Penicillin V Acylase from *Rhodotorula aurantiaca* (NCIM 3425): Studies on Biochemical and Biophysical characterization of the enzyme

Thesis submitted to the University of Pune for the Degree of
Doctor of Philosophy in Biotechnology

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OCTOBER 2008

DECLARATION BY RESEARCH GUIDE

This is to certify that the work incorporated in the thesis entitled, **'Penicillin V Acylase from *Rhodotorula aurantiaca* (NCIM 3425): Studies on Biochemical and Biophysical characterization of the enzyme',** submitted by Mr. Atul Kumar, 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.

Dr. Archana V. Pundle
(Research Supervisor)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Penicillin V Acylase from *Rhodotorula aurantiaca* (NCIM 3425): Studies on Biochemical and Biophysical characterization of the enzyme"**, submitted 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 411 008, India, under the supervision of Dr. Archana V. Pundle. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University.

Atul Kumar

(Research Scholar)

……Dedicated to my late mother

And my mentor

Gururbrahma Gururvishnu: Gururdevo Maheshwara: |

Gurussakshat Parabrahma Tasmai Shreegurave Nama: ||

ACKNOWLEDGEMENT

I take this opportunity to express my reverence to my research supervisor Dr. Archana V. Pundle who introduced me to a fascinating monarchy of biotechnology. Her inspiring guidance and constant motivation has helped me to understand better and remain optimistic. I am grateful to her for giving me liberty in the work undertaken and for continuous encouragement during the course of the present study. Although this eulogy is insufficient, I preserve an everlasting gratitude to her.

I am thankful to Dr. Asmita Prabhune for her timely suggestions and help during the course.

I am also grateful to Dr. C. G. Suresh, Dr. M.I. Khan, Dr. Absar Ahmad, Biochemical sciences Division, for their suggestions and support during the course. I am thankful to Dr. Sushma Gaikwad for her suggestions and help in fluorometric studies.

I am thankful to Dr. Vidya Gupta, the Head of Biochemistry Sciences Division for providing divisional facilities. I am thankful to all scientific and non-scientific staff members of the Biochemical Sciences Division for their help.

I would also like to extend my sincere thanks to Dr. S. Mazumdar and B. T. Kansara, Division of Biochemistry, Tata Institute of Fundamental Research, Mumbai for their help in Circular Dichroism spectropolarimeter.

I express my deep feelings and love for my labmates Dr. Ashwini, Dr. Mrudula, Dr. Sulabha, Dr. Sachin Shah, Ambrish, Sharath, Sridevi, Reetika, Asmita, Nupura, Mrs. Aparna, Vishnu, Jayshree, Malathi and Kastoori. This work would have been incomplete without their help, cooperation and discussions.

I am very much thankful to my room mates Dr. Anish, Mr. Nitin, Mr. Chetan joshi, Mr. Saravanan and the best Mr. Abu Ayoobul Ansari, for their

support. I am sure they would have enjoyed my frustration of Ph.D. work by hearing my favorite words.

I am also thankful to my numerous friends: Amit, Akhilesh, Arun, Arshad, Atul thakur, Anil, Ajit, Ashutosh, Arindam, Ashavani, Fazal, Feroz, Geetha, Maggie, Dr. Manish Chandra, Dr. Manish, Mahesh, Meera, Nagraj, Noor, Poorva, Poonam, Priya, Rohtas, Ramya, Sagar, Shabab, Shadab, Shreekanth, Sharmili, Sajid, Shashidhar, Satya, Sushim, Sameer, Surbhit, Suresh, Sanjay, Santosh, Uma, Umesh, Veer Singh, Vipul, and Vinod for their help and charming company.

My thanks are to Council of Scientific and Industrial Research, New Delhi, India for financial support, Head, Division of Biochemical Science, Dr. S. Sivaram, Director, and Dr. B. D. Kulkarni, Deputy Director NCL, Pune for allowing me to carry out my research and extending all possible infrastructural facilities at NCL; and permitting me to submit the present work in the form of thesis.

Finally, it gives me great pleasure to thank my papa and mummy who have been a constant inspiration for me. I am also thankful to my brothers Neeraj and Abhishek, sister Radha, and a small cute Master Swapnil for their love, unfailing support, tremendous patience, sacrifice and encouragement they have given in their own way during my long period of studies. They have been a constant source of strength and inspiration for me. At the end, I want to thank my fiancé Deepti for being there all the time with her love, trust, support and understanding. In fact, I am very lucky to have her as my life partner.

……..***Atul Kumar***

ABSTRACT

Chapter 1: Introduction

The ***first chapter*** is general introduction of the thesis and it gives brief review of literature on β-lactam antibiotics, penicillin acylases and the penicillin acylase producing microorganisms. The chapter deals with the Ntn hydrolase super family members, their structural similarities, catalytic behavior and the mechanism. Characteristics of penicillin V acylases produced by various microorganisms and their application in industries are described in the chapter.

Chapter 2: Screening of penicillin V acylase producing microorganisms

The ***second chapter*** deals with screening and identification of potential microorganisms producing penicillin V acylase. Three organisms, *Bacillus* sp. (isolate), *Erwinia aroideae* (DSMZ 30186) and a yeast, *Rhodotorula aurantiaca* (NCIM 3425), were found to exhibit relatively high levels of whole-cells PVA activity. The isolated *Bacillus* sp., *Erwinia aroideae* (DSMZ 30186) and yeast, *Rhodotorula aurantiaca* (NCIM 3425), cells were found to produce PVA upto 11, 16 and 20 IU/ gDW, respectively. The characterization of isolated bacterial culture was carried out by polyphasic analysis, which comprised fatty acid methyl ester (FAME) analysis and 16S r DNA sequencing. As consequence of polyphasic analysis, isolated culture was identified as *Bacillus cereus* (ATUAVP 1846). This is the first time that *Bacillus cereus* was identified as penicillin V acylase source.

Chapter 3: Erwinia aroideae penicillin V acylase: Production and effect of organic solvents on cell bound enzyme activity

The ***Chapter three*** is divided in to two sections: A and B.

Section A: Production of penicillin V acylase from Erwinia aroideae

The section deals with the production of intracellular penicillin V acylase from an efficient bacterial culture *Erwinia aroideae* (DSMZ 30186). *E. aroideae* produced high levels of intracellular penicillin V acylase. Various carbon and nitrogen sources were used to study the effect on the production of penicillin V acylase from *E. aroideae.* Amongst various carbon sources used (2.0%); fructose,

galactose, sucrose and mannitol increased enzyme production up to 579, 544, 534 and 504 IU/g dry weight (DW), respectively, compared to minimal medium (263 IU/g DW); however maximum PVA productivity (2796 IU/L) was achieved using fructose. Ammonium ions escalated the enzyme production to 1266.9 IU/g DW; however sodium glutamate was the best nitrogen source for overall productivity of enzyme (2045 IU/L). Various concentrations of cornsteep liquor and skim milk were used as supplements; 1.0 % cornsteep liquor and 3.0 % skim milk was optimum for the production of PVA up to 823 and 93 IU/g DW, respectively. Optimum cultural conditions for the production of PVA from *E. aroideae* were standardized. *Erwinia aroideae* produced 1543 IU/g DW penicillin V acylase in Erlenmeyer flasks (250 ml) containing 50 ml of minimal medium with 0.3% ammonium sulphate, pH 7.0 at 28 ^{o}C and 180 rpm when incubated for 56 h.

Section B: Effect of organic solvents on cell bound penicillin V acylase activity from Erwinia aroideae

This section deals with the effect of organic solvents on cell-bound penicillin V acylase activity. The cell bound PVA activity showed an eight fold increase upon treatment with chloroform (5 µL/mg $_{\text{dry biomass}}$) for 10 min and diethyl ether (10 µL/mg $_{\text{dry biomass}}$) for 45 min. Hexane, toluene, ethyl acetate and dichloromethane enhanced the enzyme activity up to two, six, four and two fold, respectively; whereas, PVA activity declined drastically on permeabilization with acetone, pyridine and alcohols. The physicochemical properties of the organic solvents used for permeabilization were correlated with the change in activity. It was found that solvents with high hydrophobicity (log *P* > 0.68) and lower dielectric constant (< 9) were relatively effective in increasing PVA activity. These results allow systematic selection of suitable solvent for best performance.

Chapter 4: Production of penicillin V acylase from Rhodotorula aurantiaca and permeabilization studies

As concluded from chapter one, ***chapter four*** deals with the production of intracellular penicillin V acylase from a newly identified yeast source, *Rhodotorula aurantiaca* (NCIM 3425). *R. aurantiaca* produced high levels of intracellular penicillin V acylase after 18 h at pH 8.0, and temperature 27^{o}C. Various carbon

and nitrogen sources were used to study the effect on the production of penicillin V acylase from *R. aurantiaca*. Among carbon and nitrogen sources used, fructose was the best carbon source for PVA production whereas tryptone was the best nitrogen source to produce the enzyme up to 170 and 1088 IU/L of culture, respectively. Additionally, permeabilization of *R. aurantiaca* cells was carried out to enhance cell-bound PVA activity using cationic detergent N-cetyl-N, N, N-trimethylammoniumbromide (CTAB). The cell-bound PVA activity was enhanced on treatment with cationic detergent. Whole-cell activity was found to be doubled (204%) on treatment of 0.01 g dry weight of cells with 50 μg/ml solution of CTAB, at pH 8.0 for one hour at room temperature. Atomic force microscopy (AFM) images of permeabilized cells show perturbation in the cell wall and offer first ever visual illustration of surface structure modifications that occur during permeabilization of *R. aurantiaca* cells leading to enhancement in activity of intracellular enzyme.

Chapter 5: Purification and characterization of penicillin V acylase from Rhodotorula aurantiaca

The ***fifth chapter*** deals with the purification and characterization of intracellular penicillin V acylase from newly identified yeast source, *Rhodotorula aurantiaca* (NCIM 3425). The enzyme was purified by hydrophobic interaction chromatography. The enzyme showed optimal activity at 45^{o}C and retained 80 % activity after incubation at 45^{o}C and pH 5.5 for 1 h. The enzyme showed maximum activity at pH 5.5 and was very stable between pH 5.5-6.0 with optimum stability at pH 6.0. It exhibited 50 % of its original activity after 30 min incubation at 60^{o}C. Enzyme hydrolyzed substrates with benzyl side chain but preferred penicillin V as primary substrate. N-terminally located serine supports the fact that it belongs to Ntn-hydrolase superfamily. The initial ten amino acid residues of *R. aurantiaca* PVA were identical to the initial sequence of NADH dehydrogenase (EC 1.6.99.3); however the enzyme lacks dehydrogenase activity. EGTA, EDTA, hexane and ethyl acetate stabilized the activity where as small chain alcohols inhibited it. 1-4 dioxane, THF, phenol and benzyl alcohol severely inhibited

enzyme activity while BME and DTT increased it. Tween 80 and Tween 20 highly enhanced the activity where as SDS and Triton X-100 inhibited it.

Michaelis-Menten constant (*Km*) for purified penicillin V acylase was calculated from Lineweaver-Burk plot and found to be 20 mM. *k*cat and catalytic efficiency (*k*cat/*Km*) were calculated to be 4.83 s^{-1} and 0.24 $mM^{-1}s^{-1}$. Dissociation constant (*Ki*) of POAA against Pen V has been investigated from Lineweaver-Burk plot and (*Ki*) of POAA was found to be 1.5 mM.

Chapter 6: Active site characterization and fluorometric studies of penicillin V acylase

The ***sixth chapter*** describes the active site studies of purified penicillin V acylase, performed using various amino acid specific modifiers and further characterization of tryptophan microenvironment in *Ra*-PVA. Modification with serine and tryptophan specific reagents such as PMSF and NBS showed partial loss of activity and substrate protection. *Ra*-PVA found to be a multi-tryptophan protein exhibiting one tryptophan, in native and four, in its denatured condition. The microenvironment of tryptophan residues in the *Ra*-PVA under native and denatured conditions were investigated by quenching of the intrinsic fluorescence of protein by neutral quenchers (acrylamide and succinimide), an anionic quencher (iodide ion) and a cationic quencher (cesium ion). The results obtained indicate that the tryptophan residues of *Ra*-PVA are largely buried in hydrophobic core of the protein matrix. Quenching of the fluorescence by acrylamide was collisional. Surface tryptophan residues were found to have predominantly more electropositively charged amino acids around them, however differentially accessible for ionic quenchers. Denaturation led to shift in λmax from 336, in native state, to 357 nm and more exposed to the solvent, consequently increase in fluorescence quenching was obtained with all quenchers. This was an attempt towards the conformational studies of *Ra*-PVA.

Chapter 7: Summary and conclusions

Seventh chapter summarizes the work presented in the thesis and emphasizes possible future research in this area. Studies on newly identified

bacterial source for the further use and characterization are highlighted in the chapter. The applications of methodology developed during research work of the thesis are described in this part.

CONTENTS

Introduction

Chapter 1

1.1. Introduction to enzymes

Proteins, along with carbohydrates, lipids and nucleic acids are the major biological macromolecules present in the living systems. The word 'enzyme' originates from Greek *énsymo*, *én* meaning "at" or "in" and *simo* means "leaven" or "yeast" (Stryer, 1995). Enzymes are proteins specialized to perform biological catalytic functions. They reside in living organisms to perform vital activities. Apart from the basic metabolic functions in the living organisms, some enzymes have great commercial applications. The first commercial application of enzymes was in the form of organisms that possessed them, as in leavening or brewing with yeast. More than 2500 enzymes are known and 250 commercially used (Woodley, 2000).

Enzymes accelerate reactions by providing an alternate pathway of lower activation energy for a reaction without altering reaction equilibrium. The rates or the enzyme turnover number (number of substrate molecules converted to product per second) vary from 36 million per second for carbonic anhydrase to lysozyme, which processes merely 2 molecules per second. Their lifetime varies from a few minutes to weeks. They have optimal pH and temperatures, where their efficiency is maximum. Some require cofactors or coenzymes for functioning-in such cases, the protein part is called apoenzyme and together with the cofactor, constitutes the holoenzyme. The main advantages of enzymes compared to most other catalysts are their chemoselectivity and steriospecificity. They are described by maximal velocity of the reaction they catalyze, *V*max, and the affinity they have for the substrate, measured as substrate concentration at half maximal velocity, *Km*, generally calculated using Michaelis-Menten equations proposed in 1913.

Enzymes are sensitive to molecules, which step up reaction, called activators and the inhibitors, which reduce their activity. Inhibition can be reversible or irreversible. The different types are: competitive, noncompetitive, uncompetitive, partially competitive and mixed.

Enzymes are specific, reaction conditions are mild, and no unwanted byproducts accumulate; these desirable features make the enzymatic reactions preferred system for commertial application. Immobilization of enzymes has permitted repeated use of costly enzymes. Enzyme catalyzed reactions are thus

cheaper than their chemical counterparts and the process more environment-friendly. Since enzymes help to overcome energy barrier of activation, it has viable and economical industrial applications in textiles, petrochemicals, food, animal feed, detergents, pulp and paper, leather etc. For example, β-lactam acylases such as penicillin acylases are used in the industrial production of semi-synthetic β-lactam antibiotics.

1.2. Discovery of antibiotics

In 1928, while working in his laboratory Sir Alexander Fleming, the famous British scientist, observed that the mold *Penicillium notatum* destroyed colonies of the bacterium *Staphylococcus aureus*, proving that some chemical substance produced by the mould was capable of killing the disease-causing bacteria. At that time, nobody took notice of Fleming's discovery. The use of penicillin as a medicine had to wait until 1940s when Howard Florey and Ernst Chain prepared a powdery form of the antibiotic. 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, 1991a). It was indeed a significant finding that antibiotics could attack bacteria specifically and caused no harm to the organism that produced them.

Antibiotics are chemicals produced by microorganisms or fungi that act on other microorganisms. Antibiotics kill the bacteria (bactericidal) or arrest its growth (bacteriostatic). 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 are β-lactam antibiotics, which can be classified based on their structure (nucleus) as

1. *Penicillin.* This 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. *Cephalosporin.* Generations – I, II, III and IV. Along with cephamycin, it forms a sub-group called cephems.
3. *Carbapenems* e.g. imipenem and meropenem
4. *Monobactams* e.g. Aztreonam
5. *β -lactam inhibitors* e.g. clavulanic acid, sulbactam

Penicillins and cephalosporins are widely found in organisms especially, in fungi belonging to *Penicillium* and *Cephalosporium*. The basic component of penicillins and cephalosporins is a β-lactam nucleus which is formed by the fusion of a 4-membered β-lactam ring to a thiazolidine ring or a six carbon ring to form 6-aminopenicillanic acid (6-APA) or 7-amino cephalosporanic acid (7-ACA), respectively. They are the most widely used group of antibiotics. Modification of side chains yields different penicillins and cephalosporins. It is obvious that the side chain determines the antibacterial range and pharmacological properties of the β-lactams. The penicillin β-lactam nucleus is derived from valine and cysteine via a tripeptide intermediate. The β-lactams inhibit the transpeptidylation step in peptidoglycan (murein) synthesis.

Cephalosporins are a group of broad-spectrum β-lactams. Cephalosporins and other newer penicillins are active against Gram-negative bacteria. They are used as penicillin substitutes, and in surgical prophylaxis, and in the treatment of gonorrhea, meningitis, pneumococcal, staphylococcal and streptococcal infections. Pharmacological characteristics of the antibiotic can be modified by substitution at 3 and 7 positions of its β-lactam ring (***Fig. 1.1.***). Newer generations of cephalosporin have progressively broader range of activity against Gram-negative organisms but a narrower range of activity against Gram-positive organisms than the preceding generation. They also have longer half-lives, reducing the dosing frequency. The advantages of cephalosporins over penicillins include low toxicity, resistance to β-lactamase (Abraham, 1987) and different antibacterial spectrum.

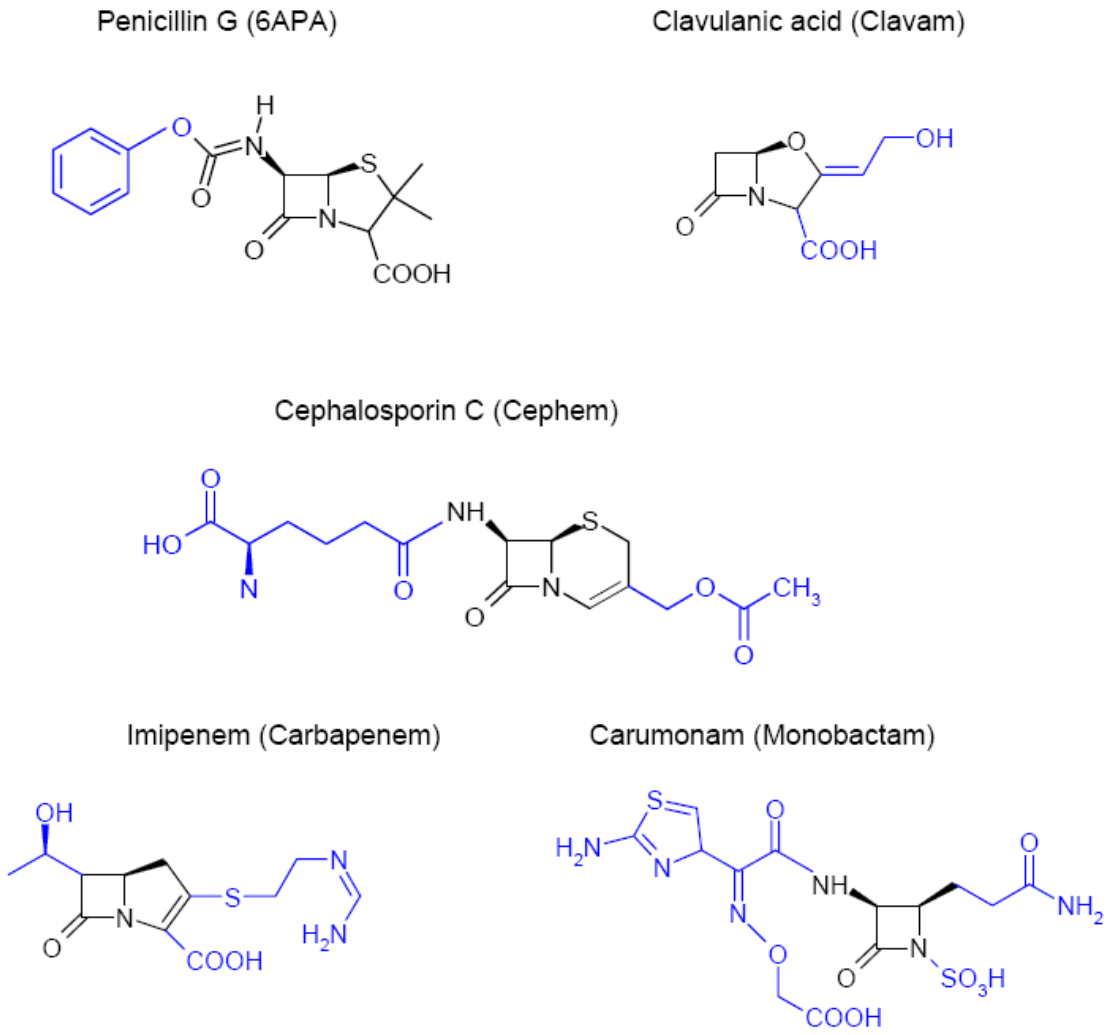

Figure 1.1: Structures of different β-lactams. The nuclei of the different antibiotics are coloured black and indicated in brackets. The side chains are in blue. In another type of classification, the antibiotics are grouped according to their side chains.

The peptidoglycan layer of bacteria is formed by cross-linked peptidoglycan chains, which are repeating alternating units of the sugars N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) with a variable peptide chain attached to the carboxyl group of the NAM-unit. Individual peptidoglycan chains are covalently cross-linked to each other via short pentapeptide bridges connecting the third position of the first peptide chain to the carboxyl group of a D-alanyl-D-alanine residue from another peptide chain, catalyzed by transpeptidases (***Fig. 1.2.***). Transpeptidases first bind to a D-alanyl-

D-alanine unit forming a covalent acyl-enzyme complex with the release of terminal D-Ala. Attack on this complex by the terminal glycine of the cross-linking pentaglycine results in the recovery of active enzyme and formation of the bond between glycine and alanine (***Scheme I, Fig. 1.3.***). Penicillin, being a structural analog of D-alanyl-D-alanine, interferes in this reaction by binding irreversibly to the enzyme (***Scheme II, Fig. 1.3.***). As a result of faulty cell wall, cells are unable to divide inspite of growing, leading to accumulation of pressure and subsequent lysis of the cell by autolysins (Abraham, 1981).

1.3. Antibiotic Resistance

Penicillin was finally prepared in large amounts after a lot of initial failures. During Second World War, it saved a lot of lives that could have succumbed to serious war wound infections. Slowly, resistant microbes began to appear (Abraham, 1981). Now, diseases like tuberculosis, which claimed to be vanished before, are making a come back with renewed resistance. Antibiotics themselves increase the prevalence of resistance by selecting naturally occurring variants of organisms that are resistant. One can contract a resistant bug due to infection or the resistance can emerge within the body during treatment due to selection pressure of the antibiotic. The resistant organisms then transfer the resistance to other non-resistant organisms. Bacteria acquire genes conferring resistance by spontaneous DNA mutation, transformation, or from plasmids. Resistance may also arise from a change in the structure of penicillin binding proteins such that the antibiotic does not bind efficiently (Essack, 2001; Antignac et al., 2003). In the case of Gram-negative bacteria, penicillins pass across the outer membrane using porins. Resistance may develop from mutation leading to modified porins. The most efficient way for bacteria to withstand penicillin action is by producing β-lactamases, which cleave β-lactams, rendering them ineffective (Jacoby & Munoz-Price, 2005) (***Scheme III, Fig. 1.3.***). Consequently, β-lactamase resistant penicillins like flucloxacillin, dicloxacillin and methicillin and later vancomycin were developed and used.

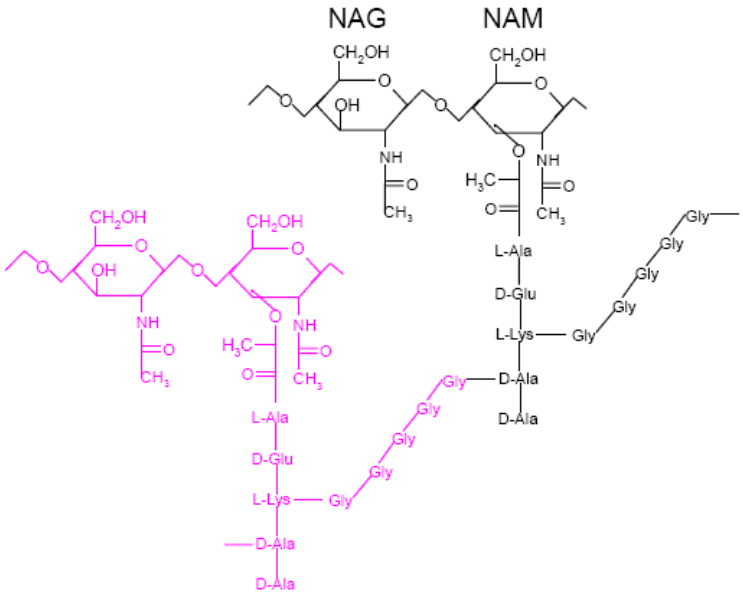

Figure 1.2: The repeating unit of bacterial cell wall. The NAM units from different chains are joined together by a pentaglycine linker.

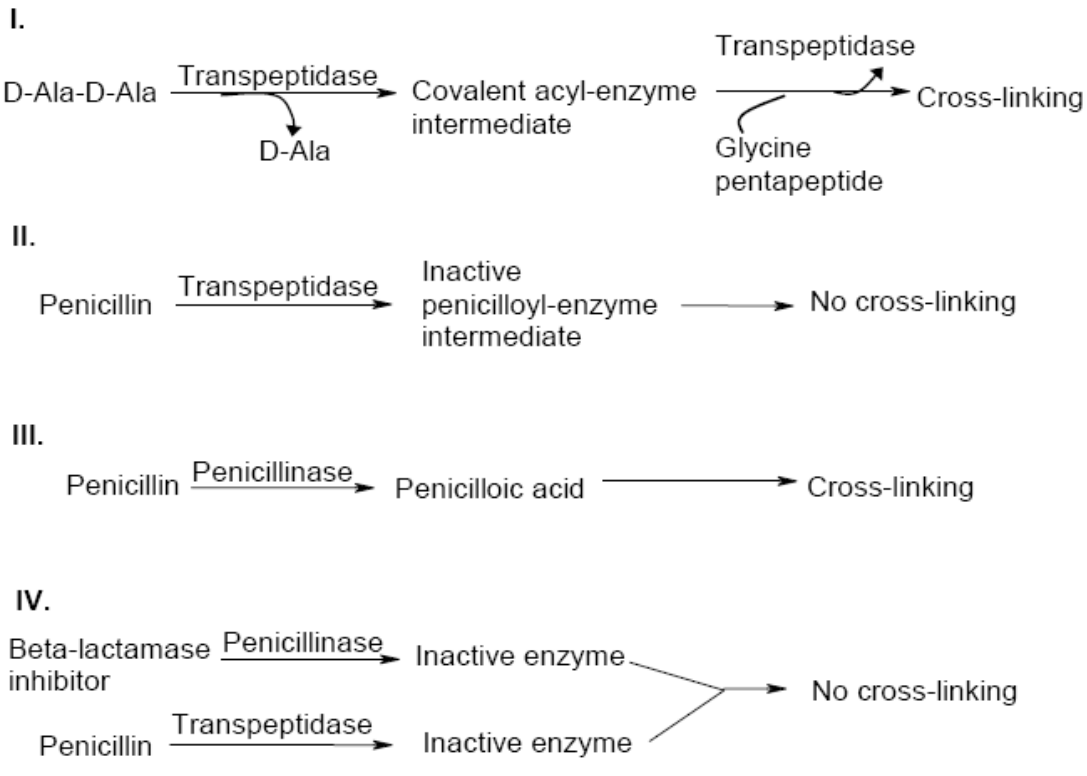

Figure 1.3: Penicillin action

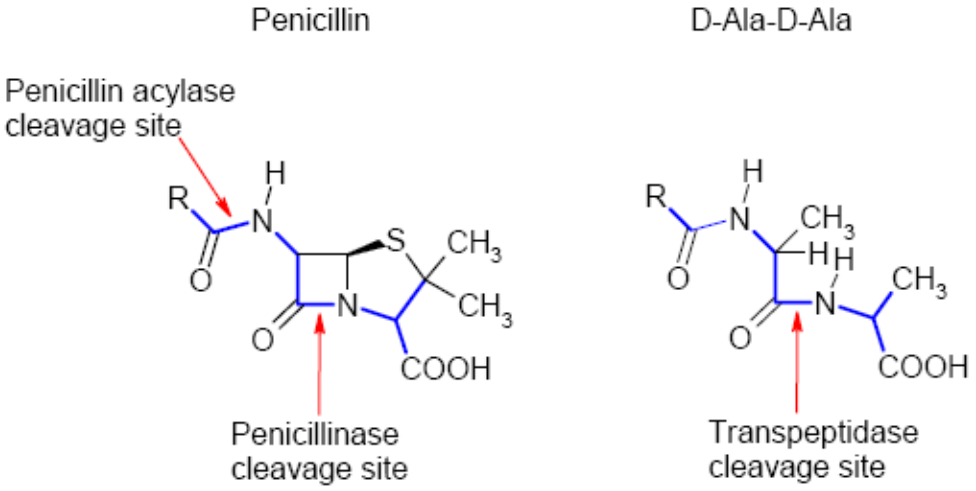

Figure 1.4: Site of enzyme

Methicillin and vancomycin resistant *Staphylococcus aureus* (referred to as MRSA and VRSA) have emerged now. This prompted the use of a group of structural analogs of β-lactams, called β-lactamase inhibitors in combination with β-lactams. Though they do not have antibacterial activity themselves, but bind tightly to β-lactamases, allowing the other β-lactam to act e.g. the natural clavulanic acid or the synthetic sulbactam. These are effective against β-lactamases commonly produced by a variety of organisms including *Staphylococcus* species, the *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Acinetobacter* species and some anaerobes. While using combination therapy with β-lactamase inhibitors, the two β-lactams act synergistically: – one competitively inhibiting β-lactamase, thus protecting the other from inactivation (***Scheme IV, Fig. 1.3.***). Although bacterial antibiotic resistance is a natural phenomenon, other factors can assist the problem. The most serious preventable cause is inappropriate antibiotic use. The need of the hour is firstly, to prevent the spread of resistance and the selection of resistant organisms, using narrow spectrum antibiotics and following proper regimen for the usage of antibiotics. Secondly, newer and effective antibiotics have to be found or developed. In the search for new antibiotics, semi-synthetic penicillins with a β-lactam nucleus and

a custom-designed side chain, look promising, though resistance to some semi-synthetic penicillins are already reported.

1.4. Semi-synthetic penicillins

Other forms of penicillins were investigated, because known natural penicillins were increasingly becoming ineffective for treatment of various pathogens. It was first observed during fermentation of *E. coli* that it produced a mixture of penicillin isoforms. Later, it was discovered that it could be preferably induced to produce Pen G by adding its side chain, phenylester, to the culture medium. It was observed that variations in the side chain alter the properties of a β-lactam antibiotic (Abraham, 1981; Vandamme & Voets, 1974) providing the first clue for more effective antibiotics. However, modifying the side chain chemically is expensive and generates by-products that have to be treated before disposal. In a multi-step reaction, natural penicillins have to be cleaved to yield the β-lactam nucleus 6-APA and in the next scheme, the nucleus has to be derivatised with the desired side chain. The amide link between the side chain and the nucleus can be hydrolyzed conventionally to yield 6-APA but β-lactam ring also gets hydrolyzed. A significant discovery was that in the absence of any precursors, a particular *Penicillium* could produce the free β-lactam nucleus (Bruggink, 2001; Vandamme & Voets, 1974). Alternatively, penicillin acylases can selectively hydrolyze the amide bond leaving the β-lactam ring intact. Penicillin acylase from *E. coli* could catalyze the breakdown of Pen G to 6-APA and its organic acid (Shewale, 1997). Later it was found that it could catalyze the second step also when it was found that under different conditions reverse reaction could occur (Vandamme & Voets, 1974). Thus the green method of semi-synthetic penicillin production was born. Other interesting discoveries included that Isopenicillin N synthase creates the bicyclic nucleus of penicillins in one step and deacetoxycephalosporin C synthase catalyses the expansion of the penicillin nucleus into the nucleus of cephalosporins.

Advances in immobilization techniques facilitated easy recovery and reuse of enzymes and increased their stability making it cost-effective (Woodley, 2000). The enzymatic method is regio-and stereo-specific and the reaction conditions are

milder. In fact, in the hydrolysis of racemic iso-propylamide of mandelic acid, immobilization improved the enantioselectivity of PGA (Rocchietti et al., 2002).

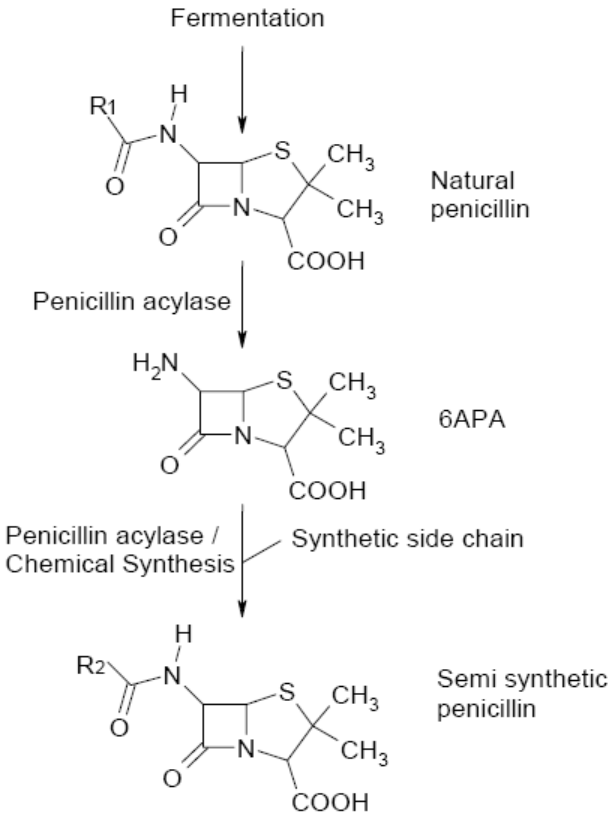

Figure 1.5: Synthesis of semi-synthetic penicillins

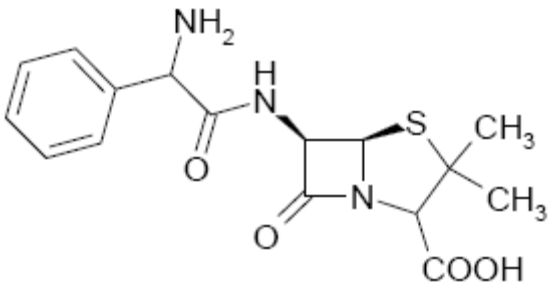

Figure 1.6: Ampicillin, a semi-synthetic penicillin

Thus, enzymatic process is cheaper and safer than chemical processes. Today, penicillin G and V are fermentatively produced at an estimated annual market volume of 16,000 tons (Bruggink & Roy, 2001) worldwide using *Penicillium* strains. About 10,000 tons of this along with 30 tons of penicillin acylases is used for production of semi-synthetic β-lactam antibiotics (Demain, 2000; Elander, 2003). Major chunk of the 6-APA produced is *via* the PGA route. The advantages of semi-synthetic penicillins are increased tolerance and diminished toxicity in humans, so they have fewer side effects; increased effectiveness due to greater selectivity against pathogens; increased resistance to β-lactamases; broader spectrum of antimicrobial activity, e.g., ampicillin is more useful than penicillin due to broader spectrum (Bruggink & Roy, 2001); improved pharmacological properties like increased stability, better absorption from the gastro-intestinal tract resulting in less dosage and lower elimination rates from the patient, thus decreasing the frequency of administration of the drugs.

The β-lactam acylases commonly used in pharmaceutical industry in the bulk manufacture of semi-synthetic β-lactams include penicillin acylases, cephalosporin acylase and glutaryl 7-aminocephalosporanic acid acylase reported from *Arthobacter viscosus, Bacillus laterosporus, Bacillus megaterium, Bacillus sphaericus, Kluyvera citrophila, Proteus rettgeri* and *Pseudomonas* sp. (Deshpande et al., 1994).

1.5. β-lactam acylases

1.5.1. Penicillin acylases (PA)

Penicillin amidohydrolases (EC 3.5.1.11) also called penicillin acylases or penicillin amidases (PA) are the enzymes that catalyze the hydrolysis of penicillin to a carboxylate and 6-aminopenicillanic acid (Sakaguchi & Murao, 1950). The EC number denotes that they are hydrolases acting on carbon-nitrogen bonds other than peptide bonds in linear amides. Apart from penicillin production, PAs are also used in other industries, in peptide synthesis or acyl group transfer reactions (Van Langen et al., 2000). Amidase from *E. coli* is used in the synthesis of artificial sweetener aspartame (Fuganti et al., 1986) and diphenyl dipeptides, whose derivatives are used as food additives, fungicidal, antiviral and anti-allergic

compounds (Van Langen et al., 2000). PAs can be used to resolve racemic mixtures of chiral compounds such as amino acids (Bossi et al., 1998), β-amino esters, amines and secondary alcohols (Svedas et al., 1996). 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. However, some PAs 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). Both PGA and PVA were found to be members of Ntn hydrolase superfamily.

1.5.1.1. Penicillin G acylase (PGA)

PGA hydrolyses Pen G to produce 6-aminopenicillanic acid (6-APA) and phenylacetic acids. PGA has been reported from *Achromobacter xylosoxidans* (Cai et al., 2004), *Alcaligenes faecalis* (Verhaert et al., 1997), *Arthrobacter viscosus* (Ohashi et al.,1988, Ohashi et al.,1989; Verhaert et al., 1997), *Bacillus megaterium* (Martin et al., 1995), *Bacillus megaterium, Escherichia coli, Kluyvera citrophila* (Barbero et al., 1986; Martin et al., 1991)*, Proteus rettgeri* (McDonough et al., 1999), *Streptomyces lavendulae*. Homologs of PAs are found throughout the whole kingdom of prokaryotes (Arroy et al., 2003). Most PGAs in gram negative organisms are usually periplasmic. PGA of *B. megaterium* is extracellular (Martin et al., 1995).

PGA from *E. coli* has been thoroughly investigated. In 1974, Kutzbach & Rauenbusch (1974) studied its general properties. The mature enzyme is a 80 kDa heterodimer of 24 kDa α-subunit and 64 kDa β-subunit comprising 209 and 566 amino acids, 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, Hewitt et al., 2000; Burtscher & Schumacher, 1992) 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 carboxy 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 non-identical subunits of 23 and 62 kDa (Barbero et al., 1986), in contrast with the previous findings (Shimizu et al., 1975). Its nucleotide sequence is 80% similar to *E. coli* ATCC 11105 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.

1.5.1.2. Penicillin V acylase (PVA)

PVA reversibly cleaves the amide bond between the side chain phenoxymethyl group and the β-lactam nucleus of Pen V, without affecting the amide bond within the β-lactam ring. Screening of penicillin acylase producing cultures indicate that the enzymes of this group are widely distributed amongst microorganisms (Vandamme & Voets, 1974; Sudhakaran & Borkar, 1985 a & b) ***Table 1.1.*** elustrates physicochemical properties of PVA from different sources . It was earlier thought that PGA is produced mainly by bacterial cultures and PVA by molds (Hamilton-Miller, 1966; Claridge et al., 1963); however, later it has been established that both PGAs and PVAs are distributed in bacteria, actinomycetes, yeasts, and fungi (Vandamme & Voets, 1974; Vandamme, 1980; Queener & Swartz, 1979; Sudhakaran & Borkar, 1985a & b; Mukherjee & Lee, 1978).

Sudhakaran & Borkar (1985) have presented a list of microorganisms producing penicillin V acylase. Different organisms that are known to produce PVA in large quantities are: *Achromobacter* sp., *Actinoplanes* sp., *Bacillus megaterium, Bacillus sphaericus, B. subtilis,* Bacterium NRRL 11240, *Beijerinckia indica* var *penicillanicum, Cryptococcus* sp., *E. aroideae, Escherichia coli* HB 101 and RR 1, *Fusarium* sp., *Micrococcus ureae, Penicillium* sp., *P. ostreatus, Pseudomonas acidovorans, P. diminuta, R. glutinis, Streptomyces lavendulae, Streptoverticillium* sp., and *Trichosporon cutaneum.* PVA is produced intracellularly by Bacterium NRRL 11240, *B. indica* var *penicillanicum, B. sphaericus, Cryptococcus* sp., *E. aroideae, Fusarium* sp., *M. ureae, P. acidovorans, Penicillium* sp., and *R. glutinis,* and extracellularly by *Fusarium* sp. SKF 235, *P. ostreatus, Streptomyces* sp., and *Streptoverticillium* sp. (Vandamme, 1988; Vandamme & Voets, 1973; Vandamme & Voets, 1975; Diers & Emborg, 1979; Oreshina et al., 1982; Vojtisek et al., 1988; Sudhakaran & Shewale, 1993; Pundle & SivaRaman, 1995; Platt & Wodznski, 1964; Cole, 1966; Vandamme & Voets, 1971 and 1972; Lowe et al., 1986; Sudhakaran & Shewale, 1990).

Generally, the nutrients are provided by complex organic nitrogen sources such as yeast extract, meat extract, peptones, and corn steep liquor; carbon sources such as glucose, sucrose, acetate, citrate, phenoxyacetic acid (POAA), glutamic acid, and starch (Vandamme, 1980; Lowe, 1981; Savidge, 1984; Stoppock et al., 1981; Lowe, 1982; Stoppock & Wagner, 1983; Pundle & SivaRaman, 1995; Lowe, 1986; Singh et al., 1988). Phenoxyacetic acid (POAA) induced the production of PVA in *Aspergillus* sp., *Epidermophyton* sp., *Fusarium* sp., *P. acidovorans, Penicillium* sp., *R. glutinis, Trichophyton* sp., and *T. cutaneum* (Cole, 1967; Vandamme, 1977; Vandamme, 1980; Lowe, 1981; Vandamme & Voets, 1973; Johanides, 1966; Sudhakaran & Shewale, 1993; Vandamme & Voets, 1971; Lowe, 1986; Thadani, 1972). Phenyl propionate or phenyl acrylate induced the PVA in *Cryptococcus* sp. CCY 17-22-1 (Vojtisek, 1988).

Table 1.1: Physicochemical properties of penicillin V acylases

Organism	pH	Temperature	Molecular Weight(kDa)	References
FUNGI				
Cephalosporium sp.	8.0	NA	NA	Cole, 1966
Coriolus versicolor	7.5	37	NA	Nara & Misawa, 1973
Emericellopsis minima	8.0	NA	NA	Cole, 1966; Cole, & Rolinson, 1961
Epidermophyton interdigitale	8.0	NA	NA	Uri et al, 1963
Fusarium sp. 7.5-5	7.0	41	107	Thadani, 1971
Fusarium sp. NTU-35	8.0	50	NA	Su et al., 1983
Fusarium Sp. SKF 235	6.5	55	83.5	Sudhakaran & Shewale, 1995
Fusarium avenaceum	7.5	37	NA	Vandergaeghe, 1975
Fusarium conglutinans	8.0	28	NA	Singh et al., 1967; Singh et al., 1969
Fusarium moniliforme	8.0	28	NA	Vandamme et al., 1971a; Vandamme et al., 1971b
Fusarium oxysporum	8.0	NA	65	Lowe et al., 1986
	7.4-7.6	45-48	NA	Sheng & Ye, 1989
Fusarium semitectum	7.5	37	67	Baumamr et al, 1971
Penicillium chrysogenum	8.0	20	NA	Cole & Rolinson, 1961
	8.5	30	NA	Erickson et al., 1965
	8.5	37	356	Meesschaert et al., 1991
Pleurotus ostreatus	8.0	50	NA	Brandl, 1965
	7.5	52	88	Schneider & Roehr, 1976
Trichophyton mentagrophytes	8.0	NA	NA	Uri et al., 1963
YEAST				
Rhodotorula glutinis	6.5	28	NA	Vandamme & Voets, 1973

BACTERIA				
Achromobacter sp.	8.5	35	NA	Savidge & Cole, 1975
Bacterium NRRL 11240	7.5	45	NA	Diers & Emborg, 1979
Beijerinckia indica	6.5	40	NA	Sudhakaran & Shewale, 1990
Bacillus sphaericus	6.8	40-60	140	Carlsen, & Emborg, 1982
	5.4-5.6	70	138	Pundle et al., 1997
Bacillus subtilis	5.8	NA	140	Olsson et al., 1985
Erwinia aroideae	5.6-5.8	28	62	Vandamme & Voets, 1975;
	7.0	36	NA	Nam & Ryu, 1984
Escherichia coli 5K (pHM 12)	8.1	54	NA	Stoppock et al., 1981
Micrococcus urea	7.4	35	NA	Nara et al., 1971
Pseudomonas acidovorans	8.0	30	NA	Lowe et al., 1981
Pseudomonas diminuta	6.4	28	NA	Carlsen & Emborg, 1982
ACTINOMYCETES				
Nocardia sp. FD 46973	8.0	28	NA	Huang et al.,1963
Nocardia globerula	7.4	35	NA	Nara et al., 1971
Streptomyces ambofaciens	7.4	35	NA	Nara et al., 1971
Streptomyces erythreus	7.5	28	NA	Haupt & Thrum, 1967
Streptomyces lavendulae	9.0-10	50	NA	Hamilton 1966; Batchelor et al., 1961; Torres et al., 1998; Torres et al., 2002
	8.0	40	-	
Streptomyces netropsis	7.5	28	NA	Haupt & Thrum, 1967
Streptoverticillium sp. 62	7.8-8.0	50	NA	Oreshina et al., 1984
Streptomyces mobaraensis	7.0	37	80	Zhang et al., 2007

Alternatively, the enzyme formation was constitutive in Bacterium NRRL 11240, *Bacterium cyclooxidans, B. indica* var *penicillanicum, B. sphaericus, E. aroideae, P. ostreatus, Streptomyces* sp., and *Streptoverticillium* sp. (Vandamme, 1980; Vandamme & Voets, 1975; Carlsen & Emborg, 1981; Ambedkar et al., 1991; Sudhakaran & Shewale, 1993; Platt et al., 1964; Lowe, 1986; Schneider & Roehr, 1976; Rudakov et al., 1978).

Most of the commercially used cultures are either mutants or genetically engineered strains. Both physical and chemical mutagenic techniques and modern genetic engineering approaches have been exploited for improved production of PVA (Sudhakaran & Borkar, 1985b; Savidge, 1984; Deshpande, 1994). *B. indica varpenicillanicum* UREMS-5 producing 168% more PVA was obtained by treating natural isolate successively with UV, γ-irradiation, and ethyl methane sulfonate (EMS) (Ambedkar, 1991). PVA production by the mutant strain was resistant to catabolite repression by glucose. Incorporation of glucose, sodium glutamate, and vegetable oil in the medium enhanced the enzyme formation to 244 IU g^{-1} dry weight (DW) of cells (Ambedkar, 1991; Sudhakaran & Shewale, 1990). High yield of PVA is obtained from genetically engineered bacteria or yeasts (Speter, 1991). PVA gene from *B. sphaericus* and *B. megaterium* UN-I has been cloned into *E. coli* and *B. subtilis* for characterization and enhanced enzyme production (Olsson et al., 1985; Gatenbeck, 1986; Olsson et al., 1986; Meevootisom & Saunders, 1987). *B. subtilis* strain 168 containing gene for PVA (pOH 38) produced the enzyme 1.7 times that of the parent strain whereas *E. coli* strain containing genes for PVA (pOH 3, pOH 35) produced less than half the activity as compared to the parent strain (Olsson, 1985; Burnett & Heckler, 1989). Gatenbeck et al. (1984) have reported the use of a recombinant shuttle vector, plasmid pOH 38, as the transforming agent for producing large amounts of PVA in *E. coli* DSM 2982 or *B. subtilis* DSM 2983. Meevootisom & Saunders (1987) observed that the transformed *E. coli* DH-1 containing the PVA gene coded plasmid pACYC 184 from *B. megaterium* UN-I produced PVA intracellularly and the genes were unstable in the absence of selection pressure for retention of vector. Highest specific activity of 3,200 Ug^{-1} DW was obtained when PVA was induced by isopropyl beta-D thiogalactopyranoside (IPTG) in the

recombinant strain of *E. coli* RR-I showing that the gene product was stable in this heterologous host (Olsson et al., 1986).

The molecular weight of penicillin V acylases ranges from 62,000 in *Erwinia aroideae* (Vandamme & Voets, 1975) to 356,000 in *Penicillium chrysogenum* (Meesschaert et al., 1991) and their subunit composition varies from monomer to tetramer. PVA differ from PGA in optimum conditions of reaction, substrate specificity, etc. The optimum pH values for PVA range between pH 5.6 - 8.5, as opposed to 6.5 - 8.5 for PGA (Margolin et al., 1980; Schumacher et al., 1986). Optimum pH of PVA from fungi range between 7.0-8.5; bacteria range between 5.5-7.5 except for PVAs from *P. acidovorans* and *Achromobacter* sp. which have pH optima at 8.0 and 8.5, respectively, and for yeasts it ranges between 6.5- 7.5 *(**Table 1.1.**).* PVAs from *Actinomycetes* exhibit a higher optimum pH range 7.4-9.0. The acidic optimum pH range of PVA can be advantageous in 6-APA production since the chemical degradation of 6-APA is less at lower pH values (Shewale & Sudhakaran, 1997). The optimum temperature of PVAs activity ranges between 28-60°C.

Generally, penicillin acylases have a low affinity toward its substrate. Pen V at higher concentrations inhibits the PVA from *Fusarium oxysporum, Fusarium* sp. SKF 235, *Malbranchea pulchella,* and *P. acidovorans* isolate E. 6-APA is a competitive inhibitor of PVAs from *B. subtilis, Fusarium* sp. SKF 235, and *P. ostreatus* NRRL 3824; it is noncompetitive for PVA from *F. oxysporum.* POAA is a competitive inhibitor of PVA from *F. oxysporum* and noncompetitive inhibitor for PVAs from *B. sphaericus* NCTC 10338, *B. subtilis,* and *Fusarium* sp. SKF 235. A mixed-type inhibition by POAA is observed for PVA from *P. acidovorans* (Shewale & Sudhakaran, 1997).

Compared to Pen G, Pen V is more stable in aqueous solutions and at the conditions of extraction. From the clinical point of view, the advantages of Pen V over Pen G are resistance to stomach acids so that they can be taken orally, resistance to penicillinase and broader range of activity against some Gram-negative bacteria, making Pen V the preferred system over Pen G (Shewale & Sudhakaran, 1997).

In case of PVA, the gene from *B. sphaericus* (Olsson et al., 1985) has been cloned and studied for biochemical and structural (Suresh et al., 1999) details. This *B. shaericus* PVA and *E. coli* PGA are very different from each other in their molecular properties. PVA is much simpler in architecture than PGA. PVA is a homotetramer of 138 kDa whereas *E coli* PGA is a 80 kDa heterodimer. PGA is produced as an inactive precursor which needs extensive post-translational modifications to form an active enzyme. PGA is usually periplasmic whereas PVA is cytoplasmic. More importantly, the two enzymes do not have detectable amino acid sequence homology. According to sequence similarity, PVA belongs to choloylglycine hydrolase family of Ntn hydrolase superfamily. PVA crystal structure demonstrated that the N-terminal nucleophile is a cysteine. DNA sequence revealed that Cys is the fourth amino acid from N-terminal methionine, indicating that the three proceeding residues including Met were removed during maturation, post-translational processing, a property shared with other Ntn hydrolases. The tree dimensional structure of PVA from *B. sphaericus* has been reorted by Suresh et al. (1999) (***Fig. 1.7***). The inactive mutants were prepared to study autocatalytic processing of precursor PVA molecule (Chandra et al., 2005).

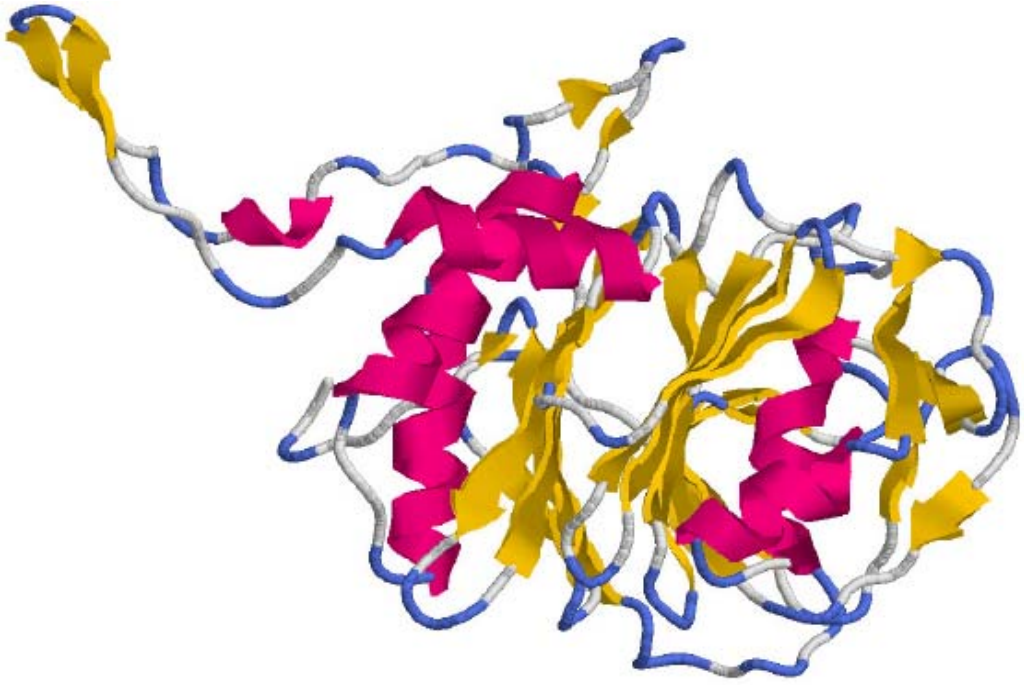

Figure 1.7: Three dimentional structure of penicillin V acylase (Suresh et al. 1999) A PVA monomer is shown with αββα core structure, a characteristic of Ntn-hydrolase family members.

1.5.1.3. Cephalosporin acylase (CA)

Cephalosporin acylases (CAs) hydrolyse β-lactams with a cephalosporin nucleus, such as cephalosporin C and/or the preferred substrate glutaryl 7-aminocephalosporanic acid to produce 7-aminocephalosporanic acid (7-ACA) and β-lactams with a charged side chain (Fritz-Wolf et al., 2002). 7-ACA is obtained from the natural antibiotic cephalosporin C, either chemically or by a two-step enzymatic process involving the enzymes D-aminoacid oxidase and glutarylamidase. The chemical production is expensive and produces harmful byproducts. Cephalosporin C acylase from *Pseudomonas* sp. strain N176 is a heterodimer of 25 and 58 kDa (Kinoshita et al., 2000). Like PGA, the enzyme is a heterodimer of two non-identical subunits, α and β, which are derived from a

nascent precursor polypeptide that is cleaved proteolytically through a two-step autocatalytic process. CA is also found to be an Ntn hydrolase.

Cephalosporin C acylases (CCA) are very interesting from an industrial point of view (Kim et al., 2000). However, the problem with enzymatic cleavage using CA is that, it has low substrate specificity for cephalosporin C than glutaryl-7ACA. Production of cephalosporin C acylase has been reported by *Arthrobacter viscosus* ATCC 53594, *Aspergillus* sp. MA-13, *Aeromonas* sp. ACY 95. *Bacillus megaterium* ATCC 53667, *Paecilomyces* sp., *Penicillium griseofulvin, Penicillium regulosm, Pseudomonas diminuta* strains N 176 and V 22, *Pseudomonas putida* and *Pseudomonas* sp. SE83 (Niwa et al. 1977; Banyu 1984; Ichikawa et al., 1987; Kawate et al., 1987; Matsuda et al., I987; Vandamme 1988; Lein, I988 & 1989; Reyes et al., 1990; Aramori *et* al., 1991a and 1991b).

Studies have been conducted on improving the performance of *P. diminuta* CA (Kim et al., 2000) and redesigning the enzyme to produce 7-ACA from cephalosporin C in a single enzymatic step (Fritz-Wolf et al., 2002). By site-directed mutagenesis relative activity of *Pseudomonas* sp. N176's CCA on cephalosporin C could be further improved to 6% as that of glutaryl 7-ACA (Ishii et al., 1995). The industrially used cephalosporin producing fungus *Acremonium chrysogenum* has provided a new tool, promoting the design of alternative biosynthetic pathways making it possible to obtain new antibiotics and to improve cephalosporin production (Diez et al., 1996).

1.6. In vivo role of penicillin acylases

The mystery of the physiological role of penicillin acylase has been obscure, since attention is diverted to its industrial applications. The question still remains essentially unanswered although evidence suggests 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 (Valle et al., 1991). The location of penicillin acylase gene in *E. coli* has been discovered near a gene encoding for an aromatic hydroxylase (Prieto et al., 1993). Later the nucleic acid sequence of a

14855 base pair region that contains the complete gene cluster encoding 4-hydroxyphenylacetic acid degradative pathway of *E. coli* ATCC 11105 was determined (Prieto et al., 1996). The gene cluster is located in a region close to the gene encoding PGA. It is therefore suggested that PGA is present in *E. coli* to improve its ability to metabolize a wider range of substrates. 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 utilisation of alternative carbon sources becomes necessary. Phenylacetic acid derivatives would be available abundantly in this kind of nonparasitic environment (Burlingame & Chapman, 1983).

PVA expression in *Vibrio cholerae* has been found to be linked with pathogenesis in response to cell density (Kovacikova et al., 2003). AphA, an activator of virulence operon, has a second binding site upstream of gene encoding PVA. It has been recognised as a negative regulator of PVA (Kovacikova et al., 2003). A high level of AphA represses the expression of PVA and activates tcpPH expression and the rest of the virulence cascade. Kovacikova et al., (2003) showed that AphA (activator of virulence operon) negatively regulates PVA gene expression in both the classical and El Tor biotypes of *V. cholerae* by binding to a site virtually identical to a promoter, which overlaps with the PVA transcriptional start site. In El Tor strain C6706, the PVA gene is also regulated by quorum sensing such that its expression is reduced at low cell density. Since PVA does not appear to play a role in virulence, the ability of AphA to oppositely regulate PVA expression compared to virulence gene expression may provide *V. cholerae* with advantages in different environmental niches.

1.7. Ntn hydrolase superfamily

The N-terminal nucleophile (Ntn) hydrolases belong to superfamily of diverse enzymes that has recently been characterized on the basis of presence of N-terminal nucleophilic residue participating in catalysis. The distinct αββα fold

results in the active site nucleophile exhibiting specific similar position (Brannigan et al., 1995). All of the proteins in this family are activated autocatalytically; they cleave an amide bond and they contain an N-terminally located catalytic nucleophile. The members of this superfamily share a strikingly similar arrangement in the catalytic environment and exhibit one of the nucleophiles such as serine/Cysteine/Threonine at their active site (PGA-Ser, GAT-Cys, Proteasome-Thr). All Ntn hydrolases catalyze amide bond hydrolysis but their substrates vary diversely. As a result, the shape, size, nature of interacting residues and their locations differ in the binding pocket. The mechanism is more or less like serine proteases, but instead of a catalytic triad, a single N-terminal residue functions as a nucleophile and the catalytic base. The list of Ntn hydrolases superfamily members is presented in ***Table 1.2.***

Post-translational autoproteolysis is a mechanism used to activate many proteins *via* self-catalyzed peptide bond rearrangements, which play an essential role in a wide variety of biological processes. They include activation cascades such as blood coagulation and fibrinolysis, cell death, embryonic development, protein targeting and degradation, viral protein processing, zymogen activation (Neurath, 1986), caspase (Salvesen & Dixit, 1999).

In all the structures elucidated so far, the two central anti-parallel β-sheets are sandwiched between two layers of anti-parallel α-helices. Between them, the β-sheets have a packing angle of from 5º in aspartyl glucosaminidase to 35º in proteasome. Eight totally conserved secondary structure units are found (Oinonen & Rouvinen, 2000). The arrangement of structural elements indicates a common ancestor. However, the composition of this core varies as do the oligomeric states. For example, PVA is a tetramer of a single type of subunit whereas proteasome comprises 28 subunits of two types α and β making up a mammoth four-layered αββα barrel. Each layer has seven subunits. The outer α- layers do not have catalytic activity. The catalytic β-chains of proteasome have a Thr as their Ntn residue.

Table 1.2: Ntn-hydrolase family members

Member	Source	Structure	Catalytic function	Activation	Nucleophile
PVA	Bacterial, fungal	Homo-tetramer	Penicillin V acylase	Peptide removal	C1
PGA	Bacterial	αβ dimmer	Penicillin G acylase	Cleavage in to subunits	S 264 beta
CA	Bacterial	αβ dimmer	Deacylation of cephalosporin	Cleavage in to subunits	S1 beta
Proteasomes	Archaea yeast	7 membered ring	Protease	Peptide removal	T1
GCA	Bacterial	$(\alpha\beta)^2$ hetero-dimmer	Deacylation of cephalosporin	Cleavage in to subunits	S 170
GAT	Bacterial	Homo-tetramer	Amido-transferase	Peptide removal	C1
Human AG	Human	$(\alpha\beta)^2$ hetero-dimmer	Glycosyl-asparaginase	Cleavage in to subunits	T 206
Bacterial AGA	Bacterial	$(\alpha\beta)^2$ hetero-dimmer	Glycosyl-asparaginase	Cleavage in to subunits	T 152

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

During post-translational activation of Ntn-hydrolases such as *Aspartylglucosaminidase* (AGA), PGA, cephalosporin acylase, and glutaryl 7-aminocephalosporanic acid acylase, the precursor polypeptide undergoes cleavage removing a spacer chain that results in the chain splitting into two parts in the processed protein, whereas in PVA, proteasome and glutamine PRPP amidotransferase, the activation is achieved by the removal of a small propeptide. This autocatalytic processing considered a common feature of Ntn-hydrolases, is

absent in the newly added member - conjugated bile salt hydrolase (BSH). Sequence shows that it is produced with the nulcleophile residue present at the second position in the N-terminus, which gets exposed after enzymatic removal of initiation formyl-methionine without the aid of any autocatalytic processing.

1.8. Applications of penicillin V acylase

The reactions catalyzed by penicillin acylases are reversible. PGAs are explored for various applications besides production of 6-APA and 7-ADCA (Shewale et al., 1990). This is due to the broader specificity of PGAs. PVAs are more specific and they find applications mainly in the production of 6-APA and 7-ADCA. Various aspects of 6-APA and 7-ADCA technology have been exhaustively reported (Vandamme, 1988; Shewale & Sivaraman, 1989; Savidge, 1984; Valle, 1991). The technical aspects of the use of immobilized PVA and PGA in the processes for production of 6-APA and 7-ADCA are not very different.

1.8.1. Production of 6-APA

PVA hydrolyses Pen V quantitatively under kinetically controlled conditions. Hydrolysis of Pen V is performed at temperatures between 28-40°C in order to avoid the degradation of Pen V and 6-APA. Control of pH during the course of hydrolysis forms a critical parameter during the operation since the side-chain acid, POAA, released during the conversion causes a decrease in pH resulting in a lower rate of forward reaction which is maintained by the addition of alkali. Therefore, strict control of pH and temperature is necessary during the process.

The concentration of Pen V in the reaction mixture varies between 5-10% and is determined by inhibition constants for Pen V, 6-APA, and POAA. Almost quantitative hydrolysis (~97%) of Pen V at concentrations between 12-20% has been reported so far (Gestrelius et al., 1983; Haagensen et al., 1983; Vanderbeek & Roels, 1984). The advantages of using Pen V in higher concentrations are many, e.g., concentration of split solution is avoided, yields of 6-APA are higher, the turnover of Pen V per batch (unit time) is higher, etc. These ultimately contribute to a lower production cost.

Pen V is hydrolysed enzymatically using both in batch and continuous modes. Industrially, a batch reactor is generally used. In a conventional stirred-tank reactor, the immobilized PVA is stirred with Pen V in a jacketed tank at a desired temperature. The pH of the reaction is maintained by the addition of alkali and controlled by a pH stat. It is necessary that the agitator should be enough to keep the immobilized PVA in suspension and allow instant distribution of incoming alkali but should not develop slow destruction of the immobilized PVA. At the end of the reaction, the hydrolysate is eliminated from the reactor. Immobilized PVA is retained in the reactor by the filter plate (fixed at bottom) for the next batch. Comparison of the productivity of immobilized PVA in stirred-tank reactor, recirculated packed-bed batch reactor, and recirculated packed-bed continuous system revealed that the stirred tank reactor is most suited for conversion of Pen V at 10% concentration provided that the immobilized PVA preparation is stable to absolute and high pressure (Mollgaard, 1987). When Pen V is used at 4% or lower concentrations, assembly of a series of packed beds is recommended (Mollgaard, 1987). At such a low concentration of Pen V, the kinetic parameters are managed better but the concentration of hydrolysate is necessary to obtain good yields of 6-APA. The final step in the 6-APA process is crystallization of 6-APA from the reaction mixture. 6-APA, being an amphoteric molecule, is precipitated at its isoelectric point, pH 4.3. The overall yield of 6-APA ranges between 85- 92%. Use of buffer concentrations above 50 mM during conversion interferes in the purification of 6-APA (Mollgaard, 1987). The interference is probably due to phosphate-catalyzed degradation of Pen V (Christensen et al., 1994).

1.8.2. Production of 7-ADCA

To produce semisynthetic cephalosporins from Pen V, the five-membered thiazolidine ring of penicillin is chemically expanded to the six-membered dihydrothiazine ring of cephalosporins forming Ceph V which is hydrolyzed chemically (a two-step process) or enzymatically (a onestep process) to generate 7-ADCA. 7-ADCA is then acylated chemically to form semisynthetic cephalosporins. The process for production of 7-ADCA from Ceph V by employing PVA is similar to the process for production of 6-APA. The yield of 7-ADCA varies

between 88- 92% (Savidge, 1984; Lowe, 1989; Matsumoto, 1993; Vandamme, 1992).

1.8.3. Production of 7-ACA

Cephalosporin C (Ceph C) as such exhibits weak antimicrobial activity but substitutions at C3 and C7 positions yield semisynthetic cephalosporins exhibiting potent antimicrobial activity; therefore, all Ceph C produced is converted either chemically or enzymatically to generate the cephem nucleus, 7-ACA, which is further converted into semisynthetic cephalosporins such as cefazolin, cefotaxime, cephalothin, etc. The enzymatic route involves a two-step process consisting of D-amino acid oxidase and glutaryl 7-ACA acylase (Matsumoto, 1993; Kumar et al., 1993; Tsuzuki et al., 1989). Alternate routes are being discoverd for the production of 7-ACA. The example of such kind of approach involves, use of PVAs from *Erwinia* sp., *Nocardia* sp., and Bacterium NRRL 11240. An acyl transferase present in *P. chrysogenum* is used to convert Ceph C to 7-phenoxyacetamidocephalosporanic acid (V-ACA) which is then hydrolyzed by PVA to obtain 7-ACA (Frederiksen & Emborg, 1984).

Table 1.3: Immobilized penicillin V acylase preparations

FUNGI	Immobilization: Matrix, principle and protocol	Reference
Fusarium SD. SKF 235	Adsorption and cross-linking of enzyme on Amberlite CG-50	Sudhakaran & Shewale, 1993
	Covalent attachment of enzyme to cyanogen bromide-activated cellulose	SivaRaman et al., 1976a, SivaRaman et al., 1976b
Fusarium fulvorum	Entrapment of cells in agar-calcium carbonate gel	Culik et al., 1965
Fusarium sp.	Entrapment of spores in polyacrylamide gel	Linko & Linko, 1983
Fusarium sp. 7.5-5	Adduct formation of enzyme by glutaraldehyde cross-linking	SivaRaman et al., 1976a, SivaRaman et al., 1976b
Fusarium oxysporum	Entrapment of cells in polyacrylamide gel	Wang & Su, 1990
	Entrapment of cells in cellulose diacetate	Sheng & Ye, 1989
Fusarium sp. NTU 35	Entrapment of cells in polyacrylamide gel	Su et al., 1984
Malbranchea pulchella	Double entrapment of mycelium in alginate and polyacrylamide	Singh, 1988
Pleurotus ostreatus	Entrapment of enzyme in cellulose acetate fibers and polyacrylic resin	Brandl et al., 1973; Brandl & Knauseder, 1975
	Entrapment of mycelium in chitosan	Khrge et al., 1982

YEASTS		
Rhodotorula glutinis	Entrapment of enzyme in polyacrylamide gel	Vandamme & Voets 1973
Cryptococcus sp. CCY-17-22-I	Cross-linking of cells with glutaraldehyde and ammonium sulfate	Vojtisek et al., 1989
ACTINOMYCETES		
Streptomyces noursei	Adsorption of enzyme on bentonite	Heuser et al., 1969
S. lavandulae ATCC 13664	Epoxy- activated acrylic beads (Eupergit C) by covalent binding	Torres et al., 2001
Streptomyces griseus	Entrapment of cells in polyacrylamide gel Adsorption of enzyme on bentonite	Chibata et al., 1974; Chibata et al., 1976 Heuser et al., 1969
Streptomyces gardeneri	Entrapment of cells in polyacrylamide gel	Chibata et al., 1974; Chibata et al., 1976
BACTERIA		
Etwinia aroideae	Entrapment of cells/enzyme in cellulose triacetate fibers	Fleming, et al, 1974; Fleming, et al, 1975
Bacterium NRRL 11240	Modification of whole broth with polyethyleneimine and cross-linking with	Gestrelius, 1980; Gestrelius, 1979
Escherichia coli 5K (pHM 12)	Entrapment in epoxy matrix, porous polyurethane foam and gel	Klein, 1981
Escherichia coli	Entrapment of cells in polyacrylamide gel	Chibata, et al., 1976
	Entrapment of cells in calcium alginatelpolymethylacrylamide	Klein & Wagner, 1980

1.9. Permeabilition

The cell wall and the cellular membrane are essential components of microbial cells. They give shapes and rigidity to the cell and they provide semipermeable barriers to chemical species that come in contact with the cell. Ideally, for a whole-cell process, starting material (nutrients, substrate, pollutants, etc.) should be transported into the cell without barrier so that the rate of production is dictated only by the metabolic functions of the cells. And once products are synthesized inside the cells and accumulated to an appropriate level, releasing products from cellular environment and allowing products to be collected outside the cell. On the contrary, bioprocesses are drastically limited by the barrier functions of cell walls and cell membranes. In recombinant protein fermentation processes, proteins are made inside the cells and have to be collected after cell disruption, and subsequently the desired proteins need to be purified from the cellular extract that contains virtually everything inside the cell. A new batch of production has to start with the growth of cells. Generally, whole-cell catalyzed reaction is slower than that catalyzed by the isolated enzymes.

In bioresearch, the capability to monitor gene expression is a precious tool to understand cell regulation. The activity of β-galacosidase (β-gal), the product of the lacZ gene of *Escherichia coli*, is one of the most widely used reporter of gene expression since its activity can be monitored using a variety of chromogenic and fluorogenic substrates in both prokaryotes and eukaryotes. However, efficient access of these substrates to β-gal enzyme is hindered by the cell membrane (Vidal-Aroca et al., 2006). Routine protocols for the quantitative determination of the *in vivo* expression of β-gal activity in any system require cell permeabilization or destruction of cell integrity to obtain crude extracts, making the assay tedious for more widespread use.

The problem of substrate permeability in whole-cell biocatalysis may be underestimated as many potential whole-cell biocatalysts are labeled as nonactive in the screening stage when the reaction of interest does not proceed at a rate that is high enough to be detected, even though cells may possess very active enzymes. Recent progresses in metabolic engineering and directed evolution have made drastically improved or novel whole-cell biocatalysts a reality (Patnaik et al. 2002; Glieder et al., 2002). These advances have made the permeability issues all the

more important as these improved biocatalysts will only be useful if they can be made accessible to substrates. Although removal of such barrier and using cell-free extract are alternatives to a whole-cell process, whole cell biocatalysts are preferred for several reasons (Duetz et al., 2001; Faber, 1995). These include the elimination of the need for tedious, expensive protein isolations and/or purification, more stable enzymes, ability to catalyze more sophisticated chemical transformations requiring multiple enzymes or pathways, and facile cofactor regenerations.

1.9.1. Molecular basis of permeabilization

Gram-negative bacteria have two cell membranes, the outer membrane and inner membrane. The inner membrane consists of phospholipid bilayer decorated with over 200 different kinds of proteins. Passage of hydrophobic molecules through the inner membrane is relatively fast because of the lipophilic bilayer; however, the passage of hydrophilic molecules has to rely on specific transport proteins that reside in the inner membrane (Neidhardt et al., 1990). The structure of the outer membrane is quite different from the inner membrane with lipopolysaccharides (LPS) as the outer leaflet. Densely packed LPS are good barriers to both hydrophobic and hydrophilic molecules due to its amphiphilicity. LPS consists of three components: lipophilic lipid A, an oligosaccharide core, and a long polysaccharides chain of a repeating unit commonly called O antigen. The hydrophilic nature of the polysaccharide component limits the penetration of hydrophobic molecules. In fact, studies show that hydrophobic molecules permeate through the LPS at about 150th to 100th of the rate through the usual phospholipid bilayers (Nikaido, 1994). And, the hydrophobic lipid A of LPS acts as a barrier to hydrophilic compounds.

Small hydrophilic compounds essential to cell growth (such as sugars, amino acids, and ions) rely on passive diffusion through special channels (or porins) in the outer membrane to cross the outer membrane barrier. However, these nonspecific transmembrane pores are of limited size. As such, they only allow the diffusion of small hydrophilic molecules within a limited size range (smaller than 600 Da; Denyer & Mailard, 2002). Hydrophilic molecules larger than 600 Da require specific protein-based permeation mechanisms. For those that do not have natural uptake system, as is the case for synthetic substrates, passive diffusion through the LPS layer is the only mechanism to get into the cell. It was also reported that the peptidoglycan may

play a role in the permeability of Gram-negative bacteria, either directly by forming a permeability barrier or indirectly by holding together the outer membrane (Burman et al. 1972).

Most Gram-positive bacteria are surrounded by a thick peptidoglycan cell wall. This structure, although mechanically strong, appears to offer little resistance to the diffusion of small molecules because the meshwork is too coarse (Nikaido, 1994).

In short, the outer membrane of Gram-negative bacteria is a good barrier for both hydrophobic and hydrophilic molecules. Hydrophilic molecules smaller than 600 Da can depend on nonspecific porins for entry and for hydrophobic molecules, neither porins nor passive diffusion through LPS is a mechanism for their rapid uptake. It is important to note that the permeability barrier does not permit unfavorable compounds in an absolute sense; it only slows down the entry of these molecules.

An important dissimilarity between drug permeability and substrate uptake rate in whole-cell bioprocesses is obseved. As substrates are reacted and transformed by intracellular enzymes, the rate of entry in relation to the rate of enzymatic reaction is important in determining the importance of permeability issue. On the other hand, if the drug is not degraded, an intracellular accumulation will result when the uptake is fast, relative to the growth rate of the cells. Therefore, in most biotechnological applications, the permeability issues are particularly important, as there is an intracellular consumption of substrate, which in most cases is the target to be maximized. The enhanced catalytic capability, compounded by the factor that biocatalysis and bioremediation deal with many hydrophobic molecules, makes the permeability one of the most important limitation in these applications.

1.9.2. Common permeabilization approaches

Though various permeabilization methods were reported in the literature, specific conditions for a given organism, substrate, or product varied widely, it is hard to generalize these methods. The common pereabilization methods, used for permeabilization of cells are follows:

1.9.2.1. Detergent treatment

Detergent treatment is a very common method used for the premeabilization of microorganisms. Among various permeabilization methods tried, Triton X-100 was most effective (van der Werf et al., 1995). The specific activity of malease was increased from 0 to 1.7 U/mg protein with 0.15% Triton X-100. Detergent concentration, duration of the treatment, and cell density are three important parameters studied for optimization. Other detergents such as N-cetyltrimethylammonium bromide (CTAB, 0.1% w/v) was also found useful as a permeabilization agent, for example, in permeabilizing *Z. mobilis* in sorbitol and gluconic acid production (Silveira & Jonas, 2002). CTAB was also an effective permeabilizer for *Rhodotorula garcilis* yeast cells, increasing D-amino acid oxidase and catalase activity by 9- and 15-fold, respectively. As observed with Triton-100, despite being effective, detergent-treated cells could not be reused due to cell lysis (Upadhya et al., 2000). This illustrates one of the major problems with these common permeabilization procedures: the unwanted excessive damage of cell membrane. Compared to solvent treatment, detergent causes more drastic change in the cell membrane and often leads to cell lysis. The extent of tolerable cell lysis may differ with a particular application, but if reuse of whole-cell catalysts is desirable, detergent permeabilization can not be selected.

1.9.2.2. Solvent treatment

Permeabilization of cells with solevent is also a common and easy method; various studies are performed and reported using solvents as a permeabilizing agent (Flores et al., 1994; Krishnan et al., 2000; Leon et al., 2003). Permeabilization study carried out with recombinant *Saccharomyces cerevisiae* expressing glyoxalase, permeabilization with 40% ethanol and isopropyl alcohol remarkably increased the initial rate of whole-cell reaction by 380 and 580 fold, respectively (Liu et al., 1999). The striking acceleration of the biocatalysis reaction was due to the increased permeability to both substrate methylglyoxal and product. The important parameters to optimize premeabilization process are nature of the solvent, concentration of the solvent, and duration of the treatment. Lower incubation temperature, typically 4°C, achieved overall better performance of the whole-cell biocatalysts. Solvent treatment was used to permeabilize cells of *Kluyveromyces lactis*. Permeabilization with 50%

ethanol for 15 min at 4°C was found to be satisfactory in the synthesis of lactulose with β-galactosidase, and final product concentration and productivity were increased by 1.3 and 2.1 fold, respectively (Lee et al., 2004). Use of 50% v/v toluene for permeabilization of *S. cerevisiae* increased the whole-cell catalyzed reaction involving catalase by six fold. Optimal conditions were 4°C and 10 min (Kubal & D'Souza, 2004), in another study.

In general, permeabilization of bacterial cells was effective as much lower solvent concentrations however for yeast cells high concentration of alcohol was most effective with. Fontanille & Larroche (2003) have repoted, only 5% v/v solvent (toluene, diethyl ether, chloroform) was used for permeabilizing *Pseudomonas rhodesiae*. The permeabilization procedures included first freeze (−20°C) and thaw (presumably at room temperature) and subsequent incubation with one of the permeabilizing solvent at 30°C for 1 hr in a biological shaker at 250 rpm. Likewise, the permeabilization of *Zymomonas mobilis* with 10% v/v toluene found to be effective (Silveira & Jonas, 2002). These measurable differences in conditions of solvent treatment reveal the differences in cell envelop between bacterial and yeast cells.

1.9.2.3. Freeze and thaw

Freeze and thaw is another simple method used to permeabilize cells. In this method, cell suspensions are alternately frozen and thawed several times. Freezing temperature varies from −20 to −80°C, and thawing usually occurs at room temperature (Matsumoto et al., 2001). Up to eight cycles was reported (Breedveld et al., 1992). Though it is a very simple and interesting approach however is a quite time-consuming process especially when multiple cycles are required.

1.9.2.4. Salt stress

Another interesting method of permeabilizing cells is salt stress, which has been used in a biocatalysis process to enhance the production of L (−) carnitine from *E. coli* cells. The whole-cell catalyzed reaction was carried out in the presence of NaCl. Product yield was increased with the increase in NaCl concentration in the reaction medium as a linear function up to 0.5 M, at which point the yield was doubled (from 40 to 80%) compared to that of the control, without the salt (Cavonas

et al., 2003). It is essential to note that *E. coli* has a native uptake system, an antiport to exchange substrate and product (Jung et al., 2002). The fact that the increase was due to improved transport indicates that the limitation of mass transfer still exists even with a natural uptake system; it is possible that transport across the outer membrane was a limiting factor in the process. Breedveld et al. (1992) have observed salt-induced permeabilization of *Rhizobium leguminosarum* for cyclic β-1, 2-glucans in the presence of 0.2 M NaCl.

1.9.2.5. Electro-permeabilization

Yang et al. (2003) have reported electropermeabilization, a method for the release of secondary metabolite from living plant cells. Release of indole alkaloids, ajmalicine, and yohimbine from *Catharanthus roseus* in tubular membrane reactors was induced by applying low-level electric current (1–5 mA). It is interesting to note that, the released product can be simultaneously collected by electrophoresis. This permeabilization was shown to maintain high cell viability. However, it is only applicable to ionic products. A related method, pulsed electric field or electroporation, more commonly employed for macromolecule uptake or killing of microbes, may also be applicable for small molecule uptake by modification of conditions. Parameters to optimize include electric field strength, energy input, pulse width, medium conductivity, pH, and cell growth phase (Muraji et al., 1999; Wouters et al., 2001). The requirement of specialized equipment and extensive sample preparations are possible limitations of this method.

1.9.2.6. Other physical and chemical approaches

Polyethylenimine (PEI) and lactic acid have also been used for permeabilization (Helander et al., 1997; Alakomi et al., 2000). EDTA as a permeabilizing agent is also commonly used method. It works by complexing divalent cations that are crucial in strengthening lateral LPS molecules, resulting in the weakening of its near-crystal structure. Alkaline treatment was reported as an effective method for permeabilizing *Achizosaccharomyces pombe* with or without 0.3% CTAB. Cell mixture with polyethylene imine (5%) was stirred slowly for about 90 min at pH 11, after which the pH was readjusted by adding an acid. Variation of susceptibility among different yeast strains was noted, highlighting the empirical

nature of the process in deriving optimal protocol (Isoai et al., 2002). Reverse micelles of AOT/hexane/water were developed for selective release of periplasmically located recombinant penicillin acylase through outer membrane permeabilization (Bansal-Mutalik & Gaikar, 2003). Recovery of protein exceeds 100% of the sonicated samples. Other methods of outer membrane permeabilization for release of periplasmic proteins were also reported in the literature. These methods typically employ a combination of two or more reagents. Examples are osmotic shock solutions with EDTA and EDTA with lysozymes (French et al., 1996; Shepard et al., 2002). In a biodiesel fuel production, recombinant *S. cerevisiae* expressing lipase was permeabilized by air-drying at 42°C for 3 h for substrates (methanol and triglyceride) to cross cell membrane barriers (Matsumoto et al., 2001).

On the other hand, the membrane permeability was modulated with the physicochemical stimuli such as osmotic pressure (Shoemaker & Vanderlick, 2002), electric field (Faurie et al., 2005), and changes in temperature and pH (Barbet et al., 1984). In addition to the above, the mechanical forces such as ultrasound (Pong et al., 2006) and shear stress (Chakravarthy & Giorgio, 1992) were applied to the liquid bulk suspending liposomes.

While these methods are effective, they are experiential and dependent on a lot of trials and errors. Even an optimal condition is identified; the process certainly adds several extra process steps, especially when want to use on large scale. For example, permeabilizing cells with 1% toluene, a commonly used procedure, would require the following steps (Dupont & Clarke, 1991): (1) harvest cells by centrifugation; (2) suspend cells in a highly buffered solution containing MgCl2; (3) incubate cells with toluene at typically 4°C for 10 min; (4) recollect cells; (5) wash to remove residual toluene. Especially when a surfactant is used, adding a permeabilizing agent, it is likely that complicates downstream processing. However in chemical permeabilizing reagents often cause extensive damage to the membrane system, even cell lysis (Fontanille & Larroche, 2003).

1.9.2.7. Molecular engineering approaches

Recombinant DNA technology provides new tools and opportunities to address cell permeability issues in a more predictable manner. Indeed, several

recent studies seem to indicate that the molecular engineering can be used to modulate permeabilization of cells.

In molecular engineering approaches, modulation of membrane permeability by varying growth conditions is also a permeabilization method. Several studies have reported the dependence of membrane compositions on various environmental growth conditions (McGarrity & Armstrong, 1975 & 1981; Arneborg et al., 1993; Shokri et al., 2002; Sololovska et al., 2003). These include growth rate, medium composition (particularly salt concentrations), growth phase, temperatures, and stress conditions. As membrane compositions determine, to a large extent, the permeability of the substrate, modulating membrane permeability could be achieved with relative ease by simply changing those conditions. The cell wall permeability of *Rhodococcus erythropolis* (an efficient hydrocarbon degrader) clearly indicated the potential of using environmental conditions as an effective way to modulate cellular membrane permeability to small molecules (Sololovska et al., 2003). Drastic changes of cellular permeability to hydrophobic and hydrophilic antibiotics were found when cells were cultivated using pristine (a branched alkane) *vs* acetate. Cell wall becomes more permeable to some hydrophobic molecules upon a shift from a hydrophilic carbon source to a hydrophobic carbon source. This is accompanied by a shift in mycolic acid profile, an important cell wall component found in cell walls of the *Mycolata* family of actinomycetes, as discussed earlier.

One study examining protein translocation to medium was carried out (Shokri et al., 2002), which shows that number of membrane changes occurred in cell grown at different growth rates. Significant changes in compositions of fatty acid and phospholipid led to a low fluidity of membrane at low growth rate, consequently a more rigid membrane that resists common cell lysis method such as sonication. In contrast, at a growth rate of 0.3 h^{-1}, the combination of high phosphatidylglycerol and low level of cyclic fatty acids and saturated fatty acids led to an optimal condition for protein transport across the outer membrane.

Cell surface display, another method, can be used to circumvent the permeation barrier of cellular membranes. In this case, displaying enzymes on the surface of microbial cells eliminates the need for the substrate to penetrate the cells (Georgiou et al., 1997; Shimazu et al., 2001). A similar method by Sroga & Dordick (2002) is to direct the heterologous enzyme to periplasmic space so that the

permeability barrier by cytoplasmic membrane can be avoided. The limitation of cell surface display or periplasmic expression is that they apply to those processes involving only one or two enzymes. In the periplasmic expression, only cytoplasmic membrane impermeability is circumvented. In addition, these methods would not be suitable for applications, involving cofactor regeneration and requiring the coupling of a significant portion of intracellular metabolic functions.

In summary, various permeabilization methods are being applied to use whole-cells as biocatalysts, however enormous difficulties are being faced to implement them in large scale. The permeabilization issues has been discussed in details in this chapter since the thesis includes various permeabilization studies, in case of bacterial source, *Erwinia aroideae* and a yeast source, *Rhodotorula aurantiaca,* to improve cell-bound PVA activity. These permeabilization studies are published in international journals. However application of these studies for scale up of the process will involve further optimization of procedure.

1.10. Conclusions and future scope of penicillin V acylase

PVA contributes low share (10-15%) in 6-APA production (Vandamme, 1988; Arroyo et al., 2003) between PGA and PVA. This is becouse the fermentation of Pen G has been a core activity in the penicillin industry, the combined efforts on PGA, and easier manipulation of the *E. coli* genes. About 85% of Pen G produced is transformed into semisynthetic penicillins and cephalosporins *via* 6-APA and 7-ADCA (Rolinson, 1988). Due to specific nature of PVA such as stability of Pen V in aqueous solution, availability of PVA preparations active between pH 6.0-7.0, ability of PVA to tolerate higher concentrations of Pen V, encourage the use of Pen V as a bulk raw material for the production of 6-APA and 7-ADCA. Even after realizing the advantages of using PVA over PGA, the major share of Pen G produced is diverted for the production of 6-APA and 7-ADCA, the use of the Pen V/PVA combination has remained low. It needs exhaustive exploration of new Pen V-producing microorganisms and effective PVA preparations to switch over from Pen G to Pen V in the pharmaceutical industries.

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Screening of Penicillin V Acylase producing microorganisms

Chapter 2

Abstract

Several isolates of bacteria, yeast and fungi from soil, air and some standard type cultures were screened for penicillin V acylase (PVA) activity in the sequence of three stages. The first stage comprised screening by *Serratia marcescens* overlay technique, the second stage comprised qualitative assay of 6-APA, produced on hydrolysis of penicillin V by aliquots of whole-cells using PDAB, and the third stage comprised the quantitative spectrophotometeric assay of 6-APA produced from the hydrolysis of penicillin V by whole-cells. Three organisms, *Bacillus* sp. (an isolate), *Erwinia aroideae* (DSMZ 30186) and yeast, *Rhodotorula aurantiaca* (NCIM 3425), were found to exhibit relatively high levels of whole-cells PVA activity. The cells of bacterial isolate, *Erwinia aroideae* (DSMZ 30186) and yeast, *Rhodotorula aurantiaca* (NCIM 3425), found to produce PVA upto 11, 16 and 20 IU/gDW, respectively. This was the first time that *R. aurantiaca,* a yeast has been reported for the production of intracellular penicillin V acylase.

Erwinia aroideae (DSMZ 30186), a bacterial culture and yeast *R. aurantiaca* (NCIM 3425) were well characterized standard type cultures however the isolated *Bacillus* sp. was an uncharacterized good source of intracellular PVA. The characterization of this isolated culture was carried out by polyphasic analysis, which comprised fatty acid methyl ester (FAME) analysis and 16S r DNA sequencing. As a consequence of polyphasic analysis, the isolated culture was identified as *Bacillus cereus* (ATUAVP 1846). This is the first time that *Bacillus cereus* was identified as PVA source.

2.1. Introduction

As described in ***chapter one,*** several bacteria, yeasts and fungi have been shown to produce PVA activity intracellularly as well as extracellularly, wide variations being reported among the strains of the various species. A preliminary survey was made in the present studies to identify wild-strains producing high levels of enzyme activity.

Microorganisms are vital source of penicillin acylase (EC 3.5.1.11), which hydrolyzes penicillins to 6-aminopenicillanic acid (6-APA), the starting material for the manufacture of semi-synthetic antibiotics. Consequently, many methods have been developed to screen for microorganisms that produce this enzyme. They include the use of benzylpenicillin as the sole source of carbon in the screening medium in which 6-APA formed is measured by bioassay after treatment with phenylacetyl chloride (Kameda et al., 1961). Walton (1964) has modified this method by incorporating colorless phenylacetyl-p-nitroaniline into the screening medium. Upon hydrolysis, the substrate gives yellow p-nitroaniline, the appearance of which is the basis for selecting acylase producers. However, this method is not very sensitive, owing to the lower solubility of the substrate. A comparable screening method was suggested by Baker (1980), using ampicillin as the substrate for enzyme hydrolysis; his method is based on the observation that ampicillin, but not 6-APA, gives a characteristic green color with the biuret reagent. This test, however, is not very practical either since the color fades rapidly. Szewczuk et al. (1980) have suggested a method which uses paper disks saturated with phenylacetyl-4-aminobenzoic acid as a substrate for such a screen. After contact with bacterial colonies, the paper disk is treated with specific reagents; red spots develop only where the paper disk has previously contacted colonies of penicillin acylase-producing bacteria. The intensity of the red spots, measured densitometrically, is reported to correlate quantitatively with the acylase activity in the bacterial colonies. The disadvantage of this method is that it involves several steps and that some solutions for color development have to be freshly prepared. Moreover, it generally works well with bacteria capable of producing only cell-bound but not extracellular acylases.

Meevootisom et al. (1983) 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) and an acidimetric method for the detection of 1-lactamase (Escamilla, 1976). Both methods are simple and require no special reagents or equipment. The microbiological method was quite sensitive and specific, and the acidimetric method was rapid and sensitive but nonspecific. Consequently, the microbiological method was 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.

2.1.1. Identification methodologies

Various methods have been used to identify isolated microorganisms. The isolated microorganisms were identified by their morphological characteristics and biochemical tests, some decades before; however polyphasic analysis has been recently introduced in the identification of microorganisms.

The three major techniques used for identification of pharmaceutically important bacteria are biochemical tests, fatty acid profiling, and DNA sequencing. Each technique has its strong points and weaknesses. Identification of isolated culture is being done by ***polyphasic analysis***, now days. The technique comprised fatty acid methyl esters (FAME) analysis and ribosomal DNA sequencing.

Fatty acid methyl esters (FAME) analysis is a common technique used for identification of microorganisms now days. For more than 15 years, a considerable part of the pharmaceutical industries have been relying on the MIDI Sherlock microbial identification system for identification in their microbiological testing laboratories. The FAME analysis identifies microorganisms based on gas chromatographic (GC) analysis of extracted microbial fatty acid methyl esters (FAMEs). Microbial fatty acid profiles are unique from one species to another, and this has resulted in creation of very large microbial libraries. The current Sherlock System libraries have over 1,500 bacterial species, along with 200 species of yeast. A combination of features makes the system attractive for use in

pharmaceutical quality control (QC) environments. These features include, but are not limited to: accurate identifications, large environmental libraries, the ability to perform presumptive “strain tracking”, high throughput, and a low cost for consumables.

The FAME analysis identifies all of the aerobic bacteria in its library using a standard sample preparation technique, so there is no need for biochemical tests or a Gram-staining. Isolated bacteria are grown on commonly used medium at 28°C for 24 hours and are harvested in their log phase of growth for FAME analysis. Some of the species that are discriminated well using FAME analysis include those of *Bacillus*, *Pseudomonas*, Gram-positive cocci and rods (such as coryneforms), Gram-negative non-fermenters (such as *Acinetobacter*), and unusual environmental organisms found in pharmaceutical facilities.

Biochemical test-based identification systems are familiar to most microbiologists and require little training to operate. Systems range from strip cards for specific groups of bacteria to large plate arrays that may be automatically scanned for changes due to pH shifts or redox reactions. The strength of identification in enterics is generally quite good and the ease of use and cost for identification is considerably less than for DNA sequencing, but higher than for FAME analysis (Cook 2003; O’Hara 2005). The use of these methods depends on choice of the correct “card” or “strip” of wells of reagents. This is typically done using information such as that gained from the Gram stain (a prerequisite step not involved in the other two major technologies). One problem with most biochemical test method is that, these methods are geared to the clinical market, and as a result, are limited in the number of environmental species they can identify.

DNA-based technology for the identification of bacteria typically uses only the 16S r DNA gene as the basis for identification. This technique has the advantage of being able to identify difficult-to-cultivate strains, and is growth and operator independent. As the 16S r DNA gene is highly conserved at the species level, speciation is commonly quite good, but as a result, subspecies and strain level differences are not shown. Problems with the 16S r DNA technology are that it requires a high level of technical proficiency as well as involve high equipment cost. As a result, the technology is not well suited for routine microbial quality

control. Technology that uses information from both the 16S r DNA and 23S r DNA genes is also used in pharmaceutical quality control, but primarily to help in strain tracking.

Isolates and standard type cultures of bacteria, yeast and fungi were screened preliminary for penicillin V acylase production before attempting the purification of enzyme and characterization of its kinetic and molecular properties. In the present chapter screening of penicillin V acylase producing microorganisms is discussed and further identification of isolated culture is described.

2.2. Material and Methods

2.2.1. Materials

Beef extract, yeast extract, tryptone and peptone were from Hi-media, India; p-dimethylaminobenzaldehyde (PDAB) and methanol were obtained from Qualigens, India.

Penicillin V (phenoxymethyl penicillin), 6-APA and corn steep liquor were gift from Hindustan Antibiotics Ltd. Pune, India. All other chemicals and reagents were commercially available high purity or analytical grade compounds. All media were prepared in distilled water and all the buffers were prepared in double-distilled water.

2.2.2. *Microorganisms*

Serratia marcescens ATCC 27117 and all other standard strains of type cultures of microorganisms were obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. *Erwinia aroideae* was obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*, a German Collection of Microorganisms and Cell Cultures, Germany.

2.2.3. Methods

2.2.3.1. Microbial isolates

Microorganisms were isolated from soil, air and drainages. Soil samples were obtained from both the open ground around the laboratory as well as the soil from the vicinity of Hindustan Antibiotics, Pune, India. The microbial contaminants in air were obtained by exposing petri plates with various nutrient media to the laboratory atmosphere.

2.2.3.2. Culture media for isolation of microorganisms

Bacterial cultures were isolated using following media:

Medium A: A complex medium used for penicillin G acylase production from *E. coli* (Prabhune & SivaRaman, 1990): (g/L) yeast extract 2, beef extract 3, peptone 2, tryptone 1, corn steep liquor (CSL) (50 % w/v) 12.5 ml; K_2HPO_4 3, KH_2PO_4 0.3, NaCl 3.5, NH_4SO_4 1 and $MgSO_4.7H_2O$ 0.2, pH adjusted to 7.5.

*Medium B***:** Nutrient broth containing (g/L) peptone 5, beef extract 3 and NaCl 5, pH adjusted to 7.0.

Actinomycetes were isolated using *Medium C*: (g/L) soluble starch 40, yeast extract 25, NaCl 20, $FeSO_4$ 0.25, KH_2PO_4 3.0, $CaCO_3$ 5, casein hydrolysate 0.4 % (w/v); pH adjusted to 6.8

Yeast cultures were isolated using *Medium D*: (g/L) glucose 20, malt extract 3, yeast extract 3 and peptone 5; pH was adjusted to 6.5. Tetracycline (500 µg/L) was added just before use to suppress bacterial growth.

Fungal isolates were obtained using potato infusion *Medium E*: (g/L) glucose 20 and potatoes (infusion from 20 g potatoes) pH adjusted to 4.5. Tetracycline (500 µg/L) was added just before use.

All the culture media contained 2.0% agar when solid media in petri pates or slants were used.

For isolation from soil samples, about 1 g soil was suspended in sterile, distilled water (10ml) and thoroughly mixed and left to settle. A loopful of the supernatant was streaked on solid medium in petri plates. The petri plates were incubated at 30^{o}C for 24-48 h. Isolated colonies of microorganisms obtained in the petri plates were sub-cultured on slants and these isolates were then screened individually for penicillin acylase production.

In addition to the isolates, both randomly selected standard cultures of microorganisms and a few known penicillin V acylase producers such as *Erwinia aroideae* and few selected strains of *Rhodotorula* were screened for enzyme production.

2.2.3.3. Screening procedure

Screening was carried out in three successive stages:

Stage one:

The preliminary screening of samples was done using the *S. marcescens* overlay technique according to the modification of the plate assay method of Oonstendrop (1972) as described by Meevotism et al. (1983). This was used as a primary screen for the isolate obtained from the various sources.

In a typical procedure, the isolate was point inoculated at the centre or within a sector of petri plate (9cm dia) containing the basal solid (2% w/v, agar) growth medium. After overnight incubation at 30^{o}C, the plate was overlaid with 5 ml of soft nutrient agar containing 10 mg/ml Pen V (w/v) and 2 ml of 24 h culture of *S. marcescens* ATCC 27117, grown in nutrient broth. After the overlaid hardened, the plate was incubated at 28^{o}C for 24 h. The diameter of the zone of clearance on the red lawn of *S. marcescens* was used as an approximate measure of PVA activity.

Stage two:

Cultures that had shown significant zone of clearance were grown in liquid media of the composition described earlier for cultivation of bacteria, yeast and fungi. A loopful of cells was inoculated in the sterile medium and was incubated for 24 h at 30^{o}C on a rotatory shaker at 150 rpm. A drop of toluene was added to the culture 4 h before harvesting the cells to arrest β-lactamase activity (Nara et al., 1971). The cells were harvested by centrifugation. Both pelleted cells and an aliquot of cell free medium was assayed for penicillin V acylase activity.

Enzyme assay of penicillin V acylase activity

Cells were resuspended in 0.5 ml of citrate buffer pH 5.8 containing penicillin V (20 mg/ml, w/v). The reaction mixture was incubated for 1 h at 40^{o}C. An aliquot was added to 1 ml citrate phosphate buffer pH 2.5 and equal volume of the colour reagent, p-dimethylaminobenzaldehyde (PDAB) (Bomstein & Evens, 1965) was added to it. The colour reagent, PDAB, was prepared by adding 1 g PDAB in 170 ml of methanol containing 0.1 % hydroquinone.

Stage three:

Depending on the isolate, bacteria, yeast or fungus, the culture medium was selected, the inoculum was developed and transferred in 250 ml Erlenmeyer flask containing same medium and incubated for 24-48 h at 30^{o}C at rotatory shaker at 180 rpm.

The cells (4.0 ml culture broth) were harvested by centrifugation at 6000 rpm for 5 min at 4 ^{o}C; washed twice with 0.01 M potassium phosphate buffer pH 6.5. The packed cells were resuspended in 0.5 ml of Pen V solution (20 mg/ml, w/v) in 0.1 M sodium citrate buffer pH 5.8 and incubated for 30 min at 40^{o}C. 0.5 ml of citrate-phosphate buffer pH 2.5 was added to the reaction mixture after incubation, to quench the hydrolysis reaction. The clear supernatant (0.25 ml) obtained by centrifugation on a microfuge was made up to final volume of 1 ml with citrate-phosphate buffer pH 2.5 to which 1 ml of PDAB reagent was added. 6-APA formed was estimated by measuring the absorbance at 415 nm of the yellow-coloured complex formed.

2.3. Results and Discussion

2.3.1. Screening of PVA producing microorganisms

The results of screening for penicillin V acylase producing microorganisms are summarized under three stages as described in ***Materials and Methods.***

Stage one:

Several microorganisms were screened for intracellular as well as extracellular activity of penicillin V acylase and some are presented here. Only three cultures, two standard cultures and one isolate, showed significant activity in

stage I of screening. None of the rendomly selected actinomycetes showed significant PVA activity. Zone of clearance produced by the bacterial isolate ATUAVP 1846 against red lawn of *S. marcescens* indicated that test microorganism is a potential PVA producer as seen in ***Fig. 2.1.***

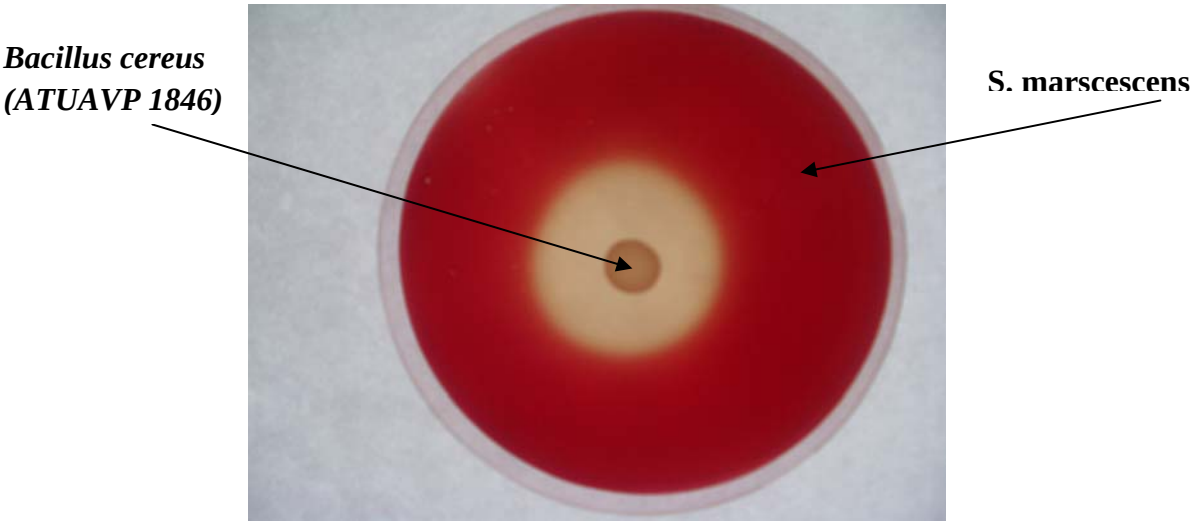

Figure 2.1: Plate assay: Zone of clearance around the colony of test organism against the red lawn of Serratia marcescens indicated positive penicillin acylase producer.

Stage Two:

The second satge activity screening of isolates and standard type cultures is depicted in ***Table 2.1***. In all cases the cell-bound PVA activity is presented in the table however cell-free medium did not contain any PVA activity. The qualitative results are presented as visual gradation from 0 to ++++ (***Table 2.1***). *Bacillus* sp. (NCIM 2451) and other *Bacillus* sp, an isolate from soil, were negative in PVA activity however another isolate a *Bacillus* sp., again isolated from soil, was found to have fairly good PVA activity. *Rhodotorula glutinis* (NCIM 3168) and (NCIM 3169) had shown negligible PVA activity however *Rhodotorula aurantiaca* (NCIM 2435) was the best source of PVA activity, amongst all the screened cultures.

Table 2.1: List of microorganisms screened for enzyme production

Microorganism	Source	Penicillin V acylase activity
Bacillus sp.	NCIM 2451	-
Bacillus sp.	Soil	-
Bacillus sp. (isolate)	Soil	+++
Erwinia aorideae	DSMZ 30186	++++
Arthrobacter viscossus 005	NCIM 2451	-
Rhodotorula glutinis	NCIM 3168	-
Rhodotorula glutinis	NCIM 3169	-
Rhodotorula aurantiaca	NCIM 3425	++++
Actinomycetes sp.	Soli	-
Aspergillus sp.	Air	-
Fusarium sp.	Soil	-
Penicillium	Soil	-

Stage Three:

The cultures which showed significant activity were finally taken to stage three screening and the results presented in ***Table 2.2.***

The following microbial cultures exhibited PVA activity:

1. Bacterial isolate
2. Bacterial culture *Erwinia aorideae* (DSMZ 30186) and
3. Yeast culture *Rhodotorula aurantiaca* (NCIM 3425)

Isolated *Bacillus, E. aroideae* and yeast, *R. aurantiaca* were found to produce intracellular penicillin V acylase up to 10, 16 and 20 IU/gDW. *E. aroideae* exhibited rather good PVA activity up to 16 IU/g DW however the cell-bound PVA activity was maximum by yeast culture, *Rhodotorula aurantiaca* which produced upto 20 IU/g DW.

E. aroiedeae was a standard type culture from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, a German Collection of Microorganisms and Cell Cultures and the yeast culture, *R. aurantica* was from NCIM, NCL, Pune. Both the cultures were well characterized standard type cultures however the isolated *Bacillus* sp. was a new source of PVA and was uncharacterized; therefore the identification of isolated culture was carried out by polyphasic analysis.

Table 2.2: Penicillin V acylase activity of whole-cells by spectrophotometric assy of 6-APA peoduced

Microorganism	Penicillin V acylase activity (IU/g DW)
Isolate	10.6
E. aroideae	16.2
R. aurantiaca	20.6

2.3.2. Identification of isolated culture

2.3.2.1. Polyphasic analysis

The identification of isolated culture was carried out by polyphasic analysis from ***Disha Institute of Biotechnology Pvt. Ltd, Nagpur and CellBioasis, Pune, India.***

Preliminary identification was done by morphological characters of the isolate and Gram-staining. The isolated culture was found to be a *Bacillus* sp., a Gram-positive strain. Further identification of culture was done by polyphasic analysis, which comprised fatty acid methyl ester (FAME) analysis and 16S r DNA sequencing to identify bacterial strain.

2.3.2.1.1. Fatty acid methyl ester (FAME) analysis

The results obtained from FAME analysis are depicted in the ***Table 2.3***. The value obtained for FAME similarity index (SI) was 0.32 which matched the best with the entry namely *Bacillus cereus*, indicating isolated culture belongs to genus *Bacillus* and species *cereus*.

Table 2.3: FAME matches

LIBRARY	Similarity Index (SI)	ENTRY NAME
RTSBA6 6.00	0.32	*Bacillus cereus*
	0.212	*Bacillus cereus*

2.3.2.1.2. 16S r DNA sequencing

16S r DNA sequence of 763 nucleotides from isolated culture is depicted in ***Figure 2.2***. The sequence was used for BLAST search of nucleotide sequence match with that available for microorganisms in the NCBI gene data bank. The sequence has shown close similarity with *Bacillus anthrasis* and *Bacillus thuringiensis,* however *Bacillus cereus* was the closest microorganism of the 16S

r DNA sequence (***Table 2.4.***) Thus, 16S r DNA sequence of isolated microorganism indicated the organism as *Bacillus cereus* (ATUAVP 1846).

Figure 2.2: 16S r DNA sequence of Bacillus cereus (ATUAVP 1846)

TTTTTAGACTTTTCGCTCGTGTCGTTACGACCAGAAAGTCGCCTTCGCCA
CTGGTGTTCCTCCATATCTCTACGCATTTCACCGCTACACATGGAATTCC
ACTTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGG
TTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTT
TACGCCCAATAATTCCGGATAACGCTTGCCACCTACGTATTACCGCGGCT
GCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCC
AGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACAGAGTTTTACGACC
CGAAAGCCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCAT
TGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC
AGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCT
TGGTGAGCCGTTACCTCACCAACTAGCTAATGCGACGCGGGTCCATCCAT
AAGTGACAGCCGAAGCCGCCTTTCAATTTCGAACCATGCAGTTCAAAATG
TTATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTATGGGCA
GGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACTTCATAAGAGCAAG
CTCTTAATCCATTCGCTCGACTTGCATGTATTAGGCACGCCGCCAGCGTT
CATCCTGAGCCAGATCACAACTCTAAAANNNNNNNNNNNNNNNNNNNNN
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNN

Table 2.4: 16S r DNA match report

Match	% Difference	Length	Library Entry Name
1	0.63	763	*Bacillus cereus*
2	0.78	763	*Bacillus anthrasis*
3	0.78	763	*Bacillus thuringiensis*

The results from both the analyses are compared in ***Table 2.5***, to finally conclude the identification of the isolate, which showed % difference of 0.63 in case of *B. cereus* however in case of *Bacillus anthrasis and Bacillus thuringiensis* the % difference was 0.78. FAME analysis similarity index has shown 0.32 value in case of *B. cereus* however it showed value of 0 in case of *Bacillus anthrasis and Bacillus thuringiensis*. Therefore, according to polyphasic analysis the isolate was identified as *Bacillus cereus* (ATUAVP 1846).

Table 2.5: Cross-library report

% Difference	Genus	Species	FAME SI
0.63	*Bacillus*	*cereus*	0.32
0.78	*Bacillus*	*anthrasis*	0
0.78	*Bacillus*	*thuringiensis*	0

2.4. Conclusions

Various microorganisms were screened for PVA production, extracellular as well as intracellular; as a result of which *E. aorideae* (DSMZ 30186), *R. aurantiaca* (NCIM 3425) and a bacterial isolate were found to be the good producer of intracellular PVA. *E. aroideae* was found to be a good producer of PVA; further studies of bacterial PVA from the identified source revealed the potential of enzyme as described in ***Chapter 3 A and B***. *R. aurantiaca* was identified as a yeast source of PVA for the first time. Further studies on eukaryotic PVA from the newly identified source *R. aurantiaca* revealed the details of enzyme as described in ***Chapter 4, 5 and 6***.

The newly isolated culture was the novel source of PVA. Identification of isolated culture was done by polyphasic analysis and the culture identified as *Bacillus cereus* (ATUAVP 1846). This was the first report of *Bacillus cereus* producing PVA. The culture can be used further for production of enzyme in large quantities; purification and characterization of enzyme might give important features of PVA and can be further used in pharmaceutical industries.

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Erwinia aroideae Penicillin V Acylase

Chapter 3

Production of Penicillin V Acylase by Erwinia aroideae

Chapter 3 A

*Part of the work presented in this chapter has been **published:***
*Atul Kumar, Vivek Lonkar & Archana V. Pundle. **(2008)** Research Journal of Biotechnology 3(2): 26-30.*

Abstract

Production of penicillin V acylase from *Erwinia aroideae* (DSMZ 30186) was standardized by media manipulation and optimizing the fermentation parameters. *E. aroideae* produced high levels of intracellular penicillin V acylase. Amongst various carbon sources used (2.0%); fructose, galactose, sucrose and mannitol increased enzyme production up to 579, 544, 534 and 504 IU/g dry weight (DW), respectively, compared to minimal medium (263 IU/g DW); however maximum PVA productivity (2796 IU/L) was achieved using fructose. Ammonium ions escalated the enzyme production to 1266.9 IU/g DW; however sodium glutamate was the best nitrogen source for overall productivity of enzyme (2045 IU/L). Various concentrations of cornsteep liquor and skim milk were used as supplements; 1.0 % cornsteep liquor and 3.0 % skim milk was optimum for the production of PVA up to 823 and 93 IU/g DW, respectively. Optimum cultural conditions for the production of PVA from *E. aroideae* were standardized. *Erwinia aroideae* produced 1543 IU/g DW penicillin V acylase in Erlenmeyer flasks (250 ml) containing 50 ml of minimal medium with 0.3% ammonium sulphate, pH 7.0 at 28 ^{o}C and 180 rpm when incubated for 56 h.

3.1. Introduction

Penicillin acylases (penicillin amidohydrolase, EC 3.5.1.11) are a group of enzymes that cleave the acyl chain of penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acid (Shewale & Sudhakaran, 1997). Penicillin V acylase (PVA) catalyzes the hydrolysis of penicillin V to yield 6-aminopenicillanic acid (6-APA) and phenoxyacetic acid. In fact, PVA contributes 12.5% of the total 6-APA produced enzymatically worldwide (Vandamme, 1988). The *in vivo* role of penicillin acylases has not yet been elucidated, but it has been suggested that these enzymes may function during the free-living mode of the organisms to metabolize aromatic compounds in order to generate a carbon source (Valle et al., 1991). Screening of penicillin acylase-producing microorganisms has shown that bacteria, actinomycetes, yeasts and fungi produce these enzymes (Sudhakaran & Borkar, 1985a & 1985b). PVA is produced intracellularly by *Bacillus sphaericus*, *Erwinia aroideae, Bacillus subtilis, Pseudomonas* sp*., Beijerinckia indica, Penicillium* sp*. Rhodotorula glutinis,* and extracellularly by *Pleurotus ostreatus, Fusarium* sp*., Streptomyces* sp*.* and *Streptoverticillium* sp. Phenoxyacetic acid induces production of enzyme in *Aspergillus, Fusarium, Penicillium* sp and *R. glutinis*. However the enzyme production is constitutive in case of *B. sphaericus, E. aroideae*, *P. ostreatus* and *Streptomyces* sp (Shewale & SivaRaman, 1989).

In the present chapter, effect of carbon and nitrogen sources as well as various media supplements on the production of intracellular penicillin V acylase from *Erwinia aroideae* (DSMZ 30186) is described. Also, optimal cultural conditions for the production of enzyme from this bacterial culture, is presented.

3.1.2. Material and Methods

3.1.2.1. Materials

Chemicals used as carbon and nitrogen sources were obtained from Himedia, India. Penicillin V potassium salt and cornsteep liquor were the kind gifts from Hindustan Antibiotics Ltd., Pune, India. p-dimethylaminobenzaldehyde (PDAB) was obtained from Merk, India and other reagents used were of analytical grade.

3.1.2.2 Microorganism

Erwinia aroideae (DSMZ 30186) was maintained on Luria Bertani medium slant. 10 ml sterile medium was inoculated with fresh agar slant culture and incubated for 24 hr at 28 ^{o}C and 180 rpm. This seed culture was used for the fermentation studies.

3.1.2.3. Cultural medium

Batch fermentation was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of medium inoculated with 10 % (v/v) of seed culture and incubated at 28 ^{o}C for 24 h at 180 rpm. The medium (minimal medium) used for enzyme production contained (g/L): Na_2HPO_4, 12.8; KH_2PO_4, 3.1; NaCl, 1.0; $MgSO_4.7H_2O$, 0.2; glucose, 4.0 and $NaNO_3$, 3.0, and the pH was adjusted to 7.0. Minimal medium was used as a basal medium for all the fermentation studies.

3.1.2.4. Optimization of cultural conditions for maximum PVA production

3.1.2.4.1. Effect of carbon source

Glucose from the minimal medium was replaced by various carbon sources (2.0%, w/v) to study the effect on PVA production from *Erwinia aroideae*. Erlenmeyer flasks (250 ml) containing 50 ml of above-mentioned medium inoculated with 10 % (v/v) of seed culture and incubated at 28 ^{o}C for 24 h at 180 rpm. The cells were harvested from the culture broth by centrifugation at 10,000 rpm for 10 min and used to determine cell-bound PVA activity as well as cell biomass.

3.1.2.4.2. Effect of nitrogen source

To determine the best nitrogen source for PVA production, the culture was grown in the minimal medium (pH 7.0). $NaNO_3$ of minimal medium was replaced by various nitrogen sources (0.3 %, w/v) while studying their effect on PVA production by *E. aroideae*. Erlenmeyer flasks (250 ml) containing 50 ml of minimal medium inoculated with 10 % (v/v) seed culture and incubated at 28 ^{o}C for 24 h at 180 rpm. The cells were harvested from the culture broth by centrifugation at

10,000 rpm for 10 min and used to determine cell-bound PVA activity as well as cell biomass.

In individual experiments, different concentrations (0.5-8.0 %, w/v) of corn steep liquor, pH 7.0, were used to study the effect on PVA production. Cornsteep liquor (CSL; 50 % dry solids content) was a gift of Hindustan Antibiotics, Pune. CSL was pretreated by suspension of 50 g of liquor in 80 ml of water; the diluted solution was adjusted to pH 7.0 with NaOH, made up to 100 ml; steamed for one h, cooled and clarified by centrifugation (Pundle & SivaRaman, 1994). Plain skim milk (2-4 %, w/v) was used to study the effect on PVA production.

3.1.2.4.3. Effect of temperature

Erlenmeyer flasks (250 ml) containing 50 ml of medium, pH 7.0 were inoculated with 10 % (v/v) seed culture and incubated at 25, 28, 30 40 and 50 ^{o}C for 24 h on rotary shaker at 180 rpm. The culture broth was centrifuged at 10,000 rpm and for 10 min at 4 ^{o}C, assayed for enzyme activity and determination of cell biomass.

3.1.2.4.4. Effect of pH of medium

To standardize optimum pH of medium, the initial pH of each flask containing 50 ml of medium was adjusted to 4.0-9.0, prior to sterilization, for PVA production and pH of medium was checked before inoculation of the culture. The pH adjusted flasks inoculated with 10 % (v/v) seed culture after readjusting pH, if necessary, were incubated at 28 ^{o}C for 24 h at 180 rpm. The cells were harvested from the culture broth by centrifugation at 10,000 rpm for 10 min and used to determine cell-bound PVA activity as well as cell biomass.

3.1.2.4.5. Effect of incubation period

To standardize the optimum incubation period for the production of PVA, *Erwinia aroideae* culture was grown in Erlenmeyer flasks (250 ml) containing 50 ml of medium and incubated at 28 ^{o}C and 180 rpm; the aliquots were removed at various time intervals. Culture broth was centrifuged at 10,000 rpm for 10 min and cells were used to determine cell biomass and whole-cell PVA activity.

3.1.2.4.6. Effect of dispensing volume

Erlenmeyer flasks (250 ml), containing different volume (25-125 ml) of medium, were used to standardize effect of aeration for PVA production. 10 % (v/v) inoculum size was used to inoculate flasks containing various volumes of medium. The inoculated flasks were kept for incubation at 28 ^{o}C and 180 rpm, for 24 h. The cells were harvested from culture broth by centrifugation at 10,000 rpm for 10 min and used for cell-biomass calculation and determination of whole-cell PVA activity.

3.1.2.5. Enzyme assay

Cell bound penicillin V acylase activity was determined by the method of Bomstein & Evans (1965), as modified by Shewale et al. (1987), measuring the amount of 6-APA formed at 40 ^{o}C. Procedure is as follows:

Cells were resuspended in 0.5 ml of 0.1 M sodium phosphate buffer pH 6.0 containing Pen V (20 mg/ml, w/v). The reaction mixture was incubated for 20 min at 40^{o}C. After the incubation, the reaction was quenched by adding 0.5 ml citrate phosphate buffer pH 2.5 and then centrifuged to get clear supernatant. An aliquot from the supernatant of the reaction mixture was added to 1 ml citrate phosphate buffer pH 2.5 and equal volume of the colour reagent, p-dimethylaminobenzaldehyde (PDAB) was added to it. The reading was taken at 415 nm exactly after 2 min. The colour reagent, PDAB, was prepared by adding 1 g PDAB in 170 ml of methanol containing 0.1 % hydroquinone. One unit (IU) of PVA activity is defined as the amount of enzyme that produces 1 μmol 6-APA per minute under the conditions defined. The product-time relationship was linear up to the mentioned period of time.

Biomass concentration was determined from optical density measurements at 600 nm and converted to dry weight with a standard curve. The biomass reported here is the dry biomass of cells and cell bound enzyme activity reported here represents IU/g dry weight (IU/g DW). All the experiments were carried out in triplicate using the freshly grown culture and the values reported here in tables and figures represent their mean value.

3.1.3. Results and Discussion

3.1.3.1. Effect of carbon source on PVA production

Various carbon sources were used to study the effect on PVA production by *E. aroideae* and the results are depicted in ***Table 3.1.1***. Production of PVA was highly influenced by fructose and galactose producing 579 and 544 IU/g DW. Sucrose and mannitol produced 534 and 504 IU/g DW penicillin V acylase from *E. aroideae*. Amongst all the carbon sources used, the maximum productivity of enzyme was achieved in case of fructose (2796 IU/L of the culture).

3.1.3.2. Effect of nitrogen source on PVA production

Various nitrogen sources were used to study their effect on the production of PVA by *E. aroideae* and the results are depicted in ***Table 3.1.2.*** Ammonium chloride, sodium glutamate and ammonium nitrate have enhanced PVA production up to 920.4, 909.15 and 921.29 IU/g DW, respectively, compared to minimal medium (188.9 IU/g DW); however maximum production (1266.9 IU/g DW) of PVA was achieved from minimal medium containing ammonium sulphate (0.3%). For the first time we are reporting 1266.9 IU/g DW production of PVA by *E. aroideae.* However among all the nitrogen sources used; maximum productivity of enzyme was achieved by using sodium glutamate up to 2045 IU/L of the culture. Torres et al. (1999) have reported maximum PVA production, 178 IU/ L of culture, by *S. lavendulae***.**

Table 3.1.1: Effect of carbon sources on the production of penicillin V acylase by Erwinia aroideae (DSMZ 30186)

Serial No.	Carbon source (2.0%)	PVA activity (IU/g DW)	Cell weight (g DW/L)	Productivity (IU/L)
1	None	263.8	2.85	751
2	Glucose	267.38	5.15	1382
3	Mannitol	504.89	5.16	2608
4	Sorbitol	318.84	3.83	122
5	Glycerol	490.97	5.2	2553
6	Fructose	579.31	4.83	2796
7	Galactose	544.38	4.91	2676
8	Maltose	124.84	1.48	185
9	Sucrose	534.07	5	2670
10	Lactose	475.01	5.25	2493
11	D-Mannose	473.86	5.83	2764
12	Inulin	84.4	1.66	140

Table 3.1.2: Effect of nitrogen sources on production of penicillin V acylase by E. aroideae (DSMZ 30186)

Serial No.	Nitrogen Source (0.3%)	PVA activity (IU/g DW)	Cell weight (g DW/L)	Productivity (IU/ L)
1	None	188.9	2.3	434
2	Sodium glutamate	909.15	2.2	2045
3	Urea	436.05	1.9	835
4	Yeast Extract	140.74	2.3	328
5	Tryptone	559	2.5	1397
6	Soyabean Casein Digest	337.14	2.2	764
7	Peptone	269.26	2.2	614
8	$NH_4H_2PO_4$	474.74	1.8	870
9	NH_4NO_3	921.29	16	1474
10	$(NH_4)_2SO_4$	1266.9	1.4	1417
11	NH_4Cl	920.4	1.6	1549
12	KNO_3	840.9	1.75	1471

Various concentrations (0.5 - 8.0%) of corn steep liquor (CSL) were used to study the effect of CSL on the production of PVA and results are depicted in ***Table 3.1.3***. PVA production was enhanced up to 823.04 IU/g DW at 1.0 % CSL concentration. *B. sphaericus* PVA production was enhanced up to 100 IU/g DW reported by Pundle & SivaRaman (1994). The cell mass of *E. aroideae* was increased at higher concentration of CSL, but the PVA production was declined; the similar results were obtained in case of *B. sphaericus* penicillin V acylase. (Pundle & SivaRaman, 1994).

Various concentrations (2-4 %, w/v) of skim milk were used to study the effect on production of PVA (***Table 3.1.3***). 3.0 % skim milk was the optimum concentration for PVA production (93.3 IU/g DW). Further increase in skim milk concentration led to increase in cell mass of the culture; however decreased enzyme production. Torres et al. (1999) have reported increased PVA production of *S. lavendulae* by using skim milk at 3.0 % concentration; the present data is in concord with the reported one.

3.1.3.3. Effect of initial pH of media

The effect of initial pH of medium, on the production of penicillin V acylase is depicted in ***Fig. 3.1.1***. The study showed that the initial pH of medium had a pronounced effect on the enzyme production. Maximum PVA activity (1125 IU/g DW) was observed at pH 7.0; at pH lower than 7.0, the PVA production dropped drastically. Penicillin V acylase from *B. sphaericus* was optimum at pH 7.5 (Pundle & SivaRaman, 1994) where as production of PVA by *S. lavendulae* was optimum at pH 6.8 (Torres et al., 1999).

Table 3.1.3: Effect of corn steep liquor and skim milk on the production of PVA by Erwinia aroideae (DSMZ 30186)

Serial No.	Concentration (%)	PVA activity (IU/g DW)	Cells weight (g DW/L)	Productivity (IU/L)
Corn steep liquor				
1	0.5	391.35	4.11	1607
2	1	823.04	2.1	1728
3	2	468.65	3.66	1712
4	4	225.18	6.66	1498.5
5	6	60.84	13	790.4
6	8	30.29	17.33	523.3
Skim milk				
7	2	30.11	1.81	54.4
8	3	93.30	1.88	175.4
9	4	25.41	3.75	95.25

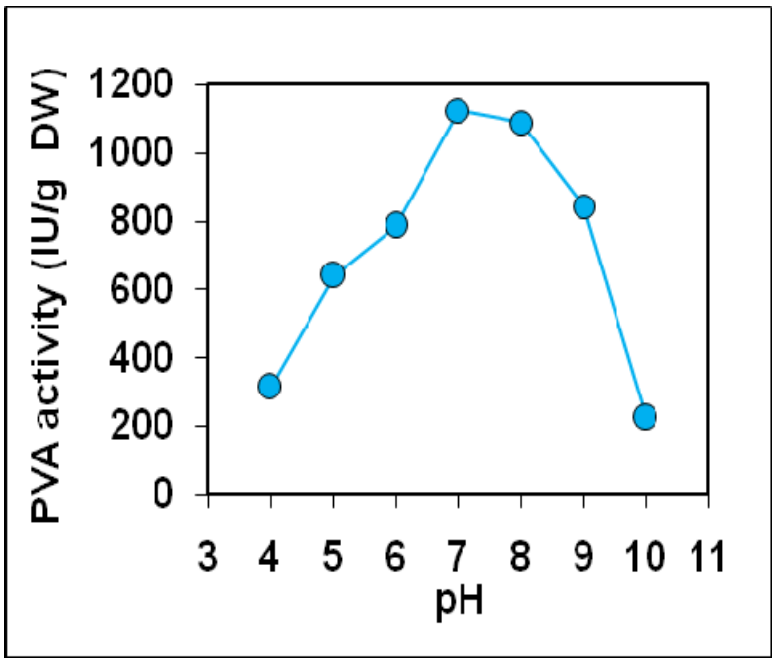

Figure 3.1.1: Effect of initial pH of medium on the production of PVA by Erwinia aroideae (DSMZ 30186)

3.1.3.4. Effect of incubation period

The growth pattern corresponding to incubation period of *Erwinia aroideae*, in production of PVA is depicted in ***Fig. 3.1.2***. The growth of *E. aroideae* increased up to 30 h and then it became constant till the period of 72 h. Production of PVA (1543 IU/g DW) constantly increased upto 56 h and drastically decreased on further incubation. Optimum incubation period for the production of PVA from *B. sphaericus* was 20 h (100 IU/g DW) (Pundle & SivaRaman, 1994). *S. lavendulae* has been reported to produce PVA optimally, at incubation period of 278 h (178 IU/l of culture) (Torres et al., 1999).

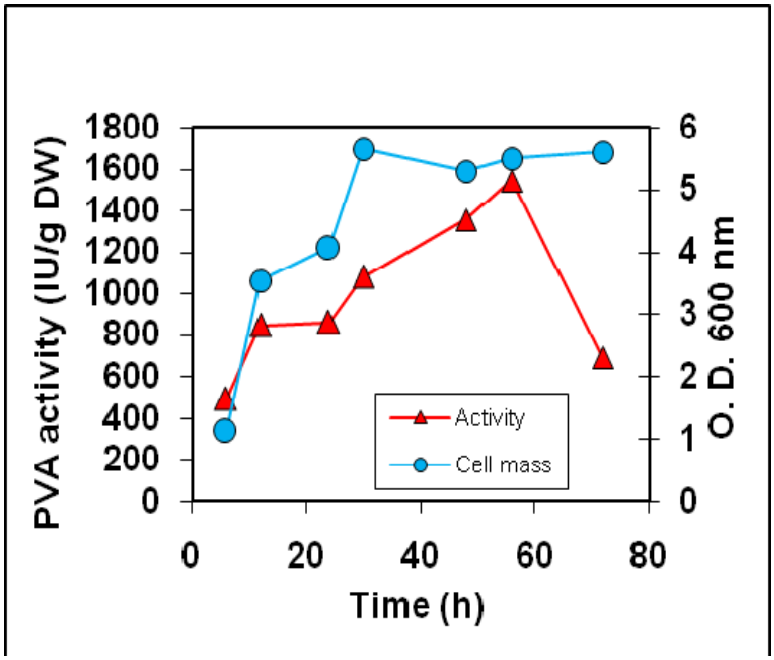

Figure 3.1.2: Effect of incubation period on the production of PVA by Erwinia aroideae (DSMZ 30186)

3.1.3.5. Effect of temperature

Effect of temperature on production of PVA from *E. aroideae* is depicted in ***Fig. 3.1.3***. Production of PVA was very much influenced by temperature. The optimum temperature for PVA production was 28 ^{o}C (1037 IU/g DW); however the production drastically decreased with increase in temperature of incubation. The optimum temperature for the production of PVA by *E. aroideae* is similar to that of *S. lavendulae* PVA (28 ^{o}C) (Torres et al., 1999); where as *B. sphaericus* produce PVA optimally, at 25 ^{o}C (Pundle & SivaRaman, 1994).

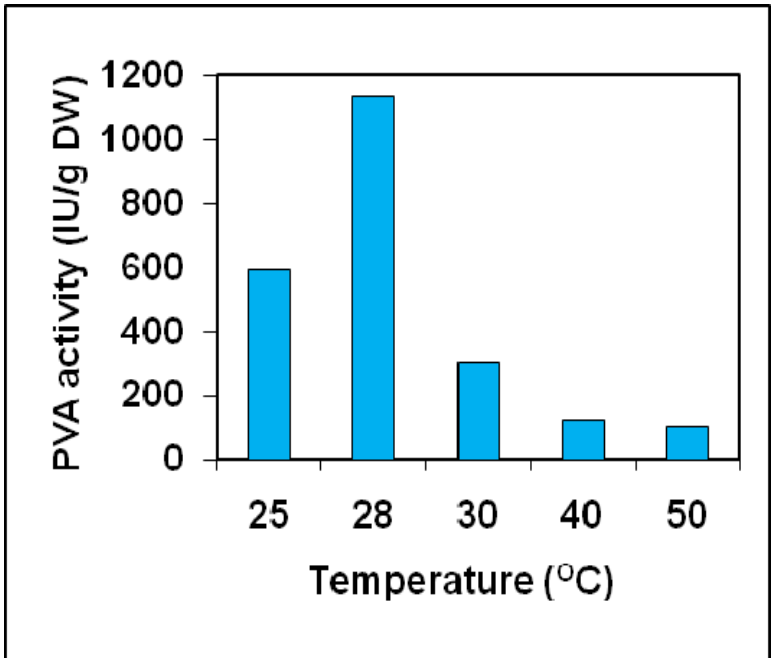

Figure 3.1.3: Effect of temperature on the production of PVA by Erwinia aroideae (DSMZ 30186)

3.1.3.6. Effect of dispensing volume

The effect of aeration on the production of PVA from *E. aroideae* is depicted in ***Fig. 3.1.4***. Erlenmeyer flasks (250 ml) containing 50 ml medium was optimum for the production of PVA (1025 IU/g DW) by *E. aroideae,* further increase in medium volume led to decrease in enzyme production.

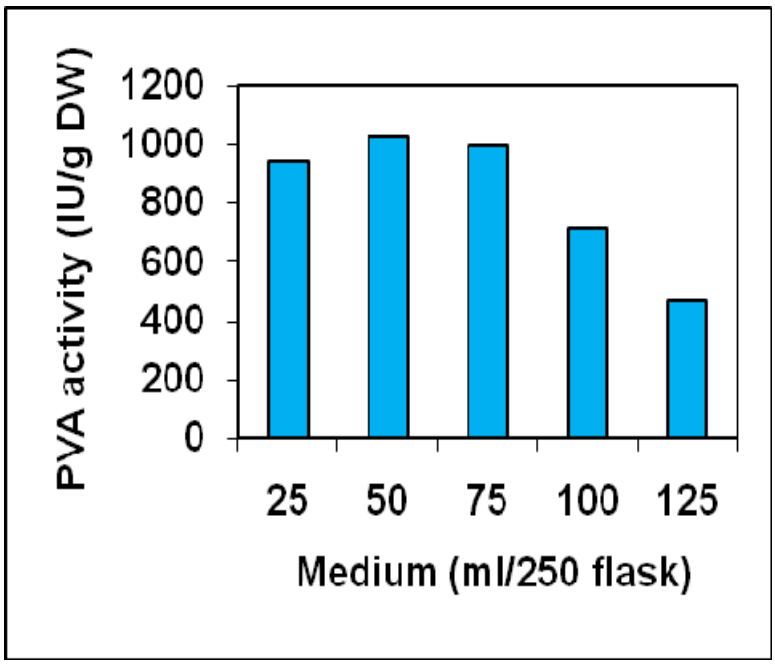

Figure 3.1.4: Effect of aeration on the production of PVA by Erwinia aroideae (DSMZ 30186)

3.1.4. Conclusions

We are reporting, for the first time, the enhanced production of PVA by *Erwinia aroideae* (DSMZ 30186). Effect of carbon sources and nitrogen sources has shown interesting results. Fructose was the best carbon source for the production and productivity of enzyme. Ammonium sulphate was the best nitrogen source for the production; however sodium glutamate was the best for overall productivity of enzyme in the given minimal medium. For the optimum production of penicillin V acylase by *E. aroideae*, cells were grown in Erlenmeyer flasks (250 ml) containing 50 ml of minimum medium with ammonium sulphate (0.3 %) at 28 ^{o}C and 180 rpm for 56 h. Enhanced production of PVA by the culture will lead to increased specific productivity of enzyme and subsequently ease to purify and characterize the enzyme followed by industrial applications.

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Effect of organic solvents on cell-bound Penicillin V Acylase activity of Erwinia aroideae

Chapter 3 B

*Part of the work presented in this chapter has been **published:***
*Atul Kumar & Archana V. Pundle **(2008)** Journal of Molecular Catalysis B: Enzymatic (**In press**).*

Abstract

Erwinia aroideae (DSMZ 30186) is a potential microbial culture to produce intracellular penicillin V acylase (PVA). Whole cell PVA activity was improved by permeabilization with various organic solvents. The cell bound PVA activity showed an eight fold increase upon treatment with chloroform (5 µL/mg $_{\text{dry biomass}}$) for 10 min and diethyl ether (10 µL/mg $_{\text{dry biomass}}$) for 45 min. Hexane, toluene, ethyl acetate and dichloromethane enhanced the enzyme activity up to two, six, four and two fold, respectively; whereas, PVA activity declined drastically on permeabilization with acetone, pyridine and alcohols. The physicochemical properties of the organic solvents used for permeabilization were correlated with the change in activity. It was found that solvents with high hydrophobicity (log *P* > 0.68) and lower dielectric constant (< 9) were relatively more effective in increasing PVA activity. These results allow systematic selection of suitable solvent for best performance.

3.2.1. Introduction

Gram-negative bacteria possess an outer membrane (OM) in addition to the plasma membrane. It functions mainly as a protective layer to prevent entry of toxic substances into the cell. The OM forms a proficient barrier against hydrophilic macromolecules and hydrophobic substances due to a lipopolysaccharide layer on the membrane surface. However, many detergents such as triton X-100 (Galabova et al., 1996), tweens (Galindo et al., 1996), N-cetyl-N, N, N-trimethylammoniumbromide (CTAB) (Prabhune et al., 1992) and organic solvents (Flores et al., 1994) are capable of disrupting the integrity of the OM. OM resists the movement of substrate and product by imposing limits on diffusion, however the drawback can be circumvented by permeabilizing the cells. Permeability issues in Gram-negative bacteria have been recently discussed by Chen (2007). Intracellular enzyme activity of wild-type microorganisms as well as recombinant strains can be enhanced by cell permeabilization. It is an easy method to improve cell bound activity keeping the viability of the cell intact. Toluene, chloroform and other organic solvents have been used successfully for permeabilization leading to enhancement in the enzyme activity in bacteria and yeast cells (Prabhune et al., 1992; Leon et al., 2003; Krishnan et al., 2000). Water-in-hexane *macro*- and micro-emulsions stabilized by sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) were used for selective permeabilization of *Escherichia coli* cells to extract and purify penicillin acylase (Mutalik & Gaikar, 2003). Cell permeabilization with organic solvents is a straightforward method to accelerate *in vivo* hydrolysis of substrate. However, little information is available on the effects of physicochemical properties of organic solvents on the cell permeabilization process. Most of the attempts involve selection of suitable solvent by trial and error method. Here we have explored the factors that affect the performance of organic solvents in permeabilizing Gram-negative cells.

Although genetic engineering methods, operational conditions, post-fermentative stages and combinations of these have been used to improve penicillin acylase production (Bhattacharya et al., 1993; Ramı´rez et al., 1994; Leon et al., 1996), higher PGA specific activities are still required to produce commercially attractive biocatalysts (Sudhakaran & Shewale, 1993; Ospina et al., 1995). Penicillin acylase is located in the periplasmic space after postranslational

processing of its polypeptide precursor (Sizmann et al., 1990). Permeabilization of cells with N-cetyl-N,N,N-trimethylammonium bromide (CTAB), prior to immobilization is, therefore, used to reduce the limitations due to insufficient permeation of substrate and product through the external membrane. Increments of 1.4 and 2-fold increase of enzyme activity have been reported (Nagalakshimi & Pai, 1994; Prabhune et al., 1992; Norouzian et al., 2002). Although media standardization, optimization of cultural conditions and genetic engineering methods have been used to improve PVA production, higher PVA activity is still required to produce commercially striking biocatalysts (Shewale & Sudhakaran, 1997). Periplasmic penicillin G acylase activity from *E. coli* was correlated with physicochemical properties of solvents recently (Leon et al., 2003), similar studies were conducted for penicillin V acylase, as both belong to the same group of enzymes, to support the previous PGA report.

Present chapter discusses the effect of various organic solvents on whole cell PVA activity of *Erwinia aroideae.* Enhancement in the activity, due to permeabilization is also correlated with physicochemical properties of the solvents, such as dielectric constant and hydrophobicity (log *P*). Effect of solvents to cell biomass ratio and kinetics of permeabilization were also studied to develop a commercially attractive biocatalyst.

3.2.2. Materials and Methods

3.2.2.1. Materials

Penicillin V potassium salt (Pen V) was a kind gift from Hindustan Antibiotics, Pune, India. Para-dimethylaminobenzaldehyde (Qualigens, India) and organic solvents such as methanol, ethanol, hexane, toluene, diethyl ether, chloroform, ethyl acetate, dichloromethane and Xylene (Qualigens, India) were of analytic grade.

3.2.2.2. Microorganism and growth conditions

Standardization of fermentation media and cultural conditions such as incubation period and temperature, initial pH as well as dispensing volume of medium for the production of penicillin V acylase from *Erwinia aroideae* (DSMZ 30186) was

carried out. *E. aroideae* cells were grown in nutrient broth (NB) with sodium glutamate (g/L; peptone 10, beef extract 10, NaCl 5 and sodium glutamate 10, pH 7.0) and incubated at 28 ^{o}C for 36 h with shaking (200 rpm) in 250 ml Erlenmeyer flasks containing 50 ml of medium. Cells were harvested by centrifugation at 10,000 rpm for 10 min, washed twice with 0.1 M sodium phosphate buffer pH 7.0 and resuspended in the same buffer and used for permeabilization. All the experiments were repeated three times with the freshly grown cells for the reproducibility of results.

3.2.2.3. Permeabilization with solvents

The amount of cell suspension required to obtain 10-20 mg biomass was centrifuged at 10,000 rpm for 3 min; the pellet was resuspended in required amount (between 5 to 100 μl/mg $_{dry\ biomass}$) of organic solvent and mixed thoroughly by vortexing. The suspension was incubated at room temperature for 15 min. Cells were harvested by centrifugation for 5 min at 10,000 rpm and 4 ^{o}C, and resuspended in 0.1 M sodium phosphate buffer pH 7.0. Treated cells were analyzed for PVA activity by standard enzyme assay; untreated cells served as control.

3.2.2.4. Enzyme assay

Cell bound penicillin V acylase activity was determined as described in the ***section 3.1.2.5*** by the method of Bomstein & Evans (1965), as modified by Shewale et al. (1987), measuring the amount of 6-APA formed at 40 ^{o}C, employing 2% w/v solution of Pen V, potassium salt, in 0.1 M sodium phosphate buffer pH 6.0**.** The 6-APA formed was estimated using 0.6% (w/v) *p*-dimethylaminobenzaldehyde (PDAB) in methanol. One unit (IU) of PVA activity is defined as the amount of enzyme that produces 1 μmol 6-APA per minute under the conditions defined. The cells were incubated for 20 min, under the assay conditions to measure the enzyme activity. The product-time relationship was linear up to the mentioned period of time.

Biomass concentration was determined from optical density measurements at 600 nm and converted to dry weight with a standard curve. The

biomass reported here is dry weight (DW) of cells and enzyme activity presented here as IU/g dry biomass. All the experiments were repeated three times with the freshly grown culture and the values reported here in tables and figures represent their mean value.

3.2.3. Results and Discussion

3.2.3.1. Permeabilization with organic solvents

Results obtained from the treatment of *Erwinia aroideae* cells with organic solvents are depicted in the ***Table 3.2.1***. Eleven different organic solvents were used to permeabilize *E. aroideae* cells. PVA activity was positively influenced by xylene, hexane, toluene, chloroform, diethyl ether, ethyl acetate and dichloromethane when the cells were incubated with the solvent at 20 μl/mg $_{dry\ biomass}$ concentration for 15 min. In contrast, solvents such as pyridine, acetone and alcohols (ethanol and methanol) drastically decreased the PVA activity. Various amounts and time intervals were used to screen the enhancement pattern of solvents and finally 20 μl/mg $_{dry\ biomass}$ and 15 min time interval was selected for data interpretation.

Enzyme activity was increased by xylene on incubation for 2 min only; further incubation led to inhibition of enzyme activity. Hexane, toluene, chloroform, diethyl ether, dichloromethane and ethyl acetate continued to enhance activity constantly even after 2 min incubation (***Table 3.2.1***). Further standardization of permeabilization conditions was carried out with those solvents, which enhanced cell bound activity.

Table 3.2.1: PVA activity of E. aroideae (DSMZ 30186) cells on permeabilization with different organic solvents. Cell bound enzyme was assayed at 40 ^{o}C for 20 min; activity of untreated cells was considered as 100 % (22.5 IU/g).

Solvents	**Incubation time (min)**	**Dielectric constants ($C^{2/}Nm^{2}$)**	**Log *P* (-)**	**Density (g/ml)**	**PVA activity (% of control)**
Hexane	15	1.89	3.5	0.659	221
Toluene	15	2.38	2.5	0.865	426
Xylene*	2	2.4	3.1	0.86	466
Diethyl ether	15	4.34	0.85	0.715	569
Chloroform	15	4.8	2	1.492	728
Ethyl acetate	15	6.0	0.68	0.894	407
Dichloromethane	15	9.1	1.2	1.326	199
Pyridine	15	12.5	0.71	0.983	56
Acetone	15	20.7	-0.23	0.791	43
Ethanol	15	24.3	-0.24	0.798	39
Methanol	15	32.6	-0.76	0.791	38
Control (Water)	15	78.5		1	100

Cells were permeabilized with 20 μl/mg biomass solvents for 15 min. Densities are adopted from Merck Index. Dielectric constants and log P values are obtained from *http://www.asiinstr.com/dcl.html and* Laane et al. (1987) respectively: P is the partition coefficient for the solvent between 1-octanol and water.

** In case of xylene, permeabilization period was restricted to 2 min as further exposure reduced PVA activity.*

No activity was detected in the supernatant of the reaction mixture indicating that there was no leakage of enzyme from the cells into the external medium due to permeabilization. Effect of temperature, on permeabilization of cells, was studied in the range of 4-37 ^{o}C, however there was no effect of temperature on permeabilization of *E. aroideae* cells to enhance whole cell PVA activity, within the tested temperature range. Consequently, all subsequent experiments were carried out at room temperature (25^{o}C).

There are many reports in which physicochemical properties of solvents are related to effects on the activity and stability of free enzymes (Affleck et al., 1992; Halling, 1987; Liepinsh & Otting, 1997; Yoshida et al., 1997). However, little information is available on the effect of solvents on enzyme activity during cell permeabilization processes. Leon et al. (2003) reported permeabilization of recombinant *Escherichia coli* cells expressing penicillin G acylase (PGA) using organic solvents and showed 380 % increase in enzyme activity. Krishnan et al. (2000) reported a three-fold increase in lactate dehydrogenase activity of *Lactobacillus plantarum* cells permeabilized with 1 % (v/v) diethyl ether (0.1 μl/mg $_{\text{dry biomass}}$ approximately); toluene and toluene-ethanol methods produced lesser improvements.

3.2.3.2. Effect of dielectric constant and hydrophobicity of solvent

It has been reported that electrostatic forces affect protein structures and their functionality (Affleck et al., 1992; Yoshida et al. 1997; Perutz, 1976). Correlation between dielectric constant values of solvents and change in PVA activity is shown in ***Fig. 3.2.1***. Permeabilization of *E. aroideae* cells with solvents of dielectric constant lower than 9 significantly increased PVA activity. Dielectric constant represents the dipole moment of the solvent molecules and directly affects the flexibility of proteins (Guinn et al., 1991). The whole cell PVA activity of *E. aroideae* was enhanced more than four folds, by the solvents exhibiting dielectric constant in between 2.0-6.0; and the highest enhancement (728 %) was observed in case of chloroform, dielectric constant 4.8.

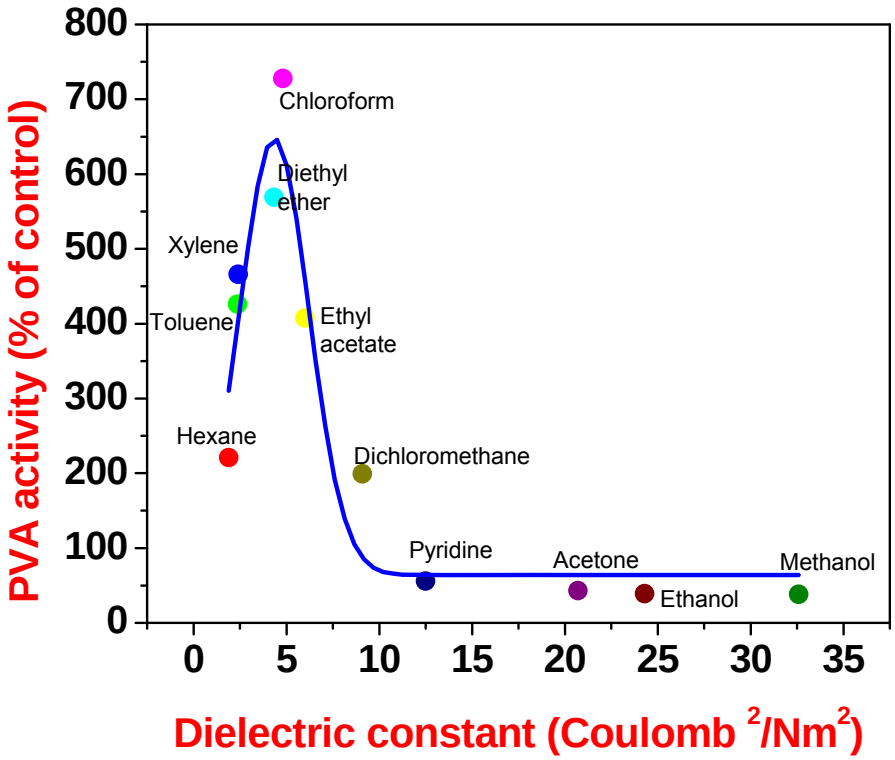

Figure 3.2.1: Correlation between dielectric constant of organic solvents and PVA activity (% of control) of E. aroideae. Cells were treated with solvent at concentration of 20 µl/mg $_{biomass}$ for 15 min except xylene (2 min). All experiments were repeated three times with the fresh batch of culture and the values reported here represent their mean value.

PVA activity dropped sharply after treatment with solvents exhibiting dielectric constant more than 9.1. Penicillin G acylase activity of *E. coli* was enhanced by the solvents with dielectric constant <5 (Leon et al., 2003). In general, enzymes are known to have a hydration shell. In reaction mixtures containing water-miscible organic solvents, distortion of the hydration shell caused by introduction of organic solvent into the enzyme solution upsets the system of interactions supporting the native conformation, thus results in the loss of catalytic activity (Simon et al., 2007). Therefore, inhibition observed in case of pyridine, acetone and alcohols was probably due to denaturation of the enzyme.

Affleck et al. (1992) have demonstrated dramatically decreased motions in the vicinity of two spin-labeled amino acids (Met-192 and Ser-195), with decreasing solvent dielectric constant, a trend consistent with changes in the electrostatic force between charged residues of the protein, might be cause of inactivation of *E. aroideae* PVA by the solvents with higher dielectric constant.

Whole cell PVA activity was also correlated with the hydrophobicity of the solvents (***Fig. 3.2.2***). Hydrophobicity is commonly measured as log *P,* where *P* is the partition coefficient of the solvent between 1-octanol and water. Solvents with higher polarity (i.e. those with low log *P* values) drastically reduced PVA activity. However, solvents with higher hydrophobicity (log *P* >0.68) such as chloroform, diethyl ether, toluene, xylene, hexane, dichloromethane and ethyl acetate, enhanced PVA activity of whole cells. Short chain alcohols and acetone have been shown to improve the activity of PVA from *Streptomyces lavendulae* (Arroyo et al., 1999); however alcohols and acetone were found to be inhibitory in the present case.

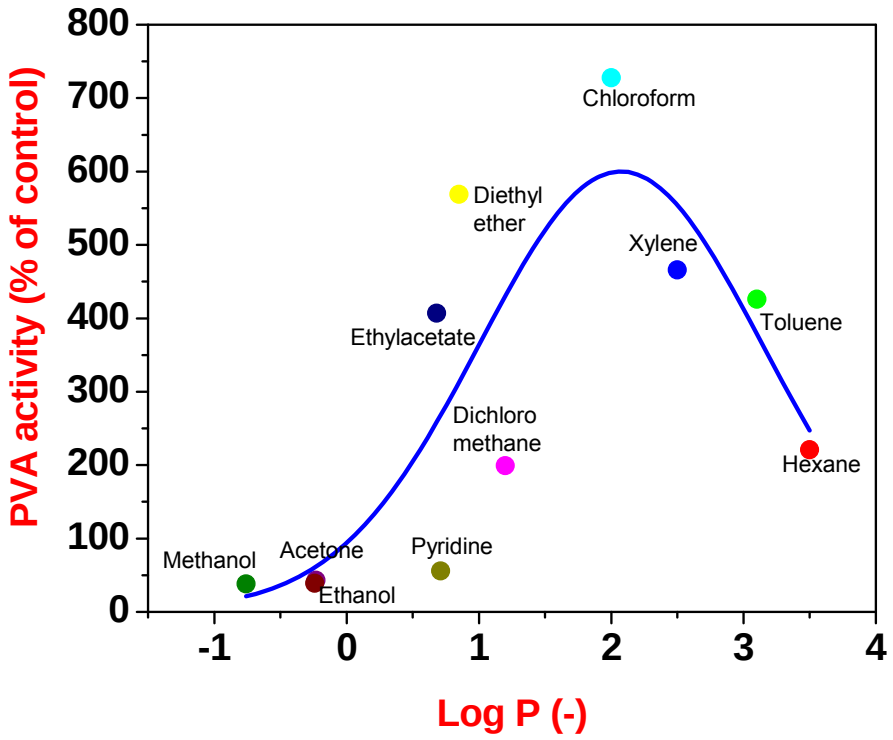

Figure 3.2.2: Correlation between hydrophobicity (log P) values of organic solvents and PVA activity (% of control) of E. aroideae. Cells were treated with 20 µl/mg $_{biomass}$ concentration of solvent for 15 min except xylene (2 min). All experiments were repeated three times with the fresh batch of culture and the values reported here represent their mean value.

The cell-bound PVA activity of *E. aroideae* was enhanced by solvents with intermediate hydrophobicity (0.68-3.5); however the maximum enhancement of the enzyme activity observed in case of chloroform, log *P* 2.0. Cell bound PGA activity of *E. coli* was enhanced by the solvents with log *P* value of 0.85-3.5 (Leon et al., 2003). Flores et al. (1994) analyzed the effect of solvent on permeabilization of *Kluyveromyces lactis* cells with chloroform, toluene and ethanol on β-galactosidase activity and found that, chloroform and toluene were more efficient than ethanol.

It is interesting to note that solvents with, dielectric constant 2.0-6.0, and hydrophobicity 0.68-2.5, have shown optima for enhancing cell-bound PVA activity from *E. aroideae;* amongst all the solvents used, chloroform of dielectric constant 4.8 and log *P* 2.0, was the best solvent to enhance whole-cell enzyme activity. Dichloromethane exhibiting higher dielectric constant and log *P values,* 9.1 and 1.2 respectively, was effective in permeabilizing and enhancing PVA activity up to 199 % of control, where as it was reported to be inhibitory in case of *S. lavendulae* PVA (Arroyo et al., 1999).

The densities of organic solvents are usually less than 1 g/ml (***Table 3.2.1***) except chloroform (1.492 g/ml) and dichloromethane (1.326 g/ml); and in water-immiscible solvents organic phase remains at the top. In case of chloroform and dichloromethane the evaporation of the solvents was not observed at permeabilization conditions due to its high density, which reduces the risk due to inflammable nature of solvent and favors scaling up of the process.

3.2.3.3. Effect of solvent concentration

Effect of organic solvents to cell mass ratio on the enhancement of PVA activity is depicted in ***Figure 3.2.3***. Different concentrations (0-100 μL/mg $_{\text{dry biomass}}$) of selected solvents were tested to observe the effect on cell permeabilization. Different optima were obtained for each solvent, might be due to the characteristic of perticular solvent. PVA activity increased steeply upto 5 μL/mg $_{\text{dry biomass}}$ of chloroform however it decreased drastically on further addition of the solvent.

PVA from *S. lavendulae* enhanced penicillin V hydrolysis when chloroform (10 % effective concentration) was used in reaction medium (Arroyo et al., 1999). 6-fold increase in PVA activity was observed when cells were incubated with diethyl ether at 5 μL/mg $_{\text{dry biomass}}$ concentration; PVA activity slightly decreased with the further increase in concentration. Toluene enhanced enzyme activity, six fold, at concentration of 20 μL/mg $_{\text{dry biomass}}$, but further addition of solvent led to decrease in activity. In case of hexane, maximum 166 % PVA activity, of control, was obtained at 20 μL/mg $_{\text{dry biomass}}$ concentration; further addition of solvent did not accelerate cell bound PVA activity. Leon et al. (2003) have reported permeabilization of *E. coli* and demonstrated increase of PGA activity upto 380 % using chloroform 30 μL/mg $_{\text{dry biomass}}$ while acetone and pyridine reduced and alcohols inactivated the enzyme activity. Similar results were obtained in the present study, however chloroform enhanced PVA activity at exceedingly lower concentration (5 μL/mg $_{\text{dry biomass}}$) than reported by Leon et al. (2003). Diethyl ether failed to improve PGA activity in the latter case while it enhanced PVA activity up to 6-fold in case of *E. aroideae* cells when incubated at 5 μL/mg $_{\text{dry biomass}}$ concentration. 1 % ethyl ether was optimal concentration to increase lactate dehydrogenase activity of *L. plantarum* (Krishnan et al., 2000).

Cell bound PVA activity was enhanced upto 421 %, of control, when cells were incubated at 5 μL/mg $_{\text{dry biomass}}$ with ethyl acetate; however further increase in proportion of the solvent led to drastically decrease in the activity. In monophasic water-organic solvent system, enzymatic activity is normally lost at high solvent concentration due to replacement of water in the protein's hydration surface layer by organic solvent (Gorman & Dordick, 1992) and that might be reason for decrease in PVA activity at higher concentration of organic solvents.

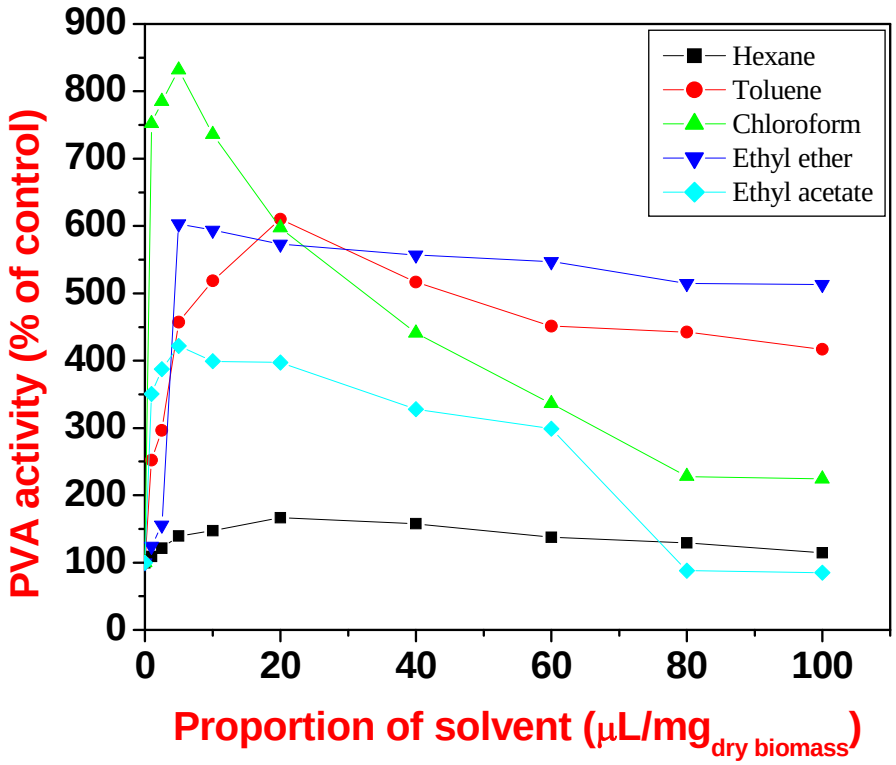

Figure 3.2.3: Effect of solvent to cell mass ratio on PVA activity of E. aroideae cells. Cells were permeabilized for 15 min in a range of solvent concentrations. The activity of untreated cells was considered as 100 % (24.0 IU/g). All experiments were repeated three times with the fresh culture and the values reported here represent their mean value.

3.2.3.4. Effect of permeabilization time

The effect of period of permeabilization for various solvents is depicted in ***Figure 3.2.4***. A tremendous increase in whole cell PVA activity was observed when cells were incubated with various selected solvents, for more than 30 min. The optimum concentration of each solvent, which showed increase, was used to achieve maximum possible permeabilization effect and, enhancement in the whole-cell enzyme activity.

839 % increase in PVA activity was observed when cells were exposed to chloroform for just 10 min at 5 μL/mg $_{\text{dry biomass}}$ concentration; and then a saturated behavior with the time was observed on further incubation, which led to decrease in the activity. Diethyl ether showed 829 % PVA activity, of control, when incubated for 45 min but a slight decrease, 798 % PVA activity, of control, was observed on further incubation. Similar results were obtained with hexane and toluene with 210 and 364 % PVA activity, of control, respectively. Ethyl acetate was found to be effective for permeabilization of *E. aroideae;* it enhanced PVA activity up to 544 % of control when the cells were incubated just for 5 min; the enzyme activity decreased drastically on further incubation. Hydrolysis of penicillin V was increased by *S. lavendulae* PVA, using chloroform in the reaction mixture (Arroyo et al., 1999); similar results are observed in the present case of whole cell PVA activity from *E. aroideae*. In the view of this, the direct binding of the solvent on specific binding sites of enzyme might be the contributing factor that determines PVA activity (Liepinsh & Otting, 1997). The prolonged exposure of cells to the solvent led to distortion of the hydration shell of enzyme, resulted in loss of the activity.

Penicillin G acylase activity of *E. coli* increased upto 334 % when cells were incubated for 10 min in chloroform Leon et al. (2003) where as, in the present study, 839 % increase in PVA activity was observed at the same incubation time. This is the first time that an 8-fold increase in PVA activity has been achieved using chloroform and diethyl ether. Krishnan et al. (2000) reported that the time period of 1 min was optimum to increase the lactate dehydrogenase activity of *L. plantarum* cells with ethyl ether at 28 ^{o}C.

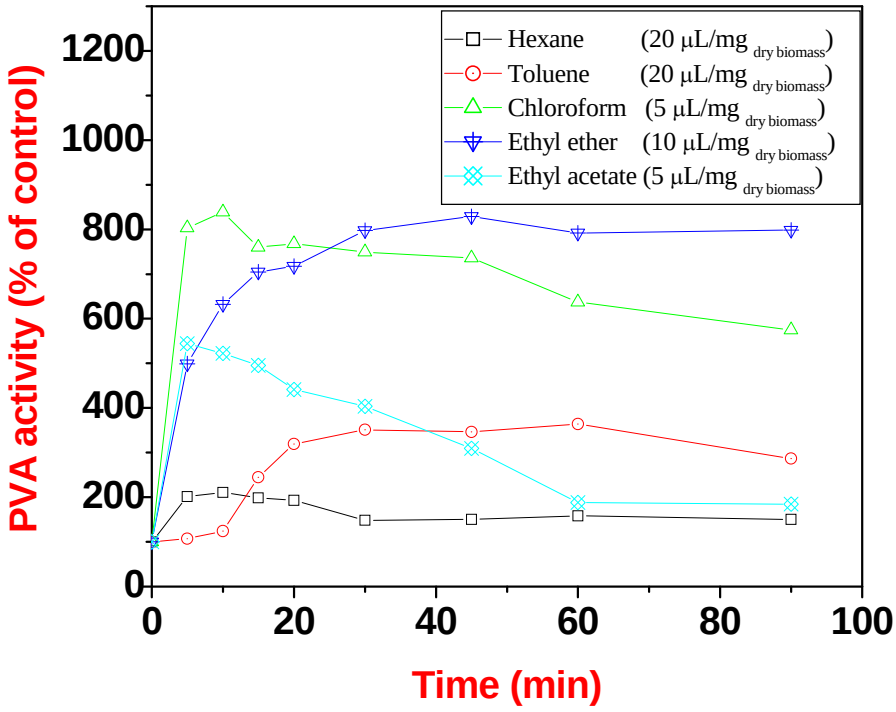

Figure 3.2.4: Effect of permeabilization time on PVA activity of E. aroideae cells. Cells were incubated with the solvent for various time intervals and sampled for PVA activity under standard assay conditions. Activity of untreated cells was considered as 100 % (26.3 IU/g). All experiments were repeated three times with the fresh culture and the values reported here represent their mean value.

3.2.4. Conclusions

Penicillin V acylase is a pharmaceutically important enzyme therefore it is relevant to enhance cell bound activity to use cells as biocatalysts on commercial scale. Solvents with low dielectric constant and high hydrophobicity are effective in improving cell bound PVA activity of *Erwinia aroideae*. Correlation between physicochemical properties of solvents and change in PVA activity provides clues for selection of a suitable solvent to improve cell bound activity in gram-negative bacteria. In this study, for the first time, we report 8-fold increase in whole cell PVA activity by employing chloroform and diethyl ether as permeabilizing agents. Solvents such as hexane and toluene were also effective in permeabilizing *E. aroideae* cells and enhancing PVA activity. Chloroform and diethyl ether are cheap reagents, and their volatile nature facilitates removal from the reaction mixture. These two reagents are effective in permeabilizing *E. aroideae* cells moreover maximum PVA activity was achieved within short period of treatment. These features make the process convenient to scale up.

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Production of Penicillin V Acylase by Rhodotorula aurantiaca and permeabilization studies

Chapter 4

*Part of the work presented in this chapter has been **published:***
*Atul Kumar, Sanjay Singh, Pankaj Poddar, Asmita A. Prabhune & Archana V. Pundle **(2008)** Applied Biochemistry and Biotechnology (**In press**)*

Abstract

Penicillin V acylase (PVA) is pharmaceutically important enzyme as it plays vital role in the manufacture of semi-synthetic β-lactam antibiotics. Standardization of fermentation parameters were carried out to enhance production of penicillin V acylase by *Rhodotorula aurantiaca* (NCIM 3425). *R. aurantiaca* produced high levels of intracellular penicillin V acylase after 18 h at pH 8.0, and temperature 27^{o}C. Various carbon and nitrogen sources were used to study the effect on the production of penicillin V acylase by *R. aurantiaca*. Among carbon and nitrogen sources used, fructose was the best carbon source for PVA production whereas tryptone was the best nitrogen source to produce the enzyme up to 170 and 1088 IU/L of culture broth, respectively. Additionally, permeabilization of *R. aurantiaca* cells was carried out to enhance cell-bound PVA activity using cationic detergent N-cetyl-N, N, N-trimethylammoniumbromide (CTAB). The cell-bound PVA activity was enhanced on treatment with cationic detergent. Whole-cell activity was found to be doubled (204 %) on treatment of 0.01 g dry weight of cells with 50 μg/ml solution of CTAB, at pH 8.0 for one hour at room temperature. Atomic force microscopy (AFM) images of permeabilized cells show perturbation in the cell wall and offer the first ever visual illustration of surface structure modifications that occur during permeabilization of *R. aurantiaca* cells leading to enhancement in activity of intracellular enzyme.

4.1. Introduction

Penicillin acylases (penicillin amidohydrolases, EC 3.5.1.11) hydrolyze the acyl side chain of penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acids, as described in the previous chapters. 6-APA is a key intermediate required in the manufacture of semi-synthetic penicillins such as ampicillin, amoxicillin etc. Penicillin acylase selectively hydrolyzes the amide bond at the side chain of the penicillin moiety, keeping β-lactam amide bond intact (Shewale et al., 1990; Valle et al., 1991; Bruggink et al., 1998). Microbial producers of penicillin V acylase (PVA) occur widely in nature (Sudhakaran & Borkar, 1985). Shewale & Shudhakaran (1997) have described the potential applications of PVA in the production of 6-APA.

The catalytic activities of intracellular enzymes may be low in whole-cell biocatalysts due to the impermeability of the cell to substrate and/or product as they diffuse to and fro the reaction medium. Perforating the natural barrier by efficient permeabilization process produces whole-cell biocatalysts with high and stable enzyme activity (Siso et al., 1992). Various methods of permeabilization of microbial cells have been reported by treating the cells with organic solvents (Flores et al., 1994; Leon et al., 2003; Krishnan et al., 2000), polyethylenimine (PEI) (Helander et al., 1997), lactic acid (Alakomi et al., 2000) and detergents such as CTAB (Prabhune et al., 1992; Canovas et al., 2004; Cheng et al., 2006) and tween (Galindo & Salcedo, 1996). Number of chemical and physical treatment methods have been described to permeabilize yeast cells (Felix et al., 1980), apart from recently discussed permeability issues by Chen (2007) in gram-negative bacteria.

Atomic Force Microscopy (AFM) is a powerful tool in microbiology which allows high resolution imaging of cell structure in the conditions close to the native state. The technique provides three-dimensional images of surface ultrastructures with molecular resolution under physiological conditions with minimal sample preparation (Dufrene, 2002).

Penicillin V acylase is not only important to the pharmaceutical industries, but it is important also because it belongs to the group of Ntn-hydrolases, the newly evolved super family. The proteins that belong to this family

characteristically possess N-terminal residue as a nucleophile, participating in the catalysis and is exposed by autocatalytic processing of the precursor (Brannigan et al., 1995). Penicillin V acylase from *B. sphaericus* has been purified and the three dimensional structure of the same has been reported by our group (Pundle & SivaRaman, 1997; Suresh et al., 1999).

Keeping in mind the potential applications of PVA, the present studies were undertaken. *Rhodotorula aurantiaca,* a psychrophilic yeast, which is a new eukaryotic source of PVA. Optimization of cultural conditions and effect of carbon and nitrogen sources on enzyme production were studied and presented here. Also, we have used cationic detergent, N-cetyl-N, N, N-trimethylammoniumbromide (CTAB), to permeabilize *R. aurantiaca* cells at extremely low concentration to enhance cell bound PVA activity; and surface changes on microbial cell wall were studied using atomic force microscopy (AFM) technique.

4.2. Materials and Methods

4.2.1. Materials

Penicillin V potassium salt was kind gift from Hindustan Antibiotics Pune, India. Peptone, yeast extract, sodium glutamate, tryptone, urea, soybean casein digest, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, ammonium chloride, ammonium nitrate, glucose, mannitol, inulin, sorbitol, glycerol and digitonin were procured from Himedia, India. N-cetyl-N, N, N-trimethylammoniumbromide (CTAB) and sodium dodecyl sulphate (SDS), triton X-100, ethanol and toluene were obtained from Merck (I) Ltd. All other reagents and chemicals used were of high purity and analytical grade.

4.2.2. Microorganisms

For isolation and screening of penicillin V acylase producers, soil samples, drainage, and waste material were collected from the vicinity of Hindustan Antibiotics, Pune and other standard type cultures were obtained from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, India. Screening for penicillin acylase producing microorganisms was carried out by plate assay method (Meevootisom et al., 1983) as described in

Chapter two. *Rhodotorula aurantiaca* NCIM 3425, NCYC 138 was obtained from NCIM, NCL, Pune, India.

4.2.3. Culture media

Batch fermentation was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of medium inoculated with 10 % v/v of seed culture developed at 28 ^{o}C for 24 h at 180 rpm. The minimal medium used for enzyme production contained (g/L): Na_2HPO_4, 12.8; KH_2PO_4, 3.1; NaCl, 1.0; $MgSO_4.7H_2O$, 0.2; glucose, 4.0 and $NaNO_3$, 3.0, the pH was adjusted to 7.0. Minimal medium was used as a basal medium for the fermentation studies.

4.2.4. Optimization of cultural conditions and effect of media supplements for maximum PVA production

4.2.4.1. Effect of carbon source

Glucose from the minimal medium was replaced by various carbon sources (2.0%, w/v) to study the effect on production of PVA from *R. aurantiaca*. A tube containing 5 ml of 24 h culture of *R. aurantiaca* grown in basal medium was used to inoculate 250 ml Erlenmeyer flasks (250 ml) containing 50 ml of above-mentioned medium. The Flasks were kept for incubation at 28 ^{o}C and 180 rpm, for 24 h. The cells were harvested from the culture broth and used to determine cell-bound PVA activity as well as cell biomass.

4.2.4.2. Effect of nitrogen source

To determine the best nitrogen source for PVA production, the culture was grown in the minimal medium, pH 7.0. $NaNO_3$ of minimal medium was replaced by different nitrogen sources (0.3 %, w/v) while studying their effect on PVA production. A tube containing 5 ml of 24 h culture of *R. aurantiaca* grown in basal medium was used to inoculate 250 ml Erlenmeyer flasks (250 ml) containing 50 ml of above mentioned medium. The flasks were kept for incubation at 28 ^{o}C and 180 rpm, for 24 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min and used to determine whole-cell enzyme activity as well as cell biomass.

4.2.4.3. Effect of temperature

To study the effect of temperature on the production of PVA by *R. aurantiaca* the culture was grown at various temperatures. Erlenmeyer flasks (250 ml) containing 50 ml of basal medium, pH 7.0 were inoculated with 10 % (v/v) seed culture and incubated at 25, 28, 30 40 and 50 ^{o}C for 24 h on rotary shaker at 180 rpm. The culture broth was centrifuged at 10,000 rpm for 10 min at 4 ^{o}C and used for determination of enzyme activity as well as cell biomass yield.

4.2.4.4. Effect of pH of medium

To standardize optimum pH of growth for PVA production, the initial pH of the medium was adjusted to 4.0-9.0, prior to sterilization, and was checked before inoculation of the culture. The flasks inoculated with 10 % (v/v) seed culture of *R. aurantiaca* were kept for incubation at 28 ^{o}C and 180 rpm, for 24 h. The cells were harvested from culture broth by centrifugation at 10,000 rpm for 10 min and used for estimation of cell biomass and whole-cell PVA activity.

4.2.4.5. Effect of incubation period

To study the effect of incubation period on the production of PVA, *R. aurantiaca* culture was grown in Erlenmeyer flasks (250 ml) containing 50 ml of medium and incubated at 28 ^{o}C and 180 rpm; and samples were removed at every six hours interval. The culture broth was centrifuged at 10,000 rpm for 10 min and harvested cells were used to determine cell-biomass and whole-cell PVA activity using standard enzyme assay.

4.2.4.6. Effect of dispensing volume

Dispensing volume of the media also play an important role in the production of enzymes. In individual experiments, Erlenmeyer flasks (250 ml), containing different volumes (25-125 ml) of basal medium, were used to standardize effect of aeration for production of PVA from *R. aurantiaca*. 10 % (v/v) inoculum size was used to inoculate flasks containing various volume of medium. The inoculated flasks were kept for incubation at 28 ^{o}C and 180 rpm, for 24 h. The cells were harvested from culture broth of *R. aurantiaca* by centrifugation at 10,000 rpm for

10 min and used for cell-biomass calculation and determination of whole-cell PVA activity.

All the experiments for optimization of cultural conditions and media constituents were carried out in triplicate with the freshly grown culture and the values reported in tables and figures represent their mean value.

4.2.5. Permeabilization procedure

R. aurantiaca cells were grown in Erlenmeyer flasks (250 ml) according to the standardized fermentation parameters and harvested by centrifugation at 10,000 rpm for 10 min. The cells were washed with 0.1 M phosphate buffer pH 6.0. 30 mg wet cells were suspended in 0.1 M Tris-HCl buffer pH 8.0 containing CTAB to the final concentration of 0.005 % (w/v). The reaction mixture was then incubated for one hour at room temperature (25^{o}C) with intermittent gentle mixing. The treated cells were recovered by centrifugation at 10,000 rpm and 4^{o}C for 5 min and washed with 0.1 M Tris-HCl buffer pH 8.0. Cell bound PVA activity was determined by standard enzyme assay. The cells treated simultaneously in absence of CTAB served as control.

4.2.6. Enzyme assay

Cell bound penicillin V acylase activity was determined by the method of Bomstein and Evans (1965) essentially as described in the previous chapter, ***section 3.1.2.5. Enzyme assay,*** measuring the amount of 6-APA formed at 40 ^{o}C, employing 2% w/v solution of penicillin V, potassium salt, in 0.1 M sodium citrate buffer pH 5.5. 6-APA formed was estimated using 0.6 % (w/v) *p*-dimethylaminobenzaldehyde (PDAB) in methanol. One unit (IU) of PVA activity is defined as the amount of enzyme that produces 1 μmol 6-APA per minute under the conditions defined.

Biomass concentration was determined from optical density measurements at 600 nm and converted to dry weight of cells (DW) with a standard curve. The biomass reported here is dry weight of cells; and enzyme activity represents IU/g DW. All the experiments were carried out in triplicate with

the freshly grown culture and the values reported in tables and figures represent their mean value.

4.2.7. Sample preparation and atomic force microscopy (AFM) analysis

For this purpose, samples were prepared by washing the CTAB treated (611 cfu/ml) as well as untreated *R. aurantiaca* cells (645 cfu/ml) with 0.1M Tris-HCl buffer, pH 8.0. These cells were immobilized on the freshly cleaved surface of mica substrate by drop-coating them followed by the air-drying of the samples at room temperature for 3 h. After fixation, the unbound cells were rinsed away with the same buffer. The mica substrate was mounted on a 6399e-piezoscanner (10µm) for AFM imaging. The images were recorded in height mode for quantitative information on sample surface topography.

The cell imaging was carried out by an atomic force microscopy operating in contact mode using a Multimode^{TM} scanning probe microscope by VEECO Instruments Inc, USA, equipped with Nano-Scope IV^{TM} controller. Standard silicon nitrate AFM cantilevers, T=0.4-0.7 micron, (Model: NP-20), with spring constant of 0.12 N/m, and a nominal tip radius of <20 nm, by VEECO Instruments, USA, were used to image both, the control and CTAB treated cells. The samples were imaged in air using the contact mode with settings of 512 pixels/line and 2.98 Hz scan rate. Some of the images were first order flattened and contrast enhanced using Nanoscope 5.30r2 for better demonstration on details.

4.3. Results and Discussion

4.3.1. Effect of cultural conditions on production of PVA

While exploring the natural microflora and the available standard cultures from national culture collection, we identified a new source of penicillin V acylase, *a* yeast, *Rhodotorula aurantiaca* (NCIM 3425). To achieve maximum production of enzyme from the organism, optimization studies of cultural conditions were carried out and the results are depicted in ***Figure 4.1.***

An incubation period of 15 -18 h found to be optimum, for the production of PVA from *R. aurantiaca*; further incubation led to drastically decreased PVA production. The *R. aurantiaca* cell mass increased constantly up to 36 h, slightly

decreased thereafter, however PVA activity reached maximum after 18 h and drastically decreased on further incubation (***Fig. 4.1a***); concluding that the enzyme was produced in the exponential phase of the yeast cell growth. Optimum parameters, for production of PVA, from *Bacillus sphaericus, Streptomyces lavendulae* and *R. aurantiaca* are compared in ***Table 4.1***. The optimum incubation period for the production of PVA from *B. sphaericus* and *Streptomyces lavendulae* was 20 and 278 h, respectively (***Table 4.1***).

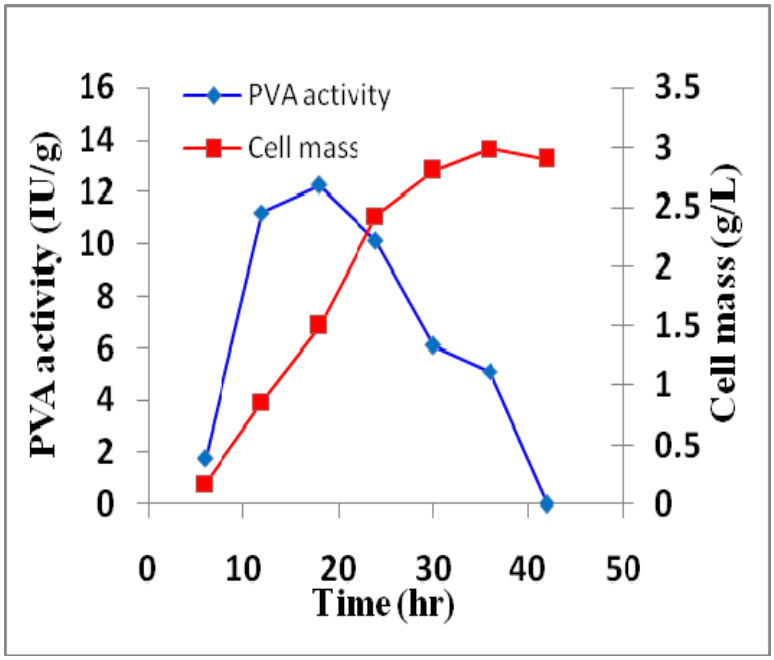

Figure 4.1a: Optimum incubation period for the production of penicillin V acylase by R. aurantiaca

R. aurantiaca PVA production was very much influenced by fermentation temperature. Maximum PVA, 12 IU/g DW, was produced at 27°C; the enzyme production lowers remarkably above and below 27°C (***Fig. 4.1b***). PVA production was dropped with the drop of cell biomass above 28°C. Production of PVA from *Bacillus sphaericus* was optimum at 25°C (Pundle & SivaRaman, 1994) whereas in the present case, 27°C was the optimum temperature for enzyme production. PVA was optimally produced by *Streptomyces lavendulae (*Torres et al., 1999), an actinomycetes, at 28°C (***Table 4.1***).

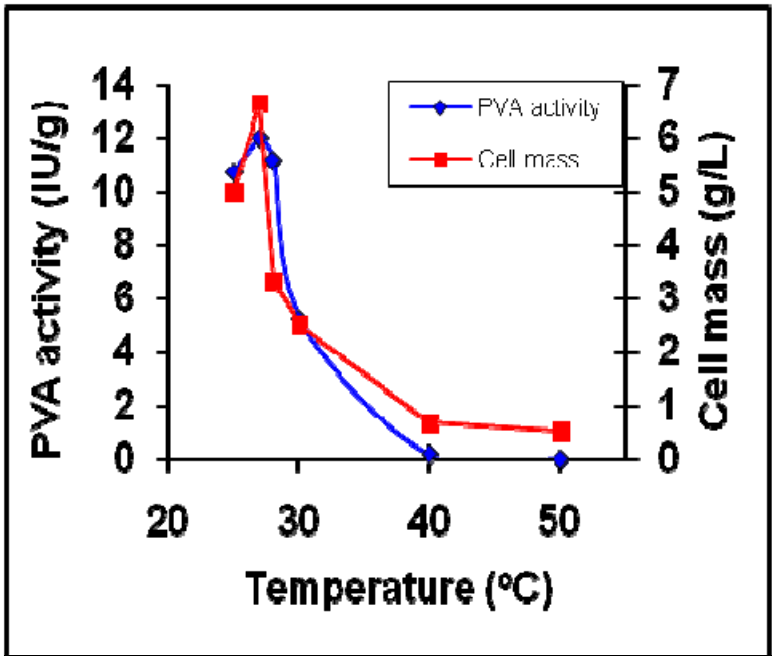

Figure 4.1 b: Temperature optimization for the production of PVA

Various volumes of media (25-100 ml) were dispensed in 250 ml Erlenmeyer flask, to study the effect of aeration, and the maximum enzyme production was found at dispensing volume of 62.5 ml medium at 180 rpm (***Fig. 4.1c***). The initial pH of medium, 8.0, was found to be the best for enzyme production, 13.25 IU/g DW (***Fig. 4.1d***). Cell growth of *R. aurantiaca* increased constantly and reached the highest, at pH 9.0, however further increase in media pH drastically decreased the cell biomass; *Rhodotorula glutinis,* a yeast, has been reported to grow near pH 9.0 (Iizuka & Goto, 1973; Kurita & Yamazaji, 2002), it is true even in case of *Rhodotorula aurantiaca.* PVA production from *B. sphaericus* was optimum at pH 7.5 where as production of PVA from *S. lavendulae* was optimum at pH 6.8 (***Table 4.1***). As a summary, optimum conditions for the production of penicillin V acylase from *R. aurantiaca* (NCIM 3425) are depicted in ***Table 4.2***.

Table 4.1: Comparison of optimum parameters for PVA production by different microorganisms

Microorganism	**Parameters**			
	Incubation period (h)	Incubation temperature (^{o}C)	pH of media	References
R. aurantiaca	18	27	8.0	*Present studies*
B. sphaericus	20	25	7.5	*Pundle & SivaRaman, 1994*
S. lavendulae	278	28	6.8	*Torres et al., 1999*

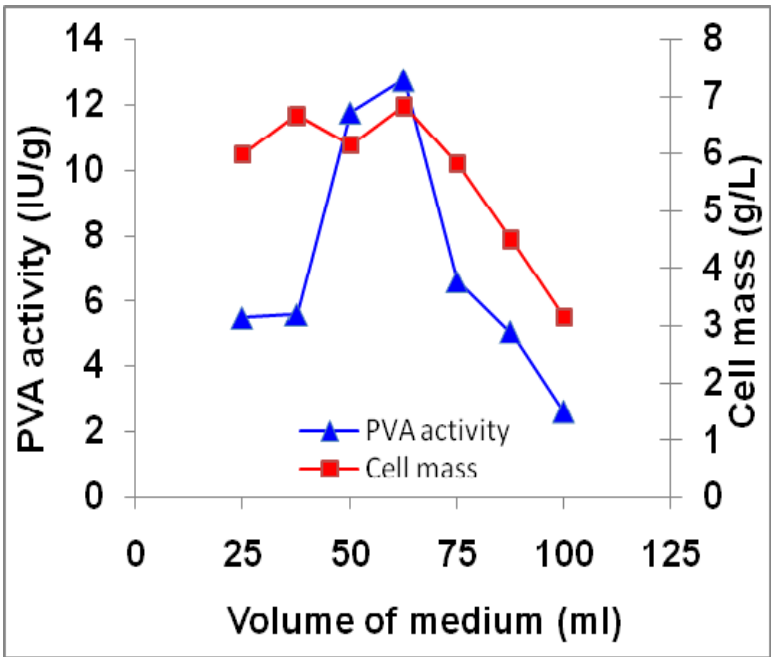

Figure 4.1 c: Optimum dispensing volume of medium for the production of PVA

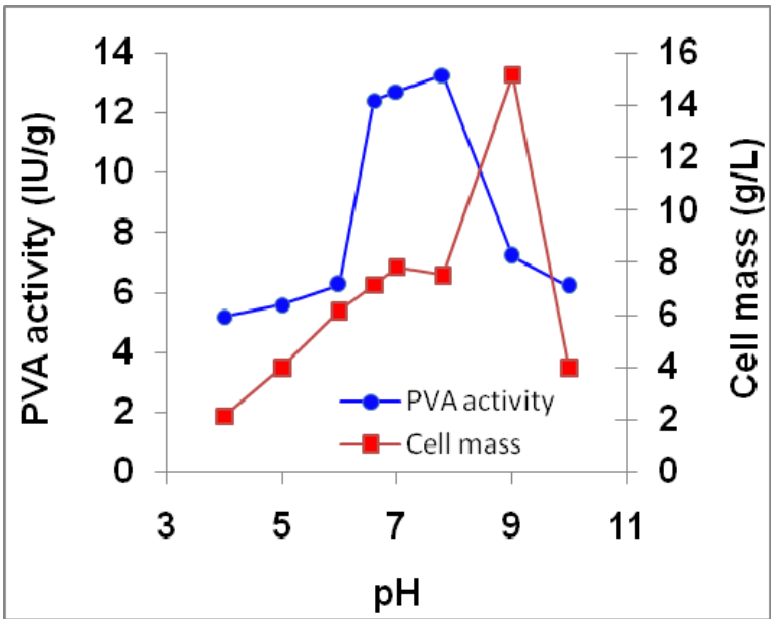

Figure 4.1 d: Optimum initial pH of medium for the enzyme production

Table 4.2: Optimum conditions for the production of penicillin V acylase by Rhodotorula aurantiaca (NCIM 3425)

Parameters	Values
Incubation period (h)	18
Incubation temperature (^{o}C)	27
Volume of medium (ml/250ml flask)	62.5
Initial pH of medium	8.0

4.3.2. Effect of carbon and nitrogen sources on production of PVA

Various carbon sources were used to study the effect on PVA production by *R. aurantiaca* and the results are provided in ***Table 4.3***. Mannitol, fructose, sorbitol and galactose produced PVA up to 103, 68.3, 60.4 and 58.8 IU/g DW, respectively however overall PVA productivity was maximum (170 IU/L of culture broth) when fructose was used in minimal medium. Mannitol, fructose and sucrose improved the penicillin G acylase (PGA) production by a 10 % from *Bacillus sp.* (Rajendaran et al., 2003). Similarly, Senthilvel & Pai (1998) reported that the production of PGA by *B. megaterium* was increased by sucrose and suppressed by glucose and fructose. In *E. coli* also, the production of PGA was suppressed by glucose, fructose, maltose and glycerol (Shewale & SivaRaman, 1989). In the present studies, addition of glucose, fructose or sucrose did not suppress but enhanced the PVA production by *R. aurantiaca.* Presence of inulin in minimal medium did not increase PVA activity where as it enhanced the cell mass and subsequently the productivity. *Streptomyces lavendulae* produced high levels of PVA (178 IU/L of culture broth) when grown in skim milk (Torres et al., 1999).

Table 4.3: Effect of carbon sources on production of penicillin V acylase by Rhodotorula aurantiaca (NCIM 3425)

Carbon sources	PVA activity (IU/g DW)	Cell weight (g DW/L)	Productivity (IU/L)
None	12.3	5.3	66
Glucose	44.1	3.3	148
Mannitol	103	1.5	154
Sorbitol	60.4	2.5	153
Glycerol	50.2	2.7	136
Fructose	68.3	2.5	170
Galactose	58.8	2.2	131
Maltose	42.2	3.4	146
Sucrose	52.2	3.1	162
Lactose	46.6	2.8	134
Mannose	45.0	3.6	162
Inulin	26.0	5.9	154

Various nitrogen sources were used to study the effect on PVA production by *R. aurantiaca* and the results are shown in ***Table 4.4***. Tryptone, sodium glutamate, peptone and ammonium phosphate produced PVA up to 89.9, 85.3, 70.9 and 58.9 IU/g DW however the specific PVA productivity was maximum (1088 IU/L) when tryptone was used in minimal medium. Tryptone and peptone increased PGA productivity without increasing the cell growth in *Bacillus* sp. (Rajendaran et al., 2003).

Table 4.4: Effect of nitrogen sources on production of penicillin V acylase by Rhodotorula aurantiaca (NCIM 3425)

Nitrogen source	PVA activity (IU/g DW)	Cell weight (g DW/L)	Productivity (IU/L)
None	26.0	18.7	488
Sodium glutamate	85.3	12.1	1033
Urea	59.3	7.9	468
Yeast extract	67.9	14.9	1012
Tryptone	89.9	12.1	1088
Soybean casein digest	46.5	21.3	990
Peptone	70.9	14.9	1061
$NH_4H_2PO_4$	58.9	19.9	1172
$(NH_4)_2SO_4$	46.6	11.5	538
NH_4Cl	64.9	11.9	775
KNO_3	52.9	9.4	498
NH_4NO_3	55.6	8.8	492

Similarly, in the present study, tryptone and peptone enhanced PVA production without increasing the cell growth. Gentina et al., (1997) have also reported, after studying various complex nitrogen sources, that casein hyrdrolysate is the best nitrogen source for PGA production by *B. megaterium* ATTC 14945. PVA production was 1.3-fold more when the cornsteep liquor was replaced with neopeptone (Sudhakan & Shewale, 1993). Production of PVA by *B. sphaericus* was achieved up to 100 IU/g DW (Pundle & SivaRaman, 1994). This is the first time we are reporting PVA production up to 1088 IU/L by *R. aurantiaca*.

4.3.3. Permeabilization of R. aurantiaca cells

Various methods were attempted to enhance cell bound PVA activity by permeabilizing *R. aurantiaca* cells. Treatment of cells with enzymes and antibiotics had no remarkable effect under the experimental conditions; the solvent treatment showed loss of PVA activity, while the detergents showed interesting results. Digitonin (0.1%) showed 15 % increase in PVA activity with respect to control (100 %, 75.1 IU/g DW). Gowda et al. (1988) used digitonin, a mild detergent, to permeabilize *Kluyveromyces fragilis* cells to obtain increased activity of intracellular enzymes like alcohol dehydrogenase, β-galactosidase etc. Triton X-100 (0.02 %) inhibited whole cell *R. aurantiaca* PVA activity (87 % residual activity) with respect to control (100 %, 78.5 IU/g DW); where as it was used to permeabilize yeast, *Yarrowia lipolytica* (Galabova et al., 1996). CTAB showed maximum escalation, 100 % increase in activity, with respect to control (80.2 IU/g DW), at very low concentration (50 μg/0.01 g DW) (***Fig. 4.2a***).

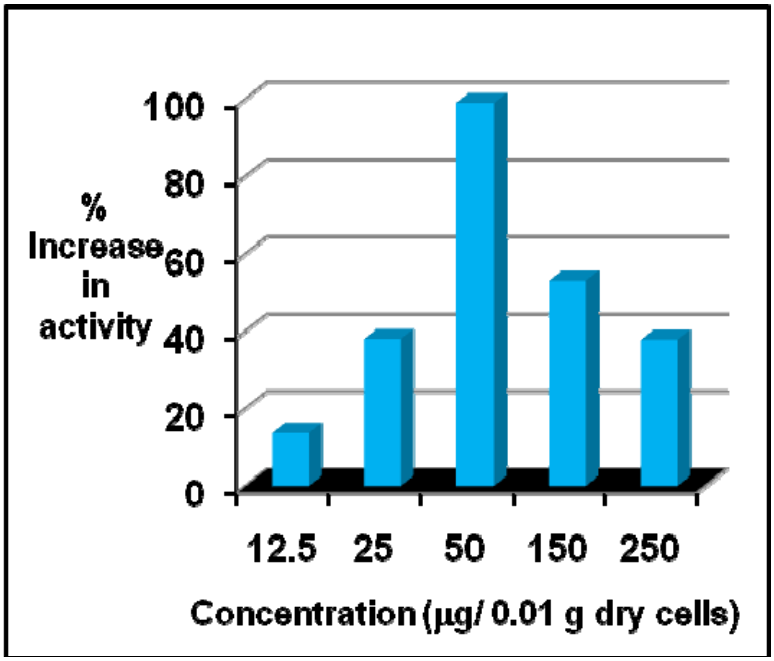

Figure 4.2 a: Effect of concentration of CTAB on the permeabilization of R. aurantiaca cells. The activity of untreated cells was considered as 100% (80.2 IU/g DW)

Hence further experiments were carried out to optimize the conditions for permeabilization to obtain maximum enhancement in activity at lowest detergent concentration. PVA activity was increased up to 165 %, with respect to control (100 %, 72.10 IU/g DW), when cells were permeabilized at room temperature, 25 ^{o}C (***Fig. 4.2b***). 192 % increase in PVA activity was obtained, with respect to control (100 %, 79.2 IU/g DW), for 60 min incubation however further incubation led to inhibition of enzyme activity (***Fig. 4.2c***).

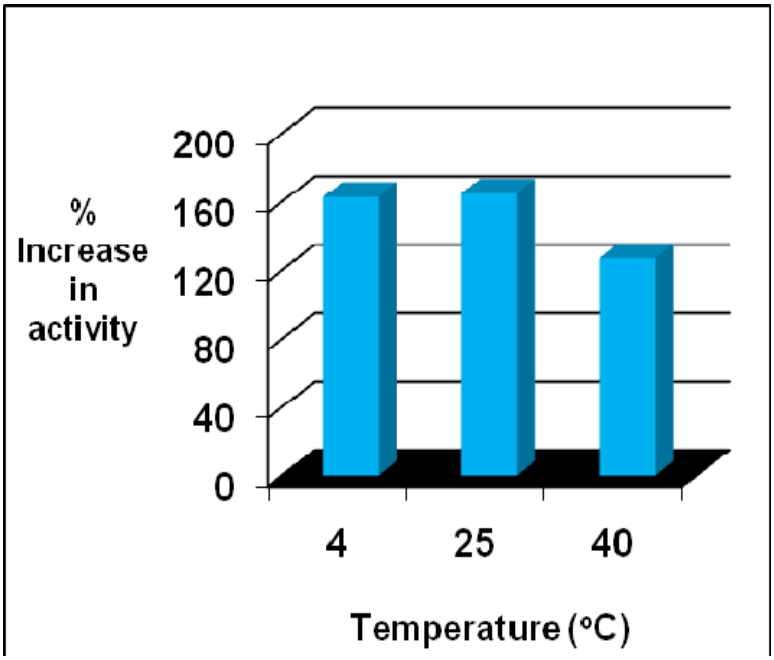

Figure 4.2 b: Effect of temperature of permeabilization on R. aurantiaca cell-bound PVA activity. The activity of untreated cells served as 100 % (72.10 IU/g DW)

There was no leakage of enzyme, in the medium, traceable during the experimental period. Cell-bound PVA activity was increased up to 204 % when the cells were treated with CTAB, suspended in Tris-HCl buffer pH 8.0 (***Fig. 4.2d***). Cell-bound PVA activity was enhanced up to 204 % when all the optimized parameters were used. Earlier we have reported enhanced production of 6-APA from benzylpenicillin by *E. coli* cells, which were sequentially treated with CTAB and glutaraldehyde then immobilized in open pore polyacrylamide beads for continuous operation (Prabhune et al., 1992).

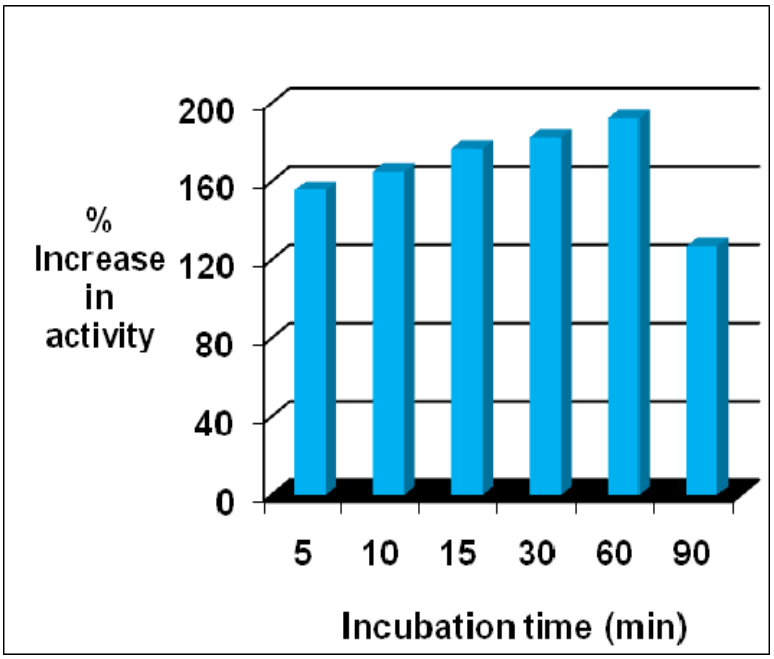

Figure 4.2 c: Effect of incubation period of permeabilization treatment on R. aurantiaca cell-bound PVA activity. The activity of untreated cells was considered as 100 % (79.2 IU/g DW)

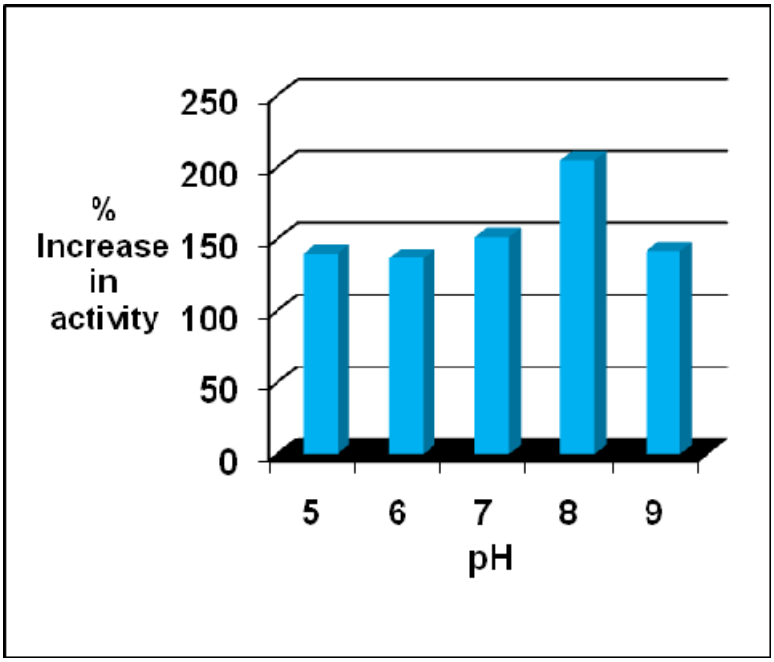

Figure 4.2 d: Effect of pH of CTAB solution on permeabilization of R. aurantiaca cells. The activity of untreated cells was considered as 100 % (85.5 IU/g DW)

4.3.4. Atomic force microscopy (AFM)

With reference to AFM image of control (untreated), the CTAB treated yeast cells showed the changes occurred on the cell surface due to permeabilization. The topography of the control as well as CTAB treated cells is depicted in ***Figure 4.3 A and B***, respectively. The length of yeast cells was around 3.6 - 4.0 microns and width around 1.5 -1.8 microns. The untreated cells show smooth surface (***Fig. 4.3 A***).

The damage to the cell surface, in case of CTAB treated cells, was clearly noticed (***Fig. 4.3 B***). However, it is assumed that the damaged surface accelerated the transport of substrate, penicillin V, and product, 6-APA, across the cell wall resulting in enhanced rate of hydrolysis.

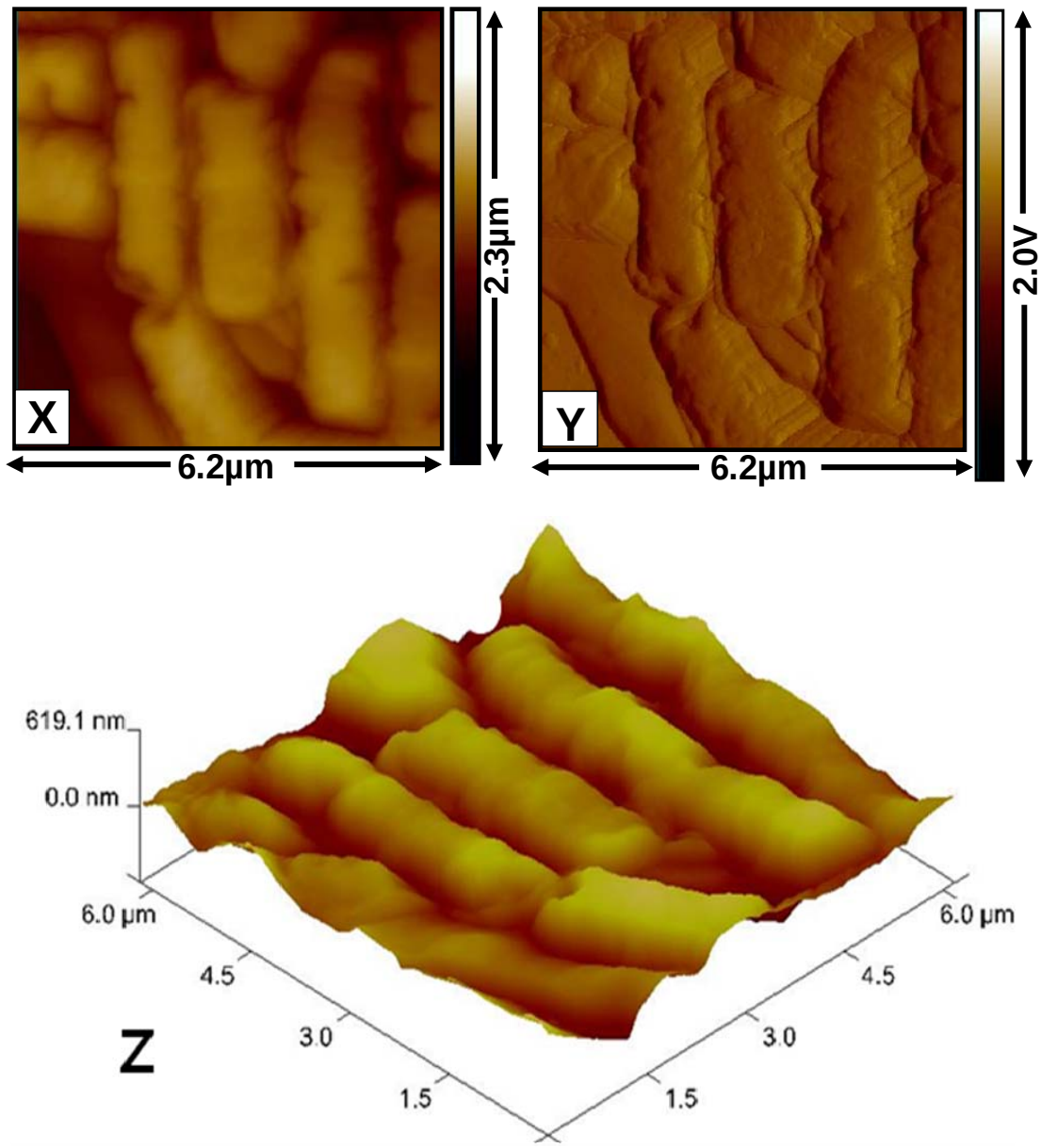

Figure 4.3 A: Control (untreated) cells (scan size 6.2 x 6.2 microns) (X) height mode image (Y) deflection mode image (Z) 3-dimensional image of height mode

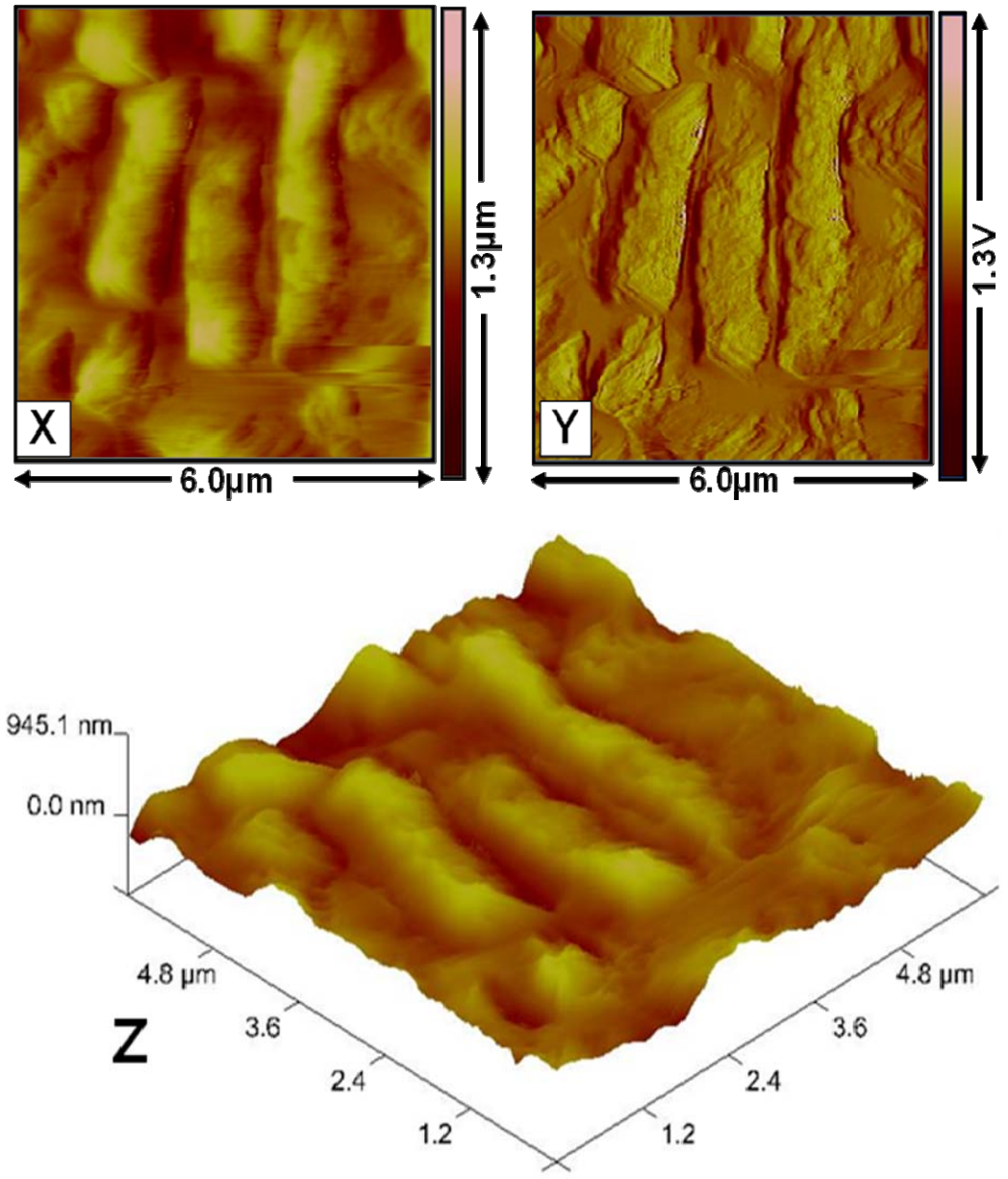

Figure 4.3 B: CTAB treated cells (scan size 6.0 x 6.0 micron) (X) height mode image (Y) deflection mode image (Z) 3-dimensional image of height mode

AFM studies of *R. aurantiaca*, a newly identified PVA producer, reveal that controlled permeabilization treatment brings about subtle structural changes in the cell wall resulting in enhancement of intracellular enzyme activity. We are reporting here for the first time the visual illustration of alterations occurring on *R. aurantiaca* cell surface during permeabilization process.

4.4. Conclusions

This is the first time that we have reported very high production of PVA from a yeast source, *R. aurantiaca* (NCIM 3425). Optimization of cultural conditions and studies of various carbon and nitrogen sources gave specific conditions for maximum production of PVA from the newly identified source. Furthermore, whole cell PVA activity was enhanced upto 204 % by treating the cells with a cationic detergent, N-cetyl-N, N, N-trimethylammoniumbromide (CTAB); so that the whole cells can be used as biocatalysts. The atomic force microscopy study gave first ever visual evidence of change in the surface topography of *R. aurantiaca* cells, due to permeabilization process, that lead to the enhancement of cell bound PVA activity.

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Purification and Characterization of Penicillin V Acylase from Rhodotorula aurantiaca (NCIM 3425)

Chapter 5

*Part of the work presented in this chapter has been **published**:*
*Atul Kumar, Asmita A. Prabhune, Suresh C. G. & Archana V. Pundle **(2008)** Process Biochemistry 43: 961-967.*

Abstract

An intracellular monomeric penicillin V acylase (PVA) of 36,000 Daltons exhibiting pI of 4.19, purified from newly identified yeast source, *Rhodotorula aurantiaca* (NCIM 3425). The enzyme was purified by hydrophobic interaction chromatography. The enzyme showed optimal activity at 45^{o}C and retained 80 % activity after incubation at 45^{o}C and pH 5.5 for 1 h. The enzyme showed maximum activity at pH 5.5 and was very stable between pH 5.5-6.0 with optimum stability at pH 6.0. It exhibited 50 % of its original activity after 30 min incubation at 60^{o}C. Enzyme hydrolyzed substrates with benzyl side chain but preferred penicillin V as primary substrate. N-terminally located serine supports the fact that it belongs to Ntn-hydrolase superfamily. The initial ten amino acid residues of *R. aurantiaca* PVA were identical to the initial sequence of NADH dehydrogenase (EC 1.6.99.3); however the enzyme lacks dehydrogenase activity. EGTA, EDTA, hexane and ethyl acetate stabilized the activity where as small chain alcohols inhibited it. 1-4 dioxane, THF, phenol and benzyl alcohol severely inhibited enzyme activity while BME and DTT increased it. Tween 80 and Tween 20 highly enhanced the activity where as SDS and Triton X-100 inhibited it.

Michaelis-Menten constant (*Km*) for purified penicillin V acylase was calculated from Lineweaver-Burk plot and found to be 20 mM. *k*cat and catalytic efficiency (*k*cat/*Km*) were calculated to be 4.83 s^{-1} and 0.24 $mM^{-1}s^{-1}$. Dissociation constant (*Ki*) of POAA against Pen V has been investigated from Lineweaver-Burk plot and (*Ki*) of POAA was found to be 1.5 mM.

5.1. Introduction

Penicillin acylases (penicillin amidohydrolases, EC 3.5.1.11) hydrolyze the acyl side chain of penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acids (Shewale & SivaRaman, 1989). The commercial importance of 6-APA in industrial synthesis of various semi-synthetic penicillins has accelerated the development of penicillin amidase research and application. Their high efficiency has resulted in the replacement of conventional chemical process in favour of enzymatic ones by the industry (Sio & Quax, 2004). Screening of penicillin acylase-producing organisms has shown that, these enzymes are produced by bacteria, actinomycetes, yeasts and fungi (Sudhakaran & Borkar, 1985). Penicillin V acylase (PVA) is produced intracellularly by few bacteria like *Bacterium* NRRL 11240, *Beijerinckia indica var penicillanicum, Bacillus sphaericus*, *Cryptococcus* sp., *Erwinia aroideae*, *Micrococcus ureae* and *Pseudomonas acidovorans;* by yeast *Rhodotorula glutinis* and by fungi *Penicillium* sp. and *Fusarium* sp. It is produced extracellularly by *Streptomyces lavendulae*, S*treptoverticillium* sp, *Fusarium* sp. SKF 235 and *Pleurotus ostreatus* (Shewale & Sudhakaran, 1997).

Penicillin acylases belong to N-terminal nucleophile (Ntn) hydrolase superfamily, which is constituted of enzymes that share a common structural fold and possess a catalytic serine or cysteine or threonine residue at the N-terminal end (Brannigan et al., 1995). PVA has been purified from *B. sphaericus, E. aroideae, B. subtilis* and *Streptomyces lavendulae* and the biochemical properties such as molecular weight, optimum pH, optimum temperature, Michaelis-Menten constant (*K*m) and inhibition kinetics are studied for various PVA preparations (Olsson et al., 1985; Pundle & SivaRaman, 1997; Vandmme & Voets, 1975; Rathinaswamy et al., 2005; Torres et al., 1998). Penicillin V acylase from *S. lavendulae* has been extensively studied by Torres et al. (1998) and Arroyo et al. (1999 & 2000).

PVA from *B. sphaericus* (NCIM 2478) (Pundle & SivaRaman, 1997) has been purified and characterized in our laboratory and subsequently the three dimensional structure was reported (Suresh et al., 1999). We have demonstrated the post-translational autoproteolytic processing of PVA from *B. sphaericus* by crystallization of three catalytically inactive mutants of the enzyme in precursor

and processed forms (Chandra et al., 2005). Cloning, purification, crystallization and preliminary structural studies of PVA from *B. subtilis* strain IG-20 (NCIMB 11621) (Rathinaswamy et al., 2005), and the evolutionary relationship between a conjugated bile salt hydrolase from *Bifidobacterium longum* and PVA described by our group (Kumar et al., 2006).

As a result of ***chapter two***, screening of PVA producing microorganisms, we discovered new source of enzyme. Yeast, *Rhodotorula aurantiaca* (NCIM 3425) found to be a good PVA producer. The studies on optimal production of PVA and further enhancement of activity through process of permeabilization have been presented in the previous chapter. In the present chapter studies on purification of the intracellular PVA from yeast, *Rhodotorula aurantiaca* (NCIM 3425), and its biochemical characterization such as molecular weight, N-terminal sequence, kinetic parameters, substrate specificity and effect of different modulators on PVA activity are discussed.

5.2. Materials and Methods

5.2.1. Materials

Octyl-sepharose CL-4B, Sephacryl S-200, ampholites pH range 3-10, native molecular weight marker kit (Sigma MW-GF-200Kit) and p-dimethylaminobenzaldehyde (PDAB) were purchased from Sigma. Low molecular weight calibration kit for SDS electrophoresis was obtained from Amersham Biosciences. Penicillin V potassium salt (Pen V) and penicillin G were gift from Hindustan Antibiotics, Pune, India. Cephalosporin C, cephalexin, cefaclor, cloxacillin, dicloxacillin, GCLH (3-Chloromethyl-8-oxo-7-phenylacetylamino-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid benzhydryl ester), GCLE (3-Chloromethyl-8-oxo-7-(2-phenoxy-acetylamino)-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid 4-methoxy-benzyl ester) and 3-VBA (8-Oxo-7-(2-phenoxy-acetylamino)-3-vinyl-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2- carboxylic acid 4-methoxy-benzyl ester) were obtained from Lupin Ltd., Pune, India. All other chemicals used were of analytic grade.

5.2.2. Enzyme assay

Penicillin V acylase activity was measured by the method of Bomstein and Evans (1965) as modified by Shewale et al. (1987). Potassium salt of phenoxymethyl penicillin (Pen V) was used as a substrate in the enzyme assay. 10 μl of the enzyme was mixed with 290 μl of substrate solution containing 20 mg/ml Pen V in 0.1 M sodium citrate buffer pH 5.5. The reaction mixture was incubated for 10 min at 40 ^{o}C with gentle shaking. The reaction was stopped by addition of 300 μl of 1.0 M citrate phosphate buffer (CPB), pH 2.5. The reaction mixture was centrifuged and an aliquot of 150 μl was processed for estimation of 6-APA by addition of 450 μl of CPB and 600 μl of 0.6% (w/v) p-dimethylaminobenzaldehyde (PDAB) in methanol. One unit (IU) of PVA activity is defined as the amount of enzyme producing 1 μmol of 6-APA per minute under the conditions defined. The substrate concentration 20 mg/ml was maintained to measure the enzyme activity in all the experiments.

5.2.3. Growth of microorganism and enzyme production

R. aurantiaca cells were grown under optimal conditions for production of penicillin V acylase as described in ***chapter four***. 500 ml Erlenmeyer flasks, containing 125 ml of minimal medium containing 0.3 % tryptone, pH 8.0, were seeded with 10 % inoculum and incubated for 16-18 h at 180 rpm and 27 ^{o}C. Cells were harvested by centrifugation at 6,000 rpm for 30 min at 4 ^{o}C, stored frozen and processed for purification of enzyme.

5.2.4. Purification of enzyme

The frozen *R. aurantiaca* cells were thawed and used for process of enzyme purification. All subsequent steps were carried out at 4^{o}C.

5.2.4.1. Preparation of cell free extract

Cell pellet was suspended in cell suspension buffer (50 mM phosphate buffer containing 1 mM EDTA and 1 mM DTT), 1 g cells in 3 ml buffer, and disrupted by sonication in ice bath four times for 2 min each at 80 amplitude with 0.8 sec. pulse on and 0.6 sec, pulse off using Branson Sonifier. Cell debris was

removed by centrifugation at 10,000 rpm for 30 min and then processed further. The cell free extract was diluted with the same cell suspension buffer and then process for ammonium sulphate precipitation.

5.2.4.2. Ammonium sulphate fractionation

The supernatant from above step was bought to 0.4 saturation by gradual addition of fine powder of ammonium sulphate, while stirring. The precipitated protein was then collected by centrifugation at 10,000 rpm for 30 min and supernatant was discarded. The precipitate was then dissolved in 50 mM phosphate buffer pH 6.0, containing 1 mM EDTA, and 1 mM DTT and this crude enzyme was then processed for hydrophobic interaction chromatography.

5.2.4.3. Hydrophobic interaction chromatography

The crude enzyme was loaded on Octyl-Sepharose column (2.5 × 25 cm) pre-equilibrated with 10 mM phosphate buffer pH 6.0, containing 24 % (w/v) ammonium sulphate. After loading enzyme, column was washed with the same buffer to remove un-bound proteins. The column was washed with 10 mM phosphate buffer, pH 6.0 containing 2 % (w/v) ammonium sulphate and subsequently with 10, 5, 2.5 mM plain phosphate buffer, pH 6.0. The enzyme was then eluted with 1 mM phosphate buffer, pH 6.0. Fractions of 3 ml each were collected on an automated fraction collector and 50 µl from each fraction was tested qualitatively for enzyme activity. Fractions containing PVA activity were pooled, concentrated with ultrafiltration using Amicon unit with PM-10 membrane. Concentrated sample then was treated with ammonium sulphate to a final concentration of 24 % (w/v) and rechromatographed as above, on another Octyl-Sepharose column (1.5 × 10 cm). Enzyme eluted with 1mM phosphate buffer, pH 6.0 was concentrated using ultrafiltration unit with PM-10 membrane. Enzyme was stored in aliquots at -20 ^{o}C.

5.2.5. Polyacrylamide gel electrophoresis

Samples were analyzed on native polyacrylamide gel electrophoresis (native-PAGE, 10 %) according to Laemmli (1970) to check the purity. Sodium

dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in slab gels using Tarson SDS-PAGE apparatus with 1 mm spacers. The enzyme was dissociated by heating in a boiling water bath in presence of SDS-PAGE loading dye. Sample (25 µl) containing 10 µg 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. (1988).

5.2.6. Characterization of penicillin V acylase

5.2.6.1. Isoelectric focusing (IEF)

Isoelectric focusing using polyacrylamide gel (10 % w/v) was performed according to the method of Vesterberg (1972). The electrophoresis was carried out in duplicate tube gels using ampholines of pH range of 3-10. For IEF, 250 µg of purified protein was used. A constant current of 12 mA and an initial voltage of 300 V for 3 h, and then 600 V were applied for 16-18 h at 10 ^{o}C. The tube gels were removed at the end of run and one was stained for protein using Coomassie Brilliant Blue R-250 and subsequently distained using a mixture of methanol:acetic acid:water (4:1:5 volume). The other one was cut into small segments which were subsequently put into tubes for elution with glass distilled water. Eluates of the gel slices were checked for pH and enzyme activity.

5.2.6.2. Determination of molecular weight of Rhodotorula aurantiaca penicillin V acylase

5.2.6.2.1. Native molecular weight determination

Molecular weight of native protein was calculated according to Andrews (1964) by size exclusion chromatography performed at 4 ^{o}C using Sephacryl S-200 column (1.5 X 100 cm) matrix. The mobile phase consisted of 10 mM sodium phosphate buffer, pH 6.0 containing 1 mM DTT, freshly prepared. The column matrix and buffer were degassed before use. The flow rate, 8 ml/h of the column, was maintained using peristaltic pump with the fraction size of 2 ml. Mr, the relative molecular weight, was determined by calibration curve using wide range

gel filtration molecular weight protein standards under identical conditions (Sigma MW-GF-200Kit), which include β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). Blue dextran was used to determine the void volume (V_o) of the column. The marker proteins (1.5 mg each) were loaded separately on the column. The elusion volume (Ve) for each marker protein was determined by measuring the buffer volume eluted upon the midpoint of the elution peak of the particular protein. The concentrated purified PVA was loaded separately under the same conditions. A standard curve was plotted for Ve/Vo *versus* log molecular weight of standard proteins. The standard curve obtained with the marker proteins gave the molecular weight of the enzyme.

5.2.6.2.2. Determination of molecular weight of subunits

Subunit molecular weight of the enzyme was determined by SDS-PAGE slab gel electrophoresis (Laemmli, 1970) using Tarson SDS-PAGE apparatus with 1 mm spacers and the samples electrophoresed alongside lower range molecular weight markers kit from Amersham Biosciences. The kit contained phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and α-lactalbumin (14.4 kDa). The enzyme was dissociated by heating in a boiling water bath in presence of SDS-PAGE loading dye. Sample (25 µl) containing 10 µg of purified PVA was loaded on 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. (1988).

The migration distance of the marker proteins, purified enzyme and dye marker on SDS-PAGE gel was measured, after the staining of the gel. Corresponding relative mobility (Rf) values of each of protein markers and enzyme was calculated by dividing migration distance of the protein with migration distance of the dye marker. A calibration curve was constructed by graphing Rf *vs* log molecular weight of the proteins and the molecular weight of the purified enzyme was determined from the curve.

5.2.6.2.3. Matrix–assisted laser desorption ionization/time-o-flight mass spectrometry (MALDI-TOF)

The mass spectrum was recorded by using time-of-flight delayed extraction MALDI mass spectrometer (Applied Biosciences). 5 μl sample was mixed in a microcentrifuge tube with 35 μl matrix solution. The matrix solution of 15 mg/ml sinapic acid was prepared in 30 % acetonitrile (ACN). About 10 μl of the mixture was applied to a stainless steel sample holder and introduced into the mass spectrometer after drying. The spectrum was obtained in reflectron mode by scanning 5 laser shots with an ion source voltage 1 of 19 kV, ion source 2 of 16.27 kv, 100 ns delay and the low mass gate at *m/z* 600.

5.2.6.3. N-terminal amino acid sequence analysis

To determine N-terminal sequence, the purified PVA was electroplated on polyvinylidene difluoride (PVDF) membrane (Bio-Rad) after electrophoresis on SDS-PAGE (Introgen NuPAGE system: 4-12 % Bis-Tris gel, MES/SDS running buffer). The enzyme band was cut out and used for N-terminal Edman sequencing. The analysis was carried out at Faculty of Biological Sciences, University of Leeds, UK.

5.2.6.4. Effect of temperature and pH on PVA activity and stability

For determination of the optimum temperature of PVA, activity was measured at different temperatures in the range of 30-70 ^{o}C at pH 6.0 in 0.1M phosphate buffer. For the thermostability, the enzyme was incubated at 25, 30, 40, 50 and 60 ^{o}C up to 2 h in 0.1 M phosphate buffer pH 6.0 and residual activity was estimated at intervals.

. To ascertain the optimum pH of enzyme, activity was determined at 40^{o}C in the pH range 4.0-9.0 using 0.1 M buffers: acetate buffer for pH 4.0 and 5.0, citrate buffer for pH 5.5, phosphate buffer for pH 6.0 and 7.0 and Tris–HCl for pH 8.0 and 9.0. For pH stability, the enzyme was incubated in 0.1 M buffers of pH range 4.0-9.0, using different buffers for 2 h at 25^{o}C. Residual activity was measured at intervals by standard enzyme assay.

5.2.6.5. Effect of potential modulators on enzyme activity

Various metal ions, chelating agents, organic solvents, reducing agents, detergents and other amino acid specific modifiers were added to enzyme solution in independent experiments, and incubated at 25 ^{o}C for 15 minutes. Relative catalytic activity of penicillin V acylase was measured. Enzyme reaction without addition of modulator was considered as control. The reaction was carried out in 100 μl volume with 50 μg of enzyme and varying concentrations of modulators, 20 μl of sample was withdrawn and employed for standard enzyme assay.

5.2.6.6. Substrate specificity

For determination of substrate specificity, 5 mg/ml of various substrates such as penicillin G, amoxicillin trihydrate, ampicillin, cephalosporin C, cephalosporin G, cephalexin, cefaclor, cloxacillin, dicloxacillin, GCLE, GCLH and 3-VBA containing amide bonds were used as substrates instead of Pen V for activity measurement by enzyme assay as described in ***section 5.2.2***.

5.2.6.7. Kinetic studies

The Michaelis-Menten constant (*Km*) with Pen V as substrate was determined by incubating the enzyme at various concentrations of Pen V (1.0-200 mM) under standard assay conditions, then fitting a linear regression curve to data points using Lineweaver-Burk plot. Catalytic turn over number *(k*cat) value was calculated from the equation

V*max* = *k*cat [E]t

5.2.6.8. Inhibition kinetics

Reaction product and substrate analogues such as phenoxyacetic acid (POAA) and taurodeoxycholic acid (TDCA), glycoldeoxycholic acid (GDCA), deoxycholic acid (DCA), taurocholic acid (TCA), phenylacetic acid (PAA), respectively were tested for their inhibitory effects on the enzyme activity at various concentrations. And finally, in an independent experiment to determine the value of dissociation constant for phenoxyacetate (*Ki,* POAA), enzyme and

different concentrations of inhibitor were pre-incubated for sufficient time to allow the system to reach equilibrium. The enzyme-inhibitor mixture was then assayed at saturated substrate concentration (100 x *Km*), which resulted in the dissociation of inhibitor and regeneration of enzyme activity. *Ki* was calculated from Lineweaver-Burk plots.

5.2.7. Protein contents

Protein was estimated by method of Lowry et al. (1951) using crystalline bovine serum albumin (BSA) as standard.

5.3. Results and Discussion

5.3.1. Purification, physical characterization and N-terminal sequencing of PVA

Hydrophobic interaction chromatography using Octyl-sepharose CL-4B allowed separation of the enzyme from undesired proteins. After elution of fractions without activity, the PVA activity was eluted with plain 1 mM phosphate buffer pH 6.0. Results of the purification procedure are summarized in ***Table 5.1***. Enzyme was purified 10 fold with 5.84 IU/mg specific activity and over all yield of 10.5 %. The enzyme was electrophoretically homogenous as seen in native-PAGE and SDS-PAGE gels (***Fig. 5.1 a and b***). Isoelectric focusing gel showed a single band at pH 4.19 (***Fig. 5.1 c***). The molecular weight (M_r) of the pure native enzyme determined to be 36,000 ± 1,000 Da by gel permeation chromatography and SDS-PAGE has shown single band of 36,000 Da, suggesting that the enzyme is a monomeric protein. The molecular weight of the enzyme was confirmed with the help of MALDI-TOF (***Fig. 5.1d***), which showed major peak at 36,693 Da. Plots for the determination of native and subunit molecular weight of *R. aurantiaca* penicillin V acylase by gel filtration chromatography and SDS-PAGE, is presented in ***Figure 5.2a and 5.2b***, respectively.

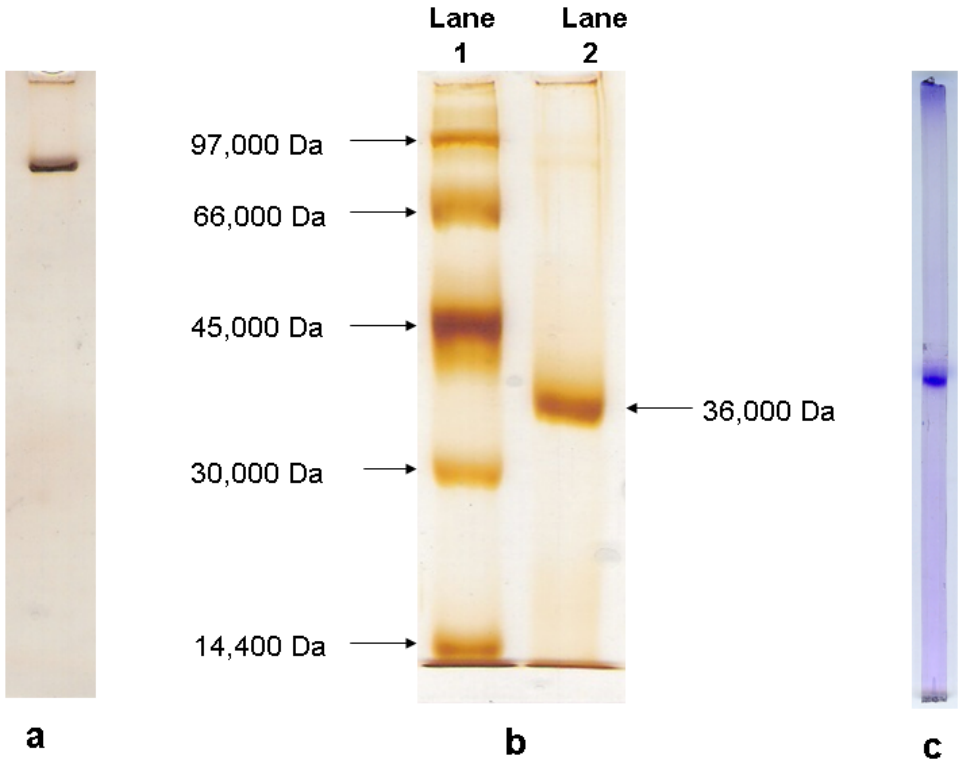


Figure 5.1:

a. Non denaturing 10 % (w/v) polyacrylamide gel electrophoresis.

b. Denaturing polyacrylamide gel electrophoresis (SDS-PAGE), 10 % (w/v) Lane 1- Molecular weight markers, Lane 2- PVA.

c. Isoelectric focusing (IEF) gel, 10 %.

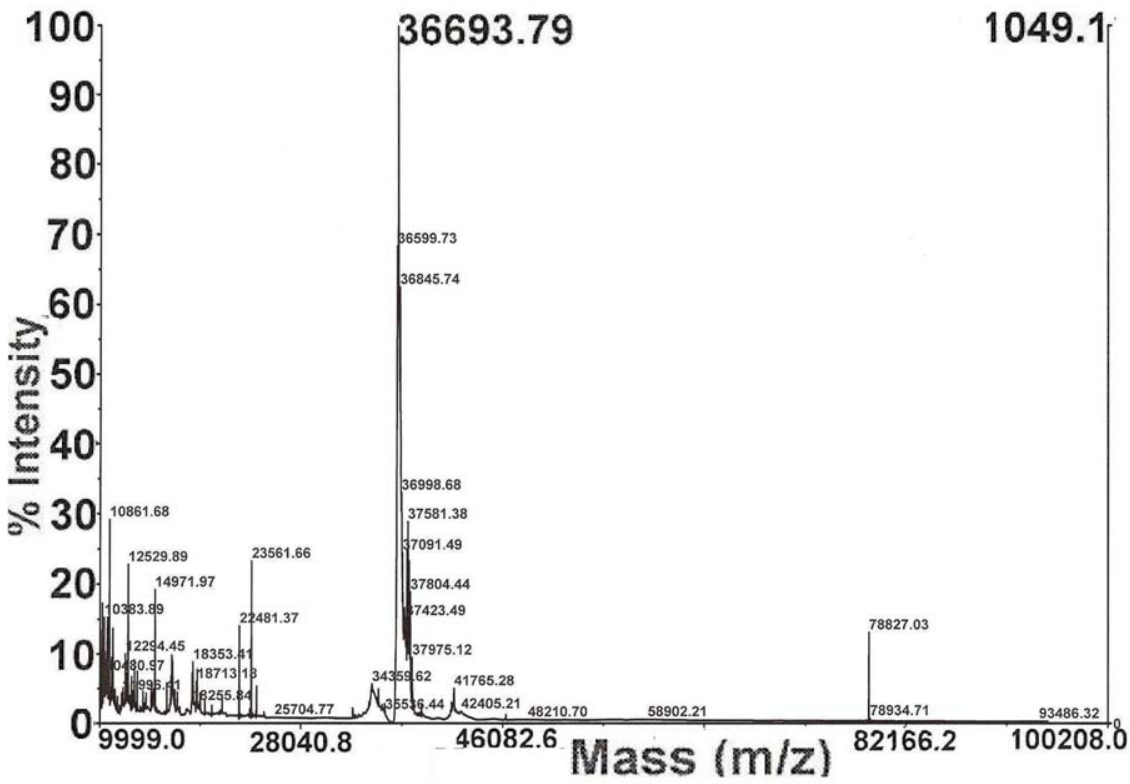


Figure 5.1d: Matrix–assisted laser desorption ionization/time-o-flight mass spectrometry (MALDI-TOF) spectrum of purified PVA.

Table 5.1: Purification summary of penicillin V Acylase from Rhodotorula aurantiaca (NCIM 3425)

Purification steps	Total protein (mg)	Total units (IU)	Specific activity (IU/mg)	Fold purification	Yield (%)
-Cell free extract	203	115.7	0.57	------	100
-Ammonium sulphate fractionation	115	101.2	0.88	1.54	88
-Octyl-Sepharose chromatography (I)	14.2	25	1.76	3.08	21.5
-Octyl-Sepharose chromatography (II)	2.1	12.26	5.84	10.2	10.5

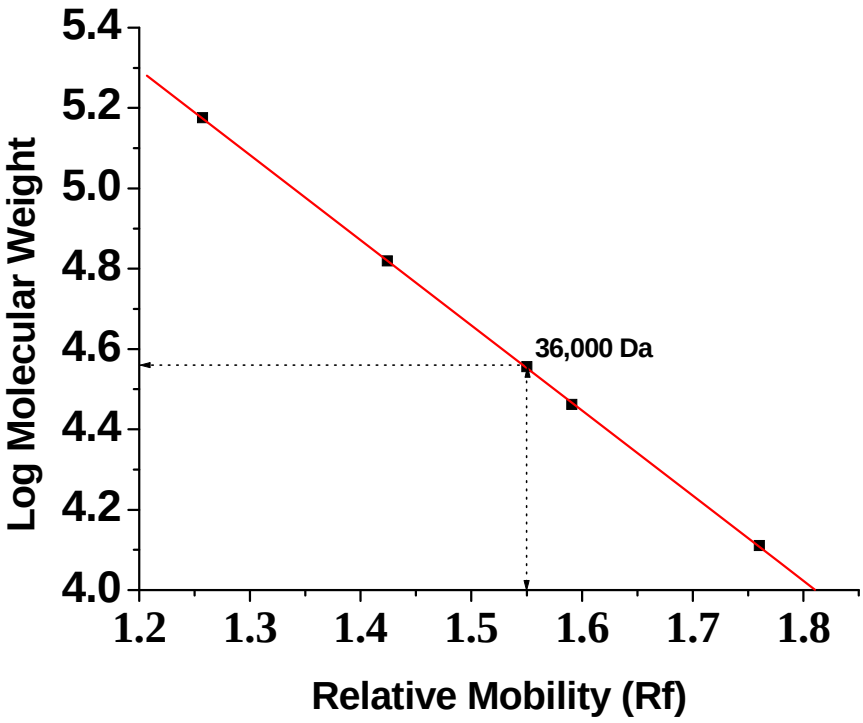


Figure 5.2a: Determination of native molecular weight of R. aurantiaca Penicillin V acylase by gel filtration chromatography. Standard curve obtained with molecular weight marker proteins such as β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

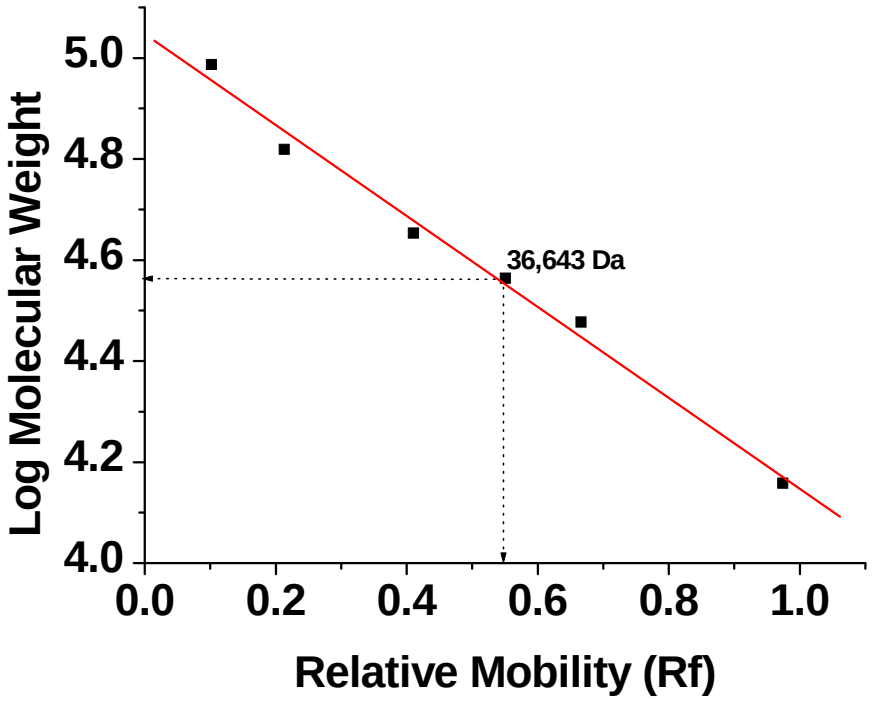


Figure 5.2b: Determination of Sub-unit molecular weight of R. aurantiaca Penicillin V acylase by SDS-PAGE. Standard curve obtained with 10 % SDS PAGE gel and molecular weight markers such as phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and α-lactalbumin (14.4 kDa).

We have purified and characterized PVA produced by a psycrophilic yeast *R. aurantiaca* (NCIM 3425). Single band on native and denaturing PAGE and the consistency of the calculated molecular mass from gel filtration chromatography with that calculated from SDS-PAGE data suggests that enzyme exists as a monomer of 36 kDa. The enzyme exhibited *pI* of 4.19. Sudhakaran & Shewale (1995) have reported a single polypeptide PVA of 83.2 kDa from *Fusarium* sp. SKF235. Vandmme & Voets (1975) reported PVA of 62 kDa. Recently Koreishi et al. (2006) reported acylase of 80 kDa from *Streptomyces mobaraensis* which is a heterodimeric enzyme with alpha and beta subunits. The enzyme was further recognized as penicillin V acylase, by Zhang et al. (2007). They cloned and the kinetic parameters were compared with the other beta-lactam acylases. We have already reported a homotetrameric PVA from *B. sphaericus* with native and subunit molecular weight of 138 and 35 kDa respectively and *pI* of 4.8 (Pundle & SivaRaman, 1997) and now we are reporting a smallest functional single unit PVA, not documented so far.

The purified enzyme consisted of a single subunit with molecular mass of approximately 36,000 Da by SDS-PAGE, as described earlier. The band was transferred to PVDF membrane for analysis of the N-terminal amino acid sequence. The resulting N-terminal sequence of *Rhodotorula aurantiaca* PVA (*Ra*-PVA) was determined to be Ser-Lys-Gln-Ile-Val-Ile-Leu-Gly-Ala-Gly.

It is interesting to note that, a BLAST search using N-terminal sequence of ten residues of *Ra*-PVA resulted in 100 % match to the N-terminal sequence of enzyme NADH dehydrogenase (EC 1.6.99.3), (pyridine nucleotide-disulfide oxidoreductase) from various *Bacillus* sp. and not corresponded to any of N-terminal sequences of beta-lactam acylases. However the enzyme did not show dehydrogenase activity. Though N-terminal sequence is homologous to NADH dehydrogenase, possibly *Ra*-PVA might have active site topology similar to penicillin acylases which will be revealed only after resolution of 3-dimensional structure of *Ra*-PVA. The present study showed that the enzyme has serine residue as a nucleophile at its N-terminus instead of cysteine as reported by Suresh et al. (1999) from *B. sphaericus* and Rathinaswamy et al. (2005) from *B. subtilis.*

5.3.2. Effect of pH and temperature on PVA activity /stability

The enzyme showed optimum activity within rather a narrow pH range of 5.0 to 6.0. Maximum activity was observed at pH 5.5 and activity dropped off rapidly above pH 6.0 with only about 20% was remaining at pH 7.0 (***Fig. 5.3a***). No loss of activity over 2 h was observed when the enzyme was incubated between pH 5.5-6.0 and 25^{o}C (***Fig 5.3b***).

PVA activity was determined from 30 to 70^{o}C. The optimum temperature of purified PVA was 45^{o}C. The activity dropped sharply beyond 50^{o}C with retaining practically no activity at 70^{o}C (***Fig. 5.4a***). The enzyme was very stable at room temperature and remained fully active for 2 h. It retained 100% activity at 40^{o}C for 30 min. The enzyme lost its 50% activity at 60^{o}C after 30 min and only 14% and 6% of the activity was left when it was incubated for 2 h at 50 and 60^{o}C, respectively (***Fig. 5.4b***).

Olsson et al. (1985) reported that the optimum pH and temperature of intracellular PVA from *B. sphaericus* was 5.8 and 37^{o}C, respectively. pH optima of *Ra*-PVA is similar but temperature optima is little higher (45^{o}C) than PVA from *B. sphaericus*. The enzyme was stable at lower pH which is favorable for the stability of penicillins and 6-APA (Shewale & Sudhakaran, 1997). PVA from *Fusarium oxysporum* has optimum temperature same as *Ra*-PVA but has higher optimum pH 7.4 -7.6 (Sheng & Ye, 1989). Temperature optima of PVA from *Erwinia aroideae*, *S. lavendulae* and *S. mobaraensis* are 36, 40 and 37^{o}C, respectively (Vandmme & Voets, 1975; Torres et al., 1998; Zhang et al., 2007) where as *Ra*-PVA has higher optimum temperature.

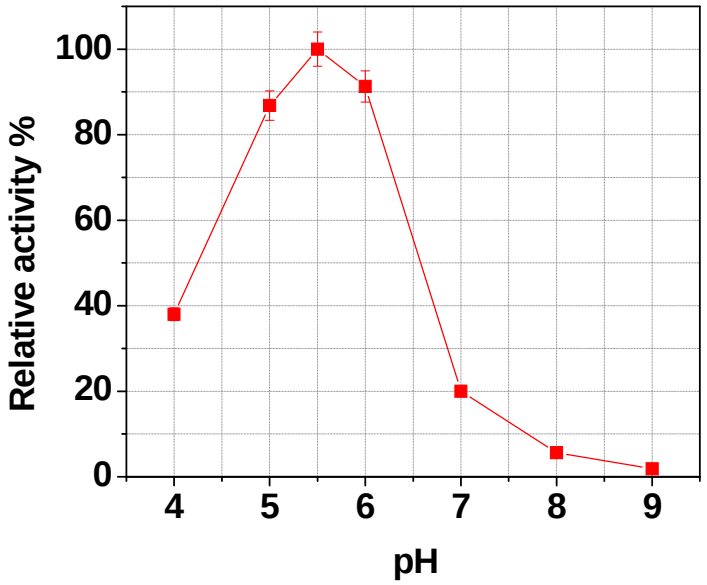


Figure 5.3a: Effect of pH on Ra-PVA activity. The activity was determined at 40^{o}C at different pH using different buffers. The activity at pH 5.5 was set as 100% (8.28 IU/ml).

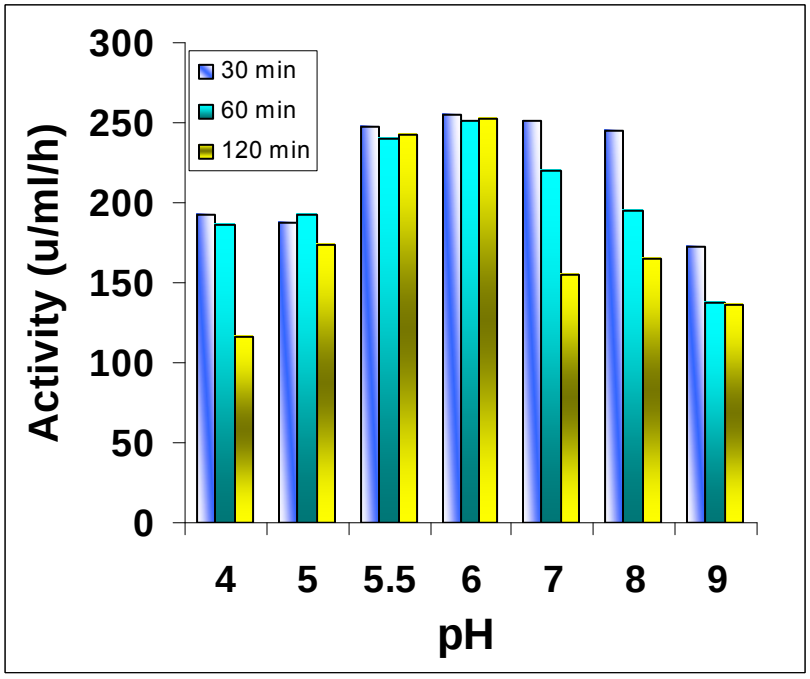


Figure 5.3 b: Stability of Ra-PVA at various pH of buffers

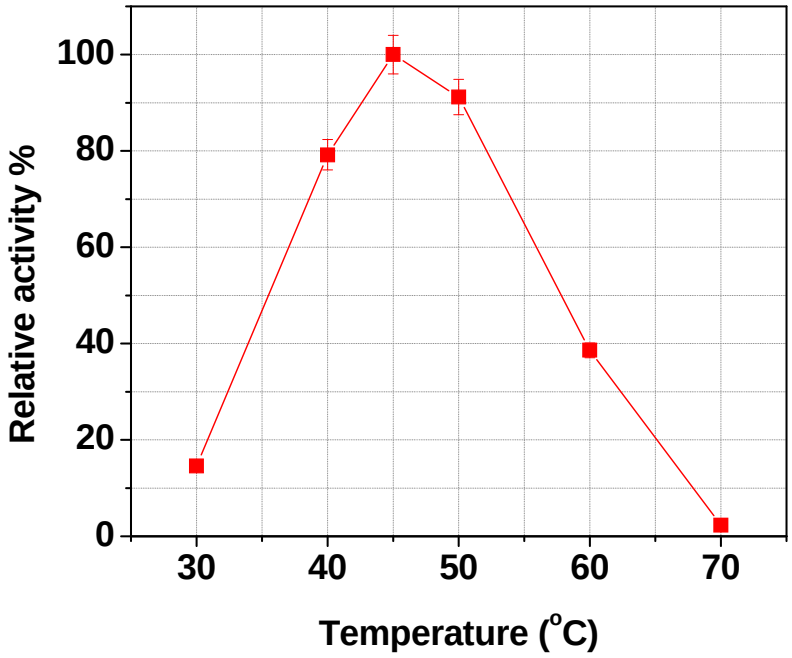


Figure 5.4a: Effect of temperature on PVA activity. The activity was determined at different temperatures at pH 5.5 in 0.1 M sodium citrate buffer. The activity at 45°C was set as 100 % (11.25 IU/ml)

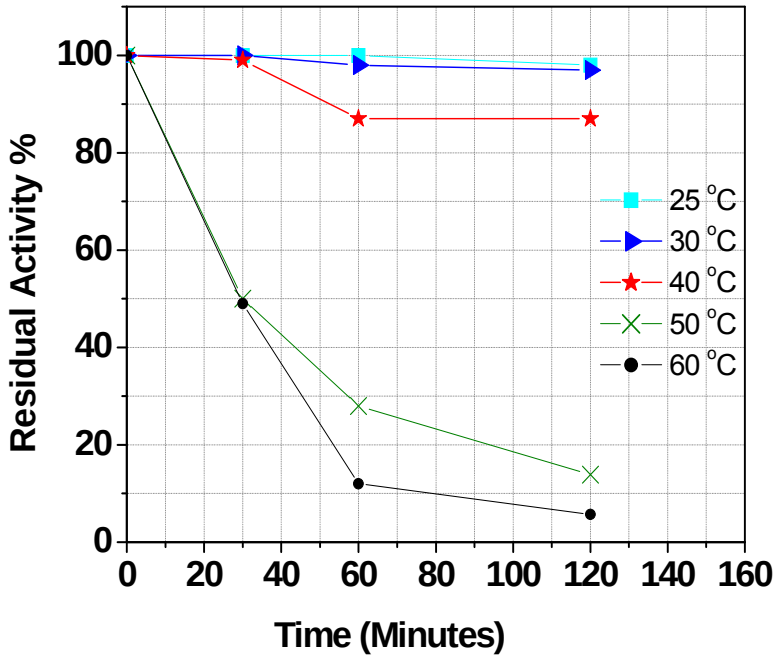


Figure 5.4b: Thermostability of PVA. Enzyme was incubated at 25, 30, 40, 50, 60°C up to 2 h in 0.1 M sodium citrate buffer, pH 5.5. Residual activity as measured at 40°C. The activity of PVA incubated at 25°C was set as 100 % (8.34 IU/ml).

5.3.3. Substrate specificity

Hydrolytic activity of the enzyme was studied on different synthetic and natural substrates as shown in ***Table 5.2***. Enzyme exhibited relatively high specificity for the side chain structure. Pen V was the best substrate for the enzyme. Enzyme hydrolyzed synthetic substrates such as GCLH, GCLE and 3-VBA to more than 10 % where as natural substrate (another analogue) such as penicillin G less than 10 %.

Substrate specificity indicated that *Ra*-PVA is different from *B. sphaericus* PVA, as the latter was incapable to cleave ampicillin, amoxicillin and cephalosporin C (Pundle & SivaRaman, 1997). The synthetic substrates such as GCLH, GCLE and 3-VBA are used in pharmaceutical industry to synthesize semi-synthetic antibiotics. These substrates were used in the present study and *Ra*-PVA hydrolyzed one of the substrates, 3-VBA, up to 30 %. This feature of enzyme becomes more interesting in the current situation of increased use of semi-synthetic cephalosporins in the medical practice.

The structure of new synthetic substrates, used in pharmaceutical industry, are given in ***Figure 5.5.***, which contained benzyl side chain with amide bond at C 7 position and various 'R' groups attached at C 4 position of cepham β lactam ring. Enzyme had very less activity on clavulanate, ampicillin and amoxicillin and cephalosporin C. It hydrolyzed cephalexin and cefaclor more than 10 % where as had no activity on cloxacillin and dicloxacillin.

Structures of new substrates showed benzyl side chain with amide bond at C 7 position and various 'R' groups attached at C 4 position of cepham β-lactam ring, amide bond cleaved by the enzyme to release the core structure for the further synthesis of antibiotics. To achieve better substrate specificity, the enzyme can be modified genetically by using mutations in this case. Zhang et al. (2007) reported PVA from *Streptomyces mobaraensis* which exhibited hydrolytic activity towards various β-lactam antibiotics. High specificity for both the side chain and the intact β-lactam ring structure was also observed in the case of penicillin V acylase from *Erwinia aroideae* (Vandmme & Voets, 1975).

Table 5.2: Substrate specificity of PVA from Rhodotorula aurantiaca. Relative activity was expressed as the percentage of the activity towards penicillin V (8.7 IU/ml). All measurements were repeated three times.

Substrate	Relative activity (%)
Penicillin V	100
Penicillin G	9.3
GCLH	13.2
GCLE	10
3-VBA	28.9
Cephalexin	10.3
Cephaclor	13.6
Clavulanate K	3.5
Amoxicillin trihydrate	5.7
Cephalosporin C	5.7
Ampicillin Na	6

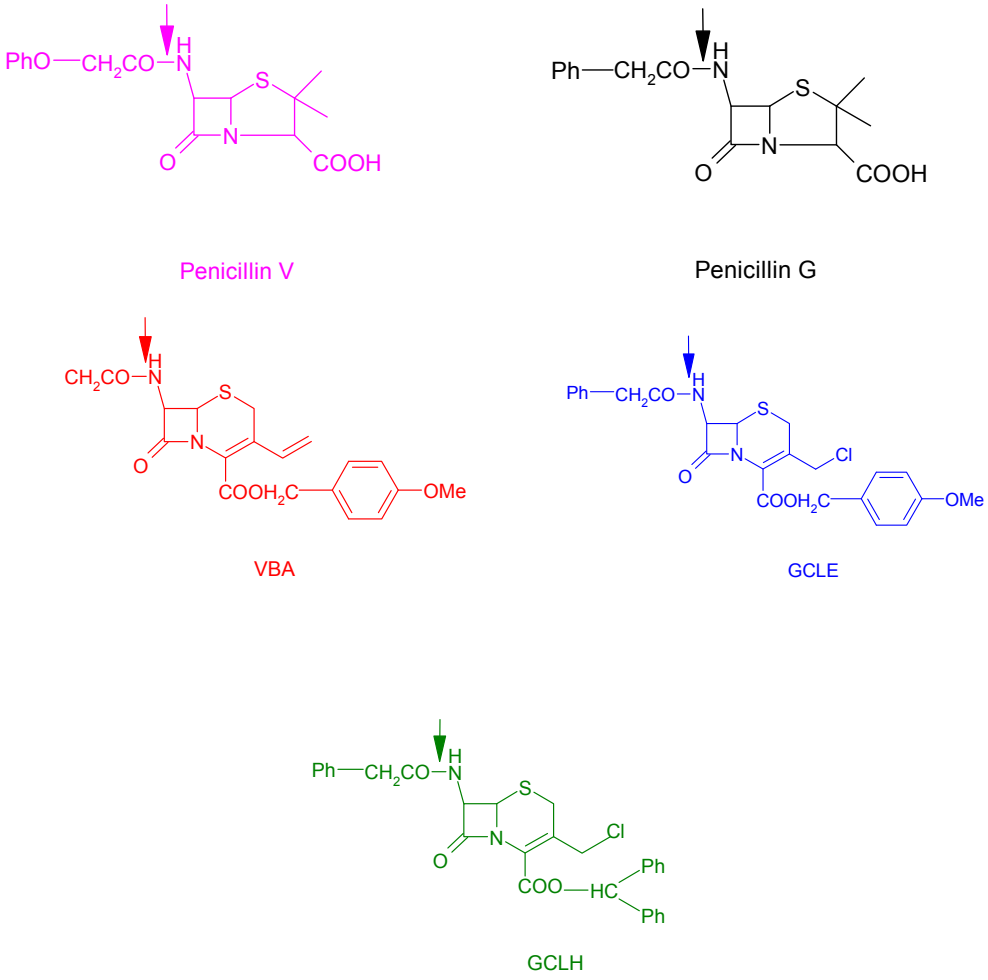


Figure 5.5: Structural formulae of substrates used for specificity test of PVA from Rhodotorula aurantiaca. Arrow indicates the site of hydrolysis.

5.3.4. Effect of potential modulators (metal ions/organic solvents/chelating agents/reducing agents/detergents and inhibitors)

Effect of metal ions and chelating agents on PVA activity is depicted in ***Table 5.3***. Amongst the metal ions tested, the PVA activity was stimulated by Mg^{++}, Mn^{++}, Ni^{++}, Co^{++}, Zn^{++}, Ca^{++} and K^{+} where as Fe^{++} and Fe^{+++} inhibited it moderately. The activity was severely inhibited by Cu^{++}, Hg^{++} and Cd^{++}. EGTA and EDTA stabilized the enzyme activity.

We have studied effect of metal ions on *Ra*-PVA activity. Inhibition of enzyme activity by the heavy metal ions was possibly due to involvement of sulfhydryl group in the hydrolysis of substrate, as earlier reports are available about the presence of cysteine residue at active site of PVA (Suresh et al., 1999).

The effect of organic solvents on PVA activity is indicated in ***Table 5.4.*** Amongst the water miscible solvents methanol, 2- propanol, n-butanol inhibited the enzyme activity where as ethanol stimulated it up to 105% at 3% (v/v) concentration. Aprotic polar solvents acetonitrile, dimethylsulphoxide (DMSO) and acetone inhibited the enzyme activity to some extent, where as 1-4 dioxane and tetrahydrofurane (THF) severely inhibited it when enzyme was incubated for 15 min with 3 and 7% (v/v) of solvents. Water immiscible solvents, such as chloroform, phenol and benzylalcohol severely inhibited the PVA activity. Ethyl acetate stimulated the activity at 3% (v/v) concentration but further addition of solvent lead to inhibition of PVA activity. Hexane stimulated PVA activity at 3 and 7% (v/v).

The effect of reducing agents, detergents and specific amino acid modifiers on PVA activity is shown in ***Table 5.5.*** Reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol (BME) enhanced the enzyme activity at 1 and 5 mM. Ascorbic acid increased PVA activity up to 105% at lower concentration of 1 mM but further addition of reagent inhibited the activity of enzyme. Tween 80 and Tween 20 enhanced the enzyme activity up to 133% and 128% respectively where as Triton X-100 inhibited it. Cationic detergent CTAB has increased the enzyme activity at 0.1% (w/v) concentration but further addition of detergent led to inhibition of enzyme activity. The enzyme activity was severely inhibited by N-Bromosuccinamide (NBS), phenylglyoxal,

phenylmethylsulphonylfluoride (PMSF) and p-hydroxymercuribenzoate (PHMB) at very low concentration of 1 mM, 10 mM, 0.5 mM and 0.5 mM, respectively.

Enzyme in presence of water organic co-solvent mixture may affect its catalytic properties and stability (Klibano, 1997). Results obtained by us for the effect of organic solvents on PVA activity are different from the results described by Arroyo et al. (1999 & 2000). They have demonstrated activation of extracellular penicillin V acylase from *S. lavendulae* in presence of organic solvents such as alcohols and aprotic polar solvents however it is not true in case of *Ra*-PVA. Direct binding of solvents to the specific binding sites of enzyme could be the contributing factor that determines modulation in the activity of protein/enzyme (Liepinsh & Otting, 1997). Enhancement of enzyme activity in presence of reducing agents implies that thiol group is contributing to the enzyme catalysis as reported by Suresh et al. (1999). Anionic detergent SDS severely inhibited the enzyme activity, possibly due to drastic changes in conformation of protein; also SDS is known to cleave disulfide bonds and denature protein. Inhibition of activity in presence of NBS, phenylglyoxal, PMSF and PHMB predict presence of tryptophan, arginine, serine and cysteine at or near the catalytic centre of enzyme. Catalytically active serine and cysteine was reported by Zhang et al. (2007) from *S. mobaraensis* PVA and Suresh et al. (1999) from *B. sphaericus* PVA, respectively.

Table 5.3: Effect of metal ions and chelating agents on PVA activity. Activity without metal ions and chelating agents was set as 100% (9.17 IU/ml).

Metal Ions	**Relative activity (%)**	
	1mM	5mM
Cu++	26.4 ± 2.1	15.6 ± 0.6
Mg++	100.5 ± 2.3	109.6 ± 1.6
Mn++	111.3 ± 1.2	108.8 ± 4.2
Fe++	41 ± 3.5	68 ± 2.0
Ni++	112.6 ± 2.7	125.6± 1.6
Hg++	7.3 ± 3.3	16.9 ± 4.5
Co++	100.8 ± 1.0	110.3 ± 1.4
Zn++	102.9 ± 1.4	107.6 ± 4.0
Ca++	98.2 ± 2.2	118.6 ± 6.1
K+	104.9 ± 3.4	107.4 ± 2.5
Fe+++	82.2 ± 3.0	59.4 ± 1.7
Cd++	55.5 ± 4.6	18.9 ± 1.8
Chelating agents	1mM	5mM
Ethylene glycol-bis (2 aminoethylether) tetra acetic acid	105.6 ± 3.1	123.5 ± 6.5
Ethylenediaminetetra acetic acid	101.3 ± 3.4	105.2 ± 1.8
Oxalate	97.2 ± 3.3	106.4 ± 1.3
Citrate	100.6 ± 1.6	99.6 ± 4.1
L-Tartarate	97.9 ± 6.2	106 ± 4.5

Table 5.4: Effect of organic solvents on PVA activity. Activity without organic solvent was set as 100 % (12.5 IU/ml).

Solvents	Relative activity (%)	
	3%	7%
Hexane	118.7 ± 2.1	112.9 ± 5.2
Dimethylsulfoxide	95.6 ± 1.2	93.7 ± 1.4
Dimethylformamide	89.2 ± 4.4	88.3 ± 1.3
Benzyl alcohol	9.3 ± 3.7	5.2 ± 4.1
Methanol	99.3 ± 3.3	90 ± 2.2
Ethanol	101.2 ± 1.1	97.9 ± 3.7
2-Propanol	27.5 ± 4.2	8.5 ± 2.9
n-butanol	82.7 ± 5.5	38.8 ± 8.8
Phenol	2.7 ± 3.0	1.6 ± 5.1
Acetonitrile	90.8 ± 3.4	93.1 ± 3.5
Tetraydrofurane	28.4 ± 4.0	3.6 ± 2.3
Acetone	98.2 ± 5.2	96.1 ± 3.6
Chloroform	93.5 ± 2.1	65.7 ± 7.6
1-4 Dioxane	18.3 ± 5.0	4.1 ± 2.4
Ethyl acetate	110.9 ± 5.8	77.4 ± 4.3

Table 5.5: Effect of reducing agents, detergents and inhibitors on PVA activity. Activity without the compounds was set as 100 % (9.57 IU/ml).

Compounds	**Relative activity (%)**	
Reducing agents	1 mM	5 mM
β-mercaptoethanol	109.2 ± 2.3	115.1 ± 2.5
Dithiothreitol	113.7 ± 4.9	119.9 ± 2.3
Ascorbic acid	105.1 ± 4.3	84.2 ± 2.1
Detergents	0.1%	0.5%
Tween 80	233.6 ± 5.6	216.8 ± 6.2
Tween 20	128.5 ± 4.6	114.9 ± 3.7
Triton X-100	5.5 ± 2.5	5.5 ± 3.2
*CTAB	130.2 ± 3.5	95.7 ± 5.2
Sodium dodecyl sulphate (SDS)	23.4 ± 4.4	1.9 ± 2.3
Inhibitors	**Concentration (mM)**	**Relative activity (%)**
N-Bromosuccinimide	1	2.7 ± 2.5
Diethyl pyrocarbonate	10	42.7 ± 5.1
Phenyl glyoxal	10	3.1 ± 2.2
N-acetylimidazole	10	67.9 ± 3.1
p-hydroxymercuribenzoate	0.5	27.2 ± 4.3
Phenylmethylsulphonylfluoride	0.5	41.3 ± 5.1
Woodward's reagent	10	47.3 ± 1.7
N-Ethyl-N- (3 dimethylaminopropyl) carbodiimide.	10	46.8 ± 4.4

*CTAB: N-cetyl-N,N,N trimethylammoniumbromide

5.3.5. *Kinetic studies*

PVA activity increased linearly with increase in substrate concentration up to 75 mM. Michaelis-Menten constant (*Km*) for purified penicillin V acylase was calculated from Lineweaver-Burk plot (***Fig. 5.6***) and found to be 20 mM. *k*cat and catalytic efficiency (*k*cat/*Km*) were calculated to be 4.83 s^{-1} and 0.24 mM^{-1} s^{-1}.

Ra-PVA has lesser *K*m and higher *k*cat and *k*cat/*K*m values than that of PVA from *Erwinia aroideae* reported by Nam et al. (Nam & Ryu, 1984). Kinetic studies revealed that enzyme is not much efficient for the commercial purpose however has immense academic importance, due to its unique monomeric structure.

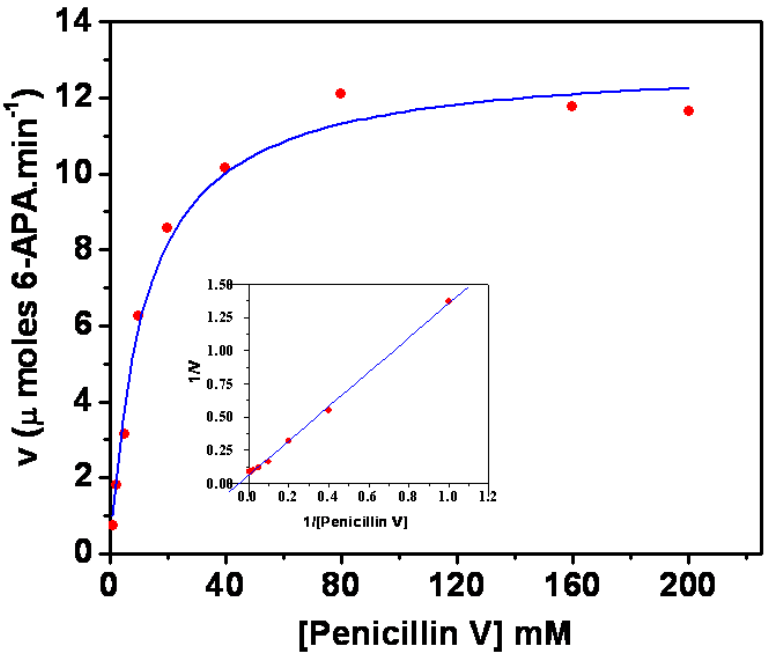


Figure 5.6: Michaelis-Menten kinetics of penicillin V acylase for the hydrolysis of penicillin V. The enzyme final concentration was 0.25 mg/ml. Inset: Lineweaver–Burk plot for the determination of Km and Vmax.

5.3.6. Ligand inhibition studies

Inhibition of enzymatic activity by conjugated bile acids (GDCA, TDCA, GCA, DCA), phenyl acetate (PAA) and phenoxyacetic acid (POAA) at different concentrations was investigated using homogeneous PVA preparation. All conjugated bile acids, PAA and POAA have shown decrease in the activity with the increase of concentrations from 10-40 mM (***Fig. 5.7***). Dissociation constant (*Ki*) of POAA against Pen V has been investigated. From Lineweaver-Burk plot, POAA showed competitive inhibition against the hydrolysis of Pen V. In order to characterize the system secondary plot (i.e. *Km'app vs* I) was also constructed. From the secondary plot, *Ki* POAA was found to be 1.5 ± 0.25 mM (***Fig 5.8 A, B and C***). Kumar et al. (2006) have shown similar results in case of conjugated bile acid hydrolase the substrate of which mimics with Pen V showing competitive inhibition.

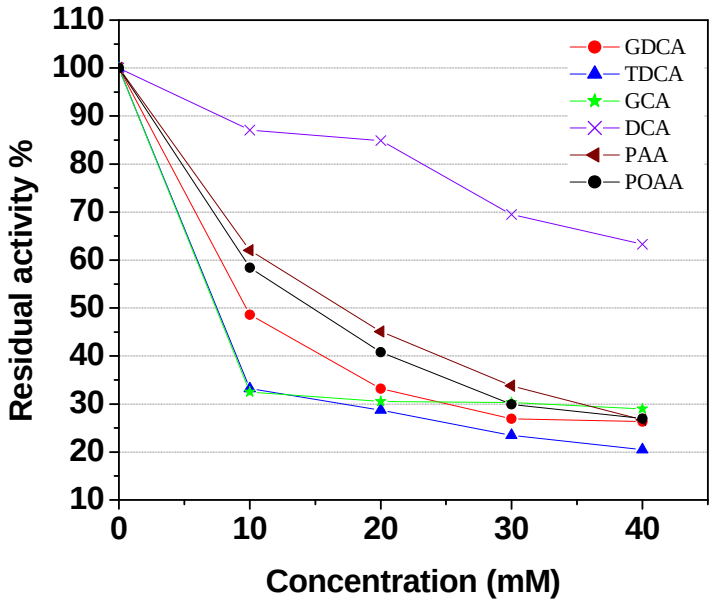
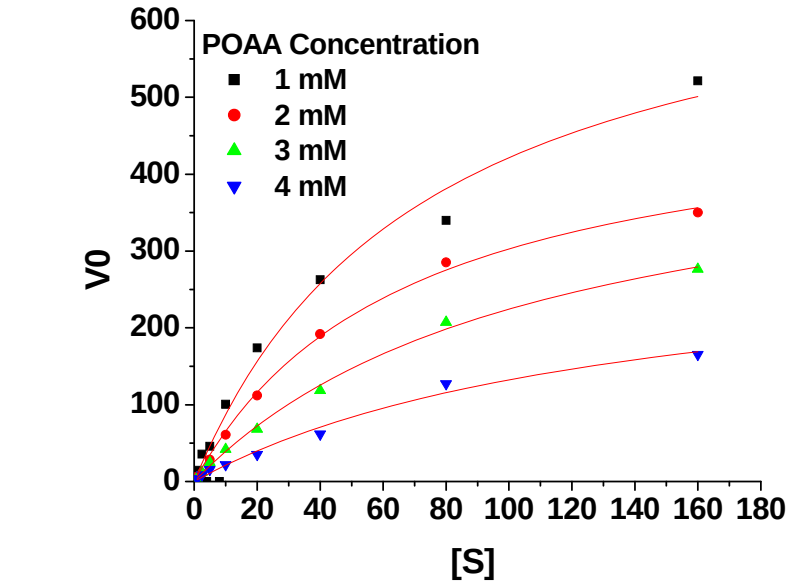
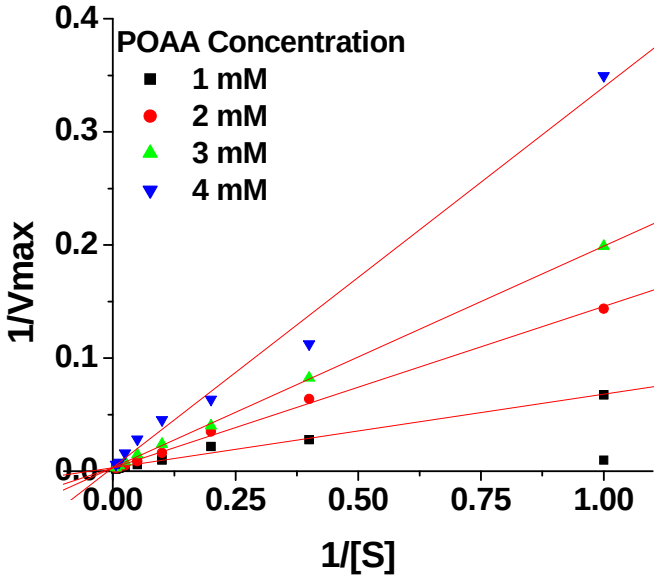


Figure 5.7: Ligands inhibition studies on PVA. GDC: Glycodeoxycholic acid, TDCA: Taurodeoxycholic acid, GCA: Glycocholic acid, DCA: Deoxycholic acid, PAA: Phenylacetic acid, POAA: Phenoxyacetic acid



A



B

Figure 5.8 A and B: Determination of inhibition constant of POAA: A) Simple curve fitting; B) L-B plot

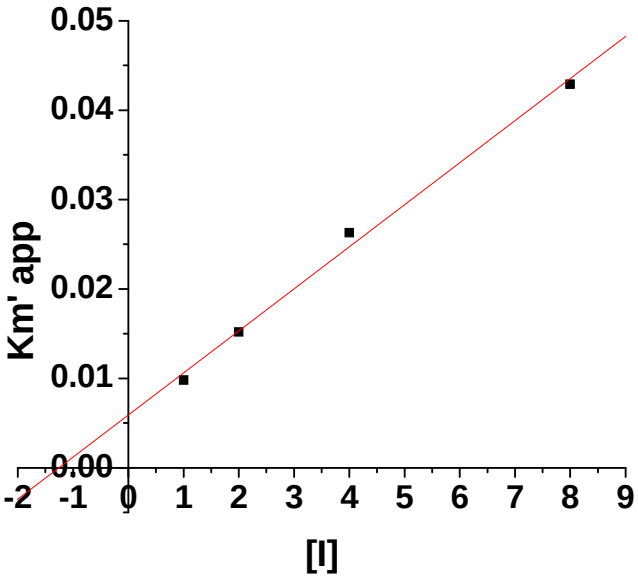


Figure 5.8 C: Determination of inhibition constant of POAA: Secondary plot

5.5. Conclusions

We purified and characterized PVA from a eukaryotic source, *Rhodotorula aurantiaca,* which is the smallest active monomeric penicillin V acylase, not reported so far. N-terminally located serine and 100 % sequence homology of ten N-terminal residues with the sequence of NADH dehydrogenase (pyridine nucleotide-disulfide oxidoreductase) from various *Bacillus* sp., but lack the activity of the same, are the interesting features of the enzyme. All penicillin acylases with N-terminally located serine as a catalytic residue have alkaline pH optima, hence it is exciting to note that despite *Ra*-PVA having N-terminally located serine, it has acidic pH optima, which is favorable for pharmaceutical industry. Though this enzyme may not be useful for the commercial purposes but the enzyme has great academic potential for the further studies. Crystallization of this enzyme is under progress, which will probably reveal the molecular details of active monomeric PVA from *R. aurantiaca*.

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Active site characterization and fluorometric studies of Penicillin V Acylase

Chapter 6

*Part of the work presented in this chapter has been **communicated**:*
*Atul Kumar, Nagaraj M. Gowda, Sushma Gaikwad & Archana V. Pundle **(2008)** Journal of Photochemistry and Photobiology*

Abstract

Penicillin V acylase (PVA), a member of newly evolved Ntn-hydrolase superfamily, is a pharmaceutically important enzyme to produce 6-animopenicilanic acid. Active site characterization of purified penicillin V acylase from the yeast source, *Rhodotorula aurantiaca* was carried out using various amino acid specific modifying reagents. Serine and tryptophan were found to be involved in the activity of *R. aurantiaca* penicillin V acylase (*Ra*-PVA). Modification of the protein with serine and tryptophan specific reagents such as PMSF and NBS showed partial loss of activity and substrate protection against inactivation. *Ra*-PVA was found to be a multi-tryptophan protein exhibiting one tryptophan, in native and four, in its denatured condition. The micro-environment of tryptophan residues in *Ra*-PVA under native and denatured conditions was investigated by solute quenching studies using neutral quenchers (acrylamide and succinimide), an anionic quencher (iodide ions) and a cationic quencher (cesium ions). The results obtained indicate that the tryptophan residues of *Ra*-PVA are largely buried in hydrophobic core of the protein matrix. Quenching of the fluorescence by acrylamide was collisional. Surface tryptophan residues were found to have predominantly more electro-positively charged amino acids around them, however differentially accessible for ionic quenchers. Denaturation led to shift in λmax from 336, in native state, to 357 nm and more exposed to the solvent, consequently increase in fluorescence quenching with all quenchers. This is an attempt towards the conformational studies of *Ra*-PVA.

6.1. Introduction

Penicillin acylases or penicillin amidohydrolases, EC 3.5.1.11 cleave the acyl side chain of penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acids (Shewale & SivaRaman, 1989). Two types of enzyme, penicillin V acylase (PVA) and penicillin G acylase (PGA), with different substrate specificities, account for enzymatic industrial production of 6-APA, the precursor of semi-synthetic penicillins. Their high efficiency has resulted in the replacement of conventional chemical process in favour of enzymatic ones by the industries (Sio & Quax, 2004). PVA is widely distributed among microorganisms, molds, yeast, bacteria and actinomycetes, intracellular as well as extracellular (Shewale & Sudhakaran, 1997). PVA from *Streptomyces lavendulae* has been extensively studied by Torres et al. (1998) and Arroyo et al. (1999 & 2000).

Penicillin acylases belong to N-terminal nucleophile (N-tn) hydrolase superfamily, which is constituted by the enzymes that share a common structural fold and possess a catalytic serine or cysteine or threonine residue at the N-terminal end (Brannigan et al., 1995). The chemical modification and the three dimensional structure of PVA from *B. sphaericus* (NCIM 2478), reported by our group, revealed catalytically active cysteine at the active site of enzyme (Pundle & SivaRaman, 1997; Suresh et al., 1999). We have also demonstrated the post-translational autoproteolytic processing of PVA from *B. sphaericus* by crystallization of three catalytically inactive mutants of the enzyme in precursor and processed forms (Chandra et al., 2005). Furthermore, the evolutionary relationship between a conjugated bile salt hydrolase from *Bifidobacterium longum* and *B. sphaericus* PVA has been described by our group (Kumar et al., 2006).

Studies on the intrinsic fluorescence properties have been widely used to obtain information about the protein structure and the confirmation changes induced by alteration of the environment and/or ligand binding (Lakawiez, 1999; Eflink & Ghiron, 1981; Grinvald & Steinberg, 1976). Tryptophan (Trp) residues present at the active site of the protein or on the exterior are relatively easy to study; and large number of studies, using chemical modification and/or fluorescence quenching, have been published, elucidating the environment and the role of these residues in such proteins (Privat et al., 1980; Peterman & Laidler,

1979 & 1980; Patanjali et al., 1984), however buried residues are much more difficult to study. Involvement of tryptophan in substrate binding was described by our group with the help of fluorescence studies in case of penicillin G acylase from *Kluyvera citrophila* (Kumar et al., 2007) however there are no reports available on florescence studies on penicillin V acylase so far.

PVA from a eukaryotic source, yeast, *Rhodotorula aurantiaca* (NCIM 3425) has recently been purified in our laboratory by hydrophobic interaction chromatography and characterized as a smallest ever reported monomeric PVA of 36 kDa (Kumar et al., 2008a). N-terminally located serine of *Ra*-PVA has given the evidence to belong to Ntn hydrolase superfamily. The treatment with different amino acid specific modifying reagents such as phenylmethylsulphonylfluoride (PMSF) and N-bromosuccinimide (NBS) showed inactivation of enzyme. The present chapter describes, for the first time, the presence of tryptophan and serine at or near active site of smallest PVA from *R. aurantiaca*. Based on the studies of steady state fluorescence, solute quenching, the exposure and environment of the tryptophan residues in the enzyme is also described in this chapter.

6.2. Materials and Methods

6.2.1. Production and purification of PVA from Rhodotorula aurantiaca

The fermentation of *R. aurantiaca* was carried out according to standardized conditions described in ***section 4.2.4.*** of the thesis, using 0.3 % tryptone in minimal medium pH 8.0. Purification of the enzyme was carried out as descried in ***Section 5.2.4.*** of the thesis.

6.2.2. Enzyme assay

PVA activity was estimated according to Bomstein and Evans method (1965), modified by Shewale et al. (1987) and as described in ***Section 5.2.2***. The released 6-APA was estimated using p-dimethylaminobenzaldehyde (PDAB). One unit (IU) of PVA activity is defined as the amount of enzyme, producing 1 µmol of 6-APA per minute under the conditions defined. The substrate concentration 20 mg/ml was maintained to measure the enzyme activity in all the experiments.

6.2.3. Protein estimation

Protein concentration was determined according to the method described by Lowry et al. (1951) using BSA as standard.

6.2.4. Active site characterization

Various amino acid specific modifying reagents were used for active site characterization of the enzyme. The list of various buffers and reagents used for modification of perticular amino acid is presented in the ***Table 6.1***.

6.2.4.1. Chemical modification of tryptophan and serine

The purified PVA (5.5 µM in 0.3 ml) and each of 50 mM, sodium acetate buffer, pH 5.0 and sodium phosphate buffer, pH 7.5, was incubated with different concentrations of NBS (10-200 µM) or PMSF (0.1-0.8 mM), respectively. The residual enzyme activity was measured under standard assay conditions by sampling (50 µL) reaction mixture at various time intervals upto 20 (2, 5, 10, 15 and 20 min) and 30 min (2, 5, 10, 20 and 30 min) for NBS and PMSF, respectively. The stock solutions of PMSF and NBS were made in absolute ethanol and milli Q water, respectively.

The number of tryptophan residues modified by NBS was calculated by measuring the decrease in the absorbance at 280 nm as described by Spande & Witkop (1967). Enzyme solution (1 ml, 13.8 µM) was titrated with aliquots of 5 µl each of freshly prepared NBS (5 mM). The NBS modification was also carried out under denaturing conditions with *Ra*-PVA. The enzyme was incubated for 16 h with 6 M guanidium hydrochloride (Gdn-HCl). The number of Trp residues modified was determined spectrophotometrically, assuming the molar extinction coefficient of 5,500 $M^{-1}cm^{-1}$ for the modified Trp at 280 nm (Spande & Witkop, 1967).

6.2.5. Substrate protection studies

Protection provided by substrate, Pen V, during modification reactions of NBS (0.2 mM) and PMSF (0.5 mM) was determined by incubating the enzyme with varying concentrations of Pen V up to the highest concentration of 50 mM, prior to treatment with modifying reagent under the reaction conditions.

Thereafter, the reaction mixture as well as the control was passed through Sephadex PD 10 desalting column and the residual activity determined under standard assay conditions. Deactivation in the column was estimated using control.

6.2.6. Circular dichroism (CD) analysis

Both the NBS and PMSF modified *Ra*-PVA samples were passed through Sephadex G 25 column to remove excess reagent and then the CD spectra were recorded. Untreated enzyme passed through the same column was used to record native spectra. The spectra were recorded on JASCO-710 spectropolarimeter from 190 to 260 nm using 1 mm path length at 25^{o}C and enzyme concentration of 0.5 mg/ml in 10 mM sodium phosphate buffer pH 6.0.

6.2.7. Fluorometric studies

6.2.7.1. Quenching of intrinsic fluorescence of Ra-PVA

Fluorescence measurements were performed for native and denatured protein with different quenchers like acrylamide (5 M), succinimide (2.5 M) (neutral quenchers), iodide (5 M) and cesium ions (5 M) (charged quenchers), on Perkin-Elmer LS 50B spectrofluorimeter at 28°C, using an excitation and emission slit width of 7 nm. 3.1 µM *Ra*-PVA in 10 mM sodium phosphate buffer, pH 6.0 was excited at 280 nm and the emission spectra were recorded in the range of wavelength 300–400 nm. Small aliquots of quencher stocks were added to protein samples and fluorescence spectra were recorded after each addition. Potassium iodide stock solution contained 200 µM of sodium thiosulfate to prevent formation of tri-iodide (I^{-3}). For quenching studies with denatured *Ra*-PVA, the protein was incubated with 6 M Gdn-HCl overnight at room temperature. Fluorescence intensities were corrected for volume changes before further analysis of quenching data.

The steady-state fluorescence quenching data obtained with different quenchers were analyzed by Stern–Volmer (Eq. 1) and modified Stern–Volmer (Eq. 2) equations in order to obtain quantitative quenching parameters (Lehrer, 1971)

$$Fo/Fc = 1 + Ksv\,(Q) \quad (1)$$

$$Fo/\Delta F = fa^{-1}1/\,[Ka\,fa\,(Q)] \quad (2)$$

Where *F*o and *F*c are the relative fluorescence intensities in the absence and presence of the quencher, respectively, (Q) is the quencher concentration. *K*sv is Stern–Volmer quenching constant; Δ*F*=*F*o−*F*c is the change in fluorescence intensity at any point in the quenching titration. *K*a is the quenching constant and *f*a is the fraction of the total fluorophores accessible to the quencher. Equation (2) shows that the slope of a plot of *F*o/Δ*F* versus $(Q)^{-1}$ (modified Stern–Volmer plot) gives the value of $(Kafa)^{-1}$ and its Y-intercept gives the value of fa^{-1}.

Table 6.1: List of various amino acid specific modifying reagents and the buffers used for chemical modification reaction

Modifying reagent	Effective concentration	Buffer	pH of Buffer 50 mM
NBS (Tryp)	1mM	Sod. Acetate buffer	pH 4.5
PHMB (Cys)	1mM	Sod. Acetate buffer	pH 5.5
PMSF (Ser)	10mM	Sod. Phosphate buffer	pH 7.5
W.R. (Carboxy grp)	10mM	Sod. Phosphate buffer	pH 6.0
DEPC (His)	10mM	Phosphate buffer	pH 7.0
Phenyl Glyoxal (Arg)	10mM	Tris/Hcl	pH 8.0
NAI (Tyro)	10mM	Sod. Borate buffer	pH 7.5

N-bromosuccinimide (NBS); P-hydroxymercuribenzoate (PHMB); Phenylmethylsulphonylfluoride (PMSF); Woodward's reagent (W.R.); diethyl pyrocarbonate (DEPC); N-acetylimidazole (NAI); trinitrobenzene sulfonate (TNBS).

6.3. Results and Discussion

The profound impact of penicillin acylases in the manufacture of antibiotics is the reason for continued interest in studying the determinants essential for catalysis of these enzymes. PVA from *Rhodotorula aurantiaca* (NCIM 3425) has been found to be a novel enzyme due to its monomeric nature and presence of serine on the N-terminal end. PMSF and NBS modified enzyme with 41.3 and 2.7 % residual activity at 0.5 mM and 1 mM, respectively, as described in the previous report (Kumar et al., 2008a).

Effect of various amino acid specific modifying reagents on *Ra*-PVA was studied by incubating the enzyme at the specific given conditions (***Table 6.1.*****)** and the results are displayed in ***Table 6.2***. The results are previously discussed in ***section 5.3.4***. of the thesis.

Table 6.2: Effect of various amino acid specific modifying reagents on penicillin V acylase by R. aurantiaca

Inhibitors	**Concentration (mM)**	**Residual activity (%)**
N-Bromosuccinimide (NBS)	1	2.7 ± 2.5
Diethyl pyrocarbonate (DEPC)	10	42.7 ± 5.1
Phenyl glyoxal	10	3.1 ± 2.2
N-acetylimidazole (NAI)	10	67.9 ± 3.1
p-hydroxymercuribenzoate (PHMB)	0.5	27.2 ± 4.3
Phenylmethylsulphonylfluoride (PMSF)	0.5	41.3 ± 5.1
Woodward's reagent (W.R.)	10	47.3 ± 1.7
N-Ethyl-N- (3 dimethylaminopropyl) carbodiimide (EDC)	10	46.8 ± 4.4

6.3.1. Serine modification by PMSF

Protein sequencing of *Ra*-PVA revealed the presence of serine at the N-terminal end (Kumar et al., 2008a). This prompted us to check the involvement of serine in catalysis hence active site characterization of purified *Ra*-PVA was carried out using PMSF as serine modifying reagent, and the results are depicted in ***Figure 6.1***. PVA being Ntn hydrolase, it is predicted that the N-terminal serine may be playing the important role of Nucleophile in the hydrolysis of Pen V. The plots of time dependent inactivation kinetics against logarithm of residual activity with various PMSF concentrations gave linear plots up to the test periods, indicating pseudo-first order kinetics of inactivation. The individual slopes of the plots were calculated to determine the respective first order constant (K_{app}) (***Fig. 6.1 A***).

The order of the reaction (n) was determined by plotting log [K_{app}] against log PMSF concentration [M] gave value n=1, indicating that the modification of a single serine residue resulted in inactivation of one mole of the enzyme (***Fig. 6.1 A Inset***). Pundle & SivaRaman (1997) and Rathinaswamy et al. (2005) have reported homo-tetramer PVA from *B. sphaericus and B. subtilis*, respectively, exhibiting cystiene as the catalytic residue. Recently, Zhang et al. (2007) have cloned and characterized PVA, from an actinomycete, *S. mobaraensis,* as a hetero-dimer exhibiting serine on its catalytic site, whereas this is the first time, we are reporting PVA from *R. aurantiaca*, a yeast, exhibiting serine at its active site.

The CD spectrum of modified *Ra*-PVA was similar to that of unmodified enzyme indicating no distinct structural changes on the treatment with PMSF have taken place (***Fig. 6.1 B***). The comparison of the CD spectra of the modified enzyme to that of untreated PVA indicated that the loss of activity is due to modification of selective residues only, and not due to disruption of the protein confirmation.

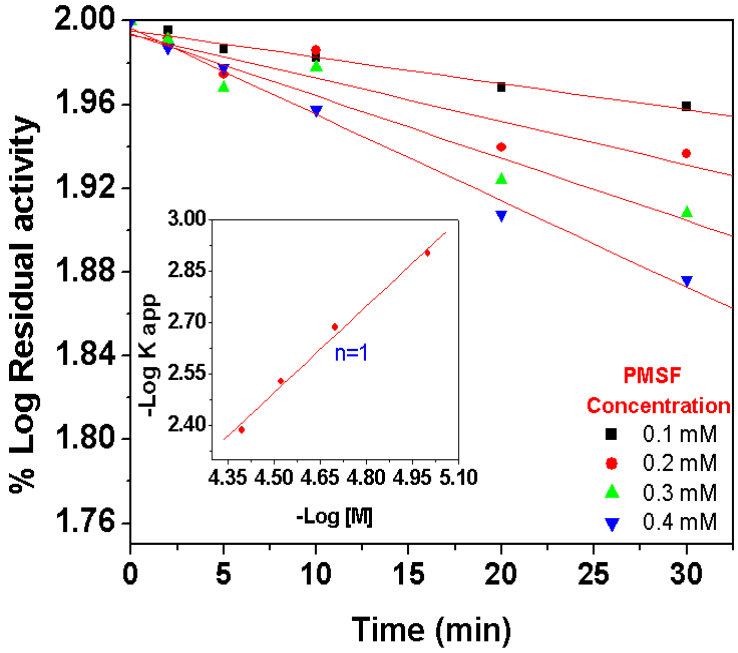


Figure 6.1 A: Time dependent inactivation kinetics of Ra-PVA, with respect to serine-specific modifying reagent, PMSF, at pH 7.5 and 25 ^{o} C. Inset: determination of order of reaction by PMSF. The pseudo-first-order rate constants (K_{app}) were plotted against log of various concentrations of PMSF.

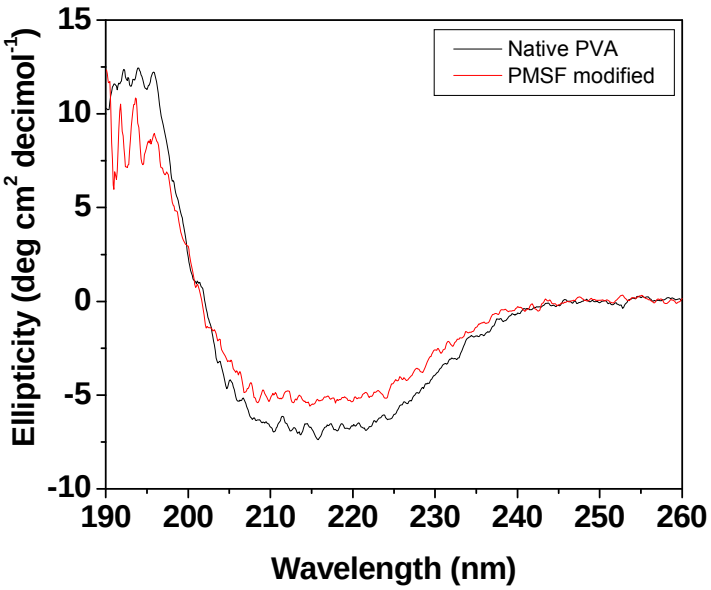


Figure 6.1 B: The Circular Dichroism (CD) spectra of native and PMSF modified Ra-PVA.

6.3.2. Tryptophan modification by NBS

NBS is a potent oxidizing agent. Due to its highly reactive source of electrophilic bromonium ions (Br^+), it is capable of adding bromine to the γ-δ carbon double bond of tryptophan which is subsequently cleaved. Treatment of *Ra*-PVA with 200 µM NBS at pH 5.0 resulted in 49 % loss of activity which indicated, involvement of tryptophan in catalytic activity of enzyme.

The plots of time dependent inactivation kinetics against logarithm of residual activity with various NBS concentrations gave linear plots up to the test periods, indicating pseudo-first order kinetics of inactivation. The individual slopes of the plots were calculated to determine the respective first order constant K_{app} (***Fig. 6.2 A***). The order of the reaction (n) was determined by plotting log [K_{app}] against log NBS concentration [M] gave value n=1, indicating that the modification of a single tryptophan residue resulted in inactivation of one mole of the enzyme (***Fig 6.2 A Inset***).

Spectrophotometric titration of *Ra*-PVA using 2 mM stock of NBS at pH 5.0 resulted in progressive decrease in absorption at 280 nm. The number of tryptophan residues, detected by NBS modification, in the native enzyme was found to be one (***Fig. 6.2 B***). The same experiment repeated with *Ra*-PVA denatured with 6 M Gdn-HCl gave four tryptophan residues per mole of enzyme.

The CD spectrum of modified *Ra*-PVA was similar to that of unmodified enzyme indicating no obvious conformational changes on the treatment with NBS have taken place (***Fig. 6.2 C***). The conformity of CD spectra of the modified enzyme to that of untreated PVA concluded that the loss of activity is due to modification of selective residues only, and not due to disruption in protein structure.

Recently, Kumar et al. (2007) have reported involvement of tryptophan residue in the substrate binding of *K. citrophila* PGA. To our knowledge, this is the first report indicating tryptophan residue to be present at or near the active site of PVA.

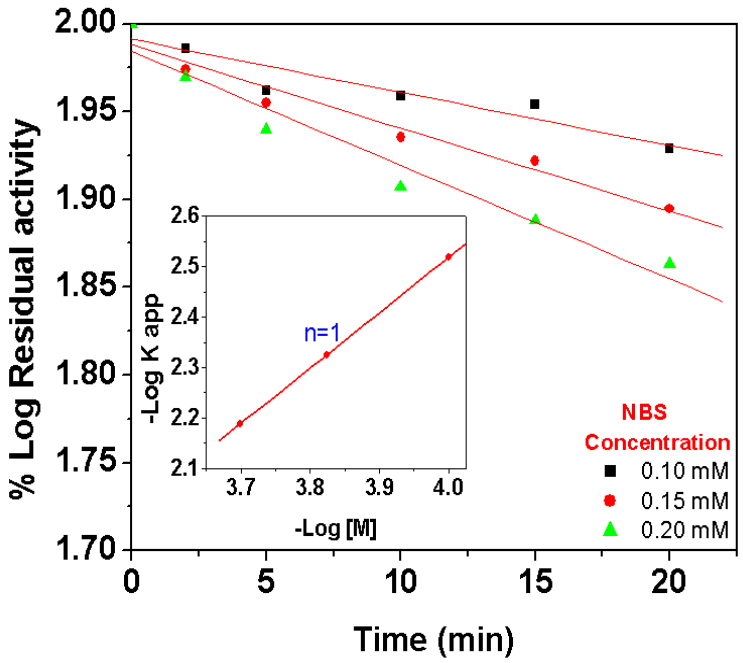


Figure 6.2 A: Time dependent inactivation kinetics of Ra-PVA, with respect to tryptophan-specific modifying reagent, NBS at pH 5.0 and 25°C. Inset: determination of order of reaction by NBS modification. The pseudo-first-order rate constants (K_{app}) were plotted against various concentrations of NBS.

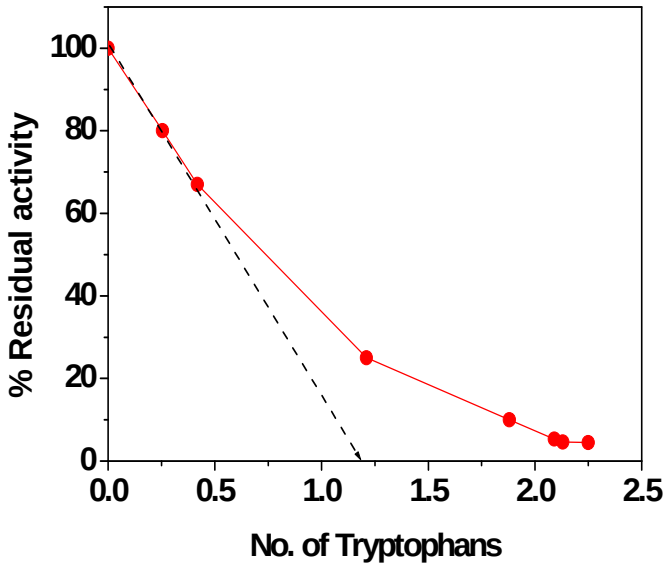


Figure 6.2 B: The titration plot of Ra-PVA with NBS to quantify tryptophan residues by the stepwise addition of NBS, as described in the text.

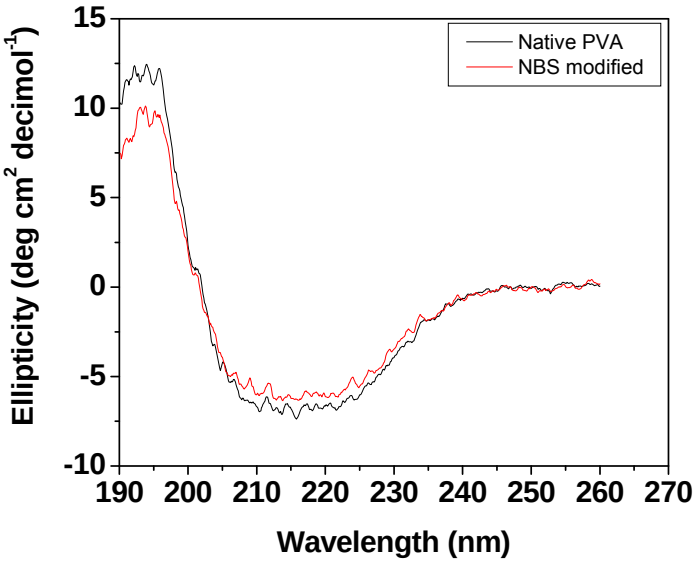


Figure 6.2 C: The Circular Dichroism (CD) spectra of native and NBS modified Ra-PVA.

6.3.3. Substrate protection studies

To confirm the catalytic residues of active center of enzyme, substrate protection studies were carried out. The protective action of the substrate, penicillin V on the inactivation of *Ra*-PVA by PMSF and NBS is shown in ***Table 6.3***. PMSF (0.5 mM) and NBS (0.2 mM) inactivated the enzyme with 37 and 49 % residual activity, respectively; whereas in the presence of 50 mM of Pen V (highest concentration tried), the percentage of enzyme activity retained was 65 and 83 in case of PMSF and NBS, respectively. Based on the observation it was confirmed that the enzyme is protected by the substrate from inactivation in presence of modifying reagents. The studies concluded that the modified residues are at the active site or close to it.

Table 6.3: Protection of Ra-PVA against inactivation by PMSF and NBS

Treatment	Enzyme activity (%)
PVA	100
PVA+ NBS (0.2 mM)	49
PVA+ Pen V (50 mM)+NBS (0.2 mM)	83
PVA+ PMSF (0.5 mM)	37
PVA+ Pen V (50 mM)+PMSF (0.5 mM)	65

6.3.4. Fluorometric studies

The exposure and environment of the tryptophan residues in *Ra*-PVA was investigated by solute quenching technique using steady state fluorescence studies. Fluorescence spectra of untreated *Ra*-PVA showed λmax at 336.0 nm upon excitation at 280 nm. Addition of PVA substrate, Pen V, resulted in progressive decrease in relative fluorescence intensity from 601 to 528 AU (12 % of original). This experiment was carried out to find out the exact concentration of substrate, Pen V, for the protection of *Ra*-PVA from NBS modification. The absorption spectra of pen V do not produce any peak in this region implying that

the quenching is purely due to interaction with tryptophan and not due to secondary absorption by ligand themselves.

Addition of small increments of NBS resulted in progressive decrease (36 % decrease) of relative fluorescence from 531 to 340 arbitrary units (AU), at final concentration of 25 μ M, without observing any shift in the wavelength (***Fig. 6.3***).

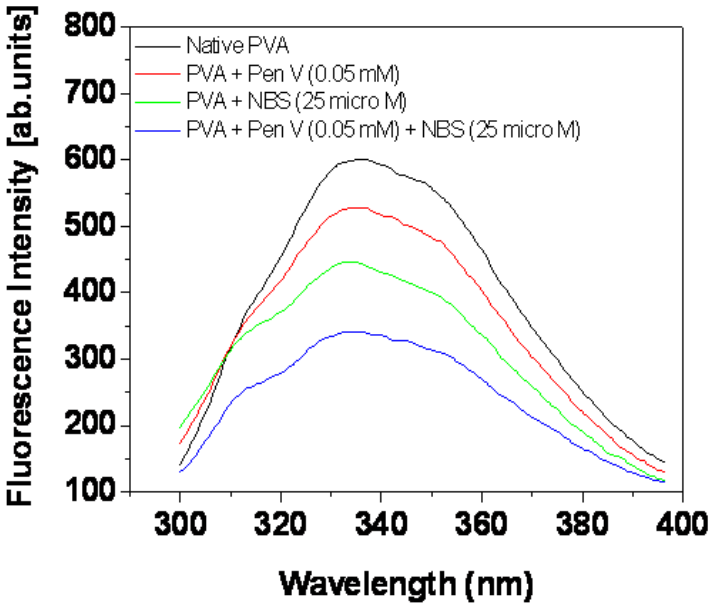
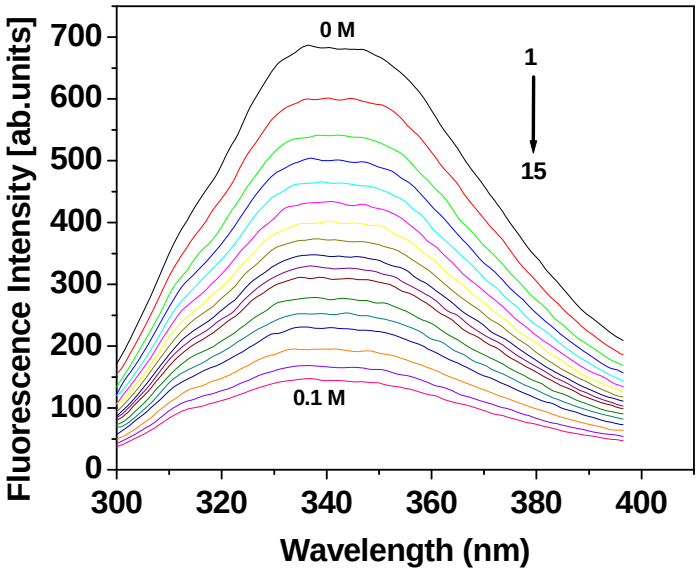


Figure 6.3: Substrate protection studies: Fluorescence spectra of Ra-PVA (2.8 μM, 2 ml), excited at 280 nm and λ max recorded at 336 nm.

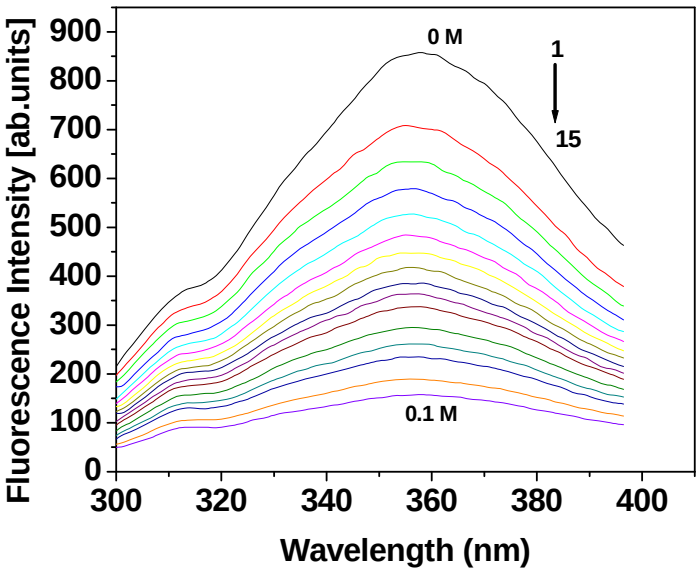
The substrate protection against fluorescence quenching by NBS was observed when *Ra*-PVA was pre-incubated with pen V (0.05 mM) before the addition of NBS. The drop in fluorescence upon addition of NBS at the highest concentration (25 μM) was only 16 % (531 to 442 AU) without alteration in the λmax (***Fig. 6.3)***. The presented results clearly indicated that of *Ra*-PVA exhibit tryptophan as a catalytic site residue which is involved in the substrate binding.

*6.3.4.1. Quenching of the intrinsic fluorescence emission of Ra-*PVA

Quenching studies were performed with *Ra*-PVA using neutral quencher (acrylamide and succinimide) an anionic quencher (iodide ions, I^-) and a cationic quencher (cesium ions, Cs^+). The fluorescence emission spectra of native *Ra*-PVA and denatured with 6-M Gdn-HCl, recorded in absence and presence of increasing concentrations of acrylamide are shown in ***Figure 6.4 A and B***, respectively. In both the cases, spectrum 1 is that of the native *Ra*-PVA in the absence of quencher and the spectra 2-15 correspond to *Ra*-PVA in the presence of increasing concentration of acrylamide, with spectrum 15 corresponding to a resultant quencher concentration of 0.1 M. The spectrum of the native enzyme showed maximum fluorescence intensity between 335 nm to 346 nm, showed λmax at 336 nm (***Fig. 6.4 A, spectrum 1***), indicating several populations of Trp, few in the hydrophobic environment and others differentially exposed to the polar environment. The denatured protein showed red shift in λmax to 357.8 nm, indicating polar environment due to exposure of the tryptophan residues. (***Figure 6.4 B, spectrum 1***).



A



B

Figure 6.4: Fluorescence spectra of Ra-PVA in the absence and presence of acrylamide. A. Under native conditions, B. under denatured conditions (6 M Gdn-HCl). Spectrum 1 corresponds to protein alone and spectra 2-15 correspond to the protein in the presence of increasing concentrations of acrylamide. The final concentration of acrylamide in both A and B is 0.1 M.

Tryptophan residues appeared to be uniquely sensitive to quenching by variety of solutes as a result of a propensity of the excited indole nucleus to donate electrons while in the excited state. Fluorescence spectra of the native and denatured PVA recorded in the absence and presence of the increasing concentrations of acrylamide displayed higher extent of quenching in the presence of 6 M Gdn-HCl, clearly revealing that unfolding results in a significant increase in the accessibility of the tryptophan residue to quencher. Also, denaturation led to significant increase in the extent of quenching with the other quenchers used in the study, namely succinimide, iodide and cesium ions (***Table 6.5***). The percentage of quenching was calculated on the basis of raw data.

Acrylamide and succinimide both are polar but uncharged quenchers. Succinimide not only has a larger molecular radius than acrylamide, but is also sterically much more rigid, so that its approach to buried fluorophores could be much more hindered. Acrylamide was the most effective quencher amongst all the quenchers used, which quenched 71.6 % of the total intrinsic fluorescence of protein, at a very less final concentration of 0.1 M, which is unusual, however succinimide quenched 42.5 %, due to its larger size and structural rigidity.

Quenching studies using negatively charged iodide ion has been quite popular for at least two main reasons. First, being charged, the ion does not probe the hydrophobic interiors of a protein and subsequently treated as a good reporter for information regarding surface tryptophan, secondly, iodide is an efficient quencher for tryptophan fluorescence (Lehrer, 1971). The ionic quenchers, iodide and cesium ions, which can not penetrate in the protein matrix and can access only surface exposed tryptophans, iodide ions (0.25 M) were found to quench 48 % of the total intrinsic fluorescence of *Ra*-PVA, which is quite higher, where as the least 23 % quenching observed in case of cationic quencher, Cs^+ (0.25 M).

Higher quenching achieved by iodide ions and lowest in case of Cs^+ with native PVA indicated surface tryptophans are in the positively charged environment. Additionally, lowest quenching achieved by Cs^+ appears to be due to the inability of the quencher to access the fluorophores. This could be due to the presence of positively charged residues in the vicinity of some of the exposed (or partially exposed) tryptophan residues, which repel the positively charged cesium ions, but allow the neutral acrylamide and succinimide and the negatively

charged iodide ion to approach the indole moieties of the tryptophan residues in their neighborhood.

The iodide ions could get concentrated in a positively charged environment in the vicinity of tryptophans which increases the probability of iodide ions colliding with them and quenching the fluorescence. Besides, low inherent quenching efficiency of Cs^+ may be partly responsible for the low quenching observed.

Denaturation of PVA resulted in a significant increase in the quenching by all the four quenchers, with extent of quenching observed being 78 %, 46 %, 25 %, and 64.5 with acrylamide (0.1M), succinimide (0.25M), Cs^+ (0.25M) and iodide ions (0.25M), respectively (***Table 6.5***). Even after the denaturation of enzyme, some residual conformation was present in the enzyme, which prevented the full access of neutral quenchers to the tryptophan therein.

Extent of quenching did not increase much in case of Cs^+ from 23 to 25, might be due to lack of electronegative environment in vicinity of tryptophans even after denaturation. Denaturation of *Ra*-PVA led to significant increase in the accessibility of tryptophan fluorescence by iodide ions indicated that the increase in electropositive environment of tryptophan residues (***Table 6.5***).

Table 6.5: Extent of fluorescence quenching of Ra-PVA with different quenchers

Quenchers	**Quenching (%)**	
	Native	In 6 M Gdn-HCl
Acrylamide (0.1M)	71.6	78
Succinimide (0.25 M)	42.5	46
CsCl (0.25 M)	23.1	25.6
KI (0.25 M)	48.3	64.5

6.3.4.2. Analysis of fluorescence quenching data

The Stern–Volmer plots for the quenching of *Ra*-PVA with different quenchers are shown in ***Figure 6.5***. The quenching profiles obtained for native and denatured protein with acrylamide and iodide follow linear dependence on the quencher concentration (***Fig. 6.5 a and d***) indicating collisional type of quenching. The profiles obtained with succinimide, and cesium ions for native PVA exhibited downwards curvature (***Fig. 6.5 b and c***) showing that certain tryptophans are selectively quenched before others in a protein. At low concentration of quencher, the slope of the Stern-Volmer plots reflects largely the quenching of the more accessible residues. At higher concentrations, the easily quenched fluorescence has been depleted, and those tryptophans having lower quenching constants become dominant.

Similar quenching patterns have been observed for several multi-tryptophan proteins (Lehrer, 1971; Teale & Badley, 1970; Sultan & Swamy, 2005). For denatured protein, all the four quenchers showed linear Stern–Volmer plots making it clear that upon denaturation, even buried tryptophans become exposed to solvent and are more accessible for quenching. These results indicate the presence of tryptophans in *Ra*-PVA in different environments where some tryptophans are partially or fully exposed to solvent while others are buried inside hydrophobic environment. These observations are in good agreement with the fluorescence spectrum of the native enzyme and also with the results of chemical modification experiments with NBS, where only one tryptophan residue in *Ra*-PVA could be modified under native condition and four tryptophan residues could be modified upon denaturation of protein. From the slopes of the two linear components of the Stern–Volmer plots, collisional quenching constants, K_{sv1} and K_{sv2} were obtained for succinimide, I^- and Cs^+ and are listed in ***Table 6.6***.

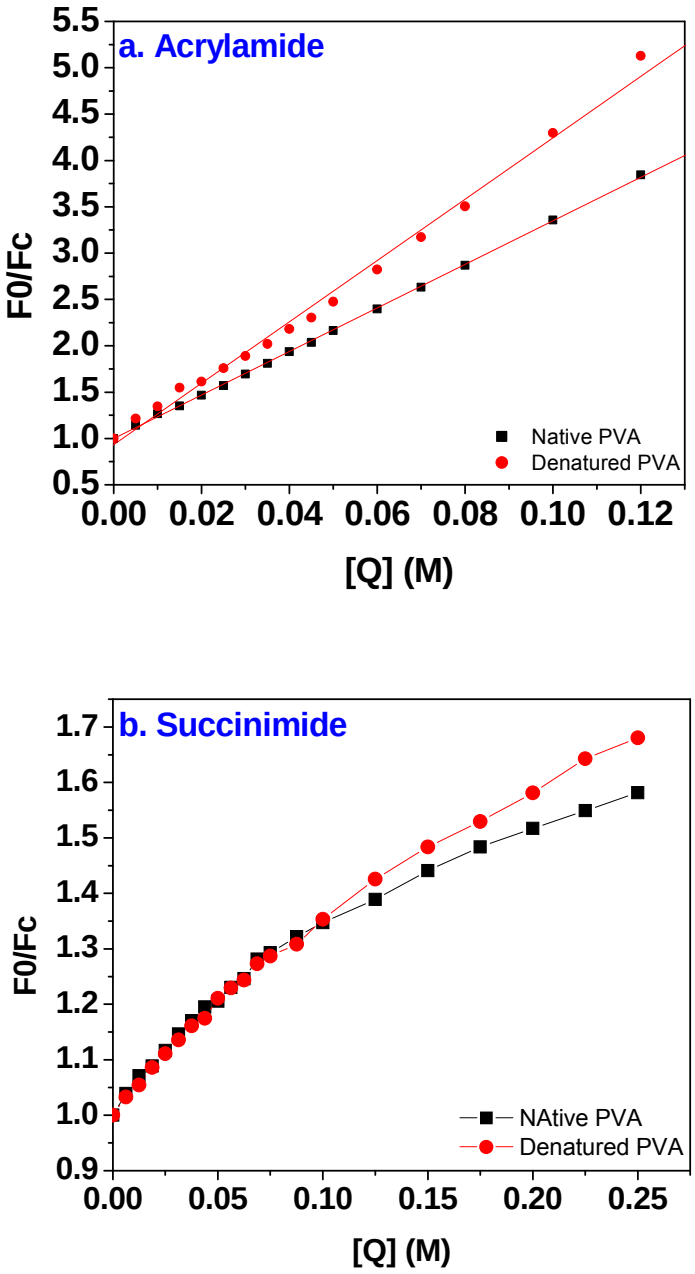


Figure 6.5: Stern-Volmer plots for the quenching of the intrinsic florescence of Ra-PVA with different quenchers a. acrylamide, b. succinimide, in native and denatured conditions (6 M Gdn-HCl).

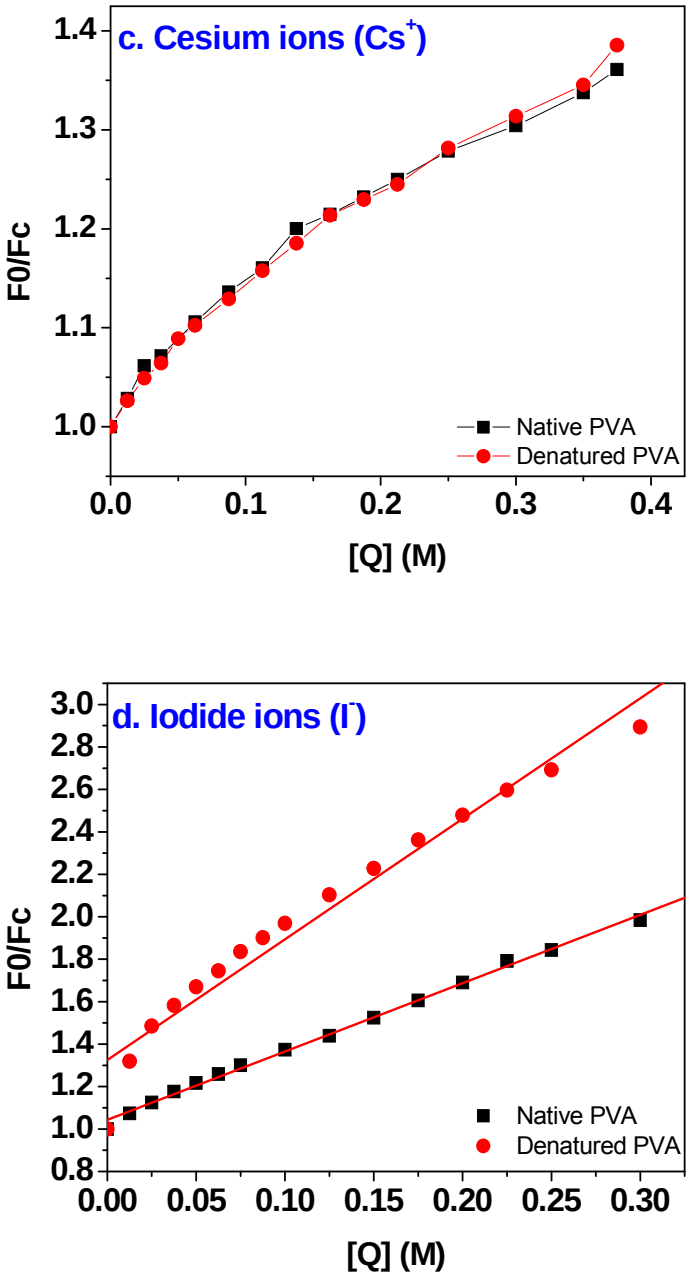


Figure 6.5: Stern-Volmer plots for the quenching of the intrinsic florescence of Ra-PVA with different quenchers c. Cs^+ ions and d. iodide ions, in native and denatured conditions (6 M Gdn-HCl).

K_{sv1} is greater than the K_{sv2} in case of succinimide and cesium ions giving substantial evidence for selective tryptophan quenching by these quenchers. Upon denaturation, the K_{sv1} increased in acrylamide from 23.6 to 31.5 and iodide ions from 3.27 to 5.9. Slight decrease in the K_{sv1} value was observed for succinimide and cesium ions (***Table 6.6***). The K_{sv1} value for Cs^+ is lower than that for I^-. The higher K_{sv} value of I^- as compared to Cs^+ indicates higher efficiency of quenching by iodide ions due to the presence of an electropositive environment around Trp residues in the enzyme.

Modified Stern–Volmer plots obtained with all four quenchers are shown in ***Figure 6.6.***, from which *fa* or fractional accessibility of the total fluorescence and *K*a, quenching constant were obtained according to Eq. 2 and listed in ***Table 6.6***. Based on the *fa* values of the four quenchers used, 100.0% and 40.0% of the total fluorescence was found to be accessible to acrylamide and succinimide respectively, and 53.0% and 32% was accessible to I^- and Cs^+, respectively. Denaturation of protein with 6 M Gdn-HCl also led to 100% accessibility with acrylamide. For succinimide, and I^- the fraction accessible increased to 49.0%, and 60.0%, respectively, however accessibility to Cs^+ almost retained same (31.3 %).

It is interesting to note that, the K_{sv} value for acrylamide has increased upon denaturation indicating the rate of quenching increased when the enzyme was denatured, however the accessibility was 100 % under both the conditions, native as well as denatured PVA. On the other hand K_{sv} value for cesium ions is decreased however the accessibility remained almost same (31.3 %) (***Table 6.6***).

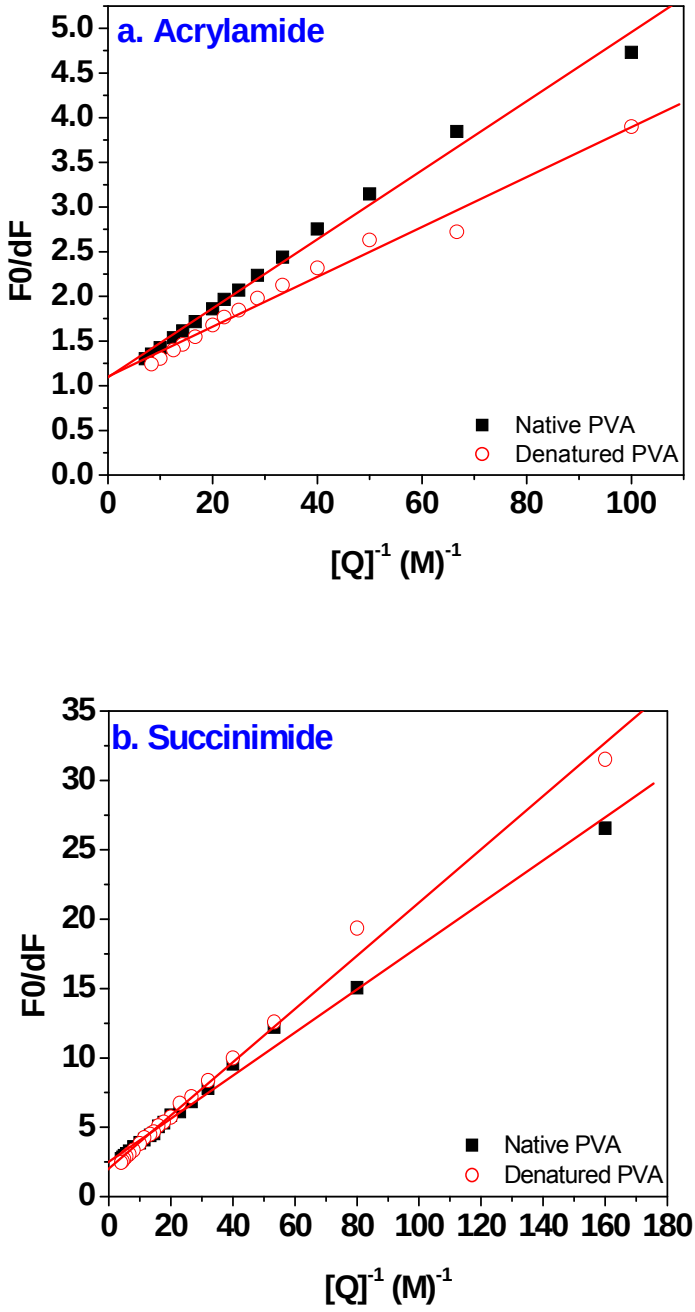


Figure 6.6: Modified Stern-Volmer plots for the quenching of the intrinsic florescence of Ra-PVA with different quenchers a. acrylamide, b. succinimide, in native and denatured conditions (6 M Gdn-HCl).

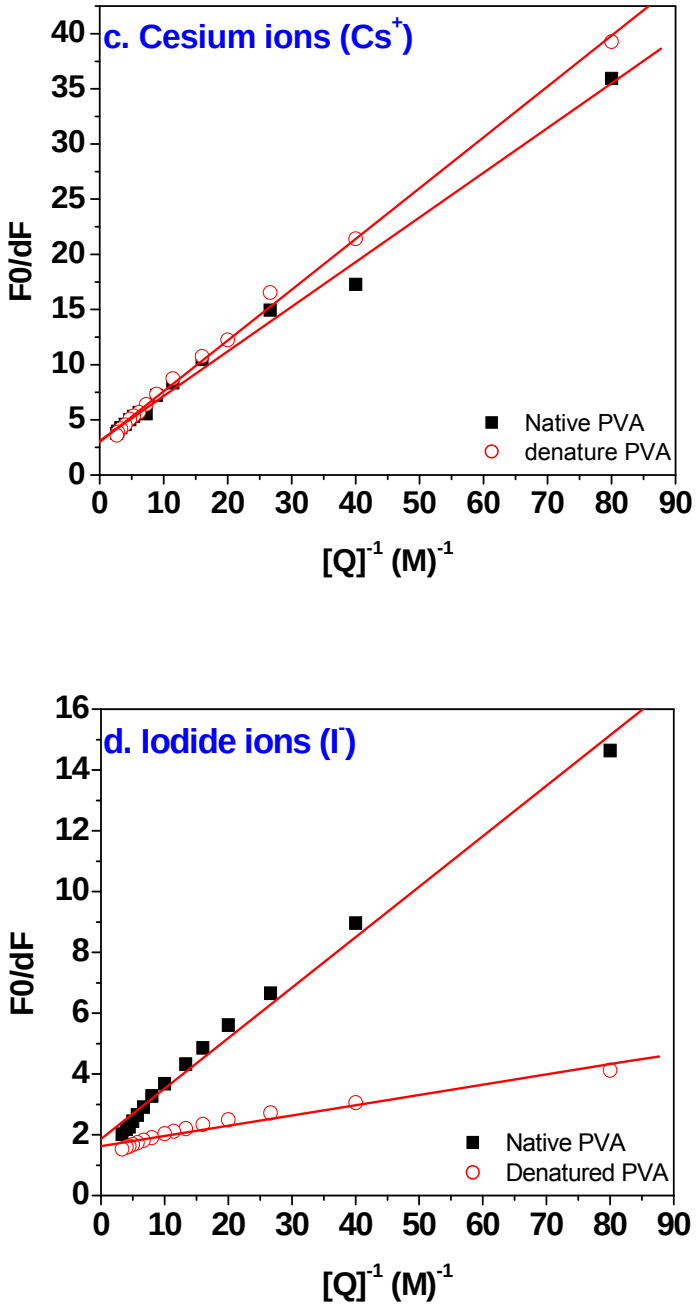


Figure 6.6: Modified Stern-Volmer plots for the quenching of the intrinsic florescence of Ra-PVA with different quenchers c. Cs + ions, and d. iodide ions, in native and denatured conditions (6 M Gdn-HCl).

Table 6.6: Summary of parameters obtained from the intrinsic fluorescence quenching of Ra-PVA with different quenchers

Quencher and condition	K_{sv1} (M^{-1})	K_{sv2} (M^{-1})	*fa*	*K*a
Acrylamide				
Native	23.6		1	23.3
Native+ Gdn-HCl	31.5		0.99	37.1
Succinamide				
Native	3.8	1.6	0.4	16
Native+ Gdn-HCl	2.7		0.49	10.5
CsCl				
Native	1.98	0.91	0.32	7.52
Native+Gdn-HCl	1.097	.	0.313	7.01
KI				
Native	3.27		0.53	11.23
Native+ Gdn-HCl	5.9		0.6	49.8

Of the quenchers used, acrylamide exhibited the maximum degree of quenching 71.6 and 78 % of the total fluorescence of the PVA, in native as well as denatured state, respectively, with increased rate of quenching. The *K*a, in case of native PVA, for succinimide is 16 M^{-1} as compared with 23.3 M^{-1} for acrylamide, which could be a reflection both of the inefficiency of quenching by the former as well as its restriction in accessibility of the tryptophans. The value of *K*a for iodide was 11.23 M^{-1} and for Cs^+ it was 7.52 M^{-1}, clearly indicating the iodide ion to be more efficient quencher than Cs^+.

6.4. Conclusions

On the whole, the chemical modification, substrate protection and fluorescence studies provide strong evidences for the presence of essential serine and tryptophan residues at the active center of *Ra*-PVA. The study supports the fact that, *Ra*-PVA belongs to Ntn hydrolase super family, due to the presence of catalytically active serine as a nucleophile at its active centre. Molecular structure of the enzyme will reveal the details of the monomeric eukaryotic PVA, however this is the first report of tryptophan residue being at or near active site of PVA. Furthermore the fluorescence studies demonstrated that tryptophan residues of *Ra*-PVA are differentially exposed to polar and non-polar environment. Quenching data obtained with I^- and Cs^+ indicate that tryptophans in *Ra*-PVA might have more electropositive environment, present in their immediate neighborhood.

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Summary and Conclusions

Chapter 7

7.1. Conclusions

Profound impact of penicillin acylases in the manufacture of semi-synthetic antibiotics is the reason for continued interest to conduct study on these enzymes. However, the exciting structural similarities and the evolutionary link among the members of Ntn-hydrolase superfamily are the core reasons for increasing research in this field. The present thesis basically emphasizes studies on penicillin V acylase from microbial cultures. Higher production of enzyme, enhancement in whole-cell enzyme activity by permeabilization and the unique characteristics of eukaryotic penicillin V acylase are the major contributions made by the thesis work to the research on penicillin acylases (PA).

7.2. Contributions made by the thesis to PA research

Screening of penicillin V acylase producing microorganisms resulted in three microbial cultures: *Erwinia aorideae* (DSMZ 30186), *Rhodotorula aurantiaca* (NCIM 3425) and a novel bacterial isolate, *Bacillus cereus* (ATUAVP 1846). This is the first time that studies on *R. aurantiaca* (NCIM 2534), a yeast source of penicillin V acylase is reported. Another major finding of the screening is *Bacillus cereus* (ATUAVP 1846) as a new bacterial source of penicillin V acylase. The culture can be used for production of enzyme in large quantities. Purification and characterization of enzyme may give important features of PVA and can be further used in pharmaceutical industries.

For the first time higher levels of penicillin V acylase production from *Erwinia aroideae* (DSMZ 30186) has been reported by the author of present thesis. An eight fold enhancement of whole-cell PVA activity of *E. aroideae* has been achieved by treatment with various organic solvents. The fundamental properties such as dielectric constant and hydrophobicity of organic solvents and their effect on cell-bound PVA activity torches light for further studies in this research area. The studies are also applicable for other systems by selecting a suitable solvent to be used for whole-cell biocatalyst with enhanced enzyme activity. Chloroform and diethyl ether are the cheap reagents, and their volatile nature facilitates removal from the reaction mixture. These two reagents are effective in permeabilizing *E. aroideae* cells to achieve maximum PVA activity

within short period of treatment. These features make the process convenient to scale up.

Production of high levels penicillin V acylase from a yeast source, *R. aurantiaca* (NCIM 3425) is also one of the prime findings of the thesis. Attempts for permeabilizing yeast, *R. aurantiaca*, led to the enhancement in cell-bound PVA activity. The studies may guide to use CTAB and its kinetics of permeabilization might be useful for application in pharmaceutical industries.

Purification of penicillin V acylase from a eukaryotic source, *R. aurantiaca (Ra*-PVA) has been reported for the first time by us in the present thesis. A unique finding of being a monomeric penicillin V acylase, not has been documented so far. The interest in studies on *Ra*-PVA got amplified when N-terminal sequencing showed presence of serine at the N-terminal end and 100 % sequence homology with the sequence of NADH dehydrogenase (pyridine nucleotide-disulfide oxidoreductase) from various *Bacillus* sp., however *Ra*-PVA was lacking the activity of the same. Owing to be an industrially important enzyme it was interesting to study the effect of different modulators on enzyme activity to know the behavior of the enzyme against various modulators, if encountered.

Active site characterization of *Ra*-PVA concluded that serine is playing important role in the catalysis as an essential active site residue. Involvement of tryptophan in the hydrolysis of substrate, Pen V, was a key feature of present work, which was reported for the first time. Micro-environment of tryptophan residues in the protein matrix has not been reported so far in case of penicillin V acylase. In the present thesis, fluorometric studies done on *Ra*-PVA using solute quenching has provided detail information on the biophysical nature or features of the protein.

7.3. Future scope of the present work

Penicillin G acylase is known to the pharmaceutical industries to produce 6-APA however despite having advantages over pen G, pen V is not figured out much in 6-APA technology. A lot more research needs to be carried out by screening new microorganisms exhibiting PVA activity with industrially favored features so that PVA can be used as much as PGA.

Stability of Pen V in aqueous solution, availability of PVA preparations active between pH 6.0-7.0, ability of PVA to hydrolyse higher concentrations of Pen V, and the specific nature of PVA, etc., encourage the use of Pen V as a bulk raw material for the production of 6-APA and 7-ADCA. Therefore, the studies can be conducted further on screening of microorganisms with features preferred by the industries. The screening studies conducted by author of the thesis led to identification of new isolate *Bacillus cereus* (ATUAVP 1846) producing intracellular PVA. The culture may give promising productivity by using media manipulation and standardization of cultural conditions. The higher production of PVA from the culture may lead to easy purification of enzyme. Attempt towards purification and characterization of enzyme can also be carried out which might be favorable to the industries. Further studies on biochemical and biophysical parameters of the enzyme may provide some additional clues to the evolutionary relationship among the members of Ntn hydrolase superfamily.

E. aroideae penicillin V acylase (*Ea*-PVA) is known since long time however its potential has not been explored so far, in terms of industrial applications. The work carried out by the author of the thesis such as optimization of *Ea*-PVA production as well as enhanced whole-cell *Ea*-PVA activity can be useful for the pharmaceutical industries. Permeabilized whole-cells with enhanced PVA activity can be used as biocatalyst by subsequent immobilization of cells using various methods. The system can, however, be further used for continuous production of 6-APA by the industries. Purification of *Ea*-PVA can be attempted to obtain homogenous preparation followed by biochemical and biophysical characterization of the enzyme. Attempts for the crystallization of the enzyme can also be carried out to get three dimensional structure of the protein.

This was the first time that eukaryotic PVA was purified and characterized. The work further can be extended by studying molecular structure of enzyme using X-ray crystallography, which can reveal the details of unique monomeric PVA.

The emphasis should be given on identification of industrially favored microorganisms which fulfill the requirement of pharmaceutical industries thus can really improve the 6-APA process and semi-synthetic penicillin technology. The work covered by the thesis gives a basic idea about the characteristics of PVA

from different organisms though the work mainly focuses on the study of yeast PVA. The enzyme, *Ra*-PVA, may not be useful for industrial application due to strong inhibition by its own product, POAA, in the hydrolysis of pen V, however the studies can proceed to understand the molecular characteristics of *Ra*-PVA.

Apart from selecting various microorganisms producing PVA, the stability of enzyme is also plays an important role in industrial process. The studies on stability of PVA should be performed by using various stabilizing agents such as solvents, detergents and additives etc. to favour the industrially useful enzymatic process. We hope the present work will serve as foundation to build up research on penicillin V acylase and help to develop improved 6-APA technology.

Publications

1. **Atul Kumar,** Vivek Lonkar & Archana V. Pundle. **(2008)** Studies on optimization of cultural conditions and effect of media constituents on production of intracellular penicillin V acylase from *Erwinia aroideae* (DSMZ 30186). **Research Journal of Biotechnology 3(2) 26-30**.
2. **Atul Kumar** & Archana V. Pundle **(2008)** Effect of organic solvents on cell-bound penicillin V acylase activity of *Erwinia aroideae* (DSMZ 30186): a permeabilization effect. **Journal of Molecular Catalysis B: Enzymatic (Article in press)**
3. **Atul Kumar**, Sanjay Singh, Pankaj Poddar, Asmita A. Prabhune & Archana V. Pundle **(2008)** 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). **Applied Biochemistry and Biotechnology (Article in press)**
4. **Atul Kumar**, Asmita A. Prabhune, Suresh C.G. & Archana V. Pundle **(2008)** Characterization of smallest active monomeric penicillin V acylase from new source: a yeast, *Rhodotorula aurantiaca* (NCIM 3425) **Process Biochemistry 43: 961-967.**
5. **Atul Kumar**, Nagaraj M. Gowda, Sushma Gaikwad & Archana V. Pundle **(2008)** *Rhodotorula aurantiaca* penicillin V acylase: Active site characterization and Fluorometric studies. **Journal of Photochemistry and Photobiology B: Biology (communicated)**

Patent File

- Archana V. Pundle, **Atul Kumar** & Asmita Prabhune A process for the production of penicillin V acylase using novel source of yeast *Rhodotorula aurantiaca* (NCIM 3425) and an improved process for 6-aminopenicillanic acid (6-APA) production through whole cell permeabilization. Indian patent application no. NCL-1 **(2005)**

Peer reviewed abstract accepted

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