccharides may block the column, so the supernatant was sonicated (80% amplitude, 10 sec on -10 sec off for 2 min) using the Branson sonicator before loading it on to the column.

A. Ion exchange chromatography on alumina column

Slurry of Alumina matrix (around 50 ml) was packed in column and then washed with distilled water. Column was equilibrated with Tris-Cl (20 mM, pH 8.0). The supernatant was passed through the column packed with alumina column to bind PGA and column was washed with Tris-Cl buffer (20 mM, pH 8.0), until the effluent was clear. The enzyme was eluted with 24% ammonium sulphate solution. Fractions of 5 ml each were collected and 10 µl from each fraction was tested qualitatively for enzyme activity.

B. Hydrophobic interaction chromatography on octyl-sepharose column

Active fractions from alumina column were pooled and directly applied to octyl-sepharose column pre-equilibrated with 24% ammonium sulphate

solution. It was washed with 24% ammonium sulphate solution and enzyme was eluted with Tris-Cl buffer (10 mM, pH 8.0). Active fractions were collected and subjected to overnight dialysis against Tris-Cl buffer (10 mM, pH 8.0).

C. Ion exchange chromatography on Q-sepharose column

The dialysate was applied to Q-sepharose column pre-equilibrated with 10 mM Tris-Cl buffer for binding the PGA. After that it was washed with 2 bed volumes of the 10 mM Tris-Cl buffer. Stepwise elution was carried out by 0.1 M, 0.15 M and 0.2 M NaCl solution. Alternate fractions were checked for any enzyme activity and it was found that enzyme eluted in 0.2 M NaCl solution. Active fractions were collected and subjected to dialysis (against Tris-HCl, 10 mM pH 8.0). The purity of enzyme was checked on 10% Native-PAGE and the enzyme was concentrated and stored at -20 ^{o}C and used for further characterization.

Polyacrylamide gel electrophoresis

Samples were analyzed on native polyacrylamide gel electrophoresis (native-PAGE, 10 %) according to Laemmli (1970) to check the purity of enzyme eluted from Q-sepharose column. Native PAGE was carried out in slab gels using Tarson PAGE apparatus with 1 mm spacers. Sample (10 µl) of protein was loaded in the well, on the top of the gel, and electrophoresed applying voltage 100 V for 1.5 h. Protein bands were visualized by staining the gel using silver staining method according to the procedure described by Rabilloud et al. (1994).

Protein estimation

The content of protein in the sample was estimated by the method Lowry et al. (1951) using crystalline bovine serum albumin as standard.

Properties of PGA from *Arthrobacter viscosus* ATCC 15294

1. pH profile:

To determine the optimum pH of the enzyme, activity was determined at 40 ^{o}C in the pH range 6 to 8.5 with 0.5 increments using 0.1 M buffers: phosphate buffer for pH 6.0 and 7.5 and Tris-Cl buffer for pH 8.0 and 8.5. The substrate, penicillin G was dissolved in different buffers as mentioned above (20 mg/ml concentration) and then standard enzyme assay was carried out.

2. pH stability:

For pH stability, the enzyme was incubated in 0.1 M buffers of pH range 4.0-10.5, using different buffers for 2 h at 25 ^{o}C. Residual activity was measured at intervals: 1 hour and 2 hours by standard enzyme assay.

3. Temperature profile:

For determination of the optimum temperature of PGA, activity was measured at different temperatures in the range of 25-70 $^{\circ}$C at pH 7.0 in 0.1M phosphate buffer.

4. Temperature stability:

For the thermostability, the enzyme was incubated from 25 to 70 ºC up to 1 h in 0.1 M phosphate buffer pH 7.0 and residual activity was estimated after every 15 mininterval.

5. Substrate specificity:

For determination of substrate specificity, 5 mg/ml of various substrates containing amide bonds such as penicillin G, penicillin V, ampicillin, amoxicillin, cephalosporin C, cephalosporin G, carbanicillin, cephalexin, and cephaclor were used as substrates for activity measurement by standard enzyme assay.

6. Enzyme kinetics:

The partially purified PGA preparations were characterized kinetically. Kinetic studies were carried out at pH 7.0 and 40 °C. The enzyme was incubated with various concentrations of the substrate (up to 20 mM) and the V*max*, K*m* values were calculated by plotting the graph of V_o (initial rate, µmole of 6-APA produced per min) against the substrate concentration.

For inhibition studies of PAA on PGA, the reactions were carried out in presence of various concentrations of PAA up to 10 mM. The reciprocal plot (Lineweaver-Burk plot) was drawn with the help of data to confirm the type of inhibition.

Molecular Weight Determination

A) SDS-PAGE

Subunit molecular weight of the enzyme was determined by running SDS-PAGE slab gel electrophoresis [Laemmli 1970] using Tarson SDS-PAGE apparatus with 1 mm spacers and samples electrophoressed alongside lower range molecular weight marker proteins comprising phophorylase b (97,000),

bovine serum albumin (66,000), ovalalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (20,000) and lysozyme (14,300). The enzyme sample (20 µL) was mixed with 5 µl SDS-PAGE denaturing dye of the following composition: (5x) 1M Tris. HCl 0.6ml, 50% glycerol 5ml,10% SDS 2ml,1% BPB 1ml, H_2O 0.9 ml, 2 mercaptoethanol 0.5 ml. Enzyme sample was heated in a boiling water bath for 2 minutes and loaded in adjacent wells in a 10% SDS-PAGE gel. Protein bands were visualized by silver staining method as stated earlier in this chapter.

B) MALDI-TOF (Matrix assisted laser desorption/ ionization time of flight) mass spectrometry

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A nitrogen laser (337nm) was used for desorption and ionization. Spectra were acquired in the range of 10 to 100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25 kV. The low mass ion gate was set at 4500 Da. The instrument was calibrated with myoglobulin and bovine serum albumin. 5 µl of enzyme (500 µg/ml) was mixed with 35 µl of matrix solution. The matrix solution of 15 mg/ml sinapic acid was prepared in 30% ACN containing. 10 µl of the mixture containing enzyme was applied to a stainless steel sample holder and introduced into the mass spectrometer after drying.

3.4 RESULTS AND DISCUSSION

Purification of PGA

The penicillin G acylase from *A. viscosus* ATCC 15294 was purified successfully by ion-exchange chromatography (alumina and Q-sepharose) and hydrophobic interaction chromatography (octyl-sepharose). This is summarized in Table 3.1. The purification fold (ratio of specific activity to the initial specific activity) obtained was 5.22 and yield (ratio of total enzyme activity to initial total activity) was 58.39%.

The purification of the enzyme was confirmed by loading the active fractions from Q sepharose column on a Native PAGE gel (10%) as seen in Figure 3.1. On staining the gel by the procedure of silver staining, one prominent band of the enzyme was seen in the successive fractions indicating proper purification of the protein. The pure fractions obtained were pooled and dialyzed against 10 mM Tris-Cl buffer containing 0.1 M NaCl and the dialyzed

sample was concentrated using 30 kDa centricon tubes. The enzyme was stored in fridge for further characterization.

Table 3.1: Chart showing purification of PGA from *Arthrobacter .viscosus*

Purification step	**Total Activity (IU)**	**Total Protein (mg)**	**Specific Activity (IU/mg)**	**Fold Purification**	**Yield (%)**
Eluent from 24% Ammonium sulfate	117.28	84.96	1.380	1	100
Octyl-sepharose	72.64	21.92	3.313	2.4	61.39
Q-sepharose	68.49	9.509	7.209	5.22	58.39

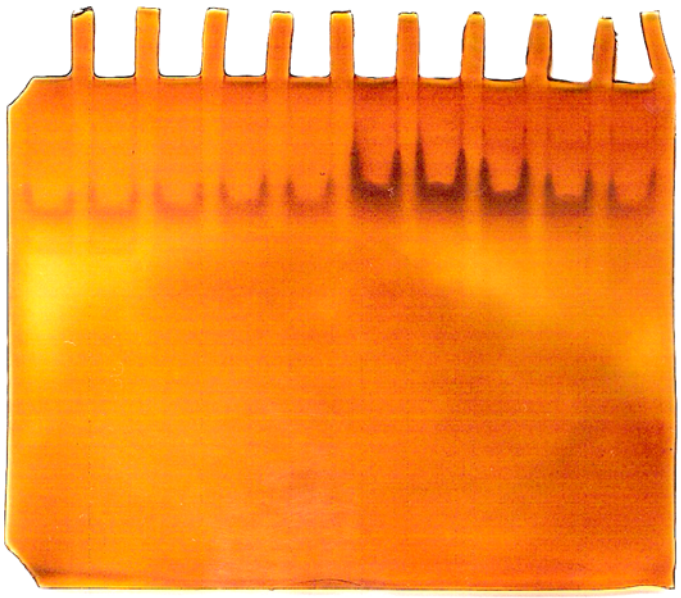


Figure 3.1: Native-PAGE of active fractions collected from Q-sepharose column.

Enzyme characterization

Subunit Molecular weight determination by SDS-PAGE

Purified PGA was loaded on SDS gel along with standard markers (Figure 3.2). The gel was silverstained and the molecular weight of the subunits was determined by Rf values calculated. The molecular weights of the subunits were found to be 26,828 for alpha subunit and 61,202 for beta subunit. This was further confirmed by the MALDI-TOF spectrophotometry which shows peaks at 26,491 and 60,722 (Figure 3.3)

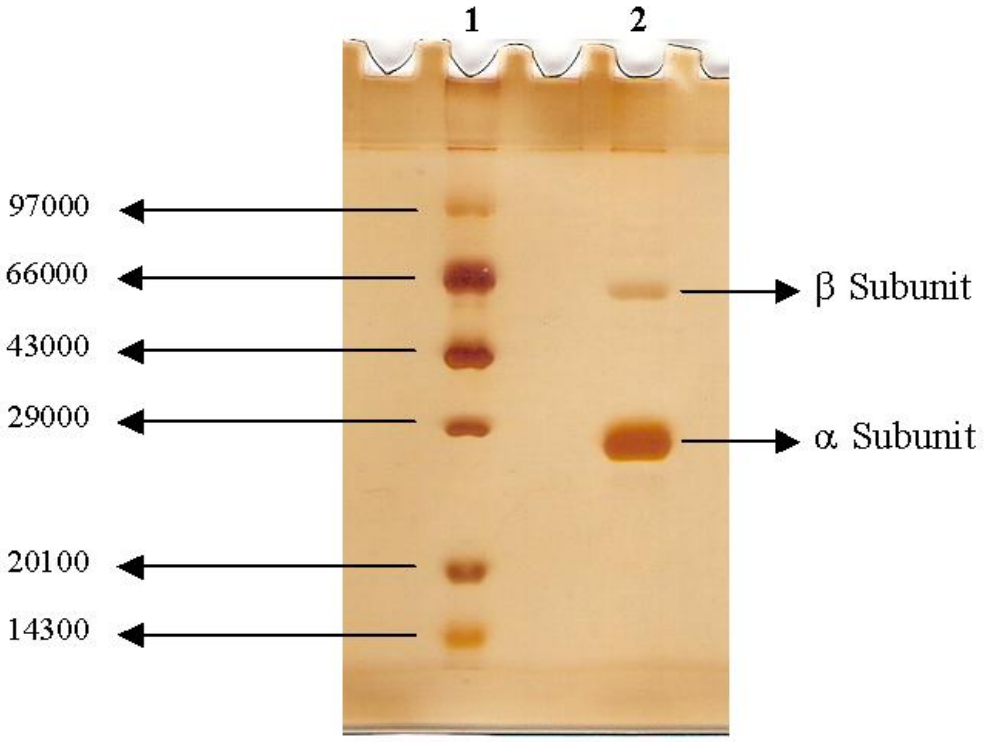


Figure 3.2: SDS-PAGE of purified PGA with molecular weight markers.

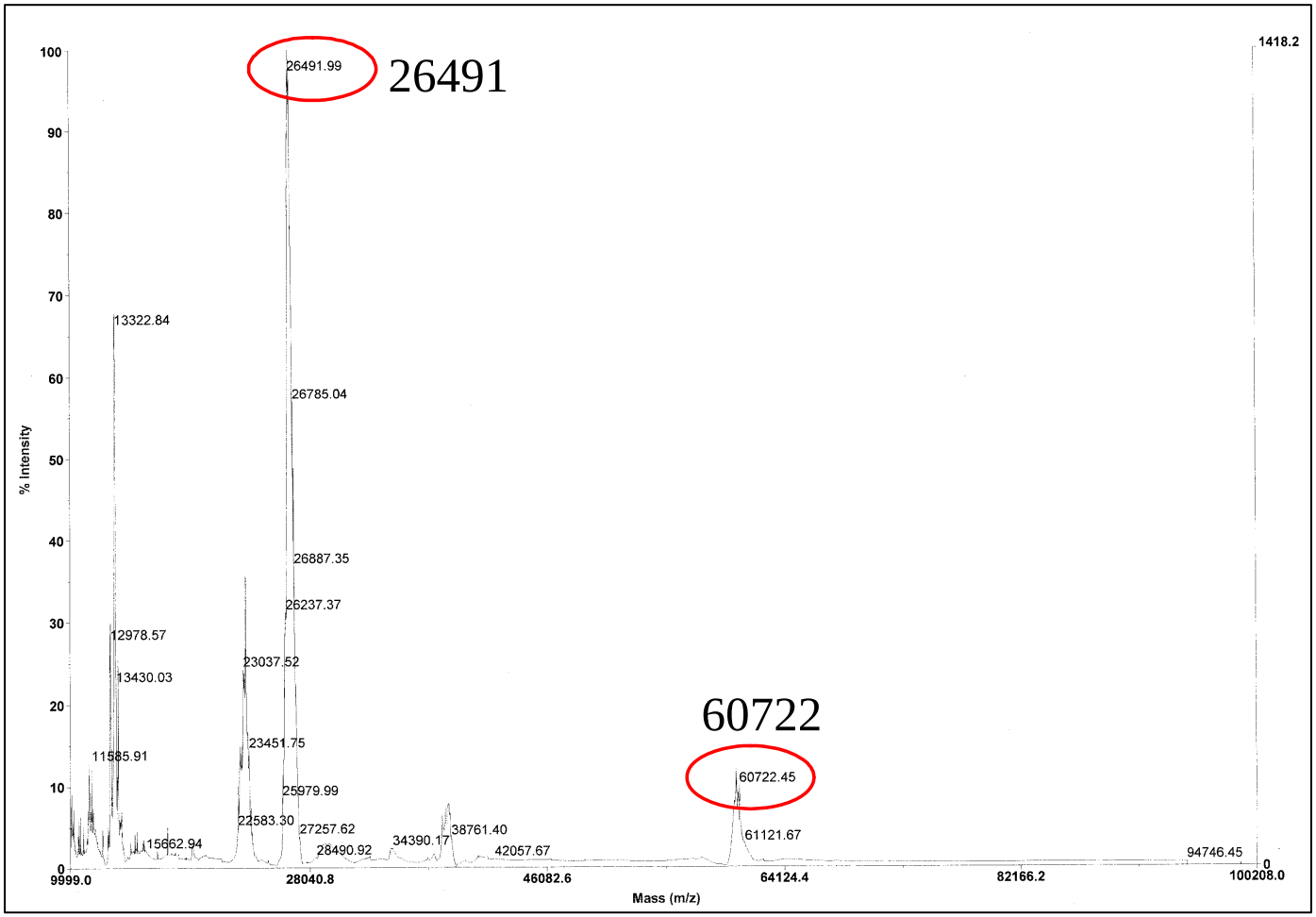


Figure 3.3: MALDI -TOF mass spectrum of Purified Penicillin G Acylase of *Arthrobacter viscosus*

1. pH profile and pH stability

The majority of the enzymes present a characteristic pH value where its activity is maximum. Above and below this pH, the activity reduces. The enzyme's three-dimensional structure, responsible for its catalytic activity, is stabilized by a hydrogen bond, hydrophobic interaction and a di-sulfide bond. Altering the hydrogen concentration modifies the equilibrium of these forces, reversibly or irreversibly deactivating the enzyme. Other factors where enzymatic activity is related to pH depend on the enzyme's acid and alkaline behavior and the substrate itself. Generally just one of the ionic substrate forms is accepted by the enzyme and its concentration (neutral or charged form) depends on the pH. Still, if an amino acid residue is directly involved in the catalysis, the charged or neutral form of this residue will be acting (Vanessa Ribeiro de Souza *et al,* 2005).

The ability of PGA from *A. viscosus* to hydrolyze substrate was measured in the range of pH 6-8.5 with 0.5 increments. The highest hydrolytic activity of PGA was observed at pH 7.5 (Figure 3.4). The stability of PGA was studied and it was found that the enzyme was quite stable over a wide range of pH (Figure 3.5).

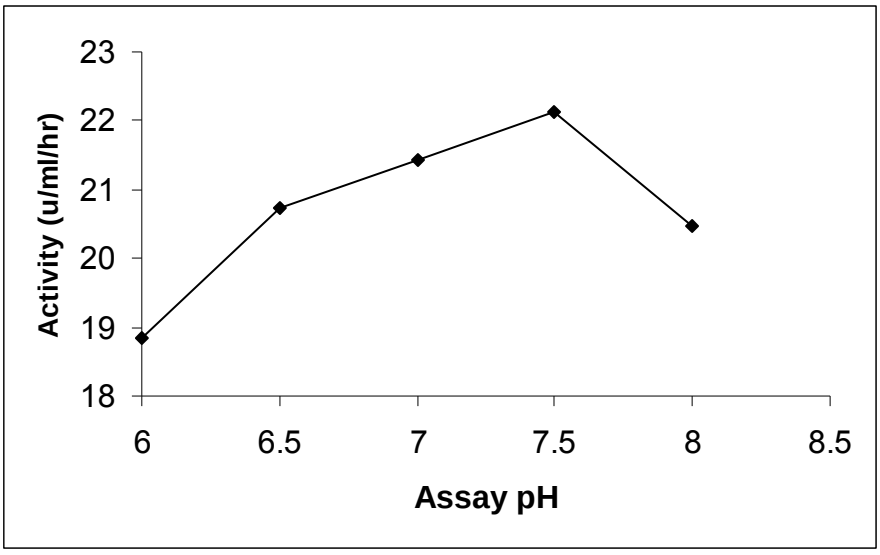


Figure 3.4: pH profile of the purified PGA. The highest activity is observed at pH 7.5

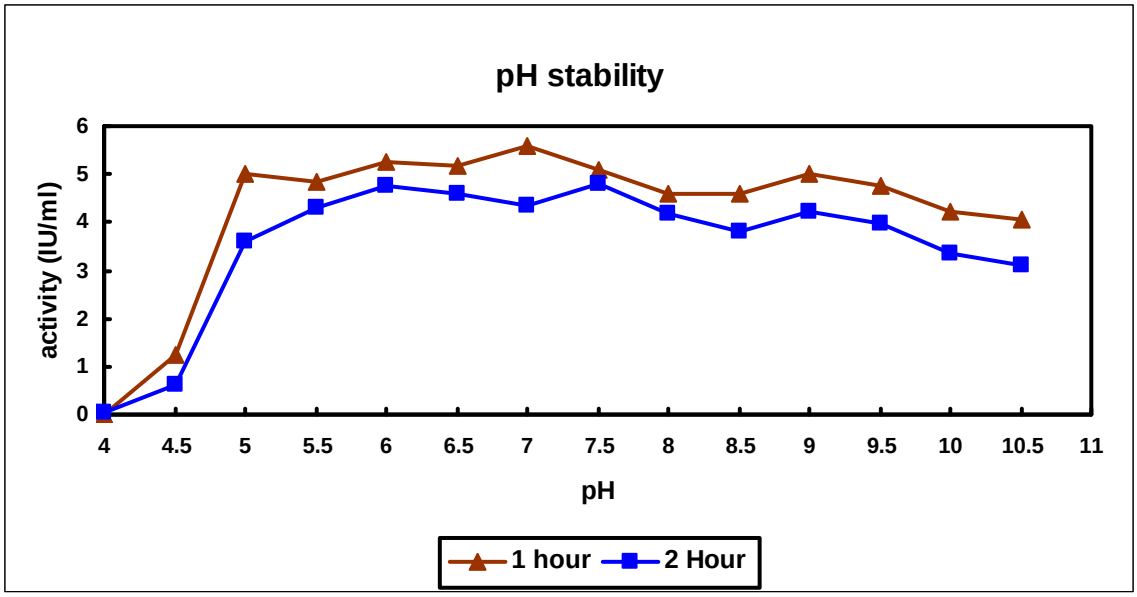


Figure 3.5: pH stability of the purified PGA from *Arthrobacter viscosus*

2. temperature profile and stability

With respect to the temperature effect, as occurs with all chemical reactions, the reaction velocity catalyzed by enzyme increases exponentially with temperature increase over a determined range in which enzyme is stable. Since enzymes are fragile protein structures projected to operate at physiological temperatures, when used at temperature above 37ºC, they lose their activity due to the denaturing of various enzyme molecules present as the temperature or the reaction time increase. Figure 3.6 shows the optimum temperature for PGA from *A. viscosus.* The optimum temperature was found to be 55°C. The temperature stability of PGA was studied from 25 to 70 degree centigrade with 5 degree increments up to 60 min and it was found that the enzyme retains its activity over a wide range of temperature. It retained its activity up to 50 degrees when kept for 1 hour. Above this temperature, the activity reduces sharply and it is almost nill at 60 °C and above (Figure 3.7). The results are a good indication for its industrial importance.

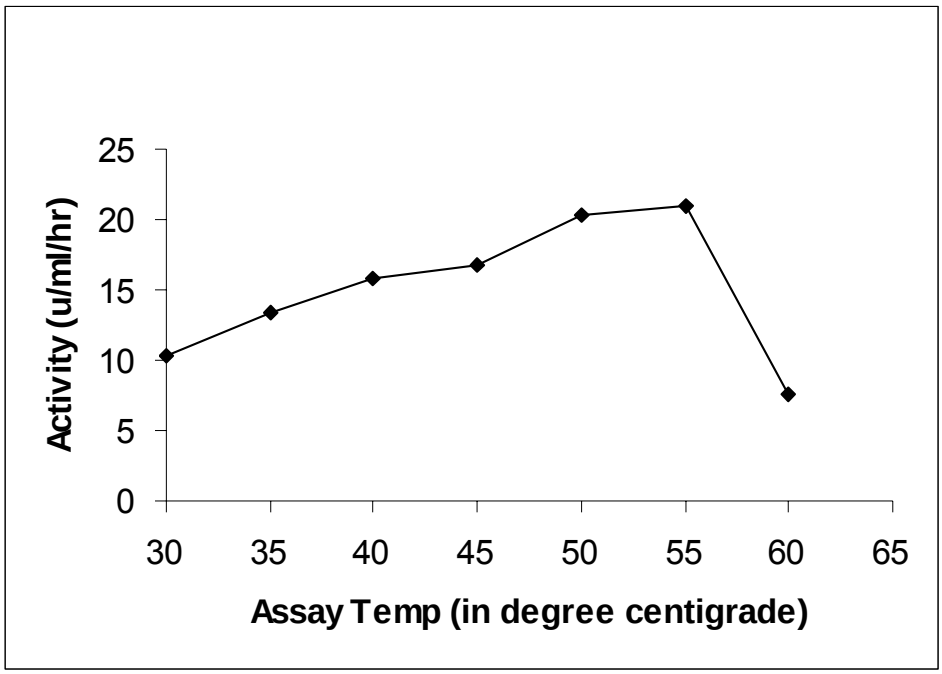


Figure 3.6: Temperature profile of the purified PGA from *Arthrobacter viscosus*

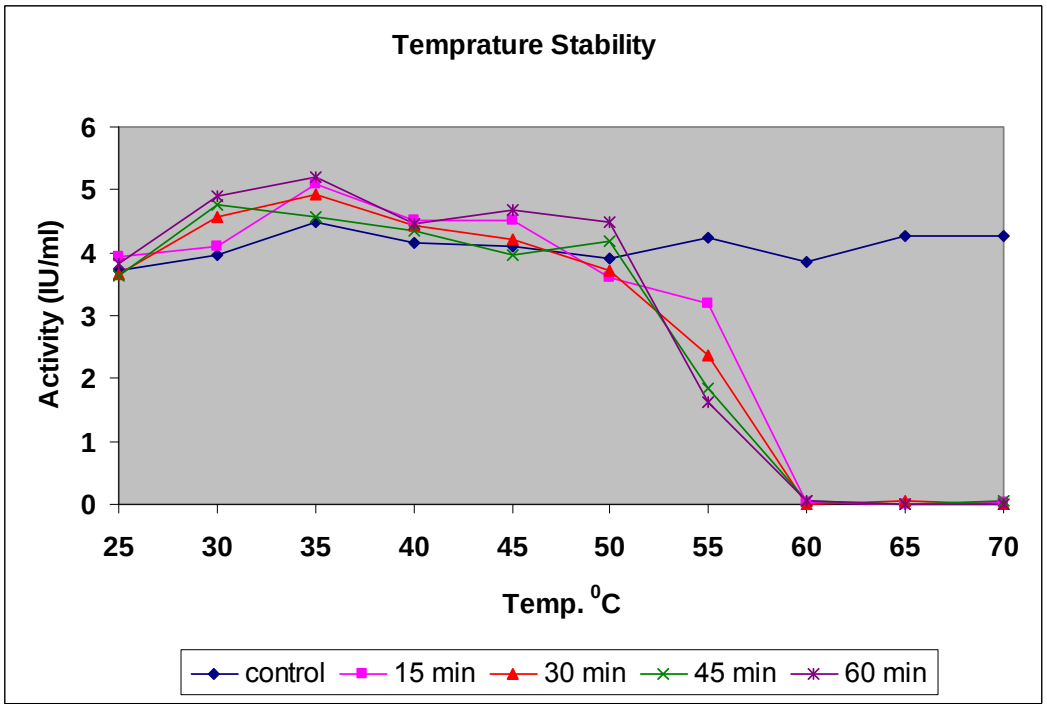


Figure 3.7: temperature stability of the purified PGA from *Arthrobacter viscosus*

3. Substrate specificity:

The purified PGA from *A. viscosus* was examined for its ability to hydrolyze natural and semi-synthetic β-lactam antibiotics under the standard conditions. The data is represented in Figure 3.8**.** Activity with pen G was considered 100% and then relative activities with other substrates were calculated. It shows that the PGA from *A. viscosus* showed a little bit greater affinity towards cephalosporin G (ceph G), as both ceph G and pen G share similar side chains (hydrophobic phenyl groups). Activity of PGA in hydrolyzing other antibiotics was lower. This may be due to structures with acyl side chains other than PAA are poor substrates for PGA.

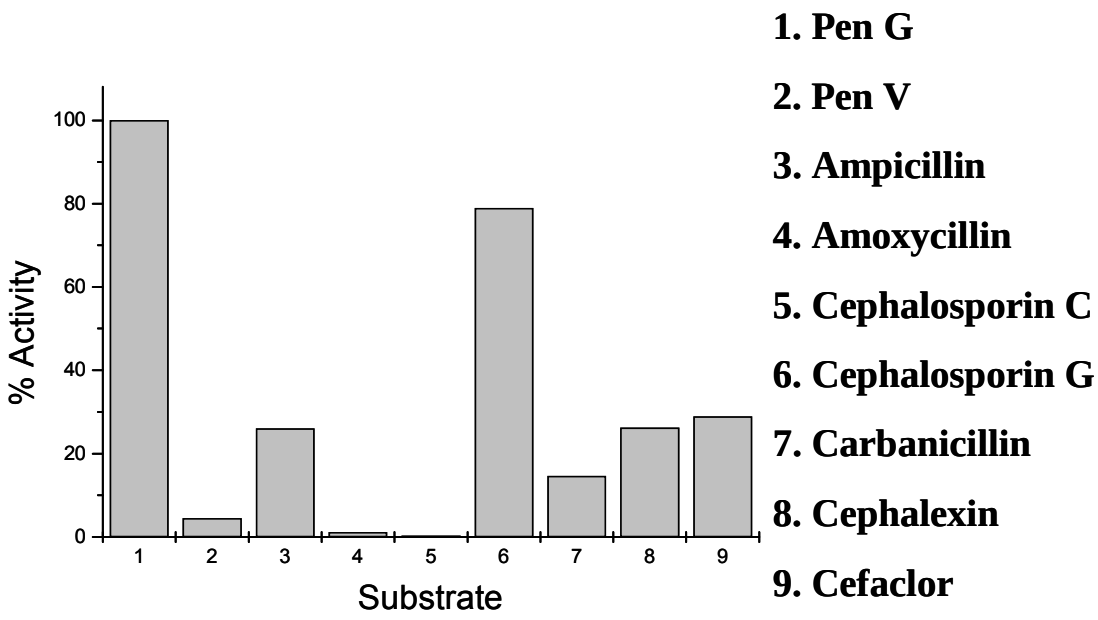


Figure 3.8: Substrate specificity profile of PGA from *Arthrobacter viscosus*

4. Enzyme kinetics:

The saturation kinetic studies were studied at 40 °C, pH 7.0. The Michaelis-Menten constant (*Km*) with pen G as substrate was determined by incubating the enzyme at various concentrations of pen G (up to 20 mM) under standard assay conditions, then fitting a linear regression curve to data points using Lineweaver-Burk plot.

PGA activity increased linearly with increase in substrate concentration up to 10 mM (Figure 3.9). Michaelis-Menten constant (K*m*) for purified penicillin G acylase was calculated from Lineweaver-Burk plot (Figure 3.10) and found to be 0.56 mM, the V*max* was found out to be 0.036 µmole/min. Inhibition kinetics study showed that the enzyme is competitively inhibited by phenyl acetic acid with an inhibition constant Ki equal to 0.86 mM (Figure 3.11).

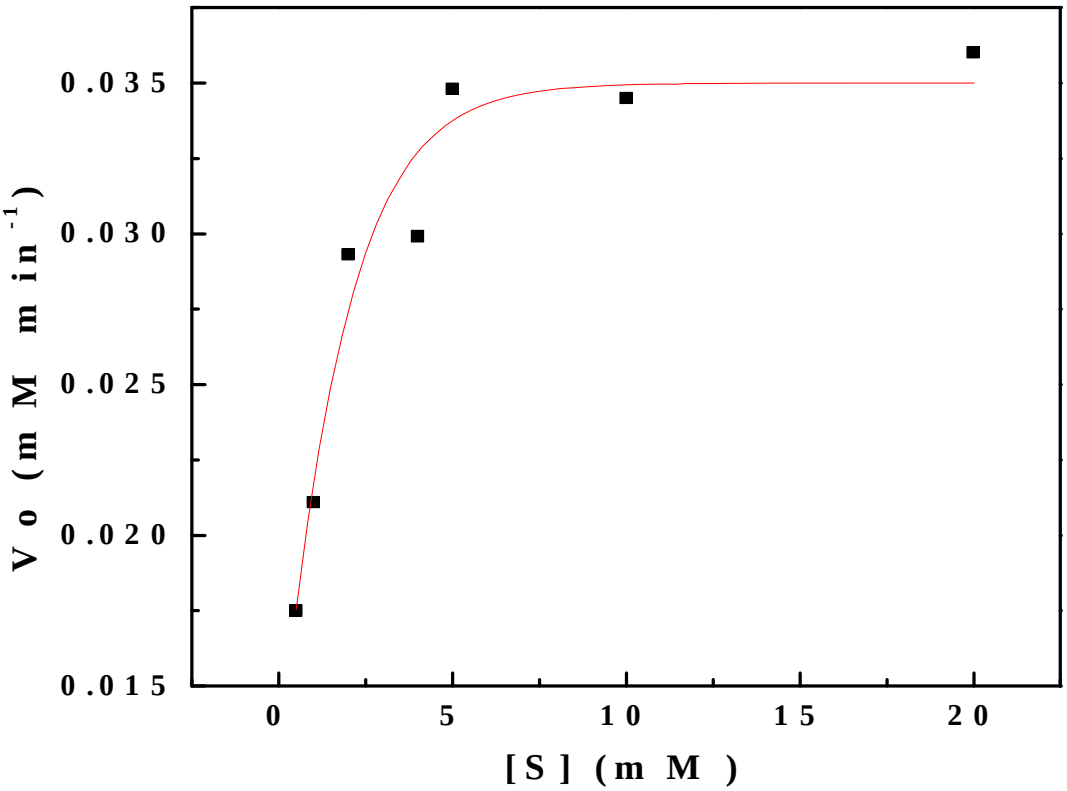


Figure 3.9: The Michaelis-Menten model adjusted to initial velocities of pen G hydrolysis, determined for various initial substrate concentrations at pH 7.5 and 37 °C

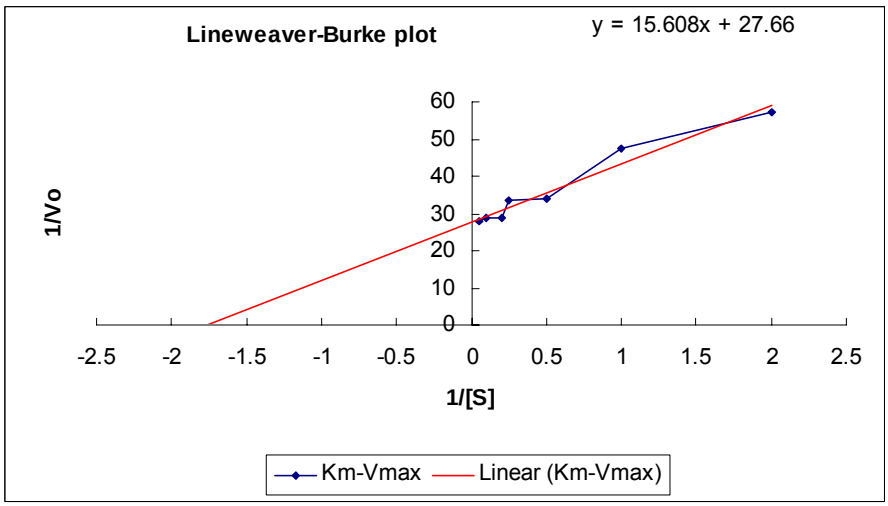


Figure 3.10: Lineweaver–Burk plot for the determination of K*m* and V*max* of PGA from *Arthrobacter viscosus*

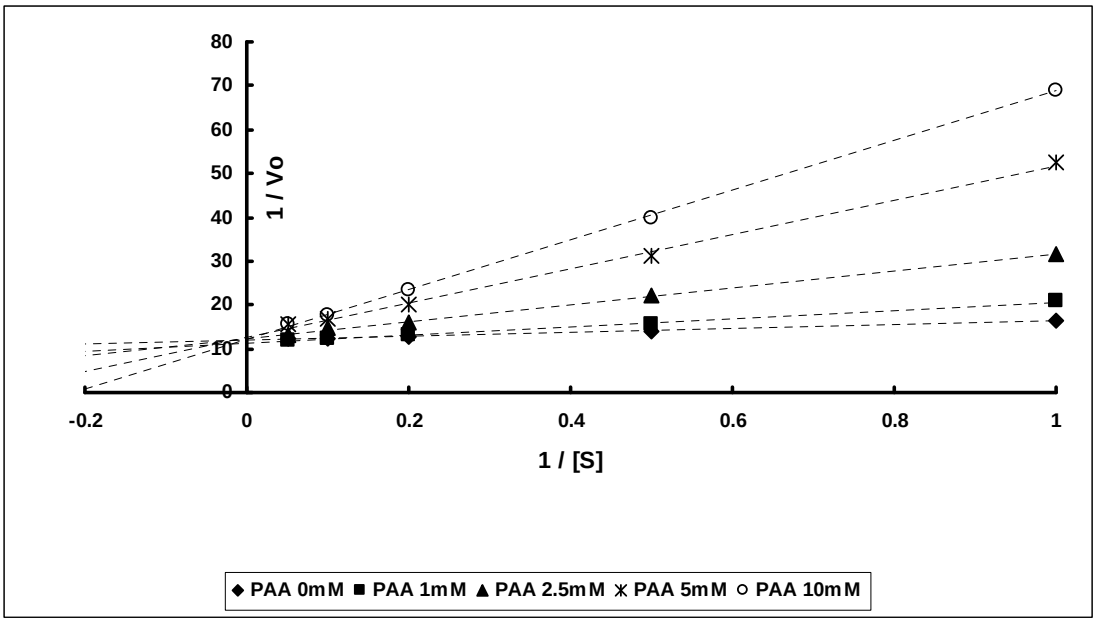


Figure 3.11: Lineweaver–Burk plot of the enzyme kinetics with various concentrations of inhibitor PAA.

CHAPTER 4

Identification and Characterization of Active site residues of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

4.1 SUMMARY

Penicillin G acylase from *Arthrobacter viscosus* ATCC 15294 was purified to homogeneity and subjected to chemical modification to identify essential amino acid residues in enzyme catalysis . Evidences are presented for the presence of an essential arginine and essential tryptophan residue in *Av*PGA. Incubation of PGA from *Arthrobacter viscosus* with phenylglyoxal results in enzyme inactivation. Benzylpenicillin (pen G) and phenylacetate protected the enzyme against inactivation, indicating the presence of arginine at or near the active site and it takes part in the catalysis reaction. The reaction follows pseudo first-order kinetics and the inactivation kinetics indicates the presence of a single essential arginine moiety. The enzyme is also inactivated by the tryptophan-modifying reagents, N-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNBBr). Benzylpenicillin (pen G) significantly lowers the extent of inactivation by the tryptophan modifying reagents. The inactivation kinetics is indicative of a single tryptophan residue in the hydrolysis of pen G by penicillin G acylase from *A. viscosus*. Protection of enzyme against modifying reagents was tested with pen G and phenylacetate.

4.2 INTRODUCTION

Penicillin G acylase is an important industrial enzyme which has profound application in the production of 6-APA and semi-synthetic β-lactam antibiotics. Enzymatic production of semi-synthetic β-lactam antibiotics has many environmental benefits compared to the classical chemical synthetic route. The active site of the enzyme has drawn special attention due to its importance in understanding the catalytic mechanism and substrate specificity for application in protein engineering (Roa et al., 1995; Martin et al., 1993). Penicillin G acylase from *E. coli* is the most studied enzyme.

The kinetics of the enzyme catalyzed hydrolysis of benzylpenicillin has indicated that the reaction proceeds via an acylenzyme intermediate (Konecy et al., 1983), a hypothesis supported by the irreversible inactivation of purified penicillin G acylase from *E. coli* (Kutzbach and Rauenbusch, 1974), *P. rettgeri* (Daumy et al., 1985) and *K. citrophila* (Martin et al., 1991) by equimolar amounts of phenylmethanesulphonyl fluoride (PMSF).

Robak and Szewczuk (1981) and Mahajan and Borkar (1983) had reported that the enzyme from *P. rettgeri* and *E. coli* are inactivated in presence of tryptophan modifying reagents, but no kinetic analysis nor estimate of the number of residues modified were reported. Kinetic evidence for the presence of an arginine residue at or near the active site of *E. coli* penicillin G acylase was provided by Prabhune and SivaRaman (1990). Martin et al. (1991) have reported the importance of serine residue in the enzyme hydrolysis. Kumar et al. (2004) have studied the *K. citrophila* PGA and demonstrated that one arginine residue is important for the catalytic reaction of PGA. Further studies on *K. citrophila* PGA have given evidence of tryptophan residue in substrate binding of the active site (Kumar et al., 2007). *Arthrobacter viscosus* PGA has potential scope in the field of semi synthetic β-lactam antibiotic industry. It has been successfully applied in the synthesis of some semi synthetic cephalosporins (Terreni et al., 2007). Information about its amino acids involved in catalysis can help in designing novel enzyme with altered substrate specificity. In this part of the thesis evidence are presented for the presence of an essential arginine and an essential tryptophan residue in *A. viscosus* PGA.

4.3 MATERIALS AND METHODS

Materials

Benzyl penicillin (pen G) and 6-APA were gifts from Hindustan Antibiotics Ltd., Pune. Phenyl Acetic aid was obtained from Aldrich. Phenylglyoxal was obtained from Fluka. N-bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNBBr) were obtained from Sigma, USA. All other chemical used were of high quality analytical grade. All buffers were prepared in glass distilled water.

Microorganism

Arthrobacter viscosus ATCC 15294 was a gift from Prof. Zoya Ignatova, Germany and routinely maintained on nutrient agar slants containing 0.1% phenyl acetic acid as an inducer.

Pencillin G acylase enzyme

Production and purification of *Arthrobacter viscosus* PGA was performed as described in chapter 3. The purity of enzyme was checked on Native-PAGE and was found to be homogenous. The enzyme activity and

residual activities were performed using pen G as substrate (Balasingham et al, 1972) and estimating 6-APA by the method of Bomstein and Evans (1965) as modified by Shewale et al. (1987).

Treatment with arginine-modifying reagent phenylglyoxal

Phenylglyoxal reacts with guanidino groups of arginine residues with high specificity under mild conditions. The reaction product with arginine contains two phenylglyoxal residues per guanidino group and is stable under mild conditions (Takahashi 1968, Yamasaki et al. 1980). Purified PGA (150 µg) from *A. viscosus* was incubated at 25 ^{o}C with different concentration of phenylglyoxal (2-20 mM) in 50 mM phosphate buffer at pH 8.0. Enzyme incubated with buffers in absence of the modifying reagent served as control. Aliquots were withdrawn at different time intervals for checking the residual activities.

Treatment with Tryptophan modifying reagents

N-Bromosuccinimide (NBS) and 2-hydroxy-5-nitrobenzyl bromide (HNBBr) were used as modifying reagents for tryptophan. NBS is a useful reagent for selectively cleaving tryptophanyl peptide bonds in peptides and proteins. It also cleaves tyrosyl peptide bonds at different pH, but more slowly than those of tryptophan (Spande and Witkop 1967). Histidine residues undergo a similar reaction, but very much slowly. NBS is a potent oxidizing agent oxidizing –SH groups more rapidly than its reaction with tryptophan. Methionine and cysteine can also be oxidized. This compound is a highly reactive source of very electrophilic bromonium ions Br^{+}. The great reactivity of HNBBr appears due to resonance stabilization of its incipient carbonium ion. Only tryptophan and to a lesser extent cysteine are able to effectively compete with water for this reactive intermediate. Purified PGA (150 µg) from *A. viscosus* ATCC 15294 was incubated at 25 °C with different concentrations of NBS (10 - 60 µM) in 50 mM sodium acetate buffer pH 5.0, or HNBBr (10 - 40 mM) in 50 mM sodium acetate buffer at pH 5.0. Aliquots were withdrawn at regular intervals of time for the residual activity. Enzyme incubated in the buffer in absence of the modifying reagents served as control.

Kinetics of Inactivation

In the chemical modification of an enzyme, the dependence of the apparent first-order rate constant of inactivation K_{app} on the concentration of the modifier is expressed by the relationship:

$$K_{app} = K\,(M)^n$$

OR

$$\log K_{app} = \log K + n \log (M)$$

where K is the second-order rate constant, (M) the molar concentration of the modifier and n is the average order of the reaction with respect to the concentration of the modifier. K_{app} can be calculated from a semi-logarithmic plot of the enzyme activity remaining as a function of time. The order of the reaction (n) can be obtained experimentally by determining K_{app} at a number of concentrations of the modifier. A plot of log K_{app} *versus* log (M) should give a straight line with a slope equal to n, where n is the number of molecules of the modifier reacting with each active unit of the enzyme to produce an enzyme-inhibitor complex (Ramkrishna and Benjamin, 1981)

Protection against Inactivation

Protection of the enzyme against inactivation by phenyl glyoxal was tested by incubating the enzyme with benzyl penicillin (pen G) or phenylacetate to a final concentration of 50 mM at pH 8.0 and was added immediately before the addition of phenylglyoxal (20 mM final concentration). Protection against inactivation by tryptophan reagents was tested with pen G or phenylacetate to a final concentration of 50 mM at pH 5.0 and was added prior to the addition of NBS (60 µM final concentration) or HNBBr (40 mM final concentration)

Titration of accessible Tryptophan Residues by NBS

The tryptophan-modifying reagent is a potent oxidizing agent which oxidizes the indole moiety of the amino acid tryptophan a chromophore absorbing at 280 nm to oxyindole, a much weaker chromophore at this wavelength. The titration of accessible tryptophan residues in PGA with NBS was followed spectrophotometrically at 280 nm. The control cuvette had 50 mM sodium acetate buffer pH 5.0 and the experimental had PGA (150 µg protein) in a total volume of 1ml of 50 mM sodium acetate buffer. Successive 10 µl aliquots of 2 mM NBS were added to both control and experimental

cuvettes at 2 minutes interval and changes in absorbance at 280 nm was recorded till no further change in absorbance was observed. After each addition, an aliquot of 10 µl was removed and the reaction arrested by the addition of 50 mM L tryptophan. The residual activity was determined under standard assay conditions. NBS mediated inactivation was monitored by measuring the decrease in absorbance at 280 nm. Tryptophan was determined by titration with NBS as described by Spande and Witkop in1967. The number of tryptophan residues oxidized (n) per mole enzyme was calculated from the relationship:

$n = (1.31 \times \Delta_{280}) / (5500 \times \text{molarity of enzyme})$

where Δ_{280} is the decrease in absorbance at 280 nm, 5500 $M^{-1}cm^{-1}$ is the molar extinction co-efficient of tryptophan at 280 nm and 1.31 is an empirical factor based on oxidation of model tryptophan peptides (Patchornik et al., 1958).

4.4. RESULTS AND DISCUSSION

Arginine modification

The treatment of *Av*PGA with various concentration of phenylglyoxal in 50 mM phosphate buffer at pH 8.0 and 25 °C reduced the activity. The inactivation was dependent on the time of treatment and concentration of the modifying agent (Figure 4.1). The rates of inactivation followed pseudo-first order kinetics, plots of the logarithm of residual activity versus time of contact with the reagents being linear throughout the test period of 1 h. The residual activitiy at the end of this period was found to be 70% of initial activity, in presence of 20 mM phenylglyoxal. The enzyme activity remained unchanged in absence of the modifier. The reaction order (n) with respect to the arginine modifying reagent was determined from the plot of the logarithm of the apparent first-order rate constant, K_{app} versus the logarithm of the reagent concentration (Figure 4.2) and the value of n=1.12 indicates that the loss of enzyme activity results from the reaction of one arginine per mole of penicillin G acylase.

Protection from the inactivation by phenylglyoxal

Blocking the active site with 50 mM pen G or 50 mM phenylacetate, the enzyme is protected against inactivation, indicating that the modified residue

is present in or near the active site (Table 4.1). Phenylacetate is known to act as a competitive inhibitor of PGA and evidence is reported for its binding at the active site of the protein (Prabhune and SivaRaman, 1990). These results indicate that the respective arginine residue is located at or near the active site of the enzyme molecule

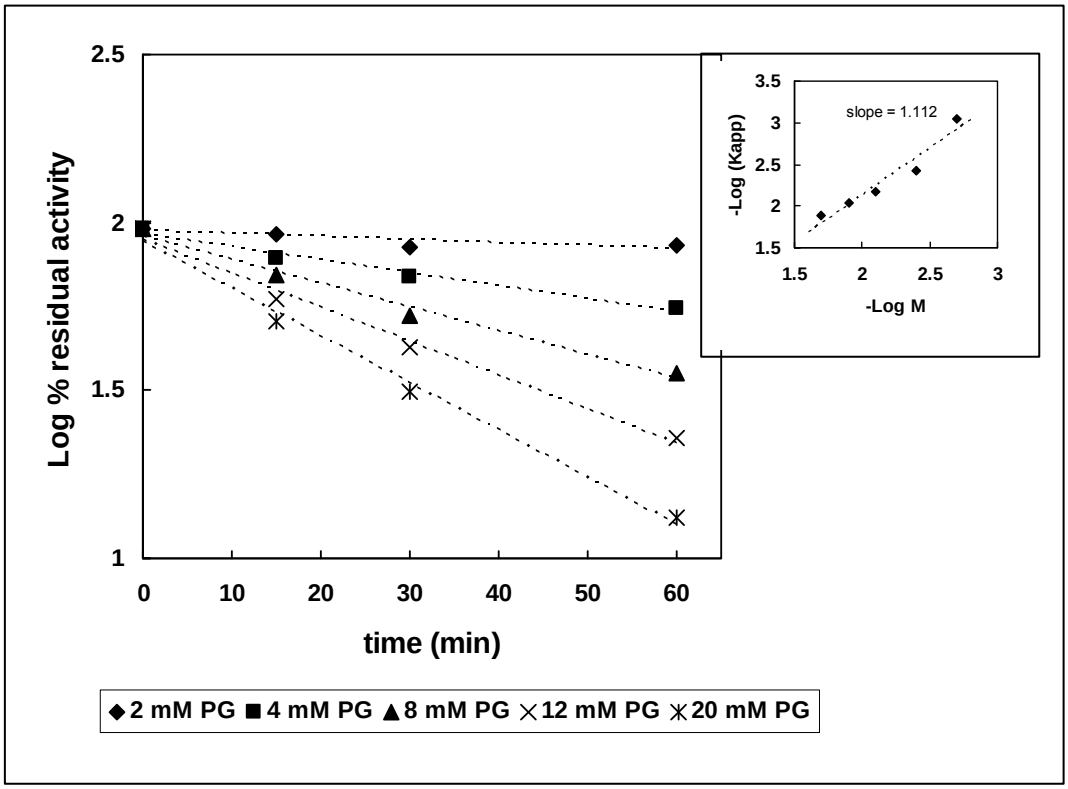


Figure 4.1: Inactivation of *Av*PGA using arginine-specific modifying reagent phenylglyoxal (PG). Inset: Determination of the order of modification with respect to arginine specific modification reagent phenylglyoxal (PG), where k_{app} is the apparent first order rate constant and M is the molar concentration of the reagent PG

Table 4.1: Protection of *Av*PGA against inactivation by arginine-specific reagent

Treatment	Enzyme activity (% initial activity)
None	100
phenylglyoxal (20 mM)	70
penG (50 mM) + phenylglyoxal (20 mM)	95
phenylacetate (50 mM) + phenylglyoxal (20 mM)	90

Inactivation of *Av*PGA by Tryptophan modifying reagents

Both NBS and HNBBr inactivated the enzyme rapidly at pH 5.0 as shown in figure 4.2 and figure 4.3. The enzyme was inactivated more rapidly by HNBBr. The enzyme activity in the presence of 60 µM NBS was 25% at the end of 40 min of test period. Residual enzyme activity in presence of 40 mM HNBBr was 30% at the end of 30 min of incubation. The plots of logarithm residual activity versus time of incubation were linear up to the end of test periods, indicating pseudo-first order kinetics of inactivation by both NBS and HNBBr.

The reaction order (n) with respect to the tryptophan modifying reagents determined from the plots of log K_{app} versus log reagent concentration gave values of n=1.06 for NBS and n=1.141 for HNBBr (Figure 4.2 inset and Figure 4.3 inset) these values indicate that the modification of a single tryptophan residue results in the inactivation of a mole of the enzyme.

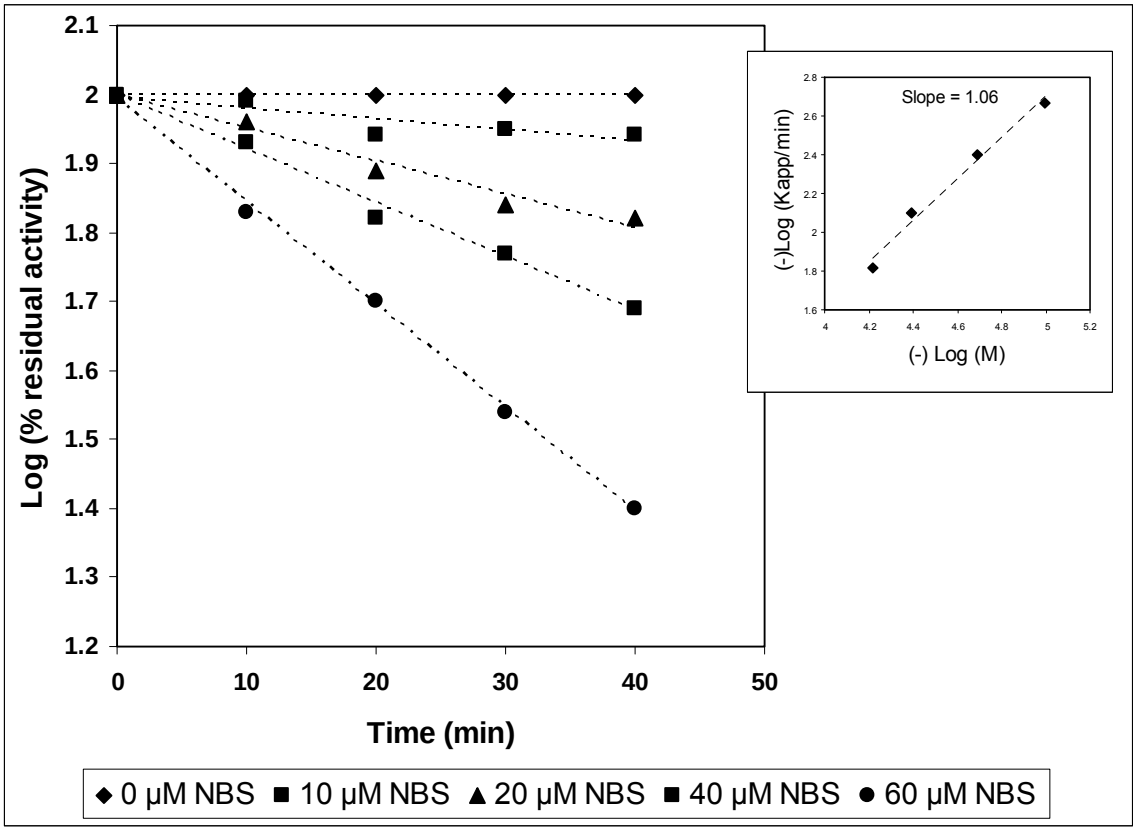


Figure 4.2: Determination of the order of modification reaction of *Av*PGA with respect to tryptophan-specific modification reagent NBS at pH 5.0 and 25 °C. **Inset**: The pseudo-first order rate constants (k_{app}) were plotted against various concentrations of NBS.

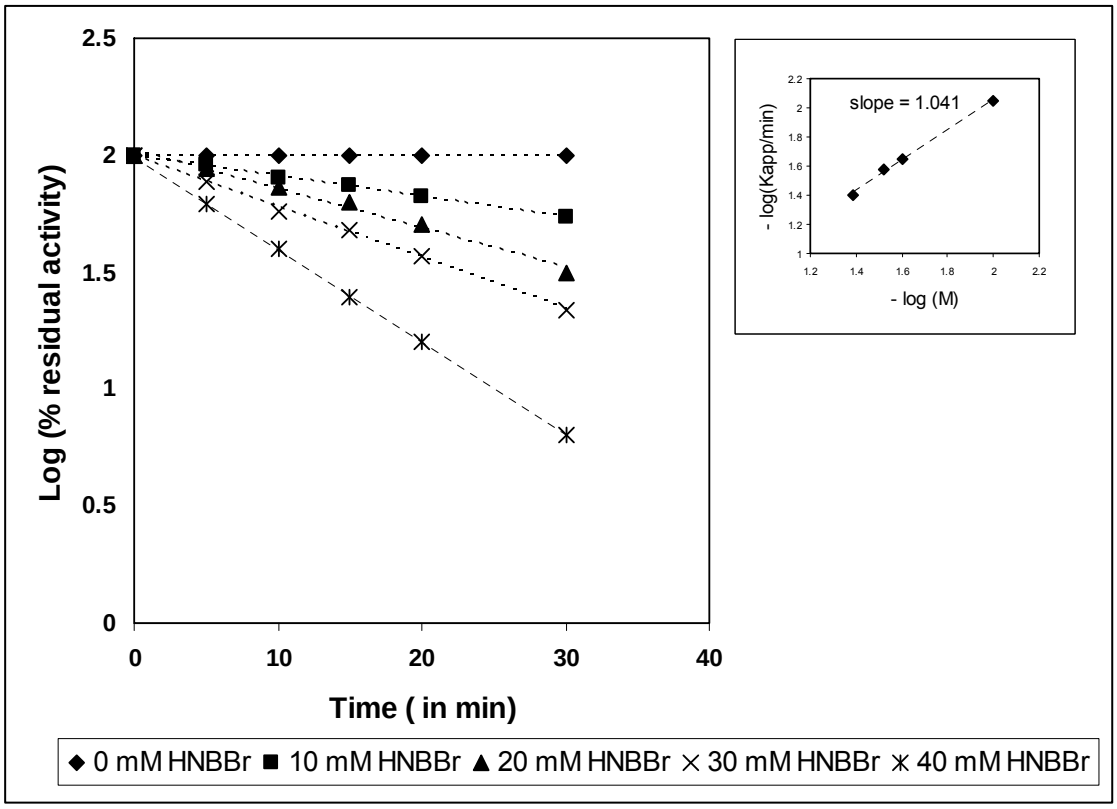


Figure 4.3: Determination of the order of modification reaction of *AvPGA* with respect to tryptophan-specific modifying reagent HNBBr at pH 5.0 and 25 °C. **Inset**: The pseudo-first order rate constants (k_{app}) were plotted against various concentrations of HNBBr.

Protection against inactivation by tryptophan reagents

The protective action of pen G (substrate) and phenylacetate (competitive inhibitor) on the inactivation of *Av*PGA by NBS and HNBBr is shown in table 4.2. In the presence of 50 mM pen G, the percentage of original activity retained by *Av*PGA was 74% after 40 min of incubation with NBS (60 µM) and 81% after incubation with HNBBr (40 mM) for 30 min. like wise phenylacetate gave corresponding values of 63% and 78% residual activity, respectively. Thus it is clear that both the substrate pen G and the inhibitor phenylacetate protect the tryptophan from getting inactivated by modifying reagent. The kinetic evidence for the involvement of a single tryptophan residue and protective action of substrate and products against

inactivation of *Av*PGA by tryptophan modifying reagent are indicative of the presence of the tryptophan moiety at or near the active site of the enzyme.

Table 4.2: Protection of *Av*PGA against inactivation by tryptophan specific reagent

Treatment	Enzyme activity (% initial activity)
None	100
NBS (60 µM)	25
Pen G (50 mM) + NBS (60 µM)	74
phenylacetate (50 mM) + NBS (60 µM)	63
HNBBr (40 mM)	30
Pen G (50 mM) + HNBBr (40 mM)	81
phenylacetate (50 mM) + HNBBr (40 mM)	78

Titration with NBS of accessible residues in *Av*PGA

The tryptophan residues in *Arthrobacter viscosus* PGA were oxidized with stepwise addition of NBS. The number of tryptophan residues oxidized was determined from absorbance at 280 nm and the molar ratio calculated using M_r value of 81,000 for the enzyme as described in MATERIALS AND METHODS. Figure 4.4 shows the effect of NBS on enzyme activity after each addition of NBS. There was a progressive decrease in absorption at 280 nm as well as increase in extent of inactivation. By extrapolating the initial linear portion of the plot to zero activity, the number of tryptophan moieties oxidized per mole of enzyme was found to be 1. This usually gives the number of residues modified when the enzyme is completely inactivated.

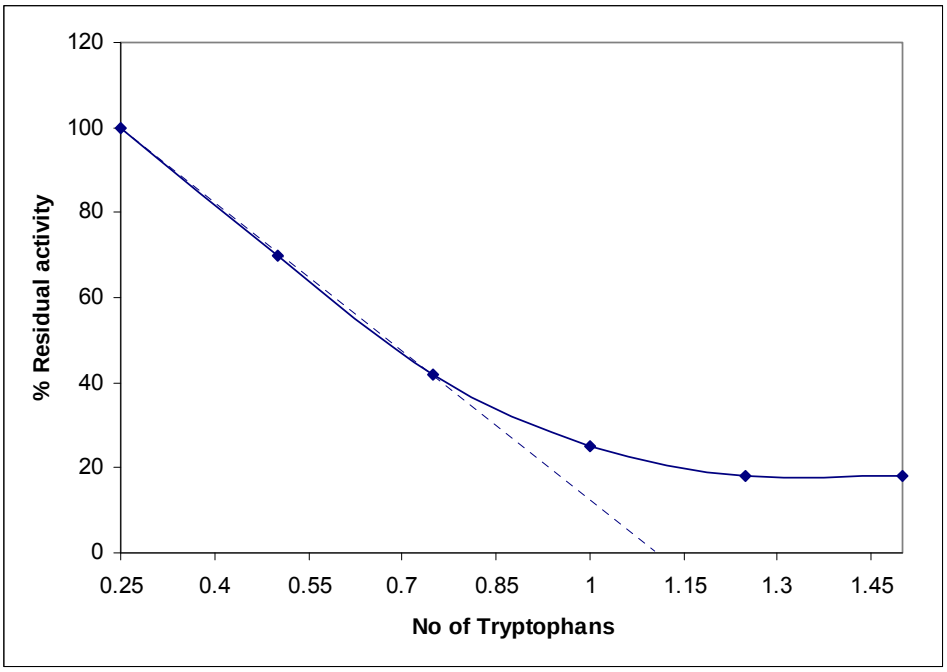


Figure 4.4: The titration of *Av*PGA with NBS is plotted. Tryptophan residues were quantified by stepwise addition of NBS as described in the text.

The profound impact of penicillin acylases in the manufacture of antibiotics is reason for continued interest in studying structural determinants of these enzymes essential for catalysis. Efforts to enhance the catalytic rate by protein engineering have been reported (Gabor and Janssen, 2004).

The results obtained from the chemical modification studies could be interpreted in terms of the reported observations on *E. coli* PGA (Prabhune and SivaRaman, 1990). The previous report concluded from inactivation studies using the arginine-specific reagents 2, 3-butanedione and phenylglyoxal that one arginine residue is involved in hydrolysis of pen G.

Structural and kinetic studies both have suggested the involvement of two charged arginine residues, Arg α145 and Arg β263, in the catalysis of PGA (Alkema et al., 2002). X-ray structure analysis of the mutant enzyme with bound pen G showed that Arg α145 displayed a large conformational change upon binding of the substrate, and moved away from the active site. The positional shift of Arg α145 as well as its neighbouring Phe α146 is necessary to create space for accommodating the β-lactam moiety of pen G. In this altered conformation, the hydrogen bonds between Arg α145: NH_2 and the main chain carbonyl oxygen of Phe β24 are replaced by hydrogen bonds

between Arg α145: NH_2 and the carboxylate group of substrate via two bridging water molecules (Done et al., 1998, Alkema et al., 2002). Arg β263 is located near the oxyanion hole, and its NH_2 group is hydrogen-bonded to Oγ of Asn β241, the putative residue which stabilizes the transition state intermediate. The presence of protonated guanidino group of Arg β263 is proposed to be obligatory for catalysis, orienting the N-terminal catalytic serine residue and contributing to a decrease in the *pK* of the α-amino group (Morillas et al., 1999).

The rate of inactivation when plotted against the concentration of the modifier indicated modification of a single tryptophan. This is in agreement with the value of Kapp obtained from pseudo-first-order kinetics.

The involvement of a hydrophobic residue like Trp in PGA catalytic activity inferred here is in agreement with the results from other investigations on the role of hydrophobic interactions in the hydrolytic activity of PGA. These include the report on the inhibitory effect of aliphatic alcohol on the activity of the enzyme from *E. coli*, the magnitude of inhibitory effects vary with the extent of hydrophobicity of the alcohol (Klyosov et al., 1977).

4.5 Conclusion

The preliminary study on the chemical modification of *Av*PGA shows involvement of arginine and tryptophan residues in the hydrolytic catalysis of pen G. These results are in good agreement with the earlier reports on other industrially used PGA producers; *E.* coli, *P.* rettgeri and *K. citrophila*. Substrate protection studies provided indication that these residues are present at or near the active site. Kumar et al in 2007 have mentioned that *Arthrobacter viscosus* PGA has very less sequence similarity with the above mentioned PGAs (less than 30 %) however the active site characteristic is similar to those of above mentioned PGAs. We have studied and reported earlier in chapter 3, the substrate specificity of *Av*PGA, which is obviously better than that of rest of PGA. This might be explained on the basis of dissimilarities in sequence homology of the enzyme and can be attributed to the substrate binding pocket. Biophysical studies and X-ray structural studies will reveal the mechanism though it's not the scope of this chapter.

CHAPTER 5

CTAB capped gold nanoparticles for colorimetric detection of hydrolysis of antibiotics by penicillin G acylase

5.1 SUMMARY

A simple method of PGA screening was developed using CTAB capped gold nanopartiicles. Gold nanoparticles capped with Cetyl trimethyl ammonium bromide (CTAB) are synthesized using chemical route. The particles could be used for detection of PGA by incorporating hydrolysis reaction with pen G. This hydrolysis reaction leads to a shift in the surface plasmon band of gold nanoparticles from 527 nm to 545 nm accompanied by a visual colorimetric change in the solution from red to blue. The process is attributed to aggregation of nanoparticles caused due to displacement of CTAB bilayer by byproducts of the hydrolysis reaction. It is proposed that the presence of 0.007 mg/ml of PGA can be detected by a color change of gold nanoparticles solution without requiring any complicated instrument or highly trained operator to conduct the test. The method could also identify the presence of different penicillins by showing different spectral shifts. Thus the work presented here would be useful not only for the detection of the pharmaceutically important drug Pen G, but also represents a general methodology for PGA detection.

5.2 INTRODUCTION

Metal nanoparticles based enzymatic assays (Laromaine et al, 2007; Guarise et al, 2006, Zhang et al, 2009; Li et al, 2010; Liu et al, 2010) are increasingly becoming popular due to their increased sensitivity as well as rapidness when compared to the conventional methods (Batchelor et al., 1961; Bomstein and Evans, 1965; Sjoberg et al., 1967; Baker 1980). The potential of gold nanoparticles in this field stems from the phenomenon of surface plasmon resonance (SPR), which is the collective oscillation of free electrons in metal nanoparticles when electromagnetic radiation is incident on them (Mulvaney, 1996). Based on metal nanoparticles, there are various sensing mechanisms (Riboh et al, 2003; Nusz et al, 2008; Storhoff et al, 2000; Kalele et al, 2005; Kalele et al, 2006; Jena et al, 2006; Hossain et al, 2009; Kneipp et al, 1999; Aslan et al, 2005) which lead to the detection of bio-molecules by monitoring changes in the localized surface plasmon resonance (LSPR) extinction. The sensing mechanisms include local refractive index changes, nanoparticle aggregation, charge-transfer interactions at nanoparticle surfaces, resonant Rayleigh scattering from nanoparticles,

surface enhanced Raman scattering and plasmon enhanced molecular florescence.

Enzyme biosensing has been achieved using gold nanoparticles (Liu et al, 2004), pH responsive hydrogels and supramolecular assemblies (Ulijn, 2006). Fischer et al. 2002 used anionically functionalized monolayer protected gold nanoclusters for inhibition of chymotrypsin. Apart from these examples, detection of enzymes such as proteases has been achieved by binding nanoparticles with peptides (Laromaine et al, 2007; Guarise et al, 2006). These peptides lead to assembly of nanoparticles due to their cross linking by long chain molecules. Enzymes specific for these peptides are then introduced which cleave the peptide bond leading to dispersion of nanoparticles. This aggregation–dispersion process leads to the colorimetric changes in the nanoparticle solution. Aggregation of gold nanoparticles leads to red shift in the plasmon band due to the electric dipole-dipole interaction leading to a coupling between the plasmon oscillations of different particles (Storhoff e al, 2005). The color of gold nanoparticle solution turns from red to blue/purple due to red shift in the plasmon band. Here the red shift refers to the shift in the plasmon band towards longer wavelength *i.e* red region of the electromagnetic spectrum. This color change has been used to detect the presence of analytes that induce selective aggregation of gold nanoparticles. The position and intensity of plasmon band is used to monitor this aggregation process.

Acylases are a group of enzymes that catalyze the cleavage of carbon–nitrogen bonds in amides. Penicillin acylases are members of the N-terminal nucleophile (Ntn) hydrolase superfamily, which share a common fold around the active site and a catalytic residue in the N-terminal position (Branningan et al, 1995; Duggleby et al, 1995). These enzymes are used mainly in the pharmaceutical industry for the production of 6-Aminopenicillinic acid (6-APA), a key intermediate in the production of semi synthetic penicillins and cephalosporins, which are now far the most widely used antibiotics. In addition, these enzymes are very useful as biocatalysts in some important reactions like peptide synthesis (Arroyo et al, 2003; Phadtare et al, 2002), and also in the resolution of clinically active compounds (Fadnavis et al, 1999; van Langen et al, 2000b). Penicillin acylases specifically catalyse hydrolysis of

penicillins. For each type of penicillin, there exists an enzyme which performs this hydrolysis reaction. Penicillin G acylase (PGA) is a specific enzyme for an important drug, penicillin G (pen G). The hydrolysis reaction of penicillins by penicillin acylases leads to breaking of its molecules into different byproducts, thereby leading to consumption of penicillins. All the enzymatic assays quantify consumption of the respective penicillins or detect the formation of byproducts over time. Enzymatic activity, which is the measure of amount of active enzymes in the solution, is then determined using such different kinds of assays.

The earliest procedures to assay penicillin acylase activity are tedious and time consuming (Bomstein and Evans, 1965; Shaikh et al., 1973; Daumy et al, 1982; Tewari et al., 1988; Szewczuk et al., 1980). Some of these include paper chromatography (Batchelor et al., 1961), gas chromatography (Chiang and Bennet, 1967), titrimetric determination using pH stat (Sjoberg et al., 1967), assays using 6-nitro-3-(phenylacetamido) benzoic acid (NIPAB) (Kutzbach and Rauenbusch, 1974), etc. Methods stated above either require expert operators or expensive instruments or reagents e.g. NIPAB

The method consists of detecting 6-aminopenicillin acid, which is a byproduct in hydrolysis of pen G, and in turn assaying PGA without any chromogenic reagent. There is a distinct color change observable in the gold nanoparticle solution with naked eyes from initial red to final blue, when the pen G hydrolysis reaction is performed by PGA in the presence of gold nanoparticles. Although various enzymes have also been detected by using nanoparticles as probes, there are no reports of detection of penicillin acylases using nanoparticles. Various sophisticated techniques like TEM, UV-VIS spectroscopy have been used in order to investigate the biosensing mechanism using gold nanoparticles. However, once this test is acceptable, in practice only colorimetric changes can be used to detect the presence of PGA.

Penicillin G acylase enzyme is added to a solution of CTAB capped gold nanoparticles and its interaction with pen G and ampicillin is studied by monitoring the surface plasmon resonance band using UV-Visible spectrometer. Specific interaction between enzyme and penicillins lead to significant shift in the absorption spectrum of gold nanoparticles thereby

leading to detection of enzyme in the solution. In the presence of penicillins, gold nanoparticle solution containing enzyme penicillin G acylase shows visual color change from red to blue. Also, for two different penicillins (penicillin G and ampicillin), different spectral changes occur. Hence, this method, apart from being simple, rapid and colorimetric, is also capable of differentiating between activities of different penicillins for a particular enzyme

5.3 MATERIALS AND METHODS

Materials

Chloroauric acid trihydrate (HAuCl4.3H2O) and CTAB was obtained from Thomas Baker, India and Molychem, India respectively. Penicillin G (pen G) was obtained from Hindustan Antibiotics Limited, Pune, India. En-zyme penicillin G acylase (PGA) was purified from the gram-positive bacteria *Arthrobacter viscosus* ATCC 15294. The enzyme was produced extracellularly in the culture broth. The cells were separated by centrifugation, and the clear broth was used for further purification by hydrophoric chromatography (octyl sepharose) followed by anion exchange chromatography (Q-sepharose). The concentration of enzyme used was 0.7 mg/ml.

Synthesis of CTAB capped gold nanoparticles

CTAB capped gold nanoparticles were synthesized by reducing aqueous solution of 5 x 10^{-4} M HAuCl4 (10 ml) containing 0.2 M CTAB with 1×10^{-2} M ice cold NaBH4 (0.6 ml). The solution was stirred for 3 hours and then kept at room temperature for 3 days. 10 μl of enzyme solution was added to 2 ml of gold nanoparticles solution followed by addition of 100 μl of substrate pen G (100 mg/ml). The solution was kept at 40 °C for 10 min. Absorption spectra were recorded after each step.

Enzyme Activity Assay

The enzyme activity of Penicillin G acylase was determined by measuring the amount of 6-aminopenicillic acid (6-APA) produced in a reaction mixture containing 10 mg ml^{-1} penicillin G in 0.1 M phosphate buffer, pH 7.0, when incubated at 40 °C (Balasingham et al, 1972). The 6-APA produced was estimated spectrophotometrically at 415 nm, after reaction with p-dimethylaminobenzaldehyde (PDAB), according to (Bomstein and Evans,

1965), modified by (Shewale et al, 1987). One unit of PGA activity is defined as the amount of enzyme required to produce 1 µmole of 6-APA per minute under the assay conditions (40 °C and pH 7.0).

Characterization

Absorption spectra of the samples were recorded in solution form on Perkin Elmer lambda 950 instruments in the range 200 to 800 nm. TEM images are acquired on Philips CM200 instrument with an accelerating voltage of 120 KV. For TEM analysis, samples were drop coated on copper grids and were allowed to dry for 45 minutes.

5.4 RESULTS AND DISCUSSION

CTAB capped nanoparticles have rarely been investigated for biosensing purposes due to the fact that CTAB bilayers are not easily displaced by biomolecules and hence are difficult to handle as far as bioconjugation is concerned. On the other hand, CTAB capped nanoparticles are chemically more stable and would have a longer shelf life. the reaction between enzyme penicillin G acylase (PGA) and pen G in the solution of CTAB capped gold nanoparticles is monitored.

Plasmon resonance band of CTAB capped gold nanoparticles was observed at 527 nm. Gold nanoparticles synthesized here are covered with a bilayer of CTAB (Nikoobakht and El-Sayed, 2007). Inner layer is bound to the gold surface via the headgroup and is connected to the outer layer through the hydrophobic interactions while the headgroups of the outer layer are in the aqueous medium. Headgroup of the CTAB chain consists of an amine group. Hence, protonated amine group is present on the outermost surface of CTAB capped gold nanoparticles. No change in the absorption peak position of the gold was observed on addition of enzyme PGA (10 µl, 0.7 mg/ml). However, on addition of 100 µl of pen G (100 mg/ml), the plasmon band shifted from 527 nm to 545 nm (Figure 5.1(a)). After the addition, the solution was kept at 40°C for 10 min to allow the reaction to occur completely. The experiment was also performed with different concentration of enzymes (Figure 5.1(b)). The red shift was found to gradually increase on increasing the concentration of enzyme from 0.007 mg/ml to 0.7 mg/ml. The concentration of pen G in this case was same as in Figure 5.1(a) *i.e* 100 mg/ml. The spectral shifts are thus sensitive for enzyme concentration as low as 0.007 mg/ml. Figure 5.1(c)

shows gradual shifts in the plasmon band as the amount of pen G is increased from 10 to 100 µl (1 to 10 mg of pen G). The extinction spectrum started changing after an addition of 10 µl of pen G and on addition of 100 µl pen G it red shifted by 18 nm. The total red shift obtained after adding 100 µl of pen G varied in the range of 17 to 23 nm on repeating the experiment several times. Also, the spectrum was recorded four hours after the addition of pen G (Figure 5.1(d)). The plasmon band was found to be gradually shifted towards the longer wavelength (572 nm) and decreased in intensity. The gradual shift in the absorption band is attributed to slow removal of remaining CTAB layers from gold nanoparticles. After the complete displacement of CTAB, the particles precipitate at the bottom of the bottle leaving clear supernatant. Peak at 322 nm is attributed to the absorption due to the penicillin G. Broadening of plasmon band along with decrease in the extinction intensity was also observed.

The spectral shifts in the surface plasmon band are accompanied by changes in the color of gold nanoparticle solution from red to blue (Figure 5.1(d) inset). Color of the solution started changing from red to dark pink after an addition of 10 µl of the pen G. However, on the addition of 25 µl Pen G, the color was purple. The purple color intensified on further addition of pen G. The figure shows the change in color after adding 100 µl of pen G.

The prominent shift in the plasmon band of gold nanoparticles can be attributed to the aggregation of nanoparticles. Figure 5.2 shows TEM images of gold nanoparticles before and after the addition of the enzyme and the pen G. Size of gold nanoparticles was found to be ~15 nm. Particles are well dispersed before the addition of enzyme and pen G. On addition of the enzyme and pen G to gold nanoparticle solution, aggregation of particles is observed. TEM image, red shift in the plasmon band and the color change in the gold nanoparticle solution together indicate aggregation of gold nanoparticles.

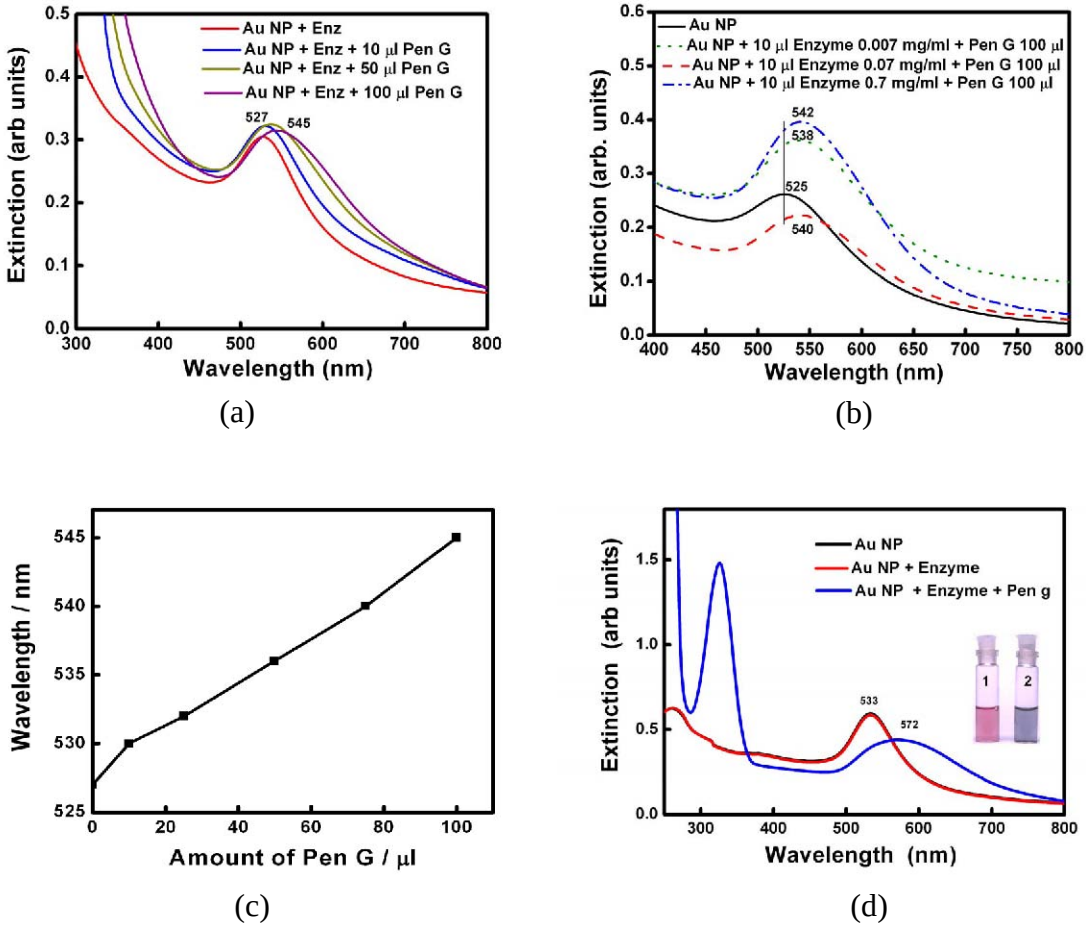


Figure 5.1: (a) changes in spectra on addition of different amounts of Pen G when the enzyme conc. is 0.7 mg/ml, (b) Changes in spectra after addition of different conc. of enzyme when the Pen G concentration is 100 mg/ml (c) Shift in extinction maximum of Au nanoparticles Vs. amount of Pen G (100 mg/ml) and (d) Spectra acquired after 4 hour of addition of Pen G. Inset shows change in the color of the Au nanoparticle solution (1) before and (2) after the enzyme-pen G addition

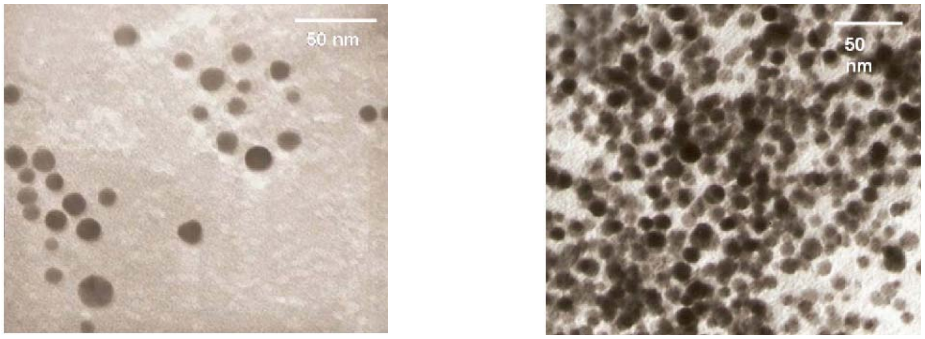
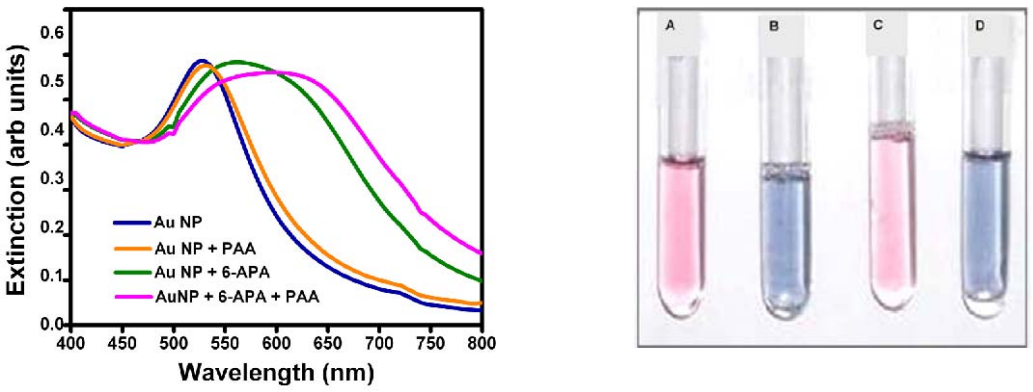


Figure 5.2: TEM images of (a) gold nanoparticles and (b) gold nanoparticles + enzyme + substrate

Reaction occurring between the enzyme and the pen G is schematically shown in Figure 1.5 in chapter 1. PGA specifically catalyses the hydrolysis of the amide bonds in pen G. Cleavage of amide bond in pen G by PGA is accompanied by the formation of 6-aminopenicillanic acid (6-APA) and phenylacetic acid (PAA).



(a) (b)

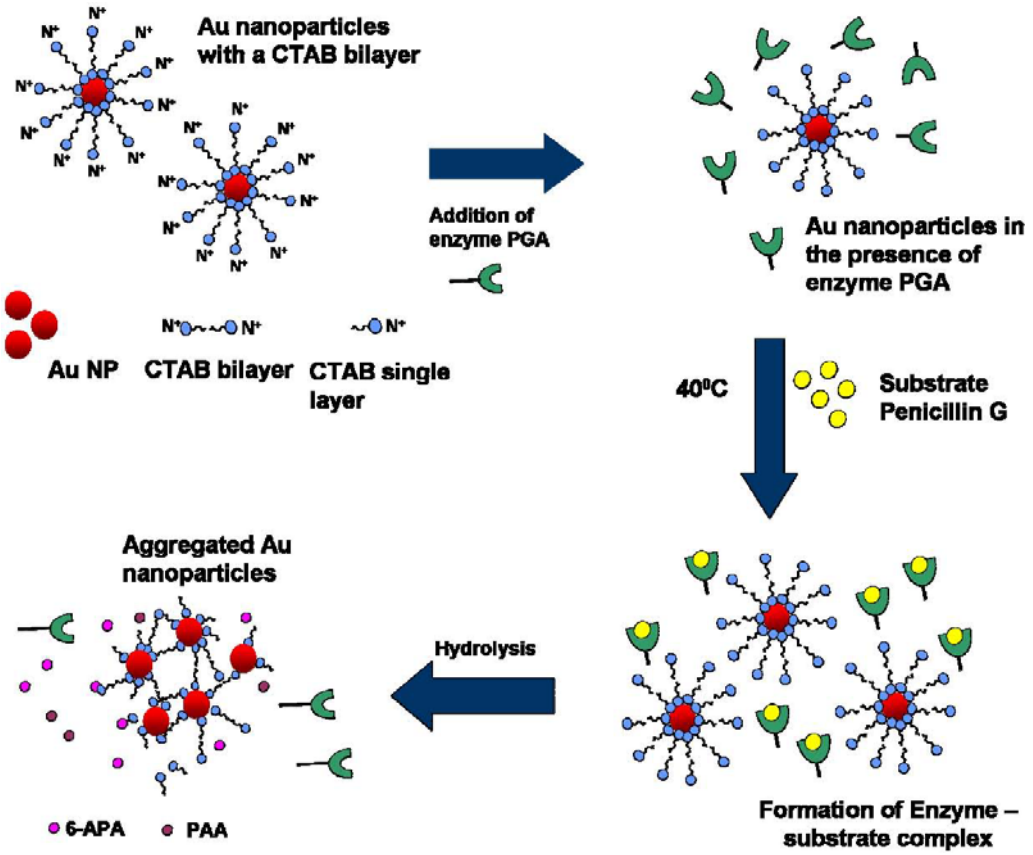
Figure 5.3: (a) UV-Vis spectra of gold nanoparticles, gold nanoparticles + PAA, gold nanoparticles + 6-APA and gold nanoparticles + 6-APA + PAA and (b) Photograph of (A) gold nanoparticles (B) gold nanoparticles + 6-APA (C) gold nanoparti-cles + PAA and (D) gold nanoparticles + 6-APA + PAA

In order to confirm the mechanism of aggregation of gold nanoparticles due to enzyme-pen G reaction, only the products of this reaction were added to the solutions. 6-APA, PAA and mixture of 6-APA and PAA were added separately to the nanoparticle solutions in the absence of enzyme and pen G, and spectral changes were recorded (Figure 5.3). Interestingly, the color changes were obtained in the presence of 6-APA only. PAA (100 µl, 2.5mM) alone was not able to induce the color changes (Figure 5.3(b)). On addition of 6-APA (100 µl, 2.5 mM), no change in the spectral peak position was obtained immediately, however the plasmon band shifted from 527 to 565 nm after keeping the solution for two hours. Addition of mixture of 6-APA and PAA (same concentrations as mentioned above) to gold nanoparticle solution caused the plasmon band to shift from 527 to 545 nm immediately and to 600 nm finally after three hours. This was also accompanied by broadening of the plasmon band. This trend is very much similar to that obtained in the

presence of enzyme and substrate pen G (see Figure 5.1). The spectral and colorimetric changes were obtained immediately when both acids were together added to the gold nanoparticle solution, which is also happening when the reaction is done in the presence of enzyme and the pen G. This indicates that the gold nanoparticles are sensing hydrolysis of the pen G. Hence, the combination of acids produced during the hydrolysis and the amount of 6-APA produced should be the key factor in determining the spectral and colorimetric changes produced in the nanoparticle solution due to the enzyme-substrate chemistry. This is further supported by the fact that the extent of red shift increases when PGA concentration is increaded (Figure 5.1(b)). This is due to the increase in the amount of 6-APA produced.

The mechanism of sensing hydrolysis reaction can be explained by Scheme 5.1. As mentioned earlier, a CTAB bilayer is present on the surface of gold nanoparticles such that N^+ group are present on both the ends. This bilayer is responsible for the stable dispersion of gold nanoparticles in the solution. When pen G is added to gold nanoparticle solution in presence of enzymes, specific interaction between enzyme and pen G occurs, leading to hydrolysis of pen G and formation of 6-APA and PAA. Since the colorimetric changes are obtained only after the addition of pen G, it is proposed here that electro-static interaction between CTAB and the products of hydrolysis (6-APA and PAA) cause partial removal of CTAB bilayer from gold nanoparticles leading to a decrease in their surface potential and hence aggregation**.** The dependence of the interparticle distance on the surface plasmon band of metal nanoparticles is well known (Storhoff et al, 2000; Lazarides and Scahtz, 2000). Aggregated or assembled nanoparticles display red shift in the plasmon band when compared to the isolated gold nanoparticles. This phenomenon is attributed to the coupling between the dipole modes of plasmons of different particles. As the interparticle distance is de-creased, more red shift in the plasmon band is observed due to an increase in the extent of coupling. A decrease in the interparticle distance after the completion of enzyme–pen G reaction is clearly observed in the TEM images (Figure 5.2). Along with the coupling of plasmon modes, contribution to the red shifting of plasmon band also comes from increased scattering of

nanoparticle aggregates in the longer wavelength region (Nikoobakht and El-Sayed, 2007).



Scheme 5.1: Schematic diagram showing the mechanism of detection of enzyme

Some of the previously reported work (Laromaine et al, 2007) have used thiol containing Cys functionalized gold nanoparticles and an assembly directing actuator to control the assembly of nanoparticles through pi-stacking interactions. Upon hydrolysis by enzymes, disassembly was observed due to increased repulsion between NH_3^+ groups and removal of the hydrophobic interactions between the actuator. This disassembly caused blue shift in the extinction spectrum of gold nanoparticles. It is noteworthy that no assembly directing ligand was used to control the assembly of gold nanoparticles. Also, in this case, the cleavage of pen G is actually leading to a red shift in the plasmon resonance band. This indicates that the products formed by the

enzyme pen G reaction decrease the electrostatic repulsion between particles causing their aggregation.

In order to check the effect in the absence of enzyme PGA, UV-Vis spectrum of gold nanoparticles was acquired when the Pen G was added to the gold nanoparticle solution without enzyme PGA in it (Figure 5.4). 100 μl of pen G was added to 2 ml of gold nanoparticle solution and spectrum was recorded. On addition of pen G, spectral shift of only 2 nm was observed indicating that the aggregation is induced only by the product of enzyme–substrate reaction and not just by the presence of pen G. No change in the color of the solution was immediately observed in this case.

The activity of enzyme was also checked by estimating the amount of 6-aminopenicillanic acid (6-APA) in the reaction. The enzyme was found to be active after the reaction.

Same experiments were performed by changing the penicillin in the reaction (Figure 5.5). Instead of pen G, ampicillin was added to the gold nanoparticle-enzyme solution. Ampicillin differs from the Pen G in the sense that one extra amino group is present on α-carbon (Figure 1.5). Enzyme penicillin acylase has less affinity towards ampicillin as compared to pen G. The reason for this lies in the action of the enzyme. The enzymes are folded in specific conformation with very unique active sites in which only particular penicillin can fit very properly. Due to one extra amino group in ampicillin, the side chain becomes little bulkier compared to that in Pen G and cannot fit into the active site properly. Hence, PGA cannot react to the same extent on amide bond in ampicillin as it does with pen G and so it has less activity. Therefore, rate of enzyme penicillin complex formation is expected to decrease significantly. Indeed, in this case, the spectral shift obtained was only 5 nm and color changes were not obtained. Since, PGA catalyses the hydrolysis of pen G more efficiently than ampicillin, higher spectral shift was obtained in case of pen G.

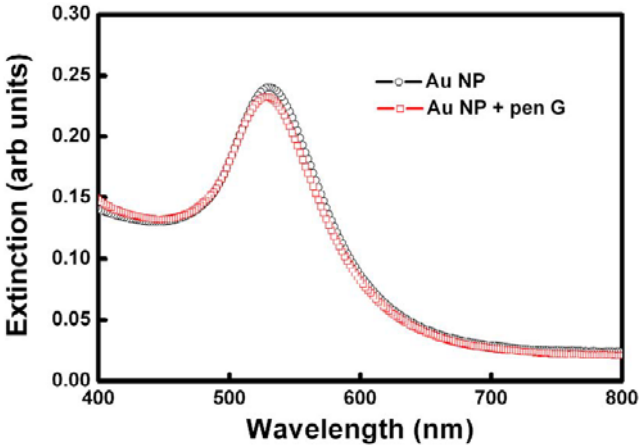


Figure 5.4: Absorption spectra of gold nanoparticles before and after addition of substrate pen G in the absence of enzyme

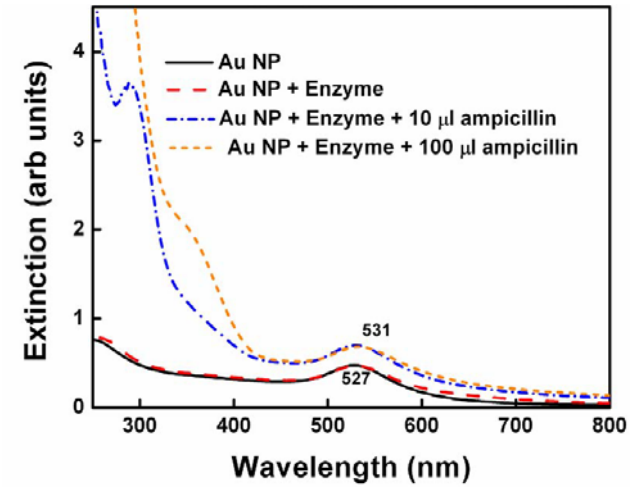


Figure 5.5: Spectral changes in gold nanoparticle-enzyme system on addition of ampicillin.

5.5 CONCLUSIONS

The colorimetric detection of Penicillin G acylase (PGA) is obtained here without adding any chromogenic agent and eventually penicillin G (pen G) using CTAB capped gold nanoparticles. Gold nanoparticle aggregation is induced by interaction between PGA and pen G causing red to blue color shift of the gold nanoparticles. Aggregation of Au nanoparticles was confirmed by TEM. The aggregation was found to be caused by 6-aminopenicillic acid (6-APA), produced as a reaction product in the reaction. Also, the above effect

was not observed in the absence of enzyme indicating that the aggregation of gold nanoparticles is caused by the enzyme-pen G reaction and not just by the presence of enzyme or pen G. To further confirm this, the experiment was also performed with another penicillin substrate (ampicillin) which is not a preferred substrate for PGA and it was observed that the spectral changes are less and slower. Quantity of enzyme, which could be detected, was 0.007 mg/ml in gold nanoparticles solution. Hence using gold nanoparticles, simple, rapid and colorimetric detection of PGA at low concentrations could be achieved by making use of enzyme-substrate chemistry. Apart from this, the system could also identify the presence of different penicillins (pen G and ampicillin) by showing different degrees of spectral shifts for them. This method will be useful to detect other impurities in the penicillins by change in the SPR.

CHAPTER 6

Immobilization studies of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

6.1 SUMMARY

PGA is an industrially important enzyme for the production of 6-APA and many semi-synthetic penicillins and cephalosporins and it is commercially used in the form of immobilized system. Magnetic iron oxide nanoparticles were used for immobilization of penicillin G acylase. PGA from *Arthrobacter viscosus* was purified and immobilized on iron oxide to test its compatibility with the matrix. The immobilized enzyme shows good activity, thus promising a potential system for industrial applications.

6.2 INTRODUCTION

Penicillin acylases are a group of enzymes that cleave the acyl chain of penicillins to yield 6-amino penicillanic acid (6-APA) and side chain acid (Shewale and ShivRaman, 1989). Penicillin G acylase (PGA) catalyses the hydrolysis of amidic bonds of penicillin G (pen G), cephalosporin G (ceph G) and related antibiotics to produce 6-amino penicillanic acid (6-APA) and 7-amino des-acetoxy cephalosporanic acid (7-ADCA), with side chain acid as a by-product (Arroyo et al., 2003; Rajendhran and Gunashekharan., 2004). Immobilized Penicillin G acylase (PGA) is widely used for the commercial production of 6-APA and 7-ADCA, which serve as the β-lactam antibiotic intermediates and are among the potential building blocks of various types of semi-synthetic penicillins (amoxicillin, ampicillin, cloxacillin) and cephalosporins (cephadroxil, cephalexin, etc.) (Arroyo et al., 2003; Elander, 2003). Currently the industrial scale demand of 6-APA is met by using PGA in immobilized from on a variety of support matrices. Immobilized form of enzyme has many advantages over employing isolated enzymes, such as easy separation of products, enhanced enzymatic stability under operational conditions and reusability.

Nanotechnology offers opportunities to interface with biology in new ways and with unprecedented precision. Considerable attention is given to nanoparticles made of magnetic materials. Magnetic nanoparticles are well-studied systems that offer:

i. controlled size,
ii. ability to be manipulated externally, and
iii. enhancement of contrast in magnetic resonance imaging (MRI) (Gao *et al.* 2009)

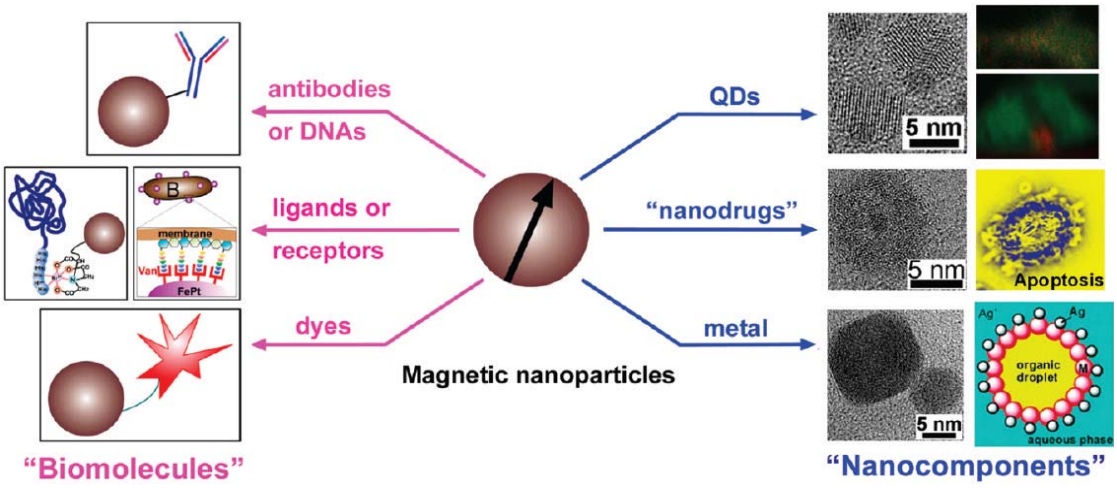


Figure 6.1 schematic representations of facets of use of magnetic nanoparticles. (Adapted from Gao et al. 2009).

With the increasing demand for functional nanoparticles, that could act as nano-devices, nano-sensors, nano-carriers, etc in different thrusts of areas. The application of the nanostructures stems from two key materials: (1) the unique properties displayed by the core nano-material and (2) the capping molecule on the surface of the nanomaterials. As explained previously, the former aspect determines what property of the nanoparticle is required in the application. Here, in this chapter, magnetic nanoparticles are used as the core nano-material. The use of magnetic nanoparticles has paved a plethora of specializations in biomedical and diagnostic applications such as cell-separation techniques, cell-labeling and targeting (Ben-David Makhluf *et al.* 2006), magnetic resonance imaging (Chun, A. L. 2009), drug delivery, magnetofection, etc. Magnetic nanoprobes have the answer to the lacking technology to assess the molecular targets of biomolecules in living cells. Jaejoon Won *et al.* developed MAGIC (Magnetism-based interaction capture) that identifies targets in living cells on the basis of induced movement of superparamagnetic nanoparticles (Won *et al.* (2005). The magnetic nanoparticles are abundantly used in biotechnological applications such as enzyme immobilsation (ValdeÌs-SoliÌs et al. 2009), drug delivery (Jain et al. 2008), or separation techniques (von Schönfeldt et al. 1999). These are exploited enormously also due to their reported low toxicity and high biocompatibility (Jain et al. 2008; Marchal, et al. 1989). Also, the latter aspect

of a proper capping molecule on the surface of the nanoparticle is more important from the functionality point of view; the molecules at the surface determine what the nanoparticle sees in the *nanocosm* and the macrocosm, and hitherto how it functions.

In this study, a general approach towards fabricating the surface of the magnetic nanoparticle to act as an immobilization substrate is suggested, so as to form a simple enzyme-substrate interaction and release the product. The objective, as shown in the schematic (Figure 6.2) was to study the activity of the enzyme capped on the magnetic nanoparticle in solution. The advantage of using magnetic nanoparticles is in the ease of separation after the reaction is over and washing is done. This idea of using magnetic nanoparticles for the separation of biomolecules and cells is already in the market; thus, if the enzyme activity in this system is not affected, it would prove to be very useful to immobilize the enzyme onto these nanoparticles.

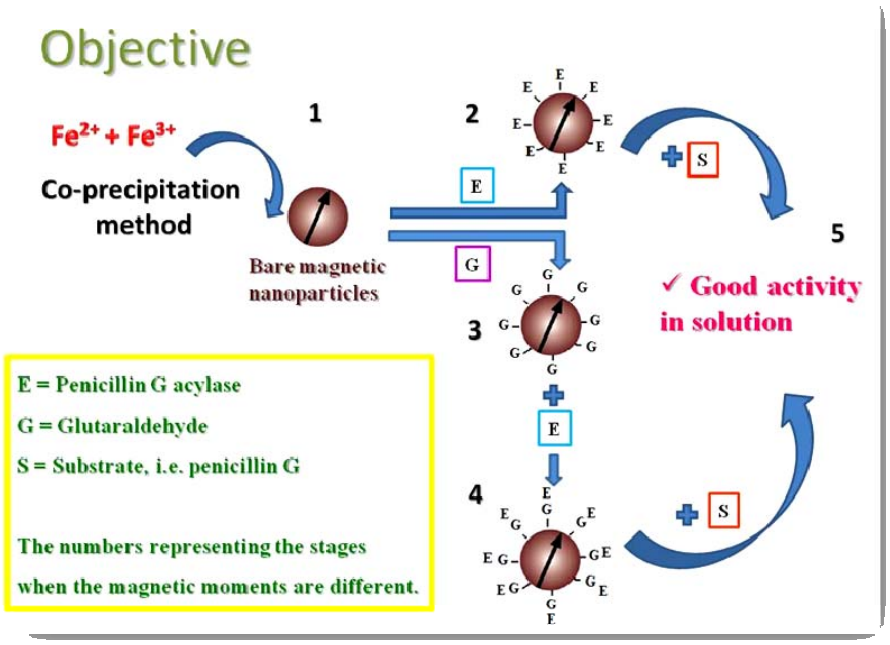


Figure 6.2 Schematic representation of the objective. Step 1 is the synthesis of magnetic nanoparticles, step 2 and 3 comprises of capping the bare nanoparticles with enzyme and glutaraldehyde respectively, step 4 is immobilizing enzyme onto the glutaraldehyde capped nanoparticles and step 5 involves determining the activity of the enzyme conjugated to these nanoparticles.

Certain nanomaterials are attractive probe candidates because of their
(1) small size (1-100 nm) and correspondingly large surface-to-volume ratio,
(2) chemically tailorable physical properties, which directly relate to size, composition and shape (Figure 6.1),
(3) unusual target binding properties and
(4) overall structural robustness.(Rosi and Mirkin, 2005)

It also presents some fresh perspectives for designing nanoparticles for industrial applications. As-synthesized nanoparticles, typically produced by wet-chemical methods or physical vapour deposition, are in most cases disordered solid solutions with a face centred- cubic (fcc) structure. The iron oxide nanoparticles synthesized in this work are chemically synthesized. And the enzyme that is used in this study is penicillin G acylase. Penicillin acylases (Penicillin amidohydrolases, PAs, EC 3.5.1.11) are a subclass of the β-lactam antibiotic acylase superfamily. These enzymes hydrolyse the relatively stable amide bonds in penicillins producing the β-lactam nucleus, 6-aminopenicillanic acid (6-APA) and the corresponding side chain. It is estimated that more than 85% of 6-APA is obtained enzymatically from PGA activity (in the form of immobilized PGA) and the remaining from penicillin V acylase (Vandamme, E. J., 1988). They are of great importance to the pharmaceutical industry for the production of semi-synthetic β-lactam antibiotics via the key intermediates 6-APA and 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) (Parmar et al*.,* 2000; Sudhakaran et al*.,* 1992). Penicillin acylase with broad substrate specificity is exploited for the commercial production of semi synthetic penicillins as well as cephalosporins (Shewale and Sivaraman, 1989). Because efficient recovery and reuse of the biocatalyst is a prerequisite for a viable process, much attention has been focused on the immobilization of penicillin G acylase. The enzyme from *Arthrobacter viscosus was* chosen as a model system to show possible activity upon immobilization onto nanoparticles.

Apart from mainly being used for the hydrolysis of natural β-lactams, penicillin acylases are also used for catalyzing the acylation of the amino group of key intermediates by appropriate esters or amides leading to the formation of semi-synthetic β-lactam antibiotics (Hernandez-Justiz, et al. 1999)). Penicillin G acylases are also useful for various other applications

such as peptide synthesis (van Langen et al., 2000a; van Langen et al., 2000b) removal of protecting groups (Waldmann et al., 1996; Alvaro et al., 2000) and kinetic resolution of racemic mixtures (van Langen et al., 2000a; Fadnavis et al., 1999).

6.3 MATERIALS AND METHODS

Synthesis of iron oxide nanoparticles

The iron oxide nanoparticles were prepared by the co-precipitation method. The co-precipitation technique is probably the simplest and most efficient chemical pathway to obtain magnetic particles. Iron oxides (either Fe_3O_4 or γFe_2O_3) are usually prepared by an aging stoichiometric mixture of ferrous and ferric salts in aqueous medium. Ferric and ferrous salts were taken at a ratio of 2:1 (Fe^{3+}:Fe^{2+}) to have a total concentration of 10^{-2} M. The mixture was heated at 80 °C for 1 h with continuous stirring under non-oxidising environment (Jolivet et al (2004)Then, 5 mL of ammonium hydroxide (30% w/w) was added rapidly and the mixture was vigorously stirred for another 1 h, after which it was cooled to room temperature. The precipitates were washed with hot water repeatedly for five times and separated by decantation before use. The considerably large numbers of nanoparticles are synthesized with good size-control.

Immobilization of penicillin G acylase

The Enzyme penicillin G acylase (PGA) used was purified from the gram-positive bacteria *Arthrobacter viscosus* (ATCC 15294). The enzyme was produced extracellularly in the culture broth. The cells were separated by centrifugation, and the clear broth was used for further purification by passing it through alumina column followed by hydrophoric interaction chromatography (octyl sepharose) and anion exchange chromatography (Q-sepharose). Purity of the enzyme was checked by native gel electrophoresis and was used as such for further studies.

The iron oxide particles were capped with glutaraldehyde (2%), by incubating at 4 °C, on shaking for proper mixing. After incubation, excess glutaraldehyde was washed off using ample phospahate buffer (pH 8). Further, the iron oxide nanoparticles, with and without glutaraldehyde capping, were suspended in phosphaste buffer (pH 7.2) and varying concentrations of penicillin G acylase (3.32 IU/mL) was added. The mixtures were incubated a shaker at 4 °C for 1

h, after which excess enzyme was washed off using the same buffer. The immobilized penicillin G acylase nanoparticles were stored in the same phosphate buffer at 4 °C. The supernatant was assayed for unbound enzyme. The quantity of enzyme bound is the difference between the amount of enzyme added to the nanoparticle sol and the amount of enzyme remaining free in the supernatant. The immobilized penicllin G acylase nanoparticles were also processed for activity determination.

Enzyme activity test

The protocol for determination of PGA activity (both soluble and immobilized) is as follows. The enzyme activity of Penicillin G acylase (PGA) was determined by measuring the amount of 6-APA produced in a 1 ml reaction mixture containing enzyme sample and 20 mg ml^{-1} Penicllin G (pen G) in 0.1 M phosphate buffer, pH 7.0, when incubated at 40 °C (Balasingham *et al*, 1972). Penicillin G was obtained from KDL Biotech. The reaction was stopped by adding 1 ml of citrate phosphate buffer (pH ~2.5). The 6-APA produced was estimated spectrophotometrically at 415 nm, after reaction with p-dimethylaminobenzaldehyde (PDAB), according to the protocol by Bomstein and Evans, 1965, modified by Shewale *et al*., 1987. 0.1 g of PDAB was dissolved in 17 mL of methanol. One unit of PGA activity is defined as the amount of enzyme required to produce 1 µmole of 6-APA in one minute under the assay conditions (40 ^{o}C and pH 7.0).

Enzyme activity, expressed in international units (IU), is defined as the quantity of the enzyme required to liberate 1 µmol of 6-APA (which corresponds to an O.D. of 0.81) produced per minute under conditions of assay.

$$Activity = \frac{\Delta O.D.}{0.81} \times \frac{1}{t} \times D.F. \times \frac{1}{vol.\,of\ enzyme\ (mL)}$$

where, *ΔO.D.* is the different in the optical density of the sample and the background,

t is the time of incubation during the activity test, and

D.F. is the dilution factor while measuring the absorbance.

6.4 Results and Discussions

The iron oxide nanoparticles synthesized by co-precipitation method was index matched (JCPDS 190629) and shown in Figure 3.28. The morphology of these nanoparticles was obtained on TEM micrographs shown in Figure 3.29. The particles synthesized were roughly around ~ 20 nm in size.

After incubation with glutaraldehyde and the enzyme PGA, the samples were tested for activity against the substrate pen G, as explained in the experimental details section.

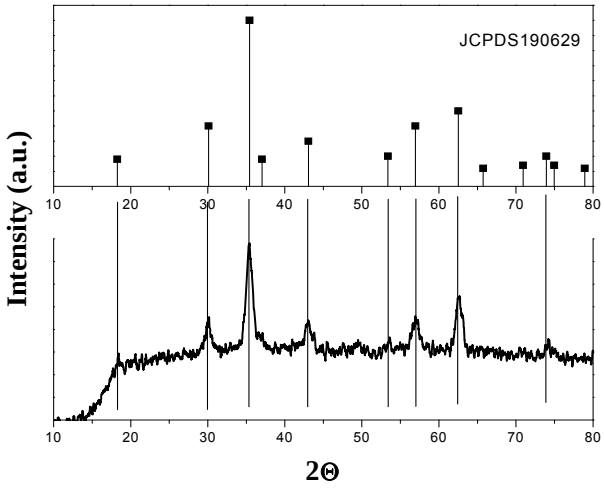


Figure 6.3: X-ray diffraction pattern of iron oxide nanoparticles synthesized by co-precipitation method

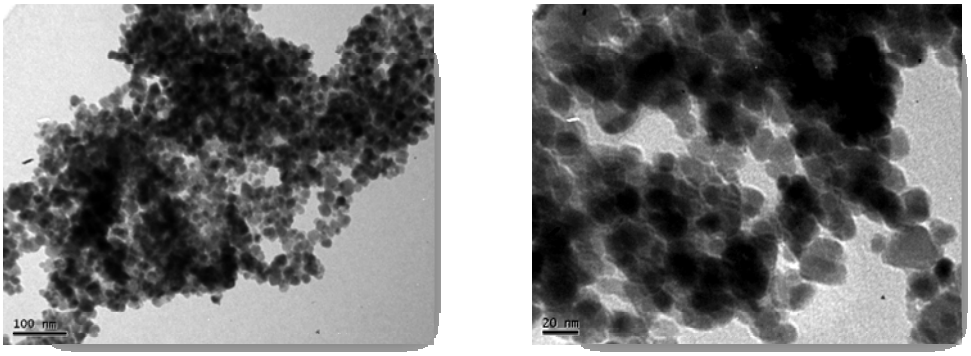


Figure 6.4: TEM micrographs of iron oxide nanoparticles.

The Figure 6.5 clearly shows that glutaraldehyde serves to be a good cross-linking agent, enabling better binding of the enzyme onto the nanoparticle surface than the enzyme bound directly onto the bare nanoparticle.

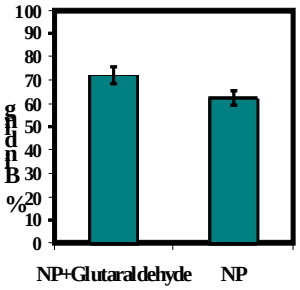


Figure 6.5: Comparision of binding percentage between enzymes immobilized on glutaraldehyde capped nanoparticles and bare nanoparticles.

Hence, for further estimation of enzymatic studies, the enzyme immobilized on the glutaraldehyde capped iron oxide nanoparticles was taken. The AFM image is shown in Figure 6.6.

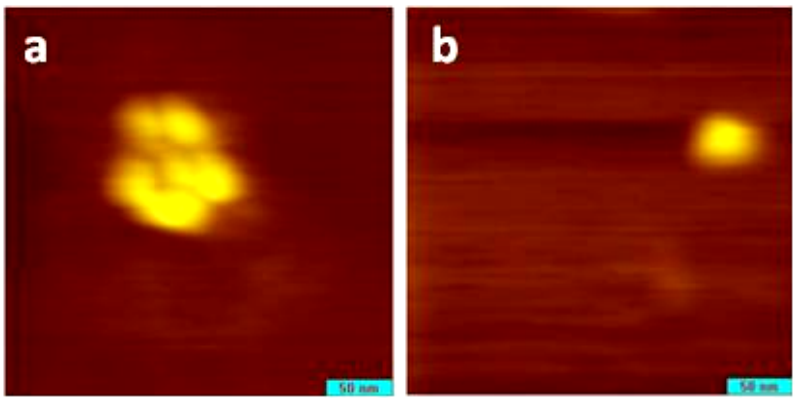


Figure 6.6 AFM contact-mode image of **(a)** unbound iron oxide nanoparticles and **(b)** enzyme bound glutaraldehyde capped iron oxide nanoparticles. A soft cantilever of spring constant = 0.2 N/m from budget sensor is used. The force set point was .03 nN.

The bare iron oxide nanoparticles are seen to be larger in size as compared to the TEM micrographs data. Clearly the lateral size of the nanoparticles imaged in AFM is large due to the convolution effect of the tip (Ebenstein et al., 2002). Nonetheless, a comparison of the unbound iron oxide nanoparticles and the enzyme bound glutaraldehyde capped nanoparticles exhibits a larger size in case of the latter due to the capping molecules on the nanoparticle surface. Thus the confirmation of capping from both activity (which was clearly absent in case of bare nanoparticles and only glutaraldehyde capped nanoparticles) and imaging is seen in the above results.

Further, saturation kinetics was performed to determine the concentration at which the saturation of immobilization was taking place for a given amount of nanoparticles as shown in Figure 6.7.

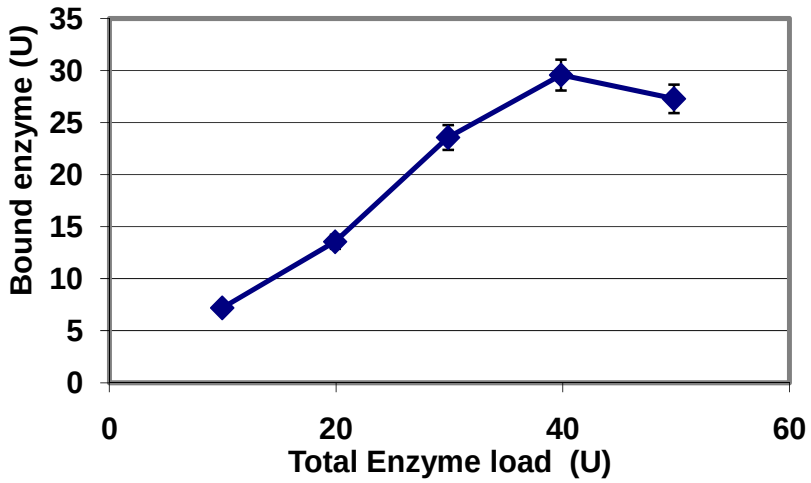


Figure 6.7 Saturation kinetics of PGA binding on nanoparticles

It was observed that a linear increase in the binding initially; after a load of 40 U of the enzyme, the plot reaches a plateau. Comparison of values for binding, immobilized enzymes (IME) activity, % expression and overall efficiency of the PGA onto glutaraldehyde capped iron oxide nanoparticles showed greater binding capacity. If the % expression values remain same

after separation and washing, this will be a potential candidate for use as a commercial carrier for immobilization of PGA.

For a given mass of nanoparticles, when incubated with different amounts of PGA (9.96 to 49.80 U/mL), a clear trend in enzyme binding, percent binding, and IME activity, and in the % expression as presented in Table 6.1. The amount of PGA bound increased from 7.20 to 27.30 U/mL, while % binding decreased from 72 to 54%. Likewise, the activity of immobilized PGA preparation (IME activity) increased from 5.28 to 24.54 IU/mL but the % expression decreased progressively.

Table 6.1: Immobilisation of PGA onto nanoparticle: effect of enzyme loading on nanoparticles

Enzyme Loaded (U/mL*)	Enzyme Bound (U/mL*)	Binding %	IME activity (IU/mL*)	Expression %
9.96	7.20	72	5.28	73
19.92	13.56	68	10.56	78
29.88	23.58	78	18.96	80
39.84	29.58	74	24.48	82
49.80	27.30	54	24.54	90

(*1 mL contains 0.125 mg of iron oxide nanoparticles); U, micomoles of 6-APA produced per hour; IME: Immobilized Enzyme activity

However, the decrease is not much and the error is minimal between the two percentages. The decrease in expression activity in the binding of PGA can be due to the following reasons:

i) Loss of activity due to leaching of bound enzyme

ii) Masking of the active site due to binding onto the nanoparticles

iii) Diffusional barriers for the substrate during activity

In addition to this, experimental errors, such as loss of nanoparticles bound with the enzyme during washes, are unavoidable and could be a major contributing factor in the difference of activity.

6.5 Conclusions

With great advantage of being able to immobilize the enzyme in its active state and efficiently form product upon reacting with the substrate at concentrations that is industrially accepted. Though further tests are needed to validate this process to the industrial level, this is a novel system where the applicability of the iron oxide nanoparticles for the immobilization and good activity has been demonstrated in the case of Penicillin G acylase. The present investigations confirm the hypothesis of the possibility of use of PGA immobilised onto glutaraldehyde capped iron oxide nanoparticles. The binding of PGA on the glutaraldehyde capped nanoparticle is due to the stable interaction of the enzyme with glutaraldehyde and this is governed by its surface properties. Studies are underway for ascertaining the re-usuability upon magnetic separation and repeated washing, and possible leaching with time.

Further refinement of the fictionalization and magnetic studies could also prove this system to be a useful biosensor. Magnetic nanoparticles have already been tested for acting as biosensors and many uses are there in the market. Superparamagnetic nanoparticles (MNPs) are biocompatible (Jain et al., 2008) making it safe from the environmental pollution point of view. Introducing these nanoparticles, as possible industrial application shown in this study, does not pose a threat to our environment.

Summary and Conclusions

Penicillin G acylase is of tremendous application in industrial production of 6-APA from natural penicillins and in the production of semi-synthetic penicillins. Due to the importance of this enzyme in pharmaceutical industry, much effort had been taken to isolate new isolates with enhanced properties. These enzymes are also used in various other application such as peptide synthesis, kinetic resolution of racemic mixture, synthesis of artificial sweetener, aspartame.

Arthrobacter viscosus PGA is produced in the culture broth and this makes it a potent candidate for industrial application. Extra cellular PGA production has many advantages over intracellularly produced enzymes, as it reduces the steps involved in down stream processing, thus reducing the cost of enzyme for industrial purposes.

The enzyme production is regulated by many factors, like media components, pH of the medium and the temperature at which the fermentation is carried out. The enzyme production by *Arthrobacter viscosus* was induced by the presence of phenyl acetic acid. Various fermentation parameters were optimized for the production of PGA by *A. viscosus*. This included initial pH of the fermentation medium. Aeration study was performed by taking different volumes of medium in 500 ml Erlenmeyer flasks. Time kinetics of the fermentation showed that maximum enzyme is produced between 48 h and 60 h of incubation.

Enzyme production was done in shake flask conditions in 1 L flasks at 28 ^{o}C. Since the enzyme is secreted in culture broth, the purification of enzyme is easier. The enzyme was purified to homogeneity by chromatography techniques including binding on Alumina column, followed by octyl-sepharose chromatography and final purification by ion exchange chromatography on Q-sepharose column. Subunit molecular weights were determined by SDS-PAGE and MALDI-TOF mass spectrometry. Biophysical parameters, like pH and temperature optima, pH and temperature stability, substrate specificity of the purified enzymes were performed. Based on these parameters characterized, the *Av*PGA is a potent candidate for industrial application.

Enzyme catalysis is an important part of any industrially important enzyme. The information of active site residues can be beneficial in

understanding its catalysis and in designing novel variant of the enzyme which can work on wide range of substrates and in turn can be useful in synthesis of novel antibiotics. With this point of view, the purified enzyme was subjected to some amino acid modifying agents. Arginine and tryptophan modification experiments showed that these amino acids play an important role in the enzyme catalysis.

CTAB capped gold nano particles were utilized for designing a simple method which can be utilized for screening novel source of PGA producers. The enzyme hydrolysis can be monitored by using these nano particles. The color of the nano particles changes from pink to blue when hydrolysis of pen G occurs. It is shown that the color change is affected by the hydrolysis product, 6-APA and not by substrate or product, phenylacetic acid.

Immobilization study is an important part for any industrially important enzyme. Since PGA is of tremendous industrial application, the study of immobilization was performed by using iron oxide nano particles. iron oxide nano particle have many advantage over other support matrices, as it provide very large surface area and also there is very less diffusional barrier in using such type of immobilization matrix.

Based on the study carried out on *Arthrobacter viscosus* penicillin G acylase, it is shown that this enzyme can be a good candidate for industrial application in semi synthetic antibiotic industry. Further research is needed on identification of amino acids residues taking part in the catalysis, which will open new vistas for this enzyme in chemical industry in designing novel semi-synthetic antibiotics. Crystallography is a strong tool to decipher the three dimensional structure of proteins. Some initial trials were performed to obtain crystals of the purified PGA from *Arthrobacter viscosus*, which is shown in appendix.

Studies on CTAB capped gold nano particles and iron oxide nano particles show promising field for an industrially important enzyme such as PGA from *Arthrobacter viscosus*. Both these matrices can be utilized for designing biosensor.

APPENDIX

Preliminary X-Ray crystallography study of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294

APPENDIX

Crystallization Trials for Penicillin G Acylase from *Arthrobacter viscosus* (*Av*PGA)

A.1 SUMMARY

Initial crystallization conditions were tried using standard screens. Penicillin G acylase from *Arthrobacter viscosus* was purified using various chromatographic techniques as stated in chapter 3 and concentrated using 30 kDa centricon up to 15 mg/mL. The hanging drop vepour diffusion method was chosen for crystallization purpose. The standard Screens CSS-I and CSS-II from Hampton Research were used for initial crystallization trials.

A.2 INTRODUCTION

X-ray crystallography is a powerful tool for three-dimensional structure determination of proteins. Although other methods like NMR and electron and atomic force microscopy are also useful tools to solve protein structures, they are still limited by the protein size and restricted structural information that can be obtained. The structure determined from X-ray crystallography provides information on structural chemistry of the interaction between protein and the cognate ligands. Thus, the knowledge of structural details allows to rationally designing novel substrates or inhibitors in modern drug design for the pharmaceutical industry. In addition, functional details are derived from protein structures, such as their biological chemistry of enzyme activity.

The flowchart (Figure A.1) describes a step-by-step protocol to proceed from a crystal to a protein structure, with the help of a known protein structure.

Crystallization of the molecule of interest is a limiting step in protein crystallography. Protein crystals are composed of approximately 50% solvent, though this may vary from 30-78% (Matthews, 1985). They are labile, fragile, and sensitive to external environments owing to their high solvent content, and the weak binding energies between protein molecules in the crystal (Littlechild, 1991). The only optimal conditions suitable for their growth are those that cause little or no perturbation in their molecular properties.

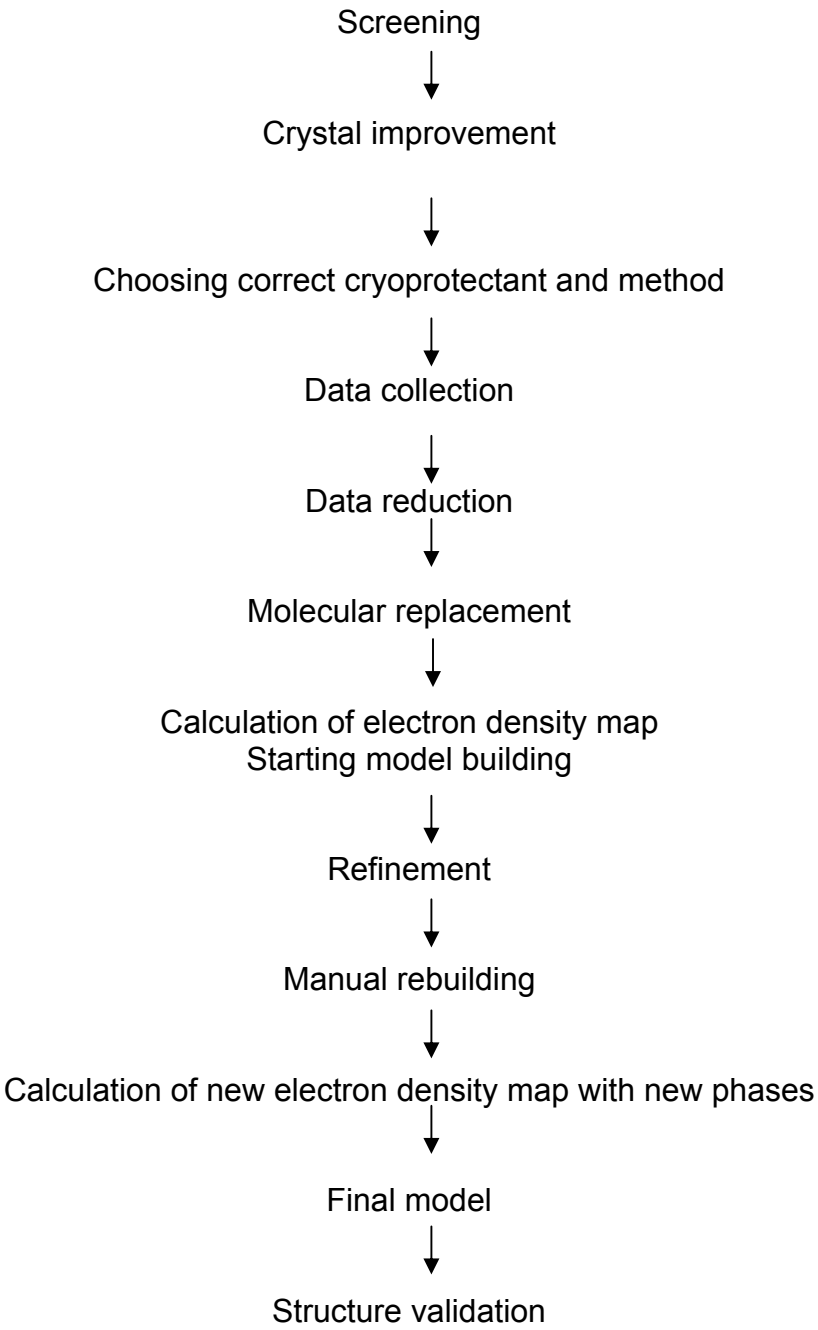


Figure A.1: General Schematic representation of steps involved in protein X-ray crystallography.

The crystallization of proteins from solution is a reversible equilibrium phenomenon. It can be considered as consisting of three stages: nucleation, growth and cessation of growth. Nucleation the necessary first step in the crystallization process, influence it decisively. Consequently, the ability to control it is of primary importance in crystallization experiments. Condition for nucleation, different from the supersaturation conditions, presents a free energy barrier which must be overcome in specific ways, and which subsequently makes crystal growth an energetically favourable process. The formation of crystals is due to decreasing free energy of the system by the formation of many new interactions which outweighs the decreasing entropy of the system, allowing for a highly organized structure. In other words the reduction in free energy of the system is the thermodynamic driving force that causes ordering in crystals (McPherson, 1982).

The basic strategy of growing protein crystals is to generate certain degree of supersaturation in the solution. At the equilibrium point, the number of protein molecules entering the solution is same as the number leaving the solution. This is referred to as the solubility limit of a protein. When the amount of protein in the solution is below this limit, the solution is undersaturated. If the amount of protein is equal to this limit, the solution is in saturated state. Crystals can grow only when the solubility exceeds the solubility limit. Every protein has a unique solubility limit (Figure A.2). Decreasing the solubility of the protein is the most effective way of creating supersaturation. Crystals will grow only from a non-equilibrium supersaturated solution. Supersaturation can be achieved by different approaches including altering the buffer pH, temperature, protein concentration, dielectric constant of the medium, and precipitant concentration.

The most popular experimental method used for crystallization is the vapour-diffusion method, both hanging-drop and sitting-drop method. The hanging-drop vapour-diffusion method is an efficient means of screening many crystallization parameters. The advantage is that it requires only a small volume of droplet, which can be as low as 1 µl per experiment, so only a small amount of sample is consumed for screening and optimization of the crystallization conditions (McPherson, 1998). The reason for the popularity of

the hanging-drop method is the ease of performing the experiment, only a 24 well-plate, grease and cover slips are required (Figure A.3).

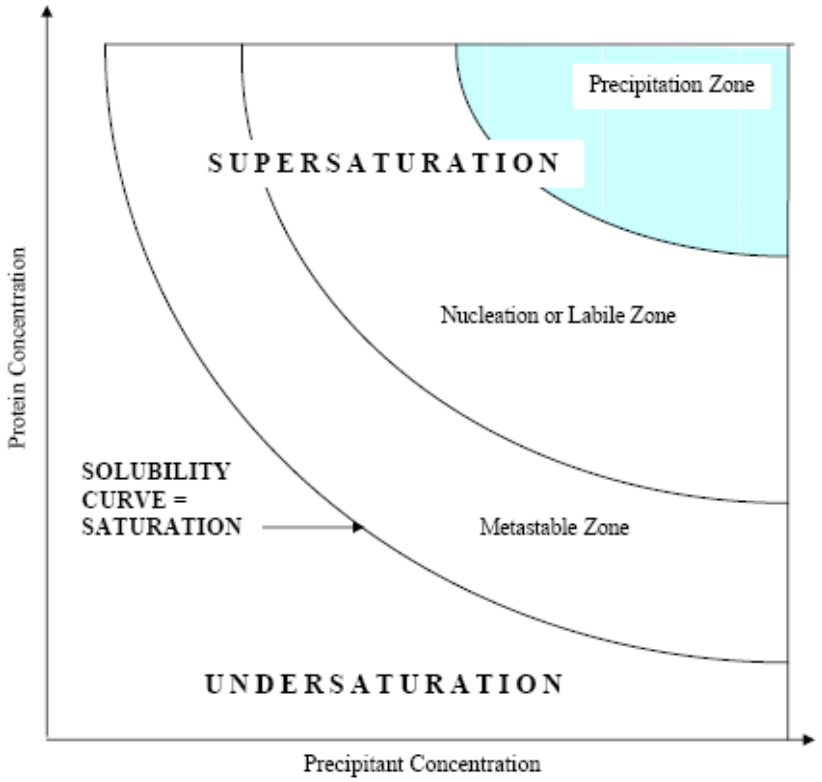


Figure A.2: The solubility phase diagram of crystallization from solution (Blow, 2002)

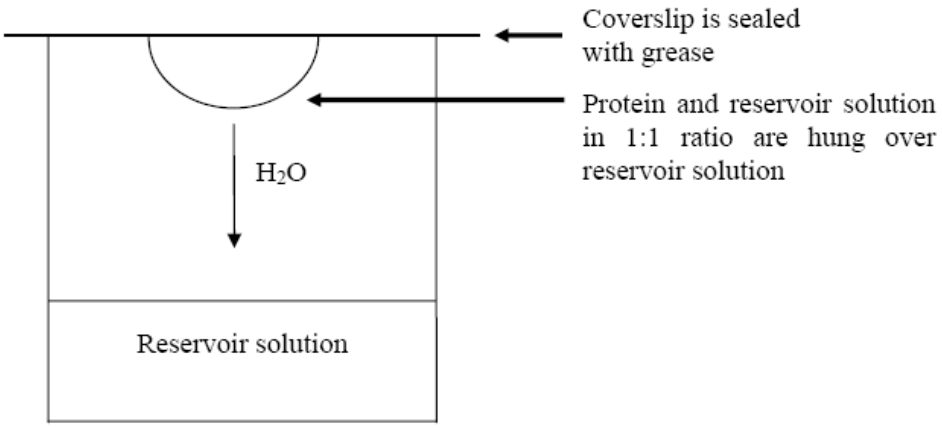


Figure A.3: The hanging-drop vapour-diffusion method for protein crystallization.

Seeding is a useful technique for growing crystals by producing a seed-stock solution in cases where spontaneous homogenous nucleation does not occur. It is generally believed that the nucleation will be initiated at a higher level of supersaturation, the labile zone (Stura and Wilson, 1991). To use seeding techniques, good quality crystal seeds have to be selected and introduced into the metastable labile zone. After seeding, the seeded crystals will continue to grow. The crystal growth conditions can also be optimized independently without the need to introduce nuclei of the protein itself (Luft and Detitta, 1999).

A.3 MATERIALS AND METHODS

Materials

The media components were obtained from HiMedia, India. Centricon tubes were purchased from Millipore. All the buffers were prepared in glass distilled water. The standard screens CSS-I and CSS-II were obtained from Hampton Research.

Purification of penicillin G acylase

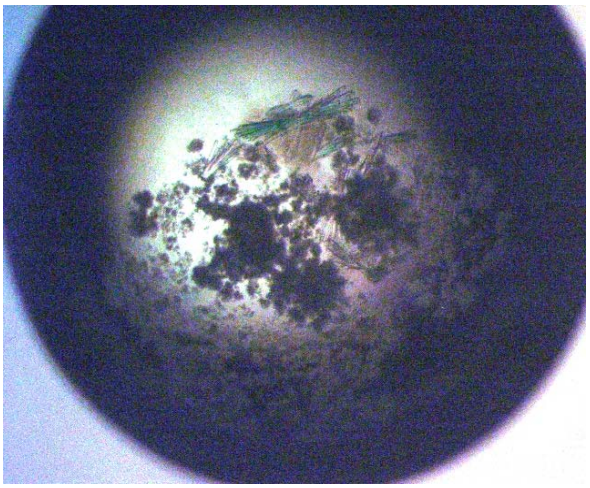
PGA from *Arthrobacter viscosus* was purified according to the method described in chapter 3. Enzyme was concentrated using using 30 kDa centricon tubes. Protein content was done using Lowery method (Lowery et al, 1951)

Crystallization trials

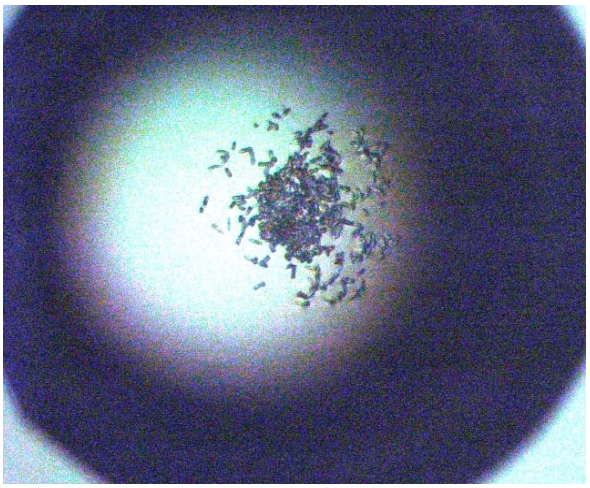
The hanging-drop vapour-diffusion method was used for initial crystallization of *Av*PGA. Precipitating solutions (1ml each), composed of precipitant, buffer solution, additive, and distilled water were pipetted into the 24 well reservoirs of the crystallization plate. Then 1 µl of protein solution was dispensed onto the centre of a clean siliconized 22 mm circular glass cover slip and mixed with 1 µl of reservoir solution instantaneously. The cover slip was then inverted using tweezers without losing the drop and sealed over the reservoir with gentle pressure to ensure proper sealing. The plate is placed on crystallization rack and maintained at 20 °C or room temperature and observed for any crystals to appear.

A.4 RESULTS AND DISCUSSION

The protein was concentrated and 15 mg/ml of protein was used for setting up initial crystallization screen. After regular observation of the plates, crystals were observed in some of the wells. The crystals are not of very good quality. The photographs of the well having some crystals are presented here. Table A.1 summarizes the various conditions in which *Av*PGA crystallized in two different shapes.



a



b

Figure A.4: (**a)** Needle shaped crystals and (**b)** rod shaped crystals obtained for *Av*PGA

Table A.1: Initial screening conditions in which crystals appeared

S. No	Condition	Time taken for crystal growth	Type of Crsytals formed
1.	0.1 M HEPES-Na pH7.5, 1.5M Lithium Sulphate monohydrate	2-3 weeks	b
2.	0.1M HEPES-Na pH 6.5, 12% PEG 20,000	2-3 weeks	a
3.	0.1M Tris-Hydrocloride pH8.5, 8%PEG 8000	2-3 weeks	a
4.	0.01M Nickel chloride, 0.1M Tris-Hydrochloride pH 7.5, 1M Lithium Sulphate monohydrate	10days	b
5.	0.01M Nickel chloride hexahydrate, 0.1M Tris-Hydrochloride pH 8.5, 1M Lithium Sulphate monohydrate	2 weeks	b
6.	0.01M Nickel chloride, 0.1M Tris-Hydrochloride pH 9.0, 1M Lithium Sulphate monohydrate	2 weeks	b
7.	0.1M Tris-HCl pH 8.5, 20% v/v Ethanol	2-3 weeks	a
8.	15% PEG 8000, 100 mM Tris-HCl pH 7.0, 120µl (0.5%) β-octyl glucopyranoside	10-12days	a
9.	15% PEG 8000, 100 mM Sodium Cacodylate pH 7.5, 120µl (0.5%) β-octyl glucopyranoside	10-12 days	a
10.	15% PEG 8000, 100 mM Sodium Cacodylate pH 8.0, 120µl (0.5%) β-octyl glucopyranoside	10-12days	a
11.	15% PEG 8000, 100 mM Sodium Cacodylate pH 7.0, 140µl (0.5%) β-octyl glucopyranoside	10-12 days	a
12.	15% PEG 8000, 100 mM Tris-HCl pH 7.5, 140µl (0.5%) β-octyl glucopyranoside	10-12 days	a
13.	15% PEG 8000, 100 mM Sodium Cacodylate pH 8.0, 140µl (0.5%) β-octyl glucopyranoside	10-12 days	a
14.	15% PEG 8000, 100 mM Tris-HCl pH 8.0, 140µl (0.5%) β-octyl glucopyranoside	10days	a

REFERENCES

Abraham, E.P.: (1981) The β-lactam antibiotics. *Sci. Am.*, **244**, 64-74.

Alicino, J. F.: (1961) Iodometric Assay of Natural and Synthetic Penicillins, 6-Aminopenicillanic Acid, and Cephalosporin C. *Anal. Chem.*, **33,** 648-649.

Alkema, W. B. L., Floris, R., Janssen, D. B.: (1999) The use of chromogenic reference substrates for the kinetic analysis of penicillin acylases. *Anal. Biochem.,* **275,** 47-53.

Alkema, W. B. L., Prins, A. K., De Vries, E., and Janssen, D. B.: (2002) Role of αArg^{145} and βArg^{263} in the active site of penicillin acylase of *Escherichia coli*. *Biochem. J.* **365,** 303-309.

Alvaro G., Feliu J. A., Caminal G., Lopezsantin J., and Clapes P.: (2000) A novel activity of immobilized penicillin G acylase: removal of benzyloxycarbonyl amino protecting group. *Biocatal. Biotrans*., **18,** 253-258.

Anon: (1992) Technomarket survey report on enzymes (Genetically engineered and immobilized), New Delhi: TIFAC, DST, pp. 125-147.

Aramori, I., Fukagawa, M., Tsumura, M., Iwami, M., Isogai, T., Ono, H., Ishitani, Y., Kojo, H., Kohasaka, M., Ueda, Y. and Imanaka, H.: (1991a) Cloning and nucleotide sequencing of new glutaryl 7-ACA and cephalosporin C acylase genes from *Pseudomonas* strain. *J. Ferment. Bioeng.,* **72,** 232-243.

Aramori, I., Fukagawa, M., Tsumura, M., Iwami, M., Ono, H., Kojo, H., Kohasaka, M., Ueda, Y. and Imanaka, H.: (1991b) Cloning and nucleotide sequencing of a novel 7β-(4-carboxybutanamido) cephalosporanic acid acylase gene of *Bacillus laterosporus* and its expression in *Escherichia coli* and *Bacillus subtilis*. *J. Bacteriol.*, **173**, 7848-7855.

Aronson, A., Sang, H. Y., and Bourne, N.: (1989) Gene structure and precursor processing of a novel *Bacillus subtilis* spore coat protein. *Mol. Microbiol.* **3**, 437-444.

Arroyo M., de la Mata I., Acebal C., and Castillón P. M.: (2003) Biotechnological applications of penicillin acylases: state of the art. *Appl. Microbiol. Biotechnol.,* **60**, 507-514.

Aslan, K., Lakowicz, J. R. and Geddes, C. D.: (2005) Metal-enhanced fluorescence using anisotropic silver nanostructures: Critical progress to

date. *Anal. Bioanal. Chem.*, **382**, 926-933.

Baker, W. L.: (1979) Estimation of 6-aminopenicillanic acid by the ninhydrin reaction. *Australian J. Pharm. Sci.* **8,** 86-90.

Baker, W. L.: (1980) A note on the detection of penicillin acylase activity in *Escherichia coli* by the reaction of ampicillin with Biuret Reagent. *J. Appl. Bacteriol*., **49**, 225-229.

Baker, W. L.: (1985) A sensitive enzymic assay for benzylpenicillin. *J. Appl. Bacteriol.*, **59**, 347-352.

Balasingham, K., Warburton, D., Dunnill, P. and Lilly, M. D.: (1972) The isolation and kinetics of penicillin amidase from *Escherichia coli*. *Biochimica et Biophysica Acta,* **276**, 250-256.

Barbero, J. L., Buesa, J. M., Gonzalez de Buitrago, G., Mendez, E., Pez-Aranda, A. and Garcia, J. L.: (1986) Complete nucleotide sequence of the penicillin acylase gene from *Kluyvera citrophila*. *Gene*, **49**, 69-80.

Batchelor, F. R., Chain, E. B., Hardy, T. L., Mansford, K. R. L., and Rolinson, G. N.: (1961) 6-Aminopenicillanic Acid. III. Isolation and Purification. *Proc. Roy. Soc. Lond. B-Biol*. Sci., **154**, 498-508.

Ben-David Makhluf, S., Qasem, R., Rubinstein, S., Gedanken, A. and Breitbar, H.: (2006) Loading Magnetic Nanoparticles into Sperm Cells Does Not Affect Their Functionality. *Langmuir,* **22**, 9480-9482.

Bennet, J. W. and Chung, K. T.: (2001) Alexander Fleming and the discovery of penicillin. *Adv. Appl. Microbiol*., **49**, 163-184.

Blow, D.: (2002) Outline of Crystallography for Biologist. *Ed.*1. New York, Oxford University Press.

Bomstein, J. and Evans, W. G.: (1965) Automated colorimetric determination of 6-aminopenicillanic acid in fermentation media. *Anal. Chem.,* **37,** 576-578.

Bossi, A., Cretich, M., Righetti, P. G.: (1998) Production of d-phenylglycine from racemic (d,l)-phenylglycine via isoelectrically trapped penicillin G acylase. *Biotechnol. Bioeng*., **60**, 454-461.

Branningan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C., Smith, J. L., Tomchick, D. R. and Murzin, A. G.: (1995) A Protien catalytic framework with an N-terminal nucleophile is capable of self-activation. *Nature*, **378**,

416-419.

Bruggink, A. and Roy, P. D.: (2001) Industrial synthesis of semisynthetic antibiotics. In Bruggink, A. (ed.) Synthesis of β-lactam antibiotics. Kluwer academic publishers, Dordrecht, pp. 12-56.

Bruggink, A., Roos, E. C. and de Vroom, E.: (1998) Penicillin acylase in the industrial production of β-lactam antibiotics. *Org. Proc. Res. Dev.*, **2,** 128-133.

Bruns, W., Hoppe, J., Tsai, H., Brüning, H. J., Maywald, F., Collins, J., Mayer, H.: (1985). Structure of the penicillin acylase gene from *Escherichia coli*: a periplasmic enzyme that undergoes multiple proteolytic processing. *J. Mol. Appl. Genet.,* **3,** 36-44.

Burlingame, R. and Chapman, P. J.: (1983) Catabolism of phenylpropionic acid and its 3-hydroxy derivative by *Escherichia coli*. *J. Bacteriol.*, **155,** 113-121.

Cabrera, Z., Gallego, F. L., Lorente, G. F., Palomo, J. M., Montes, T., Grazu, V., Guisan, J. M. and Fernandez-Lafuente, R.: (2007) Asymmetric hydrolysis of dimethyl phenylmalonate by immobilized penicillin G acylase from *E. coli*. *Enzyme Microb Technol*., **40**, 997-1000.

Cai, G., Zhu, S., Yang, S., Zhao, G. and Jiang, W.: (2004) Cloning, Overexpression, and Characterization of a Novel Thermostable Penicillin G Acylase from *Achromobacter xylosoxidans*: Probing the Molecular Basis for Its High Thermostability. *Appl. Environ. Microbiol.,* **70**, 2764-2770.

Cainelli, F., Giacomini, D., Galletti, P., DaCol, M. (1997) Penicillin G acylase mediated synthesis of the enantiopure (S)-3-aminoazetidin-2-one. *Tetrahedron Asym.*, **8**, 3231–3235.

Cao, L., Langen, L. M., Rantwijk, F., and Sheldon, R. A.: (2001) Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of β-lactam antibiotics. *J. Mol. Catal. B: Enzym*., **11**, 665-670.

Carleysmith, S. W., Dunnill, P. and Lilly, M. D.: (1980) Kinetic behaviour of immobilized penicillin acylase. *Biotech. Bioeng*., **22**, 735-756.

Chandel, A. K., Rao, L. V., Narasu, M. L. and Singh, O. V.: (2008) The realm of Penicillin G Acylase in β-lactam antibiotics. *Enzyme Microbiol. Tech.,* **42**, 199-207.

Chen, J., Li, Y., Jiang, W., Yang, Y., Zhao, G and Wang, E.: (1998) Secretary expression of GL-7ACA acylase. *Acta Biochim. Biophys. Sinica,* **30**, 393-396.

Chiang, C. and Bennet, R. E.: (1967) Purification and properties of penicillin amidase from *Bacillus megaterium*. *J. Bacteriol.,* **93**, 302-308.

Chou, C. P., Tseng, J., Kuo, B., Lai, K., Lin, M., and Lin, H.: (1999) Effect of SecB chaperone on production of periplasmic penicillin acylase in *Escherichia coli*. *Biotechnol. Prog.* **15**, 439-445.

Chou, J., Yen, C. and Lai, Y.: (2009) Penicillin G biosensor, systems comprising the same, and measurement using the systems. US patent 7501258 B2.

Chun, A. L.: (2009) Nanoparticles offer hope for TB detection. *Nature Nano.,* **4**, 698-699.

Claridge, C. A., Gourevitch, A. A. and Lein, J.: (1960) Bacterial penicillin amidase. *Nature* **187**, 237-238.

Claridge, C. A., Luttinger, J. R. and Lein, J.: (1963) Specificity of penicillin amidases. *Proc. Soc. Exp. Biol. Med.*, **113**, 1008-1012.

Cole, M.: (1964) Properties of the Penicillin Deacylase Enzyme of *Escherichia coli*. *Nature*, **203**, 519-520.

Cole, M. and Sutherland, R. S.: (1966) The role of penicillin acylase in the resistance of gram-negative bacteria to penicillins. *J. Gen. Microbiol.*, **42**, 345-356.

Cole, M.: (1967) Microbial synthesis of penicillin and 6-APA. *Process Biochem.*, **2**, 35-43.

Cole, M.: (1969) Hydrolysis of penicillins and related compounds by the cell-bound penicillin acylase of *Escherichia coli*. *Biochem. J.*, **115**, 733-739.

Cole, M., Savidge, T. and Vanderhaegde, H.: (1975) Penicillin acylase (assay). *Methods Enzymol.* **43**, 698-705.

Crawford, L., Stepan, A. M., McAda, P. C., Rambosek, J. A., Conder, M. J., Vinci, V. A. and Reeves, C. D.: (1995) Production of cephalosporin intermediates by feeding adipic acid to recombinant *Penicillium chrysogenum* strains expressing ring expansion activity. *Biotechnology N. Y.*, **13**, 58-62.

Dai, M., Zhu, Y., Yang, Y., Wang, E., Xie, Y., Zhao, G., and Jiang, W.: (2001) Expression of penicillin G acylase from the cloned pac gene of *Escherichia coli* ATCC 11105. Effects of pacR and temperature. *Eur. J. Biochem*., **268**, 1298-1303.

Daumy, G. O., McColl, A. S. and Apostolakos, D.: (1982) Repression of penicillin G acylase of *Proteus rettgeri* by tricarboxylic acid cycle intermediates. *J. Bacteriol.,* **152**, 104-110.

Daumy, G. O., Danley, D. and McColl, A. S.: (1985) Role of protein subunits in *Proteus rettgeri* penicillin G acylase. *J. Bacteriol.*, **163**, 1279-1281.

De Souza, V. R., Silva, A. C. G., Pinotti, L. M., Araujo, H. S. S., Giordano, D. L. M. C.: (2005) Characterization of the penicillin G acylase from *Bacillus megaterium* ATCC 14945. *Braz. Arch. Biol. Technol*., **48**, 105-111.

Demain A. L.: (2000) Small bugs, big business: The economic power of the microbe. *Biotecchnol. Adv.,* **18,** 499–514.

Demain, A. L. and Elander, R. P.: (1999) The β-lactam antibiotics: past, present, and future. *Antonie van Leeuwenhoek,* **75,** 5-19.

Deshpande, B. S., Ambedkar, S. S., and Shewale, J. G.: (1993) Comparative evaluation and determination of β-lactam intermediates. *Hind. Antibiot. Bull.*, **35**, 195-198.

Díaz, E., Ferrandez, A., Prieto, M. A. and García, J. L.: **(**2001) Biodegradation of aromatic compounds by *Escherichia coli. Microbiol. Mol. Biol. Rev*. **65,** 523-569.

Done, H., Brannigan, J. A., Moody, J. A., and Hubbard, R. E.: (1998) Ligand-induced conformational change in penicillin acylase, *J. Mol. Biol.* **284,** 463-475.

Duggleby H. J., Tolley S. P., Hill C. P., Dodson E. J., Dodson G., and Moody P. C. E.: (1995) Penicillin acylase has a single-amino-acid catalytic centre. *Nature*, **373**, 264-268.

Ebenstein, Y., Nahum, E. and Banin, U.: (2002) Tapping Mode Atomic Force Microscopy for Nanoparticle Sizing: Tip−Sample Interaction Effects *Nano. Lett.,* **2**, 945-950.

Economou, A.: (2000) Bacterial protein translocase: a unique molecular machine with an army of substrates. *FEBS Lett*. **476**, 18-21.

Elander R. P.: (2003) Industrial production of beta-lactam antibiotics. *Appl. Microbiol. Biotechnol.,* **61**, 385-392.

Fadnavis, N. W., Sharfuddin, M., Vadivel, S. K. and Bhalerao, U. T.: (1997) Efficient chemoenzymatic synthesis of (2S, 3S)-3-hydroxyleucine mediated by immobilized penicillin G acylase. *J. Chem. Soc. Perkin Trans.*, **1**, 3577–3578.

Fadnavis, N. W., Sharfuddin, M. and Vadivel, S. K.: (1999a) Resolution of racemic 2-amino-1-butanol with immobilised penicillin G acylase. *Tetrahedron: Asymmetry* **10**, 4495-4500.

Fadnavis, N. W., Vadivel, S. K. and Sharfuddin, M.: (1999b) Chemoenzymatic synthesis of (4*S*,5*R*)-5-hydroxy-γ-decalactone *Tetrahedron: Asymmetry*, **10**, 3675-3680.

Fernandez-Lafuente, R.; Rosell, C. M., Caanan-Haden, L. Rodes, L. and Guisan, J. M.: (1999) Facile synthesis of artificial enzyme nano-environments via solid-phase chemistry of immobilized derivatives: Dramatic stabilization of penicillin acylase versus organic solvents. *Enzyme Microb. Technol.*, **24**, 96-103.

Fischer, N. O., McIntosh, C. M., Simard, J. M. and Rotello, V. M.: (2002) Supramolecular chemistry and self-assembly special feature: inhibition of chymotrypsin through surface binding using nanoparticle-based receptors. *Proc. Natl. Acad. Sci.*, **99**, 5018-5023.

Fuganti, C. and Grasselli, P.: (1986) Immobilized penicillin acylase: Application to the synthesis of the dipeptide aspartame. *Tetrahedran lett*. **27**, 3191-3194.

Gabor, E. M., and Janssen, D. B.: (2004) Increasing the synthetic performance of penicillin acylase PAS2 by structure-inspired semi-random mutagenesis. *Protein Eng. Des. Sel.*, **17**, 571-579.

Gabor E. M., de Vries E. J., Janssen D. B.: (2005) A novel penicillin acylase from the environmental gene pool with improved synthetic properties. *Enzyme Microb.Technol.,* **36**, 182-190.

Galan, B., Garcia, J. L. and Prieto, M. A.: (2004) The PaaX Repressor, a Link between Penicillin G Acylase and the Phenylacetyl-Coenzyme A Catabolon of *Escherichia coli* W. *J. Bacteriol*., **186,** 2215-2220.

Gang, D. M., and Shaikh, K.: (1976) Regulation of penicillin acylase in *Escherichia coli* by cyclic AMP. *Biochim. Biophys. Acta.,* **425**, 110-114.

Gao, J., Gu, H. and Xu, B.: (2009) Multifunctional Magnetic Nanoparticles: Design, Synthesis, and Biomedical Applications *Acc. Chem. Res.* **42**, 1097

Gentina, J. C., Acevedo, F. and Villarga, M. P.; (1997) Effect of complex nitrogen sources on production of penicillin acylase from *Bacillus megaterium. World J. Microbiol. Biotechnol.*, **13**, 127-128.

Govardhan, C.: (1999) Crosslinking of enzymes for improved stability and performance. *Curr. Opin. Biotechnol*., **10**, 331-335.

Guarise, C., Pasquato, L., De Filippis, V. and Scrimin, P.: (2006) Gold nanoparticles based protease assay. *Proc. Natl. Acad. of Sci.*, **14**, 3978-3982.

Guisan, J. M., Alvaro, G., Fernandez-Lafuente, R., Rosell, C. M., Garcıa-Lopez, J. L. and Tagliani, A.: (1993) Stabilization of heterodimeric enzyme by multipoint covalent immobilization: Penicillin G acylase from *Kluyvera citrophila*. *Biotech. Bioeng*., **42**, 455-464.

He, J., Li, X., Evans, D. G., Duan, X. and Li, C.: (2000) A new support for the immobilization of penicillin acylase. *J. Mol. Catal. B*., **11**, 45-53.

Hernandez-Justiz O, Terreni M, Pagani G, Garcia J. L., Guisan J. M., and Fernandez-Lafuente R.: (1999) Evaluation of different enzymes as catalysts for the production of β-lactam antibiotics following a kinetically controlled strategy. *Enzyme Microb. Technol*., **25,** 336–343.

Holt, R. J. and Stewart G. T.: (1964) Production of amidase and β-lactamase by bacteria. *J. gm. Microbiol*., **36**, 203-213.

Hossain, M., Huang, G., Kaneko, T. and Ozaki, Y.: (2009) Characteristics of surface-enhanced Raman scattering and surface-enhanced fluorescence using a single and a double layer gold nanostructure. *Phys. Chem. Chem. Phys.,* **11**, 7484-7490.

Huang, H. T., English, A. R., T. A. Seto, T. A., Shulll, G. M. and SOBIN, B. A.: (1960) Enzymatic hydrolysis of the side chain of penicillins. *J. Am. Chem. Soc*., **82**, 3790-3791.

Huang, H. T., Seto, T. A. and Shull, G. M.: (1963) Distribution of substrate specificity of benzylpenicillin acylase. Appl. *Appl. Microbiol.,* **11,** 1-6.

Ignatova, Z., Mahsunah, A. A., Georgieva, M., and Kaslike, V.: (2003) Improvement of post translational bottlenecks is the production of penicillin amidase in recombinant *Escherichia coli* strain, *App. Environ. Microbiol.* **69,** 1237-1245.

Ishii, Y., Saito, Y., Fugimura, T., Isogai, T., Kojo, H., Yamashita, M., Niwa, M. and Kohsaka, M.: (1994) A novel 7β-(4-carboxybutanamido) cephalosporanic acid acylase isolated from P*seudomonas* starin C427 and its high-level production in *Escherichia coli*. *J. Ferment. Bioeng.,* **77**, 591-597.

Ishiye, M. and Niwa, M.: (1992) Nucleotide sequence and expression in *Escherichia coli* of the cephalosporin acylase gene of a *Pseudomonas* strain. *Biochim. Biophys. Acta,* **1132**, 233-239.

Isogai, T., Ono, H., Ishitani, Y., Kojo, H., Ueda, Y., and Kohsaka, M.: (1990) Structure and expression of cDNA for D-amino acid oxidase active against cephalosporin C from *Fusarium aolani*. *J. Biochem.* **108**, 1063-1069.

Isupov, M. N., Obmolova, G., Butterworth, S., Badet-Denisot, M. A., Badet, B., Polikarpov, I., Littlechild, J. A. and Teplyakov, A.: (1996) Substrate binding is required for assembly of the active conformation of the catalytic site in Ntn amidotransferases: evidence from the 1.8 Å crystal structure of the glutaminase domain of glucosamine 6-phosphate synthase. *Structure*, **4**, 801-810.

Jain, T. K., Reddy M. K., Morales M. A., Leslie-Pelecky D. L. and Labhasetwar V.: (2008) Biodistribution, Clearance, and Biocompatibility of Iron Oxide Magnetic Nanoparticles in Rats. *Mol. Pharmaceu.,* **5**, 316-327.

Jena, B. and Raj, C.: (2008) Optical sensing of biomedically important polyionic drugs using nano-sized gold particles. *Biosens. Bioelectron.*, **23**, 1285-1290.

Jolivet, J. P., Chanéac, C. and Tronc, E.: (2004) Iron oxide chemistry. From molecular clusters to extended solid networks. *Chem. Comm.*, **5**, 481-487.

Kalele, S. A., Ashtaputre, S. S., Hebalkar, N. Y., Gosavi, S. W., Deobagkar, D. N., Deobagkar, D. D. and Kulkarni, S. K.: (2005) Optical detection of antibody using silica-silver core-shell particles. *Chem. Phys. Lett.,* **404**, 136-141.

Kalele, S. A., Kundu, A. A., Gosavi, S. W., Deobagkar, D. N., Deobagkar, D. D. and Kulkarni, S. K.: (2006) Rapid detection of *Escherichia coli* by using antibody-conjugated silver nanoshells. *Small*, **2**, 335-338.

Kallenberg, A. I., van Rantwijk, F. and Sheldon, R.A.: (2005) Immobilization of Penicillin G Acylase: The Key to Optimum Performance. *Adv. Synth. Catal.*, **347**, 905-926.

Kameda Y., Kimura Y., Toyoura E., Omori T.: (1961) A method for isolating bacteria capable of producing 6-aminopenicillanic acid from benzylpenillin. *Nature*., **191**, 1122-1123.

Kasche, V., Lummer, K., Nurk, A., Piotraschke, E., Rieks, A., Stoeva, S., and Voelter, W.: (1999) Intramolecular autoproteolysis initiates the maturation of penicillin amidase from *Escherichia coli*. *Biochim. Biophys. Acta*, **1433**, 76–86.

Kaufmann, W. and Bauer, K.: (1960) Enzymatic splitting and Resynthese of penicillin. *Naturwissenschaften* **47**, 474-475.

Kaufmann, W. and Bauer, K.: (1964) Variety of Substrates for a Bacterial Benzyl Penicillin-splitting Enzyme. *Nature*, **203**, 520.

Kazan, D., Ertan, H. and Erarslan, A.: (1996) Stabilization of Penicillin G Acylase against pH by chemical cross-linking. *Process Biochem.*, **31**, 135-140.

Kheirolomoom, A., Khorasheh, F., and Fazelina., H.: (2002) Influence of external mass transfer limitation on apparent kinetic parameters of penicillin G acylase immobilized on nonporous ultrafine silica particles. *J. Biosci. Bioeng*., **93**, 125–129.

Kim, D. J. and Byun, S. M.: (1990) Purification and properties of ampicillin acylase from *Pseudomonas melanogenum. Biochim. Biophys. Acta. Protein Struct. Mol. Enzymol.,* **1040**, 12-18.

Kim, H. S., Kang, T. S., Hyun, J. S., and Kang, H. S.: (2004) Regulation of Penicillin G Acylase gene expression in *Escherichia coli* by repressor PaaX and the cAMP-cAMP receptor protein complex. *J. Biol. Chem.*, **279**, 33253-33262.

Kim, Y., Yoon, K., Khang, Y., Turley, S. and Hol, W. G.: (2000) The 2.0 Å crystal structure of cephalosporin acylase. *Structure*, **8**, 1059-1068.

Klei, H. E., Daumy, G. O. and Kelly, J. A.: (1995) Purification and preliminary crystallographic studies of penicillin G acylase from *Providencia rettgeri*. *Protein Sci.*, **4**, 433-441.

Klyosov, A. A., Svedas, V. K. and Galaev, I. Y.: (1977) Study of the active center topography of penicillin amidase from *E. coli*. II. The role of hydrophobicity in the specificity of the enzyme inhibition. *Bioorg. Khimiya*, **3**, 800-805.

Kneipp, K., Kneipp, H., Itzkan, I., Dasari, R. R. and Feld, M. S.: (1999) Ultrasensitive chemical analysis by raman spectroscopy. *Chem. Rev.*, **99**, 2957-2975.

Konecy, J., Scheneider, A. and Sieber, M.: (1983) Kinetics and mechanism of acyl transfer by penicillin acylases *Biotechnol. Bioeng.*, **25**, 451-467.

Konstantinovic, M., Marjanovic, N., Ljubijankic, G. and Glisin, V.: (1994) The penicillin amidase of *Arthrobacter viscosus* (ATCC 15294). *J. Bacteriol*., **143**, 79-83.

Kumar, A., Singh, S., Poddar, P., Prabhune, A. and Pundle, A.: (2009) Effect of Cultural Conditions and Media Constituents on Production of Penicillin V Acylase and CTAB Treatment to Enhance Whole-Cell Enzyme Activity of *Rhodotorula aurantiaca* (NCIM 3425). *Appl. Biochem. Biotechnol*., **157**, 463-472.

Kumar, K. K., Sudhakaran, V. K., Deshpande, B. S., Ambekar, S. S. and Shewale, J. G.: (1993) Cephalosporin acylases: enzyme production, structure and application in the production of 7-ACA. *Hind. Antibiot. Bull.*, **35**, 111-125.

Kumar R. S., Suresh C. G., Pundle A. and Prabhune, A.: (2004) Evidence for the involvement of arginyl residue at the active site of penicillin G acylase from *Kluyvera citrophila*. *Biotechnol. Lett.*, **26**, 1601-1606.

Kumar, R. S., Prabhune, A. A., Pundle, A. V., Karthikeyan, M. and Suresh, C. G.: (2007) A tryptophan residue is identified in the substrate binding of penicillin G acylase from *Kluyvera citrophila. Enzyme Microb. Technol.*, **40**, 1389-1397.

Kutzbach, C. and Rauenbusch, E.: (1974) Preparation and general properties of crystalline penicillin acylase from *Escherichia coli* ATCC 11105. *Hoppe-*

Seylers Z. Physiol. Chem., **354,** 45-53.

Laemmli, U. K.: (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680-685.

Laromaine, A., Koh, L., Murugesan, M., Ulijn, R. V. and Stevens, M. M.: (2007) Protease triggered dispersion of nanoparticle assemblies. *J. Am. Chem. Soc.*, **129**, 4156-4157.

Lazarides, A. and Scahtz, G. C.: (2000) Nanostructures in biodiagnostics. *J. Phys. Chem. B*, 104, 460-467.

Lee, Y. S., Kim, H. W. and Park, S. S.: (2000) The role of alpha-amino group of the Nterminal serine of beta subunit for enzyme catalysis and autoproteolytic activation of glutaryl 7-aminocephalosporanic acid acylase. *J. Biol. Chem.*, **275**, 39200-39206.

Li, Y., Chen, J., Jiang, W., Mao, X., Zhao, G. and Wang, E.: (1999) *In vivo* post-translational processing and subunit reconstitution of cephalosporin acylase from *Pseudomonas* sp. 130. *Eur. J. Biochem.,* **262**, 713-719.

Li, Y., Jiang, W., Yang, Y., Zhao, G. and Wang, E.: (1998) Overproduction and purification of glutaryl 7-amino cephalosporaninc acid acylase. *Protein Express. Purif.,* **12**, 233-238.

Li, Y., Schluesener, H. J. and Xu, S.: (2010) Gold nanoparticles based biosensors. *Gold Bulletin*, **43**, 29-41.

Lin, W., Huang, S., and Chou, C. P.: (2001) High-level extracellular production of penicillin acylase by genetic engineering of *Escherichia coli*. *J. Chem. Technol. Biotechnol.* **76**, 1030-1037.

Lindsay, C. D. and Pain, R. H.: (1991) Refolding and assembly of penicillin acylase, an enzyme composed of two polypeptide chains that result from proteolytic activation. *Biochemistry*, **30**, 9034-9040.

Littlechild, J. A.: (1991) Protein crystallization: magical or logical: can we establish some general rules? *J. Phys. D: Appl. Phys.,* **24**, 111-118.

Liu, J. and Lu, Y.: (2004) Adenosine-dependent assembly of aptazyme-functionalized gold nanoparticles and its application as a colorimetric biosensor. *Anal. Chem.*, **76**, 1627-1632.

Liu, J., Liang, L., Li, G., Han, R. and Chen, K.: (1998) H^+ISFET-based biosensor for determination of penicillin G. *Biosens. Bioelectron*., **13**, 1023-1028.

Liu, R., Teo, W., Tan, S., Feng, H., Padmanabhan, P. and Xing, B.: (2010) Metallic nanoparticles bioassay for *Enterobacter cloacae* P99 *β*-lactamase activity and inhibitor screening. *Analyst*, **135**, 1031-1036.

Livermore, D. M.: (2000) Antibiotic resistance in staphylococci. *Int. J. Antimicrob. Agents,* **16,** 3-10.

Lowe, D. A., Romancik, G. and Elander, R. P.: (1981) Penicillin acylases. A review of existing enzymes and the isolation of a new bacterial penicillin V acylase. Dev. *Ind. Microbiol*., **22**, 163-180.

Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W. and Huber, R.: (1995) Crystal structure of the 20S proteasome from the Archaeon *T. acidophilum* at 3.4 Å resolution. *Science,* **268**, 533-539.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and randall, R. J.: (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193,** 265-275.

Luft, J. R., and Detitta, G. T.: (1999) A method to produce microseed stock for use in the crystallization of biological macromolecules. *Acta Crystallogr. D Biol. Crystallogr.,* **55**, 988-993.

Mahajan, P. B. and Borkar, P. S.: (1983) Chemical modifications of *Escherichia coli* penicillin acylase I, tryptophan involvement at the catalytic site. *Hind. Antibiot. Bull.*, **25**, 6-10.

Marchal, G., Hecke P. V., Demaerel, P., Decrop, E., Kennis, C., AL Baert, and van der Schueren E.: (1989) Detection of liver metastases with superparamagnetic iron oxide in 15 patients: results of MR imaging at 1.5 T. *Am. J. Roentgen* **152**, 771-775.

Martin, J., Slade, A., Aitken, A., Arche, R. and Virden, R.: (1991) Chemical modification of serine at the active site of penicillin acylase from *Kluyvera citrophila*. *Biochem. J*., **280**, 659-662.

Martin, L., Prieto, M. A., Cortes, E. and Garcia, J. L.: (1995) Cloning and sequencing of the pac gene encoding the penicillin G acylase of *Bacillus megaterium* ATCC 14945. *FEMS Microbiol. Lett*., **125**, 287-292.

Massolini, G., Fracchiolla, G., Calleri, E., Carbonara, G., Temporini, C.,

Lavecchia A., Cosconati, S., Novellino, E. and Loiodice, F.: (2006) Elucidation of the enantioselective recognition mechanism of a penicillin G acylase-based chiral stationary phase towards a series of 2-aryloxy-2-arylacetic acids. *Chirality*, **18**, 633-643.

Matsuda, A. and Komatsu, K.: (1985) Molecular cloning and structure of the gene for 7β-(4-carboxybutanamido) cephalosporanic acid acylase from a *Pseudomonas* strain. *J. Bacteriol.*, **163**, 1222-1228.

Matsuda, A., Matsuyama, K., Yamamoto, K., Ichikawa, S. and Komatsu, K. I.: (1987) cloning and characterization of the gene for two distinct cephalosporin acylases from a *Pseudomonas* starin. *J. Bateriol.*, **169**, 5815-5820.

Matthews, B. W.: (1985) Determination of protein molecular weight, hydration, and packing from crystal density. *Methods Enzymol*. **114**, 176-187.

McPherson, A.: (1982) Preparation and analysis of protein crystals. John Wiley & Sons.

McPherson, A. (1998) Crystallization of biological macromolecules. Cold Spring Harbor Laboratory Press.

McVey, C. E., Tolley, S. P., Done, S. H., Brannigan, J. A. and Wilson, K. S.: (1997) A new crystal form of penicillin acylase from *Escherichia coli*. *Acta Crystallogr. D Biol. Crystallogr.*, **53**, 777-779.

McVey, C. E., Walsh, M. A., Dodson, G. G., Wilson, K. S., and Brannigan, J. A.: (2001) Crystal structure of penicillin acylase enzyme-substrate complexes: structural insights into the catalytic mechanism. *J. Mol. Biol.*, **313**, 139-150.

Meevootism, V., Somsuk , P., Prachaktam, R. and Flegel, J. W.: (1983) Simple screening method for isolating Penicillin Acylase-producing bacteria. *Appl. Environ. Microbiol.*, **46**, 1227-1229.

Merino E., Balbas P., Recillas F., Becerril B., Valle F. and Bolivar F.: (1992) Carbon regulation and the role in nature of the *Escherichia coli* penicillin acylase (pac) gene. *Mol. Microbiol*., **6,** 2175-2182.

Morillas, M., Goble, M. L., and Virden, R.: (1999) The kinetics of acylation and deacylation of penicillin acylase from *Escherichia coli* ATCC 11105:

evidence for lowered pK_a values of groups near the catalytic center. *Biochem. J.* **338**, 235–239

Mulvaney, P.: (1996) Surface plasmon spectroscopy of nanosized metal particles. *Langmuir*, **12**, 788-800.

Murao, S. (1955) Penicillin-amidase II. Productional conditions of penicillin-amidase. *J. Agric. Chem. Soc.* (Japan), **29**, 400, 404

Murao, S., and Kishida, Y.: (1961) Studies on Penicillin-amidase. Part V Studies on the Penicillin-amidase Producing Microorganisms *J. Agric. Chem. Soc.* (Japan), **35**, 610-615.

Nara, T., Misawa, M., Okachi, R., and Yamamoto, M.: (1971). Enzymatic synthesis of *D(-)-α*-aminobenzylpenicillin Part I Selection of penicillin acylase-producing bacteria. *Agr. Biol. Chem.,* **35**, 1676-1682.

Nayler, J. H. C.: (1991) Early discoveries in the penicillin series. *Trends Biochem. Sci.,* **16**, 195-234.

Nicholas, R. A., Hamilton, H. and Cohen, M. S.: (1995) β-Lactam antibiotics. Chapmann & Hall, New York.

Niersbach, H., Kuhne, A., Tischer, W., Weber, M., Wedekind, F., and Plapp, R.: (1995) Improvement of catalytic properties of Penicillin G Acylase from *Escherichia coli* ATCC 11105 by selection of a new substrate specificity. *Appl. Microbiol. Biotechnol.*, **43**, 679-684.

Nikoobakht, B. and El-Sayed, M. A.: (2001) Evidence for bilayer assembly of cationic surfactants on the surface of gold nanorods. *Langmuir*, **17**, 6368-6374.

Nusz, G., Marinakos, S., Curry, A., Dahlin, A., Hook, F., Wax, A. and Chilkoti, A.: (2008) Label-Free plasmonic detection of biomolecular binding by a single gold nano-rod. *Anal. Chem.,* **80**, 984-989.

Oh, B., Kim, M., Yoon, J., Chung, K., Shin, Y., Lee, D. and Kim Y.; (2003) Deacylation activity of cephalosporin acylase to cephalosporin C is improved by changing the side chain conformations of active-site residues. *Biochem. Biophys. Res. Commun*., **310**, 19-27.

Oh, S. J., Kim, Y. C., Park, Y. W., Min, S. Y., Kim, I. S., and Kang, H. S.: (1987) Complete nucleotide sequence of the penicillin G acylase gene and flanking regions, and its expression in *Escherichia coli*. *Gene*. **56**, 87-97.

Ohashi, H., Katsuta, Y., Nagashima, M., Kamel, T. and Yano, M.: (1989)

Expression *Arthrobacter viscosus* penicillin G acylase gene in *Escherichia coli* and *Bacillus subtilis*. *Appl. Environ. Microbiol*,, **55**, 1351-1356.

Oinonen, C., and Rouvinen, J.: (2000) Structural comparison of Ntn-hydrolases. *Protein Sci*., **9**, 2329–2337.

Oinonen, C., Tikkanen, R„ Rouvinen, J. and Peltonen, L.: (1995) Three Dimensional Structure of Human Lysosomal Aspartyiglucosaminidase. *Nat. Struct. Biol.,* **2**, 1102-1108.

Okachi, R. Misawa, M. and Nara, T.: (1972) Production of 6-Aminopenicillanic Acid by *Kluyvera citrophila* KY 3641. *Agr. Biol. Chem.,* **36,** 925-930.

Okachi, R., Kato, F., Miyamura, Y. and Nara, T.: (1973) Selection of *Pseudomonas melanogenum* KY 3987 as a new ampicillin-producing bacteria. *Agric. Biol. Chem*., **37**, 1953-1957.

Oliver, G., Valle, F., Rosetti, F., Gomez-Pedrozo, M., Santamaria, P., Gosset, G. and Bolivar, F.: (1985) A common precursor for the two subunits of the penicillin acylase from *Escherichia coli* ATCC11105. *Gene*, **40**, 9-14.

Olsson, A. and Uhlen, M.: (1986) Sequencing and heterologous expression of the gene encoding penicillin V amidase from *Bacillus sphaericus*. *Gene*, **45**, 175-181.

Oostendorp J.G.: (1972) A quantitative microbiological determination of 6-aminopenicillanic acid. *Antonie Van Leeuwenhoek*., **38,** 201-206.

Parmar, A., Kumar, H., Marwaha, S. S., and Kennedy, J. F.: (2000) Advances in enzymatic transformation of penicillins to 6-aminopenicillanic acid (6-APA). *Biotechnol. Adv*., **18**, 289–301.

Patchornik, A., Lawson, W. B. and Witkop, B.: (1958) Selective cleavage of peptide bonds. II. The tryptophyl peptide bond and the cleavage of glucagons 1. *J. Am. Chem. Soc.*, **80**, 4745-4748.

Phadtare, S., Parekh, P., Gole, A., Patil, M., Pundle, A., Prabhune, A. and Sastry, M.: (2002) Penicillin G acylase-fatty lipid biocomposite films show excellent catalytic activity and long term stability/reusability. *Biotechnol. Prog.*, **18**, 483-488.

Prabhune, A. A. and SivaRaman, H.: (1990) Evidence for involvement of arginyl residue at the catalytic site of penicillin acylase from *Escherichia coli. Biochem. Biophys. Res. Commun.* **173**: 317-322.

Prabhune, A. A., Rao, B. S., Pundle, A. V. and Sivaraman, H.: (1992) Immobilization of permeabilized *Escherichia coli* cells with penicillin acylase activity. *Enzyme Microb. Technol*., **14**, 161-163.

Prabhune, A. and Sivaraman, H.: (1991) Immobilization of PGA in porous beads of polyacrylamide gel. *Appl*. *Biochem. Biotechnol*., **30**, 265-272.

Pruess, D. L. and Johnson, M. J.: *(*1965*)* Enzymatic Deacylation of S^{35}-Benzylpenicillin. *J. Bacteriol.,* **90***,* 380-383.

Rabilloud, T., Vuillard, L., Gilly, C., and Lawrence, J. J.: (1994) Silver-staining of proteins in polyacrylamide gels: a general overview. *Cell. Mol. Biol. (Noisy-le-grand),* **40,** 57-75.

Rajendhran J., Krishnakumar V. and Gunasekaran P.: (2003) Production of penicillin G ayclase from *Baciilus* sp.: Effect of medium components. *World J. Microbiol. Biotechnol.*, **19**, 107-110.

Rajendhran, J. and Gunasekaran, P.: (2004) Recent Biotechnological Interventions for Developing Improved Penicillin G Acylases. *J. Biosci. Bioeng.***, 97**, 1-13.

Rajendhran, J. and Gunasekaran, P.: (2007) Molecular cloning and characterization of thermostable β-lactam acylase with broad substrate specificity from *Bacillus badius*. *J. Biosci. Bioeng.,* **103**, 457-563.

Rajendhran, J., Krishnakumar, V., and Gunasekaran, P.: (2002) Optimization of a fermentation medium for the production of penicillin G acylase from *Bacillus* sp. *Lett. Appl. Microbiol*., **35**, 523–527.

Ramkrishna, S. and Benjamin, W. B.: (1981) Evidence for an essential arginine residue at the active site of ATP citrate lyase from rat liver. *Biochem. J.*, **195**, 735-743.

Riboh, J. C., Haes, A. J., McFarland, A. D., Yonzon, C. R. and Van Duyne, R. P.: (2003) A nanoscale optical biosensor: real-time immunoassay in physiological buffer enabled by improved nanoparticle adhesion. *J. Phys. Chem. B*, **107**, 1772-1780.

Rio, G., Rodriguez, M. E., Munguia, M. E., Munguia, A. L. and Soberon, X.: (1995) Mutant *Escherichia coli* Penicillin Acylase with enhanced stability of alkaline pH. *Biotechnol. Bioeng.*, **48**, 141-148.

Roa, A., Castillon, M. P., Goble, M. L., Virden, R., and Garcia, J. L.: (1995) New insights on the specificity of Penicillin acylase, *Biochem. Biophys.. Res. Comm.,* **206,** 629 -636.

Robak, M. and Szewczuk, A.: (1981) Penicilin amidase from *Proteus rettgeri. Acta Biochem. Pol.*, **28**, 275-284.

Rocchietti, S., Urrutia, A. S. V., Pregnolato, M., Tagliani, A., Guisan, J. M., Fernandez-Lafuente, R. and Terreni, M.: (2002) Influence of the enzyme derivative preparation and substrate structure on the enantioselectivity of penicillin acylase. *Enzyme Microb. Technol.*, **31**, 88-93.

Roche, D., Prasad, K. and Repic, O.: (1999) Enantioselective acylation of β-amino esters using penicillin G acylase in organic solvents. *Tetrahedron Lett.*, **40**, 3665-3668.

Rolinson, G. N., Batchelor, F. R., Butterworth, D., Cameron-Wood, J., Cole, M., Eustace, G. C., Hart, M. V., Richards, M. and Chain, E. B.: (1960) Formation of 6-aminopenicillanic acid from penicillin by enzymatic hydrolysis. *Nature* **187**, 236-237.

Rolinson, G. N.: (1971) Bacterial resistance to penicillins and cephalosporins. *Proc. R. Soc. Lond. B Biol. Sci.*, **179**, 403-410.

Rosi, N. L., and Mirkin, C. A.: (2005) Nanostructures in Biodiagnostics. *Chem. Rev.* 105, 1547-1562.

Sakaguchi, K., and Murao, S.: (1950) A preliminary report on a new enzyme, "penicillin amidase." *J. Agr. Chem. Soc. Jpn.,* **23**, 411 only.

Savidge, T. A. and Cole, M.: (1975) Penicillin Acylase (bacterial), *Methods Enzymol.*, **43**, 705-721.

Savidge, T. A.: (1984) Enzymatic conversions used in the production of penicillins and cephalosporins. In: Vandamme E (ed) Biotechnology of Industrial Antibiotics. Marcel Dekker, New York, p 171.

Schumacher, G., Sizmann, D., Haug, H., Buckel, P., and Bock, A.: (1986) Penicillin acylase from *E. coli*: unique gene–protein relation. *Nucleic Acids Res.*, **14**, 5713–5727.

Self, D. A., Kay, G. and Lilly, M. D.: (1969) The conversion of benzyl penicillin to 6-aminopenicillinic acid using an insoluble derivative of penicillin amidase. *Biotech. Bioeng.* **11**, 337-348.

Senthilvel, S. G. and Pai, J. S.: (1996) Purification of penicillin acylase of *Bacillus megaterium*. *Biotechnol. Tech.,* **10**, 611-614.

Shaikh, K., Talati, P. G. and Gang, D. M.: (1973) Spectrophotometric Method for the Estimation of 6-Aminopenicillanic Acid. *Antimicrob. Agents Chemother.*, **3,** 194-197.

Shaw, S. Y., Shyu, J. C., Hsieh, Y. W. and Yeh, H. J.: (2000) Enzymatic synthesis of cephalothin by penicillin G acylase. *Enzyme Microb. Technol*., **26**, 142-151.

Shewale J. G. and Sivaraman H.: (1989) Penicillin Acylase: Enzyme Production and its Application in the Manufacture of 6-APA. *Process Biochem*., **24,** 146-154.

Shewale J. G., Kumar K. K. and Amberkar G. R.: (1987) Evaluation of determination of 6-aminopenicillanic acid by p-dimethyl aminobenzaldehyde. *Biotechnol. Tech.,* **1,** 69-72.

Shewale, J. G. and Sudhakaran, V. K.: (1997) Penicillin V acylase: Its potential in the production of 6-aminopenicillanic acid. *Enzyme Microb. Technol.*, **20**, 402-410.

Shewale, J. G., Deshpande, B. S., Sudhakaran, V. K. and Ambedkar, S. S.: (1990) Penicillin acylases: application and potentials. *Process Biochem.,* **25**, 97-103.

Shimizu, M., Masuike, T., Fujita, H., Kimura, K., Okachi, R. and Nara, T.: (1975) Search for microorganisms producing cephalosporin acylase and enzymatic synthesis of cephalosporins. *Agric. Biol. Chem*., **39**, 1225-1232.

Sikyta, B. and Slezak, J.: (1964) Continuous cultivation of *Escherichia coli* possessing high penicillin-acylase activity. *Biotechnol. Bioeng.*, **6**, 309-319.

Sizmann, D., Keilmann, C. and Bock, A.: (1990) Primary structure requirements for the maturation *in vivo* of penicillin acylase from *Escherichia coli* ATCC 11105. *Eur. J. Biochem.*, **192**, 143-151.

Sjoberg, G., Nathorst-Westfelt, L. and Ortengreen, B.: (1967) Enzymatic hydrolysis of some penicillins and cephalosporins by *Escherichia coli* acylase. *Acta Chemica Scandinavica*, **21**, 547-551.

Skrob, F., Becka, S., Plhackova, K., Fotopulosova, V., Kyslik, P.: (2003) Novel penicillin G acylase from *Achromobacter* sp. CCM 4824. *Enzyme Microb. Technol.*, **32**, 738-744.

Smith, J. L., Zaiuzec, E. J., Wery, J. P., Niu, L., Switzer, R. L., Zaikin, H. and Satow, Y.: (1994) Structure of the Allosteric Regulatory Enzyme of Purine Biosynthesis. *Science,* **264**, 1427-1433.

Sonawane, V. C.: (2006) Enzymatic modifications of cephalosporins by cephalosporin acylase and other enzymes. *Crit. Rev. Biotechnol.*, **26**, 95-120.

Spande, T. F. and Witkop, B.: (1967) Determination of the tryptophan content of proteins with N-bromosuccinimide. *Methods Enzymol.*, **11**, 498-506.

Storhoff, J. J., Lazarides, A. A., Mucic, R. C., Mirkin, C. A., Letsinger, R. L. and Schatz, G. C.: (2000) What controls the optical properties of DNA-linked gold nanoparticle assemblies? *J. Am. Chem. Soc.*, **122**, 4640-4650.

Storhoff, J. J., Mucic, R. C., Letsinger, R. L., and Mirkin, C. A.: (1997) Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. *Science*, **277**, 1078-1080.

Stura, E. A., and Wilson, I. A.: (1991) Applications of the streak seeding technique in protein crystallization. *J. Cryst. Growth,* **110**, 270-282.

Sudhakaran, V. K. and Borkar, P. S., (1985a) Microbial transformation of β-lactam antibiotics: enzymes from bacteria, sources and study-a sum up. *Hind. Antibiot. Bull.,* **27**, 63-119.

Sudhakaran, V. K. and Borkar, P. S. (1985b) Phenoxymethyl penicillin acylases: sources and study-a sum up. *Hind. Antibiot. Bull*., **27**, 44-62.

Sudhakaran, V. K. and Shewale, J. G.: (1995) Purification and characterization of extracellular penicillin V acylase from *Fusarium* sp. SKF 235. *Hind. Antibiot. Bull*., **37**, 9-15.

Sudhakaran, V. K., Deshpande, B. S., Ambedkar, S. S. and Shewale, J. G.: (1992) Molecular aspects of penicillin and cephalosporin acylases. *Process Biochem.*, **27**, 131-143.

Suresh, C. G., Pundle, A. V., SivaRaman, H., Rao, K. N., Brannigan, J. A., McVey, C. E., Verma, C. S., Dauter,. Z., Dodson, E. J. and Dodson, G. G.:

(1999) Penicillin V acylase crystal structure reveals new Ntn-hydrolase family members. *Nat. Struct. Biol*., **6**, 414-416.

Svedas, V. K., Savchenko, M. V., Beltser, A. I. and Guranda, D. F.: (1996) Enantioselective Penicillin Acylase-catalyzed Reactions. *Ann*. *N.Y. Acad. Sci*., **799**, 659-669.

Szewczuk, A., Siewinski, M. and Slowinska, R.: (1980) Colorimetric assay of penicillin amidase activity using phenylacetyl-aminobenzoic acid as substrate. *Anal. Biochem.*, **103**, 166-169.

Takahashi, K.: (1968) The reaction of phenyl glyoxal with arginine residue in proteins. *J. Biol. Chem.,* **243**, 6171-6179.

Terreni, M., Ubiali, D., Bavaro, T., Pregnolato, M., Fernández-Lafuente, R. and Guisán, J. M.: (2007) Enzymatic synthesis of cephalosporins. The immobilized acylase from *Arthrobacter viscosus*: A new useful biocatalyst. *Appl. Microbiol. Biotechnol.*, **77**, 579-587.

Tewari, Y. B. and Goldeberg, R. N.: (1988) Thermodynamics of the conversion of penicillin G to phenylacetic acid and 6-aminopenicillanic acid. *Biophys. Chem.*, **29**, 245-252.

Thony-Meyer, L., Bock, A., and Hennecke, H.: (1992) Prokaryotic polyprotein precursors. *FEBS Lett.*, **307**, 62-65.

Topgi, R. S., Ng, J. S., Landis, B., Wang, P. and Behling, J. R.: (1999) Use of enzyme penicillin acylase in selective amidation/amide hydrolysis to resolve ethyl 3-amino- 4-pentynoate isomers. *Bioorg. Med. Chem.,* **7**, 2221-2229.

Torres-Bacete, J., Arroyo, M., Torres-Guzman, R., de la Mata, I. Castillon, M. P. and Acebal, C.: (2001) Stabilization of penicillin V acylase from *Streptomyces lavendulae* by covalent immobilization. *J. Chem. Technol. Biotechnol.*, **76**, 525-528.

Torres-Bacete, J., Hormigo, D., Stuart, M., Arroyo, M., Torres, P., Castillón, M. P., Acebal, C., García, J. L. and de la Mata, I.: (2007) Newly discovered penicillin acylase activity of aculeacin A acylase from *Actinoplanes utahensis*. *Appl. Environ. Microbiol*., **73**, 5378-5381.

Torres-Guzman, R., de la Mata, I., Torres-Bacete, J., Arroyo, M., Castillon, M. P. and Acebal, C.: (2002) Substrate specificity of penicillin acylase from

Streptomyces lavendulae. *Biochem. Biophys. Res. Commun*., **291**, 593-597.

Trumpower, B. L., (1990) Cytochrome bc1 complexes of microorganisms. *Microbiol. Rev.* **54**, 101-139.

Ulijn, R. V.: (2006) Enzyme-responsive materials: a new class of smart biomaterials. *J. Mat. Chem.*, **16**, 2217-2225.

Uozumi, N., Sakurai, K, Sasaki, T., Takekawa, S., Yamagttta, I. I., Tsukagoshi, N., and Udaka, S.: (1989) A single gene directs synthesis of a precursor protein with beta- and alpha-amylase activities in *Bacillus polymyxa*. *J. Bactcriol*. **171**, 375-382.

Valdés-Solís, T., Rebolledo, A. F., Sevilla, M., Valle-Vigón, P., Bomatí-Miguel, O., Fuertes, A. B. and Tartaj P.: (2009) Preparation, Characterization, and Enzyme Immobilization Capacities of Superparamagnetic Silica/Iron Oxide Nanocomposites with Mesostructured Porosity. *Chem. Mat.* **21**, 1806-1814.

Valle F., Balbas P., Merino E. and Bolivar F.: (1991) The role of penicillin amidases in nature and in industry. *Trends Biochem. Sci*., **16**, 36–40.

van Langen L. M., Oosthoek N. H. P., Guranda D. T., van Rantwijk F., Svedas V. K., and Sheldon R. A.: (2000a) Penicillin acylase-catalyzed resolution of amines in aqueous organic solvents. *Tetrahedron Asym.,* **11,** 4593–4600.

van Langen L. M., van Rantwijk F., Svedas V. K., Sheldon R. A.: (2000b) Penicillin acylase-catalyzed peptide synthesis: a chemo-enzymatic route to stereoisomers of 3,6-diphenylpiperazine-2,5-dione. Tetrahedron Asym., **11,** 1077–1083.

Vandamme, E. J. and Voets, J. P.: (1973) Some aspects of the penicillin V acylase produced by *Rhodotorula glutinis* var. *glutinis*. *Z. Allg. Mikrobiol*., **13**, 701-710.

Vandamme, E. J. and Voets, J. P.: (1974) Microbial Penicillin Acylases. *Adv. Appl. Microbiol.*, **17**, 311-369.

Vandamme, E. J. and Voets, J. P.: (1975) Properties of the purified penicillin V-acylase of *Erwinia aroideae*. *Experientia*, **13**, 140-143.

Vandamme, E. J.: (1988) Immobilized biocatalysts and antibiotic production: biochemical, genetical and biotechnical aspects. In *Bioreactor immobilized*

Enzymes and cells: Fundamentals and applications, ed. Moo-Young, M. pp. 261–286. Marcel Dekker, New York.

Verhaert, R. M., Riemens, A. M., van der Laan, J. M., van Duin, J., and Quax, W. J.: (1997) Molecular cloning and analysis of the gene encoding the thermostable penicillin G acylase from *Alcaligenes faecalis*. *Appl. Environ. Microbiol*., **63**, 3412-3418.

Vojtisek, V. and Slezak, J.: (1975) Penicillinamidohydrolase in *Escherichia coli* III. Catabolite repression, diauxie, effect of cAMP and nature of the enzyme induction. *Folia Microbiol.*, **20**, 298-306.

von Schönfeldt, V., Krishnamurthy, K., Foppiani, L. and Schlatt S.: (1999) Magnetic cell sorting is a fast and effective method of enriching viable spermatogonia from Djungarian hamster, mouse, and marmoset monkey testes. *Biol. Reproduc.* **61**, 582-589.

Waldmann H, Heuser A, and Schulze S.: (1996) Selective enzymatic removal of protecting groups: the phenylacetamide as amino protecting group in phosphopeptide synthesis. *Tetrahedron Lett*., **37**, 8725-8728.

Warburton, D., Balasingham, K., Dunnill, P. and Lilly, M. D.: (1972) The preparation and kinetics of immobilized penicillin amidase from *Escherichia coli*. *Biochim. Biophys. Acta*, **284**, 278-284.

Warburton, D., Dunnill, P. and Lilly, M. D.: (1973) Conversion of benzyl penicillin to 6-aminopenicillinc acid in a batch reactor and continuous feed stirred tank reactor using immobilized penicillin amidase. *Biotech. Bioeng*., **15**, 13-25.

Weaver, S. S. and Bodey, G. P.: (1980) CI-867, a new semisynthetic penicillin: in vitro studies. *Antimicrob Agents Chemother*. **18**, 939-943.

Weber, K. and Osborn, M.: (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem*., **244**, 4406-4412.

Won, J., Kim, M., Yi, Y. W., Kim, Y. H., Jung, N. and Kim, T. K. (2005) A Magnetic Nanoprobe Technology for Detecting Molecular Interactions in Live Cells. *Science*, **309**, 121-125.

Woodley, J. M.: (2000) Advances in Enzyme Technology-UK contributions. *Adv. Biochem. Eng.*, **70**, 93-108

Yamasaki R. B., Vega A. and Feeneg R. E.: (1980) Modification of available arginine residues in protein by p-hydoxy phenylglyoxal. *Anal. Biochem.,* **109,** 32-40.

Yang, Y. Yun, D., Guan, H., Peng, H., Chen, J., He, Y. and Jiao, R: (1991) Cloning of GL-7-ACA acylase gene from *Pseudomonas* sp. 130 and its expression in *Escherichia coli*. *Chin. J. Biotech.*, **7**, 99-107.

Zhang, Q. J., Zhang, L. F., Han, H. X. and Zhang, Y. Y.: (1983) A method for screening penicillin G acylase-producing bacteria by means of 2-nitro-5-phenylacetaminobenzoic acid test paper. *Anal. Biochem.,* **156**, 413-416.

Zhang, X., Guo, Q. and Cui, D.: (2009) Recent advances in Nanotechnology applied to biosensors. *Sensors*, **9**, 1033-1053.

Zhou, H., Wei, Z., Yang, Y. and Jiao, R.: (1997) Studies on purification and properties of GL-7ACA acylase from CU334. *Acta Microbiol. Sinica*, **37**, 196-201.

Publications

- **Ambrish Rathore**, Archana Pundle, Asmita Prabhune. (2006) Effect of culture conditions on the production of Penicillin G acylase from *Arthrobacter viscosus* (ATCC 15294) *Res. J. Biotechnol*., **1**, 5-9.
- Neha R. Tiwari, **Ambrish Rathore**, Asmita Prabhune, Sulabha K. Kulkarni: (2010) Gold Nanoparticles for Colorimetric detection of hydrolysis of antibiotics by penicillin G acylase. *Adv. Biosci. Biotechnol*., **1**, 322-329.
- Ramya Jagannathan, **Ambrish Rathore**, Abhimanyu Rana, Pankaj Poddar, Asmita Prabhune: Study of magnetic nanoparticles capped with enzyme of industrial importance - Penicillin G acylase as a case study. (manuscript submitted to *Biotechnology Progress*)
- **Ambrish Rathore** and Asmita Prabhune: Purification and biophysical characterization of penicillin G acylase from *Arthrobacter viscosus* ATCC 15294 (manuscript under preparation)

Posters presented

- Penicillin G Acylase from *Arthrobacter viscosus* (Accepted in "**Biotech 2004, New Delhi, 13-15 October, 2004**")
- Pharmaceutically Important Enzyme: Penicillin G Acylase from *Arthrobacter viscosus* (Accepted in "**73rd Annual Meeting of SBC (I), Pantnagar, 21-24 November 2004**)
- Study on the Maturation of Penicillin G Acylase from *Arthrobacter viscosus* (Accepted in "**74th Annual Meeting of SBC (I), CDRI, Lucknow, India, November 7-10th, 2005**